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Antiviral Research 34 (1997) 1–15

Antiviral Research

Inhibition of human immunodeficiency virus type-1 replication in macrophages and H9 cells by free or liposome-encapsulated L-689,502, an inhibitor of the viral protease

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Received 17 December 1995; accepted 4 November 1996

Abstract

Macrophages are recognized as a major reservoir of HIV-1 in infected individuals. We examined the effect of an inhibitor of the viral protease, L-689,502, on virus production by monocyte-derived macrophages infected with HIV-1_{BaL}. Continuous treatment with L-689,502 drastically inhibited virus production in a dose-dependent manner in the range of 10–200 nM, in some cases by more than 1000-fold, compared to untreated cells. Since liposomes can be targeted to macrophages in vivo, we examined whether the inhibitor was effective following delivery in liposomes. The inhibitor encapsulated in multilamellar liposomes was more effective than the free drug in inhibiting virus production in macrophages, throughout the concentration range studied. The EC_{90} of the liposomal inhibitor was 2.9- to 4.5-fold lower than that of the free compound. L-689,502 encapsulated in sterically stabilized liposomes with prolonged circulation time inhibited virus production at a level comparable to the free inhibitor. When macrophages were infected and treated for only a limited time, L-689,502 in multilamellar liposomes was the most effective of the three treatments. In chronically infected H9 cells treated continuously, the free inhibitor was more effective than the liposome-encapsulated drug, but virus production was reduced only to 40–60% of controls. In contrast, treatment of acutely infected H9 cells with either free or encapsulated L-689,502 inhibited virus production by up to three orders of magnitude. Our results indicate that liposomes may be useful for the delivery of HIV protease inhibitors with low aqueous solubility and low oral bio-availability, and for the targeting of these drugs to lymph nodes. © 1997 Elsevier Science B.V.

Abbreviations: DME-HG, Dulbecco's Modified Eagle's Medium, high glucose; DMSO, dimethyl sulfoxide; FBS, heat-inactivated fetal bovine serum; MLV, multilamellar vesicles; PEG-DSPE, poly(ethylene glycol) distearoylphosphatidylethanolamine; PC, egg phosphatidylcholine; PG, phosphatidylglycerol; PHEPC, partially hydrogenated egg phosphatidylcholine; PI, protease inhibitor L-689,502; PI-MLV, MLV containing protease inhibitor; SSV, sterically stabilized vesicles.

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PII S0166-3542(96)01017-0

1. Introduction

Prevention of infectious virus production by cells containing the human immunodeficiency virus type 1 (HIV-1) proviral DNA in their genome is important in reducing the viral load in infected individuals. The protease of HIV-1 is crucial to the processing of the viral gag–pol precursor polyprotein. This processing is essential for the production of infectious virions (Kay and Dunn, 1990; Robins and Plattner, 1993). The protease is known to catalyze eight cleavages of the precursor, the last of which is thought to occur at the point of budding of assembled virions from the cell membrane (Robins and Plattner, 1993; Kaplan et al., 1994). In contrast to inhibitors of reverse transcriptase, which act at an early stage of the viral life cycle, inhibitors of the viral protease should be effective at later stages. Numerous inhibitors exhibiting a high specificity for the viral protease over cellular proteases have been developed (Meek et al., 1990; Ashorn et al., 1990; Erickson et al., 1990; Roberts et al., 1990; Huff, 1991; Kempf et al., 1995). For example, the protease inhibitor U-75875 was shown to be effective in reducing virus production in acutely infected monocyte/macrophages at a concentration of 1 μM, and in chronically infected cells at 10 μM (Perno et al., 1993). The inhibitor A77003 reduced HIV production by 50% in H9 and MT-4 cells in the range of 150–300 nM, while in CEM cells this range was 30–70 nM (Kempf et al., 1991). The EC_{50} for ABT-538 in peripheral blood lymphocytes infected with seven patient isolates was in the range 9–130 nM (Kempf et al., 1995). The inhibitor L-689,502 inhibited the spread of infection by more than 95%, in the range 6–50 nM in infected MT-4 cells and 12–25 nM in peripheral blood lymphocytes, as detected by viral p24 production (Thompson et al., 1992). In monocyte/macrophages, 25–50 nM L-689,502 completely prevented the spread of the virus from initially infected cells, as detected by immunofluorescence with anti-HIV-1 human serum (Thompson et al., 1992).

Macrophages are recognized to be a major reservoir of HIV-1 in infected individuals, particularly in the lymph nodes, brain, lungs, skin, liver and spleen (Gartner et al., 1986; Ho et al., 1986; Meltzer et al., 1990). Macrophage-tropic HIV-1 variants are also thought to initiate infection in humans, and to persist during the asymptomatic phase of the disease (Schuitemaker, 1994). Thus, effective delivery of protease inhibitors to macrophages is likely to reduce the viral burden and reduce the risk of virus transmission to T-cells.

Liposomes are avidly phagocytosed by macrophages both in vitro and in vivo and can be used to target drugs to these cells in vivo (Raz et al., 1981; Daleke et al., 1990; Lee et al., 1993; Poste et al., 1982; Roerdink et al., 1986; Düzgüneş et al., 1988; Gonzalez-Rothi et al., 1991). Liposome-mediated delivery or targeting of drugs can reduce toxicity and dose requirement (Düzgüneş et al., 1988, 1991; Bakker-Woudenberg et al., 1993; Tomioka et al., 1991; Van Etten et al., 1995; Kedar et al., 1994; Torre-Cisneros et al., 1993; Szoka and Chu, 1988; Szoka, 1991). Moreover, liposomes may be advantageous for the delivery of highly hydrophobic compounds, such as most of the HIV protease inhibitors.

We investigated the effect of free and liposome-encapsulated L-689,502 on virus production by human monocyte-derived macrophages infected with a monocytotropic HIV-1 strain. We also investigated whether recently developed liposomes with prolonged circulation in the bloodstream (Papahadjopoulos et al., 1991; Allen, 1994; Woodle and Lasic, 1992) could be used to effectively deliver the inhibitor to macrophages. Finally, we compared the effect of free and liposome-encapsulated inhibitor on virus production in a chronically or acutely infected T-cell line. Some of our results have been presented earlier in preliminary form (Pretzer et al., 1995).

2. Materials and methods

2.1. Reagents

The HIV-1 protease inhibitor L-689,502 (referred to as PI below) (Thompson et al., 1992) was a gift from W. Thompson and P. Anderson of Merck (West Point, PA). Dimethyl sulfoxide

Hybri-Max (DMSO) was obtained from Sigma (St. Louis, MO). Egg phosphatidylcholine (PC) and phosphatidylglycerol (PG), as well as L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (egg) (Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Polyethylene glycol (average molecular mass of 1900 Da) covalently coupled by a carbamate bond to distearoylphosphatidylethanolamine (PEG-PE) (Allen et al., 1991), as well as partially hydrogenated egg phosphatidylcholine (PHEPC), were a gift from M. Woodle (Liposome Technology, Menlo Park, CA). Cholesterol was obtained from Calbiochem (La Jolla, CA).

2.2. Liposomes

Multilamellar vesicles (MLV) were prepared at a 1:1:1 molar ratio of PC:PG:cholesterol. PI-MLV were prepared by including PI at a molar ratio of 0.4:1:1:1 (PI:PC:PG:cholesterol). Rhodamine-labeled MLV (Rh-MLV) were prepared by including Rh-PE at a molar ratio of 0.02:1:1:1 (Rh-PE:PC:PG:cholesterol). Briefly, chloroform solutions of the lipids were mixed, with or without PI, and evaporated dry under vacuum in a rotary evaporator. The dried mixture was hydrated by vortexing in argon-saturated buffer (10 mM HEPES, 140 mM NaCl, 10 mM KCl, pH 7.4, 290–300 mOsm). The osmolality of the buffer was measured in a Wescor (Logan, UT) vapor pressure osmometer. Liposomes were prepared under sterile conditions, within a week before the start of each experiment. It was determined that 96% of the PI co-pelleted with the liposomes following centrifugation, 24 h after the liposomes were prepared. Phase contrast and fluorescence microscopy indicated the liposomes were greater than 3 μm in diameter and had a heterogeneous size distribution.

Sterically stabilized vesicles with prolonged circulation time consisted of 0.15:1.85:1 molar ratios of PEG-PE:PHEPC:cholesterol (SSV), 0.4:0.15:1.85:1 molar ratios of PI:PEG-PE:PHEPC:cholesterol (PI-SSV), or 0.02:0.15:1.85:1 molar ratios of Rh-PE:PEG-PE:PHEPC:cholesterol (Rh-SSV). Chloroform solutions of lipids with or without PI were evaporated and hydrated in buffer. Liposomes were then extruded four times through two stacked 0.08 μm polycarbonate membranes (Poretics, Livermore, CA) in a custom-made high pressure extrusion chamber under argon. Dynamic light scattering measurements with a Coulter NP-4 instrument indicated that the liposomes had an average diameter of 120 ± 20 nm. The lipid concentration was quantitated by phosphate assay (Bartlett, 1959). Liposomes were serially diluted in medium for use in cell experiments.

2.3. Cells and virus

Human macrophages were isolated from HIV-seronegative buffy coats by Ficoll-Hypaque (Histopaque 1077, Sigma, St. Louis, MO) gradient centrifugation and plastic adherence. Briefly, mononuclear cells separated by centrifugation were counted and plated in (DME-HG) (Irvine Scientific, Santa Ana, CA) without serum, supplemented with L-glutamine (4 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were allowed to adhere overnight, after which non-adherent cells (approximately 90–95% of the number plated) were removed by gentle washing, and the medium was replaced with DME-HG containing 20% heat-inactivated fetal bovine serum (FBS; Sigma), 10% human AB serum (Advanced Biotechnologies, Columbia, MD) and L-glutamine, penicillin and streptomycin as above (DME 20/10). The cells were left undisturbed in this medium to differentiate for 5–6 days. Subsequent culture and experiments were carried out in DME-HG with 20% heat-inactivated FBS and the usual supplements (DME/20), in a 37°C incubator with 5% CO_2. Medium was replaced three times per week.

H9, a human T-cell line originally derived as a single-cell clone from a HUT-78 cell line (Mann et al., 1989; Popovic et al., 1984a,b), was obtained from R. Gallo through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Cells were cultured in RPMI-1640 (Irvine Scientific) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (RPMI/10), and split 1:6 every 3–4 days.

Chronically infected H9/HTLV-IIIB cells were kindly provided by T. El-Beik and J. McGrath (San Francisco General Hospital), and were cultured the same as uninfected H9 cells.

HIV-1_{BaL} was obtained from Advanced Biotechnologies, then propagated in macrophages. Cells were infected on the ninth day after seeding in T25 flasks (Costar, Cambridge, MA), and cultured in DME/20 with replacement of medium three times per week. Supernatants were collected and clarified by two centrifugations (1500 rpm ($469 \times g$), and 2000 rpm ($834 \times g$)) for 10 min each in an H1000B Sorvall rotor, and then pooled based on similar p24 content. Supernatants were aliquotted and stored at −80°C. All but one of these experiments used the same lot of this preparation (p24, 50 ng/ml; reverse transcriptase, 8.3×10^3 cpm/ml). The first experiment (Fig. 1) used the day 12 supernatants, which were pooled separately (p24, 43.6 ng/ml).

HIV-1_{IIIB} was obtained from the culture supernatant of H9/HTLV-IIIB cells. The supernatant was clarified by filtration through a 0.45 μm filter (Millipore, Bedford, MA), aliquotted and stored at −80°C (p24, 111.7 ng/ml; reverse transcriptase, 8.42×10^4 cpm/ml; $TCID_{50}$ on H9 cells, 1.17×10^4/ml).

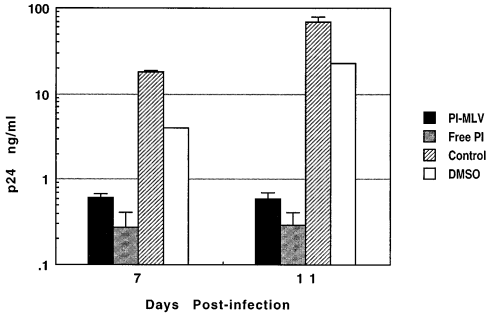

Fig. 1. Effect on viral p24 production of pre-treatment and continuous post-infection treatment of macrophages with 100 nM free or liposome-encapsulated L-689 502. Macrophages were plated at $0.5–1 \times 10^5$ cells/well in 48-well plates. After 6 days the cells were treated overnight with free or liposome-encapsulated protease inhibitor (PI). The following day the treatment medium was removed, and the cells were infected with HIV-1_{BaL} at 4.4 ng p24/well, as described in Section 2. Following infection the cells were cultured in the continuous presence of liposome-encapsulated or free PI.

2.4. Infection and treatment

Macrophages were infected with HIV-1_{BaL} 7–15 days after isolation. Medium was removed from the wells and replaced with virus stock in a volume of medium sufficient to cover the cells (100–200 μl/well in 48-well plates, 28 μl in 96-well plates). Macrophages in 48-well plates were infected with 5 ng p24/well, and in 96-well plates with 1.4 ng/well. The cells and virus were incubated 2–2.5 h at 37°C, then washed 3 times with medium and incubated in DME/20 with or without treatments, with thrice weekly medium replacement. For overnight treatment, medium was removed on the following day, the wells were washed three times and fresh medium was placed in the wells. Cells were then cultured as usual, without further treatments. For continuous treatment, the medium replacement on the following day was omitted, and fresh dilutions of treatments were added with each medium replacement. In one experiment macrophages were pretreated overnight with liposomes or free PI. Following pretreatment, the wells were washed three times and infected as usual. After infection, the treatments were replaced.

H9 cells, at a density of 2×10^7 cells/ml in RPMI/10, were infected with HIV-1_{IIIB} at a concentration of 4.2 ng p24 per 10^6 cells (MOI, 4.4×10^{-4}), for 140 min at 37°C, 5% CO_2. Cells were then washed three times in medium and plated in 24-well plates at a density of 5×10^5/ml (10^6 cells in 2 ml/well), in RPMI/10 with or without treatments. Every 3–4 days 1.5 ml supernatant was removed and replaced with fresh medium with or without treatments.

For treatment of cells with free PI, serial dilutions were made in DMSO, and the final dilution into the medium resulted in a concentration of 0.4% DMSO in the well. In addition to the untreated control, each experiment included a control containing 0.4% DMSO.

Infection was monitored by ELISA determination of p24 antigen in harvested culture supernatants (Konopka et al., 1990). With our standard curve, p24 is accurately detected down to 0.1 ng/ml. The results were expressed as the mean ± S.D. of duplicate determinations from 2 to 5 wells

for each condition. Unless specified otherwise, results were compared to untreated controls. Statistical significance was evaluated by the unpaired Student's t-test, using Stat View software (Abacus Concepts, Berkeley, CA).

2.5. Uptake of fluorescent liposomes

Macrophages were plated in 48-well plates as described above and were used 20 days after isolation, at which time they were well differentiated. Fresh medium (1 ml) was placed in wells and either Rh-MLV or Rh-SSV were added (0.2 μmol phospholipid/ml). The cells and liposomes were incubated at 37°C for 5 h. The medium was then aspirated, and the wells were washed 7 times with 0.5 ml medium to remove free liposomes. The last wash was left in place and the cells were observed by phase contrast and fluorescence microscopy, using a Nikon Diaphot inverted microscope. The cells were observed again after overnight incubation.

H9 cells were pelleted, resuspended in medium and incubated in 48-well plates, at a cell density of 1.7×10^5/ml in 1 ml. Liposomes were added and the cells were incubated as above. After 5 h, the cells were washed four times by centrifugation in 2 ml medium, and resuspended in 1 ml of medium and placed in fresh wells. The cells were observed as above.

3. Results

3.1. Continuous treatment of macrophages with PI or PI-MLV

We initially examined the effect of overnight treatment of macrophages prior to infection followed by continuous treatment after infection, with 100 nM PI or PI-MLV (Fig. 1). Viral p24 production was held at less than 1 ng/ml for up to 11 days. At 11 days these values were less than 1% of the untreated control (the value for free PI was 1.3% of the DMSO control).

Having determined that 100 nM PI was strongly inhibitory, we examined the dose range between 10 and 200 nM. When macrophages were

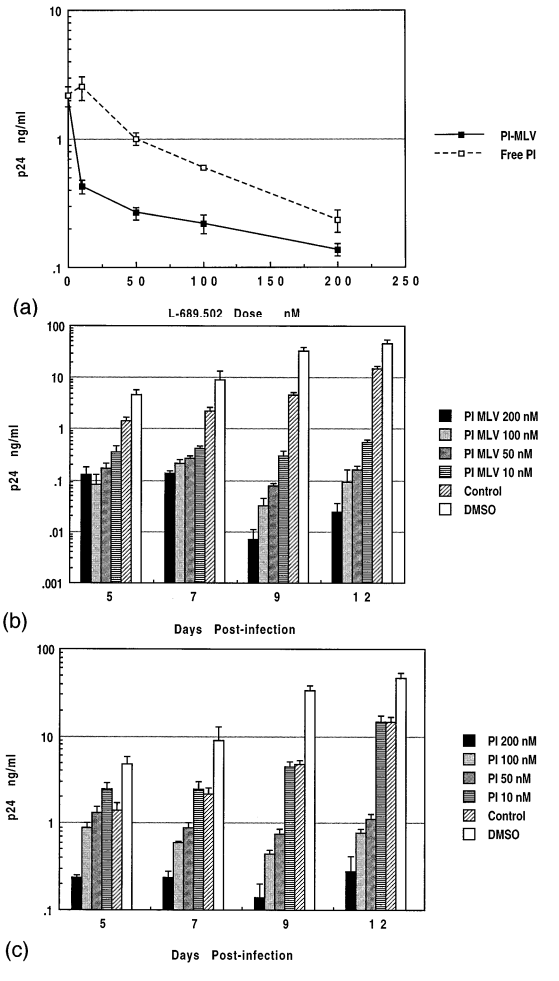

Fig. 2. Dose-dependence of viral p24 production by acutely infected macrophages treated continuously with free or encapsulated L-689 502. Macrophages were plated in 48-well plates at $1–2 \times 10^5$ cells/well, and on the 7th day were infected with HIV-1_{BaL} at 5 ng p24/well, then treated continuously with 200, 100, 50 or 10 nM PI, either free or encapsulated in MLV. (a) Dose-response at day 7 post-infection. The zero value is the untreated control. The DMSO control value was 9.05 ± 4.04 ng/ml. (b) and (c) Results of treatment with PI-MLV or free PI, respectively, on days 5–12.

continuously treated with PI or PI-MLV for 12 days following infection (pre-treatment was omitted from this and all subsequent experiments), there was dose-dependent inhibition of p24 production relative to the untreated control. At day 7, the differences between the p24 values obtained at the various doses were all statistically signifi-

cant (Fig. 2a). For example, comparison of the values for 50 and 100 nM free PI yielded $P \leq 0.005$, and that for 100 and 200 nM PI yielded $P \leq 0.0005$. The p24 values obtained throughout the experiment are shown in Fig. 2b and c, for treatments with PI-MLV and PI, respectively. While the highest doses (50–200 nM) of either treatment kept virus production at a low level, the lowest dose of free PI could not prevent virus production from increasing over time.

Treatment with PI-MLV produced lower p24 levels than with free PI throughout the concentration range studied (Fig. 2). In this experiment the p24 levels of the DMSO control were consistently higher, by at least a factor of 3, than the untreated control. It is not clear whether the higher values obtained with free PI were an effect of the DMSO, as this correlation was not consistent in other experiments. The data with free PI were also expressed as a percentage of the DMSO control, and the 50% or 90% effective concentrations (EC_{50} and EC_{90}, respectively) were determined (Table 1). While the EC_{50} values of free and liposomal PI were similar, the EC_{90} of PI-MLV was considerably lower than that of free PI (Table 1). An empty liposome control, at the same concentration of lipid as that of the highest PI-liposome concentration, was included in every experiment for each type of liposome used in this study, and the results were similar in every experiment: p24 in the empty liposome control was similar to or slightly higher than in the untreated control (data not shown).

Table 1
The EC_{50} and EC_{90} values of free and liposome-encapsulated L-689 502[a]

Day	EC_{50} (nM)		EC_{90} (nM)	
	Free PI	PI-MLV	Free PI	PI-MLV
9	6.0	5.6	28.2	9.7
12	8.1	5.2	41.9	9.4

[a]The data were taken from the experiment described in Fig. 2. The percentage reduction of viral p24 production in the case of free PI was expressed with respect to the DMSO control. Since empty liposome controls were similar to untreated controls throughout this study, the percentage reduction of p24 in the case of liposome encapsulated PI (PI-MLV) was expressed with respect to untreated controls.

3.2. Continuous treatment compared with overnight treatment of acutely infected macrophages with PI, PI-MLV or PI-SSV

In these experiments we asked whether a single treatment with free or liposome-encapsulated PI could be as effective as continuous treatment and whether SSV would provide any long term advantage over MLV. Macrophages continuously treated with 100 nM PI, free or encapsulated in MLV or SSV, had p24 levels reduced to approximately 1/10 to 1/100 of the controls (Fig. 3a). There was little or no difference in p24 production between macrophages treated with free PI or PI-SSV. However, macrophages treated with PI-MLV had p24 levels at or below 0.1 ng/ml, about 1/10 the level of the other treatments. The p24 levels of the treated wells remained relatively steady throughout the eight-day experiment, while the control p24 levels increased, so that the differences between the treated and control wells increased during the treatment period.

When macrophages were infected and then treated overnight (Fig. 3b), p24 levels rose throughout the course of the experiment (14 days), but the levels in the treated wells rose more slowly than those in the controls. The difference between untreated controls and treated wells increased over time, though less strikingly than with continuous treatment. PI-MLV was the most effective of the three treatments, keeping p24 levels at about 50% of control. PI-SSV showed little effect until day 14 (day 9: $0.01 < P \leq 0.025$; day 14: $P \leq 0.0005$, compared with untreated controls). The effect of free PI was similar to that of PI-SSV through day 9, but at day 14 both liposome treatments were more effective than free PI (PI compared to PI-SSV at day 9: $0.1 < P \leq 0.375$; at day 14: $0.0005 < P \leq 0.005$). In both of these experiments the untreated and DMSO controls were similar except at day 4–6, when the DMSO control was higher.

Although p24 levels were not kept as low by the single treatment as by continuous treatment, the longer lasting effect of liposome-encapsulated PI compared to free PI (to 14 days after a single

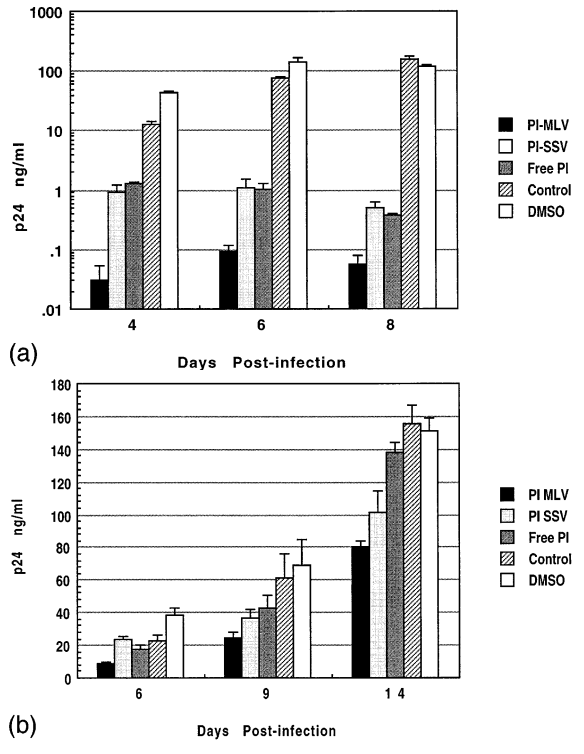

Fig. 3. Comparison of continuous and overnight treatment of macrophages with 100 nM L-689 502, free or encapsulated. Macrophages were plated at $1–2 \times 10^5$ cells/well in 48-well plates. (a) A portion of the cells was infected 8 days after plating with HIV-1_{BaL} at 5 ng p24/well, as described in Section 2. The cells were then treated continuously with the inhibitor encapsulated in either multilamellar (PI-MLV) or sterically stabilized vesicles (PI-SSV), or with the free inhibitor (PI). (b) Thirteen days after plating, additional cells were infected similarly, and treated overnight with liposomes or free PI at 100 nM. The cells were then washed and cultured without further treatments.

treatment) suggests that liposomes may provide a local reservoir of the inhibitor in macrophages.

3.3. Reproducibility of results with different macrophage preparations

In three experiments using different macrophage preparations, the cells were infected and treated overnight with 100 nM PI or PI-MLV (Fig. 4). The absolute p24 levels of the controls by day 7–8 varied considerably, from less than 10 to more than 60 ng/ml (Fig. 4a). While p24 levels in two of the preparations were roughly similar, the levels in the third preparation were much lower. In one of the experiments (Expt. 2) the DMSO control was lower than the untreated control ($0.0005 < P \leq 0.005$), while in the other two they were very similar. However, in all three experiments significant reduction of the p24 levels was produced by PI-MLV, with a lesser or insignificant reduction by free PI (Expt. 1: not significant; Expt. 3: $0.05 \geq P > 0.025$, comparing the control with free PI treatment). When the results are viewed as percent of control (Fig. 4b), the three experiments look more similar. PI-MLV was consistently in the range of 35–50% of control, and free PI ranged from >60 to $<90\%$. In Expt. 2, the only case in which free PI significantly re-

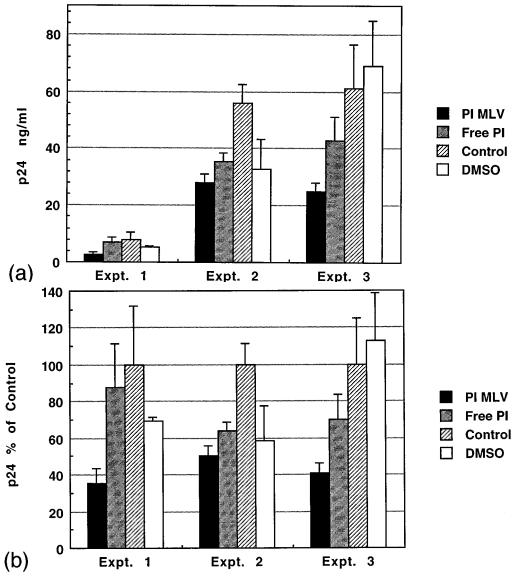

Fig. 4. Overnight treatment of macrophages with 100 nM free or liposome-encapsulated L-689 502: Comparison of 3 experiments. Macrophages were plated in 48-well plates at $1–2 \times 10^5$/well (Expts. 1 and 3) or $0.7–1.4 \times 10^5$/well (Expt. 2). In Expt. 1 the cells were infected on the 15th day after plating. In Expt. 2 they were infected on the 8th day, and in Expt. 3 on the 13th day. In all experiments cells were infected with HIV-1_{BaL} at 5 ng p24/well. Immediately following infection the cells were treated overnight with liposomes or free PI at 100 nM, washed, and cultured without further treatments. Virus production was determined on day 7 post-infection for Expt. 1 and 2, and day 8 for Expt. 3. Results are shown both as p24 (ng/ml) in culture supernatants (a) and as percent of control (b).

Table 2
The effect of L-689 502 on virus production by macrophages with established infection[a]

Treatment	p24 (ng/ml)		
	Day 4	Day 11	Day 14
None	69.8 ± 2.6	64.6 ± 8.6	95.4 ± 7.0
PI-MLV (100 nM)	49.3 ± 8.0	47.3 ± 6.9	52.9 ± 9.6
Free PI (100 nM)	41.6 ± 4.9	27.0 ± 6.8	53.9 ± 7.6

[a]Eight day old macrophages in 48-well plates were incubated with HIV-1_{BaL} (5 ng p24/0.7–1.4 × 10^5 cells/well) for 2 h. The cells were washed and incubated for 4 days before the beginning of the treatment period. The indicated days are days after the start of treatments (i.e., day 4 shown in the table corresponds to day 8 post-infection). The treatments were present throughout the indicated time period.

duced the p24 level, the DMSO control was also unusually low, and is essentially equal to the free PI value. Thus the results seen in this experiment for free PI could be partly an effect of the DMSO. In general, despite variations in p24 levels among macrophage preparations, the trend of the results was the same, and PI-MLV was more effective than free PI.

3.4. Continuous treatment of macrophages infected previously for 4 days

To examine whether free or liposome-encapsulated PI could also inhibit virus production in macrophages with a more established infection than the acute infection model, macrophages were infected for a 2 h period, and then incubated for 4 days before beginning continuous treatment. Neither free PI nor PI-MLV was very effective at reducing viral p24 production in culture supernatants (Table 2). On day 14 following the onset of treatment, the effects of free PI and PI-MLV were virtually the same (p24 levels were 56.5% and 55.5% of controls, respectively).

3.5. Continuous treatment of chronically infected H9 cells with PI or PI-MLV

We investigated the effect of PI and PI-MLV in a T lymphocyte cell line to establish whether the enhanced anti-HIV activity of PI-MLV described above was specific for macrophages, and whether the liposome-encapsulated inhibitor would be effective in cells that are not phagocytic. In two separate experiments H9 cells chronically infected with HIV-1_{IIIB} were treated continuously with 100 or 10 nM PI or PI-MLV (Fig. 5). The results are shown as percent of control at day 4. In experiment 1, PI-MLV caused no significant reduction of virus production (Fig. 5b). In experiment 2, PI-MLV at 10 or 100 nM reduced the p24 levels slightly but significantly ($0.01 < P \leq 0.025$, and $P \leq 0.0005$, respectively). In both experiments free PI at 100 nM was the most effective treatment, while at 10 nM it had no effect (Fig. 5a). At 100 nM free PI the p24 levels were 40–60% of untreated controls, despite the increased p24 levels of the DMSO controls relative to the untreated controls. These p24 levels were significantly lower than those obtained with the same concentration

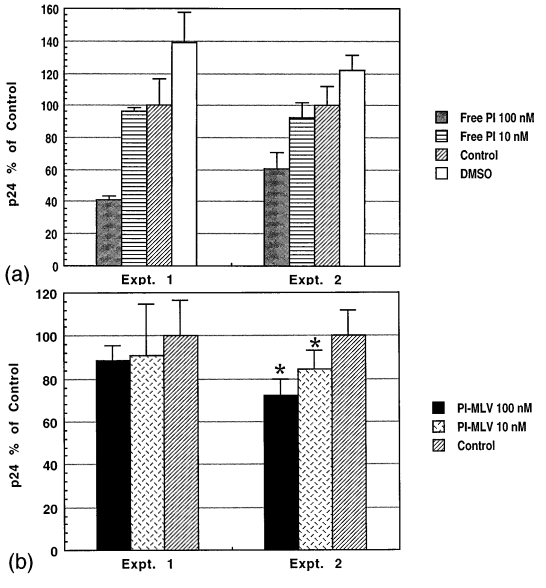

Fig. 5. Continuous treatment of chronically infected H9 cells with free or liposome-encapsulated L-689 502: Comparison of 2 experiments. In both experiments chronically infected H9 cells were washed, plated in 48-well plates (2 × 10^5 cells/well in Expt. 1, 1 × 10^5/well in Expt. 2), then continuously treated with two concentrations of the inhibitor (PI), either free (a) or encapsulated in multilamellar vesicles (PI-MLV) (b). Results are shown at day 4 after start of treatment. 100% p24 values were 84.6 ± 13.8 ng/ml for Expt. 1 and 82.2 ± 9.6 ng/ml for Expt. 2. *: Statistically significant reduction.

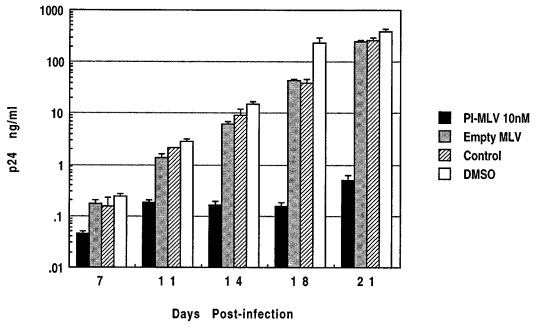

Fig. 6. Continuous treatment of acutely infected H9 cells with free or liposome-encapsulated L-689 502. H9 cells were infected as described in Section 2 and treated continuously for the indicated periods with 100 or 10 nM PI, either free (PI) or encapsulated in multilamellar vesicles (PI-MLV). Treatments were discontinued after day 18. Empty MLV: cells were treated with MLV containing no PI at a lipid concentration equal to that used for PI-MLV 100 nM. For 100 nM treatments of PI or PI-MLV, all values were zero. For 10 nM free PI, all values were zero except on day 11, when p24 was 0.027 ± 0.004 ng/ml.

of PI-MLV ($P \leq 0.0005$ in Expt. 1, and $0.01 < P \leq 0.025$ in Expt. 2).

3.6. Continuous treatment of acutely infected H9 cells

The relatively small effect of the PI on virus production in chronically infected H9 cells was interesting, and prompted us to investigate the effect of the inhibitor in acutely infected H9 cells. H9 cells were infected with HIV-1_{IIIB} and treated continuously for 14 days with 100 or 10 nM PI or PI-MLV. Culture was continued through day 21 with no further addition of treatments (most of the treatment was removed with the day 18 medium change) (Fig. 6). The infection began slowly, but reached p24 levels of >200 ng/ml in the controls by day 21. Fig. 6 also shows a control consisting of empty liposomes at the lipid concentration corresponding to the highest concentration of PI-MLV. At every time point the p24 level of this control was very similar to that of the untreated control, indicating that the liposomes themselves did not affect p24 production. The DMSO control also produced p24 levels comparable to the untreated controls. The p24 levels were drastically reduced in all of the PI-treated wells throughout the experiment. At 10 nM, free PI was effective in keeping the p24 level at 0, with the possible exception of day 11, when it was still below 0.03 ng/ml. PI-MLV at 10 nM was less effective; although the p24 level stayed below 0.2 ng/ml through day 18, it more than doubled to 0.5 ng/ml at day 21, following the removal of the treatments. At 100 nM, both free PI and PI-MLV kept the p24 levels at 0 ng/ml for 21 days, even after the removal of treatments on day 18. Thus, in marked contrast to chronic infection, acute HIV infection of H9 cells was extremely sensitive to PI treatment with or without liposomes, although free PI was more effective than PI-MLV.

3.7. Uptake of liposomes by cells

Fluorescence microscopy indicated that after 5 h or overnight incubation with Rh-MLV or Rh-SSV, almost all of the macrophages displayed rhodamine fluorescence. Few or none of the H9 cells incubated with Rh-MLV became fluorescent during this period. These observations indicate that macrophages internalized the liposomes avidly, while H9 cells did not (data not shown).

4. Discussion

Our results indicate that the HIV-1 protease inhibitor L-689,502 is highly effective in inhibiting the production of virions in acute infections of macrophages and of the $CD4^+$ T cell line H9. The first study describing this protease inhibitor had indicated that the spread of the SF162 isolate in monocyte/macrophages was completely inhibited at 25–50 nM, as measured by cell immunofluorescence (Thompson et al., 1992). Here we have found that virus production in macrophage cultures infected with the BaL isolate is inhibited by about 1000-fold over a 12-day observation period, at an inhibitor concentration of 100 nM. In H9 cells acutely infected with the IIIB isolate, virus production could be inhibited by about 10 000-fold at the same concentration. The reduction in p24 production observed in our evaluation of the antiviral effect of L-689,502 is consistent

with observations made with the protease inhibitors U-75875 and A77003 (Ashorn et al., 1990; Kageyama et al., 1994b). Although it is theoretically possible that the p24 ELISA used in our studies may also detect the uncleaved gag in defective virions, we have not investigated this issue further, particularly since viral p24 detected was reduced in the presence of the protease inhibitor.

L-689,502 was much less effective at reducing p24 production by cells with established or chronic infection, whether macrophages or H9. Although the protease acts at the final stage of virion maturation, the high level of virus production by these cells may be the reason for the lower effectiveness of the PI on chronically infected cells. The inhibitor must remain bound to the protease in order to block its function. Rosé et al. (1995) have found that a reduction of protease activity by 50-fold is sufficient to prevent infectious virion formation. It is reasonable to assume that the relative concentrations of the protease and inhibitor will determine the total amount of inhibition and any resultant effect on virion production. Although untreated acutely infected cells produce levels of viral p24 after 14 days of incubation comparable to those of chronically infected cells, the former are exposed to the inhibitor immediately after the infection period (2 h), when the virus concentration is very low. Thus, protease molecules produced during the first round of viral replication would be effectively inhibited by the PI, thereby limiting any further replicative cycles of the virus. In the case of acutely infected cells, the PI was able to block virus production even at 10 nM. It is also possible that the inhibitor L-689,502 acts both at the level of virus maturation and at earlier points in the HIV-1 life cycle. Previous studies have indicated that some protease inhibitors, including UK-88 947 and SR414 476, can inhibit DNA provirus formation when added before infection (Baboonian et al., 1991; Venaud et al., 1992).

The delivery of the inhibitor in liposome-encapsulated form to macrophages is more effective than the administration of the free drug, as indicated by the enhanced reduction of viral p24 at a particular drug concentration and the lower EC_{90} of PI-MLV compared to PI (Table 1). Previous studies from our laboratory have shown that certain liposome-encapsulated antibiotics are more effective than the free drug against the intracellular opportunistic pathogen *Mycobacterium avium* in cultured macrophages (Kesavalu et al., 1990; Majumdar et al., 1992). This advantage of liposome-encapsulated drugs is most likely due to the phagocytosis of liposomes, which then act as a reservoir providing a high local concentration of the drugs in macrophages. The enhancement of the effect of the PI by encapsulation in multilamellar liposomes also indicates that the inhibitor did not merely leak out of the liposomes in the cell culture medium but was transported into the macrophages via liposomes. Delivery of an HIV protease inhibitor, saquinavir, to macrophages in nanoparticles also resulted in an enhanced antiviral effect (Bender et al., 1996). While the free drug showed little antiviral effect at concentrations below 10 nM, the nanoparticle formulation reduced virus production by 80% even at 1 nM. The first anti-HIV drug to be tested in liposomes against HIV-1 infection in macrophages was the reverse transcription inhibitor 2′,3′-dideoxycytidine triphosphate (ddCTP) (Szebeni et al., 1990). In this case, liposome-encapsulated ddCTP did not show an enhanced effect over the free drug. The authors suggested, however, that liposome encapsulation may provide benefits, considering the short circulation half-life, neurotoxicity and mucocutaneous side-effects of ddC, and that liposomes may prolong drug action (Szebeni et al., 1990).

Experiments with acutely infected H9 cells indicated that PI-MLV are as effective as free PI in reducing virus production at the higher concentration employed (i.e. 100 nM), but are less effective than the free drug at the lower concentration (10 nM). Since, unlike macrophages, T cells do not efficiently take up multilamellar liposomes, it is likely that a sufficient amount of PI leaks out of the liposomes and becomes available to the cells at the higher concentration of the drug employed. At the lower concentration, however, any leakage must result in a free drug concentration considerably lower than 10 nM. The observation that liposome-encapsulated drugs can become avail-

able to cells that do not efficiently take up the liposomes is promising for the eventual use of this type of formulation for delivery of protease inhibitors to infected lymphocytes in lymph nodes and in the bloodstream.

The superiority of liposome encapsulation for drug delivery to macrophages may actually be enhanced in vivo, due to the localization of liposomes in the reticuloendothelial system (the mononuclear phagocyte system) following intravenous injection (Poste et al., 1982; Alving, 1983; Popescu et al., 1987; Senior, 1987; Szoka, 1991). Studies with liposome-encapsulated reverse transcriptase inhibitors have shown that the drugs localize in the reticuloendothelial system (Désormeaux et al., 1994; Makabi-Panzu et al., 1994). The plasma half-life of liposome-encapsulated 2′,3′-dideoxyinosine (ddI) was shown to be 46 times higher than that of the free drug (Harvie et al., 1995). Therapeutic ddI concentrations were achieved in lymph nodes for a period of about 3 h when administered in liposomes, compared to only 30 min in the case of the free drug. When delivered in sterically stabilized liposomes, the systemic clearance of ddI was 180 times slower than that of the free drug (Harvie et al., 1996). The systemic clearance of foscarnet encapsulated in conventional liposomes was found to be 77-fold lower than that of the free drug (Dusserre et al., 1995). Alternative routes of administration of liposomes may provide additional advantages. For example, injection of multilamellar liposomes containing ddC into the cerebrospinal fluid resulted in a 21-fold enhancement of the half-life of ddC levels over the free drug (Kim et al., 1990). Subcutaneous injection of liposomes can result in accumulation in local lymph nodes (Kaledin et al., 1982; Allen et al., 1993), which may be particularly beneficial for the treatment of high levels of infection in these tissues (Pantaleo et al., 1991; Pantaleo et al., 1993; Embretson et al., 1993).

The above observations indicate that delivery of protease inhibitors in liposomes may present certain advantages, particularly for potent compounds which do not have high oral bioavailabilities. For example, the bioavailability of the original formulation of saquinavir was reported to be 4%, while a newer formulation is expected to increase bioavailability to 12% (Ezzell, 1996). Encapsulation in liposomes may enable the delivery of prototype protease inhibitors which were not developed further because of poor oral bioavailability and which may be useful against emerging drug-resistant strains. Liposomes may also be useful as carriers of inhibitors with very low aqueous solubility. In view of the high cost of the synthesis of protease inhibitors, the large oral doses necessary to achieve therapeutic levels in the circulation, the gastrointestinal side-effects of the drugs and poor absorption in patients with gastrointestinal problems, the advantages of less frequent administration of liposomal inhibitors and their high bioavailability will have to be weighed against the necessity of intravenous or subcutaneous injection. Another consideration in evaluating the advantages of liposome-mediated delivery is the binding of protease inhibitors to serum proteins and the resulting inhibition of antiviral activity. For example, the IC_{75} of the inhibitor KNI-272 was increased by a factor of 25–100 in the presence of 80% FBS (Kageyama et al., 1994a). Delivery of certain protease inhibitors in liposomes, especially SSV, may prevent binding of the drug to serum components, and target the inhibitors to macrophages and lymph nodes.

Our experiments also show that PI encapsulated in SSV has the same inhibitory activity as the free drug. These liposomes have been shown to have prolonged circulation in the bloodstream, and are termed ‘stealth liposomes’ because they avoid immediate uptake and clearance by the reticuloendothelial system (Papahadjopoulos et al., 1991; Allen, 1994; Woodle and Lasic, 1992). The use of such liposomes in vivo may have several advantages over conventional liposomes: (i) They may increase the time of availability of antiviral drugs in the blood for delivery to peripheral blood mononuclear cells, and act as a sustained release system (Allen et al., 1992); (ii) liposomes containing poly(ethylene glycol)-phosphatidylethanolamine have been found to extravasate into tumor tissues (Papahadjopoulos et al., 1991; Gabizon, 1992) and to localize in lymph nodes, bone marrow, intestines and skin, particularly in deep tissue resident macrophages (Huang et al., 1992), and thus may be ideal vehicles to

carry antiviral agents to various tissues; and (iii) following subcutaneous injection, small stealth liposomes have been found to pass through the lymph node chains draining the site of injection and to localize partially in the bloodstream (Allen et al., 1993).

Pharmacokinetic studies with L-689,502 have revealed dose-dependent kinetics, which have been interpreted to arise from liver toxicity (Lin et al., 1992). Thus, further clinical studies with this inhibitor have not been undertaken. Nevertheless, our studies indicate that liposomes can be used to enhance the therapeutic effect of protease inhibitors, and that they can serve as a carrier for these highly hydrophobic drugs. Testing the efficacy of newer protease inhibitors encapsulated in liposomes, the application of liposome-encapsulated protease inhibitors in an animal model, and the effect of this formulation on macrophages infected with patient isolates will be subjects for further study in our laboratory.

Acknowledgements

We thank Drs W. Thompson and P. Anderson (Merck, Sharpe and Dohme) for the gift of the L-689,502, and Dr K. Konopka (University of the Pacific) and Dr D. Hoekzema (Advanced Biotechnologies) for helpful discussions. This work was supported by grant AI-32399 from the National Institute of Allergy and Infectious Diseases. The early stages of the investigation were supported by grant R91UP002 from the University of California Universitywide AIDS Research Program.

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