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Original Articles

Nelfinavir, an HIV protease inhibitor, induces apoptosis and cell cycle arrest in human cervical cancer cells via the ROS-dependent mitochondrial pathway

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ABSTRACT

HIV protease inhibitors (HIV-PIs) are a class of antiretroviral drugs designed to target the viral protease. Strikingly, these drugs have also been reported to possess antitumor effect. In this study, we evaluated the activity of one HIV-PI, Nelfinavir, against human cervical cancer cells. We found that Nelfinavir inhibited the growth of cervical cancer cell lines at the lowest micromolar concentrations clinically attainable. Nelfinavir promoted apoptosis and arrested the cell cycle at G1 phase. Apoptosis is attributed to the promotion of mitochondrial reactive oxygen species (ROS) production, which results in the translocation of mitochondrial apoptosis inducing factor (AIF) to the nucleus. We further showed that Nelfinavir increased mitochondrial ROS production by decreasing manganese superoxide dismutase (MnSOD) protein levels. Taken together, our results suggest that Nelfinavir can be repositioned as a cervical cancer therapeutic.

Introduction

Cervical cancer is the third most common type of cancer in women worldwide and a leading cause of cancer-related death for women in developing countries [1]. Conventional therapies for cervical cancer include surgery, radiotherapy and chemotherapy [2]. Surgery is the first option for patients with early-stage cervical cancer, while radiotherapy and chemotherapy have proven to be effective treatments for patients in the advanced stages. The most commonly used chemotherapy drugs in advanced cervical cancer is platinum-based chemotherapy [3]; however, the effect of these drugs is limited, and their side effects are serious [4,5]. Therefore, development of new chemotherapeutic agents is required.

HIV protease inhibitors (HIV-PIs) are a class of small-molecule drugs that were rationally designed to target the viral aspartyl protease. They are peptidomimetic drugs designed to mimic the peptide bond targeted by viral protease, but not by any other mammalian endopeptidase [6], which means they have a good specificity of action with tolerable side effects. Currently, nine FDA-approved HIV-PIs, Nelfinavir, Indinavir, Ritonavir, Amprenavir, Saquinavir, Lopinavir, Tipranavir, Atazanavir and Darunavir, are available. These drugs, given in combination with reverse transcriptase inhibitors, are the mainstays of the current therapeutic regimens for HIV-infected patients. In recent years, accumulating evidence supports that HIV-PIs can be promising antineoplastic agents. Early support for this came from a report of a patient with regression of Kaposi's sarcoma (KS) following therapy with HIV-PIs. Such antitumor mechanism was attributed to HIV load reduction or CD4 T cell gain [7]. However, several recent studies support that HIV-PIs have direct antitumor activities that are independent of their antiviral activity, including leukemia [8], lung cancer [9], breast cancer [10], glioblastoma [11], multiple myeloma [12], melanoma [13] and ovarian cancer [14]. Studies also show that treatment of HIV-infected women with Saquinavir, an HIV-PI, can reduce the onset of uterine cervical intraepithelial neoplasia (CIN) and halt its progression to cervical carcinoma [15], but the direct effect and mechanism of HIV-PIs on cervical carcinoma remains unknown.

In this study, we aimed to identify an HIV-PI-based anti-HIV drug with the most potent inhibitory activity on the growth of cervical cancer cells and investigate its direct effect on cervical cancer cells and elucidate the underlying mechanisms of action.

Abbreviations: HIV-PIs, HIV protease inhibitors; ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; KS, Kaposi's sarcoma; CIN, cervical intraepithelial neoplasia; DCFH-DA, dichloro-dihydro-fluorescein diacetate; NAC, N-acetylcysteine.

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Materials and methods

Cell viability assays

Cell viability was determined by XTT array as previously described [16]. Briefly, the cervical cancer cell lines (Hela, SiHa and CaSki) were seeded in triplicate in 96-well plates at 10^4 cells per well and cultured in the presence of HIV-PIs as indicated for 3 days, the medium was exchanged, and the cells were incubated for 4 h with 1 mg/mL XTT reagent and PMS. Absorbance was measured at 450 nm in the microplate reader.

BrdU/7-AAD staining

For assessment of *in vitro* proliferation, the APC BrdU flow kit (BD Biosciences) was used. Briefly, cells (2×10^5 cells/mL) were pulsed with 10 μM BrdU for 30 min and then washed and stained with a dilution of 1:50 APC-labeled antibodies directed against BrdU. 7-AAD was mixed with the cells. Fluorescence-activated cell sorting (FACS) analysis was performed on a FACS (BD Biosciences). Fluorescence was detected at 670 nm for 7-AAD and at 660 nm for APC, and data were analyzed with FlowJo software.

Apoptosis

Detection was performed by the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen). Briefly, cells were seeded in 12-well plates and incubated for 24 h by various drug concentrations as indicated. After treatment, approximately 2×10^5 cells were harvested, washed once with PBS, and then stained with Annexin V-FITC and PI according to the manufacturer's instructions. The resulting fluorescence was detected by flow cytometry at 530 nm for FITC and at 585 nm for PI, and analyzed with FlowJo software.

Assays to measure levels of total cellular and mitochondrial ROS

Total cellular ROS and mitochondrial ROS levels in the cervical cancer cell lines were measured by dichloro-dihydro-fluorescein diacetate (DCFH-DA) and MitoSOX™ Red assays based on the manufacturers' instructions. Briefly, cells were incubated in HBSS with DCFH-DA or MitoSOX™ Red at a final concentration of 10 μM or 5 μM for 15 min at 37 °C and then washed with HBSS. The intensity of fluorescence was recorded using a flow cytometer (at 530 nm for DCFH-DA and 585 nm for MitoSox, and imaged using confocal microscopy (Zeiss 510 Meta).

Measurement of mitochondrial transmembrane potential ($\Delta\Psi m$) loss

Mitochondrial depolarization was monitored with Rhodamine 123 (Life Technologies, South San Francisco, CA). After incubation with 1 μL of Rh-123 (10 mmol/l), cells were kept in a 5% CO_2 incubator for 15 min. Then, the cells were washed with PBS and viewed under confocal microscopy using a blue filter (450–490 nm), followed by measuring the intensity of fluorescence at 530 nm by flow cytometry.

Immunofluorescence

Cells were washed twice with ice-cold PBS before fixation with ice-cold methanol. After blocking with 5% bovine serum albumin in PBS containing 0.3% Triton X-100 for 1 h, cells were incubated with the primary antibody against AIF for 1 h. Cells were washed with PBS three times and incubated with the secondary antibody conjugated with FITC (Molecular Probes, Eugene, OR) for 1 h. The nuclei were stained with propidium iodide (Sigma) for 5 min after secondary antibody incubation and three rinses with PBS. After washing three times with PBS, coverslips were mounted onto microscope slides using ProLong® Gold Antifade Mountant (Molecular Probes). The slides were analyzed using confocal microscopy (Zeiss 510 Meta).

Western blot

Briefly, cell lysates were prepared by extracting proteins with RIPA Lysis Buffer (Millipore, Temecula, CA) supplemented with protease inhibitors (Millipore). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence procedures (Bio-Rad) according to the manufacturer's recommendations.

Preparation of mitochondrial and nuclear fractions

The cells were washed with ice-cold PBS, left on ice for 10 min, and then resuspended in isotonic homogenization buffer [250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L Na-EDTA, 1 mmol/L Na-EGTA, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonylfluoride, and 10 mmol/L Tris–HCl (pH 7.4)] containing a proteinase inhibitor mixture (Roche). After 80 strokes in a Dounce homogenizer, the unbroken cells were spun down at 30 *g* for 5 min. The nuclei and heavy mitochondrial fractions were fractionated at 750 *g* for 10 min and 14,000 *g* for 20 min, respectively, from the supernatant. The nuclei fraction was washed three times with homogenization buffer containing 0.01% NP40.

Small interfering RNA transfection

RNA interference of AIF lentivirus was obtained commercially from Santa Cruz Biotechnology (Santa Cruz, CA). The lentivirus was transfected into HeLa cells according to the manufacturer's protocol. At 48 h after transduction, the medium was replaced, and cells were harvested. Assays were performed 48 h after transfection. The protein level of AIF was detected in the cell lysate by Western blot and immunofluorescence analysis.

In vivo *tumor experiments*

For tumor implantations, single tumor cell suspensions (1×10^7) were prepared in a 100 μL of PBS mixture and injected subcutaneously at the back of nude mice (6–8 weeks old). Then the mice were divided into two groups (n = 5). One group received an i.p. injection of 200 μL of saline solution of 10% DMSO as control, and the other group received a treatment consisting of i.p. injection (once daily) of Nelfinavir (1 mg per mouse) dissolved in 200 μL of an injectable saline solution of 10% DMSO. Treatment for all groups commenced on day 2 after implantation. Mouse tumors were measured three times a week. Tumor growth was scored daily by two cross-sectional measurements, and tumor size was calculated using the formula described by Kyriazis et al. [17] as follows: tumor volume = $\text{width}^2 \times \text{length} \times 0.4$. The experiments were terminated when the tumors reached 200–300 mm^3, and the mice were sacrificed under anesthesia. All of the procedures involving animals were approved by the Laboratory Animal Center, Xinqiao Hospital, Third Military Medical University, Chongqing, China. Mouse care and use were performed in accordance with local ethical guidelines.

Immunohistochemical staining

Tumor tissue specimens were fixed in 10% neutral formalin and embedded in paraffin after collection from the expired mice. Tissue sections of 5 μm thick were dewaxed and incubated with 0.01 M natrium citricum for antigen retrieval. The slides were rinsed with PBS and incubated overnight at 4 °C with diluted Ki67, TUNNEL, AIF and SOD-2 antibodies. The following experiments were performed using the immunostaining kit according to the manufacturer's instructions.

Statistical analysis

All data from quantitative assays were expressed as mean ± standard deviation (SD). Statistical analyses were performed using the independent-samples t-test or one-way ANOVA. The difference was considered statistically significant when $P < 0.05$. All statistical analyses were carried out with SPSS 13.0 software.

Results

Effect of HIV-PIs on the growth of cervical cancer cell lines

We first systematically investigated the effectiveness of 9 HIV-PIs (Nelfinavir, Indinavir, Ritonavir, Amprenavir, Saquinavir, Lopinavir, Tipranavir, Atazanavir and Darunavir) on the growth of cervical cancer cell line HeLa. Cell viability was assessed using XTT assay after treating HeLa cells with different concentrations (1–1000 μM) of HIV-PIs for 72 h. We found that exposure of HeLa cells to the 9 HIV-PIs showed very different activity against HeLa cells. Nelfinavir was the most active drug in that it significantly inhibited cell viability in a dose-dependent manner ($P < 0.001$), with an IC_{50} of 9.71 μM. Lopinavir, Atazanavir, Ritonavir and Saquinavir were less active than Nelfinavir with IC_{50} in a range of 23–30 μM, whereas Indinavir, Darunavir and Amprenavir had essentially no activity at concentrations up to 100 μM (Fig. 1A). Thus, these HIV-PIs can be ranked from most to least potent as follows: Nelfinavir > Lopinavir > Atazanavir > Ritonavir > Saquinavir > Tipranavir > Amprenavir > Darunavir > Indinavir. Because Nelfinavir showed the highest activity against the HeLa cell line and the concentration at IC_{50} of 9.7 μM, which is attainable in the plasma of patients (5–12 μM) who take Nelfinavir at standard dosage, we focused our subsequent investigations on this drug. To confirm the effect of Nelfinavir on cervical cancer, we used two other cervical cancer cell lines (SiHa and CaSki) as controls. As shown in Fig. 1B, Nelfinavir could also effectively inhibit the growth of cervical cancer cell lines