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Protopine, a novel microtubule-stabilizing agent, causes mitotic arrest and apoptotic cell death in human hormone-refractory prostate cancer cell lines

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ABSTRACT

In this study, we investigated the anticancer effect of protopine on human hormone-refractory prostate cancer (HRPC) cells. Protopine exhibited an anti-proliferative effect by induction of tubulin polymerization and mitotic arrest, which ultimately led to apoptotic cell death. The data suggest that protopine increased the activity of cyclin-dependent kinase 1 (Cdk1)/cyclin B1 complex and that contributed to cell apoptosis by modulating mitochondria-mediated signaling pathways, such as Bcl-2 phosphorylation and Mcl-1 down-regulation. In conclusion, the data suggest that protopine is a novel microtubule stabilizer with anticancer activity in HRPC cells through apoptotic pathway by modulating Cdk1 activity and Bcl-2 family of proteins.

1. Introduction

Prostate cancer is the second leading cause of cancer death of men in the United States. In 2011, an estimated 240,890 cases of prostate cancer will be newly diagnosed and about 33,720 men will die from this disease [1]. Until recently, mitoxantrone plus prednisone was the only drug treatment approved by the US FDA for the treatment of advanced prostate cancer. Mitoxantrone treatment was well tolerated and provided palliation for some patients, but did not prolong survival [2]. In 2004, two clinical trials showed that the combined use of docetaxel (an anti-mitotic agent) plus prednisone or estramustine improved survival better than mitoxantrone plus prednisone treatment [3,4]. It triggered a lot of effort on the strategy of developing microtubule-stabilizing drugs for advanced prostate cancer [5,6]. Moreover, the ability of taxanes to inhibit tumor growth has made them key components of chemotherapeutic regimens for a variety of solid tumors, including those derived from ovary, breast, head and neck, and lung as well as malignant melanoma [7].

Microtubules, dynamic polymers of α/β-tubulin heterodimers, are key components of the mitotic spindle and are essential for chromosome segregation during the cell division of eukaryotes. Microtubule ends undergo stochastic switching between a polymerized state and a depolymerized state, and numerous spindle-related regulators control this process. A large body of evidence indicates that microtubule-targeting agents (MTAs) are effective anticancer drugs because they interfere with the dynamics of the mitotic spindles, leading to cell cycle arrest and ultimately to death of the cancerous cells [8]. There are two main groups of microtubule-targeting anti-mitotic drugs: microtubule-stabilizing agents (e.g., taxanes, epothilones and

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discodermolide) and microtubule-destabilizing agents (e.g., *Vinca* alkaloids, estramustine, and cryptophycins). MTAs act as inhibitors in G2/M phase transition and subsequently induce the cell-cycle arrest and apoptosis in tumor cells. Cyclin-dependent kinase 1 (Cdk1) and cyclin B1 form a heterodimer in the nucleus, whose activity determines the progression of the cell cycle from G2- to M-phase. Numerous factors regulate Cdk1 activity, including the level of cyclin B1, phosphorylation of Cdk1 on different residues, Cdk1 inhibitor $p21^{Cip1/Waf1}$, and Cdc25 phosphatase activity [9]. Cdk1 plays a pro-apoptotic role in the mechanism of anti-mitotic drugs. It has been reported that MTAs are able to increase Cdk1 activity and induce apoptosis in prostate cancer cells [9,10]. Recently, several lines of evidence suggest that mitochondria-mediated pathways contribute significantly to apoptosis-related cell cycle arrest in diverse types of cancer cells. MTAs can promote Cdk1 activation and that triggers mitochondrial membrane permeabilization by targeting on Bcl-2 family proteins, such as Bcl-2 phosphorylation and Mcl-1 down-regulation [10–12].

Protopine (tetrahydro-5-methyl *bis*-[1,3]benzdioxide-[4,5-C:5′,6]-azecin-13(5H)-one) is an isoquinoline alkaloid purified from the Chinese herb, *Corydalis* tubers. In traditional Chinese herbal medicine, it has been used as a remedy for cardiovascular diseases such as hypertension, cardiac arrhythmia and thromboembolism [13]. Protopine contains a unique nitrogen-containing 10-membered cyclic ketone (dibenzazocin ring) and has been shown to exhibit a number of pharmacological activities, including anti-arrhythmic effects, anti-thrombotic effects and anti-platelet aggregation effects [13]. In the present study, we observed significant anticancer activity of protopine on HRPC cells. We also report the novel effect of protopine to promote tubulin polymerization and that resulted in mitotic arrest of the cell cycle and apoptosis through Cdk1 activation and the alternation of Bcl-2 family of proteins. Unlike the complicated structures of taxanes and *Vinca* alkaloids that restrict chemical modification, protopine has a relatively simple structure and chemical synthesis has been achieved [14], making it a potential pharmacophore for further development.

2. Materials and methods

2.1. Materials

Protopine (purity of $\geqslant$98%), sulforhodamine B (SRB), EGTA, EDTA (disodium salt), leupeptin, dithiothreitol, propidium iodide (PI), PMSF, ribonuclease A, roscovitine, FITC-conjugated antimouse IgG, RPMI 1640 medium and all of the other chemical reagents were obtained from Sigma (St Louis, MO). Fetal bovine serum (FBS), penicillin, streptomycin and all other tissue culture regents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). 4′,6-Diamidino-2-phenylindole (DAPI) was from Roche Molecular Biochemicals (Mannheim, Germany). Trizol reagent was from Invitrogen (Carlsbad, CA); random primer and M-MLV RT were purchased from Promega (Madison, WI). The following antibodies were used: phospho-Cdc2 (Tyr^{15}), phospho-Cdc2 (Thr^{161}) and phospho-Bcl-2 (Ser^{70}) (Cell Signaling Technologies, Boston, MA); cyclin B1, Cyclin dependent kinase1 (Cdk1), Bax, Bak, Bcl-2, Bcl-xL, Bim, HA, Mcl-1, PARP (Santa Cruz Biotechnology, Santa Cruz, CA), MPM-2 antibody (Upstate Biotechnology, Lake Placid, NY), caspase-3 (Imgenex, San diego, CA), pan-actin (Chemicon International, Temecula, CA), phospho-Cdk1 substrate antibody (Calbiochem, Samdoegp, CA) and mouse anti-β-tubulin (from Sigma, St Louis, MO).

2.2. Cell culture

Human prostate cancer cell lines (PC-3, DU-145) were purchased from the American Type Culture Collection (Manassas, VA, USA). NCI/ADR-RES cell line was obtained from the DTP Human Tumor Cell Line Screen (Developmental Therapeutics Program, NCI). Both types of cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS (v/v) and penicillin (100 U/ml)/streptomycin (100 μg/ml)/amphotericin B (0.25 μg/ml). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

2.3. SRB assay

The cells were inoculated into 96-well plates in complete media and incubated overnight for attachment. Cells were then fixed *in situ* with 10% trichloroacetic acid (TCA) to represent a measurement of the cell population at the time of drug addition (T_0). After an additional 48 h incubation with or without protopine in medium with 5% FBS, the assay was terminated by 10% TCA. SRB dye at 0.4% (w/v) in 1% acetic acid was added to stain the cells. Unbound dye was removed by washing with 1% acetic acid twice and the plates were air dried. Bound dye was subsequently solubilized with 10 mM trizma base, and the absorbance was read at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control group (C), and cell growth in the presence of protopine (T_i), the percentage growth was calculated as: $[(T_i - T_0)/(C - T_0)] \times 100$. Growth inhibition of 50% (IC_{50}) is calculated as the drug concentration, which caused a 50% reduction in the net protein increase in control cells during the drug incubation.

2.4. Flow cytometric analysis

After drug treatment, the cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS), then pellets were resuspended and fixed in ethanol (70%, v/v) at −20 °C overnight, and washed once with PBS. After centrifugation, the cells were incubated for 15 min at room temperature in 0.1 ml of phosphate-citric acid buffer (0.2 M $NaHPO_4$, 0.1 M citric acid, pH 7.8). Cells were stained with PI staining buffer containing Triton X-100 (0.1%, v/v), RNase A (100 μg/ml) and PI (80 μg/ml) for 30 min in the dark. Cell cycle distribution was analyzed by flow cytometry with CellQuest software (Becton Dickinson, San Jose, CA).