Study on liver targeting and hepatocytes permeable valaciclovir polybutylcyanoacrylate nanoparticles *

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Subject headings   valaciclovir; polybutylcyan-oacrylate nanoparticles; liver targeting; hepatocytes permeability

Abstract
AIM   To prepare valaciclovir polybutylcyan-oacrylate nanopa rticles (VACV-PBCA-NP) with liver targeting and hepatocyte permeable characte ristics.
METHODS   Emulsion polymerization method was employed to prepare VACV-PBCA-NP. The formula and preparation conditions were optimized by using the uniform design. The organ distribution of the intravenously injected VACV-PBCA-NP and VACV in animal was determined using HPLC. The hepatocytes permeability of VACV-PBCA-NP was demonstrated by cell uptake experiment *in vitro*.
RESULTS   The drug loading and the drug embedding ratio of VACV-PBCA-NP were 11.20% and 84.85% respectively, with an average diameter of 10 4.77nm±11.78nm. The releasing characteristics *in vitro* fitted the two-phase kinetics. 74.49% of the drug was found to localize in the liver 15min after the administration of VACV-PBCA-NP in the mice. Compared with VACV, VACV-PBCA-NP showed distinct characteristic of sustained-*release in vivo* and the drug entering hepatocytes were also greatly increased.
CONCLUSION   VACV-PBCA-NP has the char-acteristic of liver targeting and can increase the permeability of VACV to hepatocytes.

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INTRODUCTION
Valaciclovir (VACV), the L-valyl ester of acyclovir (ACV), is a new antiviral drug which can be hydrolyzed into ACV and L-valyl rapidly in the presence of enzyme *in vivo*. Compared with ACV, VACV has the advantages of better solubility and higher bioavailability[1,2]. It has been demonstrated that ACV is effective for hepatitis B[3-6], but not so effective as for herpes virus infection. This is chiefly due to the poor penetration of ACV into the liver cells[7]. One approach solving this problem is to use drug carriers capable of enhancing the liver and liver intracellular drug delivery.

Nanoparticles (NP) is a new drug carrier[8,9]. We have investigated the action of VACV-loaded NP and demonstrated that the amount of drug increased 2. 99 fold in the liver and decreased 5.46 fold in the kidney. Significant increase of intracellular drug was also observed.

MATERIALS AND METHODS
Materials
Both VACV obtained from Sichuan Institute for Antibiotics Industry and ACV supplied by Hubei Institute for Medicinal Industry, meet the USP reference standard. Butyl-cyanoacrylate (BCA) monomer was purchased from Shenzhen Nanguang Medicinal Colla Co. Ltd. and collagenase type I from Sigma Co. VACV-PBCA-NP injection was self-made.

Kunming mice, white Japanese rabbits and Wistar rats were all provided by Laboratory Animal Center, West China University of Medical Sciences.

Methods
Preparation of VACV-PBCA-NP VACV-PBCA-NP was prepared by emulsion polymerization method due to the good water solubility of VACV. An optimum procedure was developed based on the uniform design. Briefly, VACV, Dextran 70, pluronic F-68 and NaHSO3 were weighed and dissolved in water. The pH of the solution was adjusted to 2.2 by adding 0.1N- HCl, and then BCA was added slowly into the solution under electromagnetic stirring, the solution was stirre d for additional 2h at room temperature. Then the pH value of this colloidal solution was adjusted to 5-7 with 0.1 N NaOH. The solution was filtered

through a microfilter membrane (0.3mm), filled into ampoules, freeze-dried and st ored for later use.

Determination of embedding ratio and drug loading
The embedding ratio and the drug loading of VACV were determined by HPLC at 254nm. The HPLC conditions consisted of Shimpack CLC-ODS column (5μm, 150 mm × 4.6 mm id), mobile phase CH_3OH -0.02mol·L^{-1} KH_2PO_4 (20:80), and flow rate 1mL·min^{-1}. The standard curve equation was A= 2704.72 + 26406.30 C (r = 0.9999). The mean recovery was 97.41% ± 1.57% . The colloidal solution of VACV-PBCA-NP was freezingly ultracentrifuged, and the content of VACV in the supernatant was assayed. The embedding ratio (ER%) and the drug loading (DL%) were calculated as follows:

$$ER\% = \frac{(\text{VACV added-VACV in supernatant})}{\text{VACV added}} \times 100\%$$

$$DL\% = \frac{(\text{VACV added-VACV in supernatant})}{\text{BCA added}} \times 100\%$$

Drug release from the VACV-PBCA-NP *in vitro*
Dynamic dialysis bag technique was used to observe the drug release from VACV-PBCA-NP *in vitro*. The freeze-dried powder of VACV-PBCA-NP was dispersed in physiological saline and the dispersion was transferred into a dialysis bag suspended in a con ical container containing physiological saline solution. The container was shaken at 37℃±1℃. Samples were withdrawn at predetermined time, adjusted to pH 9-11 with 0.1N-NaOH, boiled for 1 h and determined. The HPLC conditions were the same as mentioned above, the standard curve equation was A = 3046.73 + 62647.64 C (r = 0.9999). The accumulative drug release percentage was calcul ated to describe the drug release.

Measurement of drug in blood and viscera of mice
Thirty Kunming mice were randomly divided into VACV-PBCA-NP group and VACV group, fifteen in each group. Each mouse was intravenously given VACV-PBCA-NP or VACV at a dose of 25mg/kg body weight. The mice were killed and anatomized 15 minutes after the administration, and the heart, liver, lungs, kidneys and blood were taken out. Plasma of 0.5mL or viscera homogenate were piped accurately and 1mL chloroform and 0.5mL 6% perchloric acid were added respe ctively. The mixture was vortexed and centrifuged, and 20μL supernatant was taken to determine VACV by HPLC.

Isolation and culture of hepatocytes The livers of Wistar rats (200 g ± 20 g in weight, fasted overnight) were taken out under aseptic condition, perfused with Hank's solution until the blood

washed out, cut into tiny pieces, digested with 0.1% collagenase at 37℃ for 45min and filtered thro ugh a stainless steel mesh (mesh size: 100μm). Hepatocytes were purified after being washed three times with Hank's solution and one more time with RPMI -1640 solution. Cells were seeded at a density of 5×10^5 cells/each culture dish and incubated with 5% CO_2 at 37℃ for 18h. Then the medium was replaced with fresh RPMI-1640 solution, VACV-PBCA-NP or VACV was added at various concentrations for further incubation. Six, 12 and 24h after the culture, the hepatocytes were taken out, washed three times with physiological saline and broken, then VACV in the cell was determined by HPLC.

Determination of blood concentration in rabbits Ten white Japan ese rabbits were randomly devided into VACV-PBCA-NP group and VACV group, five in each group. Each rabbit was intravenously given VACV-PBCA-NP or VACV at a dose of 15mg/kg body weight, 2mL blood was taken at different time points after the injection and ACV in plasma was detected at 254nm by HPLC method including Shimpack CLC-ODS analytical column (5μm, 150mm×4.6mm id) and mobile phase methanol-water-acetic acid (1:99:0.5).

RESULTS

Morphology (Figure 1)
The surface of the VACV-PBCA-NP is regular and non-adhesive. The average, the maximum and the minimum diameter of VACV-PBCA-NP is 104.77nm, 141nm and 76nm respectively. Its diameter is not abnormally distrib uted.

Drug loading characteristics   Table 1 shows the embedding ratio and drug loading of VACV-PBCA-NP.

Table 1 Embedding ratio and drug loading of VACV-PBCA-NP

Batch No.	Embedding ratio (%)	Drug loading (%)
961010	85.10	12.15
961012	83.75	10.51
961015	85.70	10.93
Average	84.85	11.20

Stability
Freeze-dried powder of VACV-PBCA-NP was stored at 3℃ - 5℃, 20℃ - 25℃ and 37℃ (RH 75%) respectively for 3 months. There were no noticeable changes in the appearance, morphology, pH and VACV content under the condition of 3℃ - 5 ℃ and 20℃ - 25℃, but changes were found at 37℃ (Table 2).

Drug release characteristics

Figure 2 shows the drug release profile of VACV-PBCA-NP freeze-dried injection. The curve (Figure 2) corresponded to the two-phase kinetics equation: $1-Q=0.3663e^{-0.0015t}+0.3000e^{-0.0524t}$.

Table 2 Stability results of VACV-PBCA-NP

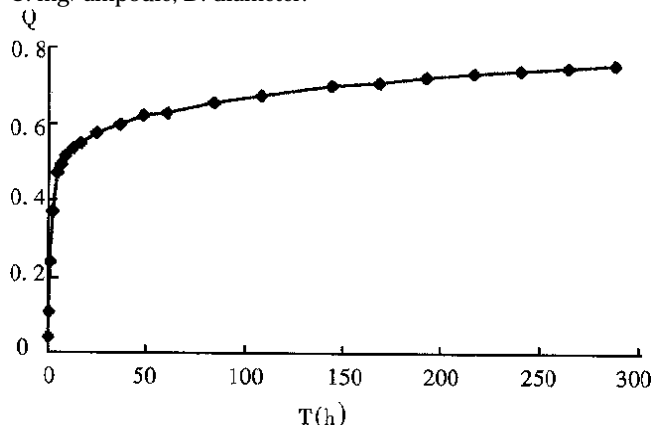| Temperature (℃)	0 month				3 months			
	D/nm	C	pH	Color	Color	D/nm	C	pH
3-5	107.28	0.49	5.34	white	110.42	0.47	5.25	white
15-25	107.28	0.49	5.34	white	109.37	0.48	5.05	white
37(RH75%)	107.28	0.49	5.34	white	105.61	0.43	4.10	white

C: mg/ampoule; D: diameter.

Figure 1 Transmission electron micrographs of VACV- PBCA-NP.
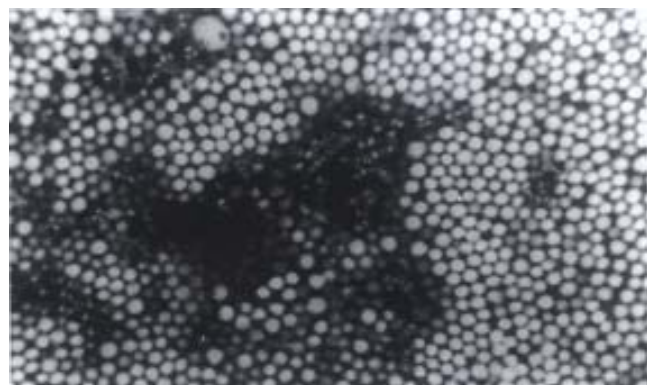

Figure 2 Release profile of VACV-PBCA-NP freeze-dried injection. Q: Percentage of accumulative drug release.

Distribution of VACV-PBCA-NP in viscera and blood of mice

The amount of ACV in each organ recorded as ACV_i and ACV_t was obtained by adding ACV_i in all viscera at different time points. The ratio of $ACV_i/ACV_t \times 100\%$ represented the relative content of VACV-PBCA-NP in viscera and blood (Table 3). Table 3 shows that the relative content of VACV-PBCA-NP in liver was 74.49%, 2.99 times higher than that of VACV, and in kidney was 9.36%, 5.46 times lower than that of VACV.

Table 3   Relative content in various organs 15min after iv administra tion of VACV-PBCA-NP and VACV respectively (%, *n*=3)

Sample	Heart	Liver	Spleen	Lung	Kidney	Blood
VACV-PBCA-NP	0.99	74.49	2.46	2.89	9.36	9.82
VACV	2.14	24.92	2.44	3.01	51.15	16.54

The permeability of VACV-PBCA-NP to hepatocytes

Because of the same amount of the cells added to each culture dish (5×10^5), the peak area of ACV was used to represent the effect of the VACV taken in by rat liver cell, the results showed that the drug amount of VACV-PBCA-NP in rat hepatocytes group was 28.77, 21.90 and 5.22 times that of VACV control group at 6 h, 12 h and 24 h, respectively.

The pharmacokinetic parameters of VACV-PBCA-NP and VACV in rabbit after iv administration

The concentration-time data of the two groups both fitted the two-compartment model, the equation was $C = 20.88e^{-7.653t} + 0.63e^{-0.0001t}$ and $C=10.19e^{-2.67226t} + 0.88e^{-0.3789t}$. The main pharmacokinetic parameters were analyzed by the single factor variance method. The results are shown in Table 4.

Table 4 The results of single factor variance analysis of the main pharmacokinetic parameters of VACV-PBCA-NP and VACV

Factors	A	B	DF	F (test)	F (criterion)	P
AUC	223.34	6.40	1.8	35.87	11.30	<0.01
MRT(h)	244.58	0.95	1.8	28.83	11.30	<0.01

A: VACV-PBCA-NP; B: VACV; DF: Degree of freedom.

Table 4 shows that there is a significant difference in the main pharmacokinetic parameters between the two groups.

DISCUSSION

The VACV-PBCA-NP freeze-dried injection stored at 37℃/RH75% would change in the appearance, pH and the drug content. The results implied that temperature and humidity affect the stability of VACV-PBCA-NP. The reason may be that higher temperature and humidity would speed up the generation of L-valyl and ACV. The refore, VACV-PBCA-NP should be preserved at low temperature and humidity.

VACV will degrade into ACV at 37℃. The experiment showed that VACV would be completely turned into ACV when heated for 1h at 100℃, but ACV was stable in this situation. Therefore, in the

in vitro experiment of the drug release from nanoparticles, the samples were treated by the method mentioned above. The released amount of VACV could be calculated by measuring ACV.

VACV will turn into ACV rapidly and completely *in vivo* because of the presence of enzyme. Therefore, we determined ACV in blood and viscera of animal after i.v. VACV and VACV-PBCA-NP by a HPLC method of good recovery.

REFERENCES

1   Beauchamp LM, Orr GF, Mirunda P. Amino and ester prodrugs of acyclovir. *Antiviral Chem Chemother*, 1992;3:157-164
2   Stemphen W, Robert BM, Marna D, Thimysta B, Donna MC, Paulo M. Pharmacokinetics of the acyclovir prodrug valaciclovir after escalating single and multiple dose administration to normal volunteer. *Clin Pharmacol Ther*, 1993,54:595-605
3   Yang SS, Guan MY, Yang LH. Comparison of acyclovir and interferon in the treatment of chronic hepatitis B. *Hubei Yixueyuan Zazhi*, 1987;8:144-147
4   Zhou JL, Xu LZ, Wu H, Min J. The effect of acyclovir to chronic active hepatitis B. *Tianjin Yiyao*, 1989;17:687-689
5   Weller IV, Carreno V, Fowler MJ. Acyclovir hepatitis B antigen positive chronic liver disease: inhibition of viral replication and transient renal impairment with iv bolus administration. *J Antimicrob Chemother*, 1983;11:223-225
6   Smith CI, Scullard GH, Gregory PB. Preliminary studies of acyclovir in chronic hepatitis B. *Am J Med*, 1982;73(1A):267-269
7   Zhang ZR, Liao GT, Hou SX. Study on mitoxantrone polybutyl cyanoacrylate nanoparticles. *Zhongguo Yaoxue Xuebao*, 1994;29:544-549
8   Marty JJ, Oppenheim RC, Speiser P. Nanoparticles: a new colloiclal drug delivery system. *Pharm Acta Helv*, 1978;53:17-23
9   Kreuter J. Nanoparticles and nanocapsules-new dosage forms in the nanometer size range. *Pharm Acta Helv*, 1978;53:33-39

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