KEAP1 Is a Redox Sensitive Target That Arbitrates the Opposing Radiosensitive Effects of Parthenolide in Normal and Cancer Cells

Yong Xu\textsuperscript{1}, Fang Fang\textsuperscript{2}, Sumitra Miriyala\textsuperscript{1}, Peter A. Crooks\textsuperscript{3}, Terry D. Oberley\textsuperscript{4}, Luksana Chaiswing\textsuperscript{4}, Teresa Noel\textsuperscript{1}, Aaron K. Holley\textsuperscript{1}, Yanming Zhao\textsuperscript{1}, Kelley K. Kiningham\textsuperscript{5}, Daret K. St. Clair\textsuperscript{1†} and William H. St. Clair\textsuperscript{2†}

\textsuperscript{1}Graduate Center for Toxicology, \textsuperscript{2}Department of Radiation Medicine, University of Kentucky, Lexington, KY 40536; \textsuperscript{3}Department of Pharmaceutical Sciences, UAMS College of Pharmacy, Little Rock, AR 72205; \textsuperscript{4}Department of Pathology, University of Wisconsin, Madison, WI 53705; and \textsuperscript{5}Department of Pharmaceutical Sciences, Belmont University School of Pharmacy, Nashville, TN 37212

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† Corresponding Authors: William H. St. Clair, M.D., Ph.D., Department of Radiation Medicine, University of Kentucky, College of Medicine, Lexington, KY 40536; Phone: 1-(859) 257-4931; Fax: 1-(859) 323-6486; Email: stclair@uky.edu. Daret K. St. Clair, Ph.D., Graduate Center for Toxicology, University of Kentucky, College of Medicine, Lexington, KY 40536; Phone: 1-(859) 257-3720; Fax: 1-(859) 323-1059; Email: dstcl00@uky.edu.

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Abstract

Elevated oxidative stress is observed more frequently in cancer cells than in normal cells. It is therefore expected that additional exposure to a low level of reactive oxygen species (ROS) will push cancer cells toward death, whereas normal cells might maintain redox homeostasis through adaptive antioxidant responses. We previously demonstrated that parthenolide enhances ROS production in prostate cancer cells through activation of NADPH oxidase. The present study identifies KEAP1 as the downstream redox target that contributes to parthenolide's radiosensitization effect in prostate cancer cells. *In vivo*, parthenolide increases radiosensitivity of mouse xenograft tumors but protects normal prostate and bladder tissues against radiation-induced injury. Mechanistically, parthenolide increases the level of cellular ROS and causes oxidation of thioredoxin (TrX) in prostate cancer cells, leading to a TrX-dependent increase in a reduced state of KEAP1, which in turn leads to KEAP1-mediated PGAM5 and Bcl-xL (BCL2L1) degradation. In contrast, parthenolide increases oxidation of KEAP1 in normal prostate epithelial cells, leading to increased Nrf2 (NFE2L2) levels and subsequent Nrf2-dependent expression of antioxidant enzymes. These results reveal a novel redox-mediated modification of KEAP1 in controlling the differential effect of parthenolide on tumor and normal cell radiosensitivity. Further, they show it is possible to develop a tumor-specific radiosensitizing agent with radioprotective properties in normal cells.
Introduction

It is well-documented that cancer cells are usually under more oxidative stress than normal cells are, in part due to a hyperactive metabolism that fuels their rapid growth (1, 2). Thus, a therapy designed to increase reactive oxygen species (ROS) to a level above the threshold for cancer cell death, but to an adaptable level for normal cells, would be an attractive strategy to selectively kill cancer cells (3, 4). Redox homeostasis is thought to regulate many cellular processes that are essential for maintenance of normal physiological conditions but is aberrantly modulated in cancers (5, 6). The functions of ROS are both beneficial and deleterious due to their dual role in the prosurvival and antisurvival pathways. As a secondary messenger in cell signaling, ROS are required for normal development and can initiate adaptive responses in cellular defense (7, 8). On the other hand, ROS cause structural damage and functional decline in DNA, proteins and lipids, and consequently act as an anti-tumorigenic factor by inducing cell senescence and apoptosis (9, 10). Indeed, ROS-mediated cell death is an important basis for radiotherapy and many chemotherapeutic treatments (11, 12). Currently, these therapeutic strategies are being used to kill cancer cells without benefit of a rational design that exploits the intrinsic differences in the cellular redox status of normal cells and cancer cells.

Antioxidant defense systems are essential for the regulation of ROS levels, which have an important function in the maintenance of cellular redox hemostasis. Mounting evidence demonstrates that a decline in antioxidant function may be involved in tumorigenesis due to prooxidant conditions that result from ROS accumulation. For example, manganese superoxide dismutase (MnSOD) is down-regulated in many types of cancer, and overexpression of MnSOD results in suppression of tumorigenesis (13-15). High levels of antioxidants caused by therapy-mediated activation of pro-survival pathways, such as NF-κB and Nrf2, are thought to protect
cancer cells against treatment (16-18). Thus, inhibition of prosurvival pathways has been a traditional strategy to enhance therapeutic efficacy.

Increasing evidence demonstrates that certain mild prooxidant compounds derived from natural herbal medicines might enhance some anticancer treatments by modulating the redox state of cancer cells to high prooxidant levels (19, 20). Parthenolide, an active ingredient derived from the traditional anti-inflammatory medical plant feverfew (*Tanacetum parthenium*), belongs to the family of sesquiterpene lactones containing an α-methylene-γ-lactone moiety and an epoxide group, which is able to conjugate thiols of proteins through a Michael addition reaction (21). In addition to its anti-inflammatory effect, parthenolide appears to be toxic to a variety of cancer cells (22-25). Importantly, parthenolide has no cytotoxic effect on normal cells (25). Mechanistically, parthenolide has been shown to increase apoptosis in cancer cells through inhibition of multiple prosurvival pathways, such as NF-κB and PI3K-AKT (19, 26). However, these findings do not explain why parthenolide is not toxic to normal cells.

Post-translational modification is a key mechanism by which proteins dramatically increase their functional diversity. Reversible redox modification of protein cysteine residues plays an important role in vital cell signaling pathways related to many physiological and pathogenic processes (27, 28). The Keap1-Nrf2 pathway is one of the main cellular defense mechanisms against oxidative stress (29, 30). The present study examines the role of cysteine modifications in modulating radiation responses in prostate cancer cells versus normal prostate epithelial cells. It elucidates the functional link between redox modulation and cell signaling transduction pathways, and it provides evidence for the differential effect of parthenolide on cellular redox status in normal and cancer cells. The results reveal that parthenolide-mediated redox modification of Keap1 serves as a central regulator of differential responses to radiation...
therapy in normal and tumor cells. The present study provides a proof-of-concept for utilizing an intrinsic difference in cellular redox conditions to kill tumor cells while protecting normal cells from the unwanted side effects of radiation.
Materials and Methods

Cell culture, cell transfection, treatment, and cell survival analysis. Human prostate carcinoma/adenocarcinoma LNCaP, PC3 and DU145 cell lines as well as human prostate epithelial viral transformed PZ-HPV-7 (PZ) and RWPE-1 cell lines were obtained from American Type Culture Collection (ATCC). Normal prostate epithelial PrEC cells were purchased from Lonza. All cell lines were cultured and maintained in the media recommended in the manufacturer’s protocols. Plasmid cloned Keap1 cDNA and Bcl-xL cDNA (OriGene) and siRNAs for knocking down Keap1, Nrf2, thioredoxin (TrX), PGAM5, and Bcl-xL (Dharmacon) were transfected into cultured cells prior to treatment. Parthenolide and its water soluble prodrug, dimethylamino-parthenolide (DMAPT), were synthesized as previously described (31). The cells were treated with 0-5 µM parthenolide followed by irradiation by a 250 kV X-ray machine (Faxitron X-ray Corp.) with peak energy of 130 kV, 0.05 mm Al filter, at a dose of 0 to 6 Gy. Cell survival rates were quantified by colony survival fraction, Trypan blue exclusion assay, and MTT assay, as previously described (25, 32).

Animals. Four- to five-week-old male NCRNU (nu/nu athymic nude) mice were obtained from Taconic (Hudson). For formation of xenograft tumors, 1.8 x 10^6 cells mixed in Matrigel (BD Biosciences) were subcutaneously injected into the right flank of the mice. Tumor volumes were routinely measured and their sizes calculated based on a protocol described elsewhere (33). Animals with an average tumor size of 500 mm^3 were randomized into several groups for DMAPT and radiation treatments. The tumors were treated five times with 10 mg/kg DMAPT and 3 Gy IR. The tumor tissues were collected, and 100 µg of tissue were lysed to quantify amounts of oxidized or reduced Keap1 and levels of downstream proteins. To determine the protective effect of parthenolide against radiation damage, mice were pretreated with DMAPT
followed by radiation treatment (5 x 3Gy). Prostate and bladder tissues were fixed, embedded and processed for routine Electron microscopy (EM). The embedded blocks were sectioned and transferred to copper grids and counterstained with uranyl acetate, followed by lead citrate. Grids were observed in an electron microscope (Hitachi H-600) operated at 75 kV. Mitochondrial damage was quantified by a pathologist (TDO) using identified ultrastructural changes including swelling, vacuolization, myelination, disorganization and loss of cristae, lysosomal degradation, and membrane disruption. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky, Approval Protocol No. 01077M2006.

**Quantification of intracellular superoxide and prooxidant.** Dihydroethidium (DHE, Invitrogen), which exhibits blue fluorescence in the cytosol until oxidized, was used to estimate the levels of superoxide after parthenolide treatment. To confirm the level of superoxide induced by parthenolide, the cells were pretreated with 50 μg PEG-SOD (Sigma) for 1 h followed by parthenolide treatment. Antimycin (Sigma) was used as a positive control because it has been shown to increase superoxide in all tested cell lines. A dichlorofluorescein (DCF) assay was used to quantify the levels of intracellular ROS after parthenolide treatment. The cells were labeled by both carboxy-H2DCFDA (sensitive to oxidation, Invitrogen) and carboxy-DCFDA (insensitive to oxidation, Invitrogen). The H2DCFDA:DCFDA ratio was used to optimize the controls of cell number, dry uptake, and ester cleavage. The procedures for both DHE and DCF were performed by the University of Kentucky Flow Cytometer Facility using a FACScan protocol provided by Dr. Douglas R. Spitz (34).

**Measurement of oxygen consumption rates (OCR).** To determine how parthenolide changes mitochondrial function in cancer and normal cells, a Seahorse Bioscience XF24 Extracellular
Flux Analyzer was used to measure OCR after parthenolide treatment. The XF24 creates a transient, 7-μl chamber in specialized microplates, which allows determination of oxygen and proton concentrations in real time. To allow comparison between experiments, data are expressed as the OCR in pmol/min or the rate of extracellular acidification in mpH/min. Reserve capacity, an important index that indicates capacity of mitochondrial respiration, is calculated by subtracting baseline OCR from maximal OCR.

**Detection of oxidized and reduced forms of Keap1 protein.** 3-N-maleimido-propionyl biocytin was used to selectively label sulphydryl (SH) and then was detected by biotin-streptavidin integration on the blots, as previously described (35). To quantify disulfide (S-S) bonds, the SH form was stabilized by treating with N-ethylmaleimide and then the S-S bonds were reduced by treating with 2-mercaptoethanol. To identify SH and S-S moieties of Keap1 protein, the labeled proteins were immunoprecipitated by Keap1 antibody (abcam) and subjected to SDS-PAGE, followed by detection with horseradish peroxidase-conjugated streptavidin (Sigma).

**Immunoblots and immunoprecipitation.** Homogenized cells and tumor tissues were electrophoresed on an 8% (w/v) SDS-PAGE gel, transferred onto a nitrocellulose membrane, and subsequently incubated with primary antibodies against Keap1 (abcam), Nrf2 (abcam), MnSOD (Upstate Biotech), CuZnSOD (eBiosci), Gpx (abcam), catalase (Millipore), TrX (BD Sciences), PGAM5 (Santa Cruz Biotech.), Bcl-xL (Santa Cruz Biotech.), LC3B (cell signaling), β-actin (sigma), and pCNA (Santa Cruz Biotech.). All secondary antibodies were obtained from Santa Cruz Biotech. Immunoblots were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech.). For immunoprecipitation, cell extracts were incubated overnight with one primary antibody at 4°C and integrated with a protein A/G agarose (Santa
Cruz Biotech). Immunocomplexes were precipitated and fractionated on an SDS-PAGE gel. Interacting proteins were detected by immunoblotting with their primary antibodies.

**SOD enzymatic assay.** MnSOD activities were measured by the nitroblue tetrazolium (NBT)-bathocuproin sulfonate (BCS) reduction inhibition method. Sodium cyanide (2 mM) was used to inhibit CuZnSOD activity (36).

**Statistical data analyses.** Multiple independent experiments were performed for each set of data presented. Images in Immunoblots were quantified using Carestream Molecular Imaging software (Carestream Health Inc.). Statistical significance was analyzed using one-way ANOVA and Tukey's Multiple Comparison Test, followed by data analysis with Graphpad Prism.
Results

Parthenolide enhances radiosensitivity of prostate tumors but protects normal tissues from radiation. Our previous studies demonstrated that parthenolide is able to selectively enhance the radiosensitivity of prostate cancer cells without injury to normal prostate epithelial cells (24, 25). To determine whether parthenolide enhances radiotherapy in vivo, human prostate cancer PC3 cells were subcutaneously implanted into the right flank of nude male mice. When tumor size reached a volume of 500 mm³, animals were randomized into groups according to treatment consisting of saline or 10 mg/kg DMAPT. One hour after saline or DMAPT was administered, the tumors were treated with fractionated radiation of 1 Gy or 2 Gy per day for 5 days, followed by routine measurement of tumor volume. The tumor growth curves are shown in Fig. 1A. Mice were humanely killed when a tumor reached the maximum size of 2000 mm³. Tumor growth was clearly delayed in the treatment groups, particularly when the drug and radiation were combined, compared to growth in the untreated group. The tumor growth rates after treatment were compared according to the days needed for tumor volume to reach 2000 mm³. DMAPT significantly enhanced radiotherapeutic efficiency compared to the effects of radiation treatment alone (Fig. 1B). A separate group of nude male mice that had no cancer cell implantation was treated with DMAPT and radiation to determine the toxicity of DMAPT to organs that can be affected by radiation therapy of prostate cancer. Prostates and bladders of the animals were examined by light and EM. At 60 days after irradiation, no gross pathology was observed (data not shown). However, ultrastructural damage was clearly observed by EM (Fig. 1C). Mitochondrial damage was most pronounced and this was morphometrically analyzed. The number of damaged mitochondria in prostate and bladder was proportional to radiation exposure. Pretreatment with DMAPT significantly reduced the number of damaged mitochondria in both
organs compared to the group without DMAPT treatment (Fig. 1C and D). These results indicate that DMAPT, the water soluble prodrug of parthenolide, is a promising agent for selectively enhancing the sensitivity of prostate cancer cells to radiation while protecting normal tissues from damage caused by radiation.

**Parthenolide differentially modulates cellular ROS levels in cancer and normal cells.** To determine the effect of parthenolide on redox homeostasis in cancer and normal cells, the levels of superoxide and total ROS after parthenolide treatment were measured using flow cytometry. The mean fluorescence intensity of DHE and the H$_{2}$DCFDA:DCFDA ratio were higher in prostate cancer PC3 cells than in normal prostate PZ and PrEC cells, indicating higher basal levels of superoxide and total ROS, respectively (Fig. 2A-B). Following parthenolide treatment, DHE and DCF levels increased further in PC3 cells but declined slightly in both types of non-cancerous cells. Combining PEG-SOD with parthenolide treatment restored the basal level of DHE fluorescence. As positive controls, addition of equivalent concentrations of the ROS stimulating agents antimycin (Fig. 2A) and PMA (Fig. 2B) caused high levels of ROS in all the tested cell lines.

The levels of antioxidant proteins were also quantified (Fig. 2C). Parthenolide altered the protein level of the antioxidant enzymes, in particular mitochondria-localized antioxidant enzymes. MnSOD and glutathione peroxidase (GpX) were significantly reduced in all three parthenolide-treated prostate cancer cell lines. Intriguingly, parthenolide had the opposite effect in the three normal prostate cell lines. However, neither cancer nor normal cells showed any obvious changes after parthenolide treatment for the major cytosolic superoxide removal protein, copper and zinc-containing SOD (CuZnSOD). This observation was confirmed by quantification of the corresponding enzyme activity (Fig. 2D). These results suggest that parthenolide-mediated
alteration of cellular redox status is mediated, at least in part, by changing the activities of antioxidant enzymes in mitochondria.

To probe whether altering cellular redox status is associated with a change in mitochondrial respiration, the OCR in the parthenolide-treated cells was measured using a Seahorse Bioscience FX OxygenFlux Analyzer. The basal and maximal OCR in normal cells was higher than in cancer cells (Fig. 2E). Importantly, parthenolide was able to increase the OCR and reserve capacity in PZ cells, whereas parthenolide had no effect on PC3 OCR. Finally, the cytotoxicity of parthenolide was tested in all the cell lines using an MTT assay, which requires active mitochondria. As shown in Fig. 2F, parthenolide was toxic to all the cancer cells but not to the normal cell lines. Taken together, these results suggest that changes in cellular redox status and mitochondrial function may be a cause for the differential biological effects of parthenolide on cancer and normal cells.

**Keap1 is susceptible to parthenolide-mediated redox modification.** Keap1, a redox sensitive protein, has been reported to play an important role in cell survival under oxidative stress (29). To investigate whether parthenolide modifies Keap1 function, a Keap1 antibody linked to biotin was used to immunoprecipitate redox-modified Keap1 protein, and the presence of oxidized (-S-S-) and reduced (-SH) cysteine residues was detected using a secondary antibody linked to streptavidin. In the three normal cell lines, parthenolide increased the oxidized form of Keap1 but decreased the reduced form of Keap1 (Fig. 3A). Interestingly, the results from the three cancer cell lines appeared to be completely opposite to results observed in normal cells treated with parthenolide: the level of the oxidized form was decreased, but the level of the reduced form was increased (Fig. 3B). To verify that the observed increase in reduced Keap1 also occurred *in vivo*, mouse xenograft tumor tissues with and without DMAPT treatment were also
used for determination of Keap1 redox status. Consistent with data obtained from cultured tumor cells treated with parthenolide, the *in vivo* results show that parthenolide decreased the oxidized form of Keap1 but increased the reduced form of Keap1 in the tumors (Fig. 3C). Changes in antioxidant proteins in mouse xenograft tumor tissues treated with DMAPT are also consistent with the result obtained from *in vitro* studies (Fig. 3D), indicating that parthenolide decreases the level of mitochondrial antioxidant proteins in prostate tumors.

**Oxidization of Keap1 leads to activation of the Nrf2 pro-survival pathway in normal cells.** Activation of the Nrf2 signaling pathway through dissociation with Keap1 resulting in Nrf2 nuclear translocation is considered to be a primary pro-survival pathway in response to oxidative stress (30, 37). To examine whether parthenolide changes Nrf2 nuclear translocation, the levels of Nrf2 in nuclei were measured. As shown in Fig. 4A, the nuclear levels of Nrf2 were increased in the three normal cell lines treated with parthenolide, but no changes were observed in the three cancer cell lines. To examine whether activation of the Nrf2 pathway is a major mechanism by which parthenolide protects normal cells against radiation injury, Keap1 and Nrf2 were silenced in PZ cells by transfecting their siRNA (Fig. 4B, left panel). Cell survival decreased when Nrf2 was silent. IR significantly reduced cell survival but the cell survival was restored when Keap1 was silenced (Fig. 4B, right panel). These results suggest that oxidation of Keap1 and subsequent activation of Nrf2 by parthenolide are essential for normal cell survival after radiation treatment.

**Thioredoxin is necessary for parthenolide-mediated reduction of Keap1 in cancer cells.** TrX is highly expressed in cancer cells and stimulates cell growth. We previously reported that parthenolide decreases the reduced form of TrX but increases the oxidized form of TrX in prostate cancer cells (25). In the present study, we verify that TrX was expressed at a high level in all three cancer cell lines whereas a low level was observed in the three non-cancer cell lines.
(Fig. 5A). Immunoprecipitation of Keap1 protein from PC3 cell extracts using a TrX antibody suggests an interaction between Keap1 and TrX that is increased by parthenolide (Fig. 5B). To detect whether the parthenolide-influenced reduction of Keap1 in cancer cells is dependent on TrX, we selectively silenced TrX by transfecting its siRNA prior to parthenolide treatment (Fig. 5C, left panel). As expected, the reduced form of Keap1 was decreased, but the oxidized form of Keap1 was increased when TrX was silent (Fig. 5C, middle and right panels). The results suggest that TrX is interacting with Keap1 to keep Keap1 in a reduced state in parthenolide-treated cells.

To further confirm that the function of Keap1 leads to cell death in parthenolide-treated cancer cells, a Keap1 expression construct was transfected into PC3 cells, followed by parthenolide and IR treatments. Overexpression of Keap1 resulted in increases in cell death in both treated and untreated cells (Fig. 5D, top panel). The levels of mitochondrial phosphoglycerate mutase 5 (PGAM5), a protein serine/threonine phosphatase that interacts with Bcl-xL in the mitochondrial membrane (38), and Bcl-xL were clearly decreased in the Keap1 transfected cells, but no changes were observed in Nrf2, Ikka and IkBa (Fig. 5D, bottom panel). These results suggest that the parthenolide-increased reduced form of Keap1 facilitates Keap1-mediated ubiquitin/proteasome-dependent degradation of PGAM5 and Bcl-xL, which is an established mechanism for parthenolide-mediated cell death in cancer cells.

**Keap1 triggers PGAM5-mediated Bcl-xL ubiquitin degradation in parthenolide-treated cancer cells.** To further investigate the mechanism by which parthenolide enhances the radiosensitivity of prostate cancer cells, we determined the interactions between Keap1, PGAM5 and Bcl-xL. The results demonstrate that a reduced form of Keap1, which is increased in parthenolide-treated PC3 cells, enhanced interaction between Keap1 and PGAM5, as detected by immunoprecipitation using a PGAM5 antibody (Fig. 6A). Bcl-xL, a prosurvival mitochondrial
protein, was also increased in the pulled down complex (Fig. 6A). Interestingly, the proteins that are associated with Keap1 were decreased in whole cell extracts (Fig. 6B). A time course of parthenolide treatment shows that PGAM5 and Bcl-xL proteins were slightly increased at 12 h but decreased at 24 and 48 h after treatment (Fig. 6C). Proteins in different cellular fractions were also quantified (Fig. 6D). The mitochondria-associated proteins PGAM5 and Bcl-xL were reduced by the parthenolide treatment, but no change was observed in Hsp75, a control for mitochondrial protein. Parthenolide had no major effect on the levels of Nrf2 and Ikkα in treated cells. These results suggest that parthenolide enhances Keap1-mediated ubiquitin/proteasome-dependent degradation of PGAM5 and Bcl-xL (39). In addition, parthenolide increased the level of mitochondria-associated autophagic protein LC3B, suggesting that parthenolide may enhance the radiation sensitivity of prostate cancer cells partially through triggering the autophagy pathway.

Because Keap1 interacts with PGAM5/Bcl-xL/Nrf2, we decided to determine the effect of PGAM5/Bcl-xL/Nrf2 in mediating parthenolide’s effect on cancer cells. PGAM5, Bcl-xL and Nrf2 were silenced using their siRNAs, followed by parthenolide treatment (Fig. 6E, bottom panel). The cell survival fraction was decreased when PGAM5 or Bcl-xL was silent, which is similar to the effect of parthenolide (Fig. 6E, top panel). No significant additive effects were observed when parthenolide was combined with PGAM5 or Bcl-xL siRNA. In contrast, a significant effect was observed when Nrf2 was silent. These results suggest that Keap1-mediated PGAM5/Bcl-xL degradation, but not Nrf2 degradation, is important for parthenolide-induced cancer cell death.

To further determine whether the function of Bcl-xL plays a major role in protecting cancer cells against parthenolide-induced cell death, a plasmid carrying Bcl-xL cDNA was
transfected into PC3 cells followed by parthenolide treatment (Fig. 6F, bottom panel). The results show that expression of Bcl-xL efficiently protects cells from cytotoxicity caused by parthenolide (Fig. 6F, top panel). Taken together, these results suggest that parthenolide enhances the radiosensitivity of prostate cancer cells, in part, by triggering ubiquitin/proteasome-based degradation of Bcl-xL.

In summary, parthenolide provides radiosensitization in prostate cancer cells but radioprotection in normal cells, and the observed differential effects are mediated, in part, by redox modification of Keap1, i.e., reducing Keap1 in cancer cells but oxidizing Keap1 in normal cells. The distinct redox modification of Keap1 initiates different signaling pathways that affect mitochondrial function, leading to cell survival or cell injury in response to radiation, as illustrated in Fig. 7.
Discussion

The majority of anticancer therapies fail because cancers develop phenotypes that are treatment resistant and because treatments cause unwanted and/or detrimental side effects to normal cells or to untargeted tissues. While conventional adjuvant therapies improve tumor response to radiation therapy, they generally cause additional damage to normal tissues. Thus, the focus of the present study is to identify adjuvant therapeutics that can reduce the side effects of radiation therapy. Our study provides a proof-of-concept for improving the efficacy of radiation therapy while protecting against injury to normal tissues. It has been demonstrated that parthenolide, the anti-inflammatory phytochemical, is able to suppress tumor growth in many organs (22-25). In addition, parthenolide appears to synergically enhance chemotherapeutic efficiency when it is combined with taxol or cisplatin to treat lung and gastric cancer cells (23, 40). Parthenolide also sensitizes radioresistant osteosarcoma cells to radiotherapy (41). Here, we demonstrate that DMAPT, a parthenolide prodrug, sensitized prostate cancer cells to radiotherapy in vivo and protected normal prostate and bladder against radiation-induced tissue injury. These results extend our previous survival studies in prostate cancer cell lines and normal prostate epithelial cells.

ROS, as products of cell metabolism, play a dual role in tumorigenesis and tumor suppression. The “two-faced” character of ROS has emerged as a potential source for discovering anticancer drugs. Redox homeostasis is frequently deregulated in cancers as it is constantly exposed to high levels of ROS compared to normal counterparts. Our data demonstrate that constitutively elevated levels of oxidative stress in cancer cells represent a specific vulnerability that can be selectively targeted by direct- or indirect-acting prooxidants and antioxidants or redox modulators. Theoretically, the differential redox status of cancer cells
compared to normal cells should provide a therapeutic window for selective redox intervention via additional increases in ROS. In this context, normal and cancer cells should respond differently to the same level of prooxidant action generated either by direct production of oxidative species or by modulation of specific cellular targets involved in redox regulation. In this study we demonstrate that parthenolide serves as a prooxidant and displays a selective redox modification capability that differentially modulates cellular redox signals and targets. The Michael acceptor reacts with a thiol group of target proteins through covalent adduction (21). Parthenolide contains electrophilic α-methylene-γ-lactone, a bifunctional Michael acceptor, and displays a potential for bifunctional target alkylation and crosslinking. The present study demonstrates the inverse effects of parthenolide on redox modification in cancer cells compared to normal cells. Remarkably, observations of the cytotoxic and cytoprotective effects of parthenolide are consistent with its action in the modulation of ROS levels in both cancer and normal cells. Alteration of cellular ROS by parthenolide is attributed to functionally up- or down-regulating antioxidant enzymes in mitochondria, which consequently regulates mitochondrial respiration. Parthenolide is able to selectively reduce the activity of several enzymes involved in oxidative stress removal in cancer cells, which in turn can cause ROS levels to rise above the threshold for cell death. This finding predicts that antioxidant proteins and mitochondria are feasible therapeutic targets.

It has been reported that parthenolide is a potent inhibitor of NF-κB, which is a ROS-responsive transcriptional factor involved in both tumor progression and tumor resistance to treatment through upregulation of anti-apoptotic genes, such as Bcl-2, Bcl-xL, survivin, and XIAP (42). We previously demonstrated that NADPH oxidase-mediated inactivation of the Foxo 3 signaling pathway is involved in the parthenolide-enhanced radiosensitivity of prostate cancer.
(25). However, previous studies did not explain how parthenolide exerts such an opposing effect in tumor and normal cells. The present study identifies Keap1 as a redox signaling sensor that plays a pivotal role in the differential regulation of the downstream signaling targets in response to radiation-mediated cytotoxicity in prostate cancer and normal cells. Keap1, an adaptor protein for ubiquitin-based processing by the CUL3/RBX1-dependent E3 ubiquitin ligase complex, functions as a sensor for thiol-reactive redox modification (43). The present study demonstrates that stabilization of Nrf2 by oxidation of Keap1 serves as a major mechanism by which parthenolide protects normal tissues against radiotoxicity through up-regulation of antioxidant enzymes in mitochondria. However, Nrf2 transcriptional activation did not play a major role in parthenolide-treated prostate cancer cells. Thus, it is interesting to note that unlike traditional chemotherapeutic agents, parthenolide is unable to enhance resistance of prostate cancer to radiation treatment by stimulating Nrf2 target genes.

In addition to regulating the Nrf2 signaling pathway, Keap1 is able to bind other proteins such as p62 and PGAM5 (44). Interaction between Keap1 and p62 facilitates release of Nrf2 from the complex, which is considered to be a noncanonical cysteine-independent mechanism for the autophagy deficiency-activated Nrf2 pathway (45). The N-terminus of PGAM5 interacts with the Kelch domain of Keap1 and its C-terminus binds to Bcl-xL. Keap1-dependent ubiquitination results in proteasome-dependent degradation of PGAM5 and Bcl-xL (38). Bcl-xL, an important member of the Bcl-2 family, is a potent antiapoptotic factor that plays a crucial role in cell survival by maintaining the electrochemical and osmotic homeostasis of mitochondria (46). The present study demonstrates that parthenolide increases the level of reduced Keap1 and consequently induces Keap1-dependent degradation of PGAM5 and Bcl-xL in cancer cells,
suggesting that formation of the Keap1-PGAM5-Bcl-xL complex is a mechanism underlying the effect of parthenolide on radiosensitization of prostate cancer cells.

Although a high rate of aerobic glycolysis in tumors, known as the Warburg effect, has been observed in various types of cancer, cancers have functional mitochondria, and mitochondrial respiration is necessary for cancer cell proliferation (47). Cancer cells depend on a hyperactive metabolism to fuel their rapid growth and also on antioxidants to quench potentially toxic ROS generated by such a high metabolic demand (48). Our results demonstrate that parthenolide not only suppresses MnSOD and GpX, two major antioxidant enzymes in mitochondria, but also activates Bcl-xL degradation in cancer cells, which suggests that mitochondria are a feasible target for anticancer treatment. The present study also shows that parthenolide may maintain normal cell survival through induction of MnSOD and GpX activity. Thus, a more efficient and safe therapy may involve modification of cellular redox signaling by alteration of the antioxidant response coupled to selective degradation of prosurvival members of the Bcl2 family in cancer cells, because conventional anticancer therapy mainly causes cell growth arrest or cell death by raising cellular ROS, which oxidizes and damages DNA, proteins and lipids. Optimizing prototype redox chemotherapeutics from natural sources provides an exciting opportunity to further develop even better candidates to enhance therapeutic efficacy with less off-target toxicity.
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Figure Legends

Figure 1. The effect of parthenolide on radiosensitivities of prostate cancer and normal cells. (A) Prostate cancer PC3 cells were injected into the flanks of nude male mice. The resulting tumors were treated with DMAPT and IR. Tumor volume was measured and tumor growth was calculated. (B) Time needed for tumor growth to reach 2000 mm$^3$ volume after treatment was calculated and plotted. (C) Mice without cancer were treated with radiation alone (5 x 3 Gy) and DMAPT (10 mg/kg) with radiation. Prostate and bladder tissues were removed for pathological analysis using EM. Asterisks indicate normal mitochondria and arrows indicate mitochondria with myelin figures. M, normal mitochondria; Ly, lysosome; and V, mitochondria with vacuoles. (D) Quantification of mitochondrial damage in mice prostate and bladder tissues.

Figure 2. The effect of parthenolide on redox homeostasis in prostate cancer and normal cells. (A-B) Cells were treated with parthenolide and then labeled with DHE or DCF. The mean fluorescence intensity of DHE (A) and the ratio of H$_2$DCFDA to DCFDA (B) were determined using flow cytometry. Concentrations of cellular superoxide and total ROS were estimated by quantification of fluorescence intensity. Antimycin and PMA were used as positive controls for generation of ROS. PEG-SOD was used as a control to remove superoxide generated by DHE. PN, parthenolide. (C) The levels of antioxidant proteins were quantified by western blots. (D) The activities of the corresponding enzymes were measured. (E) Quantification of the basal oxygen consumption, ATP-linked oxygen consumption, the maximal OCR after the addition of FCCP, and the reserve capacity of the cells. NS, not significant. (F) Three cancer cell lines and three non-cancer cell lines were treated with parthenolide (PN) at the indicated concentrations. Cell survival fraction was determined by MTT assay.
Figure 3. **Keap1 redox modification by parthenolide.** (A-B) Three normal cell lines (A) and three prostate cancer cell lines (B) were treated with saline or parthenolide (PN). Keap1 was immunoprecipitated with its antibody and SH- and S-S moieties of Keap1 were demonstrated by ECL detection. (C) Tumor tissues were homogenized and incubated with MPB. Reduced (SH-) and oxidized (S-S) forms of Keap1 in the tissues were detected. (D) Tumor tissues were homogenized and antioxidant proteins were quantified by western blots. The right panel shows representative blots and the left panel shows the average of multiple blots.

Figure 4. **Activation of Keap1-Nrf2 pathway by parthenolide in normal cells.** (A) Parthenolide (PN) increases nuclear levels of Nrf2 in normal cells but not in cancer cells. Nuclear proteins extracted from the parthenolide-treated cell lines were immunoblotted to quantify nuclear levels of Nrf2 using PCNA as loading control. (B) The effect of Keap1 and Nrf2 in normal cells. PZ cells were transfected with siRNAs to knock down Keap1 or Nrf2, respectively. After treatment with parthenolide (PN) and IR, the cell survival fraction was quantified using Trypan blue exclusion assay (right), and the knocked-down Keap1 and Nrf2 were confirmed by western blots (left).

Figure 5. **TrX-dependent Keap1 reduction by parthenolide in prostate cancer cells.** (A) The levels of TrX in normal and cancer cells before and after treatment with parthenolide (PN) were detected by western blots. (B) After the indicated treatment, Keap1 was immunoprecipitated using a TrX antibody. (C) PC3 cells were transfected with TrX siRNA prior to the indicated treatment (top). SH- and S-S bands in Keap1 protein were detected as described in Fig. 3 (bottom). (D) A Keap1 cDNA construct was transfected into PC3 cells. The levels of related proteins were detected by western blots (bottom). The effect of Keap1 on radiosensitivity was analyzed using a Trypan blue exclusion assay (top).
Figure 6. Degradation of PGAM5-Bcl-xL caused by parthenolide-mediated reduction of Keap1 in prostate cancer cells. (A) PC3 cells were treated with parthenolide (PN). Keap1 and Bcl-xL were immunoprecipitated using a PGAM5 antibody. (B) The total levels of Nrf2, PGAM5, and Bcl-xL were quantified by western blots. (C) The levels of mitochondria-associated proteins after parthenolide treatments. (D) Proteins in various cellular fractions were identified with antibody specific for each protein. (E) PC3 cells were transfected with siRNA to knock down PGAM5, Bcl-xL and Nrf2, respectively (bottom). Cell survival fraction was quantified by Trypan blue exclusion assay (top). (F) A Bcl-xL expression construct was transfected into PC3 cells and the expression of Bcl-xL was monitored by western blots (bottom). Cell survival fraction was quantified by Trypan blue exclusion assay (top).

Figure 7. A proposed mechanistic model for parthenolide-mediated inverse therapeutic effects on radiosensitivity of prostate cancer and radioresistance of normal cells.

Parthenolide sensitizes cancer cells to radiation, in part, by maintaining Keap1 in a reduced state and enhancing its interaction with PGAM5 and Bcl-xL, resulting in degradation of Bcl-xL in mitochondria. In contrast, parthenolide protects normal cells against radiation via oxidation of Keap1 and release of the Nrf2 transcription factor for activation of mitochondrial antioxidant enzymes.
Figure 1

A

Tumor volume (mm$^3$) vs. Day after treatment for different treatments: untreated, DMAPT, 1 Gy, 1 Gy + DMAPT, 2 Gy, and 2 Gy + DMAPT.

B

Number of days when tumor volume reaches 2000 mm$^3$ for each treatment condition.

C

Micrographs showing prostate and bladder tissue treated with 5 x 3 Gy and DMAPT + 5 x 3 Gy.

D

Bar chart showing the percentage of damaged mitochondria/total mitochondria in prostate and bladder tissue treated with 5x3 Gy and DMAPT/5x3 Gy.
Figure 2

A. DHE assay

B. DCF assay

C. Western blot analysis of cancer and normal cells

D. Enzyme activity

E. OCR and Reserve Capacity

F. Cell survival (%)
Figure 3

A

B

C

D

* p<0.05
Figure 4

A

PN (5 μM) -+ -+ -+ -+ -+ -+ -+ -+

PC3 DU-145 LNCaP PZ PrEC RWPE-1

Nrf2

PCNA

B

ctrl siRNA Keap1 siRNA Nrf2 siRNA

PN (5 μM) -+ -+ -+ -+ -+ -+ -+

Keap1

Nrf2

β-actin

Cell survival (%) Untreated 6Gy PN 5uM PN + 6Gy

P<0.01

P<0.01
KEAP1 Is a Redox Sensitive Target That Arbitrates the Opposing Radiosensitive Effects of Parthenolide in Normal and Cancer Cells

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