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The anti-herpes simplex virus activity of *n*-docosanol includes inhibition of the viral entry process

Laura E. Pope [a,*], John F. Marcelletti [a], Lee R. Katz [a], Janet Y. Lin [a], David H. Katz [a], Mary Lynn Parish [b], Patricia G. Spear [b]

[a] *LIDAK Pharmaceuticals, 9393 Towne Centre Drive, Suite 200, San Diego, CA 92121, USA*
[b] *Northwestern University Medical School, Department of Microbiology—Immunology, 303 East Chicago Avenue, Chicago, IL 60611-3008, USA*

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Abstract

n-Docosanol-treated cells resist infection by a variety of lipid-enveloped viruses including the herpesviruses. Previous studies of the mechanism of action demonstrated that *n*-docosanol inhibits an event prior to the expression of intermediate early gene products but subsequent to HSV attachment. The studies reported here indicate that *n*-docosanol inhibits fusion of the HSV envelope with the plasma membrane. Evidence suggests that antiviral activity requires a time-dependent metabolic conversion of the compound. Cellular resistance to infection declines after removal of the drug with a $t_{1/2}$ of approximately 3 h. Reduced expression of viral genes in *n*-docosanol-treated cells was confirmed by a 70% reduction in expression of a reporter gene regulated by a constitutive promoter inserted into the viral genome. Inhibited release in treated cells of virion-associated regulatory proteins—an immediate post entry event—was indicated by a 75% reduction in the expression of β-galactosidase in target cells carrying a stably transfected *lacZ* gene under control of an HSV immediate—early promoter. Finally, the fusion-dependent dequenching of a lipophilic fluorescent probe, octadecyl rhodamine B chloride, inserted into the HSV envelope was significantly inhibited in treated cells. Inhibition of fusion between the plasma membrane and the HSV envelope, and the subsequent lack of replicative events, may be the predominant mechanism for the anti-HSV activity of *n*-docosanol.

Keywords: *n*-Docosanol; Fatty alcohol; Herpes simplex virus; Antiviral activity; Mechanism of action; Entry

1. Introduction

n-Docosanol formulated as *n*-docosanol 10% cream is under clinical investigation as a new drug

Abbreviations: FBS, fetal bovine serum; Pur, puromycin; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

* Corresponding author. Tel.: +1 619 5580364; fax: +1 619 4535845.

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for the topical treatment of herpes simplex infections. Efficacy in reducing the healing time of recurrent oral—facial herpes simplex infections has been demonstrated in Phase II (Habbema et al., 1996) and in recently completed Phase III placebo-controlled clinical trials (manuscript in preparation). Positive results were also obtained in a Phase I/II pilot study using *n*-docosanol 10% cream as a topical treatment for cutaneous Kaposi's sarcoma lesions in HIV-1 positive patients (Scolaro et al., 1997). *n*-Docosanol topical cream prevented vaginal transmission of SIVmac251 in rhesus macaques (Miller et al., 1995), suggesting that the compound has antimicrobial functions that may be useful as a prophylactic to prevent the transmission of HIV in humans.

n-Docosanol exhibits antiviral activity in vitro against a wide range of lipid-enveloped viruses. Susceptible human viruses include HSV-1 and HSV-2 (including acyclovir-resistant strains and clinical isolates), influenza A, respiratory syncytial virus, cytomegalovirus, varicella zoster virus, human herpesvirus 6 and HIV-1. The ID_{50} values (concentration where 50% inhibition is observed) ranged from 3 to 12 mM for these susceptible viruses. Non-enveloped viruses and enveloped viruses that are endocytosed have an apparent resistance to the effects of *n*-docosanol (Katz et al., 1991; Marcelletti et al., 1992 and unpublished data, LEP). For in vitro efficacy studies, the insoluble *n*-docosanol is formulated by suspending the molecule in the inert and non-toxic surfactant Pluronic F-68, a block copolymer of polyethylene oxide and polypropylene oxide, or a related molecule, Tetronic 908 (Schmolka, 1991). The relatively high concentrations of *n*-docosanol required for in vitro activity may be a result of the physiochemical nature of the surfactant-stabilized particles as detailed in Section 4. However, because *n*-docosanol concentrations as high as 300 mM are not cytotoxic (Katz et al., 1991) the therapeutic index for the drug is favorable.

Previously reported studies, generally conducted with HSV, demonstrated that *n*-docosanol does not directly inactivate virus since virus preparations can be mixed with the compound without loss of infectivity (Katz et al., 1991). Instead, the drug apparently modifies the target cell in a manner that inhibits viral replication. Studies have demonstrated that radiolabeled *n*-docosanol is extensively incorporated into host cells and metabolized to phospholipids with the chromatographic properties of phosphatidylcholine and phosphatidylethanolamine (Katz et al., 1994; Pope et al., 1996). Furthermore, conditions that increase the amount of *n*-docosanol metabolism increase the amount of antiviral activity, suggesting that this intracellular metabolic conversion of the drug is required for antiviral activity (Pope et al., 1996).

n-Docosanol inhibits HSV-induced plaque formation and production of viral particles as judged in a secondary plaque assay. It also inhibits, as determined by ELISA, the production of HSV core and envelope proteins and the number of cells expressing the intranuclear HSV-1 specific immediate—early protein ICP4 (Katz et al., 1991; Marcelletti et al., 1992). These observations suggested that *n*-docosanol interferes with an early step in HSV infection.

In this paper we describe studies conducted to investigate further the mechanism of action for the anti-HSV activity of *n*-docosanol utilizing (1) an HSV recombinant virus which expresses β-galactosidase on entry of the viral genome into the nucleus of a susceptible host cell; (2) a host cell transformed to express β-galactosidase upon entry of HSV virion proteins into the cell; and (3) HSV-2 fluorescently labeled with octadecyl rhodamine B chloride.

2. Materials and methods

2.1. Formulation of n-docosanol

n-Docosanol (98% pure; M. Michel, New York) was suspended in Tetronic 908 (poloxamine 908, M_r 25000; BASF; Parsippany, NJ) generally as follows. Tetronic 908 was diluted to 1.6 mM in 37°C sterile saline, and the solution was then heated to 50°C. *n*-Docosanol was added to 300 mM to the Tetronic in saline and the mixture was sonicated (Branson 450 sonifier; Danbury, CT) for 21 min at an initial output of 65 W; this warms the mixture to 86°C. The resulting suspen-

sion consists of very fine globular particles with an average size of 0.1 microns as measured by transmission electron microscopy.

2.2. Other reagents

Heparin and NP-40 were obtained from Sigma (St. Louis, MO) and octadecyl rhodamine B from Molecular Probes (Eugene, OR). Anti-gD neutralizing monoclonal antibody (III-174) was generated as described (Para et al., 1985).

2.3. Cell lines

Plaque reduction assays were typically performed in Vero cells (African Green monkey kidney; ATCC no. CCL-81). The HEp-2 (human epidermoid carcinoma; ATCC no. CCL-23) cell line and NC-37 human B cells (ATCC No. CCL-214) were obtained from the American Type Culture Collection. The CHO-IEβ8 cell line was developed in one of our laboratories (Parish, Montgomery and Spear, unpublished studies). It was selected by transfection of Chinese hamster ovary cells (CHO-K1; ATCC no. CCL-61) with a plasmid carrying a puromycin (Pur) selectable marker and *lacZ* under control of the HSV-1 ICP4 promoter. The cell line was selected in Pur and screened for expression of β-galactosidase after HSV infection but not in the absence of infection.

2.4. Viruses

The MacIntyre strain of HSV-1 (VR-539) and the MS strain of HSV-2 (VR-540) were obtained from the American Type Culture Collection. HSV-2 (333), a wild-type strain, was obtained from Dr Fred Rapp. Stock preparations were titered for levels of plaque-forming units (PFU) in Vero cells and stored frozen at -80°C. HSV-1(KOS)gL86 is a replication-defective mutant in which the gL ORF is replaced with *lacZ* under control of the CMV promoter. This mutant is propagated in gL-expressing Vero cells and is fully infectious but can undergo only one round of replication in non-complementing cells. It was obtained using a method described in a manuscript in preparation by Novotny and Spear.

2.5. Viral plaque assays in cell culture

Cultured cells were plated in 35-mm wells (2 ml; 3×10^5 cells/ml) in DMEM containing L-glutamine, pennstrep (cDMEM) and supplemented with 5% FCS. *n*-Docosanol or the corresponding control vehicle (lacking *n*-docosanol) was added at the outset of the culture. All cultures were then inoculated with 175 p.f.u. of HSV-1 or HSV-2. The cultures were incubated for an additional 42–44 h, washed once with fresh medium, stained and fixed (the staining/fixative consists of 1.25 mg/ml of carbol-fuchsin plus 2.5 mg/ml of methylene blue in methanol) and then scored for HSV-induced plaques using a dissecting microscope ($10\times$ magnification). The data are averages of duplicate cultures, which varied by no more than 5–10%.

2.6. Assays for viral entry

Twenty-four hours before infection, cultured cells were seeded into 24-well (16-mm) plates at 2.5×10^6 cells/well in 0.5 ml cDMEM supplemented with 10% fetal bovine serum (FBS). After cell attachment (4–6 h later) heparin, *n*-docosanol-surfactant, or surfactant alone was added to the cells in 0.5 ml DMEM/10% FBS. The agents were dissolved in the medium at two times the desired final concentration. For infection, 0.7 ml of medium was removed from each well and 25 μl of virus suspension was added to the remaining 0.3 ml to give a virus dose of at least 20 p.f.u./cell. The plates were rocked at 37°C for 3 h and then put in a 37°C CO_2 incubator for another 2–3 h. At 5–6 h after infection, the cells were fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde, washed, then permeabilized with 0.02% NP-40, 0.01% deoxycholate and 2 mM $MgCl_2$. After washing again, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added for development of blue product. The substrate was removed and replaced with 50% glycerol. Plates were photographed. To quantify the amount of color in each well the glycerol was removed from all wells that were then washed $3\times$ with distilled H_2O. DMSO (0.6 ml) was added to solubilize the dye and, after transferring 100 μl of

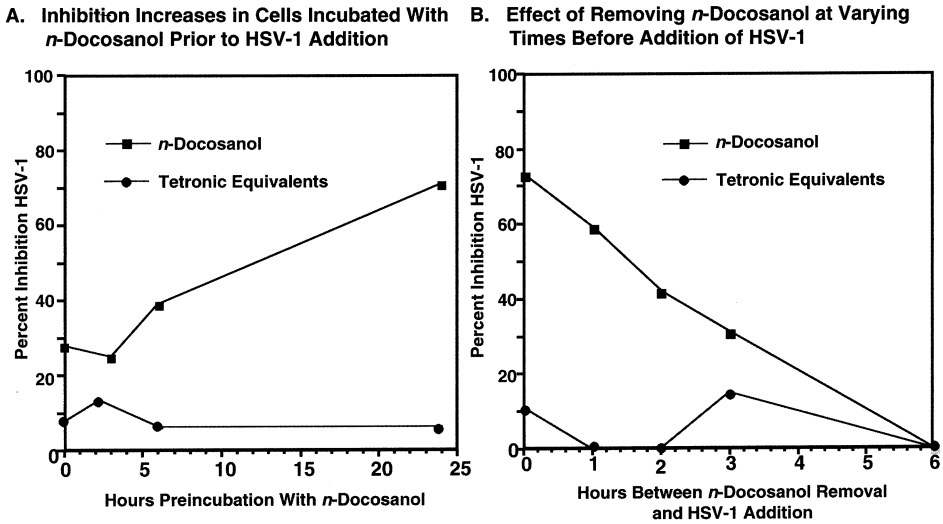

Fig. 1. The inhibition of HSV-1 increases when cells are incubated with *n*-docosanol before viral addition and this inhibitory effect has a half-life of approximately 3 h. (A) Vero cells were plated as described in Section 2 and incubated with 9 mM *n*-docosanol, the corresponding control vehicle or no addition for 0, 3, 6, or 24 h prior to the addition of HSV-1. The viral plaque assay was continued as described in Section 2 and the number of p.f.u. determined. The data are expressed as % inhibition compared to wells receiving no treatment. (B) Vero cells were plated, *n*-docosanol or the corresponding control vehicle was added and cells were incubated at 37°C in 10% humidified CO_2. After 21, 24, 25, 26, and 27 h (6, 3, 2, 1, and 0 h before the addition of HSV-1), media containing drug was removed and the cells were washed with media. After a total of 27 h, HSV-1 was added to all wells. Two hours later virus-containing media was removed and replaced with fresh media lacking virus or drugs. The cultures were incubated and processed for determination of the number of HSV-induced plaques as in (A).

each sample well from the 24-well plate to a 96-well plate, the OD_{600} was recorded using a 96-well plate reader.

2.7. Fluorescence assay for fusion

The HSV envelope was labeled with octadecyl rhodamine B chloride (R-18) as previously described (Hoekstra et al., 1984). NC-37 human B cells were inoculated at 2.5×10^5 cells/ml, 25 ml per flask. Cells were incubated overnight at 37°C with no addition or in the presence of 15 mM *n*-docosanol or the corresponding concentration of Tetronic 908. Cells were harvested by centrifugation and resuspended to 1×10^6 cells/ml. Aliquots (0.2 ml in test tubes) were chilled for 20 min at 4°C before the addition of 100 μl R-18 labeled HSV-2. After 3 h at 4°C, 3 ml media containing *n*-docosanol or Tetronic 908 at the original concentrations were added and the samples were incubated at 37°C for various times. Cells were centrifuged at 4°C, washed with saline, centrifuged, and resuspended in 10% formalin in saline (3 ml). The cells were washed with saline and resuspended in PBS containing 10% FCS. Fluorescence intensity was measured by using a fluorescence-activated cell sorter (FACScan; Becton-Dickinson).

3. Results

3.1. The active form of the drug has a finite lifetime in the cell membrane with a half-life of approximately 3 h

Antiviral activity is increased in target cells incubated with *n*-docosanol prior to the addition of HSV. This is illustrated in Fig. 1A which shows the effect of incubation time of Vero cells with 9 mM *n*-docosanol on inhibition of HSV-1-induced plaques. In this experiment, 9 mM *n*-docosanol inhibited plaque formation in Vero cells 28% when added simultaneously with, or 3 h prior to

virus addition; this was increased when cells were treated with drug 6 h prior to inhibition, but the greatest inhibition occurred in cells treated 24 h before HSV-1 addition. Intermediate time intervals were not examined.

To establish the length of time Vero cells remain resistant to HSV infection after optimal time of incubation with *n*-docosanol, Vero cells were incubated with 9 mM *n*-docosanol for 21–27 h. Media containing unincorporated drug was then removed and replaced with fresh media. Drug was not replaced. HSV-1 was added immediately, or following a 1-, 3-, or 6-h period of incubation at 37°C. Two hours following addition of HSV-1, excess virus was removed and the plaque reduction assay was continued as described in Section 2. As shown in Fig. 1B, the antiviral activity observed (% inhibition plaque formation) decreased gradually as the time between drug removal and viral addition increased. With a 3-h interval between drug removal and HSV-1 addition, 50% of the inhibitory activity was lost; with a 6-h interval no inhibition of HSV-1 plaque formation was observed.

3.2. Attachment of HSV-1 to specific cell surface receptors is unaffected in n-docosanol-treated cells

Previous studies (Katz et al., 1991) have verified that attachment of HSV-1 to specific cell surface receptors is unaffected in *n*-docosanol-treated cells. Vero cells incubated with 15 mM *n*-docosanol bound normal levels of [^{3}H]HSV-1 added at 2 p.f.u./cell. Heparin inhibited this interaction 96%. The specificity of the binding assay was confirmed using mouse HSV-1 immune sera which reduced binding by 96% compared to normal mouse sera which did not inhibit [^{3}H]HSV binding.

3.3. Production of β-gal is inhibited in n-docosanol-treated HEp-2 cells infected with HSV-1(KOS)gL86

To investigate the effects of *n*-docosanol treatment on entry of HSV into target cells, we utilized a viral construct HSV-1(KOS)gL86. In this replication-defective mutant, in which *lacZ* expression is under control of the CMV promoter, β-galactosidase is expressed after entry of the viral genome into a susceptible host cell nucleus. Addition of X-gal results in the development of blue color proportional to the number of cells infected. The intensity of the signal is inhibited by agents such as heparin which block viral binding (WuDunn and Spear, 1989; Shieh et al., 1992; see also Fig. 2) or agents which prevent entry including neutralizing monoclonal antibodies to gD, an HSV-specific protein required for entry (Fuller and Spear, 1985, 1987; Highlander et al., 1987). This signal is not inhibited by acyclovir or other agents that inhibit DNA replication.

The effect of *n*-docosanol treatment of HEp-2 cells at doses ranging from 0.9 to 9.9 mM (0.33–3.3 mg/ml) on the entry of HSV-1(KOS)gL86 was examined. HEp-2 cells were incubated for 24 h with the indicated concentrations of *n*-docosanol suspended in Tetronic 908 prior to addition of the mutant virus. At 5–6 h after infection the cells were fixed and permeabilized and X-gal was added. *n*-Docosanol treatment resulted in the visibly apparent production of fewer blue cells at

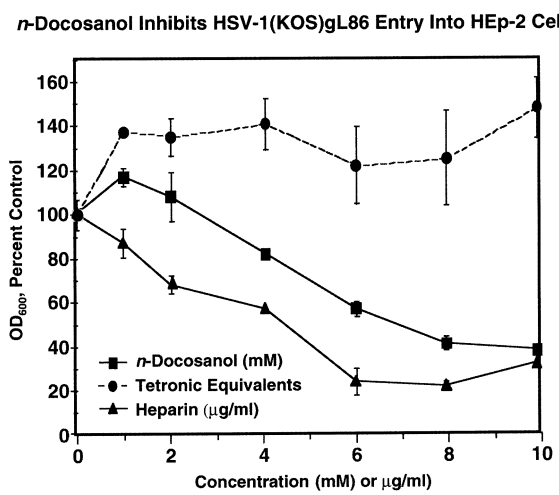

Fig. 2. Uptake of HSV-1(KOS)gL86 into HEp-2 cells is inhibited in *n*-docosanol-treated cells. After attachment of HEp-2 cells to culture wells, *n*-docosanol-vehicle, vehicle alone, or no agent (control) was added. Five to six hours following infection with HSV-1(KOS)gL86, cells were processed, X-gal was added and blue color development was quantified by measuring the absorbance at 600 nm.

n-docosanol concentrations as low as 4 mM. Almost no color development occurred in cells treated with 8 and 10 mM *n*-docosanol, respectively. To quantify the inhibition of viral infectivity, the substrate within the HEp-2 cells was solubilized by the addition of DMSO and the OD_{600} was recorded as shown in Fig. 2. The ID_{50} for *n*-docosanol was approximately 7 mM, roughly equivalent to the ID_{50} values, 4 and 9 mM, for inhibition of HSV production and plaque formation, respectively, in Vero cells (Katz et al., 1991). The vehicle, Tetronic 908, without *n*-docosanol, was not inhibitory to viral entry. In fact, treatment of cells with equivalent volumes of vehicle enhanced the blue color development as much as 40%. Heparin was examined at concentrations between 1 and 10 μg/ml; inhibition appeared to be complete at 6 μg/ml.

These results established that the HSV genome does not effectively enter the nucleus in *n*-docosanol-treated cells. Combined with the failure of *n*-docosanol to inhibit viral attachment, this experiment indicates that a step of viral entry is blocked by *n*-docosanol treatment and that this event occurs subsequent to viral attachment but prior to nuclear entry of the viral genome.

3.4. n-Docosanol inhibits HSV-2(333) infectivity of CHO-IEβ8 cells

To further narrow the point of inhibition of viral entry in *n*-docosanol-treated cells, we investigated the effects of the drug on entry of HSV-2 into CHO-IEβ8 cells selected by transfection of CHO cells with a plasmid carrying a Pur selectable marker and *lacZ* under control of the HSV-1 ICP4 promoter. In this cell line, β-gal expression is induced upon entry of HSV virion proteins into the cell, an event which occurs immediately upon viral entry into the cellular cytoplasm and which is not dependent on virion transport to the nucleus. Color development is proportional to the number of cells infected and, as in the previous assay, is effectively inhibited by agents such as heparin which block viral attachment and by agents which inhibit entry (such as antibodies to gD) but not by acyclovir and other inhibitors of DNA replication.

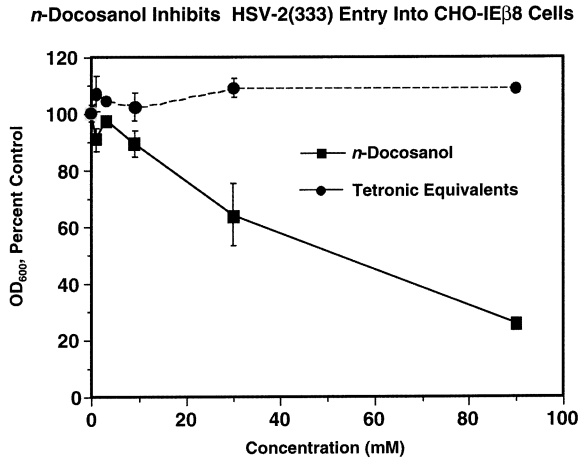

Fig. 3. *n*-Docosanol suspended with Tetronic 908 inhibits the entry of HSV-2 (333) into CHO-IEβ8 cells. CHO-IEβ8 cells were seeded into 24-well plates as described in Section 2. After cell attachment, heparin, *n*-docosanol-vehicle, vehicle alone, or no agent (control) was added. Five to six hours after infection, the cells were processed as described in Section 2, X-gal was added and the absorbance at 600 nm determined.

As illustrated in Fig. 3, *n*-docosanol inhibited β-galactosidase expression in this assay. Whereas treatment of CHO-IEβ8 cells with vehicle alone resulted in a slight increase in OD_{600} ($\sim$10%), *n*-docosanol treatment of cells results in a concentration-dependent decrease in the color development signifying infected cells. In this experiment 30 mM *n*-docosanol inhibited color production 40% compared to untreated cells and 55% compared to Tetronic 908-treated cells. The maximal observed inhibition in comparison to untreated cells was approximately 75%. This, in combination with the lack of inhibition in the binding assay, narrows a point of inhibition to an event after viral attachment but prior to release of virion proteins and manifestation of VP16 transactivator activity (an immediate post-entry event not dependent on virion transport to the nucleus).

3.5. n-Docosanol-treated NC-37 human B cells exhibit decreased fusion with octadecyl rhodamine B chloride-labeled HSV-2

Because of the selectivity of the inhibitory effects of *n*-docosanol for lipid-enveloped fusion-

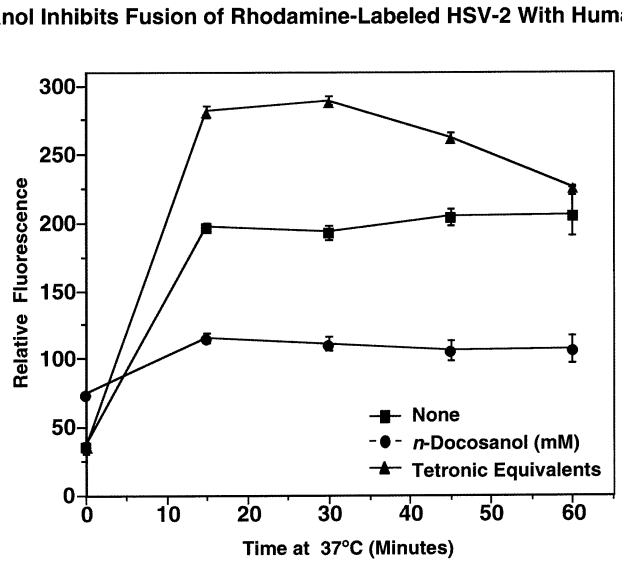

Fig. 4. *n*-Docosanol-treated NC-37 human B cells exhibit decreased fusion with octadecyl rhodamine B chloride-labeled HSV-2. NC-37 human B cells were inoculated in the presence of 15 mM *n*-docosanol, the corresponding concentration of Tetronic 908 (0.1 mM) or without addition. As described in Section 2, cells were harvested and R-18-labeled HSV-2 was added to aliquots in the presence of compounds at their original concentration. Following incubation at 37°C for the times indicated, cells were fixed and fluorescence intensity determined by FACScan.

dependent viruses and the absence of viricidal effects, we considered the possibility that *n*-docosanol may inhibit viral entry by altering target cell membranes to prevent effective fusion of viral particles with target cells. To investigate the effects of *n*-docosanol on HSV fusion with cellular membranes we conducted fluorescence dequenching assays. The membranes of intact HSV-2 virions were labeled with octadecyl rhodamine chloride (R-18) and added to human B cells. In this model, if viral fusion with the cellular membrane occurs, the tightly packed rhodamine molecules diffuse into the larger membrane of the host cell. This relieves fluorescence self-quenching and causes an increase in signal intensity.

NC-37 human B cells were treated with 15 mM *n*-docosanol 24 h before the addition of R-18 labeled HSV-2. As shown in Fig. 4, this concentration of *n*-docosanol inhibited the relative increase in fluorescence intensity occurring with viral/cell fusion by approximately 50% compared to cells receiving no treatment. Treatment of NC-37 cells with Tetronic control suspensions was not inhibitory, and instead caused a noticeable increase in fluorescence intensity, reminiscent of the observation made with the β-gal expressing systems discussed above (Figs. 2 and 3). Compared to the effect observed with the Tetronic control alone, *n*-docosanol inhibited the fluorescent response by as much as 76%. *n*-Docosanol was not inhibitory if added only during the fusion process; a prior incubation period of the compound with cells was necessary. This is consistent with the requirement for metabolic conversion in the antiviral process. The observation also establishes that the presence of *n*-docosanol does not itself quench or otherwise inhibit fluorescence. Anti-gD monoclonal antibody (a specific inhibitor of penetration) at a 1:40 dilution completely blocked the increase in fluorescent signal (not shown) confirming that the experimental protocol is an appropriate measure of viral penetration. These results indicate that fusion of HSV viral particles to the host membranes is significantly inhibited in *n*-docosanol-treated cells.

4. Discussion

Most available antiviral therapeutic compounds block replication processes shared by the virus and infected target cell and hence are toxic, mutagenic, and/or teratogenic and can potentially induce drug-resistant viral mutant substrains. Therefore, the identification of new antiviral compounds, particularly those with new mechanisms of action, is important. The 22-carbon, saturated, primary alcohol, *n*-docosanol, lacks any toxic, mutagenic, or teratogenic properties (Johnson, 1988). In contrast to the mode of action of conventional antiviral agents, the predominant mechanism for the anti-HSV activity of *n*-docosanol appears to be inhibition of fusion between the plasma membrane and the HSV envelope and, as a result, the blocking of entry and subsequent viral replication. The mechanism of action explains the effectiveness of *n*-docosanol against all tested lipid-enveloped viruses that employ fusion as the sole or major means of entry into the cell and contrasts its mode of action to other antiviral agents that target a single viral protein. Based on this mechanism of action the emergence of HSV strains resistant to the antiviral effects of *n*-docosanol may be unlikely.

Previous results had suggested that *n*-docosanol may be specific for lipid-enveloped viruses and that lipid-enveloped viruses which primarily enter cells by fusion with the plasma membrane are effectively blocked by *n*-docosanol. In contrast, the drug generally exerts no detectable activity against viruses that are either non-enveloped, or are enveloped and endocytosed. One exception to this general pattern is influenza A, an enveloped virus that has been reported to enter cells via receptor-mediated endocytosis (Matlin et al., 1982) but which is effectively inhibited by *n*-docosanol. The reasons for this anomaly are currently unclear.

The in vitro doses (mM) required for antiviral inhibition with *n*-docosanol are high compared to results with existing therapeutic compounds such as acyclovir. This may result from the nature of the surfactant-stabilized suspensions of *n*-docosanol. Due to the insolubility of *n*-docosanol, the particles are thermodynamically stable making transfer to cultured cells an inefficient process. As determined using radiolabeled *n*-docosanol, less than 1 out of 1000 molecules of *n*-docosanol added to culture enters the cell (Pope et al., 1996).

Optimal inhibition of viral replication was observed in Vero cell cultures to which HSV was added 6–24 h after addition of *n*-docosanol. This observation can be explained by a time-dependent uptake and metabolism of *n*-docosanol by host cells, an event apparently required for antiviral activity (Pope et al., 1996). The rate of this metabolic conversion in vivo is likely to be faster than that observed in the artificial milieu of the tissue culture system especially considering the thermodynamic stability of the surfactant-stabilized particles. The gradual loss of resistance to HSV in *n*-docosanol-treated cells reported herein would also be predicted due to rapid turnover not only of a required lipid metabolite (Dawidowicz, 1987) but of the plasma membrane itself which is constantly being internalized and replaced (Pearse and Bretscher, 1981). However, even with this rapid turnover, viral entry was reduced for several hours following removal of unincorporated drug. Furthermore, the topically applied cream remains on the skin surface acting as a constant reservoir of *n*-docosanol. Available data demonstrated that *n*-docosanol exerts an effect on the host cell that inhibits early events in viral replication but does not inhibit the amount of HSV which attaches to cells. The effect of *n*-docosanol on progressively earlier events in viral entry was therefore examined.

Penetration of HSV-1(KOS)gL86 into HEp-2 cells was inhibited by *n*-docosanol with a concentration dependence ($ID_{50} = 7$ mM) roughly equivalent to inhibition of HSV-1 or HSV-2 production ($ID_{50} = 4$ mM) or plaque formation ($ID_{50} = 9$ mM) in Vero cells (Fig. 2) confirming that *n*-docosanol inhibits an early event in the viral replication cycle. The inhibitory activity of *n*-docosanol on β-galactosidase expression must counteract the apparent stimulatory action of the vehicle alone, the mechanism for which is unclear.

n-Docosanol inhibition of HSV-2 entry was also evidenced by reduced release into treated cells of virion-associated regulatory proteins (Fig. 3). *n*-Docosanol treatment caused as much as an

80% reduction in the expression of β-galactosidase in target cells containing a stably transfected *lacZ* gene under control of an HSV immediate–early promoter (ICP4). This observation, in combination with the lack of inhibition of viral attachment in *n*-docosanol-treated cells, confirms that *n*-docosanol blocks an event occurring after viral attachment but prior to release of tegument proteins. This is an immediate post-entry event and is not dependent upon virion localization in the nucleus. The inhibitory concentrations were higher than that generally required for in vitro anti-HSV activity. Additional early events in viral replication may also be inhibited by *n*-docosanol.

n-Docosanol appears to inhibit the biophysical process of viral/cell fusion. The fusion-dependent dequenching of octadecyl rhodamine B chloride, inserted into the HSV envelope was significantly inhibited in *n*-docosanol-treated cells (Fig. 4). The concentration dependence of fluorescence inhibition correlated to that observed for inhibition of HSV-1 replication by *n*-docosanol in other in vitro assays. Incorporation of *n*-docosanol, or its metabolites and resulting perturbations of normal membrane composition may alter the biophysical properties of the plasma membrane in such a way as to inhibit fusion of attached virions. The compound may inhibit the function of normally occurring cellular mediators of entry (Montgomery et al., 1996; Geraghty et al., 1998).

In summary, inhibition of fusion between the plasma membrane and the HSV envelope, and the subsequent lack of replicative events, may be the predominant mechanism for the anti-HSV activity of *n*-docosanol. This mechanism of action may be generally applicable to the spectrum of viruses susceptible to the inhibitory effect of *n*-docosanol; however, further experimentation will be necessary to verify this.

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