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Combination of Carmustine and Selenite Inhibits EGFR Mediated Growth Signaling in Androgen-Independent Prostate Cancer Cells

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ABSTRACT

Although aberrant androgen receptor (AR) signaling is a central mechanism for castration resistant prostate cancer (CRPC) progression, AR-independent growth signaling is also present in CRPC. The current therapeutic options for patients with CRPC are limited and new drugs are desperately needed to eliminate these crucial growth signaling pathways. We have previously shown that combination of carmustine and selenite effectively induces apoptosis and growth inhibition by targeting AR and AR-variants in CRPC cells. High levels of EGFR expression present in the CRPC cells mediates the cell proliferation via AR-independent growth signaling mechanisms. Therefore, in this study, we investigated whether the combination of carmustine and selenite could inhibit EGFR mediated growth signaling and induce apoptosis in androgen independent-AR negative prostate cancer cells. EGF exposure dose and time dependently increased phospho-EGFR (Tyr845, Tyr1068, and Tyr1045), pAkt (Ser473), and pERK1/2 (Thr204/Tyr202) protein expression levels in AIPC cells. Combination of carmustine and selenite treatment markedly suppressed EGF-stimulated proliferation and survival of AIPC cells and effectively induced apoptosis. The ROS generated by the combination of carmustine and selenite exhibited a strong inhibition on EGF stimulated EGFR and its downstream signaling molecules such as Akt, NF-kB, ERK1/2, and Cyclin D1. Individual agent treatment showed only partial effect. Overall, our findings demonstrated that the combination of carmustine and selenite treatment dramatically inhibits EGFR signaling, proliferation, and induces apoptosis in AIPC cells, suggesting a potential candidate for the treatment of CRPC. The results of the study further suggest that the combination of carmustine and selenite treatment can overcome EGFR mediated AR-independent growth response in CRPC during anti-androgen therapy. J. Cell. Biochem. 118: 4331–4340, 2017.

KEY WORDS: CASTRATION RESISTANT PROSTATE CANCER; ANDROGEN-INDEPENDENT PROSTATE CANCER; CARMUSTINE; SELENITE; PC-3 CELLS; EGFR; CHEMOTHERAPY; Akt; ERK1/2

Prostate cancer is the most common cancer and second leading cause of cancer related mortality in men in the United States. The American Cancer Society has projected that in 2017 the number of new cases of prostate cancer in the United States will exceed 161,360 and about 26,730 prostate cancer deaths are anticipated. Although androgen ablation therapy is the primary and standard treatment for localized metastatic prostate cancer, tumors overcome androgen blockade, and develop a hormone-independent phenotype within 24–36 months that become resistant to ADT therapy [Harris et al., 2009; Lamont and Tindall, 2011]. Although current next generation therapies including abiraterone acetate and enzalutamide and chemotherapeutic regimens have improved the quality of life by reducing circulating androgen levels [de Bono et al., 2011] or blocking the binding of androgens to androgen receptor [Scher et al., 2012], these therapies are culminating in the survival rate by 12–19 months for patients with androgen-independent, hormone refractory prostate cancer [Mimeault et al., 2007; Harris et al., 2009]. These androgen-AR blockers displayed limited patient responses due to

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resistance mechanisms caused by abnormal activation of AR and non-AR signaling mediate cell growth [Mulholland et al., 2011; Al Nakouzi et al., 2015]. Therefore, it is reasonable to hypothesize that if androgen receptor independent mechanisms of growth and/or survival are present in prostate cancer then targeting androgen receptor pathway alone is likely to be ineffective over time. Therefore, anti-cancer drugs are desperately needed to eliminate prostate cancer by targeting not only AR signaling pathways, but also targeting AR-independent signaling pathways.

The signaling network of distinct oncogenic pathways initiated by hormones, cytokine, and growth factors through their cognate receptors is typically involved in the progression of PC from androgen dependent states into highly metastatic and androgen independent forms [Mimeault and Batra, 2006]. Among the oncogenic molecules, epidermal growth factor receptor (EGFR) is the most frequently activated gene and enhanced phosphorylation of EGFR by its ligand results in increased cell growth via activation of its downstream PI3K/Akt and Ras/Raf/Mek/ERK1/2 signaling pathways. These oncogenic molecules interact with each other to promote the sustained growth, survival, invasion and metastasis of AIPC [Mimeault and Batra, 2006]. Elevated levels of EGF/EGFR expression have been reported in metastatic human prostate tumors [De Miguel et al., 1999] and other cancers including breast, bladder, colon and lung [Sharma et al., 2007]. Therapies are being evaluated to treat cancers that express high levels of EGF/EGFR by blocking the binding sites for EGF or by inhibiting the EGFR tyrosine kinase activity [Mendelsohn and Baselga, 2000].

The phosphorylated Akt promote cell survival by phosphorylation and inactivation of downstream targets including IKK, which in turn phosphorylates IkB-alpha leading to its dissociation from NF-kB [Lokeshwar et al., 2010]. The activated NF-kB then translocate to the nucleus and promotes transcription of target genes [Zollo et al., 2012]. Phosphorylation of ERK1/2 is a hallmark of MAPK activation and highly activated in numerous human tumors including prostate cancer [Price et al., 1999]. ERK1/2 pathway activated through receptor tyrosine kinases mediated activation of Ras by triggering the exchange of GDP bound to Ras for GTP. The Ras phosphorylates Raf-1, which in turn sequentially phosphorylates MEK1/2 and ERK1/2. The ERK1/2 then induces gene transcription through the activation of various transcription factors [Dhillon et al., 2007; Rodriguez-Berriguete et al., 2012].

Reactive oxygen species (ROS) play an important role in the induction of apoptosis under physiological and pathological conditions. Tumors exhibit higher basal levels of ROS and altered redox environment compared to normal cells. Even though increase in the amount of ROS above the threshold levels of cancer cells are required for the effective induction of apoptosis, tumors are well-adapted to such oxidative stress by developing an enhanced, endogenous antioxidant capacity. In addition, this adaptation makes the cancer cells resistant to ROS inducing anticancer agents [Thamilselvan et al., 2012, 2016]. Targeting these adaptive mechanisms of cancer cells with redox modulating strategies by increasing ROS and decreasing antioxidants with combination therapy could be a feasible therapeutic approach to selectively kill cancer cells without causing toxicity to normal cells. We have previously demonstrated that low dose selenite (2.5 μM) in combination with carmustine (20 μM) effectively induced apoptosis in CRPC cells without causing genotoxicity to normal prostate epithelial cells via induction of reactive oxygen species [Thamilselvan et al., 2012, 2016]. Selenite treatment not only generates superoxide, but at the same time consumes cellular GSH, an antioxidant. Carmustine is an alkylating chemotherapeutic agent which has the property to induce ROS indirectly by inhibiting glutathione reductase and thioredoxin reductase [Thamilselvan et al., 2012, 2016]. It is remarkable that combination of carmustine and selenite completely reduced tumor growth both in vitro and in vivo by reducing androgen receptor (AR) full length and AR-variants in AR-positive prostate cancer cells [Thamilselvan et al., 2016]. Since, AR independent mechanisms of cell survival are operative in CRPC, in the present study we investigated whether combination of carmustine and selenite inhibits EGFR-mediated growth signaling and triggers apoptosis in AR-negative prostate cancer cells.

MATERIALS AND METHODS

CELL CULTURE

Androgen-independent prostate cancer cell line, PC-3 (American Type Culture Collection, Manassas, VA) was cultured in F-12K media (Gibco-BRL Life Technologies Inc, Gaithersburg, MD), respectively, at 37°C with 5% CO2 in a humidified incubator. Media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Prior to the beginning of the experiment, PC-3 cells were switched from FBS-F-12K medium to serum free (SF) F-12K medium for 24 h.

COMBINATION AGENTS, GROWTH FACTOR, AND INHIBITORS

Based on our previous concentration dependent studies on prostate cancer cells, we selected 2.5 μM sodium selenite (selenite) and 20 μM carmustine (Sigma–Aldrich, St. Louis, MO) for cell culture studies [Thamilselvan et al., 2012, 2016]. These concentrations showed dramatic induction of apoptosis in AIPC and CRPC cells without causing genotoxicity (DNA double strand break) in normal prostate cells [Thamilselvan et al., 2012, 2016]. Selenite was dissolved in distilled water, whereas carmustine was dissolved in ethanol, and the final concentration of ethanol in the culture medium was kept at 0.05% (v/v) in cells treated with carmustine and control cells treated with ethanol. EGF was dissolved in distilled water (10–100 ng/ml) and used for dose-dependent study. The optimal dose of EGF 50 ng/ml was selected from the dose dependent experiments performed in the present study and used for the following analysis. EGFR tyrosine kinase inhibitor, AG1478 was dissolved in DMSO and used at a concentration of 10 μM. A superoxide scavenger, MnTMPyP was dissolved in distilled water and used at a concentration of 10 μM.

CELL VIABILITY

PC-3 cells were seeded in 48-well plates at a density of 5×10^4 cells per well and allowed to grow for 48 h. The cells were treated with carmustine and/or selenite in the presence or absence of EGF for 48 h. Control cells were treated with ethanol. Cell viability

(survival) was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as we described previously [Thamilselvan et al., 2011].

CELL GROWTH

PC-3 cells were seeded in 48-well plates at a density of 3×10^4 cells per well and allowed to grow for 24 h. After 24 h, the cells were washed once with serum free media and then treated with carmustine and selenite in the presence or absence of EGF for 48 h. Additional set of experiments were conducted in cells treated with or without AG1478 in the presence of absence of EGF for 48 h. Cell growth was then determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as we described previously [Thamilselvan et al., 2011].

APOPTOSIS

The cells were treated with carmustine and/or selenite in serum free media for 48 h in the presence or absence of EGF. The apoptosis was then quantitated using acridine orange/ethidium bromide staining as we described previously [Thamilselvan et al., 2011].

WESTERN BLOT

(i) PC-3 cells were treated with a different concentration of EGF (0, 5, 10, 20, 50, 100 ng/ml) for 5 min. (ii) PC-3 cells were treated with 50 ng/ml EGF for different time periods (0, 2, 5, 10, 30, and 60 min). (iii) The PC-3 cells were treated with carmustine and/or selenite in serum free F-12K media for 48 h. EGF (50 ng/ml) was added 5 min before the termination of the experiment. (iv) PC-3 cells were treated with or without AG1478 in serum free F-12K media for 48 h. EGF (50 ng/ml) was added 5 min before the termination of the experiment. The cell lysates were then used for Western analysis as we described previously [Thamilselvan et al., 2016] using the primary antibodies directed against total EGFR (Santa Cruz), phospho-EGFR (Tyr845, Tyr1045, Tyr1068), phospho-Akt (Ser473), total Akt, phospho-ERK1/2 (Thr204/Tyr202), total ERK, cyclin D1 (Cell Signaling Technology, Danvers, MA), phospho-IkB (Ser32), and total IkB (Santa Cruz). The membranes were reprobed with antibodies to α-tubulin to ensure equal protein loading [Thamilselvan et al., 2016].

REACTIVE OXYGEN SPECIES

The effect of superoxide scavenger, MnTMPyP, a SOD mimetic on ROS generated by combination of carmustine and selenite was studied in AIPC cells. PC-3 cells were treated with or without the combination of 20 μM carmustine and 2.5 μM selenite in the presence of 10 μM MnTmPyP (EMD Millipore, MA) for 48 h. EGF (50 ng/ml) was added 5 min before the termination of the experiment. The cell lysates were then used for Western analysis as described above using primary antibodies directed against p-EGFR (Tyr845, Tyr1068), p-Akt (Ser473), and ERk1/2 (thr204/Tyr202).

STATISTICAL ANALYSIS

The Student's *t*-test was used to evaluate differences between treated and untreated cells. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

DOSE AND TIME DEPENDENT EFFECT OF EGF ON EGFR SIGNALING PROTEINS IN PC-3 CELLS

The EGFR is a transmembrane tyrosine kinase receptor which is stimulated by growth factors. EGF bind to the extracellular domain of the receptor. Ligand binding induces receptors to dimerize, and dimer formation undergoes autophosphorylation on multiple tyrosine residues located in the *C*-terminal non-catalytic sequence which include Y992, Y1068, Y1086, Y1148, and Y1173. EGFR could also be phosphorylated by other kinases including Src (Y845, Y891, Y920, Y1045, Y1101, Y1173), which in turn induce cell proliferation via activation of Akt and ERK1/2 signaling pathways [Sato, 2013]. To identify the optimal concentrations of EGF at which EGFR and its downstream signaling molecules are activated in PC-3 cells, we studied the dose-dependent effect of exogenous addition of EGF. The effect of EGF at 5, 10, 20, 50, and 100 ng/ml for 5 min on EGFR and its downstream signaling proteins expression in PC-3 cells were determined by Western blot analysis (Fig. 1A). EGF treatment of PC-3 cells results in a phosphorylation of EGFR (Tyr845, Tyr1045, and Tyr 1068), Akt (Ser473), and ERK1/2 (Thr204/Tyr202) in a concentration dependent manner. EGF at 10 ng/ml did not increase phosphorylation of EGFR, Akt, and ERK1/2 in PC-3 cells. EGF at 20 ng/ml increased phosphorylation of EGFR, Akt, and ERK1/2. The 20 ng/ml EGF stimulated PC-3 cells demonstrated variable responses. The increased phosphorylation of EGFR at Tyr845 and Tyr 1068 with 20 ng/ml EGF were significantly higher when compared to phosphorylation of EGFR at Tyr1045. Addition of EGF at a concentration of 50 ng/ml significantly increased phosphorylation of all the above proteins studied, and the phosphorylation was further increased with increasing concentration of 100 ng/ml of EGF. This experiment demonstrated that 50 ng/ml EGF represented the optimal concentration, primarily involved in the activation of EGFR singling pathway in PC-3 cells.

We next monitored the time dependent (0, 2, 5, 10, 30, 60 min) effect of 50 ng/ml of EGF on EGFR and it downstream signaling proteins expression by Western blotting to determine whether prolonged exposure to EGF resulted in an increase in basal protein phosphorylation. As shown in Figure 1B, EGF treatment of PC-3 cells, results in a rapid phosphorylation of EGFR (Tyr845, Tyr1045, and Tyr1068) that peaks at 5 min and gradually returns to baseline by 60 min, whereas, the phosphorylation of Akt and ERK1/2 peaks at 10 min and gradually returns to baseline by 60 min. The optimal concentration of EGF (50 ng/ml) and the time of maximal activation (5 min) identified will be used for subsequent experiments, to study the effect of carmustine and/or selenite on EGF induced EGFR signaling and its downstream signaling mediated cell proliferation.

EFFECT OF CARMUSTINE AND/OR SELENITE ON EGF-INDUCED SURVIVAL AND GROWTH OF PC-3 CELLS

The effect of 20 μM carmustine and/or 2.5 μM selenite in the presence or absence of 50 ng/ml EGF on cell viability of PC-3 cells was measured using MTT by assessing the percentage of viable cells. As shown in Figure 2A, treatment of PC-3 cells with selenite alone for 48 h reduced cell viability to $75.0 \pm 3.0\%$ of untreated cells, and the viability was not altered further by EGF treatment. Carmustine

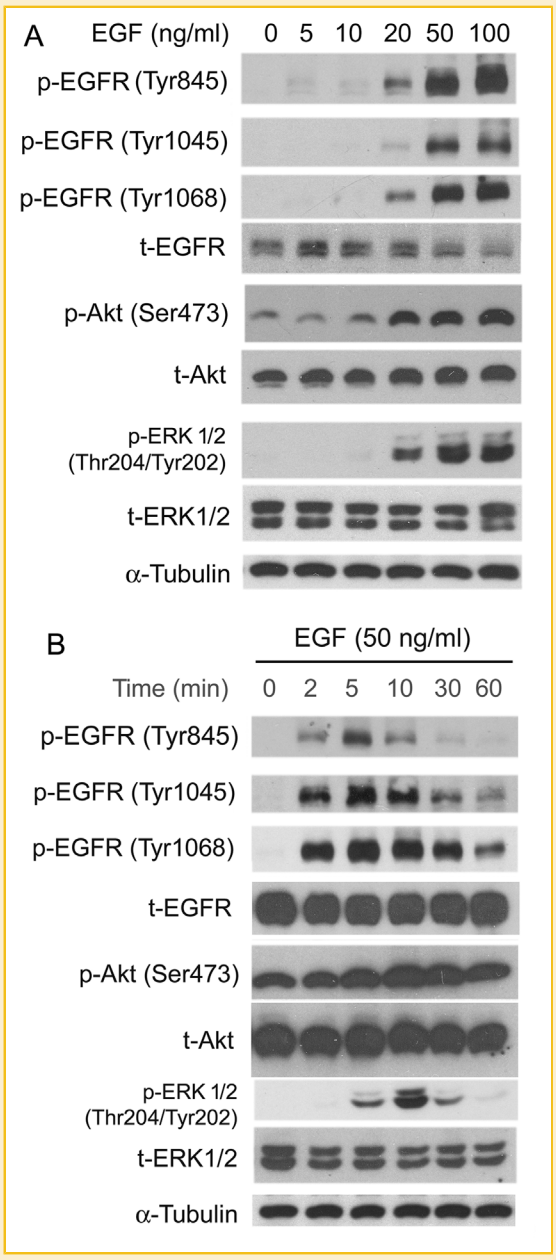

Fig. 1. EGF dose and time dependently activates EGFR and its downstream signaling proteins in PC-3 cells. (A) PC-3 cells were exposed to serum free medium containing indicated concentration of EGF (5, 10, 20, 50, and 100 ng/ml) for 5 min. (B) PC-3 cells were exposed to serum free medium containing EGF (50 ng/ml) for different time periods (2, 5, 10, 30, and 60 min). Cell lysates from each sample were used for Western blot analyses performed as described in the Materials and Methods section with antibodies directed against pEGFR, tEGFR, pAKT, tAkt, pERK1/2, and tERK1/2. α-tubulin was used as a loading control. A typical Western blot from 1 of 3 experiments is shown.

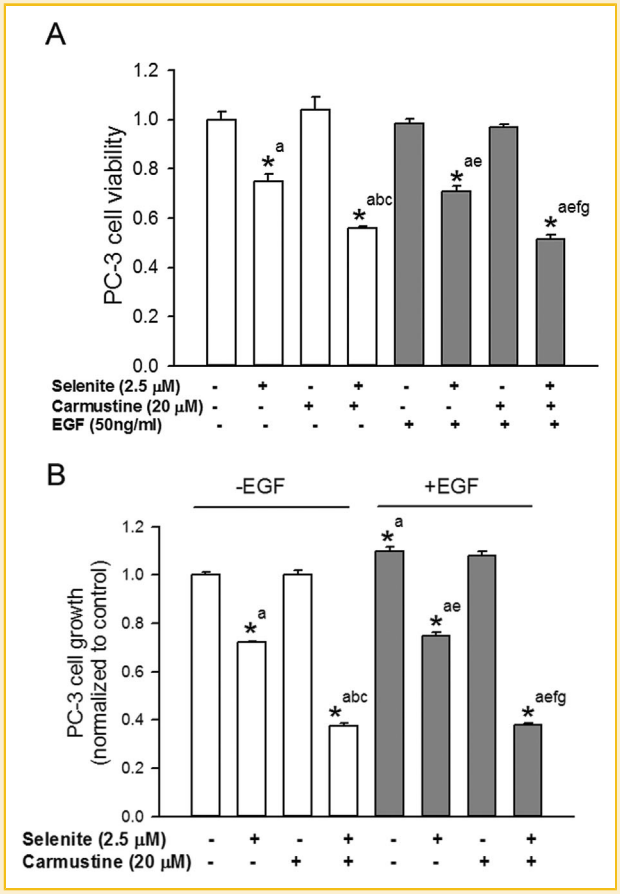

Fig. 2. Combination of carmustine and selenite treatment inhibits cell viability and growth in EGF stimulated PC-3 cells. (A) PC-3 cells were treated with 20 μM carmustine and/or 2.5 μM selenite in the presence or absence of EGF (50 ng/ml) in serum free media for 48 h. Cell viability was then determined by MTT assay as described in the materials and methods section. Data were normalized to the control and presented as the mean ± SD ($^*P < 0.05$; n = 4) and expressed as a percent of viable cells. (B) PC-3 cells were exposed to serum free medium containing 20 μM carmustine and/or 2.5 μM selenite in the presence or absence of EGF (50 ng/ml) for 48 h. Cell growth was determined as described in method section. Data were normalized to the control and presented as the mean ± SD ($^*P < 0.05$; n = 4) of viable cells. Comparisons shown: a: significant compared with vehicle-treated control; b: significant compared with 2.5 μM selenite-treated cells; c: significant compared with 20 μM carmustine-treated cells; d: significant compared with 2.5 μM selenite and 20 μM carmustine-treated cells; e: significant compared with 50 ng/ml EGF-treated cells; f: significant compared with 50 ng/ml EGF and selenite- treated cells; g: significant compared with 50 ng/ml EGF and carmustine-treated cells.

alone treatment showed no changes in viability of PC-3 cells with or without EGF stimulation. However, both agents in combination in the presence or absence of EGF significantly decreased viability (in the absence of EGF: 55.75 ± 1.0%; in the presence of EGF: 51.40 ± 2.0%) when compared to the cells treated with vehicle or individual agents alone.

To ensure that reduction in cell viability reflected in growth inhibition we further analyzed antiproliferative effect of 20 μM carmustine and/or 2.5 μM selenite in PC-3 cells stimulated with or without EGF for 48 h. Addition of EGF alone increased the proliferation of PC-3 cells compared to vehicle treated control cells (Fig. 2B). Proliferation of PC-3 cells stimulated with or without EGF was partially inhibited by selenite treatment when compared to control cells. The proliferation of cells stimulated with or without EGF were unaffected by carmustine treatment. Whereas,

combination of carmustine and selenite treatment significantly reduced the proliferation of PC-3 cell in the presence or absence of EGF when compared to individual agent treatment. These data suggest that carmustine and selenite in combination are potentially cytotoxic as well as antiproliferative activity against PC-3 cells in the presence or absence of EGF.

COMBINATION TREATMENT INDUCES APOPTOSIS IN EGF STIMULATED PC-3 CELLS

We next determined whether the reduction in cell viability and growth inhibition observed in PC-3 cells were accompanied by induction of apoptosis. The PC-3 cells stimulated with or without 50 ng/ml EGF were treated with 20 μM carmustine and/or 2.5 μM selenite for 48 h. The apoptotic cells were then detected using acridine orange and ethidium bromide and observed under fluorescence microscope. As shown in Figure 3, EGF treatment alone did not induce apoptosis. While selenite showed partial induction of apoptosis, carmustine alone showed no induction of apoptosis in EGF stimulated PC-3 cells. It was interesting to note that the carmustine and selenite in combination dramatically induced apoptosis in EGF stimulated PC-3 cells as evidenced from yellow and orange color stained apoptotic cells are indicated by white arrows.

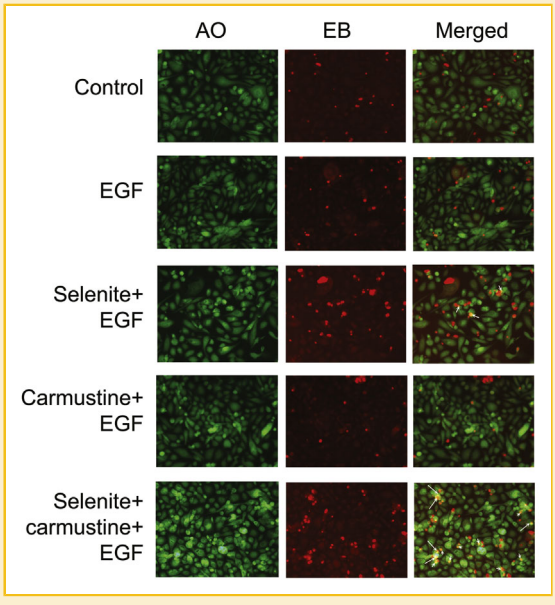

Fig. 3. Combination of carmustine and selenite treatment induces apoptosis in EGF stimulated PC-3 cells. PC-3 cells were exposed to serum free medium containing 20 μM carmustine and/or 2.5 μM selenite in the presence or absence of EGF (50 ng/ml) for 48 h. Morphological assessment of cell death was then investigated using the acridine orange and ethidium bromide (AO/EB) staining. The cells were then examined and photographed using a fluorescence microscope (200×). Solid arrow represents early apoptotic cells (yellow fluorescence). Dashed arrow represents late apoptotic cells (orange or red fluorescence). Green fluorescence represents viable cells. Photographs shown are representative of one of three similar experiments.

EFFECT OF CARMUSTINE AND/OR SELENITE ON EGF-INDUCED EGFR AND ITS DOWNSTREAM SIGNALING PROTEINS ACTIVATION IN PC-3 CELLS

To further understand the molecular mechanism of carmustine and/or selenite induced growth inhibition in PC-3 cells, protein expression levels of EGFR mediated growth signaling machinery were investigated. We first evaluated the effect of carmustine and/or

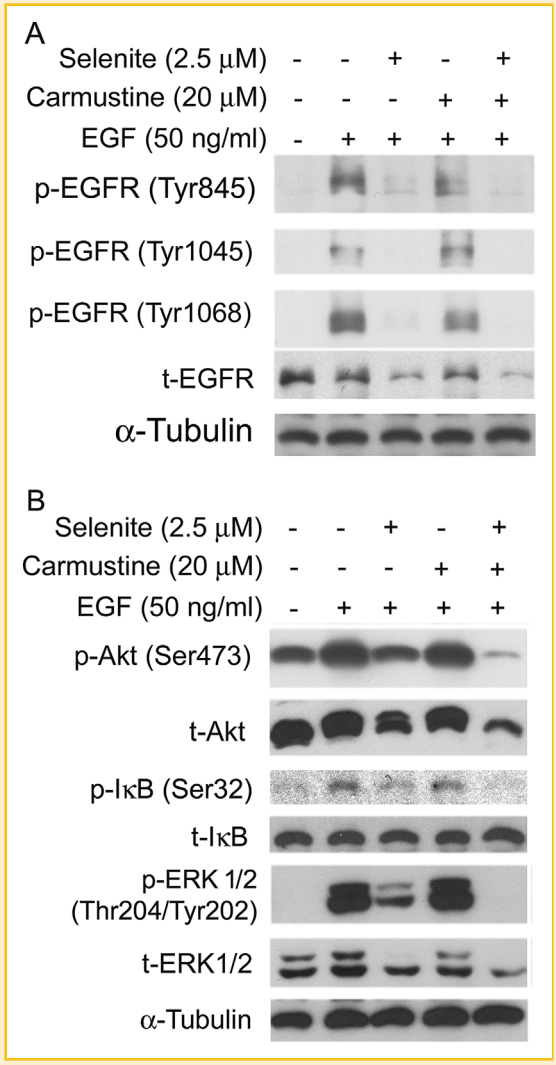

Fig. 4. Combination of carmustine and selenite treatment inhibits EGF induced phosphorylation of EGFR and its downstream signaling proteins in PC-3 cells. (A) PC-3 cells were treated with 20 μM carmustine and 2.5 μM selenite for 48 h. EGF (50 ng/ml) was added 5 min before the termination of the experiment. Control experiment without EGF was also included. Cell lysates from each sample were used for Western blot analyses performed as described in the Materials and Methods section with antibodies directed against pEGFR, tEGFR. (B) PC-3 cells were treated with 20 μM carmustine and 2.5 μM selenite for 48 h. EGF (50 ng/ml) was added 5 min before the termination of the experiment. Control experiment without EGF was also included. Cell lysates from each sample were used for Western blot analyses performed as described in the Materials and Methods section with antibodies directed against pEGFR, tEGFR, pAKT, tAkt, pIkBα, tIkBα, pERK1/2, and tERK1/2. α-tubulin was used as a loading control. A typical Western blot from 1 of 3 experiments is shown.

selenite on EGF-induced phosphorylation of EGFR. The PC-3 cells were treated with 20 μM carmustine and/or 2.5 μM selenite for 48 h. The cells were then stimulated with 50 ng/ml EGF for 5 min. The control cells without EGF treatment were also included for comparison. The cell lysates were then subjected to Western blotting analysis for EGFR protein expression (Fig. 4A). Western blot analysis demonstrated low/undetected basal levels of phosphorylation of EGFR in PC-3 cells, and when stimulated with EGF resulted in dramatically increased the levels of EGFR phosphorylation at Tyr845, Tyr1045, and Tyr1068. Carmustine alone treatment did not have any effect on EGFR phosphorylation. Whereas, selenite alone or carmustine+ selenite treatment dramatically reduced EGF induced EGFR phosphorylation and markedly decreased total EGFR protein expression levels.

As Akt and ERK1/2 signaling downstream of EGFR plays an important role in survival and proliferation of prostate cancer cells, we next determined whether Akt and ERK1/2 activation was altered by combination treatment (Fig. 4B). Western blot analysis demonstrated basal levels of p-Akt and low/undetected basal levels of p-ERK1/2 in PC-3 cells, and when stimulation with EGF resulted in a significantly increased levels of Akt and ERK1/2 phosphorylation in PC-3 cells. Carmustine alone did not decrease the phosphorylation, but selenite alone markedly decreased AKT and ERK1/2 phosphorylation. However, combination treatment completely abolished both Akt and ERK1/2 phosphorylation. Total Akt and total ERK protein expression levels were also dramatically decreased in combination treatment. This data confirms that combination treatment inhibits EGF-induced EGFR mediated Akt and ERK1/2 growth signaling pathways in PC-3 cells.

Akt regulates transcriptional activity of the NF-κB by inducing phosphorylation and degradation of IκB-α leading to its dissociation from NF-κB. Once activated, NF-κB translocates to nucleus and regulates transcriptional activities of survival genes [Bai et al., 2009]. We determined the effect of EGF on IκB-α phosphorylation (Fig. 4B) by Western blot analysis. As expected EGF treatment increased IκB-α phosphorylation. selenite partially decreased phosphorylation of IκB-α, while carmustine did not affect IκB-α. Whereas, combination completely prevented IκB-α phosphorylation in EGF stimulated PC-3 cells. These data correlated very well with decreased viability and growth in PC-3 cells treated with the combination of carmustine and selenite.

EFFECT OF AG1478 OR COMBINATION AGENTS ON EGF-INDUCED EGFR DEPENDENT ACTIVATION OF AKT AND ERK SIGNALING IN PC-3 CELLS

To confirm that Akt and ERK1/2 activation is dependent on EGF-induced phosphorylation of EGFR, the effect of AG1478, a specific inhibitor of EGFR, on EGF activated EGFR, Akt, ERK1/2, cyclin-D1 and cell proliferation were investigated. The effect of combination agents was compared against the effect of AG1478. The PC-3 cells were treated with or without 10 μM AG1478 for 48 h followed by 50 ng/ml EGF for 5 min. The cells lysates were then subjected to western blot analysis (Fig. 5A). Western blot analysis demonstrated low/undetected basal levels of phospho-EGFR (Tyr845, Tyr 1045 and Tyr 1068) protein expression. Treatment of PC-3 cells with AG1478 completely reduced the basal and EGF-induced EGFR

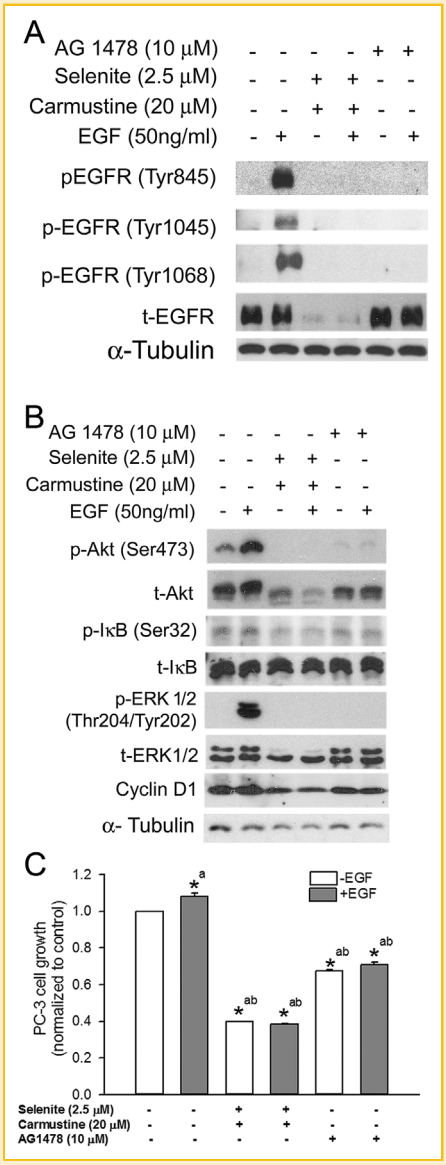

Fig. 5. Combination of carmustine and selenite or AG1478 inhibited EGFR activation and EGFR mediated activation of Akt/NF-kB and ERK1/2 signaling mediated growth in PC-3 cells. (A) The PC-3 cells were treated with or without the combination of 20 μM carmustine and 2.5 μM selenite or AG1478 (10 μM) for 48 h. EGF (50 ng/ml) was added 5 min before the termination of the experiment. Cell lysates from each sample were used for Western blot analyses performed as described in the Materials and Methods section with antibodies directed against pEGFR, tEGFR. (B) The PC-3 cells were treated with 20 μM carmustine and/or 2. μM selenite in the presence or absence of AG1478 (10 μM) for 48 h. EGF (50 ng/ml) was added 5 min before the termination of the experiment. Cell lysates from each sample were used for Western blot analyses performed as described in the Materials and Methods section with antibodies directed against pEGFR, tEGFR, pAKT, tAkt, plkBα, tlkBα, pERK1/2, and tERK1/2. α-tubulin was used as a loading control. A typical Western blot from 1 of 3 experiments is shown. (C) The PC-3 cells were treated with or without the combination of 20 μM carmustine and 2.5 μM selenite or 10 μM AG1478 in the presence or absence of 50 ng/ml EGF for 48 h. Cell growth was determined as described in method section. Data were normalized to the control and presented as the mean ± SD (*$P < 0.05$; $n = 4$) of viable cells. Comparisons shown: a: significant compared with vehicle-treated control; b: significant compared with 50 ng/ml EGF-treated cells.

phosphorylation. Similarly, combination of carmustine and selenite treatment also completely abolished p-EGFR protein expression in PC-3 cells stimulated with EGF. Although AG1478 dramatically reduced phospho-EGFR expression, the total EGFR was not affected by AG1478 treatment. However, the combination treatment of carmustine and selenite greatly reduced total EGFR protein expression levels both in EGF treated and untreated cells.

Even though, treatment of PC-3 cells with AG1478 completely reduced the basal and EGF stimulated EGFR protein expression, the levels of p-Akt and p-IκB-α expression (Fig. 5B) were not completely reduced. These data indicate that the Akt is constitutively active in the absence of p-EGFR, and the activation is further enhanced in the presence of p-EGFR. AG1478 or combination of carmustine and selenite completely abolished the phosphorylation of ERK1/2 (Fig. 5B) in EGF stimulated PC-3 cells. In addition, combination treatment dramatically decreased total Akt and total ERK1/2 protein expression levels when compared to that of AG1478 treatment.

Since overexpression of cyclin-D1 promotes tumor cell growth [Yamamoto et al., 2006] we determined the effect of combination treatment or AG1478 on cyclin-D1 protein expression by western blotting. As shown in Fig. 5B, cyclin D1 protein expression was not changed in basal and EGF stimulated PC-3 cells. AG1478 treatment partially reduced cyclin D1 whereas combination of carmustine and selenite treatment substantially reduced cyclin D1 when compared to AG1478 treatment in EGF stimulated PC-3 cells.

To confirm whether inhibition of EGFR signaling and its downstream Akt and ERK1/2 phosphorylation resulted in growth inhibition, the PC-3 cells were treated with the combination of 20 μM carmustine and 2.5 μM selenite or 10 μM AG1478 in the presence or absence of 50 ng/ml EGF for 48 h. The cell proliferation was determined by MTT assay (Fig. 5C). Treatment of PC-3 cells with AG1478 significantly reduced proliferation of both untreated control ($67.61 \pm 0.4\%$) and EGF stimulated ($70.80 \pm 1.3\%$) cells. Similarly, combination of carmustine and selenite treatment dramatically reduced proliferation of both untreated control ($39.72 \pm 0.3\%$) and EGF stimulated ($38.30 \pm 0.5\%$) cells and the decrease was greater when compared to that of AG1478 treated cells. These data indicate that the combination of carmustine and selenite decreased cell growth by inhibiting EGFR and EGFR mediated activation of Akt/NF-kB and ERK1/2 signaling in AIPC cells.

COMBINATION OF CARMUSTINE AND SELENITE TREATMENT INHIBITS EGFR MEDIATED GROWTH SIGNALING VIA ROS GENERATION

Since, our previous study demonstrated that the combination of carmustine and selenite-induced ROS triggers apoptosis in 22Rv1 and PC-3 cells [Thamilselvan et al., 2012, 2016], in the present study we determined whether ROS generated by the combination of carmustine and selenite can inhibit EGF activated EGFR and its downstream signaling in PC-3 cells (Fig. 6). The PC-3 cells were treated with 20 μM carmustine and 2.5 μM selenite in the presence or absence of 10 μM MnTMPyP for 48 h. The cells were then stimulated with 50 ng/ml EGF for 5 min. The cells lysates were subjected to Western blot analysis. As shown in Figure 6, combination of carmustine and selenite treatment dramatically reduced EGF-induced p-EGFR (Tyr845 and Tyr 1068), p-Akt, and p-ERK1/2. It is intriguing to note that the decreased levels of these phospho-proteins observed in the combination of carmustine and selenite treatment was completely restored to EGF treated control levels by MnTMPyP treatment. MnTMPyP alone did not have any effect on the expression of EGF induced p-EGFR (Tyr845 and Tyr 1068), p-Akt, and p-ERK1/2. These data indicate that the combination of carmustine and selenite treatment inhibited EGFR and its downstream growth signaling via generation of ROS.

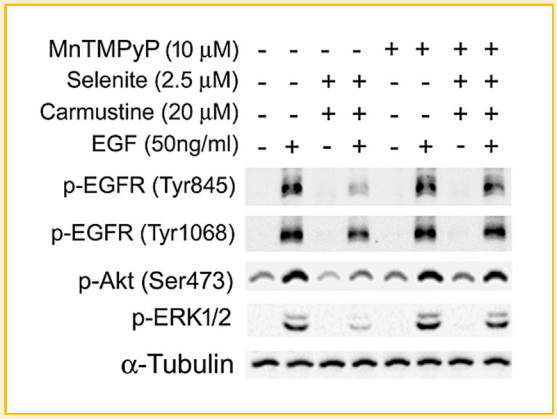

Fig. 6. ROS scavenger completely restores downregulated EGFR and its downstream signaling proteins in combination of carmustine and selenite treated PC-3 cells. PC-3 cells were treated with 20 μM carmustine and 2.5 μM selenite in the presence or absence of MnTmPyP (10 μM) for 48 h. EGF (50 ng/ml) was added 5 min before the termination of the experiment. Control experiment without EGF was also included. Cell lysates from each sample were used for Western blot analyses was performed as described in the Materials and Methods section with antibodies directed against pEGFR, pAKT, pERK1/2. α-tubulin was used as a loading control. A typical Western blot from 1 of 3 experiments is shown.

DISCUSSION

The major obstacle for the treatment of advanced prostate cancer is the development of resistance to androgen deprivation therapy leading to androgen independent prostate cancer due to activation multiple signaling pathways including signaling mechanisms independent of AR, as we now called as castration resistant prostate cancer (CRPC). During androgen withdrawal, EGFR plays a critical role in tumor growth, and the prostate cancer tissue becomes more susceptible to the growth-promoting action of EGF family of growth factors. Elevated expressions of both EGFR and its ligands have been reported in prostate cancer tumor tissues [Traish and Morgentaler, 2009]. It has been reported that the increased expression of EGFR was observed in 75.9% of the patients who received ADT before radical prostatectomy, when compared to EGFR expression observed in 41.4% of the patients who received radical prostatectomy as a first line therapy. Whereas, the increased expression was observed in 100% of the patients with metastatic castration resistant prostate cancer, suggesting that the EGFR expression was increased when the disease progresses from androgen dependent to castration resistant

disease [Di Lorenzo et al., 2002]. Given the frequency at which elevated EGFR signaling is present in tumors progressed from androgen responsive tumors to castration resistant tumors, EGFR is one of the major target for therapeutic approach.

In the present study, our data demonstrates that the combination of carmustine and selenite downregulates EGFR by receptor dephophorylation and degradation and inhibits cell growth and survival in AR-negative prostate cancer cells. In addition, combination blocks EGFR mediated downstream growth signaling cascades including Akt, ERK1/2, and NF-kB signaling pathways via increased ROS generation.

Binding of EGF to EGFR activate Akt via activation of PI3K and the PTEN is the negative regulator of PI3K/Akt signaling pathway. Studies have shown that long-term ADT for prostate cancer resulted in constitutively activated PI3K/Akt pathway, and progression of prostate cancer to androgen-independent state, with ~33% of surgically removed human prostate tumors showed weekly expressed or loss of PTEN [Dreher et al., 2004; Pfeil et al., 2004]. Thus, loss of PTEN results in increased activation of Akt and causes resistance to EGFR targeted chemotherapeutic agents [Festuccia et al., 2005], and EGFR inhibitor was shown to be effective only in prostate cancers with intact PTEN. In the present study, combination of carmustine and selenite completely inhibited the activation of Akt in PTEN null PC-3 cells. We have previously demonstrated that the combination of carmustine and selenite dramatically inhibited Akt signaling in PTEN positive 22Rv1 and VCaP cells [Thamilselvan et al., 2016]. Therefore, the present data demonstrates that the combination agent abolishes Akt activation regardless of the presence or absence of a functional PTEN.

Activated Akt regulates the NF-kB, a cell survival factor via phosphorylation and activation of molecules in the NF-kB pathway [Ozes et al., 1999]. NF-kB is activated by TNF-α, EGF, UV radiation [Thanos and Maniatis, 1995]. Activated NF-kB is translocated to nucleus and promotes the expression of several antiapoptotic genes including Bcl-2 and survivin [Mimeault et al., 2012]. In the present study, suppression of NF-kB activation by combination of carmustine and selenite indicates a strong inhibition of Akt mediated oncogenic transformation. Similarly, others have shown that curcumin downregulates transcription factors necessary for cell growth through modulation of NF-kB and PI3K/Akt pathways [Reuter et al., 2008] and suppresses tumor growth in murine melanoma by selectively downregulates catalytic subunit of IKK [Yang et al., 2006; Godwin et al., 2013].

ERK1/2 is a key signaling molecule regulates proliferation, differentiation, survival, and migration through the phosphorylation of phosphatases, transcription factors, and cytoskeletal proteins [Dhillon et al., 2007]. Elevated levels of MAPK was detected in recurrent prostate tumors, and growth factor signaling can regulate androgen responsive genes via AR dependent and androgen independent mechanism [Gioeli et al., 1999]. Our previous findings and current findings demonstrates that combination of carmustine and selenite dramatically decreased cellular and secreted PSA in CRPC and inhibition of ERK1/2 signaling in AIPC cell lines [Thamilselvan et al., 2016]. Consistent with our findings, inhibition of ERK1/2 activation has been shown to prevent cell proliferation induced by EGFR in PC-3 and LNCaP cells [Guo et al., 2000]. ERK1/2 can also promote cell survival by phosphorylating MCl-1 and enhancing its anti-apoptotic activity, and inhibition of ERK1/2 causes downregulation of MCl-1 [McCubrey et al., 2007] which is in agreement with our previous findings that combination of carmustine and selenite decreased Bcl-1 and Mcl-1 in PC-3 cells. MEK1/2 and ERK inhibitors has been shown to potentiates Tyrphostin AG825 and radiation induced apoptosis in androgen-independent prostate cancer cells [Murillo et al., 2001]. In addition, ERK activation is required for the induction of immediate-early genes and the induction and maintenance of the increased expression of cyclin D1 which play an essential role in driving cell-cycle progression. Thus growth factor stimulated ERK activation may function to ensure G1 phase progression by upregulating proliferation promoting genes and downregulating antiproliferative genes [Yamamoto et al., 2006]. In the present study, downregulation of cyclin D1 by the combination correlated with decreased EGFR mediated growth signaling and increased apoptosis in AIPC cells.

Current androgen and AR blockers do not provide a clinical benefit for patients with CRPC due to activation of multiple growth signaling mechanisms including mechanisms independent of AR. Additionally studies have shown that EGFR expression was increased in enzalutamde resistant and docetaxel resistant CRPC tumors and cell lines [Hour et al., 2015; Shiota et al., 2015]. In our previous studies we have demonstrated that the combination of carmustine and selenite inhibits cell growth and induces apoptosis by downregulating AR and AR-variants via increased generation ROS in AR-positive prostate cancer cells [Thamilselvan et al., 2016]. Similarly, in the present study, combination of carmustine and selenite inhibited cell growth and induces apoptosis by

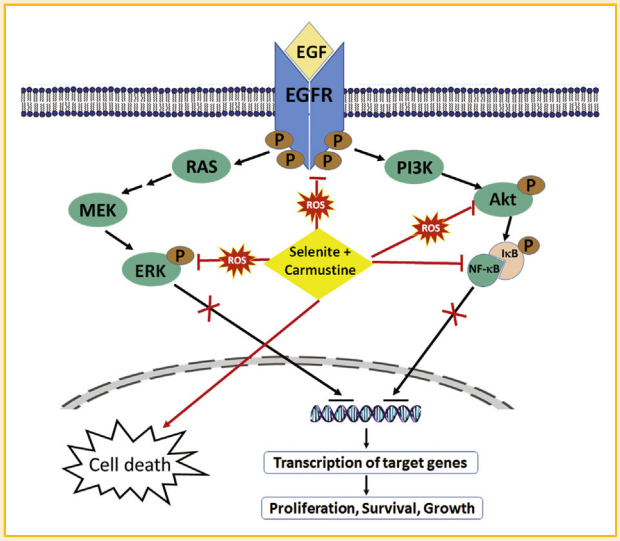

Fig. 7. Schematic model depicting the mechanism by which combination of carmustine and selenite inhibits EGFR signaling and induce apoptosis via ROS generation in PC-3 cells. Combination treatment inhibits phosphorylation of EGFR and suppressing downstream Akt/NF-kB and ERK1/2 cell survival and growth signaling mechanisms. Stimulatory signaling events are indicated in black lines with black arrow. Inhibitory mechanisms are indicated in red lines with a block at the end.

downregulating EGFR mediated growth signaling via induction of ROS in AR-negative prostate cancer cells. A diagrammatic overview of EGFR and its downstream PI3K/Akt/NF-kB and Ras/Raf/MEK/ERK signaling pathway of cell proliferation and its inhibition by carmustine and selenite via ROS generation in AR-negative PC-3 cells is presented in Figure 7.

In conclusion, the present study demonstrates that combination of carmustine and selenite induce apoptosis and inhibit cell proliferation by downregulating EGFR and its downstream PI3K/Akt/NF-kB and ERk1/2 signaling pathways in androgen independent, AR-negative prostate cancer cells. However, additional experiments are needed to establish the precise mechanisms of action of combination agents. Combination treatment completely abolished both constitutively activated and EGFR activated Akt. Given these profound results, further preclinical studies (in vitro and in vivo) on the mechanisms of action are warranted. Taken together, our findings suggest that the combination of carmustine and selenite shows a promising chemotherapy for successful treatment, improved survival, and better quality of life for patients with castration resistant prostate cancer. The results from this study further suggest that the combination agents can be effective in clinical situations involving several other cancers that are resistant to conventional chemotherapy due to increased EGFR mediated growth signaling.

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