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Sustained and specific in vitro inhibition of HIV-1 replication by a protease inhibitor encapsulated in gp120-targeted liposomes

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ABSTRACT

Selective delivery of antiretrovirals to human immunodeficiency virus (HIV) infected cells may reduce toxicities associated with long-term highly active antiretroviral therapy (HAART), may improve therapeutic compliance and delay the emergence of resistance. We developed sterically stabilized pegylated liposomes coated with targeting ligands derived from the Fab' fragment of HIV-gp120-directed monoclonal antibody F105, and evaluated these liposomes as vehicles for targeted delivery of a novel HIV-1 protease inhibitor. We demonstrated that the immunoliposomes were selectively taken up by HIV-1-infected cells and localized intracellularly, enabling the establishment of a cytoplasmic reservoir of protease inhibitor. In antiviral experiments, the drug delivered by the immunoliposomes showed greater and longer antiviral activity than comparable concentrations of free drug or drug encapsulated in non-targeted liposomes. In conclusion, by combining a targeting moiety with drug-loaded liposomes, efficient and specific uptake by non-phagocytic HIV-infected cells was facilitated, resulting in drug delivery to infected cells. This approach to targeted delivery of antiretroviral compounds may enable the design of drug regimens for patients that allow increased therapeutic adherence and less toxic treatment of HIV infection.

1. Introduction

Approximately thirty-three million people worldwide are infected by the human immunodeficiency virus (HIV) (UNAIDS, 2008). Although highly active antiretroviral therapy (HAART), combining several antiretroviral drugs, has had a dramatic impact upon long-term survival rates (Pomerantz and Horn, 2003), there are many associated toxicity issues covering a wide range of serious adverse events including central nervous system toxicity, hypertriglyceridemia and lipodystrophy (Montessori et al., 2004), presumably due to exposure of uninfected cells to drug. Selective delivery of HIV-1 inhibitors to infected cells would reduce the exposure of uninfected cells to toxic effects, enable delivery of higher concentrations of inhibitor to infected cells, reduce the daily pill burden, and hence potentially increase adherence to antiviral regimens. Furthermore, since toxicities are dose-limiting, selective delivery of an inhibitor to infected cells resulting in higher local doses may delay the development of drug resistance.

The concept of drug targeting has been used to improve the therapeutic index of drugs, notably in cancer therapy. Specific drug delivery involves the association of a drug with a carrier system, e.g. nanotechnology and polymer therapeutics (Duncan, 2003; Ferrari, 2005). Liposomes are vesicles with a lipid membrane surrounding an aqueous core, and represent the archetypical form of a nanovector (Torchilin, 2005). Early research revealed that uncoated liposomes were quickly cleared from the blood by the reticuloendothelial system, which led to the development of liposomes coated with polyethylene glycol (PEG), resulting in steric stabilisation and prolonged circulation in the bloodstream (Blume and Cevc, 1990; Klibanov et al., 1990). A notable example of this strategy for drug delivery is pegylated liposomal doxorubicin that is approved for clinical use as an anticancer agent (Gabizon et al., 2003).

Delivery of a HIV-1 protease inhibitor with pegylated liposomes has been reported previously (Pretzer et al., 1997). As liposomes are avidly phagocytosed by macrophages (Allen et al., 1991), the main target cells of this approach were HIV-infected macrophages. To deliver drugs to other infected cells in addition to macrophages, a more specific targeting approach was necessary. The most obvious binding partner for targeted liposomes on HIV-1-infected cells is gp120. This glycoprotein is located on the viral envelope as

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trimers of gp41-gp120 heterodimers constituting the envelope spikes located at the surface of virus particles. Such spikes are also present on the plasma membrane of HIV-1-infected cells where they are acquired by the newly formed virus during the budding process. Non-pegylated liposomes coupled to a soluble form of the natural host ligand of gp120, i.e. sCD4, proved indeed to be associated with HIV-1-infected but not with uninfected cells (Flasher et al., 1994). However, sCD4 is a whole protein molecule. Compared to peptides or antibodies, whole proteins are generally more challenging to produce and have more liabilities for being immunogenic which may decrease the life time of the labelled liposome. Peptides derived from CD4 were successfully tested as targeting moieties for liposomes (Slepushkin et al., 1996). The proof of principle of antibody-based targeted liposomal technology for drug delivery in HIV infection has been demonstrated using anti-HLA-DR coated pegylated liposomes containing the HIV-1 protease inhibitor indinavir (Gagné et al., 2002).

An excellent candidate moiety for targeting drug-loaded liposomes to HIV-1-infected cells is a human monoclonal immunoglobulin G 1κ (IgG1κ) antibody, monoclonal antibody F105, that specifically binds to the viral gp120 (Posner et al., 1991). In a previous study (Clayton et al., 2007), we demonstrated the high affinity of F105 for its ligand gp120, its selective binding to HIV-1-infected cells and the rapid cellular uptake and accumulation in the Golgi. Hence, gp120-targeting by F105 offers an opportunity to discern HIV-1-infected cells from uninfected cells. Moreover, this strategy was reported in a recent *in vivo* study where siRNAs were delivered to HIV-infected cells via targeting with an F105 Fab fragment (Song et al., 2005).

In this work, we report the specific targeting and delivery of an antiretroviral drug, the protease inhibitor PI1, encapsulated in pegylated liposomes that are coated with a F105 Fab' fragment as a targeting moiety. The anti-HIV immunoliposome was shown to enable selective delivery of PI1 to HIV-1-infected cells. It was also demonstrated that the effect of the targeted PI1 on viral replication was greater than the effect of a comparable concentration of free drug or non-targeted drug.

2. Materials and methods

2.1. Cells and reagents

F105 was produced as described before (Clayton et al., 2007), and the F105 IgG was pepsin digested in 0.5 M citric acid pH 3.0 containing 150 U of enzyme per mg of IgG to produce Fab'2 fragments. The reaction was incubated at 37 °C for 3.5 h and then stopped by adjusting the pH to 7.2 with 2.5 M Tris buffer. Separation of Fab'2 from larger Fc fragments and whole IgG was achieved by applying the digest sample to an XK 16/10 Mabselect column (Amersham Biosciences, Piscataway, NJ). F105 Fab'2 was further reduced to Fab', using 9 mM 1,4-dithioerythritol (DTE; Sigma, St. Louis, MO) at pH 5 and 40 °C for 30 min. Following reduction, DTE was removed by passing the mixture through a PD10 desalting column, followed by elution with 0.9% NaCl and adjustment to pH 6.0 with a stock of 1 M sodium phosphate dibasic buffer prior to the liposome-conjugation step.

Recombinant HIV-1 LAV gp120 was produced by Protein Sciences Corporation (Meriden, CT) in insect cells using the baculovirus expression system.

The HIV-1 protease inhibitor PI1 (Fig. 1), a substituted benzimidazole sulfonamide, was synthesized in-house following procedures described before (De Kock et al., 2006) and stored in dimethylsulfoxide (DMSO) (Sigma).

The human T-cell lines HUT78 and PM1, kindly provided by the Medical Research Council (London, UK) and SRA Life Sciences (Rockville, MD) respectively, were cultured in RPMI 1640 medium

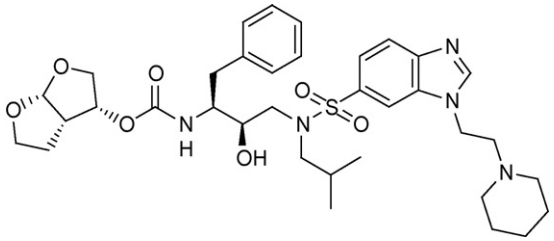

Fig. 1. Chemical structure of PI1.

(Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Highclone, Logan, UT). Chronically HIV-1-BaL-infected PM1 cells (PM1-BaL) and HIV-1-IIIB-infected HUT78 cells (HUTIIIB) were produced and characterized as previously described (Clayton et al., 2007). The MT4-LTR-EGFP cell line, containing an enhanced green fluorescent protein (EGFP) marker gene, was generated as previously described (Ivens et al., 2005). Briefly, MT4 cells (kindly provided by Naoki Yamamoto, National Institute of Infectious Diseases, AIDS Research Center, Tokyo, Japan) were transfected with pLTR-EGFP using lipofectamine (Invitrogen). The pLTR-EGFP plasmid is based on pEGFP1-C1 (BD Biosciences Clontech, Palo Alto, CA) where the CMV immediate-early promoter was replaced by the 5′-LTR promoter of pHXB2D (NIBSC, Hertfordshire, UK). Based on fluorescence, transfected cells were selected with geneticin (G418). The MT4-LTR-EGFP cell line needs to be maintained in medium (RPMI 1640 supplemented with 2 mM L-glutamine, 0.1% $NaHCO_3$, 10% heat-inactivated fetal calf serum, 0.02% gentamycin) containing 0.8% G418 for stable expression of EGFP. G418 is omitted from the medium prior to use in antiviral experiments. The resulting cell line expresses EGFP upon induction by HIV-1 Tat.

2.2. Preparation of drug-loaded pegylated liposomes

Liposomes were composed of hydrogenated soy phosphatidylcholine (HSPC; Lipoid K.G., Ludwigshafen, Germany), cholesterol (Croda, Goole, UK), and methoxypolyethyleneglycol-di-stearoyl-phosphatidylethanolamine (mPEG–DSPE, with mPEG MW 2000 Da; Sygena, Liestal, Switzerland). The lipids, at a HSPC:cholesterol:mPEG–DSPE molar ratio of 55:40:5, were dissolved in absolute ethanol at 65 °C and subsequently mixed for 1 h with an hydration buffer at the same temperature, at an ethanol to hydration buffer volume ratio of 1–9. This process led to the spontaneous formation of liposomes. Next, the liposomes were down-sized by high-pressure extrusion at 65 °C, using five passes through 0.2 μm pore-size polycarbonate membranes and then passed through 0.08 μm pore-size polycarbonate membranes (Whatman/GE Healthcare, Piscataway, NJ), to obtain liposomes with a mean diameter of ~100 nm.

Liposomes containing the fluorophore Alexa488 (Al^{488}; Molecular Probes, Eugene, OR) were prepared as described above with the hydration buffer consisting of 25 mg/ml 10K dextran conjugated Al^{488}. The non-encapsulated Al^{488} was removed by size exclusion chromatography (SEC) using a Sepharose 4B column. A 10 mM citrate solution containing 150 mM NaCl at pH 5.5 was used to equilibrate the column and to separate post-extrusion materials. The liposomal fraction was collected and concentrated using a Vivaspin centrifugal concentrator (100 kDa molecular weight cut-off; Vivascience, Hanover, Germany). The pH of the solution was subsequently adjusted to 6.0.

Liposomes containing PI1 were prepared as described above with the hydration buffer containing 150 mg/ml dextran sulfate ammonium salt (DSAS) at pH 5.5. After the extrusion step, the non-encapsulated DSAS and the ethanol were removed by diafiltration of the liposome solution, using a 300,000 molecular weight cut-off

polysulfone hollow fiber cartridge (GE Healthcare, Piscataway, NJ) and 12 volume exchanges of a 10 mM citrate solution containing 150 mM NaCl at pH 5.5.

PI1 loading in liposomes was achieved by mixing a solution of PI1 at 5 mg/ml with a liposome solution at 20 mM total lipid at 63 °C for 1 h (pH 5.5). The drug spontaneously loaded inside the liposomes as a result of the ammonium gradient (Abra et al., 2002). Following drug loading, any non-encapsulated drug was removed and the liposome fraction was concentrated, as described above for Al^{488}-loaded liposomes.

2.3. Antibody-conjugation of liposomes

Maleimide-derivatized pegylated phospholipid (malPEG–DSPE) was inserted into the lipid bilayer of liposomes containing PI1 or Al^{488} by incubation for 1 h at 62.5 °C in a pH 6.0 solution (10 mM citrate solution containing 150 mM NaCl), using a calculated ratio of ~700 malPEG–DSPE molecules per liposome (assuming 80,000 phospholipids per liposome) and a total lipid concentration of approximately 20 mM. A volume of Fab' solution (pH 6.0) that previously resulted in a final ratio of 70 Fab' molecules per liposome was then added to the post-inserted liposomes. The mixture was allowed to conjugate at room temperature overnight. Unreacted maleimide groups were subsequently quenched by adding cysteine. The quenched liposomal solution was run on a Sephacryl-300 SEC column (using a solution containing 10 mM histidine and 150 mM sodium chloride at pH 6.5 for elution) to remove excess cysteine and unconjugated protein. The liposomal fraction was collected and concentrated using a Vivaspin centrifugal concentrator.

Liposomes containing PI1 or Al^{488} are hereafter designated L-PI1 or L-Al^{488} and the F105 Fab'-conjugated liposomes are designated F105-L-PI1 and F105-L-Al^{488}.

2.4. Characterization of liposomes

To measure Al^{488} concentration, Al^{488}-loaded liposomes were incubated in isopropyl alcohol (0.75 M HCl/isopropyl alcohol, 1:9 v/v), which destroyed any liposomes present and released encapsulated Al^{488}. Fluorescent emission at 519 nm was measured after excitation at 495 nm in order to determine Al^{488} concentration.

PI1-loaded liposomes were incubated in ethanol/glacial acetic acid/water, 90:1:9 (v/v) to release encapsulated drug. Next, absorbance was measured at 265 nm in order to determine PI1 concentration.

To determine the percentage of encapsulated Al^{488} or PI1, samples were separated into liposomal-encapsulated and free Al^{488} or PI1 by SEC, using a Sepharose 4B column. Peaks were identified visually or by absorbance or fluorescence readings of individual fractions from the column and then pooled together. Al^{488} or PI1 content of the peaks was assayed as described above.

To estimate the average number of Fab' fragments conjugated per liposome, the number of liposomes per ml of sample and the concentration of lipid-conjugated Fab' were estimated separately. The number of liposomes per ml was determined by assaying the phospholipid concentration via a phosphorus assay (Bartlett, 1959), and assuming that 80,000 phospholipid molecules per liposome (based on a liposome diameter of 100 nm). The concentration of lipid-conjugated Fab' was estimated by first determining the fraction of conjugated Fab' in a post-conjugation liposome sample, using polyacrylamide gel electrophoresis (NuPage® Novex® 4–12% Bis-Tris gel [Invitrogen, San Diego, CA] and MOPS running buffer) with Sypro Ruby (Molecular Probes, Eugene, OR) staining overnight and band quantitation by densitometry using Typhoon 9400 (GE Healthcare, Piscataway, NJ). The fraction of lipid-conjugated Fab' was then multiplied by the total protein concentration of the sample in order to obtain the concentration of lipid-conjugated Fab'.

The retention of PI1 inside liposomes was evaluated by incubation of F105-L-PI1 and L-PI1 liposomes under conditions mimicking antiviral experiments in cell based virus replication assays, i.e. up to 3 days of incubation at 37 °C in a medium of 90% RPMI 1640 and 10% FBS. Samples were taken after days 1 and 3, and drug encapsulation in liposomes was determined by SEC (Sepharose 4B column with 0.9% saline elution solution) and UV/visible spectrophotometry (absorption at 265 nm). The 90% RPMI 1640 and 10% FBS medium itself, as well as free PI1 (no liposome) incubated for 1 and 3 days in this medium, did not show any absorption at 265 nm in the range of SEC retention times characteristic of the drug-loaded liposomes. Therefore, the percent encapsulation calculation is a true representation of the percent of liposome-encapsulated drug (by opposition to released, or free, drug). Comparison with the drug encapsulation of the initial sample (no incubation with 90% RPMI 1640 and 10% FBS) enabled assessment of drug leakage resulting from incubation conditions.

2.5. Kinetic analysis of the interaction of F105 IgG, Fab'2 and Fab' with recombinant HIV-1 gp120 using BIAcore technology

Interactions between recombinant gp120 and F105 IgG, Fab'2 or Fab' were measured by surface plasmon resonance (SPR) on a BIAcore S51 instrument (Biacore, Uppsala, Sweden). Recombinant gp120 was immobilized on a CM5-certified sensor chip (Biacore) using standard amine coupling chemistry according to the manufacturer's instructions. Briefly, a mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) was used to activate the dextran matrix on the sensor chip surface. After activation, the resulting reactive succinimide esters on the chip surface reacted spontaneously with uncharged amino groups and other nucleophilic groups of gp120 (50 μg/ml in 10 mM sodium acetate buffer at pH 4.5) that was passed over the surface at a flow rate of 10 μl/min and a contact time of 15 min, linking gp120 covalently to the dextran matrix. The amount of immobilized protein was measured in resonance units (RU), with one RU representing about 1 pg protein bound per square millimeter of the sensor surface. Following this procedure, gp120 immobilization levels of 4000–7000 RU were obtained. The kinetic interaction studies were performed in phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.005% P20 surfactant (Biacore) at 20 °C and a flow rate of 30 μl/min. A reference flow cell was used to correct refractive index changes due to bulk effects. Serial dilutions of F105 IgG, Fab'2 and Fab' in running buffer were injected over the gp120 surface for 600 s and dissociated for a period of 1000 s. A duplicate sample and a zero concentration sample were used as a positive and a negative control. After each sample injection, the flow system was washed with running buffer containing 50% DMSO, and the immobilized surface was regenerated with two consecutive injections of 10 mM glycine pH 2.5 (for 30 s) and 10 mM HCl (30 s). Each concentration series was analyzed by simultaneous non-linear regression analysis (global fitting) using BIAcore T100 software. The analysis of the F105-gp120 interaction was based on a mathematical model assuming heterogeneous ligand binding (IgG and Fab'2) or homogeneous 1:1 binding (Fab').

2.6. Analysis of uptake and binding of F105-L-Al^{488} to cells by FACS

PM1 cells (uninfected and HIV-1-BaL-infected) were washed in PBS and incubated in cell culture medium or medium containing F105-L-Al^{488} for 24 h at 37 °C in a 5% CO_2 atmosphere. To investigate the specificity of binding of the F105-L-Al^{488} immunoliposomes due to the targeting moiety F105, incubations were done in the presence or absence of 15 μg/ml unconjugated F105 IgG. After incubation, cells were washed three times in PBS to remove free

liposomes, and analyzed for fluorescence of Al^{488} in a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

2.7. Analysis of uptake of F105-L-Al^{488} by confocal microscopy

Uninfected and HIV-1-BaL-infected PM1 cells were incubated in 200 μl of cell culture medium as a negative control, or medium containing 8 μg/ml F105-L-Al^{488} or 10 μg/ml of the Golgi marker wheat germ agglutinin (WGA) for 24 h at 37 °C in a 5% CO_2 atmosphere. After incubation, cells were centrifuged and resuspended in 200 μl PBS and placed in the wells of an 8-well plate (Nalge Nunc, Rochester, NY) and were analyzed using a Leica SP2 confocal microscope (Leica, Wetzlar, Germany) equipped with an Ar/Kr laser, using a 60× oil objective and a 1024 × 1024 pixel resolution. Image processing was carried out with Adobe Photoshop 7.0 software (Adobe, San Jose, CA). To investigate the specificity of binding and uptake due to the targeting moiety, cells were incubated with F105-L-Al^{488} immunoliposomes with and without 15 μg/ml unconjugated F105 IgG.

2.8. Analysis of the antiviral effect of F105-L-PI1

HUTIIIB cells were incubated for 2 h with F105-L-PI1, L-PI1, and free PI1 (final DMSO concentration 0.5%) at drug concentrations of 10, 1, 0.1 and 0.01 μM. As negative controls, HUTIIIB cells were incubated with F105-L-Al^{488} or L-Al^{488} in separate experiments, at equivalent lipid concentrations as the corresponding drug containing formulations (F105-L-PI1 and L-PI1, respectively). Next, the cells were washed and incubated at 37 °C in a 5% CO_2 atmosphere for 1, 2, 3 and 4 days. The supernatant was collected and the release of infectious virus was monitored by titration on MT4-LTR-EGFP cells as previously described (Jochmans et al., 2006). The level of inhibition by the different PI1 formulations was calculated by comparing EGFP expression in MT4 cells infected with the supernatants from treated and untreated HUTIIIB cells.

3. Results

3.1. Immunoliposome preparation and characterisation

Immunoliposomes loaded with either the fluorophore Al^{488} or the antiretroviral compound PI1 were prepared by two different methods. For the dye-loaded liposomes, a dye solution was used during lipid hydration and liposome formation. For the drug-loaded liposomes, the antiretroviral compound was added after liposome formation and was loaded inside the liposomes using an ammonium gradient-based loading method. In both cases, free unincorporated compound (not encapsulated) was removed by size exclusion chromatography. A sulfydryl-reactive lipid (MalPEG–DSPE) was then inserted into the liposome bilayer, which enabled the surface conjugation of the HIV-gp120-targeting F105 Fab' antibody through the formation of a disulfide bond. Conjugation of Fab' to liposomal surface was achieved by adding reduced Fab' to the liposomes. The reduced Fab'2 mixture contained mainly Fab', in addition to low concentrations of heavy chain (HC) and light chain fragments (LC), as well as unreduced Fab'2. This conjugation methodology resulted in efficient ligand incorporation, as shown in Fig. 2. After incubation of the Fab' mixture with the PI1- or Al^{488}-containing liposomes, and subsequent SEC purification of the resulting immunoliposomes, the main F105-related component was the Fab'-MalPEG–DSPE conjugate (FabCG). Other, minor, components included MalPEG–DSPE conjugates of LC and HC (named LCCG and HCCG, respectively), as well as residual HC and LC.

Several parameters were measured to characterize the final drug-loaded or dye-loaded immunoliposomes (Table 1). PI1 concentrations of antibody-conjugated and control liposomes were around 2 mg/ml, while the total lipid concentration varied between 11.2 and 14.5 mM. The Fab'-conjugated liposomes contained 83–91 antibody molecules per liposome, a value similar to that reported for other antibody-coated liposomes (Lukyanov et al., 2004). Based on yields obtained with previous trials, the expected antibody to liposome ratio was ~70. Higher values were obtained due to a higher-than-usual percentage of Fab' formed during the Fab'2 reduction process.

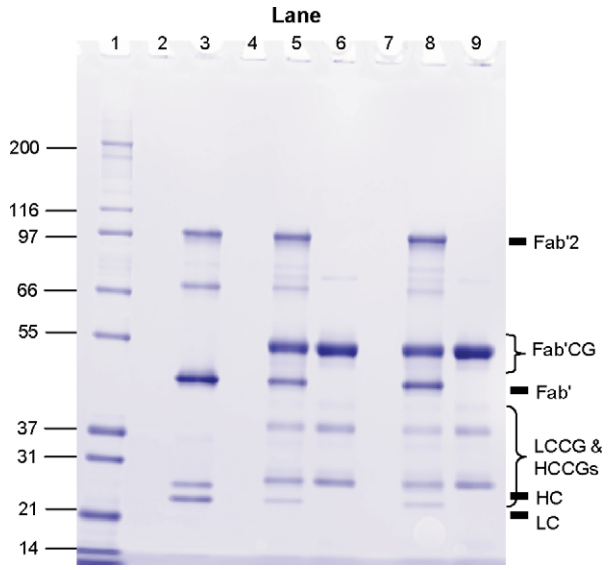

Fig. 2. Conjugation materials during the process of conjugating F105 Fab' to liposomes. Lanes: 1, molecular weight marker; 3, Fab'2 post-reduction/pre-conjugation; 5, F105-L-PI1 pre-SEC; 6, F105-L-PI1 post-SEC; 8, F105-L-Al^{488} pre-SEC; 9, F105-L-Al^{488} post-SEC. CG, conjugate; LC, light chain; HC, heavy chain; LCCG and HCCG, MalPEG–DSPE conjugates of LC and HC, respectively.

Table 1
Characterisation of the respective liposome preparations.

Liposome	PI1 conc. (mg/ml)	Total lipid conc. (mM)[a]	# F105 per liposome
F105-L-Al^{488}	–	11.2	83
F105-L-PI1	1.87	12	91
L-Al^{488}	–	13.4	–
L-PI1	2.26	14.5	–

Targeted liposomes are indicated with a leading F105 notation; liposomes were loaded with Al^{488} or PI1, respectively.

[a] Total lipid concentration (TLC) is based on determination of phospholipid (HSPC and malPEG–DSPE) concentration (PC) with a phosphorus assay (see Section 2.4) and the assumption that the original HSPC:cholesterol:malPEG–DSPE molar ratio of 55:40:5 is maintained through the liposome preparation process. Thus, TLC = PC × 1.67.

PI1 retention experiments under conditions mimicking antiviral experiments in cell-based virus replication assays indicated minimal PI1 leakage over time at 37 °C. There was only ~0.5% PI1 leakage at day 1 and ~1% leakage at day 3 for both F-105 coated (F105-L-PI1) and non-coated (L-PI1) liposomes (data not shown).

3.2. Kinetic analysis of the interaction of F105 IgG, Fab'2 and Fab' with recombinant HIV-1 gp120 using BIAcore technology

The binding kinetics of F105 IgG, Fab'2 and Fab' to gp120 were analyzed by SPR. After immobilization of recombinant gp120 on a CM5 sensor surface, serial dilutions of F105 IgG, Fab'2 and Fab' were injected over the gp120 surface. A heterogeneous ligand binding model best described the binding of the bivalent entities F105 and the F105 Fab'2 fragment, to gp120. This model uses two bind-

Table 2
Association and dissociation rates (k_{on} and k_{off}) and derived affinity constants (K_D) for the interaction of F105 IgG ($n = 8$), F105 Fab'2 ($n = 3$) and Fab' ($n = 1$) with recombinant gp120.

	F105 IgG	F105 Fab'2	F105 Fab'
k_{on1} ($M^{-1}\,s^{-1}$)	$(4.8 \pm 0.6) \times 10^4$	$(2.9 \pm 1.1) \times 10^4$	0.7×10^4
k_{off1} (s^{-1})	$(7.8 \pm 8.7) \times 10^{-5}$	$(2.6 \pm 0.9) \times 10^{-5}$	2.7×10^{-5}
K_{D1} (M)	$(2.3 \pm 2.6) \times 10^{-9}$	$(1.1 \pm 0.7) \times 10^{-9}$	3.9×10^{-9}
k_{on2} ($M^{-1}\,s^{-1}$)	$(3.9 \pm 0.8) \times 10^5$	$(3.5 \pm 1.5) \times 10^5$	–
k_{off2} (s^{-1})	$(7.4 \pm 9.5) \times 10^{-5}$	$(2.5 \pm 0.3) \times 10^{-5}$	–
K_{D2} (M)	$(3.2 \pm 4.3) \times 10^{-10}$	$(7.9 \pm 3.2) \times 10^{-11}$	–

The data were analyzed based on the heterogeneous (IgG and Fab'2) or 1:1 (Fab') ligand binding model.

ing modes described by two pairs of rate constants (k_{on1}/k_{off1} and k_{on2}/k_{off2}). When describing binding of the monovalent F105 Fab' fragment to gp120, the contribution of the second pair of rate constants became negligible and the binding could be described with the simple bimolecular 1:1 binding model.

The overall affinity constants (K_D) were calculated from the association (k_{on}) and dissociation (k_{off}) values (Table 2). The F105 IgG and both F105 fragments had comparable affinities. The K_D for binding to gp120 was 2.3 nM for F105 IgG, 1.1 nM for the F105 Fab'2 fragment, and 3.9 nM for Fab', in line with the previously observed lack of influence of antibody valence on affinity (Cavacini et al., 1994). The K_D for binding of soluble CD4 (sCD4), the natural ligand of gp120, to gp120 was 35 nM (Clayton et al., 2007). F105 IgG and its fragments had a higher binding affinity for gp120 in comparison with sCD4.

3.3. Analysis of binding and uptake of immunoliposomes

Specificity of binding and uptake of the immunoliposomes by HIV-1-infected cells was measured by FACS with F105-L-Al^{488} (Fig. 3). As expected from the specificity of monoclonal antibody F105 for the gp120, the F105-L-Al^{488} was bound and taken up extensively by HIV-1-infected cells, whereas there was no discernible binding or uptake in uninfected PM1 cells. When F105-L-Al^{488} was incubated with PM1-BaL cells in competition with an excess of F105 IgG (15 μg/ml), F105-L-Al^{488} binding to the cells was markedly reduced.

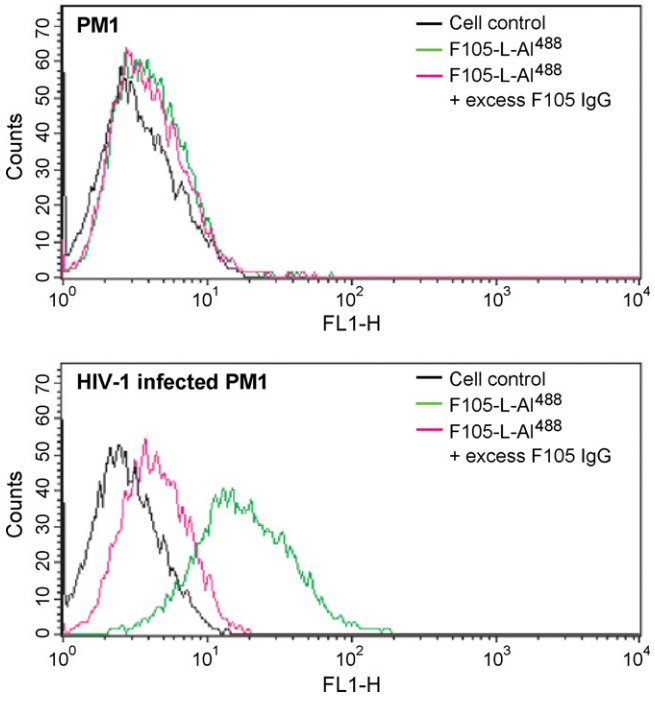

Fig. 3. FACS histograms showing binding and uptake of F105-L-Al^{488} immunoliposomes by uninfected and HIV-1-BaL-infected PM1 cells.

These experiments indicated the specificity of the uptake between the targeted liposomes by HIV-1-infected cells, and verified the preservation of the specific binding of F105 Fab' to gp120 after conjugation and incorporation into liposomes. The internalisation of F105-L-Al^{488} at the single cell level was investigated by confocal microscopy to explore further trafficking of the liposome after cellular uptake had occurred (Fig. 4). Following binding and internalisation of the F105-L-Al^{488}, fluorescent label was detected at the membrane and in the cytoplasm of HIV-1-BaL-infected PM1 cells. Co-localisation of WGA with F105-L-Al^{488} indicated that the liposomes accumulate in the Golgi (Parkkinen et al., 1997). When F105-L-Al^{488} was incubated with PM1-BaL cells and an excess of free F105 IgG, the binding and uptake of F105-L-Al^{488} was inhibited (Fig. 3), demonstrating again the specificity of the liposome internalisation.

Binding and internalisation of F105-L-Al^{488} was assessed on persistently HIV-1-BaL-infected PM1 cells, as protocols were optimised in this system. Binding and uptake experiments with F105-L-Al^{488} were repeated with HIV-1-IIIB-infected cells and as expected, since F105 binding is not dependent on co-receptors (Clayton et al., 2007), results for both systems were comparable (data not shown).

3.4. Antiviral effect of targeted, non-targeted and free PI1

The antiviral effect of L-PI1, F105-L-PI1 and free PI1 was evaluated on persistently HIV-1-IIIB-infected cells as used in our screening program (Guillemont et al., 2005). At a PI1 concentration of 1 μM, F105-L-PI1 provided sustained 100% inhibition of viral replication over 4 days (Fig. 5A), where the same concentration of free PI1 and non-targeted L-PI1 resulted in approximately 60% and 80% inhibition, respectively. At a reduced PI1 concentration of 0.1 μM (Fig. 5B), the influence of the drug delivery vehicle on the antiviral effect of PI1 was more profound. The F105-L-PI1 formulation enabled approximately 80% inhibition of viral replication for the entire duration of the experiment. However, at a PI1 concentration of 0.1 μM within L-PI1 or as free PI1, approximately 20% and 10% inhibition, respectively, was observed at day 4. This difference in inhibition of viral replication clearly demonstrated that the targeted liposome delivery of a known concentration of PI1 to infected cells enabled a greater and more sustained antiviral effect than the delivery of the free PI1 or untargeted liposomal drug.

The dose-response relationship at 24 h post-infection (Fig. 6) confirmed the increased antiviral effect of F105-L-PI1 compared with L-PI1 and free PI1. F105-L-PI1 demonstrated full antiviral potency at a concentration (0.1 μM) where L-PI1 and free PI1 were ineffective. Furthermore, there was approximately 30% inhibition of viral replication at the lowest concentration of 0.01 μM with the F105-L-PI1 formulation, whereas no inhibition was observed with the free PI1 and L-PI1 at this concentration. As expected, the non-drug containing L-Al^{488} and F105-L-Al^{488} formulations did not show any antiviral effect.

4. Discussion

The current standard of care for HIV-1-infected individuals generally results in long-term suppression of HIV-1 replication and prevention of disease progression. However, toxicities associated with prolonged combination therapy are known to cause serious adverse events that may impede therapeutic adherence, leading to development of HIV-1 resistance. Since it is presumed that toxici-

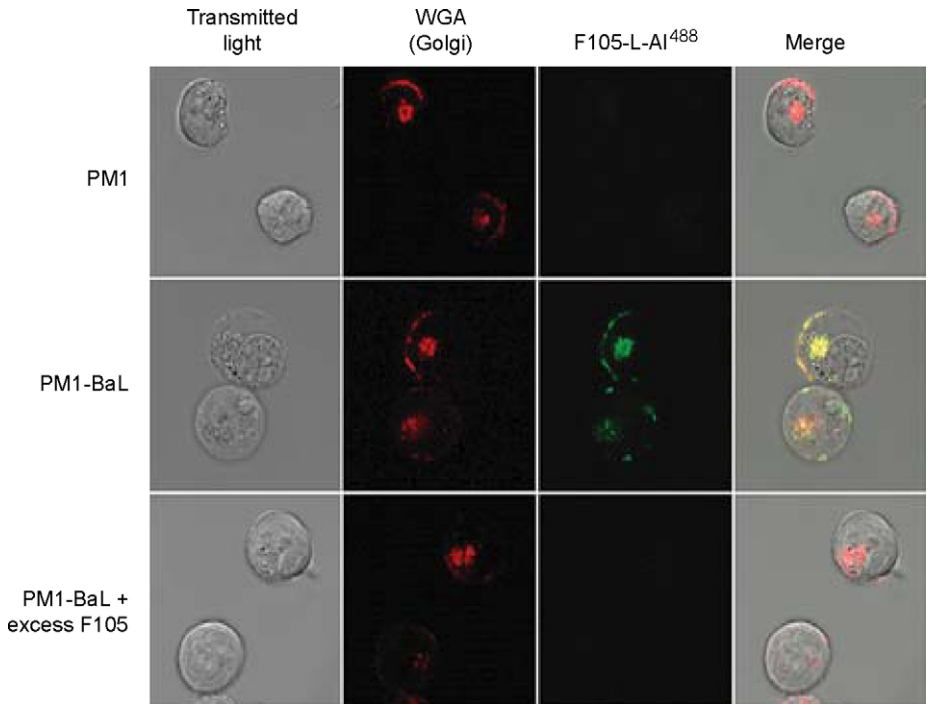

Fig. 4. Confocal microscopy pictures showing uptake of F105-L-AI^{488} immunoliposomes by HIV-1-infected PM1 cells. PM1: parent PM1 cell line; PM1-BaL: PM1 cells stably infected with HIV-1 BaL; WGA is a Golgi marker.

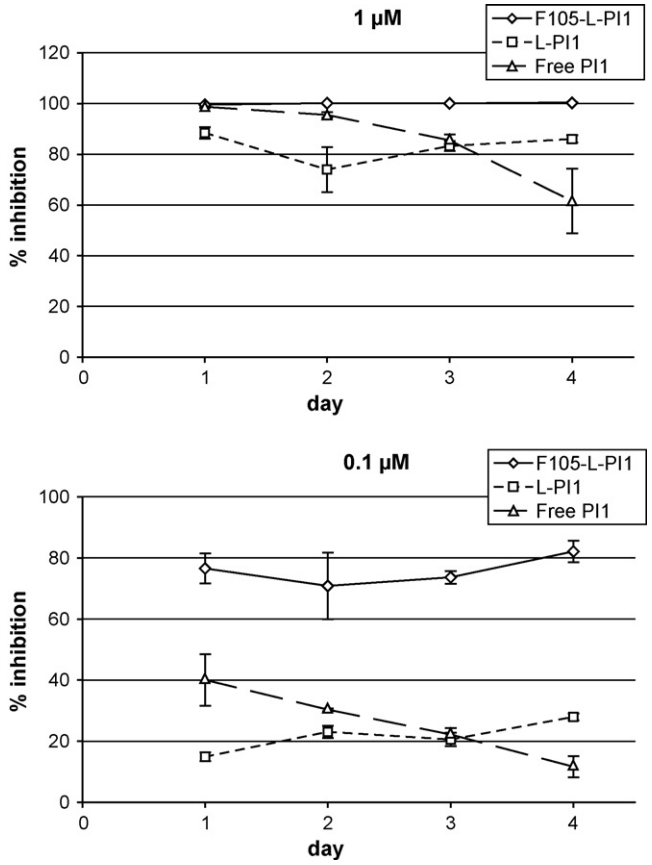

Fig. 5. Antiviral effect of different liposomal preparations (F105-L-PI1 and L-PI1) and free PI1. Antiviral activity was measured over a 4-day period in persistently HIV-1-IIIB-infected HUT78 cells, at inhibitor concentrations of 0.1 and 1 μM. Values are mean ± standard deviation of 3 replicate measurements in one representative experiment.

ties mainly result from drug exposure of uninfected cells, targeting antiretrovirals specifically to HIV-infected cells would likely reduce these effects. Moreover, targeted therapy would enable delivering higher concentrations of inhibitors to infected cells, thus reducing daily pill burden. In a previous publication (Clayton et al., 2007), we proposed to use the gp120-specific monoclonal antibody F105 as a targeting moiety in the selective delivery of antiretrovirals to HIV-1-infected cells. Here the F105 Fab' fragment was linked to sterically stabilized pegylated liposomes containing a protease inhibitor payload, and this construct was used to verify the feasibility of our targeting concept.

For the selective drug delivery to HIV-infected cells to be successful, the F105 Fab' covalently attached on the surface of the liposomes must be accessible for gp120 binding. To ensure that

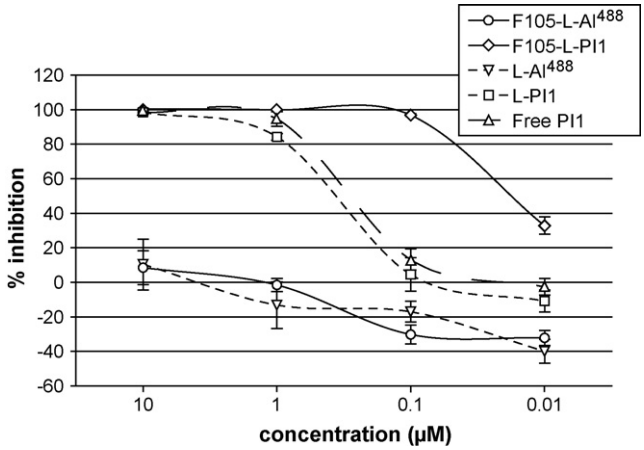

Fig. 6. Dose response curves for the antiviral effect of different liposomal preparations (F105-L-PI1, L-PI1, F105-L-AI^{488}, and L-AI^{488}) and free PI1. Antiviral activity was measured in persistently HIV-1-IIIB-infected HUT78 cells, at 24 h post-infection. Values are mean ± standard deviation of 3 replicate measurements in one representative experiment.

the Fab' ligands are not masked by the PEG coating of the stabilized liposomes, a methodology for efficiently conjugating the Fab' to the tip of a pegylated lipid already inserted in the liposome bilayer was developed. The success of the ligand-targeted approach also relies on the drug remaining encapsulated inside the liposomes until these liposomes are taken up inside the cells, as a result of F105-gp120 binding. In this regard, the use of pegylated liposomes is probably critical, since it is expected to significantly reduce the rate of uptake by cells of the reticuloendothelial system, in comparison with non-pegylated liposomes (Drummond et al., 1999). In addition, remote drug loading based on ammonium gradient ensures efficient retention of the drug inside liposomes (Bolotin et al., 1994; Gabizon et al., 1993).

F105 and its fragments Fab'2 and Fab' bind tightly to recombinant HIV-1 gp120 with higher affinity than the natural ligand-derived soluble CD4. Therefore, these targeting moieties can compete with the natural ligand of gp120. Using F105 fragments instead of the whole IgG is presumed to lower the immunogenicity of the targeted immunoliposomes. The monovalent Fab' fragment was selected for use as a targeting moiety as this monovalent approach was employed successfully in two previous examples of targeted therapy in HIV (Gagné et al., 2002; Song et al., 2005).

The protocols used for Fab'2 reduction and subsequent conjugation resulted in an efficient coating of the liposomes with F105 Fab'. The superior and sustained antiviral activity of F105-L-PI1 when compared with free PI1 is consistent with an efficient retention of the drug inside the ligand-targeted pegylated liposomes (in the assay conditions) as well as an appropriate accessibility of the Fab' for binding to gp120. The very low drug leakage observed upon incubation of the F105-L-PI1 in cell culture medium at 37 °C (only ~ 1% leakage at day 3) confirms that stable drug encapsulation was achieved. Ultimately, pharmacokinetics and tissue distribution (and potentially efficacy) studies in preclinical models will be necessary to assess further the viability of the ligand-targeted liposomes approach for the treatment of HIV infection.

The FACS and confocal imaging studies showed that the F105-L-Al^{488} bound efficiently to HIV-infected cells but not to uninfected cells. Free F105 IgG successfully competed the F105-L-Al^{488} from binding to infected cells, offering further evidence that the F105 Fab' at the surface of the liposomes was responsible for this selective binding. Following binding, F105-L-Al^{488} and F105-L-PI1 immunoliposomes were readily taken up by HIV-1-infected cells, as demonstrated by the green fluorescence pattern of the confocal images and the immediate high-level antiviral effect of PI1, respectively. Internalised immunoliposomes localised in the Golgi apparatus over time, an observation consistent with previous data on uptake and internalisation of the F105 IgG (Clayton et al., 2007). The plasma membrane–Golgi endocytosis and recycling pathway, is used by the Golgi marker WGA (Vetterlein et al., 2002). During this multi-step process, receptor-bound WGA is internalised, cycled through vesicular endosomes and the endocytic *trans*-Golgi networks and finally taken up in the Golgi. A similar pathway is likely to be the mode employed by the immunoliposomes to gain entry inside the cell after binding gp120 on the plasma membrane; this is in line with the natural recycling pathway for gp120 (Blot et al., 2003). Uptake of F105-L-PI1 immunoliposomes in the Golgi enables the establishment of a reservoir of antiviral drug in the cytoplasm of an HIV-infected cell. However, because of liposome destabilization and drug release, PI1 is expected to diffuse from the Golgi and distribute throughout the cytoplasm over time. The strong and sustained antiviral activity of ligand-targeted liposomal PI1 confirms that the protease inhibitor is released from liposomes, and subsequently reaches the sites of HIV-1 protease activity inside the cell. The reproducible but markedly less potent antiviral activity of non-targeted L-PI1 is possibly due to a very low level of non-specific binding of the non-targeted liposomes to the surface of the HIV-infected cells. This binding could persist even after cell wash, at 2 h after start of incubation, and lead to residual antiviral activity through: (i) some degree of liposome internalisation, or (ii) slow extracellular release of some of the drug from the membrane-bound liposomes over the 4 days of the assay.

Drug delivery by targeting a viral glycoprotein and exploiting the retrograde transport pathway may offer a viable alternative to targeting via more established routes that include receptor-mediated endocytosis which has been employed for numerous therapeutic areas including cancer, Alzheimer's disease and lysosomal storage diseases (reviewed in Bareford and Swaan, 2007). Following receptor-mediated endocytosis, the environment of the late endosomes changes upon fusion with the pre-lysosomal vesicles, creating a lysosome with a slightly lowered pH of around 5.5–6, ensuring appropriate conditions for the activity of the lysosomal acid hydrolases. Hence, the classical endocytosis route and subsequent lysosomal environment may be detrimental to the drug or delivery vehicle, possibly resulting in degradation. By avoiding the lysosome and ultimately targeting the Golgi, as we have shown in this study, it is less likely that the delivered drug and immunoliposomes will be degraded.

The targeting of the gp120 on the plasma membrane of infected cells seems an attractive strategy, but the intracellular retention in the Golgi over longer periods of time may be an issue. As more gp120 is produced by and circulated through the infected cell, there is the possibility that the immunoliposomes become displayed on the plasma membrane where drug is likely to diffuse away from the target cell and therefore be less likely to deliver a local antiviral effect. Longer term studies of retention and efficacy in in vitro systems and animal models would be required to address this concern. However, we may speculate that the integrity of the immunoliposome particle is more likely to be preserved in the Golgi, in comparison with the more destructive lysosome, thereby ensuring retention of the drug in the infected cell, and avoiding degradation of the immunoliposome to a non-targeted particle by cleavage of the conjugated targeting antibody.

Recent examples of ligands used to enable delivery to cellular compartments through alternative endocytic mechanisms include the shigella toxin B subunit (STB), which binds to the trisaccharide domain of globotriaosylceramide and enters endocytic vesicles and transit to the trans-Golgi network and endoplasmic reticulum (Cosson and Letourneur, 1997). It was shown that STB coupled with model antigen Mage 1, was internalised by peripheral blood mononuclear leucocytes and accumulated in the endoplasmic reticulum and Golgi apparatus (Haicheur et al., 2000), further highlighting the potential of alternative endocytic pathways for drug delivery. Additionally, the penetration by diphtheria toxin directly into the cytosol from the endosome offers a targeting moiety and route of delivery that has been utilized in delivering genes (Fisher and Wilson, 1997) and peptides (Stenmark et al., 1991).

The dose response of the F105-L-PI1 and free PI1 at 24 h post-incubation revealed that the antiviral effect of the F105-L-PI1 is about 20-fold higher than that of free drug at this time point, since the PI1 concentration leading to 50% inhibition was about 20-fold higher for free PI1. The most remarkable property of the F105-L-PI1 was that, in contrast to free PI1, the antiviral activity did not decrease with time over the 4-day period assessed. Such sustained intracellular drug release effect, probably related to the mode of specific uptake and retention inside the cell, is a favourable sign for *in vivo* use of this immunoliposome formulation for HIV therapy.

The presence of a targeting moiety on drug-loaded targeted immunoliposomes may potentially add an additional level of resistance development. However, the local delivery of higher drug doses could provide a higher barrier for the development of drug resistance to the antiviral immunoliposome payload.

By attaching the F105 Fab'-targeting moiety to the drug-loaded liposomes, efficient liposome uptake by non-phagocytic cells was facilitated, as demonstrated in a T-cell based assay. This is advantageous for treating HIV-1 infection, as T-cells are the prime viral targets. However, it remains to be seen if all HIV-1-infected cells express sufficient gp120 at the plasma membrane to bind the immunoliposomes. One concern of this approach in targeting drug delivery to HIV-1-infected cells is that latently infected cells, such as resting T-cells, do not express gp120, and therefore would be unlikely to uptake F105-L-PI1. However, as HIV-1 in latently infected cells does not actively replicate, this hidden virus is also insensitive to non-targeted antiretrovirals (Geeraert et al., 2008). Moreover, it remains unclear whether all relevant body compartments would be penetrated by the immunoliposome, for instance, assessment of the penetration of the immunoliposomes into the central nervous system would be an important future study. Recent studies showing the delivery of AZT across the blood-brain barrier in transferrin-coated PEG nanoparticles (Mishra et al., 2006) offer some insight into the potential use of targeting to deliver payloads to the central nervous system. Additionally, it may be possible that circulating virions could saturate the targeting moieties of the liposomes and hence further reduce their efficacy as a drug delivery system in the body.

Clearly, the potential of targeting antiretroviral drugs in liposomes via virus-targeted antibody fragments to reduce pill burden, increase adherence and reduce unwanted toxicities of drug regimens remains an attractive but challenging opportunity.

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