Anti-herpes simplex virus activity of *n*-docosanol correlates with intracellular metabolic conversion of the drug

Laura E. Pope,[1,*] John F. Marcelletti,[*] Lee R. Katz,[*] and David H. Katz[*,†]

LIDAK Pharmaceuticals* and Medical Biology Institute,† 11077 North Torrey Pines Road, La Jolla, CA 92037

Abstract The 22-carbon fatty alcohol, *n*-docosanol, exhibits in vitro antiviral activity against several lipid-enveloped viruses including herpes simplex viruses 1 and 2 by a mechanism that interferes with normal viral entry into target cells. We previously reported that mammalian cells incorporate significant quantities of radiolabeled *n*-docosanol. Herein, we report that cells extensively metabolize the internalized fatty alcohol. This is evidenced by incorporation of up to 60% of cell-associated radiolabel into phospholipids that copurify with phosphatidylcholine and phosphatidylethanolamine. Analysis by chemical (Vitride) reduction suggests that a significant portion of *n*-docosanol is oxidized to *n*-docosanoic acid and then incorporated as an acyl group on polar lipids. A measurable amount of radiolabel, however, is resistant to Vitride reduction, consistent with incorporation of *n*-docosanol into ether lipids. The rate and extent of metabolic conversion of *n*-docosanol vary with the cell type and surfactant used to suspend the compound. Furthermore, the anti-HSV activity of *n*-docosanol is quantitatively proportional to the amount of metabolism observed. These findings suggest that the anti-HSV activity of *n*-docosanol involves cellular uptake and metabolism of the drug.–**Pope, L. E., J. F. Marcelletti, L. R. Katz, and D. H. Katz.** Anti-herpes simplex virus activity of *n*-docosanol correlates with intracellular metabolic conversion of the drug. *J. Lipid Res.* 1996. **37:** 2167–2178.

Supplementary key words long-chain alcohols • viral inhibition • lipid-enveloped viruses • herpes simplex virus • phosphatides

We have previously established that *n*-docosanol, a 22-carbon saturated fatty alcohol, inhibits herpes simplex virus in vitro and HSV-mediated disease in vivo (1, 2). In addition, it has been documented that *n*-docosanol is nontoxic, nonmutagenic, and nonteratogenic (3). These characteristics suggest unique clinical benefits of the compound for the treatment of viral diseases. A topical formulation of the compound has shown efficacy in humans with recurrent herpes labialis in a double-blind, placebo-controlled phase II clinical trial (4) and the drug is currently under investigation in phase III clinical trials for the same indication.

For use in tissue culture or for systemic in vivo studies, we suspend the water-insoluble molecule in the inert, nonionic, and nontoxic surfactant Pluronic F-68 (Plu) or in Tetronic 908 (Tet), both block copolymers of polyethylene oxide and polypropylene oxide. The long-chain alcohol can also be solubilized with more common laboratory surfactants such as Triton X-100, but these surfactants are not physiologically compatible. In contrast, polyethylene oxide/polypropylene oxide copolymers have a long history of safe use by the pharmaceutical industry (5). When appropriately formulated, the suspensions are extremely homogenous with a mean size of 0.1 microns and a size-range of 0.045 to 0.3 microns with a bell-shaped distribution. We have established that *n*-docosanol, when formulated as described herein, inhibits the replication of a number of lipid-enveloped viruses including herpes simplex viruses 1 and 2 and certain retroviruses in vivo and in vitro (1, 2) as well as respiratory syncytial virus, cytomegalovirus, and HIV in vitro (1, 2). Control solutions of Plu or Tet without *n*-docosanol exhibit no antiviral activity.

Considering the therapeutic potential of *n*-docosanol, it is important to understand its mode of action. The compound is not directly viricidal (1). Non-enveloped viruses appear resistant to the effects of *n*-docosanol as do most enveloped viruses that enter cells by receptor-mediated endocytosis (1; L. E. Pope, J. F. Marcelletti, L. R. Katz, D. L. Barnard, and D. H. Katz, unpublished results). Moreover, the finding that *n*-docosanol must be present at the time of viral addition, but is no longer required after 30 min of viral–cell interaction, suggests

Abbreviations: Plu, Pluronic F-68; HSV, herpes simplex virus; DMEM, Dulbecco's high glucose modified Eagle's medium; FCS, fetal calf serum; pfu, plaque-forming units; Tet, Tetronic 908.

[1]To whom correspondence should be addressed.

that *n*-docosanol acts very early in the infectious process (1, 2). Although binding of HSV to cells is not inhibited by *n*-docosanol, the compound substantially reduces the rate of viral entry into the cytosol and prevents localization of virus in the nucleus (1, 2). These results indicate that *n*-docosanol interferes with the normal process of viral entry into host cells.

Optimal viral inhibitory activity in vitro requires incubation of *n*-docosanol/Plu suspensions with target cells for at least 8 h before addition of virus. This pre-incubation requirement indicated that time-dependent interactions of *n*-docosanol with the target cell are essential for its antiviral activity. Thus, to discern the means by which *n*-docosanol exerts its antiviral activity, we have performed studies on the uptake, distribution, and metabolism of *n*-docosanol from surfactant-stabilized suspensions. As shown herein, the results of these studies with cultured cell lines indicate that *1)* significant amounts of the alcohol are incorporated into cell membranes; *2)* the molecule is extensively metabolized; and *3)* the rate and extent of metabolism vary with both the characteristics of the surfactant used to solubilize the alcohol and the nature of the cell type treated. As predicted, based on the known enzymology of long chain fatty alcohols (6–10), some of the resulting metabolic products exhibit characteristics of two of the major phosphatide classes, phosphatidylcholine and phosphatidylethanolamine. Most importantly, evidence from these studies suggests that metabolic conversion of *n*-docosanol is correlated with the antiviral activity of the compound.

MATERIALS AND METHODS

Formulation of *n*-docosanol in Plu or Tet

n-Docosanol (98% pure; M. Michel, New York) was suspended in Plu (poloxamer 188; M_r 8400; BASF, Parsippany, NJ) or in Tet (Tetronic 908; M_r 25,000; BASF) as follows. Unless otherwise indicated, Plu or Tet were diluted to 8.3 mM or 2.8 mM, respectively, in sterile DMEM at 37°C, and the solution (125 ml in an Erlenmeyer flask) was then heated to 50°C. *n*-Docosanol (60 mM, final concentration) was added to the Plu (or Tet) in DMEM and the mixture was sonicated (Branson 450 sonifier; fitted with a ½ inch tapped horn and a flat tip) for 21 min at an initial output of 65 W; this warmed the suspension to 86°C. The resulting suspension consists of very fine globular particles with an average size of 0.1 μm as measured by transmission electron microscopy and by laser light scattering analysis performed by Delta Analytical, North Huntington, PA on a Horiba LA-900. Where indicated, the concentrations of surfactant or *n*-docosanol were varied without other changes in the procedure. Control suspensions lacking *n*-docosanol were sonicated under identical conditions.

Preparation of *n*-[^{14}C]docosanol suspensions

Radioactively labeled suspensions were prepared by including *n*-[1-^{14}C]docosanol (55 mCi/mmol in ethanol, American Radiolabeled Chemicals, St. Louis, MO) during preparation of suspensions. Typically, 4 μCi of the radioactive compound per ml of suspension to be prepared was placed into a vessel. The ethanol was evaporated under a stream of nitrogen and then *n*-docosanol-containing Plu or Tet suspensions were prepared in this vessel as described for nonlabeled *n*-docosanol.

Viruses and cell lines

Cell lines obtained from the American Type Culture Collection were: *i)* Vero (African Green monkey kidney; ATCC no. CCL 81), *ii)* MDBK (epithelial-like bovine kidney; ATCC no. CCL 22), *iii)* normal human skin fibroblasts (ATCC no. CRL 1900), *iv)* human fetal foreskin (Hs68, ATCC no. CRL 1635), and *v)* human fetal lung (HFL-1, ATCC no. CCL 159). All cell lines were maintained and cultures for viral assays were performed in DMEM supplemented with 1 mM Na pyruvate, 4 mM L-glutamine, 50 units/ml penicillin plus 50 μg/ml streptomycin, and 10 mM HEPES buffer (denoted DMEM) and further supplemented with 5% (simian and bovine cells) or 10% (human cell lines) fetal calf serum (FCS; HYCLONE Laboratories, Logan, UT). All cultures were maintained at 37°C in humidified 10% CO_2.

The viruses HSV-1 (MacIntyre strain, no. VR-539) and HSV-2 (MS strain, no. VR-540) were obtained from the American Type Culture Collection. Stock preparations were generated and titered for levels of plaque-forming units (pfu) in Vero cells and stored frozen at -70°C.

Virus assays in cell culture

Cultured cells were plated either in 16-mm (1 ml; 1.5×10^5 cells/ml) or in 35-mm wells (2 ml; 3×10^5 cells/ml) in DMEM supplemented with 5% (simian and bovine cells) or 10% (human cell lines) FCS. Varying concentrations of *n*-docosanol suspension or the corresponding control vehicle (lacking *n*-docosanol) were added at the outset of the culture. All cultures were then inoculated with 50 μl containing 50 (16-mm wells) or 175 (35-mm wells) pfu 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 × magnification). The data are averages of duplicate cultures, which varied by no more than 5–10%.

n-Docosanol uptake by intact cells

Adherent cell cultures in 35-mm wells were incubated with various quantities of *n*-[1-^{14}C]docosanol in Plu or in Tet. For incubation times greater than 20 h, *n*-docosanol-containing suspensions were added at the onset of culture; for shorter incubation periods, the cells were incubated first for 18–20 h to allow for adherence. After the required time of incubation, the suspension was removed, the wells were washed extensively with saline, and the cells were recovered by treatment with 10 μg/ml of trypsin for 10 min at 37°C. Cell-associated *n*-[1-^{14}C]docosanol was quantified after the addition of 6 ml Scintiverse BD (Fisher Scientific) to the total sample using a Beckman LS6000 scintillation counter. Values were corrected for nonspecific binding observed in wells lacking cells, typically less than 200 cpm/well.

Cell fractionation of *n*-[1-^{14}C]docosanol-treated Vero cells

n-[1-^{14}C]docosanol was added at the onset of culture to Vero cells (3×10^5 cells/ml, 50 ml) at the indicated concentrations. After 37°C/CO_2 incubation for the desired time, cells were extensively washed with saline and harvested with trypsin–EDTA (0.5 mg/ml trypsin; 0.2 mg/ml EDTA). After suspension in hypotonic buffer (1 mM $NaHCO_3$, 2 mM $CaCl_2$, 5 mM $MgCl_2$, pH = 7.8) and lysis by sonication, cells were fractionated into membrane or cytoplasm by differential centrifugation as described by Storrie and Madden (11). Nuclei were isolated by centrifuging through sucrose after lysis of whole cells with Nonidet P-40 (12).

Lipid extraction and thin-layer chromatographic analysis of *n*-docosanol-treated cells

Cells cultured in 35-mm wells with *n*-[1-^{14}C]docosanol were washed extensively with saline before trypsinization with 0.6 ml of trypsin–EDTA (0.5 mg/ml trypsin; 0.2 mg/ml EDTA) for 10 min. Lipids were extracted with slight modifications of the procedure of Folch, Lees, and Sloane Stanley (13) by vigorous shaking with an equal volume of chloroform–methanol 3:2 (v/v) followed by centrifugation for 10 min 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 that was applied to silica thin-layer chromatogram sheets (Eastman Kodak) and developed with hexane–diethyl ether–acetic acid 40:60:1 (v/v). The chromatograms were cut into 0.5-cm strips, 6 ml Scintiverse BD was added and the samples were then analyzed by scintillation counting. The migration positions of *n*-docosanol (R_f = 0.55) and *n*-docosanoic acid (R_f = 0.81) standards were also determined.

Cellular lipid fractionation

Confluent cultures of *n*-[1-^{14}C]docosanol-treated cells (in T-175 flasks) were washed, harvested, and extracted with chloroform–methanol as described above. Cells from one flask were resuspended in 5.0 ml saline before extraction (2×) with an equal volume of chloroform–methanol 3:2 (v/v). The 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, as described by Kates (14), with 4 ml chloroform, 16 ml acetone, and 4 ml methanol to separate the lipids into major classes.

Fractionation of phospholipids

The phosphatide-containing methanol eluate from the silica cellular lipid fractionation described above was further analyzed by TLC as described previously (15). A portion (0.5 ml) was evaporated under nitrogen, resuspended in 20 μl chloroform–methanol 3:2 (v/v) and applied to a plastic-backed silica sheet. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine standards (Sigma, St. Louis, MO) were also included. After development with chloroform–methanol–acetic acid–water 60:50:1:4 (v/v) the sheet was divided into 5-mm strips, the positions of the standards were determined by staining with iodine vapor, and the cpm per fraction were determined by scintillation counting.

Vitride reduction

Phosphatides were reduced with Vitride [$NaAlH_2(OCH_2CH_2OCH_3)_2$, 70% in benzene; Kodak, Rochester, NY] as described by Snyder, Blank, and Wykle (16). Methanol eluates (3.5 ml/flask) obtained using the above fractionation procedure were evaporated to dryness and the remaining material was redissolved in 1.25 ml diethyl ether containing 20% (v/v) benzene. The samples were heated in closed tubes at 37°C for 60 min. After adding 3 ml of 4% acetic acid, the products were recovered by extracting 3 times with 3 ml diethyl ether and analyzed by TLC chromatography on silica developed with diethyl ether–acetic acid 100:0.5 (v/v). This system separates the products of Vitride reduction of phospholipids–glycerides (R_f = 0.49) and fatty alcohols (R_f = 0.90). Fatty alcohols produced were formerly acyl moieties of the phospholipids. Ether-linked groups are resistant to reduction and are not liberated from the glycerides. No attempt was made to distinguish alkyl ethers from plasmalogens (alk-1-enyl lipids).

FIGURE 1

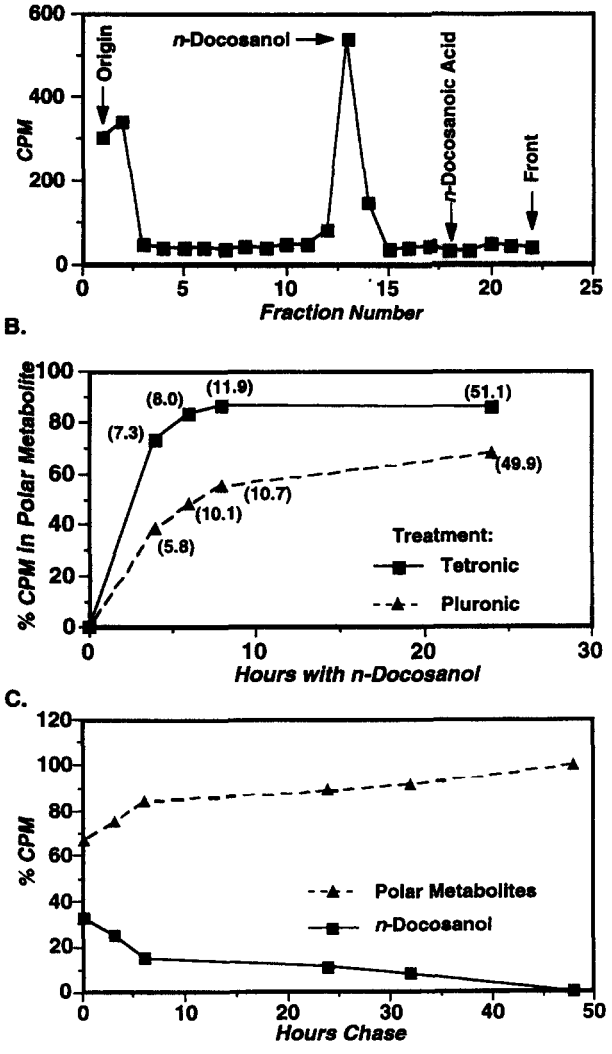

Fig. 1. A: Radioactivity from *n*-[1-^{14}C]docosanol is incorporated into polar metabolites. Vero cells (3×10^5 cells/ml; 2 ml total in DMEM with 5% FCS) were plated in 6-well dishes and incubated for 24 h with 6 mM *n*-[1-^{14}C]docosanol (0.83 mM Plu). The cells were washed and harvested and then extracted with an equal volume of chloroform–methanol as described in Materials and Methods. The chloroform layers were evaporated under nitrogen and the samples were resuspended in 20 µl of chloroform–methanol 3:2 (v/v) before application to silica sheets and development with hexane–diethyl ether–acetic acid 60:40:1 (v/v). B: *n*-[1-^{14}C]docosanol is metabolized more rapidly from a Tet than from a Plu suspension in Vero cells. Vero cells (5×10^5 cells/ml DMEM containing 5% FCS; 2 ml total volume) were allowed to adhere to 6-well plates during a 12-h incubation at 37°C in 10% humidified CO_2. Media were removed and replaced with 6 mM *n*-[1-^{14}C]docosanol suspended with either 0.83 mM Plu or with 0.28 mM Tet (equivalent weights). Cultures were incubated at 37°C/CO_2 for the indicated times. Values in parentheses indicate total nmoles *n*-docosanol equivalents bound/well, expressed as the average of duplicates that varied by less than 5%. An identical set of cells was washed, harvested, extracted with chloroform–methanol and analyzed on TLC as described for Fig. 1A. The percentage of cpm remaining at the origin is referred to as percent cpm in polar metabolite. C: Essentially all *n*-[1-^{14}C]docosanol is metabolized after a 48-h chase. Vero cells (3×10^5 cells/ml DMEM + 5% FCS) were added to 6-well dishes and incubated 24 h with 6 mM *n*-[1-^{14}C]docosanol (0.83 mM Plu). Cell monolayers were washed extensively and then incubated for the indicated times with fresh DMEM containing 5% FCS. Cells were washed, harvested, extracted with chloroform–methanol, and analyzed by TLC as described for Fig. 1A.

HPLC analysis of *n*-alcohols liberated by Vitride reduction

The *n*-alcohol-containing fraction was cut from a preparative silica TLC plate developed as described above. Radioactively labeled sample was eluted with a minimal volume of chloroform–methanol 1:1 (v/v). The sample was evaporated to dryness under nitrogen, resuspended in 100 µl acetonitrile, and injected onto a µBondapak Fatty Acid Analysis (Waters; Milford, MA) column 3.9 × 300 mm in length containing 10 µ particles and following a Waters µBondapak phenyl guard column. Samples were eluted with acetonitrile–water–tetrahydrofuran 55:25:20 (v/v) at 1 ml/min using a Waters HPLC 600E pumping system equipped with U6K injector. Radioactivity was monitored with a Radiomatic A-140A flow scintillation counter (Packard; Meriden, CT) equipped with a 1.0 ml cell.

RESULTS

Cellular uptake and distribution of *n*-[1-^{14}C]docosanol by simian (Vero) cells

Our previous work documented that *n*-[1-^{14}C]docosanol solubilized with Plu is taken up gradually by adherent cell monolayers. Incorporation of *n*-docosanol into Vero cells after a 24-h incubation increased linearly as a function of concentration between 0 and 3 mM; 3 mM was saturating (1). In a separate experiment, at saturating conditions, Vero cells incorporated 3.3 nmol *n*-docosanol/10^6 cells in 4 h. This is less than 1% of the total *n*-docosanol added to culture and is equivalent to 2×10^9 molecules/cell. Binding of *n*-docosanol appeared to be irreversible as it could not be removed even with extensive washing at 37°C with DMEM or saline solutions with or without 0.2 M CsBr, which removes nonspecifically-associated particles (17). The time dependence of uptake in a monolayer of Vero cells is complicated by the fact that the cells are proliferating. Thus, while uptake on a per cell basis peaks between 8 and 12 h, the total number of molecules bound to the cell population increases dramatically between 32 and 48 h, because the cells are growing exponentially (not shown). These studies were performed in cell monolayers for the times indicated in order to study incorpora-

tion of *n*-docosanol under conditions similar to those used in HSV viral plaque assays.

To determine the intracellular distribution of *n*-docosanol, Vero cells were incubated with 3 mM *n*-[1-^{14}C]docosanol suspended in Plu. After 24 h, the cells were extensively washed, harvested, ruptured by sonication, and the membrane and cytoplasmic components were separated by differential centrifugation. In a typical experiment, most of the radioactivity (73%) localized in membranous fractions; much less (27%) was associated with the high-spin soluble (cytoplasmic) fraction. Total radioactivity recovered was 86%. Nuclei from a parallel culture of Vero cells similarly labeled with *n*-[1-^{14}C]docosanol were isolated by treatment with Nonidet-P40 and subsequent centrifugation through sucrose. Less than 1% of the incorporated radioactivity was found associated with the nuclear fraction.

Metabolism of *n*-docosanol in Vero cells

To assay for possible metabolic conversions of cell-associated *n*-docosanol, Vero cells were incubated with 6 mM *n*-[1- ^{14}C]docosanol suspended in Plu. After 24 h, the cells were washed and harvested. Lipids were extracted, applied to silica sheets, and analyzed as described in Materials and Methods. The chromatograms were developed with hexane–diethyl ether–acetic acid 40:60:1 (v/v) which separates nonpolar lipids including fatty alcohols and fatty acids (14) from more polar lipids, including the phosphatides, which do not migrate in this system and thus remain at the origin. As shown in **Fig. 1A,** almost all the extracted radioactivity localized either at the position of the *n*-docosanol standard (53%) or at the origin of the chromatogram (47%). No radioactivity above background was detected eluting with *n*-docosanoic acid. This elution profile is highly reproducible and indicates that Vero cells metabolize a large portion of the incorporated *n*-docosanol.

n-[1- ^{14}C]docosanol is metabolized in Vero cells more rapidly from a Tet than from a Plu suspension

Uptake and metabolism of *n*-docosanol was studied as a function of the surfactant used to suspend and deliver the molecule, Plu versus Tet. Plu is a difunctional polymer (M_r = 8,400) produced by addition of propylene oxide and ethylene oxide to propylene glycol; Tet is a tetrafunctional copolymer (M_r = 25,000) produced by adding the same components to ethylenediamine (5, 18). The total amount of radiolabel from *n*-[1- ^{14}C]docosanol incorporated by Vero cells is not significantly affected by the surfactant used to solubilize the compound. These values appear in parentheses in Fig. 1B. However, the rate and extent of metabolism of the compound to the more polar material are significantly higher with the Tet than the Plu suspension (Fig. 1B). At 3 h after the addition of *n*-[1- ^{14}C]docosanol suspensions, the proportion of radioactivity in the polar fractions of cells treated with Tet suspensions of the fatty alcohol is approximately twice the proportion appearing in the same fractions from cells treated with Plu suspensions of the same molecule. Vero cells transformed 50% of the *n*-docosanol in 3 h when it was added in a Tet suspension, whereas it took 7 h for 50% conversion when it was added in a Plu suspension.

Essentially all *n*-[1- ^{14}C]docosanol is metabolized after a 48-h chase

As described previously, we have been unable to wash out radioactive *n*-docosanol from treated cells. It appeared to become irreversibly associated with the cells. In the pulse/chase study shown in Fig. 1C, cells were incubated with radiolabeled drug for 24 h. The drug was then removed, fresh media were added, and the cells were incubated for the indicated times. Essentially all the radiolabel from *n*-[1-^{14}C]docosanol was transformed into the more polar material during the prolonged chase period without added drug.

Metabolic inhibitors block both cellular uptake and conversion of *n*-[1- ^{14}C]docosanol to polar compounds

As illustrated in **Fig. 2A,** the amount of radiolabeled *n*-docosanol incorporated into Vero cells is reduced 77% and 75% after 3 and 6 h, respectively, by the inclusion of 5 mM sodium azide and 50 mM 2-deoxyglucose (effective metabolic inhibitors) in the culture. Furthermore, of the percentage of *n*-docosanol that is absorbed, metabolism into the more polar fraction is inhibited by these agents 67% and 75% after 3 and 6 h, respectively, (Fig. 2B). Metabolic inhibitors thus inhibit both uptake and metabolism of *n*-docosanol independently.

Radiolabeled polar metabolites of *n*-docosanol exhibit chromatographic properties of phosphatidylcholine and phosphatidylethanolamine

A predictable fate for long chain fatty alcohols is their incorporation into phospholipids through either an ether or, after oxidation, an ester linkage (19, 20). To characterize the metabolic products remaining at the origin in the TLC system described above, polar metabolites were isolated and subjected to further analysis as follows. Vero cells were incubated with 3 mM *n*-[1-^{14}C]docosanol (suspended in Plu) and, after 72 h, the cells were washed, harvested, and extracted. To isolate the polar fraction, the chloroform extract was applied to a silica column that was then eluted sequentially with chloroform, acetone, and methanol. Radioactivity was recovered in the chloroform fraction, containing neutral metabolites including underivatized *n*-docosanol (38%), or in the methanol fraction (61%), containing phosphatides. The methanol eluate was further ana-

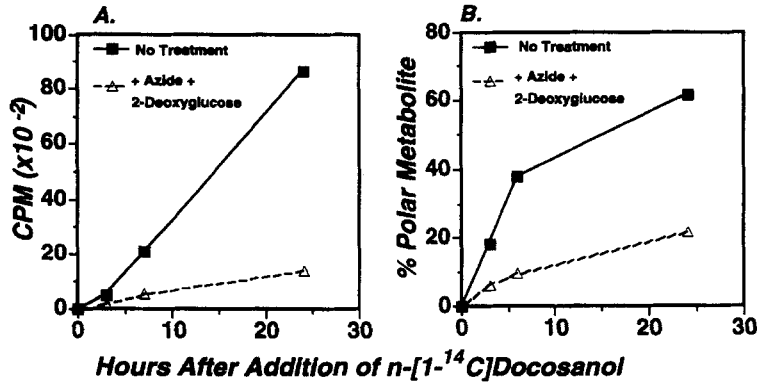

Fig. 2. Sodium azide and 2-deoxyglucose block *n*-docosanol uptake and metabolism. Vero cells (5×10^5 cells/ml DMEM containing 5% FCS; 2 ml total volume) were allowed to adhere to 6-well plates during a 12-h incubation at 37°C in 10% humidified CO_2. The monolayers were washed with saline before the addition of fresh media containing 6 mM *n*-[1- ^{14}C]docosanol and 0.83 mM Plu. Sodium azide (5 mM) and 2-deoxyglucose (50 mM) were added to the cultures as indicated. After incubating for the indicated times at 37°C in humidified CO_2, half of the cell cultures was washed with saline and total radioactivity incorporated was determined by scintillation counting as described in Materials and Methods (Fig. 2A). To quantify polar metabolites (Fig. 2B), a parallel set of treated Vero cells was washed and harvested and then extracted with an equal volume of chloroform–methanol. The chloroform layers from duplicate wells were combined, evaporated under nitrogen, and the samples were dissolved in 20 µl of chloroform–methanol 3:2 (v/v) before application to silica sheets and development with hexane–diethyl ether–acetic acid 60:40:1 (v/v).

lyzed by TLC on silica gel with a chromatography system [chloroform–methanol–acetic acid–water 60:50:1:4 (v/v)] optimized for the separation of major classes of phospholipids. In a typical experiment (**Fig. 3**), 60% and 40% of recovered cpm migrated in the region of phosphatidylcholine and phosphatidylethanolamine, respectively. When *n*-docosanol was suspended with Tet instead of Plu, 60–70% of counts migrated with an R_f value equivalent to phosphatidylcholine, 20–25% with R_f values equivalent to phosphatidylethanolamine, and 5–10% with an R_f value equal to that of phosphatidylserine. Thus, the radioactive portion (carbon 1) of *n*-docosanol suspended either with Tet or Plu is incorporated into molecules possessing the properties of the phosphatides.

Vitride reduction of polar metabolites releases material with the HPLC retention time of *n*-docosanol

To further define the chemical nature of the radioactive products, Vero cells (in T-175 flasks) were incubated with 9 mM *n*-docosanol for 24 h. Lipids were extracted and subjected to silica fractionation. Polar metabolites eluting in the methanol fraction were concentrated and reduced with Vitride as described in Materials and Methods. After this treatment and analysis by TLC, 60–70% of radioactivity was associated with the liberated fatty alcohol fraction. This represents material that had formerly been linked to the glycerol backbone as an acyl group. Most of the remaining counts (20–30%) migrated as did alkyl glycerol standards (e.g., chimyl alcohol; average R_f = 0.49) and consistent with radioactivity incorporated through ether lipids. No attempt was made to differentiate non-hydrolyzable products of plasmalogens (alk-1-enyl-lipids) from those of alkylglycerols. To verify that the Vitride reaction had gone to completion and that the glyceride fraction was resistant to reduction, it was eluted from the silica TLC plate, concentrated, and subjected to a second treatment with Vitride and TLC analysis. More than 70% of radioactivity migrated again with the glyceride-containing fraction, not the alcohol.

As described in Materials and Methods, the alcohol-containing fraction released by Vitride reduction was eluted from preparative TLC and analyzed in an HPLC system optimized to separate fatty alcohols of varying chain lengths. The elution times of *n*-octadecanol (C-18, peak 1) and *n*-docosanol (peak 2) standards are illustrated in **Fig. 4A.** A chromatogram of the radiolabeled fatty alcohols is shown in Fig. 4B. A significant proportion of radioactivity (21.5% of the released alcohols) eluted with the retention time of *n*-docosanol. The remaining counts eluted in a broad peak with a shorter retention time characteristic of shorter chain fatty alcohols or acids. Palmitic acid, for example, exhibits a retention time of 5.5–6.0 min in this HPLC system.

Role for the polar metabolites in the antiviral activity of *n*-docosanol

The metabolic processes described above could be required for the antiviral activity of *n*-docosanol. Alternatively, metabolism could reduce the amount of bioactive species. We therefore conducted experiments to

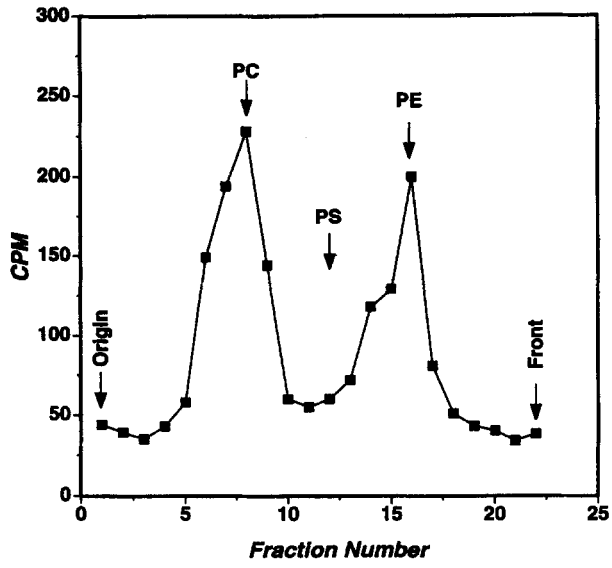

Fig. 3. Radioactive metabolites of *n*-[1-^{14}C]docosanol display the properties of phosphatidylcholine and phosphatidylethanolamine. A portion (0.5 ml) of the methanol eluate of the silica fractionation described in Materials and Methods was evaporated under nitrogen, resuspended in 20 µl chloroform–methanol 3:2 (v/v), and analyzed by TLC with chloroform–methanol–acetic acid–water 60:50:1:4 (v/v) as the solvent. The migration positions of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine standards (PS) are indicated.

investigate the relationship between enzymatic conversion and antiviral activity. As shown below, results of such studies have demonstrated that the efficiency of metabolic conversion correlates with the magnitude of the antiviral activity of *n*-docosanol.

n-Docosanol suspended in Tet exhibits greater antiviral activity than similar Plu suspensions. To investigate the role of metabolism in antiviral activity we utilized the previously described observation that *n*-docosanol is metabolized in Vero cells more rapidly from a Tet than from a Plu suspension (Fig. 1B). When antiviral efficacy with the two types of suspensions was compared as assayed by plaque formation in Vero cells, we found that the EC_{50} for inhibition of HSV replication by *n*-docosanol is reduced from an average of 15.3 mM when suspended with Plu to approximately 5.6 mM in the corresponding Tet suspension (**Table 1**) with the same conditions. Hence *n*-docosanol exhibits a 2.7-fold lower EC_{50} (Table 1) and a 2-fold greater rate and extent of metabolism (Fig. 1B) when suspended with Tet than with Plu.

Additionally, the requisite preincubation (before the addition of HSV) of Vero cells in order to obtain significant inhibitory activity with *n*-docosanol/Plu suspensions is not so critical when the alcohol is suspended with Tet. Thus, *n*-docosanol at 18 mM in Tet inhibited HSV-1 plaque production by 64% when added concurrently with virus (not shown). Although this is less than the 99% inhibition seen with a 24-h preincubation, it is substantially more than the limited inhibitory activity (less than 20%) observed at this *n*-docosanol concentration with Plu suspensions without preincubation. Plu and Tet controls lacking *n*-docosanol exhibited no significant levels of antiviral activity.

n-Docosanol is a more efficient inhibitor of HSV-1 plaque formation in Vero cells than in MDBK cells; Vero cells metabolize more n-docosanol than MDBK cells. We also examined the relationship between observed antiviral activity and drug metabolism in the epithelial-like bovine kidney cell line, MDBK. This cell line exhibits an interesting apparent resistance to the anti-HSV activity of *n*-docosanol. The highest concentration of *n*-docosanol (suspended in Tet) examined in this system, 15 mM, completely inhibits plaque production in Vero cells, whereas only 30% inhibition is observed with the same concentration and surfactant in MDBK cells (not shown). The EC_{50} value in MDBK cells listed in Table 1 was estimated by extrapolation of the data and indicates that approximately 4× more *n*-docosanol would be required in MDBK as compared to Vero cells to elicit a 50% reduction in HSV plaque production.

Total cellular uptake and relative conversion to polar metabolites were determined in Vero and MDBK cells. Both parameters were higher in Vero cells (**Table 2**) and the differences increased with longer incubation times. MDBK cells metabolize, proportionally, 10%, 17%, and 19% less *n*-docosanol at 24, 48, and 72 h, respectively, than Vero cells. The ultimate effect of decreased uptake combined with decreased metabolism is illustrated in the last column of Table 2 and in **Fig. 5,** which shows that after 72 h Vero cells contained 4.6-fold higher amounts of phosphatide-like metabolites than do MDBK cells. Small amounts of radioactivity retained the R_f of *n*-docosanol and an additional peak, more pronounced in Vero than in MDBK cells, was observed with an R_f of approximately 0.4. (The identity of this species has not been determined; the R_f value is consistent with observed values for diglycerides.) Therefore, 4-fold lower amounts of *n*-docosanol (in Tet) required in Vero as compared to MDBK cells for 50% reduction in HSV plaque formation (Table 1) correlates closely with the increased production of metabolites in Vero cells.

Antiviral activity corresponds to the level of metabolites but not to the level of n-docosanol in host cells. Radioactively labeled *n*-docosanol is also incorporated into monolayers of several human fibroblast lines including cells derived from normal skin (ATCC no. CRL 1900), fetal foreskin (Hs68, ATCC no. CRL 1635), and fetal lung (HFL-1, ATCC no. CCL 159). Human fibroblasts incorporate much more labeled *n*-docosanol than do the same number of simian Vero cells; however, the amount

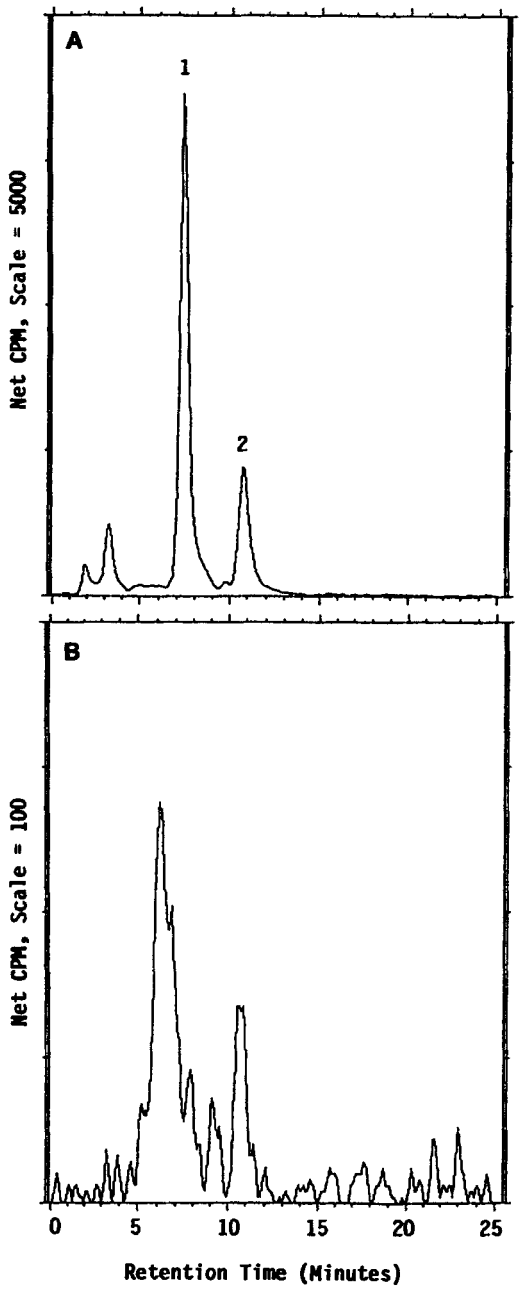

Fig. 4. *n*-Docosanol is a major component of radiolabeled *n*-alcohols released by Vitride reduction of the polar lipid fraction of Vero cells incubated with *n*-[1- ^{14}C]docosanol. Vero cells (in T-175 flasks) were incubated with 9 mM *n*-docosanol (0.06 mM Tet) for 24 h. Lipids were extracted and subjected to silica fractionation. Polar metabolites eluting in the methanol fraction were concentrated and reduced with Vitride; the resulting *n*-alcohol-containing fraction was obtained after preparative TLC; and the alcohols were analyzed by HPLC as described in Materials and Methods. Elution patterns of fatty alcohol standards are illustrated in panel A: peak 1 = *n*-octadecanol; peak 2 = *n*-docosanol. Peak 1 was integrated from 6.4 to 9.4 min with a retention time of 7.3 min. Peak 2 was integrated from 10.0 to 13.7 min with a retention time of 10.7 min. The elution pattern of the fatty alcohol fraction liberated by Vitride reduction of the sample is shown in panel B. The majority of radioactivity eluted from 5.5 to 7.5 min (60.82%) with a retention time of 6.2 min or from 9.9 to 11.8 min with a retention time of 10.5 min (21.49%).

of metabolites formed per cell is roughly equivalent (Table 2). For example, by 48 h, human skin fibroblasts (CRL 1900) accumulated almost twice the total radioactivity as incorporated by the same number of Vero cells; at 72 h, the human skin cells contained almost four times the total radioactivity per cell. Additionally, after 72 h, the human skin cells began to appear highly vacuolized when examined by light microscopy. In contrast, the proportion of radioactivity in the polar fraction was consistently and significantly greater in Vero cells than in CRL 1900 cells, more than 2 times greater at 48 h and 4 times greater at 72 h. As a result, the absolute amount of radiolabeled metabolites per cell was roughly equivalent in the two cell types.

Although there is greater accumulation of *n*-docosanol in human fibroblasts as compared to Vero cells, the EC_{50} for *n*-docosanol inhibition of HSV replication is approximately the same in both cell types (Table 1). Thus, antiviral activity does not correlate with the total amount of *n*-docosanol accumulated but does correlate with the amount of polar metabolites formed.

DISCUSSION

Because of its potential clinical effectiveness, we have conducted studies designed to delineate the mechanism by which *n*-docosanol exerts its antiviral activity (1, 2). These studies suggest that the compound appears to interfere with one or more of the pathways of viral entry into the cell and subsequent migration to and entry into the nucleus of infected target cells. The key points of evidence supporting this hypothesis are summarized as follows. First, unlike shorter chain-length alcohols (21) the compound has no direct viricidal activity, as virus can be mixed with a *n*-docosanol suspension, then recovered from the suspension and shown to retain normal infectivity (1). Second, the compound does not interfere with binding of herpes virus to heparan sulfate receptors on target cells (1, 2); 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 (2). Third, migration of radiolabeled virus to the nucleus is significantly inhibited in *n*-docosanol-treated cells resulting in substantially diminished viral DNA and protein synthesis and progeny virions (2). Finally, susceptibility to the antiviral effects of *n*-docosanol is generally confined to lipid-enveloped viruses that enter cells by fusion with the plasma membrane (L. E. Pope, J. F. Marcelletti, L. R. Katz, D. L. Barnard, and D. H. Katz, unpublished observations). One exception to this pattern is influenza A, an enveloped virus that has been reported to enter cells via receptor-mediated endocytosis (22) but which is effectively inhibited by *n*-do-

TABLE 1. Antiviral effectiveness of *n*-docosanol varies with cell type and surfactant

Cell Type	Surfactant	HSV EC_{50}	SD	n
		mM	mM	
Vero	Pluronic	15.3	3.3	2
Vero	Tetronic	5.6	1.7	3
MDBK	Tetronic	>20	N.A.	3
Normal skin fibroblasts	Pluronic	10.9	2.9	3

EC_{50} refers to the concentration required for 50% inhibition of HSV-2 plaque production in the cell type indicated as described in Materials and Methods. Cells were incubated 24 h at 37°C/CO_2 with varying concentrations of *n*-docosanol in Tet or Plu before the addition of virus. Values listed are the averages of multiple determinations (n).

cosanol. Reasons for this apparent anomoly remain to be determined.

We previously reported that a substantial incubation (at least 8 h) of target cells with *n*-docosanol/Plu suspensions was required before introduction of virus to observe optimal antiviral activity. The pre-incubation requirement suggested that the antiviral activity of *n*-docosanol involves an interaction of drug and target cells. Therefore, to further elucidate the mechanism of the antiviral activity of *n*-docosanol, we conducted studies on the cellular uptake, distribution, and metabolism of *n*-docosanol from surfactant-stabilized suspensions under conditions that duplicate the conditions of the in vitro viral plaque assay.

Adherent monolayers of Vero cells readily incorporate significant quantities of *n*-docosanol. We previously reported that Vero cells exhibit biphasic kinetics of uptake of 3 mM *n*-docosanol (1); an initially rapid rate during the first 30 min (0.12 nmol/min) is followed by a more gradual rate (0.4 nmol/h). The inefficiency in *n*-docosanol transfer from thermodynamically stable particles that remain suspended in media to viral host cells at the bottom of the tissue culture wells explains the relatively high doses (mM) required for in vitro efficacy. However, as more than 300 mM *n*-docosanol is nontoxic for a variety of cultured cell types, an EC_{50} of 2–3 mM equates to a favorable selectivity ratio of approximately 100.

The cellular incorporation of fatty alcohols up to 18 carbons in length has been described (23, 24) and the results are comparable to those reported here. For example, Ehrlich ascites cells incorporated, also into a non-exchangeable form, approximately 2×10^9 molecules of hexadecanol (solubilized by binding to albumin) per cell in 60 min (24). Cellular uptake of lipid molecules from particles such as those used here (as demonstrated by electron microscopy studies) generally occurs by one of four mechanisms: *a)* fusion, *b)* endocytosis, *c)* stable adsorption, or *d)* lipid transfer (25). Further study would be necessary to define which mode(s) is (are) operational and to determine the fate of the surfactant. The differing relative amounts incorporated per cell in Vero versus normal skin cells (Table 2) and the appearance of translucent vacuoles in the latter suggest that different modes may predominate in different cell types. Metabolic inhibitors block independently both the uptake and metabolism of radiolabeled *n*-docosanol by Vero cells. The combination of sodium azide and 2-deoxyglucose is commonly used to inhibit endocytosis (26), which would also be inhibited in this system.

The distribution and metabolism of *n*-docosanol in cultured cells suggests that it is incorporated into the cellular lipid pool and undergoes expected anabolic and catabolic reactions. Within 3 h in Vero cells, more than 30% (from a Plu suspension) or more than 70% (from a Tet suspension) of radioactivity is incorporated into molecules possessing the properties of phosphatides (Fig. 1B). Based on the release of radioactivity as *n*-docosanol after Vitride reduction, a significant proportion of the *n*-docosanol incorporated is apparently oxidized to the acid and incorporated onto the glycerol backbone (Fig. 4). The acid, however, does not accumulate to any extent as TLC analysis of lipid extracts from Vero cells treated with *n*-docosanol did not reveal any counts migrating with an R_f equivalent to *n*-docosanoic acid. A smaller percentage of radioactivity in the polar fraction is resistant to Vitride reduction. This is consistent with assimilation of *n*-docosanol into polar metabolites with an ether linkage without oxidation to the fatty acid equivalent. The incorporation of fatty alcohols into ether lipids is known to occur in the peroxisomes of a wide variety of mammalian cells (27–31). For example, *n*-[1- ^{14}C]hexadecanol and *n*-[1- ^{14}C]octadecanol incorporate into the phospholipids of Ehrlich ascites cells with the proportion of alkyl versus acyl linkages varying with the concentration of hexadecanol added to the culture media (27). Although these types of metabolites are normal components of cells, incorporation of the 22-carbon alcohol will predictably alter the normal membrane lipid distribution, although this has not yet been quantified experimentally.

The rate and extent of metabolism of *n*-docosanol appear to be determining factors in the antiviral activity of the drug. Conditions that optimize antiviral activity

TABLE 2. Uptake and metabolism of *n*-[1- ^{14}C]docosanol differs with cell type

Cell Type	Hours Incubation	Total Uptake	% CPM in Polar Fraction	Polar Metabolites
		nmol/10^5 cells		*nmol/10^5 cells*
Experiment 1				
MDBK	24	3.3	24	0.79
MDBK	48	2.5	32	0.80
MDBK	72	2.4	37	0.89
Vero	24	4.6	34	1.6
Vero	48	4.3	49	2.1
Vero	72	7.3	56	4.1
Experiment 2				
CRL 1900	24	3.2	20	0.64
CRL-1900	48	5.8	16.5	0.96
CRL-1900	72	19.2	9.7	1.9
Vero	24	4.2	25	1.1
Vero	48	3.1	46	1.4
Vero	72	4.9	51	2.5

In experiment 1, cells were plated in 6-well plates, 2 ml per well, at 3 × 10^5 cells/ml with 6 mM *n*-[1-^{14}C]docosanol (sp act = 85,000 cpm/μmol) suspended with 0.28 mM Tet. In experiment 2, Vero cells or normal skin fibroblasts were incubated in 6-well dishes (5 × 10^5 cells in 2 ml media) at 37°C for the indicated times with 3 mM *n*-[1- ^{14}C]docosanol (sp act = 108,000 cpm/μmol) in 0.5 mM Plu. In both experiments, cells were washed, harvested, extracted, and analyzed by TLC with hexane–diethyl ether–acetic acid 40:60:1 (v/v) as described in Materials and Methods. Cell number was determined in identical wells treated with equivalent non-radioactive *n*-docosanol; cells were harvested and the number of viable cells (determined by trypan blue exclusion) were counted with a hemocytometer. All measurements were made on duplicate wells which generally varied from 2 to 5%.

tend to increase the rate and/or extent of metabolism. For instance, higher viral inhibition and a faster rate of metabolism were observed with *n*-docosanol delivered in Tet suspensions as opposed to that from Plu suspensions even though the total amount of incorporated radioactivity was not significantly different. The same relationship is not apparent with respect to the amount of unmodified *n*-docosanol. Greater accumulation of *n*-docosanol was observed in the human skin cell lines than in the Vero cell line (Table 2), but inhibition of HSV replication by *n*-docosanol and the amount of polar metabolites formed were similar in the two lines. (It is noted that the cellular distribution of polar metabolites was not determined; as Vero cells are much smaller than CRL 1900 cells the density of polar metabolites in the plasma membrane may be less than in Vero cells.) Moreover, a lower amount of both uptake and metabolism of *n*-docosanol in MDBK versus Vero cells also correlates with the relative insensitivity of MDBK cells to the anti-HSV activity of the compound. Thus far, all the available evidence support the hypothesis that antiviral activity with *n*-docosanol requires cellular uptake and metabolism of the compound.

Observations of Roos and Choppin (32, 33) provide precedence that alterations in normal membrane lipid composition may affect fusion events. They 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 some of the products obtained via metabolic conversion of *n*-docosanol as described above. Subsequently, Roos et al. (34) demonstrated that the same cells exhibited an increased susceptibility to virally induced fusion. Viral/cell fusion is thought to be essential for HSV entry into cells, and activities of this sort would be consistent with the observed antiviral properties of this drug.

Lemay, Ceccarelli, and Epand (35) observed in 1994 that long-chain aliphatic alcohols, including *n*-docosanol, do not inhibit fusion of influenza to liposomes. We have demonstrated, however, that *n*-docosanol treatment of Vero cells renders them resistant to infection by influenza A (2). As liposomes are not metabolically active, *n*-docosanol metabolism does not occur in this system. Thus inhibition of viral/cell fusion after *n*-docosanol metabolism remains a likely mechanism for its antiviral activity.

Numerous different lipid metabolites could be responsible for the observed anti-HSV activity. A wide range of radiolabeled metabolic products are expected including those resulting from β-oxidation and perhaps re-incorporation of radiolabel from released 2-carbon units. Isolation of the radiolabeled 22-carbon fatty alco-

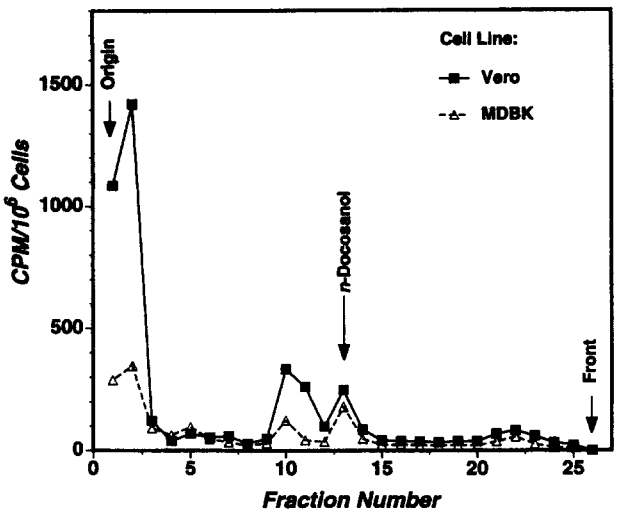

Fig. 5. Vero cells metabolize more *n*-docosanol than MDBK cells. Vero or MDBK cells were plated as described in Fig. 1A. *n*-[1-^{14}C]Docosanol was added to 6 mM (0.28 mM Tet) and the cultures were incubated 72 h at 37°C/CO_2. Cells were extracted and analyzed on TLC with hexane–diethyl ether–acetic acid 20:30:1 (v/v) as the developing solvent as described in Fig. 1. Duplicate plates were treated with an identical suspension lacking the radioactive label. After extensive washing with 0.9% saline and incubation with trypsin–EDTA (0.5 mg/ml trypsin; 0.2 mg/ml EDTA) for 15 min at 37°C, the number of cells/well excluding trypan blue were enumerated by counting on a hemocytometer.

hol as the major identifiable species after Vitride reduction, however, suggests that significant levels of anabolic products are formed during incubation of *n*-docosanol with cultured cells.

The results described herein are also consistent with the possibility that metabolism of *n*-docosanol occurs concomitantly with other processes required for antiviral activity. For example, it could induce a cellular metabolic pathway that results in inhibition of HSV replication. The amount of induction could vary with cell type or the surfactant used to suspend the compound. The most convincing evidence that metabolism is required for antiviral activity would be to demonstrate such activity with a fraction containing metabolites but lacking *n*-docosanol. Concentrated fractions of cellular lipids eluted from silica with chloroform, acetone, or methanol were tested in a plaque assay as described in Materials and Methods. At the attainable concentrations (limited due to cellular toxicity of the solvent), no antiviral activity was detected for any of these fractions. Specific bioactive product(s) may have to be generated from *n*-docosanol in situ. Alternatively, the membrane lipid composition could be altered after incorporation and metabolism of added *n*-docosanol in such a way as to make the cell membrane resistant to viral/cell fusion. This again would be consistent with the observations of Roos and Choppin (32, 33) discussed above. Further characterization of the metabolism of *n*-docosanol should not only help to elucidate the mechanism for its antiviral activity, but might also allow us to develop more effectively its therapeutic potential as a treatment for diseases resulting from host infection by lipid-enveloped viruses.

Ms. Regina R. McFadden and Mr. Phillip M. Douglass are gratefully acknowledged for expert technical assistance. We thank Drs. Carol O. Cowing, Ronald T. Ogata, and Frank M. Huennekens for critically reviewing the manuscript. The assistance of Ms. Beverly Burgess in the preparation of the manuscript is greatly appreciated. This work was supported by LIDAK Pharmaceuticals. This is publication number 7 from LIDAK Pharmaceuticals and publication number 243 from the Medical Biology Institute.

Manuscript received 10 April 1996 and in revised form 12 July 1996.

REFERENCES

1. Katz, D. H., J. F. Marcelletti, M. H. Khalil, L. E. Pope, and 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.
2. Marcelletti, J. F., L. E. Pope, M. H. Khalil, R. R. McFadden, L. R. Katz, and D. H. Katz. 1992. Lidakol. *Drugs Future.* **17:** 879–882.
3. Johnson, W., Jr. 1988. Final report on the safety assessment of cetearyl alcohol, cetyl alcohol, isostearyl alcohol, myristyl alcohol and behenyl alcohol. *J. Am. Colloid Toxicol.* **7:** 359–413.
4. Katz, D. H., L. Habbema, K. De Boulle, and G. A. Roders. 1994. *n*-Docosanol cream is an effective topical treatment for recurrent herpes labialis. *Antiviral Res.* **23:** 75.
5. Schmolka, I. R. 1991. Poloxamers in the pharmaceutical industry. *In* Polymers for Controlled Drug Delivery. P. J. Tarcha, editor. CRC Press, Boston. 189–214.
6. Snyder, F., T-C. Lee, and R. L. Wykle. 1985. Ether-linked glycerolipids and their bioactive species: enzymes and metabolic regulation. *In* The Enzymes of Biological Membranes. Volume 2. A. N. Martenosi, editor. Plenum Publishing Corp., New York. 1–58.
7. Van den Bosch, H., R. B. H. Schutgens, R. J. A. Wanders, and J. M. Tager. 1992. Biochemistry of peroxisomes. *Annu. Rev. Biochem.* **61:** 157–197.
8. Rizzo, W. B., D. A. Craft, A. L. Dammann, and M. W. Phillips. 1987. Fatty alcohol metabolism in cultured human fibroblasts. *J. Biol. Chem.* **262:** 17412–17419.
9. Mangold, H. K., and N. Weber. 1987. Biosynthesis and biotransformation of ether lipids. *Lipids.* **22:** 789–799.
10. Rizzo, W. B., A. L. Dammann, and D. A. Craft. 1988. Sjögren-Larsson syndrome. *J. Clin. Invest.* **81:** 738–744.
11. Storrie, B., and E. A. Madden. 1990. Isolation of subcellular organelles. *Methods Enzymol.* **182:** 203–225.
12. Ucker, D. S., and K. R. Yamamoto. 1984. Early events in the stimulation of mammary tumor virus RNA synthesis by glucocorticoids. *J. Biol. Chem.* **259:** 7416–7420.
13. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.

14. Kates, M. 1986. Separation of lipid mixtures. *In* Laboratory Techniques in Biochemistry and Molecular Biology. Volume 3. R. H. Burdon and P. H. van Knippenberg, editors. Elsevier, New York. 186–278.
15. Christie, W. W. 1959. The analysis of complex lipids. *In* Lipid Analysis. Pergamon Press, New York. 186–278.
16. Snyder, F., M. L. Blank, and R. L. Wykle. 1971. The enzymic synthesis of ethanolamine plasmalogens. *J. Biol. Chem.* **246:** 3639–3645.
17. Lyte, M., and M. Shinitzky. 1985. A special lipid mixture for membrane fluidization. *Biochim. Biophys. Acta.* **812:** 133–138.
18. Wyandotte Chemicals Corp. 1968. The Plu® Grid, 6th edition. Wyandotte Chemicals Corp., Wyandotte, MI. 27 pp.
19. Mukherjee, K. D., N. Weber, H. K. Mangold, M. Volm, and I. Richter. 1980. Competing pathways in the formation of alkyl, alk-1-enyl and acyl moieties in the lipids of mammalian tissues. *Eur. J. Biochem.* **107:** 289–294.
20. Bandi, Z. L., H. K. Mangold, G. Holmer, and E. Aaes-Jorgensen. 1971. The alkyl and alk-1-enyl glycerols in the liver of rats fed long-chain alcohols or alkyl glycerols. *FEBS Lett.* **12:** 217–220.
21. Sands, J., D. Auperin, and W. Snipes. 1979. Extreme sensitivity of enveloped viruses, including herpes simplex, to long-chain unsaturated monoglycerides and alcohols. *Antimicrob. Agents Chemother.* **15:** 67–73.
22. Matlin, K. S., H. Reggio, A. Helenius, and K. Simons. 1982. The entry of enveloped viruses into an epithelial cell line. *Prog. Clin. Biol. Res.* **91:** 599–611.
23. Franks, N. P., and W. R. Lieb. 1986. Partitioning of long-chain alcohols into lipid bilayers: implications for mechanisms of general anesthesia. *Proc. Natl. Acad. Sci. USA.* **83:** 5116–5120.
24. Spector, A. A., and J. M. Soboroff. 1972. Studies on the cellular mechanism of free fatty acid uptake using an analog, hexadecanol. *J. Lipid Res.* **13:** 790–796.
25. Pagano, R. E., and J. N. Weinstein. 1978. Interactions of liposomes with mammalian cells. *Annu. Rev. Biophys. Bioeng.* **7:** 435–468.
26. Sleight, R. G., and R. E. Pagano. 1985. Transbilayer movement of a fluorescent phosphatidylethanolamine analogue across the plasma membranes of cultured mammalian cells. *J. Biol. Chem.* **260:** 1146–1154.
27. Cabot, M. C., and F. Snyder. 1980. Manipulation of alkylglycerolipid levels in cultured cells. Fatty alcohol versus alkylglycerol supplements. *Biochim. Biophys. Acta.* **617:** 410–418.
28. Brown, A., and F. Snyder. 1992. Alkyldihydroxyacetonephosphate synthase. *Methods Enzymol.* **209:** 377–384.
29. Datta, N. S., G. N. Wilson, and A. K. Hajra. 1984. Deficiency of enzymes catalyzing the biosynthesis of glycerol–ether lipids in Zellweger syndrome. *N. Engl. J. Med.* **311:** 1080–1083.
30. Snyder, F., W. T. Rainey, Jr., M. L. Blank, and W. H. Christie. 1970. The source of oxygen in the ether bond of glycerolipids. *J. Biol. Chem.* **245:** 5853–5856.
31. Hajra, A. K., C. L. Jones, and P. A. Davis. 1978. Studies on the biosynthesis of the O-alkyl bond in glycerol ether lipids. *In* Enzymes of Lipid Metabolism. S. Gatt, L. Freysz, and P. Mandel, editors. Plenum Publishing Corp., New York. 369–378.
32. Roos, D. S., and P. W. Choppin. 1985. Biochemical studies on cell fusion. I. Lipid composition of fusion-resistant cells. *J. Cell Biol.* **101:** 1578–1590.
33. Roos, D. S., and P. W. Choppin. 1985. Biochemical studies on cell fusion. II. Control of fusion response by lipid alteration. *J. Cell Biol.* **101:** 1591–1598.
34. Roos, D. S., C. S. Duchal, C. B. Stephensen, K. V. Holmes and P. W. Choppin. 1990. Control of virus-induced cell fusion by host cell lipid composition. *Virology.* **175:** 345–357.
35. Lemay, C. T. Y., D. F. J. Ceccarelli, and R. M. Epand. 1994. Fusion of influenza to liposomes is not inhibited by aliphatic primary alcohols. *Biosci. Rep.* **14:** 33–42.