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The phytoalexin camalexin mediates cytotoxicity towards aggressive prostate cancer cells via reactive oxygen species

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Abstract

Camalexin is a phytoalexin that accumulates in various cruciferous plants upon exposure to environmental stress and plant pathogens. Besides moderate antibacterial and antifungal activity, camalexin was reported to also exhibit antiproliferative and cancer chemopreventive effects in breast cancer and leukemia. We studied the cytotoxic effects of camalexin treatment on prostate cancer cell lines and whether this was mediated by reactive oxygen species (ROS) generation. As models, we utilized LNCaP and its aggressive subtype, C4-2, as well as ARCaP cells stably transfected with empty vector (Neo) control or constitutively active Snail cDNA that represents an epithelial to mesenchymal transition (EMT) model and displays increased cell migration and tumorigenicity. We confirmed previous studies showing that C4-2 and ARCaP-Snail cells express more ROS than LNCaP and ARCaP-Neo, respectively. Camalexin increased ROS, decreased cell proliferation, and increased apoptosis more significantly in C4-2 and ARCaP-Snail cells as compared to LNCaP and ARCaP-Neo cells, respectively, while normal prostate epithelial cells (PrEC) were unaffected. Increased caspase-3/7 activity and increased cleaved PARP protein

shown by Western blot analysis was suggestive of increased apoptosis. The ROS scavenger *N*-acetyl cysteine (NAC) antagonized the effects of camalexin, whereas the addition of exogenous hydrogen peroxide potentiated the effects of camalexin, showing that camalexin is mediating its effects through ROS. In conclusion, camalexin is more potent in aggressive prostate cancer cells that express high ROS levels, and this phytoalexin has a strong potential as a novel therapeutic agent for the treatment of especially metastatic prostate cancer.

Keywords

Camalexin; Reactive oxygen species; Prostate cancer; Apoptosis

Introduction

Prostate cancer is the leading cause of non-cutaneous malignancy in men in Western industrialized nations and originates almost always from the glandular epithelia of the organ [1]. Although prostate cancer mortality has been declining in recent years, it is still the second leading cause of cancer death in men in the United States [2]. With current treatment options, including surgical or medical castration (leuprolide and flutamide), the 5-year survival rate for men diagnosed with organ-confined prostate cancer is approaching 100 %; however, the prognosis becomes worse once the cancer extends beyond the prostatic capsule [1]. Once cancers metastasize to other organs, a majority of patients die from their tumors as opposed to other causes [3], and is due, in part, to the tumor ultimately becoming androgen-independent at a median of 18–24 months after castration [4]. Taxanes and estramustine have been increasingly used for cytotoxic chemotherapy in these patients [5], but, unfortunately, their therapeutic efficacy is limited and most hormone refractory prostate cancer patients treated with these chemotherapeutic agents manifest resistance and eventually succumb to the disease [5]. Therefore, metastatic prostate cancer remains the main cause of prostate cancer-related death in men.

Current interest in epithelial to mesenchymal transition (EMT) stems from its developmental importance and its involvement in several adult pathologies, including prostate cancer [6, 7], where it provides a mechanism for tumor cells to become more motile, invasive, and metastatic [8–11]. This involves loss of epithelial characteristics, such as decreased expression of E-cadherin and cytokeratins, and acquisition of mesenchymal markers, such as increased expression of vimentin and N-cadherin, as the cells become more invasive and metastatic [7]. Snail transcription factor, a member of the Snail superfamily, is a zinc finger protein that can down-regulate E-cadherin by binding several E-boxes located in the promoter region [8], thus, promoting EMT. Human cancer development is associated with chronic inflammation with the release of reactive oxygen species (ROS) by inflammatory cells, resulting in DNA damage [12, 13]. ROS has been associated with EMT [14, 15] and it has been shown that TGF- β induces EMT via the upregulation of ROS species, hydrogen peroxide (H₂O₂), and MAPK ERK signaling in proximal tubular epithelial cells [16]. We have previously established a human prostate cancer EMT cell model by overexpressing Snail transcription factor in AR-CaP cells, and have found that Snail-mediated EMT is partly regulated by increased ROS and ERK signaling and leads to increased tumorigenicity [17]. Hydrogen peroxide has been shown to increase progressively in the lesser aggressive LNCaP cells to its more aggressive sublines C4, C4-2, and C4-2B, with concomitant increase in tumorigenic and metastatic potential [18]. Since increased levels of ROS in cancer cells is seen as inducing survival [14, 15, 19, 20], many therapies in development are focused on reducing ROS levels. However, more recently, it appears that the opposite approach is being taken. Since ROS levels are already high in cancer cells, increasing the levels further may tip the balance and trigger cell death instead, by producing so much

damage that the cell undergoes apoptosis or even necrosis (<http://www.ageingcancer.wordpress.com>; accessed August 21, 2010) [21–23].

Numerous epidemiological studies have shown an association between reduced cancer risk and intake of a diet rich in phytochemicals from fruits and vegetables [24–27]. Camalexin [3-(thiazol-2-yl)-1H-indole] (Fig. 1) is a natural phytoalexin and the principal one found in *Arabidopsis thaliana*, *Camelina sativa*, and *Capsella bursa-pastoralis*, where it accumulates in response to environmental stress and reportedly displays antimicrobial activities against various plant pathogens [28–30]. This compound has been researched predominantly with regard to plant chemical defense mechanisms, and has been shown to exert cytostatic/cytotoxic effects on the human protozoan pathogen *Trypanosoma cruzi* [31] and on the human breast cancer cell line *SKBr3*, where it displays higher potency than the traditional anticancer agents cisplatin and melphalan [32]. Mezencev et al. [33] showed that camalexin induced apoptosis in Jurkat T-leukemia cells by increasing the concentration of ROS and activation of caspases. However, its cytotoxicity against eukaryotic cells and potential as a human disease-modifying drug has been examined in a limited context and its role in prostate cancer has never been investigated. Hence, for this reason, we have further investigated the cytotoxic effect of this compound on prostate cancer cells. We have found that the more aggressive prostate cancer cells that express high levels of ROS are more sensitive to camalexin as compared to the lesser aggressive prostate cancer cells.

Materials and methods

Reagents and antibodies

Growth media RPMI 1640 (1× with L-glutamine, and without L-glutamine and phenol red) and penicillin–streptomycin were from Mediatech, Inc. (Manassas, VA). G418 was from EMD Corp Biosciences (Brookfield, WI). Fetal bovine serum (FBS) and charcoal/dextran-treated FBS (DCC-FBS) were from Hyclone (South Logan, UT). Trypsin/EDTA was from Mediatech, Inc. (Manassas, VA). MTS CellTiter 96® Aqueous One Solution reagent was from Promega Corporation (Madison, WI). Camalexin (40 mM in absolute ethanol) was kindly obtained from our collaborator Dr. Roman Mezencev of the Georgia Institute of Technology, Atlanta, GA, and was synthesized as described previously [34]. N-acetyl cysteine (NAC) and mouse monoclonal anti-human actin antibody were from Sigma-Aldrich, Inc. (St. Louis, MO). Rabbit monoclonal anti-cleaved PARP primary antibody was from Cell Signaling Technology, Inc. (Danvers, MA). Rabbit polyclonal anti-caspase-3 primary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated sheep anti-mouse, donkey anti-rabbit secondary antibodies, and the enhanced chemiluminescence (ECL) Western blotting detection reagent were from GE Healthcare UK Limited and nitrocellulose membranes were from Bio-Rad (item number 162-0115, Germany). Hydrogen peroxide (H₂O₂) ACS 30 % was from Acros Organics (Fairlawn, NJ). Dichlorofluorescein (H₂DCFDA) and the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit were from Invitrogen (Eugene, OR). The Dual Sensor MitoCasp™ Kit was from Cell Technology (Mountain View, CA).

Cell lines and culture

Human prostate cancer cell line ARCaP stably transfected with constitutively active Snail cDNA (ARCaP Snail representing an aggressive cell line) or an empty vector Neo (ARCaP Neo representing the lesser aggressive cell line) has been previously described to represent an EMT model [17] and was utilized in these experiments. Additionally, we utilized LNCaP cells with lesser metastatic potential obtained from the American Type Culture Collection (ATCC) and its more aggressive subline C4-2 cells (a kind gift from Dr. Leland Chung, Cedar-Sinai Medical Center, Los Angeles, CA). Normal prostate epithelial cells (PrEC)

were obtained from the ATCC and maintained in prostate epithelial growth media (PrEBM) containing supplements and growth factors (BPE, hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin B, and retinoic acid) from Cambrex Bio Science (Walkersville, MD). Reagents trypsin/EDTA, HEPES-buffered saline solution (HEPES-BSS), and trypsin neutralizing solution (TNS) used in splitting PrEC cells were also obtained from Cambrex Bio Science (Walkersville, MD). LNCaP and C4-2 cells were routinely cultured in RPMI 1640 medium supplemented with 10 % (v/v) FBS, 2 mM L-glutamine, 50 µg/ml penicillin, and 100 µg/ml streptomycin. ARCaP Snail and Neo cell lines had an additional 400 µg/ml G418 added to the growth media. They were grown to 70 % confluence in 95 % air, 5 % CO₂ humidified incubator at 37 °C, and routinely passaged using 0.05 % trypsin/EDTA solution. For all experimental conditions, RPMI 1640 without L-glutamine and phenol red and reconstituted with 5 % dextran charcoal stripped FBS (DCC-FBS) was used.

Detection of reactive oxygen species

Cells were plated at a density of 1×10^4 cells per well in 96-well black, clear bottom plates and left to attach overnight. Baseline ROS levels were assessed in untreated ARCaP Neo, ARCaP Snail, LNCaP, and C4-2 cells. Prior to treatment, ARCaP Snail and C4-2 cells were starved in serum-free and phenol red-free RPMI. The cells were then exposed to 25 or 50 µM camalexin plus or minus 10 mM NAC, a H₂O₂ antagonist, and 100 µM H₂O₂ as a positive control. Procedures were performed following the manufacturer's protocol (Molecular Probes®, Invitrogen), with slight modifications. Briefly, cells were loaded with 10 µM (final working concentration) of H₂DCFDA in pre-warmed Hanks Balance Salt solution (HBSS) for 45 min. This was replaced with experimental media with various treatments, as well as untreated controls, for 3 days. H₂DCFDA oxidation was measured on a spectrofluorometer (BioTek Synergy HT, BioTek Instruments, Inc., Winooski, VT) with excitation at 485 nm and emission at 528 nm at desired increments.

Migration assay

The migratory potentials of ARCaP Neo, ARCaP Snail, LNCaP, and C4-2 cells were assayed in vitro. Cells (5×10^4) in 0.1 % BSA were plated in the upper chamber of Costar 24-well plates containing 8-µM pore size polycarbonate filter inserts coated with 3.67 µg/µl of rat tail collagen, while the lower chamber contained 10 % BSA. After 5 h (for ARCaP Neo and ARCaP Snail cells) or 24 h (for LNCaP and C4-2 cells), cotton swabbing was performed to remove cells that did not migrate. Those that had migrated into the lower chamber were collected, fixed, stained with crystal violet, and then counted.

Cell viability assay

PrEC, ARCaP Neo, ARCaP Snail, LNCaP, and C4-2 cells were plated at a density of 2,000 cells per well in 96-well plates and allowed to attach overnight. Camalexin (10, 25, and 50 µM) were added to cells and allowed to incubate at 37 °C in a humidified 5 % CO₂ atmosphere for 0 through 5 days and then viability was assessed daily using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay according to the supplier's protocol. For ROS inhibition studies, aggressive cell lines ARCaP Snail and C4-2 were treated with 10 mM NAC plus 25 µM camalexin for 3 days and cell viability was assessed daily. LNCaP and ARCaP Neo cells (less aggressive cells) were similarly exposed to 25 µM camalexin, and 25 and 50 µM H₂O₂ respectively, and in combination with 25 µM camalexin, to induce oxidative stress, and, again, cell viability was assessed daily.

Western blot analysis

Western blot was performed as described previously [10]. Briefly, cells were plated at a density of 2×10^6 cells in 75-cm² flasks, and allowed to attach overnight. Prior to treatments, ARCaP Neo and ARCaP Snail cells were serum-starved in serum-free and phenol red-free RPMI medium overnight, while LNCaP and C4-2 cells were serum-starved for 4 h. Cells were then lysed with buffer containing 1× RIPA, with protease inhibitors (aprotinin 0.1 mg/ml, PMSF 1 mM, leupeptin 0.1 mM, pepstatin A 0.1 mM) and phosphatase inhibitor (10 mM sodium orthovanadate) and centrifuged at 13,000 rpm. The total protein content was determined using the BCATM Protein Assay Kit (Thermo Scientific, Rockford, IL), following the protocol as per the manufacturer's specifications. Cell lysates (40–50 µg) were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and then subsequently transferred to pure nitrocellulose membrane (Bio-Rad Laboratories). Western blot analysis for Snail, caspase-3, and cleaved PARP protein expression was then performed. Anti-mouse and anti-rabbit IgG horseradish peroxidase in blocking solution (5 % nonfat milk in TBS-TB) was employed as secondary antibodies for chemiluminescent detection. Blots were visualized with a chemiluminescence ECL detection system (Pierce, Rockford, IL) and analyzed using a FujiFilm LAS-3000 imager and darkroom X-ray film to observe protein expression. To evaluate protein loading, membranes were immediately stripped with RestoreTM Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and reprobbed for β-actin as a loading control.

Measurement of caspase-3/7 activation

Caspases are generally accepted as the executioners of apoptosis [35–37]. We treated ARCaP Snail cells with 25 µM camalexin alone and in combination with 10 mM NAC for 3 days and performed FACS analysis of caspase-3/7 activity in ARCaP Snail cells using the Dual Sensor: MitoCaspTM assay (MITCAP 200-1 kit, Cell Technology, Inc., Mountain View, CA), according to the manufacturer's camalexin, protocol. Stained cells were evaluated in an Accuri® C6 Flow Cytometer (Accuri Cytometers, Inc., Ann Arbor, MI) and analyzed using FlowJo software.

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) and Tukey's multiple comparison test as a post-hoc test from GraphPad Prism3 software (San Diego, CA); * $p < 0.05$ was considered to be significant. Multiple experiments were performed in replicates of 3.

Results

ROS is higher in aggressive prostate cancer cells as compared to less aggressive prostate cancer cells and is increased further by camalexin treatment

ROS has been associated with increased prostate cancer, so we sought to confirm previous studies that more aggressive cancer cells express more ROS. ARCaP cells overexpressing Snail have been shown to increase tumorigenicity [17] as compared to ARCaP Neo control cells, and we confirmed that ARCaP Snail cells are more migratory on collagen as compared to ARCaP Neo (** $p < 0.001$, Fig. 2a). LNCaP-C4-2 cells portray a progression model of prostate cancer [18], and we confirmed that C4-2 cells were more migratory than LNCaP cells (** $p < 0.001$, Fig. 2a). Baseline determination of levels of ROS in untreated prostate cancer cells showed that ARCaP Snail and C4-2 (more aggressive cells) had increased endogenous ROS versus the less aggressive cells ARCaP Neo and LNCaP (** $p < 0.001$, Fig. 2b). Since camalexin has been reported to increase ROS in leukemic cells [38], we examined whether it would do the same in prostate cancer cells. ARCaP Snail and C4-2

cells were treated with 25 or 50 μM camalexin, and 100 μM H_2O_2 was included as a positive control. For C4-2 cells, 25 or 50 μM camalexin increased ROS by approximately $130 \pm 5\%$ ($*p < 0.05$) and $150 \pm 5\%$ ($**p < 0.01$) of untreated controls, respectively (Fig. 2c). The addition of ROS scavenger, NAC, significantly decreased camalexin-mediated ROS (Fig. 2c). As was expected, the positive control 100 μM H_2O_2 significantly increased ROS in cells. Similarly, 25 or 50 μM camalexin increased ROS by approximately 210 % ($*p < 0.05$) or $350 \pm 25\%$ ($***p < 0.001$) in ARCaP Snail cells, respectively, as compared to untreated controls, which was abrogated by NAC (Fig. 2d). Therefore, ARCaP Snail and C4-2 cells are more migratory and express more ROS than ARCaP Neo and LNCaP cells, respectively, and camalexin can further increase ROS levels.

Camalexin treatments induced a more significant decrease in cell proliferation in the more aggressive prostate cancer cells as compared to the lesser aggressive cells

In order to test whether camalexin can affect cell viability, we utilized the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS assay) on camalexin-treated prostate cancer cells (ARCaP Neo, ARCaP Snail, LNCaP, C4-2) and normal prostate epithelial cells (PrEC) from days 0 through 5. We have represented here the results for day 0 and day 3 only, as camalexin treatment of cells produced a concentration-dependent decrease in cell viability. For day 0, both ARCaP Neo and ARCaP Snail cells showed no change in viability, as expected (Fig. 3a); however, on day 3, camalexin treatment of ARCaP Snail decreased cell viability by approximately 25 % (10 μM , $p < 0.05$) and 35 % (25 and 50 μM , $p < 0.001$), while ARCaP Neo cells remained unaffected (Fig. 3b). Similarly, at day 0 for both LNCaP and C4-2 cells, viability was unaffected (Fig. 3c), but on day 3, 50 μM camalexin decreased cell viability in LNCaP by approximately $40 \pm 2\%$ ($p < 0.01$), while 10, 25, and 50 μM camalexin decreased C4-2 cell viability by approximately $40 \pm 5\%$ ($p < 0.01$) (Fig. 3d). Interestingly, normal prostate epithelial cells (PrEC) were not significantly affected by camalexin treatments at days 0 and 3 (Fig. 3e). Hence, the normal prostate epithelial cells (PrEC) and the lesser aggressive prostate cancer cells (LNCaP and ARCaP Neo) are less sensitive to camalexin treatments as compared to the more aggressive cancer cells (C4-2 and ARCaP Snail).

Camalexin treatment induces caspase expression/activity and cleaved PARP in the aggressive cell lines without altering Snail expression

Because we were impressed with this compound's ability to significantly decrease viability especially in the more aggressive prostate cancer cells, we wanted to further demonstrate its apoptotic cytotoxic effect and also find out if camalexin could affect Snail expression. First, we conducted Western blot analysis of lysates from untreated and 3-day camalexin-treated (10, 25, and 50 μM) ARCaP Neo and LNCaP cells (less aggressive), and ARCaP Snail and C4-2 cells (more aggressive) for the detection of Snail and total caspase-3 protein expression. In ARCaP Neo cells, no detectable levels of Snail protein expression were noted for untreated control and camalexin treatments, while Snail was expressed in ARCaP Snail cells and was not affected by camalexin treatments ($***p < 0.001$, Fig. S1a). LNCaP cells expressed only very low levels of snail protein, while C4-2 cells expressed more Snail and, generally, camalexin treatment did not affect Snail expression except in 10 μM camalexin-treated LNCaP cells, which produced a moderate expressing band for Snail protein (Fig. S1c). Interestingly, camalexin induced an increase in total caspase-3 protein levels in the more aggressive ARCaP Snail ($**p < 0.01$ for the 50- μM treatment) and C4-2 cells, but not in the less aggressive ARCaP Neo or LNCaP cells (Fig. S1a–d). In fact, in the lesser aggressive LNCaP cells, the 10- μM -treated cells showed a significant decrease in protein expression ($***p < 0.001$, Fig. S1c, d). In addition to looking at total caspase levels, we next investigated caspase-3/7 activity upon camalexin treatment and whether NAC could antagonize the effects of camalexin on caspase activity.

We conducted FACS analysis of caspase-3/7 activity in ARCaP Snail cells. Subsequent to 3-day camalexin treatments, a greater fraction of ARCaP Snail cells showed increased caspase-3/7 activity (34.2 %) versus the untreated control cells (21.7 %) (Fig. 4). Interestingly, the addition of the H₂O₂ scavenger, NAC, to the camalexin-treated cells abrogated the percent increase in caspase-3/7 activity, almost returning it to the untreated control levels (Fig. 4). Overall, these results suggest that camalexin can increase cell apoptosis by activating caspase levels and activity in ARCaP Snail and C4-2 cells; these effects are mediated through ROS, since NAC could inhibit this activity.

Increased caspase-3/7 activity induced cleavage of one of its downstream targets, PARP

The cleavage of known caspase-3/7 substrate PARP protein expression (89 kDa) was determined via Western blot analysis of treated and untreated ARCaP Neo, ARCaP Snail, LNCaP, and C4-2 cells. Following camalexin exposure, ARCaP Snail cells (more aggressive) showed marked increase in the levels of cleaved PARP protein expression at all treatments as compared to the untreated control, with significance at the 25- and 50- μ M camalexin treatments (* p < 0.05, ** p < 0.01 respectively, Fig. 5a, b). However, ARCaP Neo cells (lesser aggressive) comparatively produced very low levels of cleaved PARP protein expression (Fig. 5a, b). Likewise, the more aggressive C4-2 cells showed marked increase in PARP protein cleavage at all 3 camalexin treatments versus the lesser aggressive LNCaP cells (Fig. 5c). Therefore, camalexin treatments can induce PARP cleavage more significantly in ARCaP Snail and C4-2 cells than in the lesser aggressive ARCaP Neo and LNCaP cells.

NAC antagonizes camalexin-mediated decrease in cell viability in aggressive prostate cancer cells

Next, we performed experiments to confirm that camalexin is mediating its effects on cell viability through ROS. Since we had observed that camalexin can decrease cell viability, we tested whether treatment with the ROS scavenger, NAC, would abrogate this effect and preserve viability in camalexin-treated ARCaP Snail and C4-2 cells. We observed, after 3 days, that camalexin-mediated decrease in cell viability was blocked by co-treatment with 10 mM NAC in ARCaP Snail, which showed approximately 15 % increased viability in response to NAC versus 25 μ M camalexin-only treatment (Fig. 6a). Although no statistical significance (ns) was noted for ARCaP Snail cells, the data strongly indicate a trend towards the preservation of viability subsequent to the addition of NAC to camalexin-treated cells. Similar results were obtained for C4-2 cells; there was approximately 25 % increased viability in the 25 μ M camalexin plus 10 mM NAC as compared to 25 μ M camalexin-only treatment (** p < 0.01), indicating that the anti-oxidant did significantly protect viability (Fig. S2a). We also evaluated cleaved PARP protein expression via Western blot analysis. Our results indicated that increased cleaved PARP protein expression in camalexin-treated ARCaP Snail cells could be inhibited by co-treatment with NAC (Fig. 6b). Therefore, camalexin decreases cell viability via ROS.

Addition of exogenous ROS potentiates the effects of camalexin in less aggressive prostate cancer cells

Alternatively, we examined whether adding exogenous ROS to less aggressive cells that had been relatively resistant to camalexin would now render them sensitive to camalexin. ARCaP Neo and LNCaP cells were exposed for up to 3 days to 25 μ M camalexin, 25, 50 μ M H₂O₂, and 50 μ M H₂O₂ in combination with 25 μ M camalexin. For ARCaP Neo, after 3 days exposure to treatments, only 50 μ M H₂O₂ and 25 μ M camalexin plus 50 μ M H₂O₂ decreased viability by approximately 50 and 60 %, respectively (** p < 0.001) (Fig. 6c). Similar results were obtained for LNCaP cells (combination treatment with H₂O₂ and camalexin was more effective at decreasing cell viability by approximately 35 $\pm$ 1 % for 25

μM camalexin plus 25 μM H_2O_2 ($p < 0.001$) and 40 % for 25 μM camalexin plus 50 μM H_2O_2 ($p < 0.001$) (Fig. S2b). Moreover, camalexin potentiated H_2O_2 -mediated PARP cleavage, as shown by Western blot analysis in ARCaP Neo cells (Fig. 6d) and LNCaP cells (Fig. S2c). Hence, from our results, the addition of H_2O_2 now rendered the less aggressive cells sensitive to camalexin, as shown by decreased cell viability and increased PARP cleavage.

Discussion

Inherent oxidative stress can affect several functions in cancer cells, including proliferation, promotion of mutations, alteration in sensitivity to anticancer agents, invasion, and metastasis [39, 40]. Studies conducted by Kumar et al. [40] on several prostate cancer cell lines have shown that inherent ROS generation is directly proportional to aggressive phenotype. We have previously shown that ARCaP prostate cancer cells stably transfected with Snail cDNA (ARCaP Snail) display more ROS (hydrogen peroxide and superoxide) as compared to ARCaP cells transfected with empty vector control (ARCaP Neo), both in vitro and in vivo [17]. Additionally, Snail has been associated with chemoresistance and resistance to radiotherapy [41]. We, therefore, utilized the ARCaP Neo/Snail model to test for compounds that could target cancer cells overexpressing Snail. We also utilized the LNCaP/C4-2 established prostate cancer progression model in which C4-2 cells have been previously shown to express more ROS than LNCaP cells [18]. We confirmed that ARCaP Snail and C4-2 had a greater migratory potential and level of endogenous ROS versus ARCaP Neo and LNCaP cells, respectively. Camalexin has been extensively investigated as regards to its biosynthesis and anti-microbial activities against various plant pathogens [28–30], and, in a limited context, in eukaryotic cells, where it has displayed cytotoxic/cytostatic effects [31, 32]. Mezencev et al. [33] have shown that camalexin induced apoptosis in leukemic cells by increasing ROS and activating caspases. However, here, for the first time, our data demonstrate that this compound is more deleterious in the more aggressive ARCaP Snail and C4-2 prostate cancer cells as compared to the less aggressive ARCaP Neo and LNCaP cells. Furthermore, addition of the ROS scavenger, NAC, to the camalexin-treated cells significantly decreased ROS generation and alleviated the cytotoxic effects of camalexin. When we examined the effects of camalexin treatment on the viability of the prostate cancer and normal epithelial prostate cell lines for up to 5 days, we found that the more aggressive ARCaP Snail and C4-2 cells displayed greater sensitivity to this compound than the lesser aggressive ARCaP Neo and LNCaP. Normal prostate epithelial cells (PrEC) were not significantly affected by camalexin treatments. The decreased viability observed in treated cells was concentration-dependent, and, to our surprise, the ARCaP cells transfected with an empty vector (Neo) remained insensitive to camalexin treatment over the 5 days, in comparison to the Snail-overexpressed cells (ARCaP Snail). From these results, we inferred that the addition of camalexin to the more aggressive prostate cancer cells already having high levels of ROS led to the generation of even more ROS, which may have tipped the balance and now triggered less cell viability and possibly increased cell death. It is believed that cancer cells utilize increased ROS to promote signal transduction associated with increased cell survival and migration. For example, ROS can inactivate PTEN and promote constitutive PI3-K signaling, which is associated with cell survival [38, 42]. Additionally, ROS can induce epithelial to mesenchymal transition (EMT), a process associated with cell migration [16]. However, if ROS levels are too high, it can promote apoptosis [21–23, 43, 44]. Therefore, there is usually a balance suggested between ROS levels used in cell signaling and ROS levels that promote apoptosis.

Caspases-3 and -7 are responsible for the majority of limited proteolytic events that drive apoptosis [45–47] and, hence, utilizing our camalexin-treated prostate cancer cells, we showed a consistent increase in total caspase-3 protein expression in our more aggressive

cells. FACS analysis provided evidence of caspase-3/7 increased activity in camalexin-treated ARCaP Snail cells, which was abrogated by the addition of NAC, the ROS scavenger, thus, again, providing evidence of oxidative stress-induced apoptosis. Caspase substrates that are candidates for effectors of neuronal apoptosis include poly-ADP ribose polymerase (PARP), DNA-dependent protein kinase, U1-soluble nuclear RNA polymerase (U1-snRNP), spectrin, lamin A, actin, and protein kinase C [47]. The best characterized is PARP, which is cleaved by caspase to generate 89-kDa and 24-kDa fragments from the 113-kDa holoenzyme [47]. Western blot analysis of our camalexin-treated cells showed PARP cleavage that was more pronounced in ARCaP Snail and C4-2 cells versus the lesser aggressive ARCaP Neo and LNCaP cells, respectively. Interestingly, we observed that camalexin treatment did not alter the expression of Snail in our cell lines, except in LNCaP 10 μ M camalexin treatment, which showed increased protein expression. Taken together, these results suggest that camalexin mediates its cytotoxic effects downstream of Snail. It is not clear why 10 μ M camalexin increased Snail expression in LNCaP cells, but it underscores the complexity of ROS signaling; it is possible that camalexin-induced ROS at low concentrations may be utilized by cancer cells to signal growth and survival. Therefore, our data suggest that camalexin as a treatment for aggressive prostate cancer cells will work better if utilized in conjunction with other chemotherapeutic agents that target the less aggressive prostate cancer cells.

Abrogation of camalexin-induced apoptosis and decreased cell viability by NAC further supports our hypothesis that camalexin mediates its effects through ROS. Conversely, when we induced oxidative stress in ARCaP Neo and LNCaP cells by the addition of H₂O₂, the cells became more sensitive to camalexin-mediated decrease in cell viability and increased cleaved PARP protein expression. Taken together, our data showed, for the first time, that camalexin significantly decreases cell viability in prostate cancer cells, and that the more aggressive ARCaP Snail and C4-2 cells displayed greater sensitivity to treatment than the lesser aggressive ARCaP Neo and LNCaP cells. Additionally, this compound induced apoptosis via increased generation of ROS. FACS analysis performed in camalexin-treated ARCaP Snail cells to verify caspase-3/7 activity displayed a strong propensity towards caspase-dependent apoptosis. However, mechanisms for camalexin-induced ROS generation in these prostate cancer cells need to be elucidated. Understanding the components of the signaling pathways that are regulated by camalexin may result in the development of new therapeutic strategies in combating metastatic prostate cancer that carries the highest mortality rate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EMT	Epithelial to mesenchymal transition
ROS	Reactive oxygen species
NAC	<i>N</i> -acetyl cysteine
H2 DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H₂O₂	Hydrogen peroxide

Camalexin structure

Fig. 1. The structural formula of camalexin

Fig. 2.

Camalexin increases reactive oxygen species (ROS). Baseline migratory potentials were assessed for ARCaP cells stably transfected with empty vector (Neo) or Snail cDNA (ARCaP Snail) and plated on collagen-coated inserts for 5 h, or LNCaP and C4-2 cells allowed to migrate for 24 h (a). ROS levels were determined in prostate cancer cells (LNCaP, C4-2, ARCaP Neo, and ARCaP Snail) using oxidation of H₂DCFDA (b). ROS was detected in C4-2 (c) and ARCaP Snail (d) cells subsequent to 3 days exposure to 25 or 50 μM camalexin, 100 μM H₂O₂, 25 μM camalexin plus 10 mM N-acetyl Cysteine (NAC), and 50 μM camalexin plus 10 mM NAC, respectively. Statistical analysis was done using analysis of variance (ANOVA) and Tukey's multiple comparison test as a post-hoc test. Values were expressed as mean ± standard error of the mean (SEM) normalized to untreated controls, 25 μcamalexin versus 25 μM camalexin +10 mM NAC, and 50 μcamalexin versus 50 μcamalexin +10 mM NAC (**p* < 0.05, ***p* < 0.01, ****p* < 0.001) (*N* = 3)

Fig. 3.

Camalexin decreases cell viability in more aggressive prostate cancer cells (ARCaP Snail and C4-2). Viability was determined using MTS proliferation assay at day 0 (**a**) or day 3 (**b**) for ARCaP Neo and ARCaP Snail; at day 0 (**c**) or day 3 (**d**) for LNCaP and C4-2; and PrEC (**e**) cells treated with 10, 25, and 50 μM camalexin. The degree of sensitivity of lesser aggressive cells (ARCaP Neo and LNCaP), versus more aggressive cells (ARCaP Snail and C4-2) to camalexin treatments was also noted. Statistical analysis was done using ANOVA and Tukey's multiple comparison test as a post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Values were normalized to untreated controls and expressed as mean $\pm$ SEM ($N = 3$).

Fig. 4. Camalexin increases caspase-3/7 activity. Determination of caspase-3/7 activation via the MitoCasp assay in ARCaP Snail cells untreated and treated with 25 μ M camalexin and 25 μ M camalexin plus 10 mM NAC for 3 days was done as described in the “Materials and methods” section. The percentages of cells undergoing caspase-3/7 activation in untreated, camalexin-treated, and camalexin-treated plus NAC were noted. Data are representative of at least 3 independent experiments. Statistical analysis was done using ANOVA and Tukey's multiple comparison test as a post-hoc test (** $p < 0.01$, *** $p < 0.001$). Values were normalized to untreated controls and expressed as mean $\pm$ SEM ($N = 3$)

Fig. 5.

Camalexin leads to increased PARP cleavage in ARCaP Snail and C4-2 cells. Western blot analysis was performed to examine cleaved PARP protein expression following the exposure of ARCaP Neo and ARCaP Snail to various doses of camalexin (a). Western blot results for cleaved PARP were quantified by densitometry with normalization to β -actin levels using the Quantity One quantification software (Bio-Rad) (b). LNCaP and C4-2 were treated with 10, 25, and 50 μ M camalexin for 3 days and the cleaved PARP examined by Western blot analysis (c). β -actin was used as a loading control. The data are representative of at least 3 independent experiments and statistical analysis was done using ANOVA and Tukey's multiple comparison test as a post-hoc test (* p < 0.05, ** p < 0.01)

Fig. 6.

NAC abrogates the effects of camalexin, while the addition of exogenous ROS potentiates camalexin-mediated decrease in cell viability. ARCaP Snail cells (a) were treated with 10 mM NAC, 25 μM camalexin, and 25 μM camalexin plus 10 mM NAC for up to 3 days and cell viability assayed using the MTS proliferation assay. Western blot analysis was done to examine cleaved PARP protein expression (89 kDa) in untreated, camalexin-treated, and camalexin plus NAC-treated ARCaP Snail cells (b). Treatment for 3 days indicated that NAC could inhibit camalexin-mediated increase in cleaved PARP. ARCaP Neo cells were treated with camalexin, H₂O₂, or camalexin plus H₂O₂ for up to 3 days and cell viability was analyzed by the MTS proliferation assay (c). Cell lysates were subjected to Western blot analysis to assess cleaved PARP protein expression following the treatment of ARCaP Neo cells with camalexin, H₂O₂, or camalexin plus H₂O₂ for 3 days. Western blot results for cleaved PARP were quantified by densitometry with normalization to β-actin levels using the Quantity One quantification software (Bio-Rad) (d). β-actin was used as a loading control and the data are representative of at least 3 independent experiments. Statistical analysis was done using ANOVA and Tukey's multiple comparison test as a post-hoc test. Values were expressed as mean ± SEM normalized to untreated controls, and 25 μM camalexin versus 25 μM camalexin + 10 mM NAC (**p* < 0.05, ***p* < 0.01, ****p* < 0.001) (*N* = 3)