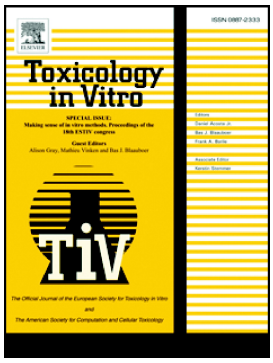

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REDUCED CELL VIABILITY AND APOPTOSIS INDUCTION IN HUMAN THYROID CARCINOMA AND MESOTHELIOMA CELLS EXPOSED TO CIDOFOVIR.

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RUNNING TITLE

Cidofovir action against human thyroid carcinoma and mesothelioma cells.

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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 occurence 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.

KEYWORDS:

Cidofovir; cancer cells; apoptosis induction; thyroid carcinoma; mesothelioma.

ABBREVIATIONS

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

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, 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, 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 et al., 2002a, 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.

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 tumour 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 hours 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 hours 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 hours of incubation, WST-8 was added to each well, and cells were further incubated at 37°C up to 4 hours. 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 (10000/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 hours, 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 analysed 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 (10000/cm^2) were seeded in sterile flasks and treated for 96 hours 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 hours 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 hours 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 (SantaCruz, 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 hours 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 (**Figures 1 and 2**). In accord to the prolonged half-life of the drug within the cell (17 to 65 hours) (Safrin et al., 1999), decreased cell viability was mainly evidenced following exposure of tumor cells to CDV for 72 to 120 hours, possibly indicating that cells need to accumulate sufficient drug-induced stress before reduced cell viability take place (Andrei et al., 1998; Andrei et al., 2000;

Hadaczek et al., 2013). Following CDV administration, cancer cells were also daily monitored microscopically to observe possible morphological changes related to necrosis; however, no alterations suggesting primary necrosis were evidenced. These findings seemed to confirm previous studies on glioblastoma cells treated with the same range of CDV concentrations (10-1000 μM) and for the same time period (24-120 h), in which necrosis induction by CDV exposure was not revealed (Hadaczek et al., 2013).

On the basis of IC_{50} values (**Table 1**), mesothelioma cancer cells resulted the most sensitive to CDV treatment; interestingly, CDV action appeared to be independent of the presence of the virus SV40, as also sustained by the subsequent evaluation of apoptotic cell death. In detail, Ist-Mes1 (SV40+), Ist-Mes2 (SV40+) and NCI-H2425 (SV40-) were analyzed for apoptosis induction after 120 hours upon CDV administration (10 μM) by western blot analysis. The decreased expression of full-length procaspase-3 (the inactive precursor of caspase-3) and the concomitant increased expression of PARP p85 fragment (indicating PARP cleavage by activated caspase-3) revealed that CDV induced apoptosis in mesothelioma cells in a virus-independent manner (**Figure 3**).

Taking into consideration the effects of CDV exposure on the remaining cancer cell lines, virus-infected HeLa cells (HPV18+) resulted the most responsive to the drug among carcinoma cells (**Table 1**), thus confirming previous findings on CDV efficacy against human HPV-harboring cell lines (Andrei et al., 1998; Andrei et al., 2000; De Schutter et al., 2013; Mertens et al., 2016). Among the uninfected carcinoma cell lines, FTC-133 follicular thyroid carcinoma cells proved to be the most sensitive to CDV treatment; in contrast, Caco-2 colorectal adenocarcinoma cells were the most resistant to the drug. As previously reported, the different sensitivity of uninfected carcinoma cells to CDV administration might be related to cancer cell mitotic rate; indeed, CDV primarily affects rapidly dividing cells, sparing cells that replicate their DNA at a lower rate (Hadaczek et al., 2013; Mertens et al., 2016). Accordingly, FTC-133 cells are characterized by a fast growth rate (doubling time of approximately 27 hours), while Caco-2 cells have a very slow mitotic rate (doubling time of 60-80 hours).

Whishing to focus on CDV effects on still unexplored uninfected carcinoma cells, FTC-133 cells were chosen to further explore the mechanisms underlying CDV action. We observed that, other than cell viability, the drug also inhibited the colony formation capacity of FTC-133 cells; indeed, a significant inhibition of clonogenic survival was found after CDV administration (100 µM) as compared to untreated cells (-55% vs. CTR) (**Figure 4**). The evidence that the reduction of single cell survival occurred at CDV concentrations that did not affect the survival of subconfluent cell cultures was already reported (Liekens et al., 2015), and might be explained taking into consideration the prolonged half-life of the drug within the cell. In fact, even if FTC-133 cells were pre-treated with CDV for only 24 hours in the clonogenic assay, the drug still remained functional inside the cell (up to 65 hours) and could continue to exert its action leading, as a final event, to a reduced colony formation capacity.

Finally, the induction of apoptotic cell death by CDV was investigated in FTC-133 cells. Apoptosis was explored by an apoptosis array detecting the expression profile of 35 apoptosis-related proteins. As reported in **Figure 5**, after 96 hours of FTC-133 incubation with CDV (100 µM), a significant increment of the main pro-apoptotic proteins was observed as compared to untreated cells, namely cytochrome c (+130%), phospho-p53 (S15) (+49%), and cleaved caspase-3 (+46%). At the same time, a significant decrement of the anti-apoptotic protein Bcl-x was found (-57% versus untreated cells). The increment of Bax/Bcl-2 ratio in CDV-treated cells (+30% as compared to control) also suggested an increased susceptibility to apoptosis. In fact, the intrinsic pathway of apoptosis is regulated by Bcl-2 protein members and the balance between pro-apoptotic (such as Bax) and anti-apoptotic (such as Bcl-2) proteins determines the sensitivity of the cells to apoptotic stimuli (Siddiqui et al., 2015).

The apoptosis array also evidenced that claspin, which is activated in response to DNA damage and replication stress (Freire et al., 2006), resulted significantly upregulated in FTC-133 cells after CDV exposure (**Figure 5**). Indeed, being the drug a nucleoside analog, CDV incorporation into cellular DNA may lead to DNA double-stranded breaks and/or stalling of the DNA replication fork, and

subsequent activation of programmed cell death (Andrei et al., 2015). As regards other apoptosis-related proteins, clusterin and livin significantly increased, whereas p21/CIP1/CDNK1A decreased in CDV-exposed cells. Clusterin and p21 can have both anti-apoptotic and pro-apoptotic activity depending on cell type and cellular conditions (Abbas and Dutta, 2009; Chen et al., 2004); while livin is considered a survival protein. However, overall the prevalence of pro-apoptotic stimuli made FTC-133 cells sensitive to apoptosis, as also confirmed by the presence of nuclear DNA fragmentation (or DNA laddering) after 96 and 120 hours of incubation with CDV (100 µM) (**Figure 6**).

In conclusion, we showed for the first time the in vitro effects of CDV on human mesothelioma and follicular thyroid carcinoma cells, evidencing reduced cancer cell viability and apoptosis induction in a virus-independent manner, as recently reported for other CDV-exposed malignant cells (Hadaczek et al., 2013; Liekens et al., 2015, Mertens et al., 2016). These findings further suggest that CDV may have therapeutic potential as antineoplastic agent; in particular, considering its mode of action, CDV combination with radiation therapy and chemotherapeutics may be expected to result in synergistic antitumor activity (Deutsch et al., 2016).

CONFLICTS OF INTEREST

The authors confirm that there are no conflicts of interest.

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FIGURE LEGEND

Figure 1: Effect of CDV treatment on carcinoma cell viability. Increasing doses of CDV (10-1000 μM) were administered to tumor cells for 24 to 120 hours. Data are expressed as mean ± SD of three independent experiments. *p<0.05 vs. untreated cells.

Figure 2: Effect of CDV treatment on mesothelioma cell viability. Increasing doses of CDV (10-1000 μM) were administered to cancer cells for 24 to 120 hours. Data are expressed as mean ± SD of three independent experiments. *p<0.05 vs. untreated cells.

Figure 3: Procaspase-3 and PARP p85 expression upon CDV administration to mesothelioma cells. Cells were treated for 120 hours with CDV 10 μM or PBS (CTR). γ tubulin was examined as a loading control. Data are expressed as mean ± SD of three independent experiments. *p<0.05 vs. untreated cells.

Figure 4: FTC-133 colony formation capacity upon CDV treatment. One thousand viable cells, pretreated for 24 hours with PBS (CTR) or CDV (10 and 100 μM), were allowed to grow for 14 days and then stained by methylene blue solution. Data are expressed as mean ± SD of three independent experiments. *p<0.05 vs. untreated cells. Representative pictures of cell colonies on culture dish are shown.

Figure 5: Apoptosis-related protein expression upon CDV administration to FTC-133 cells. Cells were treated for 96 hours with CDV 100 μM or PBS (control) and membrane arrays were performed. Data are expressed as changes related to control (means±SD). *p<0.05 vs. untreated cells. Representative pictures of the arrays are shown.

Figure 6: DNA fragmentation analysis upon CDV administration to FTC-133 cells. Cells were treated for 96 and 120 hours with CDV 100 μM or PBS (CTR). M: molecular marker; 1-2: 96 h-CTR and CDV-treated cells; 3-4: 120 h-CTR and CDV-treated cells. A representative picture of agarose gel electhroforesis is shown.

TABLES

Table 1: IC_{50} values (µM) after 24 to 120 hours of cell incubation with increasing concentrations of CDV (10-1000 µM). The mean of three independent experiments is shown.

	24h	48h	72h	96h	120h
Mesothelioma cell lines					
NCI-H2452	>1000	118	32	31	23
Ist-Mes1 (SV40+)	>1000	196	39	32	34
Ist-Mes2 (SV40+)	>1000	234	25	20	27
Carcinoma cell lines					
HeLa (HPV18+)	>1000	>1000	423	90	47
FTC-133	>1000	>1000	570	200	74
Hep-G2	>1000	>1000	893	270	89
PC-3	>1000	>1000	903	359	225
MDA-MB-231	>1000	>1000	>1000	449	225
NCI-H1975	>1000	>1000	>1000	>1000	369
Caco-2	>1000	>1000	>1000	>1000	>1000

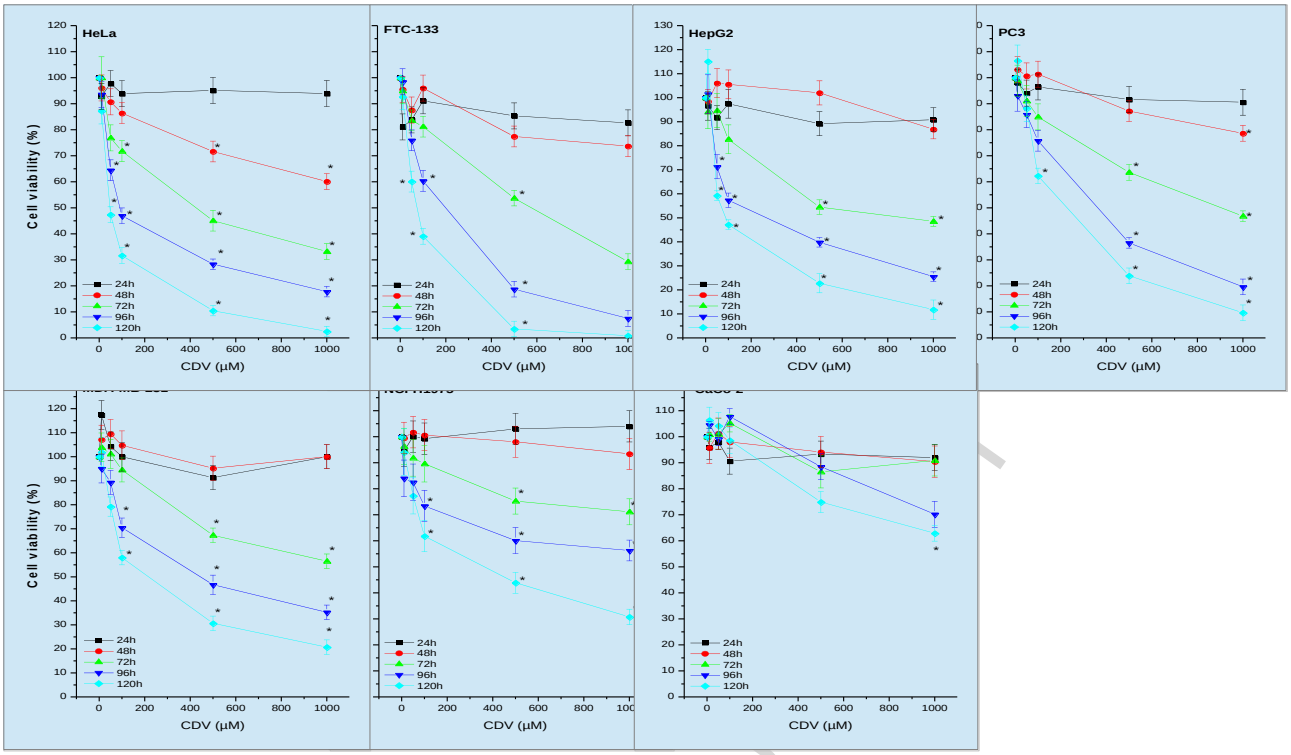


Fig. 1

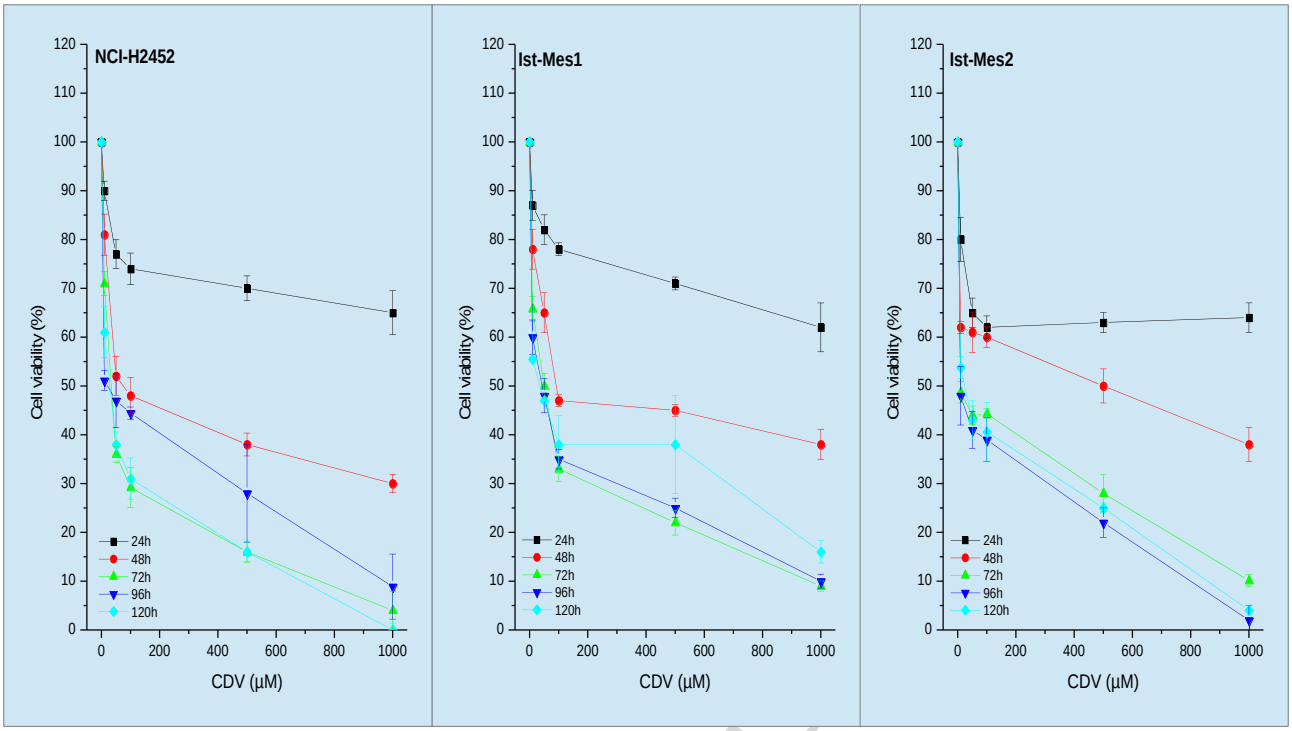
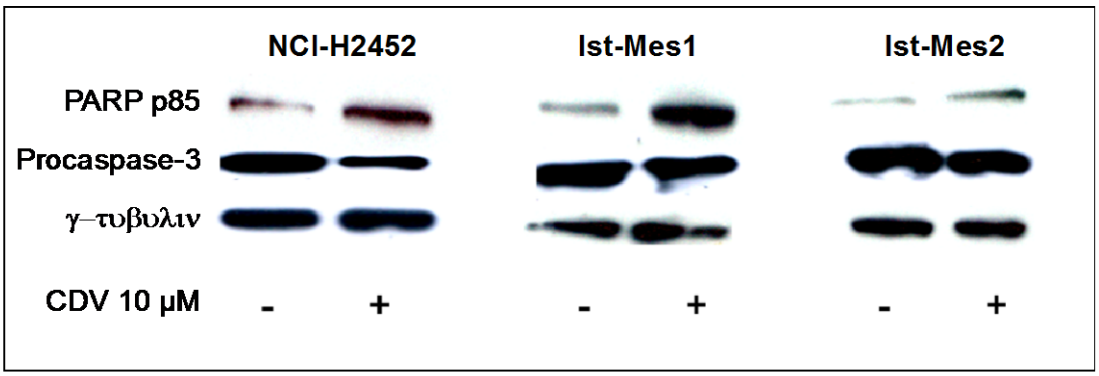


Fig. 2



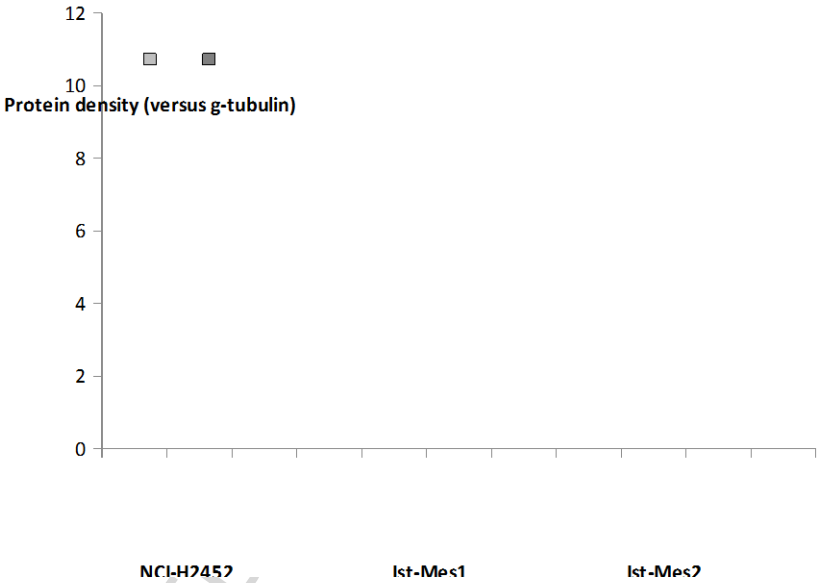


Fig. 3

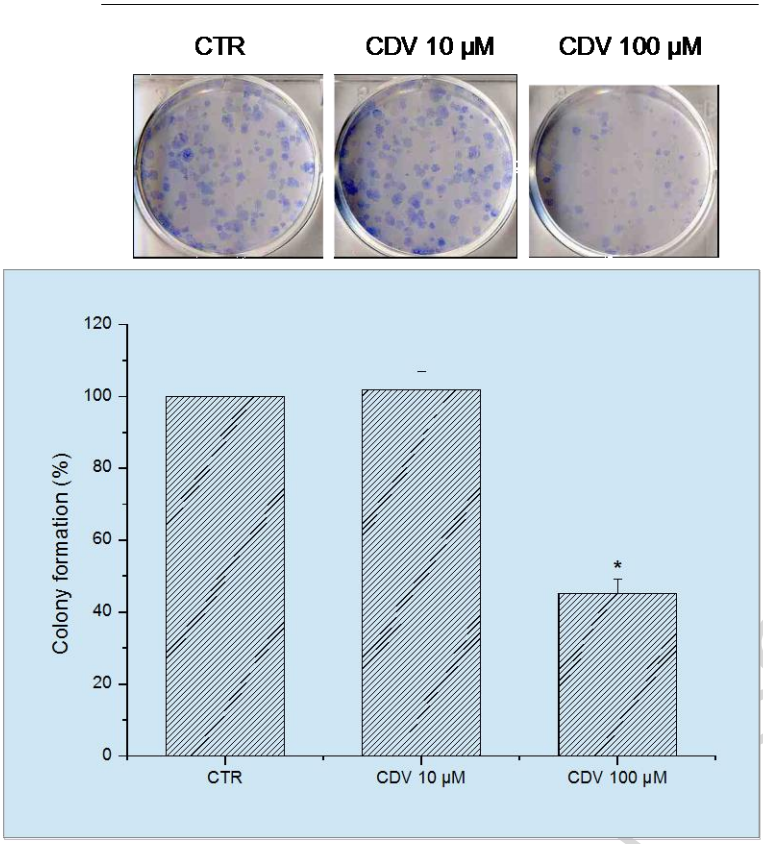


Fig. 4

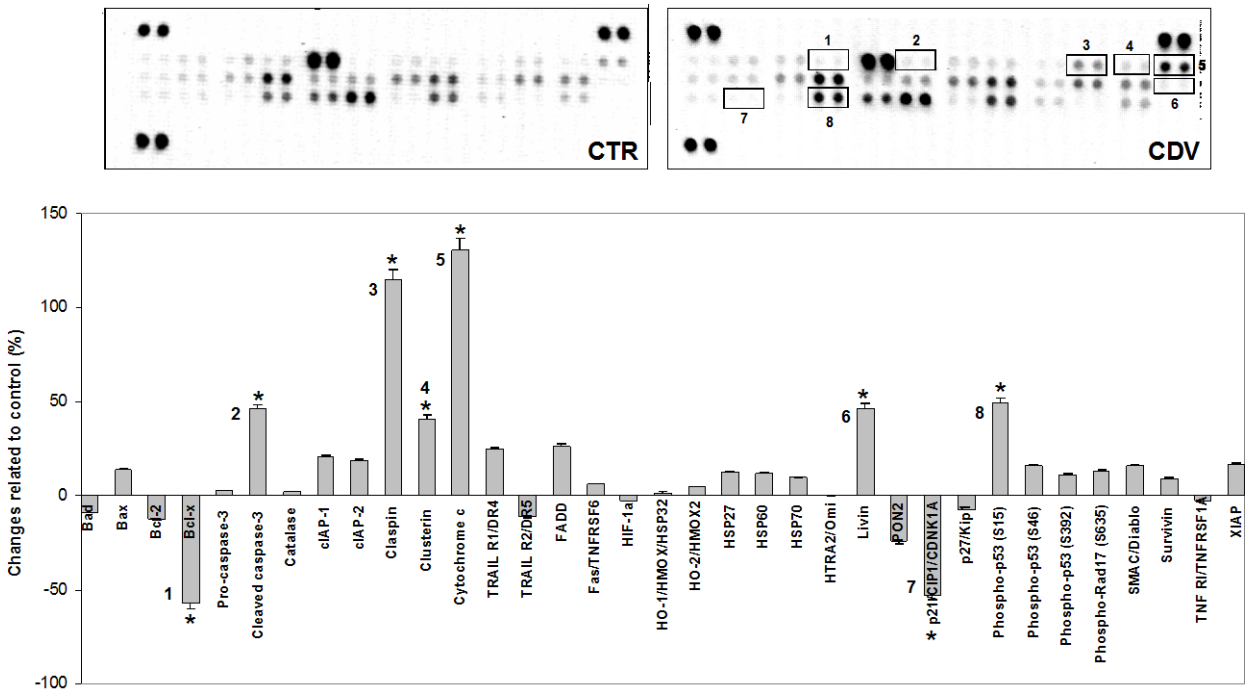


Fig. 5

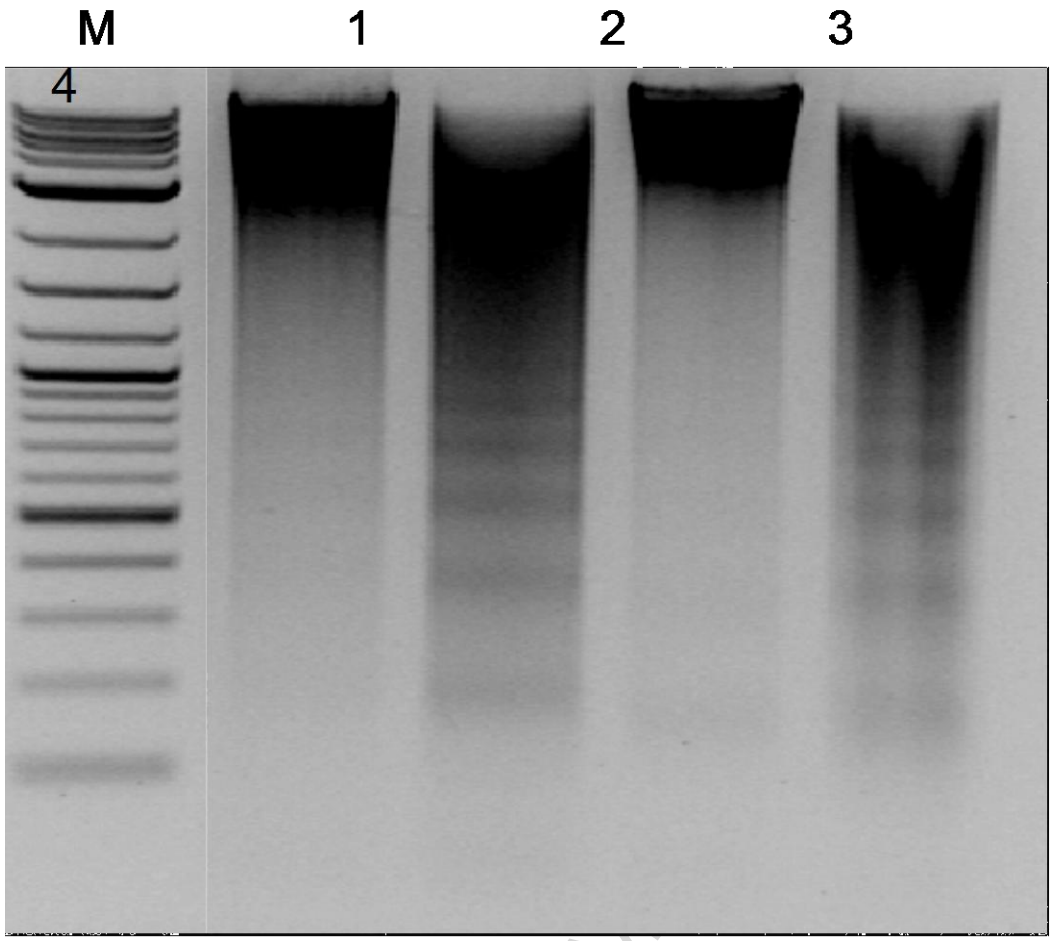


Fig. 6

HIGHLIGHTS

- CDV showed antitumor activity against virus-infected and -uninfected cancer cells.
- CDV induced intrinsic apoptosis in human thyroid carcinoma and mesothelioma cells.
- Thyroid carcinoma and mesothelioma could benefit from CDV treatment.