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Fenretinide inhibits HIV infection by promoting viral endocytosis

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Abstract

HIV fusion is mediated by the sequential interaction of the viral envelope glycoprotein with cellular receptors at the plasma membrane. We have previously reported that the upregulation of cellular ceramide levels following fenretinide treatment inhibits HIV fusion. As ceramide facilitates the internalization of a variety of microbes, we hypothesized that it may also promote the engulfment of HIV virions. Hence, we analyzed the effect of fenretinide treatment on virus binding and uptake. We observed that virus binding is not altered by fenretinide treatment. The distribution of HIV receptors was also unchanged. In contrast, virus uptake showed a significant increase. We have determined that fenretinide treatment promotes the internalization of virions from the plasma membrane and the accumulation of virus in the endocytic fraction of HeLa cells. This effect of fenretinide appears to be specific for virus as the endosomal accumulation of gp120, transferrin and horse-radish peroxidase was not increased. Notably, fenretinide increased the infectivity of influenza virus, which fuses in the endosomal compartment upon low pH activation. Our data suggest that fenretinide treatment effectively inhibits HIV infection by re-directing the virus to the endocytic pathway.
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Keywords: HIV; Ceramide; Fenretinide; Endocytosis

1. Introduction

Enveloped viruses infect cells following the merger of the viral and the target cell membranes. Fusion is catalyzed by conformational changes in the envelope protein that projects through the viral membrane. Two distinct strategies are employed in triggering these conformational changes. Viruses such as influenza enter cells via endocytosis with productive infection initiated from within the endosome. The acidic pH of this intracellular compartment triggers a series of conformational changes in the envelope glycoprotein leading to exposure of the fusion peptide and subsequent membrane fusion (reviewed by Earp et al., 2005). In contrast, a neutral pH-fusing virus such as HIV-1 initiates infection following receptor engagement at the plasma membrane. In this case the sequential engagement of receptor proteins induces conformational changes in the envelope protein, ultimately culminating in viral-cell fusion (Gallo et al., 2003). HIV has been well documented to enter cells via the endocytic pathway, but in contrast to low-pH-fusing viruses like influenza, this pathway generally results in viral inactivation (Marechal et al., 1998).

We have previously reported that fenretinide (*N*-(4-hydroxyphenyl)retinamide, 4-HPR) treatment of HIV-1 receptor positive cells inhibits HIV-1 fusion (Finnegan et al., 2004). Fenretinide, a synthetic retinoid, has well described anti-tumor and chemopreventative activities and can kill cancer cells by inducing apoptosis. This mechanism of apoptosis induction is not completely understood. It has been suggested that increasing cellular ceramide levels may play an important role, as fenretinide stimulates two key enzymes in the ceramide biosynthetic pathway, serine palmitoyl transferase and ceramide synthase. It has been shown that fenretinide treatment enhances cellular ceramide levels (Reynolds et al., 2004; Wang et al., 2001). We have confirmed that fenretinide increases ceramide levels in our experimental system (Finnegan et al., 2004) and have proposed this elevation as mediating anti-HIV activity. In support of this hypothesis we have described other mechanisms that increase ceramide levels, and have confirmed that these approaches also inhibit HIV infection.

Ceramide generally constitutes a very small percentage of plasma membrane lipids and is an important regulator of diverse cellular events (Gulbins et al., 2004). Many of these functions have been attributed to the biophysical properties of ceramide as it is cone shaped and relatively poorly hydrated. As a result of this, increasing ceramide levels has been shown to affect both the structure and curvature of membranes. Studies have shown

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that increasing ceramide levels in macrophages and fibroblasts results in endocytic vesicle formation (Holopainen et al., 2000; Zha et al., 1998). It was determined that up to 30% of the plasma membrane can be consumed in this process. Also, the addition of exogenous ceramide (C6) induces endocytic vesicle formation in fibroblasts, which is reversible upon ceramide removal (Li et al., 1999). In model systems, studies have documented that the generation of ceramide promotes vesicle formation and aggregation (Holopainen et al., 2000).

This property of ceramide to facilitate vesicle formation has been exploited by a variety of microbes to facilitate entry and infection. It has been reported that Neisseria gonorrhoeae infection is entirely dependent on the activity of an acid sphingomyelinase at the cell surface. Sphingomyelinase, which cleaves sphingomyelin, is a rapid mechanism to increase ceramide levels at the cell membrane. This, in turn, triggers phagocytosis of the bacterium into mucosal epithelial cells (Grassme et al., 1997). Likewise, the generation of ceramide induces the formation of large raft signaling platforms that are required to internalize *Pseudomonas aeruginosa* (Grassme et al., 2003). Sindbis virus and *Plasmodium falciparum* entry have also been suggested to involve ceramide upregulation (Jan et al., 2000; Hanada et al., 2000). Collectively, these pathogens appear to use a common mechanism to facilitate entry. Increasing ceramide levels induces the formation of ceramide-enriched platforms that cluster together due to the tendency of ceramide to self-aggregate. This clustering concentrates the receptors for these pathogens, and the biophysical properties of ceramide facilitate internalization (Gulbins et al., 2004).

We show in this study that fenretinide treatment, most likely due to ceramide upregulation, has adverse effects on HIV infection. We have determined that fenretinide treatment of cells promotes the endocytic uptake of HIV in HeLa cells which have been engineered to express CD4 and coreceptor. This effect appears to be specific for virus as the endocytic accumulation of gp120 (HIV envelope protein), transferrin (a marker protein for receptor-mediated uptake) and horse-radish peroxidase (a marker protein for non-absorbtive endocytosis) are not increased. This effect is CD4-independent as increased endocytic accumulation was also observed in parental HeLa cells. Likewise, fenretinide does not appear to specifically target HIV as increased infectivity of influenza is also detected. Collectively, these studies indicate that fenretinide treatment promotes viral uptake. This has adverse effects for HIV, as entry into cells via the endocytic pathway generally results in viral inactivation. Hence, fenretinide effectively inhibits HIV infection.

2. Materials and methods

2.1. Reagents and cells

The TZM-bl indicator cell line, obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc., is a HeLa cell line derivative that expresses high levels of CD4 and CCR5 along with endogenously expressed CXCR4. TZM-bl cells contain HIV LTR-driven β-galactosidase and luciferase reporter cassettes that are activated by HIV tat expression. TZM-bl cells are routinely subcultured every 3–4 days by trypsinization and are maintained in DMEM supplemented with 10% fetal bovine serum and 1X penicillin–streptomycin (complete media) at 37 °C with 5% CO_2 in a humidified incubator. Fenretinide [*N*-(4-hydroxyphenyl)retinamide] was purchased from Biomol Research Labs (Plymouth Meeting, PA). Streptavidin-HPR, horse-radish peroxidase, DEAE-dextran and FITC-cholera toxin were obtained from Sigma (St. Louis, MO). PE-conjugated anti-CD4, CXCR4 or CCR5 monoclonal antibodies were from BD-Pharmingen (San Jose, CA). The anti-H3N2 FITC antibody and isotype control were obtained from Virostat (Portland, ME). Gp120-biotin was obtained from Intracell (Issaquah, WA), biotinylated transferrin, anti-transferrin receptor and Alexa conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR). Bicinchoninic acid (BCA) protein assay kit was from Pierce Chemical Co. (Rockford, IL). The P24 ELISA kit was obtained from the AIDS Vaccine Program NCI-Frederick, MD, and was performed as described in the manufacturer's protocol.

2.2. Fenretinide treatment

For all experiments using fenretinide, TZM-bl cells were incubated with the indicated concentration of fenretinide for 48 h at 37 °C in complete media.

2.3. Gp120 binding assay

TZM-bl cells were harvested with cell dissociation buffer without trypsin and resuspended at 10^6 cells/ml in PBS. They were then incubated with gp120 (IIIb isolate; 5 μg/ml) for 2 h at 4 °C. Cells were washed and directly fixed with 2% PFA. Cells were stained with the gp120 specific Mab 2G12 (5 μg/ml) (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Hermann Katinger) to determine gp120 binding. Following staining, samples were resuspended in 1 ml of PBS and read by a FACScalibur (Becton Dickinson, San Jose, CA) at 10,000 events/sample with respect to unlabeled cells.

2.4. Viral binding and entry assays

TZM-bl cells were harvested with cell dissociation buffer without trypsin, washed twice with phosphate-buffered saline (PBS) and re-suspended at 3×10^6 cells in 2 ml of complete medium. Purified HIV-1 (40 ng of p24; ADA and NL4-3 isolates) was added to the cells in 40 μg/ml DEAE-dextran for 3 h at 4 or 37 °C. Following this incubation, the cells were spun down and unbound virus was aspirated. The cells were washed five times with cold PBS to remove unbound material and treated with trypsin to remove surface bound virions or with a control treatment. The cells were then washed twice with PBS and cell lysates were prepared by resuspending the pellet in 300 μl of lysis buffer (PBS containing 1% Triton-X 100) and freezing at −20 °C. Viral attachment (4 °C, no trypsin) and internalization (37 °C

with trypsin) was monitored by measuring the amount of p24 in the supernatant of cell lysates by enzyme-linked immunosorbent assay (ELISA). Total cell protein was calculated using BCA and all samples were normalized for protein content. The average was taken from three separate experiments that were normalized to 100% relative to the untreated control sample.

2.5. Analysis of receptor extraction by Triton-X 100

TZM-bl cells were harvested with cell dissociation buffer without trypsin and resuspended at 0.5×10^6 cells/ml in PBS. Following incubation in PBS with 5% FBS and 5% NMS for 20 min, phycoerythrin-conjugated mouse IgG anti-CD4 (RPA-T4), anti-CXCR4 (12G5), anti-CCR5 (2D7) or anti-transferrin receptor MAbs were added at 5 μg/ml. GM1 expression was assayed by incubation with FITC-conjugated Cholera Toxin at 5 μg/ml. Following incubation at 4 °C for 1 h the cells were washed three times with ice-cold PBS and samples were split into duplicates. To one set of samples 300 μl of ice-cold 1% Triton-X 100 in PBS was added for 30 min on ice. Control samples received PBS alone. The cells were subsequently pelleted to remove the Triton-X 100 and washed twice with PBS. The samples were maintained on ice and analyzed by a FACScalibur (Becton Dickinson) at 10,000 events/sample with respect to unlabeled cells.

2.6. Biochemical measurement of fluid-phase uptake

Fluid-phase endocytosis was quantitated as described previously (Marsh and Helenius, 1980). In brief, TZM-bl cells were plated on six-well plates 48 h prior to the assay and treated with fenretinide. Cells were washed once with DMEM and incubated at 37 °C with 0.5 ml prewarmed media containing 10 μg/ml HRP (Type 2; Sigma Chemical Co., St. Louis, MO). Following incubation for the specified time, dishes were placed on ice and washed 10 times with ice-cold PBS. Subsequently the cells were trypsinized, washed an additional 2 times and finally resuspended in 0.5 ml of 0.25% Triton-X 100. Total cell protein was calculated using BCA and all samples were normalized for protein content. Horse-radish peroxidase (HRP) activity was determined using 3-3′,5,5′-tetramethylbenzidine (TMB) a soluble chromogen substrate for horse-radish peroxidase.

2.7. Cell fractionation assays

TZM-bl cells were incubated with biotin-conjugated transferrin (transferrin-Bt, 250 ng/ml), biotin-conjugated gp120 (gp120-Bt, 1 μg/ml) or HIV-1 (40 ng of p24; ADA isolate or NL4-3 in 40 μg/ml DEAE-dextran) for 3 h at 37 °C. To remove surface bound protein/virions, cells were washed three times with ice-cold PBS and trypsinized for 5 min at room temperature. Trypsin activity was quenched by the addition of serum containing media and cells were then washed twice in ice-cold PBS. The cells were resuspended in 2 ml of hypotonic solution (20 mM Tris–HCl [pH 8], 10 mM KCl, 1 mM EDTA) for 15 min at 4 °C and disrupted by Dounce homogenization (15 strokes, 7 ml B pestles). Nuclei and cell debris were pelleted by centrifugation (3000 rpm for 5 min at 4 °C). The postnuclear extracts were centrifuged at 22,000 rpm for 30 min at 4 °C. The pellet representing the vesicular fraction including endosomes was resuspended in lysis buffer (0.5% Triton-X 100) and p24gag levels were quantitated by ELISA. The vesicular pellet stained positive by Western blot for both early endosome antigen 1 (EEA1) and mannose-6-phosphate receptor (M-6-P-R), marker proteins of early and late endosomes, respectively. Total cell protein was calculated using BCA and all samples were normalized for protein content. Transferrin and gp120 content were quantitated by immunoblot analysis.

2.8. Immunoblot analysis

Cellular proteins were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Blots were incubated for 1 h in TBST (10 mM Tris–HCl [pH 8.0], 150 mM NaCl, 0.2% Tween 20) containing 5% powdered skim milk. After four washes with TBST, membranes were incubated for 1 h with the primary antibody (SA-HRP diluted 1:3000) in TBST with 3% BSA. Immunoreactivity was detected by using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ).

2.9. Virion internalization assay

TZM-bl cells were harvested with cell dissociation buffer without trypsin and resuspended at 10^6 cells/ml in PBS. They were then incubated with pre-chilled HIV virus (40 ng of p24; NL4-3 isolate in 40 μg/ml deae-dextran) for 2 h at 4 °C. Cells were washed five times with ice-cold PBS and fixed in 4% PFA directly or following incubation at 37 °C for 15 min. The cells were then stained with the gp120 specific Mab 2G12 (5 μg/ml) (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Hermann Katinger) to quantitate surface bound virions. Following staining, the samples were resuspended in 1 ml of PBS and read by a FACScalibur (Becton Dickinson) at 10,000 events/sample with respect to unlabeled cells.

2.10. Influenza infection

HeLa cells were incubated with 20 μl of the X31 strain influenza virus in 2 ml of DMEM. Infection was allowed to proceed for 2 h at which time the unbound virus was aspirated and complete media was added. Infection continued for 24 h, the cells were then dissociated and stained as described previously for flow cytometry analysis. The anti-HA antibody (anti-H3N2 FITC labeled, ViroStat, ME) was employed at a 1:10 dilution.

3. Results

3.1. Fenretinide treatment does not influence envelope or viral binding

As described previously, fenretinide treatment of cells inhibits HIV-1 infection at the level of viral-cell membrane

Table 1
Fenretinide does not influence gp120 or viral binding

	0 μM	1 μM	5 μM
Gp120	100	102 ± 22	109 ± 26
Virus	100	110 ± 7	102 ± 11

TZM-bl cells were treated with fenretinide for 48 h at 37 °C. Cells were incubated with gp120 (5 μg/ml) or prechilled virions for 3 h at 4 °C. After extensive washing, cells were stained with the anti-gp120 specific Mab 2G12 and binding was detected by flow cytometry. Viral binding was detected by p24 ELISA. The results represent an average of three independent experiments with standard deviations indicated.

fusion (Finnegan et al., 2004). As fenretinide stimulates ceramide upregulation, which alters the topography of the plasma membrane, a likely hypothesis to explain such inhibition would be an effect on HIV-1 receptor engagement or distribution. To assess whether fenretinide treatment influences HIV-1 receptor engagement we incubated fenretinide-treated TZM-bl cells at 4 °C with either the viral envelope protein gp120, or pre-chilled HIV-1 virions. Following extensive washing gp120 engagement was assayed using flow cytometry by employing the gp120 specific Mab 2G12, while viral binding was determined by p24 ELISA. As seen in Table 1, fenretinide treatment did not effect gp120 binding to target cells. Equal binding of the gp120-specific Mab 2G12 was detected in the presence or absence of drug treatment. Quantitative analysis of viral binding also revealed similar values for p24 content, indicating that fenretinide has little effect on this initial step in the fusion cascade (Table 1). Results are shown for the CCR5 tropic isolate ADA, similar results were obtained with the CXCR4 isolate NL4-3.

3.2. Fenretinide treatment does not influence HIV receptor distribution, as determined by Triton-X 100 extraction

Experimental results have indicated that the integrity of lipid rafts is important for HIV-1 entry. CD4, the initial receptor for HIV-1 localizes primarily in lipid rafts (Popik et al., 2002; Nguyen et al., 2005), ordered lipid domains that are characterized by insolubility to Triton-X 100 extraction. Data on coreceptor localization is more controversial, although recruitment of coreceptor to lipid raft domains to form an env-CD4-coreceptor tricomplex is thought to be necessary for fusion (Sorice et al., 2001; Manes et al., 2000). Ceramide generally constitutes a minor fraction of the lipid content of cells and strongly promotes domain formation. Very small amounts of ceramide can promote phase separation when mixed with phospholipids (Veiga et al., 1999; Carrer and Maggio, 1999). Increasing ceramide levels would therefore be expected to influence existing microdomain organization with possible consequences for the localization of CD4 and/or of the coreceptors CXCR4 and CCR5. Altering the partitioning of the receptors could likely influence HIV fusion due to the required sequential engagement of CD4 followed by coreceptor (Gallo et al., 2003).

We investigated this possibility by determining Triton-X 100 extraction of the main HIV-1 receptors, CD4, CXCR4 and CCR5 following fenretinide treatment. We compared the extractability of these proteins with GM1, a characterized raft marker and the transferrin receptor, a well characterized non-raft marker protein. As seen in Fig. 1, following Triton-X 100 treatment, transferrin receptor is predominantly extracted (97%) as expected for this protein. In contrast, GM1, a raft localized ganglioside, remains predominantly cell associated (10% extracted). In agreement with previous reports (Percherancier et al., 2003; Nguyen and Taub, 2002; Nguyen et al., 2005), the coreceptors CXCR4 and CCR5 are almost entirely extracted (96%) under our assay conditions; notably, the percent extraction remains relatively unchanged following 1–5 μM fenretinide treatment. CD4, which is generally accepted to be raft localized protein (Sorice et al., 1997; Popik et al., 2002; Nguyen et al., 2005), is also largely extracted (75%). This result most likely reflects the overexpression of this protein in our HeLa cell line. Following 1 μM fenretinide treatment CD4 extraction decreases slightly from 75% to 69% and 5–10 μM treatment decreases the extractability of this protein to approximately 65%. This is most likely due to an increase in the rigidity of the membrane where CD4 is localized, as would be predicted based on prior experimental data (Holopainen et al., 1998; Huang et al., 1998).

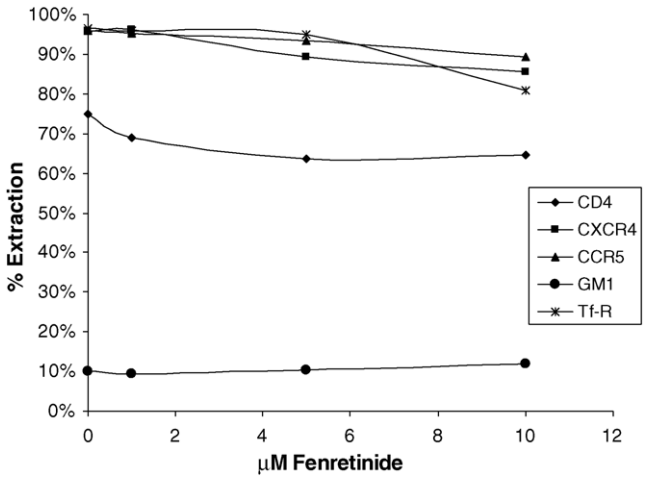

Fig. 1. Fenretinide has minimal effects on detergent extraction of CD4, CXCR4 or CCR5. TZM-bl cells were treated with fenretinide for 48 h at 37 °C. Expression of CD4, CXCR4, CCR5 and transferrin receptor were analyzed by addition of the respective PE-conjugated antibodies, while GM1 was detected by addition of FITC-conjugated cholera toxin B subunit. Following antibody or cholera toxin labeling, the TZM-bl cells were subjected to Triton-X 100 extraction at 4 °C, as described in Section 2. Antibody binding was quantitated using flow cytometry. Reactivity was compared to control samples and percent extraction was calculated. The results shown are from one experiment; similar results were obtained in three independent experiments.

We also investigated the distribution of these receptors by confocal microscopy. However, on our reporter cell line the HIV receptors are overexpressed, resulting in a continuous plasma membrane staining pattern. The limits of resolution and sensitivity of confocal microscopy cannot resolve any subtle changes in such a staining pattern. Following fenretinide treatment no alteration in staining was observed, indicating that no gross membrane effects such as capping were induced (data not shown). Taken together, these results suggest that fenretinide treatment has minimal effects on the localization of the HIV receptor proteins CD4, CXCR4 and CCR5.

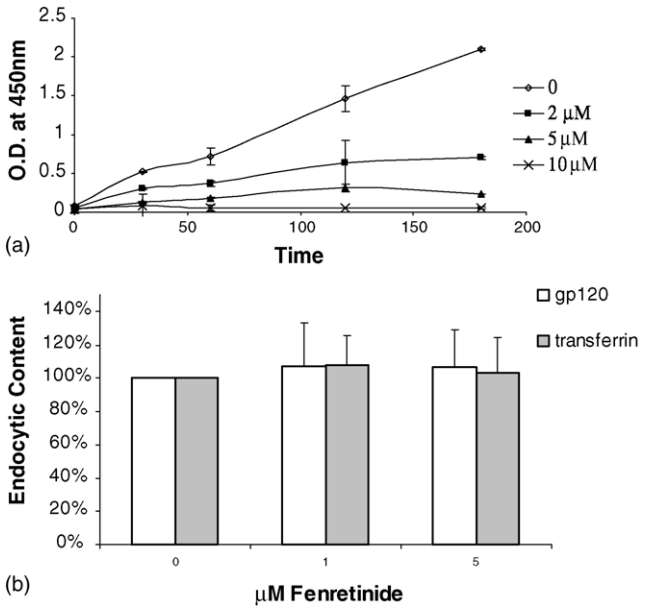

Fig. 2. Fenretinide inhibits fluid phase but not receptor-mediated endocytosis. (a) Confluent dishes of fenretinide-treated TZM-bl cells were incubated in medium containing HRP (10 μg/ml). At the times indicated the cells were placed on ice, washed extensively with PBS and trypsinized to remove cell associated protein. Cells were lyzed and the amount of internalized HRP was assayed by addition of TMB. The results represent an average of three independent experiments, (b) TZM-bl cells were incubated with biotin-conjugated transferrin or biotin-gp120 for 3 h at 37 °C and subjected to subcellular fractionation as described in Section 2. The endosomal fraction was isolated and biotin-labeled content was quantitated by immunoblot analysis with streptavidin conjugated HRP. Results were normalized to control samples and represent an average of three independent experiments.

3.3. Fenretinide inhibits fluid phase but not receptor-mediated endocytosis

In addition to altering the topography of the plasma membrane due to the formation of ceramide-enriched domains, increasing ceramide has been shown to influence the endocytic pathway. To investigate the possibility that fenretinide modulates endocytic pathways we initially measured the rate of endocytosis of a soluble protein HRP, a marker protein for fluid phase, non-receptor-mediated uptake. Following fenretinide treatment, cells were incubated with HRP for increasing times up to 3 h at 37 °C, and then assayed for internal accumulation. As observed in Fig. 2a, fenretinide treatment has a dose-dependent inhibitory effect on HRP uptake. This is in agreement with a previous report which showed that HRP uptake is decreased in a dose-dependent manner following incubation of cells with C6-ceramide. This effect was also observed following the addition of other ceramide analogues and application of sphingomyelinase (Chen et al., 1995). Following 2 μM fenretinide treatment we calculate 42% inhibition of HRP uptake after 30 min which increases to 66% by 3 h. Treatment with 5 and 10 μM fenretinide results in a more pronounced inhibition. We have determined that at these higher concentrations HRP uptake is inhibited from 89% to 97% (Fig. 2a).

We next investigated the effect of fenretinide on receptor-mediated endocytosis, as HIV engages specific receptors on the cell surface. To this end we employed transferrin, the soluble ligand for transferrin receptor, a classical marker protein for this pathway. Endocytic uptake was analyzed by incubating cells with media containing biotin-conjugated transferrin following either fenretinide or control treatment. After 3 h at 37 °C the cells were trypsinized to remove cell surface ligand, and subjected to subcellular fractionation. The vesicular endosome fraction was isolated from the cellular lysate and analyzed for transferrin content by Western blot. Densitometric analysis provided the data shown in Fig. 2b. As observed, fenretinide treatment does not influence the amount of transferrin in the endocytic fraction.

Given that productive viral entry is mediated by specific envelope–receptor interactions, we also determined if the viral envelope protein gp120 undergoes increased endocytosis following fenretinide treatment. To test this we quantitated endocytic uptake of biotin conjugated gp120 as described previously for transferrin. Target TZM-bl cells were incubated with biotin-conjugated gp120 and subjected to subcellular fractionation as described. Gp120 content was then analyzed by Western blot. Densitometric analysis determined that the gp120 content of the endocytic fraction is unaltered by fenretinide treatment (Fig. 2b).

3.4. Fenretinide increases accumulation of internalized virus

As discussed previously, HIV entry into target cells occurs either via productive fusion at the plasma membrane or via endocytosis, which generally results in virus inactivation. Given that fenretinide treatment inhibits HIV infection by preventing viral fusion at the plasma membrane (Finnegan et al., 2004) but not viral attachment (Fig. 1), we next investigated the possibility that fenretinide increases endocytosis of HIV virions. We observed, however, that fenretinide treatment, while inhibiting HRP uptake did not influence the accumulation of transferrin or gp120. In agreement with these results, the internalization rate of the viral envelope protein gp120 was also unaltered by fenretinide treatment (data not shown). To investigate internalization of HIV, we cocultured fenretinide-treated target TZM-bl cells with virus. Following incubation at 37 °C, cells were washed extensively and treated with trypsin to cleave cell membrane associated virus. Control experiments confirmed the efficiency of trypsin cleavage (data not shown) and internal virus was quantitated by p24 ELISA. We determined that 1 μM fenretinide-treated cells resulted in approximately 48% more internal virus over a 3 h time period than untreated control cells. This value was increased to 75% following 5 μM drug treatment (data not shown). To confirm that virus is accumulating in the endocytic pathway we again employed subcellular fractionation. We analyzed the vesicular endosomal fraction for p24 content in control and fenretinide-treated TZM-bl cells. As shown in Fig. 3a, 1 μM fenretinide-treated cells accumulated 40% more virus in the endosomal fraction than control cells in agreement with the quantitation of intracellular P24 (data not shown). Following 5 μM drug treatment the endocytic p24 content was further increased over 100%.

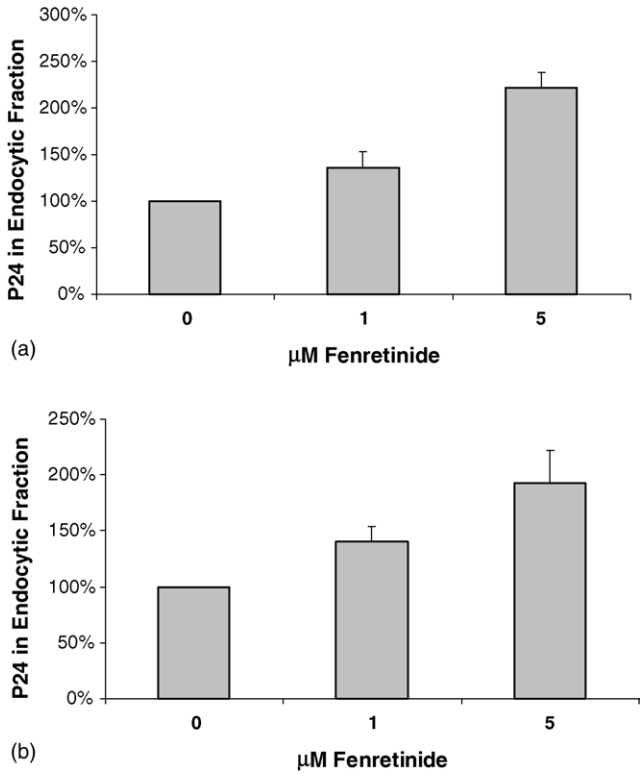

Fig. 3. Fenretinide treatment increases endocytic viral content in a CD4 independent manner. (a) TZM-bl cells were incubated with fenretinide for 48 h at 37 °C. Cells were then incubated with HIV virions for 3 h at 37 °C and subjected to subcellular fractionation as described in Section 2. The endosomal fraction was isolated and viral content was quantitated by P24 ELISA. The results represent an average of three independent experiments. (b) HeLa CD4-negative cells were incubated with fenretinide for 48 h at 37 °C. Cells were then incubated with HIV for 3 h at 37 °C and subjected to subcellular fractionation as described in Section 2. The endosomal fraction was isolated and viral content was quantitated by P24 enzyme-linked immunosorbent assay. The data shown were obtained with ADA, a CCR5 tropic HIV-1 isolate. Similar results were obtained with NL4-3, a CXCR4 tropic HIV-1 isolate. The results represent an average of three independent experiments.

3.5. Accumulation of virus is CD4-independent

To determine if the increased internalization of virus is receptor-dependent, we investigated the effects of fenretinide on virion accumulation in parental HeLa cells (CD4 negative). Using subcellular fractionation we quantitated the virion content in the endocytic fraction of fenretinide-treated HeLa cells. Interestingly, we quantitated similar levels of p24 in HeLa cells following fenretinide treatment (Fig. 3b) when compared to the CD4 receptor positive HeLa cells (Fig. 3a). Hence, it appears that fenretinide stimulates receptor-independent accumulation of virions in the endocytic pathway.

3.6. Fenretinide increases viral internalization

As shown in Fig. 3a, fenretinide treatment increases the amount of virus detected in the vesicular endosomal fraction of cells. To determine if the rate of viral endocytosis is increased, we directly quantitated HIV-1 internalization using flow cytometry. We incubated cells with pre-chilled virions for 2 h at 4 °C, washed extensively and then cocultured virus and TZM-bl cells at 37 °C for 15 min. We monitored viral internalization by decreased accessibility of the gp120 specific Mab 2G12. As seen in Fig. 4, fenretinide-treated cells show a dose-dependent increase in the percentage of virions internalized. Relative to untreated control cells 1 μM fenretinide treatment results in a 25% increase in the internalization of virions within 15 min. TZM-bl cells treated with 5 μM fenretinide internalize 45% more virus. Taken together, these results strongly suggest that fenretinide increases HIV endocytosis.

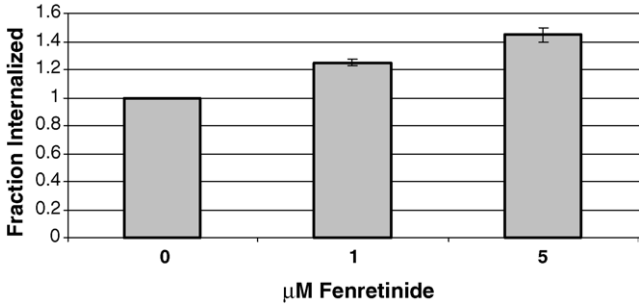

Fig. 4. Fenretinide treatment increases viral internalization. TZM-bl cells were treated with fenretinide for 48 h at 37 °C, and then incubated with prechilled NL4-3 virions for 2 h at 4 °C. After extensive washing, the cells were incubated at 37 °C for 15 min. To monitor viral internalization cells were stained with the anti-gp120 specific Mab 2G12 and binding was detected by flow cytometry. The results represent an average of three independent experiments.

3.7. Fenretinide enhances influenza infection

In contrast to HIV which fuses at the plasma membrane, influenza fuses in the endocytic compartment of cells. As fenretinide treatment increases HIV endocytosis, we reasoned that such treatment should increase the infectivity of a low pH-fusing virus such as influenza. To test this we infected fenretinide-treated TZM-bl cells with influenza virus, and investigated the infection efficiency by monitoring expression of HA, the influenza envelope protein. As seen in Fig. 5, 1 μM fenretinide treatment increases influenza infection by 50% while 5 μM treatment results in 350% increase. These results suggest that fenretinide treatment promotes general viral endocytosis. For low pH-fusing viruses this enhances infection efficiency while for neutral pH-fusing viruses such as HIV, fenretinide treatment inhibits infection.

4. Discussion

Treatment of cells with fenretinide alters the biosynthesis and metabolism of ceramides, which have been implicated as signaling molecules in diverse cellular processes. Given that viruses usurp a variety of cell biological mechanisms to enter cells (Smith and Helenius, 2004) and that ceramide has been implicated in modulating many intracellular signaling pathways (Hannun and Luberto, 2004), it is entirely possible that perturbing cellular ceramide levels may alter viral entry.

Previously, we proposed that fenretinide treatment inhibits HIV infection by perturbing the local membrane structure and

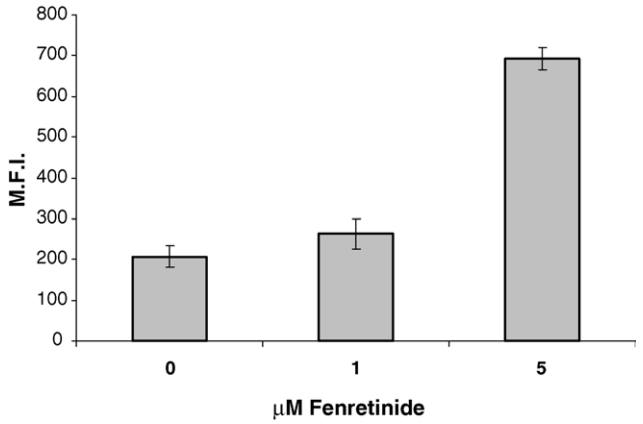

Fig. 5. Fenretinide treatment increases influenza virus infection. TZM-bl cells were treated with fenretinide for 48 h at 37 °C, and then infected with influenza (X31) virus overnight. To monitor infection, cells were stained with the anti-HA (H3N2) specific antibody and binding was detected by flow cytometry. Staining was compared to an isotype antibody and results are shown with isotype binding subtracted. The results represent average values for four independent experiments.

organization inhibiting crucial early events in the HIV fusion process (Finnegan et al., 2004). In this report we examined this hypothesis by measuring viral attachment to TZM-bl cells and possible changes in receptor localization. As shown in Table 1, equal binding of gp120 to both control and fenretinide-treated cells was observed. Likewise, virion attachment as determined by p24 quantitation was also unaffected by fenretinide treatment. We investigated the localization of the primary HIV receptor, CD4 and of the coreceptors CXCR4 and CCR5 using two approaches. We quantitated extraction of these proteins in 1% Triton-X 100 at 4 °C, as extraction of proteins under these conditions is classically used to define proteins that are localized in non-raft domains (Brown and Rose, 1992). As observed in Fig. 1, fenretinide treatment has minimal effects on the domain localization of these receptors as very little change in extraction was observed. We therefore conclude that fenretinide treatment neither causes changes in HIV-1 binding nor in HIV-1 receptor disposition.

In HeLa cells approximately 90% of virion entry is mediated by endocytosis in a CD4- and coreceptor-independent manner (Marechal et al., 1998). Entry via this pathway does not generally result in productive infection as the virus is inactivated upon encountering the low pH of the endosomal compartment. Moreover, it has been demonstrated in T cells and PBMC that there is a dynamic balance between viral fusion and endocytosis (Schaeffer et al., 2004). We therefore examined whether fenretinide treatment of TZM-bl HeLa cells inhibits infection by increasing viral endocytosis, diverting HIV from productive fusion at the plasma membrane. We determined that endocytosis of the classical markers HRP and transferrin, as well as the HIV envelope protein gp120 are not increased by fenretinide treatment of HeLa cells. In fact, fluid-phase endocytosis undergoes dose-dependent inhibition, in agreement with previous studies (Chen et al., 1995; Sallusto et al., 1996). In contrast, HIV-1 exhibited a significant increase in endocytic viral accumulation and increased virus internalization following fenretinide treatment in a CD4-independent manner. Additionally, this effect did not appear to be specific for HIV as infection of influenza, which fuses upon encountering the low pH of the endocytic compartment, is increased following treatment (Fig. 5). Increasing HIV-1 endocytosis would alter the dynamic balance between fusion and endocytosis. In agreement, it has been demonstrated that perturbing the endocytic pathway using vacuolar ATPase inhibitors or by employing weak lysomotrophic agents increases the productive fusion pathway resulting in cytosolic delivery of the viral nucleocapsid (Fredericksen et al., 2002; Schaeffer et al., 2004). As entry is so heavily weighted toward the endocytic pathway in HeLa cells, even minor increases in magnitude would be expected to have significant consequences for the direct fusion route.

We hypothesize that virion morphology and avidity of receptor–ligand interactions play a role in the virion specific endocytosis observed here. HIV is approximately 90–100 nm in size with on average 8–10 envelope trimers projecting from the viral membrane (Zhu et al., 2003). The initial interaction between virus and cells allows for multiple interactions between cell adhesion proteins on the surface of HIV-1 virions and their receptors on the target membrane. Additionally charge–charge interactions, between basic structures on the virus (e.g. the V3 loop) and polyanionic heparin sulfate proteoglycans on the cell surface, play an important role in mediating the initial interaction (Mondor et al., 1998). Recent data describing the interaction of influenza virus with cells shed some light on possible mechanisms of virus-induced endocytosis. It was observed that influenza virus internalization is mediated by the de novo formation of clathrin coated pits (CCPs) indicating that virus engagement generates a signal that is transduced across the PM initiating the formation of CCP specifically at these sites (Rust et al., 2004). Given our disparate results observed with HIV-1 versus monomeric gp120 molecules we suggest that multivalent HIV binding to cells results in similar signaling events, which are enhanced due to ceramide upregulation.

Understanding the mechanism by which fenretinide inhibits HIV infections has important implications for the development of therapeutic approaches to both inhibit and treat HIV infection. Inhibiting viral infection by diverting the virus from productive fusion at the plasma membrane to the endocytic pathway is a novel approach targeting the viral entry process. We anticipate that this knowledge can be further exploited for the development of antiviral agents.

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