IKK inhibitor suppresses epithelial-mesenchymal transition and induces cell death in prostate cancer

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Abstract. IkB kinase (IKK)/nuclear factor kB (NF-kB) pathway activation is a key event in the acquisition of invasive and metastatic capacities in prostate cancer. A potent small-molecule compound, BMS-345541, was identified as a highly selective IKKa and IKKb inhibitor to inhibit kinase activity. This study explored the effect of IKK inhibitor on epithelial-mesenchymal transition (EMT), apoptosis and metastasis in prostate cancer. Here, we demonstrate the role of IKK inhibitor reducing proliferation and inducing apoptosis in PC-3 cells. Furthermore, BMS345541 inhibited IkBo phosphorylation and nuclear level of NF-kB/p65 in PC-3 cells. We also observed downregulation of the N-cadherin, Snail, Slug and Twist protein in a dose-dependent manner. BMS-345541 induced upregulation of the epithelial marker E-cadherin and phosphorylated NDRG1 at protein level. Moreover, BMS-345541 reduced invasion and metastasis of PC-3 cells in vitro. In conclusion, IKK has a key role in both EMT and apoptosis of prostate cancer. IKK inhibitor can reverse EMT and induce cell death in PCa cells. IKK was identified as a potential target structure for future therapeutic intervention in PCa.

Introduction

Prostate cancer (PCa) is one of the most common malignancies and one of the leading causes of cancer deaths in men in the Western world. There are many therapeutic options against localized prostate cancer including prostatectomy and radiation therapy (1). However, in advanced cancer, most tumors ultimately relapse after a period of initial response to therapy and progress to metastatic cancer (2,3). It is still a clinical challenge to deal with advanced prostate cancer in patients. Unfortunately, many aspects remain unknown of the cellular and molecular mechanisms for metastatic disease (4). As is known, epithelial-mesenchymal transition (EMT) plays essential roles in development, invasion and migration of prostate cancer. EMT is characterized by loss of homotypic adhesion and cell polarity (5). The most familiar change that occurs during EMT is the downregulation of surface E-cadherin expression and increased expression of N-cadherin. Many signaling pathways, including TGF-β, Wnt, notch, PI3K/AKT, and hedgehog, have been intricately connected to the onset of EMT (6). A number of studies have reported that EMT-inducing transcription factors, such as Snail, Slug, Twist, and Zeb, are directly or indirectly involved in cancer cell metastasis through different signaling cascades and pathways (7).

Nuclear factor kB (NF-kB) signaling has been previously identified as an important pathway in the regulation of EMT in tumor progression (8). The NF-kB family is composed of five proteins, including Rel-A/p65, Rel-B, C-Rel, p52, and p50. In unstimulated cells, uninduced NF-kB dimers are restrained in the cytoplasm by complex formation with a member of the IkB family (9). On stimulation, IkB proteins are phosphorylated by the multisubunit IkB kinase (IKK) complex, subsequently ubiquitinated and degraded through the proteasomal pathway. Then, the liberated NF-kB heterodimer rapidly translocates into the nucleus, where it binds to the kB site and induces transcription of a wide variety of target genes involved in cancer development and progression (10). The IKK complex consists of IKKa/IKK1, IKKβ1/IKK2, and IKKγ/NEMO. Recently, two protein kinases called IKKe/IKKi and TBK1 (TANK-banding kinase) were identified that exhibit structural similarity to IKKa and IKKβ (11). However, it remains unclear what is the regulation relationship between upstream NF-kB activator IKK family members and EMT in prostate cancer.

Because IKK is a key molecular complex specifically regulating IkB proteins and subsequently targeting NF-kB, we speculated that IKK would be a potential therapeutic target for prostate cancer. A potent small-molecule compound, BMS-345541, was identified as a highly selective IKKa and IKKβ inhibitor to inhibit kinase activity (12). To determine whether IKK inhibitor manipulates the process of EMT and cell death, we delivered the BMS-345541 drug to human prostate cancer PC-3 cell in vitro. Here we investigated the detailed effect of IKK inhibitor on EMT, apoptosis, and metastasis in prostate cancer cells.
Materials and methods

Biological reagent. BMS-345541 (4(2’-aminoethyl) amino-1, 8-dimethylimidazo(1,2-α) quinoxaline) were obtained from Calbiochem (San Diego, CA, USA). BMS-345541 was dissolved in DMSO to produce a 50-mmol/l stock solution for experiments. All phosphospecific or total antibodies used in this study were purchased from Cell Signaling Tech (Denver, MA, USA) and Santa Cruz Biotech (Santa Cruz, CA, USA).

Cell culture. PC-3 and LNCaP cells were obtained directly from the American Type Culture Collection (Manassas, VA, USA) and Santa Cruz Biotech (Santa Cruz, CA, USA). All cell lines were grown in RPMI-1640 medium (Gibco) both supplemented 10% heat-inactivated FBS (fetal bovine serum), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, 0.2 mM glutamine, and 1 mM pyruvate and incubated at 37°C in a 5% CO2 incubator. Cells were treated with BMS-345541 in the following experiments. Cell treated with DMSO were used as controls. Each experiment was repeated three times.

Cell viability assay by MTT. The cells were seeded in a 96-well culture plate and cultured overnight. The MTT assay was used to determine cell viability. BMS-345541 was added to the cells in different time and concentration. The MTT regent (5 mg/ml) was added and the cells then incubated for a further 4 h. The reduced MTT crystals were dissolved in DMSO and the absorbance was detected on BioTek ELISA reader (Winooski, VT, USA) at 570 nm wavelength.

Cell invasion assay. The cell invasion assay was performed using Boyden chambers with 8 μm porosity polycarbonate filters coated with 50 μg/ml Matrigel solution. The cells in 24-well plates at a concentration of 5×10^4/2 incubator. - After lysis, the lysates were centrifuged for 10 min at 13,000 g at 4°C. Total protein samples (25-50 μg) were transferred onto PVDF membrane after electrophoretic separation in 12% SDS polyacrylamide gel. After blocked with 5% non-fat milk in Tris-buffered saline for 1 h at room temperature, the membranes were incubated overnight with the primary antibody at 4°C and washed three times in PBS containing 0.1% Tween-20, then incubated with the horseradish peroxidase conjugated secondary antibodies at room temperature for 1 h. The membranes were washed three times in PBS, and then developed with a horseradish peroxidase chemiluminescence detection reagent and exposed to X-ray film.

TUNEL apoptosis assay. A quantitative evaluation method was applied by using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (TdT) nick-end labelling (TUNEL) kit to examine apoptotic cells. Briefly, coverslips with adherent cells treated with BMS-345541 (5 μmol/l) for 72 h were fixed in 4% paraformaldehyde at room temperature for 30 min. Then they were rinsed in distilled water and incubated in PBS containing 0.2% Triton X-100. DNA fragments were labeled with TUNEL-enzyme (Boehringer Mannheim). The kit was used according to the manufacturer’s instructions, with the addition of incubation in TdT reaction buffer for 10 min before TUNEL reaction. The coverslips were then incubated in TdT reaction mixture for 60 min at 37°C in humidified chamber, rinsed in stop wash buffer for 10 min and washed by PBS for 3 times. The reaction was detected by incubating coverslips with streptavidin-HRP in PBS for 30 min at room temperature. Then washed by PBS 3 times, and the sections were incubated with DAB solution for 10 min.

Assessment of the apoptotic index. Positive signal was defined as the presence of a dark brown staining on the nuclei of the neoplastic cells or on apoptotic bodies as morphologically defined. Cells were defined as apoptotic if the whole nuclear area of cells labeled positively. Apoptotic bodies were defined as small, positively labeled, globular bodies in the cytoplasm of the tumor cells that could be found either singly or in groups. The apoptotic index was determined by the percentage of apoptotic cells divided by the number of tumor cells in x400 magnification. A total of ≥1,000 neoplastic nuclei were counted based on 10 randomly chosen fields at x400 magnification. Apoptotic cells were identified by TUNEL in conjunction with characteristic morphological changes, such as cell shrinkage, membrane blebbing, and chromatin condensation.

Statistical analysis. Data are expressed as mean ± standard deviation (SD) from three independent experiments. All statistical analyses were performed using the SPSS 19.0 for Windows software system. Data with two groups were analyzed by Student’s t-tests, and data with multiple groups were analyzed by one-way ANOVA. A significant difference was considered when the P-value from a two-tailed test was <0.05.

Results

BMS-345541 inhibits the growth of prostate cancer cells. Since inappropriate regulation of IKK/NF-κB correlates with prostate cancer progression, IKK inhibitor might be a
potential therapeutic agent (13). Hereby, we first assessed the effects of BMS-345541 on cell viability in PC-3 and LNCaP prostate cancer cells, respectively. PC-3 (1x10^5) and LNCaP (5x10^3) cell lines were cultured in medium with BMS-345541 at 0, 2.5, 5, 10, 15, 20 and 25 µmol/l concentrations. We then measured cell viability at different time points (8-96 h). From the results of MTT assay, the inhibition rate of prostate cancer cells treated with varying doses showed a dose-dependent increase. (B) Cells were treated with BMS-345541 at different times (8-96 h). BMS-345541 reduced cell viability in a time-dependent manner.

Figure 1. Effect of BMS-345541 on the growth of prostate cancer cells. Cell viability was assessed by MTT assay. (A) PC-3 and LNCaP cells were treated with various concentrations of BMS-345541 (0-25 µmol/l). From the results of MTT assay, the inhibition rate of prostate cancer cells treated with varying doses showed a dose-dependent increase. (B) Cells were treated with BMS-345541 at different times (8-96 h). BMS-345541 reduced cell viability in a time-dependent manner.

Figure 2. Effect of BMS-345541 on cytoplasmic level of pIkB and nuclear level of NF-κB/p65 in human prostate cancer PC-3 cells. (A) BMS-345541 dose-dependently inhibited the phosphorylation of IkBα as detected by western blotting. Cells treated with BMS-345541 for 12 h were used for preparation of cytoplasmic extracts. Actin served as the loading control. (B) BMS-345541 inhibited NF-κB/p65 as detected by immunoblotting. Cells treated with BMS-345541 for 12 h were used for preparation of nuclear extracts. LaminA/C served as the loading control.

BMS-345541 inhibited IkBα phosphorylation and nuclear level of NF-κB/p65 in PC-3 cells. BMS-345541 was identified as a selective inhibitor of the catalytic subunits of IKK (IKKβ IC_{50}=0.3 micron, IKKα IC_{50}=4 micron). It binds to similar allosteric sites on IKKα and IKKβ, which then affects the active sites of subunits differently (14). The IkBα is constitutively phosphorylated in PC-3 cells by IKK (15). To evaluate the effect of the IKK inhibitor BMS-345541 on phosphorylation of IkBα, we performed western blotting using cytoplasmic extracts from PC-3 cells treated with DMSO or BMS-345541 (final concentration, 0.5, 2.5 and 5 µM) in DMSO for 12 h. As shown in Fig. 2, treatment of PC-3 cells with 0.5, 2.5 and 5 µM doses of BMS-345541 for 12 h resulted in a significant decrease in p-IkBα level in a dose-dependent
In addition, p50/p65 dimer is considered to be the most important of NF-κB proteins, and nuclear translocation of p50/p65 is required for their transactivation potential (16). Western blot analysis of nuclear protein from PC-3 cells treated with BMS-345541 showed a dose-dependent inhibition of the NF-κB/p65 level (Fig. 2). Compared with DMSO-treated control, a 60% inhibition in NF-κB/p65 protein expression in the nucleus was observed at 10 µM dose of BMS-345541 for 12 h.

**BMS‑345541 reverses EMT in PCa cells.** Accumulated evidence suggests that prostate cancer cells can activate the process of EMT, and epithelial cells undergo multiple biochemical changes including expression of mesenchymal biomarkers, induction of angiogenesis and resistance to apoptosis (17-19). To characterize the effect of IKK inhibitor on EMT, we used BMS-345541 with varying concentration in PC-3 cells. As shown in Fig. 3, BMS-345541 induced upregulation of the epithelial marker E-cadherin at protein level. Nevertheless, we observed downregulation of the N-cadherin, Snail, Slug and Twist proteins in a dose-dependent manner.

N-myc downstream-regulated gene 1 (NDRG1) is a potent metastasis suppressor that has been demonstrated to inhibit the TGF-β-induced EMT in prostate cancer cells (20). To elucidate the molecular role of NDRG1 in EMT and the IKK inhibitor effect on NDRG1, immunoblot analysis was used to measure the level of phosphorylation of NDRG1 in PC-3 treated by BMS-345541. There was a significant increase in phosphorylated NDRG1 by blocking IKK in a dose-dependent manner (Fig. 3).

**BMS‑345541 decreases invasion and metastasis of PC‑3 cells in vitro.** We investigated whether BMS-345541 has an impact on invasion and migration of PC-3 cells, which is usually associated with the propensity to metastasis. The invasion ability of PC-3 cells was assessed by transwell invasion assay, and BMS-345541 significantly decreased the cell invasion through matrigel-coated filters by 40% at 24 h (Fig. 4). We further examined the cell migration by wounding cells plated on the cell culture plates. The result of wound healing assay showed that PC-3 cells treated by BMS-345541 were unable to recolonize the denuded zone as fast as the control cells did at 48 h after removal (Fig. 5).

The above results demonstrate that IKK inhibitor exerts a control on the invasion and migration capacities of prostate cancer cells and suggests that, besides its effect on the cell proliferative rate, it could also contribute to both tumor aggressiveness and metastasis.
**BMS-345541** induces cell apoptosis of PC-3 cells. PC-3 cells after exposure to BMS-345541 for 72 h were examined by TUNEL assay. As shown in Fig. 6, apoptotic nuclei and fragmented DNA were stained dark brown in treated cells, but not in the control cells. The apoptotic index is significantly higher in the BMS-345541 treated cells than in the control (Table I).

To gain better understanding of the mechanism leading to cell death, we measured the combined effect of IKK inhibitor on the expression of Bcl-2 and Bax proteins. As shown in Fig. 7, the decreases in Bcl-2 expression were significantly greater in samples treated with BMS-345541 than those in control samples. Moreover, IKK inhibitor resulted in greater increases in Bax protein expression than untreated control.
Table I. Effect of BMS-345541 on apoptosis in prostate cancer PC-3 cells (TUNEL assay).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic index (%)</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.8±0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMS-345541</td>
<td>30.5±1.2</td>
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Discussion

Several lines of evidence suggest that IKK/NF-κB pathway activation is a key event in the acquisition of invasive and metastatic capacities in prostate cancer (21). Furthermore, NF-κB expression is upregulated in patients with castration-resistant prostate cancer (CRPC) who progress more rapidly (13). The kinase subunits of IKK complex have previously been shown to be involved either directly or indirectly in the regulation of cellular proliferation (22). Hence, both IKKα and IKKβ are considered to be therapeutic targets for development of anticancer agents (23,24). However, the precise mechanisms how it is achieved in prostate cancer are only partly understood. In this study, we characterized a highly selective IKK small-molecule kinase inhibitor-BMS-345541, and demonstrated a key role of IKK in both EMT and apoptosis of prostate cancer. Unlike other reported IKK inhibitors, BMS-345541 was found to bind to an unidentified allosteric site of the catalytic subunits, and so behaves as an ATP-non-competitive inhibitor. The high selectivity of BMS-345541 for IKKα and IKKβ suggests that the allosteric site is unique to the IKKs, although it cannot be excluded that the site may also be present within other kinases not yet tested for selectivity (14). Especially, BMS-345541 is a potent and selective inhibitor of IKKα and IKKβ. It displays 10-fold greater selectivity towards IKKβ than IKKα with no activity towards IKKε or other protein kinases, even at concentrations as high as 100 µM.

In our study, we showed that BMS-345541 treatment results in a concentration-dependent suppression of prostate cancer cell survival in vitro. In addition, BMS-345541 could induce cell apoptosis and cause inhibition of tumor cell migration and invasion in PC-3 cells. Thus, we demonstrate the significant inhibitory effect on CRPC of IKK inhibitor. This phenomenon may involve several biologic properties such as EMT and programmed cell death existing in tumor cells. We found that BMS-345541 treatment resulted in inhibition of cytoplasmic pIκBα and reduction of NF-κB p65 nuclear translocation. Thus, significant blockade of NF-κB pathway was achieved by IKK inhibitor. Based on the complexity of signaling networks that regulate induction of EMT, and the plasticity of these transitions, it is important to focus on the most promising methods toward safe and effective reversal of EMT. IKK/NF-κB appears to be a potential pathway in the regulation of EMT. Our results showed that IKK inhibitor reduced the critical EMT markers and transcription factors including N-cadherin, Snail, and Slug in PC-3 cells. Nevertheless, the level of E-cadherin protein increased in a dose-dependent manner with BMS-345541 treatment.

N-myc downregulated gene 1 (NDRG1) is a known metastasis suppressor in multiple cancers, being also involved in cell growth and differentiation, apoptosis, stress responses, angiogenesis and EMT (25,26). However, the relationship of IKKs, EMT and NDRG1 is unclear. Here, we first confirmed the effects of IKK inhibitor on NDRG1 protein in prostate cancer cells. The results showed BMS-345541 induced upregulation of membrane pNDRG1 in a dose-dependent manner. It has been reported that NDRG1 modulated EMT through upregulation of the E-cadherin expression, but downregulation of the N-cadherin, Snail, Slug, and Vimentin (27). We predict that there is a complex signaling network among IKK, NDRG1 and EMT which needs further studies in the future.

Several lines of evidence demonstrated that NF-κB activation and NDRG1 downregulation can maintain tumor cell viability, and regulation targeting these factors is sufficient to induce apoptosis (28,29). In our research, the TUNEL assay revealed IKK inhibitor regulated both NF-κB and NDRG1, and resulted in a dramatic induction of prostate cancer cell apoptosis. Molecules belonging to the B-cell lymphoma (Bcl-2)/Bax system play a crucial role in the regulation of the apoptotic process. In particular, Bcl-2 is an intracellular protein that inhibits apoptosis while Bax counteracts the anti-apoptotic function of Bcl-2 by binding to this molecule. Furthermore, we confirmed that the cell apoptosis was mediated through Bcl-2 downregulation and Bax overexpression. The Bax/Bcl-2 ratio appears more important than the individual Bax or Bcl-2 level in determining cell apoptosis, and high Bax/Bcl-2 ratio leads to greater apoptotic activity.

In conclusion, the IKK inhibitor BMS-345541, significantly suppresses the growth, invasion, migration of prostate cancer cells in vitro, as well as induces cell apoptosis. The mechanism may involve in the blockade of IKK/NF-κB pathway and EMT, and NDRG1 plays an important role in this process. Our studies provide a rationale and molecular basis for IKK inhibitor in the clinical treatment of prostate cancer. IKK inhibitors have the potential as novel therapeutic agents to deal with the advanced prostate cancer in the future.

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References


