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Vinca alkaloids cause aberrant ROS-mediated JNK activation, Mcl-1 downregulation, DNA damage, mitochondrial dysfunction, and apoptosis in lung adenocarcinoma cells

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ABSTRACT

Vinca alkaloids are clinically used to inhibit the growth of malignancy by interfering with microtubule polymerization. The purpose of this study was to identify the molecular mechanisms underlying growth inhibition as well as apoptosis in vinca alkaloid-treated lung adenocarcinoma cells. Consistent with nocodazole, treatment with vinorelbine (VNR) caused mitotic prometaphase arrest in a time-dependent manner, accompanied by cell apoptosis, dependent on both dose and time. VNR sequentially induced mitochondrial transmembrane potential (MTP) loss and caspase-dependent apoptosis following myeloid cell leukemia (Mcl) 1 downregulation. Prolonged activation of c-Jun N-terminal kinase (JNK) was required for vinca alkaloid- and nocodazole-induced apoptosis but not cell cycle arrest. Vinca alkaloids and nocodazole caused glutathione/reactive oxygen species (ROS) imbalance, and inhibiting ROS prevented prolonged JNK activation, decreased Mcl-1 levels, MTP loss, and apoptosis. Notably, cell size and granularity were enlarged in stimulated cells; unexpectedly, many ROS-producing mitochondria were accumulated followed by aberrant JNK-mediated mitochondrial dysfunction. Unlike cisplatin, which causes DNA damage in each phase of the cell cycle, VNR and nocodazole induced aberrant JNK-regulated DNA damage in prometaphase; however, inhibiting ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) did not reverse mitotic arrest or apoptosis. These results demonstrate an essential role of ROS in vinca alkaloid-induced aberrant JNK-mediated Mcl-1 downregulation and DNA damage followed by mitochondrial dysfunction-related apoptosis but not mitotic arrest.

1. Introduction

Lung cancer is one of the leading causes of death worldwide. In 2010, there were approximately 222,520 newly diagnosed cases and 157,300 deaths from lung and bronchus cancer in the United States [1]. Many chemotherapy agents have shown therapeutic benefits in lung cancer, e.g., microtubule inhibitors (taxenes and vinca alkaloids), antimetabolites (pemetrexed and gemcitabine), etc. Vinca alkaloids, including vinorelbine (VNR), vincristine, vinblastine, and vindesine, bind to tubulin as potent inhibitors of mitotic microtubule polymerization. VNR, a semi-synthetic vinca alkaloid, is used both as a single agent and in combination with cisplatin for first-line treatment of advanced and metastatic non-small cell lung cancer (NSCLC). The lipophilic feature ensures that VNR is rapidly distributed over the peripheral tissues, so the peak plasma concentration of VNR is about 1 μM. The levels decay to 100 nM at 1 h and to 10 nM at 24 h after the intravenous infusion of 20–30 mg/m^2 [2,3]. The concentration of VNR that is used in *in vitro* studies is thought to be equal to the levels in plasma [4].

In addition to causing cell cycle arrest by targeting microtubules, VNR causes apoptosis of leukemia and lymphoma cells

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through caspase-3 activation [5]. Combination treatment of vinca alkaloids and paclitaxel induces apoptosis with caspase-8 activation, independent of CD95 in human colon cancer cells [6]. Phosphorylated Bcl-2 and activated caspase-8, -9, and -3 are observed in tumor tissues of NSCLC patients with VNR treatment [7]. VNR causes apoptosis by increasing Bax and Bcl-xs expression, decreasing Bcl-2 and Bcl-xL, releasing cytochrome *c*, and augmenting caspase-9 and caspase-3 activities in lung carcinoma cells [8]. Furthermore, VNR-induced apoptosis is enhanced by the activation of caspase-8 via caspase-9-mediated activation of caspase-3 but not through CD95 in Jurkat cells [9]. Therefore, an intrinsic pathway of mitochondrial dysfunction-related apoptosis is usually induced by VNR.

Myeloid cell leukemia (Mcl) 1 localizes in the mitochondria, promotes cell survival by suppressing cytochrome *c* release from mitochondria and is regulated at the transcriptional and post-translational levels. The mitogen-activated protein kinase c-Jun N-terminal kinase (JNK) phosphorylates Ser121 and Thr163 of Mcl-1 and then induces Mcl-1 inactivation after stimulation with hydrogen peroxide [10]. Mcl-1 is rapidly degraded through two main routes by caspase-mediated and proteasome-dependent degradation. Activation of caspase-3 cleaves Mcl-1 at Asp127 and Asp157 to expose a C-terminal domain with a death-promoting activity [11,12]. Additionally, glycogen synthase kinase-3 causes Ser159 and Thr163 phosphorylation to facilitate Mcl-1 ubiquitylation and degradation in response to apoptotic stimuli [13]. Vinblastine induces JNK-mediated phosphorylation and the consequent inactivation of Bcl-2 and Bcl-xL prior to caspase-3 activation [14]. However, the molecular basis for JNK activation remains unknown.

Oxidative stress-activated apoptosis signal regulating kinase 1/JNK is important for apoptosis [15]. Chemotherapy, radiotherapy, and photodynamic therapy can often promote reactive oxygen species (ROS) generation while killing tumor cells by the generation of ROS directly in tumor cells or by inhibiting the anti-oxidative enzymes of tumor cells [16]. VNR can cause vascular toxicities. Notably, it has been demonstrated that VNR induces oxidative stress by increasing ROS generation to cause depolarization of mitochondrial membranes in porcine aorta endothelial cells [4]. However, the oxidative stress caused by VNR lacks a mechanism study. In this study, we demonstrated an essential role of ROS in vinca alkaloid-induced aberrant JNK-mediated Mcl-1 downregulation followed by mitochondrial dysfunction and apoptosis in lung adenocarcinoma cells. We further examined a possible mechanism for ROS generation caused by VNR. The effects of aberrant ROS/JNK on vinca alkaloid-induced mitotic arrest and DNA damage were also investigated.

2. Materials and methods

2.1. Drugs

VNR was purchased from Sigma–Aldrich (St. Louis, MO). The broad-spectrum caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoro methyl ketone (z-VAD-fmk), caspase-3 inhibitor benzyloxycarbonyl-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me)-fluoromethyl ketone (z-DEVD-fmk), caspase-9 inhibitor benzyloxycarbonyl-Leu-Glu(O-Me)-His-Asp(O-Me)-fluoromethyl ketone (z-LEHD-fmk), and caspase-2 inhibitor benzyloxycarbonyl-Val-Asp(O-Me)-Val-Ala-Asp(O-Me)-fluoromethyl ketone (z-VDVAD-fmk) were also purchased from Sigma–Aldrich and dissolved in dimethyl sulfoxide (DMSO). Nocodazole, cathepsin B inhibitor benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone (z-FA-fmk), cathepsin D inhibitor pepstatin A, GSK-3 inhibitors lithium chloride (LiCl), 6-bromoindirubin-3′-oxime (BIO), SB216763, and SB415286, proteasome inhibitor MG132, and ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) inhibitor caffeine were purchased from Calbiochem (San Diego, CA). MEK inhibitor PD98059, JNK inhibitor SP600125, p38 MAPK inhibitor SB202190, and antioxidant diphenylene iodonium (DPI) were obtained from Sigma–Aldrich and dissolved in DMSO prior to dilution with PBS. Rabbit anti-human MPM2, α-tubulin, lamin A/C, PARP, caspase-3, Bcl-2, Mcl-1, Bcl-xL, phospho-apoptosis signal regulating kinase (ASK) 1 (Thr845), ASK1, phospho-JNK (Thr183/Tyr185), JNK, and γ-H2AX were purchased from Cell Signaling Technology (Beverly, MA). β-actin antibodies and horseradish peroxidase-conjugated anti-rabbit IgG were obtained from Chemicon International (Temecula, CA). All drug treatments in cells were assessed for their cytotoxic effects using cytotoxicity assays before experiments. Non-cytotoxic dosages were used in this study.

2.2. Cell culture

Human lung adenocarcinoma AS2 cell line was established from ascites that had been generated by PC14PE6 cells (a gift from Isaiah J. Fidler) (MD Anderson Cancer Center, Houston, TX) in nude mice [17]. AS2 and human lung adenocarcinoma A549 cells were routinely grown on plastic in Dulbecco's modified Eagle's medium (Gibco-BRL; Grand Island, NY) with L-glutamine and 15 mM HEPES, supplemented with 10% fetal bovine serum (Gibco-BRL), 100 units of penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in 5% CO_2. Other chemical drugs used for cell culture were purchased from Sigma–Aldrich.

2.3. Cytotoxicity assay

To evaluate cell damage, lactate dehydrogenase (LDH) activity was assayed using a colorimetric assay (Cytotoxicity Detection kit; Roche Diagnostics, Lewes, UK) according to the manufacturer's instructions. Aliquots of the culture media were transferred to 96-well microplates. A microplate reader (Spectra MAX 340PC; Molecular Devices, Sunnyvale, CA) was used to measure the absorbance at 620 nm with a reference wavelength of 450 nm, and the data were analyzed with Softmax Pro software.

2.4. Cell cycle and apoptosis assay

The cell cycle and apoptosis was analyzed using nuclear propidium iodide (PI; Sigma–Aldrich) staining as previously described [18] and then analyzed using flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA) with excitation set at 488 nm and emission detected at the FL-2 channel (565–610 nm). For cell cycle analysis, the distribution of cells in different phases of the cell cycle was calculated using the MetaMorph software (Molecular Devices, Downingtown, PA). For apoptotic analysis, the samples were analyzed using CellQuest Pro 4.0.2 software (Becton Dickinson), and quantification was performed using WinMDI 2.8 software (The Scripps Institute, La Jolla, CA). Apoptosis levels were reported as percentages of sub-G_1. Annexin V plus PI staining was also performed using a commercial kit (Sigma–Aldrich) according to the manufacturer's instructions. To observe nuclear condensation, 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich)-stained cells were observed using a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

2.5. Immunostaining

The cells were fixed in 3.7% formaldehyde (Sigma–Aldrich) in PBS for 10 min. After washing twice with PBS, the cells were incubated with primary antibodies against MPM2, α-tubulin, lamin A/C, phospho-JNK, and γ-H2AX in antibody diluent (DAKO Corporation, Carpinteria, CA) with or without PI for DNA content