# *In Vitro* and *in Vivo* Antitumor Efficacy of Berberine-solid Lipid Nanoparticles Against H22 Tumor

## Xiang-ping Meng[1,a], Yi-fei Wang[2,b], Zhi-ping Wang[3,c*]

[1]Medical Technology and Engineering College, Henan University of Science and Technology, Luoyang 471003, China

[2]Institute of biological medicine, jinan university, guangzhou 510632, China

[3]School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006, China

[a]mxiangping@163.com; [b]twangyf@jnu.edu.c; [c]wzping_jshb@126.com

*Corresponding author

**Keywords:** solid lipid nanoparticles, berberine, antitumor efficacy, H22 tumor

**Abstract** Hepatocarcinoma, a malignant cancer, threaten human life badly. It is a current issue to seek the effective natural remedy from plant to treat cancer due to the resistance of the advanced hepatocarcinoma to chemotherapy. Berberine (Ber), an isoquinoline derivative alkaloid, has a wide range of pharmacological properties and is considered to have anti-hepatocarcinoma effects. However its low oral bioavailability restricts its wide application. In this report, Ber loaded solid lipid nanoparticles (Ber-SLN) was prepared by hot melting and then high pressure homogenization technique. Both *in vitro* and *in vivo* anti-hepatocarcinoma effects of Ber-SLN relative to efficacy of bulk Ber were evaluated. The particle size and zeta potential of Ber-SLN were $154.2 \pm 0.8$ nm and $-18.63 \pm 0.99$ mV, respectively. MTT assay showed that Ber-SLN effectively inhibited the proliferation of H22 cells, and the corresponding $IC_{50}$ values were 11.6 µg/ml (18.3 µg/ml of bulk Ber). *In vivo* studies also showed higher antitumor efficacy, and inhibition rates was 65.1% (41.4 % of bulk Ber) at 100mg/kg intragastric administration in H22 solid tumor bearing mice. These results suggest that the delivery of Ber-SLN is a promising approach for treating tumors.

## 1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and the third most common cause of cancer death[1]. In last decades, most patients diagnosed with hepatoma have low recovery rates, and conventional and modified therapies currently available are rarely beneficial [2]. Moreover, the limited responses of hepatoma, mainly hepatocellular carcinoma, to these agents are often due to its multidrug resistance (MDR) to them. Thus, developing new therapeutic agents for hepatocellular cancer becomes an urgent need to reduce the mortality caused by this disease[3]. At present, the demands for more effective and safer therapeutic agents for cancer have greatly increased. Natural products from medical plants are valued as an important source to find innovative agents for treatment of cancer. In addition, most extracts from medical plants have relatively low toxicity, which usually could rival the defect of chemotherapeutic drugs. Thus, traditional Chinese medical plants have been widely used in Asian countries as therapeutic agents for cancer due to their significant antitumor effects with lower toxicity[4].

Berberine (Ber), an isoquinoline derivative alkaloid isolated from several Chinese medicines, such as berberidis radix (Chinese name: Sankezhen), phellodendri chinensis cortex (Chinese name:

Huangbo), coptidis rhizoma (Chinese name: Huanglian) and mahoniae caulis (Chinese name: Gonglaomu), is commonly used as a quality control marker[5]. Ber has been reported to exhibit promising pharmacological activities including diarrhea, bellyache, and microbial infection[6]. In recent decades, much focus has been put on its significant anti-cancer activities[7-9], especially in anti-hepatoma activities[10-15]. Despite the promising biological effects, Ber is poorly absorbed, resulting in low bioavailability after oral administration. It has been reported that the oral bioavailability of Ber in rats was 0.68%[16]. The low absorption and bioavailability of Ber is still not fully understood. Recently, several studies have proposed some interpretations, since Ber is a lipophobic compound, it is restrained from passing through the membranes of intestinal cells. Secondly, Ber acts as a substrate of several ATP-binding cassette transporters, such as P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP). To circumvent these pitfalls, several strategies like absorption enhancer, self-microemulsion and solid lipid nanoparticles, have been used to increase its bioavailability[16-19].

Solid lipid nanoparticles (SLNs), i.e. nanoparticles composed of a mixture of a solid lipid which lipid matrix is solid at room and body temperature, are novel generation of solid lipid-based colloidal carriers, with improved stability and drug encapsulation ability and bioavailability. SLNs have all pre-requisites to enter the market faster than other colloidal carriers-among the reasons it is the use of starting materials that are biodegradable and already in use in pharmaceutical and cosmetic products, many of them of GRAS status (generally regarded as safe). Several cosmetic products based on SLNs are already marketed. Secondly, providing an advantage over liposomes or polymeric nanoparticles, preparation methods feasible at industrial scale are available, and these methods avoid the use of organic solvents[20-22]. Furthermore, *in vitro* tolerability of SLNs appears to be much higher than that of polymeric nanoparticles. Toxicology of nanomaterials is becoming an important issue nowadays, especially with regard to nanomaterials present in environment and nanomaterials intended for medical use. The biggest concern about nanomaterial safety is focused on nanomaterials with at least one dimension smaller than 100 nm. Although SLN typically do not reach this size (z-average data are most often reported over 100 nm), it was suggested SLN be called "submicron particles" rather than nanoparticles, facilitated cellular uptake of lipid nanoparticles themselves or their drug payload have been reported repeatedly. Indeed during the first 10 years of development very encouraging results were reported. However, for approval in use in medicinal products, more evidence will be required. Research reports reflect this challenge, even despite expected low toxicity or no toxicity at all. Up to date, considerable amount of data on SLNs behaviour in cell culture is available[23]. A few techniques have been used to prepare drug loaded SLNs. The high-pressure homogenization (HPH) method with a high productivity and a lower level contamination which is favorable for implementation of industrial products has shown great superiority over other methods[20]. In this report, Ber loaded SLN (Ber-SLN) was prepared by hot melting and then HPH technique. Both *in vitro* and *in vivo* anti-hepatocarcinoma effects of Ber-SLN relative to efficacy of bulk Ber were evaluated.

## 2. Materials and Methods

### 2.1. Preparation of the Ber-SLN

HPH technique was applied to prepare Ber-SLN. Briefly, The glyceryl behenate ( Compritol 888 ATO, GATTEFOSSE SAS, France) of 2.0 % was heated to about 75 $^0$C (above melting point), and cremophor EL (Kolliphor EL, BASF, Germany）of 1.0 % was dissolved in distilled water and heated to the same temperature. Ber (chloride form, purchased from Aladdin industrial corporation, Shanghai, China) powder of 0.3 % was dispersed in melted glyceryl behenate solution using high

speed homogenization 5000 rpm for 15 min (IKA T18 basic ULTRA-TURRAX®, Germany). Then the pre-mix was passed through a Lab HPH (APV-2000, Germany), 10 cycles were performed at 500 bar, and 20 cycles at 1500 bar.

## 2.2. Characterization of the Ber-SLN

The particle size, polydispersity index, and Zeta potential measurements were performed on a Nano-ZS90 (Malvern Instruments Ltd., Malvern, UK) thermostated at 25 $^0$C. The sample was diluted 50 times with bi-distilled water before the measurements. All values were measured at an analysis angle of 90 $^0$C in a 10-mm diameter cell. Each value reported is the average of three measurements.

## 2.3. Cell viability assay

H22 cells were treated with different concentrations (0, 0.1, 1, 10, and 100 μg/ml) of Ber-SLN and Ber solution respectively. And then, the effect of Ber-SLN on the viability of cells was determined by the colorimetric MTT assay. The inhibition rate was expressed as following formula:

Inhibition rate (%) = [1− (absorbance of experimental group / absorbance of control group)]×100.

## 2.4. Determination of in vivo antitumor effect

Antitumor activity against a solid tumor mass was evaluated in Kunming mice. Ten days after receiving tergal s.c. inoculation of $1\times10^6$ H22 cells prepared as described in Section 2.1. The H22 cell suspension was inoculated to the right armpit of the mice subcutaneously for 0.2 ml per mouse on day 0. The tumor-bearing mice were divided into four groups (10 mice each group), including negative control group (normal saline), positive control group [cyclophosphamide (CTX) 60 mg/kg], and two groups for Ber-SLN and Ber administration with dosages of 100 mg/kg, respectively. After administered orally by gastric intubation once a day for continuous 21 days, the mice were sacrificed, and solid tumors were excised and weighed [24]. The antitumor activity was expressed as following formula:

Inhibition rate (%) = [1–(tumor weight of experimental group/tumor weight of control group)]×100.

## 2.5. Statistical analysis

Results were expressed as mean ± standard deviation (SD). Student's t-test was used to compare the mean differences between samples using the statistical software SPSS version 16.0 (SPSS, Chicago). In all cases $P < 0.05$ was considered statistically significant.

# 3. Results

## 3.1. Particle size analysis and Zeta potential

The mean particle size and polydispersity index (PDI) were measured immediately after the preparation of the SLN. The mean particle size with PDI $0.527 \pm 0.103$ was $154.3 \pm 4.1$ nm (Figure 1). The PDI is a measure of particles size distribution. The values less than 0.3 indicate a high degree of homogeneity in particle size and vice versa. The zeta potential of Ber-SLN was $-19.7 \pm 0.46$ (Figure 2).

## 3.2. Cytotoxicity of Ber-SLN

To determine whether Ber-SLN has growth-inhibitory effects, H22 cells were exposed to different concentrations of Ber-SLN for 72 h. The data showed that the growth of H22 cells were significantly inhibited by Ber-SLN, the $IC_{50}$ was 10.7 μg/ml (22.1 μg/ml of bulk Ber).

## 3. 3. Antitumor activity of Ber-SLN on solid tumor in vivo

H22 tumor-bearing mice were used to evaluate the antitumor activity of Ber-NLS *in vivo*. Due to the fast growth of tumor, the transplanted tumor model mice in the control group gradually exhibited a series of weak appearance, such as the lost of appetite, the reduced activity and the body weight with dim hairs. At later stages, ulceration in some tumors was observed as a result of the

tumor perforation out of the skin, and six mice died at day 12 and day 18. After treated orally with Ber-NLS, the growth of H22 tumors in the model mice was significantly suppressed compared with control group (p<0.05). Inhibiting ratios of H22 tumor cells were 66.8 % (41.4 % of Ber solution) at concentrations of 100 mg/kg intragastric administration (Table 1), which indicated Ber-SLN possessed excellent antitumor activity. Furthermore, the body weights of Ber-SLN-treated group were increased significantly when compared with the negative control group during the 21 day-experimental period. The frequently used chemotherapy drug CTX, exhibited a high antitumor activity (79.1 %). However, CTX considerably reduced the body weight of tumor-bearing mice, indicating the strong side effect to the body.
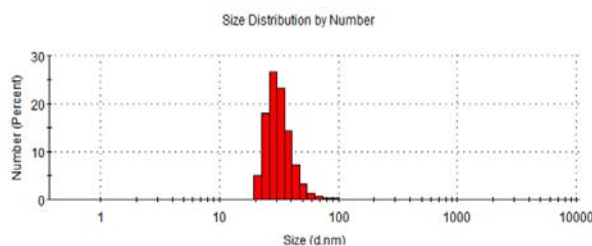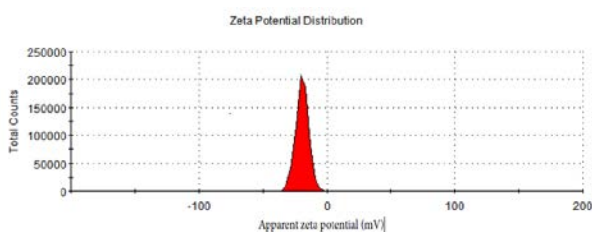


Figure 1: The particles size of Ber-SLN



Figure 2: The zeta potential of Ber-SLN

Table 1:    *In vivo* antitumor activities of Ber-SLN on H22-bearing mice.

| Sample | Dose | Mice | | Body weight (g) | | Tumor weight | Inhibition | *P* value |
|--------|------|------|------|------|------|------|------|------|
| | （mg/kg） | Before | After | Before | After | x±SD | rate（%） | |
| Control | | 10 | 9 | 31.7±5.2 | 35.5±8.9 | 2.92±1.07 | | |
| CTX | 60 | 10 | 6 | 32.6±5.9 | 21.9±4.8 | 0.61±0.32 | 79.1 | 0.004 |
| Ber-SLN | 100 | 10 | 10 | 31.3±4.2 | 34.7±4.4 | 0.97±0.91 | 66.8 | 0.041 |
| Ber | 100 | 10 | 9 | 29.9±2.8 | 34.9±3.7 | 1.71±0.49 | 41.4 | 0.053 |

## 4. Conclusion and Discussion

In present study, we demonstrated that Ber-SLN effectively inhibited the growth of H22 *in vitro*, and possessed excellent antitumor activity *in vivo* models. Moreover, Ber-SLN produced less adverse effects compared with CTX, greatly prolonged the life span of tumor-bearing mice. Therefore, Ber-SLN may be explored as a novel potential antitumor agent for the functional food and pharmaceutical purpose. This study also provides evidences to support the therapeutic effects of compound for treatment of cancer in China. Despite of the promising results from our current investigation, there are still a plethora of practical issues which may be difficult to reconcile for the ultimate use of Ber-SLN for the novel target-therapy in cancer management.

Until recently, there is no multicenter, well controlled, long-term clinical trial to evaluate the efficacy of Ber in the treatment of cancer, due to its low bioavailability. There were a few reports focusing on the development of new dosage forms of Ber to increase its bioavailability, such as

using the intestinal absorption enhancer, self-microemulsion and solid lipid nanoparticles[16-19]. In this investigation, we first studied the effect of Ber-SLN on H22 tumor to assess if SLN could enhance Ber anti-cancer effects. The present study showed that Ber reduced tumor weight in H22 tumor-bearing mice, which were consistent with other studies[25]. The therapeutic effects of Ber-SLN were obviously improved when prepared with SLN. It showed that the ability of SLN to enhance the efficacy of Ber was most probably by improving its bioavailability and increasing its blood level.

More than 95 % of the Ber-SLN particles showed a small particle size (154.3 nm) (Figure 1). SLN were prepared using a HPH method which results in small particles. Small particle size less than 200 nm is desired for being usually invisible to the reticulo-endothelial system and for circulating over a prolonged period of time *in vivo*. Moreover, a zeta potential of $\geq \pm 25$ mV is recommended for achieving stable dispersions. This is attributed to the existence of repulsive forces between the particles, preventing them from contacting each other and agglomerating. Neutral particles obtained presently can thus be considered intrinsically stable as no interparticulate molecular interactions (both attractive and repulsive) are expected from these particles. In contrast to the neutral and negatively charged particles, the positively charged nanoparticles are taken up more rapidly by the cell membranes[20].

## Acknowledgment

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