Capsaicin: A Novel Radio-Sensitizing Agent for Prostate Cancer

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INTRODUCTION. Radio-sensitizing agents sensitize tumor cells to the lethal effects of radiotherapy (RT) allowing for use of lower doses of radiation to achieve equivalent cancer control, while minimizing adverse effects to normal tissues. Given their limited toxicity and ability to easily integrate into the diet, compounds occurring naturally in the diet make ideal potential radio-sensitizing agents. In this study, we have examined whether capsaicin, the active compound in chilli peppers, can modulate the response to RT in preclinical models of prostate cancer (PCa).

METHODS. The effects of RT (1–8 Gy) and/or capsaicin (1–10 μM) on colony formation rates in human PCa cells were assessed using clonogenic assays. Mechanistic studies were performed by Western Blot, immunocytochemistry, and flow cytometry. Athymic mice (n = 40) were inoculated with human LNCaP cells. Once tumors reached 100 mm³, animals were randomized into four groups: control, capsaicin alone (5 mg/kg/d), RT alone (6 Gy), and capsaicin and RT.

RESULTS. Capsaicin reduced colony formation rates and radio-sensitized human PCa cells (Sensitizer enhancement ratio = 1.3) which corresponded to the suppression of NFκB, independent of TRP-V1 receptor. Cell cycle modulation occurred following RT and capsaicin treatment independently. In vivo, oral administration of capsaicin with RT resulted in a ‘greater than additive’ growth delay and reduction in the tumor growth rate greater than capsaicin (P < 0.001) or RT (P < 0.03) alone. Immunohistochemical analysis revealed a reduction in proliferation and NFκB expression, and increase in DNA damage.

DISCUSSION. Our findings suggest that capsaicin acts as a radio-sensitizing agent for PCa through the inhibition of NFκB signalling. Prostate 75:113–125, 2015.

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INTRODUCTION

Radiotherapy (RT) is a very commonly used treatment for prostate cancer (PCa) [1,2]. Conventionally, RT is administered to the prostatic region on weekdays in 2 Gy fractions resulting in a cumulative dose of 70–80 Gy. Radiotherapy mediates its lethal effects on tumor cells by inducing DNA damage [3]. At a molecular level, cells respond to this damage by either undergoing cell death, and/or repairing the damaged DNA through the upregulation of repair proteins and survival factors, such as. NFκB [4]. Targeting such repair pathways and survival factors chemically or pharmacologically can sensitize cells to the lethal effects of RT [4]. If successful, such radiosensitizing agents achieve their effect by increasing tumor cell kill while minimizing adverse effects on normal tissues [4,5]. Compounds occurring naturally in the human diet are ideal radio-sensitizing agents given their ability to be easily integrated into the diet and minimal toxicity at conventional doses [5].

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is the natural compound found in the Capsicum sp. plants and is responsible for the burning sensation experienced on the contact of red and chilli peppers. It displays both analgesic and anti-inflammatory properties and has been widely used topically for treatment of various chronic pain syndromes, through the binding and activation of the vanilloid receptor, TRPV1. More recently capsaicin demonstrated anti-carcinogenic properties in several cancers, including PCa [6–9]. These anti-cancer properties have been reported to act through a number of mechanisms including the NFκB pathway inhibition [7,10].

NFκB resides in the nucleus in an inactive state bound to the IκB complex [11]. In the classical NFκB activation pathway, after the cell undergoes an insult (e.g., stress, radiation), the IKK complex enzyme degrades IκB proteins through the ubiquitinization process, causing the dissociation of the NFκB- IKK complex. At this point, NFκB can be phosphorylated and translocated into the nuclear compartment, where it can bind to various DNA binding sites resulting in the upregulation of genes involved in a number of processes including cell cycle regulation, cell survival, and radioresistance [11]. Hence, targeting the NFκB pathway has sought to be an effective strategy to overcome resistance and improve radiation therapy [11–14].

In the present study, we have examined whether capsaicin, the active compound found in chilli peppers, can modulate the response to radiation through the inhibition of NFκB pathway in PCa in both in vitro and in vivo model systems. We observed capsaicin to enhance prostate cancer radio-sensitivity though the suppression of radiation-induced NFκB signalling in the in vitro model. The radio-sensitizing effect is independent of cell cycle inhibition and TRPV1 activation. We also found that in the LNCaP xenograft model, capsaicin fed animals treated with radiotherapy experienced a significant delay in tumor growth. This effect was ‘more than additive’ effect compared to capsaicin or radiation alone.

METHODS

Cell Lines

Three human prostate cancer cell lines DU145, LNCaP, and PC3 were obtained from the American Type Culture Collection (ATCC) (Rockville, Maryland). LNCaP cells were cultured in RPMI 1,640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% foetal bovine serum (FBS; Gibco, Grand Island, NY), 0.3 mg/ml l-glutamine and 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Burlington, ON, Canada). PC3 and DU145 cells were cultured in Dulbecco’s minimal essential medium/F12 (Invitrogen, Burlington, ON, Canada) with 10% FBS supplemented with 0.3 mg/ml l-glutamine and 100 IU/ml penicillin and 100 µg/ml streptomycin. PC3AR2 cells are PC3 cells, which have been transfected with a full-length functional androgen receptor (AR). PC3AR2 cells were obtained as a generous gift from Dr. Ted Brown, Mount Sinai Hospital, Toronto, ON, Canada. Human prostate epithelial cells (RWPE-1) were purchased from ATCC, and cultured in Keratinocyte Serum Free Medium (K-SFM), supplemented with 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml human recombinant epidermal growth factor (EGF). All cell lines were subcultured at a ratio of 1:3–1:5, as described by the supplier. All cells were maintained at 37°C in a 5% CO2 incubator under sterile conditions.

Chemicals

Capsaicin [(E)-N-[(4-Hydroxy-3-methoxyphenyl)m-ethyl]-8-methyl-6-nonenamide] was obtained from Tocris Bioscience (Bristol, UK). Capsaicin was dissolved in dimethyl sulfoxide (DMSO, Sigma) to create a stock concentration of 0.1 M and stored at –20°C. All compounds were prepared and stored with minimal exposure to light to avoid oxidation. All other chemicals were purchased from Sigma unless otherwise specified.

Clonogenic Assays

Prostate cancer cells were seeded at densities ranging from 400 to 4,000 cells per 10 cm petri dish, depending on the cell line. Twenty-four hours after plating, cells were treated either with capsaicin alone
(0.01–10.0 μM), radiation alone (0–8 Gy), or in combination. In the combination treatment regimen, cells were treated with capsaicin 1 hr prior to irradiation. The total treatment period was for 5 days without media change. Capsaicin alone colony formation assays were mock irradiated. Colonies developed over 9–14 days and were stained with crystal violet and manually counted. Each assay was internally controlled using untreated cells (cell media or 0.01% DMSO carrier). Relative plating efficiencies were expressed as percentages relative to the plating efficiency of untreated cells. The Sensitizer Enhancement Ratio (SER) was calculated by dividing Area Under the Curve (AUC) for the control dimethyl sulfoxide (DMSO) by the AUC of the capsaicin treated cells. All experiments were performed in triplicates (at a minimum) and statistical analysis carried out as detailed below.

**Experimental Design for TRP-VI Experiments**

Capsazepine (CZP), the TRP-V1 inhibitor, was purchased from Santa Cruz Biotechnology (CA). CZP was dissolved in DMSO (Sigma) to create a stock concentration of 0.1 M and stored at −20°C. Working solutions of CZP were prepared fresh in appropriate cell media. For clonogenic assay experiments, cells were treated with capsaicin and/or CZP simultaneously as detailed in the clonogenic assay protocol. Clonogenic assays experiments were carried out in duplicates for at a minimum of three biological replicates per cell line.

**Flow Cytometry**

Cell cycle arrest pattern and S phase were determined by flow cytometry in LNCaP cells labeled with anti-bromodeoxyuridine (BrdU) fluorescein isothiocyanate (FITC) and propidium iodide (PI) as per the published manufacturer protocol. Briefly, asynchronously growing cells (5 × 10^5 cells/plate) were plated using 10 cm petri dishes and treated with capsaicin (10 μM) for 48 hr followed by a dose of 2 Gy of radiation. Control plates were treated with vehicle alone (cell culture media or 0.01% DMSO) and mock irradiated. Cells were pulse labeled with BrdU for 2 hr prior to harvesting. As a negative control, a no-BrdU control was included. Cells were trypsinised, fixed in ice cold 70% ethanol and stored at −20°C until further analysis. Cells were subsequently washed in buffer (PBS [Invitrogen, Burlington, ON, Canada] and 0.5% Tween-20) and treated with 2N HCl for 20 min to expose labeled DNA. Cells were incubated for 1 hr on ice with anti-BrdU conjugated FITC (DAKO, Burlington, ON, Canada). Cells were washed, centrifuged, and resuspended in 10 μg/ml PI, and allowed to incubate for 30 min on ice. Samples were filtered through a nylon mesh and cell cycle analysis performed on the FACS Calibur flow cytometer using the Cell Quest Pro software package (Becton Dickinson, San Jose, CA).

**Western Blot Analysis**

Western Blot analyses of lysates from LNCaP treated cells were carried out as described [15]. Briefly, LNCaP cells were plated at a density of 1 × 10^6 cells per 10 cm plate. Twenty-four hours after plating, adherent cells were treated with capsaicin (10 μM) and irradiated (2 Gy) after 1 hr of incubation. Control wells were treated with vehicle alone (DMSO 0.01%). After treatment for 24 hr, the cells were lysed using NP-40 lysis buffer containing inhibitors (leupeptin/pepsstatin, apro tinin and phenylmethane sulfonylfluoride), sodium dodecyl sulfate (SDS), deoxycholate and ethylenediaminetetraacetic acid (EDTA). Protein was quantified using the Bradford protein assay technique prior to loading into 12% SDS gels for electrophoresis. Antibodies phosphor-NFκB p65 (Ser 536), NFκB p65, IkBα (L35A5), phosphor- IkBα (Ser 32) were purchased from Cell Signaling Technology (Beverly, Massachusetts) unless specified otherwise. Image quantification software (ImageJ, US National Institute of Health, Bethesda, MD) was used to semi-quantitatively determine protein expression levels, relative to β-actin.

**Immunocytochemistry Analysis**

LNCaP and PC3 cells were trypsinized and seeded in triplicate at a 2 × 10^5 cells per well in a 24-well cell culture plate on round slide cover (Thermo Fisher Fisher 100411-9), and left to adhere overnight. After 24 hr, cells were treated with capsaicin (10 μM), and/or irradiated with 6 Gy (with vehicle alone). At 24 hr post-radiation and/or capsaicin treatment, cells were fixed in 4% paraformaldehyde for 10 min at room temperature. After washing by 200 μl PBS-TX three times, cells were blocked in 100 μl PBS with 0.1% Triton 1% BSA and 1% goat serum at room temperature (RT). Blocking buffer was then removed and incubated with anti-phospho histone H2AX (ser139) antibody (Millipore) in blocking buffer overnight at 4°C. The well was washed with 200 μl PBS three times and incubated with alexafluor 488 labeled goat-anti-mouse secondary antibody (Invitrogen) at room temperature in the dark for 1 hr. Wells were washed with 200 μl PBS, and stain nuclear with 0.5 μg/ml DAPI in PBS for 10 min in the dark. After washing three times with 200 μl PBS wells were covered with mounting medium and coverslip. All images were processed identically and analyzed using the Zeiss}

*The Prostate*
Imager M1 microscope with Stereo Investigator software (MicroBrightField, Williston, VT) at 20× or 100× magnifications.

Effect of Capsaicin and Radiation on Prostate Cancer Cells in Vivo

Ethical approval for the in vivo component of this work was obtained from the University of Toronto Animal Research Ethics Board and all work was conducted in accordance with established guidelines and protocols approved by the Canadian Council on Animal Care (CCAC). Six-week old male nu/nu athymic nude mice (Harlan Laboratories Mississauga, ON, Canada) were used in this study. 1×10⁶ LNCaP cells resuspended in 100 μL matrigel solution (BD Biosciences, CA) were inoculated subcutaneously unilaterally into the flank of each mouse. Mice were monitored each day for tumor growth. When tumors achieved an average volume of 100 mm³ (considered as day 1) mice were randomized into four treatment groups: control (n=15), capsaicin (n=15), radiation alone (n=15) or capsaicin and radiation (n=15). Capsaicin was administered orally by gavage on alternating days over a period of two weeks at a dose of 5 mg/kg body weight. Capsaicin in ethanol (0.2 M) was then diluted fresh in saline solution prior to administration. Mice randomized to RT groups received one 6 Gy fraction of RT (Faxitron machine) in sterile cages under general anesthesia (GA) [16], following the third administration of capsaicin or saline. Control animals were mock irradiated. Mice were weighed thrice weekly with simultaneous tumor measurement. Mice with tumors exceeding 17 mm diameter were culled in accordance with CACC guidelines. Serum samples were obtained by saphenous vein puncture during treatment period. At experiment termination (day 54), serum samples were collected by direct cardiac puncture. Liver samples were also obtained at the termination of the study and fixed for histological analysis by a pathologist for any toxicities associated with the study.

Serum Extraction and Liquid Chromatography-Mass Spectrometry (LC-MS) for Capsaicin Analysis

Twenty microliters of serum from each sample of the in vivo studies was thawed and transferred to individual eppendorf tubes. 5 μL of 1 μg/ml deuterated testosterone (d3T, C/D/N Isotopes) was then added followed by 50 μL of acetonitrile after which samples were vortexed for 5–10 sec and centrifuged as for 5 min at 20,000 g to sediment precipitated protein. The clarified supernatant was transferred to LC vials for analysis. Standards were prepared in 50% methanol and QC samples with capsaicin spiked blank mouse serum similar to sample prep above. Optima grade (Fisher) solvents and 18 MΩ water (Millipore) were used for sample preparation and subsequent LC-MS analysis.

Analysis was carried out with an Acuity UPLC coupled in series with a Quattro Premier (Waters). A 100 mm BEH C18, 1.7 μ column (Waters) was used for separations with a 40–85% acetonitrile (ACN) gradient from 0.2 to 3 min, ramped 0.5 min to 98% ACN for flushing (2.5 min) followed by a 2 min re-equilibration for an 8 min run length (0.1% formic acid present throughout). All MS data was collected in ES+ at unit resolution with the following instrument parameters: capillary, 3.0 kV; extractor and RF lens, 3 V and 0 V; source and desolvation temperatures, 120°C and 300°C; desolvation and cone gas (N2), 1,000 L/hr and 50 L/hr; collision gas (Ar), 0.15 ml/min (6.2e– m bar). Compounds were detected using multiple reaction monitoring (MRM) with m/z 306 > 137 and 306 > 182 for capsaicin and m/z 292 > 97 for d3T (22 V/17 V, 22 V/11 V and 32 V/21 V cone/collision volt combinations used respectively for the three transitions) with 0.1 sec dwell each. Retention times (RT) for d3T and capsaicin were 2.35 and 2.8 min, respectively.

Quanlynx (Waters) was used for analysis of data using peak area ratios of capsaicin/d3T for calibration and quantification. Calibration standards ranged from 0.002 to 1 μg/ml (6 points) with R2 > 0.99 and all % deviation from nominal <15% above 0.01 μg/ml (omitting 1 μg/ml due to curvature). Comparison of spiked serum with QC samples indicated little to no matrix interference with extraction efficiencies of 95%.

A peak with RT 1.9 min was also observed in the capsaicin MRM’s only in the serum from mice with capsaicin in diet, likely a capsaicin metabolite. Characterization of this potential metabolite was not possible however; a parent m/z could not be observed due to the low serum levels of both capsaicin and metabolite.

Immunohistochemical Analysis

Immunohistochemical analysis was performed as previously described [17]. Briefly, deparaffinized sections were incubated with the primary antibody [anti-p27Kip1 rabbit polyclonal anti-body (Santa Cruz Biotechnology) diluted 1:100 (200 μg/mL) in PBS; PCNA rabbit polyclonal antibody (Santa Cruz Biotechnology) diluted 1:50 (200 μg/mL) in PBS; and anti-γH2AX (Cell Signaling), phosphor-NFκB p65 (Ser 536) monoclonal antibody (Cell Signaling) diluted 1:100 (200 μg/mL) in PBS; NFκB p65 (Cell Signaling) diluted 1:100 (200 μg/mL) in PBS; IκBα (L35A5) (Cell Signaling) diluted 1:100 (200 μg/mL) in PBS; phosphor- IκBα (Ser 32) (Cell
Signaling) diluted 1:100 (200 μg/mL) in PBS respectively, overnight at 4°C. Slides were then reacted with biotin-labeled anti-rabbit IgG/anti-mouse IgG and incubated with preformed avidin-biotin peroxidase complex (Vector Laboratories). Metal-enhanced diaminobenzidine substrate (Vector Laboratories) was added and sections were counterstained with hematoxylin and eosin.

Statistics
All in vitro clonogenic experiments were analyzed using Graph Prism. Between group variations for all in vitro experiments were assessed using Student’s t-test. Analyses of the in vivo results were performed using either Student’s t-testing or repeated measures ANOVA techniques. Statistical analysis was performed using SAS software, version 8 (SAS Institute Inc., Cary, NC).

RESULTS
Capsaicin Treatment Reduces Colony Formation in Prostate Cancer Cells
The colony forming assay was used to test the proxy proliferative rate of capsaicin on the various prostate cancer cells: LNCaP (AR positive, p53 wild-type), PC3 (AR negative, p53 negative) PC3AR2 (PC3 cells transfected with full length androgen receptor, AR positive, p53 negative), and DU145 (AR negative, p53 mutated). In each of the cell lines tested, increasing concentrations of capsaicin treatment significantly reduced the proxy proliferation rate of the cancer cells. In the LNCaP cell line, cells were treated with 1, 10, and 20 μM of capsaicin, and resulted in a 29%, 72%, and 92% reduction in colony formation, respectively. Treating the PC3AR2 cell line with these same concentrations of capsaicin resulted in a 30%, 83%, and 99% reduction in colony formation, a similar dose-response to LNCaP cells. The PC3 cell line was treated with 1, 10, and 50 μM of capsaicin and had 11%, 35%, and 85% reduction in colony formation, respectively. The DU145 cell line was treated with 50, 100, and 200 μM of capsaicin, a significantly higher dose compared to the other cell lines, and resulted in a 25%, 75%, and 99% reduction in colony formation. The concentrations of capsaicin were selected to determine the IC50 concentration of capsaicin for each cell line (Fig. 1a–d).

Capsaicin Treatment Sensitizes Prostate Cancer Cells to Radiation
In order to assess the sensitizing effect of capsaicin on radiation we generated radiation survival curves for LNCaP, PC3, and RWPE-1 cells using 0.1, 1.0, and 10 μM of capsaicin, respectively. The RWPE-1 epithelial cell line was assessed to compare the sensitizing effect on malignant and non-malignant cells. Curves were normalized for cell kill with capsaicin alone. As depicted in Figure 2a-b, PC3 cells were sensitized by capsaicin at 0.1 μM (SER = 1.2, SD = 0.195), 1 μM (SER = 1.2, SD = 0.099) and 10 μM (SER = 1.2, SD = 0.099).
LNCaP cells had a similar sensitization effect at the 0.1 μM (SER = 1.2, SD = 0.075), 1 μM (SER = 1.2, SD = 0.027) and 10 μM (SER = 1.2, SD = 0.022). These results suggest that there is not a dose-dependent response with increasing concentrations of capsaicin. Interestingly, RWPE-1 cells did not respond in the same manner, at concentrations of at 0.1 μM (SER = 0.9, SD = 0.11), 1 μM (SER = 0.9, SD = 0.33), capsaicin had no effect, while at 10 μM, capsaicin sensitized cells (SER = 1.3, SD = 0.149). Since we did not see a dose-dependent response with increasing doses of capsaicin, we decided to perform subsequent mechanistic studies using only one concentration of capsaicin, which was found to be effective (10 μM). Immunocytochemical analysis of LNCaP and PC3 cells revealed that treatment of LNCaP cells and PC3 cells with capsaicin and radiation increases the expression of DNA damage marker, phosphor-H2AX. Morphological changes to the nuclei of these cells also reflected an increase in cell death in the treatment groups (Supplementary Fig. S1).

**Capsaicin Treatment Alters the Cell Cycle Distribution in LNCaP Cells**

To determine whether the alterations in cell cycle contributed to the sensitization effect of radiation, LNCaP cells were treated with capsaicin (10 μM) and radiation, collected and fixated, and assessed for cell cycle alterations at increasing time points (10 min, 6, 24, 48, and 72 hr) (Fig. 3 and Supplementary Table S1). We observed a significant reduction in the percentage of cells in the S-phase of the cell cycle when cells were treated with capsaicin and/or radiation compared to their respective controls at 24, 48, and 72 hr (P < 0.05). However, we did not see a significant difference in the percentage of cells in the S-phase after radiation alone when compared to cells that were treated with both capsaicin followed by radiation. This data thus suggests that the inhibition of the cell cycle is not the primary mechanism mediating the sensitization effects of capsaicin.

**Capsaicin Treatment Suppresses NfκB Expression in LNCaP Cells by Western Blot Analysis**

To better understand the mechanisms of radiosensitization after capsaicin treatment, we examined the NfκB pathway by Western Blot analysis. Analysis of the lysates after the treatment revealed a reduction in phosphorylated NfκB (pNfκB) expression after capsaicin treatment alone or in combination with radiation. However, the levels of pNfκB expression increased in lysates that were treated with radiation alone suggesting that capsaicin suppressed the upregulation of pNfκB following radiation exposure (Fig. 4a). Representative

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**Fig. 2.** Low doses capsaicin sensitizes prostate cancer cells to radiation. Survival curves for were generated using various concentrations of capsaicin (0.1, 1.0, and 10.0 μM) in (a) LNCaP, (b) PC3, and (c) Normal Prostate Epithelial cell lines (RWPE-1). The sensitizer enhancement ratio (SER) was calculated using Area Under Curve (AUC) DMSO Control/Area Under Curve with Capsaicin, with corresponding standard deviation (SD). Each of the following experiments was performed a minimum of three times for each cell line.
Western Blot of pNFκB, NFκB is depicted in Figures 4a, 4b and 5c, supporting the suppressive effects of capsaicin on NFκB pathway. Correspondingly, Western Blot analysis on LNCaP cells treated with capsaicin and/or radiation demonstrated an up-regulation of the tumor suppressor proteins p21 and p27kip1 in a time-dependent manner (data not shown) consistently across all treatment groups (Supplementary Table S1).

**Capsaicin Treatment Sensitizes Cells to Radiation Independent of the Vanilloid Receptor, TRP-V1**

A number of studies have suggested that the transient receptor potential-vanilloid 1 receptor (TRP-V1)—also commonly referred to as the capsaicin receptor—may be involved in mediating some of the anti-proliferative effects of capsaicin [18]. To understand whether TRP-V1 was involved in mediating the radio-sensitization effects on PC3 cells, we used one of the most commonly used pharmaceutical inhibitors to TRP-V1, CZP. CZP was added to cells and clonogenic assays performed concurrently by treatment with capsaicin to assess whether the inhibitory effect of capsaicin on colony formation could be reversed with CZP treatment. Results of these experiments demonstrated that the inhibition of the TRP-V1 receptors by CZP did not reverse the effects of capsaicin as depicted by no alteration on colony formation (Fig. 5a). Furthermore, mechanistic studies analyzing NFκB signaling by Western Blot analysis revealed that treating PC3 cells with capsaicin and CZP does not reverse the phosphorylated NFκB (pNFκB) suppression induced by capsaicin. Unexpectedly, CZP treatment exerted independent effects on pNFκB inhibition, which prevented from determining the role of TRP-V1 receptor in sensitizing cells to radiation [19–21]. Taken together, we found that treating PC3 cells with the TRP-V1 inhibitor, CZP, did not reverse the sensitizing effects of capsaicin, suggesting that capsaicin is not sensitizing cells predominately through TRP-V1 signalling. Furthermore, we have found that CZP alters clonogenic survival and exert effects on NFκB signaling independent of capsaicin.

**Capsaicin and Radiation Treatment Reduced Tumor Growth in a Mouse Xenograft Model**

We tested the radiosensitizing effect of capsaicin in vivo by using the LNCaP xenograft model (described above) (Fig. 6). Tumors were measured thrice weekly during the duration of the study. We observed
a significant difference in the overall tumor growth rates between each of the groups overtime ($P < 0.001$). Mice in the capsaicin treatment group ($P < 0.001$), radiation group and capsaicin and radiation group had a significantly slower tumor growth rate compared to the control group ($P < 0.001$ and $P < 0.001$). Furthermore, capsaicin and radiation group had a significantly slower growth rate than either the capsaicin alone group ($P < 0.001$) and radiation alone group ($P < 0.001$) suggesting that capsaicin might be potentiating the effect of radiation by sensitizing the tumor cells. Growth delay calculations depict a ‘greater than additive’ effect for the capsaicin and radiation group compared to either treatment alone. Mice had no significant change in body weight or food consumption (data not shown). Capsaicin was well tolerated with no liver toxicities as assessed histologically. Two mice experienced mild to moderate inflammation of the esophagus/upper stomach area. Interim serum analyses revealed the presence of capsaicin and an associated metabolite in the treatment groups 1 hr after capsaicin administration (data not shown).

**Tumors of Mice Treated With Capsaicin and Radiation Display Markers of Persistent DNA Damage ($\gamma$H2AX), Reduction in Proliferative Marker, Ki67, and NFκB**

At the termination of the study, tumors were extracted from the mice and processed for pathological analyses. Immunohistochemical staining for Ki67 of prostate cancer tumors showed a reduction in the levels of Ki67 expression in all of the treatment groups (Fig. 7a). Next, we studied to see if there was any occurrence of DNA damage as a consequence of the treatment using the DNA damage marker, $\gamma$H2AX (Fig. 7d). We demonstrated the presence of positively stained nuclei only in the capsaicin and radiation combination group, suggesting sustained and persistent DNA damage in this treatment group. Our in vitro studies suggested that capsaicin mediated its radio-sensitizing effects through the inhibition of radiation-induced NFκB expression. To determine whether capsaicin altered the expression of NFκB expression in vivo, prostate tumors were stained for...
NFκB expression. Expression levels were positive in the control group, highly expressed in the radiation group, and weak in both the capsaicin group, and the capsaicin and radiation group, recapitulating the effects observed in our in vitro experiments.

**DISCUSSION**

Enhancing the effect of radiotherapy through the use of successful radio-sensitizing agents is a promising strategy for the treatment and management of prostate cancer [4,5].

In our current study, we have demonstrated that capsaicin leads to preferential radio-sensitization of prostate cancer cells compared to non-malignant epithelial cells through the suppression of the NFκB signalling. Our in vivo analyses revealed that the oral administration of physiologically relevant doses of capsaicin, when combined with radiation, is well tolerated, significantly reduces the tumor growth rate, and correspondingly alters NFκB and γH2AX expression in LNCaP xenograft tumors. These findings provide pre-clinical evidence supporting capsaicin as a novel radio-sensitizing agent for prostate cancer.

Targeting the androgen signaling pathway has been one approach to enhancing the effect of radiation for prostate cancer [22]. The 5-alpha-reductase inhibitors, dutasteride and finasteride, have been intensely investigated for their chemopreventive effects and are often used as an adjunct to radiotherapy [23]. To understand whether capsaicin altered the effects of radiation through various androgen-signaling pathways we generated survival curves for both the androgen-sensitive LNCaP cell line as well as the androgen-Insensitive PC3 cell line. Results of these studies indicated that although capsaicin alone was more...
effective at reducing the colony formation in androgen-sensitive cell lines, as previously reported [15], the SER was altered independent of androgen status, suggesting that the radio-sensitizing effect of capsaicin is not mediated via the androgen signaling pathway in vitro. We did not observe a dose-dependent effect, showing similarity in the sensitizing effects in both PC3 and LNCaP cells with capsaicin concentrations at 1.0 and 10.0 μM. It is possible that treatment with higher concentrations of capsaicin we would have seen dose-dependent effect, however, these concentrations are not deemed to be physiologically relevant.

A number of agents that target cell cycle regulation have been investigated as potential radio-sensitizing agents [4,24]. As radiotherapy induces cell cycle arrest predominately in the G2/M phase of the cell cycle allowing for an increase in the percentage of cells in the G1-phase and a reduction in the percentage of cells in the S-phase, targeting alternate phases of the cell cycle has been used to sensitize tumors to radiation [25]. Since capsaicin inhibits cell cycle through G1/S phase inhibition, we anticipated that cell cycle inhibition might be responsible for the radio-sensitizing effects of capsaicin. However, our results suggest that although capsaicin and radiation causes significant reduction in the percentage of cells in the S-phase of the cell cycle, this effect however, was not significantly greater than either agent alone suggesting that a similar mechanism may be affecting cells after treatment with radiation or capsaicin. Protein analyses of the tumor suppressor proteins p21 and p27 Kip1 demonstrated up-regulation in a time-dependent manner, when treated with either capsaicin or radiation alone; however, the combination did not demonstrate a ‘greater than additive’ effect; hence, indicating that capsaicin does not enhance the effect of radiation primarily through cell cycle inhibition.

The nuclear transcription factor kappa-B (NFκB) signalling pathway is upregulated following radiation in prostate cancer cells to promote cell survival and proliferation [11–14]. Such responses have been attributed to clonogen survival and resistance; hence, targeting this pathway has been proposed to enhance the effect of radiation by combating NFκB-induced resistance [12]. Several studies have found that impairing the NFκB activation through the inhibition of TNF activation or IκB phosphorylation significantly enhances the lethality of radiation [11,25–30]. Mori et al. have shown that capsaicin has also been shown to down regulate NFκB activity through IκBα degradation [7]. Our studies reveal that at capsaicin inhibits radiation-induced NFκB expression in a time-dependent manner. Furthermore, the 24 hr time point, LNCaP cells treated with capsaicin and radiation have a reduction in a number of proteins involved in the NFκB signalling pathway, indicating that NFκB signalling plays a role in mediating the radio-sensitizing response induced by capsaicin. In our in vivo studies using the LNCaP xenograft model, we observed similar results as radiation-induced NFκB expression.

**Fig. 6.** Capsaicin and Radiation significantly reduce tumor growth rate. Variation in tumour volume (mm3) over time in four different treatment groups; Control (vehicle alone), Capsaicin (5 mg/kg body weight), Radiation (6 Gy) or Capsaicin and Radiation in combination (indicated by broken arrow). Capsaicin was administered on Day 12–24, animals were irradiated (6 Gy) on Day 16. Tumours growth will be continued to be monitored overtime. Error bars represent standard deviation (SD). The ‘ symbol denotes significance (P < 0.001) ‘ relative to control; ‘” denotes significance (P < 0.05) relative to radiation alone.
was suppressed by capsaicin administration, and resulted in sustained DNA damage, marked by the presence of phosphorylated H2AX, at the termination of the study. Hence, it is likely that inhibiting the survival factor, NFkB, may act to inhibit cell survival factors allowing for more DNA damage, cell death, and tumor control, thereby suggesting that the inhibition of NFkB is responsible for radio-sensitizing prostate cancer cells to radiation. Several reports have reported that inhibition of NFkB by natural compounds can sensitize prostate cancer cells to radiation [10,31].

The vanilloid receptor, TRP-V1, belongs to the family of transient receptor potential (TRP) cation-
selective receptor that modulates the concentration of intracellular calcium upon activation by capsaicin or other stimuli (i.e., heat, acidity, etc.) [32,33]. Few studies have reported that TRP-V1 mediates the anti-carcinogenic potential of capsaicin; nonetheless, this mechanism still remains unclear [18,34]. To understand whether capsaicin acts to enhance the effect of radiation through TRP-V1 activation, we used the TRP-V1 inhibitor, CZP, and found that it did not reverse the radio-sensitizing effects of capsaicin. Interestingly, CZP independently altered a number of proteins in the NFκB pathway, suggesting that CZP may have effects independently, which has been established previously [19–21]. Hence, we were unable to fully understand the role of the TRP-V1 receptors in terms of its radio-sensitizing potential in prostate cancer cells. It is also likely that CZP may alter other receptors in the TRP family [35,36]. Further detailed analyses with more specific TRP-V1 inhibitors or knock-out models are necessary to understand how the TRP-V1 receptor affects the radio-sensitizing effect of capsaicin in prostate cancer.

To study the effects of capsaicin and radiation in vivo we used an LNCaP xenograft model. Results of our study revealed that oral administration of capsaicin administered three times a week over a two-week period significantly reduced the tumor growth rate, prolonged the tumor growth delay, decreased the proliferative index, weakened NFκB expression, and induced sustained DNA damage, marked by the presence of phosphorylated form of H2AX, γH2AX. Sanchez et al., have reported that capsaicin administered subcutaneously can reduce the growth rate of PC3 xenograft tumor [6].

The bioavailability of capsaicin in mice is not well documented; limited clinical studies have found that consumption of chili peppers can result in serum concentrations of capsaicin in the nanomolar range [37,38]. In our study, mice had detectable levels of serum in the blood, with no indication of toxicity, after the oral administration of capsaicin (5 mg/kg b.w.), suggesting that capsaicin is bioavailable and well tolerated. Furthermore, mice administered capsaicin alone also experienced a significant reduction the growth rate and proliferative index of LNCaP tumors, and when combined with one 6 Gy fraction of radiation tumors demonstrated a further reduction in tumor growth rate and growth delay. Immunohistochemical studies of these tumors revealed the presence of DNA damage only in tumors treated with both capsaicin and radiation, indicating sustained DNA damage, as tumors were collected nearly four weeks after treatment at the termination of the study [16,39]. Similar to our in vitro mechanistic studies, tumors treated with capsaicin and radiation resulted in the suppression of radiation-induced NFκB expression, suggesting that this mechanism may be driving radio-sensitizing effects of capsaicin.

In summary, we have revealed for the first time, that capsaicin enhances the effect of radiation in pre-clinical model systems with no significant toxicities on normal tissue. Furthermore, at low doses of capsaicin treatment differentially affects cancerous and normal epithelial cells through the suppression of NFκB activity in vitro. Our in vivo analyses revealed that oral administration of capsaicin is well tolerated and significantly reduce the tumor growth rate, proliferative index, NFκB expression, resulting induce sustained DNA damage. Based on our present findings, it would be important to conduct clinical trials to assess the radio-sensitizing effects of capsaicin and determine whether it can be used as an adjunct to current radiotherapy regimens.

REFERENCES


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