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Synergistic inhibition of herpesvirus replication by docosanol and antiviral nucleoside analogs

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Abstract

Interactions between docosanol (*n*-docosanol, behenyl alcohol) and nucleoside or pyrophosphate analogs were investigated in vitro. The anti-HSV activity of acyclovir (ACV) was synergistically enhanced by treatment of cells with docosanol as judged by inhibition of progeny virus production and plaque formation. This drug interaction between ACV and docosanol was observed with laboratory strains of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), oral and genital clinical isolates of HSV, cytomegalovirus (CMV), and varicella zoster virus (VZV). Near optimal concentrations of docosanol plus ACV inhibited HSV replication >99% more than either drug alone, including emergence of ACV-resistant variants. The response was observed with African Green Monkey kidney cells, normal human foreskin cells, and normal human lung cells. Treatment of cells with docosanol also synergistically intensified the inhibition of HSV production by all tested nucleoside analogs, including trifluorothymidine (TFT), adenine arabinoside (Ara-A), and ribavirin. An additive anti-HSV effect was observed with docosanol and phosphonoformate (PFA). No evidence was found for either synergistic inhibition of cellular DNA synthesis or induction of overt cellular toxicity when docosanol was combined with ACV, TFT, Ara-A, ribavirin, PFA, 8-azaguanine, or 5-fluorouracil. The ability of docosanol treatment to increase the antiviral activities of nucleoside analog antiviral drugs, coupled with a lack of toxic interactions, translates to substantial improvements in drug selectivity ratios.

Keywords: Herpesvirus; Docosanol; Acyclovir; Drug–drug interactions

1. Introduction

n-Docosanol (docosanol) is a primary 22 carbon straight chain alcohol that inhibits replication of herpesviruses in vitro and in vivo (Katz et al., 1991, 1994; Marcelletti et al., 1992; Pope et al., 1998, 1996). The mechanism of action appears to involve modification of cellular metabolism such that treated cells are resistant to infection by certain lipid-enveloped viruses. Mechanistic studies with herpes simplex virus (HSV) revealed that the compound is not directly viricidal as are shorter chain-length alcohols (Snipes et al., 1977). Rather, metabolism of docosanol by cells appears to be required for antiviral activity (Pope

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et al., 1996). HSV binds equally well to untreated and docosanol-treated cells but, in the latter population, viral penetration, genome localization to the nucleus, gene expression, and productive infection are inhibited (Katz et al., 1991, 1994; Pope et al., 1998). Since HSV entry is the earliest event to be inhibited in docosanol-treated cells, and since antiviral activity is not observed when the compound is added after penetration, it was considered that alteration of the entry process is the primary antiviral mechanism of docosanol. It is likely that a common mechanism results in the inhibition of replication by human and murine cytomegalovirus (CMV) and varicella zoster virus (VZV).

Docosanol does not exhibit appreciable toxicity when (a) added to cultured cells up to 300 mM (Katz et al., 1991), (b) administered systemically to experimental animals up to 2000 mg/kg (Katz et al., 1994), or (c) applied to normal or HSV-infected human or guinea pig skin (Habbema et al., 1996; Marcelletti et al., 1992; Sacks et al., 2001). Topical application of a 10% docosanol cream inhibited cutaneous HSV disease in guinea pigs (Katz et al., 1994; Marcelletti et al., 1992). Recent phase II and III double-blind, placebo-controlled clinical trials confirmed 10% docosanol cream to be safe and effective in the treatment of recurrent HSV labialis in humans (Habbema et al., 1996; Sacks et al., 2001). Docosanol 10% cream has recently been approved by the FDA for the treatment of recurrent herpes simplex labialis. Additional indications for docosanol are being pursued, such as CMV-mediated retinitis, HSV genitalis, and shingles caused by VZV.

Acyclic guanosine analogs such as acyclovir (ACV) are a leading class of antiviral drug for the treatment of primary and recurrent HSV oral-facial and genital diseases. ACV is selectively metabolized by herpesvirus-infected cells resulting in the generation of the triphosphorylated ACV moiety that can inhibit viral DNA synthesis (Elion, 1993). This antiviral mechanism of action is distinctly different from that of docosanol, suggesting that combining these two drugs could improve therapeutic efficacy in the treatment of mucocutaneous HSV diseases. For this reason, possible interactions between docosanol and such drugs were evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Purchased chemicals and their source of supply were: docosanol (>98% pure, MW 326), M. Michel, New York, NY; Pluronic F-68 (poloxamer 188, M_r 8400) and Tetronic 908 (M_r 25 000), BASF, Mount Olive, NJ; ACV powder, Burroughs Wellcome, Research Triangle Park, NC; adenine 9-β-D-arabinofuranoside (Ara-A), 8-azaguanine (8-AG), 5-fluorouracil (5-FU), PFA, ribavirin, trifluorothymidine deoxyriboside (TFT), Sigma, St. Louis, MO; ^{3}H-thymidine (^{3}H-TdR), American Radiolabeled Chemicals; Pluronic F-68 and Tetronic 908, BASF, Parsippany, NJ.

2.2. Formulation of docosanol

Docosanol was suspended in Pluronic F-68 (M_r 8400) or Tetronic 908 (M_r 25 000) for use in the tissue culture experiments as previously described (Katz et al., 1991; Pope et al., 1996). Briefly, Pluronic F-68 or Tetronic 908 was diluted in sterile saline and heated to 70 °C. Docosanol was added to the solution and the mixture was sonicated. The resulting suspensions consisted of very fine globular particles with an average size of 0.1 μm as measured by transmission electron microscopy and laser light scattering analysis performed by Delta Analytical, North Huntington, PA using a Honda LA-900 (Pope et al., 1996). The control vehicles for these suspensions contained only Pluronic F-68 or Tetronic 908 in saline.

2.3. Preparation of viral stocks and in vitro assays for viral replication and DNA synthesis

The following strains of virus were obtained from the American Type Culture Collection (ATCC), Manassas, VA: MacIntyre strain of HSV-1, #VR-539; MS strain of HSV-2, #VR-540; human CMV, Towne strain, #VR-977; VZV, Ellen strain, #VR-1367. Clinical isolates of HSV

were obtained from active lesions of patient volunteers with recurrent herpes labialis or genitalis as previously described (Katz et al., 1991). Stock HSV preparations were generated in Vero cell (African Green monkey kidney, ATCC #CCL-81) cultures, characterized for levels of plaque-forming units (PFU) in Vero cells, and stored at −85 °C. Stocks of CMV and VZV were obtained cryopreserved from ATCC and were stored at −85 °C before use without further culture. Additional cell lines used in these studies included MRC-5 normal human lung cells (ATCC# CCL 171) and Hs68 newborn human foreskin fibroblasts (ATCC# CRL 1635).

The direct plaque assay was conducted with cells plated in 2 cm^2 wells (24-well plate, 1.5×10^5/ml, 1 ml/well) in DMEM supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin as described (Katz et al., 1991). Unless indicated otherwise, docosanol suspension and the corresponding control vehicle (lacking docosanol) were added at the outset of the culture period. After 24 h incubation, varied concentrations of ACV were added to certain cultures, and all of the cultures were inoculated with the required PFU of HSV. The cultures were incubated (10% CO_2 in air; humidified) for an additional 44 h, stained (the staining/fixative consisted of 1.25 mg/ml of carbol-fuchsin plus 2.5 mg/ml of methylene blue in methanol), and scored for HSV-induced plaques using a dissecting microscope (10 × magnification).

The assay for PFU production used the same culture medium with cells plated in 1 cm^2 wells (48-well plate, 10^5/ml, 0.5 ml/well). Unless indicated otherwise, docosanol and the control vehicle were added at the outset of the culture period. After 24 h incubation, varied concentrations of test drug and required PFU of virus were added to the cultures (see legends to figure and tables). After 72 h incubation, the culture supernatant fluids were harvested and assayed for PFU content in fresh cell cultures. Each of the initial triplicate cultures/determination were titered in triplicate in 0.32 cm^2 wells (96-well plate, 10^5/ml, 0.1 ml/well) to identify the dilution of virus that exhibited infection of 50% of inoculated wells within 4 additional days incubation.

The assays for infection of cells by CMV and VZV were conducted with MRC-5 cells plated in 2-cm^2 wells as described for the direct plaque assay. After 24-h culture in the presence or absence of docosanol or Pluronic F-68, ACV was added to certain cultures before infection with 500 PFU of CMV or VZV. After 48 h incubation, the cultures were washed with saline and fed fresh medium lacking inhibitor. After an additional 2 days incubation, the cells were harvested by trypsinization and assayed for infected cell content in fresh MRC-5 cell cultures. Cells from each of the initial triplicate cultures/determination were assayed at various cell densities in quadruplicate (0.32-cm^2 wells) to identify that concentration of cells that exhibited 50% infection of inoculated wells within 6 days incubation.

DNA synthesis was assayed by tritiated thymidine (^{3}H-TdR) incorporation to assess drug toxicity. Vero cells were cultured in 0.32-cm^2 wells at a concentration of 5×10^4/ml, 0.1 ml/well. After 24 h incubation in the presence or absence of docosanol or Pluronic F-68, the cultures were inoculated with 25 μl medium alone or medium containing the indicated analog drug at final concentrations ranging from 0.08 to 50 μg/ml. After 48 h incubation, the cultures were inoculated with 50 μl containing 1 μCi ^{3}H-TdR. After 24 h additional incubation, the cultures were washed with saline and extracted with 0.5 M perchloric acid to eliminate acid-soluble nucleotides and nucleosides. Residual DNA was solubilized with 1 M NaOH and radioactivity was assessed by liquid scintillation counting. Fifty percent inhibitory concentrations (IC_{50}) were determined for each of the analog drugs from triplicate determinations for each drug and treatment combination.

3. Results

3.1. Docosanol can enhance the activity of ACV against HSV-1 PFU production

The effects of docosanol on the anti-HSV activity of ACV were tested on production of infectious PFU. As shown in panel A of Fig. 1, Vero cells were cultured with varied concentra-

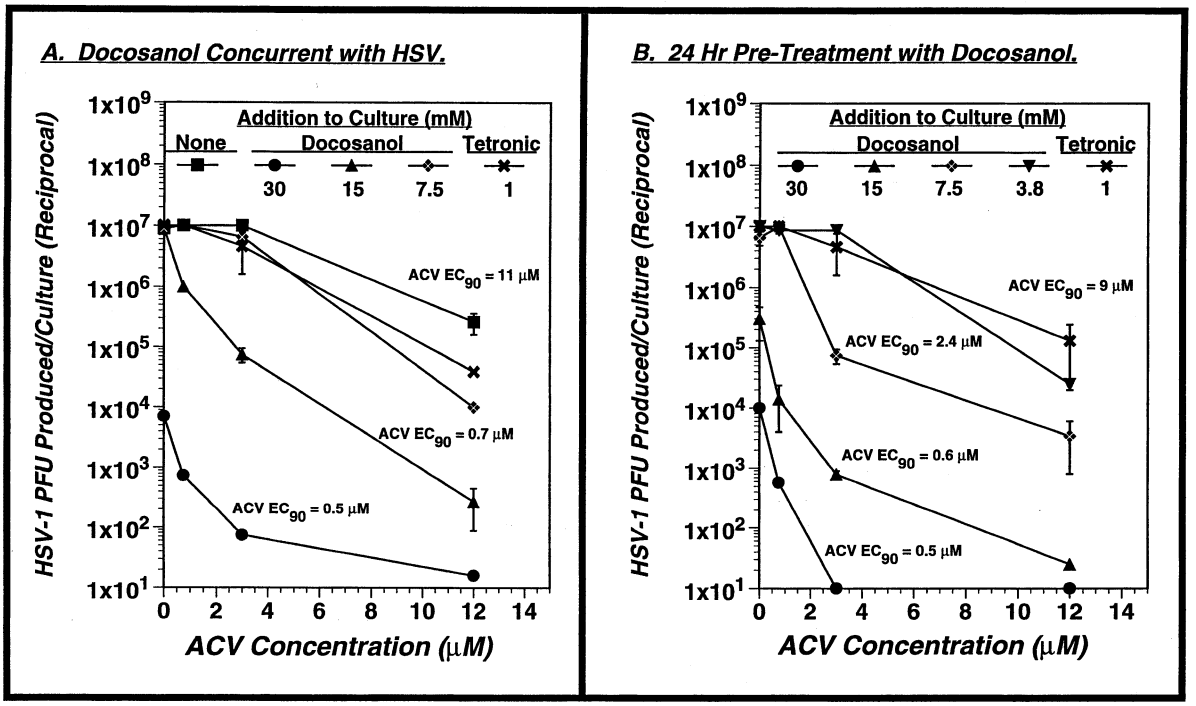

Fig. 1. Docosanol can enhance the activity of ACV against HSV-1 PFU production. The PFU assay was conducted as described in Section 2 and in the text with Vero cells treated with docosanol or Tetronic 908 concurrently with HSV-1 (500 PFU) infection (panel A) or after 24 h pre-treatment (panel B). The data are expressed as means and standard errors of HSV-1 PFU detected in triplicate cultures for each drug combination.

tions of ACV and infected with 500 PFU of HSV-1 (multiplicity of infection, MOI = 0.002). Immediately thereafter, certain of the cultures were treated with the required amounts of docosanol or that amount of Tetronic 908 vehicle contained in the highest docosanol-containing cultures. Progeny HSV-1 PFU were measured 3 days later. Untreated cultures exhibited $\sim 1 \times 10^7$ HSV-1 PFU and ACV inhibited PFU production in such cultures with a 90% effective concentration (EC_{90}) of ~ 11 μM. As shown at the extreme left of the X-axis, i.e. zero ACV, cultures exposed to 30 mM docosanol at the time of infection exhibited $>99\%$ fewer HSV-1 PFU (8×10^3). The efficacy of ACV was increased in parallel cultures containing docosanol and the ACV EC_{90} on the remaining virus was reduced to 0.5 μM (96% or 25-fold reduction). Docosanol at 15 mM did not substantially inhibit PFU production, although it did lower the ACV EC_{90} to 0.7 μM (94% or 17-fold reduction). Docosanol at 7.5 mM and the Tetronic 908 control did not inhibit PFU production or substantially lower the ACV EC_{90}.

It has been previously shown that a 12–24 h pre-treatment of cells with docosanol substantially improves the antiviral activity of the compound (Katz et al., 1991; Pope et al., 1996). As illustrated in panel B of Fig. 1, pre-treatment of cells with the compound only modestly improved the enhancement of ACV against HSV-1 PFU production. Vero cells were cultured in parallel with those described in panel A, in the presence or absence of docosanol or Tetronic 908. After 24 h incubation, the cultures were treated exactly as those described in panel A. The values for the medium alone group therefore apply to both panels. Docosanol at 30 mM inhibited PFU production $>99\%$ (1×10^4) and lowered the ACV EC_{90} 96% (25-fold). Pre-treatment of the cells with 15 mM docosanol reduced PFU production by 96% (3×10^5) and

lowered the ACV EC_{90} 95%. While pre-treatment with 7.5 mM docosanol did not inhibit PFU production, the ACV EC_{90} was reduced 79% (4.8-fold) under such conditions. The lowest docosanol concentration (3.8 mM) and the Tetronic control did not alter PFU production or ACV efficacy even with pre-treatment. The combination of ACV and pre-treatment with 7.5–30 mM docosanol resulted in an approximate 2-log greater decrease in virus yield as contrasted to parallel cultures that did not receive docosanol pre-treatment. Cellular toxicity was not visually apparent in cultures treated with docosanol or ACV, either alone or in combination. This experiment was also conducted with HSV-2 and comparable results and conclusions were obtained (data not shown). The remaining studies to be presented used a 24-h pre-treatment step.

We have demonstrated that docosanol suspended in Tetronic 908 inhibits HSV replication more potently than docosanol in Pluronic F-68 (Pope et al., 1996). Although not shown, limited studies were conducted comparing the two preparations side by side and no appreciable differences were observed with respect to the synergistic interaction between docosanol and ACV. This point is illustrated by a comparison of the data shown in Panel B of Fig. 1 using docosanol in Tetronic 908 with the data presented by the upper three bars of Fig. 4 using docosanol in Pluronic F-68. In both experiments, Vero cells were pre-treated with 15 mM docosanol and infected with 500 PFU of HSV-1. Comparable 15–20-fold reductions of ACV EC_{90s} were observed with docosanol in either Tetronic 908 or Pluronic F-68.

Multiple independently conducted experiments involving combination anti-HSV activity of docosanol and ACV were plotted in an isobologram as described (Elion et al., 1954; Greco et al., 1995). The resulting plots revealed synergistic anti-HSV activity with the combination of docosanol plus ACV (not shown). These data were also analyzed using the fractional product method as described (Greco et al., 1995) and consistent Bliss synergy was observed (not shown). These mathematical models confirmed that the reduction of the ACV effective concentration values caused by treatment of cells with docosanol is greater than what would be expected based on additivity.

3.2. Docosanol and ACV can synergistically inhibit HSV-2 plaque formation in Vero cell cultures

The effects of ACV and docosanol, alone or in combination, against direct plaque formation by HSV-2 are presented in Fig. 2. Vero cells were cultured in medium alone or in medium containing 3 mM docosanol or that amount of Pluronic F-68 (0.4 mM) contained in the docosanol cultures. After 24 h incubation, the cultures were inoculated with varied amounts of ACV and infected with 50 PFU of HSV-2. The cultures were scored for HSV-2 plaques 44 h thereafter. Untreated cultures (medium alone) exhibited a mean of 46 plaques and ACV inhibited plaque formation in such cultures with an EC_{50} of 5 μM. Pluronic F-68-treated cultures exhibited a similar mean number of plaques and ACV EC_{50}. Cultures treated with docosanol exhibited $\sim$40% fewer plaques than the control cultures, a reflection of the antiviral activity of the compound. Moreover, docosanol-treated cultures exhibited a 25-fold reduction of the ACV EC_{50} (0.2 μM). Theoretical additivity was calculated by multiplying the untreated control value by the remaining fraction obtained with drug #1 alone, the product of which was multiplied by the remaining fraction obtained with drug #2 alone; this was done for each drug combination to obtain a line of theoretical additivity. As shown in Fig. 2, it is clear that the combined antiviral action of docosanol plus ACV do not coincide with the line of theoretical additivity, particularly at the higher end of the ACV concentration curve where 100% inhibition of HSV-2 replication was observed in the presence of docosanol. This experiment was also conducted with HSV-1 with comparable results.

3.3. It requires approximately 500-fold more HSV-1 PFU to induce a plaque in the presence of docosanol (15 mM) plus ACV (44 μM) than it does with either compound alone

Because complete inhibition of plaque formation was observed in Fig. 2 with certain combina-

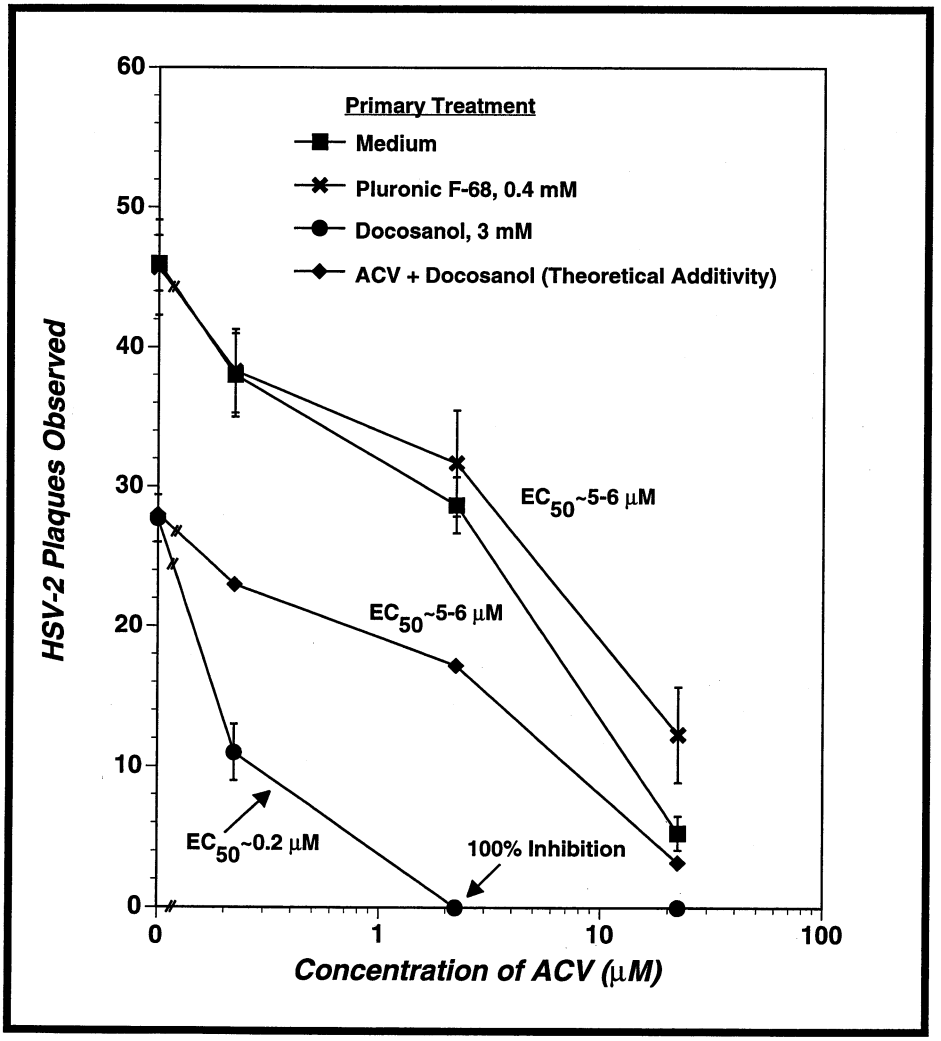

Fig. 2. Docosanol and ACV can synergistically inhibit HSV-2 plaque formation in Vero cell cultures. The direct plaque assay was conducted with Vero cells as described in Section 2 and text. Groups of cultures contained the indicated concentration of docosanol or Pluronic F-68 for 24 h before infection with 50 PFU of HSV-2. The data are expressed as means and standard errors of plaques observed in triplicate wells/determination.

tions of docosanol plus ACV, it was of interest to determine how many HSV PFU would be required to induce a plaque when both compounds were present in near optimal amounts. Vero cells were cultured in medium alone or in medium containing 15 mM docosanol or 4 mM Pluronic F-68. After 24 h incubation, certain of the cultures were inoculated with ACV (44 μM) and HSV-1 was added in varied amounts to the cultures as indicated in Table 1. After 48 h incubation, the cultures were washed, fed fresh medium without inhibitor, and incubated an additional 24 h to let plaques fully develop. Cells cultured in medium alone exhibited a mean of 8.6 plaques following infection with 10 PFU of HSV-1, and there were too many plaques to count (TMTC) following infection with 100 or more PFU. As presented in the bottom line of Table 1, approximately 1.16 PFU were required to generate 1 plaque in such cultures. ACV alone inhibited HSV-1 infection

Table 1
It requires approximately 500-fold more HSV-1 PFU to induced a plaque in the presence of docosanol (15 mM) plus ACV (44 μM) than it does in the presence of docosanol or ACV alone

PFU added/culture	HSV-1-induced plaques observed in:					
	Medium	Medium+ACV	Docosanol	Docosanol+ACV	Pluronic F-68	Pluronic F-68+ACV
10	8.6 (0.9)	0.67 (0.3)	0.33 (0.3)	0	7.8 (2)	1 (0)
100	TMTC*	2.6 (0.3)	2.3 (0.3)	0	TMTC	7.8 (2)
1000	TMTC	25 (1.2)	17.3 (2.4)	0	TMTC	29 (1.9)
10 000	TMTC	TMTC	TMTC	0.67 (0.3)	TMTC	TMTC
100 000	TMTC	TMTC	TMTC	3 (0)	TMTC	TMTC
PFU needed/plaque	1.16	31	44	24 129	1.28	19

Vero cells were cultured (16-mm wells, 10^5 cells/ml, 1 ml/well) in medium alone, or in medium containing 15 mM docosanol or 4 mM Pluronic F-68. After 24 h incubation, ACV was added to 44 μM and the cultures were infected with the indicated number of HSV-1 PFU. After 2 days incubation, the cultures were washed and inoculated with fresh medium lacking docosanol, Pluronic F-68, or ACV. After an additional 1 day incubation, the cultures were stained and scored for HSV-1-induced plaques. The data are expressed as means and standard errors derived from triplicate cultures per determination.

* TMTC, too many to count.

such that means of 2.6 and 25 plaques were observed in cultures infected with 100 and 1000 PFU, respectively. These data equate to a requirement of 31 PFU to generate 1 plaque in the presence of 44 μM ACV. Although not shown, the PFU generated in the presence of 44 μM ACV exhibited comparable replication in the presence and absence of 44 μM ACV indicating selection of ACV-resistant HSV. Very similar PFU requirements were observed with cells cultured in docosanol alone, i.e. 44 PFU required to generate 1 plaque. However, potent inhibition of HSV infection was observed with the combination of docosanol plus ACV and it required ~24 000 PFU to generate 1 plaque under such conditions. The Pluronic F-68 control did not alter HSV-1 infection or ACV activity beyond that seen with medium alone.

3.4. The synergistic antiviral interaction with docosanol plus ACV can be demonstrated in human foreskin fibroblasts infected with clinical isolates of HSV

It was important to verify that the synergistic antiviral effects of docosanol plus ACV were not an artifact resulting from the use of an immortal cell line (Vero) or HSV strains that have been passed for many years in the laboratory setting. To address this issue, the ability of docosanol to enhance the anti-HSV activity of ACV was tested against PFU production using normal human foreskin fibroblasts and clinical isolates of HSV. The MacIntyre strain of HSV-1 was also tested for comparison. As shown in Fig. 3, the MacIntyre strain of HSV-1 exhibited a sensitivity to ACV alone in foreskin fibroblasts like that seen in Vero cells. Moreover, the ability of docosanol to enhance the activity of ACV in foreskin fibroblast was also like that seen with Vero cells. This same analysis was conducted using the MS strain of HSV-2 and MRC-5 normal human lung cells with comparable results and conclusions (not shown).

The remaining data shown in Fig. 3 concern the sensitivity of clinical isolates to the synergistic antiviral effects of docosanol plus ACV. It is apparent the replication of these clinical isolates is as sensitive as that of the MacIntyre strain of HSV-1 to inhibition by ACV, either alone or in combination with docosanol. The first two clinical isolates were derived from genital lesions and the other two were derived from oral lesions. These results also confirm that the anti-HSV interaction

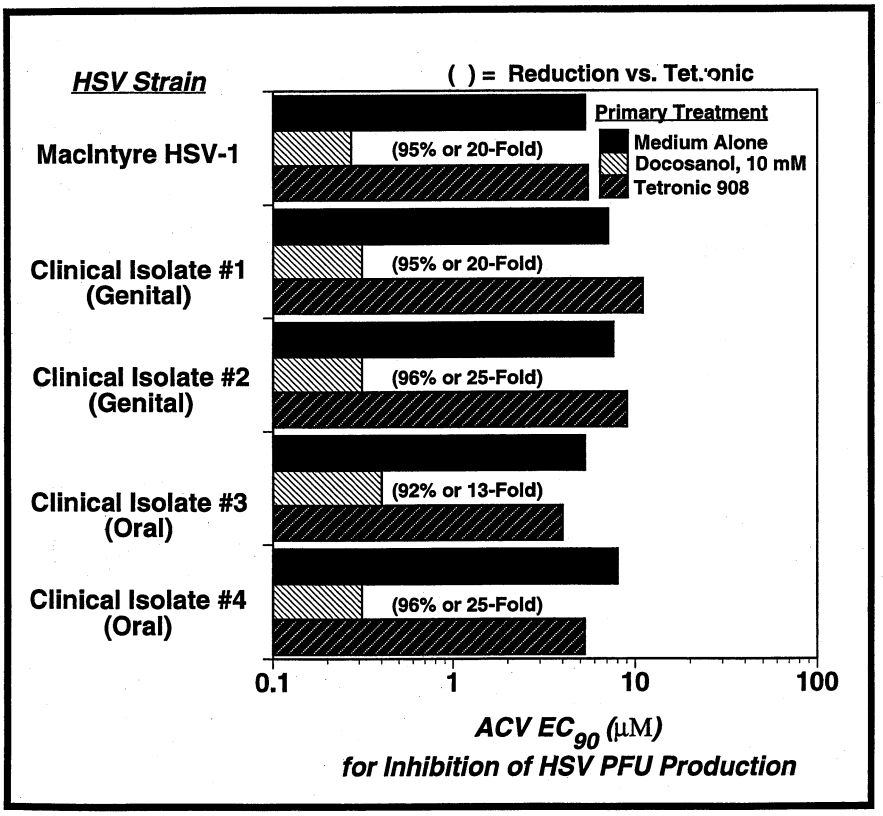

Fig. 3. The synergistic antiviral interaction with docosanol plus ACV can be demonstrated in human foreskin fibroblasts infected with clinical isolates of HSV. Foreskin fibroblasts were cultured 24 h in the presence or absence of docosanol or Tetronic 908 as described in the legend to Fig. 1, before addition of varied amounts of ACV and infection with 500 PFU of HSV. Culture fluids were assayed for progeny HSV PFU 72 h later in foreskin fibroblast cultures as described in Section 2. The data are expressed as ACV EC_{90} values for inhibition of HSV production derived from triplicate initial cell cultures/drug combination.

with docosanol plus ACV is fully operational with normal human cells and clinical HSV isolates.

3.5. *Docosanol and ACV synergistically inhibit replication of human varicella zoster virus (VZV) and cytomegalovirus (CMV) in MRC-5 cell cultures*

VZV and CMV exhibit sensitivity to the antiviral effects of ACV, so it was possible that docosanol could stimulate such effects to inhibit replication of these viral genera. This issue was investigated as shown in Table 2. As described for Group I, MRC-5 cells cultured in medium alone exhibited ~350 000 VZV-infected cells (approximately one-half of total cells/culture) 4 days following infection with 500 PFU of VZV. ACV inhibited VZV infection in parallel cultures with an EC_{50} of 3 μM and an EC_{90} of 10 μM. As shown by the data in the third column for Groups II-IV, fewer VZV-infected cells were generated in the presence of docosanol and 10 mM was an approximate EC_{50}. Parallel cultures that also contained ACV demonstrated that treatment with docosanol substantially enhanced ACV anti-VZV efficacy. The high docosanol concentration of 30 mM reduced the ACV EC_{50} to 0.3 μM (90% or 10-fold reduction) and the EC_{90} to 2 μM (80% or 5-fold reduction). Lower docosanol levels also enhanced ACV activity against VZV in a concentration-dependent manner. The Pluronic F-68 control did not alter VZV infected cell numbers

Table 2
Docosanol and ACV synergize to inhibit replication of varicella zoster virus (VZV) and cytomegalovirus (CMV) in MRC-5 cell cultures

A. VZV-infected groups	Primary treatment	IC/culture w/o ACV	ACV ECs in parallel cultures	
		( ) = % Inhibition	EC_{50} (μM)	EC_{90} (μM)
I	Medium only	35×10^{-4}	3	10
II	Docosanol, 30 mM	7×10^{-4} (80%)	0.3	2
III	Docosanol, 10 mM	15×10^{-4} (57%)	0.6	4
IV	Docosanol, 3.3 mM	21×10^{-4} (40%)	1	6
V	Pluronic F-68	33×10^{-4} (6%)	3	10
B. CMV-infected groups	Primary treatment	IC/Culture w/o ACV	EC_{50} (μM)	EC_{90} (μM)
VI	Medium only	20×10^{-4}	30	250
VII	Docosanol, 30 mM	3×10^{-4} (85%)	2	6
VIII	Docosanol, 10 mM	8×10^{-4} (60%)	2	25
IX	Docosanol, 3.3 mM	16×10^{-4} (20%)	8	35
X	Pluronic F-68, 8 mM	20×10^{-4} (0%)	30	250

MRC-5 cell cells were cultured in medium alone or in medium containing the indicated concentration of docosanol or Pluronic F-68 as described in Section 2, before addition of varied amounts of ACV and infection with 500 PFU of VZV (Gps I–V) or CMV (Gps VI–X). Infected cells (IC) content was determined 4 days later as described in Section 2. Infected cell data are expressed as mean infected cells/culture derived from triplicate wells per initial culture group. ACV EC_{50s} and EC_{90s} values were derived from inhibition curves in the presence of the indicated primary treatment.

or ACV efficacy. Although not shown, anti-VZV synergy with the combination of docosanol plus ACV was confirmed using the fractional product model as described by others (Greco et al., 1995).

More substantial reductions of the ACV effective concentrations were observed with CMV (Groups VI–X, Table 2). Untreated (Group VI) and Pluronic F-68-treated (Group X) MRC-5 cell cultures exhibited ~200 000 CMV-infected cells 4 days following inoculation with 500 PFU of CMV. ACV inhibited infection of such cultures with an EC_{50} and EC_{90} of 30 and 250 μM, respectively. As shown by the third column for Groups VII–IX, docosanol inhibited CMV replication with an approximate EC_{50} of ~10 mM. Docosanol at 30 mM reduced the ACV EC_{50} to 2 μM (93% or 14-fold reduction) and EC_{90} to 6 μM (98% or 50-fold reduction). Docosanol at 3.3 and 10 mM also substantially increased the activity of ACV against CMV replication. The Pluronic F-68 control did not display activity against CMV. Although not shown, anti-CMV synergy with the combination of docosanol plus ACV was confirmed using the fractional product model as described by others (Greco et al., 1995).

3.6. Docosanol can synergistically interact with nucleoside, but not pyrophosphate, analogs to inhibit HSV-1 replication in vitro

Antiviral interactions with docosanol and analogs other than ACV were also investigated with HSV-1 PFU production in Vero cell cultures (Fig. 4). Untreated, docosanol-treated (15 mM) and Pluronic F-68-treated Vero cells were infected with 500 PFU/culture of HSV-1 and exposed to varied concentrations of the indicated analog antiviral drugs. Three days later, the culture supernatant fluids were harvested and analyzed for progeny HSV-1 PFU. A typical ACV anti-HSV-1 EC_{90} of ~20 μM was observed in control cultures, which was reduced to 0.9 μM (20-fold reduction) in the presence of docosanol. The nucleoside analog Ara-A exhibited an EC_{90} of ~20 μM in the absence of docosanol and, when the compound was present, an EC_{90} of ~1.3 μM (15-fold reduction) was observed. TFT exhibited an EC_{90} of ~7 μM in the absence of docosanol and an EC_{90} of ~1.3 μM (5-fold reduction) when both drugs were present. Ribavirin in medium only or Pluronic inhibited HSV-1 replication with an EC_{90}

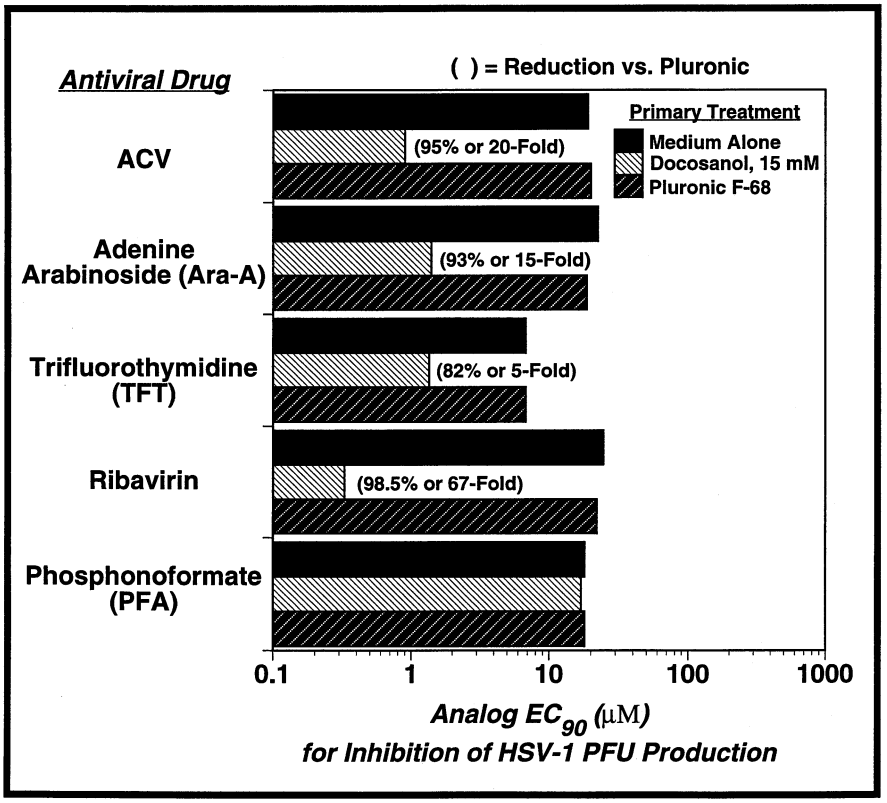

Fig. 4. Docosanol can synergistically interact with nucleoside, but not pyrophosphate, analogs to inhibit HSV-1 replication in vitro. Vero cells were cultured 24 h in the presence or absence of docosanol or Pluronic F-68 as described in the legend to Fig. 1 before addition of varied amounts of the indicated analog drug and infection with 500 HSV-1 PFU. Culture fluids were assayed for progeny HSV PFU 72 h later in Vero cell cultures as described in Section 2. The data are expressed as analog drug EC_{90} values for inhibition of HSV-1 production derived from triplicate initial cell cultures/drug combination.

of $\sim$22 μM, which was reduced to $\sim$0.33 μM (67-fold reduction) when docosanol was present. PFA exhibited an EC_{90} of 18 μM in medium only and Pluronic F-68-treated cultures and an EC_{90} of 17 μM with docosanol-treatment. However, docosanol alone reduced PFU yield $\sim$90% and the inhibitory activity of PFA was expressed on the remaining virus such that the additive effects of the two compounds resulted in greater viral inhibition than that observed with either drug alone (not shown).

3.7. The effects of docosanol on Vero cell ^{3}H-TdR incorporation in the presence of various analog drugs

The ability of docosanol to enhance nucleoside or pyrophosphate analog cellular toxicity in the absence of virus infection was tested on ^{3}H-TdR incorporation (DNA synthesis) by Vero cells (Table 3). As described in the legend to Table 3, Vero cells were cultured 24 h in the presence or absence of docosanol (15 mM) or Pluronic F-68 (2

Table 3
The effects of docosanol on Vero cell ^{3}H-TdR incorporation in the presence of various analog drugs

Analog drug	Analog drug IC_{50} (μM) with the following primary culture treatments		
	Medium	Docosanol (15 mM)	Pluronic F-68 (4 mM)
ACV	> 222	> 222	> 222
PFA	> 167	> 167	> 167
Ara-A	64	75	81
Ribavirin	49	41	61
TFT	7	7	8
5-FU	13	11	12
8-AG	4	3	3

Vero cell incorporation of ^{3}H-TdR was monitored in the presence or absence of analog drugs, docosanol, or Pluronic F-68 as described in Section 2. The data are expressed as the micromolar concentration of analog drug needed to inhibit incorporation of ^{3}H-TdR by 50% (IC_{50}). Such values were derived from triplicate determinations of ^{3}H-TdR incorporation for each concentration of analog drug in the presence or absence of docosanol or Pluronic F-68.

mM), before exposure to various concentrations of analog drugs. DNA synthesis (^{3}H-TdR incorporation) was measured 48–72 h thereafter and the IC_{50s} for the drugs determined given the various conditions. ACV at the highest concentration (50 μg/ml) inhibited DNA synthesis 35–38% in control cultures and 29% in cultures treated with docosanol; this can be extrapolated to an approximate IC_{50} of ~1300 μM in either case. Toxicity was not visually apparent in any of these ACV-containing cultures. PFA did not substantially inhibit DNA synthesis at the high 50 μg/ml concentration regardless of the presence or absence of docosanol or Pluronic F-68; toxicity was not visually apparent when these cultures were examined microscopically. The remaining drugs (Ara-A, ribavirin, TFT, 5-FU, and 8-AG) exhibited inhibitory activities at the concentrations tested, but such activities did not fluctuate substantially in the presence or absence of docosanol or Pluronic F-68. Microscopic evaluation of the Ara-A- and ribavirin-treated cultures revealed minimal toxicity regardless of treatment at the high 50 μg/ml concentration and no toxicity at the next lowest level (10 μg/ml). Obvious cytopathology was apparent with cultures exposed to 50 or 10 μg/ml TFT, 5-FU, and 8-AG. However, as documented with the IC_{50s} for these drugs, such toxicity was visually comparable regardless of the presence or absence of docosanol or Pluronic F-68.

4. Discussion

Combination drug therapy has emerged as a leading-edge concept for the treatment of viral diseases and the results reported herein suggest that docosanol could have an important role in this strategy. Since docosanol and nucleoside or pyrophosphate analogs have disparate antiviral mechanisms of action, a combination would be predicted to be more efficacious than either drug alone. However, it was observed that the combination of docosanol plus the tested nucleoside analogs displayed greater antiviral activities than what would be expected based on additivity. Treatment of cells with docosanol did not enhance the antiviral activity of PFA, but an additive anti-HSV effect was observed. Importantly, interactions leading to synergistic inhibition of cell DNA synthesis and overt toxicity were not observed when docosanol was combined with these nucleoside or pyrophosphate analogs, even with analogs that have a high degree of reactivity with uninfected cells, i.e. 5-FU or 8-AG. These results suggest that docosanol could markedly increase the selectivity ratio (i.e. efficacy vs. toxicity) of nucleoside analog antiviral drugs. Additional studies of this sort using other cell lines, and in particular normal human cells like foreskin fibroblasts, are needed to establish this latter supposition.

It has been necessary to suspend docosanol in surfactants for use in tissue culture or for systemic animal studies (Katz et al., 1994). Tetronic 908 and Pluronic F-68 have been identified as suitable for such purposes. These surfactants are of a class of compound referred to as poloxamers, which have a long history of safe use by the pharmaceutical industry (Schmolka, 1991). Pluronic F-68 is a difunctional polymer produced by coupling propylene oxide and ethylene oxide to propylene glycol (Schmolka, 1991). Tetronic 908 is a tetrafunctional copolymer produced by coupling the aforementioned oxides to ethylenediamine (Schmolka, 1991). In spite of very similar chemical properties, metabolism and antiviral activity of docosanol is greater with Tetronic 908 than with Pluronic F-68 (Pope et al., 1996). The limited comparison described herein suggests that the two surfactants have very similar ability to facilitate synergistic antiviral interactions between docosanol and ACV. Thus, 15 mM docosanol in Tetronic 908 enhanced the activity of ACV ~95% in HSV-1-infected Vero cell cultures (Fig. 1), as did the same concentration of docosanol in Pluronic F-68 in similarly infected Vero cells (Fig. 4). Detailed analysis is required to make a definitive assessment, but the present work indicates that the two surfactants are for the most part interchangeable with respect facilitating the anti-herpesvirus interaction between docosanol and ACV.

Passage of HSV in the presence of ACV allowed for the emergence of ACV-resistant variants as described relative to Table 1. This was expected based on the findings that many laboratory and clinical strains of HSV can contain appreciable frequencies (1–20%) of ACV-resistant variants without previous exposure to ACV (Parris and Harrington, 1982; Smith et al., 1980). It has previously been concluded that the MacIntyre HSV-1 strain contains 4.8% such variants (Smith et al., 1980). The plating efficiency shown in Table 1 for this HSV-1 strain indicates 1-3% variants that can replicate in the presence of 44 μM ACV. The plating efficiency for HSV-1 observed in Table 1 in the presence of docosanol plus ACV suggest that the expected numbers of ACV-resistant variants were effectively inhibited from replication. The disparate antiviral mechanisms of action of the two compounds likely contributed to these results, allowing for dual inhibition of wild-type HSV and antiviral effects by one drug on variants resistant to the other. Most HSV variants that can replicate in 10–25 μM ACV are inhibited from replication in 100 μM ACV (Parris and Harrington, 1982; Smith et al., 1980), i.e. they are partially-resistant variants. The ability of docosanol to increase the effective (rather than absolute) ACV concentration that can target both fully ACV-sensitive and partially-resistant virions may also contribute to the substantially better antiviral inhibition observed with the combination versus either drug alone.

Docosanol substantially enhanced the antiviral activity of ACV against all tested herpesviruses. Considering that such activity was initially documented with the MS strain of HSV-2 and the MacIntyre strain of HSV-1 that were established in the laboratory some 30 years ago, it was important to verify that the phenomena could be observed with clinical isolates of HSV. As presented in Fig. 3, clinical HSV isolates displayed sensitivity to docosanol-mediated enhancement of ACV activity like the aforementioned laboratory HSV strains. While limited numbers of clinical isolates were evaluated, it appears to make little difference whether the virus was isolated from the oral or genital region. This might seem obvious considering that the MS strain is HSV-2 and the MacIntyre strain is HSV-1, but both of these strains were isolated from human brain tissue. CMV and VZV demonstrated sensitivity to the antiviral interaction between docosanol and ACV. CMV and VZV may also display enhanced sensitivity to other nucleoside analogs in the presence of docosanol, but this has yet to be tested. The extent to which this interaction will operate against other viruses will have to be empirically determined, but likely candidates include ACV-resistant HSV mutants and other genera of the herpesviridae family, HIV, influenza virus, respiratory syncytial virus, and vaccinia virus.

Docosanol enhanced the activity of ACV with all cell lines tested, including Vero monkey kidney cells, normal human lung cells, and foreskin cells. These latter results confirm that the phenomena is

not limited to an immortal cell line that has been passed for many years in the laboratory, but may also be expressed in normal diploid human cells with the typical finite life-span. We have previously shown that docosanol can inhibit replication of human herpesvirus-6 and HIV in human peripheral blood mononuclear cells (PBMC; Marcelletti et al., 1996). Such results indicate that PBMC can effectively metabolize docosanol. Therefore, it is quite possible that docosanol can enhance or augment the antiviral activities of nucleoside or pyrophosphate analogs against these and other viruses that infect PBMC.

Treatment of cells with docosanol synergistically enhanced the anti-HSV activity of all tested nucleoside analogs that could inhibit HSV replication. This would be expected to some degree since the different nucleoside analogs tend to use similar cellular and viral mechanisms for transport across the plasma membrane, metabolic activation, and antiviral expression. An analysis of this response with the various drugs should give clues as to the synergistic mechanism of docosanol. The 67-fold increase in ribavirin activity with docosanol treatment versus the 5-fold increase of TFT activity lends credence to this notion, but too little information is presently available to draw conclusions. The finding that docosanol exhibited an additive effect with PFA against HSV replication is indicative of some degree of specificity in the synergistic response observed with nucleoside analogs, i.e. synergy is likely not due to a generalized increase in cellular metabolism. This supposition is further indicated by the lack of interactions between docosanol and the nucleoside or pyrophosphate analogs when virus is not present, as in the inhibition of cellular DNA synthesis. The antiviral interaction between docosanol and nucleoside analogs, and lack thereof with the pyrophosphate analog PFA, suggests a mode of action involving nucleoside metabolism as observed with other agents that can synergistically increase the antiviral activity of ACV (e.g. Neyts et al., 1998; O'Brien et al., 1990; Reardon and Spector, 1991).

These substantial increases in drug selectivity and efficacy illustrate how combination drug therapy, and in particular that involving docosanol, could be used to increase the potency of nucleoside or pyrophosphate analogs such that effective antiviral concentrations are substantially lower than concentrations that elicit toxicity. Moreover, as exemplified with the increase in ACV efficacy, combination therapy with docosanol was associated with substantially reduced emergence of progeny virus in general, including ACV-resistant variants. Combination antiviral drug therapy using docosanol plus nucleoside or pyrophosphate analogs could therefore allow for decreased patient exposure to potentially toxic or allergenic analog compounds, while at the same time increase efficacy resulting in shorter duration of disease and less likelihood for selection of drug-resistant mutants.

The practical applications of a docosanol plus nucleoside analog combinations for the treatment of herpesvirus-mediated diseases are numerous. A topical formulation could target any number of external herpesvirus lesions, including herpes labialis, genitalis, and Kaposi's sarcoma. With appropriate formulations additional disease targets could be investigated. These include oral, nasal, ocular, vaginal, pulmonary, and potentially systemic herpesvirus infections. However, the practicality of docosanol plus nucleoside analog combinations must yet be confirmed in animal models for human viral diseases, studies that are presently ongoing.

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