YC-1 suppresses constitutive nuclear factor-\(\kappa\)B activation and induces apoptosis in human prostate cancer cells

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Abstract

Although the indazole compound, YC-1, is reported to exert anticancer activities in several cancer cell types, its target and mechanism of action have not been well explored. The objectives of this study were to ascertain whether YC-1 directly induces apoptosis in prostate cancer cells and to explore the mechanism(s) whereby YC-1 causes cell death. Hormone-refractory metastatic human prostate cancer PC-3 cells were selected for this study. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay indicated that YC-1 suppresses growth of PC-3 cells in a concentration-dependent and time-dependent manner. Apoptosis was determined using 4,6-diamidino-2-phenylindole staining, and cell cycle progression was examined by FACSscan flow cytometry. YC-1 treatment showed chromatin condensation and increased the percentage of PC-3 cells in the hypodiploid sub-G0-G1 phase, indicative of apoptosis. Additionally, exposure to YC-1 was found to induce activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase. Translocation and activation of nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) were determined by immunofluorescent staining and ELISA, respectively. The results showed that YC-1 abolished constitutive nuclear translocation and activation of NF-\(\kappa\)B/\(p65\). Furthermore, inhibition of inhibitor of \(\kappa\)B\(_{\alpha}\) (\(\kappa\)B\(_{\alpha}\)) phosphorylation and accumulation of \(\kappa\)B\(_{\alpha}\) were observed. The antitumor effects of YC-1 were evaluated by measuring the growth of tumor xenografts in YC-1-treated severe combined immunodeficient mice. The volumes of PC-3 tumors produced in severe combined immunodeficient mice were observed to decline significantly after treatment with YC-1 compared with vehicle controls. We concluded that the antitumor effects of YC-1 in PC-3 cells include the induction of apoptosis and the suppression of NF-\(\kappa\)B activation. Given these unique actions, further investigations of the effects of YC-1 against hormone-refractory prostate cancer are warranted. [Mol Cancer Ther 2005;4(10):1628–35]

Introduction

According to estimates of the American Cancer Society, ~232,090 new cases of prostate cancer are expected in the United States in 2005, and ~30,350 men will die of this disease (1). Prostate cancer is also prevalent in Taiwan, where it is ranked as one of the top 10 most common cancers. Patients diagnosed with prostate cancer that is localized or androgen sensitive are treated by surgery, radiation, and androgen ablation therapy. However, recurrence is rapid in most of these patients who later develop invasive or metastatic, hormone-refractory, and apoptosis-resistant prostate cancer.

The progression of prostate cancer from a localized and androgen-sensitive form to an invasive, metastatic, hormone-refractory, and apoptosis-resistant form is commonly associated with loss of androgen dependence. This loss is often correlated with overexpression of several anti-apoptotic and cell survival genes that render the cancer cells resistant to apoptosis (2). The importance of nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) in the development and progression of cancer has recently become widely recognized (3). Additionally, the NF-\(\kappa\)B/RelA complex has been shown to be constitutively activated in prostate tissues from human patients with prostate cancer, in androgen-insensitive human prostate carcinoma cells, and in prostate cancer xenografts (3, 4). Numerous studies have shown that suppression of constitutive NF-\(\kappa\)B activation by certain small molecules or by genetic manipulation can induce apoptosis, enhance radiosensitization and chemosensitization, suppress invasion, and inhibit metastatic growth in cancer cells, including prostate cancer cells (5). Agents capable of suppressing NF-\(\kappa\)B activation are therefore anticipated to be potentially useful in the management of prostate cancer.

The NF-\(\kappa\)B/Rel family of transcription factors is composed of five mammalian homologues: Rel (also known as c-Rel), RelA (also known as p65 and NF-\(\kappa\)B3), Rel-B, p50/p105 (also known as NF-\(\kappa\)B 1), and p52/p100 (also known as NF-\(\kappa\)B2). These transcription factors are recognized to

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serve important roles in the processes of inflammation and tumorigenesis. Members of the NF-κB/Rel family are known to form both homodimers and heterodimers, such as the classic NF-κB transcripational activator p50/p65 dimers. The transcriptional activity of NF-κB factors is tightly regulated by the inhibitor of κB (IκB) proteins. Binding with IκB serves to retain NF-κB in the cytoplasm in an inactive state. In response to various signals, IκB is phosphorylated by specific protein kinases that target the inhibitor for ubiquitination followed by 26S proteosomal degradation. NF-κB is released from IκB during its degradation. The transcription factor is subsequently translocated from the cytoplasm to the nucleus, where it binds to cognate sequences in the promoter regions of multiple genes and directly targets specific genes for expression leading to increased cell proliferation and antiapoptotic responses (6).

Aberrant proliferation and impairment of apoptosis, a distinct form of cell death with defining morphologic and biochemical characteristics, including membrane blebbing, chromatin condensation, caspase activation, and nuclear fragmentation (7), are both critical to oncogenesis and to the pathogenesis of cancer. In view of the importance of NF-κB-activated pathways in proliferative and antiapoptotic responses, agents that suppress these pathways are anticipated to be useful in the prevention and/or treatment of cancer. To date, however, such agents have not become available.

YC-1 was studied initially in this laboratory and found to possess antiplatelet properties. The antiproliferative and antiangiogenesis effects of this drug were subsequently identified in this and other laboratories (8–10). The ability of YC-1 to promote cell cycle arrest was shown more recently (11). The present study was undertaken to ascertain whether YC-1 directly induces programmed cell death in prostate cancer cells and to define the mechanism(s) whereby this agent exerts its antitumor effects.

Materials and Methods

Materials

YC-1 was obtained from Yung-Shin Pharmaceutical Industry Co., Ltd. (Taichung, Taiwan). RPMI 1640, fetal bovine serum, penicillin, streptomycin, and all other tissue culture reagents were obtained from Invitrogen (Carlsbad, CA). EGTA, EDTA, leupeptin, DTT, phenylmethylsulfonyl fluoride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, and β-isopropanol were purchased from Sigma Chemical (St. Louis, MO). 4,6-Diamidino-2-phenylindole was from Roche Diagnostics Corp. (Indianapolis, IN). Antibodies of NF-κB/p65, poly(ADP-ribose) polymerase (PARP), nucleolin (also designated C23), FITC-conjugated antirabbit IgG antibody plus 1 μg/mL 4,6-diamidino-2-phenylindole in PBS for 30 minutes at room temperature. The slides were examined by Leica TCS SP2 Confocal Spectral Microscope using a ×63 oil immersion objective to detect the presence of chromatin condensation/fragmentation as a marker of apoptosis. Approximately 100 cells were counted to calculate the percentage of changed cells.

Western Blotting

Total cell lystate was lysed with lysis buffer as previously described (12). The nuclear protein fractions were separated as described previously (14). Cells homogenates were diluted with loading buffer and boiled for 5 minutes for detecting phosphorylation, expression, and cleavage of proteins. For Western blot analysis, proteins (30–60 μg) were separated by electrophoresis in a 6% to 15% polyacrylamide gel and transferred to a nitrocellulose membrane. After incubation at room temperature in PBS/5% nonfat milk for 1 hour, the membrane was washed with PBS/1% Tween 20. Then the membrane was immunoreacted with mouse antihuman caspase-3 and nucleolin monoclonal antibodies or the rabbit antihuman NF-κB/p65, PARP, and IκB polyclonal antibody for overnight at 4°C. After four washings with PBS/1% Tween 20, horseradish peroxidase–conjugated antirabbit or antirabbit IgGs (diluted 1:2,000) were applied to the membranes for 1 hour at room temperature. Finally, the membranes were visualized with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, United Kingdom).

Measurement of Caspase-3 Activity

The caspase-3 activity assay kits purchased from BioVision (Mountain View, CA) were used for the detection of caspase-3 activity in commercial protocol as described.
previously (12, 15). In brief, proteolytic reactions were done containing cytosolic extracts, 2× reaction buffer containing DTT, and caspase-3 colorimetric substrate (DEVD-p-nitroanilide). The reaction mixture was incubated at 37°C for 1 to 2 hours, and then the formation of p-nitroanilide was measured at 405 nm using an ELISA reader.

**Tumor Xenografts Implantation**

Male severe combined immunodeficient (SCID) mice were obtained from the Laboratory Animal Center of Medical College, National Taiwan University. SCID mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. PC-3 cells (5 × 10⁶ cells) were implanted s.c. in the flank of each SCID mice. Mice were randomly assigned into three groups (nine mice per group), whereas tumors reached an approximate volume of 60 mm³. Then 0.5% carboxymethyl cellulose alone (control) or YC-1 (10 and 30 mg/kg; dissolved in 0.5% carboxymethyl cellulose) was administered orally daily, and the tumor volume as well as the body weight was measured 3 to 4 days. Tumor volume was determined by measuring the largest diameters (l) and the smallest diameters (s), and the volumes were calculated (V = 0.5ls²).

**In situ Detection of Tumor Tissue Sections by Immunohistochemistry and Immunofluorescence**

Prostate tumors harvested at autopsy were processed for immunohistochemistry using an antibody that recognizes an epitope overlapping the nuclear localization sequence of the activated form of NF-kB/p65 subunit (Chemicon International; ref. 16). In brief, 5-μm paraffin sections were deparaffinized and endogenous peroxidase was destroyed with 3% H₂O₂ in 100% methanol. Nonspecific antigenic sites were blocked with 3% bovine serum albumin in PBS for 30 minutes at room temperature. Tissues were incubated with a monoclonal antibody, which recognized the NF-kB/p65, overnight at 4°C. Negative controls were done using nonspecific IgG. A standard LSAB technique (DAKO, Glostrup, Denmark) was used to detect the reaction products. *In situ* detection of apoptotic cells was carried out using terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) reaction mixture according to manufacturer’s protocol (Promega, Madison, WI) as described previously (15).

**Statistics and Data Analysis**

Data are presented as the mean ± SE for the indicated number of separate experiments. Statistical analysis of data was done with Student’s *t* test. *P* values < 0.05 were considered significant.

**Results**

**Concentration-Dependent and Time-Dependent Induction of PC-3 Cell Death by YC-1**

PC-3 cells, which are hormone-refractory prostate carcinoma (HRPC) cells constitutive for NF-kB activation (17), were selected for these studies. Cells were treated with YC-1 at increasing doses and for varying times, and cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay (Fig. 1). YC-1 was found to induce cell death in a concentration-dependent and time-dependent manner. During morphologic examination of the cells on culture plates, YC-1-treated PC-3 cells were observed to exhibit both rounding and blebbing (data not shown), consistent with imposition of apoptotic cell death.

**Effects of YC-1 on Cell Cycle Progression**

Studies were done to ascertain whether YC-1-induced cytotoxicity was associated with a disturbance of cell cycle regulation. The cell cycle distributions of YC-1-treated and vehicle-treated preparations were therefore analyzed by flow cytometry. As illustrated in Fig. 2A, most vehicle-treated PC-3 cells accumulated at the G₀/G₁ phase. YC-1 treatment resulted in an increase in the percentage of cells in the sub-G₁ phase. This increase was concentration dependent and indicative of apoptosis, consistent with induction of cell death. The mode of induction of YC-1-induced cell death was also examined by 4′,6-diamidino-2-phenylindole staining using immunofluorescence microscopy (Fig. 2B). Unlike untreated control preparations (left), the chromatin of YC-1-treated cells (right, ~60% cells) was found to be condensed further supporting the occurrence of apoptosis.

**Suppression of Constitutive NF-kB Activation and Nuclear Translocation of NF-kB/p65 Subunits by YC-1**

Because NF-kB is known to be constitutively activated in PC-3 cells, the possibility that YC-1-induced apoptosis was attributable to the suppression of NF-kB activation was considered (18). The NF-kB activities of PC-3 cells treated with 0, 50, 75, or 100 μmol/L YC-1 for 18 hours were therefore measured. As illustrated in Fig. 3A, YC-1 treatment suppressed constitutive NF-kB activity in a dose-dependent manner. The extent of NF-kB/p65 inhibition at 50, 75,
and 100 μmol/L YC-1 was found to be 24%, 38%, and 53%, respectively. The effects of YC-1 on NF-κB signaling were further explored by examining the nuclear translocation of the NF-κB/p65 subunit in control versus treated PC-3 preparations. Immunofluorescence confocal microscopy revealed that YC-1 treatment abolished the nuclear translocation of the p65 subunit (Fig. 3B). Thus, YC-1 inhibited nuclear translocation of NF-κB/p65 subunit followed suppression of NF-κB activity. Likewise, YC-1 treatment resulted in a time-dependent decrease in nuclear translocation of NF-κB/p65 protein as shown in Western blotting (Fig. 3C).

**Inhibition of IκBα Phosphorylation and Accumulation of IκBα in YC-1-Treated Cells**

Translocation of NF-κB to the nucleus is normally regulated by IκBα degradation (6). We examined whether the inhibition of NF-κB activation by YC-1 was due to decreased degradation of IκBα. Western blotting for IκBα was done as an index of total inhibitor expression levels. When cells were treated for varying time periods with 75 μmol/L YC-1, significant time-dependent increases in total IκBα expression levels were observed (Fig. 4). Because degradation of IκBα normally requires that the inhibit
phosphorylated (6), it was of interest to examine the extent of \( \text{IkB}\alpha \) phosphorylation in YC-1-treated compared with nontreated preparations. When cells were treated for varying times with 75 \( \mu \text{mol/L} \) YC-1 followed by Western blotting for the phosphoprotein (Fig. 4), time-dependent decreases in expression of the phosphorylated form of \( \text{IkB}\alpha \) were observed. These results are indicative that YC-1 suppressed \( \text{NF-\kappa B} \) activity is through dephosphorylation and accumulation of \( \text{IkB}\alpha \).

### Involvement of Caspases in YC-1-Induced Apoptosis

Activation of caspases during apoptosis is correlated with the cleavage and activation/inactivation of a range of critical cellular substrates, including activation of the DNA repair enzyme known as PARP (7). Death attributed to the suppression of \( \text{NF-\kappa B} \) activity has been reported to be associated with the activation of caspase-3-like proteases and cleavage of PARP (19, 20). Potential effects of YC-1 on the activations of caspase-3 and cleavage of PARP in PC-3 cells were therefore explored. Preparations were treated with varying concentrations of YC-1. To identify changes in caspase-3 activation, the expression and the quantitative activity of the enzyme were determined by Western blotting and ELISA assays, respectively. Western blotting for PARP was done to assess PARP cleavage. A YC-1 concentration-dependent cleavage (Fig. 5A) and time-dependent activation (Fig. 5B) of caspase-3 and a concentration-dependent cleavage of PARP (Fig. 5A) were observed.

### Evaluation of Tumor Growth in a Xenograft Animal Model

To extend studies with YC-1 in cell culture to in an \textit{in vivo} system, the effects of the drug on growth of PC-3 tumors generated after s.c. inoculation of PC-3 cells into SCID mice were examined. YC-1 administered orally at doses of 10 and 30 mg/kg was observed to inhibit tumor growth markedly. The data suggest a decreasing trend in the mean volume of tumors of YC-1-treated mice compared with that of tumors of vehicle-treated controls (Fig. 6A). Additionally, no loss of body weight was observed in YC-1-treated mice at the dosages used (Fig. 6B). Furthermore, staining with antibody that recognizes an epitope overlapping the nuclear localization sequence of the activated form of NF-\( \kappa \)/p65 subunit, and TUNEL of tissue sections (Fig. 7) revealed that most cells in the control tumors were positively stained by NF-\( \kappa \)/p65 and very few cells were stained by TUNEL. In contrast, tumors treated with YC-1 contained fewer NF-\( \kappa \)/p65 cells, indicating the inhibition of activated NF-\( \kappa \), and more TUNEL–positive cells, indicating the induction of apoptosis.

### Discussion

YC-1 has been shown in several studies to inhibit cell growth, promote cell cycle arrest, and decrease angiogenesis (8, 9, 11). The present report shows for the first time that YC-1 directly induces apoptosis in HRPC PC-3 cells. Evidence that constitutive \( \text{NF-\kappa B} \) activity suppresses apoptosis in HRPC cell lines has been previously provided (3, 18). Inhibition of constitutive \( \text{NF-\kappa B} \) activation has therefore been proposed to result in induction apoptosis.
However, controversy currently exists regarding the effects of YC-1 on NF-κB activity and the consequences of these effects. For example, YC-1 is reported to induce cyclooxygenase-2 expression via an increase NF-κB activity (23), whereas other reports indicate that YC-1 inhibits inducible nitric oxide synthase expression via suppression of NF-κB activity (13, 24). Despite this controversy, the suppression of NF-κB activity has been shown to be closely correlated with the induction of apoptosis in prostate cancer cells (3, 5, 21, 22). In agreement with this observation, YC-1 was observed in the present study to suppress NF-κB activity and to induce apoptosis in PC-3 cells. Findings were consistent with a cause-effect relationship between suppression of NF-κB activity and induction of apoptosis.

The constitutive activation of NF-κB in HRPC cells has been suggested to be due to an aberrantly high degree of IκB phosphorylation and degradation in these cells (4). IκB of cancer cells is recognized to serve as a substrate for a variety of protein kinases, such as NF-κB-inducing kinase, phosphoinositide-3 kinase, protein kinase C, mitogen-activated protein/extracellular signal-regulated kinase kinase kinases, and interleukin-1R (IL-1R)–associated kinase. Development of agents that influence the IκB/NF-κB pathway to induce apoptosis in HRPC cells was therefore suggested to represent a rational therapeutic objective (5). In the present study, YC-1 was observed to inhibit the phosphorylation and degradation of IκBα. Accordingly, YC-1 represents a potential therapeutic candidate for targeting the IκBα-NF-κB pathway in prostate cancer cells. Activation of NF-κB is associated with a wide range of signaling events, including changes in the expression of c-IAP-1, c-IAP-2, Bel-2, IL-1, IL-6, IL-8, granulocyte macrophage colony-stimulating factor, vascular endothelial growth factor, cyclooxygenase-2, matrix metalloproteinase-9, and cyclin D1. These events are consistently linked with increased proliferation, angiogenesis, invasion, metastasis, evasion of apoptosis, and resistance to radiation and chemotherapy in cancer cells (3, 5, 6). Previous studies have clearly shown that YC-1 decreases angiogenesis in association with a decrease in hypoxia-inducible factor-1α (HIF-1α) concentration in conditionally hypoxic cancer cells (9). However, the mechanism for the change in HIF-1α concentration is undefined. It is noteworthy that YC-1 inhibited the hypoxic induction of erythropoietin and vascular endothelial growth factor in Hep3B cells (25), and that NF-κB plays a key role in HIF-1-regulated erythropoietin gene expression in the same cells has been reported (26). Activation of an NF-κB-dependent pathway in normoxic cells has been shown to stabilize HIF-1α (27). Furthermore, modulation of
HIF-1α expression by the phosphoinositide-3 kinase/Akt/mammalian target of rapamycin pathway, which was linked to changes in NF-κB activity, in prostate cancer cells could have important implications regarding cancer progression (28). The possibility that YC-1 exerts additional antitumor effects, such as the inhibition of HIF-1α expression, in PC-3 cells deserves exploration. Additionally, blockade of NF-κB activity by transfection with a mutated IκBα caused suppression of angiogenesis, invasion, and metastasis in vitro and in vivo in highly metastatic prostate cancer cells (29). In the present study, YC-1 was observed to accumulate IκBα. Accordingly, YC-1 may therefore prove useful in suppressing both invasion and metastasis of prostate cancer.

The inactivation of NF-κB is well established to enhance radiation-induced apoptosis in cancer cells (3, 6). Radiation was found to promote activation, as opposed to suppression, of NF-κB in a manner potentially attributable to production of reactive oxygen species and suppressed NF-κB activity enhanced radiosensitization (6). Moreover, Moeller et al. showed the existence of a connection among free radicals, HIF-1α, and radiosensitivity in cultured cells and observed that YC-1 enhanced the therapeutic effects of radiotherapy in a xenograft model (30). It remains to be established whether YC-1 will be fully effective in suppressing NF-κB activity when used in combination with radiation or with other agents for the management of human prostate cancer. However, there was evidence of down-regulation of serum IL-6, downstream NF-κB effector, in phase I trial of the proteasome inhibitor bortezomib in patients with HRPC (31). Preclinical studies also indicated bortezomib suppressed NF-κB and induced apoptosis in cell culture and tumor xenographs (32, 33). This phase I trial and preclinical studies support bortezomib in combination with radiation or chemotherapy as a potential treatment for prostate cancer (34). Nonetheless, given the unique mechanism of action of YC-1 in prostate cancer cells, investigations of the efficacy of therapies involving YC-1 in combination with anticancer treatments effective through different mechanisms of therapies involving YC-1 in combination with anti-angiogenesis, inhibition of tumor metastasis, and radiosensitization (6).

The indazole drug, YC-1, directly induces apoptosis in HRPC PC-3 cells. YC-1 suppresses the translocation and activation of NF-κB activity in these cells, a novel drug effect. Treatment with YC-1 also significantly reduces the volume of s.c. PC-3 tumors produced in SCID mice. Considering the unique actions of YC-1, further investigations of the effects of this agent against HRPC are warranted.

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References


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