n-Docosanol: Broad Spectrum Anti-Viral Activity against Lipid-enveloped Viruses[a]

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INTRODUCTION

Earlier work of others demonstrated the existence of virucidal activity of medium length (10–12 carbons) saturated alcohols; such activity was complicated by coexisting toxicity, and appeared to be lost as chain length increased up to 18 carbons or longer.[1] Recently, we re-examined alcohol compounds for activity against various viruses, specifically focusing on higher chain lengths with the aim of identifying potential broad spectrum anti-viral activity among such compounds. This work confirmed that saturated alcohols of 18 carbons or longer, like the 22-carbon compound *n*-docosanol, exhibit very potent anti-viral activity both *in vitro* and *in vivo*.[2–5]

n-Docosanol is derived from hydrogenation of fatty acid mixtures extracted from various plant sources or fossil fuels, yielding corresponding fatty alcohol mixtures from which *n*-docosanol can be purified by fractional vacuum distillation. The purified compound is highly lipophilic and extremely insoluble, prompting conventional wisdom to dictate that this molecule and others like it should be biologically inert. Our previous studies[2–5] and those presented herein document that conventional wisdom is absolutely wrong in this case. Thus, appropriately formulated *n*-docosanol exhibits broad-spectrum anti-viral activity with apparent specificity for lipid-enveloped viruses including, among others, types 1 and 2 herpes simplex and respiratory syncytial virus,[2–4] murine and human cytomegalovirus, and certain retroviruses (unpublished), but no activity against the non-enveloped poliovirus. Importantly, unlike alcohols of 20 carbons or less, which exhibit

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varying degrees of toxicity, *n*-docosanol is essentially devoid of toxic properties for cultured cell lines, experimental animals, or human volunteers. This combination of broad-spectrum anti-viral activity with minimal or no toxicity suggests that *n*-docosanol possesses very significant therapeutic potential for use in human and animal diseases.

MATERIALS AND METHODS

Viruses and Cell Lines

The MacIntyre strain of HSV-1, the MS strain of HSV-2, the Davis strain of human CMV, and the Towne strain of human CMV were obtained from the American Type Culture Collection (ATCC; VR-559, VR-540, VR-807, and VR-977, respectively), Rockville, MD. Stock preparations of these strains were generated in Vero cells (African Green Monkey kidney, ATCC #CCL-81) and stored at −85°C. Murine CMV was also obtained from ATCC (VR-194) and was passed in SC-1 murine embryo fibroblasts (ATCC CRL-1404). The Friend virus stock was the gift of Dr. Philip Furmanski, New York University. Other cell lines included in these studies were an isolate of human fetal lung fibroblasts, MRC-5 (ATCC CCL-171), the MDBK cell line (ATCC CCL-22) and the X-C cell line (ATCC CCL-165). The IIIB strain of HIV was obtained from an infected H9 cell line provided by the AIDS Repository of NIH.

Formulation of n*-Docosanol in Pluronic F-68 or Tetronic 908*

n-Docosanol (98% pure; M. Michel, New York) was suspended in Pluronic F-68 (poloxamer 188; M_r 8400; BASF, Parisppany, NJ) or in Tetronic 908 (M_r 25,000; BASF) generally as follows. Pluronic F-68 or Tetronic 908 were diluted to 8.3 mM or 11 mM, respectively, in 37°C sterile Dulbecco's high glucose modified Eagle's medium (DMEM), and the solutions were then heated to 50°C. *n*-Docosanol was added to 60 mM to the Pluronic or Tetronic in DMEM and the mixture was sonicated (Branson 450 sonifier) for 21 min at an initial output of 65 W; this warms the mixture to 86°C. The resulting suspension consists of very fine globular particles with an average size of 0.1 microns as measured by transmission electron microscopy. Where indicated, the concentrations of surfactant or *n*-docosanol were varied without other changes in the procedure.

Preparation of n*-[^{14}C]Docosanol Suspensions*

Radioactively labeled suspensions were prepared by including *n*-[1-^{14}C]docosanol (specific activity, 55 mCi/mmole in ethanol; American Radiolabeled Chemicals, St. Louis, MO) as a tracer in preparing radioactive suspensions. Typically, 4 μCi of the radioactive compound per ml of suspension to be prepared were placed into a vessel. The ethanol was evaporated under a stream of nitrogen and then Pluronic F-68 or Tetronic 908 suspensions were prepared in this vessel exactly as described for nonlabeled *n*-docosanol.

Cell Fractionation of n-*[^{14}C]Docosanol-treated Vero Cells*

n-[^{14}C]Docosanol was added to 3 mM at the onset of culture to Vero cells (3×10^5 cells/ml, 50 ml). After 24 hours incubation at 37°C, cells were extensively washed with saline and harvested with trypsin-EDTA (0.5 mg/ml trypsin; 0.2 mg/ml EDTA). After suspending in hypotonic buffer and lysing by sonication, cells were fractionated into membranous or cytoplasmic fractions by differential centrifugation as described.[6] Nuclei were isolated by centrifuging through sucrose after lysis of whole cells with NP-40.[7]

Lipid Extraction and Thin Layer Chromatographic Analysis of n-*Docosanol-treated Cells*

Cells cultured in 35-mm wells with *n*-[^{14}C]docosanol were washed extensively with saline before trypsinization with 0.6 ml of trypsin-EDTA for 10 minutes. Lipids were extracted with slight modifications of the classical Folch procedure (8) by vigorous shaking with an equal volume of chloroform:methanol (3:2; v:v) followed by a 10-minute centrifugation in an Eppendorf centrifuge. The resulting chloroform layer was evaporated to dryness under nitrogen, resuspended in 100 μl chloroform:methanol (3:2; v:v), evaporated to dryness again and resuspended in a final volume of 20 μl which was spotted onto silica thin layer chromatogram sheets (Eastman Kodak) and developed with hexane:diethyl ether:acetic acid (40:60:1; v:v:v). The chromatograms were cut into 0.5 cm strips that were analyzed by scintillation counting. The migration positions of *n*-docosanol (R_f = 0.55) and *n*-docosanoic acid (R_f = 0.42) standards were determined in the same chromatogram.

Cellular Lipid Fractionation

Cells from confluent flasks (T-175) of *n*-[^{14}C]docosanol-treated Vero or MDBK cells were washed, harvested and extracted with chloroform:methanol as described above. Cells were resuspended in 5.0 ml saline before extraction (2×) with an equal volume of chloroform:methanol (3:2; v:v). The resulting chloroform layer was reduced to 1.25 ml and applied to a column of silica (0.4 ml, Kieselgel 60, 230–400 mesh; EM Sciences, Gibbstown, NJ). The column was eluted sequentially with 4 ml chloroform, 16 ml acetone, and 4 ml methanol to separate the lipids into major classes. Neutral lipids, including *n*-docosanol, elute with chloroform. The acetone would elute such compounds as cerebrosides, sulfatides and ceramide polyhexosidases. The methanol fraction contains the phosphatides.

Fractionation of Phospholipids

The phosphatide-containing methanol elute from the silica fractionation described above was further analyzed on thin layer chromatography. A portion (0.5 ml) was evaporated under nitrogen, resuspended in 20 μl chloroform:methanol (3:2; v:v) and spotted on a plastic-backed silica thin layer chromatography sheet. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine standards (Sigma, St. Louis) were also spotted. After development with chloroform:methanol:acetic acid:water (60:50:1:4; v:v:v:v), the sheet was divided into 5

mm strips, the positions of the standards were determined by staining with iodine vapors and the cpm per fraction were determined by scintillation spectrometry.

In Vitro *Virus Assays*

HSV-1, HSV-2, and human CMV were assayed in cultures of either Vero cells or the human fetal lung fibroblast cell lines. The cells were plated at 3×10^5/ml in either 16-mm (1 ml) or 35-mm wells (2 ml) in DMEM medium supplemented with 5% fetal calf serum (HYCLONE Laboratories, Logan, UT), 4 mM L-glutamine, 50 Units/ml penicillin, 50 μg/ml streptomycin, and 10 mM HERPES buffer. Varying concentrations of *n*-docosanol suspension, the corresponding control vehicle (lacking *n*-docosanol), or acyclovir were added at the outset of the culture; untreated controls contained medium only. All cultures were then inoculated with 50 μl containing the required PFU of HSV-1, HSV-2, or CMV. The cultures were incubated (10% CO_2 in air; humidified) for an additional 42–44 hours or 6 days (CMV), 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 virus-induced plaques using a dissecting microscope (10× magnification). Murine CMV was assayed in the same manner, except 16-mm cultures of SC-1 cells was used instead.

The *in vitro* infectivity assay for HIV was conducted in activated human peripheral blood mononuclear cells (PBMC). Human PBMC were isolated from heparinized peripheral blood samples by Ficoll-Hypaque flotation. The blood donors were from a pool of individuals screened for HIV, EBV, CMV and HBV. The cells were cultured in 35-mm dishes at a density of 10^6 cells/ml with RPMI 1640 plus 10% FCS, 5 units/ml IL-2, and 5 μg/ml PHA as culture medium. After 48 hours incubation, the activated T-cell blasts were infected with HIV. The viral stock contains 10^5 median tissue culture infectious dose ($TCID_{50}$) using human PBL target cultures with p24 antigen expression as an endpoint. The p24 assay was performed using the Coulter p24 ELISA assay kit (Coulter Immunology, Hialeah, FL) according to the manufacturer's instructions. Briefly, 200 μl of diluted p24 antigen standard or culture supernatant fluid was added to wells of a 96-well plate coated with antibody to HIV-1 p24 antigen. After addition of 20 μl/well of lysis buffer, the walls were covered and incubated 1 hour at 37°C. The wells were then washed extensively, inoculated with 200 μl/well of biotinylated anti-p24 antibody, and incubated 1 hour at 37°C. After extensive washing, the wells were inoculated with 200 μl of horseradish peroxidase-coupled streptavidin and incubated 1 hour at 37°C. Thereafter, the wells were washed, inoculated with 200 μl of tetramethylbenzidine substrate, and incubated at room temperature for 30 minutes. The wells were then read at 450 nm using a Microplate Autoreader (Bio-Tek Instruments). Relative p24 antigen levels were calculated using a standard curve with purified p24 supplied by the manufacturer.

HSV-mediated Encephalitis in Adult Mice

Adult female CAF_1 mice were inoculated i.v. with 10^5 tissue culture PFU of HSV-1 as described by others;[9] HSV-1 PFU content was determined in Vero cell cultures. Groups of such animals were either untreated or injected i.v. with saline containing the indicated dosage of acyclovir, *n*-docosanol/Pluronic F-68, or with Pluronic F-68 vehicle control only. Such treatments were given once daily, with

the first being given one hour after virus, and the subsequent three respective injections being administered on days 1, 2, and 4 post-virus; thus, 4 treatments with the dose of drug indicated in the figure. The animals were monitored for death over an experimental period of 21 days, the time at which HSV-mediated mortality subsides. The data are expressed as percent of animals remaining alive at the termination of the experiment, with 5-6 animals per group.

RESULTS AND DISCUSSION

n-*Docosanol Suspensions Inhibit Infectivity of Herpes Simplex Viruses* in Vitro

One of the more difficult hurdles to overcome in the study of the biological activity of *n*-docosanol was the development of an appropriate formulation which allowed acceptable delivery of the compound to biological systems. Initially, this was accomplished by formulating a suspension of the hydrophobic molecule in the inert and non-toxic cationic surfactant, Pluronic F-68. Such suspensions proved to be homogeneous in quality consisting of *n*-docosanol containing particles averaging 0.10 microns in size.[2] Suspended in this way *n*-docosanol exerts excellent inhibitory activity *in vitro* against both type 1 and 2 herpes simplex virus infectivity of both simian and human cell lines.[2-4] Significantly, *n*-docosanol/Pluronic suspensions are equally effective against both wild-type and acyclovir-resistant mutants of HSV. Thus, as shown in FIGURE 1A, both acyclovir and *n*-docosanol inhibit plaque formation by wild type HSV-2 equally. FIGURE 1B illustrates that an ac-

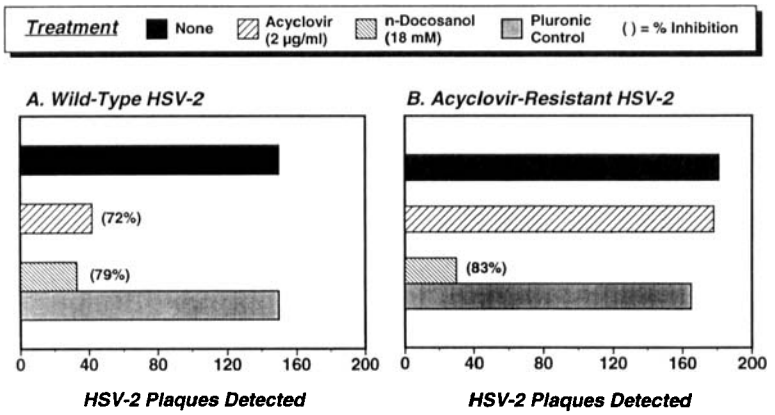

FIGURE 1. *n*-Docosanol/Pluronic inhibits Acyclovir-resistant HSV-2. Vero Cells were cultured in 35-mm wells (6×10^5 cells per well) in medium alone (= none) or in the presence of the indicated concentration of acyclovir, *n*-docosanol/Pluronic F-68 suspension or control suspension (Pluronic F-68 only). The cultures were inoculated 24 hours later with 150 PFU of either wild-type HSV-2 or an acyclovir-resistant laboratory isolate from the wild-type HSV-2 that was plaque-purified and passaged in 20 µg/ml acyclovir 44 hours later, the plates were incubated fixed, stained, and scored for numbers of plaques. The data presented are means of plaques scored from duplicate cultures. The percent inhibition observed in cultures treated with acyclovir or *n*-docosanol relative to untreated control cultures is denoted in parentheses.

yclovir-resistant HSV-2 mutant fails to respond to acyclovir, as expected, but is very clearly susceptible to the inhibitory activity of *n*-docosanol. The last bar in both panels illustrates that the Pluronic surfactant alone lacks any anti-viral activity.

n-*Docosanol Resolves Cutaneous Herpes Lesions in Guinea Pigs*

Anti-viral activity in a tissue culture system does not always translate into drug efficacy in whole animal studies or in man. Therefore, we formulated *n*-docosanol into a topical emulsion which was specifically designed to maximize skin penetration and minimize potential local irritation reactions. As shown in FIGURE 2, this topical formulation of *n*-docosanol exerts excellent anti-viral activity in the treatment of HSV-1-induced cutaneous lesions in experimental animals. HSV-1 was

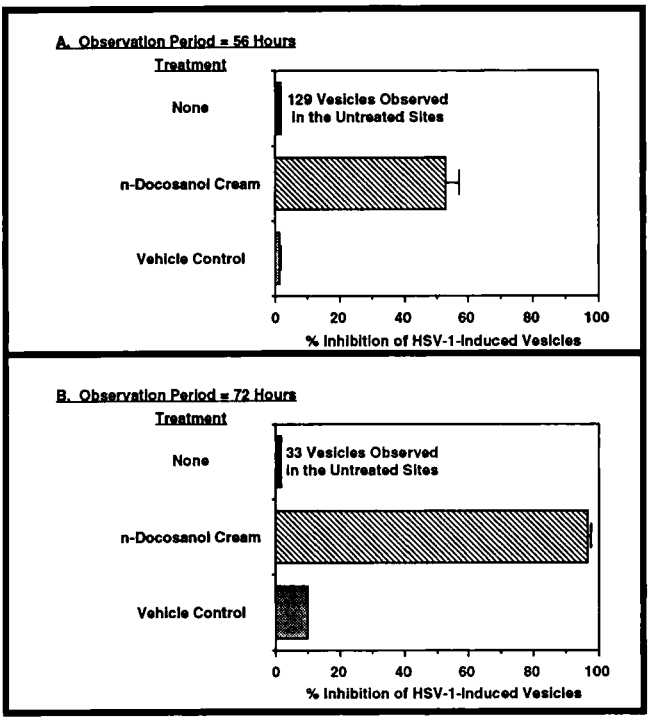

FIGURE 2. *n*-Docosanol in a cream formulation inhibits HSV-1-induced cutaneous lesions in hairless guinea pigs. The backs of hairless guinea pigs were cleaned and inoculated with purified HSV-1 by puncture of the skin with a tattoo instrument. Two hours later the virus inoculation sites were either untreated or treated with 100 μl of *n*-docosanol-containing cream (10%) or control vehicle; parallel control sites were left untreated. Additional treatments were applied 24, 30, and 48 hours after virus inoculation. Vesicle numbers at each inoculation were determined at the 56 and 72 hour time points after inoculation. The data are expressed as means and standard errors (derived from duplicate sites per determination) of percent inhibition of vesicle number at treated sites as compared to the untreated sites.

inoculated with a tattoo instrument into the skin on the backs of hairless guinea pigs. The sites were either left untreated or treated with *n*-docosanol-containing cream or the control vehicle. Vesicle formation was enumerated at the inoculation sites at the 56- and 72-hour time points after inoculation, representing peak and resolution phases, respectively, of the disease. As shown, treatment with *n*-docosanol cream inhibited vesicle number by 51% and 99% at the 56- and 72-hour timepoints, respectively, compared to the untreated sites. Treatment of sites with the vehicle control exhibited no appreciable inhibition in vesicle number.[3] Although not shown, comparable results have been obtained with HSV-2-induced cutaneous lesions in this hairless guinea pig model.

Clinical Studies of n-*Docosanol in Man*

Preclinical toxicology studies with the topical formulation of *n*-docosanol proved that the compound is devoid of toxic, mutagenic, or teratogenic properties when examined by standard procedures.[10] Under an Investigational New Drug (IND) application approved by the United States Food and Drug Administration (August, 1991), Phase I Clinical Safety Studies in human subjects have been conducted and verified that the topical formulation of *n*-docosanol is safe for human use.[11] Phase II clinical trials of *n*-docosanol as topical treatment for both oral and genital herpes infections were initiated in late 1992 in both the United States and Europe. The results of these double-blind, placebo-controlled studies are expected in late 1993 and, if warranted, expanded multicenter Phase III trials will commence in early 1994.

Mechanism of Anti-Viral Action of n-*Docosanol*

We have conducted extensive studies designed to delineate the mechanism by which *n*-docosanol exerts its anti-viral activity. Although not yet totally complete, the collective implications of the results of the studies conducted to date are that the compound appears to interfere with one or more of the common pathways of viral entry into, and migration to, the nucleus of infected target cells.[2-5] The key points of evidence supporting this notion can be summarized as follows: First, the compound has no direct virucidal activity, since virus can be mixed with *n*-docosanol suspension, then recovered from the suspension and shown to retain normal infectivity.[2] Second, the compound does not interfere with binding of herpes virus to HSV-specific receptors on target cells;[2-4] however, in the presence of *n*-docosanol HSV virions which have bound to target cell receptors remain on the cell surface for a prolonged time period.[4]

The latter point is illustrated in the experiment summarized in FIGURE 3.[4] In this experiment, HSV-2 was incubated with Vero cells in the absence or presence of *n*-docosanol at 4°C to allow for receptor binding of the virus. At the end of 3 hours, all cultures were washed and then replated at 37°C in order to initiate the viral entry process. At 20 minute intervals thereafter, the various cultures were exposed to pH 3.0 citrate buffer under conditions which remove and inactivate surface-bound, but not internalized, HSV virions and then re-cultured the full 44 hour period required to develop optimal HSV plaques. All cultures exposed to citrate buffer at time-0 failed to develop plaques, as expected. As shown by the uppermost lines on the graph, internalization of HSV-2 is virtually complete within 20 minutes after the shift to 37°C in the untreated and Pluronic control-treated

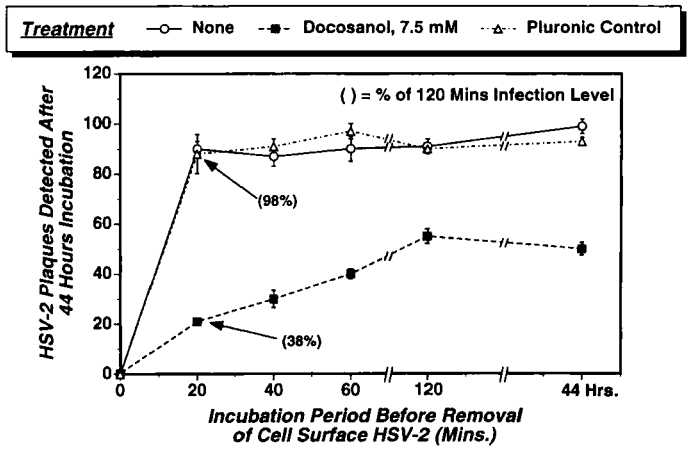

FIGURE 3. HSV-2 remains on the surface of *n*-docosanol-treated Vero cells for prolonged times. Vero cells were cultured as described in the legend to FIGURE 1 and incubated overnight. The cultures were then chilled at 4°C, inoculated with 100 PFU of HSV-2, and incubated 3 hours at 4°C. At time zero the cultures were washed with medium, inoculated with fresh medium (containing the indicated inhibitor) and incubated at 37°C. At each indicated time period, the cultures were washed with citrate buffer (pH 2.5) and reinoculated with fresh medium (lacking inhibitor). After a total of 44 hours incubation the cultures were stained and scored for HSV-2-induced plaques. The data are expressed as geometric means and standard errors derived from triplicate cultures per group.

cultures. In contrast, internalization of HSV in the *n*-docosanol treated cultures was less than 40% complete by 20 minutes and required more than 1 hour to reach completion.[4] These results clearly indicate that the kinetics of viral fusion and transmembrane migration are delayed in some way by *n*-docosanol.

Even after internalization reaches completion in *n*-docosanol-treated cells, subsequent viral migration to the cell nucleus is significantly inhibited; this has been shown by greater than 80% decreases in both the amounts of HSV core and envelope protein antigens detectable by ELISA and the numbers of infected cells expressing the intranuclear HSV-specific immediate-early protein, ICP-4, as detected by immunofluorescence. Finally, and not unexpectedly, the replication of infectious virions as measured in secondary plaque assay cultures is markedly diminished by 99% or more in *n*-docosanol-treated cells.[2]

Thus, the presence of *n*-docosanol has no effect on the initial steps of viral binding and allows eventual entry of virus into the target cell cytoplasm, although this process is considerably delayed for some yet-to-be determined reason. However, the process of migration to and localization in the nucleus is substantially blocked having the ultimate effect of a marked decrease in productive viral replication.

In order to better define the precise mechanism by which *n*-docosanol exerts its anti-viral activity, we have recently studied the cellular uptake, distribution and metabolism of *n*-docosanol from surfactant-stabilized suspensions.[5] The results of such studies have provided some interesting insight on the metabolic basis of the compound's anti-viral action. First, we have been able to show that radioactively labeled *n*-docosanol is progressively incorporated into cultured Vero cells, reach-

ing a peak uptake per cell between 6 and 12 hours after exposure. The process is irreversible since once the compound is cell-associated it cannot be removed even with extensive washing with cesium bromide, which effectively removes non-specifically associated cell-bound particles.

Second, at saturating concentrations less than 1% of the total *n*-docosanol added to cultures becomes cell-associated within 24 hours. Nonetheless, this corresponds to nearly 8×10^9 molecules per cell—an astounding amount in view of the number of lipid molecules typically found in plasma membranes. The fact that such a small fraction of *n*-docosanol in the suspension added to cultures becomes cell-associated indicates that the actual bioactive dose is orders of magnitude less than the amount of drug added to the cultures.

Cellular distribution studies examining subcellular fractions recovered by differential centrifugation of sonication-disrupted cells demonstrated that after 12 hours of exposure 75% of the radioactive compound is contained in cell membranes, and less than 1% is associated with nuclear fractions; the balance of radioactivity was found associated with the soluble cytoplasmic fraction.[5]

Analyses of the metabolic conversions of *n*-docosanol have shown that the compound is progressively metabolized to polar compounds, which were demonstrated by thin layer chromatography to be phosphatides that must be generated either via anabolic (ether linkages) or catabolic (oxidative) reactions. FIGURE 4 demonstrates a thin layer chromatographic analysis of a methanol eluted (phosphatide-containing) fraction from a silica gel column of an extract of *n*-docosanol-treated Vero cells. Non-metabolized *n*-docosanol was previously eluted from the silica with chloroform. As shown, approximately 62% of the counts migrated in the region of phosphatidylcholine and 38% migrated in the region of phosphatidylethanolamine.[5] Our studies have also documented that such metabolic conversions can be blocked by appropriate metabolic inhibitors. Thus, the effective energy poisons sodium azide and 2-deoxyglucose reduce both uptake of *n*-docosanol by Vero cells by 90% and metabolic conversion into polar metabolites by 80%.[5] It is probable that the combination of sodium azide and 2-deoxyglucose mainly inhibits cellular uptake of *n*-docosanol by inhibiting endocytosis. However, such uptake

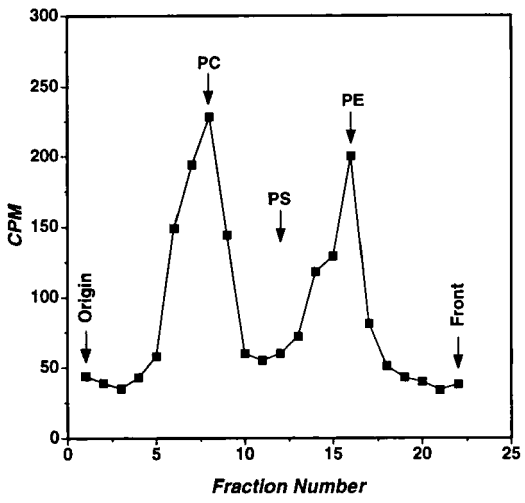

FIGURE 4. Radioactive metabolites of *n*-[^{14}C]docosanol display the properties of phosphatidylcholine and phosphatidylethanolamine. A portion (0.5 ml) of the methanol eluate of the silica lipid fractionation described in MATERIALS AND METHODS was evaporated under nitrogen, resuspended in 20 μl chloroform:methanol (3:2; v:v) and spotted on a silica thin layer chromatography (TLC) sheet. After development with chloroform : methanol : acetic acid:water (60:50:1:4; v:v:v:v), the positions of standards were determined by staining with iodine vapors and the cpm per fraction determined by scintillation spectrometry after cutting the plastic-backed sheet into 5 mm strips.

could also occur by a fusion mechanism which may also require energy and would therefore be inhibited by these energy poisons. In addition, a passive diffusion mechanism of *n*-docosanol entry could also be facilitated by subsequent metabolism—in other words higher metabolic activity would favor higher levels of entry of the molecule. Thus even though initial uptake may occur through a non-energy-dependent mechanism it would be inhibited by energy poisons if subsequent metabolism were prevented.

The most interesting aspect of these studies is the indication of a possible role for the polar metabolites of *n*-docosanol in the anti-viral activity of the compound. Recently, Roos and Choppin[12,13] demonstrated that resistance of mouse fibroblasts to polyethylene glycol–induced fusion correlated with an increase in both free fatty alcohols and an elevation in glycerides, including an ether-linked compound that would be analogous to the products obtained via metabolic conversion of *n*-docosanol as described above. We therefore conducted experiments to investigate the possibility that the enzymatic conversion of *n*-docosanol is a necessary prerequisite for its anti-viral activity. The results of such studies have demonstrated, firstly, that the rate and extent of metabolic conversion—but not that of cellular uptake—of *n*-docosanol to its polar metabolites is determined by the nature of the surfactant used to suspend the compound and, indeed, that efficiency of metabolic conversion directly correlates with the magnitude of anti-viral activity of *n*-docosanol.

An initial step in conducting such studies involved switching to a different surfactant for suspending *n*-docosanol. Tetronic 908 is a closely related cousin to Pluronic F-68, since both are block copolymers of ethylene oxide and propylene oxide. However, in contrast to Pluronic which is a difunctional polymer with a molecular weight of 8,400, Tetronic 908 is a tetrafunctional copolymer produced by adding propylene oxide and ethylene oxide to ethylenediamine resulting in a molecule with an average molecular weight of 25,000.

Among other things, when Vero cells are exposed to equivalent doses of *n*-docosanol suspended in Tetronic versus Pluronic, the rate and extent of metabolism of the compound to polar metabolites is significantly higher with the Tetronic than the Pluronic suspension.[5] This is shown in FIGURE 5. It should be emphasized, however, that the total uptake of radioactive *n*-docosanol was equivalent from the two different suspension formulations; it was the metabolic conversion that differed significantly. Correlating with this higher metabolic conversion from Tetronic than Pluronic suspensions is the finding that the ED_{50} for inhibition of HSV replication by *n*-docosanol is 5-10 mM in Tetronic and around 3 times higher in Pluronic (ref 5; not shown). This appears to relate to the 3-fold higher levels of metabolic conversion in cells treated with *n*-docosanol in Tetronic illustrated in FIGURE 5.

To eliminate the possibility that these findings are peculiar to the Vero cell culture system, we made a reciprocal analysis taking advantage of the fact that, relative to Vero cells, the epithelial-like bovine kidney cell line, MDBK, exhibits an interesting apparent resistance to the anti-HSV activity of *n*-docosanol. This difference is significant in that *n*-docosanol is 3-4 fold more effective in inhibiting HSV-induced plaques in Vero cells than in MDBK cells.[5] When we compared total cellular uptake and relative metabolism, the results were strikingly clear. First, both the total amounts of *n*-docosanol uptake and the relative amounts of metabolic conversion were 3–4 times higher in Vero than in MDBK cells. The ultimate effect of decreased uptake combined with decreased metabolism in MDBK versus Vero cells is graphically illustrated in FIGURE 6, which shows quite clearly that after 72 hours, Vero cells contain almost 4-fold higher amounts of

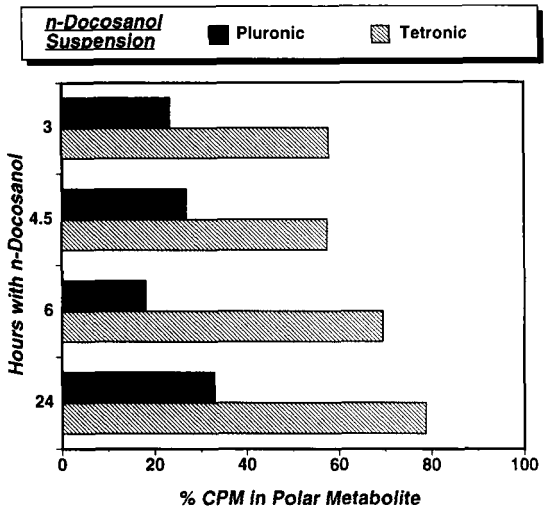

FIGURE 5. *n*-[^{14}C]-Docosanol is metabolized more rapidly from a Tetronic than from a Pluronic suspension in Vero cells. Vero cells were plated in 6-well dishes as described in MATERIALS AND METHODS. After the cells were adherent, media was removed and replaced with 6 mM *n*-[^{14}C]docosanol suspended with either 0.83 mM Pluronic F-68 or with 0.28 mM Tetronic 908 (equivalent weights). Cultures were incubated at 37°C/CO_2 for the indicated times. They were then washed, harvested, extracted with chloroform:methanol, resuspended in 20 μl of chloroform:methanol (3:2; v:v) before spotting on thin layer chromatography sheets and developing with hexane:diethyl ether:acetic acid (60:40:1; v:v:v). The chromatograms were cut into 0.5 cm strips and analyzed by scintillation counting. With this solvent system the polar phosphatides remain at the origin. The percentage of cpm remaining at the origin is referred to as % cpm in polar metabolite.

the phosphatide metabolite which remains at the origin in this solvent system. Nonetheless, of the counts that are metabolized in two cells lines, the relative amounts in the major classes of phosphatides—phosphatidylcholine and phosphatidylethanolamine—that are formed are **not** different in the two cell lines. Moreover, pulse-chase experiments showed that both lines eventually convert all of the incorporated counts into the more polar form.[5] Such results suggest that MDBK cells may effectively regulate uptake and/or metabolism of *n*-docosanol through a feedback type mechanism that is either less effective or non-operative in Vero cells.

Broad Spectrum Anti-Viral Activity of n-*Docosanol against Several Lipid-enveloped Viruses*

Consistent with the mechanistic observations summarized above, we predicted that *n*-docosanol would have potential for interfering with a variety of different viruses—specifically those which contain lipid in their outer envelopes and which use fusion mechanisms for entering susceptible target cells. TABLE 1 summarizes the human and murine lipid-enveloped viruses that, to date, have been shown to be

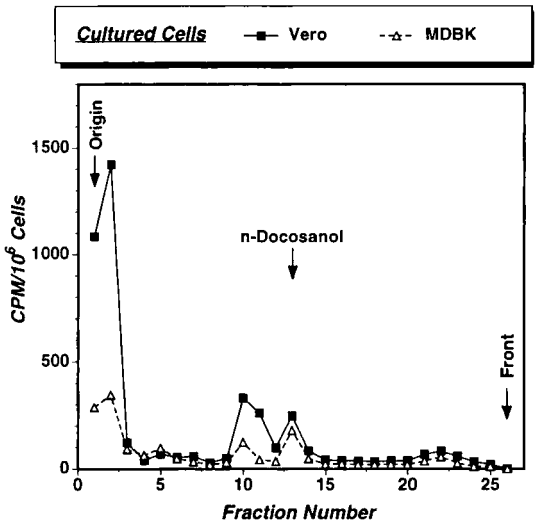

FIGURE 6. *n*-[^{14}C]Docosanol is metabolized more by Vero cells than by MDBK cells. Vero or MDBK cells were plated as described in FIGURE 4. *n*-[^{14}C]Docosanol was added to 6 mM (0.24 mM Tetronic 908) and the cultures were incubated 72 hours at 37°C/CO_2. Cells were extracted and analyzed on TLC with hexane:diethyl ether :acetic acid (20:30:1; v:v:v) as the developing solvent as described in FIGURE 5. With this solvent system the polar phosphatides remain at the origin. The position of migration of *n*-docosanol is indicated. Duplicate plates were treated with an identical suspension lacking the radioactive label, and the numbers of cells in these duplicate plates were determined by counting cells excluding trypan blue with a hemocytometer.

susceptible to the anti-viral activity of *n*-docosanol. Indeed, every lipid-enveloped virus that we have tested can be effectively blocked by this drug. In contrast to its uniform effectiveness against lipid-enveloped viruses, the drug exerted no detectable activity against poliovirus, the one non-enveloped virus that we have examined for susceptibility to the compound.[2]

The experiment depicted in FIGURE 7 illustrates the substantial anti-viral activity of *n*-docosanol suspended in Tetronic against human cytomegalovirus infectivity for cells of the MRC-5 line of human fetal lung fibroblasts. As shown, the anti-viral activity of the compound was generally consistent regardless of the input quantity of CMV, and there was no discernible activity exerted by the Tetronic control alone. Comparable results illustrating anti-viral activities of *n*-docosanol have been obtained with murine CMV (unpublished) and human respiratory syncytial virus[2] in culture assay systems.

TABLE 1. Spectrum of Anti-Viral Activity of *n*-Docosanol against Lipid-enveloped Viruses

Human	Murine
• Herpes simplex-1	• Cytomegalovirus
• Herpes simplex-2	• Friend leukemia virus
• Human herpesvirus-6	• LP-BM5
• Respiratory syncytial virus	
• Cytomegalovirus	
• HIV-1	

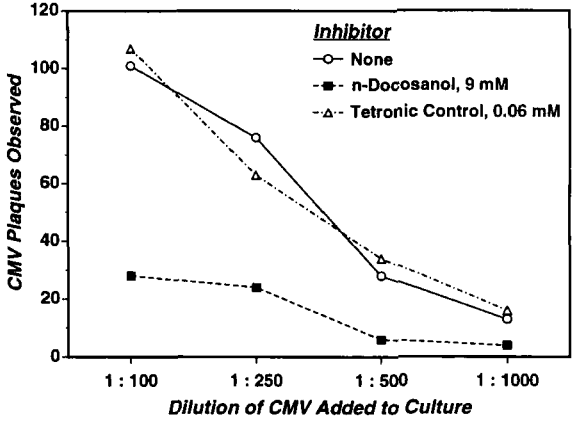

FIGURE 7. *n*-Docosanol/Tetronic 908 inhibits replication of human CMV in MRC-5 cells. MRC-5 fibroblast cells were cultured in the presence or absence of *n*-docosanol/Tetronic or Tetronic control, as described in the legend to FIGURE 1. After 24 hours, the cultures were inoculated with the indicated amount of CMV stock and incubated an additional 44 hours. The resulting CMV-induced plaques were developed and visualized as described in the legend to FIGURE 1. The data are presented as means of duplicate cultures per determination.

n-*Docosanol Inhibits Activity of Retroviruses*

In addition to the results with herpes virus, CMV and RSV this drug also has anti-retroviral activity both *in vitro* and *in vivo*. A formulation possessing anti-retroviral activity and lacking toxicity would have substantial usefulness in treating a variety of retroviral diseases in humans and domestic animals. Notwithstanding the implications for treatment of AIDS, availability of a treatment regimen for diseases caused by retroviruses like feline leukemia virus, bovine leukemia virus, as well HTLV-1 and -2 would have substantial benefits in humanitarian terms. Our studies have established that *n*-docosanol does inhibit replication of murine retroviruses *in vitro* and *in vivo*.[3]

Initial studies focused on the murine Friend leukemia virus (FV 14). Inoculation of adult mice with FV results in the induction of a leukemia of erythroid progenitors, specifically the basophilic erythroblast. This erythroleukemia is characterized by the rapid proliferation of virus-infected erythroid cells, viremia, immunosuppression, and ultimately death of the animal. Intravenously injected FV will circulate through hematopoietic organs, such as the spleen, and infect erythroid cells. If such infected spleens are fixed day 10 after virus injection, discrete macroscopic nodules can be seen on the surface of the organ; these represent clones of leukemic cells and form the basis of the spleen focus assay.[14]

The experiment summarized in FIGURE 8 illustrates that *n*-docosanol inhibits Friend Virus-induced leukemia and viremia in adult mice injected intravenously with 75 focus-forming units of Friend Virus. Treated groups were injected intravenously with the varying doses of *n*-docosanol or Pluronic vehicle alone intravenously on the same day as virus inoculation and once daily for the next 3 days. After 10 days, half of the animals in each group were sacrificed and examined for the presence of leukemic foci in their spleens, while the remaining animals were

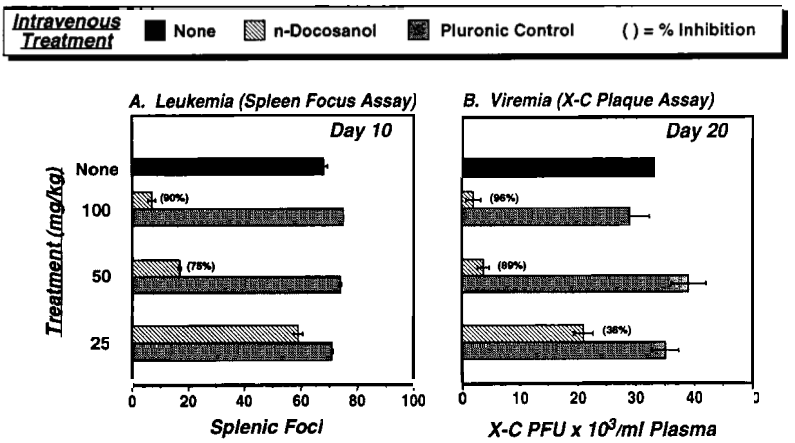

FIGURE 8. *n*-Docosanol inhibits *in vivo* Friend virus-induced leukemia and viremia. Adult BALB/c mice were injected intravenously with 75 spleen focus-forming units of FV. Treated groups were injected intravenously with the indicated doses of *n*-docosanol or Pluronic vehicle alone on the same day as virus inoculation and once daily for the next 3 days. After 10 days, half of the animals in each group were sacrificed and examined for leukemic foci in their spleens **(A)**. The remaining mice were retained 10 more days and bled for viremia determinations **(B)**. Viremia was measured using the X-C plaque assay. Briefly, primary fibroblast cultures were derived by digestion of 14-day BALB/c embryos with trypsin and cultured in DMEM plus 10% fetal calf serum. After 72 hours, the cells were transferred into 16 mm dishes (10^5/well), pretreated with 5 μg/ml polybrene and then infected with 75 X-C plaque-forming units of Friend virus stock or dilution of test plasma. After incubation for 7 days, the cultures were irradiated and overlaid with X-C cells (3×10^5/well). Three days later, the cultures were washed, stained and scored for plaques of multinucleated giant cells. The data presented are geometric means and standard errors of splenic foci or X-C plaque-forming units derived from three animals per group.

retained for 10 additional days to monitor viremia. Treatment with *n*-docosanol exerted a very clear dose-related inhibitory affect on both the development of leukemic foci, shown in panel A, and the development of viremia, shown in panel B. In contrast, treatment with comparable amounts of the Pluronic vehicle alone as control exerted no discernible effect. We believe that these results reflect the inhibitory activity of *n*-docosanol on viral replication, since corollary *in vitro* studies have documented a very potent activity of this drug against replication of Friend Virus in primary embryo fibroblast cultures.[3]

Moreover, we have recently made the more important observation that *n*-docosanol inhibits *in vitro* replication of HIV-1 and human herpes virus 6 (unpublished). Our initial studies on HIV were conducted in collaboration with Dr. Paolo Lusso in Dr. Robert Gallo's laboratory and one of several experiments of this type is summarized in FIGURE 9. Normal human peripheral blood mononuclear cells were activated with 1 μg/ml PHA plus 5 units/ml of IL-2 in medium alone or in the presence of the *n*-docosanol, Pluronic F-68 control vehicle, or phosphonoformic acid (PFA). The next day, the cultures were inoculated with HIV-1 and examined 4 days later for evidence of viral replication by detection of the p24 viral antigen. Substantial levels of HIV-1 replication occurred in the control-treated cultures which corresponds comparably to the untreated group. As shown, *n*-docosanol

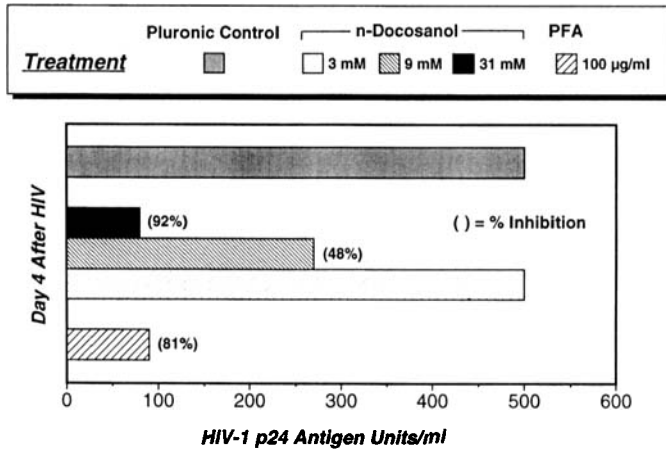

FIGURE 9. *n*-Docosanol inhibits *in vitro* replication of HIV-1 in cultures of PHA/IL-2-stimulated human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were cultured in medium containing 1 µg/ml PHA plus 5 units/ml IL-2 alone or also containing 100 µg/ml PFA, the indicated dosage of *n*-docosanol/Pluronic, or the amount of Pluronic F-68 control vehicle contained in the high dose of *n*-docosanol/Pluronic. After overnight incubation, the cultures were inoculated with HIV-1 at a multiplicity of infection of 1 virion/cell. After 24 hours incubation at 37°C, the cultures were washed and inoculated with fresh medium containing PHA and IL-2, but lacking inhibitor. Replication of HIV-1 was determined 4 days later by quantitation of virla antigens by a p24-specific ELISA for HIV-1 as described in MATERIALS AND METHODS.

exhibited a dose-related inhibitory activity against HIV-1 in cultures of PHA/IL-2-stimulated human peripheral blood mononuclear cells, exhibiting activity at the highest dose comparable to that of the very potent anti-viral compound phosphonoformic acid or PFA. Since these initial experiments were conducted, we have reproduced these observations in our own laboratory, showing even higher levels of anti-viral HIV activity using the more potent formulation of *n*-docosanol suspended in Tetronic. The dose response of HIV-1 to *n*-docosanol indicates an ED_{50} of about 6–9 mM.

Finally, given the subject matter of this symposium it is particularly appropriate that we present a representative example of the capacity of *n*-docosanol to protect mice against the uniformly lethal encephalitis induced by systemic infection with HSV-1. As illustrated by the two separate experiments summarized in FIGURE 10, most untreated adult mice inoculated intravenously with suitable quantities of HSV-1 died within 21 days. Only 15–20% of such inoculated mice remained alive at that time. Intravenous treatment (starting 4 hours after virus inoculation and repeated once daily on days 1, 2, and 4 after inoculation of HSV) with sufficient doses of either acyclovir or *n*-docosanol markedly altered the fatal outcome resulting in 80–90% survival at day 21 at the most effective doses employed. Conversely, treatment with the Pluronic vehicle control alone had no effect on the fatal outcome of this experimental model. The cumulative experimental data that we have generated in this model indicates that the ED_{50} for *n*-docosanol is around 50 mg/kg compared to the ED_{50} of 40 mg/kg for acyclovir.

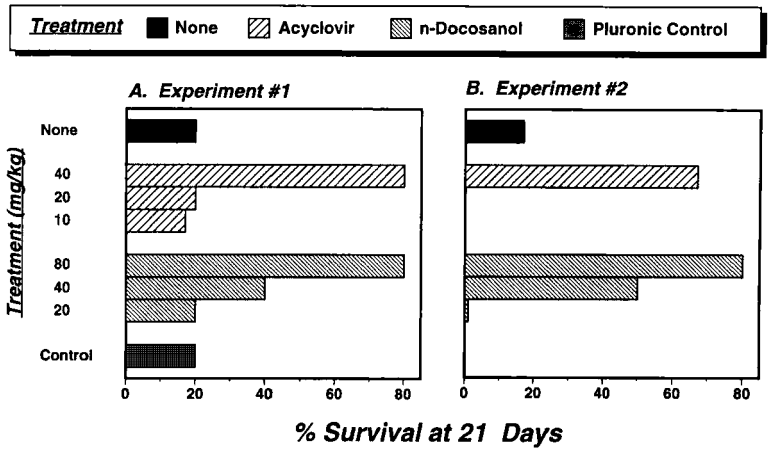

FIGURE 10. Inhibition of lethal HSV-1 encephalitis in mice by systemic administration of *n*-docosanol/Pluronic. Adult female CAF_1 mice were inoculated intravenously with 10^5 tissue culture plaque-forming units of HSV-1. Groups of such animals were either untreated or treated intravenously with the indicated dosages of acyclovir, *n*-docosanol/Pluronic F-68, or with 6.7 mg Pluronic F-68 vehicle control only (the amount contained in the high 80 mg/kg dose of *n*-docosanol); the stock *n*-docosanol preparation contained 30 mg *n*-docosanol and 100 mg Pluronic F-68. Treatments were given once daily, starting one hour after virus inoculation; three additional injections were administered on days 1, 2, and 4 post-virus (a total of 4 treatments). The animals were monitored for death over a period of 21 days, the time at which HSV-mediated mortality subsides. The data are expressed as percent of animals (5–6 per group) remaining alive at the termination of the experiment.

CONCLUSIONS

The studies summarized in this presentation document that the 22 carbon-long saturated alcohol known as *n*-docosanol exerts substantial inhibitory activity against infectivity and replication of several different lipid-enveloped viruses of both human and animal origin. Moreover the anti-viral activity of the compound can be verified in both tissue culture and after either topical or systemic administration into intact, living beings. The remarkable broad spectrum characteristics of *n*-docosanol coupled with the equally remarkable absence of toxicity of the compound indicate its very promising potential as a therapeutic drug against a number of viral diseases in both humans and animals. Moreover, the observations described here which point to a non-specific mechanism of action of the drug—namely by interfering with early post-entry events in the virus/target cell replication cycle—suggest that an even wider variety of viruses than those examined to date might be susceptible to the anti-viral activity of *n*-docosanol.

Finally, and perhaps most importantly, this mechanism of action indicates that drug-induced mutations resulting in resistant virus variants—a particular problem of great importance in diseases caused by chronic viruses such as those focused on in this symposium and in disorders such as AIDS and certain forms of hepatitis—would be very remote as a result of the therapeutic use of this drug. Future studies of this compound in the laboratory and in the clinic will no doubt provide definitive answers on these issues.

ACKNOWLEDGMENTS

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REFERENCES

1. SANDS, J., D. AUPERIN & W. SNIPES. 1979. Extreme sensitivity of enveloped viruses, including herpes simplex, to long-chain unsaturated monoglycerides and alcohols. Antimicrob. Agents Chemother. **15:** 67–73.
2. KATZ, D. H., J. F. MARCELLETTI, M. H. KHALIL, L. E. POPE & L. R. KATZ. 1991. Antiviral activity of 1-docosanol, an inhibitor of lipid-enveloped viruses including herpes simplex. Proc. Natl. Acad. Sci. USA. **88:** 10825–10829.
3. MARCELLETTI, J. F., L. E. POPE, M. H. KHALIL, R. R. MCFADDEN, L. R. KATZ & D. H. KATZ. 1992. Lidakol®. Drugs of the Future. **17:** 879–882.
4. MARCELLETTI, J. F., L. E. POPE, R. R. MCFADDEN, M. H. KHALIL, L. R. KATZ & D. H. KATZ. 1994. The anti-herpes virus activity of *n*-docosanol involves alteration of viral entry into the cell. Submitted for publication.
5. POPE, L. E., J. F. MARCELLETTI, L. R. KATZ & D. H. KATZ. 1994. Metabolism of the anti-viral agent *n*-docosanol by target cells. Submitted for publication.
6. STORRIE, B. & E. A. MADDEN. 1990. Isolation of subcellular organelles. Methods Enzymol. **182:** 203–225.
7. UCKER, D. S. & K. R. YAMAMOTO. 1984. Early events in the stimulation of mammary tumor viruses RNA synthesis by glucocorticoids. J. Biol. Chem. **259:** 7416–7420.
8. FOLCH, J., M. LEES & G. H. SLOANE STANLEY. 1957. Simple methods for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. **226:** 497–509.
9. RENIS, H. E., E. E. EDISON, J. MATHEWS & J. E. GRAY. 1976. Pathogenesis of herpes simplex virus types 1 and 2 in mice after various routes of inoculation. Infect. Immun. **14:** 571–578.
10. Toxicology Reports on file with the U.S. Food and Drug Administration in Investigational New Drug (IND) Application #37,321.
11. Phase I Clinical Trial Reports on file with the U.S. Food and Drug Administration in Investigational New Drug (IND) Application #37,321.
12. ROOS, D. S. & P. W. CHOPPIN. 1985. Biochemical studies on cell fusion. I. Lipid composition of fusion-resistant cells. J. Cell Biol. **101:**1578–1590.
13. Roos, D. S. & P. W. Choppin. 1985. Biochemical studies on cell fusion. II. Control of fusion response by lipid alteration. J. Cell Biol. **101:** 1591–1598.
14. AXELRAD, A. & R. A. STEEVES. 1964. Assay for Friend leukemia virus: Rapid quantitation method based on enumeration of macroscopic spleen foci in mice. Virology **24:** 513–517.