Food and Chemical Toxicology 59 (2013) 281–288

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

# Proteomic-based identification of multiple pathways underlying n-butylidenephthalide-induced apoptosis in LNCaP human prostate cancer cells

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ARTICLE INFO

*Article history:*
Received 18 February 2013
Accepted 29 May 2013
Available online 12 June 2013

*Keywords:*
Butylidenephthalide
Cell cycle
Apoptosis
LNCaP human prostate cancer
Proteomics

ABSTRACT

Although numerous studies have shown the cancer-preventive properties of butylidenephthalide (BP), there is little report of BP affecting human prostate cancer cells. In the present study, proteomic-based approaches were used to elucidate the anticancer mechanism of BP in LNCaP human prostate cancer cells. BP treatment decreased the viability of LNCaP human prostate cancer cells in a concentration- and time-dependent manner, which was correlated with G0/G1 phase cell cycle arrest. Increased cell cycle arrest was associated with a decrease in the level of CCND1, CDK2, and PCNA proteins and an increase in the level of CDKN2A, CDKN1A, and SFN proteins. Proteomic studies revealed that among 48 differentially expressed proteins, 25 proteins were down-regulated and 23 proteins were up-regulated and these proteins fall into one large protein protein interaction network. Among these proteins, FAS, AIFM1, BIK, CYCS, SFN, PPP2R1A, CALR, HSPA5, DDIT3, and ERN1 are apoptosis and endoplasmic reticulum (ER) stress associated proteins. Proteomic data suggested that multiple signaling pathways including FAS-dependent pathway, mitochondrial pathway, and ER stress pathway are involved in the apoptosis induced by BP.


## 1. Introduction

Based on GLOBOCAN 2008, prostate cancer is the second most frequently diagnosed cancer (14% of the total new cancer cases) and the sixth leading cause of cancer death (6% of the total) in worldwide males (Jemal et al., 2011). The incidence rate for prostate cancer varies by more than 25-fold worldwide, for example Australia/New Zealand has the highest incidence rate, whereas, Asia has the lowest (Ferlay et al., 2010). These differences may be due to some of the genetic, hormonal, and dietary factors. Epidemiological and laboratory studies have suggested that diet-based naturally occurring agents, due to their cost-effectiveness and human acceptability could be ideal candidates for the treatment and prevention of human prostate cancer (Khan et al., 2010; Syed et al., 2007). Therefore, there is a growing interest in identification new and effective anti-cancer compounds in the dietary, as well as to elucidate the mechanisms of cancer prevention.

n-Butylidenephthalide (BP) is one of the major compounds isolated form the extracts of *Angelica sinensis* (Oliv.) Diels (also known as dong quai or danggui) which is a popular traditional Chinese

*Abbreviations:* 2DE, two-dimensional gel electrophoresis; ABC, ammonium bicarbonate; ACN, acetonitrile; BAD, BCL2 antagonist of cell death; BCL2, B-cell lymphoma 2; BP, n-butylidenephthalide; CCND1, cyclin D1; CDC25A, cell division cycle 25 homolog A; CDK, cyclin dependent kinase; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTE, dithiolerythritol; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; IEF, isoelectric focusing; IPG, immobilized pH gradient; LC/MS/MS, liquid chromatography tandem mass spectrometry; MTT, 3-(4,5-dimethyl thizol-2-yl)-2,5-diphenyl tetrazolium bromide; NL, non-linear; PAGE, polyacrylamide gel electrophoresis; PANTHER, protein analysis through evolutionary relationships; PBS, phosphate-buffered saline; PI, propidium iodide; RPMI, Roswell Park Memorial Institute; SDS, sodium dodecyl sulfate; TBST, tris-buffered saline Tween-20; STRING, search tool for the retrieval of interacting genes/proteins; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling, other proteins which are not mentioned above are denoted as their gene symbols as listed in Table 2.

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http://dx.doi.org/10.1016/j.fct.2013.05.045

herbal medicine and has been used to treat menstrual disorders, modulate the immune system, and acts as an anti-oxidant (Circosta et al., 2006; Shen et al., 2005; Yang et al., 2006). BP has been shown to have anti-tumor activity against colon cancer, glioblastoma multiforme brain tumors, hepatocellular carcinoma, and lung cancer (Chen et al., 2008; Kan et al., 2008; Lin et al., 2008; Wei et al., 2009). However, there is little report of BP affecting human prostate cancer cells.

Previously oligodeoxynucleotide-based microarray was used to screen the anti-tumor mechanism of BP in glioblastoma multiforme brain tumor cells. It was found that the induction of Nur77 gene expression using BP induced the cancer cell death (Lin et al., 2008). A later report assessed the possible role of Nur77-induced apoptosis in hepatocellular carcinoma cells and the result implicated the PI3K/AKT/GSK3β signaling pathways may be involved in the regulation of BP-induced apoptosis in hepatocellular carcinoma (Chen et al., 2008). However, the mRNA expression of Nur77 did not obviously change after treatment with BP in lung cancer cells; instead, the mRNA expression of hTERT was found to be inhibited (Wei et al., 2009). To date, the precise mechanism of the BP action in cancer is still unclear.

The purpose of this study was to investigate the effects of BP in an androgen-sensitive human prostate adenocarcinoma cell line (LNCaP) human prostate cancer cells including cytotoxicity, cell-cycle arrest, proteins involved in apoptosis and the molecular anti-tumor mechanism. Proteins instead of genes are important regulators of the intracellular environments. Investigations of proteins will help researchers to understand the mechanism of drugs. Because the mechanism often involves changes in the expression of multiple proteins rather than a single protein, a global analysis of the protein alterations can result in valuable information to understand the drug action mechanism.

In this study, proteomic techniques were used to investigate protein changes associated with LNCaP upon treatment with BP. Two-dimensional gel electrophoresis (2DE) coupled with nano-liquid chromatography tandem mass spectrometry (LC/MS/MS) was used to characterize the differentially expressed proteins in the BP treated LNCaP cells. Functional studies and network analysis of the differentially expressed proteins were used to induce the possible molecular anti-tumor mechanism of BP in prostate cancer.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

LNCaP human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA). LNCaP cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% sodium pyruvate, and 2 mM L-glutamine at 37 °C in a humidified atmosphere with 5% $CO_2$. Cells were plated in 6-well plates at a seeding density of approximately $2 \times 10^5$ cells/well.

### 2.2. Chemicals and reagents

BP (MW: 188.23) was purchased from Lancaster Synthesis Ltd. (Newgate Morecambe, UK) and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/mL and stored at −20 °C as a stock solution. RPMI 1640 medium, fetal bovine serum, penicillin, streptomycin, L-glutamine, sodium pyruvate, trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, CA). The In Situ Cell Death Detection kit was from Roche Applied Science (Mannheim, Germany). All other chemicals, except otherwise noted, were purchased from Sigma Chemical Co. (St. Louis, MO).

### 2.3. Growth inhibition assay

Cell viability was assayed by using 3-(4,5-dimethyl thizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric dye reduction assay. Briefly, the LNCaP cells ($2 \times 10^5$/well) were incubated in 6-well plates containing 2 mL of serum-containing medium. Cells were allowed to adhere for 18–24 h then washed with phosphate-buffered saline (PBS). Freshly prepared 0.2% DMSO or freshly diluted BP solution (0–100 μg/mL in culture medium) was then added to LNCaP cells. After 24 or 48 h of exposure, the DMSO or BP-containing medium was removed, washed with PBS, and replaced by fresh medium. The cells in each well were then incubated in culture medium containing 300 μg/mL MTT for 1 h at 37 °C. After the medium was removed, 2 mL of DMSO were added to each well. Absorbance at 570 nm of the maximum was detected by a PowerWave X Microplate ELISA (enzyme-linked immuno sorbent assay) Reader (Bio-Tek Instruments, Winooski, VT). The absorbance for DMSO-treated cells was considered as 100%. The results were determined by three independent experiments and each experiment was performed in triplicate.

### 2.4. Cell cycle analysis

Approximately $5 \times 10^5$ cells/well of LNCaP cells were incubated with 0–100 μg/mL BP for the indicated time. Cells were harvested with trypsin/EDTA, collected, washed with PBS, fixed with cold 100% ethanol overnight, and then stained with a solution containing 45 μg/mL propidium iodide (PI), 10 mg/mL RNase A, and 0.1% Triton X-100 for 1 h in the dark. Cells were analyzed with a FACScan™ flow cytometry (Becton–Dickinson, Franklin Lakes, NJ) equipped with an argon ion laser at 488 nm wavelength. The data were then analyzed using CellQuest 3.0.1 (Becton–Dickinson) and ModFit LT V2.0 (Verity Software House, Topsham, ME). The results were determined by three independent experiments and each experiment was performed in triplicate.

### 2.5. Apoptosis assay

Apoptosis of cells was determined with an In Situ Cell Death Kit based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction. The cells were treated as described above then incubated with TUNEL reaction mixture according to the manufacturer's instructions. To quantify cell apoptosis, the slides were viewed under microscopy. The percentage of cells undergoing apoptosis was determined by three independent experiments and each experiment was performed in triplicate.

### 2.6. Protein extractions

Cells cultured with DMSO (control) or 70 μg/mL of BP for 24 h were harvested, washed twice with ice-cold PBS, and then extracted with lysis buffer containing 7 M urea, 4% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 M thiourea, 0.5% pH 3–10 non-linear (NL) immobilized pH gradient (IPG) buffer (GE Healthcare, Piscataway, NJ), and the protease inhibitor cocktail. After a 3-h incubation at 0 °C, the cell lysates were centrifuged for 15 min at 16,000 rpm. The protein concentration of the resulting supernatants was measured using the BioRad Protein Assay (BioRad, Hercules, CA).

### 2.7. 2DE

Total proteins (250 μg) were mixed and denatured in a 2D sample buffer (8 M urea, 4% CHAPS, 0.002% Bromophenol Blue, 65 mM dithiolerythritol (DTE) and 0.5% IPG buffer). Then the sample solution was loaded onto an IPG strip ($180 \times 3 \times 0.5$ mm, GE Healthcare) in a strip holder for the first-dimension isoelectric focusing (IEF). IEF was performed using Ettan IPGphor horizontal electrophoresis system (GE Healthcare). The electrophoresis conditions were set at 20 °C rehydration: 30 V 12 h, step 1: 500 V 1 h, step 2: gradient 1000 V 8 h, step 3: gradient 8000 V 3 h, step 4: 8000 V 4 h. After IEF, the IPG strips were first equilibrated with a solution containing 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 0.002% bromophenol blue and DTE (1% w/v) for 20 min, followed by a second equilibration for 15 min with the same solution containing iodoacetamide (2.5% w/v) instead of DTE. Finally, the strip was applied to the second dimension 12.5% SDS–polyacrylamide gel electrophoresis (PAGE) in a running buffer containing 25 mM Tris, 0.192 M glycine and 0.1% SDS using an EttanDaltsix (GE Healthcare) electrophoresis system. Gels were run at 5 W/gel for 15 min and followed by 17 W/gel for 4–5 h. After separation the gels were visualized with Sypro Ruby.

### 2.8. Image analysis

The stained gels were scanned with ImageQuant 5.2 software (GE Healthcare) on a Typhoon 9200 scanner (GE Healthcare) with a resolution of 300. The spot-intensity calibration, spot detection, background abstraction, matching, 1D calibration and the establishment of average gel were performed with ImageMaster 2D Platinum 5.0 analysis software (GE Healthcare). Protein staining spots were quantified by calculation of spot volume after normalization of the image using the total spot volume normalization method multiplied by the total area of all spots. The calculation of the theoretical molecular weight and pI values of the identified protein spots were based on the algorithms included in the ImageMaster 2D Platinum 5.0 analysis software package. Statistical analysis was carried out with Excel 2010 by three independent experiments and each experiment was performed in triplicate.