Activating Stress-Activated Protein Kinase–Mediated Cell Death and Inhibiting Epidermal Growth Factor Receptor Signaling: A Promising Therapeutic Strategy for Prostate Cancer

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Abstract
Epidermal growth factor receptor (EGFR) activation is an important event that regulates mitogenic signaling, such as the Raf, mitogen-activated protein kinase (MAPK), and extracellular signal–regulated kinase 1/2 cascades. EGFR activation has been implicated in the transition of prostate cancer from androgen dependence to independence. Therefore, inhibition of EGFR may effectively suppress prostate cancer growth and progression. The goal of this study was to determine whether the natural compound psoralidin alters EGFR-mediated signaling resulting in the inhibition of prostate cancer growth. Results suggest that inhibition of EGFR alone (by serum deprivation) fails to induce stress-mediated protein kinases (SAPK), namely, Jun NH2-terminal kinase/c-Jun signaling, in androgen-independent prostate cancer (AIPC) cells. Treatment with psoralidin, however, inhibited both constitutive and EGF-induced EGFR activation and simultaneously triggered SAPK signaling, resulting in the induction of apoptosis in AIPC cells. In addition, psoralidin downregulated EGFR-regulated MAPK signaling and inhibited cell proliferation in AIPC cells. Oral administration of psoralidin effectively suppressed PC-3 xenograft tumors in nude mice. Compared with control tumors, inhibition of pEGFR expression and an increase in the phosphorylation, activation, and nuclear translocation of c-Jun were observed in psoralidin-treated tumor sections. Our studies suggest that psoralidin may be a potent therapeutic agent that modulates EGFR-mediated key epigenetic events in AIPC.

Introduction
Epidermal growth factor receptors (EGFR) play an important role in the pathogenesis of prostate cancer and in the transition of prostate cancer from an androgen-dependent to an androgen-independent state (1). One of the major alterations in androgen-independent prostate cancer (AIPC) is the overexpression of EGFR and its cognate ligand EGF (2); EGFR is frequently overexpressed in prostate cancer tumors, especially in patients treated with hormone therapy (3). Hence, inhibition of EGFR signaling could be a promising approach for the treatment of prostate cancer.

In response to EGF and/or various growth factors, EGFR regulates many signaling pathways, including the mitogen-activated protein kinase (MAPK) and the stress-activated protein kinase (SAPK) cascades (4). The MAPK pathway plays a pivotal role in the molecular network that governs cell growth, proliferation, differentiation, survival, and apoptosis. The effects of MAPK signaling are based on the stimuli and cell type (5). In normal physiologic conditions, MAPK is tightly regulated. However, activation of the MAPK pathway is often associated with an increased prostate cancer Gleason score and tumor stage (6). The MAPK superfamily is divided into three subgroups, namely, the extracellular signal–regulated kinase (ERK), c-Jun NH2-terminal protein kinase (JNK), and p38. EGFR, either through MAP/ERK kinase (MEK) or directly, can phosphorylate and activate ERK (7), which in turn regulates growth, proliferation, the cell cycle, and apoptosis in various cell types. The p38 MAPK is well known for its function as a tumor suppressor because of its involvement in the inhibition of cell proliferation and cell cycle progression, induction of the mitochondrial apoptotic pathway, and regulation of oncogene-induced senescence in many cancer types (8, 9). These studies suggest a dual role for MAPK signaling where, based on the external stimuli, the fate of a cell can be determined.

JNKs belong to the MAPK family of proteins. Based on cell type and stimuli, JNK signaling pathways are
important for regulating both cell proliferation and apoptosis (10, 11). Several external stimuli, such as cytokines, stress factors, and agents that induce genotoxic and cytotoxic stress, have been known to activate JNKs (12). In response to these stimuli, activated JNKs initiate one or both of the following downstream signaling: a nuclear event and/or a mitochondrial event. In the nucleus, activated JNK gains entry into the nucleus where it transactivates c-Jun and other transcription factors to increase expression of proapoptotic genes (13). In the mitochondria, activated JNK translocates to the mitochondria and antagonizes the antiapoptotic function of Bcl-2 and Bcl-xL. In addition, JNK enhances the release of cytochrome c, thereby initiating the caspase cascade (14). Eventually, JNK signaling leads to the induction of apoptosis in many cancer types. Thus, modulating the activity of JNKs may be relevant to cancer therapy.

Treatment of AIPC is often challenged because of the inability to achieve complete remission of the tumor. As a result, the development of new therapeutic strategies is essential. Psoralidin is a natural compound isolated from the leaves of *Psoralea corylifolia*. Psoralidin is extensively used in traditional medicine for the treatment of various ailments. We previously reported that psoralidin inhibits the phosphatidylinositol-3 kinase (PI3K)/Akt pathway leading to the inhibition of cell proliferation and the induction of apoptosis in AIPC cells (15). In the present study, we determine whether psoralidin inhibits EGFR signaling, which in turn may suppress AIPC growth and progression. Our results suggest that psoralidin inhibits EGFR-mediated signaling events and induces SAPK-mediated apoptotic signaling. Psoralidin treatment thereby leads to the induction of apoptosis in AIPC cells and the inhibition of tumor growth in xenograft models.

Materials and Methods

**Cell lines and plasmids**

PC-3, DU-145, LNCaP, and C4-2B cells were purchased from the American Type Culture Collection. The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% glutamine, and antibiotics. Psoralidin was isolated and purified (99.8%) in Dr. Rohr’s laboratory at the University of Kentucky. EGF and JNK inhibitor (SP600125) were purchased from either Cell Signaling Technology, Santa Cruz Biotechnology, or R&D Systems.

**Western blot analysis**

PC-3, DU-145, LNCaP, and C4-2B cells (at 70–80% confluency) were grown either in complete medium or were serum deprived for 48 hours. Whole cell lysates were obtained and Western blot analysis was done using pEGFR (Tyr 1173) and EGFR antibodies. PC-3 and DU-145 cells were grown in complete medium and were treated with the IC50 of 60 and 45 μmol/L psoralidin, respectively. Cells were also serum deprived for 48 hours and stimulated with EGF (100 ng/mL) alone or treated concurrently with EGF and psoralidin for 30 and 60 minutes with the above mentioned concentrations. Whole cell lysates were subjected to Western blot analysis as described previously (16) using pEGFR (Tyr 1173), EGFR, Raf1, MEK-1, MEK4, MEKK-1, pMEK1/2, pMEK3/6, ERK, pERK1/2, pElk (ser 383), Elk, pJNK1/2 (p54, p46), JNK 1/2, pc-Jun (ser 73), c-Jun, Bcl-2, Bax, caspase-3, and survivin antibodies purchased from either Cell Signaling Technology, Santa Cruz Biotechnology, or R&D Systems.

**Kinase assay**

PC-3 and DU-145 cells (at 70–80% confluence) were treated with vehicle (DMSO) or psoralidin for 24 hours. Cell lysates were immunoprecipitated using an ERK antibody or c-Jun fusion beads, and ERK or JNK kinase assays were respectively done as previously described (17) using ERK and JNK kinase kits purchased from Cell Signaling Technology.

**Transient transfection and promoter assays**

PC-3 (at 80–90% confluence) were transiently transfected using Lipofectamine Plus reagents (Life Technologies) with 4 μg of either c-Jun promoter-luciferase construct or control vector (pGL3.4) containing Renilla luciferase to normalize transfection efficiency, and promoter analysis was done as described previously (18).

**Apoptosis assay**

PC-3 and DU-145 cells (at 70–80% confluence) were treated with the above mentioned concentrations of psoralidin or vehicle (DMSO) in the absence or presence of 5 μmol/L of a JNK inhibitor. An apoptotic assay (Annexin V-FITC) was done as described earlier (19, 20). Tumor sections from control and psoralidin-treated animals were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay as described previously (15).

**Xenograft studies**

The effect of oral administration of psoralidin on PC-3 xenografts was tested in accordance with the University of Kentucky Animal Care and Use Committee guidelines. In our pilot studies we orally administered various doses (25, 50, or 100 mg/kg body weight) of psoralidin to PC-3 xenograft animals and determined that 50 mg/kg body weight psoralidin was the optimal dose that suppresses prostate cancer growth with no toxicity to the study animals (data not shown). Tumor injection, measurement of tumor size, and growth inhibition studies were done as described previously (15, 18). Briefly, PC-3 cells (1 × 106) were s.c. injected into 5- to 6-week-old male nude (nu/nu) mice from Harlan laboratories. The tumors were allowed to reach a 50 mm3 volume and the animals were randomized into two groups with 10 animals each. Psoralidin was dissolved in sunflower oil. The animals in one group were orally fed with sunflower oil alone whereas the others received 50 mg/kg body weight.
psoralidin for 4 weeks. Tumor volume in the animals was monitored and measured daily for the entire study period.

**Immunohistochemical analysis**
The effect of psoralidin on activated levels of EGFR and c-Jun in PC-3 xenografts was assessed by immunohistochemical analysis using pEGFR (Tyr 1173) and pc-Jun antibodies as described previously (16).

**Statistical analysis**
ANOVA was used to calculate statistical significance between the samples. Densitometry analysis was carried out using UN-SCAN-gel software.

**Results**

**Basal expression of EGFR in androgen-dependent and androgen-independent prostate cancer**
EGFR is highly expressed in prostate cancer (2, 3); hence, we intended to determine the basal levels of activated (phospho) EGFR in prostate cancer cells lines (PC-3, DU-145, LNCaP, and C4-2B). PC-3 and DU-145 cells had higher basal expression of pEGFR compared with LNCaP and C4-2B (Fig. 1A). In serum-starved conditions, a significant decrease in pEGFR expression was seen (Fig. 1A), but we found that serum starvation alone failed to increase the expression of JNK or c-Jun in both PC-3 and DU-145 cells (Fig. 1B). Next, to establish EGF-induced EGFR signaling; serum-starved AIPC cells were stimulated with EGF, and pEGFR expression was studied. These results collectively suggest that although serum starvation decreases pEGFR expression, it fails to induce the expression of stress-mediated JNK/c-Jun in AIPC cells. In addition, we found that treatment of serum-starved AIPC cells with EGF caused a rapid induction of pEGFR expression at 60 minutes following stimulation (Fig. 1C), suggesting that EGFR signaling can be modulated in AIPC cells.

**Psoralidin downregulates EGF-induced EGFR signaling in AIPC**
Next, we determined whether psoralidin inhibits EGFR signaling in AIPC cells. Previously we published that psoralidin, a natural compound, inhibits the growth of AIPC cells with IC50 values of 60 μmol/L in PC-3 and 45 μmol/L in DU-145 cells. Our results show that psoralidin inhibits EGF-induced pEGFR expression without altering total EGFR levels in both PC-3 and DU-145 cells (Fig. 2A). Upon stimulation by EGF, EGFR may activate the downstream Ras-dependent ERK/MAPK pathway (21). Thus, we analyzed the expression pattern of the...
MAPK family of proteins. We observed that EGF significantly induced the expression of MEK-4 and pMEK-1/2 in PC-3 and DU-145 cells when compared with Raf, MEK-4, and MEKK-1. This EGF-induced activation of MAPK (MEK-4 and pMEK-1/2) was overcome by psoralidin (Fig. 2B). Interestingly, in AIPC cells grown in complete medium psoralidin downregulated the expression of Raf-1, MEK-4, MEKK-1, and pMEK1/2 within 60 minutes (data not shown).

Next we found that EGF induced high expression of pERK but no significant change in pElk expression in PC-3 cells. On the other hand, in DU-145 cells, EGF did not change expression of pERK but significantly increased expression of pElk (Fig. 2C). We also noted that in EGF-induced cells, psoralidin downregulated pERK and pElk-1 expression at the earlier time points (30 and 60 minutes) in DU-145 but not in PC-3 cells (Fig. 2C).

Psoralidin induces SAPK (JNK/c-Jun) and inhibits EGF-mediated survival pathways in AIPC

Recent studies suggest that SAPKs induce apoptosis in prostate cancer cells (22, 23). Based on this, we investigated the effect of psoralidin on SAPK-mediated proapoptotic signaling pathway in AIPC cells. EGF treatment reduced the expression of pJNK and pc-Jun in both PC-3 and DU-145 cells. However, psoralidin restored this EGF-mediated inhibition in both AIPC cells (Fig. 3A). Previous studies had reported that EGFR induced prosurvival signaling by inducing expression of survivin and Bcl-2, resulting in the suppression of apoptosis (24); hence, we determined whether psoralidin overcomes EGF-mediated prosurvival signaling in AIPC cells. Our results suggest that psoralidin inhibited EGF-induced survivin and Bcl-2, and upregulated expression of Bax and cleaved caspase-3 in both EGF-induced AIPC cell lines (Fig. 3B). These results suggest that psoralidin induces SAPK-mediated apoptotic signaling and simultaneously overcomes EGF-mediated survival signaling in AIPC.

Inhibition of JNK/c-Jun partially abrogates psoralidin-mediated apoptosis in AIPC cells

Because psoralidin induces expression of pJNK and pc-Jun, we studied whether treatment with psoralidin induces JNK kinase activity. We found a significant increase in JNK kinase activity (an approximately 4.5-fold and 3-fold increase in PC-3 and DU-145 cells, respectively) in psoralidin-treated AIPC cells when compared with control (Fig. 4A). These results suggest that psoralidin increases JNK kinase activity and thereby activates downstream c-Jun in AIPC cells. Next, considering the effect of psoralidin on JNK/c-Jun, we determined whether inhibition of JNK kinase abrogates psoralidin-mediated effects...
on AIPC cells. As expected, pharmacologic inhibition of JNK significantly decreased pJNK expression (data not shown) and psoralidin-mediated apoptosis in PC-3 cells, but no significant difference in apoptosis was observed in DU-145 cells (Fig. 4B). Similarly, inhibition of c-Jun using siRNA significantly abrogated the ability of psoralidin to induce apoptosis in AIPC cells (data not shown). Additionally, we found that psoralidin mediates its function by regulating the c-Jun promoter in AIPC cells (Fig. 4C). These results collectively suggest that psoralidin mediates its apoptotic function, at least in part through the JNK/c-Jun pathway. Thus, inhibition of the JNK/c-Jun pathway interferes with psoralidin-mediated apoptosis in AIPC cells.

Figure 3. Psoralidin overcomes EGF-mediated inhibition of SAPK signaling and modulates proapoptotic machinery in AIPC cells. A, serum-deprived PC-3 and DU-145 cells were stimulated with 100 ng/mL of EGF and treated concurrently with psoralidin for 30 or 60 minutes. Whole cell lysates were subjected to Western blot analysis using pJNK1/2, JNK1/2, pc-Jun, and c-Jun antibodies. B, PC-3 and DU-145 cells were serum deprived for 48 hours, stimulated with 100 ng/mL of EGF, and treated concurrently with EGF and psoralidin for up to 12 hours. Whole cell lysates were subjected to Western blot analysis using survivin, Bcl-2, Bax, and cleaved caspase-3 antibodies. β-Actin was used as the internal loading control.

Figure 4. Psoralidin increases JNK kinase activity and potentiates JNK-mediated apoptosis in AIPC cells. A, PC-3 and DU145 cells were treated with 60 and 45 μmol/L (IC50) psoralidin respectively for 24 hours. Whole cell lysates were subjected to immunoprecipitation (IP) using c-Jun fusion beads. JNK kinase activity was assayed by Western blot analysis using a pc-Jun antibody (top). Bars, fold increase in JNK kinase activity following the treatment with psoralidin (bottom). B, pc-Jun and c-Jun antibodies. C, PC-3 cells were transfected with the c-Jun promoter-luciferase reporter construct, and renilla control vector (pGL3.4) was used to normalize transfection efficiency followed by treatment with psoralidin or vehicle. Luciferase reporter assay was done to determine c-Jun promoter activity. Bars, fold change in c-Jun promoter activity ± SD.
Psoralidin inhibits tumor growth in PC-3 xenografts

We had previously reported that intratumoral injection of psoralidin inhibits prostate cancer growth in nude mouse models (20). In our pilot studies we orally administered various doses (25, 50, or 100 mg/kg body weight) of psoralidin to PC-3 xenograft animals and determined that 50 mg/kg body weight psoralidin was the optimal dose that suppresses prostate cancer growth with no toxicity to the study animals. As seen in Fig. 6A, oral administration of psoralidin (50 mg/kg), five days a week for four weeks, significantly suppressed further PC-3 tumor growth compared with control animals. Moreover, we observed the animals and monitored tumor sizes up to two weeks after termination of psoralidin treatment. We found that the tumors in psoralidin-treated animals failed to regrow. Gross pathology and histopathology studies revealed no significant toxicity in psoralidin-treated animals (data not shown). Immunohistochemical analysis of psoralidin-treated tumor tissues showed a significant reduction in pEGFR expression, and induced and increased nuclear entry of pc-Jun (Fig. 6B). We also found a significant increase in the number of apoptotic cells in psoralidin-treated tumor sections when compared with control tumor sections (Fig. 6B).

Figure 5. Psoralidin inhibits EGFR-mediated MAPK and induces SAPK signaling in AIPC cells grown in complete medium. PC-3 and DU145 cells were grown in complete medium containing 10% fetal bovine serum and treated with 60 and 45 μmol/L (IC50) psoralidin for up to 12 hours. Whole cell lysates were subjected to Western blot analysis using pEGFR and EGFR antibodies (A); MEK-1, MEK-4, pMEK3/6, pMEK1/2, and MEKK-1 antibodies (B); and pERK and ERK antibodies (C, top). C, PC-3 and DU145 cells were treated with 60 and 45 μmol/L psoralidin for 24 hours. Whole cell lysates were subjected to immunoprecipitation using ERK antibody. ERK kinase activity was assayed by Western blot analysis using a pElk-1 antibody. Total ELK-1 antibody was used as control (bottom). D, PC-3 and DU145 cells were grown in complete medium containing 10% fetal bovine serum and treated with 60 and 45 μmol/L psoralidin for up to 12 hours. Whole cell lysates were subjected to Western blot analysis using pJNK1/2, JNK1/2, pc-Jun, and c-Jun antibodies. β-Actin was used as the internal loading control.
These results collectively suggest that psoralidin effectively suppresses prostate tumor growth through the inhibition of EGFR-mediated survival signaling with a simultaneous induction of SAPK-mediated apoptotic events.

**Discussion**

Overexpression of EGFR plays an important role in the pathogenesis of prostate cancer (25) and is correlated with Gleason score and androgen independence (26). In this study we show that psoralidin, a natural compound, effectively alters EGFR-mediated signal transduction pathways and inhibits prostate tumor growth in an *in vivo* model.

EGFR signaling, being one of the major signaling cascades in prostate cancer, has been the focus of growing interest in therapeutic targeting in both the laboratory and clinical trials (27). Our results suggest that AIPC cells (PC-3 and DU-145) express high levels of phosphorylated EGFR compared with LNCaP and C4-2B cells. Although serum deprivation significantly inhibits pEGFR expression, it fails to trigger SAPK-mediated apoptotic signaling in prostate cancer cells. Similar results were observed by other investigators, with inhibition of EGFR by gefinitib failing to significantly inhibit cell viability in cancer models (28). These studies imply that EGFR inhibition alone may be insufficient and that a secondary signal may be required to initiate apoptosis in prostate cancer cells.
In response to EGF and/or various growth factors, EGFR regulates several signaling pathways, including PI3K/Akt and MAPK/ERK1/2/ELK in AIPC (6, 29, 30). Here we show that EGF stimulation results in robust pEGFR expression within 60 minutes in both PC-3 and DU-145 cells. We also observed a concomitant increase in the expression of several downstream of EGFR, namely, MAPK (MEK-4 and pMEK-1/2). These data suggest that EGFR, through EGF stimulation, governs many prosurvival signaling pathways in prostate cancer, and psoralidin overcomes the EGFR-induced prosurvival signals as was seen by a complete inhibition in the expression of prosurvival molecules downstream of EGFR. Similar observations have been made by other research groups (31).

Prostate stromal tissue produces EGF (32), which stimulates several survival pathways converging at the level of ERK. ERK is involved in cell survival or death depending on a cell type and stimuli-dependent manner (27). ERK is shown to be activated in premalignant lesions and early-stage prostate cancer (33). In our study, we found no significant change in the expression of pERK, but reduced kinase activity of ERK in AIPC as seen by a reduction in the expression of pElk in both AIPC cells. These results suggest that psoralidin not only inhibits EGFR activation but also inhibits the entire downstream prosurvival signaling of EGFR in AIPC cells.

JNK/SAPK signaling plays a dual role, both in the oncogenic process and as a tumor suppressor (11). As mentioned above, serum starvation fails to induce pJNK/p-c-Jun activation in AIPC cells. These data suggest that inhibition of pEGFR expression alone may be insufficient to induce apoptosis in AIPC cells. Psoralidin activates the JNK/c-Jun pathway in both AIPC cell lines. These results suggest that psoralidin-mediated apoptosis may be through the activation of the JNK pathway in AIPC cells. Kinase assays were done to determine the effect of psoralidin on JNK kinase activation. We observed a robust induction in JNK kinase activity in both AIPC cell lines. Additionally, we found that inhibition of JNK kinase activity using a pharmacologic inhibitor significantly reduced psoralidin-mediated apoptosis in both AIPC cell lines. Similarly, inhibition of c-Jun using siRNA also abrogated psoralidin-mediated apoptosis in AIPC cells (data not shown). These results imply that activation of JNK/c-Jun is essential at least in part for psoralidin-mediated effects on PC-3 and DU-145 cells. NF-κB inhibits JNK-mediated apoptosis (34), which correlates with our findings. We had previously reported that psoralidin inhibits Akt-mediated NF-κB activation in AIPC cells (15). Inhibition of the Akt/NF-κB pathway by psoralidin may lead to the induction of the JNK pathway and resultant apoptosis in AIPC cells. Similar studies reported that dietary compounds, like phenethyl isothiocyanate, curcumin, and epifallocatchin gallate, induced apoptosis by activating JNK-mediated cytochrome c release and caspase activation in many cancer models (35–37).

Additionally, we had previously reported that psoralidin altered the Bax/Bcl-2 ratio, induced caspase-9 and -3, and caused poly (ADP-ribose) polymerase cleavage in both AIPC cells (38). Interestingly, we found that EGF treatment induced survivin and Bcl-2 and inhibited expression of Bax and caspase-3 cleavage, and psoralidin treatment overcame these EGF-mediated effects in both AIPC cell lines.

Oral administration of psoralidin (50 mg/kg) effectively suppresses the growth of PC-3 xenograft tumors in nude mouse models. Our results show that psoralidin failed to completely regress the tumors, but it inhibited further tumor growth when compared with the control animals. The inability of psoralidin to cause tumor regression may be due to insufficient duration of psoralidin treatment in our animal studies. Similar observations were made using other dietary compounds polyphenol Epigallocatechin-3-gallate, and isothiocyanate sulforaphane (39, 40). Our immunohistochemical analysis suggests an inhibition of pEGFR expression and an increase in p-c-Jun expression and nuclear entry of p-c-Jun in psoralidin-treated tumors compared with control tumors. These findings correlate with results from our cell culture models.

In conclusion, our findings suggest that psoralidin may be a potential therapeutic agent that targets key signaling pathways in AIPC cells. Based on our data, psoralidin may provide a translational tool that may be relevant to the treatment of prostate cancer with a defined mechanism of action.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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