Research Article

Design and Characterization of Novel Peptide-Coated Lipid Nanoparticles for Targeting Anti-HIV Drug to CD4 Expressing Cells

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Abstract. Human immunodeficiency virus (HIV) persists in lymph nodes and lymphoid tissues even during aggressive drug treatment, likely due to insufficient drug concentrations at this site. Therefore, to eliminate this residual virus, methods that enhance lymph node drug concentrations are currently being evaluated. Although enhanced drug concentrations in tissue have been achieved with drug-associated lipid nanoparticles, targeting these particles to CD4⁺ cells may provide specific delivery of drug to HIV target cells and further enhance drug efficacy. We have evaluated four candidate peptides with reported binding specificity to CD4 for anchoring on lipid nanoparticle preparations previously shown to localize in lymph nodes. Terminal cysteine containing candidate peptides were conjugated to lipid nanoparticles through maleimide-linked phospholipids for targeting to CD4 cells. Using fluorescently labeled lipid nanoparticle binding to cells with varying degree of CD4 expression (CEMx174, Molt-4, Jurkat, and Ramos), we identified two peptide sequences that provided CD4 selectivity to nanoparticles. These two peptide candidates on lipid nanoparticles bound to cells corresponding to the degree of CD4 expression and in a peptide dose dependent manner. Further, binding of these targeted lipid nanoparticles was CD4 specific, as pre-exposure of CD4⁺ cells to anti-CD4 antibodies or free peptides inhibited the binding interactions. These results indicate targeting of lipid nanoparticles for specific binding to CD4 can be accomplished by tagging CD4 binding peptides with peptides, and these results provide a basis for further evaluation of this targeted delivery system to enhance antiviral drug delivery to CD4⁺ HIV host cells, particularly those in lymph nodes and lymphoid tissues.

KEY WORDS: CD4 targeting; HIV; lipid nanoparticle; peptide; targeted drug delivery.

INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) infections continue to be a major global concern with 33.4 million people infected; the majority of whom are in developing countries (1). Highly active anti-retroviral therapy (HAART) combination treatment has been effective in reducing viral loads to undetectable levels in the blood of many patients and prolonging survival for many years (2). However, because patients are on these therapies long-term, drug failure is becoming a more serious concern, with recent reports of up to 50% failure (3). A major tissue pool of HIV during HAART therapy is in the lymph nodes and lymphoid tissues where the majority of HIV target cells reside. Virus isolated from lymphoid tissues, blood CD4⁺ T cells, and plasma are all equally sensitive to anti-HIV drugs (4,5), particularly protease inhibitors (6). These data suggest that even at effective plasma drug concentrations, insufficient drug exposure to lymphoid tissue may be one of the key factors in the inability to completely eliminate residual virus. In support of this hypothesis, we and others have shown that intracellular levels of the protease inhibitor indinavir in mononuclear cells of

lymphoid tissues were significantly less than those cells in blood (7–9). As 98% of circulating lymphocytes reside in the lymphatic system (10), these lower and likely sub-therapeutic drug levels in the lymphatic system may allow low and persistent levels of viral replication and increase the probability of developing and harboring drug resistant virus.

To address the apparent drug insufficiency in the lymph node and lymphoid tissues, we have previously developed a lipid nanoparticle (LNP) preparation modified with polyethelene glycol (PEG) containing the HIV protease inhibitor indinavir and have achieved high levels of lymph node accumulation (over 2,000% more than free drug) (11). After subcutaneous administration to HIV-2-infected macaques, LNPs rapidly accumulate first in local lymph nodes and then to all nodes throughout the body and deliver much higher concentrations of drug than either oral or subcutaneously administered soluble drug for at least 24 h. Additionally, these LNP drug carriers show 50% indinavir release at pH 5.5 (similar to the pH of an endosome (12)), which may be a primary release mechanism. When tested in an HIV-2₂₈₇-infected macaque model, lipid complexes containing indinavir were found to have enhanced drug delivery and paralleled reductions in virus load and rate of CD4⁺ T cell decline (8).

Although we have achieved significantly increased accumulation of drug in lymphoid tissue, targeted enhancement of drug accumulation may be possible. Within a lymph

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node, less than 30% of the total cells are HIV target cells expressing the major HIV receptor CD4 (13); however, all cells are likely exposed to drug with our current formulations. Therefore, it may be possible to increase drug selectivity to HIV host cells in lymphoid tissue by directing drug loaded nanoparticles to HIV host CD4⁺ T cells. This could result in enhanced drug accumulation and prolonged residence time in CD4⁺ T cells, which are the major host of HIV infection and replication. In addition, dendritic cells and macrophages, both low-level carriers of HIV, also express low but detectable levels of CD4, and may take up targeted LNPs phagocytically.

Previous efforts targeting an HIV drug carrier *in vitro* to infected cells with soluble CD4 (14,15), CD4-derived peptides (16), gp120 antibody fragments (17), mouse anti-HLA-DR antibody Fab fragments (18,19), and mannan or mannose (20–22) have been able to enhance accumulation of carriers on target cells, and in some cases, increase concentrations of antiviral drugs. However, the combination of targeting drug-associated nanoparticles and examining the effects on cellular HIV has not been systematically studied, particularly those for *in vivo* targeting within the cells in lymphoid tissues.

Therefore, we have designed and evaluated targeted LNPs utilizing four peptides previously reported to bind selectively to CD4 molecules (23,24). These peptides conjugated to lipid head groups are incorporated into fluorescent LNPs and were characterized based on size, peptide incorporation, indinavir association, stability, and binding to cell lines expressing varied levels of CD4 *in vitro*. Our results show that CD4 binding peptides can be efficiently incorporated onto drug-associated LNPs. Evaluation of candidate peptides for binding to CD4⁺ cells suggests that binding of nanoparticles to target cells is dependent on peptide inclusion and sequence, and exhibit selectivity and specificity for CD4-expressing cells.

MATERIALS AND METHODS

Materials

Cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[4-(p-maleimidophenyl)butyramide] (MPB-PE), Egg-phosphatidyl choline (EPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), 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) were purchased from Avanti Polar Lipids (Alabaster, AL). CD4 binding peptide (CD4-BP) characteristics are listed in Table I

and were synthesized and purified to >95% by Genscript (Piscataway, NJ). Molt-4 and CEMx174 cell lines were obtained from American Type Culture Collections (Manassas, VA). Ramos cell lines were obtained from NIH AIDS Research and Reference Reagent Program (Germantown, MD). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum purchased from Invitrogen (Carlsbad, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen; Carlsbad, CA). Indinavir was kindly provided by Merck and Company (Whitehouse Station, NJ), RPA-T4 CD4 antibody was purchased from Pharmingen (San Diego, CA). Other reagents were analytical grade or higher.

Lipid Nanoparticle Preparation

Anti-HIV LNPs were prepared by sonication as previously described (8). Briefly, lipids and drug were dissolved in methanol and chloroform in a glass vial. The lipid/drug was then dried under nitrogen gas into a thin film, which was vacuum-desiccated overnight at room temperature. The lipid drug mixture was then rehydrated in 0.9% NaCl, 10 mM NaHCO₃ buffer at pH 7 for 20 min, and the lipid complex diameter was reduced to approximately 100 nm by sonication in a bath type sonicator for 15 min. Mean nanoparticle size distribution was determined by photon correlation spectroscopy with a Zetasizer 5000 (Malvern Instrument; Worcester-shire, 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 with a validated liquid chromatography and mass spectrometry method as described previously (11). Data are presented as mean ± standard deviation. Maintained stability was defined as no significant difference in subsequent measurements compared with those made upon preparation.

Lipid Nanoparticle with Peptide for Cell Targeting

MPB-PE and DSPE-Mal-mPEG 2000 contain a maleimide linker attached to the head group of the phospholipid, which allows for covalent coupling to antibodies or peptides with a terminal cysteine amino acid to form a thioether linkage. Covalent coupling of lipid to peptide occurred optimally at pH 6.5 and was not enzymatically degraded or affected by reducing agents (Fig. 1). Covalent coupling of peptides to LNPs was accomplished under aqueous conditions using a 0.9% NaCl, 10 mM NaHCO₃ aqueous buffer after the nanoparticle preparation described above.

For peptide coupling to preformed LNPs, 100 µg of peptide and 1 µM LNPs were combined in 100 µL of buffer at

Table I. Peptides with CD4 Recognition Sequences for Targeting of Lipid Nanoparticles

CD4-BP	A.A.	Peptide sequence	Description
1	16	CKSIHIGPGRAFYTGTG	HIV-IIIB gp120 V3 ^a
2	16	CKGIRIGPGRAYAAE	Consensus sequence gp120 V3 ^a
3	28	CARRPKFYRAPYVKNHPNVWGPWVAYGP	ST40 CD4 antibody mimetic with linker from P-28 to K-13 ^b
4	28	CARRPKFYRAPYVKNHPNVWGPWVAYGP	ST40 CD4 antibody mimetic ^b

CD4-BP CD4 binding peptide, A.A. amino acids

^a (24)

^b (23)

Fig. 1. Covalent coupling of peptides to MPB-PE containing lipid-nanoparticles. Schematic representation of peptides containing a terminal cysteine with $-SH$ group coupling to nanoparticles containing a proportion of lipid with a maleimide linker. LNPs are prepared with 1.1–2.4 mol% of MPB-PE. Peptides are coupled to nanoparticles at pH 6.5 through covalent binding of the cysteine SH to the maleimide on the lipid, forming a stable thioether linkage. Not drawn to scale

pH 6.5 at room temperature and were agitated for 8 h. To remove any unbound peptide, nanoparticles were dialyzed against 1 L of buffer. The dialysis buffer was replaced every hour, four times total. Peptide incorporation efficiency was measured by DC protein assay according to the manufacturer's protocol (Bio-Rad; Hercules, CA) before and after dialysis.

Cell Binding

To determine the binding profile of LNPs to cells, 10–50 nmol (total lipid or equivalent) of targeted and untargeted LNP preparations with 1 mol% of fluorescent lipid (NBD-PE) or 2 μ L of RPA-T4 CD4 antibody were incubated with 5×10^4 Molt-4 cells in 50 μ L of phosphate-buffered saline (PBS) for 2 h at room temperature with agitation every 15 min. Cells were then washed four times in PBS with 1% fetal bovine serum and qualitative nanoparticle binding was determined by fluorescent microscopy and imaging analysis software (Zeiss; Thornwood, NY).

Concentration Dependent Binding of Nanoparticles to Cells

Targeted and untargeted fluorescent LNPs at concentrations from an estimated 0 to 0.5 nmol peptide were incubated with 8×10^5 cells per well in triplicate of Molt-4 (high CD4), CEMx174 (high CD4), Jurkat (very low CD4), or Ramos (CD4 negative) in 100 μ L of PBS in triplicate in a 96-well plate for 30 min at 4°C. Cells were then washed four times in PBS and resuspended in 100 μ L and fluorescence was measured. Fluorescence values were converted to bound peptide concentrations using the specific activity of the targeted LNPs (fluorescence value per nanomole of peptide offered). The binding affinity of each peptide-targeted LNP formulation was determined by fitting the data to a single site $B_{\max}$ binding model using SigmaPlot (Systat Software; San Jose, CA). Estimated total binding sites were calculated by converting the estimated $B_{\max}$ value to picomoles of bound peptide per cell.

Inhibition of CD4-Mediated Targeted Nanoparticle Binding to Cells

To determine CD4 dependent nanoparticle binding to cells, cells expressing CD4 were pre-incubated with excess amounts of either RPA-T4 or Sim2 CD4 monoclonal antibody or tenfold molar excesses of free CD4-BP2 or CD4-BP4 (in 100 μ L) for 1 h at 4°C. After blocking, cells were washed once and subsequently incubated with 15 nmol

of either CD4-BP2 or CD4-BP4-targeted LNPs in 100 μ L for 30 min with agitation at 4°C. Cells were washed four times and inhibition of binding was quantified by differences in fluorescence densitometry.

CD4-Peptide-Mediated Indinavir Nanoparticle Binding and Anti-HIV Activity

To determine the peptide-mediated binding of indinavir loaded nanoparticles and subsequent anti-HIV activity, 2×10^5 CEMx174 cells were incubated with culture media containing 6.26 or 25 μ M indinavir either in soluble or targeted nanoparticle formulations for 30 min at 37°C. For anti-HIV activity, 10^5 cells were washed twice in PBS, resuspended in media and infected with HIV-2₂₈₇ at 0.1 Multiplicity of Infection for 1 h. Cells were then washed three times to remove unadsorbed virus, resuspended in media, and eight 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 microscopic observation of the development of syncytia (giant cells), which has been validated with HIV-2 p27 antigen production detected by enzyme-linked immunosorbent assay as described previously (8). The percent infected relative to the total was calculated for the eight replicates of each treatment or control condition.

To quantify cell-associated indinavir, 10^5 CEMx174 cells were exposed to drugs for 30 min at 37°C, then washed three times to remove unassociated drug. 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 and centrifugation to pellet cell debris, 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 mounted on a liquid chromatography–mass spectrometer (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 mode, and the analytes were detected using selected ion monitoring at m/z 603.7–623.7 to detect indinavir. The final drug concentration is estimated as described previously (8).

Statistical Analysis

Data were analyzed for statistical significance using Student's two-tailed t test with significance at $p < 0.05$.

RESULTS

Design and Characterization of Lipid-Drug Nanoparticles for Peptide Coupling

We first evaluated two LNP preparations for size, peptide coupling efficiency, drug association efficiency and stability. These LNPs were prepared in the presence of the HIV protease inhibitor indinavir with MPB-PE (2.5 mol%) to allow subsequent attachment to peptides with terminal cysteines (Fig. 1). As shown in Table II the diameters of LNPs composed of indinavir (1:3 drug-lipid molar ratio), EPC, cholesterol, and MPB-PE (7:3:0.25 molar ratio) were determined. This formulation (EPC/cholesterol/MPB-PE) has been characterized previously and has been shown to be pH sensitive as well as efficient in encapsulation of protease inhibitors (approaching 100%) (8,11). This composition (EPC/cholesterol/MPB-PE) produced particles of 112 ± 13 nm and efficiently incorporated peptides at $87 \pm 5\%$ for CD4-BP2 and $93 \pm 4\%$ for CD4-BP4. These particles with or without MPB-PE incorporated $90 \pm 2\%$ indinavir and were stable for more than 2 months at 4°C .

Although the EPC/cholesterol/MPB-PE LNPs appeared to have properties adequate for initial cell specificity and selectivity experiments, in preparation for *in vitro* infection experiments leading towards *in vivo* testing of targeted LNPs, we have also characterized a pegylated-targeted nanoparticle formulation: DSPC/DSPE-mPEG 2000/DSPE-Mal-mPEG 2000 (8:0.8:0.2 molar ratio) that we have shown in untargeted form to be pH sensitive, efficiently associate drug, and enhance drug concentrations within the lymph nodes of macaques (11). Similarly to the EPC/cholesterol/MPB-PE LNP diameter was determined to be 113 ± 7 nm. This preparation efficiently incorporated peptide to a similar extent as EPC/cholesterol/MPB-PE at $82 \pm 2\%$ and $89 \pm 7\%$ for CD4-BP2 and BP4, respectively, as well as associated indinavir at $99 \pm 2\%$. In addition this formulation was similarly stable. It should also be noted that maleimide functionalized lipid was required for coupling of peptides as preparations of nanoparticles not including this lipid had no quantifiable peptide bound (data not shown).

Evaluation of Candidate Peptides for Binding Selectivity and Affinity to CD4⁺ Cells

Based on reported data, four promising candidate peptides with reported CD4 binding affinity (listed in Table I) were selected for covalent attachment to anti-HIV nanoparticles (23,24). Two are derived from consensus sequences of the HIV envelope gp120 V3 domain (CD4-BP1 and BP2), and two of

which are peptide mimetics based on computer aided structural design that reduced the IgG binding site from the CD4 antibody ST40 to the residues critical for binding (CD4-BP3 and BP4). CD4-BP3 and CD4-BP4 are identical except that CD4-BP3 incorporates a linker from the carboxy terminal proline to lysine at position 13. Including the linker conformationally constrains the peptide such that the amino acids required for binding CD4 might be presented more naturally to the receptor. Peptide-coated EPC/cholesterol/MPB-PE nanoparticles were then evaluated for binding affinity to the CD4⁺ Molt-4 cell line. Cells were incubated with fluorescent LNPs targeted with each of the candidate peptides, untargeted fluorescent LNPs, or fluorescent CD4 antibody. Figure 2 shows the relative binding of each preparation to the CD4⁺ cell line. No cellular autofluorescence was observed (Fig. 2a) and no binding was detectable after incubation with untargeted LNPs (Fig. 2c). Figure 2b shows binding of the CD4 antibody RPA-T4 to the CD4⁺ cells. However, there were distinct differences in the binding profiles of the peptide coated, CD4-targeted LNP preparations. Of the V3 peptides (CD4-BP1 and CD4-BP2) only nanoparticles coated with CD4-BP2 bound significantly to the cells (Fig. 2d, e) despite significant sequence similarity (Table I). The antibody mimetic peptides CD4-BP3 and CD4-BP4 exhibited similar binding profiles as shown in Fig. 2f, g, both with similar binding to cells.

CD4-Dependent Binding of Targeted Nanoparticles to Cells

To evaluate CD4-dependent binding of targeted and untargeted LNPs, we used cells reported to express varying density of CD4 (25): they are (1) Molt-4 and CEMx174 that express high density of CD4⁺ (2), Jurkat that expresses very low CD4, and (3) Ramos (CD4⁻ control B cell line). These cells were used to test the dependence of binding on CD4 content and to compare targeting peptide candidates CD4-BP2 and CD4-BP4. As shown in Fig. 3, we found extensive fluorescence LNPs label on all healthy, high-expression CD4⁺ cells incubated with either CD4-BP2 or CD4-BP4-targeted LNPs (Fig. 3e, f, i, and j). More specifically, it is apparent that CD4 antibody binding to the cells was similar to the binding profile of LNP preparations coated with the candidate CD4 peptides CD4-BP2 and CD4-BP4 (Fig. 3a, b, e, f, i, and j).

Both LNPs coated with either CD4-BP2 or CD4-BP4, referred to as CD4-BP2-LNP or CD4-BP4-LNP bind equally well to high CD4-positive cells, Molt-4 and CEM-174 (Fig. 3e, f, i, and j); the binding levels (based on fluorescence intensity) were similar to that of CD4 selective monoclonal antibody (RPA-T4) (Fig. 3a, b). In contrast, control LNPs did not exhibit any detectable binding to these high CD4-positive cells under

Table II. Characterization of Lipid Nanoparticle Formulations

Lipid Composition (m/m)	MPB (% total lipid)	Diameter (in nm)	Binding peptide-2 (% conjugation)	Binding peptide-4 (% conjugation)	Indinavir (% incorporation)	Stability (months at 4°C)
EPC/chol	2.5	112 ± 13	87 ± 5	93 ± 4	90 ± 2	>2
DSPC/PEG	2.5	113 ± 7	82 ± 2	89 ± 7	99 ± 2	>2

Nanoparticles were prepared by thin film rehydration and sonication. The diameter of the nanoparticles was measured by dynamic light scattering, peptide conjugation leading to its incorporation to lipid nanoparticles was measured by Bradford protein assay, indinavir drug association to lipid nanoparticles was determined by LC/MS, and stability by remeasurement of nanoparticle properties. Data expressed were mean and a standard deviation (SD) of triplicate samples from at least three sets of preparations

Fig. 2. Microscopic analysis of candidate peptides coupled to targeted LNPs in binding to CD4⁺ Molt-4 cells; 5×10^4 cells were incubated with 50 nmol of nanoparticles or 2 μ L of RPA-T4 CD4 antibody for 2 h at room temperature, washed, and observed for fluorescence. **a** Untreated Cells, **b** CD4 antibody, **c** Untargeted nanoparticles, **d** CD4-BP1 targeted nanoparticles (TNP), **e** CD4-BP2 TNP, **f** CD4-BP3 TNP, and **g** CD4-BP4 TNP. Images are representative of at least four repeat experiments

the same conditions (Fig. 3m, n). In the very low CD4-positive Jurkat cells, CD4-BP2-NP and CD4-BP4-NP bind poorly (Fig. 3g, k), which coincides with the binding of RPA-T4 CD4 antibody (Fig. 3c). Again, control LNP did not exhibit any detectable binding (Fig. 3o). In the CD4-negative control Ramos cells, no binding was apparent with any of the tested preparations (Fig. 3d, h, l, and p). In addition, nanoparticles without peptides, and peptide nanoparticles with randomly scrambled CD4-BP2 and CD4-BP4 peptide do not bind to any cells regardless of CD4 expression (data not shown).

Collectively, these data indicate that both CD4-BP2 and CD4-BP4 are able to mediate CD4 selective binding of LNP to cells that express CD4 molecules but not to cells lacking this marker.

Concentration-Dependent Binding of Targeted Nanoparticles to CD4 cells

To differentiate the binding characteristics of the two peptide candidates, CD4-BP2-LNP or CD4-BP4-LNP formulations, we incubated increasing concentrations of these fluorescently labeled LNP with the CD4⁺ cells. Both CD4-BP2-LNP and CD4-BP4-LNP bind extensively to CD4 high positive cells, Molt-4 and CEM-174 (Fig. 4a, b) and the binding levels were similar for each peptide (4.96–4.05 times higher than control in Molt-4 and 8.1–6.07 times higher than control in CEMx174). In contrast, control LNPs had very little detectable binding to these cells under the same conditions (Fig. 4a, b). In the very low CD4-positive Jurkat cells, CD4-BP2-LNP and CD4-BP4-LNP binding was significantly decreased, although still 2.12 and 1.86 times greater than untargeted nanoparticle binding (Fig. 4c). In the CD4-negative control Ramos cells, no differences in binding between untargeted and targeted nanoparticles was observed (Fig. 4d). In all cases of binding to CD4⁺ cell lines, it appears

that CD4-BP2-targeted LNPs bind to cells to a slightly greater extent than LNPs targeted with CD4-BP4.

Taken together, these results suggest that both candidate peptide nanoparticle preparations provide CD4 selective binding, as the degree of CD4 expression (high, low, or none) correlates with binding of targeted nanoparticles and untargeted nanoparticles does not show significant binding to CD4⁺ cells.

The binding data were further analyzed to determine an apparent nanoparticle binding affinity and estimate total cellular binding sites. The apparent binding affinity of CD4-BP2 and CD4-BP4-targeted nanoparticles for each of the high CD4⁺ cell lines is presented in Table III. The binding affinity is expressed as K_d (disassociation constant) for CD4-BP2-LNP and CD4-BP4-LNP exposed to two cell lines Molt-4 and CEMx174. Estimates were not made for Jurkat cells as these data point to non-saturable, nonspecific binding. Although the calculated values are statistically different between the two CD4⁺ cells, both the K_d values are in a low single digit micromolar range. However, CD4-BP4-NP appears to have slightly higher affinity in each cell line tested ($K_d=1.3$ vs. 3, 3.3 vs. 5.8 μ M, respectively for Molt-4 and CEMx174 cells). These results indicate that although CD4-BP2-LNP bind to both CD4⁺ cells with higher capacities, CD4-BP4-LNP may exhibit a significantly higher affinity for CD4⁺ cells.

Targeted Nanoparticle Binding Inhibition

To further characterize the CD4 binding specificity of CD4-BP2-LNP and CD4-BP4-LNP in regards to their likely interaction with cellular CD4, we pre-incubated CEMx174 cells with several blocking agents, which included unbound CD4-BP2 and CD4-BP4, as well as CD4 antibodies RPA-T4 and Sim2 (which bind to separate locations on CD4). Figure 5 shows the results from pre-blocking cells with these agents with subsequent binding of each candidate targeted LNP, and then determining fluorescent density of binding relative to

Fig. 3. Qualitative fluorescence microscopic analysis of differential binding of targeted or untargeted LNPs or RPA-T4 antibody to cell lines variably expressing CD4. 5×10^4 cells were incubated with 10 nmol of nanoparticles or 2 μ L of RPA-T4 CD4 antibody for 2 h at room temperature, washed, and observed for fluorescence. Columns **a** Molt-4, **b** CEMx174, **c** Jurkat, and **d** Ramos. Rows **a** CD4 antibody, **e** CD4-BP2 nanoparticles (NP), **i** CD4-BP4 NP, and **m** untargeted control NP. Images are representative of at least four separate repeat experiments

unblocked control. In the case of CD4-BP2-targeted nanoparticles (Fig. 5a), significant inhibition of binding compared with unblocked control is observed after pre-incubation with all agents. Total blocking, defined as not significantly different from untargeted nanoparticle binding, was observed after pre-incubation with free CD4-BP4 and Sim2 antibody. Interestingly, only partial blocking was seen with free CD4-BP2 and RPA-T4. For blocking of binding of CD4-BP4-targeted LNPs a slightly different pattern is obtained (Fig. 5b). Whereas total blocking is demonstrated when pre-incubated with free CD4-BP4 or RPA-T4, only partial blocking is seen after free CD4-BP2 or Sim2 incubation. These results suggest that the two peptide candidates, CD4-BP2 and CD4-BP2 conjugated to LNPs exhibit comparable but distinct binding selectivity and specificity to CD4 at possibly different sites within CD4 molecules expressed on cell surface.

DISCUSSION

We have previously shown that lipid-associated drug formulations administered subcutaneously localize to lymph nodes and significantly increased drug concentration, which resulted in lower viral loads and preservation of CD4⁺ cells (8). Whether further targeting CD4⁺ HIV target cells within lymph nodes and lymphoid tissue could be achieved with CD4 binding peptides, it is not known if this targeting could further enhance drug efficacy in this tissue site. To address this question, we have identified and evaluated four peptides with reported CD4 receptor binding specificity, in the context of their expression on nanoparticles. We found that two of the candidate peptides possessed desirable CD4⁺ binding properties and would be suitable for further characterization of enhancement of antiviral activity of an anti-HIV drug in an infected cell model.

Fig. 4. Analysis of LNP binding dependent upon candidate peptide and CD4 content of cell lines. Increasing concentrations of targeted LNP preparations were incubated with cell lines expressing a varying degree of CD4 (MOLT-4, CEMx174, Jurkat) or no CD4 (Ramos) for 30 min at 4°C, washed, and plated in a 96-well plate for fluorescence quantification. The specific activity of targeted nanoparticles was calculated and fluorescence values were converted to corresponding peptide values. Results are the mean values from three experiments. **a** MOLT-4, **b** CEMx174, **c** Jurkat, and **d** Ramos. Filled circles, CD4-BP2 LNPs; open circles, CD4-BP4 LNPs; and triangles, untargeted LNPs

Targeted LNPs for enhancing antiviral potency has previously been explored, but no complete characterization of a target LNP drug formulation for specific delivery to HIV host cells in lymph nodes of humans or primates and subsequent reduction in markers of disease progression has been done. One of the earliest attempts to target HIV host cells was to attach peptides from the CDR-2 domain of CD4 to liposomes that would recognize HIV envelope sequences on the surface of infected cells. These targeted liposomes showed threefold greater binding to infected cells compared with the uninfected cells; however, they were only evaluated for inhibition of syncytia (which they were unable to prevent) rather than a drug loaded version (16). Whole soluble CD4 has also been coupled to liposomes and was able to target liposomes to infected macrophages which was accompanied by a tenfold increase in efficacy of protease inhibitor (14). Two studies examining Fab fragments from a mouse anti-HLA-DR antibody coupled to

drug loaded liposomes were shown to increase accumulation of drug in mouse lymph node target cells by at least twofold over untargeted liposomes and at least 120-fold over soluble drug after subcutaneous injection (18,19). These studies established the potential of targeting to enhance drug concentrations but neglected to test antiviral effect. Most recently, a study targeting infected cells with drug loaded liposomes targeted with fragments of an antibody raised against gp120 showed that in *in vitro* infection experiments, intracellular drug concentrations were significantly increased and drug efficacy was significantly enhanced over free drug or untargeted liposomes (17). Although it is an encouraging result, it is unlikely that the magnitude of effect would translate *in vivo*, as it is known that gp120 is shed in large quantities from infected cells, and this would likely decrease the effectiveness of these particles (26).

Although antibodies or antibody fragments such as Fab (50 KD) or Fab₂ (100 KD) are generally used for nanoparticle

Table III. Quantification of Affinity (K_d) of Targeted Lipid Nanoparticles on Cell Lines Variably Expressing CD4

Lipid nanoparticles coated with peptide	Binding affinity to CD4 ⁺ Cells K_d (μ M)			Estimated total binding sites per cell ($\times 10^6$)		
	CEMx174	Molt-4	Jurkat	CEMx174	Molt-4	Jurkat
CD4-BP2 LNP	3.0 $\pm$ 0.4 ^{a, c}	5.8 $\pm$ 0.7 ^{b, c}	ND	1.53	1.12	ND
CD4-BP4 LNP	1.3 $\pm$ 0.2 ^{a, d}	3.3 $\pm$ 0.3 ^{b, d}	ND	0.77	1.36	ND

^a BP2 and BP4 LNP significantly different in CEMx174 cells $p < 0.05$

^b BP2 and BP4 LNP significantly different in Molt-4

^c BP2 LNP significantly different in CEMx174 and Molt-4 cells

^d BP4 LNP significantly different in CEMx174 and Molt-4 cells. $n = 3$

Fig. 5. Competitive inhibition of targeted LNP binding to CD4⁺ cells. CD4 was blocked by pre-incubating cells with saturating amounts of either RPA-T4 or Sim2 CD4 antibody or tenfold molar excesses of free CD4-BP2 or CD4-BP4 for 1 h at 4°C. After blocking, cells were washed once and subsequently incubated with 15 nmol of either CD4-BP2 or CD4-BP4-targeted LNPs in 100 μ L for 30 min with agitation at 4°C. Cells were washed four times and inhibition of binding was quantified by differences in fluorescence densitometry in quintuplicate. **a** Inhibition of CD4-BP2-targeted nanoparticle binding and **b** inhibition of CD4-BP4-targeted nanoparticle binding. Asterisks, significantly different from targeted LNP binding $p < 0.05$

targeting purposes, peptides (<5 KD) offer several advantages over antibodies, including: reduced potential of protein denaturation, heterogeneity in chemical conjugation, and potentially reduced immunogenic response. In addition, peptides with more defined physical and chemical properties have improved shelf life over that of antibody or antibody fragments. In our design, peptide-coated LNPs are prepared synthetically without cellular or biological product and can be produced at a much lower cost than antibodies or fragments.

We have shown that attachment of peptides on LNPs can be accomplished efficiently by conjugating peptide with a terminal cysteine to maleimide lipids in two formulations. Both the EPC/cholesterol/MPB-PE and DSPC/DSPE-mPEG

2000/DSPE-Mal-mPEG 2000 were able to provide almost complete drug association and peptide conjugation into the LNPs and will be used for further *in vitro* studies.

Results from evaluating the four candidate peptides coated onto LNPs by examining binding to CD4⁺ Molt-4 cells allowed selection of two peptide candidates with potential CD4 binding affinity. Surprisingly, and despite significant sequence similarity between CD4-BP1 and CD4-BP2, the extent of cellular binding of LNPs targeted with CD4-BP2 was far greater than CD4-BP1-targeted LNPs. Regarding the CD4 antibody mimetic peptides, we observed no difference in binding to CD4⁺ cells of targeted LNPs prepared with these two peptides, which suggests the unconstrained peptide is able to bind in a competent conformation and that the linker is unnecessary. Therefore, only CD4-BP2 and CD4-BP4 were evaluated further.

The CD4-targeted nanoparticle binding to cell lines appeared to relate to the levels of CD4 expression (Fig. 3). Literature reports of CD4 expression indicate that Molt-4 and CEMx174 have high expression, Jurkat has very low CD4 expression, and Ramos, a B cell line, has none (25). Of the highly expressing cell lines Molt-4 and CEMx174, it has been reported that Molt-4 express approximately 50% more CD4 than CEMx174. Jurkat CD4 expression is approximately 5% to 10% that of Molt-4 and CEMx174, respectively. In general, we observed that binding of the targeted LNPs coincided with the relative content of CD4 reported to be on the cells as well as the pattern of binding after incubation of cells with CD4 antibody (Fig. 3). In addition, targeted nanoparticles with scrambled CD4-BP2 and CD4-BP4 peptide were unable to bind to CD4⁺ cells (data not shown). Interestingly, contrary to the literature report of higher expression of CD4 in Molt-4 cells, we found that after incubation with CD4 antibody or targeted nanoparticles that more binding is evident on the CEMx174 cells. Whether this difference is due to cell passage or specific lineage differences affecting CD4 expression is not clear and warrants further investigation. Regardless, these data indicate that CD4 binding peptides expressed on LNPs exhibit selectivity and affinity for CD4⁺ cells according to their levels of expression.

The selection of these CD4 cell lines was driven not only by relative CD4 expression levels but also by reported expression of the HIV coreceptors CXCR4 and CCR5. The expression pattern of these coreceptors was of primary interest because of the origin of CD4-BP2 having been derived from the V3 loop on HIV gp120. In general, this region on the virus is primarily thought to interact with CXCR4 or CCR5, more so than CD4. Although literature data indicate affinity of CD4-BP2 peptide for CD4 (24) and the intensity of targeted nanoparticles containing this peptide binding to cell lines correlated well to cellular CD4 expression, it is still possible, although unlikely, that this peptide could show affinity for the coreceptors. As each cell line has a distinct expression pattern for CD4, CXCR4, and CCR5 (25) we can qualitatively determine if these targeted LNPs have potentially any affinity to these receptors. Molt-4 cells are reported to have high expression of all three receptors. CEMx174 cells have high expression of CD4, low expression of CXCR4, and no expression of CCR5. Finally, Jurkat have very low CD4, but high expression of the coreceptors. Had the targeted nanoparticles bound significantly to Molt-4 and Jurkat

Table IV. Enhanced Indinavir Localization and Anti-HIV Effect in CD4⁺-CEMx174 Cells after Treatment with CD4-Targeted Lipid Nanoparticles

Indinavir-lipid nanoparticle formulation ^a		Cell-associated indinavir (% bound) ^b		HIV infection (% positive wells) ^c	
Peptide	Lipid composition	(6.25 μ M) ^d	(25 μ M) ^d	(6.25 μ M) ^d	(25 μ M) ^d
Soluble drug	None	62.4 $\pm$ 1.2	21.2 $\pm$ 6.0	100	75 $\pm$ 18
None	DSPC/PEG	0.8 $\pm$ 0.2 ^e	0.6 $\pm$ 0.1 ^e	100	100
CD4-BP2	DSPC/PEG	69.8 $\pm$ 3.7	20.1 $\pm$ 0.3	75 $\pm$ 18	62.5 $\pm$ 13
CD4-BP4	DSPC/PEG	80.5 $\pm$ 1.4 ^e	37.0 $\pm$ 1.8 ^e	0	0

^a Indinavir, either in soluble form or in DSPC: mPEG-DSPE (DSPC/PEG) associated form were exposed to HIV host, CEMx174 cells for evaluation

^b Cell-associated indinavir was determined after cells were incubated with drug preparations for 30 min and then washed to remove unassociated drug. Subsequently, cells were infected with 0.1MOI HIV-2₈₇ for 1 h and washed to remove unadsorbed virus

^c Percentage of infection was determined based on HIV infected CEMx174 cells detected at day 4 post-HIV infection

^d The data expressed were mean $\pm$ standard deviation of quadruplicates and presented according to their initial indinavir concentrations

^e Significantly different from soluble drug ($p < 0.05$)

cells, it would be possible that CXCR4 may be involved in cell binding. Likewise, if CCR5 were involved, extensive binding to CEMx174 would not be seen. Based on the cell binding data of the candidate targeted LNPs, combined with literature reports, it is unlikely CD4-BP2 nanoparticle binding is mediated through the HIV coreceptors.

When candidate peptides were coated on fluorescently labeled LNPs, extensive and specific fluorescence was observed on cells dependent on CD4 content (Fig. 3). To quantify the extent of association, we performed several sets of experiments in these cell lines with increasing concentrations of the candidate targeted LNPs as well as untargeted nanoparticles. Overall, these data complemented the single dose qualitative binding experiments, showing similar extents of binding of nanoparticles to the cell lines. Total binding of the targeted LNPs decreased significantly as CD4 expression declined, as seen in Jurkat and Ramos cell experiments. Evaluation of the nonspecific binding of untargeted nanoparticles showed that there is only a minor association with the tested cell lines.

Pre-existing anti-gp120 antibodies in chronically infected patients may theoretically interfere with CD4-targeted nanoparticles, particularly those constructed with sequences derived from gp120 of HIV envelope (CD4-BP2). However, given the polyclonal nature of this antibody response, and much higher concentrations of the antibody needed to completely neutralize this response, the interference may be manageable. Furthermore, the impact of the antibody concentrations in the lymphatic system would be expected to be much lower than in the plasma for binding to these particles (which are intended for subcutaneous route of administration that provide first-pass lymphatic exposure and preferential accumulation in lymph nodes and lymphoid tissues).

Although CD4-BP2-targeted nanoparticles exhibited a higher total level of binding, their apparent affinity was slightly less than that of the CD4-BP4-targeted nanoparticles. Nevertheless, the apparent K_d values calculated for the nanoparticles were in a similar micromolar range across both CD4⁺ cell lines. Based on the high (up to mg/L) concentration of LNPs found in primate lymph nodes after subcutaneous 10 mg/kg indinavir LNP administration in primates (8), the CD4-targeted nanoparticles are within the millimolar binding affinity to CD4 cells within the lymph nodes and lymphoid tissues.

It is interesting to note that we did not observe a linear reduction in total binding to Jurkat cells compared with the higher CD4 expressing cells. Again, literature values indicate that Molt-4 and Jurkat have approximately 125,000 and 1,000 CD4 receptors per cell, respectively (25). Therefore, one might expect the total binding of targeted nanoparticles to Jurkat cells to be about 1% that of Molt-4, for example. However, it is important to note that although total bound peptide is measured, each 100 nm nanoparticle contains hundreds of targeting peptides, but only a low percentage will likely be interacting with cellular receptors. Considering that Jurkat and Molt-4 cells are approximately the same size, the density of potential targets will be vastly different. It is conceivable that in an interaction of the targeted nanoparticles with a Jurkat cell, target density dictates that fewer peptides per nanoparticle will be directly bound to the cell receptor. Conversely, on a cell with a higher density of targets, the nanoparticle will likely have more of its peptides bound to the cell of the total available and compete with other nanoparticles for binding. This phenomenon may also be a possible reason why we were unable to obtain definitive saturation of binding on these CD4 expressing cell lines and a more robust estimation of K_d in line with previously reported values. As nanoparticle concentration increases, competition for each cellular receptor increases, and although from the cell perspective receptor occupancy remains at or near 100%, more nanoparticles can be bound at increasing concentrations because fewer peptides per nanoparticle are binding to the cellular receptors.

In further characterization of the binding of targeted LNPs to HIV target cells, and their interaction and dependence on CD4, we tested whether molar excesses of free peptides or CD4 antibodies could block binding of the targeted nanoparticles when preincubated with cells. In general, the specific binding locations of the antibodies and peptides have yet to be fully characterized; however, it is known that the RPA-T4 antibody binds to the CDR1 and CDR3 regions on CD4, and the ST40 antibody, from which the CD4-BP4 peptide is derived, binds to the CDR3 region of CD4. Further, both CD4 antibodies are reported to inhibit HIV gp120 binding, from which CD4-BP2 is derived (23,27). Therefore, these antibodies should prevent binding of targeted nanoparticles. In blocking of CD4-BP2-targeted

nanoparticles, we were able to achieve total blocking (*i.e.*, not significantly different from untargeted control) with free BP2 and BP4 peptides, as well as with Sim2 antibody (Fig. 5). In the case of CD4-BP4, we were able to achieve total blocking with free BP4 and RPA-T4 antibody, but only partial blocking with the others. Overall, it appears that the binding of both targeted nanoparticles is sensitive to CD4 receptor occupancy and that CD4-BP4-targeted LNPs are more resistant to blocking than CD4-BP2; whether this is due to CD4 binding preferences or other factors is not known and it remains to be tested.

Finally, we evaluated the ability of indinavir-associated targeted LNPs to enhance drug localization and anti-HIV effect in the CD4⁺, HIV susceptible cell line, CEM-174. Our initial data presented in Table IV indicate that both CD4-BP2 and CD4-BP4-targeted LNP preparations significantly enhanced indinavir association to CEM-174 cells after 30 min of exposure, particularly at low drug concentrations. In parallel, significant enhancement of anti-HIV activity was observed (as shown by a reduction in the percent of HIV-positive cells after treatment), particularly for indinavir-LNPs coated with CD4-BP4 peptides, compared with soluble or control LNP formulated indinavir (Table IV). These initial data demonstrate the potential utility of CD4-specific binding peptides in enhancing drug localization and antiviral activity on HIV target cells.

We recognize that preferential targeting of an anti-HIV drug treatment to an uninfected, healthy cell with a nonspecific antiviral drug may pose off-target toxicity concerns. Particularly, CD4 expression has been reported to be down regulated in infected T cells (28), thus drug delivery via a CD4-targeted LNP may potentially bind and deliver drug to uninfected T cells with equal or greater efficiency. However, the anti-viral efficacy and host-cell toxicity would be limited due to the selectivity of anti-viral compounds that attack viral enzyme targets (*i.e.*, viral but not host protease or reverse transcriptase). As the normal T cell does not express these viral enzymes, these anti-HIV drugs will have minimal cytotoxic effects on uninfected host cells. In addition, this strategy may be able to compete with HIV for CD4 binding, as well as pre-load drug into the cell perhaps preventing production of new virions.

CONCLUSIONS

In summary, we have designed, characterized, and evaluated targeted LNP formulations using two peptides selective for HIV host cells expressing CD4. These candidate peptide-coated targeted nanoparticle preparations are selective for CD4⁺ cell lines in relation to their relative CD4 expression. The peptide-mediated binding of LNPs with drugs was inhibited by pre-incubation of cells with CD4 antibody or free peptide. Collectively, these results provide a basis for evaluating this delivery system to enhance efficacy in HIV infected CD4⁺ cells.

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