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Enhanced anti-HIV efficacy of Indinavir after inclusion in CD4 targeted lipid nanoparticles

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Abstract

Background—Combination drug therapy has reduced plasma HIV to undetectable levels; however, drug sensitive virus persists in patients' lymphoid tissue. We have reported significant lymphoid tissue drug localization with Indinavir-associated lipid nanoparticles (LNPs). Our current objective is to evaluate whether additional enhancement is achievable by targeting these particles to CD4⁺-HIV host cells.

Methods—We characterized two peptide-coated (CD4-BP2 and CD4-BP4) drug-associated LNPs and demonstrated CD4-cell specificity. Drug-associated LNPs expressing polyethyleneglycol were exposed on HIV-2 infected cells under dynamic conditions that emulated lymph node physiology for 15, 30, and 60 minutes at concentrations from 0–25 μ M and evaluated for antiviral activity and cell-associated drug concentrations. The specificity of CD4-mediated enhancement of Indinavir-LNPs antiviral activity was evaluated by blocking with anti-CD4 antibody.

Results—Inclusion of CD4 binding peptides on LNPs enhanced antiviral activity for all incubation conditions, compared to control particles or soluble drug (e.g., 60 min exposure, EC₅₀=0.12–0.12 vs. 0.46 μ M for targeted nanoparticles vs. soluble drug). The CD4-BP4 peptide exhibited higher efficiency in eliciting antiviral activity than CD4-BP2 coated particles (EC₅₀=7.5 μ M vs. >25 μ M at 15 min drug exposure). This enhancement appears to be driven by CD4 availability and cell-associated Indinavir concentrations, as blocking of CD4 significantly ablated Indinavir efficacy in targeted particles and Indinavir concentrations reflected the observed anti-HIV activity.

Conclusions—We constructed CD4-targeted LNPs that provide selective binding and efficient delivery of Indinavir to CD4⁺-HIV host cells. Inclusion of polyethyleneglycol in LNPs would minimize immune recognition of peptides. The enhancement of anti-HIV effects is effective even under limited time exposure.

Keywords

targeted drug delivery; HIV; CD4; nanoparticles

Introduction

Although highly active anti-retroviral therapy (HAART) combination treatment is effective in reducing viral loads to undetectable levels in the blood of human immunodeficiency virus (HIV)-infected patients and prolonging survival,¹ significant amounts of drug sensitive HIV remain in the lymphoid tissues.^{2–4} We and others have demonstrated that insufficient drug

exposure to the susceptible cells in lymphoid tissue is likely one of the key factors in the inability of traditional HAART to completely eliminate virus from this tissue.⁵⁻⁷

We previously developed a lipid nanoparticle (LNP) preparation containing HIV protease inhibitors that rapidly accumulate in lymph nodes of HIV-2 infected macaques, first, near the injection site and subsequently through all nodes, which delivers significantly higher drug concentrations in lymph nodes, far exceeding those given by oral or subcutaneous soluble drugs.⁸ LNP-mediated enhanced drug delivery significantly reduced virus load and slowed the rate of CD4⁺ T-cell decline in primate HIV models.⁶

Although we have significantly increased accumulation of drug in lymphoid tissue, this enhancement is only tissue specific. Less than 30% of the lymph node cells are HIV-target CD4⁺ cells;⁹ however, all cells in lymph nodes are likely exposed to drug with our current formulations. Consequently, it is desirable to enhance drug exposure to HIV-infected cells within lymphoid tissue by directing HIV drug only to virus host cells, which could be achieved by selectively directing LNP to CD4⁺ T-cells. Targeting nanoparticles for specific anti-HIV drug delivery has previously shown to enhance accumulation of particles on target cells and, in some cases, increased concentrations of antiviral drugs to those cells *in-vitro*, but the combination of targeting drug-associated particles and examining the effects on HIV-infected cells has to be fully characterized.¹⁰⁻¹⁸

Therefore, we have designed and characterized drug loaded LNP targeted to CD4 cells. Our results suggest that CD4 binding peptides incorporated onto nanoparticles provides binding selectivity to CD4⁺ cells. Further, we evaluated these CD4-targeted lipid nanoparticles (TLNP) loaded with Indinavir for effects on anti-HIV efficacy as compared to untargeted LNP and soluble drug preparations. Our results suggest that the antiviral potency of TLNP is dependent on CD4 availability on host cells and is mediated by enhanced localization of the HIV protease inhibitor, Indinavir, to elicit antiviral effects. This anti-HIV TLNP delivery system also reduced the cell contact time required to elicit antiviral effects. These results demonstrate the potential of this strategy to further enhance delivery of HIV drugs to CD4⁺ HIV-host cells in the lymph nodes and lymphoid tissues.

Materials and Methods

Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy-polyethylene glycol-2000 (DSPE-mPEG-2000), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-maleimide-polyethylene glycol-2000 (DSPE-Mal-mPEG-2000), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). CD4 Binding Peptide (CD4-BP) characteristics are listed in Table 2 and were synthesized and purified to >95% by Genscript (Piscataway, NJ). CEMx174 cell lines were obtained from American Type Culture Collections (Manassas, VA). RPMI-1640 medium, phosphate buffered saline (PBS), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Indinavir was kindly provided by Merck and Company (Whitehouse Station, NJ). Other reagents were analytical grade or higher.

Lipid Nanoparticle Preparation

Briefly, anti-HIV LNP were prepared by sonication as previously described.⁶ Lipids and drug were dissolved together in methanol and chloroform (1:1 v/v) in a sterile glass test-tube. The mixture was then dried under nitrogen gas into a thin film, which was vacuum desiccated overnight at room temperature. The DSPC:DSPE-mPEG-2000:DSPE-Mal-

mPEG-2000 (8:0.8:0.2), with and without 1mol percent NBD-PE, and Indinavir (3:1 total lipid to drug) mixture was then rehydrated in 0.9% NaCl, 10mM NaHCO₃ buffer at pH 7 for 20 minutes and the lipid complex size was reduced to approximately 100nm by sonication in a bath type sonicator. Mean nanoparticle size distribution was determined by photon correlation spectroscopy with a Zetasizer 5000 (Malvern Instrument; Worcestershire, UK) with an argon laser operating at 633 nm. Drug loading was evaluated after separation of lipid associated and free drug by dialysis and followed by measuring drug concentration with a validated liquid chromatography and mass spectrometry (LC-MS) method as described previously.⁸

Lipid Nanoparticle with Peptide for Cell Targeting

To prepare CD4-targeted LNP, we employed the two candidate peptides with proven affinity to CD4 molecules (see table, Supplemental Digital Content 1). To coat the peptides on LNP, we used DSPE-Mal-mPEG-2000 which contains a maleimide linker attached to PEG and allows for covalent coupling to peptides with a terminal cysteine to form a thioether linkage (see figure, Supplemental Digital Content 2).

Covalent coupling of lipid to peptide occurs optimally at pH 6.5 and is not enzymatically degraded or affected by reducing agents. Covalent coupling of peptides to lipid nanoparticles containing DSPE-Mal-mPEG-2000 is accomplished in rehydration buffer after the nanoparticle preparation described above.

For peptide coupling to LNP with DSPE-Mal-mPEG-2000, 100 µg of peptide and 1 µm of lipid nanoparticles are combined in 100 µL of rehydration buffer at pH 6.5 at room temperature and are agitated for 8 hours. To remove any unbound peptide, nanoparticles were dialyzed against 1L of buffer. The dialysis buffer was replaced 4 times every hour. Peptide incorporation efficiency was 82±2 and 89±7 percent for CD4-BP2 and BP4 as measured by DC protein assay (Bio-Rad; Hercules, CA).

Evaluation of Anti-HIV Efficacy In Vitro

To determine the anti-HIV efficacy of TLNP, 10⁵ CEMx174 cells were incubated with 0–25 µM (0, 0.02, 0.05, 0.1, 0.2, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, and 25) Indinavir suspension either in soluble or TLNP formulation for 15, 30, or 60 minutes at 37°C. Cells were then washed twice in PBS, resuspended in media and infected with HIV-2₈₇ at 0.1 Multiplicity of Infection for 1 hour. Cells were then washed 3 times to remove unadsorbed virus, resuspended in media, and 8 replicates were plated into a 96-well tissue culture plate and incubated at 37°C for 4 days. The presence of virus-infected cells in each replicate well was determined by the development of syncytia and percent infected relative to the total was calculated. This assay has been validated with a p24 HIV antigen assay for viral infectivity.⁸

Inhibition of Targeted Lipid Nanoparticle Mediated Enhancement of Indinavir Efficacy

To determine the specificity of the enhanced antiviral effect of the TLNP-associated Indinavir formulation 10⁵ cells were infected with HIV-2 and washed as described above, then incubated with 5 µM Sim2 CD4 antibody (NIH AIDS Research and Reference Reagent Program; Germantown, MD) for 1 hour at 37°C. Subsequently, cells were washed and incubated for 30 minutes with 6.25, 12.5, and 25 µM of Indinavir, either soluble or TLNP-associated, washed, and infection was monitored as described above.

Cell Associated Indinavir Concentration

10⁵ CEMx174 cells were resuspended in 1mL of RPMI-1640. Cells were incubated with 6.25 or 25µM Indinavir, either soluble or TLNP-associated, for 30 minutes at 37°C. Cells were washed and half of the cells were immediately extracted, half were incubated at

37°C and extracted on day 4 post-drug incubation. To extract drug, cells were pelleted, then lysed in 50 μ L of acetonitrile in the presence of 50 μ g/mL ritonavir as an internal control. After lysis, 50 μ L of water was added to each sample. 10 μ L of each sample was injected onto a 5 μ m, 2.1 $\times$ 50-mm Zorbax SB-C18 column (Agilent Technologies, Palo Alto, CA). The mobile phase consisted of a 0.1% acetic acid solution in methanol. The flow rate was set at 0.25 mL/minute. The mass spectrometer (Applied Biosystems 3200 Q Trap; Foster City, CA) was operated in atmospheric pressure ionization-electrospray ionization (API-ESI⁺) mode, and the analytes were detected using selected ion monitoring at m/z 603.7–623.7 to detect Indinavir.

Evaluation of Temperature Dependent Binding of Targeted Nanoparticles

To determine the temperature dependent binding profile of TLNP to cells, 10 nmol (total lipid or equivalent) of TLNP preparations or 2 μ L of RPA-T4 CD4 antibody were incubated with 5×10^4 CEMx174 cells in 50 μ L of PBS for 2 hours at 4, 22, or 27°C with agitation every 15 minutes. Cells were then washed 4 times in PBS with 1% FBS and qualitative nanoparticle binding was determined by fluorescence microscopy and imaging analysis software (Zeiss; Thornwood, NY). Images are representative of at least four separate experiments.

Statistical Analysis

Data was analyzed for statistical significance using Student's 2- sided *t*-test with significance at $p < 0.05$.

RESULTS

Evaluation of Drug Associated Targeted Nanoparticle Antiviral Efficacy

To evaluate the effect of Indinavir in TLNP, LNP, and soluble drug on the inhibition of HIV infection, we exposed infected CEMx174 cells to drug formulations for varying amounts of time and monitored the development of infection at day 4. The TLNPs were prepared with two CD4-binding peptides, CD4-BP2 or CD4-BP4. As shown in Table 1, TLNP prepared with these peptides are selective for CD4⁺ cells. Control or irrelevant peptide coated LNP bind to CD4 cells with lower efficiency. In addition, the TLNP binding was inhibited with anti-CD4 antibody Sim2. CD4-BP2 and CD4-BP4 empty TLNP were also included as controls to test if occupancy of the CD4 receptor by these preparations would have drug-independent antiviral effects. To simulate the dynamic conditions within lymph nodes, we exposed HIV infected cells for 15 (Figure 1A), 30 (B), or 60 minutes (C).

At 60 minutes of exposure, Indinavir-associated with TLNP was approximately three times as potent as soluble drug (0.12–0.13 compared to 0.49 μ M EC₅₀; see table, Supplemental Digital Content 3) and no differences between targeted Indinavir-associated LNP anti-HIV effects were apparent. However, as drug exposure times decreased, greater differences between the preparations were discernable. Figure 1A shows that after a 15 minute incubation of drug preparations with cells, only the CD4-BP4 TLNP had a significant anti-HIV effect from which an EC₅₀ of 7.52 ± 0.76 μ M could be estimated. This is also the case after a 30 minute incubation (panel B), with only the drug associated CD4-BP4 TLNP having sufficient HIV inhibition to allow calculation of an EC₅₀ value of 6.47 ± 0.21 μ M.

Although soluble drug and drug associated CD4-BP2 TLNP show some viral inhibition at higher concentrations of 12.5 and 25 μ M after 30 minute incubations, neither reached lower than 50% uninfected cells (Figure 1). Collectively, these data indicate that after incubation of cells with soluble Indinavir and TLNP for 60 minutes, significant viral inhibition was detected. Between the two Indinavir-associated TLNP, only the TLNP with CD4-BP4, and

not CD4-BP2, provided a significant degree of reduction in HIV infection within 0–25 μM Indinavir range after short 15–30 minute incubations. Soluble Indinavir, Indinavir-associated CD4-BP2-TLNP and control LNP formulations of Indinavir were ineffective in this concentration range.

Anti-CD4 Antibody Mediated Inhibition of Targeted Nanoparticle Enhancement of Indinavir Efficacy

To determine whether the observed enhancement in Indinavir efficacy is due to CD4 recognition by the TLNP, we used anti-CD4 monoclonal antibody, Sim2 to inhibit TLNP effects on antiviral activity. Sim2 antibody has been reported to inhibit HIV infection and syncytial development in CD4⁺ HIV host cells when continually exposed in culture.¹⁹ Figure 2 compares the difference between drug treated cells at three concentrations (Panels A–C) in the presence or absence of Sim-2. As expected, all untreated but HIV infected cells were 100% positive for viral infection, as were cells that were infected and then blocked with antibody but left untreated. At the three soluble Indinavir concentrations tested, there is no statistically significant difference in HIV infection between cells blocked with Sim-2 antibody or unblocked. In contrast, in Indinavir-associated TLNP, pre-blocking HIV-infected cells with Sim-2 antibody reverse the nanoparticle-mediated enhanced antiviral effect (Figure 2). This is most dramatically observed in panel A where unblocked CD4-BP4 TLNP at 25 μM Indinavir prevented infection in 100% of cells tested; however, by pre-blocking with antibody this is reduced to only about 13% negative for infection. Pre-blocking with anti-CD4 antibody did not significantly change the antiviral effect of soluble Indinavir, as soluble drug entry is not mediated by CD4. Pre-blocking with anti-CD4 antibody produced a consistent and significant reduction in TLNP-mediated enhanced Indinavir effect across all studied concentrations (Figure 2). As shown in Figure 2A, CD4-BP2-TLNP effect is completely inhibited by antibody blocking. In Panel B, the magnitude of the effect is reduced but significant after incubation of 12.5 μM Indinavir. CD4-BP4-TLNP was able to inhibit infection in 50% of the cells without blocking and 0% after blocking. Approximately 75% of cells were infected after CD4-BP2-TLNP incubation without blocking and 100% with blocking. Lastly, Panel C shows that at 6.25 μM Indinavir, soluble Indinavir had no effect on infection. CD4-BP4-TLNP infected cells moved from approximately 63–100% and CD4-BP2-TLNP from 75–100%. Taken together, this data suggests that the observed enhancement of drug efficacy is mediated by access to CD4 as blocking of CD4 with antibody Sim-2 abrogated the antiviral enhancement mediated by the TLNP.

Targeted Nanoparticle Mediated Enhanced Cell Associated Indinavir Concentrations

To determine if the TLNP mediated enhancement of Indinavir efficacy was due to increased cell-associated drug concentrations, we incubated uninfected cells with low (6.25 μM) and high (25 μM) concentrations of soluble Indinavir, Indinavir-associated LNP, or Indinavir-associated CD4-BP2 or CD4-BP4 TLNP for 30 minutes or 4 days. Cell-associated drug concentrations were measured by mass-spectrometry.

In all cases, the TLNP significantly enhanced delivery of drug to cells compared to LNP (Table 2). With a 30 minute incubation and wash, very little cell associated drug was detected after incubation of LNP at both low (6.25 μM) and high (25 μM) concentrations 34.24 ng/10⁵ cells and 101.92 ng/10⁵ cells, respectively. In contrast, cell associated drug levels were comparable between the two TLNP preparations and soluble drug (approximately 2700–6600 ng/10⁵ cells). Compared to LNP, soluble drug delivered 82 and 36 times more drug to cells. In the case of TLNP, CD4-BP2 particles delivered 91 and 35 times more Indinavir to cells while CD4-BP4 particles delivered 105 and 65 times as much. CD4-BP4 coated TLNP exhibit approximately 2 times higher cellular drug concentrations at

25 μM , which extends to 4 days post exposure. These relative results were similar after incubation of cells for 4 days, although a statistically higher percentage of drug may stay associated with cells when drug was associated to TLNP compared to soluble drug alone.

Evaluation of Temperature Dependent Binding of Targeted Lipid Nanoparticles

To investigate the potential mechanism of Indinavir entry into cells, we examined the binding of fluorescently labeled TLNP and CD4 antibody to CD4⁺ cells after incubation at low, moderate, and physiological temperatures. At 4°C, little binding of the TLNP was evident as cellular activity and membrane fluidity is low (Figure 3A). However, at room temperature, concentrated or punctated regions of TLNP were observed, suggesting active binding and capping of TLNP to the pole of the cell (Panel C). At 37°C, TLNP appears generally diffuse with concentrated punctation, possibly indicating internalization (Panel E). In contrast, no observable difference was observed after incubation of CD4 antibody at the three temperatures, suggesting a different binding mechanism and no apparent internalization of antibody into cells (Panels B, D, and F).

DISCUSSION

Although current antiretroviral drug combinations are effective in eliminating detectable virus from the blood, active virus persists in tissues with limited blood perfusion, such as lymphoid tissues. We have previously shown that directing HIV protease inhibitors to these sites via LNP increases concentrations of drug in these tissues *in-vivo*, which correlated with a reduced rate of loss of lymphocytes and decreased tissue viral loads.⁸

These encouraging results led us to further probe the therapeutic potential of drug delivery to lymph nodes by LNP. Currently, we have assessed whether adding an additional level of recognition, in the form of peptides that bind CD4, could further enhance drug efficacy in HIV host CD4⁺ cells. We have previously demonstrated the feasibility of selective localization to CD4 expressing lymphocytes and that these TLNP preparations are specific to CD4 *in-vitro* with significant binding affinity to CD4⁺ cells.

In this report we have evaluated enhancement of antiviral efficacy under conditions modeled to more closely reflect the dynamic environment in lymph nodes *in-vivo*. Our examination of the therapeutic potential of Indinavir-associated TLNP was with variable and limited times of drug exposure to simulate *in-vivo* conditions with flow and circulation. These conditions are significantly different from typical antiviral assays where drugs are statically exposed for the duration of the assay (e.g., 3–7 days) to determine antiviral activity.²⁰ We found that under conditions of dynamic drug exposure, CD4-BP4 TLNP provided significantly higher antiviral effect, compared to CD4-BP2 TLNP and soluble drug (Figure 1 and Supplemental Digital Content 3). The calculated EC₅₀ for soluble Indinavir after an hour incubation is similar to the value we have obtained previously⁸ when the tenfold increase in administered virus is accounted for; however, it appears that for LNP mediated effects to manifest, a longer incubation time is required. As enhanced antiviral effect in target cells is our primary goal, for subsequent *in-vivo* testing of these TLNP preparations, CD4-BP4 will be the primary candidate while CD4-BP2 will be the secondary.

To confirm that increased cell associated drug concentrations due to active delivery of drug by TLNP was the primary explanation for enhanced Indinavir efficacy, we examined cell-associated Indinavir concentrations after incubation of cells with drug in soluble form, or associated with LNP or TLNP. We found that cellular drug concentrations paralleled anti-HIV effect for all drug preparations at the dose range studied. Interestingly, it appears that approximately 94% of the dose of drug at 6.25 μM was recovered from cells incubated with CD4-BP4 TLNP versus 81% from CD4-BP2 TLNP, and 72% after soluble incubations,

respectively. This could account for the significant differences in antiviral efficacy in these preparations.

To ascertain whether these accumulation profiles were maintained after incubation, we also examined cellular Indinavir levels after cells had incubated for 4 days. Although all cellular drug concentrations are significantly lower, indicative of redistribution of a portion of the initially bound drug to the surrounding space and several rounds of cell division, the proportion of drug remaining cell-associated from the TLNP preparations is significantly higher than soluble drug. This suggests that in addition to enhanced delivery of drug to the cell, that cellular retention may also be enhanced; however, this requires verification. This enhanced association and accumulation of TLNP and delivery of drug to the cell is likely a major mechanism of the observed enhanced anti-viral effect of TLNP. To begin to examine the potential mechanism of drug accumulation in cells we performed targeted lipid nanoparticle binding at increasing temperatures to capture changes in binding profile (Figure 3). As incubation temperatures increased, the pattern of TLNP association to cells significantly changed. At low temperatures, cell activity and membrane fluidity are low, and as a result, a low level of dispersed binding of TLNP was observed. At room temperature, TLNP appeared to accumulate at the poles of cells, suggesting multivalent binding and a capping response by the cells. At physiological temperatures, capping is not evident; however, areas of punctate and diffuse fluorescence are observed, possibly indicating internalization of the lipid nanoparticles after capping. Given the previously described pH sensitive drug release of Indinavir, these data may assist in explaining the mechanism of enhanced drug accumulation seen in the TLNP preparations.

In contrast to our previously published studies of untargeted drug associated LNP *in-vitro* and *in-vivo*, we did not observe any antiviral effect attributable to these particles in these current experiments. This is explained *in-vitro*, by differences in the method of exposing drug to the target cells. In the previous studies, soluble or lipid associated drug treatments were applied to infected cells and remained for the duration of the experiment, while in the current study as discussed, cells were washed before incubation. Because an increase in drug accumulation is observed *in-vivo* after drug associated LNP administration, we can hypothesize that although flow through the lymph node tissue may be an important factor in drug disposition and accumulation, especially when considering specific targeting to HIV host cells, “trapping” of the LNP is also likely an important factor in general accumulation in the tissue. The impact of trapping as it relates to the disposition and accumulation of the TLNP to the CD4 cell target remains to be determined.

Although peptides expressed on lipid particles can exhibit significant immunogenicity *in-vivo*, we have included a polyethylene glycol (PEG) conjugated to lipid, DSPE-mPEG-2000 (10 mole percent), in our TLNP design to address this issue. Unlike typical preparations of peptide expressed on the surface of lipid nanoparticles that enhance immunogenicity, lipid nanoparticles with surface PEG are not taken up by phagocytic cells, such as macrophages and dendritic cells, thus greatly reducing immunogenicity potential. The role of PEG in reducing recombinant protein immunogenicity has been documented clinically with a number of therapeutic enzymes including adenosine deaminase (PEGADEMASE)²¹ and L-asparaginase.²² Thus, peptide coated TNP immunogenicity, while probable, would likely be minimal.

While the exact mechanism of CD4-targeted nanoparticle mediated enhancement of drug efficacy is not clear, it is likely that the anti-HIV drug loaded in these particles requires cell interaction and CD4 binding with subsequent internalization and release of Indinavir to inhibit HIV protease function. A possible schematic for this process is presented in Figure 4; however, this and other potential mechanisms remain to be investigated. We envision that

TLNP accumulation and effect is mediated first by the binding of the particles to multiple CD4 molecules on the surface of an HIV host cell (1). Once bound, these particles are then internalized through endocytosis to an endosome (2). Due to the pH dependent lipophilicity and lowered pH of an endosome, significant drug release from the nanoparticles occurs (3). Intracellular free drug then acts to inhibit the viral protease and prevent maturation of virus particles in infected cells (4). Alternatively, drug release can occur due to lowered extracellular pH during active virus infection. This drop in pH could also allow Indinavir release and subsequent cell penetration.

In summary, we have constructed CD4 targeted LNP that provide selective binding to CD4⁺ cells and efficiently delivers drugs to these cells, which significantly enhances anti-HIV effects even when subjected to dynamic washout conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Concentration and time-dependent effects of CD4-targeted LNP in enhancing anti-HIV efficacy of Indinavir-associated LNP. Targeted and untargeted, Indinavir associated and empty lipid nanoparticles, and soluble Indinavir were incubated with cells for 15 min (Panel A), 30 min (Panel B), 60 min (Panel C) in quadruplicate in 96-well plates. Cells were then washed and infected at 0.1 MOI HIV-2. Unadsorbed virus was washed away and cells were incubated for 4 days at 37°C. The percentage of HIV-2 infected cells was evaluated on day 4 as described in the Materials and Methods. Data expressed were mean $\pm$ SD of quadruplicate samples from 4 experiments.

Figure 2.

CD4 specific inhibition of targeted lipid nanoparticle enhancement on Indinavir anti-HIV efficacy. To determine CD4-dependent enhancement of targeted Indinavir LNP, 10^5 CD4-positive cells infected with HIV-2₂₈₇ were preincubated with indicated CD4 antibody Sim2 for 1 hour at 37°C. Cells were then incubated for 30 minutes with 25 (Panel A), 12.5 (Panel B), and 6.25 μ M (Panel C) of Indinavir in soluble and targeted LNP. The percentage of infected cells was determined as described in the Materials and Methods. Data expressed were Mean $\pm$ SD of quadruplicate samples from four experiments. Unblocked CD4-BP4-LNP, CD4-BP2-LNP, and soluble Indinavir mediated antiviral effect is shown in white bars; Antibody blocked CD4-BP4-LNP, CD4-BP2-LNP, and soluble Indinavir mediated effects are shown in black bars. *: Significantly different from unblocked treatment at $p < 0.05$

Figure 3.

Qualitative fluorescence microscopic analysis of temperature dependent binding of targeted lipid nanoparticles or RPA-T4 antibody to CD4⁺ Molt-4 cells. 5×10^4 cells were incubated with 10 nmol of nanoparticles and 2 μ L of RPA-T4 CD4 antibody for 2 hours at 4°C, 22°C, or 37°C, washed, and observed for fluorescence. **Column A:** RPA-T4 Ab, **B:** Targeted Nanoparticles (TNP). Images are representative of at least four separate repeat experiments.

Figure 4.

Proposed mechanism of targeted lipid-Indinavir nanoparticle mediated cellular accumulation and inhibition of HIV in CD4⁺ cells. It is possible that CD4 targeted peptides coated on lipid nanoparticles can bind to CD4 receptors and some particles may be endocytosed while others could release drug at the cell surface in a pH dependent manner. The fraction of release will depend on the pH at the cell surface. Endocytosed nanoparticles can release stable drug either in the endosome or lysosome. The lower pH conditions protonate Indinavir and allow release from lipid into the cytoplasm. In virally infected cells, HIV protease inhibitors will mediate antiviral effects while no effect will occur in uninfected cells lacking HIV protease. Specific proposed steps as follows: 1. Binding of Indinavir (INV)-associated targeted lipid nanoparticles (TNP) to CD4 receptors. 2. Endocytosis of targeted LNP to endosome. 3. pH dependent release of Indinavir from LNP. 4. Indinavir inhibits HIV protease and prevents virus maturation. Inset: Scheme of pH-sensitive release from lipid-Indinavir nanoparticles (Indinavir, stars). Not drawn to scale.

Table 1Specific binding of nanoparticles to CD4⁺ cells^a

Percentage of Nanoparticles Bound to CD4 ⁺ cells decorated with respective peptides (in arbitrary fluorescence units)				
Inhibitor	Control (No peptide)	Untargeted peptide	CD4-BP2 peptide	CD4-BP4 peptide
None	42.3±0.1	43.8±0.6	85.8±14.6 [*]	59.7±6.6 [*]
CD4 Antibody (Sim2)	ND	ND	43.7±1.0	47.5±2.0

^aTo determine the extent of CD4 dependent nanoparticle binding to cells, 10⁵ CEMx174 cells were pre-incubated with 10-fold molar excess of anti-CD4 antibody Sim2 for 1 hour at 4°C, washed, then incubated with 15 nmol of fluorescent labeled nanoparticle preparations in 100 µL for 30 minutes at 4°C. Cells were washed and inhibition of binding was quantified by changes in fluorescent quintuplicate.

^{*} Significant at p<0.05 compared to untreated control cells.

Table 2Intracellular Indinavir concentrations after incubation with soluble or lipid associated Indinavir nanoparticle^a.

Indinavir Formulation	Intracellular Indinavir (ng/10 ⁵ CEMx174 cells)			
	Day 0		Day 4	
	6.25 μ M	25 μ M	6.25 μ M	25 μ M
Soluble	2,774 $\pm$ 53	3,772 $\pm$ 1,066	53 $\pm$ 1	124 $\pm$ 4
<u>DSPC: DSPE-mPEG</u>				
Untargeted	34 $\pm$ 10 *	102 $\pm$ 3 *	7 $\pm$ 0.1 *	5 $\pm$ 0.4 *
CD4-BP2-LNP	3,105 $\pm$ 164	3,584 $\pm$ 62	93 $\pm$ 1 *	148 $\pm$ 1 *
CD4-BP4-LNP	3,584 $\pm$ 62 *	6,586 $\pm$ 328 *	162 $\pm$ 4 *	282 $\pm$ 5 *

^aTo quantify Indinavir levels after enhanced delivery to cells with LNPs, triplicate 10⁵ CEMx174 cells were incubated with 6.25 or 25 μ M Indinavir formulations, for 30 minutes at 37°C, washed, pelleted, then lysed in 100 μ L of acetonitrile and 100 μ L of water was added to each sample. 10 μ L of each sample in duplicate was injected onto a Zorbax SB-C18 column with mobile phase consisting of a 0.1% acetic acid solution in methanol. The mass spectrometer was operated in atmospheric pressure ionization-electrospray ionization (API-ESI⁺) mode, and the analytes were detected using selected ion monitoring at m/z 603.7–623.7 to detect Indinavir.

* Significant at p<0.05 compared to soluble drug.