PREPARTION AND *IN VITRO* ANTIVIRAL ACTIVITY OF LIPOSOMES OF LIPOPHILIC ESTERS OF ACYCLOVIR

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ABSTRACT The long chain acyclovir such as the acyclovir laurate and acyclovir palmitate were prepared directly from acyclovir by application of the usual esterification methods with appropriate acyl chlorides. The lipophilic prodrugs were found to be retained easier by liposomes whereas acyclovir escaped readily from liposomes. When assayed in African green monkey cell cultures against herpes simplex virus type I strain, the acyclovir palmitate liposomes proved to be more active compared with the parent drug and its liposome, suggesting an enhanced compatibility between the ester and liposomal lipids and an increased uptake of encapsulated prodrug by infected cells.

Key words Acyclovir; Liposomes; Lipophilic prodrug; Antiviral activity

The biodegradable and biocompatible liposome has been suggested as a pharmacological carrier because of its ability to accommodate both water soluble and lipid soluble compounds in their native state and to deliver them to different tissues or cell types after administration. Great efforts have been made to approach the problem of entrapment and stability of drugs in liposomes. These efforts were mainly centered on the liposomal lipid compositions, physicochemical properties of liposomes and manufacturing techniques. With regard to the influence of the entrapped drugs on encapsulation and stability of liposomes, some promising results have been reported by modifying the chemical structure of the drug without altering the activity[1~3]. These results were based on the fact that water soluble compounds or any small molecule not having too many hydrogen bonds can leak rapidly through bilayer structures while lipophilic drugs ensure a successful entrapment through the incorporation of the hydrocarbon chains with the liposome bilayer[4].

The antiviral acyclovir {9-[2-hydroxyethoxy)methyll guanine}[5] is one of the well established antiherpes compounds. Its potentials in treatment of chronic hepatitis B have also been explored[6, 7]. Because of its limited solubility in water (~ 0.2%, 25 ℃), acyclovir cannot be given as eye drops or intravenous injections. The poor absorption requires a larger dose which in turn results in an acute kidney failure.

We have endeavored at using liposome as a pharmaceutical carrier to promote the uptake of acyclovir in both topical and systemic application in treating both herpes infections and hepatitis. To overcome the shortcoming of low encapsulation and rapid leakage of acyclovir liposomes, we modified the structure of acyclovir by esterifying the 2′-OH as laurate and palmitate. The incorporation of long chain derivatives into liposomes was

investigated and preliminary antiviral experiment in cell culture was carried out to demonstrate the enhanced uptake of drug through liposome – cell interaction.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (EPC) was extracted from egg yolk as described[8]. The lipid was purified on a neutral aluminum oxide column and shown to be pure by thin – layer chromatography. The lipid oxidation index was below 0.2. The obtained EPC was loaded into an air – tight bottle and sealed with acetone at – 20 ℃. Cholesterol (Chol) was a Merck product. Lauroyl chloride and palmitoyl chloride were kindly prepared by Dr. X Cheng of the Department of Organic Chemistry, Beijing Medical University. Acyclovir (ACV) was purchased from Pharmaceutical Industry Research Center, Wuhan, China and was recrystallized from water.

Diethyl ether and pyridine were distilled before use. All other chemicals were of analytical grade.

Preparation of acyclovir laurate (C_{12} – ACV) and acyclovir palmitate (C_{16} – ACV)

The two ester derivatives were prepared by similar method as follows. Take acyclovir palmitate as example: acyclovir 1.112 g (5 mmol) was suspended in 50 ml pyridine at room temperature (21 ℃), 1.52 ml (5 mmol) palmitoyl chloride was added dropwise, the mixture was sealed and stirred with a stirring bar at 21 ℃ for 48 h, then the solution was poured into 300 ml water while stirring and acidified to pH 2 with 2 mol/L HCl. The white preciptate was filterted and washed thoroughly with cold water, and then with 5% $NaHCO_3$ (10 ml × 3), and was recrystallized from methanol. The resultant solid was dissolved in $CHCl_3$ — MeOH (2:1, V/V) and dried ($MgSO_4$). When the solvent was evaporated, the crude product (1 g) was dissolved in 5 ml chloroform and applied to a column (3.5 × 50 cm) of 100 g silica gel, the column was eluted with $CHCl_3$ — MeOH (9.5:0.5, V/V). Fractions (8 ml) were collected and a portion of each fraction was taken for TLC test. After pooling the appropariate fractions and evaporating the solvent, about 0.415 g (37%) colorless ester was obtained. An analytical sample of C_{16} – ACV was obtained by crystallization from MeOH: mp 200 ~ 202 ℃; UV λ_{max} 250 nm; IR (KBr) cm^{-1} 1730 (COOR), 3200 (NH_2); TLC Rf 0.65 ($CHCl_3$ — MeOH, 9:1).

For C_{12} – ACV, the yield was 1.49 g (64%), mp 162 ~ 164 ℃: UV λ_{max} 250nm; IR (KBr) cm^{-1} 1735 (COOR), 3200 (NH_2); TLC Rf 0.62 ($CHCl_3$ — MeOH, 9:1).

Determination of octanol – buffer partition coefficient (P)

C_{12}–ACV and C_{16}–ACV were dissolved in *n*–octanol at a concentration of 0.5 mg/ml, and ACV was dissolved in octanol – saturated phosphate buffer solution (PBS, pH7.21) at 1.0 mg/ml. Three ml of each of the above solutions were transferred to a test tube, then 3 ml PBS were added to each of the two octanol tubes, and 3 ml *n*–octanol were added to the ACV buffer tube. All tubes were plugged and shaken on a vortex mixer for 1 h. The mixtures were allowed to stand overnight at room temperature. The UV absorbances of

both octanol phase and buffer phase were measured at 250 nm with the corresponding solvent as blank. The partition coefficient was determined as:

$$P = \frac{A_{oct}}{A_{PBS}}$$

Liposome preparation

ACV liposomes (LUV) The procedure was followed as described by Szoka et al[19]. EPC (12.5 μmol) and Chol (12.5 μmol) were dissolved in 10 ml chloroform. The solvent was removed under reduced pressure on a rotary evaporator. The lipid film was dissolved in 3 ml ether. One ml ACV PBS (6.67 μmol/ml) was added. The system was purged with N_2 and sonicated for 5 min in a bath-type sonicator. The dispersion was placed in a rotary evaporator to remove most of the ether. An additional 3 ml PBS was added and the suspension evaporated at 21 ℃ to remove the remaining organic solvent.

C_{12}-ACV liposomes and C_{16}-ACV liposomes (SUV) EPC (12.5 μmol) and Chol (12.5 μmol) together with C_{12}-ACV or C_{16}-ACV (6.67 μmol) in 10 ml chloroform were evaporated to give an even film on the wall of flask. After removing the remaining trace of chloroform with N_2, PBS was added to a volume of 4 ml. The flask was agitated on a vortex mixer at 21 ℃ for 30 min in iced water to give a homogeneous suspension of SUV.

Determination of encapsulation efficiency (EN %)

One ml liposome suspension was carefully added into a dialysis bag (6 mm × 8 cm, Merck) which had been previously boiled in distilled water. Each of the two ends of the bag was fixed by a dialysis clip. The bag was dialyzed against 100 volumes of PBS for three times at 40 ℃, each for 1 h to remove the nontrapped drug. The liposomes in the bag were then washed thoroughly into a 10 ml volumetric flask with mixed solvents (isopropanol — ether — water, 2:4:4, V/V), and diluted to volume with the mixed solvents. UV absorbance was measured at 250 nm against the mixed solvents as blank (A_D).

Another 1 ml liposome suspension was transferred into 10 ml volumetric flask without being dialysyzed and was diluted as above. Its absorbance against mixed solvents blank was recorded (A_{ND}).

To eliminate the absorbance caused by liposomal lipids (A_B), blank liposomes were prepared according to the REV method described above, with PBS to replace the drug solution. The EN% could be calculated as:

$$EN\ \% = \frac{A_D - A_B}{A_{ND} - A_B} \times 100$$

Leakage of Drugs from Liposomes

Leakage was assessed by further dialysis of liposomes against a 100-fold PBS at 4℃ after the separation of nontrapped drug. The UV absorbance was measured at various time intervals and the sample solution was poured back to the dialysate every time after the measurement to continue the dialysis. The dialysis was carried on in a flask which was covered tightly with a paraffin film to prevent the evaporation of water. After 3 days dialysis at 4℃, the bags were taken out. Half aml 10% Triton X-100 solution was added to each of the bags, and another 0.5 ml 2 mol/L HCl was added to bags of C_{12}-ACV

and C_{16} – ACV liposomes. Dialysis was carried out at 37 ℃ on a water bath for a week to make sure the liposomes have been damaged and esters have been hydrolyzed. The leakage percentage was calculated according to the ratios between the absorbances before and after the incubation.

Antiviral activity experiment

Antiviral assays Herpes simplex virus type I, strain HK_2PK_2 was passaged for 3 times in Vero (African green monkey) cells. $TCID_{50}$ (50% tissue culture infection dose) was titrated as 10^{-7}. Virus (10^{-5}/0.1 ml) solutions were exposed to the test compounds of varying concentrations in Hank's solution for 30 min. 0.05 ml such mixture was added to Vero cell cultures which had grown for 3 days. Minima essential medium (MEM) supplemented with 10% calf bovine serum was added to a volume of 0.2 ml. The viral CPE (cetopathic effect) was completed and recorded at five to seven days after viral inoculation.

Control tests were set by adding only Hank's solution (negative control) or only viral solution (positive control) to cell cultures.

Test compounds: ACV was given in PBS solution, while C_{16}–ACV were dispersed in 0.5% CMC solution and sonicated to give a homogeneous suspension. Liposome preparation was carried on in a biological purifying table with all containers sterilized. All compounds, both in free form and encapsulated in liposome (without separating the nontrapped drug), were tested under the concentrations of 0.044, 0.222, 2.222 and 22.22 μmol/L.

RESULT AND DISCUSSION

Properties of the prepared liposomes

The octanol – water partition coefficient (P) is one of the most extensively determined properties of organic compounds. It had been used as a measurement of hydrophilic-lipophilic balance which was thought to be related to drug entrapment[10]. Table 1 shows that the encapsulation efficiency of acyclovir was dramatically increased by esterification bacause of the higher values of P of the ester derivatives. It is clear that the acyclovir palmitate has a relatively greater EN % than the acyclovir laurate, which can be explained by the better compatibility of palmitoyl residue with the acyl chains of lecithin. According to Fildes and Oliver[3], when liposomes containing long chain esters are formed by dispersing a mixed film, a given amount of phospholipid will have a limited affinity for a particular ester. In our experiment, although this limit was not determined, it can be estimated that the present concentrations of derivatives in forming liposome were well below the saturation concentration, since no sediment was observed as a separate phase for hours after preparation.

Tab 1. Octanol－buffer partition coefficient (P) and encapsulation efficiency (EN %) of acyclovir and ester derivatives

Compound	P	Liposome type	EN %
ACV	0.392	LUV	29.9
C_{12}－ACV	397	SUV	95.6
C_{16}－ACV	1015	SUV	97.1

As shown in Fig 1, while more than half of the encapsulated acyclovir was released from liposomes within 60 h at 4℃, acyclovir lauratate was retained by nearly 70% in liposomes, and acyclovir palmitate as high as 85%. We can expect that acyclovir ester derivatives liposomes might be more stable under physiological conditions than acyclovir liposomes.

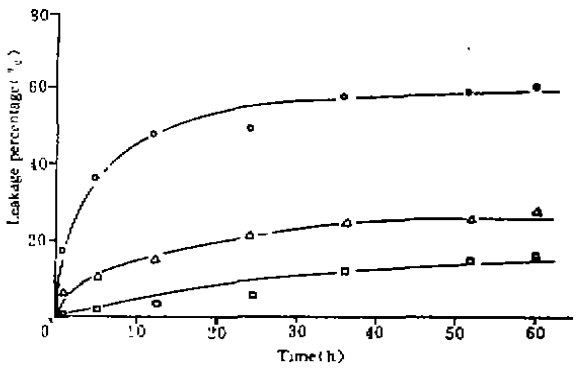

Fig 1. Leakage curve of ACV○, C_{12}－ACV△ and C_{16}－ACV □ from liposome at 4℃ (1.1×10^{-3} mmol/25 μmol lipids).

In vitro **antiherpes efficacy**

Liposome encapsulation enhanced the activity of acyclovir and its ester derivatives in inhibiting HSV－1. At the lowest concentration of 0.044 μmol/L, acycluvir showed no effect, which corresponded to the result of Grumpacker et al (ID_{50} 0.06～0.35 μmol/L)[1], while acyclovir liposomes showed a 25% cell protection at the same concentration (Fig 2).

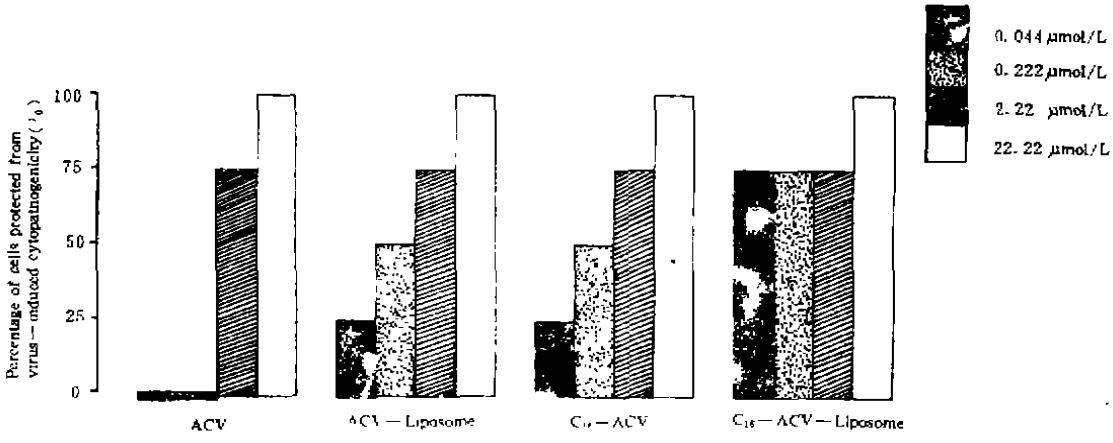

Fig 2. Inhibition effect of acyclovir, acyclovir palmitate and their liposomes on the HSV－1 virus－induced cytophogenicity of Vero cells at different concentrations. Average values for two separate determinations.

The suspension of acyclovir palmitate showed a similar efficacy with ACV liposome, this is probably resulted by the greater penetration ability of the lipophilic prodrug into the infected cells. Acyclovir must be phosphorylated inside the cell by the virus－coded dThd kinase to be active as an inhibitor of herpesvirus replication[5]. So in *in vitro* studies, the

antiherpes activity is apparently influenced by the ability of antiherpes compounds to enter the cells. The liposome－cell membrane fusion and endocytosis which dominate in liposome－cell interaction helps efficiently the delivery of drugs into cells. This explains the highest protection percentage of infected cells by liposome encapsulated acyclovir palmitate. The antiviral test also suggests that the acyclovir palmitate is readily hydrolized to release the parent compound, although further experiment is needed to confirm it.

From the result of this study, it is indicated that incorporation of acyclovir into liposomes could be improved by introducing long chain substitunt group on 2′－OH. Such a liposome system may allow controlled release of acyclovir to the topically HSV infected site, or may enhance the absorption of acyclovir to liver cells in the treatment of chronic hepatitis B.

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15-21 R978.7 无环鸟苷，脂质体，前体药物，抗病毒

无环鸟苷亲脂性前体药物脂质体的制备及体外抗病毒活性

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提要 本文通过将无环鸟苷（acyclovir，简称 ACV）2′位羟基分别与月桂酰氯或棕榈酰氯进行酯化反应，制得亲脂性前体药物无环鸟苷月桂酸酯和无环鸟苷棕榈酸酯（分别简称为 C_{12}－ACV 和 C_{16}－ACV），使脂质体包封率从 ACV 的 29.9% 提高到 C_{12}－ACV 的 95.6% 和 C_{16}－ACV 的 97.1%；漏泄实验表明在 4 ℃ 透析 60 h 后，一半以上的 ACV 从脂质体中漏泄，而 C_{12}－ACV 和 C_{16}－ACV 的滞留率分别为 70% 和 80%；体外抗疱疹病毒的试验中，在最低试验浓度 0.044 μmol/L 时，ACV 不显示抗病毒活性，而 C_{16}－ACV 脂质体抑制细胞病变率达 75%，说明前体药物通过与脂质体脂膜的结合增加了药物的进入细胞能力，从而提高了 ACV 的抗病毒能力。

关键词 无环鸟苷；脂质体；亲脂性前体药物；抗病毒活性