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Reduced cell viability and apoptosis induction in human thyroid carcinoma and mesothelioma cells exposed to cidofovir

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ABSTRACT

Besides its well-recognized antiviral activity, Cidofovir (CDV) has been shown to exert anticancer properties both within in vitro and in vivo models. The aim of this study was to evaluate the effects of CDV on still unexplored cultured cancer cells from human mesothelioma as well as breast, colon, liver, lung, prostate, and thyroid carcinomas. Overall, a dose- and time-dependent inhibition of cell viability was observed after CDV exposure. To clarify the mechanisms underlying CDV action, apoptotic cell death was investigated in two infected cell lines [Ist-Mes1 and Ist-Mes2 mesothelioma cells (SV40+)] and in two uninfected cell lines (NCI-H2425 mesothelioma cells and FTC-133 thyroid cancer cells), which resulted the most sensitive to CDV treatment. Reduced expression of procaspase-3 and increased expression of PARP p85 fragment were observed in both infected and uninfected mesothelioma cells, indicating apoptosis induction by CDV in a virus-independent manner. Similarly, the increase of the pro-apoptotic proteins p53, cytochrome *c* and caspase-3, the decrease of the survival protein Bcl-x, and the increment of Bax/Bcl-2 ratio revealed the occurrence of apoptosis in CDV-treated FTC-133. The presence of nuclear DNA fragmentation confirmed apoptotic cell death by CDV. Overall, our findings warrant further investigations to explore the therapeutic potential of CDV for human mesothelioma and follicular thyroid carcinoma.

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1. Introduction

Cidofovir (CDV) is an acyclic nucleoside phosphonate analog with a broad-spectrum anti-DNA virus activity, including herpes-, papilloma-, polyoma-, and adenoviruses. Its mechanism of action is based on a higher affinity of the active diphosphate metabolite (CDVpp) for viral DNA polymerases compared to cellular DNA polymerases (De Clercq and Holý, 2005).

Besides its well-recognized antiviral activity, CDV has been shown to exert in vitro antiproliferative effects against papillomavirus (HPV)-positive cervical carcinoma cells (Andrei et al., 2000) and cytomegalovirus (CMV)-infected glioblastoma cells (Hadaczek et al., 2013). Interestingly, cancer cell death after CDV administration has been observed even in the absence of viral infection (Andrei et al., 1998; Hadaczek et al., 2013), suggesting that CDV can inhibit cellular DNA synthesis and tumor cell proliferation regardless of endogenous viral presence. In accord, CDV does not depend upon a viral gene product to become phosphorylated and activated, but rather is phosphorylated by the human cellular cytidine kinase enzyme (De Clercq and Holý, 2005).

In vivo, CDV has been shown to inhibit cancer growth in virus-associated tumors, such as nasopharyngeal and cervical carcinomas, hemangiomas and hemangiosarcomas (Liekens et al., 1998; Liekens et al., 2001; Tristram et al., 2014; Yoshizaki et al., 2008), as well as in virus-independent tumors, such as basal cell carcinoma, cutaneous squamous cell carcinoma, and glioblastoma (Calista, 2002a; Calista et al., 2002b; Hadaczek et al., 2013). Recently, CDV has been also demonstrated to inhibit lung metastasis of virus-independent, fibroblast growth factor-2-driven tumors (Liekens et al., 2015). All these findings confirm that CDV may show therapeutic efficacy in cancer beyond the setting of viral infection and warrant further studies to select those tumor types that are most likely to benefit from CDV therapy.

With this in mind, in the present paper we evaluated the effects of CDV on still unexplored cultured cancer cells from human breast, colon, liver, lung, prostate, and thyroid carcinomas, and mesothelioma. One HPV-positive cervical carcinoma cell line and two Simian Virus 40 (SV40)-positive mesothelioma cell lines were also included as controls to confirm CDV efficacy against virus-infected tumor cells.

Abbreviations: CDV, Cidofovir; CDVpp, Cidofovir diphosphate; HPV, human papillomavirus; CMV, cytomegalovirus; SV40, Simian Virus 40.

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2. Materials and methods

2.1. Cell culture

The effects of CDV administration were investigated in the following cancer cell lines: Caco-2 (colorectal adenocarcinoma), FTC-133 (follicular thyroid carcinoma), HeLa (cervix carcinoma), Hep-G2 (liver hepatoblastoma), MDA-MB-231 (breast carcinoma), NCI-H1975 (non-small cell lung adenocarcinoma), PC-3 (prostate carcinoma), and NCI-H2452, Ist-Mes1, and Ist-Mes2 (mesothelioma). HeLa cells were positive to HPV18, while Ist-Mes1 and Ist-Mes2 were positive to SV40, a DNA tumor virus that has been implicated in the causation of mesothelioma (Pershouse et al., 2006).

Caco-2, HeLa, MDA-MB-231 and PC-3 cells were available within the Department of Biomolecular Sciences, University of Urbino "Carlo Bo"; FTC-133, Hep-G2, NCI-H1975, Ist-Mes1 and Ist-Mes2 cells were from Interlab Cell Line Collection (ICLC, Genova, Italy); NCI-H2452 cells were from the American Type Culture Collection (ATCC, Rockville, MD).

NCI-H1975 and PC-3 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin 100 U/mL. HeLa and FTC-133 cells were grown in DMEM medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin 100 U/mL; while Caco-2, MDA-MB-231 and Hep-G2 were grown in the same conditions but with the addition of 1% non-essential amino acids (NEAA). Mesothelioma cells were expanded as previously described (Stoppoloni et al., 2008). NCI-H2452 cells were cultured as monolayers in flasks using ATCC complete growth medium; Ist-Mes1 and Ist-Mes2 were cultured in DMEM with pyruvate supplemented with 10% FBS, 2 mM glutamine, 1% NEAA and antibiotics (0.02 U/mL penicillin and 0.02 mg/mL streptomycin). All cells were maintained in a CO_2 incubator at 37 °C and 5% CO_2. Cell culture materials and reagents were from VWR International (Milan, Italy).

The drug CDV, obtained from Sigma-Aldrich (Milan, Italy), was resuspended in phosphate buffer saline (PBS) and sterilized using a 0.45 μm syringe-filter before use. Increasing doses of CDV (from 10 to 1000 μM) were administered to cancer cells up to 120 h of incubation (medium was not refreshed during CDV exposure). Untreated cells receiving PBS served as controls (CTR).

2.2. Cell viability evaluation

Cell viability was analyzed at 450 nm by the WST-8 reagent [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Sigma-Aldrich, Milan, Italy). The assay was based on the cleavage of the tetrazolium salt WST-8 by cellular dehydrogenases in viable cells. Briefly, cancer cells were seeded in 96-well plates at 1000–3000 cells/well. After 24 h of incubation, the medium was replaced with fresh medium containing increasing doses of CDV (up to 1000 μM), as previously reported (Hadaczek et al., 2013); untreated controls were also included. After 24, 48, 72, 96 and 120 h of incubation, WST-8 was added to each well, and cells were further incubated at 37 °C up to 4 h. Colour development was monitored at 450 nm in a multiwell plate reader (Thermo Fisher Scientific, Milan, Italy).

2.3. Clonogenic assay

Colony formation capacity after CDV administration was measured using the clonogenic assay. Briefly, FTC-133 cells (10,000/cm^2) were seeded in sterile flasks and pre-treated with PBS (CTR) or with increasing concentrations of CDV (10 and 100 μM). After 24 h, 1000 viable cells per well were plated in 6-well plates and allowed to grow for about 14 days. Colonies were then fixed and stained for 90 min at room temperature with a 0.25% methylene blue in a 50% ethanol solution. Pictures were captured digitally and analyzed using a software for densitometric analysis (Quantity One 4.0.1, Bio-Rad Laboratories, Milan, Italy) to evaluate the colony volumes. Data were expressed as a percentage of the control.

2.4. Apoptosis antibody array

Apoptosis induction by CDV in FTC-133 cells was investigated using the Human Apoptosis Array Kit (R&D Systems, Milan, Italy) according to the manufacturer's instructions. Briefly, cancer cells (10,000/cm^2) were seeded in sterile flasks and treated for 96 h with PBS (CTR) or with CDV 100 μM. Cells were detached, lysed and protein levels were determined using the Bradford method (Bradford, 1976). Arrays were incubated overnight with 400 μg of cell lysates to detect the expression profile of 35 apoptosis-related proteins exploiting capture and control antibodies spotted on nitrocellulose membranes. After washing to remove unbound proteins, membranes were incubated with a cocktail of biotinylated detection antibodies. Streptavidin-Horseradish peroxidase (HRP) was then applied. Signals were developed using chemiluminescent reagents and then exposed to X-ray films. Pictures were analyzed using a software for densitometric analysis (Quantity One 4.0.1, Bio-Rad Laboratories, Milan, Italy) to evaluate the amount of protein levels. All spots were in duplicate.

2.5. DNA fragmentation analysis

Genomic DNA fragmentation was monitored in FTC-133 cells as a marker of late apoptosis after 96 and 120 h upon CDV administration (100 μM) by agarose gel electrophoresis, as previously described (Catalani et al., 2013). DNA laddering was visualized on a UV transilluminator by ethidium bromide staining. Images were obtained using a Gel Doc 2000 (Bio-Rad Laboratories S.r.l, Milan, Italy).

2.6. Western blot analysis

Apoptosis induction upon CDV administration (10 μM) to mesothelioma cells was evaluated after 120 h of incubation, as previously described (Stoppoloni et al., 2010). Briefly, 25–50 μg of proteins from cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked and blotted with relevant antibodies: poly (ADPribose) polymerase (PARP) (Promega, diluted 1:500), caspase-3 (Santa Cruz, diluted 1:200) and γ-tubulin (Sigma, diluted 1:5000). In detail, anti-PARP p85 Fragment pAb is a polyclonal antibody directed against the N-terminus of the 85 kDa fragment (p85) of human PARP that results from caspase cleavage, and does not detect the 116 kDa intact PARP molecule. As regards caspase-3, the antibody used is recommended for the detection of caspase-3 p20 and p17 subunits and full length procaspase-3 (inactive precursor). HRP-conjugated secondary antibodies were detected by Enhanced ChemiLuminescence (ECL, Amersham Biosciences). Anti-mouse or rabbit IgG HRP-conjugated secondary antibodies (1:3000) (Bio-Rad Laboratories, Hercules, CA, USA) were used.

2.7. Statistical analysis

The data are presented as the mean ± standard deviation of three independent experiments and analyzed using Student's *t*-test. Significance level was set at $p < 0.05$ for all analysis.

3. Results and discussion

In the present study, increasing concentrations (10–1000 μM) of the antiviral drug CDV were administered up to 120 h to a wide variety of cultured cancer cells from human breast, cervix, colon, liver, lung, prostate, and thyroid carcinomas, as well as from human mesothelioma.

Overall, a dose- and time-dependent inhibition of cell viability was observed after CDV exposure (Figs. 1 and 2). In accord to the prolonged half-life of the drug within the cell (17 to 65 h) (Safrin et al., 1999),

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