

Enhanced antiproliferative effects of alkoxyalkyl esters of cidofovir in human cervical cancer cells *in vitro*

Karl Y. Hostetler,¹ Steffney Rought,¹
Kathy A. Aldern,¹ Julissa Trahan,¹
James R. Beadle,¹ and Jacques Corbeil²

¹Department of Medicine, University of California, San Diego, La Jolla, California and ²Université Laval, CHUQ Research Centre, Québec, Canada

Cidofovir

- nucleotide analogue of deoxycytidine monophosphate (dCMP)
- must undergo cellular phosphorylation to its active diphosphate form.

Received 6/17/05; revised 10/19/05; accepted 11/4/05.

Grant support: National Cancer Institute grant CA105039; National Institute of Allergy and Infectious Disease grant AI29164 (K.Y. Hostetler); the Veterans Medical Research Foundation, San Diego, CA; University of California, San Diego Center for AIDS Research Genomics Core Laboratory (J. Corbeil), and Canada Research Chair program (J. Corbeil).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: K. Y. Hostetler has an equity interest and is a consultant to Chimerix, Inc. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies.

Requests for reprints: Karl Y. Hostetler, Department of Medicine, University of California, San Diego, Mail Code 0676, 9500 Gilman Drive, La Jolla, CA 92093-0676. Phone: 858-552-8585, ext. 2616; Fax: 858-534-6133. E-mail: khostet@ucsd.edu

Copyright © 2006 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-05-0200

Introduction

Cidofovir (Vistide) is an acyclic phosphonate analogue of dCMP approved for the treatment of cytomegalovirus retinitis in HIV-infected persons (1). Cidofovir also inhibits poxvirus replication *in vitro* and is active in lethal poxvirus challenge models in mice (2, 3). However, cidofovir is not orally bioavailable and must be administered by i.v. infusion. Its dose-limiting toxicity in man is nephrotoxicity (4). To address the need for an orally bioavailable drug, we synthesized a series of alkoxyalkyl esters of cidofovir: hexadecyloxypropyl-cidofovir (HDP-CDV), octadecyloxyethyl-cidofovir (ODE-CDV), and oleyloxyethyl-cidofovir (OLE-CDV) (5, 6). The structures of cidofovir and oleyloxyethyl-cidofovir are shown in Fig. 1. These compounds were previously shown to be >100 times more active than cidofovir against cytomegalovirus, herpes simplex virus, adenovirus, and poxviruses *in vitro* (5–8). The remarkable increase in antiviral activity was due to greatly increased cellular uptake and conversion to cidofovir-diphosphate, the active inhibitor of viral DNA synthesis (9). Because these analogues resemble lysophosphatidylcholine, a natural lipid that is readily absorbed intact from the small intestine, HDP-CDV and ODE-CDV are highly orally bioavailable and, in contrast to cidofovir, do not concentrate in the kidney, the site of dose-limiting toxicity (4, 10).

Cidofovir has also been shown to selectively inhibit human papillomavirus (HPV) DNA⁺ cancer cell proliferation *in vitro* (11–14) and has antiproliferative activity against HPV-related tumors both in animals (11) and in man (15). This is due, at least in part, to cidofovir-induced reductions in cellular levels of the E6 and E7 proteins, which allows cellular levels and function of p53 and retinoblastoma (pRb) to recover, therefore exerting their normal functions (11). In light of the >100-fold increase in

antiviral activity of alkoxyalkyl esters of cidofovir against a variety of DNA viruses, we hypothesized that the compounds would have increased activity versus cidofovir against HPV-positive (HPV⁺) cervical cancer cells. Because Andrei et al. (12, 13) and Abdulkarim et al. (11) have shown that the antiproliferative activity of cidofovir is associated with increased p53 and pRb and decreased levels of E6 and E7, we compared the activity of cidofovir, HDP-CDV, ODE-CDV, and OLE-CDV in normal human foreskin fibroblasts (HFF), C33A cervical cancer cells that lack HPV, and in three HPV⁺ cervical cancer lines, CaSki, HeLa, and Me-180. We found that ODE-CDV and OLE-CDV are 500 to 17,000 times more active than unmodified cidofovir as inhibitors of cervical cancer cell proliferation *in vitro*.

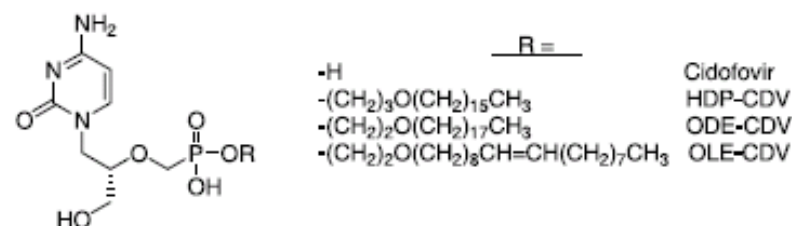
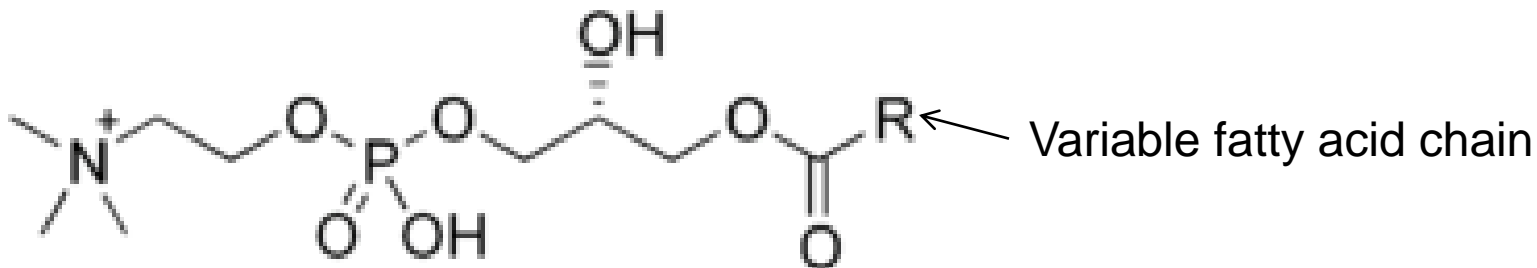
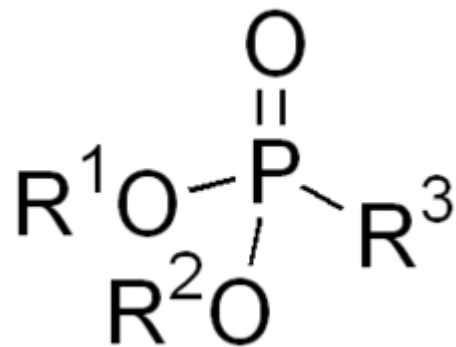


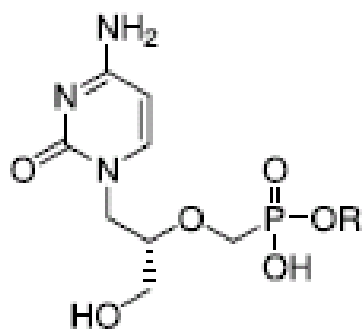
Figure 1. Structures of the alkoxyalkyl esters of cidofovir.



lysophosphatidylcholines



Phosphonic acid



<u>R =</u>		
-H		Cidofovir
-(CH ₂) ₃ O(CH ₂) ₁₅ CH ₃		HDP-CDV
-(CH ₂) ₂ O(CH ₂) ₁₇ CH ₃		ODE-CDV
-(CH ₂) ₂ O(CH ₂) ₈ CH=CH(CH ₂) ₇ CH ₃		OLE-CDV

Figure 1. Structures of the alkoxyalkyl esters of cidofovir.

Why do the study? (Look in Intro)

- Cidofovir: effective against cytomegalovirus & poxvirus, as well as HPV⁺ cancer cells (proliferation)
 - However, 1) not orally bio-available and must be administered via infusion
 - And, 2) has dose-limiting nephrotoxicity (kidney) in man
- Therefore, analogues synthesized which resemble a natural lipid (lysophosphatidylcholine)
 - 1) easily absorbed from the small intestine and
 - 2) do not concentrate in the kidney



Now have structures that are orally bio-available & not toxic to kidneys

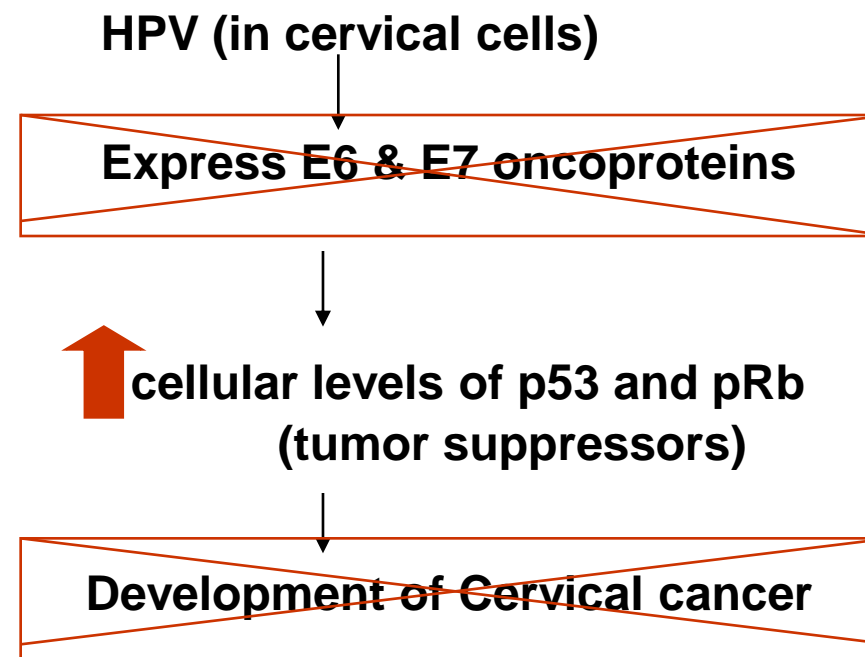


Analyse effect on HPV⁺ cervical cancer cells

Abstract

Nearly all cervical cancers are associated with the high-risk subtypes of human papillomavirus (HPV) expressing the E6 and E7 oncoproteins. The E6 and E7 oncoproteins reduce cellular levels of the p53 and the retinoblastoma (pRb) tumor suppressors, respectively, and represent an important component of the malignant phenotype. Several groups have shown that treatment with cidofovir suppresses levels of E6 and E7, restoring cellular p53 and pRb levels, in turn slowing cell replication and increasing the susceptibility of the cancer cells to radiation and apoptosis. Recently, our group synthesized alkoxyalkyl esters of cidofovir, which were found to be > 100 times more active than unmodified cidofovir *in vitro* against various double-stranded DNA viruses, including cytomegalovirus, herpes simplex virus, adenoviruses, cowpox, vaccinia, and variola viruses. We compared the activity of octadecyloxyethyl-cidofovir (ODE-CDV) and oleyloxyethyl-cidofovir (OLE-CDV) with that of unmodified cidofovir against both HPV-negative and HPV-positive cervical cancer cells. We compared the antiproliferation activity in CaSki, HeLa, and Me-180 cells, prototypical HPV-positive cell lines bearing the HPV-16, HPV-18, and HPV-68 high-risk subtypes, with the activity in C33A cells, a cervical cancer cell line

Alkoxyalkyl esters of Cidofovir: >100 times more active against other double-stranded DNA viruses than Cidofovir alone.



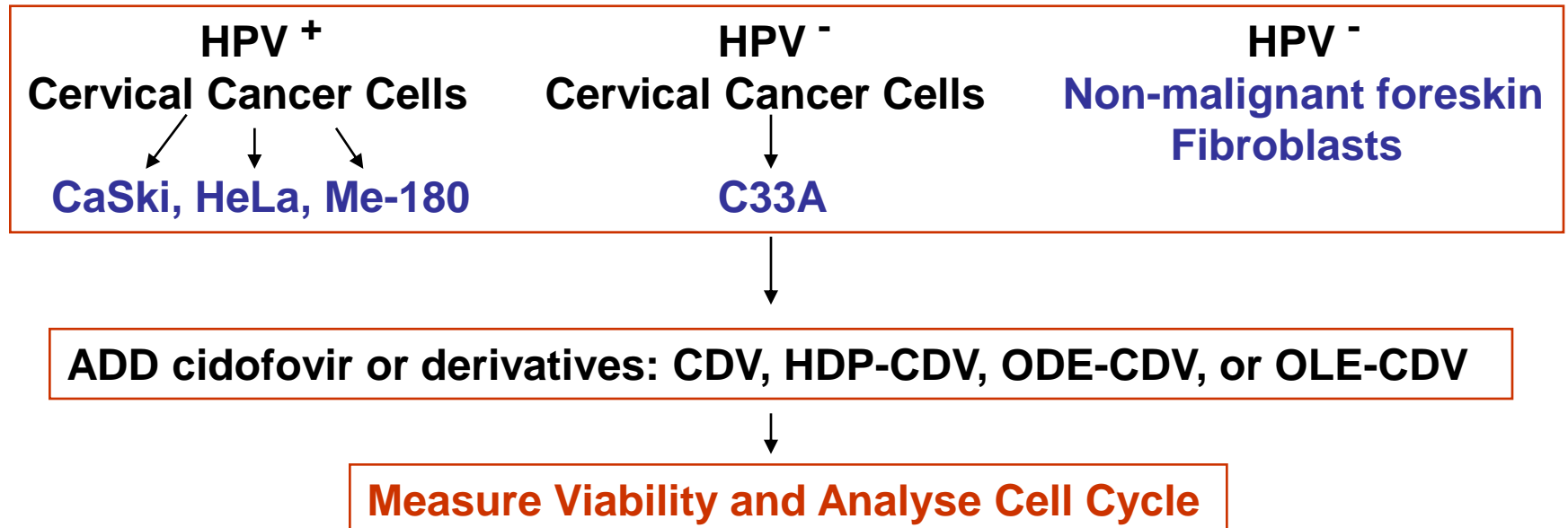
lacking HPV, and in nonmalignant primary human foreskin fibroblast cells. OLE-CDV and ODE-CDV were several logs more potent than cidofovir in CaSki, Me-180, HeLa, and C33A cervical cancer cells as determined by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt proliferation assay. Cell cycle analysis indicates that the cidofovir analogues interfere with passage of dividing cells through the S phase. ODE-CDV and OLE-CDV were 500 to 17,000 times more active than cidofovir in inhibiting the growth of cervical cancer cells. ODE-CDV and OLE-CDV showed selectivity for cervical cancer cells versus nonmalignant human foreskin fibroblast cells and warrant further investigation as potential therapies for cervical cancer. [Mol Cancer Ther 2006; 5(1):156 – 9]

?

Are these analogues of Cidofovir also more effective (than Cidofovir alone) on:

- HPV⁺ cervical cancer cells
- HPV⁻ cervical cancer cells

Methodology



- Cell viability

- 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt proliferation assay: yellow to orange in viable cells: plate reader
- **RESULT:** OLE-CDV and ODE-CDV both more potent than CDV alone at decreasing viability (and therefore proliferation) of all cervical cancer cells (CaSki, HeLa, Me-180, C33A)

- Cell cycle

- Looked at S phase (synthesis of new DNA): propidium iodide stained-nuclei: flow cytometer
- **RESULT:** OLE-CDV and ODE-CDV both more active than CDV alone in inhibiting progression through S phase of all cervical cancer cells (CaSki, HeLa, Me-180, C33A)

Looks like no specific effect on HPV⁺ versus HPV⁻ lines

Materials and Methods

Cidofovir Analogues

HDP-CDV, ODE-CDV, and OLE-CDV were synthesized, purified, and characterized as previously described (5, 8). Briefly, a mixture of cyclic cidofovir, hexadecyloxypropanol, octadecyloxyethanol, or oleyloxypropanol and triphenylphosphine was treated with diisopropylazodicarboxylate to give the cyclic ester. Base-catalyzed hydrolysis with 1 N NaOH gave HDP-CDV, ODE-CDV, or OLE-CDV as the sodium salt. Purity was assessed at >98%. The proton nuclear magnetic resonance and mass spectrometry data for these compounds have been reported previously (5, 8). The structures of cidofovir and OLE-CDV are shown in Fig. 1.

Cells

Normal HFFs (CRL-1634 and Hs27) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in MEM containing 10% fetal bovine serum (Life Technologies, Grand Island, NY). Cervical cancer cell lines associated with the HPV high-risk subtypes 16 and 18, CaSki (CRL-1550), Me-180 (HTB-33), HeLa (CCL-2), and C33A cells were also obtained from the American Type Culture Collection and maintained, respectively, in RPMI 1640, McCoy's 5A, or MEM containing 10% fetal bovine serum.

Cell Proliferation Assays

The various cell lines were plated at 5×10^3 per well in 96-well plates using media with 10% fetal bovine serum added and incubated for about 24 hours. A 10 mmol/L stock solution of cidofovir was prepared in water; 10 mmol/L stocks of HDP-CDV, ODE-CDV, and OLE-CDV were prepared in 10% DMSO in distilled water. Serial drug dilutions were made in media containing 2% fetal bovine serum to give a final 6% fetal bovine serum

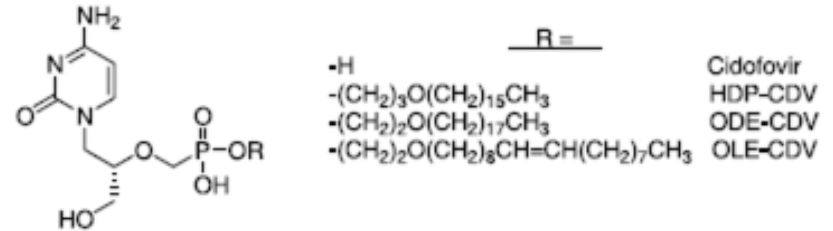
concentration, added to the wells, and incubated at 37°C for 5 days. Determination of cell proliferation was done using a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt Cell Proliferation kit II (Roche Molecular Biochemicals, Mannheim, Germany). This assay relies on the metabolism of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt, a yellow tetrazolium salt, to formazan, an orange dye, which is quantified in a multiwell scanning spectrophotometer (ELISA plate reader). This conversion occurs only in viable cells (16). Briefly, the reagents were mixed and added to the wells and placed on a shaker for 15 minutes followed by incubation at 37°C for ~30 minutes. The A_{450} was determined using an ELISA plate reader (Biotek Instruments, Winooski, VT). The data were plotted, and the IC_{50} was assessed using graphing software (Sigmaplot 8.01).

Cell Cycle Analysis

HFF, HeLa, Me-180, and CaSki cells were treated with cidofovir or OLE-CDV as above for 5 days. On the fifth day, the cells were washed twice in PBS and resuspended in 30% ethanol in PBS and stored overnight at 4°C for subsequent cell cycle analysis. To detect S-phase arrest and apoptosis (sub- G_1 phase), cells fixed in ethanol were washed $1 \times$ in PBS and stained with 50 μ g/mL propidium iodide + RNase for 20 minutes and then analyzed on a Coulter Epics Elite Flow Cytometer using Expo 32 software (Beckman Coulter, Fullerton, CA). The different cell cycle regions were set to those defined by the untreated control cells.

Materials and Methods

- Cells: ATCC
 - 5×10^3 cells per well; 96 well plates
- Media: MEM, McCoy's 5A, RPMI 1640
- Serum: FCS (10%)



- CDV Derivatives
 - Soluble in 10% DMSO; insoluble in water (hydrophobic side chains)
 - Incubation time: 5 days
- Tetrazolium salt: yellow to orange in live cells
 - really a proliferation assay? (viability?)
- Cell cycle: flow cytometry
 - Propidium iodide

Results

In normal HFF cells, HDP-CDV, ODE-CDV, and OLE-CDV were 68 to 200 times more active as antiproliferatives than unmodified cidofovir (Table 1). In cervical cancer cells, HDP-CDV was the least active of the three compounds with IC₅₀ values ranging from 0.42 μmol/L (HeLa) to 8.7 to 34 μmol/L (C33A, CaSki, and Me-180 cells), 18 to 1,230 times more active than cidofovir itself. ODE-CDV and OLE-CDV were 500 to 17,000 times more active than cidofovir as antiproliferative agents, confirming that the increased antiviral activity noted against cytomegalovirus, adenoviruses, and poxviruses in our earlier studies (5–8) also results in a greater antiproliferative activity in both HPV⁻ and HPV⁺ cervical cancers. The increased activity versus cidofovir was highly significant at the $P < 0.001$ level. ODE-CDV and OLE-CDV were significantly more active as antiproliferatives in CaSki cells, Me-180 cells, and C33A cells (Table 1). The selectivity of the antiproliferative effects of the two most active compounds was compared in normal primary HFF cells and in the various cervical cancer cell lines. ODE-CDV and OLE-CDV were 2.5- to 4.2-fold more selective in CaSki cells, 10.8- to 12.7-fold in Me-180 cells, 2,700- to 17,000-fold more selective in HeLa cells, and 9.0- to 12.3-fold more selective in C33A cells compared with normal primary HFF cells.

Wrong: 20-140

Table 2: Cell cycle

Most notable change: **S phase**
- Cells do not undergo division

IC₅₀: drug concentration that reduces viable cell number by 50%

HFF cells

HDP-CDV

Table 1: cell viability

Fold diff vs CDV column:

ODE-CDV and OLE-CDV

-HFF cells: All derivatives **68-200 times** more active than cidofovir
-Cancer cells: **HDP-CDV** least active; **ODE-CDV & OLE-CDV** 500-17000x more active than CDV

The cell lines were exposed to increasing concentrations of cidofovir and OLE-CDV, and cell cycle analysis was carried out. The presence of the compounds alters cell cycle regulation, and the most notable change was S phase arrest (Table 2). This may be through recognition of DNA damage, which occurs when sufficient drug levels are present to reestablish appropriate level of p53. Alternatively, cidofovir (or cidofovir diphosphate) may alter pool sizes of the deoxynucleotide triphosphates or inhibit cellular DNA polymerases or become incorporated into the nascent DNA chain and interfere with elongation. Cells do not undergo a division and remain in the S phase of the cell cycle. This is especially true for HPV⁺ cell lines. The molar amount of cidofovir required to cause a similar response in S phase arrest is ~ 100-fold greater than that of OLE-CDV, regardless of the cell line.

As low as possible

p<0.05 = significant

Table 1. Antiproliferative activity of CDV, HDP-CDV, ODE-CDV, and OLE-CDV in primary HFFs and in CaSki, Me-180, HeLa, and C33A cervical cancer cells *in vitro*

Cell type/HPV subtype/drug	IC ₅₀ (μmol/L)	P vs CDV	P vs HDP-CDV	Fold difference vs CDV	Selectivity vs HFF cells
Primary HFF cells (no HPV)					
CDV	853 ± 254 (3)	—	—	—	—
HDP-CDV	12.4 ± 13.2 (4)	<0.001	—	69 = 853/12.4	—
ODE-CDV	4.2 ± 4.7 (4)	<0.001	>0.05	203	—
OLE-CDV	3.8 ± 2.6 (4)	<0.001	>0.05	225	—
CaSki cells (HPV 16)					
CDV	910 ± 123 (4)	—	—	—	0.94
HDP-CDV	14.5 ± 7.0 (4)	<0.001	—	64	0.86
ODE-CDV	1.7 ± 1.2 (4)	<0.001	<0.01	535	2.5
OLE-CDV	0.91 ± 1.1 (4)	<0.001	<0.01	1000	4.2
Me-180 cells (HPV 68)					
CDV	627 ± 315 (6)	—	—	—	1.4
HDP-CDV	34 ± 9.5 (4)	<0.001	—	18	0.36
ODE-CDV	0.39 ± 0.19 (4)	<0.001	<0.001	1608	10.8
OLE-CDV	0.30 ± 0.12 (4)	<0.001	<0.001	2090	2.7
HeLa cells (HPV 18)					
CDV	516 ± 301 (5)	—	—	—	1 = 3.8/0.3
HDP-CDV	0.42 ± 0.35 (4)	<0.01	—	1230	30
ODE-CDV	0.03 ± 0.01 (4)	<0.01	>0.05	17200	140
OLE-CDV	0.19 ± 0.34 (5)	<0.01	>0.05	2720	20
C33A cells (no HPV)					
CDV	266 ± 29 (3)	—	—	—	3.2
HDP-CDV	8.7 ± 2.4 (3)	<0.001	—	31	1.4
ODE-CDV	0.34 ± 0.30 (3)	<0.001	<0.001	782	12.3
OLE-CDV	0.42 ± 0.33 (3)	<0.001	<0.001	633	9.0

NOTE: The data are mean ± S.D. The numbers in parentheses are the number of replicates. Statistical analysis by one-way ANOVA Tukey-Kramer multiple comparisons test (Instat, GraphPad Software, San Diego, CA). IC₅₀, drug concentration that reduces viable cell number by 50% in 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt cell proliferation assay. Selectivity of the various drugs for cancer cells versus primary HFF cells (selectivity = IC₅₀ HFF / IC₅₀ cancer cell). Fold difference versus CDV is the fold increase in antiproliferative activity versus CDV. Abbreviations: HDP-CDV, hexadecyloxypropyl-cidofovir; ODE-CDV, octadecyloxyethyl-cidofovir; OLE-CDV, oleyloxyethyl-cidofovir.

Table 2. Effect of CDV and OLE-CDV on the proportion of cells in the S phase

Drug	Concentration ($\mu\text{mol/L}$)	% S phase			
		HFF	HeLa	Me-180	CaSki
CDV	1,000	ND	35.8	55	31.1
	100	15	29.6	32.9	41.9
	10	5.0	18.2	14.9	22.5
OLE-CDV	1.0	12.6	75	20.9	56.3
	0.1	37	23.8	31.3	30.2
	0.01	13.3	14.0	13.4	31.9
No drug	0	3.1	17.4	9.3	20.1

NOTE: Cells were exposed to drugs at concentrations indicated for 5 days and subjected to cell cycle analysis as described in Materials and Methods. Abbreviations: OLE-CDV, oleyloxyethyl-cidofovir; ND, not determined.

Need far less OLE-CDV to achieve effect

Note difference in dose

Note how different cells respond differently at diff doses!

Under basal conditions

Why is most notable change in S phase?

- 1) Recognition of DNA damage (sufficient drug levels allow re-establishment of p53 levels)
- 2) Perhaps CDV/derivatives alter pool size of dNTP's
- 3) Perhaps they inhibit the polymerase?
- 4) Perhaps they become incorporated into nascent chain?

Another reason for S phase changes

Discussion

The HPV high-risk subtypes encode the E6 oncoprotein which interacts with p53 (17) and accelerates its degradation through ubiquitination (18). Inactivation of p53 results in uncontrolled growth, a prerequisite for oncogenesis. HPV protein E7 from the high-risk HPV subtypes interferes with the activity of the retinoblastoma protein, pRb, which regulates the entry of the cell into the S phase (19). The activation of p53 through phosphorylation is responsible for the activation of panoply of genes associated with apoptosis induction, including Bax, GADD45, NOXA, Fas, PERP, and others (20).

The alkoxyalkyl esters of cidofovir, particularly ODE-CDV and OLE-CDV, are selectively active in cervical cancer cells as shown above. There are many possible reasons and mechanisms for the increased anticancer activity of OLE-CDV, and this class of molecules in HPV DNA⁺ and HPV⁻ cancer cells. First, these compounds may be antiproliferative because they inhibit cellular DNA polymerase enzymes after anabolic phosphorylation to cidofovir-diphosphate (an analogue of dCTP). Increased activity of the alkoxyalkyl esters of cidofovir could be due to increased cellular uptake and conversion to cidofovir-diphosphate as we have previously shown in MRC-5 human lung fibroblasts (9). Johnson and Gangemi (14) have suggested that HPV DNA⁺ cancer cells may have a viral pathway that favors the anabolic phosphorylation of cidofovir, leading to its observed antiproliferative selectivity in cervical cancer. An equally likely scenario, already shown by Andrei et al. (12, 13), Johnson and Gangemi (14), and Abdulkarim et al. (11), is that

cidofovir down-regulates the HPV oncoproteins, E6 and E7, leading to up-regulation of the key cell cycle regulators, p53 and pRb. Higher levels of p53 allow the cells to regain control of the cell cycle and induce apoptosis when assessing extensive the DNA damage found in cancer cells. Preliminary Western blotting studies in HeLa cells showed that OLE-CDV up-regulates p53 at much lower concentrations than cidofovir. Our analysis of the cell cycle by flow cytometry indicated that OLE-CDV is substantially more active than cidofovir in inducing S-phase arrest (Table 2). However, up-regulation of p53 is not likely to be the only mechanism, because the enhanced effects of ODE-CDV and OLE-CDV were also noted in HPV⁻ C33A cervical cancer cells. There may be other as yet unrecognized mechanisms of the antiproliferative action of ODE-CDV and OLE-CDV, including metabolic differences, possible nucleotide pool imbalances, inhibition of DNA polymerases, and/or arrest of DNA synthesis. Further studies are needed to determine the molecular mechanisms by which the alkoxyalkyl analogues of cidofovir exert their action.

In summary, we have identified a family of highly active cidofovir analogues, which are several logs more active than unmodified cidofovir in HPV⁻ C33A cervical cancer cells and in CaSki, Me-180, and HeLa cervical cancer cells, which contain the high-risk HPV subtypes 16, 68, and 18, respectively. There are very few specific chemotherapeutic agents for treatment of cervical cancer. ODE-CDV and OLE-CDV are highly active and may prove useful for therapy of cervical cancers. Additional studies with ODE-CDV and OLE-CDV are indicated to elucidate their uptake, metabolism, and mechanisms of action in cervical cancer cells and to evaluate their activity in cervical cancer-bearing animals. Alkoxyalkyl esters of cidofovir may be useful in treatment of cervical cancer.

Discussion

- How do CDV & derivatives decrease proliferation?
 - Inhibition of cellular DNA polymerases
 - Down-regulation of E6/7 oncoproteins leads to increased p53 and Rb – this leads to increased arrest at S phase and apoptosis (not measured)
 - Other unrecognised mechanisms
- What has the study shown?
 - Derivatives of CDV are much more active than CDV against cervical cancer
 - Needs to be taken further in animals and then in humans
 - This is important as there are not many agents for the treatment of this cancer