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THE RADIOSENSITIZATION EFFECT OF PARTHENOLIDE IN PROSTATE CANCER: IMPLICATIONS FOR SELECTIVE CANCER KILLING BY MODULATION OF INTRACELLULAR REDOX STATE

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ABSTRACT OF DISSERTATION

Yulan Sun

The Graduate School
University of Kentucky
2010
THE RADIOSENSITIZATION EFFECT OF PARTHENOLIDE IN PROSTATE CANCER: IMPLICATIONS FOR SELECTIVE CANCER KILLING BY MODULATION OF INTRACELLULAR REDOX STATE

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School at the University of Kentucky

By

Yulan Sun

Lexington, Kentucky

Director: Dr. Daret K. St. Clair, Professor of Toxicology

Lexington, Kentucky

2010

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ABSTRACT OF DISSERTATION

THE RADIOSENSITIZATION EFFECT OF PARTHENOLIDE IN PROSTATE CANCER: IMPLICATIONS FOR SELECTIVE CANCER KILLING BY MODULATION OF INTRACELLULAR REDOX STATE

Parthenolide (PN), a major active component of the traditional herbal medicine feverfew, has been shown to have anti-inflammatory and anti-tumor properties. More remarkably, the cytotoxicity of PN seems selective to tumor cells but not their normal cell counterparts. In the present study, we investigate whether and how PN selectively enhances tumor sensitivity to radiation therapy by using prostate cancer cells LNCaP, DU145 and PC3, as well as normal prostate epithelial cells PrEC.

Our study demonstrates that inhibition of NF-κB pathway and suppression of its downstream target MnSOD are common mechanisms for the radiosensitization effect of PN in prostate cancer cells. The differential susceptibility to PN in two radioresistant cancer cells, DU145 and PC3, is due, in part, to the fact that in addition to NF-κB inhibition, PN activates the PI3K/Akt pro-survival pathway in both cell lines. The presence of PTEN in DU145 cells enhances the radiosensitization effect of PN by suppression of the steady state level of activated p-Akt.

We also demonstrate that PN selectively exhibits a radiosensitization effect on prostate cancer PC3 cells but not on normal prostate epithelial PrEC cells. PN causes oxidative stress in PC3 cells but not in PrEC cells, as determined by the oxidation of the ROS-sensitive probe H2DCFDA and intracellular reduced thiol and disulfide levels. In PC3 but not PrEC cells, PN activates NADPH oxidase leading to a decrease in the level of reduced thioredoxin, activation of PI3K/Akt and consequent FOXO3a phosphorylation, which results in the downregulation of FOXO3a targets, antioxidant enzyme MnSOD and catalase. Importantly, when combined with radiation, PN further increases ROS levels in PC3 cells, while it decreases radiation-induced oxidative stress in PrEC cells, possibly by increasing GSH level.

Overall, our data support the concept that increasing oxidative stress in cancer cells, which are already under high constitutive oxidative stress, will lead to cell death, while the same stress may allow normal cells to maintain redox homeostasis through adaptive
response. Thus, modulating cell redox status may be a novel approach to efficiently and selectively kill cancer cells.

KEYWORDS: Parthenolide, radiation, prostate cancer, oxidative stress, redox signaling

Yulan Sun
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January 28, 2010
Date
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This dissertation is dedicated to my parents.
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Chapter One
Introduction

Prostate cancer has a high incidence in the United States. It is estimated that about one man in six will be diagnosed with prostate cancer during his lifetime. It accounted for about one-fourth of newly diagnosed cancer cases and causes 10% of cancer deaths in men in 2008 [1]. The treatment of prostate cancer commonly involves surgery, radiation, and hormone therapy. Radiation can be used to treat patients with any stage of prostate cancer. Patients with localized early-stage prostate cancer can be effectively treated with radiation, which is also used after surgery to destroy the cancer cells that may remain in the original area, and thus preventing cancer recurrence. For the advanced stage of prostate cancer, radiation treatment may be used to reduce the tumor size and help relieve pain. However, according to a multi-institutional pooled study, in patients with pretreatment PSA levels of 10-20, 20-30 and more than 30 ng/ml, the 5-year biochemical recurrence free rates (no evidence of disease on the basis of the PSA value) were 68%, 51%, and 31%, respectively [2]. One possibility for the recurrence of prostate cancer after radiation treatment is that cancer cells develop radioresistance. Therefore, finding agents that sensitize prostate tumor cells to radiation therapy would enhance tumor response and possibly prevent tumor recurrence.

Radiation therapy

Radiation therapy works mostly by the generation of free radicals. Ionizing radiation, such as X-ray and gamma ray, ejects electrons from atoms when they are absorbed by biological material. The loss and gain of electrons generate free radicals. The free radicals initially formed in the presence of O$_2$ may include hydroxyl radical (OH$^-$), superoxide (O$_2^-$), and organic radicals (R$^\cdot$) [3] (Figure 1.1). The reactions of these free radicals generate more reactive species, such as hydrogen peroxide (H$_2$O$_2$) and organic hydroperoxide (ROOH). The biological effect of ionizing radiation may be due to direct action and indirect action [4]. Direct action refers to that radiation directly ionizes the atoms of some critical targets in the cells, such as DNA, leading to the damage of DNA. Alternatively, radiation can ionize other molecules, especially water, which constitutes
about 80% of cell content, to produce free radicals, such as hydroxyl radical OH· (Figure 1.1). The free radicals can then diffuse to damage the DNA, protein and lipid targets. This is called indirect action, which is dominant for ionizing radiation. The damaging effect of radiation is greatly enhanced if O₂ is present during radiation, which is called the oxygen effect. One possible mechanism for the oxygen effect is that, in the presence of oxygen, the radical formed in the biological targets (R·) can react with oxygen to form organic hydroperoxide (RO₂·), which cannot be regenerated to the original compound (R) and is considered irreparable, thus “fixing” the damage [4]. Another possibility is that superoxide radical (O₂·⁻), which is formed in the presence of oxygen during radiation, contributes to the oxygen effect. This is supported by the fact that higher superoxide dismutase (SOD) activity reduces the oxygen enhancement ratio [5]. Since using a free radical scavenger can reduce the effect of ionizing radiation by a factor of three, it is estimated that about two-thirds of the biological damage by radiation is due to the free radical mediated mechanism [4].

In addition to the free radicals generated immediately after radiation by direct ionization, radiation also induces reactive species production by other sources. Leach et al. showed that radiation-induced ROS/RNS (Reactive oxygen species/ Reactive nitrogen species) generation within 10 minutes after radiation is inhibited by mitochondrial permeability transition inhibitor cyclosporin A, and is absent in the mitochondria-deficient ρ₀ cells [6], indicating that mitochondria are important source for radiation-induced reactive species. Nitric oxide synthase is also activated several minutes after radiation treatment, leading to the generation of nitric oxide (NO·⁻) [7]. NADPH oxidase is shown to be involved in radiation-induced ROS generation at a later time point (up to 12 hours) [8]. The free radicals generated from different sources after radiation may act later at different time points and amplify or prolong the deleterious effect of radiation, leading to cell death, the bystander effect [9], chronic oxidative stress and normal tissue injury [10].

The major biological target of X-ray is DNA. Radiation induces diverse types of DNA lesions including both base modifications and strand breaks, mainly through the free radical-mediated mechanism. Double-stranded breaks are regarded as the most lethal lesions. Unrepaired DNA lesions lead to genetic instability and increased frequency of
mutations and chromosomal aberrations. Lethal mutations or dysfunctional chromosomal aberrations eventually lead to the loss of replicative potential, usually after several mitotic cycles. This is called the mitotic cell death pathway, which is the predominant mechanism by which radiation kills mammalian cells. The oxidative DNA damage caused by radiation also triggers apoptotic cell death, which is usually p53 dependent. However, apoptosis is not the major form of cell death in irradiated solid tumors, including prostate tumors [11].

In addition to oxidative damage to DNA, the reactive species generated by radiation can also activate multiple intracellular signaling pathways involved in the control of cell death and survival, leading to either apoptotic and cell death response, or adaptive and repair response. For example, radiation can alter cytoplasmic Ca\(^{2+}\) homeostasis [12] or act on the cellular membrane to induce ceramide production [13], leading to apoptosis. Radiation-induced mitochondria damage [14] and the activation of Fas, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) and TNF-\(\alpha\) (tumor necrosis factor alpha) death receptor pathways also contribute to radiation-induced cell death [15]. In addition to the induction of cell death pathways, X-ray has also been shown to activate a number of pathways and transcription factors involved in cell cycle arrest, DNA damage repair, anti-apoptosis and cell growth such as ATM (ataxia telangiectasia mutated), p53, EGFR (epidermal growth factor receptor), PI3K (phosphatidylinositol 3-kinase)/AKT, MAPK (mitogen activated protein kinase), and NF-\(\kappa\)B (nuclear factor kappa B) [16]. The induction of DNA damage repair and reactive species detoxification processes by radiation has been implicated in radioadaptive response [17, 18] and may also contribute to induced resistance to radiation.

**Radioresistance**

The mechanisms for the development of radiation resistance in human tumor are not yet fully understood. Mutation or constitutive activation of some prosurvival pathways has been correlated with resistance to X-ray-induced cell killing [19]. A proteomics study performed by Skvortsova and colleagues has shown that radiation resistant prostate cancer cells [20] exhibit higher levels of androgen receptor and epidermal growth factor receptor (EGFR) and activation of their downstream pathways, such as the Ras-mitogen-
activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-Akt and Jak-STAT pathways. Activation of NF-κB pathway has also been associated with the development of radioresistance in prostate cancer cells [21].

Since radiation mainly kills cells by inducing oxidative stress, cellular antioxidant status also affects sensitivity to radiation. It has been shown that compared with the radiation-sensitive mice, radiation-resistant mice have higher SOD and catalase enzyme activities [22]. Antioxidant enzyme MnSOD upregulation is implicated in low dose ionizing radiation (≤10 cGy) and fractionated ionizing radiation-induced adaptive response, leading to radioresistance [18, 23]. Our previous study found that selective inhibition of RelB-induced MnSOD after radiation treatment can sensitize prostate cancer cells to radiation treatment [24]. Radiation also induces antioxidant enzyme peroxiredoxin II [25, 26] expression and protects cancer cells from oxidative stress-induced damage.

In addition to the tumor cell genetic abnormality and intracellular stress response, the microenvironment of a tumor, such as hypoxia, elevated growth factor expression, cell adhesion molecule [27] and tumor angiogenesis [28], also contributes to the development of radioresistance. Further understanding of the mechanisms of radioresistance in tumor cells will help to improve the outcome of radiation therapy.

**Oxidative stress**

Oxidative stress refers to an imbalance between pro-oxidant and antioxidant that favors the former, leading to potential damage. Both the increase in production of reactive species (RS) and the decrease in antioxidants can lead to oxidative stress.

Reactive species, including ROS (reactive oxygen species) and RNS (reactive nitrogen species), are free radicals that contain one or more unpaired electrons, such as superoxide (O$_2^-$), hydroxyl radical (OH·), nitric oxide (NO·), and non-radicals, such as hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$). Reactive species can be produced either endogenously or exogenously. The most important endogenous source of ROS is the mitochondrial electron transport chain. Aerobic organisms use oxygen to facilitate the energy production in the form of ATP, most often by mitochondrial oxidative phosphorylation. During mitochondrial oxidative phosphorylation, electrons pass through
the electron transport chain on the mitochondrial inner membrane and eventually reduce the oxygen to water. However, some components of the mitochondrial electron transport chain, such as complex I (NADH-dehydrogenase) and complex III (ubiquinone-cytochrome b), can leak electrons directly to O\(_2\), leading to the one electron reduction of O\(_2\) and generation of O\(_2^•\). It is estimated that 1-3\% of the O\(_2\) reduced in mitochondria may form O\(_2^•\). Superoxide can be dismutated by superoxide dismutase (SOD) to yield hydrogen peroxide. In the presence of transition metal ions, in particular iron, hydrogen peroxide is subsequently converted through Fenton and Haber-Weiss reactions to a hydroxyl radical. Reactive species can also be generated by other enzymes, such as xanthine oxidase (XO), membrane-associated NADPH oxidase and cytochrome P450 in endoplasmic reticulum, oxidases in peroxisomes, NOS (nitric oxide synthase), and auto-oxidation of small molecules such as dopamine, epinephrine and flavins [29, 30]. The exogenous sources of reactive species include irradiation (i.e., UV radiation, X-ray, \(\gamma\)-ray), cigarette smoke and redox cycling of some chemicals, such as quinone and aromatic nitro compound.

To counterbalance the production of reactive species, biological organisms develop the antioxidant system (Figure 1.2). The enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), peroxiredoxin (Prx) and glutathione S-transferase (GST). There are three forms of SODs in mammals: cytoplasmic copper zinc SOD (SOD1), mitochondrial manganese superoxide dismutase (SOD2) and extracellular copper zinc SOD (SOD3), which catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Catalase, located in peroxisomes, is responsible for the removal of hydrogen peroxide by direct decomposition of H\(_2\)O\(_2\) to O\(_2\). GPxs, localized in the cytosol, mitochondria and extracellular fluids, remove hydrogen peroxide by coupling its reduction to H\(_2\)O with oxidation of glutathione. Prxs, which are widely distributed both intracellularly and extracellularly, also reduce hydrogen peroxide, organic peroxides and peroxynitrite. The typical 2-cysteine Prxs are thioredoxin-dependent peroxide reductases. GST catalyzes the conjugation of glutathione with electrophilic centers on a wide variety of substrates, including lipid hydroperoxide. GST has also been reported to conjugate with 4-hydroxynonenal (4-HNE), the highly toxic endproduct of lipid peroxidation. In addition to these antioxidant enzymes, small thiol-
containing peptides, such as glutathione (GSH), glutaredoxin (Grx) and thioredoxin (Trx) systems also help to scavenge reactive oxygen species and maintain other proteins in their reduced state. The non-enzymatic antioxidants include, among others, Vitamin E, Vitamin C, β-carotene and coenzyme Q.

It has been shown that the balance between prooxidant and antioxidant plays an important role in cell survival and death. When the redox balance is shifted in favor of prooxidants, oxidative damage to nucleic acids, lipids, and proteins can lead to changes in cell function and cell viability. Depending on the cell type and the extent of the resulting oxidative stress, the consequences of oxidative stress can be very different, from elevated proliferation, induction of adaptive response, to cell injury, senescence and cell death. A wide range of diseases and pathological conditions is associated with oxidative stress, such as neurodegenerative diseases and cancer.

**Oxidative stress and cancer**

It has been postulated that oxidative stress is associated with tumor formation, progression and metastasis. Reactive species can attack DNA, leading to various oxidative DNA damages, such as 8-hydroxy-deoxyguanosine (8OH-dG). Nuclear genomic DNA and mitochondrial DNA are both susceptible to oxidative damage. Unrepaired DNA damage may result in mutations in oncogenes and tumor suppressor genes, leading to carcinogenesis. Mutation and alteration of mitochondrial DNA also contribute to the process of carcinogenesis. Reactive species can also act as signaling molecules and regulate cell signaling pathways that are involved in cell growth and apoptosis. For example, in response to oxidative stress, MAPK (mitogen activated protein kinase) / AP-1 (activator protein-1) and NF-κB pathways are activated. The altered gene expression downstream of these pathways leads to the stimulation of cell proliferation and suppression of cell apoptosis, contributing to tumor progression [30]. Oxidative stress may also promote tumor metastasis by altering the tumor microenvironment and stimulating angiogenesis [31].

Prostate cancer cells have been shown to have increased level of oxidative stress compared to normal prostate cells and the level of oxidative stress is proportional to the aggressive phenotype [32]. The increased oxidative stress level in prostate cancer cells
may result from an increase of ROS generation and/or a decrease in the antioxidants. The increased ROS generation may come from mitochondria [33] or the NADPH oxidase (NOX) system [32]. Prostate cancer has also been reported to have a lower antioxidant defense system compared to normal prostate tissue. For example, lower antioxidant enzymes MnSOD, CuZnSOD, catalase [34, 35], and defects in several classes of GSTs [36], are observed in prostate cancer cells compared with benign prostate cells. The antioxidant Nrf2 [Erythroid 2p45 (NF-E2)-related factor 2 (Nrf2)] pathway [37] is also downregulated in prostate cancer cells.

Oxidative stress is not only involved in tumorigenesis, but also implicated in tumor therapy. The small shift in cell redox status toward prooxidation will usually increase the proliferation of tumor cells and be involved in tumorigenesis and tumor progression. A highly oxidizing condition is strongly cytotoxic and is a primary mechanism for tumor cell killing by radiation therapy and some chemotherapeutics, such as taxol and adriamycin. Since tumor cells are under more oxidative stress than normal cells, inhibition of antioxidant systems or exposure to further exogenous ROS insults would cause more ROS-mediated damage in them. Therefore there is presumably a therapeutic window for modifying cell redox status, which allows normal cells to maintain redox homeostasis through adaptive response, but pushes the tumor cells, which are already under high constitutive oxidative stress, beyond their tolerance, leading to selective targeting of tumor cells in cancer therapy (Figure 1.3). A number of studies support this hypothesis. Increasing cell H\textsubscript{2}O\textsubscript{2} level by SOD mimics-mediated superoxide dismutation has a differential effect on non-transformed NIH3T3 cells and CT26 colon and Hepa 1-6 liver tumor cells [38]. In NIH 3T3 cells, which have low levels of ROS, slightly elevated H\textsubscript{2}O\textsubscript{2} induces cell proliferation, but the same level of H\textsubscript{2}O\textsubscript{2} leads to cell death in CT26 and Hepa 1-6 tumor cells, which have high levels of endogenous ROS. Regulating intracellular redox state is also a good target to selectively sensitize cancer cells to oxidative stress-inducing therapy, such as radiotherapy. For example, 2-Deoxy-D-glucose (2-DG), a potent inhibitor of glucose metabolism, has been shown to preferentially induce radiosensitization in transformed cells but not non-transformed cells via perturbations in thiol metabolism [39]. Selenite sensitizes prostate cancer cells to
radiation by altering the intracellular redox state and preferentially inducing apoptosis in cancer cells [40].

Parthenolide
Parthenolide is a sesquiterpene lactone derived from the leaves of the traditional herbal medicine feverfew (Tanacetum parthenium). Parthenolide has been traditionally used in Europe to treat inflammatory diseases, such as fever, migraine, and arthritis [41]. More recently, it has been found to be a potent NF-κB inhibitor [42]. The biological activity of parthenolide is thought to be mediated through the α-methylene-γ-lactone moiety and the epoxide in its structure (Figure 1.4). These functional groups can react with nucleophiles, especially with cysteine thiol groups in a Michael addition reaction.

Recently, the anti-tumor property of parthenolide has attracted great interest among researchers. Parthenolide has been shown to inhibit growth or induce apoptosis in a number of tumor cell lines [43-47]. More remarkably, it has been shown that parthenolide is cytotoxic to hepatoma cells and leukemia cells while sparing normal liver cells and hematopoietic cells [43, 46], suggesting that the cytotoxic effect of parthenolide is selective for tumor cells. Many mechanisms have been postulated as being involved in the anti-tumor effect of parthenolide, including inhibition of nucleic acid synthesis [48, 49], depletion of thiols, induction of oxidative stress [43, 45, 46], induction of mitochondria dysfunction [43], disruption of intracellular calcium equilibrium [45], induction of cell cycle G2/M phase arrest [43, 44], depletion of HDAC1 (histone deacetylase 1) [50], inhibition of tubulin carboxypeptidase activity [51], sustained activation of JNK (c-Jun N-terminal kinase) [52, 53], inhibition of MAPK (mitogen-activated protein kinase) activity [54], activation of p53 [46, 55], suppression of STAT3 (signal transducer and activator of transcription 3) [56] and inhibition of NF-κB [46, 53] activation. It has also been demonstrated that parthenolide sensitizes cancer cells to various apoptosis-inducing agents mainly through inhibition of NF-κB [53, 57-60]. Inhibition of the NF-κB pathway by parthenolide is a well-established consequence of its direct interaction with Cys residues in IKKβ and p65 [61-63].

Parthenolide has also been shown to play a dual role in regulating the intracellular redox state. Direct reaction with the Cys thiols by parthenolide may lead to depletion of
intracellular GSH and protein thiols and induction of ROS in some cancer cells [43, 45], but another study suggests that parthenolide possibly increases GSH levels by activation of the Nrf2-ARE (antioxidant/electrophile response element) pathway [64] in hippocampal HT22 cells. Li-Weber et al. reported that parthenolide at low dose (up to 5 µmol/L) neutralizes H₂O₂ generated by the TCR (T cell receptor) signaling pathway in Jurkat T cells and protects cells from CD3-induced apoptosis; at high dose (10 µmol/L), it induces O₂⁻ and generates oxidative stress, leading to an increase of dead cells [65]. Therefore, depending on the cell type and concentration, parthenolide may act as either a prooxidant or an antioxidant under different conditions.

Since parthenolide not only inhibits NF-κB, which is known to be an important mediator of radioresistance, but also regulates intracellular redox state, a determinative factor for cell sensitivity to radiation, I hypothesize that parthenolide may sensitize cancer cells to radiation treatment.

**Thiol chemistry and biological importance**

Thiols (-SH) are important in integrating intracellular redox changes with cellular signaling transduction pathways. Thiols are easily oxidized and usually the oxidation products can be reversibly reduced, making them a good candidate for signal transduction. Thiols can react with almost every physiological oxidant, including superoxide, hydrogen peroxide, nitric oxide and peroxynitrite. Different oxidants may induce different sets of target thiol oxidation. The oxidation of thiols can form disulfide (-S-S-, including mixed disulfides with glutathione or glutathionylation, -S-SG), sulfenic acid (-SOH), sulfenic acid [-S(O)OH], sulfonic acid [-S(O)₂OH], thiol-sulfinate [-S(O)-S-], thiyl radical (-S·), nitrosothiol (-SNO). Some of these oxidation products are not stable and are highly reactive. For example, sulfenic acid or thiyl radical may further react to form disulfide. The reactivities of thiol proteins and non-protein thiols are related to their structures, hydrophobicity, pH, the availability of thiol group, abundance and competition for oxidants, and also the pKa of the cysteine residue. Cysteine pKa values span a wide spectrum from as high as 8.8 for cysteine in GSH, to 6.5 for thioredoxin and as low as 5.4 for the phosphatase PTP1 (protein tyrosine phosphatase 1). The reactivity of thiol protein is generally inversely related to the pKa value. The lower the pKa is, the more reactive
the thiol protein is. The cellular thiol redox state is controlled by two major systems, the glutathione and thioredoxin. The glutathione/ glutathione reductase/ glutaredoxin (Grx) system catalyzes the reduction of glutathionylated proteins, while thioredoxin/ thioredoxin reductase has greater specificity for protein disulfides. Both these systems utilize NADPH as the ultimate source of electrons.

A number of regulatory proteins have cysteines on their active sites. The oxidation and reduction of the cysteine thiols on these proteins have important effect on their functions or act as the molecular switch for their downstream signaling cascades [66]. For example, the cysteines on the active site of some kinases and phosphatases are sensitive to reactive species induced oxidation. Phosphatases such as PTEN (phosphatase and tensin homologue deleted from chromosome-10), PTP1B (protein tyrosine phosphatase 1B), and SHP-2 (Src homology 2 domain (SH2)-containing tyrosine phosphatase 2) have been shown to form a sulfenic acid intermediate and to be inactivated by H$_2$O$_2$ mediated oxidation. On the other hand, protein kinases and GTPases, such as Src tyrosine kinase and Ras, are activated by cysteine oxidation. Many transcription factors also contain redox sensitive cysteine residues. AP-1 (activator protein-1), Ref-1 (Redox factor-1), NF-$\kappa$B, and p53 all contain redox sensitive cysteine residue in their DNA binding domain. The oxidation of these cysteine residues often leads to a decrease in DNA binding activity. Proteins control activation and localization of transcription factors can be oxidized as well, such as Keap1 (Kelch-like ECH-associated protein-1), which regulates Nrf2, and IκB, which regulates NF-$\kappa$B. Oxidation can also significantly change the tertiary structure of proteins; thereby influencing their functional properties and interactions with other proteins. For example, the oxidation of thioredoxin leads to its dissociation from ASK-1 (apoptosis signal-regulating kinase 1), resulting in ASK-1 activation and induction of apoptosis.

Thiols could play a significant role in the resistance of tumor cells to chemotherapeutic agents [67]. Modulation of thiol redox-related enzymes may be an effective means of enhancing anticancer therapies. Depleting cellular thiols has been shown to enhance radiation-induced cell death [68]. Parthenolide contains an $\alpha$-methylene $\gamma$-lactone moiety, which is electrophilic and apt to react with biological nucleophiles such as the thiol groups in proteins via a Michael addition reaction (Figure
1.5). The chemical properties of parthenolide might make it a good candidate for modifying cellular redox signaling and endow it with great potential in cancer therapy.

**Research objectives**

This study aims to address whether and how parthenolide can sensitize prostate cancer cells to radiation treatment.

In chapter two, we report our investigation of whether parthenolide can sensitize prostate cancer cells to radiation treatment by using different prostate cancer cell lines. The studies were focused on investigating the role of NF-κB in the radiosensitization effect of parthenolide. As we compared the effect of parthenolide in different prostate cancer cell lines, we found that inhibition of NF-κB is a common but not exclusive mechanism for the effect of parthenolide, which led us to study how parthenolide affects the PI3K/Akt pathway and how PTEN influences cell sensitivity to parthenolide.

In chapter three, we report our comparison of the effect of parthenolide on prostate cancer cells and normal prostate epithelial cells. Here, our study focused on how parthenolide differentially regulates intracellular redox status and redox-related signaling pathways in cancerous and noncancerous prostate cells.

The overall objective of this research is to investigate how parthenolide sensitizes prostate cancer cells to radiation treatment and how the selective cancer killing effect is achieved. The data also provide some experimental evidence for the potential clinical use of parthenolide in cancer therapy.
Figure 1.1 Possible reactions for the free radicals generated by radiation.

\[
\begin{align*}
\text{H}_2\text{O} & \rightarrow \text{H}_2\text{O}^+ + e^- \\
\text{H}_2\text{O}^+ + \text{H}_2\text{O} & \rightarrow \text{H}_3\text{O}^+ + \text{OH}^- \\
\text{OH}^- + \text{R-H} & \rightarrow \text{H}_2 + \text{R}. \\
e^{-}_{\text{aq}} + \text{O}_2 & \rightarrow \text{O}_2^{-}. \\
\end{align*}
\]

\( e^{-}_{\text{aq}} \), hydrated electrons, ‘aq’ stands for aqueous.
Figure 1.2 Scheme of cellular reactive species generation and antioxidant system.
Sources of reactive species: ETC, mitochondria electron transport chain; NOX, NADPH oxidase; XO, xanthine oxidase; P450, cytochrome P450; NOS, nitric oxide synthase.
Antioxidant system: SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; TrxR, thioredoxin reductase; TRXox, oxidized thioredoxin; TRXre, reduced thioredoxin; Prx, peroxiredoxin.
Figure 1.3 The therapeutic window for selective targeting of tumor cells by modulating cell redox status.

Under normal physiologic conditions, cell redox status is kept in balance. A small shift in cell redox potential toward an oxidizing condition will activate repair or adaptive signaling, leading to upregulation of the antioxidant system and increased oxidative damage repair pathway, as well as promotion of cell proliferation. However, further shift to an extreme oxidizing condition will activate the apoptotic or necrotic pathway, leading to cell death. Tumor cells, compared to normal cells, are usually under oxidative stress and have increased proliferation. Therefore, a further increase in oxidative stress will easily disrupt the redox balance of tumor cells, leading to cell death. However, an adaptive response enables normal cells to maintain redox balance. Therefore, a therapeutic window which is determined by the redox difference between normal and tumor cells exists for selective targeting of tumor cells by redox intervention.
Figure 1.4 Chemical structure of parthenolide
Figure 1.5 Parthenolide reacts with thiol group in a Michael addition reaction.
Chapter Two

The radiosensitization effect of parthenolide in prostate cancer cells is mediated by NF-κB inhibition and enhanced by the presence of PTEN

Synopsis

Parthenolide has been shown to have anti-inflammatory and anti-tumor properties. However, whether and how parthenolide enhances tumor sensitivity to radiation therapy remain unknown. In this study, we demonstrate that inhibition of the NF-κB pathway is a common mechanism for the radiosensitization effect of parthenolide in prostate cancer cells LNCaP, DU145 and PC3. Parthenolide inhibits radiation-induced NF-κB DNA binding activity and the expression of its downstream target Sod2, the gene coding for an important anti-apoptotic and antioxidant enzyme (MnSOD) in the three prostate cancer cell lines. Different susceptibilities to parthenolide’s effect were observed in two radioresistant cancer cells, DU145 and PC3, with DU145 cells showing higher sensitivity. This differential susceptibility to parthenolide is due, in part, to the fact that in addition to NF-κB inhibition, parthenolide activates the PI3K/Akt pro-survival pathway in both DU145 and PC3 cell lines. However, the activated Akt in DU145 cells is kept at a relatively low level compared to that in PC3 cells due to the presence of functional PTEN. Transfection of wild-type PTEN into PTEN null cells, PC3, confers the enhanced radiosensitization effect of parthenolide in PTEN-expressing cells. When PTEN expression is knocked down in DU145 cells, the cells become more resistant to parthenolide’s effect. Taken together, these results suggest that parthenolide inhibits the NF-κB pathway and activates the PI3K/Akt pathway in prostate cancer cells. The radiosensitization effect of parthenolide is due, in part, to the inhibition of the NF-κB pathway. The presence of PTEN enhances the radiosensitization effect of parthenolide, in part, by suppressing the absolute amount of activated p-Akt.
Introduction

Prostate cancer is the most common cancer type and the third leading cause of cancer deaths of U.S. men in 2006 [69]. Radiation therapy is frequently used to treat early stage and inoperable locally advanced prostate cancer. The outcome of radiation therapy can be greatly improved if higher doses of radiation are applied, especially for patients with unfavorable tumors (i.e., Stage T3 lesions, pretreatment PSA levels greater than 10 ng/ml, or biopsy Gleason scores ≥7). For these patients, the 5-year cancer-free survival rate increased from 41% to 75% when radiation dose was increased to more than 7200 cGy [70]. However, the side effects and late complications resulting from high dose radiation increase to an unacceptable level, which limits the use of high doses of radiation. Another problem in radiation therapy is that tumor cells develop radioresistance. Therefore, it is important to find agents that can effectively sensitize malignant tumor cells to radiation therapy at low doses while minimizing radiation toxicity to surrounding organs.

Many mechanisms are involved in the development of radioresistance in tumor cells. One possible mechanism is activation of the nuclear factor kappa B (NF-κB) signaling pathway. It has been shown that about two-thirds of the biological damage caused by radiation is due to free radical mediated indirect action [4]. NF-κB is a redox sensitive transcription factor family that regulates cell survival and death. In response to radiation-induced reactive oxygen species (ROS), the NF-κB pathway is activated [71]. There are five members in the NF-κB family: RelA (p65), RelB, c-Rel, NF-κB1 (p105/ p50) and NF-κB2 (p100/ p52). NF-κB is normally sequestered in the cytoplasm by its inhibitor IκB family members in an inactive complex. Two NF-κB activation pathways exist. The classical pathway activates the IκB kinase complex (IKC), which consists of IKKα, IKKβ and IKKγ, leading to IκBα phosphorylation, ubiquination and further degradation by the 26S proteasome. As a result, the p50:RelA dimer is released and translocated into the nucleus. The alternative pathway activates the IKKα homodimer, leading to the partial degradation of p100 and activation of the p52:RelB dimer. Russell and Tofilon reported that radiation activates the classical NF-κB pathway by selective degradation of plasma membrane-associated IκBα [72]. Our previous study shows that RelB is also activated by radiation [24]. Activation of NF-κB induces transcription of its target genes, which are involved in anti-apoptosis and tumor metastasis [73]. Among numerous NF-κB
downstream targets, manganese superoxide dismutase (MnSOD) has been identified as a constitutively and immediately accessible NF-κB target [74]. MnSOD is a nuclear-encoded primary antioxidant enzyme localized in mitochondria. The known function of MnSOD is to remove superoxide radicals in mitochondria [75]. Overexpression of MnSOD is protective against radiation-induced cell death [24, 76-78].

We and others have demonstrated that NF-κB is constitutively activated in aggressive prostate cancer [24, 79, 80], and may be responsible for the intrinsic radioresistance of some prostate cancer cells. Thus, inhibition of the NF-κB pathway represents a target to enhance the sensitivity of prostate cancer to radiation therapy.

Parthenolide is a major active component of the herbal medicine feverfew (Tanacetum parthenium), which is conventionally used in Europe to treat inflammatory diseases, such as fever, migraine, and arthritis [41]. Parthenolide has been shown to inhibit growth or induce apoptosis in a number of tumor cell lines [43, 46, 47, 81]. More remarkably, it has been shown that parthenolide is cytotoxic to hepatoma cells and leukemia cells while sparing normal liver cells and hematopoietic cells [43, 46], suggesting that the cytotoxic effect of parthenolide may be selective for tumor cells. Many mechanisms are postulated as being involved in the anti-tumor effect of parthenolide, including inhibition of nucleic acid synthesis [48], depletion of thiols, induction of oxidative stress [43, 45], induction of mitochondria dysfunction [43], disruption of intracellular calcium equilibrium [45], induction of cell cycle G2/M phase arrest [43, 44], sustained activation of JNK [52, 53], and inhibition of NF-κB [53]. It has also been demonstrated that parthenolide sensitizes cancer cells to various apoptosis-inducing agents mainly through inhibition of NF-κB [57]. However, whether parthenolide sensitizes cancer cells to radiation-induced cell death and whether inhibition of NF-κB is sufficient for the radiosensitization effect of parthenolide remain unknown.

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumor suppressor protein with dual-specificity protein phosphatase activity [82]. It dephosphorylates focal adhesion kinase (FAK) [83, 84], a major regulator of the integrin signalling pathway, as well as the Src-homology collagen protein (Shc), and thus inhibits the growth factor-induced mitogen-activated protein (MAP) kinase signalling pathway [85, 86]. PTEN also functions as a lipid phosphatase and dephosphorylates the second
messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) to antagonize the phosphatidylinositol 3-kinase (PI3K) signalling pathway. Loss of chromosome 10q, which harbors the PTEN gene, is found in about 60% of advanced prostate cancer [87]. Functional loss of PTEN and subsequent activation of the PI3K/Akt (v-akt murine thymoma viral oncogene homologue 1, also called protein kinase B, PKB) pathway have been widely implicated in prostate cancer progression to metastasis. In this study, we used three prostate cancer cell lines, including the radiosensitive LNCaP cells and two radioresistant cell lines, DU145 (PTEN wild-type) and PC3 (PTEN null), to investigate whether PTEN cooperate with NF-κB in the radiosensitization effect of parthenolide.

**Materials and Methods**

**Cell culture and treatment.** Human prostate cancer cell lines LNCaP, DU145, and PC3 were obtained from American Type Culture Collection (Manassas, VA). LNCap and PC3 cells were cultured in RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin mixture, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 1% NEAA mixture (Cambrex), 1% MEM vitamin mixture (Cellgro) and 2 mmol/L L-glutamine. DU145 cells were cultured in MEM medium (Sigma) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin mixture, 1 mmol/L sodium pyruvate and 0.1 mmol/L non-essential amino acids. Cells were grown in a 5% CO2 atmosphere at 37°C. Parthenolide stock solution (5 mmol/L) was prepared in DMSO and diluted in culture medium to the indicated final concentration for cell treatment. DMSO (0.1%) diluted in medium was used as vehicle control. A 130 kv X-ray machine (Faxitron X-ray Corporation) was used to radiate cells, with a dose rate of 89.7 cGy/min.

**Clonogenic survival assay.** Cells were trypsinized and plated in triplicate into 6-well plates at different densities based upon cell types and doses of radiation. LNCaP, DU145 and PC3 cells were plated at the density of 2,000-10,000 cells/well, 100-600 cells/well, and 100-500 cells/well respectively. Cells were treated with indicated concentrations of parthenolide or vehicle control for 24 hours prior to exposure to indicated doses of radiation. Twenty-four hours after radiation treatment, the medium containing parthenolide was removed and cells were maintained in normal culture medium. Twelve
days after plating, cells were washed and stained with crystal violet, and the colonies containing more than 50 cells were counted. Plating efficiency (PE) was calculated by dividing the average number of cell colonies per well by the amount of cells plated. Survival fractions were calculated by normalization to the plating efficiency of appropriate control groups. The dose modifying factor (DMF) is calculated by the ratio of the dose of radiation in the absence or presence of the drug to achieve the same cell survival.

**MTT assay.** Cells were plated at the density of 5,000 cells/well into 96-well plates and grew overnight. Then cells were pretreated with indicated concentrations of parthenolide for 3 hours, and exposed to 6 Gy radiation or were sham-irradiated. Twenty-four hours after radiation, parthenolide-containing medium was replaced with normal culturing medium. Five days after radiation, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 50 μg/well) was added and incubated at 37°C for 1 hour. After removal of medium, DMSO 200 μL was added to each well to dissolve the purple formazan crystal. The absorbance was measured at 540 nm. The cell survival was referenced to the control group.

**Nuclear extracts preparation and electrophoretic mobility shift assay (EMSA).** Cells were collected and suspended in 500 μL of buffer A (10 mmol/L HEPES-KOH with 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.2 mmol/L phenylmethylsulfonyl fluoride [PMSF], 500 μmol/L of DTT, and protease inhibitors). The samples were kept on ice for 15 minutes and vortexed vigorously for 15 seconds. The lysate was then centrifuged at 14,000 rpm for 30 seconds. The pellet was dissolved in 100 μL of buffer B (20 mmol/L HEPES/KOH with 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 25% glycerol, 0.2 mmol/L PMSF, 500 μmol/L DTT, 0.2 mmol/L EDTA [pH 8.0], and protease inhibitors) and kept on ice for 20 minutes followed by centrifugation at 12,000 rpm for 2 minutes. The supernatant, identified as the nuclear extract, was frozen at −80°C. Protein concentration was determined by the Bradford method. Double-stranded oligonucleotides corresponding to the consensus sequence of the NF-κB binding site (5'-GAGACTGGGAATACCCCAGT-3') were labeled with [³²P]ATP. Reaction solution (20 μL) containing 5 μg of the nuclear extract, 4 μL of 5× binding buffer (50 mmol/L Tris-HCl [pH 7.5], with 20% glycerol, 5 mmol/L MgCl₂, 2.5 mmol/L EDTA, 5 mmol/L
DTT, and 0.25 mg/mL poly dI-dC) and 1 µL [\(^{32}\)P]-labeled probe, was incubated at room temperature for 20 minutes. Samples were separated on 4% polyacrylamide gel and visualized by phosphorimaging.

**Western blot analysis.** For each treatment group, a certain amount of the whole cell lysate was separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. After blocking in 5% milk for 1 hour, the membrane was incubated with the primary antibody and then the corresponding secondary antibody. The signals were detected by enhanced ECL system and quantified by Quantity One® (Bio-Rad). Anti-MnSOD antibody was purchased from Upstate, anti-actin antibody from Sigma, Akt and phosphor-Akt (Ser473) antibody from Cell Signaling Technology, PTEN antibody from Santa Cruz Biotechnology.

**SOD activity gel electrophoresis.** Cellular SOD activities were measured based on the inhibition of the reduction of nitroblue tetrazolium (NBT) by SOD as described by Beauchamp and Fridovich [88]. Briefly, 50 µg protein samples in 0.05 mol/L phosphate buffer were loaded on 12.5% native polyacrylamide gel. Electrophoresis was conducted overnight at 4 °C. Following electrophoresis, the gel was stained in the dark for SOD activity with 2.43 mmol/L NBT for 20 minutes, riboflavin-TEMED solution for 15 minutes, and then exposed to light. The achromatic bands demonstrated the presence of SOD activity.

**Transfection of cells with plasmid DNA and siRNA and determination of cell survival by trypan blue exclusion assay.** The green fluorescent protein (GFP)-PTEN expression construct was kindly provided by Dr. Vivek M. Rangnekar (University of Kentucky). PTEN siRNA and scrambled control siRNA were purchased from Santa Cruz Biotechnology. Cells were plated into 12-well plates, and transiently transfected with GFP-PTEN expression plasmid and GFP control plasmid, PTEN siRNA and control siRNA by using Lipofectamine™ 2000 (Invitrogen, CA) according to the manufacturer's instructions. Briefly, 1 µg plasmid DNA or 36 pmol siRNA was mixed with 50 µL incomplete medium without fetal bovine serum and antibiotics and then complexed with a mixture of 3 µL of Lipofectamine and 50 µL of incomplete medium for 20 minutes at room temperature. The mixture was diluted with 500 µL of incomplete medium and added to the cells. After 5 hours, the medium was replaced with 1ml of complete medium.
containing 10% fetal bovine serum and incubated overnight. Parthenolide was added to the medium, and 24 hours later, cells were treated with 6 Gy radiation or were sham-irradiated. After another 24 hours, the cells were processed for trypan blue exclusion assay and collected for Western blot analysis. Cell suspension (20 μL) was mixed with 20 μL 0.04% trypan blue solution and loaded on to a hemocytometer. Cells were counted under a light microscope. Dead cells retained the dye while the viable cells excluded trypan blue and appeared bright. Cell survival was calculated against the relative control group.

**Statistical analysis.** Statistical analysis was done by using either Student's t test (for two-group comparison) or one-way ANOVA (for multiple-group comparison). Data are reported as mean ± SD.

**Results**

**Parthenolide’s efficiency in sensitizing LNCaP, DU145 and PC3 cells to radiation treatment differs.**

To determine whether parthenolide can enhance the sensitivity of prostate cancer cells to radiation treatment, three human prostate cancer cell lines LNCaP, DU145 and PC3, were plated for clonogenic cell survival assay. In the absence of parthenolide, LNCaP cells were much more sensitive to radiation treatment than DU145 and PC3 cells were (Figure 2.1 A), which is consistent with our previous result [24]. Radiation, 2 Gy, killed 46% of LNCaP cells but only approximately 10-25% of DU145 and PC3 cells. Parthenolide sensitized all three prostate cancer cell lines to radiation treatment in a dose-dependent manner. LNCaP cells were most sensitive to parthenolide treatment. The lowest concentration of parthenolide to show a radiosensitization effect at 2 Gy radiation was 0.25 μmol/L in LNCaP cells; 0.5 μmol/L in DU145 cells and 1.5 μmol/L in PC3 cells. Since LNCaP cells showed high sensitivity to radiation alone, we focused our study of the radiosensitization effect of parthenolide on the radioresistant aggressive prostate cancer cell lines DU145 and PC3. In these two cell lines, DU145 showed significantly greater sensitivity to parthenolide treatment. The DMF of 1.0 μmol/L parthenolide at 0.37 survival fraction (D$_{1}$) was 1.8 in DU145 cells and 1.3 in PC3 cells. For 1.5 μmol/L parthenolide, the DMF was 2.6 in DU145 cells and 1.6 in PC3 cells. The different
radiosensitization efficiency of parthenolide in DU145 and PC3 cells was further demonstrated by the MTT assay (Figure 2.1 B). Due to a higher cell density used in MTT assays compared to clonogenic assays, higher concentrations of parthenolide were used. Consistent with the results of clonogenic survival assay, the radiosensitization effect of parthenolide was dose-dependent in both DU145 and PC3 cells (Figure 2.1 B), with 5 µmol/L parthenolide showing the most dramatic effect. There was no significant difference between the radiosensitivity of DU145 cells and PC3 cells in the absence of parthenolide. The relative cell survival after 6 Gy radiation was 0.62±0.03 in DU145 cells, and 0.68±0.03 in PC3 cells (p>0.05) in the absence of parthenolide. Parthenolide showed higher toxicity and higher radiosensitization effect in DU145 cells than in PC3 cells. On the fifth day after 5 µmol/L parthenolide treatment, the relative cell survival fraction was only 0.12 in DU145 cells, whereas it was 0.65 in PC3 cells. After 6 Gy radiation, the relative cell survival decreased from 0.12 to 0.02 (decrease of 83.3%) in the presence of 5 µmol/L parthenolide in DU145 cells, while in PC3 cells it decreased from 0.65 to 0.21 (decrease of 67.8%). Due to the high cell density for determining biochemical and molecular biology endpoints, we used 5 µmol/L parthenolide in all subsequent studies.

**Parthenolide inhibits radiation-induced NF-κB activation in prostate cancer cells.**

Nuclear extracts from cells treated with radiation in the absence or presence of parthenolide were analyzed for NF-κB DNA-binding activity by EMSA. NF-κB DNA binding activity was clearly increased by 6 hours after radiation in all three cell lines (Figure 2.2 A). DU145 and PC3 showed higher NF-κB DNA-binding activity compared to LNCaP cells (Figure 2.2 B), which is consistent with the aggressive and radioresistant characteristics of these two cell lines. Parthenolide inhibited radiation-induced NF-κB DNA binding activity in all three cancer cell lines, especially the p65/p50 hetero-dimer binding activity, which is shown as the top band on the gel since it can be super-shifted by both p65 and p50 antibodies (Figure 2.2 B). This result is consistent with previous reports that parthenolide can inhibit NF-κB activity by direct targeting at IκB Kinase Complex (IKC) [61, 89], thus inhibiting NF-κB nuclear translocation and targeting p65 [62, 63] and directly inhibiting its DNA binding activity. Since NF-κB activation has been well established as a mediator of radioresistance [90], these results suggest that
parthenolide may exert its radiosensitization effect in prostate cancer cells by inhibiting radiation-induced NF-κB activation, thereby suppressing the transcription of NF-κB target genes involved in regulating cell survival and death.

**Parthenolide suppresses MnSOD induction by radiation in prostate cancer cells.**

Radiation exerts its effect largely by inducing oxidative stress. MnSOD is an important antioxidant enzyme induced by radiation. Therefore, we determined the effect of parthenolide on the expression of MnSOD, a well-established radiation-induced NF-κB target gene [24, 91]. Radiation induced MnSOD protein levels of all three prostate cancer cell lines at 24 and 48 hours after radiation (Figure 2.3 A). In DU145 and PC3 cells, the induction of MnSOD was higher than in LNCaP cells (Figure 2.3 B), which is consistent with the relative radioresistance of these two cell lines. Parthenolide suppressed radiation-induced MnSOD in all three cancer cell lines used. This further demonstrated that the transcription activity of NF-κB was inhibited in all three cancer cell lines. Consistent with the changes in MnSOD protein levels, the increase in MnSOD activity by radiation, determined by SOD activity gel electrophoresis, was also suppressed in the presence of parthenolide in PC3 cells (Figure 2.3 C). CuZnSOD activity was used as a loading control which did not change significantly with treatment. Suppression of MnSOD will expose cells to radiation-induced oxidative stress; thus, this result suggests that the radiosensitization effect of parthenolide may be partially mediated through inhibition of radiation-induced MnSOD expression.

**Parthenolide activates Akt.**

A greater radiosensitization effect of parthenolide was observed in DU145 cells than in PC3 cells (Figure 2.1). Both DU145 cells and PC3 cells are androgen-independent radioresistant aggressive prostate cancer. Neither has any functional p53 and they are known to have comparable levels of NF-κB [92]. One of the major differences of these two cell lines is their PTEN status. DU145 cell is known to have functional PTEN, whereas PC3 cell is PTEN null. The major function of PTEN is to dephosphorylate the second messenger PIP3 and to antagonize the PI3K/Akt, a pro-survival pathway. To elucidate the effects of PTEN on the differential radiosensitization efficiency of
parthenolide in these two prostate cancer cell lines, we determined the effect of parthenolide on Akt activation. When we loaded 30 µg of proteins from both PC3 and DU145 whole cell lysates, as expected, PTEN-competent DU145 cells expressed a much lower level of activated p-Akt compared with PC3 cells at baseline (Figure 2.4 A). However, in order to ensure the detection of p-Akt in DU145 cells, protein was overloaded in PC3 cells, and therefore the changes in p-Akt level caused by radiation and parthenolide were not in the linear range for densitometry. To demonstrate a better trend of treatment-induced changes in the p-Akt levels in these two cell lines, we loaded different amounts of protein for the two cell lines so that the levels of p-Akt were in the linear range. Radiation induced the phosphorylation of Akt in both cell lines, with the induction peaking at 1 hour after radiation (Figure 2.4 B). A previous study showed that activation of Akt after radiation is very rapid (10~15 min) [93], and our data confirm that this is an early event. The differences in peak times appear to be cell-type specific. Parthenolide alone increased the phosphorylation of Akt. When radiation was combined with parthenolide, greater induction of Akt phosphorylation was observed in both cell lines. In DU145 cells, which have wild type PTEN, the phosphorylation of Akt was kept at a relatively low level compared to that in PC3 cells. Even with combined radiation and parthenolide treatments, the level of p-Akt in DU145 cells remained lower than the basal level in PC3 cells (Figure 2.4 A). Consistent with the pro-survival role of the p-Akt pathway, the radiosensitization effect of parthenolide was lower in PC3 cells, which have much higher levels of p-Akt after parthenolide treatment compared to DU145 cells similarly treated.

**PTEN enhances the radiosensitization effect of parthenolide.**

To directly test the role of PTEN in the radiosensitization effect of parthenolide in the two prostate cancer cell lines, we overexpressed PTEN in the PTEN null cell line PC3 and knocked down PTEN expression in PTEN-expressing cell line DU145 and determined cell survival after radiation and parthenolide treatment. We transfected GFP control plasmid and GFP-PTEN expression plasmid into PC3 cells. The transfection efficiency was approximately 85% based upon the percentage of GFP positive cells under fluorescent microscope (data not shown). Compared to the cells transfected with GFP
control, in the presence of GFP-PTEN expression, the normalized cell survival level was decreased by 44% when treated with 5 µmol/L parthenolide alone, 16% when treated with 6 Gy radiation alone and 68% when treated with parthenolide combined with radiation (Figure 2.5 A). Overexpression of GFP-PTEN in PC3 cells conferred the cells more susceptible to parthenolide and radiation toxicity, and also enhanced the radiosensitization effect of parthenolide. Consistent with the role of PTEN, the phosphorylation of Akt induced by parthenolide, radiation and combined treatment was inhibited in the presence of GFP-PTEN (Figure 2.5 A). In a complementary experiment, PTEN was knocked down in DU145 cells by using PTEN siRNA. Compared to the cells transfected with control siRNA, the normalized cell survival when PTEN expression was knocked down, was increased by 53% when treated with 5 µmol/L parthenolide alone, 54% when treated with 6 Gy radiation alone, and 74% when treated with parthenolide combined with radiation (Figure 2.5 B). Abrogation of PTEN expression in DU145 cells rendered the cells more resistant to the combined effect of parthenolide and radiation. Consistently, the phosphorylation of Akt induced by parthenolide, radiation and combined treatment was further increased when PTEN expression was knocked down (Figure 2.5 B). Together, these results suggest that the presence of PTEN enhances the radiosensitization effect of parthenolide.

**Discussion**

Our results demonstrate that parthenolide sensitize prostate cancer cells to radiation treatment. Among the three prostate cancer cell lines used in our study, LNCaP cells express androgen receptor (AR) and have wild-type p53. Both DU145 and PC3 cells are AR negative and have no functional p53 [94]. The androgen responsive LNCaP cells showed higher sensitivity to radiation treatment, which is consistent with previous studies [24, 95]. Several factors can contribute to the fact that LNCaP cells are sensitive to radiation: 1) LNCaP cells have functional p53; 2) The activity of NF-κB in LNCaP cells is low, and RelB level is especially low [24]. 3) The presence of AR in LNCaP cells is antagonistic to NF-κB function [96, 97]. Since LNCaP cells are sensitive to radiation treatment, our studies of the radiosensitization effect of parthenolide have been focused on the radioresistant cell lines, DU145 and PC3.
It has been shown that parthenolide inhibits the NF-κB pathway by targeting IKK [61, 89] or directly modifying p65 [62]. The α-methylene-γ-lactone functional group in parthenolide can react with nucleophiles, such as cysteine sulfhydryl groups, in a Michael addition reaction [98]. Inhibition of the NF-κB pathway by parthenolide is considered to be a consequence of alkylation of cysteine 179 of IKKβ or cysteine 38 of p65. Previous studies have shown that parthenolide sensitizes cancer cells to various apoptosis-inducing agents through inhibition of NF-κB [57]. In our study, inhibition of NF-κB and its downstream target, MnSOD, appears to be a common mechanism for the radiosensitization effect of parthenolide in the prostate cancer cell lines studied. NF-κB activation induces many genes with anti-apoptotic activities, including MnSOD, a mitochondria antioxidant enzyme that scavenges ROS. Inhibition of the NF-κB pathway has been shown to sensitize prostate cancer cells to radiation treatment. Kim et al [21] demonstrated that using proteosome inhibitor-1 to inhibit NF-κB activation can increase the radiosensitivity of Ki-Ras transformed human prostate epithelial 267B1/K-ras cells. Our previous finding demonstrated that selective inhibition of RelB by dominant negative p100 significantly sensitizes prostate cancer cells to ionizing radiation [24]. The present study extends those findings to demonstrate that parthenolide inhibits radiation-induced NF-κB activation in all prostate cancer cell lines tested (Figure 2.2 A) and sensitizes them to radiation treatment.

MnSOD is a key antioxidant enzyme that regulates cell transformation, tumor growth, and cell response to stress-inducing therapeutic regimens [99]. Previous studies have shown that radiation induces cellular ROS levels and MnSOD expression. Inhibition of MnSOD expression by antisense MnSOD or selective inhibition of RelB can enhance the radiosensitivity of tumor cells [23, 24]. In the present study, we show that parthenolide inhibits radiation-induced MnSOD expression in three prostate cancer cell lines (Figure 2.3). Since MnSOD is capable of removing superoxides generated by radiation, the radiosensitization effect of parthenolide is likely to be mediated, in part, by the inhibition of MnSOD expression and activity.

Although our results show that parthenolide sensitizes both radioresistant prostate cancer cells, DU145 and PC3, to radiation in a dose-dependent manner (Figure 2.1 A), the efficiencies of parthenolide’s effect are different in the two cell lines. Higher toxicity
and a higher radiosensitization effect of parthenolide were observed in DU145 cells. Similar activities (Figure 2.2 B) and levels [92] of NF-κB, and a similar level of inhibition of the NF-κB target gene, MnSOD, by parthenolide (Figure 3.3 B) were detected in the two radioresistant prostate cancer cell lines. Therefore, the difference in the susceptibility of the two cell lines to parthenolide suggests that, in addition to inhibition of NF-κB, other mechanisms may be involved in the effect of parthenolide. We tested this hypothesis by examining the effect of parthenolide on the PI3K/Akt pathway in the two cell lines with different PTEN status. Our results suggest that the PI3K/Akt pathway is activated by parthenolide and the different cellular status of PTEN makes a difference in cell susceptibility to parthenolide’s effect. The PI3K/Akt pathway is activated in response to growth factors and adhesion to matrix or other cells. It promotes normal cell growth and proliferation. Activated PI3K converts PI(4,5)P_2 to PI(3,4,5)P_3 (PIP_3), which is a lipid second messenger that activates many downstream molecules by binding to their pleckstrin-homology (PH) domains. PIP_3 recruits Akt to the cell membrane and allows phosphatidylinositol-dependent kinase 1 (PDK1) and a second kinase (termed PDK2, though not yet conclusively identified) to phosphorylate and activate Akt at thr308 and ser473, respectively. Activated Akt promotes both cell growth and cell survival by phosphorylation and inactivation of its downstream substrates including glycogen synthase kinase 3 (GSK3), the proapoptotic protein BCL2-antagonist of cell death (BAD), and the forkhead (FOXO) family of transcription factors, which promotes expression of p27-Kip1, a cell-cycle inhibitor. It also activates the mammalian target of rapamycin (mTOR) by phosphorylating and inactivating tuberous sclerosis complex 2 (TSC2), thus promoting protein synthesis. The pro-survival role of Akt accounts for its transforming potential and for the resistance of cancer cells to some chemotherapeutic agents and ionizing radiation [100, 101]. Radiation-induced activation of the PI3K/Akt pro-survival pathway is considered to be an important contributor to radioresistance in cancer cells. Gottschalk et al [102] demonstrated that PI3K inhibitor LY294002 sensitized prostate cancer cells to radiation through inactivation of Akt. Cao et al. [103] showed that the mTOR inhibitor RAD001 sensitized prostate cancer cells to radiation treatment. Our results demonstrate (Figure 2.4 B) that the PI3K/Akt pathway is activated by radiation in two radioresistant prostate cancer cell lines. This may contribute
to their radioresistance. When parthenolide activates the PI3K/Akt pathway, its radiosensitization effect resulting from inhibition of the NF-κB pathway may be counteracted. Thus, the activation of the PI3K/Akt pathway by parthenolide participates in determining cell susceptibility to its radiosensitization effect. Significant radiosensitization effect was observed in the two radioresistant prostate cancer cell lines in spite of the activation of the PI3K/Akt pathway, and this fact suggests that parthenolide’s inhibition of the NF-κB pathway overwhelms its effect of activating the PI3K/Akt pathway in determining cell response to radiation.

PTEN is a tumor suppressor which antagonizes PI3K by degrading PI(3,4,5)P_3 back to PI(4,5)P_2. PTEN mutations have been identified in 10-15% of all prostate cancers [104] and in up to 60% of advanced prostate cancers with multiple metastases or in prostate cancer cell lines [105]. Haploinsufficiency of the PTEN gene has been shown to promote prostate cancer progression in a transgenic mouse model [106]. The PI3K/Akt pathway can be activated by the deletion of PTEN [102]. Loss of functional PTEN and the subsequent activation of the PI3K/Akt pathway may render cells more resistant to radiation treatment in advanced prostate cancer. Rosser et al. [107] showed that adenoviral-mediated PTEN transgene expression sensitizes prostate cancer cells to radiation. Our results confirm the result from the earlier study and extend to demonstrate that the presence of PTEN enhances the radiosensitization effect of parthenolide, in part, by suppressing the absolute amount of activated p-Akt. Our data suggest that the radiosensitization effect of parthenolide will be less effective in aggressive prostate cancer cells lacking wild-type PTEN.

In summary, the present study demonstrates that parthenolide exerts its radiosensitization effect in prostate cancer cells, in part, by inhibiting the NF-κB pathway and its downstream target MnSOD. However, parthenolide also activates the PI3K/Akt pro-survival pathway, which might also affect a cell’s susceptibility to parthenolide. The presence of PTEN enhances the radiosensitization effect of parthenolide by antagonizing the PI3K/Akt pathway. Understanding the mechanisms that are involved in the radiosensitization effect of parthenolide will enhance our ability to further improve the use of parthenolide as an effective adjuvant in radiation therapy of prostate cancer.
Figure 2.1 Parthenolide sensitizes prostate cancer cells to radiation treatment.

A. Clonogenic survival assay. Cells were treated with the indicated concentrations of parthenolide (PN) for 24 hours, and then exposed to indicated doses of radiation. Twenty-four hours after radiation, the media containing PN was removed. Cells were then maintained for 9 days. The cultures were stained and the colonies containing more than 50 cells were counted. Survival fraction was determined by dividing the plating efficiency of radiated cultures by the plating efficiency of non-radiated cultures. Values shown are the means ± SD for triplicates. * p<0.05 compared with DMSO treated counterpart.
B. MTT assay. Cells were pretreated with the indicated concentrations of parthenolide for
3 hours, and then exposed to 6 Gy radiation. Twenty-four hours after radiation, the media
containing PN was removed. Cells were then maintained for 4 days.

DU145

PC3
Figure 2.2 Parthenolide inhibits radiation induced NF-κB DNA binding activity in prostate cancer cells.

A. Cells were treated with DMSO or 5 µmol/L parthenolide for 3 hours before ionizing radiation (IR). Nuclear extracts (NE) were prepared at 6 hours after 6 Gy IR for electrophoretic mobility shift assay (EMSA) with radiolabeled NF-κB probes.

<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>DU145</th>
<th>PC3</th>
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<tbody>
<tr>
<td></td>
<td>PN (0µM)</td>
<td>PN (5µM)</td>
<td>PN (0µM)</td>
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<tr>
<td>50X non self</td>
<td>50X self</td>
<td>without IR</td>
<td>6h after 6 Gy IR</td>
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<td>50X non self</td>
<td>50X self</td>
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<td>6h after 6 Gy IR</td>
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<td>50X non self</td>
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<td>6h after 6 Gy IR</td>
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<td>50X non self</td>
<td>50X self</td>
<td>without IR</td>
<td>6h after 6 Gy IR</td>
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![Image of gel electrophoresis](image.png)
B. Anti-p50 and anti-p65 antibodies were used for supershift.

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<tr>
<th></th>
<th>LNCaP</th>
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<th>PC3</th>
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<tbody>
<tr>
<td>without Ab</td>
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<tr>
<td>Anti-p50 Ab</td>
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<td>Anti-p65 Ab</td>
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Supershift

- p50/p50
- p65/p50
Figure 2.3 Parthenolide suppresses MnSOD induction by radiation in prostate cancer cells.

Cells were treated with DMSO or 5 µmol/L parthenolide for 3 hours before radiation. Whole cell lysates were prepared at indicated times after 6 Gy radiation for detection of MnSOD protein levels and activities.

A. Representative Western blots.

<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>DU145</th>
<th>PC3</th>
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<tbody>
<tr>
<td>Time after 6 Gy radiation</td>
<td>DMSO</td>
<td>PN (5µM)</td>
<td>DMSO</td>
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<tr>
<td></td>
<td>c 24h 48h</td>
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<tr>
<td>MnSOD</td>
<td><img src="image" alt="LNCaP MnSOD" /></td>
<td><img src="image" alt="DU145 MnSOD" /></td>
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<tr>
<td>Actin</td>
<td><img src="image" alt="LNCaP Actin" /></td>
<td><img src="image" alt="DU145 Actin" /></td>
<td><img src="image" alt="PC3 Actin" /></td>
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B. Quantitation of MnSOD protein levels from 3 independent experiments. *p<0.05 Compared with radiation untreated control; # p<0.05 Compared with DMSO treated counterpart.
C. Representative SOD activity gels of PC3 cells from 4 independent experiments.
**Figure 2.4 Parthenolide activates Akt in PC3 and DU145 cells.**

Cells were pretreated with DMSO or 5 μmol/L parthenolide for 3 hours before radiation. Whole cell lysates were prepared at indicated times after 6 Gy radiation. The fold changes of p-Akt/Total Akt were normalized to no treatment controls.

A. Protein samples (30 μg) from both cell lines were loaded on the same gel to compare the relative protein level of these two cell lines.

<table>
<thead>
<tr>
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<td>Akt</td>
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<td>PTEN</td>
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<td>Actin</td>
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B. Protein samples from PC3 cells (15 µg) and from DU145 cells (50 µg) were loaded separately to demonstrate changes in protein levels after treatments.

**PC3**

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<th>Time after 6 Gy radiation</th>
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<td>DMSO</td>
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<td>PN (5µM)</td>
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 Fold change of p-Akt/Total Akt  

| 1 | 3.4 | 1.9 | 2.1 | 3.1 | 3.2 |

**DU145**

<table>
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<tr>
<th>Time after 6 Gy radiation</th>
<th>C</th>
<th>1h</th>
<th>6h</th>
<th>C</th>
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<tr>
<td>DMSO</td>
<td></td>
<td></td>
<td></td>
<td>PN (5µM)</td>
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</table>

 Fold change of p-Akt/Total Akt  

| 1 | 3.3 | 3.0 | 3.2 | 7.6 | 4.3 |

Figure 2.5 The presence of PTEN enhances the radiosensitization effect of parthenolide.

A. PC3 cells were transiently transfected with GFP control plasmid and GFP-PTEN expression plasmid. Parthenolide was added to the medium, and 24 hours later, cells were treated with 6 Gy radiation or were sham-irradiated. After an additional 24 hours, the cells were processed for trypan blue exclusion assay and collected for Western blotting. Cytotoxicity was normalized to the corresponding control group. * p<0.05 compared with the cells transfected with GFP control plasmid. The fold changes of p-Akt/Total Akt were normalized to no treatment control.
B. DU145 cells were transiently transfected with control siRNA and PTEN siRNA. Cells were treated in the same way indicated in A for cytotoxicity assay and Western blot. * p<0.05 compared with the cells transfected with control siRNA.
Figure 2.6 Summary of chapter two
Chapter Three

A NADPH oxidase dependent redox signaling pathway mediates the selective radiosensitization effect of parthenolide in prostate cancer cells

Synopsis

We previously reported that parthenolide enhances the radiation sensitivity of prostate cancer cells, in part, by inhibiting the NF-κB pathway. However, we have also found that parthenolide activates the PI3K/Akt pathway. Using survival indexes determined by MTT and growth rate assays, we found that parthenolide selectively exhibits a radiosensitization effect on prostate cancer PC3 cells but not normal prostate epithelial PrEC cells. Parthenolide selectively induces oxidative stress in PC3 cells but not in PrEC cells, as determined by the oxidation of ROS sensitive probe H$_2$DCFDA and the intracellular reduced thiol and disulfide levels. Importantly, when combined with radiation, parthenolide further increases ROS levels in PC3 cells, while it slightly decreases radiation-induced oxidative stress in PrEC cells. Parthenolide activates NADPH oxidase, an important source of ROS, in PC3 cells but not in PrEC cells. The reduced thioredoxin, an important antioxidant, is significantly decreased after parthenolide treatment in PC3 cells as a downstream event of NADPH oxidase activation. The activation of NADPH oxidase also leads to the PI3K/Akt activation and consequent FOXO3a phosphorylation. The NADPH oxidase dependent phosphorylation of FOXO3a leads to the down-regulation of its targets, antioxidant enzyme MnSOD and catalase, in PC3 cells. Together, our data suggest that NADPH oxidase mediates intense oxidative stress by both increasing ROS generation and decreasing antioxidant defense capacity. This results in a dramatic enhancement of oxidative stress and the selective radiosensitization effect of parthenolide in prostate cancer cells.
Introduction

Selective cancer killing without harming normal tissues is a fundamental challenge in cancer therapy. Cancer cells are usually under increased oxidative stress compared with normal cells. For example, prostate cancer cells often have increased ROS generation from mitochondria [33] or the NADPH oxidase (NOX) system [32], and decreased antioxidant enzymes, such as MnSOD, CuZnSOD and catalase [34, 35]. In response to increased oxidative stress, cells may show elevated proliferation, induction of adaptive response, cell injury, or even cell death depending on the intensity of the stress. It is hypothesized that exposure to further exogenous ROS would push the tumor cells, which already have high constitutive oxidative stress levels to cell death, while normal cells may still maintain redox homeostasis through adaptive responses. Therefore, regulating intracellular redox state represents an ideal target to selectively sensitize cancer cells to oxidative stress-inducing therapy, such as radiotherapy. Radiation has been used extensively to treat prostate cancer, either alone or in combination with other types of cancer treatment. About 70% of the biological damage caused by radiation is due to the generation of ROS [4]. Oxidative stress has also been shown to be a major mechanism for parthenolide [43] induced cell death. Our previous study showed that parthenolide sensitizes human prostate cancer cells to radiation treatment through inhibiting the NF-κB pathway [108]. However, whether the radiosensitization effect of parthenolide is selective to prostate cancer cells but not normal prostate cells, and whether parthenolide regulates intracellular redox state differentially in cancer and normal cells, are unknown.

NADPH oxidase is an important source of ROS. It accounts at least partially for the increased levels of ROS in prostate cancer [32, 109]. The first discovered NADPH oxidase is phagocyte NADPH oxidase. It is a multisubunit enzyme localized to cell membranes, consisting of membrane-bound components (gp91phox and p22phox) and cytosolic components (p47phox, p67phox, p40phox and Rac) that translocate to the membrane upon activation. Homologues of gp91phox (Nox2), including Nox1-5, Duox1 (dual oxidase) and Duox2, have been identified and named as Nox (NADPH oxidase) proteins in non-phagocytic cells. Their activation requires p47phox paralog Nox01 (Nox organizer 1) and p67phox paralog Noxa1 (Nox activator 1), or calcium binding [110]. In general, Nox proteins have binding sites for NADPH, FAD (flavin adenine dinucleotide) and
hemes, and catalyze the transfer of an electron to molecular oxygen to generate superoxide anion ($O_2^-$), which is then enzymatically dismutated to $H_2O_2$. Eighty percent of human prostate tumor samples show markedly increased Nox1 protein and mRNA levels [109]. NADPH oxidase-mediated generation of ROS has been shown to be an important contributor to X-ray induced cell death [8, 111]. However, whether parthenolide activates NADPH oxidase in prostate cancer cells is unknown.

Glutathione (GSH) and thioredoxin (Trx) are important intracellular thiol-containing antioxidants. Disrupting GSH and Trx systems will impair cellular antioxidant activity and exacerbate radiation-induced oxidative stress. Parthenolide is a sesquiterpene lactone, which can react with cysteine thiol groups via a Michael addition reaction. It has been reported that parthenolide can deplete intracellular GSH and induce oxidative stress in hepatoma cells [43]. However, the effect of parthenolide on Trx has not been elucidated.

In the previous study, we found that parthenolide activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in prostate cancer cells and that the cellular p-Akt level might affect cell sensitivity to parthenolide [108]. Activated Akt is known to inhibit apoptosis and promote cell survival by activation of the mammalian target of rapamycin (mTOR) or direct phosphorylation and inactivation of its downstream targets, including glycogen synthase kinase 3 (GSK3), the proapoptotic protein BCL2-antagonist of cell death (BAD), and Forkhead box class O (FOXO) transcription factors. In contrast to the well-known pro-survival role of Akt, some recent studies suggest that the PI3K/Akt pathway may induce oxidative stress and trigger cell death under certain conditions [112]. The mechanisms by which Akt induces ROS may involve: 1) stimulation of mitochondrial oxidative metabolism; 2) reduction of antioxidant defense via FOXO suppression.

The FOXO transcription factors are mammalian homologues of DAF-16, which regulates longevity in Caenorhabditis elegans. There are four members of the FOXO family: FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6. Among them, FOXO1 and FOXO3a are the most highly expressed FOXO proteins in human prostate [101]. FOXO factors perform diverse functions by regulating the transcription of their downstream targets which are involved in a variety of cellular processes, including cell cycle and cell death regulation, differentiation and development, cellular stress response, and energy metabolism control [113, 114]. In prostate cancer, FOXOs have
been shown to inhibit tumorigenesis. FOXO1 deletion is detected in more than 30% of prostate cancer specimens [115]; FOXO3a hyper-phosphorylation and inactivation has been associated with the progression of prostate cancer to androgen independence [116]. Overexpression of FOXO1 and FOXO3a in the prostate cancer cell line provokes apoptosis [117]. However, FOXOs also extend mammalian lifespan by protecting cells against oxidative stress-induced cell death [118]. As the cellular functions of FOXOs are diverse and in some cases appear to be antagonistic, it is postulated that the activity of FOXO transcription factors is differentially regulated in specific tissues in response to various types or intensities of external stimuli [119]. The regulation of FOXOs is mostly achieved by changes in posttranslational modifications on the FOXO proteins, including phosphorylation, acetylation, mono- and poly-ubiquitination. External stimuli may regulate FOXO subcellular localization through phosphorylation. The phosphorylation of FOXO by Akt is inhibitory phosphorylation, which allows the chaperone protein 14-3-3 to bind to FOXO factors in the nucleus, enhances FOXO nuclear export and decreases FOXO nuclear reentry, leading to cytoplasmic sequestration. On the contrary, the phosphorylation of FOXO by oxidative stress-activated MST1 (mammalian Ste20-like kinase) and JNK (c-jun terminal kinase) pathways disrupts 14-3-3 binding and triggers the relocalization of FOXO from the cytoplasm to the nucleus, and therefore it can be regarded as an activation phosphorylation. Radiation induces FOXO3a nuclear translocation and activates FOXO3a activity [120, 121]. Activation of FOXO3a by radiation may induce cell apoptosis by upregulating its targets, FasL and Bim [120]; but on the other hand, it also inhibits cell cycle progression and protects cells against oxidative damage and genotoxic stress [118, 121-123] by upregulating cell cycle inhibitors and antioxidant enzymes, and by promoting DNA damage repair.

In this study, we explored the effect of parthenolide on intracellular redox status in both prostate cancer and normal prostate cells, and investigated how parthenolide regulates NADPH oxidase, GSH and Trx, and the redox-related PI3K/Akt/FOXO3a pathway and how they are linked to each other to contribute to the radiosensitization effect of parthenolide.
Materials and Methods

Cell culture and treatment. Human prostate cancer cell lines PC3 and DU145 were obtained from American Type Culture Collection (Manassas, VA) and cultured as previously described. Human normal prostate epithelial PrEC cells were purchased from Lonza (Walkersville, MD) and maintained in Prostate Epithelial Cell Growth Medium (PrEGM, Lonza). All cells were grown in a 5% CO₂ atmosphere at 37°C. Parthenolide stock solution (5 mmol/L) was prepared in DMSO and diluted in culture medium to the indicated final concentration for cell treatment. DMSO (0.1%) diluted in medium was used as vehicle control. A 130 kV X-ray machine (Faxitron X-ray Corporation) was used to radiate cells, with a dose rate of 89.7 cGy/min. NADPH oxidase inhibitor diphenylene iodonium (DPI, Sigma) and PI3K inhibitor wortmannin (Cell Signaling) were dissolved in DMSO. The final concentrations used to treat cells were 0.5 µmol/L and 1 µmol/L respectively.

MTT assay. Cells were plated into 96-well plates at different densities according to their plating efficiencies (PC3 cells at 1,000 cells/well, PrEC cells at 5,000 cells/well) and grew overnight to achieve similar confluence. Then, cells were treated with parthenolide for 24 hours, and exposed to the indicated dose of radiation or sham-irradiated. Twenty-four hours after radiation, parthenolide-containing medium was replaced with normal culturing medium so that the total time for parthenolide treatment was 48 hours. After four cell doubling times [124] (around 4 days for PC3 cells and 8 days for PrEC cells), 3-(4,5-methylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (MTT, 50 µg/well) was added and incubated at 37°C for 1 hour. After removal of medium, DMSO 200 µL was added to each well to dissolve the purple formazan crystal. The absorbance was measured at 540 nm. Cell survival was normalized to the untreated control group.

Cell growth curve. PC3 and PrEC cells were plated into 12-well plates at different densities according to their plating efficiencies and grew overnight. Cells were treated in the same way as described above for MTT assay. Twenty-four hours after radiation (day 1), parthenolide-containing medium was replaced with normal culturing medium. Cells from each well of the triplicates were trypsinized and counted, and the mean number of cells/well was obtained every other day from the triplicate average. The results were
plotted on a log-linear scale and fitted into exponential growth curve fit. The growth rate constant in each treatment group was compared.

**DCF assay.** Cells were plated into 48-well plates and grew overnight. Twenty-four hours after parthenolide or DMSO treatment, cells were washed twice with PBS and preloaded with 20 μmol/L carboxy-H₂DCFDA (invitrogen) or the oxidized carboxy-DCFDA (invitrogen, insensitive to oxidation, used to normalize cell number, as well as uptake, efflux, and ester cleavage of H₂DCFDA) diluted in PBS by incubation at 37°C for 30 minutes. At the end of the incubation period, the cells were again washed twice with PBS, then irradiated while they were covered with 100 μL PBS per well. After the radiation exposure, the plates were incubated at 37°C for 20 minutes and read with a Gemini XPS fluorescence microplate reader at excitation and emission wavelengths of 485 and 528 nm, respectively. The fluorescence level of cells preloaded with carboxy-H₂DCFDA was normalized to that of cells preloaded with carboxy-DCFDA (the ratio of H₂DCFDA /DCFDA) to eliminate the cell number and dye uptake differences between different treatment groups. The PBS used in this assay was supplemented with 1 mmol/L CaCl₂ and MgCl₂.

**Detection of reduced thiols and disulfides.** The protein thiols were labeled by 3-(N-maleimido-propionyl) biocytin (MPB) and detected by avidin-biotin technology on the blots as previously described by Bayer et al [125]. Cells were harvested by scraping in SEE buffer (0.1 mol/L sodium phosphate, pH 7.0, 5 mmol/L EDTA, 5 mmol/L EGTA) and homogenized with 50 strokes in a Dounce homogenizer on ice. Protein samples were then treated with 10 μg/mL MPB (Sigma) for 30 minutes and dialyzed for 3 hours. Samples were kept on ice throughout and dialysis was conducted at 4°C. Labeled proteins were subjected to SDS-PAGE, followed by detection with HRP-conjugated streptavidin and ECL® Plus (GE Healthcare). To detect protein disulfides, samples were first treated with 10 mg/mL N-ethylmaleimide (NEM; Sigma) for 1 hour and dialyzed for 3 hours against PBS. Then 2-mercaptoethanol (ME; Sigma) was added at a final concentration of 2% (v/v). After 30 minutes incubation, protein was dialyzed overnight with 3 buffer changes. The NEM-blocked, ME-reduced protein was then treated with MPB as described above for direct thiol labeling.
NADPH oxidase activity assay. This assay was performed as described previously by Cui and Douglas [126]. After treatment, cells were washed and scraped in ice-cold PBS, and centrifuged at 750×g for 10 minutes. The cell pellet was resuspended in buffer containing 20 mmol/L KH$_2$PO$_4$, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail, and homogenized with 50 strokes in a Dounce homogenizer on ice. After determining protein concentration, the homogenate was used immediately to assay NADPH oxidase activity. To start the assay, 30 µg of homogenate in 20 µL volume was added into 180 µL reaction buffer (50 mmol/L phosphate buffer, pH 7.0, containing 1 mmol/L EGTA, 150 mmol/L sucrose, 500 µmol/L lucigenin and 500 µmol/L NADPH). Photoemission in terms of RLU was measured by Xenogen IVIS Spectrum every minute for 15 minutes. The reaction velocity was calculated as the change of RLU per minute per µg protein. The Km and Vmax values were calculated from the respective Lineweaver-Burk plots by using various concentrations of substrate NADPH in the assay.

GSH assay. Cells were collected for glutathione assays (total GSH, GSSG, and reduced GSH levels) using the Glutathione A assay Kit (Cayman Chemical). Briefly, cell pellets were homogenized in 500 µL cold PBS buffer, and centrifuged at 10,000 ×g for 15 minutes at 4 °C. The supernatant was measured for protein concentration and then deproteinated by adding equal volume of the 10% metaphosphoric acid to precipitate protein. Before the assay, 50 µL of 4 mol/L triethanolamine was added into per mL supernatant to adjust the pH. For GSSG assay, 100 µL of supernatant was incubated with 1 µL of 1 mol/L 2-vinylpyridine at room temperature for 60 minutes to derivatize GSH. Total GSH (GSHt) and GSSG were measured using recycling assay involving the reaction of DTNB (5,5’-dithio-bis-2-nitrobenzoic acid) and glutathione reductase, and then normalized to cellular protein level. The amount of reduced GSH was calculated by subtracting the amount of GSSG from total GSH (GSHt - 2GSSG).

Preparation of whole cell extracts, cytoplasmic and nuclear fractions. Cell pellets were suspended in 100 µL cell lysis buffer and incubated for 30 minutes on ice. The samples were then centrifuged at 13,000 rpm for 1 minute. The supernatant was collected as whole cell extract. Cytoplasmic and nuclear fractions were isolated using the Nuclear
Extract Kit (Active Motif). Protein concentration was determined by Bradford assay (Bio-Rad).

**Western blot analysis.** Western blot analysis was performed as previously described [108] using corresponding antibodies against Akt, Phospho-Akt (Ser 473), FOXO3a and Phospho-FOXO3a (Ser 253) (Cell Signaling), actin (Sigma), Nox1 (Santa Cruz), Lamin A/C (Santa Cruz), catalase (Santa Cruz) and MnSOD (Upstate). Representative blots and quantification from three independent experiments are shown.

**Knocking down Nox1 using siRNA.** Cells were plated into 6-well plates, and transiently transfected with Nox1 siRNA (Santa Cruz, final concentration 80 nmol/L) and control siRNA by using Oligofectamine™ (Invitrogen, 5µL/well) according to the manufacturer's instructions.

**Electrophoretic mobility shift assay (EMSA).** Double-stranded oligonucleotides corresponding to the MnSOD promoter region containing consensus FOXO3a binding element (DBE, Daf-16 family protein binding element) [118] (5’-TTCTGACGTCTGTAAACAAGCCAGCCTTT-3’) were labeled with [32P] ATP. The assay was performed as previously described [108].

**Chromatin immunoprecipitation (ChIP assay).** Cells were collected and processed by using the ChIP-IT kit (Active Motif) after treatment. Briefly, protein/DNA complexes were fixed, and DNA were sheared using an enzymatic shearing cocktail (200 U/mL) at 37°C for 10 minutes to generate DNA fragments around 150-1000 bp in length. Samples were precipitated using anti-FOXO3a antibody (Cell Signaling). The DNA was reverse cross-linked, purified and then analyzed by quantitative PCR. Taq DNA polymerase (Promega) was used to amplify the MnSOD promoter fragment containing DBE. The sequences of primer set were: upper-strand primer, 5’-CACCCCAACACGTAGCCCTAGTTACATTC-3’; and lower-strand primer, 5’-CTAGGCTTTCCGGTAAAGTGGAATGGGAAAAAC-3’.

**SOD mimetic treatment and colony survival assay.** PC3 cells were trypsinized and plated in triplicate into 6-well plates at the concentrations of 100 cells/well for the no radiation treatment group and 200 cells/well for the 4 Gy radiation treatment group. Parthenolide or DMSO was used to treat cells for 24 hours. SOD mimetic (MnTE-2-PyP5+) was added at the same time as parthenolide. Then, the cells were exposed to 4 Gy
radiation or were sham-irradiated. Twenty-four hours after radiation treatment, the media containing parthenolide was replaced with growing media with or without SOD mimetic. Twelve days after the cells were plated, they were washed and stained with crystal violet, and the colonies containing more than 50 cells were counted. Plating efficiency (PE) was calculated by dividing the average number of cell colonies per well by the amount of cells plated. Survival fractions were calculated by normalization to the plating efficiency of appropriate control groups.

**FOXO3a transient transfection and determination of cell survival by trypan blue exclusion assay.** The plasmids HA-FOXO3a WT (wild-type) and HA-FOXO3a TM (triple mutant) were obtained from Addgene [113]. In HA-FOXO3a TM, the three key regulatory sites of Akt phosphorylation, Thr32, Ser253, and Ser315, were converted to alanine such that Akt could no longer phosphorylate these sites. Cells were plated into 12-well plates and transfected with HA-FOXO3a WT, HA-FOXO3a TM expression plasmids or pECE vector control plasmid, using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 1.5 µg plasmid was mixed with 50 µL incomplete medium without fetal bovine serum and then complexed with a mixture of 2.5 µL of Lipofectamine and 50 µL of incomplete medium for 20 minutes at room temperature. The mixture was diluted with 500 µL of incomplete medium and added to the cells. After 6 hours, the medium was replaced with 1 mL of complete medium with or without parthenolide and incubated overnight. Cells were then treated with 6 Gy radiation or were sham-irradiated. Forty-eight hours after radiation, the cells were processed for trypan blue exclusion assay and collected for Western blot analysis. Cell suspension (10 µL) was mixed with 10 µL 0.04% trypan blue solution and then loaded onto a hemocytometer. Cells were counted under light microscope. Dead cells retained the dye while the viable cells excluded trypan blue and appeared bright. The cell survival was calculated against the relative untreated control group.

**Statistical analysis.** Statistical analysis was performed using either Student's t test (for two-group comparison) or one-way ANOVA (for multiple-group comparison). Data were reported as mean ± SE.
Results

The radiosensitization effect of parthenolide is selective to prostate cancer PC3 cells but not normal prostate epithelial PrEC cells.

Previously, we showed that parthenolide synergistically enhances the sensitivity of prostate cancer cells to radiation treatment using colony survival assay [108]. In the present study, we compared the effect of parthenolide in prostate cancer PC3 cells and normal prostate epithelial PrEC cells. Because PrEC cells did not form colony in vitro, we performed MTT assays. PrEC cells were more resistant to parthenolide-induced cytotoxicity compared with PC3 cells (Figure 3.1 A). The sub-cytotoxic dose of parthenolide (1μmol/L) was chosen to study the parthenolide and radiation combination effect on cell survival. As shown in Figure 3.1 B, radiation 6 Gy decreases cell viability similarly in PC3 cells (by 53%) and in PrEC cells (by 41%). In the presence of parthenolide 1μmol/L alone, the viability of PC3 cells is 74.4% of control. When combined with 6 Gy radiation, cell viability is further decreased to 23.6% of control. However, 1μmol/L of parthenolide alone does not reduce cell viability in PrEC cells and does not enhance radiation-induced cytotoxicity. Comparisons of growth curves for these two cell lines also reveal a selective radiosensitization effect of parthenolide (Figure 3.1 C) in PC3 cells. Radiation 6 Gy decreases cell growth rate by approximately 50% in both cell lines (Table 3.1). However, in the presence of parthenolide, different effects were observed in prostate cancer PC3 and noncancerous PrEC cells after radiation. In PC3 cells, parthenolide alone decreases cell number without affecting cell growth rate. A combination of parthenolide and radiation showed radiosensitization in PC3 cells by both decreased cell start number and lower growth rate, leading to much fewer cells compared with the radiation-treated group at 7 days after radiation. In PrEC cells, parthenolide alone slightly decreases cell number without much change in cell growth rate. Combination treatment, however, shows a smaller cell start number but the same growth rate as the control group, leading to similar cell numbers compared with the radiation-treated group at 7 days after radiation, which is consistent with the MTT result after 4 cell doubling times.
**Parthenolide induces oxidative stress in PC3 cells but not in PrEC cells.**

The differential effect of parthenolide in prostate cancer PC3 cells and normal prostate epithelial PrEC cells led us to investigate the determinant factors for the selectivity of parthenolide’s effect. Since oxidative stress has been shown to be the major mechanism for both parthenolide [43] and radiation induced cell death, we compared the effect of parthenolide on cellular ROS level in PC3 and PrEC cells by DCF assay. As shown in Figure 3.2 A, neither parthenolide nor radiation changes the fluorescence in cells loaded with oxidized carboxy-DCFDA, which means the dye uptake is not affected by the treatment. Radiation significantly increases the normalized carboxy-H$_2$DCFDA fluorescence, a general indicator of cellular ROS level, in both PC3 and PrEC cells. However, parthenolide alone only increases the normalized carboxy-H$_2$DCFDA fluorescence in PC3 cells but not PrEC cells, indicating parthenolide selectively induces oxidative stress in prostate cancer PC3 cells but not normal prostate PrEC cells. When combined with radiation, parthenolide further elevates the cellular ROS level in PC3 cells, consistent with the radiosensitization effect. Interestingly, in PrEC cells, parthenolide even decreases radiation-induced ROS level (significant at 5 µmol/L), showing an antioxidant property.

Parthenolide exerts its effect mainly by targeting Cys thiol groups (-SH) in a Michael addition reaction, and this direct reaction with the Cys thiols has been shown to lead to the depletion of intracellular GSH and protein thiols and induction of ROS in some cancer cells [43, 45]. We therefore detected the protein thiols and oxidized disulfides in PC3 and PrEC cells by MPB labeling. As shown in Figure 3.2 B, parthenolide 5 µmol/L significantly decreases the reduced protein thiols and increases the oxidized disulfides staining in PC3 cells. The decrease in the reduced protein thiols may result from the direct reaction of parthenolide with protein thiols or the oxidation of thiol groups due to parthenolide-induced oxidative stress. However, in PrEC cells, parthenolide does not significantly change the protein thiols and disulfides, which is consistent with the selective induction of oxidative stress in PC3 cells but not PrEC cells.
Parthenolide activates NADPH oxidase in PC3 cells but not in PrEC cells.

Oxidative stress is the imbalance between prooxidants and antioxidants. Increases in the production of ROS and decreases in antioxidants can both lead to oxidative stress. One of the major sources of ROS generation in prostate cancer cells is the NADPH oxidase system [32, 109]. Therefore, following parthenolide treatment we measured NADPH oxidase activity to see whether it is involved in the parthenolide-induced oxidative stress in prostate cancer PC3 cells. Our results (Figure 3.2 C) show that parthenolide enhances NADPH oxidase activity dose-dependently in PC3 cells, which can be inhibited by DPI, a NADPH oxidase inhibitor. However, in normal prostate PrEC cells, NADPH oxidase activity is not affected by parthenolide treatment, consistent with the selective induction of oxidative stress in PC3 cells but not PrEC cells. The enzyme kinetics study (Figure 3.2 D) performed in PC3 cells indicates that parthenolide activates NADPH oxidase without changing the Vmax but decreasing Km (Table 3.2).

Parthenolide decreases reduced Trx in PC3 cells as a downstream event of NADPH oxidase activation, but increases GSH in PrEC cells.

Since parthenolide targets thiols, we also analyzed two important thiol-containing small molecule antioxidants, GSH and Trx. Total GSH and oxidized GSSG levels were measured after parthenolide, radiation and combination treatment. As shown in Figure 3.3 A, total GSH level is slightly increased in PC3 cells and significantly increased in PrEC cells after parthenolide treatment. The reduced GSH/GSSG ratio is not significantly changed by parthenolide in PC3 cells. But in PrEC cells, it is increased 2.4 fold by parthenolide, which may lead to the protective effect against radiation-induced oxidative stress we observed in DCF assay. Globally decreased protein thiols were observed in PC3 cells but not in PrEC cells after parthenolide treatment (Figure 3.2 B). We then specifically looked at reduced Trx, which is the active form of Trx, by using Trx antibody to pull down the thiol-labeled protein sample. Parthenolide significantly decreases reduced Trx in PC3 cells without altering the Trx protein amount (Figure 3.3 B). However, in the presence of DPI, the decrease caused by parthenolide in the reduced Trx is abolished, suggesting that this is a downstream event of NADPH oxidase activation. It is likely that Trx was oxidized by NADPH oxidase derived reactive species.
Activation of NADPH oxidase by parthenolide is upstream of the PI3K/Akt activation in PC3 cells.

Our previous study found that parthenolide activates the PI3K/Akt pathway in PC3 cells. PI3K/Akt pathway has been known to be activated by growth factors and adhesion to matrix or other cells. It has also been shown to be activated in response to oxidative stress [127], so we tested whether the activation of PI3K/Akt pathway is a downstream event of NADPH oxidase activation in PC3 cells. The PI3K inhibitor wortmannin 1µmol/L prevents the activation of Akt by parthenolide, as indicated by the decrease of p-Akt/Akt ratio. However, activation of NADPH oxidase by parthenolide is not affected (Figure 3.4 A). We then tried to identify the specific subtype of NOX in PC3 cells and knock down the subtype. We examined the expression of Nox1-5 and Duox1, 2 by real-time PCR and found that PC3 cells expressed the mRNA of Nox1, Nox4, Duox1 and Duox2. Nox1 is the major Nox isoform in PC3 cells. Knocking down Nox1 by siRNA inhibits both parthenolide-induced NADPH oxidase activation and Akt activation (Figure 3.4 B), suggesting Nox1-dependent NADPH oxidase activation is upstream of PI3K/Akt activation by parthenolide. This was further confirmed by using NADPH oxidase inhibitor, DPI, which can significantly inhibit NADPH oxidase activity as shown in Figure 3.2 C. In the presence of DPI, Akt activation by parthenolide and the downstream target of Akt kinase, FOXO3a phosphorylation, are both prevented (Figure 3.4 C).

Activation of Akt by parthenolide induces FOXO3a inhibitory phosphorylation and suppresses its downstream targets, antioxidant enzymes MnSOD and catalase in prostate cancer cells, but not in PrEC cells.

FOXO3a is one of the main targets of activated Akt. There are three conserved Akt phosphorylation sites on FOXO3a: Thr32, Ser253 and Ser315 [128]. Consistent with our previous study [108], parthenolide increases Akt Ser473 phosphorylation in prostate cancer PC3 and DU145 cells (Figure 3.5 A). Consequently, the phosphorylation on Ser253 in FOXO3a is increased by parthenolide in a dose-dependent manner. Radiation slightly increases both Akt and FOXO3a phosphorylation, but not as dramatically as parthenolide does. This may be explained by the fact that radiation only induces transient activation of Akt, as shown by our previous study, which showed that Akt
phosphorylation peaks at 1 hour after radiation and then drops at 6 hours after radiation [108]. In DU145 cells, the total FOXO3a level is increased after radiation treatment, consistent with Yang’s observation in osteosarcoma cells [120]. Parthenolide treatment decreases total FOXO3a level in DU145 cells, which may be due to Akt activation-induced FOXO3a degradation by proteasome [129]. While FOXO3a phosphorylation increases in prostate cancer cells, in normal prostate epithelial PrEC cells, the combination of parthenolide with radiation does not enhance but slightly decreases FOXO3a phosphorylation.

Akt-mediated phosphorylation has been known to induce FOXO3a relocalization from the nucleus to the cytoplasm. To verify whether parthenolide-induced FOXO3a phosphorylation would affect FOXO3a nuclear-cytoplasmic shuttling, we isolated cell nuclear fractions after parthenolide and radiation treatment. Nuclear protein Lamin A/C was used as markers for nuclear fraction. Our data (Figure 3.5 B) show that in both PC3 and DU145 cells, nuclear FOXO3a level is increased at 6 hours after radiation treatment, in spite of radiation-induced Akt-mediated inhibitory phosphorylation of FOXO3a (Figure 3.5 A). This might be due, in part, to the fact that radiation also activates JNK [16], which can induce activatory phosphorylation of FOXO3a. Parthenolide induces FOXO3a phosphorylation and decreases nuclear FOXO3a levels in both cell lines (Figure 3.5 B). In PC3 cells, as FOXO3a is excluded from nucleus, more FOXO3a is detected in the cytoplasmic fraction after parthenolide treatment, consistent with the stable FOXO3a level in PC3 whole cell lysate (Figure 3.5 A). However, in DU145 cells, as the FOXO3a level in whole cell lysate is decreased by parthenolide treatment, we observe a decrease in FOXO3a level both in nuclear and cytoplasmic fraction.

FOXO3a is a transcription factor which binds DNA at the consensus sequence DBE (5’-TTGTTTAC-3’) [130]. It has been shown that radiation can increase FOXO3a transcriptional activity [120], which depends not only on the level of FOXO3a available in the nucleus, but also on its DNA binding activity. We then detected the DNA binding activity of FOXO3a after parthenolide treatment. Using gel mobility shift assay, we found that radiation enhances FOXO3a DNA binding activity in PC3 cells. Parthenolide inhibits FOXO3a DNA binding dose-dependently (Figure 3.5 C). However, parthenolide does not change FOXO3a DNA binding activity in PrEC cells. To verify that FOXO3a
indeed binds to the promoter of its target gene, we also performed a chromatin immunoprecipitation assay using anti-FOXO3a antibody to pull down the DNA protein complex. We found that radiation enhances FOXO3a binding to the MnSOD promoter region containing the FOXO3a consensus sequence (Figure 3.5 C). In the presence of parthenolide, FOXO3a binding at the MnSOD promoter is suppressed, consistent with the EMSA result. Our data suggest that FOXO3a DNA binding activity is reduced by parthenolide because of the decreased nuclear FOXO3a level.

FOXO3a has been shown to regulate a wide range of target genes involved in cell cycle arrest, apoptosis, stress response and DNA repair. Since radiation kills cells largely through inducing oxidative stress, we detected FOXO3a targets, antioxidant enzymes catalase and MnSOD, in prostate cancer PC3 and DU145 cells (Figure 3.5 D). MnSOD protein level was increased 24 hours after radiation treatment in both cell lines, but was suppressed by parthenolide. Parthenolide also decreases catalase levels dose-dependently in both cell lines. Consistent with FOXO3a DNA binding activity, MnSOD and catalase protein levels are not changed by parthenolide in PrEC cells.

### Suppression of antioxidant enzymes by parthenolide contributes to its radiosensitization effect.

The suppression of antioxidant enzymes by parthenolide in prostate cancer cells may contribute to the induction of oxidative stress and the selective radiosensitization effect of parthenolide. To confirm the role of antioxidant enzymes in the selective radiosensitization effect of parthenolide, we treated PC3 cells with an antioxidant SOD mimic, MnTE-2-PyP5+. As shown in Figure 3.6 A, the survival fraction of PC3 cells treated with 4 Gy radiation in combination with 1.0 µmol/L parthenolide is 0.25, which is significantly lower than the survival fraction of cells treated with 4 Gy radiation or 1.0 µmol/L parthenolide alone. However, the survival fraction increases to 0.39 in the presence of SOD mimic. SOD mimic reduces the radiosensitization effect of parthenolide in PC3 cells, confirming the role of antioxidants in the radiosensitization effect of parthenolide.

We then overexpressed FOXO3a in PC3 cells to investigate whether overexpression of FOXO3a can rescue cells from parthenolide-induced radiosensitization effect by
induction of antioxidant enzymes. We used two FOXO3a expression plasmids, HA-FOXO3a WT and HA-FOXO3a TM, which bears three mutations at the Akt phosphorylation sites leading to a constitutively active FOXO3a. Cell survival was determined 48 hours after radiation and parthenolide treatments. Overexpression of FOXO3a WT and FOXO3a TM decreases survival of untreated cells, possibly due to the induction of apoptotic targets of FOXO3a [119]. To better compare the effect of radiation and parthenolide in FOXO3a-overexpressing cells and empty vector transfected cells, we normalized all untreated cell viability to 100% to eliminate the basal survival differences among three different transfection groups. After normalization, we observed that FOXO3a overexpression does not significantly affect cell sensitivity to radiation treatment. However, compared with the cells transfected with vector control, overexpression of FOXO3a WT slightly increases cell survival after parthenolide treatment alone and combined treatment. Overexpression of constitutively active FOXO3a TM in PC3 cells significantly confers cellular resistance to parthenolide’s effect (Figure 3.6 B). The expression of exogenous HA-FOXO3a was confirmed by Western blot. Consistent with the role of FOXO3a, the basic levels of antioxidant enzymes, catalase and MnSOD are higher when active FOXO3a TM is overexpressed (Figure 3.6 C). These data demonstrate that FOXO3a plays an important role in maintaining cellular antioxidant enzymes, catalase and MnSOD, that are involved in the radiosensitization effect of parthenolide.

**Discussion**

Parthenolide has been shown to cause cell death in acute myelogenous leukemia (AML) cells and prostate cancer stem cells without affecting hematopoietic stem cells (HSC) and normal prostate stem cells [46, 131], suggesting the selectivity of its cytotoxic effect to cancer cells. Consistent with this finding, in the present study, we found that normal prostate epithelial PrEC cells are more resistant to parthenolide-induced cytotoxicity compared with prostate cancer PC3 cells. Furthermore, the radiosensitization effect of parthenolide, which we discovered in our previous study [108], is selective to prostate cancer cells but not normal prostate epithelial PrEC cells, as shown by MTT and cell growth assays (Figure 3.1). Our results also indicate that parthenolide “rejuvenates”
irradiated normal prostate cells since the cell growth rate after radiation is restored to the untreated control level when combined with parthenolide treatment. This suggests that parthenolide may facilitate repair of radiation-induced damage in normal cells. However, the possible mechanisms involved need to be further investigated.

The selective targeting of cancer cells by parthenolide is of great interest. Cancer cells and normal cells have a different redox status, which may be targeted for selective cancer killing. Parthenolide has been shown to induce oxidative stress in a number of cancer cells [43, 46]. We therefore investigated whether the selective effect of parthenolide on cancer cells is due to differential regulation of intracellular redox status in cancer and normal cells. Our data show parthenolide selectively induces oxidative stress in prostate cancer PC3 cells but not normal prostate PrEC cells (Figure 3.2). When combined with radiation, parthenolide further increases ROS levels in PC3 cells, while it slightly decreases radiation-induced oxidative stress in PrEC cells. This may explain the selective radiosensitization effect of parthenolide in PC3 cells and the “rejuvenation” of irradiated PrEC cells.

NADPH oxidase is a major source of ROS in prostate cancer cells [32]. Several studies have shown a variable expression profile of the seven Nox family members (Nox1-5, Duox1, 2) in prostate cancer cells. A study by Arnold and colleagues using a National Cancer Institute (NCI) prostate cancer tissue microarray (CPCTR) showed that prostate tumor is significantly more likely (86%) to have Nox1 staining than benign prostate tissue (62%) [109, 132]. Our results also show Nox1 is the major Nox isoform in PC3 cells. Parthenolide activates NADPH oxidase in PC3 cells but not in PrEC cells (Figure 3.3), consistent with the selective induction of oxidative stress in prostate cancer cells. The enzyme kinetic constant Vmax, which is the maximal velocity available from the amount of enzyme in the reaction mixture, is not changed by parthenolide. Since Vmax is directly proportional to the catalytic constant of the enzyme (Kcat), which describes the frequency at which the enzyme-substrate complex is converted to product, and the total enzyme concentration ([E]), these data suggest that the amount of enzyme is not increased when parthenolide activates NADPH oxidase. This is confirmed by Western blot, as shown in Figure 3.4 B. However, the Michaelis constant Km, which is the substrate concentration at which the reaction rate reaches half of Vmax, is decreased.
approximately 50% by parthenolide (Table 3.2), suggesting that parthenolide may increase the affinity of NADPH oxidase for the substrate NADPH or increase the stability of the enzyme-substrate complex. The fully activation of Nox1 requires the p22phox, Nox01, Noxa1 and Rac1 [110]. Parthenolide may facilitate the assembly of the multisubunit enzyme complex and thus promote substrate binding. Further study is needed to determine how parthenolide activates NADPH oxidase.

Parthenolide is a sesquiterpene lactone, which can react with biological nucleophiles such as the thiol groups via a Michael addition reaction. The depletion of intracellular GSH and protein thiols by parthenolide may contribute to the induction of oxidative stress in some cancer cells [43, 45]. In contrast to this possibility, our data show parthenolide increases intracellular GSH level, especially in PrEC cells (Figure 3.3). This is possibly due to the activation of the Nrf2/ARE (antioxidant/electrophile response element) pathway [64]. The increase of GSH may partially account for the antioxidant activity of parthenolide observed in PrEC cells. Since the reactivities of thiol groups are inversely related to their pKa, it is reasonable to assume that parthenolide more readily reacts with certain protein thiols which have low pKa than with GSH which has a high pKa of 8.8. Parthenolide decreases reduced protein thiols globally in PC3 cells but not in PrEC cells (Figure 3.2). This may due to the direct reaction of parthenolide with protein thiols or the oxidation of reduced thiols by parthenolide-induced oxidative stress. For an example, parthenolide decreases reduced thioredoxin (Trx), an important intracellular antioxidant, as a result of oxidation by NADPH oxidase derived ROS (Figure 3.3). Thioredoxin (Trx) has a redox-active dithiol within the conserved active site: -Trp-Cys32-Gly-Pro-Cys35-Lys- [133]. The Trx/TrxR system has multiple functions in cell growth, defense against oxidative stress, and apoptosis [134]. Overexpression of Trx protects cells not only from oxidative stress-induced apoptosis [135], but also from cytotoxic and DNA-damaging effects of many chemotherapeutic drugs [136]. Since thioredoxin is an important intracellular redox buffering system and its proper function depends on the redox-sensitive cysteine thiol at its active site, decrease of reduced thioredoxin by parthenolide will likely sensitize cancer cells to oxidative stress induced cell death.

In addition to direct oxidative damage, NADPH oxidase derived ROS plays an important role in redox signaling due to its highly regulated activation. We have
previously found that parthenolide activates the PI3K/Akt [108], an oxidative stress responsive pathway [127]. In the present study, we further demonstrate that the activation of NADPH oxidase is upstream of PI3K/Akt activation (Figure 3.4). The oxidation and inactivation of phosphatases that directly dephosphorylate PI3K or AKT kinase by NADPH oxidase-derived ROS may contribute to PI3K/Akt activation. As a downstream target of Akt kinase, FOXO3a is phosphorylated and excluded from the nucleus by parthenolide treatment, and the transcriptional activity of FOXO3a is suppressed by parthenolide in prostate cancer cells (Figure 3.5). FOXOs have been implicated as important mediators of cellular response to oxidative stress. Silencing FOXO3a results in defective ionizing radiation-induced G1/S and G2/M checkpoints [121]. In addition to inducing cell cycle arrest in response to oxidative stress, FOXO3a mediates the detoxification of ROS by upregulation of antioxidant proteins, such as MnSOD, catalase, peroxiredoxin III (Prx III) and sestrin 3 [112, 118, 137, 138]. DNA damage repair is also triggered by the capability of FOXO3a to induce the transcription of Gadd45a, which is involved in the maintenance of genomic stability and DNA repair [139, 140]. Recently, Tsai and colleagues reported that FOXO3a can promote DNA repair in a transcription-independent manner. They showed that exposure of LNCaP prostate cancer cells to ionizing radiation leads to direct interaction of FOXO3a and ataxia telangiectasia mutated (ATM) protein, resulting in autophosphorylation of ATM on Ser1981 and prompting the repair of damaged DNA [121]. Based on the capabilities of FOXO3a to induce cell cycle arrest, stress resistance and DNA repair, our observation that parthenolide inhibits FOXO3a function by phosphorylation and nuclear exclusion suggests that suppression of FOXO3a may contribute to the radiosensitization effect of parthenolide. This possibility is supported by the finding that overexpression of constitutively active FOXO3a abolishes the radiosensitizing effect of parthenolide. Our data show that FOXO3a transcriptional targets, antioxidant enzymes MnSOD and catalase, are downregulated by parthenolide (Figure 3.5). MnSOD is a mitochondria antioxidant enzyme that removes superoxide radicals. Inhibition of MnSOD expression by antisense MnSOD or selective inhibition of RelB can enhance radiosensitivity of prostate cancer cells [23, 24]. It is well established that MnSOD is a FOXO3a target [118] and also a NF-κB target [141]. Our previous study showed that the NF-κB pathway is inhibited by parthenolide. Thus, it is
likely that both NF-κB and FOXO3a are contributors to the suppression of radiation-induced MnSOD expression by parthenolide. Further study is needed to investigate whether these two transcription factors cooperate with each other or act independently in response to oxidative stress. Catalase is a FOXO3a target with a number of DBEs between -2339 and -1667 of the promoter [137]. It is a ROS scavenger which reduces hydrogen peroxide to water. Mitochondria-targeted catalase overexpression has been shown to increase the survival of irradiated human pancreatic cancer cells [142], suggesting a role for catalase in protecting cells against radiation-induced cell death. Overexpression of constitutively active FOXO3a renders PC3 cells more resistant to a parthenolide-induced radiosensitization effect by up-regulating its targets MnSOD and catalase (Figure 3.6), confirming the role of FOXO3a in promoting cellular antioxidant defense.

To summarize, our present study demonstrates that in prostate cancer cells, parthenolide induces dramatic oxidative stress via NADPH oxidase activation. On one hand, NADPH oxidase activation by parthenolide increases ROS generation. The NADPH oxidase derived ROS may decrease reduced Trx or act as a second messenger to activate the PI3K/Akt/FOXO3a pathway, leading to a decrease in antioxidant defense capacity. On the other hand, parthenolide increases GSH levels but does not activate NADPH oxidase in normal prostate epithelial cells. Thus, the selective induction of oxidative stress by parthenolide in prostate cancer cells accounts for the selective radiation sensitization effect of parthenolide. Our results also imply that modulating intracellular redox state might be an ideal way to achieve selective cancer killing in cancer therapy.
Figure 3.1 The radiosensitization effect of parthenolide is selective to prostate cancer PC3 cells but not normal prostate epithelial PrEC cells.

A. MTT assay. PC3 and PrEC cells were treated with indicated concentrations of parthenolide for 48 hours. Cell viability was measured by MTT assay and normalized to untreated control after four cell doubling times.

MTT assay after 4 cell doubling time
B. The combination effect of parthenolide and radiation on cell viability was compared in PC3 and PrEC cells by MTT assay after four cell doubling times as described in Materials and Methods. * p<0.05 compared with DMSO treated control.
C. Cell growth curve. The cell numbers from triplicate samples were plotted on a log-linear scale as mean ± SE. Equations derived from exponential growth curve fit [Y=Start*exp(K*X)] are shown for each growth curve. Cell number begins at Y=Start and increases exponentially with rate constant K.
Table 3.1 Comparison of the growth rate constant in PC3 and PrEC cells after parthenolide and radiation treatment.

The growth rate constant K was obtained from the exponential growth curve fit of the two cell lines (Figure 3.1 C) and normalized to their relative DMSO treated control groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PC3</th>
<th>PrEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
<td>% of control</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.8277</td>
<td>100.00</td>
</tr>
<tr>
<td>PN 1μM</td>
<td>0.8575</td>
<td>103.60</td>
</tr>
<tr>
<td>IR 6 Gy</td>
<td>0.4223</td>
<td>51.02</td>
</tr>
<tr>
<td>PN 1μM + IR 6 Gy</td>
<td>0.4285</td>
<td>51.77</td>
</tr>
</tbody>
</table>
Figure 3.2 Parthenolide induces oxidative stress in PC3 cells, but not in PrEC cells.
A. DCF assay. Cells were first treated with parthenolide or DMSO for 24 hours, and then preloaded with carboxy-DCFDA (insensitive to oxidation) or carboxy-H$_2$DCFDA (sensitive to oxidation) in PBS and irradiated. After 20 minutes incubation, the plates were read. Normalized carboxy-H$_2$DCFDA (the ratio of carboxy-H$_2$DCFDA/carboxy-DCFDA) was compared. # p<0.05 compared with no radiation control. * p<0.05 compared with DMSO treated no radiation control. § p<0.05 compared with indicated group.
**DCF assay in PC3 cells**

![Graph showing relative fold change of H2DCFDA/DCFDA in PC3 cells with and without IR (6Gy).]

**DCF assay in PrEC cells**

![Graph showing relative fold change of H2DCFDA/DCFDA in PrEC cells with and without IR (6Gy).]
B. detection of protein thiols and disulfides. PC3 and PrEC cells were treated with DMSO or parthenolide (5 µmol/L) for 24 hours. Then cells were harvested for thiols and disulfides labeling by MPB as described in Materials and Methods. Labeled protein thiols were then separated by SDS-PAGE and detected by HRP-streptavidin. 1. DMSO; 2. PN 5 µmol/L.
C. NADPH oxidase activity assay. Cells were treated with DMSO or parthenolide for 24 hours in the absence or presence of 0.5 μmol/L DPI. Cell homogenates were then collected and used for analysis. Photoemission generated by the reaction of superoxide radical and lucigenin were monitored every minute for 15 minutes. The reaction velocity (V) was calculated as the change of RLU per minute per μg protein. * p<0.05 compared with DMSO treated no DPI control. # p<0.05 compared with no DPI control.
D. Lineweaver-Burk plot of NADPH oxidase activity in PC3 cell. The reciprocal of the enzymatic reaction velocity (1/V) was plotted against the reciprocal of the substrate concentration (1/[NADPH]) as a straight line with y-intercept equivalent to 1/Vmax and x-intercept representing -1/Km. Equations derived from Lineweaver-Burk equation of enzyme kinetics $1/V = (Km/Vmax) \times (1/[S]) + 1/Vmax$ are shown for each treatment group.

**NADPH oxidase activity**

![Graph showing NADPH oxidase activity](image)

- DMSO
- PN 1 µM
- PN 5 µM

Equations:
- $y = 0.8063x + 0.0015$ with $R^2 = 0.9942$
- $y = 0.7724x + 0.0016$ with $R^2 = 0.9882$
- $y = 0.5248x + 0.0018$ with $R^2 = 0.9701$
Table 3.2 The Vmax and Km of NADPH oxidase in PC3 cells after parthenolide treatment.

The enzyme kinetic constants Vmax and Km of NADPH oxidase in PC3 cells were obtained from the Lineweaver-Burk plot (Figure 3.5 B).

<table>
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<tr>
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<th>PN 5 µM</th>
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<tbody>
<tr>
<td>Vmax</td>
<td>666.67</td>
<td>625.00</td>
<td>555.56</td>
</tr>
<tr>
<td>Km</td>
<td>537.53</td>
<td>482.75</td>
<td>291.56*</td>
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</table>
Figure 3.3 Effect of parthenolide on thiol-containing antioxidants GSH and Trx.
A. GSH assay. PC3 and PrEC cells were treated with DMSO or parthenolide (5 µmol/L) for 24 hours. Then cells were sham-irradiated or subjected to 6 Gy radiation (IR). Cells were harvested at 24 hours after radiation for GSH detection. * p<0.05, **p<0.001 compared with DMSO control.
B. Detection of reduced Trx (TRXre) by immunoprecipitating MPB labeled protein sample with Trx antibody, followed by avidin detection.

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<tr>
<td>TRXre (12KD)</td>
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</table>

1. DMSO; 2. PN 5μM
Figure 3.4 Activation of NADPH oxidase by parthenolide is upstream of PI3K/Akt activation in PC3 cells.

A. PI3K inhibitor wortmannin does not suppress parthenolide-induced NADPH oxidase activation. PC3 cells were treated with DMSO or parthenolide for 24 hours. Wortmannin (1 µmol/L) was added 1 hour before and throughout the parthenolide or DMSO treated period. Then cell homogenates were collected for NADPH oxidase activity measurement and Western blot analysis. * p<0.05 compared with indicated groups.
B. Knocking down Nox1 suppresses parthenolide-induced NADPH oxidase activation and Akt activation. Control siRNA and Nox1 siRNA were transfected into PC3 cells. Cells were then treated with DMSO or parthenolide for 24 hours and collected for NADPH oxidase activity measurement and Western blot analysis. * p<0.05 compared with indicated groups.
C. NADPH oxidase inhibitor DPI suppresses parthenolide-induced Akt activation and FOXO3a phosphorylation. PC3 cells were treated with DMSO or parthenolide for 24 hours in the absence or presence of 0.5 μmol/L DPI. Then cell homogenates were collected for Western blot analysis.
Figure 3.5 Activation of Akt by parthenolide induces FOXO3a inhibitory phosphorylation and suppresses its downstream targets, antioxidant enzymes MnSOD and catalase in prostate cancer cells, but not in PrEC cells.

A. Cells were treated with DMSO or parthenolide for 24 hours before being subjected to 6 Gy radiation. Whole cell lysates were prepared 6 hours after radiation for Western blot analysis.

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<tr>
<td>PN 5µM</td>
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B. Western blots analysis of cytoplasmic and nuclear fractions.

**PC3**

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<th>Nuclear Fraction</th>
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<tr>
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<tr>
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<td>IR (6 Gy)</td>
<td>- + - + - + - +</td>
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**DU145**

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<tr>
<td>Actin</td>
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<tr>
<td>IR (6 Gy)</td>
<td>- + - + - + - +</td>
<td>DMSO PN 5μM DMSO PN 5μM</td>
</tr>
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</table>
C. Detection of FOXO3a DNA binding activity by EMSA and ChIP assay. Cells were treated with DMSO or parthenolide for 24 hours before IR. Six hours after radiation, cells were processed for EMSA or ChIP assay. For ChIP assay, input samples were prepared for each group before adding antibodies as an indicator of the samples input. Anti-RNA polymerase II antibody was used and GAPDH promoter region was amplified as negative control.
D. Cells were treated with DMSO or parthenolide for 24 hours before being subjected to 6 Gy IR. Whole cell lysates were prepared 24 hours after radiation for Western blot analysis.

**PC3**

<table>
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<tr>
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<th>0.8</th>
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</table>

**DU145**

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**PrEC**

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<th>1.2</th>
<th>1.1</th>
<th>1.1</th>
<th>1.1</th>
</tr>
</thead>
</table>
Figure 3.6 Suppression of antioxidant enzymes by parthenolide is involved in its radiosensitization effect.

A. SOD mimetic partially abolishes the radiosensitization effect of parthenolide in PC3 cells. Colony survival assay was performed with SOD mimetic in PC3 cells as described in Materials and Methods. * p<0.05 compared with untreated control. ** p<0.05 compared with PN alone and radiation alone. # p<0.05 compared with PN in combination with radiation.
B. Overexpression of FOXO3a protects PC3 cells against the effect of parthenolide. PC3 cells were transiently transfected with pECE vector control, HA-FOXO3a WT or HA-FOXO3a TM expression plasmids. Parthenolide was added to the medium. Cells were grown overnight, then treated with 6 Gy radiation or were sham-irradiated. After 48 hours, the cells were processed for trypan blue exclusion assay and collected for Western blot analysis. Both unnormalized and normalized cell viability are shown. * p<0.05 compared with pECE vector control plasmid transfected cells under the same treatment condition.

![Trypan Blue Exclusion Assay in PC3 cells](image1)

![Trypan Blue Exclusion Assay in PC3 cells](image2)
C. Western blots to confirm overexpression of FOXO3a in PC3 cells.

1. pECE vector control
2. FoxO3a WT
3. FoxO3a TM
Figure 3.7 Summary of chapter three

Prostate Cancer Cells

Normal Prostate Cells
Chapter Four
Summary and overall discussion

Overcoming treatment resistance and improving selectivity are two major challenges in cancer treatment. Better understanding of cancer cell characteristics and the mechanisms for the development of resistance will help us to target cancer cells more efficiently and more selectively. Elevated oxidative stress and aberrant redox homeostasis are frequently observed in cancer cells compared to their normal cell counterparts. Persistent high ROS in cancer cells often leads to adaptive responses which include the upregulation of antioxidants, inhibition of apoptosis and increased cell proliferation. Such adaptive responses may contribute to tumorigenesis, metastasis and treatment resistance. I hypothesize that modulating cell redox status and the redox-related signaling pathway may be an ideal way to kill cancer cells selectively. The study presented here is a good example in support of this hypothesis. Our data demonstrate that parthenolide regulates prostate cancer cell redox status through multiple mechanisms, including both induction of ROS generation and inhibition of the antioxidant defense system. The differential modulation of intracellular redox status by parthenolide in cancer and normal prostate cells leads to the selective radiosensitization effect of parthenolide. This study confirms that modulating cell redox status is an efficient and selective way to kill cancer cells, and identifies parthenolide as such an agent.

Parthenolide enhances radiation sensitivity in prostate cancer cells through multiple mechanisms

The sensitivity of cancer cells to radiation treatment is determined by numerous factors. For example, the activation of oncogene Ras [143] and some prosurvival pathways such as the PI3K/Akt, NF-κB pathway increases radiation resistance. DNA damage repair capacity is also correlated with radiation sensitivity. Since induction of oxidative stress is an important mechanism for radiation induced cell killing, cancer cells may exhibit radiation resistance as a result of adaptive response to the increased intrinsic ROS level in cancer cells or the exogenous ROS derived from radiation. Thus, a combination of radiotherapy with some agents that modulate the intracellular redox status
in cancer cells will theoretically exacerbate the oxidative stress induced by radiation and enhance radiosensitivity. Although other mechanisms may also be involved, our study focused on how parthenolide modulates cell redox status, leading to the induction of oxidative stress and radiosensitization effect.

1) Parthenolide suppresses radiation induced NF-κB activation and its downstream target, antioxidant enzyme MnSOD induction, in prostate cancer cells. NF-κB is a redox sensitive transcription factor, which is activated by radiation through either the nuclear or the cytoplasmic pathway [144]. The nuclear pathway depends on the ATM activation induced by DNA damage. The cytoplasmic pathway may involve the ROS-mediated NIK (NF-κB-inducing kinase) or PI3K/Akt activation. The NF-κB downstream targets are involved in cell cycle regulation (such as cyclin B1, cyclin D1), prevention of apoptosis [such as XIAP (X-chromosome-linked inhibitor of apoptosis), Bcl-XL], DNA damage repair [such as GADD45β (growth arrest and DNA damage-inducing protein β) and Ku], and detoxification of ROS (such as MnSOD). It has been shown that MnSOD is critical in the radiation-induced adaptive response, which not only acts as an antioxidant, but also mediates the expression of genes that participate in radiation-induced adaptive responses [23]. Previous studies from our lab demonstrated that using p100 mutant, RelB siRNA [24], SN52 [145] or 1,25-dihydroxyvitamin D3 [146] can suppress RelB-mediated MnSOD expression and sensitize prostate cancer cells to radiation. The present study demonstrates that parthenolide is an effective NF-κB inhibitor that suppresses MnSOD induction and mediates the radiosensitization effect. In addition to MnSOD, other NF-κB targets may also be involved in the radiosensitization effect of parthenolide. Watson et al. reported that suppression of radiation-induced NF-κB activity by parthenolide leads to the radiosensitization through inhibition of split-dose repair [147], implying that NF-κB targets related to cell cycle progression and DNA damage repair are also involved.

2) Parthenolide activates NADPH oxidase, which is an important source of ROS and can mediate redox signaling. The NOX protein is a transmembrane protein. The subcellular localization of NOX protein includes plasma membrane, nuclear membrane, endosome and endoplasmic reticulum [148]. Therefore, the NOX can generate ROS both extracellularly and intracellularly. The superoxide radical generated by NADPH oxidase is short-lived and highly reactive, and due to its negative charge, it is unable to cross the
membrane. The hydrogen peroxide derived from superoxide radical is more stable and can cross biological membranes freely, so it is more important in NOX-dependent redox signaling transduction. Due to the short-lived nature and the limited diffusion distance of ROS, it is speculated that the production of ROS by NOX is localized and can only modulate the redox-sensitive targets in its proximity, for example inactivation of phosphatases or activation of kinases. Our study shows that parthenolide induced NADPH oxidase activation is upstream of PI3K/Akt activation. Both the NOX1 protein [149] and PI3K kinase [150] has been reported to be located in the caveolae, where numerous signaling molecules are concentrated. Thus, the caveolae may be a platform where NOX initiates downstream signaling. The mechanism by which parthenolide activates NADPH oxidase needs further investigation. Our data show that parthenolide may increase the affinity of NADPH oxidase with its substrate, NADPH. One possibility is that parthenolide increases the NADPH oxidase complex assembly. The activation of NADPH oxidase needs the activation of Rac1, which is a member of the Ras superfamily of GTPases. It has been shown that the modulation of the cysteine thiol can activate Ras [151, 152]. It is possible that parthenolide may modify the cysteine thiol on Rac1 and increase its activity.

3) Parthenolide globally decreases reduced protein thiols (-SH) in cancer cells; specifically, it markedly decreases reduced thioredoxin as a downstream event of NOX activation. Protein thiols often play an important role in redox signaling transduction. Thioredoxin catalyzes the reduction of protein disulfides to thiols, thus maintaining their proper function. One critical pathway regulated by Trx is the Ref-1/AP-1 pathway. Ref-1 (protein redox factor-1) functions as an apurinic (apyrimidinic) endonuclease to repair DNA damage, and to regulate the DNA binding activity of several nuclear transcription factors, including AP-1. In response to radiation, Trx has been shown to translocate into the nucleus and interact with Ref-1 to activate AP-1 DNA binding activity [153], which then initiates the protective or reparative responses to the damaging effects of radiation. Whether a decrease of the reduced form of Trx by parthenolide sensitizes cancer cells to radiation by interrupting the Ref-1/AP-1 signal needs further study.

4) The activation of NADPH oxidase by parthenolide leads to the activation of PI3K/Akt kinase and the inhibitory phosphorylation of FOXO3a. Antioxidant enzymes
MnSOD and catalase are inhibited as a result of the suppression of the FOXO3a transcriptional function. Our data show that both NF-κB and FOXO3a contribute to the inhibition of the antioxidant enzyme MnSOD. NF-κB may play a more important role because NF-κB is constitutively active in most tumor cells, while FOXO3a activity is usually low in aggressive tumor cells. Our screening by real-time PCR of 12 putative FOXO3a targets (Table 4.1) in PC3 cells that are involved in stress response and DNA repair (reviewed from [118, 119, 137-139, 154]) has identified SEPP1 (selenoprotein P) and FEN1 (flap structure-specific endonuclease 1) (Figure 4.1) as also being targets of parthenolide, in addition to the two-well established FOXO3a targets, MnSOD and catalase. Consistent with MnSOD protein level, SOD2 mRNA levels are induced 1.5 fold after radiation treatment. In the presence of parthenolide, induction by radiation is completely suppressed. Radiation slightly increases the mRNA levels of antioxidant protein SEPP1 and DNA repair enzyme FEN1, but the increase is not significant. Parthenolide significantly decreases the mRNA levels of CAT, SEPP1 and FEN1 compared with radiation-treated group by approximately 20%, 70% and 30%, respectively. Selenoprotein P is predominantly an extracellular protein containing 10 selenocysteines. It reduces phospholipid hydroperoxide in a manner similar to Gpx (Glutathione peroxidase) using thioredoxin as the preferred electron donor [155, 156]. Tran et al. identified selenoprotein P as a FOXO3a target [139]. Kabuyama reported that selenoprotein P suppresses lipid hydroperoxide both inside and outside the myofibroblasts and functions as an anti-apoptotic factor against oxidative stress [157]. The phospholipid bilayer of cell membranes is susceptible to attack by the radicals generated following the interaction of water with radiation [158]. The resulting lipid hydroperoxides and lipid hydroperoxide breakdown products (e.g., α, β unsaturated aldehydes) contribute to altered plasma membrane lipid composition and cell damage. Inhibition of SEPP1 by parthenolide may exacerbate the damage of cell membrane following ionizing radiation, leading to radiosensitization. FEN1 is a structure-specific nuclease which recognizes and cleaves 5’ overhang or flap DNA structures. Exposure of DNA to ROS causes the formation of several different lesions, including oxidized AP sites. The altered sugar phosphate backbone prevents base excision repair (BER) at the AP site. FEN1 promotes the repair of an oxidized AP site generated by γ-irradiation by
5.1 fold through cleavage of an intermediate reaction generated by template strand displacement during gap-filling [159]. The inhibition of FEN1, a putative FOXO3a target [154] by parthenolide, may lead to accumulation of oxidized DNA damage and promote radiation-induced cell death. Our combined data demonstrate that suppression of FOXO3a by parthenolide mainly affects proteins involved in oxidative defense and repair of oxidative DNA damage through transcriptional suppression.

**Selective targeting cancer cells by parthenolide**

Parthenolide has been previously shown to induce cell death in hepatoma cells, leukemia cells and prostate cancer stem cells while sparing normal liver cells, hematopoietic cells and normal prostate stem cells [43, 46, 131]. However, the mechanisms involved in its selective cytotoxicity to cancer cells remain unknown. Our study confirms the results of others by showing that the radiosensitization effect of parthenolide is selective to prostate cancer cells. Our data extend to demonstrate that parthenolide selectively induces oxidative stress in prostate cancer cells but not in normal prostate cells. The inhibition of the NF-κB pathway (Figure 4.2), activation of NADPH oxidase, suppression of FOXO3a transcription function, and the inhibition of antioxidant enzymes MnSOD and catalase are all observed only in the prostate cancer cells PC3 but not in the normal prostate cells PrEC. More interestingly, parthenolide can even partially protect normal prostate cells from radiation-induced oxidative stress and recover the growth rate of irradiated cells, showing some protective effect to the normal cells.

The possible mechanisms for the differential effect of parthenolide in cancer and normal cells may involve:

1) Cancer cells including prostate cancer cells usually have constitutively activated NF-κB, which is involved in cancer growth and treatment resistance, compared to normal tissue. Therefore, cancer cells are more sensitive to parthenolide-induced NF-κB inhibition than normal cells are, which have relatively low NF-κB. In our cell model, prostate cancer cells DU145 and PC3 have higher levels of NF-κB members compared to normal prostate PrEC cells (Figure 4.3). Gu et al. showed that parthenolide specifically inhibits the growth of antiestrogen-resistant breast cancer cells with high NF-κB activity, but not antiestrogen-responsive cells with low NF-κB activity [160].
2) Normal prostate cells have higher antioxidant capacity compared with prostate cancer cells, including the major antioxidant enzymes catalase and MnSOD, and also GSH content (Figure 4.4). Since induction of oxidative stress is the major mechanism for the effect of parthenolide, cellular antioxidant status will likely affect sensitivity to parthenolide. Wang et al. found that in catalase-knockdown multiple myeloma (MM) cells, parthenolide induces more cell death compared with wild-type MM cells [161], which is consistent with our speculation that cells with higher antioxidant capacity are more resistant to parthenolide.

3) Parthenolide may differently affect GSH level in cancer and normal cells. A previous study by Wen et al. showed that parthenolide depletes GSH in hepatoma cells but increases GSH in normal liver cells [43]. The reactivities of thiols are related to their pKa and abundance. The pKa of the cysteine in GSH is as high as 8.8, so it is usually less reactive. GSH is the most abundant thiol in a cell, so parthenolide may conjugate with GSH inside the cell. However, due to the much higher concentration of GSH inside cells compared to the parthenolide concentration we used, it is unlikely that parthenolide will deplete intracellular GSH by direct reaction. Our result indicates that in both prostate cancer and normal prostate cells, GSH level is increased after parthenolide treatment, especially in the normal prostate cells PrEC. This may be due to the activation of the Nrf2 pathway, which can induce the transcription of $\gamma$-glutamylcysteine synthetase (GCS), the rate-limiting enzyme in the synthesis of GSH. The Nrf2 activity is regulated by Kelch-like ECH-associated protein-1 (Keap1). The human Keap1 protein contains 27 cysteines. Nine of them are predicted to have low pKa values and thus relatively high reactivities [162]. Parthenolide may target these cysteines to activate the Nrf2 pathway and induce GSH. The Nrf2 pathway [37] is usually downregulated in prostate cancer compared with normal prostate, which may explain the more remarkable increase of GSH in normal prostate cells. The dramatic increase of GSH in normal prostate cells may lead to the protective effect against oxidative stress.

4) Another possibility is that glutathione-S-transferase (GSTs)-mediated parthenolide metabolism is different in normal and cancer cells. GSTs catalyze the conjugation of xenobiotics, such as electrophiles, with reduced glutathione. The conjugate is then transported outside the cells. Parthenolide is an electrophile and may conjugate with GSH
The dual role of Akt

Our study found that parthenolide activates the PI3K/Akt pathway as a downstream event of NADPH oxidase activation. Through phosphorylation, activated Akt mediates the activation or inhibition of several targets involved in the regulation of cell survival and death [100]. For example, the phosphorylation and inactivation of the proapoptotic protein BCL2-antagonist of cell death (BAD) promote cell survival. The phosphorylation of glycogen synthase kinase 3 (GSK3) inhibits its kinase activity and thus prevents downstream cyclin D1 phosphorylation and degradation, leading to cell proliferation. Akt also activates the mammalian target of rapamycin (mTOR) and promotes protein synthesis. IKK can also be phosphorylated by Akt, leading to the activation of NF-κB prosurvival pathway. Although parthenolide activates the PI3K/Akt pathway, the NF-κB pathway is inhibited by parthenolide through direct interaction with IKKβ or p65. Another important target of Akt is the forkhead (FOXO) family of transcription factors. The phosphorylation of FOXO by Akt prevents its nuclear localization and target gene transcription. The FOXO targets include both pro-apoptotic proteins, such as BIM and FAS ligand, and also the proteins involved in the oxidative stress resistance and DNA damage repair, such as MnSOD, catalase and GADD45. Therefore, activation of Akt may promote cell survival by inhibiting the pro-apoptotic target, but also may sensitize cells to oxidative stress-induced cell death [112] by suppressing stress resistant proteins.

The activation of Akt plays a dual role in the radiosensitization effect of parthenolide. In chapter two, we showed that PTEN enhances the radiosensitization effect of parthenolide by keeping the activated p-Akt at a low level and thereby abrogating its prosurvival function. In chapter three, we showed that Akt mediated FOXO3a phosphorylation leads to the suppression of antioxidant defense enzymes and contributes to the radiosensitization effect of parthenolide. In DU145 cells, parthenolide activates
Akt, even when p-Akt is kept to a relatively low level due to PTEN function, and it inhibits FOXO3a function and its downstream targets MnSOD and catalase. This indicates that PTEN cannot prevent parthenolide-induced Akt activation and its function in sensitizing cells to oxidative stress, but can only inhibit the basal p-Akt level and the prosurvival role of Akt.

The prosurvival role of the PI3K/Akt pathway is well known. Cancer cells usually have constitutively active PI3K/Akt and inhibition of the PI3K/Akt pathway has often been targeted for cancer therapy. However, a recent study shows that in acute myelogenous leukemia patients, overall survival and relapse-free survival are better in patients with constitutive PI3K (56% and 72%) than patients without PI3K activation (33% and 41%) [163], suggesting that constitutive activation of PI3K/Akt can represent a favorable prognostic factor in cancer patients, consistent with the dual role of Akt.

To summarize, our study demonstrates for the first time that parthenolide can differentially regulate intracellular redox status in prostate cancer and normal prostate cells, and selectively sensitize prostate cancer cells to radiation-induced cell death. Parthenolide modulates cell redox status through multiple mechanisms, including activation of NADPH oxidase, suppressing NF-κB-, and FOXO3a-dependent expression of antioxidant enzymes, and altering cellular thiol buffering systems, i.e. GSH and Trx. To further elucidate how parthenolide regulates intracellular redox status, the effect of parthenolide on mitochondria function should be studied since mitochondria are an important source of ROS generation.

The differential effect of parthenolide in cancer and normal cells makes it a promising agent for sensitizing cancer tissues to the radiation or other oxidative stress-inducing chemotherapies while protecting normal tissues from oxidative stress-induced damage. Our preliminary data confirm that the selective radiosensitization effect of parthenolide to cancer cells is not only observed in prostate cancer, but also in lung cancer (Figure 4.5). Further studies need to be done to prove whether the selective effect of parthenolide is cell-type specific. It is also important to test the effect of parthenolide in vivo. Sweeney et al. [58] reported that, due to its poor solubility, the serum concentration of parthenolide is 200 nmol/L in mice when given at the maximal attainable dose (40 mg/kg), which is
much lower than the dose we used in vitro. To improve the serum level of parthenolide, a water soluble derivative of parthenolide, LC-1 [164] can be used in a future in vivo study. LC-1 can be metabolized into parthenolide in mice and reach a concentration of more than 10 µmol/L in plasma, according to our preliminary data (Figure 4.6).

Overall, our data support the concept that increasing oxidative stress in cells with intrinsically heightened oxidative stress levels leads to cell death while the same stress in cells with low intrinsic oxidative stress confers adaptive response and cell survival. Thus, selective enhancement of cancer therapy with oxidative stress-inducing agents is a possibility and can be particularly effective in cancer cells with high intrinsic oxidative stress levels.
Table 4.1 List of FOXO3a target genes detected by real-time

<table>
<thead>
<tr>
<th>Short Name</th>
<th>Gene Name</th>
<th>probe</th>
<th>Primer For. (5'-&gt;3')</th>
<th>Primer Rev. (5'-&gt;3')</th>
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</thead>
<tbody>
<tr>
<td>CAT</td>
<td>catalase</td>
<td>67</td>
<td>cgcagttcggcttccac</td>
<td>gggtccgaactgtgca</td>
</tr>
<tr>
<td>SOD2</td>
<td>superoxide dismutase 2</td>
<td>22</td>
<td>ctggacaaaacctcgcccct</td>
<td>tgatggcctcggcaactc</td>
</tr>
<tr>
<td>PA26</td>
<td>Sestrin-1 (p53-regulated protein PA26)</td>
<td>46</td>
<td>ggggcgttaaccctacatta</td>
<td>tcactaagtaggacgactgtgctt</td>
</tr>
<tr>
<td>PRDX3</td>
<td>peroxiredoxin 3</td>
<td>62</td>
<td>agcaaaattttcagacccagt</td>
<td>ctttaaaatagggctcatcgtg</td>
</tr>
<tr>
<td>SEPP1</td>
<td>selenoprotein P, plasma, 1</td>
<td>38</td>
<td>gpaacgtccagagtaagca</td>
<td>acatgctggtgtgtcagc</td>
</tr>
<tr>
<td>TXNIP</td>
<td>thioredoxin interacting protein</td>
<td>85</td>
<td>acgcttctctggaggaccca</td>
<td>aagctcaagccgacactggt</td>
</tr>
<tr>
<td>WIP1</td>
<td>protein phosphatase Wip1</td>
<td>25</td>
<td>cccatgctcactaccaccagt</td>
<td>tggtctgtaaatccccccttg</td>
</tr>
<tr>
<td>FEN1</td>
<td>flap structure-specific endonuclease 1</td>
<td>82</td>
<td>acggcgaaccacgacgaggtgtag</td>
<td>gggccacatcagcaatgtag</td>
</tr>
<tr>
<td>GADD45A</td>
<td>growth arrest and DNA-damage-inducible, alpha</td>
<td>37</td>
<td>agagcgaagaaggacggaaggg</td>
<td>tggctcaggtgcttgcagta</td>
</tr>
<tr>
<td>MLH3</td>
<td>Homo sapiens mutL homolog 3</td>
<td>67</td>
<td>tcggttcaccaaggagtaag</td>
<td>tcatcacaaccgcaataaaacttg</td>
</tr>
<tr>
<td>p21</td>
<td>cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>82</td>
<td>cgaagctgcttcctgagggag</td>
<td>catgctgctctgcaggaacat</td>
</tr>
<tr>
<td>p27</td>
<td>Cyclin-dependent kinase inhibitor 18 (p27Kip1)</td>
<td>39</td>
<td>cctcagagagccagtagctgag</td>
<td>agtagaaccctagggaagctg</td>
</tr>
</tbody>
</table>
Figure 4.1 Parthenolide suppresses FOXO3a target genes, SOD2, catalase, SEPP1 and FEN1 transcription in prostate cancer cells PC3.

PC3 cells were treated with DMSO or 5 µmol/L parthenolide for 24 hours before radiation. Cells were collected at 6 hours after 6 Gy radiation and mRNAs were isolated using TRIZOL reagent (Invitrogen) according to the instructions provided by the manufacturer. Reverse transcriptase reaction was performed using the Superscript™ III First-Strand synthesis system (Invitrogen) to synthesize template cDNA. Quantitative real-time PCR was performed using the LightCycler 480 PCR system (Roche). The PCR primers and matched probes for each gene were designed using Roche Universal Probe Library software (Applied, Roche). The relative changes of gene expression were calculated and normalized to GAPDH by using the $2^{-\Delta\Delta Ct}$ method. *p<0.05 compared with DMSO treated control; #p<0.05 compared with IR treated group.
Figure 4.2 Parthenolide inhibits radiation induced NF-κB DNA binding activity in PC3 prostate cancer cells but not in normal prostate PrEC cells.

Cells were treated with DMSO or 5 μmol/L parthenolide for 3 hours before radiation. Nuclear extracts (NE) were prepared at 6 hours after 6 Gy IR for electrophoretic mobility shift assay (EMSA) with radiolabelled NF-κB probes. Anti-p50 and anti-p65 antibodies were used for supershift.
Figure 4.3 The comparison of basal NF-κB levels in prostate cancer cells DU145, PC3 and normal prostate PrEC cells.

1. DU145
2. PC3
3. PrEC
Figure 4.4 Normal prostate cells PrEC have higher antioxidant capacity compared with prostate cancer cells.

A. Comparison of antioxidant enzyme MnSOD and catalase level in three prostate cell lines.

<table>
<thead>
<tr>
<th></th>
<th>Catalase</th>
<th>MnSOD</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. DU145  
2. PC3  
3. PrEC

B. Comparison of total GSH (GSHt) and GSH/GSSG ratio in PC3 and PrEC cells.

![Bar chart showing GSHt and GSH/GSSG ratios for PC3 and PrEC cells]
Figure 4.5 Parthenolide sensitizes lung cancer cells A549 but not normal lung cells NL-20 to radiation treatment.

Clonogenic survival assay was performed as described in Materials and Methods in chapter two. For NL-20 cells, a feeder layer was used for colony formation. To make the feeder layer, cells were first treated with 30 Gy radiation, which is lethal to the cells, then plated into the 6-well plates and grew overnight. Cells were then plated on the feeder layer to form a colony. Briefly, cells were treated with the indicated concentrations of parthenolide (PN) for 24 hours, and then exposed to indicated doses of radiation. Twenty-four hours after radiation, the media containing PN was removed. Cells were then maintained for 9 days. The cultures were stained and the colonies containing more than 50 cells were counted. Survival fraction was determined by dividing the plating efficiency of radiated cultures by the plating efficiency of non-radiated cultures. Values shown are the means±SD for triplicates.
Figure 4.6 Pharmacokinetics study of LC-1

Nude mice were injected with LC-1, a water soluble derivative of parthenolide, at the dose of 40 mg/kg i.p. Then plasma samples were collected at 0.5 hour (3 samples), 1 hour (3 samples) and 3 hours (3 samples) after injection. Each sample is approximately 500 μl. The concentrations of LC-1 and parthenolide in the plasma were determined using LC/MS by the Brunswick Laboratories.
Appendix: List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>Acute myelogenous leukemia</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant/electrophile response element</td>
</tr>
<tr>
<td>ASK-1</td>
<td>Apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2-antagonist of cell death</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DBE</td>
<td>Daf-16 family protein binding element</td>
</tr>
<tr>
<td>2-DG</td>
<td>2-Deoxy-D-glucose</td>
</tr>
<tr>
<td>DMF</td>
<td>Dose modifying factor</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenylene iodonium</td>
</tr>
<tr>
<td>Duox</td>
<td>Dual oxidase</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ETC</td>
<td>Mitochondria electron transport chain;</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>Fas</td>
<td>Fibroblast-associated cell surface antigen</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box class O transcription factors</td>
</tr>
<tr>
<td>GADD45β</td>
<td>Growth arrest and DNA damage-inducing protein β</td>
</tr>
<tr>
<td>GCS</td>
<td>γ-Glutamylcysteine synthetase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>Grx</td>
<td>Glutaredoxin</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-Hydroxynonenal</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>IKC</td>
<td>IκB Kinase complex</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein-1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
</tbody>
</table>
MPB  3-(N-maleimido-propionyl) biocytin
MST1  Mammalian Ste20-like kinase
mTOR  Mammalian target of rapamycin
NADPH oxidase  Nicotinamide adenine dinucleotide phosphate oxidase
NEM  N-ethylmaleimide
NF-κB  Nuclear factor kappa B
NIK  NF-κB-inducing kinase
NOS  Nitric oxide synthase
NOX  NADPH oxidase
Noxa1  Nox activator 1
Nox01  Nox organizer 1
Nrf2  Erythroid 2p45 (NF-E2)-related factor 2
PDK  Phosphatidylinositol-dependent kinase
PE  Plating efficiency
PI3K  Phosphatidylinositol 3-kinase
PIP3  Phosphatidylinositol-3,4,5-trisphosphate
PN  Parthenolide
Prx  Peroxiredoxin
PSA  Prostate specific antigen
PTEN  Phosphatase and tensin homologue deleted from chromosome-10
PTP  Protein tyrosine phosphatase
Ref-1  Redox factor-1
RLU  Relative light unit
RNS  Reactive nitrogen species
ROS  Reactive oxygen species
RS  Reactive species
SHP-2  Src homology 2 domain (SH2)-containing tyrosine phosphatase 2
SOD  Superoxide dismutase
STAT  Signal transducer and activator of transcription
TCR  T cell receptor
TNF-α  Tumor necrosis factor alpha
TRAIL  Tumor necrosis factor-related apoptosis-inducing ligand
Trx  Thioredoxin
TrxR  Thioredoxin reductase
TSC2  Tuberous sclerosis complex 2
XIAP  X-chromosome-linked inhibitor of apoptosis
XO  Xanthine oxidase
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116. Lynch RL, Konicek BW, McNulty AM, et al. The progression of LNCaP human prostate cancer cells to androgen independence involves decreased FOXO3a


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Kentucky Opportunity Fellowship, University of Kentucky, 2006
Young Investigator Award, SFRBM, 2005
Kentucky Opportunity Fellowship, University of Kentucky, 2005
Research Challenge Trust Fund Fellowship, University of Kentucky, 2004
Excellent Graduate Student Award, Sichuan University, 2002
Excellent Graduation Dissertation Award, WCUMS, 2000
Medical Education Fellowship of United Laboratories, WCUMS, 1999

Publications

Sun Y, St Clair DK, Xu Y, Crooks PA, St Clair WH. A NADPH oxidase dependent redox signaling pathway mediates the selective radiosensitization effect of parthenolide in prostate cancer cells. (submitted)
Xu Y, Fang F, St Clair DK, Sun Y, St Clair WH. Cytotoxic effect of Gleevec (imatinib mesylate) on survival of prostate cancer cells: the role of RelB in radio-sensitization. (submitted)


Sun Y, St Clair DK, Fang F, Warren GW, Rangnekar VM, Crooks PA, St Clair WH. The radiosensitization effect of parthenolide in prostate cancer cells is mediated by NF-κB inhibition and enhanced by the presence of PTEN. Mol Cancer Ther. 2007, 6(9):2477-86.

**Presentations and Abstracts**

Sun Y, St Clair DK, Xu Y, Crooks PA, St Clair WH. A NADPH oxidase dependent redox signaling pathway mediates the selective radiosensitization effect of parthenolide in prostate cancer cells. Presented at the 16th annual meeting of SFRBM (Society of Free Radical Biology and Medicine), 2009.

Sun Y, St Clair DK, Xu Y, Crooks PA, St Clair WH. Suppression of FOXO3a as a Novel Mechanism for the Radiosensitization Effect of Parthenolide. Awarded at the 15th annual meeting of SFRBM (Society of Free Radical Biology and Medicine), 2008.

Sun Y, St Clair DK, Xu Y, Crooks PA, St Clair WH. Suppression of FOXO3a as a Novel Mechanism for the Radiosensitization Effect of Parthenolide. Poster presentation and selected for oral presentation at Cancer Research Day, Markey Cancer Center, University of Kentucky, 2008.

Sun Y, St Clair DK, Xu Y, Crooks PA, St Clair WH. Differential radiosensitization of malignant prostate cancer and noncancerous prostate epithelial cells by parthenolide is mediated by transcriptional regulation of manganese superoxide dismutase. Presented at the 14th annual meeting of SFRBM (Society of Free Radical Biology and Medicine), Washington DC, 2007.

Sun Y, St Clair DK, Xu Y, Crooks PA, St Clair WH. Differential radiosensitization of malignant prostate cancer and noncancerous prostate epithelial cells by parthenolide is mediated by transcriptional regulation of manganese superoxide dismutase. Poster presentation and selected for oral presentation at Cancer Research Day, Markey Cancer Center, University of Kentucky, 2007.

Sun Y, Warren G, St Clair DK, Crooks PA, St Clair WH. Parthenolide selectively sensitizes prostate cancer cells but not normal prostate epithelial cells to radiation treatment through inhibition of NF-κB activation. Presented and awarded at the 12th annual meeting of SFRBM (Society of Free Radical Biology and Medicine), Austin, TX, 2005.