Ribonucleotide reductase inhibitors enhance cidofovir-induced apoptosis in EBV-positive nasopharyngeal carcinoma xenografts

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TITLE

SHORT REPORT: RIBONUCLEOTIDE REDUCTASE INHIBITORS ENHANCE CIDOFOVIR-INDUCED APOPTOSIS IN EBV-POSITIVE NASOPHARYNGEAL CARCINOMA XENOGRAFTS

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RUNNING TITLE

ANTIVIRAL AGENTS AND APOPTOSIS IN NPC

FOOTNOTES

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ABSTRACT

In nasopharyngeal carcinoma (NPC), Epstein-Barr virus (EBV) infection is mainly latent, and the tumor cells contain episomal viral DNA. We have shown that the acyclic nucleoside phosphonate analog, cidofovir [(*S*)-1-[3-hydroxy-2-(phosphonylmethoxy)-propyl]cytosine] (HPMPC), inhibits growth of NPC xenografts in nude mice by causing apoptosis. The ribonucleotide reductase (RR) inhibitors, hydroxyurea and didox (3,4-dihydroxybenzohydroxamic acid), have been demonstrated to inhibit neoplastic growth and are used as antiviral and anticancer agents. Here, we show that RR inhibitors enhance the antitumor effect of cidofovir in EBV-transformed epithelial cells. MTT assays indicate that hydroxyurea and didox enhance cidofovir-induced cell toxicity in NPC-KT cells, an EBV-positive epithelial cell line derived from NPC. The effect is due to enhancement of apoptosis through the caspase cascade as shown by pronounced cleavage of poly (ADP-ribose) polymerase. Finally, hydroxyurea strikingly enhanced the cidofovir-induced growth-inhibitory effect on NPC grown in athymic mice. The results suggest that RR inhibitors should enhance the antitumor effect of acyclic nucleoside phosphonate analogs on NPC.

KEY WORDS

Epstein-Barr virus, cidofovir, ribonucleotide reductase inhibitor, hydroxyurea, apoptosis

INTRODUCTION

Cidofovir, [(*S*)-1-(3-hydroxy-2-phosphonyl-methoxypropyl) cytosine] (HPMPC) belongs to a new class of antiviral molecules, the acyclic nucleoside phosphonate analogs. The activity spectrum of cidofovir encompasses virtually all members of the human herpesviruses: herpes simplex virus type 1 and 2, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus (EBV) and human herpes viruses 6, 7, and 8, as well as polyomavirus, papillomavirus, adenovirus, and poxvirus infections[1]. This class of molecules is characterized by a stable phosphonate linkage between the acyclic nucleoside and the phosphate moiety[1,2]. As a consequence, in contrast to antiviral drugs such as acyclovir and ganciclovir, cidofovir bypasses the first phosphorylation step by herpesvirus-encoded kinases[1,2]. Thus, cidofovir is effective against thymidine kinase (TK)-deficient HSV and VZV strains and protein kinase-deficient CMV strains that are resistant to acyclovir and ganciclovir[1,2]. Cidofovir enters the cell by endocytosis[3] and is then metabolized to the diphosphorylated form by cellular kinases[4,5]. Diphosphorylated cidofovir acts as both a competitive inhibitor and an alternative substrate of dCTP in the viral DNA-polymerase reaction[6]. Two consecutive cidofovir molecules must be incorporated at the 3' end of the DNA chain to effectively terminate DNA polymerization[7].

Cidofovir also inhibits growth of tumors in which virally encoded DNA polymerases are not expressed and therefore through a different mechanism. Cidofovir was effective for treatment of extensive HPV-infected squamous papillomatous lesions of the hypopharynx and esophagus[8]. Intratumoral injections of cidofovir in patients with recurrent laryngeal papillomatosis produced complete and permanent responses[9,10].

Ribonucleotide reductases (RR) play a central role in DNA biosynthesis furnishing a continuous and balanced supply of the four deoxyribonucleoside triphosphates (dNTPs). The reaction catalyzed in mammalian cells represents the rate-limiting step of the *de novo* synthesis pathway of dNTPs and ultimately governs cell proliferation[11]. RR activity also seems to play an important role in DNA repair[12]. The primary mechanism of action of hydroxyurea is through the inhibition of RR. This mechanism accounts for the cell-cycle-specificity of hydroxyurea: the drug is active in S phase, producing arrest of proliferating cell populations in the G1/S phase of the cell cycle[13]. Hydroxyurea has been demonstrated to inhibit neoplastic growth *in vitro* and *in vivo* and is used in cancer chemotherapy[14].

Nasopharyngeal carcinoma (NPC) is universally associated with EBV infection[15]. In NPC, EBV infection is mainly latent, and the tumors contain episomal viral DNA rather than linear genomes. Episomal DNA is replicated by host DNA polymerase, whereas the linear genomes produced in cytolytic infection are replicated by EBV DNA polymerase. Previously, we showed that cidofovir induced rapid cell death through apoptosis in EBV-positive NPC grown in athymic nude mice[16]. Intratumoral injection of the related drugs 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) or 9-2-(R)-(phosphonomethoxy)propyladenine (PMPA) also slowed tumor growth, but only moderately[17]. The effect of the drugs on NPC is independent of EBV replication.

Neyts *et al.* subsequently demonstrated that hydroxyurea potentiated the anti-herpesvirus activities of purine and pyrimidine nucleoside phosphonate analogs[18]. We show here that RR inhibitors also enhance the antitumor effects of cidofovir in EBV-transformed epithelial cells and in xenografts of NPC grown in athymic mice.

MATERIALS AND METHODS

Reagents. Cidofovir (HPMPC) was generously provided by Gilead Science. Hydroxyurea and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Didox (3,4-dihydroxybenzohydroxamic acid) was a generous gift from Dr. Howard L. Elford.

Cells. NPC-KT is an EBV-positive epithelial cell line derived from fusion between EBV-negative nasopharyngeal epithelial Ad-AH cells and EBV-positive NPC tissue[19]. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics.

MTT Assays. NPC-KT cell proliferation was analyzed by MTT assay[17,20]. Administration of cidofovir, RR inhibitors (hydroxyurea or didox), or combinations of drugs was done once at the beginning of culture. Cells were treated with phosphate-buffered saline solution (PBS) or drug(s); then the MTT assay was performed on days 1, 3, and 5. Each well was rinsed twice with PBS and incubated for 5 hours in medium with 20 μl of 5 mg/ml MTT dissolved in PBS. After removal of medium from each well, 200 μl of DMSO were added to each well and pipetted repeatedly to dissolve crystals. Spectrophotometric absorbance at 595 nm (for formazan dye) was measured with absorbance at 655 nm for reference.

Western Blot Analysis. NPC-KT cells were treated with PBS or drug(s) for 72 hours. The cleavage of poly (ADP-ribose) polymerase (PARP) during apoptosis was described previously[21,22]. The cleavage of the protein was analyzed by rabbit polyclonal anti-PARP antibody with Western blot analysis as described[17].

NPC Xenografts Grown in Athymic Nude Mice. The NPC xenograft C15 was passaged in athymic nude mice as described previously[16,17].

Intratumoral Treatment. Cidofovir was prepared as a 20 mg/ml solution in PBS that was neutralized to pH 7.4 and sterilized through filtration. Hydroxyurea was prepared in an 80 mg/ml solution in PBS. Mixed solutions of cidofovir and hydroxyurea were also prepared. The NPC xenograft C15 was grown subcutaneously until the size of the tumor reached 0.1 cm^3. Intratumoral injection with 75 μl of drug(s) or PBS daily for 21 consecutive days was then carried out (Table I). Tumor size was estimated weekly by measuring the dimensions of the tumors with calipers.

RESULTS

Effects of Cidofovir and RR Inhibitors on NPC-KT cells. We first used the MTT assay to detect the effects of cidofovir, hydroxyurea, and didox on the proliferation of NPC-KT cells, an EBV-positive epithelial cell line. NPC-KT cells (10^3) were treated with either PBS or drug in each well. The viability of the cells was measured by MTT assay at days 1, 3, and 5. The absorbance values indicate the degree of cell viability. At 1 mM, cidofovir strongly reduced the viability of NPC-KT cells compared with the effects at 0.1 mM (Fig. 1 A). The cytotoxic effect of hydroxyurea on NPC-KT cells was maximal at concentrations greater than 0.125 mM (Fig. 1 B). Didox, another RR inhibitor, significantly reduced NPC-KT cell-viability at concentrations of 0.1 mM or more (Fig. 1C).

RR Inhibitors enhance Cidofovir-induced Cell Toxicity in NPC-KT cells. Cidofovir (1 mM) is cytotoxic for NPC-KT cells *in vitro*[17]. The cytotoxic effects of RR inhibitors at concentrations greater than 0.125 mM and 0.1 mM of hydroxyurea and didox, respectively, were pronounced. We next tested the effects of combinations of 0.125 mM and 0.0625 mM of hydroxyurea or 0.1 mM and 0.05 mM of didox with 1 mM cidofovir. Combination of 1 mM cidofovir and 0.125 mM hydroxyurea exaggerated the cytotoxic effect of each RR inhibitor alone at day 5 (Fig. 2 A). The cytotoxic effect of the drug combination appeared to be mainly additive. The combination of 1 mM cidofovir and 0.0625 mM hydroxyurea also enhanced the cytotoxic effect in an additive fashion, but the effect was more limited (Fig. 2 B). Didox also appeared to have some additive effect on the cytotoxic effect of cidofovir (Fig. 2 C and D). These results suggest that RR inhibitors enhance the cytotoxic effect of cidofovir in NPC-KT cells.

Combination of Cidofovir and RR Inhibitors Enhances Apoptosis. Previous studies have shown the correlation between PARP cleavage during apoptosis and caspase activation[22,23]. PARP cleavage produces a C-terminal fragment (containing the catalytic domain) with 85 kDa and an N-terminal fragment of 24 kDa with the DNA-binding domain[22]. Thus, detection of the 85 kDa product of PARP indicates the involvement of caspase-mediated apoptosis. Previously, we showed that treatment of NPC-KT cells with 1 mM cidofovir produced a cleaved form of PARP at day 4[17]. Here we checked the production of cleaved-PARP at day 3 and could detect faintly the 85 kDa cleaved form with 1 mM cidofovir, but more weakly than at day 4 as shown previously[17] (Fig. 3). NPC-KT cells cultured with 0.125 mM hydroxyurea clearly induced cleavage of PARP. Didox (0.1 mM) induced only faintly visible cleavage of PARP. Combination of 0.125 mM hydroxyurea or 0.1 mM didox with cidofovir produced more pronounced PARP-cleavage than did 1 mM cidofovir alone. These data support the impression that RR inhibitors enhance cidofovir-induced cytotoxic effects through caspase-mediated apoptosis.

Combination of Cidofovir and Hydroxyurea enhances suppression of Growth of NPC Xenografts. Intratumoral injections of drug(s) were started when the size of the tumor reached 0.1 cm^3. Intratumoral injection of cidofovir and hydroxyurea together enhanced the growth-inhibitory effect of either drug alone on C15 NPC xenografts (Fig. 4). Small changes (average loss ~10%) in body weight were observed in mice treated with the combination of cidofovir and hydroxyurea (Table 1). Only the combination of cidofovir and hydroxyurea decreased tumor size below the basal level. In a single mouse there was complete remission. These results suggest that hydroxyurea enhances cidofovir-induced growth inhibition of NPC.

DISCUSSION

NPC is an epithelial malignancy with extraordinarily high incidence in southern China and in Chinese who migrate elsewhere from this area[24]. In NPC, EBV infection is mainly latent, and the tumors contain episomal viral DNA rather than linear genomes. The episome is the basis for latent EBV infection, and its replication is accomplished by cellular DNA polymerase, whereas replication of linear genomes in the virus-productive state is accomplished by a virally encoded DNA polymerase[25]. NPC C15 is an EBV-positive tumor, which when grown in athymic mice retains many of the characteristic features of NPC including histology, cell markers, maintenance of a fixed EBV episomal copy number, type 2 EBV latency antigens, and lack of viral replication[26].

Cidofovir (HPMPC) belongs to a new class of antiviral molecules, the acyclic nucleoside phosphonate analogs[1,2,25]. The drug inhibits herpesvirus-encoded DNA polymerases[27,28]. The compound is a potent inhibitor of the cytolytic replication of EBV, but does not affect EBV episomal copy number or the growth of latently infected lymphocytes[25,29]. Cidofovir has a uniquely broad spectrum of indications for clinical use, encompassing DNA-virus infections, as well as various experimental forms of cancer of both viral and non-viral origin including vascular tumors, such as hemangiomas and hemangiosarcomas grown in SCID mice[30,31,32]. For NPC the growth inhibition by cidofovir was attributable to induction of apoptosis as shown in NPC-KT cells by a DNA-fragmentation assay and by PARP-cleavage[17]. Here, we confirmed that cidofovir has growth-inhibitory effects both *in vitro* and *in vivo*. The inhibitory effect of cidofovir on tumor growth was consistently through induction of apoptosis.

RR plays a central role in DNA biosynthesis furnishing a balance of the deoxyribonucleoside triphosphates (dNTPs). The reaction catalyzed in mammalian cells represents the rate-limiting step of the *de novo* synthesis pathway of dNTPs. RR can be separated into 2 dissimilar protein components named R1 (large subunit) and R2 (small subunit), both necessary for activity[11].

Here, we show that two RR inhibitors, hydroxyurea or didox, induced apoptosis in NPC-KT cells through PARP-cleavage, which indicates that the caspase cascade is activated. RR inhibition following exposure to hydroxyurea decreases the size of dATP and dGTP pools and increases the dTTP pool-size, resulting in inhibition of DNA synthesis[33]. The clinical use of hydroxyurea as an anticancer agent is based on the drug's ability to block DNA synthesis *via* inhibition of RR activity. Didox is a polyhydroxy-substituted benzohydroxamic acid derivative that belongs to a new class of RR inhibitors[34]. It has been suggested that didox-induced RR inhibition is due to the reversible destruction of the R2 tyrosil free radical, as demonstrated for hydroxyurea[11]. However, other results suggest that didox has some other properties[11,35,36]. A hydroxyurea-resistant cell line overexpressing R2 was not cross-resistant to didox[35], and the nucleotide imbalance caused by didox is different from that caused by hydroxyurea[35,36]. Compatible with our results, Grusch *et al.* showed that didox induced apoptosis though caspase 8 or 9 cascades, which resulted in cleavage of the caspase substrate PARP in HL60 and K562 leukemia cells[37].

Abdulkarim *et al.* showed that cidofovir induced a downregulation of the EBV oncoprotein latent membrane protein-1 (LMP1) associated with a decrease of the antiapoptotic Bcl-2 and an increase of the proapoptotic Bax protein in NPC C15[38]. Previously, we showed that local or systemic administration of cidofovir produced striking effects on growth of LMP1-expressing NPC C15 xenografts. Similar effects

were produced in C17 and C18 xenografts which are EBV-positive, but do not express detectable LMP1 protein; unlike most primary NPC the latter NPCs have mutations in the *p53* gene[16]. Widespread apoptosis was produced in all three types of tumors. Thus the data suggest that apoptosis was dependent on neither p53 nor LMP1 expression in contrast to the results of Abdulkarim *et al.* [16,38]. We had also reported previously that PARP-cleavage was induced by cidofovir in NPC-KT cells, which do not express LMP1[17]. Although LMP1-signaling might be partially involved, the main pathway of cidofovir-induced apoptosis may be LMP1-independent.

Chodosh *et al.* showed that hydroxyurea-treated primary B lymphocytes immortalized by EBV ceased to proliferate as episomes were lost, which suggests that eradication of EBV with hydroxyurea is an appropriate therapeutic strategy for some EBV-associated lymphoid malignancies[39]. Clinical application of low-dose hydroxyurea based on *in vitro* anti-EBV activity indicates the potential for hydroxyurea as a therapeutic agent in EBV-associated lymphoid disease[40,41]. Huyghe *et al.* demonstrated that the anti-apoptotic protein Bcl-2 and the tumor suppressor p53 were involved in the development of hydroxyurea-mediated apoptosis in lymphoblastoid cell lines. Contrary to our findings, the hydroxyurea-induced cell death was limited, and no activation of the caspase cascade was detected in the lymphoblastoid cells[42]. Thus, these observations suggest that induction of apoptosis by hydroxyurea should be through a pathway independent of LMP1 and p53 in epithelial cells, and that the mechanism of hydroxyurea-induced apoptosis in EBV-positive epithelial cells is likely to be different than in lymphoblastoid cell lines. The mechanism of hydroxyurea-induced apoptosis *via* the caspase cascade in epithelial cells needs to be elucidated.

Hydroxyurea potentiates the antiherpesvirus activities of purine and pyrimidine nucleoside and nucleoside phosphate analogs[18]. The potentiating effect of hydroxyurea may stem from depletion of the intracellular dNTP pools, thus favoring the triphosphates or diphosphates of the nucleoside analogs in their competition with the natural nucleotides at the viral DNA-polymerase level. Cidofovir competes in its diphosphate form with native dCTP. Hydroxyurea brings about a decrease in the level of the intracellular pools of the different dNTPs. Of all dNTP pools, dATP levels are most efficiently depleted by hydroxyurea[43]. Hydroxyurea (0.05 mM) enhances the inhibition of human immunodeficiency virus by PMEA and PMPA both in wild-type virus and in clinical isolates resistant to the compounds. These antiviral activities should be due to imbalances of dNTP pool sizes[44,45]. The influence of dNTP imbalance on various forms of cancer of both viral and non-viral origin needs more investigation.

The pronounced effects of the combination of an acyclic nucleoside phosphonate analog and a RR inhibitor might be due to cell-cycle arrest in S phase. This mechanism accounts for the cell cycle-specificity of hydroxyurea, which produces arrest of proliferating cell populations in the G1/S phase of the cell cycle[46]. Hatse *et al.* examined the effects of PMEA on the cell-cycle distribution pattern in K562 and THP-1 cell lines and concluded that cell differentiation and apoptotic cell death are two cell-specific alternative responses to PMEA-induced cell-cycle arrest in S phase[47]. Although many pathways could be involved in cidofovir and hydroxyurea-induced apoptosis, the mechanism of the additive or synergistic effect of the drugs is unclear. Each step in apoptosis will need to be examined to dissect the apoptotic effect of each drug on EBV-positive epithelial cells.

Systemic treatment with cidofovir has a nephrotoxic potential in human beings, which is lessened when combined with probenecid[48]. Toxicity of hydroxyurea occurs primarily through myelosuppression[49]. Nude mice treated with a combination of cidofovir and hydroxyurea lost ten per cent of their body weight on average in our system. It should be possible to devise more effective and less toxic combinations of the acyclic nucleoside phosphonate analogs and RR inhibitors that may have potential for therapy of patients with NPC, for which effective new agents are needed.

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FIGURE LEGENDS

Fig. 1. Effects of cidofovir and RR inhibitors on NPC-KT cells. NPC-KT cells (10^3) were cultured with (A) cidofovir, (B) hydroxyurea, or (C) didox at indicated concentrations, followed by measurement of absorbance at day 1, 3, and 5 in MTT assays. Absorbance with SD is shown.

Fig. 2. RR inhibitors enhance cidofovir-induced cell toxicity in NPC-KT cells. The effects of combination of cidofovir and hydroxyurea or didox on NPC-KT cell viability were evaluated with MTT assays. Absorbance with SD is shown. The effect of cidofovir was evaluated at 1 mM and hydroxyurea at (A) 0.125 mM or (B) 0.0625 mM. Concentrations of didox were (C) 0.1 mM or (D) 0.05 mM.

Fig. 3. Combination of cidofovir and RR inhibitors enhances apoptosis. NPC-KT cells were cultured at indicated conditions; then cells were collected at day 3. Protein extracts (100 μg) were analyzed for PARP expression by Western blot analysis. Bands at 116 kDa and 85 kDa show uncleaved and cleaved forms of PARP, respectively. Arrow indicates PARP cleaved by 1 mM cidofovir.

Fig. 4. Combination of cidofovir and hydroxyurea enhances suppression of growth of NPC xenografts. NPC C15 growing in athymic nude mice were treated with cidofovir, hydroxyurea, or the two drugs combined. Changes in tumor volume in animals injected intratumorally daily with 75 μl of a 2% cidofovir solution, 80 mg/ml hydroxyurea, the combination, or PBS. Treatment started when the tumors had reached a size of 0.1 cm^3 and continued for 21 days. Tumor volumes were estimated weekly by measuring the dimensions of the tumors with calipers. Relative volumes

with SDs are shown for each condition. Significance of “cidofovir alone *versus* cidofovir + hydroxyurea” ($p=0.0063$) and “hydroxyurea alone *versus* cidofovir + hydroxyurea” ($p=0.0040$) were confirmed statistically with t-test.

Table 1 *Relative body weight changes after intratumoral injections with 75 μl of drug(s) or PBS daily for 21 consecutive days*

Treatment	No. of mice treated	Relative body weight changes after treatment (SD)
cidofovir (HPMPC)	9	1.029 (0.020)
hydroxyurea	10	0.989 (0.106)
cidofovir + hydroxyurea	13	0.901 (0.032)
PBS	12	1.021 (0.123)

[a] PBS, phosphate-buffered saline solution.

[a] SD, standard deviation.

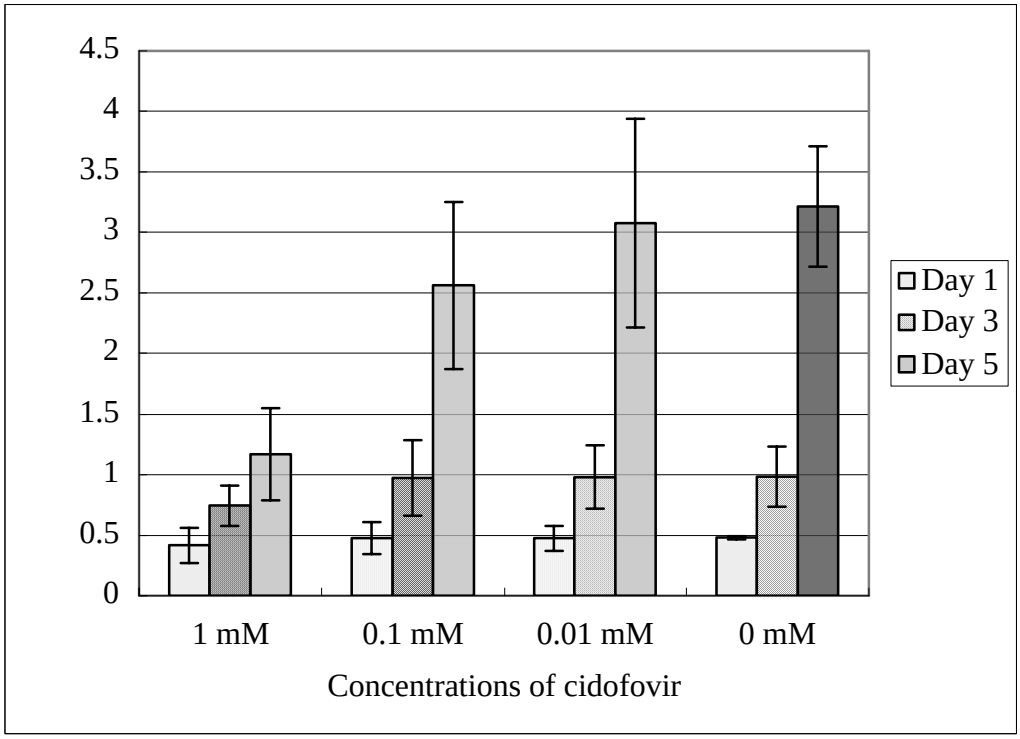
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1 mM
0.1 mM
0.01 mM
0 mM
Concentrations of cidofovir
Day 1
Day 3
Day 5

Fig. 1A

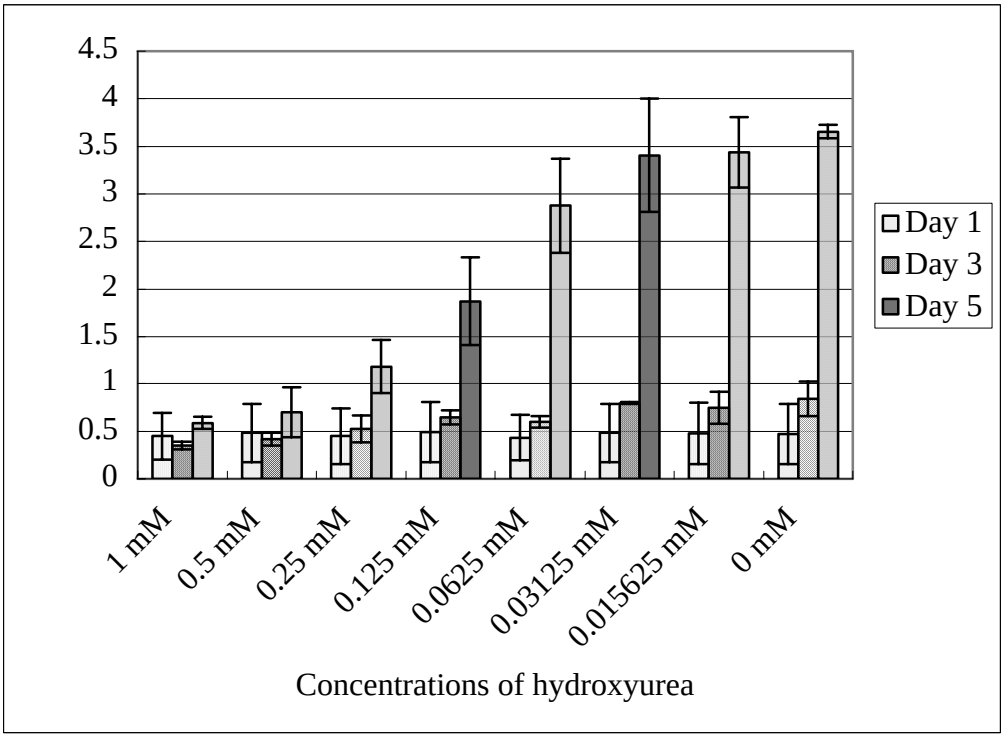
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Day 1
Day 3
Day 5
1 mM
0.5 mM
0.25 mM
0.125 mM
0.0625 mM
0.03125 mM
0.015625 mM
0 mM
Concentrations of hydroxyurea

Fig. 1B

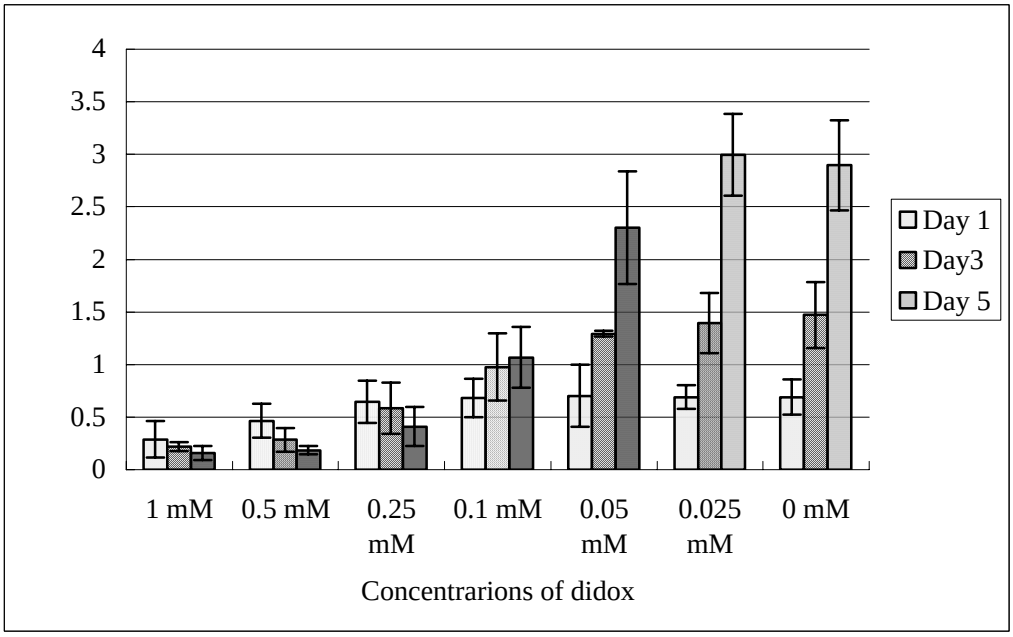
4
3.5
3
2.5
2
1.5
1
0.5
0
1 mM
0.5 mM
0.25 mM
0.1 mM
0.05 mM
0.025 mM
0 mM
Day 1
Day3
Day 5
Concentrarions of didox

Fig. 1C

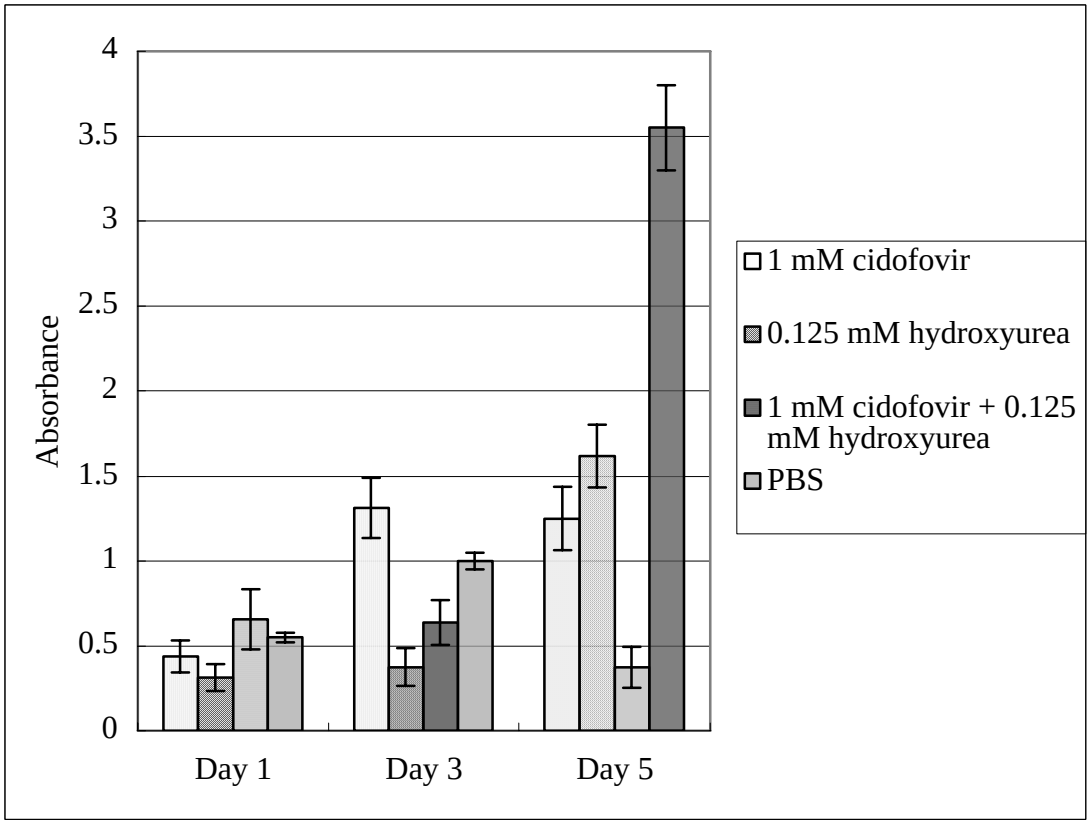
4
3.5
3
2.5
2
1.5
1
0.5
0
Absorbance
Day 1
Day 3
Day 5
1 mM cidofovir
0.125 mM hydroxyurea
1 mM cidofovir + 0.125 mM hydroxyurea
PBS

Fig. 2A

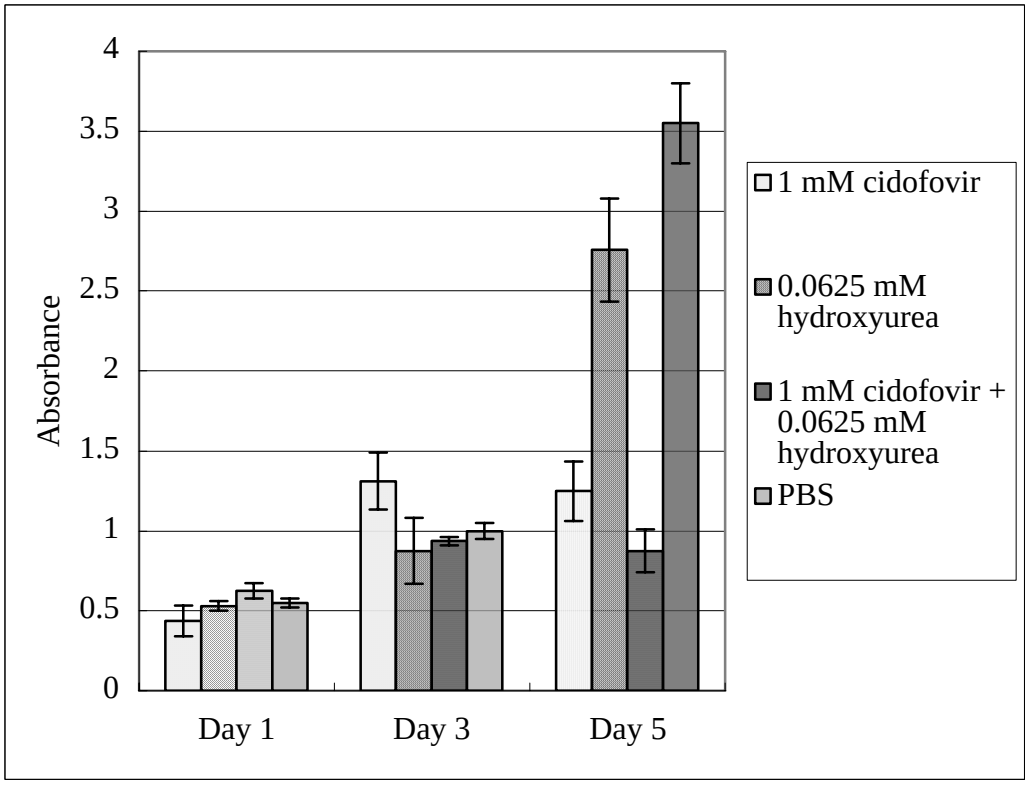
4
3.5
3
2.5
2
1.5
1
0.5
0
Absorbance
Day 1
Day 3
Day 5
1 mM cidofovir
0.0625 mM hydroxyurea
1 mM cidofovir + 0.0625 mM hydroxyurea
PBS

Fig. 2B

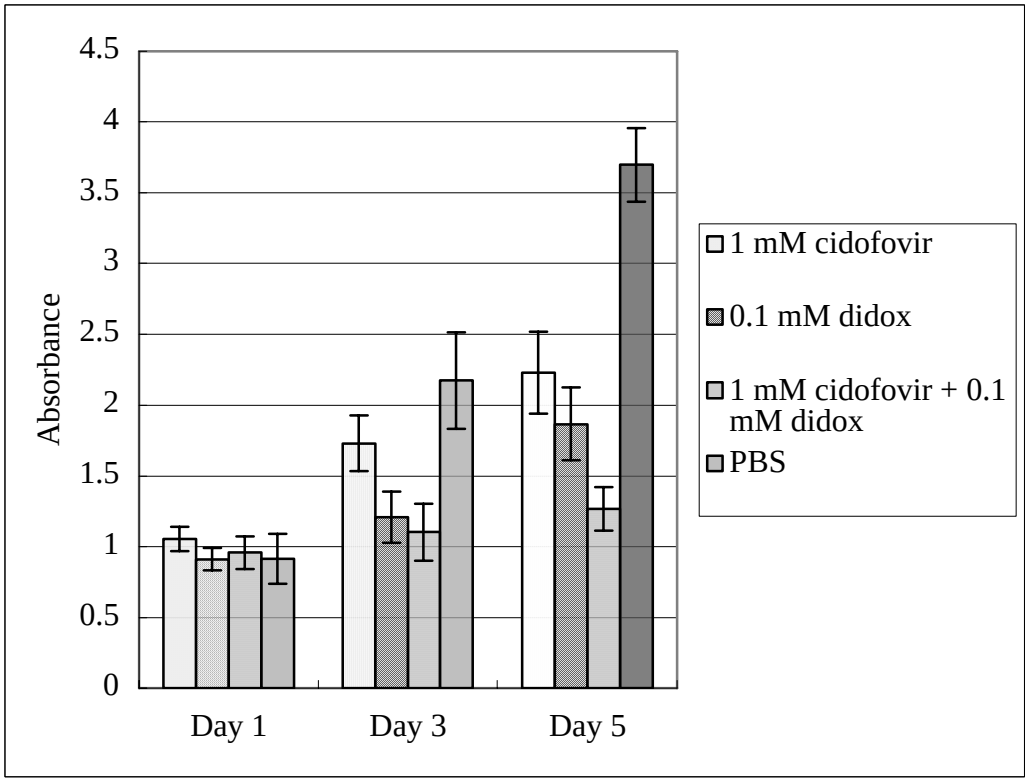
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Absorbance
Day 1
Day 3
Day 5
1 mM cidofovir
0.1 mM didox
1 mM cidofovir + 0.1 mM didox
PBS

Fig. 2C

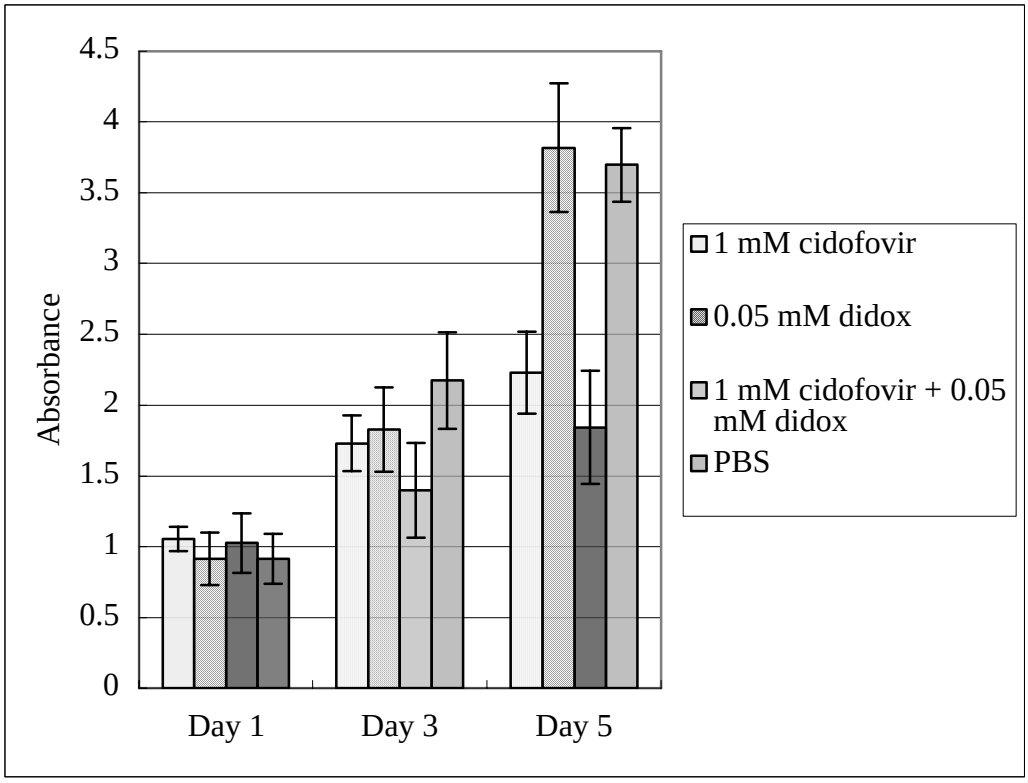
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Absorbance
Day 1
Day 3
Day 5
1 mM cidofovir
0.05 mM didox
1 mM cidofovir + 0.05 mM didox
PBS

Fig. 2D

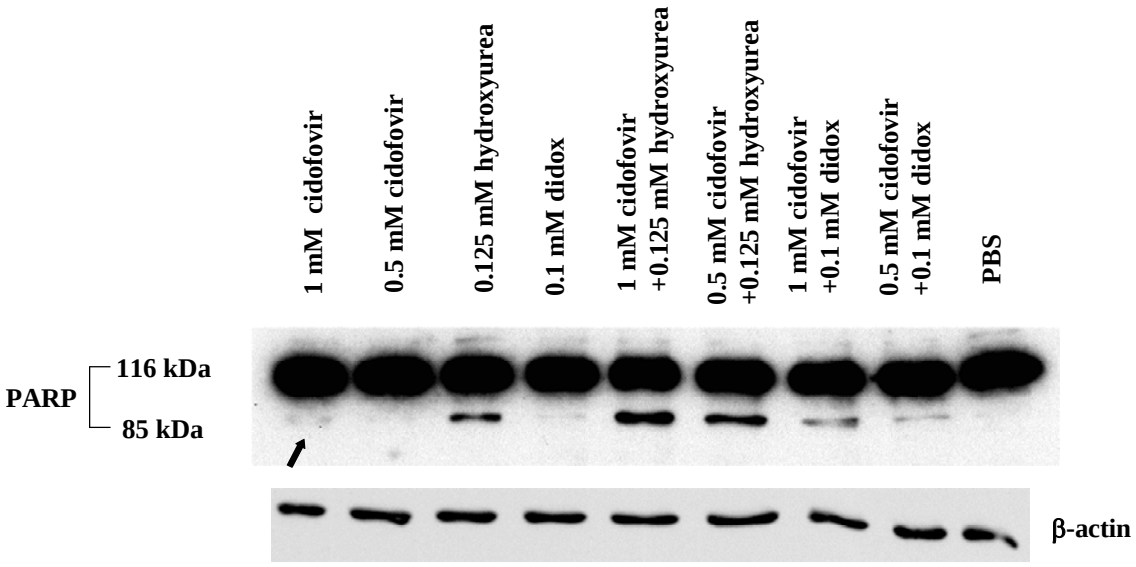
1 mM cidofovir
0.5 mM cidofovir
0.125 mM hydroxyurea
0.1 mM didox
1 mM cidofovir +0.125 mM hydroxyurea
0.5 mM cidofovir +0.125 mM hydroxyurea
1 mM cidofovir +0.1 mM didox
0.5 mM cidofovir +0.1 mM didox
PBS
PARP
116 kDa
85 kDa
β-actin

Fig. 3

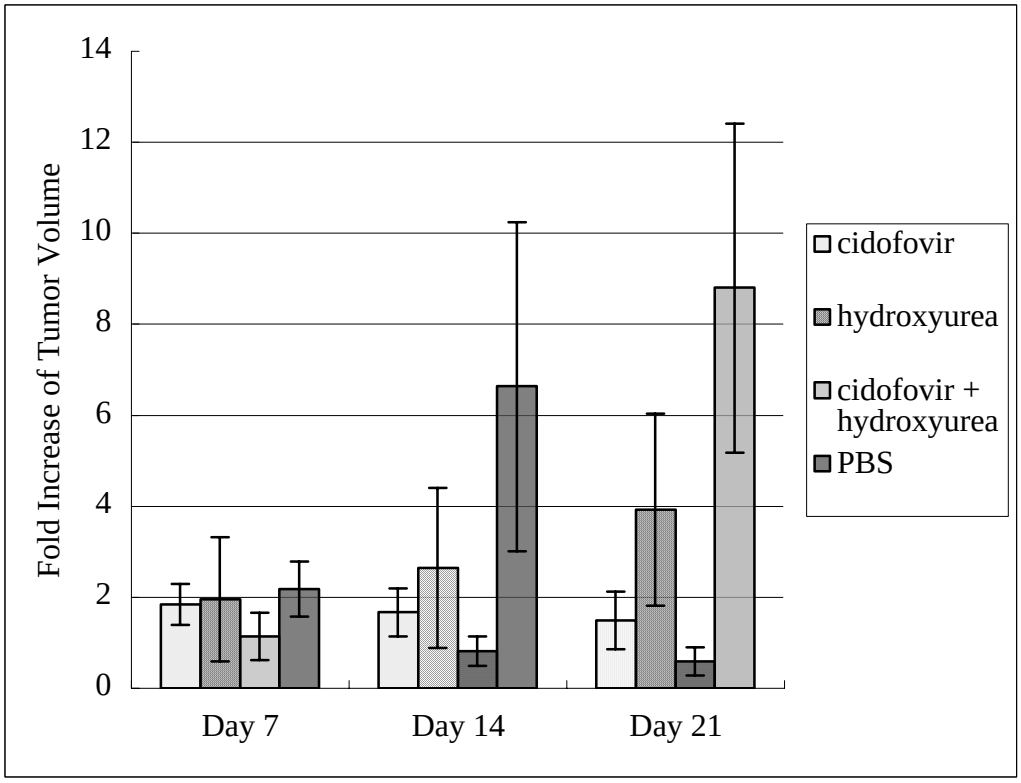
14
12
10
8
6
4
2
0
Fold Increase of Tumor Volume
Day 7
Day 14
Day 21
cidofovir
hydroxyurea
cidofovir + hydroxyurea
PBS

Fig. 4