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Characterization, pharmacokinetics, tissue distribution and antitumor activity of honokiol submicron lipid emulsions in tumor-burdened mice

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Received March 8, 2012, accepted June 15, 2012

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Pharmazie 68: 41–46 (2013) *doi: 10.1691/ph.2013.2062*

Honokiol, isolated from the Chinese traditional herb magnolia, is a poorly water-soluble component and has been found to have anti-tumor properties. In the current study, honokiol submicron lipid emulsions (HK-SLEs) were prepared by high pressure homogenization technology. After HK-SLEs were physically characterized, their pharmacokinetics, tissue distribution and antitumor activity after intravenous (IV) administration to tumor-burdened mice were examined, using honokiol solution (HK-SOL) as the control. The results showed that the mean particle size, zeta potential, pH value, osmolality, drug loading (DL)% and entrapment efficiency (EE)% of HK-SLEs were 186.6 ± 1.7 nm, -35.65 ± 0.67 mV, 7.22 ± 0.26, 298 ± 2.3 mOsm/L, 7.1 ± 0.2% and 95.5 ± 0.2%, respectively. HK-SLEs were stable for at least 12 months when stored at $4 \pm 2\,^{\circ}$C. The pharmacokinetic results showed that the drug concentration-time curves of HK-SLEs and HK-SOL could both be described by an open two-compartment model. The half-life of HK-SLEs ($t_{1/2(\alpha)} = 8.014$ min, $t_{1/2(\beta)} = 35.784$ min) was remarkably prolonged compared to that of HK-SOL ($t_{1/2(\alpha)} = 4.318$ min, $t_{1/2(\beta)} = 15.522$ min). HK-SLEs exhibited a greater AUC and reduced plasma clearance. The tissue distribution results indicated that HK-SLEs have better targeting properties to lung and tumor tissues compared with those of HK-SOL. Both HK-SLEs and HK-SOL tended to accumulate in brain tissue. *In vivo* study showed that HK-SLEs treatment caused significant inhibition of mouse sarcoma S180 tumor growth compared to HK-SOL. These results suggest that HK-SLEs might be an effective parenteral carrier for honokiol delivery in cancer treatment.

1. Introduction

Honokiol (3,5-di-(2-propenyl)-1,1-biphenyl-2,2-diol) is a bioactive component isolated and purified from the Chinese traditional herb magnolia (Chen et al. 2007). Honokiol has been found to have anti-microbial, anti-thrombotic (Pyo et al. 2002), anti-inflammatory (Liou et al. 2003), anti-oxidant (Haraguchi et al. 1997), anti-anxiety (Kuribara et al. 2000), and anti-tumor properties (Kay et al. 2003) without appreciable toxicity. Additionally its anti-tumor properties were extensively studied. Honokiol has shown to be effective against cancer cells *in vitro* or *in vivo*, including brain cancer cells (Lin et al. 2012), breast cancer cells (Singh et al. 2011), hepatocellular carcinoma cells (Rajendran et al. 2012), human colorectal cell (Ponnurangam et al. 2012) and human lung cancer cells (Yang et al. 2002) et al. The antitumor activity of honokiol may be realized by inducing apoptosis (Wang et al. 2004), inhibiting angiogenesis (Bai et al. 2003) and causing cell cycle arrest (Hahm et al. 2007). These findings have generated interest for honokiol as a novel chemotherapeutic agent.

Unfortunately, the clinical use of honokiol is greatly limited by its poor water solubility. Many methods were reported to overcome this problem, such as MPEG–PLA nanoparticles (Zheng et al. 2010), honokiol-in-HP-β-CD-in-liposome (Wang et al. 2011a), micelles/hydrogel (Gong et al. 2009), PCL-PEG-PCL nanoparticles (Gou et al. 2010) and chitosan microparticles (Li et al. 2009). However, some of these drug delivery systems had low entrapment efficacies for honokiol.

To our knowledge, little work has been done to prepare honokiol submicron lipid emulsions (HK-SLEs). SLE, an oil-in-water emulsion, has been used as an IV carrier for lipophilic and amphiphilic drugs for more than 40 years (Venkateswarlu et al. 2001). Many parenteral lipid emulsions are currently available for nutritional and therapeutic applications, including preparations with etomidate, diazepam, prostaglandin E1 and β-elemene (Levy et al. 1989; Shibata et al. 2009; Wang et al. 2005). The basic structure of an SLE is a neutral lipid core stabilized by a monolayer of amphiphilic lipid. The oil phase of the submicron lipid emulsion allows "solubilization" of these drugs without the use of organic solvents, therefore avoiding potential side effects. At the same time, contact of the drug with body fluids and blood vessels can also be largely avoided, potentially reducing the rate of phlebitis. Compared to other particulate carriers, such as liposomes and polymeric nanoparticles, SLE possesses a number of advantages. SLE is composed of accepted excipients and can be produced on a large scale using existing production lines for parenteral nutrition emulsions. Additionally, they can be terminally sterilized by autoclaving and are physically stable for long periods of time (Zhao et al. 2011). In conclusion, SLE is a ready-to-use, low-cost product (Lundberg 2003).

In this study, HK-SLEs were prepared by high pressure homogenization technology. The particle size, zeta potential, pH value, osmolality, entrapment efficiency (EE), drug loading % (DL %) and long-term stability of HK-SLEs were examined. Pharmacokinetics, tissue distributions and antitumor activity of

HK-SLEs after intravenous administration to tumor-burdened mice were compared to tumor-burdened mice treated with a honokiol solution (HK-SOL). This study shows that HK-SLEs have potential clinical applications as a new dosage form.

2. Investigations, results and discussion

2.1. Preparation of HK-SLEs

The effects of the percentage of soybean oil, the percentage of emulsifier, the ratio of phospholipids and poloxamer and the ratio of drug and phospholipids were examined in this study. Additionally, the influences of the emulsification temperature, emulsification time, homogeneous stress and homogenization times on the emulsion quality were studied. Then, based on the results of single factor analysis, the formulation and the preparation techniques were optimized by orthogonal design. At last, the optimized formula of the submicron emulsion contained 1.2 g honokiol, 11.0 g soybean oil, 2.0 g soybean lecithin, 1.0 g synperonic F68 (poloxamer 188), 2.5 mL glycerol and 82.3 mL water. Honokiol could be dissolved in the soybean oil without the addition of organic solvents or increasing the homogenization time and pressure.

2.2. Characterization of the HK-SLEs

The mean intensity particle size of HK-SLEs was 186.6 ± 1.7 nm. The particle size distribution spectrum showed that all the particles were smaller than 342 nm, and 90% were less than 220.2 nm. The polydispersity index of size was approximately 0.165 ± 0.05. The zeta potential was -35.65 ± 0.67 mV. The pH value and osmolality of the HK-SLEs were determined to be 7.22 ± 0.26 and 298 ± 2.3 mOsm/L, respectively. The DL% of the HK-SLEs was $7.1 \pm 0.2\%$ and the EE was $95.5 \pm 0.2\%$.

The zeta potential measurement was performed to predict the stability of the colloidal aqueous dispersion (Komatsu et al. 1995). Charged particles with absolute values of zeta potential larger than 30 mV are less likely to aggregate (Levy et al. 1994). However, this is true only when some requirements are met. One requirement is that the medium surrounding the particles is not changed (Washington et al. 1990). In this study, emulsion was diluted with water, so although the zeta potential was larger than 30 mV, it is only one characteristic of the HK-SLEs. The pH of the HK-SLEs may decrease upon storage due to the hydrolysis of phospholipids and triglycerides. Thus, adjusting the initial pH to a stable value (pH 7.4) is important to minimize fatty acid formation. The required osmolality for parenteral preparations is 285–310 mOsm/L (Ganta et al. 2008). All the results showed that the characteristics of HK-SLEs could fulfill the requirements for IV administration.

2.3. Long-term stability of the HK-SLEs

The investigation of long-term stability was conducted over 12 months. The results from Table 1 showed that the parameters applied to evaluate the physicochemical stability of HK-SLEs did not change significantly during the 12 month storage at $4 \pm 2\,^{\circ}C$ ($p < 0.05$), indicating excellent physical and chemical stability. Therefore, it can be concluded that HK-SLEs is an excellent candidate for intravenous administration and can undergo real-life storage conditions for at least 12 months.

2.4. Validation of analytical methods

A HPLC method was developed to measure the amount of honokiol in plasma and tissue samples. The results showed that honokiol retention times were approximately 7.0 min. No peaks interfered with the analysis in the sample chromatograms. The linear range of the equation for the calibration curves of honokiol in plasma and tissue samples was between 0.0892 and 31.25 μg/mL with a correlation coefficient greater than 0.99. The extraction recovery of honokiol in plasma and tissue samples were 77.78–98.50% and the method recovery was 91.71–110.53%. The intra-day and inter-day relative standard deviations were both less than 5.01%.

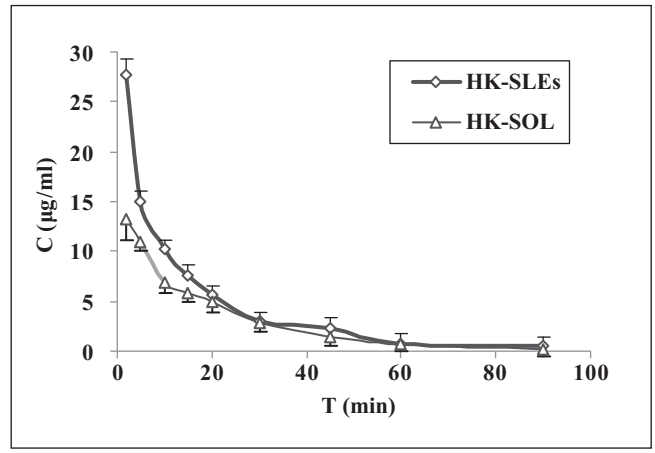

Fig. 1: The mean plasma concentration-time curves of honokiol after IV administration of HK-SLEs and HK-SOL at a dose of 31.5 mg/kg to tumor-burdened mice (n = 6)

2.5. Pharmacokinetic studies

The mean plasma concentration–time curves of honokiol in tumor-burdened mice after IV administration of HK-SOL and HK-SLEs at a single dose of 31.5 mg/kg body weight are shown in Fig. 1. The concentrations of honokiol in the plasma declined bi-exponentially and were higher for HK-SLEs than HK-SOL at the initial time points. Both curves fit the open two-compartment model. The mean pharmacokinetic parameters are listed in Table 2. The results showed that there were significant differences in most of these parameters between the two dosage forms. The plasma concentrations detected at 2 min for HK-SLEs and HK-SOL were 27.71 μg/ml and 13.18 μg/ml, respectively. Although both profiles exhibited a rapid distribution phase followed by a slower elimination phase, the half-lives after IV administration of HK-SLEs ($t_{1/2(\alpha)} = 8.014$ min, $t_{1/2(\beta)} = 35.784$ min) were remarkably prolonged compared to those after IV administration of HK-SOL ($t_{1/2(\alpha)} = 4.318$ min, $t_{1/2(\beta)} = 15.522$ min). The plasma AUC for HK-SLEs was approximately 1.5 times that of HK-SOL. Moreover, apparent volumes of distribution of the central com-

Table 1: Characterization of HK-SLEs over 12 months at $4 \pm 2\,^{\circ}C$ (n = 3)

Characterization	0 days	12 months
Physical appearance	Good	Good
Particle size (nm)	186.6 ± 1.7	189.3 ± 2.2
Polydispersity Index	0.165 ± 0.05	0.172 ± 0.09
Zeta potential (mV)	-35.65 ± 0.67	-36.52 ± 0.81
pH value	7.22 ± 0.26	7.31 ± 0.41
Osmolality (mOsm/L)	298 ± 2.3	299 ± 2.8
DL (%)	7.1 ± 0.2	7.0 ± 0.3
EE (%)	95.5 ± 0.2	94.9 ± 0.8

Table 2: Pharmacokinetic parameters of honokiol after IV administration (n = 6)

Parameters	Unit	HK-SOL	HK-SLEs
V(c)	(mg/kg)/(μg/ml)	2.048 ± 0.017	1.287 ± 0.081*
$t_{1/2(\alpha)}$	min	4.318 ± 2.336	8.014 ± 1.266*
$t_{1/2(\beta)}$	min	15.522 ± 0.929	35.784 ± 18.545*
CL(s)	[mg/(kg·min)]/(μg/ml)	0.119 ± 0.018	0.082 ± 0.010*
AUC_{0-t}	(μg/ml)·min	265.626 ± 38.314	386.273 ± 46.734*
MRT	min	19.409 ± 0.509	18.646 ± 0.724

V(c): apparent volumes of distribution of the central compartments; $t_{1/2(\alpha)}$: half-life of distribution phase; $t_{1/2(\beta)}$: half-life of elimination phase; CL(s): clearance; AUC: area under the plasma concentration-time curve; MRT: mean residence time
*$P < 0.05$ vs. HK-SOL group

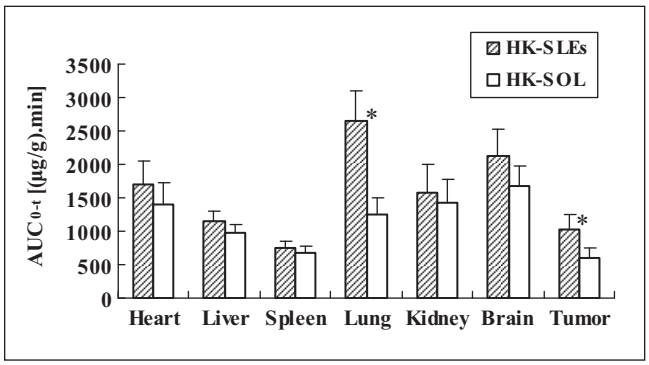

Fig. 3: The area under curve (AUC_{0-t}) of honokiol in various tissues after IV administration of HK-SLEs and HK-SOL at a dose of 31.5 mg/kg to tumor-burdened mice (n = 6)
*$P < 0.05$ vs. HK-SOL group

partments (Vc) and clearance (CL) after administration of HK-SLEs were as large as 0.63 and 0.69 times as those of HK-SOL, respectively. However, the mean residence time (MRT) of honokiol did not differ significantly between these two formulations. Therefore, the results indicate that the free honokiol in HK-SOL was rapidly cleared in the bloodstream, which was in accordance with the literature (Wang et al. 2011). Conversely, the HK-SLEs had a higher plasma drug concentration at the first few minutes and withstood clearance. This implies that the honokiol in HK-SLEs circulates in the blood stream for a longer time, resulting in better therapeutic effects.

2.6. Tissue distribution study in tumor-burdened mice

The mean concentrations of honokiol in different tissues at 2, 5, 10, 15, 20, 30, 45, 60 and 90 min after IV administration of HK-SLEs and HK-SOL are shown in Fig. 2. Honokiol distributed into the organs rapidly and was also eliminated rapidly, which corresponded to blood levels. Two minutes after administration,

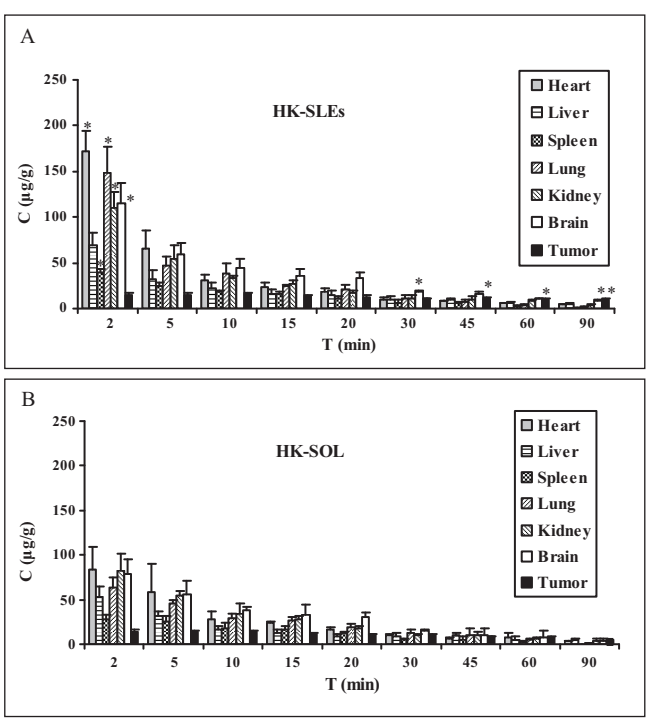

Fig. 2: Concentrations of honokiol in different tissues at 2, 5, 10, 15, 20, 30, 45, 60 and 90 min after IV administration of HK-SLEs and HK-SOL at a dose of 31.5 mg/kg to tumor-burdened mice (n = 6) A) HK-SLEs; B) HK-SOL
*$P < 0.05$ vs. HK-SOL group

honokiol was observed in all collected tissues, with the highest concentrations being found in the heart, followed by lung, brain, kidney, liver, spleen and tumor tissues. The AUC of honokiol in various tissues was also calculated (Fig. 3). The drug concentration at 2 min and the AUC of HK-SLEs were both higher than those of HK-SOL for the same tissues. The AUC of HK-SLEs declined in the order of lung, brain, heart, kidney, liver, tumor and spleen tissues. Meanwhile, the biggest AUC of HK-SOL was in the brain, followed by kidney, heart, lung, liver, tumor and spleen tissues.

The results in normal tissue indicated that the drug tended to accumulate into tissues with their own rich blood supply, such as the brain, lung, and heart. Free honokiol in HK-SOL accumulated in the brain, which might be explained by its solubility and small size. Lipid soluble small molecules with a molecular weight (MW) of < 400 Daltons (Da) can cross the BBB via lipid-mediation (Pardridge 2005). There are many reports on the ability of emulsions to accumulate in brain (Pan et al. 2002). The lung AUC for HK-SLEs was as 2.1 times that of HK-SOL, which further confirmed the uptake of emulsion droplets by the mononuclear phagocyte system (MPS) in the lung (Ganta et al. 2003; Takino et al. 1994). However, the honokiol concentration and AUC of both formulations were low in the liver and spleen which are both highly penetrated by the MPS. With respect to the liver, a recent investigation established that glucuronidation and sulfation are the main metabolic pathways for honokiol in rat and human liver tissue (Böhmdorfer et al. 2011). The hepatic extraction ratio and clearance of honokiol was high in rat liver, resulting in low bioavailability. This study suggested that although HK-SLEs have a strong tendency to accumulate in the liver, most of them were bio-transformed and eliminated by the liver. Therefore, the honokiol level detected was extremely low. There are significant difference between the honokiol concentrations of HK-SLEs and HK-SOL in the plasma and most tissues detected in the initial (≤ 2 min). Such differences reduced or disappeared quickly later. Plasma protein binding ratio may play an important role to influence the distribution and clearance of HK-SOL and HK-SLEs. Many drugs will demonstrate non-specific binding in plasma and tissues to constituent proteins, such as albumin, α1-acid glycoprotein, and lipoproteins, as well as circulating cells, including red blood cells and platelets (Mager 2006). The plasma protein binding ratio of honokiol was 60% to 65% with its concentration 0.5 to 20 mg/L (Wang et al. 2011b). The lipid solubility of HK-SOL and HK-SLEs may cause differences in their plasma protein binding ratios. Honokiol in HK-SOL may be both bound more by red blood cell and the plasma protein, compared with HK-SLEs. The unbound honokiol concentration in the plasma of HK-SOL was lower than that of HK-SLEs. Therefore, whole honokiol concentra-

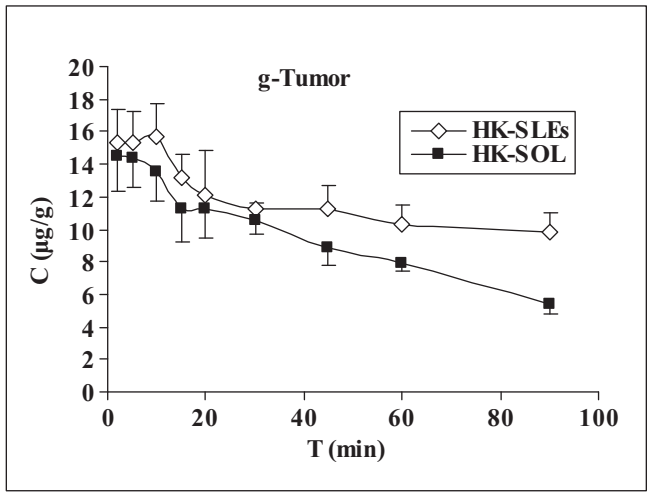

Fig. 4: The mean tumor concentration-time curves of honokiol after IV administration of HK-SLEs and HK-SOL at a dose of 31.5 mg/kg to tumor-burdened mice (n = 6)

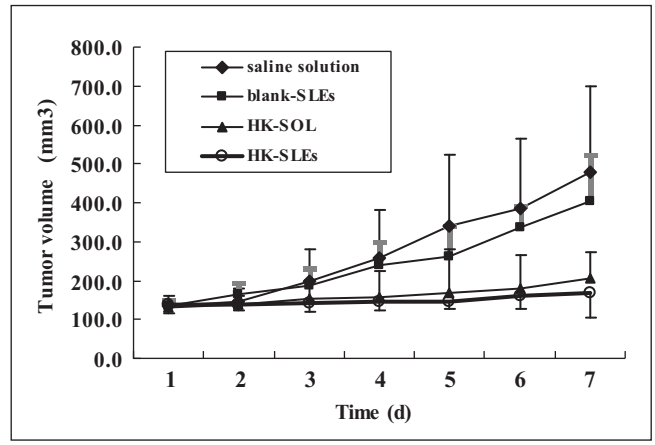

Fig. 5: Tumor volume after injection of different groups (n = 10)

tion (bound and unbound) of HK-SOL in plasma was lower than that of HK-SLEs at the beginning. Only unbound drug is hypothesized to cross membranes and distribute through the body. The lower concentration of unbound honokiol in HK-SOL group may help to explain the lower honokiol concentration of HK-SOL group in most tissues detected, compared with that of HK-SLEs group. With time goes on, more honokiol bound by the red blood cell and the plasma protein in HK-SOL group was quickly released, while the released honokiol in HK-SLEs was less. It will make the difference between the honokiol concentration of HK-SOL and that of HK-SLEs in plasma and tissues narrow or disappeared.

In tumor tissue, honokiol concentration declined more slowly when compared to normal tissues. The mean tumor concentration–time curves of honokiol in tumor-burdened mice after IV administration of HK-SOL and HK-SLEs at a single dose of 31.5 mg/kg body weight are shown in Fig. 4. The honokiol concentration for HK-SLEs was higher than that of HK-SOL at the same time points. The exception was at the 2 min and 5 min time points. The $AUC_{0-\infty}$ of HK-SLEs (5595.98 μg/g·min) was approximately 4.2 times that of HK-SOL (1335.74 μg/g·min). These results show that SLE may be a better drug carrier than SOL to target honokiol to solid tumors.

2.7. In vivo antitumor efficacy

The *in vivo* antitumor activity of HK-SLEs was investigated and compared with those of HK-SOL using S180 bearing mice as the model animals. Fig. 5 shows that the tumor volume of blank-SLEs group was similar to the saline group, which indicated that the vehicle control (without any drug) did not show any effect on tumor weight and progression. Both tumor volumes of HK-SLEs group and HK-SOL group were significantly smaller than those of the saline group and blank-SLEs group. This result indicates that HK-SLEs and HK-SOL could effectively inhibit tumor growth.

The tumor growth inhibition rates (TGI) are listed in Table 3. TGI of HK-SLEs and HK-SOL were higher than 40%, which clearly demonstrated that both HK-SLEs and HK-SOL exhibited marked antitumor activity against S180. At the same dosage, the HK-SLEs group showed more significant TGI than those HK-SOL group did ($P < 0.05$). This result suggested that HK-SLEs exhibited increased antitumor activitity against S180, compared with HK-SOL.

There is no significant difference between the tumor volume of HK-SOL group and that of HK-SLEs group. This may be caused by the irregular shape of the tumors between different mouse groups. The volume was calculated by the formula of $V = 1/2 \times L \times W^2$, where length (L) is the longest diameter and width (W) is the shortest diameter of the tumor. Some tumors in HK-SOL group were higher than those of the HK-SLEs group, although they have a similar value of $1/2 \times L \times W^2$. It could cause a large deviation between the calculated volume and the real one. Tumor weight measured was more accurate than the tumor volume calculated. So we think that HK-SLEs exhibit higher tumor inhibition rate than HK-SOL.

It has been shown that parenteral emulsions with particle sizes < 400 nm localize more in tumor cells (Mizushima and Hoshi 1993). Colloidal systems such as emulsions, liposomes and polymeric nanoparticles could increase the drug accumulation in solid tumors, compared to low molecular compounds through the Enhanced Permeability and Retention (EPR) effect (Gabizon 1992).

In conclusion, the novel HK-SLEs, which have advantages in safety, production and long-term stability, could not only improve pharmacokinetics behavior of honokiol, compared with HK-SOL, but also seems to have better targeting properties for lung, brain and tumor tissues. HK-SLEs also demonstrated increased therapeutic activity compared with HK-SOL in S180 tumor-burdened mice, suggesting that it might be a potential and valuable delivery system for clinical applications.

3. Experimental

3.1. Materials

Honokiol, with a purity of 98.31%, was purchased from Rongsheng Bio-Technology Co., Ltd. (Xi'an, China). Soybean lecithin (SPC) S75 was obtained from Lipoid (Ludwigshafen, Germany). Purified soybean oil for parenteral use was purchased from Jin hai tang Pharmaceutical Co. (Jiangxi, China). Synperonic F68 was obtained from BASF Co. (Germany). Glycerol and dehydrated alcohol (analytical grade) were purchased from Shuangshuang Chemicals (Yantai, China). Methanol (chromatographic quality) was purchased from Concord Co. (Tianjin, China). Water were purified using a Millipore Ultrapure Water System. All other chemicals and reagents in this study were of analytical or chromatographic grade.

3.2. Preparation of HK-SLEs

HK-SLEs containing honokiol, soybean oil, soybean lecithin, synperonic F68 and glycerol were successfully prepared for this study. Briefly, soybean lecithin, synperonic F68 and glycerol were dissolved in water as the water phase of the emulsion. Honokiol was dissolved in the soybean oil as the oil phase of the emulsion. Both phases were heated separately to 65 °C, and the coherent phase was slowly injected into the oil phase. The mixture was emulsified by a high-speed stirrer (IKA T25 basic) at 21,500 rpm for 8 min, after which a coarse emulsion was obtained. Afterwards, a fine emulsion was prepared by passing the coarse emulsion through a microfluidics processor (M-110 L, MFIC, USA) (Pinnamaneni et al. 2003). Microfluidics conditions

Table 3: Tumor growth inhibition rate of the HK-SLEs groups and control group (n = 10)

Groups	Dose (mg/kg)	Mouse number (start/end)	Average tumor weight (g)	TIR(%)
Saline solution	–	10/10	1.06 ± 0.15	–
blank-SLEs	–	10/10	0.89 ± 0.16	6.96 ± 11.98
HK-SOL	10	10/10	0.51 ± 0.14	$51.74 \pm 12.64^{\#}$
HK-SLEs	10	10/10	0.33 ± 0.13	$68.88 \pm 7.65^{*\#}$

* $P < 0.05$ vs. HK-SOL group
$^{\#}$ $P < 0.05$ vs. Saline solution group

were typically 13 kpsi for 5 cycles followed by 18 kpsi for 10 cycles at 25 °C. The pH was adjusted to 7–8 with 0.1 N sodium hydroxide solution. Finally, the emulsion was packed in 5 mL sterile glass vials under nitrogen. The vials were sealed and the emulsion was sterilized by autoclaving (autoclaving sterilization cabinet, YXQ-LS-305, stainless steel sterilization apparatus, Shang hai) at 121 °C for 15 min.

3.3. Preparation of HK-SOL

Ten milliliters of honokiol solution was prepared by dissolving 31.5 mg honokiol in a water solution, composed of 8% dehydrated alcohol, 1.25% synperonic F68 and 2.5% glycerol. In detail, honokiol was dissolved in dehydrated alcohol. Then, synperonic F68 and glycerol were added to the solution. At last, ultrapure water was added to final volume of ten milliliters. The preparations were made fresh before drug administration.

3.4. Characterization of HK-SLEs

3.4.1. Particle size and zeta potential measurement

Particle size and zeta potential measurements were performed using a Malvern Zetasizer (Nano-ZS90, Malvern Instruments, UK) at 25 °C. HK-SLEs was diluted with 19 times the volume of water and was then added into the size and potential folded capillary cell. Size measurements were performed first, followed by a measurement of the zeta potential. All data presented in the results are the mean of 3 test runs.

3.4.2. PH and osmolality measurements

The pH of HK-SLEs samples was measured using a pH meter (model 868, Aolilong, China). Measurement of the emulsion osmolality was based on the freezing-point method as described in the user's manual (Advanced Instruments). Briefly, after calibration of the osmometer (STY-1A, Tianjin, China) with reference standards (100 and 3000 mOsm/kg, Advanced Instruments), the osmolality was recorded using 0.1 ml of the sample.

3.4.3. Drug loading %

The drug loading % (DL %) of honokiol refers to the percentage of honokiol found in HK-SLEs with respect to the total amount of honokiol, lipid and surfactant through the SLEs preparation process. DL % was represented as a percentage, according to the following formula:

$$\mathrm{DL}\% = \frac{\text{Amount of honokiol found in SLEs}}{\text{Total initial amount of honokiol} + \text{lipid} + \text{surfactant used in SLEs}} \times 100\%$$

3.4.4. Entrapment efficiency

The entrapment efficiency (EE) of the HK-SLEs was determined using an ultrafiltration method utilizing Millipore filter (molecular weight cut-off 10 000 Da, USA) (Yue et al. 2009). The sample was placed in the inner chamber, and the unit was centrifuged at 4000 rpm for 30 min. The emulsion, along with the encapsulated drug, remained in the inner chamber, and the aqueous phase moved into the sample recovery chamber through the filter. The concentration of honokiol in the HK-SLEs and the free drug in the aqueous solution (the non-incorporated drug) were assayed by HPLC after dilution with methanol. EE could be calculated using the following formula:

$$\mathrm{EE} = \frac{\text{Amount of non-incorporated honokiol found in SLEs}}{\text{Total amount of honokiol founded in the SLEs}} \times 100\%$$

3.4.5. Long-term stability investigation

The stability of the HK-SLEs was monitored over 12 months at 4 ± 2 °C. Their physical and chemical stability were evaluated by physical appearance, particle size, pH value, osmolality, DL % and EE.

3.5. HPLC

The concentrations of honokiol in the preparations and biological samples were determined using an HPLC instrument (Angilent 1100, USA). A solvent delivery system, equipped with a column heater, a plus autosampler and detection, was used with the UV detector set at 294 nm. Chromatographic separations were conducted on a reversed-phase column (Hypersil BDS C18 column, 150 × 4.6 mm, 5 μm) at 25 °C. Samples were eluted using methanol and a 0.05% phosphoric acid solution (73:27, v/v) at a flow rate of 1.0 ml/min. The injection volume was 20 μl. Agilent Chem Station software was used for data acquisition and analysis.

3.6. Animals

For the formation of *in vivo* tumors, S180 cell line ($1 \times 10^6 - 1 \times 10^7$) were injected into the flank of KM mice with an average body weight of 20 g (20 ± 2 g). Mice and cell were obtained from the Laboratory Animal Center of Zhengzhou University (Zhengzhou, China). This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocols were approved by our Institutional Animal Care and Use Committee.
When tumors became visible at approximately 1 week after inoculation, these mice were given the drug preparations. The mice were pathogen free and were kept in environmentally controlled quarters for at least 1 week before use. The mice had free access to a standard diet and water. Food was withdrawn 18 h prior to the experiment. No weight loss or other toxicities were observed in honokiol treated or control mice.

3.7. In vivo pharmacokinetic and biodistribution studies

Tumor-burdened KM mice (n = 108) were randomly divided into 18 groups, with 6 mice in each group. HK-SOL and HK-SLEs diluted with a 5% glucose solution (V:V = 1:3), were intravenously administrated to the tumor-burdened mice as a single dose of 31.5 mg/kg via the dorsal tail vein. HK-SOL was given to the first nine groups (HK-SOL groups), while HK-SLEs was given to the remaining nine groups (HK-SLEs groups). Blood samples were taken from the retro-orbital plexus of the mice with heparinized tubes at predetermined times (2, 5, 10, 15, 20, 30, 45, 60 and 90 min) after injection. After immediate centrifugation at 5000 rpm for 5 min at 4 °C, plasma samples were obtained and stored at −20 °C until they were analyzed. The mice were immediately sacrificed after collection. Samples of tissues (heart, liver, spleen, lung, kidney, brain, and tumor) were excised and stored at −20 °C until they were analyzed.
Accurately weighed tissue specimens were placed in a homogenizing tube with a double volume of water and were homogenized. Each tissue homogenate (0.2 mL) or the plasma sample (0.2 mL) was accurately transferred to a clean 1.5 mL test tube and 0.4 mL acetonitrile was added. After vortex mixing for 1 min, the mixture was centrifuged at 10 000 rpm for 10 min. Supernatant (20 μl) was injected into the HPLC for analysis.

3.8. In vivo antitumor efficacy

In vivo antitumor efficacy of HK-SLEs was evaluated in KM mice, bearing S180. S180 cell line was subcutaneously injected into the mice at the right axillary region, and the tumor was allowed to grow. When the tumor volume reached about 50 mm^3, the mice were randomly assigned to 4 groups, based on the tumor size, with 10 mice in each group (day 0). The first two groups were given HK-SLEs and HK-SOL at dose of 10 mg/kg via the tail vein injection once a day for 7 days, while the remaining two groups were given honokiol free lipid emulsion (blank-SLEs) and saline solution as a negative control, respectively.
The tumor volumes were measured once a day using a Vernier's caliper across its two perpendicular diameters. The volume was calculated by the formula of $V = 1/2 \times L \times W^2$, where length (L) is the longest diameter and width (W) is the shortest diameter. Each mouse was weighed at the time of treatment to make sure the dosage used according to the mg/kg amount reported. After seven days, all the mice were sacrificed by cervical dis-

location. The tumors were collected and weighed to calculate TGI using the formula: TGI% = [(Wc-Wt) × 100%]/Wc. (Wc and Wt, the final tumor weight of the negative control group and the treated groups, respectively (Zhang et al. 2009)).

3.9. Statistical analysis

The pharmacokinetic and tissue distribution results were analyzed for statistical significance using the practical pharmacokinetic program version 97 (3P97), edited by the Committee of the Mathematic Pharmacology, the Chinese Society of Pharmacology. The compartment model was established using the survival square sum (SUM), the Akaike's information criterion (AIC) and the goodness of fit and the fitted degree (r^2). Significant differences between the two groups were evaluated using Student's t-test for independent samples and expressed as a one-way P-value. The statistical analyses were performed using the statistical package for social science (SPSS, version 12.0). In all analyses, a P-value of < 0.01 or 0.05 was considered statistically significant. All descriptive parameters were expressed as the mean ± SD.

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