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Original article

Podophyllotoxin acetate enhances γ-ionizing radiation-induced apoptotic cell death by stimulating the ROS/p38/caspase pathway

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ABSTRACT

To develop a new radiosensitizer against non-small cell lung cancer cells, we screened a natural product library for growth-inhibitory compounds. PA was found to be cytotoxic toward NCI-H460 cells, and its IC_{50} value was determined. The radiosensitizer effects of PA were tested at its IC_{50} value in clonogenic and cell-counting assays. The intracellular mechanism underlying this effect was determined by immunoblotting and by measuring propidium iodide uptake and ROS generation. The radiosensitizer activity of PA *in vivo* was tested in nude mice by treating with PA and IR, and measuring tumor volume and assessing apoptosis. PA, tested at its experimentally determined IC_{50} value (12 nM), enhanced IR-induced death of NCI-H460 cells by increasing apoptosis, yielding a mean calculated dose-enhancement ratio of 1.67. Combination with PA and IR also increased the production of ROS, which subsequently induced phosphorylation of p38, suppressed phosphorylation of ERK, and activated caspase-3, -8, and -9. Notably, inhibition of ROS production prevented p38 phosphorylation, and inhibition of ROS production or p38 activation blocked caspase activation and apoptosis. In a xenograft assay, combination with PA and IR delayed tumor growth by 11.4 days compared with controls, yielding an enhancement factor of 1.48. Collectively, these results indicate that PA functions as a radiosensitizer by enhancing apoptosis through activation of a ROS/p38/caspase pathway and suppression of ERK.

1. Introduction

Lung cancer is among the most lethal diseases worldwide. Non-small cell lung cancer (NSCLC), in particular, is associated with a low 5-year survival rate [1]. NSCLC therapy typically involves surgery, radiotherapy, and/or drug treatment. Unfortunately, drugs and radiation therapy, when used individually, frequently result in therapeutic resistance—the primary treatment obstacle for most cancers, including NSCLCs. Therefore, the development of new therapeutic combinations of drugs and radiotherapy is an essential element of strategies to improve patient survival. The rationale for combining radiation and drug therapies is based on the concept that combinations of therapeutic modalities with different mechanisms of actions will be more effective at eradicating the cancer than a single agent [2]. Although various conventional anticancer drugs have been used as radiosensitizers, there remains a need for new and more effective radiosensitizers [3,4].

PA is a derivative of podophyllotoxin that is an abundant lignan isolated from podophyllin, a type of resin from Podophyllum. Lignans are a family of natural products and secondary metabolites, generated via the shikimic acid pathway, consisting of two bound phenylpropane units. Podophyllotoxin exhibits the aryltetralin structure of cyclolignans, which are lignans containing a carbocycle between the two phenylpropane units created by two single carbon–carbon bonds through the side chains, one of which is between the β-β'positions. Podophyllotoxin is known to exert antiviral effects against herpes, measles, influenza and venereal warts, and has also been shown to exhibit immunosuppressive activity [5]. PA mediates its antitumor activity by reversibly binding to tubulin and interrupting tubulin polymerization. This disruption of the dynamic equilibrium of microtubules prevents formation of mitotic-spindles microtubules, resulting in cell cycle arrest. To improve the antitumor effects of PA, many investigators have synthesized various derivatives, including three representative semi-synthetic epipodophyllotoxin derivatives: etoposide,

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teniposide and etopophos. The anticancer activity of these drugs is derived from their inhibitory activity toward DNA topoisomerase, especially topoisomerase II. DNA topoisomerases are ubiquitous enzymes responsible for controlling the topological state of DNA in cells. There are two forms of DNA topoisomerase; type I cleaves a single strand of DNA, and type II cleave both strands. These podophyllotoxin derivatives prevent the re-ligation of DNA, but do not inhibit microtubules because of the presence of a bulky glucoside moiety in their chemical structure. Podophyllotoxin analogs composed of a nucleic acid–drug–enzyme complex induce single- and double-stranded DNA breaks, leading to cell death or apoptosis [6].

In this study, we isolated PA from a natural compound library and tested whether it functions as a radiosensitizer to enhance the efficacy of IR. Our results demonstrate a radiosensitizer effect of PA *in vitro* and *in vivo*, and show that this effect is attributable to the enhancement of apoptosis through activation of a ROS/p38/caspase pathway and suppression of ERK.

2. Materials and methods

2.1. Cell culture and chemical reagents

The NCI-H460 human NSCLC cell line was purchased from American Type Culture Collection (Rockville, MD). SB203580, N-acetyl-L-cysteine (NAC), 2′,7′-dichlorofluorescin diacetate (DCF-DA) and z-VAD-fmk were obtained from Calbiochem (La Jolla, CA). PA and the Natural Product Collection were from MicroSource Discovery Systems, Inc. (Gaylordsville, CT).

2.2. MTT assay and IC_{50} determination

NCI-H460 cells (4×10^3 cells/well) were seeded and treated with different concentrations of PA. After incubating for 72 hours, 50 μL of a 3-(4,5-dimethylthiazol-2-yle)-2,5-diphenyltetrazolium-bromide (MTT) solution (2 mg/mL) was added to each well, and the plates were incubated at 37 °C for 2 hours. The formazan crystals formed by living cells were dissolved in 200 μL/well of dimethyl sulfoxide (DMSO), and the absorbance of individual wells was read at 545 nm using a microplate reader (Original Multiscan; Thermo Co. Waltham, MA, USA). The 50% inhibitory concentration (IC_{50}) was calculated from a concentration-response analysis performed using Softmax Pro software (Molecular Devices, Sunnyvale, CA).

2.3. Clonogenic assay

Clonogenic assays were performed as described previously [7]. NCI-H460 cells were seeded in triplicate 60-mm dishes at densities estimated to yield 20–100 colonies/dish (100, 200, 400, 600 and 1000 cells/dish). After 24 hours of incubation, cells were pre-incubated with or without 10 nM PA for 16 hours and then exposed to different doses of IR (1, 3, 5, 7 Grey [Gy]) using ^{137}Cs as a radiation source (Atomic Energy of Canada, Ltd., Ontario, Canada). Treated cells and controls were cultured for 10–14 days, and colonies larger than 200 μm in diameter were counted using a colony counter (Imaging Products, Chantilly, VA).

2.4. Immunoblot analysis

Immunoblot analyses were performed as described previously [8]. Membranes were probed with antibodies against caspase-3, -8, and -9; phospho-p38, p38, phosphorylated extracellular signal-regulated kinase (ERK), and total ERK (Cell Signaling Technology, Inc., Beverly, MA). An anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO) was used a control for equal loading. Relative band densities of targets, determined densitometrically and normalized to that of β-actin, p38 or ERK, were analyzed using Image J software (NIH, USA).

2.5. ROS detection assay

ROS production was detected as described previously [9]. Cells were seeded at a density of 1.5×10^5 cells and treated under various conditions as described in the text. Treated cells were incubated with 20 μM DCF-DA for 20 minutes and then trypsinized. ROS was detected and analyzed using a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.6. Propidium iodide uptake assay

Cells were seeded at a density of 1×10^5 cells and incubated with or without 10 nM PA. After 16 hours of incubation, cells were exposed to 3 Gy IR, and then incubated for 48 hours. Treated cells were harvested by trypsinization, washed twice with cold PBS, and resuspended in 300 μL of a 5-μg/mL propidium iodide (PI, Sigma-Aldrich) solution. The apoptotic fraction was evaluated using a FACSort flow cytometer (Becton Dickinson).

2.7. Xenograft size determination and TUNEL assay

All animal experiments were performed under approved protocols of our Institutional Animal Care and Use Committee. The *in vivo* effects of PA were evaluated in a xenograft model, created by injecting NCI-H460 cells (1×10^7) subcutaneously into 6-week-old BALB/cAnNCrj-nu/nu mice (Charles River Japan Inc., Japan). Mice were divided into four groups (5 mice/group): control (mock treated), IR-only, PA-only, and PA and IR (combination). When xenografts reached ~100–120 mm^3, PA (5 mg/kg) was subcutaneously injected into tumor sites in mice in PA-only and combination groups. Tumors in IR-only and control groups were injected with an equal volume of vehicle solution (DMSO). After 6 hours, mice in IR-only and combination groups were locally irradiated (5 Gy) using a ^{60}Co γ-ray source (Theratrom 780; AECL Ltd., Mississauga, Ontario). This protocol was repeated three times at 5-day intervals for 35 days. Tumor dimensions (long and short axes) were measured periodically, and tumor volumes were calculated as (short $axis^2 \times$ long axis/2). For terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL) assays, tumors were extracted, fixed with formaldehyde, and then embedded in a paraffin block. Sliced tissues were stained and analyzed with the Apoptag TUNEL assay kit (Merck KGaA, Darmstadt, Germany), as described by the manufacturer. Tumor growth delay values were calculated as described previously [7].

2.8. Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA), and the significance of differences between experimental groups was determined using Student *t*-test. *P*-values < 0.05 were considered significant; individual *P*-values in figures are denoted by asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). The numbers above each point or bar in graphs are the means of three independent experiments, and error bars signify standard deviations (SD).

3. Results

3.1. PA acts as radiosensitizer by enhancing apoptotic cell death in vitro

PA (Fig. 1A) was screened from a natural product library as an anticancer drug candidate. The IC_{50} value of PA in NCI-H460 cells