Increased Efficacy of Phosphonoformate and Phosphonoacetate Inhibition of Herpes Simplex Virus Type 2 Replication by Encapsulation in Liposomes

FRANCIS C. SZOKA, JR.,* AND CHUN-JUNG CHU

Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446

Received 29 October 1987/Accepted 21 March 1988

Phosphonoformate and phosphonoacetate encapsulated in liposomes have substantially greater activity against herpes simplex virus type 2 in Vero cell tissue culture than the nonencapsulated compounds at the same dose. Encapsulation of phosphonoformate in liposomes resulted in a 30-fold increase of the antiviral effect with no increase in cytotoxicity measured by inhibition of thymidine incorporation into normal Vero cells. Thus, the selectivity of the liposomal drug increased 27-fold compared with the nonencapsulated compound. Liposome encapsulation of phosphonoacetate at a ratio of 0.3 $\mu\text{mol}/\mu\text{mol}$ of lipid resulted in a 150-fold increase of antiviral activity with a concomitant 250-fold increase in cytotoxicity. However, the selectivity of phosphonoacetate could be increased by reducing the drug-to-lipid ratio. Liposome uptake by Vero cells, measured by the cell association of a nonexchangeable radiolabeled lipid, plateaued after 24 h of incubation and saturated at 60 nmol of lipid per mg of cellular protein at a lipid concentration of 300 μM . The saturation of liposome uptake on the Vero cells may account for the 27-fold increase in selectivity observed with the liposomal phosphonoformate. The greater activity of the encapsulated phosphono compounds is most likely due to their increased transport into the cytoplasm; this occurs subsequent to the uptake and processing of the liposome in the lysosomes of the cell. Liposome encapsulation of these agents may result in superior efficacy against viral infections residing in endocytotically and phagocytically active cells such as macrophages.

Phosphonoformate (PF) and phosphonoacetate (PA) have been shown to be effective inhibitors of viral replication for a variety of DNA-containing (PF and PA) (14, 22-24) and RNA-containing (PF) viruses (22, 27, 29). These compounds are potent inhibitors of cell-free DNA polymerases (22, 23) and reverse transcriptase (27, 29). However, greater than 100-fold more drug is necessary to obtain antiviral effects in tissue culture and animals (22, 23).

The reason for the decreased activity in intact cells is due to the low permeability of the phosphono compounds. The pK_a s of the three titratable groups of PF are 7.3, 3.4, and 0.5 (34), while the analogous values for PA are 8.2, 5.0, and 2.6 (20). The poor penetration can be attributed to the multianionic nature of the phosphono compounds at physiological pH. Attempts to enhance their transport properties by chemical modifications have failed to produce more active analogs, even though a large number of congeners have been synthesized and tested (8, 15, 20).

An alternative approach to increase the transport of PF and PA into cells that are endocytotically active is to encapsulate the drugs in liposomes, a versatile biodegradable drug carrier (31, 33). Endocytosis of the liposome-encapsulated agent places the liposome and its contents into the low pH environment of the lysosome (pH 4.0 to 5.5) (5, 30) where the charge on the drug will be decreased owing to titration (Fig. 1). Neutralization of charge should enhance the penetration of the drug from the lysosome into the cytoplasm. This approach substantially increases the efficacy of aspartylmethotrexate (13) and fluoroorotic acid (12). Agents showing enhanced efficacy when encapsulated in liposomes and applied to cell cultures under steady-state conditions have been designated liposome-dependent drugs

(12, 13). In this study, we demonstrated that PF and PA behave as liposome-dependent drugs in their inhibitory action against herpes simplex virus type 2 (HSV-2) replication in tissue culture.

MATERIALS AND METHODS

Materials. Phosphonoformate trisodium salt (PF), PA, and cholesterol were obtained from Sigma Chemical Co., St. Louis, Mo. Egg phosphatidylcholine and egg phosphatidylglycerol were purchased from Avanti Polar Lipids, Birmingham, Ala. The *p*-hydroxybenzamidinium dihexadecylphosphatidylethanolamine was synthesized and iodinated as described previously (1). [^3H]thymidine was a product of Amersham Corp., Arlington Heights, Ill.

Drug and liposome preparation. Drug solutions were prepared at 80 mM. The pH was adjusted to 7.4 with either HCl or NaOH, and the osmolality was adjusted to 300 mosM with NaCl before encapsulation. Reverse-phase evaporation liposomes (REV) composed of egg phosphatidylcholine-egg phosphatidylglycerol-cholesterol in a molar ratio of 9:1:8 were prepared and extruded through a 0.2- μm polycarbonate membrane as described previously (32). Nonencapsulated drugs were removed by dialysis against a 2×100 -fold excess of 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-140 mM NaCl-0.1 mM diethylenetriamine-pentaacetic acid (pH 7.4) buffer. Non-drug-containing (NDC) liposomes were prepared by encapsulating the HEPES buffer. The concentrations of encapsulated drugs and phospholipids were determined by assaying the phosphate content after acid digestion by the method of Bartlett (2). The vesicle sample was extracted in a two-phase system by the method of Bligh and Dyer (3). The methanolic aqueous phase contained the drug, and the chloroform phase contained the phospholipid. Liposome diameter was deter-

* Corresponding author.

FIG. 1. Concept of a liposome-dependent drug illustrated with PF. PF has three titratable groups. At pH 7.4, it will exist predominantly as the trianionic compound; thus, it will have poor membrane permeability. The liposome-encapsulated drug can be internalized into the lysosome of an endocytotically or phagocytically active cell. In this acidic compartment, the drug will be partially protonated upon release from the liposome. In this form it will have a greater membrane permeability. In the cytoplasm it will be deprotonated again, hence effectively trapped in this compartment.

mined with a laser light-scattering apparatus (NS-4; Coulter Electronics, Inc., Hialeah, Fla.).

Virus and cell culture. HSV-2 strain G and Vero cells were obtained from D. Eppstein, Palo Alto, Calif. Vero cells were routinely grown in Eagle minimum essential medium supplemented with 5% heat-inactivated fetal calf serum obtained from the cell culture facility at the University of California, San Francisco. The virus was propagated in Vero cells, and titers of various viral preparations were determined by plaque assay in Vero cells (21).

CPE assay. A modified version of the cytopathic effect (CPE) assay developed by Epstein et al. (7) was performed as follows. Vero cells (10^4) were cultured in a 96-well plate for 2 days at 37°C. After removal of culture fluid, cells were infected by adsorbing HSV-2 (800 PFU per well) to cells at 37°C for 1 h before drug treatment. Various concentrations of drugs, liposomal drugs, and drugs plus NDC liposomes (NDC-REV) were added to each well. Each dose was tested in quadruplicate. Controls included in each plate comprised a cell control and a virus control. After 48 h, cells were rinsed with phosphate-buffered saline and then fixed and stained with Armstrong solution (0.5% [wt/vol] crystal violet, 50% (vol/vol) ethanol, 5% (vol/vol) Formalin, 0.85% [wt/vol] NaCl [7]). Excess dye was washed off, and the dye incorporated by the viable cells was eluted with dimethyl sulfoxide. The optical densities (OD) were read at 550 nm with a multichannel spectrophotometer (Titertek-Multiskan). The percentage of dye uptake was calculated as $(OD_{\text{treatment}} - OD_{\text{virus control}}) / (OD_{\text{cell control}} - OD_{\text{virus control}}) \times 100$. The ED_{50} is the concentration of the drug at which the test well demonstrates 50% of the control well dye uptake.

Virus yield assay. Vero cells (10^6) cultured in a 6-well (35-mm-diameter) plate were inoculated with HSV-2 with a multiplicity of infection of 0.05. Drug treatment started 1 h after virus adsorption. Two days later, cell suspensions were subjected to three cycles of freezing and thawing to release virus. The number of viruses in the supernatant of the cell cultures was determined by plaque assay in Vero cells with

a 1.5% SeaPlaque agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) overlay. After virus plaques had developed (2 days), cells were stained with a 0.01% neutral red solution (6).

Cytotoxicity assay. Vero cells without virus infection were exposed to drug treatment for 44 h under the same culture conditions as described above for the CPE assay. Then, 1 μCi of [^3H]thymidine was added to each well for 6 h. At the end of the pulse, the cells were lysed and collected onto glass fiber filters with a multiple-channel cell harvester (Skatron Inc., Sterling, Va.). Radioactivity (counts per minute) associated with each filter disk was counted in a Beckman liquid scintillation spectrometer. The percentage of [^3H]thymidine incorporation was expressed as $(\text{counts per minute}_{\text{treatment}} / \text{counts per minute}_{\text{control}}) \times 100$. The IC_{50} is the concentration of the drug required to reduce thymidine incorporation to 50% of the control value.

A second measure of drug cytotoxicity was cell growth in the presence of drug or encapsulated drug. Vero cells (10^5) were plated in 35-mm culture dishes. Liposome-encapsulated and nonencapsulated drugs were diluted in growth medium to various concentrations and then added to cell cultures in duplicate at time zero. Cells were removed from dishes, treated with 0.05% trypan blue, and counted with a hemacytometer at the indicated times.

Uptake of liposomes by Vero cells. A trace amount of ^{125}I -labeled *p*-hydroxybenzamidinium dihexadecylphosphatidylethanolamine was incorporated in liposomes as a marker for uptake studies as described previously (1). Vero cells (1.2×10^6) in 35-mm culture dishes were incubated with various amounts of ^{125}I -labeled liposomes. After incubation for the times indicated, nonattached liposomes were removed by rinsing the cultures three times with phosphate-buffered saline, and the cells were then lysed with 0.5 N NaOH. Radioactivities associated with the cell lysate were measured in a Beckman gamma scintillation spectrometer. The concentrations of cellular protein in the lysate were determined by the method of Lowry et al. (19).

RESULTS

Liposome preparations. PF and PA are highly water soluble compounds that can be readily encapsulated in liposomes at high concentrations. Liposome encapsulation resulted in 0.20 μmol of PF per μmol of lipid and 0.31 μmol of PA per μmol of lipid. Owing to the negative charges on the phosphono compounds at pH 7.4, there is little leakage (less than 1% per week) of either PF or PA from the liposomes upon storage at 4°C. The diameter of the liposomes as measured by dynamic light scattering ranged between 0.16 and 0.22 μm for various preparations.

Antiviral effects. To measure the antiviral efficacy of the liposome-encapsulated compounds, we used a quantitative dye-binding CPE assay (7). In this assay, nonencapsulated PA had an EC_{50} of about 60 μM , and the addition of NDC liposomes did not change this value (Fig. 2A; Table 1). Liposome encapsulation of PA resulted in a 150-fold increase in efficacy ($EC_{50} = 0.4 \mu\text{M}$) (Fig. 2A; Table 1). Nonencapsulated PF in the presence or absence of NDC liposomes had an EC_{50} of about 210 μM . Encapsulation of PF resulted in about a 30-fold increase in efficacy, $EC_{50} = 7 \mu\text{M}$ (Fig. 2B; Table 1). Plaque reduction assays in the presence of liposome-encapsulated drug could not be done because the agarose overlay restricted the diffusion of the liposome. However, virus yield assays confirmed the increased effectiveness of the liposome-encapsulated PF. In

FIG. 2. Antiviral effects of encapsulated and nonencapsulated phosphono compounds. (A) Comparison of the antiviral effect of PA and liposomal PA assessed by the CPE assay. Vero cells were infected with HSV-2 for 1 h before drug treatment. At 48 h after drug treatment, viable cells were fixed and stained and the percentage of dye uptake was calculated as described in Materials and Methods. Symbols: ■, PA; ○, PA plus NDC-REV; □, PA-REV. Values are the mean $\pm$ standard deviation (SD) (bars); $n = 4$. (B) Comparison of the antiviral effect of PF and liposomal PF assessed by the CPE assay. Symbols: ■, PF; ○, PF plus NDC-REV; □, PF-REV. Values are the mean $\pm$ SD (bars), $n = 4$.

replicate virus yield assays, a 6- to 100-fold increase in efficacy was seen with the liposome-encapsulated PF compared with the nonencapsulated PF and a 100- to 500-fold increase in efficacy compared with free PA was obtained with liposome-encapsulated PA.

Cytotoxicity. The inhibition of incorporation of thymidine into cellular DNA and effect on cell growth were used as a measure of the cytotoxicity of the drugs. Thymidine incorporation assays were performed under the same tissue culture conditions as for the CPE assay.

PA had an IC_{50} of 507 μ M which was considerably reduced to 2 μ M when the drug was encapsulated in liposomes (Fig. 3A; Table 1). In contrast, PF exhibited an IC_{50} of 880 μ M which was not appreciably reduced by liposome encapsulation (Fig. 3B; Table 1). Measurement of cell growth in the presence of the drugs, either encapsulated or nonencapsulated, confirmed the results of the cytotoxicity measured by thymidine incorporation (Fig. 3C and D). Liposomal PA was more effective than free drug at reducing cell growth; an approximately 50% reduction was observed at 10 μ M (Fig. 3C). Nonencapsulated PA at 500 μ M reduced cell growth to about 50% of control cell growth (Fig. 3C), in close correspondence to its effect on thymidine incorporation. Liposomal PF at 1,000 μ M did not inhibit cell growth at 48 h and slightly depressed cell growth at 72 h, whereas nonencapsulated PF at 1,000 μ M reduced cell growth to less than 50% of control (Fig. 3D).

Although PA in liposomes was approximately 150-fold more active as an antiviral agent, it was also 250-fold more cytotoxic. Therefore, its selectivity ratio ($IC_{50}/EC_{50} = 5.3$) was less than that for the free drug (Table 1). Reducing the encapsulation ratio (moles of PA/moles of lipid) from 0.31 to 0.05 decreased the cytotoxic effects so that the IC_{50} was closer to that of the free drug; however, the antiviral effects were not concomitantly reduced. At the lower drug/lipid ratio, the $IC_{50} = 150 \mu$ M and the $EC_{50} = 0.4 \mu$ M, so that the selectivity increased to 375. Thus, varying the amount of

drug in the liposome modulates the selectivity ratio, which could be an important factor in the formulation of the liposome-encapsulated drug. Encapsulation of PF in liposomes resulted in a 30-fold increase in antiviral activity but no increase in cytotoxicity. Thus, the selectivity ratio of the PF was increased from 20 to approximately 100 by liposome encapsulation. This unexpected increase in the selectivity ratio for liposomal PF was replicated in five separate experiments.

Liposome uptake by Vero cells. A possible explanation for the increase in the selectivity ratio for liposomal PF but not for liposomal PA at the highest encapsulation ratio tested is that liposome uptake by the Vero cells is saturable. The maximum amount of liposome-encapsulated drug that becomes cell associated is a function both of the saturation level of cell-associated liposomes and of the concentration of the drug in the liposome. When the drug does not leak from the liposome until it is internalized by the cell, intracellular

TABLE 1. Comparison of the efficacy and toxicity of liposome-encapsulated PF and PA with that of the nonencapsulated compounds

Agent	EC_{50} (μ M $\pm$ SD) ^a	IC_{50} (μ M $\pm$ SD) ^a	Selectivity (IC_{50}/EC_{50})	Enhancement ^b
PA	60 $\pm$ 25 (2) ^c	507 $\pm$ 162 (3)	8.4	
Lipo PA ^d	0.4 $\pm$ 0.1 (2) ^c	2.1 $\pm$ 0.1 (2) ^c	5.3	0.63
PF	210 $\pm$ 84 (4)	880 $\pm$ 57 (5)	4.2	
Lipo PF ^d	7 $\pm$ 2.9 (4)	>800 (5) ^e	114	27

^a The value in parentheses is the number of independent determinations.

^b Enhancement = the ratio of the selectivity of the liposomal drug to the selectivity of the nonencapsulated drug.

^c These values are the range, rather than the SD.

^d Lipo PA, Liposome-encapsulated PA; Lipo PF, liposome-encapsulated PF.

^e In one experiment, the liposomal PF was as cytotoxic as the free PF. In four additional experiments, the liposomal PF was less cytotoxic than the free PF and the IC_{50} was not reached.

FIG. 3. Inhibition of thymidine incorporation and cell growth by encapsulated and nonencapsulated phosphono compounds. (A) Inhibition of thymidine incorporation by PA and liposomal PA in Vero cells. Vero cells were incubated with the drug for 44 h, and then thymidine incorporation was measured as described in Materials and Methods. Symbols: ■, PA; □, PA liposomes. Values are the mean $\pm$ SD (bars), $n = 4$. (B) Inhibition of thymidine incorporation by PF and liposomal PF in Vero cells. Incubation conditions were the same as described for panel A. Symbols: ■, PF; □, PF liposomes. Values are the mean $\pm$ SD (bars), $n = 4$. (C) Inhibition of Vero cell growth by PA and liposomal PA. Symbols: - - -○- - -, control; ●, PA, 100 μ M; ▲, PA, 500 μ M; ■, PA, 1,000 μ M; ○, liposomal PA, 0.4 μ M; △, liposomal PA, 10 μ M; □, liposomal PA, 50 μ M. Each point is the mean of duplicate cell counts that agreed to within 10%. (D) Inhibition of Vero cell growth by PF and liposomal PF. Symbols: - - -○- - -, control; ▲, PF, 500 μ M; ■, PF, 1,000 μ M; △, liposomal PF, 300 μ M; □, liposomal PF, 1,000 μ M. Each point is the mean of duplicate cell counts that agreed to within 10%.

drug concentrations that inhibit viral replication but not cellular DNA synthesis can be attained.

Measurement of the cellular association of a nonexchangeable radiolabeled lipid marker demonstrated that liposome uptake plateaued after 24 h (Fig. 4A). Moreover, the uptake saturated at a liposome dose of about 300 μ M (Fig. 4B). If the liposomes retain the encapsulated drug during the cellular uptake process, the amount of drug that becomes cell associated is the product of the amount of drug per liposome times the number of liposomes that are cell associated (Fig. 5). Varying the amount of drug per liposome will then affect the amount of drug that becomes cell associated. The difference in the computed cell-associated drug between PF and PA is due to a higher encapsulation ratio for PA. For comparison, uptake of nonencapsulated PF is assumed to be a linear function of the external drug concentration (Fig. 5). It is evident that a majority of drug uptake from the liposome form occurs at low levels of added liposome-encapsulated drug. The consequence of this for drug action is discussed below.

DISCUSSION

The order of magnitude increases in efficacy against HSV-2 achieved by the encapsulation of PF or PA in

liposomes illustrates the concept of a liposome-dependent drug (Fig. 1) (12, 13). For the nonencapsulated compounds, the EC_{50} s for both PA (60 $\pm$ 25 μ M) and PF (210 $\pm$ 85 μ M) were consistent with previously published values (PA = 60 to 100 μ M and PF = 60 to 200 μ M) against various strains of HSV (8, 14, 15, 20, 22, 23, 24). Thus, the increase in efficacy of the liposome-encapsulated compounds (PA = 0.4 μ M, PF = 7 μ M) could be important for in vivo antiviral activity of PA and PF.

For PF, the increased efficacy was achieved without any substantial increase in cytotoxicity as measured by inhibition of thymidine incorporation. Thus, the selectivity ratio of liposomal PF was significantly better than that of the nonencapsulated compound. In contrast, at 0.3 μ mol of drug per μ mol of lipid, PA exhibited a 150-fold increase in efficacy but also had a 250-fold increase in cytotoxicity so that the selectivity of the drug was slightly decreased compared with the free drug. The selectivity ratio could be increased by decreasing the drug-to-lipid ratio in the liposome. For both drugs, the cytotoxicity of the nonencapsulated compounds, as measured by either thymidine incorporation or growth rates, was similar to previously reported values (22, 23). In purified DNA polymerase alpha preparations, PA (K_i = 35 μ M) has a lower inhibition constant than PF (K_i = 50 μ M),

FIG. 4. Uptake of liposomes by Vero cells. (A) ^{125}I -labeled liposomes ($300\ \mu\text{M}$) were added to the cell culture for various periods. At the end of each incubation, non-cell-associated liposomes were washed off and cell-associated radioactivity was counted. (B) Various concentrations of ^{125}I -labeled liposomes were incubated with Vero cells for 24 h. Cell-associated radioactivity was measured as described in Materials and Methods. Values are the mean $\pm$ SD (bars), $n = 3$.

so that the rank order effect of the two nonencapsulated agents on thymidine incorporation observed in this study was consistent with the inhibition constants (22).

Thus, the possibilities of manipulating the selectivity ratio of these compounds by varying the drug/lipid ratio provides

an alternative to drug modification for improving drug therapy. We postulate that the difference between the two phosphono compounds at the high drug/lipid ratios relates to three factors: (i) the difference in the encapsulation ratio, that is, the micromoles of drug per micromoles of lipid,

FIG. 5. Comparison of uptake of PF and PA by Vero cells after delivery in liposomes. The amount of cell-associated drugs was estimated by measuring the amount of cell-associated liposomes at 24 h, assuming all the liposomal drug contents remained cell associated. Symbols: □, PF; ■, PA. The dashed line originating at the origin and proceeding through the points marked B, C, and E is the curve relating extracellular free drug concentration to intracellular drug concentration. See Discussion for a detailed explanation. Point A is the intracellular drug concentration at the EC_{50} for the liposomal PF. Point B is at the same intracellular concentration as point A but is attained at an extracellular free drug concentration of $210\ \mu\text{M}$. Point C is the intracellular PF concentration at the IC_{50} . Point D represents the intracellular drug concentration obtained with the liposomal PF. Point E is the intracellular drug concentration at the IC_{50} for free PA. Point F is the intracellular PA concentration for the liposomal PA. Notice that the liposomal PF is not able to attain the necessary intracellular drug concentration to reach the IC_{50} , whereas the liposomal PA attains an inhibitory intracellular concentration at a much lower level of added compound than the free PA.

achieved in the preparation of the liposomes; (ii) the relative sensitivity of the Vero cell to the cytotoxic effects of the two drugs; and (iii) the fact that liposome uptake by the Vero cells saturates (Fig. 4 and 5).

In Fig. 5 this concept is illustrated graphically. It is assumed that drug uptake for the encapsulated compound is directly related to liposome uptake and that no leakage of the drug from the liposome or liposome-cell complex into the medium occurs. Another assumption is that uptake of the nonencapsulated drug is linearly proportional to the amount of drug in the culture medium. Finally, it is assumed that the antiviral effect and the cytotoxic effect occur at the same intracellular drug concentration, regardless of whether the drug is delivered in liposomes or as the nonencapsulated compound.

For PF, the drug concentration delivered in liposomes that inhibits viral replication (EC_{50}) can be estimated from the liposome uptake data, since the fraction of the liposomes that become cell associated at any lipid concentration is known. This inhibitory level of PF can be achieved intracellularly by the nonencapsulated drug, albeit at a higher external concentration (Fig. 5). The assumption of a linear relationship between external and internal drug levels allows construction of the dashed line in Fig. 5 which passes through the origin and point B (the inhibitory drug level for nonencapsulated compound at $EC_{50} = 210 \mu M$). Given that the same intracellular drug concentration would exist at EC_{50} for liposome-encapsulated or nonencapsulated compound, point B can be obtained from knowing the inhibitory drug level at EC_{50} for the liposome-encapsulated drug (point A). At the IC_{50} (880 μM) of free PF, the intracellular concentration (point C) exceeds that attainable by the liposomal form (point D). Thus, the liposome can increase the efficacy without increasing cytotoxicity of the encapsulated PF. If the same dashed line (Fig. 5) is used to represent the intracellular concentration attained by the nonencapsulated PA, then the extracellular concentration of PA that caused cytotoxicity (point E) results in an intracellular concentration of PA that can be achieved by a low level of liposomal PA (point F). Thus, both the efficacy and toxicity would be increased for PA when it is encapsulated at the highest drug-to-lipid ratio.

Based on this model, a decrease of 10-fold in the PA concentration encapsulated in liposomes should still result in an enhancement of the antiviral effect but should limit the maximal concentration of PA in the cell to below the cytotoxic range. When this was done, the efficacy was not changed, while the cytotoxicity decreased 15-fold from that obtained at the highest drug-to-lipid ratio. We believe the present data are consistent with the model. A direct test of this model by measuring the intracellular concentration of the phosphono compounds is not possible at this time owing to the absence of a simple, sensitive assay for the phosphono compounds or radiolabeled derivatives.

As discussed by Heath et al. (12, 13), for a drug to be considered a liposome-dependent drug several conditions must be met. First, the free drug must be relatively impermeable owing to a potentially reversible chemical feature. Second, the target cell must be able to internalize the liposome through an active endocytotic pathway that terminates in a low pH compartment. Third, the liposome must retain the drug until it is internalized by the cell. Liposomes that are too fragile will release the drug prior to internalization, and no enhancement of activity will be observed. All these conditions for a liposome-dependent drug appear to have been met in the system tested here for PF and PA. In

regard to the first point, it is important to realize that a freely permeable drug will redistribute from the cell, even if the liposome is initially able to concentrate it in the cytoplasm. Such compounds must be contrasted with PF and PA, which have low permeabilities in their ionized forms. Once protonated in the lysosome, these two compounds can more readily diffuse into the cytoplasm. The pH of the cytoplasm is about 7; thus, the phosphono compounds would revert to the ionized form and become trapped in this compartment.

The optimal use of liposome-dependent drugs *in vivo* will depend on targeting of the liposome. This can be done either by an active scheme in which a targeting ligand is employed or by exploiting the known capacity of macrophages to take up liposomes. If the latter approach is taken, viral diseases that infect the macrophage would be ideal targets for such therapy (16). Clearly, PF and PA appear to be good candidates for liposome encapsulation for therapy of virally infected macrophages.

The antiviral potency of the phosphono compounds is hindered by their inability to cross membranes. Nonetheless, PF has been used to treat cytomegalovirus infections in recipients of bone marrow and renal transplants (25). Moreover, the potent anti-reverse transcriptase activity of PF (27, 29) has already been exploited in a limited clinical trial to treat human immunodeficiency virus infections (9, 11). In these studies, up to 15 g of PF per day has been infused on a continuous basis for a period of 21 days. The large dose and continuous intravenous infusion is necessary because PF is cleared rapidly from the blood and is poorly absorbed when taken orally. A 100-fold enhancement of efficacy would permit liposome-encapsulated PF to be given as an intravenous bolus rather than as a continuous infusion. In addition, human immunodeficiency virus is known to infect macrophages (4, 10, 17, 18, 26), and the macrophage has been suggested by some to be a significant factor in the persistence of the virus and its transport into the brain *in vivo* (4, 28; M. Popovic, S. Gartner, and R. C. Gallo, Abstr. 3rd Int. Conf. AIDS, p. 214, 1987). The liposome effect demonstrated here is not virus specific. We would expect that other viral infections sensitive to the phosphono drugs in endocytotically active cells would behave in a fashion similar to that of the HSV system. Thus, it could be of considerable therapeutic importance to extend these results to other viral systems, including a model human immunodeficiency virus macrophage infection (4).

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