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# Cellular uptake mechanism and clearance kinetics of fluorescence-labeled glycyrrhetinic acid and glycyrrhetinic acid–modified liposome in hepatocellular carcinoma cells

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ABSTRACT

Glycyrrhetinic acid (GA) is a natural pentacyclic triterpene derivative that exerts significant effects in the suppression of liver cancer. The receptors of GA on liver cells and hepatocellular carcinoma (HCC) cells have drawn broad attention. The effects of GA might depend on its transport into and out of cells. However, the question has not been previously addressed despite its obvious and fundamental importance. In this paper, GA and GA-modified liposome (GA-Lip) were labeled with fluorescein isothiocyanate (FITC) or coumarin 6 (Cou6) using chemical or pharmaceutical techniques. The transport courses of FITC-GA and GA-Cou6-Lip were studied in HepG2 cells in vitro. We found that the fluorescence labeled GA and GA-Lip uptake and clearance were time-dependent. FITC-GA uptake involved passive diffusion and active transport, and the receptors were in the cytomembrane proteins. GA-Cou6-Lip uptake was mediated by caveolae-dependent endocytosis. In addition, FITC-GA and GA-Cou6-Lip clearance of the HCC cells fitted exponential decay and second-order processes, respectively. These findings provide new insights into the anti–HCC actions of GA.

## 1. Introduction

Hepatocellular carcinoma (HCC) is a complex and heterogeneous tumor, which is the primary cancer of the liver with an occurrence rate of approximately up to 90% and a 5-year overall survival rate of less than 10% (Goyal et al., 2013). It is the fifth type of cancer worldwide and the third cause of cancer related deaths (Jemal et al., 2011). Apart from surgical resection and transplantation, chemotherapy is an important treatment strategy for cancer. Although various chemotherapeutic agents are used to cure HCC as single or combinational agents, the low specificity and systemic cytotoxicity are the two main problems that chemotherapeutic agents encountered. Because of the satisfactory efficacy, the application of natural compounds in the treatment of cancer is a new trend in modern clinical medicine.

Glycyrrhetinic acid (GA), a pentacyclic triterpene derivate as one of the main chemical component of *Glycyrrhiza uralensis* Fisch (licorice) can be generated from glycyrrhizin acid. It has same pharmacological effects to glycyrrhizic acid (Li et al., 2014) and has been widely used in the traditional Chinese medicine prescription.

GA also plays an important role in the treatment of liver cancer. On one side, GA exerts an active effect to inhibit HCC cell proliferation. It was found to arrest the cell cycle in the G1-phase and was accompanied by activation of caspase-8 and reduction of the anti-apoptotic proteins, Bcl-2 and Bcl-$_x$L of HepG2 cells (Satomi et al., 2005). Meanwhile, GA triggered a protective autophagy in HepG2 cells via the activation of the extracellular regulated protein kinase (ERK). The MEK inhibitors could reverse GA-triggered autophagy by decreased expression of LC3-II and formation of autophagosomes. (Tang et al., 2014). Additionally, GA was also found to decrease gap junction via reducing the expression level of connexin 32 and actin in junctionally proficient HepG2 cells, indicating that it induced the decrease of HCC metastases with anticancer activity (Cai et al., 2016).

On the other side, GA mediates nano-carriers to achieve targeted delivery to liver and HCC cells. The specific binding partners for GA on the cellular membrane of hepatocytes have been confirmed (Negishi et al., 1991). Protein kinase C α (PKCα) was proved to be the target of GA, which was expressed at higher level in HCC cells than that in the adjacent non-tumor liver cells (He et al., 2010). Therefore, we hypothesize that there are more GA receptors on HCC cells.

Liposomes have caught intensive attention during the past 40 years, which has led to the approval of several liposomal drugs for clinical application. Many therapeutic drugs were encapsulated in GA modified

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liposomes, such as doxorubicine (Chen et al., 2016), cisplatin (Zhang et al., 2015a,b), wogonin (Tian et al., 2014), brucine (Chen et al., 2012) and plasmid DNA (He et al., 2010), which displayed potential targeting ability to hepatocytes and HCC cells with better anti-HCC efficacy than unmodified liposomes.

Fluorescence labeling may offer invaluable benefits such as drug delivery, drug targeting, biomedical imaging, protein tracking and various clinical applications (Valizadeh et al., 2012). So, in this paper, GA and GA-modified liposome (GA-Lip) were labeled with fluorescein isothiocyanate (FITC) or coumarin 6 (Cou6) using chemical or pharmaceutical techniques. The transport characteristics and possible mechanisms of FITC-GA and GA-Cou6-Lip were investigated in HCC cells.

## 2. Materials and methods

### 2.1. Materials

18β-glycyrrhetinic acid (18β-GA) and coumarin 6 (Cou 6) were obtained from J & K scientific Ltd. (Beijing, China). Fluorescein isothiocyanate (FITC) was obtained from Sigma-Aldrich Co. (St. Louis, Missouri, USA). DSPE-PEGylated 18β-glycyrrhetinic acid (18β-GA-DSPE) was synthesized and characterized in our lab. Phospholipids and cholesterol were purchased from Tywei pharmaceutical Co. (Shanghai, China). Phenylmethylsulfonyl fluoride (PMSF, purity ≥ 98%) was obtained from Amresco Co. (Solon, Ohio, USA). Bovine serum albumin (BSA), the radioimmunoprecipitation assay (RIPA) lysis buffer, the enhanced bicinchoninic acid (BCA) protein assay kit, and the membrane and cytosol protein extraction kit were purchased from Beyotime institute of biotechnology (Jiangsu, China).

Human HCC (HepG2) cells were incubated in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, California, USA) with 10% fetal bovine serum (FBS, Gibco, Waltham, Massachusetts, USA) under standard cell culture conditions of 37 °C in a humid atmosphere with 5% $CO_2$. All tests were carried out during the exponential phase.

### 2.2. Synthesis of fluorescence labeled glycyrrhetinic acid

FITC-labeled 18β-glycyrrhetinic acid (FITC-GA) was synthesized by conjugating FITC with aminated GA (Fig. 1) (Zhang et al., 2013). Firstly, 0.942 g (2 mmol) 18β-GA in 25 ml of dichloromethane was reacted with 0.576 g (3 mmol) EDC·HCl. 0.346 g (3 mmol) NHS was added sequentially to the solution with stirring at ambient temperature, and 1 ml of ethylenediamine and 0.5 ml of triethylamine were then added. After reaction overnight, the precipitant was filtered off and evaporated to dryness. The intermediate was dissolved with dichloromethane and poured into ice-cold ether, crystallized, and dried. Secondly, 21.0 mg (0.04 mmol) reactant and 19.5 mg (0.05 mmol) FITC were dissolved in 1 ml dimethyl sulfoxide with 60 µl pyridine in a vial for 6 h reaction. And then, the solution was precipitated in cold acetone. After 24 h, the solvent was separated and the precipitate was washed twice with acetone to remove excess FITC. The residue was freeze-dried to obtain FITC-GA as a dark red crystal. The yield of product was 55.8%.

The FT-IR spetra of GA and FITC-GA were recorded as KBr pellets in the spectral range of 4000–400 $cm^{-1}$ using a Bruker vector 22 infrared spectrometer (Bruker, Switzerland). In addition, the $^1H$ NMR (DMSO-*d*6) spectra was characterized at 300 Hz with an ARX-300 (600) NMR spectrometer (Bruker, Switzerland).

### 2.3. Transmembrane transport of FITC-labeled GA

HepG2 cells were seeded in 6-well plates at $1 \times 10^6$ cells per well. The cells were treated with 20 µM FITC-GA in DMEM without FBS for 0.25, 0.5, 1.0, or 2.0 h (Xintaropoulou et al., 2015). Other HepG2 cells were incubated in the above medium for 2 h, and the medium was then removed. Fresh DMEM without FITC-GA was added and co-incubated.

After incubation with FITC-GA or blank DMEM, the medium was removed and the cells were washed 3 times with cold PBS. Cells were lysed with 100 µl of lysis buffer and harvested with 900 µl of PBS. The cell suspension was then shaken and centrifuged (12,000 rpm for 10 min). The fluorescence intensity of the 200 µl supernatant was measured ($\lambda_{ex}$ = 490 nm, $\lambda_{em}$ = 520 nm) with a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific, Waltham, Massachusetts, USA). The content of FITC-GA was calculated from the fluorescence intensity which was normalized by the cell protein quantity. The protein concentration was determined with a BCA protein assay kit. In addition, the uptake course was viewed with an IX71 fluorescent microscope (Olympus, Japan). Using an OriginPro 10.0 software, the clearance curve was fitted as the first order, the second order, the exponential decay and the polynomial kinetics, respectively.

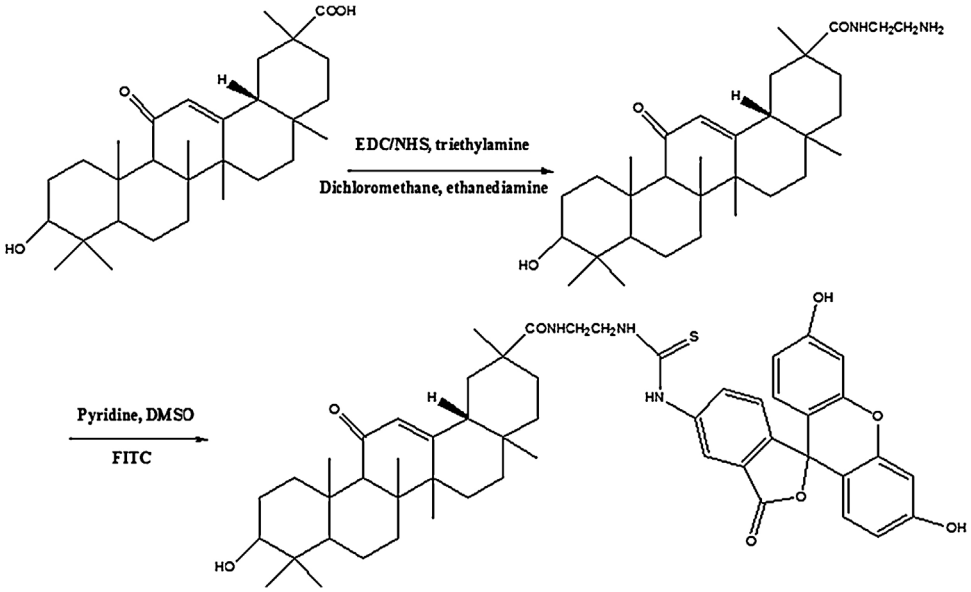


**Fig. 1.** Schematic representation of synthesis of FITC-GA. GA was amidated 30-carboxyl with ethylenediamine. FITC-GA was synthesized via GA-$NH_2$ isothiocyanate reaction.