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Preparation and Body Distribution of Freeze-Dried Powder of Ursolic Acid Phospholipid Nanoparticles

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Ursolic acid (UA) is a poor soluble natural triterpenoid. It has a wide variety of antitumor activities. We extracted it from *Crataegus pinnatifida* for the first time. To achieve a high bioavailability, targeting effect, stability, and an intravenous (i.v.) administration, the UA phospholipid nanopowders (UA-PL-NP) were prepared, characterized, and evaluated. With soybean phospholipid as the carrier and poloxamer 188 as emulsifier, the UA nanoparticle suspension was prepared by solvent emulsification–evaporation and ultrasonic dispersion. The UA-PL-NP was obtained by freeze drying. The body distribution in mice was studied after i.v. administration of UA-PL-NP and an UA control solution (UA-Sol). The entrapment efficiency (EE) and UA concentration in vitro and in vivo were analyzed by high-performance liquid chromatography (HPLC). The results showed that the UA-PL-NP had an average diameter of 273.8 nm with a zeta potential of –23.2 mV. The EE was up to 86.0%, and the drug loading (DL) was 12.8%. After i.v. administration of UA-PL-NP with low, middle and high doses, UA concentration in the livers of mice obviously increased during tested period and was highest in tested organs at 4 h. The AUC_{0-12} ratio of UA-PL-NP in liver to that in plasma was much higher than that of UA-Sol, and the liver AUC_{0-12} ratio of UA-PL-NP to UA-Sol was 8.6. These results indicate the UA-PL-NP have a good targeting to the liver after i.v. administration. Therefore, the UA-PL-NP is demonstrated to be available as an i.v. and liver targeting system for lipophilic antitumor triterpenoids.

Keywords ursolic acid; triterpenoid; phospholipid nanoparticles; freeze-dried powder; body distribution

INTRODUCTION

In recent years, the potential of natural products as anticancer agents from plants, notably from medicinal plants used in traditional Chinese medicine (TCM), has been recognized by the scientific community. Ursolic acid (UA, 3β-hydroxy-urs-12-en-28-oic acid) is a poor soluble triterpenoid in nature. It widely exists in food products (Price, Johnson, & Fenwick, 1987) and in more than 120 plant species (Hutchings, Scott, Lewis, & Cunningham, 1996), many of which are used as medicinal plants in traditional medicine (Simon, Najid, Chulia, Delage, & Rigaud, 1992). For example, *Crataegus pinnatifida* (Hawthorn) is a well-known herb rich in UA and widely used as medicine and food in China. UA has a wide range of pharmacological activity, such as anti-inflammation (Saraswat, Visen, & Dayal, 1996), hepatoprotection (Martin-Aragon, de las Heras, Sanchez-Reus, & Benedi, 2001; Young et al., 1994), and antitumor (Niikawa, Hayashi, Sato, Nagase, & Kito, 1993). Numerous studies show that the mechanisms of antitumor effects for UA are multiple, including resistance of various carcinogens and tumor promoters (Hsu, Kuo, & Lin, 2004; Huang et al., 1994), inhibition of the formation and growth of various tumor cells (Kim et al., 2004; Lee, Chung, Kim, Lee, & Kim, 1994), the induction of tumor cell differentiation (Cardenas, Quesada, & Medina, 2004), an antiangiogenic effect (You et al., 2001), and modulation of body defense systems, such as antioxidant potential and immune functions (Liu, 1995). The result of experiment on acute and subacute of UA shows that it is relatively nontoxic (Xiong, Chen, & Li, 2001). It probably becomes a new anticancer medicine with low toxicity and high efficiency. Although UA has been used in cosmetics and health products (Chacon, Molpeceres, Berges, Guzman, & Aberturas, 1999), there is no antitumor pharmaceutical preparation reported in the current literature up to now. So it is very necessary to develop an anticancer product of UA.

In the last decade, significant effort has been made to develop nanoparticles for drug delivery (Brigger, Dubernet, & Couvreur, 2002; Fessi, Devissaguet, & Puisieux, 1992). These systems in general can provide targeted (cellular/tissue) delivery of drugs to sustain drug effect in target tissue, to solubilize drugs for intravascular delivery, and so on (Desai, Labhasetwar, Amidon, & Levy, 1996; Panyam & Labhasetwar, 2003). Generally, nanoparticles are initially obtained as a milky

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suspension, which is stable in short periods. However, particle aggregation may occur after long periods of storage. Lyophilization represents a good alternative to get a long-term product stability (Perez-Camino & Cert, 1999).

Phospholipids have been extensively investigated, that is, as drug carriers in the form of liposomes and as emulsifier in emulsions and solid lipid nanoparticles (Mehnert & Mader, 2001). In particular, phosphatidylcholines (PCs) are the most prevalent among the various phospholipids for the preparation of such nanoparticles, as they have the advantage of chemical inertness, biocompatibility, biodegradability, and intravascular delivery (Fumiyoshi & Tomoko, 2005).

The purpose of this research was to develop UA phospholipid nanopowders (UA-PL-NP) with a high bioavailability, targeting effect, stability, and an intravenous (i.v.) administration. We have done an initial study and prepared UA nanoparticle suspension in laboratories (Zhou & Yi, 2004). In this research, the medium amplified preparations of UA-PL-NP were prepared and their physicochemical properties were evaluated. The body distribution and liver targeting in mice after i.v. administration of UA-PL-NP were studied.

MATERIALS AND METHODS

Materials

UA (99.6%) was extracted from *C. pinnatifida* (Chinese Hawthorn), soybean phospholipid (purity >90%, for injection) was purchased from Shanghai Taiwei Pharmaceutical Company (Shanghai, China). Poloxamer 188 (injection grade) was purchased from BASF (Shanghai, China). Glucose for injection was purchased from Chongqing Daxin Pharmaceutical Company (Chongqing, China). Mannitol for injection was purchased from Shandong Rizhao Pharmaceutical Company (Shandong, China). Methanol (HPLC grade) was purchased from Fisher Scientific International Inc. (NJ, USA). All other chemicals were of analytical grade.

Preparation of UA Nanoparticles Suspension

An optimized result of orthogonal design experiments based on the effect of single factors was obtained. UA-PL-NP was prepared by solvent emulsification–evaporation and ultrasonic dispersion. Briefly, soybean phospholipid, UA, and poloxamer 188 were dissolved in the cosolvent of ethanol and ethyl acetate. This solution was then injected through a 22G needle into 60°C phosphate-buffered saline (PBS, pH 6.5) with magnetic stirring. The organic solvents were removed under reduced pressure using a rotavapor at 42°C. The resulted emulsion was put in ultrasonic bath for 15 min and then diluted with distilled water to supplement the losing water because of vapor. The final suspension was filtered through a 1.0-μm cellulose ester millipore filter to separate nanoparticles or agglomerates. UA-Sol was prepared by dissolving UA (15.0 mg) in a 10-mL mixture of ethanol and polyethylene glycol 400 (1:1).

Freeze Drying of UA Nanoparticles Suspension

At first, 5% glucose and mannitol as cryoprotectants were added into the UA nanoparticles suspension, then the dispersion was filled into 10-mL glass vials, and the vials were frozen at −70°C for 24 h. After, the samples were immediately placed into a Christ model Alpha freeze dryer (type 1050; Van Der Heyden, Brussels, Belgium), the first drying step was performed at a shelf temperature of −50°C with a pressure below 1 mbar for 24 h. Second, the temperature was increased to the range of 25–50°C for several hours. Then the UA-PL-NP were successfully prepared.

Transmission Electron Microscopy

The UA-PL-NP were dispersed into distilled water. The morphology of the TET–SLN was examined by transmission electron microscopy (TEM) (JEM-100CXII, Japan). Samples were prepared by placing a drop of UA nanoparticles suspension onto a copper grid and air dried, followed by negative staining with a drop of 2 M aqueous solution of sodium phosphotungstate for contrast enhancement. The air-dried samples were then directly examined under the transmission microscope.

Average Diameter and Zeta Potential

The nanoparticles were characterized for their size and zeta potential with a Zeta-sizer (Brookhaven Instrument corporation, Brookhaven, NY, USA). Three batches of UA-PL-NP samples were prepared according to the method described previously. Each sample was diluted with distilled water until the appropriate concentration of particles was achieved, and each sample was measured three times to calculate the average diameter and zeta potential.

Entrapment Efficiency and Drug Loading

Powdered UA-PL-NP were dispersed into distilled water. About 1 mL of UA-PL-NP suspension was diluted to 10 mL with methanol. UA-PL-NP were separated from the liquid medium by centrifuging (802,400×g, 1 h). The nonentrapped UA remaining in the supernate was analyzed by high-performance liquid chromatography (HPLC). Entrapment efficiency (EE) and drug loading (DL) of UA was calculated.

Body Distribution of UA-PL-NP

Male Kunming mice weighing 20–30 g (provided by Central Animal Laboratory of Tianjing Medical University) were used for body distribution studies. The mice were fasted overnight but had free access to water. The experiments were approved by Tianjing Medical University Institutional Animal Care and Use Committee in accordance with the National Institute of Health 1996 Guide for the Care and Use of Laboratory Animals.

The UA-PL-NP were dispersed into distilled water, and the nanoparticles suspension were injected intravenously into the tail vein. Animals were administrated with a single dose of 10, 15, and 20 mg UA/kg for UA-PL-NP. At predetermined time periods (5, 60, and 240 min) after injection, seven animals at each time point from each group were treated. The blood samples were collected from the ocular artery after eyeball removal. Then, the mice were killed and the heart, liver, spleen, lung, kidney, stomach, intestine, testis, and brain were excised, rinsed with saline, and blotted with filter paper. Blood samples were anticoagulated with heparin and centrifuged at 3,750×g for 10 min to obtain plasma. The plasma and the tissue homogenates were stored in a freezer (−20°C) until assayed. Tissue extracts were prepared by adding 2 mL of ethyl acetate to 1 g of homogenized tissue. The mixtures were vortexed for 5 min followed by centrifugation at 3,500 × *g* for 15 min. The organic phases were removed and evaporated to dryness under a gentle stream of nitrogen at 60°C. The residue was reconstituted in 100 μL of mobile phase, and 20 μL was injected into HPLC column.

Other animals were treated with a single dose of 15 mg UA/kg for UA-PL-NP and with a single dose of 15 mg UA/kg for UA-Sol. At predetermined time periods (0.083, 1, 4, 8, and 12 h) after injection, the blood and liver samples were collected and treated with the above method.

HPLC Analysis of Ursolic Acid

The concentrations of UA in vitro were assayed based on the reversed-phase HPLC methods described previously (Zhou & Yi, 2004). UA was analyzed by HPLC with an Eclipse XDB C_{18} column (5 μm, 250 × 4.6 mm^2) at room temperature (25°C). The samples were eluted with methanol–water–triethylamine (90:10:0.03, vol/vol/vol) at a flow rate of 0.6 mL/min and detected at 210 nm.

The concentrations of UA in vivo were determined by another reversed-phase HPLC method. The HPLC system consisted of a Shimadzu LC-6A solvent delivery pump equipped with a 20-μL loop and rheodyne sample injector, SPD-6AV UV-Visible detector set at 220 nm. The column used was C_{18} Kromasil analytical column (5 μm, 250 × 4.6 mm^2) at room temperature. The mobile phase consisted of methanol–water–acetic acid (400:60:5, vol/vol/vol), and the flow rate was 1.3 mL/min.

Data Analysis

The plasma and tissue concentration data of UA obtained from mice were pooled to mean concentration data. AUC_{0-12} was calculated using the linear trapezoidal rule. The drug targeting efficiency (*Te*) and relative targeting efficiency (*Re*) were calculated based on the reported method (Gupta & Hung, 1989). The *Te* and *Re* were defined as follows:

$$Te = \frac{\text{AUC of UA-PL-NP in targeting organ}}{\text{AUC of UA-PL-NP in Plasma}}$$

$$Re = \frac{\text{AUC of UA-PL-NP in targeting organ}}{\text{AUC of UA-Sol in targeting organ}}$$

RESULTS AND DISCUSSION

Characterization of Freeze-Dried Powder of UA-PL-NP

The final production was white finely dispersed powders. Under TEM, most of the nanoparticles look spherical and regular as shown in Figure 1. The nanoparicles had an average diameter of 273.8 ± 2.3 nm (n = 3). The surface carried negative charge with a zeta potential of −23.2 ± 1.5 mV (n = 3), the EE was 86.0 ± 0.4% (n = 3), and the DL of powdered UA-PL-NP was 12.8 ± 0.2% (n = 3) (Table 1).

In this study, soybean phospholipid was chosen as carrier, poloxamer 188 was chosen as emulsifier, phosphate-buffered saline (pH 6.5) was chosen as aqueous phase, and the UA-PL-NP were successfully prepared by solvent emulsification–evaporation and ultrasonic dispersion. The preparation method is based on the solvent emulsification–evaporation method (Siekmann & Westesen, 1996; Sjöström & Bergenstahl, 1992). The lipid matrix is dissolved in a water-immiscible organic solvent (e.g., chloroform) that is emulsified in an aqueous phase. Upon evaporation of the solvent under reduced pressure, a nanoparticle dispersion is formed by precipitation of the lipid in the aqueous medium. Depending on the fat load and emulsifier used, particles with average diameters of 30–100 nm can be obtained. But an important disadvantage of this technique is toxicological problems possibly arising from solvent residues. In this study, the drug nanosuspension was prepared from emulsion containing partially water-miscible solvent mixture with low toxicity by a solvent diffusion technique. The process is based on the water miscibility of these solvents. Upon rapidly injecting the partially water-miscible solvent

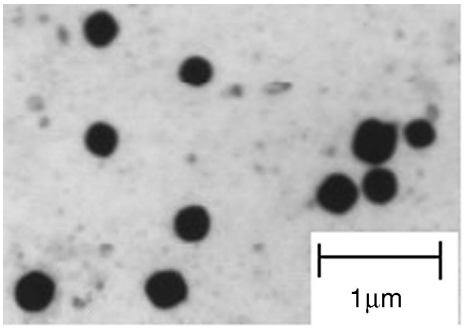

FIGURE 1. Micrograph of ursolic acid phospholipid nanopowders (UA-PL-NP) by transmission electron microscope (×19,000).

TABLE 1
Characterization of Ursolic Acid Nanopowders

Batch No.	Diameter (nm)	Zeta Potential (mv)	Entrapment Efficiency (%)	Drug Loading (%)
040110	272.1	–22.54	85.6	12.8
040114	276.4	–22.14	86.0	12.6
040118	273.0	–24.88	86.3	13.0
Average ± *SD*	273.8 ± 2.3	23.19 ± 1.48	86.0 ± 0.4	12.8 ± 0.2

mixture into water, the drug dissolved in the organic solvent mixture solidifies instantly because of diffusion of the organic solvent from the droplets to the continuous phase (Schubert & Muller-Goymann, 2003; Trotta, Gallarate, Pattarino, & Morel, 2001). But the nanoparticles obtained by solvent emulsification–evaporation had a broad size distribution and easily aggregated to form precipitation. Studies proved that further ultrasonication could make the nanoparticle size more uniform and phosphate-buffered saline (pH 6.5) as aqueous phase could increase the stability of phospholipid nanoparticles, which was coincidence with the report of literature (Wang & Sun, 1998). This preparation method offers clear advantages such as the use of pharmaceutically acceptable organic solvents, no need for high pressure homogenization, easy handling, and a fast production process without technically sophisticated equipment.

Freeze drying is a convenient unit operation to dry nanocrystal dispersions (Goldblith, Rey, & Rothmayr, 1975; Konan, Gurny, & Allémann, 2002). But freeze drying may generate many stresses that could destabilize colloidal suspension of nanoparticles, especially, the stress of freezing and dehydration (Abdelwahed, Degobert, & Fessi, 2006b). For these reasons, special excipients must be added to the suspension of nanoparticles before freezing to protect these fragile systems (Abdelwahed, Degobert, & Fessi, 2006a). These excipients are usually added to protect the product from freezing stress (cryoprotectant) or drying stress (lyoprotectant) and also to increase its stability upon storage. In this study, lactose, glucose, mannitol, and sucrose were selected as the cryoprotectants. Results showed that the changes of particle size and drug encapsulation percentage were very slight when the UA-PL-NP were freezed in the presence of 5% glucose and 5% mannitol.

Body Distribution

Figure 2 shows the concentration of UA in the tissues at different time points after i.v. administration (I, II, and III represents different doses of UA-PL-NP). Figure 3 shows the concentration of UA in the tissues at 4 h after i.v. administration of different doses. The concentration of UA in the liver increased steadily with the extension of time over the 4-h sampling period and was the highest among the tested organs at 4 h after i.v. administration with high, middle, and low dose of UA-PL-NP. In contrast, at 5 min after i.v. administration of UA-PL-NP, the concentration of UA in the intestines with the

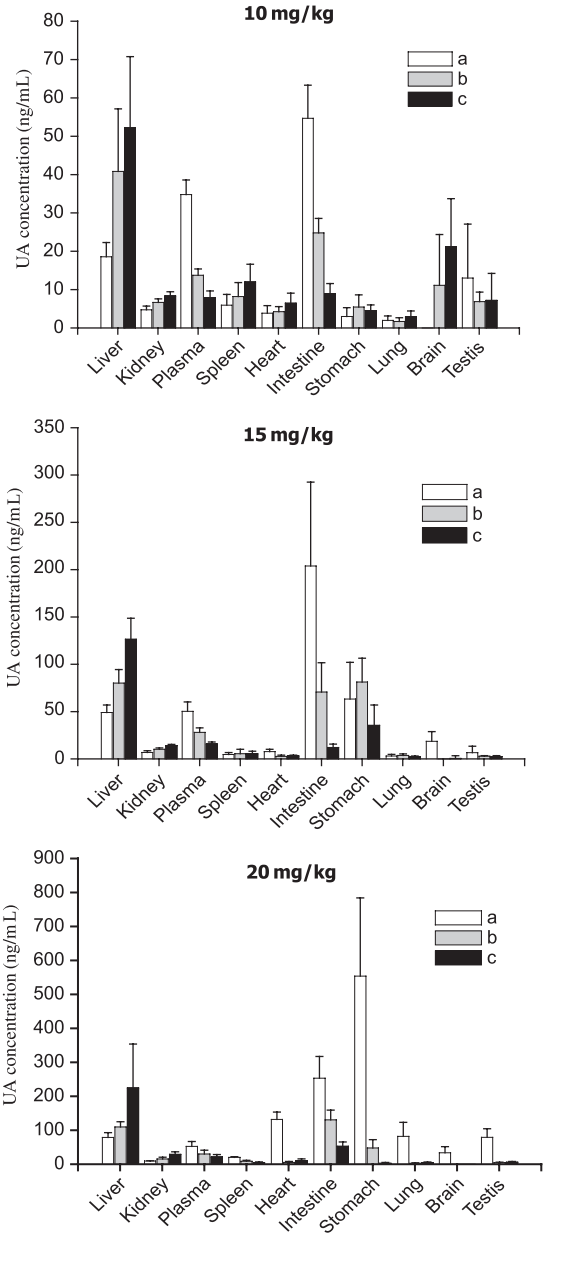

FIGURE 2. Ursolic acid concentration in the tissues of mice at different times after intravenous administration of ursolic acid phospholipid nanopowders (UA-PL-NP) (a, 5 min; b, 60 min; c, 240 min) (n = 7, $M \pm SD$).

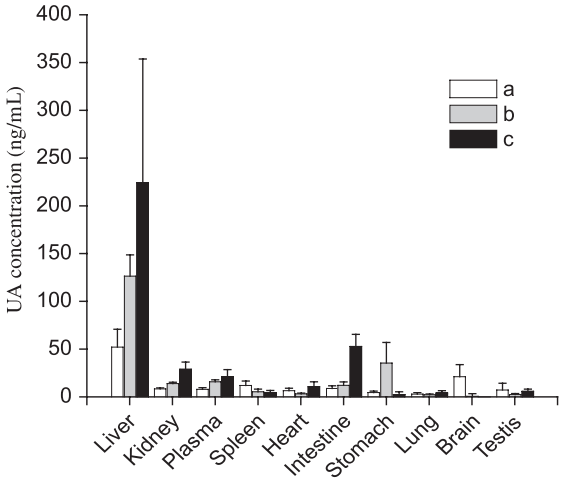

FIGURE 3. Ursolic acid concentration in the tissues of mice at 4 h after intravenous administration of the nanoparticle formulation with the different doses of ursolic acid phospholipid nanopowders (UA-PL-NP) (a, 10 mg/kg; b, 15 mg/kg; c, 20 mg/kg) ($n = 7$, $M \pm SD$).

doses of 10 and 15 mg UA/kg was highest in the tested organs, while that in the stomach is highest at the doses of 20 mg UA/kg and then decreased synchronously with the decline in plasma concentration. At 4 h after i.v. administration, liver concentration of UA increased continuously while other tissues concentration were relatively low. The UA concentration in most tissues were very good reproducibility in different dose groups as time changed.

Table 2 shows the UA concentrations in plasma and liver after i.v. administration of UA-PL-NP and UA-Sol. The results of index of evaluation for liver targeting in vivo are shown in Table 3. The AUC_{0-12} ratio of UA-PL-NP in liver to that in plasma (*Te*) was 5.8 and in spleen to that in plasma was 0.27 while that of UA-Sol was 1.3 and 0.16, respectively. The liver AUC_{0-12} ratio of UA-PL-NP to UA-Sol (*Re*) was 8.6 and spleen was 1.7. The high *Te* and *Re* of UA-PL-NP for liver and spleen indicated that UA-PL-NP mainly accumulated in RED organs, especially in liver.

Intravenously injected particulated substances of drug carriers with an average size below 7 μm are normally taken up by the macrophages of the RES, particularly by the Kupffer cells of the liver (Yang et al., 1999). The RES has a major role in removing small foreign particles from blood by coating them with serum components, the opsonins, which act as labels to passively target the nanoparticles by certain phagocytic cells (Couvreur, Dubernet, & Puisieux, 1995; Moghimi, 1995). These results indicated that UA-PL-NP had successfully targeted UA to liver and could greatly improve the efficacy of UA to treat liver cancers and hepatitis. The UA concentration in the stomach and intestine was higher than that in other tested organs at 5 min after i.v. administration and then decreased rapidly over time. These results indicate UA exists bile

TABLE 2
Concentration of Ursolic Acid (ng/mL Organ) in Mice After Intravenous Administration of UAPL-NP and UA-Sol (15 mg/kg Body Weight, $n = 7$)

	Plasma				Liver				Spleen			
	UA-PL-NP		UA-Sol		UA-PL-NP		UA-Sol		UA-PL-NP		UA-Sol	
Time (h)	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
0.083	50.26	9.99	63.25	10.53	49.08	7.89	32.71	13.62	4.72	1.94	4.87	2.68
1	28.06	4.74	10.37	5.15	80.15	14.25	18.86	7.19	5.46	4.67	3.84	2.24
4	15.75	2.12	5.65	2.08	126.42	22.19	10.35	4.31	5.38	2.79	2.93	1.44
8	5.66	1.94	2.51	1.55	67.33	16.25	4.25	1.67	3.91	1.06	1.77	1.21
12	2.07	2.24	0.82	0.79	22.85	11.32	1.33	0.85	1.74	1.54	0.81	0.82

UA-PL-NP, ursolic acid phospholipid nanopowders; UA-Sol, ursolic acid control solution.

TABLE 3
Results of Index of Evaluation for Liver Targeting In Vitro

				Te		*Re*	
Preparation	$(AUC_{0-12})_{plasma}$	$(AUC_{0-12})_{liver}$	$(AUC_{0-12})_{spleen}$	Liver	Spleen	Liver	Spleen
UA-PL-NP	161.99	939	43.53	5.8	0.27	8.6	1.7
UA-Sol	83.39	109.18	25.27	1.3	0.16	1	1

UA-PL-NP, ursolic acid phospholipid nanopowders; UA-Sol, ursolic acid control solution.

excretion and enterohepatic cycle which are verified by the later excretion studies of UA.

CONCLUSION

In this study, the i.v. UA-PL-NP were prepared by modified emulsification–evaporation. The powder of UA-PL-NP obtained by freeze drying had high EE and stability and a good targeting to the liver after i.v. administration. It is anticipated to be used as a novel nanocontrolled release antihepatitis and antihepatoma medicine. Further studies such as pharmacokinetics studies and antitumor activity tests in vitro and in vivo should be performed to evaluate the applicability of UA-PL-NP as liver targeting preparation.

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