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A novel anticancer agent, retigeric acid B, displays proliferation inhibition, S phase arrest and apoptosis activation in human prostate cancer cells

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ABSTRACT

Retigeric acid B (RB), a naturally occurring pentacyclic triterpenic acid, has been noted for its antifungal properties *in vitro*. Here, we observed that RB inhibited prostate cancer cell proliferation and induced cell death in a dose-dependent manner, but exerted very little inhibitory effect on noncancerous prostate epithelial cell viability. Treatment of androgen-independent PC-3 cells with RB caused a moderate increase in $p21^{Cip1}$, and enforced the cell cycle arrest in the S phase. A block of S phase was accompanied with decreases in cyclin B, and increases in cyclin E and cyclin A proteins and phosphorylated retinoblastoma protein (pRb), whereas the expression of cdk2 remained almost unchanged in PC-3 cells exposed to RB. Moreover, RB significantly inhibited DNA synthesis with a dose-dependent reduction in the incorporation of BrdU into DNA, and enhanced apoptosis of PC-3 cells with induction of a higher ratio of Bax/Bcl-2 proteins, and activation of caspase-3 which, in turn, promoted the cleavage of poly (ADP-ribose) polymerase (PARP). However, pretreatment with the pan-caspase inhibitor z-VAD-fmk only partially alleviated RB-triggered apoptosis in PC-3 cells, suggesting the involvement of both caspase-dependent and caspase-independent pathways. Additionally, treatment of androgen-sensitive LNCaP cells with RB led to a reduction in the expression of androgen receptor (AR), and subsequently decreased the transactivity of AR. These observations help to support the search for promising candidates to treat prostate cancer.

1. Introduction

Prostate cancer (PCa) is one of the most common malignant tumors in man and hormonal withdrawal therapy remains an effective treatment for advanced PCa. However, the development of hormone-refractory prostate cancer (HRPC) occurs within a few years after hormonal deprivation therapy and is associated with poor prognosis and few therapeutic options [1,2]. Therefore, novel strategies targeting the molecular basis of PCa progression are crucial to the development of more effective regimes for this disease. Androgen withdrawal decreases cell proliferation and promotes apoptotic cell death. Compelling evidence suggests that progression of PCa to metastatic HRPC is associated with its chemoresistant nature and dysregulation of normal apoptotic mechanisms [3,4]. Basic and clinical research studies link altered expression of several apoptosis-regulatory proteins with the development of HRPC, including Bcl-2, Bcl-xL, and IAP family genes [5,6]. Bcl-2 and/or Bcl-xL specific antisense oligonucleotide treatment leads to activation of caspase and subsequent apoptosis, and thus enhancement of chemosensitivity [7]. Bax overexpression results in apoptotic cell death in PC-3 and DU145 PCa cells, which are known to offer resistance to chemical proapoptotic agents such as staurosporine [6,8]. Therefore, enhancement of the apoptotic process emerges as a significant therapeutic strategy for the effective inhibition of androgen-independent PCa cells.

Recently, docetaxel, a derivative from the naturally occurring compound taxol, has emerged as an active chemotherapeutic agent to improve quality of life and survival conditions in patients with metastatic HRPC [9,10]. The promising results, together with epidemiologic studies and numerous basic laboratory findings, provide evidence that natural products and derivatives with diverse pharmacologic properties play a potential role in the prevention and treatment of various cancers including PCa [11,12]. An increased effort has been made to isolate various chemicals and

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investigate the mechanisms of action of bioactive compounds for the development of chemopreventive and/or therapeutic agents [13].

Naturally occurring triterpenic acid derivatives, such as boswellic acids, exhibit potent antitumoral activities in many carcinoma cells [14–16]. Recently, we described the identification of retigeric acid B (RB), a pentacyclic triterpenic acid isolated from the lichen *Lobaria kurokawae* Yoshim, as a novel bioactive principle noted for its antifungal activity [17,18]. Although many natural chemicals with triterpenic structures such as boswellic acids, have chemotherapeutic properties against cancer, RB has received little attention for its anticancer properties. In this study, we used two analogues of pentacyclic triterpenic acids, retigeric acid A (RA) and retigeric acid B (RB), isolated from the *Lobaria kurokawae* Yoshim, and for the first time tested their efficacy against carcinoma cell growth. *In vitro* screening assays led to the identification of RB as a potent analog which showed a broad range of antitumoral activity against multiple cancer cell-types, including PCa cells. Further investigations demonstrated that RB was able to suppress androgen receptor (AR) expression and transcriptional activity in LNCaP cells, and inhibition of PC-3 cell growth was accompanied by induction of the S-phase cell cycle arrest as well as apoptosis.

2. Materials and methods

2.1. Chemicals

Retigeric acid A (RA) and retigeric acid B (RB) were isolated from the lichen *L. kurokawae* in our laboratory. The purity and chemical structure were described previously [17,18]. Acetyl-11-keto-β-boswellic acid (AKBA) was isolated and purified by reverse phase high performance liquid chromatography as described previously [16]. The RA, RB and AKBA were prepared in dimethyl sulfoxide (DMSO) and stored as small aliquots at −20 °C. Mibolerone (Mib, New England Nuclear) was dissolved in ethanol. Caspase inhibitor Z-VAD-FMK was purchased from Enzo Life Sciences.

2.2. Cell culture and treatments

Human PCa LNCaP (The American Type Culture Collection, Rockville, MD), PC-3 and DU145 cells (The Cell Bank of Chinese Academy of Sciences, Shanghai), human epidermoid cancer KB and vincristine resistant KB (KB/VCR) cells, human ovarian cancer 3-AO and cisplatin-resistant 3-AO (3-AO/CDDP) cells (a gift from Dr Beihua Kong, Qilu Hospital of Shandong University) were cultured in RPMI 1640 medium (HyClone) supplemented with 10% fetal bovine serum (HyClone). Human benign prostate epithelial RWPE1 cells were obtained from The American Type Culture Collection, Rockville, MD and maintained in Keratinocyte1 medium (K-SFM) supplemented with bovine pituitary extract and epidermal growth factor (EGF) (Gibco, Grand Island, NY). Human hTERT-RPE1 cells (a gift from Dr. Changjun Zhu, School of Medicine, Shandong University) were cultured in DMEM-F12 medium (HyClone) containing 10% fetal bovine serum (HyClone). Human breast cancer MCF-7 cells were cultured in DMEM medium (HyClone) supplemented with 10% fetal bovine serum (HyClone). Human osteosarcoma U2OS and Saos2 cells were cultured in McCOY's 5A with 10% fetal bovine serum (HyClone). The cells were maintained in 5% CO_2 at 37 °C until reaching approximately 50–70% confluence, and then treated with different amounts of chemicals as indicated. LNCaP cells were maintained in serum-free RPMI 1640 medium for further 24 h to deplete endogenous steroid hormones, and then treated with Mib (1 nM) with or without chemicals in the medium containing 5% charcoal stripped serum (JRH Biosciences). Control cells were subjected to DMSO and/or ethanol treatment.

2.3. Cell proliferation assays

Cell proliferation in the presence of RA and RB was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) colorimetric assay. The LNCaP cells were propagated in 24-well culture plates and grown under the conditions described above, and then treated with vehicle, Mib alone or Mib combined with desired concentrations of RA or RB for a further 24 h. All other cells were seeded in 96-well culture plates, and challenged with the indicated concentrations of RA or RB. Each test dose was performed in triplicate on each plate. After treatment with RA or RB for 24 h, cells were incubated with 10 μl MTT for 4 h and the cell growth response to the chemicals was detected by measuring the absorbance at 570 nm on a plate reader (Bio-Rad, USA). Three replicates were used for each treatment.

2.4. Cytotoxicity assay

Cytotoxicity was quantified by the release of lactate dehydrogenase (LDH) from PC-3, LNCaP and RWPE1 cells after treatment with RB. Cells were incubated in a 24-well microplate (Costa) and exposed to different concentrations of RB for 24 h, and LDH activities in medium were determined using a LDH detection kit (Ortho-Clinical Diagnostics) on a Vitros 250 (Ortho-Clinical Diagnostics). Percentage of cytotoxicity was calculated by the following formula: Cytotoxicity (%) = (mean LDH activity of RB-treated cells-blank)/(mean LDH activity of untreated control-blank).

2.5. Cell cycle analyses

The PC-3 cells were seeded in 75 ml flasks and cultured under the conditions described above. After treatment with RB for 48 h, cells were collected, washed with ice-cold PBS, and then fixed in 70% ethanol. Cell cycle analyses and apoptosis were determined with propidium iodide staining and flow cytometry using the Becton Dickinson FACScan. The analyses were performed in triplicate to allow for statistical evaluation.

2.6. DAPI staining

Morphological changes of apoptosis were determined by staining PC-3 cell nuclei with DAPI. Cells were cultured on chamber slides and treated with RB (5, 10, and 20 μM) for 24 h. Following two washes with PBS, the cells were fixed with 90% ethanol/5% acetic acid for 1 h at room temperature, washed with PBS, and then incubated with DAPI solution (1.5 mg/ml in PBS) for 30 min at room temperature. The cells were washed with PBS and mounted. Images of DAPI fluorescence were collected using a Nikon phase-fluorescence microscope.

2.7. Western blot assay

After treatment with RB at the desired concentrations or AKBA (30 μM) for 24 h, cells were washed twice with ice-cold PBS and cell lysates were prepared using RIPA buffer containing fresh protease inhibitor mixture (50 μg/ml aprotinin, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM β-glycerolphosphate). Proteins were quantified using the BCA protein assay (Biocolor BioScience & Technology, Shanghai, China). Samples containing equal amounts of protein (120 μg) from the lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (PALL). The membranes were blocked with 5% non-fat milk in TBST buffer (20 mM Tris–HCl, 137 mM NaCl, and 0.1% Tween 20, pH 8.0) for 1 h at room temperature prior to incubation with specific antibodies against Bcl-2, Bax, total PARP and cleaved PARP, phospho-cdc2 (Tyr15),