

Full Length Research Paper

Synthesis and characterization of folic acid-conjugated human serum albumin (HSA) nanoparticles for isoalantolactone cellular uptake in HeLa

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Folic acid was previously demonstrated to mediate intracellular nanoparticle uptake. Isoalantolactone (IAL) was demonstrated to possess a variety of pharmacological activities *in vivo* and *in vitro*, including cytotoxic, diuretic and immunosuppressive activity. Here, we developed folic acid-conjugated human serum albumin nanoparticles for IAL (FHNS-IAL) encapsulation to improve the targeted activity, water solubility and to reduce untoward effects. Human serum albumin nanoparticles for IAL (HNS-IAL) were prepared by desolvation and stabilized by chemical cross-linking with glutaraldehyde. Folic acid was covalently coupled to amino groups on the surface of HNS-IAL by carbodiimide reaction. The average diameter of spherical FHNS-IAL was 118.7 ± 11.6 nm and the IAL encapsulation efficiency was $36.1 \pm 3.3\%$. The cytotoxic activity *in vitro* and the cellular uptake of FHNS-IAL were examined by HeLa cells. The results suggested that covalent conjugation of folic acid to HNS-IAL increased IAL uptake into cancer cells. Moreover, the cytotoxic effects of IAL as monotherapy on HeLa cells were smaller than those encapsulated with FHNS. The experiments *in vivo* also confirmed a superior anti-tumor effect of FHNS-IAL by human tumor xenograft animals. These data suggested that covalent linkage of folic acid could specifically increase the cellular uptake of FHNS-IAL by cancer cell. The FHNS-IAL exhibited good property to improve the uptake of HeLa cells and could become a potential targeted drug delivery system for the future cancer chemotherapy. Therefore, folic acid-conjugated human serum albumin (HAS) nanoparticles for IAL encapsulation would be highly beneficial for biomedical and pharmaceutical applications.

Key words: Isoalantolactone, nanoparticles, serum albumin, folic acid, drug delivery system.

INTRODUCTION

Traditional Chinese medicines have been used to treat human diseases in China for centuries. The dried roots of *Inula helenium* L. and *Inula racemosa* Hook f. are used commonly as folk medicine under the name of 'Tumuxiang'. Several experimental studies demonstrated that the major active component in *I. helenium* L. and *I. racemosa* Hook f. was isoalantolactone (IAL) (Khvorost

and Komissarenko, 1976; Wang et al., 2010; Zhang et al., 2010). Pharmacological investigations showed IAL possessed the effects of anticancer (Cho et al., 2004; Zhang et al., 2005; Pal et al., 2010; Konishi et al., 2002), significant anti-inflammatory and hepatoprotective activity similar to that of silymarin (Wang et al., 2000), anti-dematophytic and antifungal activity (Cantrell et al.,

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1999; Liu et al., 2001). However, the clinical application was restricted by its insolubility and toxicity. To maintain natural drug's high activity against many kinds of cancers and overcome the problems associated with its formulation, some new formulations, including liposomes, micelles, and polymeric nanoparticles were created to develop its local drug delivery methods (Yoshizawa et al., 2011; Watanabe et al., 2008; Saad et al., 2008; Patil et al., 2009). Among these formulations, nanoparticles (NPs) were developed to enhance the therapeutic activity of anticancer agents (Wang et al., 2010, 2010).

To increase the therapeutic effect on tumors, various targeting ligands were investigated as tumor targeted drug carriers. Folic acid was a low-weight vitamin that could selectively bind to folate receptors, which were frequently overexpressed on the surfaces of many human cancer cell types but highly restricted in most normal tissues (Toffoli et al., 1997). Therefore, liposomes functionalized with folic acid could specifically promote their cancer cellular uptake through folate receptor mediated endocytosis (RME), and the nonspecific binding to extracellularplate components was observed as well (Gabizon et al., 1999; Park et al., 2005).

Human serum albumin (HSA) is a natural material, and therefore potentially used as a biocompatible and biodegradable carrier with anticancer drugs (Chen et al., 2010). The anticancer agent, paclitaxel, was conjugated to HSA with further modifications by folic acid via N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) (Dosio et al., 2009). Folic acid could be covalently coupled to amino groups on the surface of HSA NPs by carbodiimide reaction, specifically increasing NPs uptake into cancer cells but not into normal cells (Ulbrich et al., 2011).

In the present study, folic acid-conjugated human serum albumin nanoparticles via glutaraldehyde were prepared for IAL (FHNs-IAL) encapsulation. The preparation process related characteristics were investigated (Nasri et al., 2012), and these would be used to lay the foundation for further study, including determining the mechanism of the nanoparticles uptake by tumor cells and their cytotoxicity (Alam et al., 2012; Asgarpanah et al., 2012).

MATERIALS AND METHODS

Reagents and chemicals

The IAL was isolated from the dried roots of *I. helenium* (Figure 1). Its structure was characterized by chemical and spectroscopic methods (proton nuclear magnetic resonance [^1H NMR], carbon nuclear magnetic resonance (^{13}C NMR) and mass spectrometry (MS)) (Konishi et al., 2002). Analysis showed that its purity was above 99% [high-performance liquid chromatography (HPLC)]. HSA, glutaraldehyde 8% solution, EDC, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and folic acid were purchased from (Sigma-Aldrich, USA). HPLC-grade methanol was purchased from Baker Company (Baker Inc., USA). Ultrapure water was prepared by a Millipore-Q SAS 67120 MOLSHEIM (France).

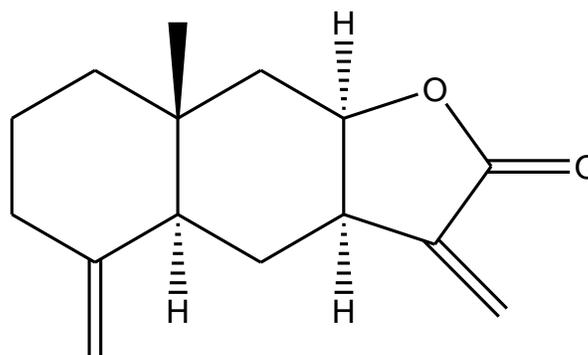


Figure 1. Molecular structure of isoalantolactone.

Perparation of HeLa cells

HeLa (Human cervical carcinoma) cells were obtained as a gift from the Fourth Military Medical University, Xi'an, China. All the cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium without folate and supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air until they reached confluence.

Perparation of experimental animals

Female BALB/c mice (body weight = 18 to 22 g) were purchased from the Experimental Animal Center of the Fourth Military Medical University, which were used in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines of Animal Care. The study was approved by the ethical committee of the Fourth Military Medical University. These mice were maintained in an aseptic environment. Tumor implantation was carried out by injecting 0.1 ml of the HeLa cell suspension (1.0×10^6 cells) into the right limb armpits of each mouse. Subcutaneous tumor growth was monitored daily until 10 to 12 days after implantation. Afterwards, tumor-bearing mice were randomly assigned to groups used for *in vivo* anti-cancer studies.

Preparation and characterization of FHNs-IAL

The folic acid-conjugated human serum albumin nanoparticles for IAL (FHNs-IAL) encapsulation were prepared by an established desolvation process as reported previously (Ulbrich et al., 2009) and modified as follows. Briefly, 200 mg HSA in 2.0 ml 10 mM NaCl solution was adjusted to pH 8.4 and added into 8.0 ml ethanol under constant stirring at room temperature. Subsequently, 250 μl of an 8% glutaraldehyde solution was added to achieve particle cross-linking. 1 ml of folic acid solution (20 mg/ml) in 0.1 M sodium hydroxide was incubated with 200 μl EDC under constant shaking in the dark for 15 min at 20°C. Subsequently, 1 ml HSA nanoparticle suspension (content 15 mg/ml) was added, and shaking continued for 1 h. Reaction was stopped by adding 100 μl hydroxylamine (500 mg/ml). The folate-conjugated nanoparticles suspensions were put in pretreated dialysis bags to remove any conjugate unassociated with NPs and dispersed the pellet to the original volume in water again. After the external pH was adjusted to pH 9.0, FHNs were incubated in 150 ml ethanol with IAL (drug: NPs = 1:10) and stirred

for 30 min. 50 μ l glutaraldehyde was added slowly with continuous stirring and cured for 12 h. The reaction solution was centrifuged at 7,000 rpm, and the precipitate was washed three times with phosphate buffered saline (PBS) (pH 7.4), and freeze-dried under vacuum. Finally, reserve samples were maintained as described.

Final IAL and folic acid concentration after loading was determined by high-performance liquid chromatography (HPLC, Agilent LC1200) at 230 and 360 nm with mobile phase (V/V, PBS/methanol = 9:1). The elution rate was 1.0 ml/min. The retention time of IAL and folic acid was 1.5 and 3.6 min under these conditions, respectively. The drug encapsulation efficiency (EE) was calculated according to the following equation:

$$EE\% = (IAL_{FHN} \div IAL_{Theoretic}) \times 100$$

Cytotoxicity assay

The cytotoxicity assay was performed by the MTT method modified from the one previously described (Dorn et al., 2006). Cells (2×10^3 /well) that were seed in 96-well plates were incubated for 12 h. The freeze-dried FHNS-IAL was dissolved into PBS buffer at the concentration of 20, 40 and 60% (w/v). The culture medium was then replaced with an equal volume of fresh medium containing different drug formulations of IAL and the different concentrations of FHNS-IAL. All of the samples were ultra violet (UV) sterilized. After 24 h or 48 h incubation, 20 μ l MTT solution was added to each well of 96-well plates, which were incubated for additional 4 h at 37°C. MTT solution in the medium was aspirated off and 150 μ l of dimethyl sulphoxide (DMSO) were added to each well and shaken softly for 10 min to solubilize the formazan crystals formed in viable cells. The optical density (OD) was read at a wavelength of 550 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co, Sunnyvale, CA). Data were averaged from the six different wells per condition and plotted as mean \pm standard error.

Cellular uptake assay

In vitro cellular uptake assay was performed following the general protocol previously reported (Wang et al., 2010). To visualize cellular uptake of FHNS-IAL, HeLa cells were reseeded in the Lab-Tek chambered slide (Miles Laboratories, U.S.A.) and incubated for 24h at 37°C. The culture media were then replaced with 1 ml of medium with Oregon green labeled FHNS-IAL for 3 h at 37°C. Cells were washed three times with PBS after treatment and then fixed by 4% (w/v) paraformaldehyde solution. The fluorescent images were viewed by fluorescent microscope (Olympus BX51, Japan).

Apoptosis assay

Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC, Sigma-Aldrich, USA) as previously described (Dorn et al., 2006). Cells (1×10^5 /well) were plated in 24-well plates and incubated with different drug formulations of IAL and various FHNS-IAL (20, 40, and 60% FHNS) at the concentration of 10 μ g/ml for 24 h. Thereafter, cells were collected and washed twice with 400 μ l cold PBS and resuspended in 100 μ l binding buffer with 5 μ l of Annexin V-FITC and 1 μ l propidium iodide (PI). After 15 min of incubation at room temperature in the dark, the cells were diluted with 400 μ l of binding

buffer and immediately analyzed by Epics Elite flow cytometer, and the data were analyzed using Expo32 software (Beckman Coulter).

In vivo anticancer activity

Forty tumor-bearing mice were divided into five groups randomly. After the tumor inoculation, IAL solution and various FHNS-IAL (dose of IAL = 10 mg/kg body weight) suspended in PBS were injected intravenously via the tail vein of animals at 5 days intervals. At predetermined time, the width and length of tumors were measured with a caliper and tumor volumes were then determined by the following equation:

$$V_{Tumor} = \frac{length \times Width^2}{2}$$

Therefore, the tumor control rate (TCR) was calculated according to the following equation:

$$TCR(\%) = \left(1 - \left(\frac{V_{ds} - V_{de}}{V_{cs} - V_{ce}} \right) \right) \times 100$$

Where V_{ds} is the tumor volume at the beginning of drug administration; V_{de} is the tumor volume at the end of drug administration; V_{cs} is the tumor volume of the control group at the beginning; V_{ce} is the tumor volume of the control group at the end.

Statistical analysis

Data are expressed as mean \pm standard deviation (SD). Statistical analyses were assessed using the Student's t-test for two groups and one-way ANOVA for multiple groups. Differences were considered to be significant at $P < 0.05$.

RESULTS

Characterization of FHNS-IAL

The physicochemical properties of IAL-nanoparticles prepared were assessed and the results were listed in Figure 2 and Table 1. The results of the FHNS-IAL prepared in the this work showed that particle sizes were around 100 nm and the zeta potential of surface charges for the FHNS-IAL were negative.

Cytotoxicity assay

IAL and various FHNS-IAL (20, 40, and 60% FHNS) were screened for *in vitro* cytotoxicity against HeLa cells for 24 and 48 h by applying MTT assay (Figure 3). The highest lethality of cancer cells occurred at the highest concentration of FHNS formulations after treatment and for the longest period of time. The orders of all HeLa cells

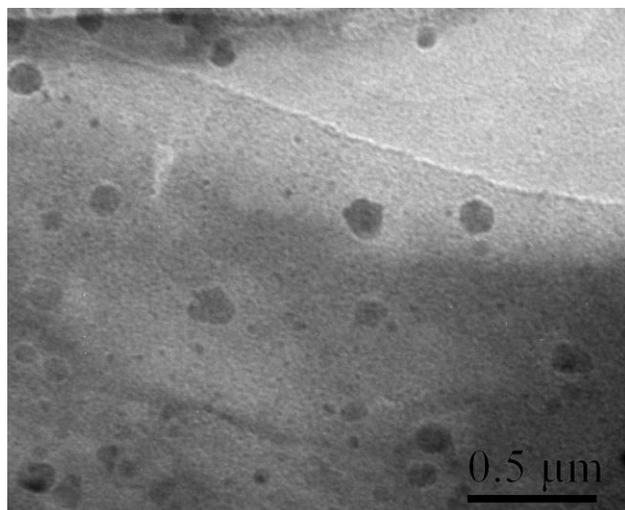


Figure 2. Scanning electron microscope (SEM) image of 40% FHNS.

viabilities examined to different drug-loaded NPs were 60% FHNS-IAL > 40% FHNS-IAL > 20% FHNS-IAL > 0% FHNS.

Cellular uptake assay

Cellular uptakes of FHNS-IAL by HeLa cells were visualized by a fluorescent microscope as shown in Figure 4. Cellular uptake extent of 60% FHNS-IAL was significantly higher than those of free IAL, 20% FHNS-IAL and 40% FHNS-IAL under the same condition. Furthermore, 60% FHNS-IAL could be uptaken fast by endocytosis process and induced apoptosis of cells.

Apoptosis assay

The results of IAL and FHNS-IAL induced apoptosis indicated that the proportion of annexin V/PI-stained cells, signifying the apoptotic cells, increased with the concentration of FHNS-IAL (Figure 5). In addition, the proportion of late apoptotic cells was extremely greater than that of early apoptotic cells (Table 2). The proportion of late apoptosis induced by 60% FHNS-IAL was 80.7% compared to 64.6% of IAL solution at concentration of 10 $\mu\text{g/ml}$.

In vivo anticancer activity

In vivo anti-tumor activity of FHNS-IAL was evaluated by female BALB/c mice. As shown in Figure 6, the growth rate of tumor were inhibited for all the treated groups after injection of various formulations compared with the PBS control group. The TCRs of IAL, 20, 40 and 60% FHNS-IAL

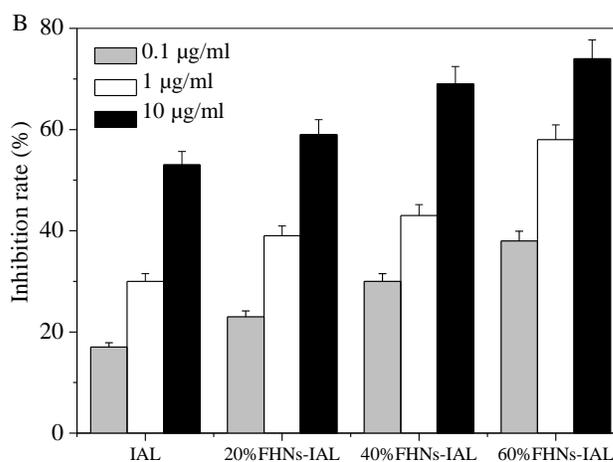
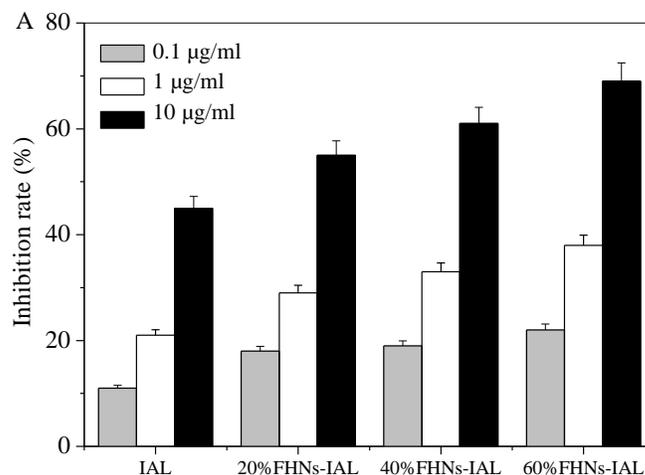


Figure 3. Cell inhibition rate of HeLa cells treated with IAL and various IAL-loaded FHNS (20, 40, and 60% FHNS). Three IAL concentrations (0.1, 1, and 10 $\mu\text{g/ml}$) were used here. (A) HeLa cells 24 h after treatment. (B) HeLa cells 48 h after treatment.

were 41.4, 47.8, 57 and 63.9%, respectively.

DISCUSSION

The results listed in Figure 2 and Table 1 indicated that the folate binding was 7.9% with the drug encapsulation efficiency (EE) of 36.1%. The EE of FHNS-IAL did not show any significant difference compared with that of HNS-IAL (35.5%). Therefore, the physicochemical properties of IAL formulations prepared in this work would be adequate. Moreover, the mean particle size of HNS-IAL increased from 112.5 to 118.7 nm by the coupling of folic acid alone (EDC activated HSA NP). The EE and the folate loading of FHNS-IAL were only reduced to 33.9 and 6.3%, respectively, when it was stored for 3 months

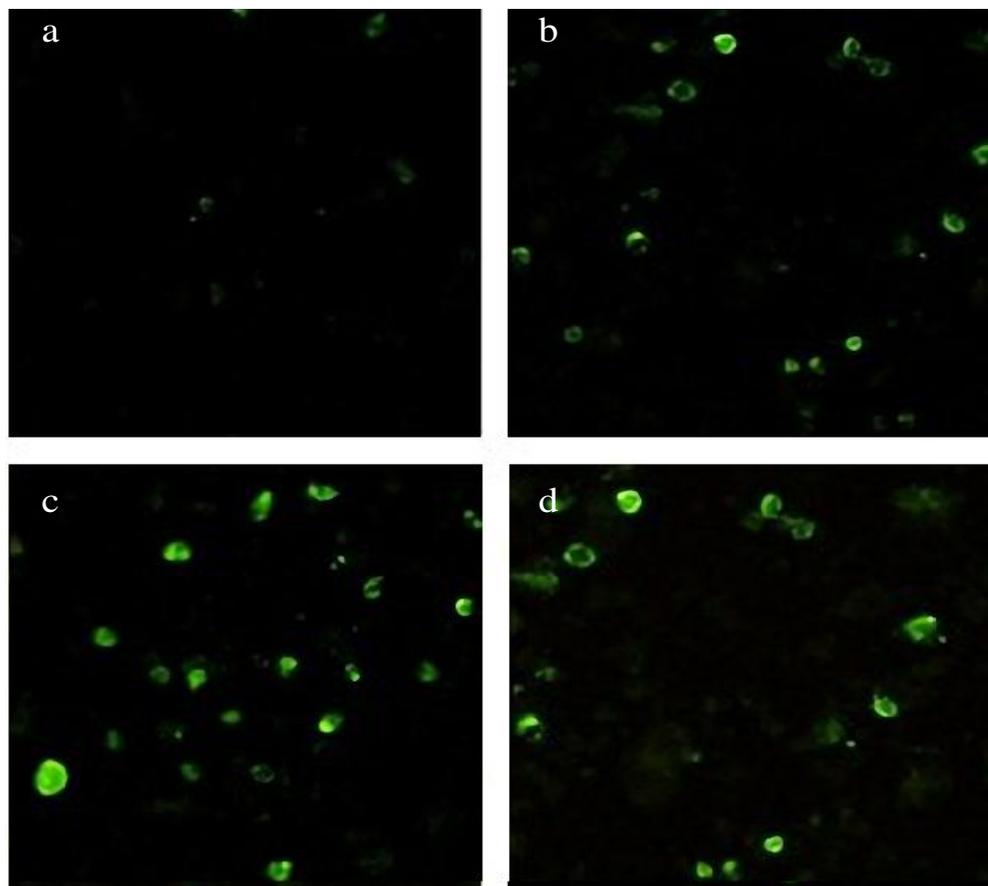


Figure 4. Fluorescent microscopic pictures of HeLa cells incubated with (a) IAL at concentrations of 10 µg/ml, (b) 20% FHNS-IAL, (c) 40% FHNS-IAL and (d) 60% FHNS-IAL for 3 h at 37°C.

at 4°C. Therefore, these data suggested FHNS-IAL were excellent stability.

The results of Figure 3 demonstrated that the cytotoxicity of FHNS-IAL to cancer cells was improved by the folic acid component, which has been reported to bind to folate receptors with high affinity, thus mediating in cellular uptake via receptor-mediated endocytosis. In addition, it was suggested that the cytotoxic effect of IAL or FHNS-IAL was related to the induction of apoptosis. The results of Figure 4 could be visualized that FHNS-IAL were primarily located on the surface of cell membrane due to their preferential binding to the folate receptors on the membrane. The fluorescent results directly indicated that FHNS-IAL were taken up by a folate-receptor-mediated endocytosis process. In contrast to free IAL, FHNS-IAL were mainly distributed in the cytoplasm for their higher solubility without exhibiting much accumulation in the nucleus after 3 h of incubation. However, most of IAL molecules transported inside the cells were likely to still be in an aggregated state in the cytoplasm region with little chance to be solubilized in the cytoplasmic fluid. It was suggested that IAL probably could induce apoptosis through a mitochondria-dependent pathway in HeLa cells (Zhang et

al., 2005).

The results of Figure 5 and Table 2 showed that the apoptosis of HeLa cell induced by FHNS-IAL was obviously dose-dependent, which also indicated that folate component improved the cytotoxicity of IAL. The results obtained in the *in vivo* antitumor studies clearly indicated that FHNS could serve as a novel formulation of IAL for targeting to the tumor site, enhancing cellular uptake, and achieving sustained release within cells. Compared with IAL solution, 60% FHNS-IAL increased the TCR over 20%. Hence, FHNS was one of the efficient anticancer drug carriers.

In this study, IAL-loaded FHNS was successfully synthesized and characterized as a new carrier for tumor-targeted drug delivery. The *in vitro* cytotoxicity assay of FHNS-IAL showed that NPs with folate components had higher cytotoxicity to cancer cells. It revealed that 60% FHNS-IAL had greater cellular uptake compared with IAL as monotherapy applied. *In vivo* experiment employing a human tumor xenograft animal also confirmed a superior anti-tumor effect of FHNS-IAL. Our present study clearly indicated that FHNS would serve as a potent IAL delivery vehicle for the future cancer chemotherapy.

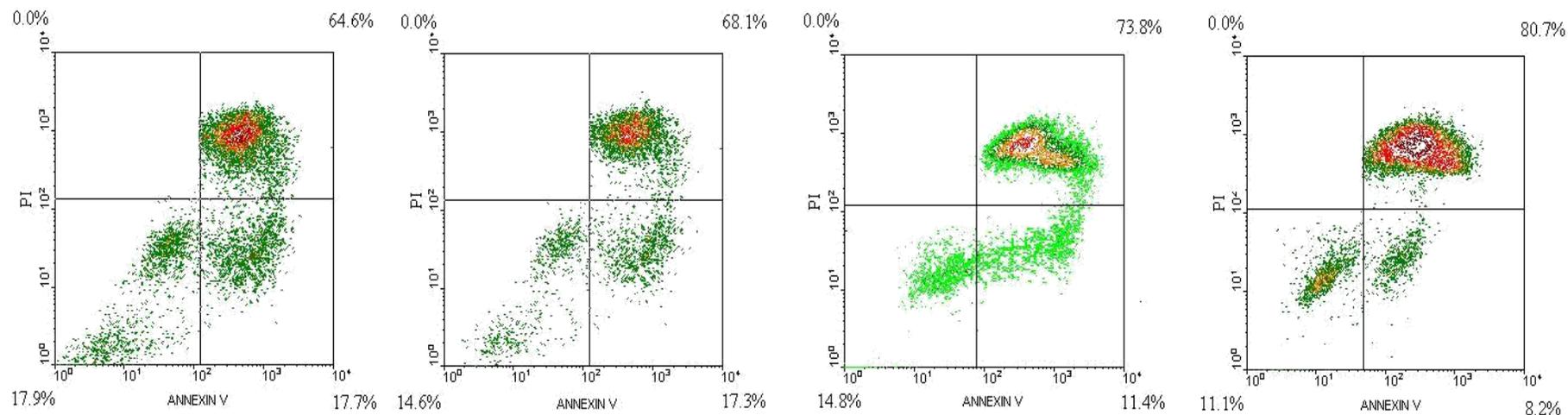


Figure 5. Detection of apoptosis of the HeLa cells induced by IAL at the concentration of 10 $\mu\text{g/ml}$ and various FHNs-IAL (20, 40, and 60% FHNs) for 24 h with annexin V/PI staining method. A: HeLa cells were treated by IAL as monotherapy. B1 area-mechanical damage Annexin V⁻/PI⁺, B2 area-late apoptosis Annexin V⁺/PI⁺, B3 area-normal cell Annexin V⁻/PI⁻, B4 area-early apoptosis Annexin V⁺/PI⁻. B-D: HeLa cells were treated by various IAL-loaded FHNs (20, 40, and 60% FHNs), respectively.

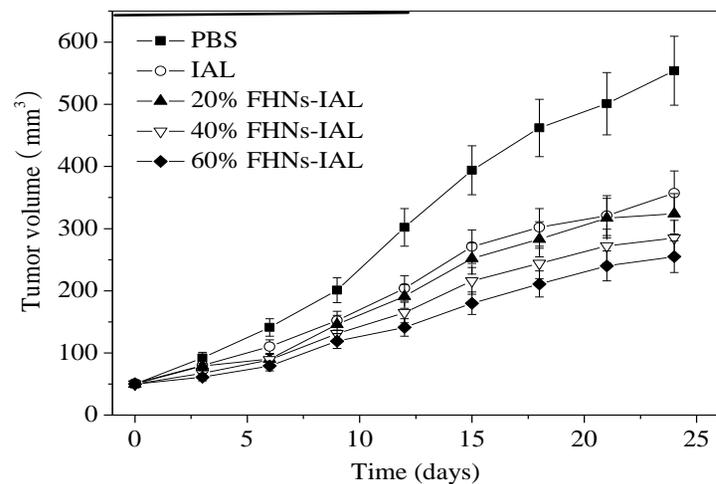


Figure 6. *In vivo* anti-tumor effects of IAL solution and various FHNs-IAL (20, 40, and 60%). BALB/c nude mice bearing HeLa tumors were intravenously injected with various formulations of IAL and with PBS as the control group.

Table 1. Physicochemical properties of FHNs-IAL. Results are expressed as the mean \pm SD (n = 3).

Formulation	Property	0 month	3 months
HNs-IAL	Particle size (nm)	112.5 \pm 14.3	134.8 \pm 24.5
	Zeta potential (mV)	-21.1 \pm 5.9	-18.4 \pm 7.2
	Folate loading (%)	—	—
	IAL EE (%)	35.5 \pm 6.9	31.2 \pm 5.1
FHNs-IAL	Particle size (nm)	118.7 \pm 11.6	129.1 \pm 19.6
	Zeta potential (mV)	-24.1 \pm 8.8	-20.1 \pm 4.4
	Folate loading (%)	7.9 \pm 0.8	6.3 \pm 0.3
	IAL EE (%)	36.1 \pm 3.3	33.9 \pm 3.9

Table 2. Apoptosis rate of the HeLa cells was detected by flow cytometer with annexin V/PI staining methods (P < 0.01).

Formulation	Annexin V/PI (%)		
	Early apoptosis	Late apoptosis	Total apoptosis
IAL	17.7 \pm 3.62	64.6 \pm 5.35	82.3 \pm 7.70
20% FHNs-IAL	17.3 \pm 4.28	68.1 \pm 6.36	85.4 \pm 8.11
40% FHNs-IAL	11.4 \pm 2.85	73.8 \pm 8.49	85.2 \pm 11.5
60% FHNs-IAL	8.2 \pm 2.60	80.7 \pm 10.49	88.9 \pm 8.6

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