

Original Article

PEGylated gambogenic acid nanostructured lipid carriers enhance intracellular delivery in A549 cells via clathrin-, caveolae-mediated endocytosis and macropinocytosis

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Received March 24, 2026; Accepted July 10, 2026; Epub September 15, 2026; Published September 30, 2026

Abstract: Objective: To investigate the transport mechanism of gambogenic acid (GNA) and PEGylated gambogenic acid nanostructured lipid carriers (P-GNA-NLC) in A549 cells and to quantify their intracellular contents. Methods: The GNA content in A549 cells was determined by high-performance liquid chromatography (HPLC). The mechanisms of GNA and P-GNA-NLC entry were elucidated using various experimental conditions, including different temperatures, concentrations, times, and endocytosis inhibitors. Initially, A549 cells were obtained through cell culture, and HPLC was employed to establish the quantitative analysis of GNA inside the cells. Subsequent experimentation involved the determination of cellular uptake profiles, the execution of carrier saturation experiments, investigations into the effects of varying temperatures and concentrations on uptake efficiency, and the implementation of endocytosis inhibitor treatment experiments. In the latter, the inhibitor's potential for inducing cellular toxicity was determined by means of an MTT assay, and the inhibitor concentration that exhibited minimal effect on cellular activity was selected for subsequent experimentation. Results: Both GNA and P-GNA-NLC entered A549 cells via active transport. GNA entered mainly through caveolae-mediated endocytosis (Cav-ME) and macropinocytosis, whereas P-GNA-NLC could enter the cells through clathrin-dependent endocytosis (CDE) pathway in addition to Cav-ME and macropinocytosis. This may account for the stronger antitumor effect of P-GNA-NLC than GNA. Furthermore, the entry of P-GNA-NLC into cells in the form of nanolipid carriers may have improved the intracellular pharmacokinetics of GNA, thus enhancing the efficacy of GNA. Conclusion: P-GNA-NLC enters cells via the Cav-ME, macropinocytosis, and CDE pathways. These findings provide a preliminary understanding of the intracellular transport mechanisms of GNA and P-GNA-NLC and provide an experimental basis for the clinical application of GNA antitumor drugs.

Keywords: Gambogenic acid, pegylated gambogenic acid nanostructured lipid carriers, intracellular uptake mechanisms

Introduction

Malignant tumors represent a significant global health burden, and the development of effective treatment strategies has been a major focus of medical research [1, 2]. Despite the significant advancements in surgical techniques, chemotherapy and radiotherapy, the high recurrence rate and drug resistance of tumors continue to present significant chal-

lenges in clinical treatment [3, 4]. In recent years, traditional Chinese medicine (TCM) has demonstrated considerable promise in the field of anticancer therapy, largely due to its distinctive theoretical and practical approaches, particularly the anti-tumor efficacy of certain TCM monomers [5, 6]. Gambogenic acid (GNA), the primary active ingredient in *Garcinia hanburyi*, has been shown in multiple studies to exert inhibitory effects on various tumor cells [7]. Liu

et al. [8] have demonstrated that GNA induces oxidative stress in the P53/SLC7A11/GPX4 axis, which ultimately results in apoptosis in osteosarcoma cells. This process suppresses cancer proliferation and invasion. Wu et al. [9] discovered that GNA can exploit the JNK signaling pathway in prostate cancer cells to induce apoptosis and autophagy in cancer cells by means of endoplasmic reticulum stress. Nevertheless, the clinical potential of GNA is constrained by a number of inherent limitations, including poor water solubility, a short biological half-life, vascular irritation, and sensitivity to light and temperature. These factors have impeded the clinical translation of GNA.

PEGylated gambogic acid nanostructured lipid carriers (P-GNA-NLC) is a nanostructured lipid carrier that has been modified with PEG on the surface [10]. It has been specifically designed for the encapsulation and delivery of GNA. The P-GNA-NLC formulation has been shown to enhance the antitumor effect of GNA [11]. This is achieved by increasing the circulation time, bioavailability and targeting in vivo of GNA, while simultaneously reducing its systemic distribution and rapid clearance by the reticuloendothelial system [10]. However, the mechanism of its intracellular transport remains unclear, which is crucial for understanding and optimizing its anti-tumor effects. The P-GNA-NLC formulation offers new possibilities for the clinical application of GNA.

Cellular pharmacokinetics is the scientific discipline concerned with the study of the dynamic characteristics of drugs within a cellular environment [12]. In the context of clinical anticancer drug treatment, the concentration of the drug within the target cell is regarded as a key indicator of the drug's antitumor efficacy [13]. However, the pharmacokinetic studies of GNA and its nanocarriers in cells remain incomplete. Consequently, it is imperative to develop a comprehensive understanding of the intracellular uptake, distribution and metabolism mechanisms of GNA and P-GNA-NLC to optimize their anti-tumor effects and promote their clinical applications.

This study aimed to elucidate the differences in the cellular uptake pathways of GNA and P-GNA-NLC by quantitative analysis, and to further explore the impact of these disparities on anti-tumor effects. Through these experiments,

it is expected that the mechanisms of uptake of GNA and P-GNA-NLC in cells will be revealed, a reference will be provided for the study of novel GNA formulations, and an experimental foundation will be established for their clinical applications. Concurrently, this study will furnish a theoretical foundation for the design and optimization of the nano drug delivery system, thereby promoting the utilization and advancement of Chinese medicinal preparations in the domain of anti-tumor therapy.

Materials and methods

Drugs, reagents and cells

GNA was isolated from the resin of *Garcinia hanburyi* in our laboratory according to a previously established protocol [10]. Its purity (> 99.0%) was verified by HPLC (methanol-0.1% phosphoric acid, 83:17, v/v; detection wavelength 360 nm) using the area normalization method and confirmed by melting point determination (108-109°C) [10]. Genistein was purchased from Aladdin (Shanghai, China). Sodium azide was obtained from Sinopharm Chemical Reagent (Beijing, China). Chlorpromazine was supplied by ZHENWU Biomedical (Shanghai, China). Cytochalasin D was purchased from Melonepharma (Dalian, China). 2-deoxyglucose was obtained from Macklin (Shanghai, China). Human lung adenocarcinoma A549 cells were obtained from Shanghai Fu Dan University (Shanghai, China). RIPA Lysis Buffer was purchased from Beyotime (Shanghai, China). High-glucose Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (USA). Fetal bovine serum (FBS), penicillin G and streptomycin were standard cell culture supplements. Phosphate-buffered saline (PBS, pH 7.27.4) was prepared in-house. Methanol was purchased from Shanghai Xingke High-Purity Solvent Co., Ltd. (Shanghai, China). Ethyl acetate was obtained from Jiangsu Qiangsheng Functional Chemical Co., Ltd. (Jiangsu, China). A549 cells were cultured in high-glucose DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin G and 100 U/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

HPLC quantification of intracellular GNA

A validated HPLC method (Shimadzu LC-15C) was used throughout. Chromatographic condi-

Table 1. Inhibitors used for different endocytic pathways

Pathway	Inhibitor	Mechanism
Active transport	Sodium/2-deoxyglucose	Inhibit cell respiration
Clathrin-mediated endocytosis (CDE)	Chlorpromazine	Interacts with clathrin to block CDE pathway
Caveolae-mediated endocytosis (cav-Me)	Genistein	Tyrosine-kinase inhibitor, to block cav-Me pathway
Macropinocytosis	Cytochalasin D	Inhibit macropinocytosis

tions: Cosmosil C18 column (4.6 × 250 mm, 5 µm), methanol-0.1% phosphoric acid (90:10, v/v), 1.0 mL min⁻¹, 30°C, 360 nm. Gambogic acid (GA, 20 µg mL⁻¹) served as the internal standard. Cell lysate (100 µL) was spiked with GA (10 µL), extracted with 1 mL ethyl acetate, vortexed for 3 min, centrifuged at 3,500 rpm for 10 min; 0.9 mL supernatant was dried under N₂ at 37°C, reconstituted in 100 µL methanol, centrifuged at 15,000 rpm for 10 min, and 20 µL was injected. The assay was linear over the range of 0.05-30 µg mL⁻¹ (R² ≥ 0.999); intra- and inter-day precision RSD values were < 8%; LLOQ was 0.05 µg mL⁻¹. Protein content was determined by BCA kit (Beyotime); results are expressed as µg GNA per mg protein.

Intracellular drug concentration determination

Precisely 100 µL of cell lysate was taken, 10 µL of gambogic acid standard solution was added, then 1 mL of ethyl acetate was added and vortexed for 3 min to extract GNA from cell lysate. After centrifugation at 4,000 r for 10 min, 0.9 mL of the supernatant was collected and concentrated with nitrogen, and finally the dried samples were re-dissolved in 100 µL of methanol. After high-speed centrifugation for 10 min, 20 µL of sample was taken for HPLC. The protein concentration of the samples was determined using a BCA kit, and the intracellular drug concentration was calculated based on the ratio of the GNA content to the protein concentration of the cell lysate.

Cellular uptake kinetics

A549 cells were seeded at 2 × 10⁵ cells/well in 6-well plates and cultured for 24 h. A pilot linearity test (5 × 10⁴ to 4 × 10⁵ cells) confirmed r > 0.99 at 2 × 10⁵ cells; this density was used thereafter. Medium was replaced with 2 mL fresh DMEM containing GNA or P-GNA-NLC (10 µg mL⁻¹) and incubated 0, 5, 30, 60, 90, 120 or 240 min (37°C, 5% CO₂). At each time-point wells were rapidly washed 3× with ice-cold

PBS, lysed, and analysed as in 2.2. Three independent experiments were performed on different days; each experiment contained six replicate wells per formulation (total n = 18). Uptake saturation was examined at 5, 10, 20 and 30 µg mL⁻¹ for 60 min; temperature-dependence was assessed at 4°C versus 37°C under identical conditions (n = 18 each).

Carrier-saturation experiment

To verify whether P-GNA-NLC enters as an intact carrier, cells were pre-incubated with blank P-NLC (lipid concentration equal to that in 10 µg mL⁻¹ P-GNA-NLC) for 60 min, washed, and then exposed to P-GNA-NLC (10 µg mL⁻¹) for a further 60 min. Control cells received P-GNA-NLC without P-NLC pre-treatment. Uptake was quantified as above (n = 18).

Endocytosis inhibition

Cells were pre-treated for 30 min with energy inhibitors NaN₃ (10 mM) + 2-deoxy-D-glucose (50 mM), clathrin inhibitor chlorpromazine (28.1 µM), caveolae inhibitor genistein (18.5 µM), or macropinocytosis inhibitor cytochalasin D (30 µM) - concentrations were selected by MTT. GNA or P-GNA-NLC (10 µg mL⁻¹) was then added for 60 min. Each inhibitor group consisted of three independent experiments (different days) with six wells per experiment (total n = 18). After three PBS washes, intracellular GNA was quantified and expressed as percentage of untreated control. The inhibitors used, their targeted pathways, and mechanisms are summarized in **Table 1**.

Statistical analysis

All data were statistically analyzed using SPSS 21.0 (IBM, Armonk, NY, USA). Continuous variables that followed a normal distribution (assessed by Shapiro-Wilk test) were described as mean ± standard deviation (SD). For comparisons involving multiple independent groups, one-way analysis of variance (ANOVA) was

GNA uptake in A549 cells

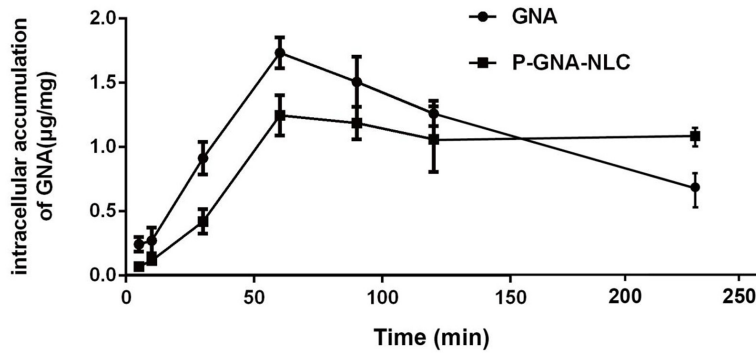


Figure 1. Time-dependent intracellular uptake kinetics of GNA and P-GNA-NLC in A549 cells. Notes: GNA, gambogic acid; P-GNA-NLC, PEGylated gambogic acid nanostructured lipid carriers.

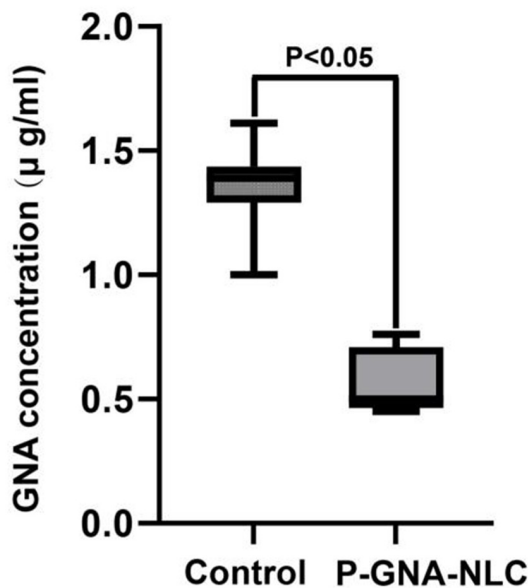


Figure 2. Intracellular accumulation of P-GNA-NLC in A549 cells pre-treated with blank P-NLC. Notes: P-GNA-NLC, PEGylated gambogic acid nanostructured lipid carriers; P-NLC, PEGylated nanostructured lipid carriers.

performed, followed by Dunnett's post hoc test for comparisons against the control group. For concentration-dependent and time-dependent uptake experiments within each formulation group, one-way ANOVA was performed followed by Tukey's HSD post hoc test for pairwise comparisons between different time points or concentrations. Comparisons between two formulations at the same time point or concentration were performed using independent Student's t-test. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Uptake curves of GNA and P-GNA-NLC in A549 cells

The intracellular uptake curves of GNA and P-GNA-NLC in A549 cells are shown in **Figure 1**. Both formulations reached peak uptake at 60 min, after which intracellular GNA content declined. Notably, the intracellular GNA content declined more slowly for P-GNA-NLC than for free GNA after 60 min (1.10 ± 0.07

vs. 0.70 ± 0.11 $\mu\text{g}/\text{mg}$ protein at 240 min). This observation may reflect differences in intracellular disposition, although reduced drug efflux or incomplete drug release from the nanocarrier cannot be excluded based on the current data.

Intracellular accumulation of P-GNA-NLC

The results of the carrier saturation assay showed that the amount of GNA in A549 cells was significantly lower in the experimental group that was first incubated with P-NLC and then with P-GNA-NLC, compared with the control group that was only incubated with P-GNA-NLC ($P < 0.05$) (**Figure 2**). Specifically, pre-treatment with blank P-NLC reduced the uptake from 1.36 ± 0.17 $\mu\text{g}/\text{mg}$ protein to 0.57 ± 0.13 $\mu\text{g}/\text{mg}$ protein, representing a significant inhibition of approximately 58.1%.

Effect of different concentrations on uptake efficiency of GNA and P-GNA-NLC

Concentration-dependent uptake of GNA and P-GNA-NLC was evaluated at 5, 10, 20, and 30 $\mu\text{g}/\text{mL}$ (**Figure 3**). For both formulations, cellular uptake increased with concentration in the lower range but plateaued at higher concentrations (≥ 20 $\mu\text{g}/\text{mL}$), indicating a saturable transport mechanism.

Effect of different temperature on uptake efficiency of GNA and P-GNA-NLC

We incubated the cells at 4°C and 37°C , respectively, and determined the uptake efficiency of GNA and P-GNA-NLC. The results showed that the uptake at 4°C was significantly

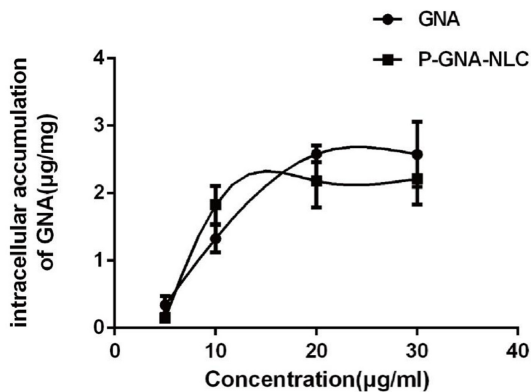


Figure 3. Concentration-dependent cellular uptake efficiency of GNA and P-GNA-NLC in A549 cells. Notes: GNA, gambogic acid; P-GNA-NLC, PEGylated gambogic acid nanostructured lipid carriers.

lower than that at 37°C, indicating that the intracellular transport of GNA and P-GNA-NLC requires energy expenditure (**Figure 4**). For GNA, the intracellular concentration decreased from 2.56 ± 0.26 µg/mg protein at 37°C to 1.69 ± 0.37 µg/mg protein at 4°C, representing a significant reduction of 34.1% ($P < 0.01$). For P-GNA-NLC, uptake decreased from 2.46 ± 0.27 µg/mg protein at 37°C to 1.71 ± 0.39 µg/mg protein at 4°C, a reduction of 30.5% ($P < 0.01$).

Effect of endocytosis inhibitors on the activity of A549 cells

The toxicity of endocytosis inhibitors and GNA to A549 cells was evaluated by MTT assay. The minimum cytotoxic concentrations of each inhibitor were determined. Based on the MTT experiment, the inhibitor concentration that ensures a cell survival rate of more than 80% was selected to guarantee that the drug concentrations used in the following experiments were safe. The results are shown in **Figure 5**. The optimal concentrations of the inhibitors were confirmed as sodium azide/2-deoxy-D-glucose (10 mM/50 mM), chlorpromazine (28.1 µmol/L), genistein (18.5 µmol/L), and cytochalasin D (30 µmol/L), with cell survival rates all above 80%, avoiding the interference of inhibitor cytotoxicity on the uptake experiment results.

Effect of endocytosis inhibitors on uptake efficiency of GNA and P-GNA-NLC

The effects of endocytosis inhibitors on GNA and P-GNA-NLC uptake are summarized in

Figure 6. For free GNA (**Figure 6A**), uptake was significantly inhibited by genistein (Cav-ME inhibitor, 50.4% inhibition, $P < 0.01$) and cytochalasin D (macropinocytosis inhibitor, 53.4% inhibition, $P < 0.01$), but not by chlorpromazine (CDE inhibitor, 12.3% inhibition, $P > 0.05$). For P-GNA-NLC (**Figure 6B**), genistein, cytochalasin D, and chlorpromazine all significantly reduced uptake (35.0%, 51.9%, and 49.6% inhibition, respectively, $P < 0.01$), indicating that P-GNA-NLC enters cells via Cav-ME, macropinocytosis, and also CDE.

Cellular uptake mechanisms of GNA and P-GNA-NLC

Through different temperature, concentration and energy inhibition experiments as well as specific receptor inhibitor experiments, the results showed that GNA and P-GNA-NLC enter A549 cells mainly through active transport. Quantitative analysis revealed distinct uptake profiles: GNA enters cells primarily through caveolae-mediated endocytosis (Cav-ME, 50.4% inhibition by genistein) and macropinocytosis (53.4% inhibition by cytochalasin D), with minimal contribution from clathrin-mediated endocytosis (CDE, 12.3% inhibition by chlorpromazine, not significant). In contrast, P-GNA-NLC utilizes multiple pathways, including caveolae-mediated endocytosis (Cav-ME, 35.0% inhibition by genistein), macropinocytosis (51.9% inhibition by cytochalasin D), and clathrin-mediated endocytosis (CDE, 49.6% inhibition by chlorpromazine). The involvement of clathrin-mediated endocytosis as an additional pathway for P-GNA-NLC, which is not utilized by free GNA, may explain the enhanced intracellular pharmacokinetics and stronger antitumor effect of the nanocarrier formulation. **Figure 7** schematically illustrates the distinct cellular uptake mechanisms of free GNA and P-GNA-NLC in A549 cells.

Discussion

The current investigation focused on analyzing the cellular uptake of GNA and P-GNA-NLC in A549 lung cancer cells, the mechanisms involved in their cellular entry, and the effects of varying concentrations, temperatures, and endocytosis inhibitors on uptake efficiency. Our findings reveal that both GNA and P-GNA-NLC are capable of undergoing active cellular transport; P-GNA-NLC, in particular, demonstrates superior cellular uptake ability when

GNA uptake in A549 cells

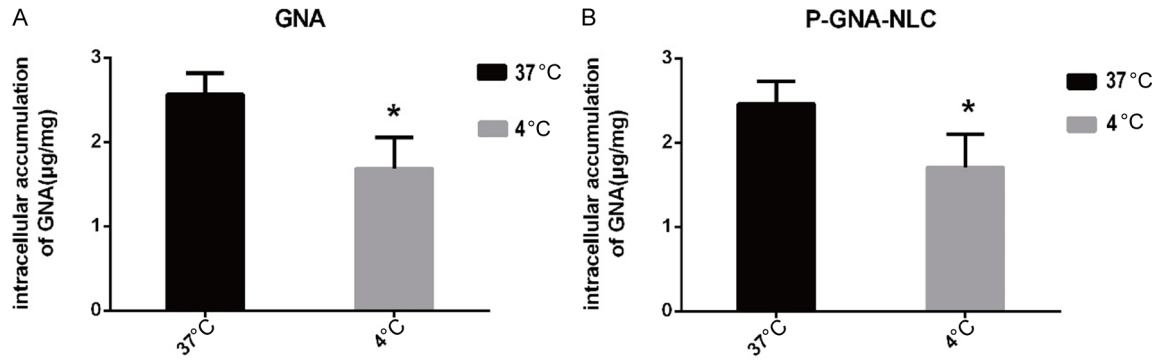


Figure 4. Temperature-dependent intracellular uptake of GNA and P-GNA-NLC in A549 cells. A. GNA (37 °C vs 4 °C). B. P-GNA-NLC (37 °C vs 4 °C). Notes: *P < 0.05. GNA, gambogenic acid; P-GNA-NLC, PEGylated gambogenic acid nanostructured lipid carriers.

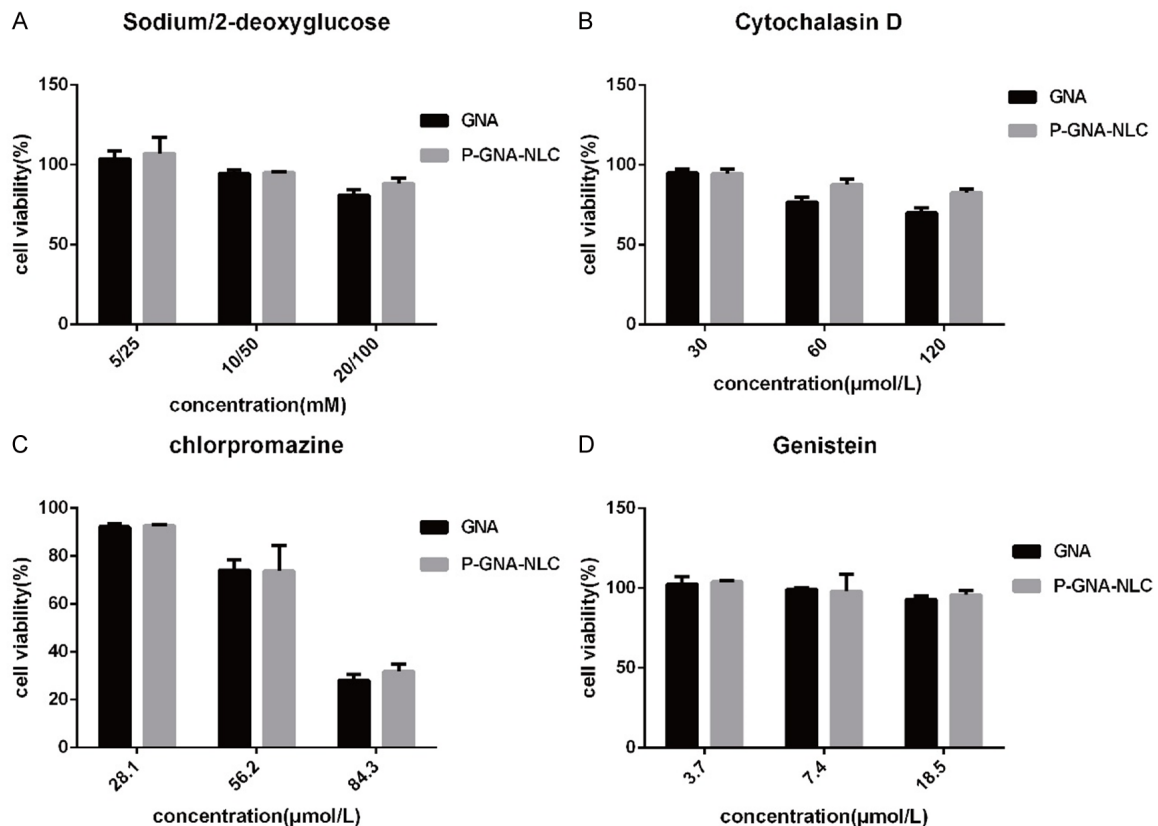


Figure 5. Cytotoxicity evaluation of endocytosis inhibitors in A549 cells. A. Sodium/2-deoxyglucose. B. Cytochalasin D. C. Chlorpromazine. D. Genistein. Notes: GNA, gambogenic acid; P-GNA-NLC, PEGylated gambogenic acid nanostructured lipid carriers.

compared to GNA. This finding is critical for understanding how P-GNA-NLC enhances the bioavailability of GNA and its anti-tumor action.

A549 cells exhibited time-dependent uptake of both GNA and P-GNA-NLC, with uptake increasing progressively before reaching a plateau,

suggesting the involvement of a saturable uptake mechanism. Notably, P-GNA-NLC demonstrated significantly greater cellular uptake than free GNA at all investigated time points. This difference may be attributed to the encapsulation of GNA within the nanolipid carrier. Previous studies have shown that nanocarrier

GNA uptake in A549 cells

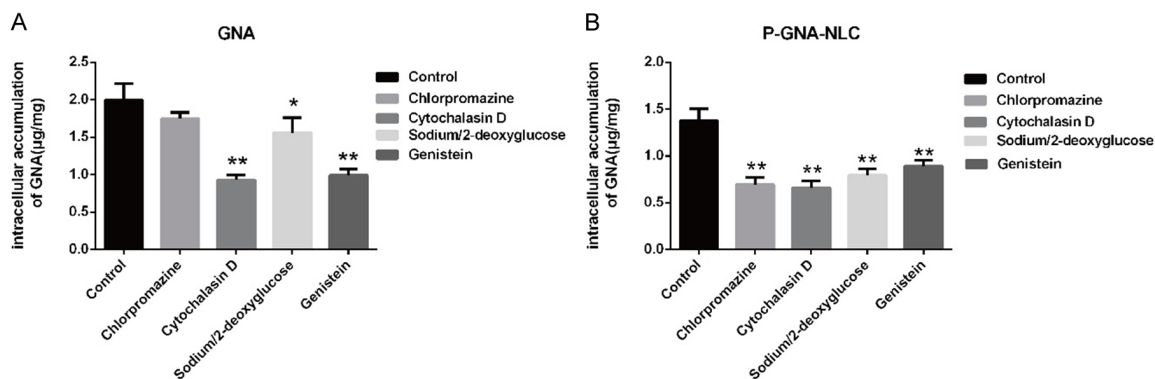


Figure 6. Effects of different inhibitors on the uptake of GNA and P-GNA-NLC by A549 cells. A. GNA. B. P-GNA-NLC. Notes: GNA, gambogenic acid; P-GNA-NLC, PEGylated gambogenic acid nanostructured lipid carriers. * $P < 0.05$, ** $P < 0.01$, compared with control (untreated) within each formulation.

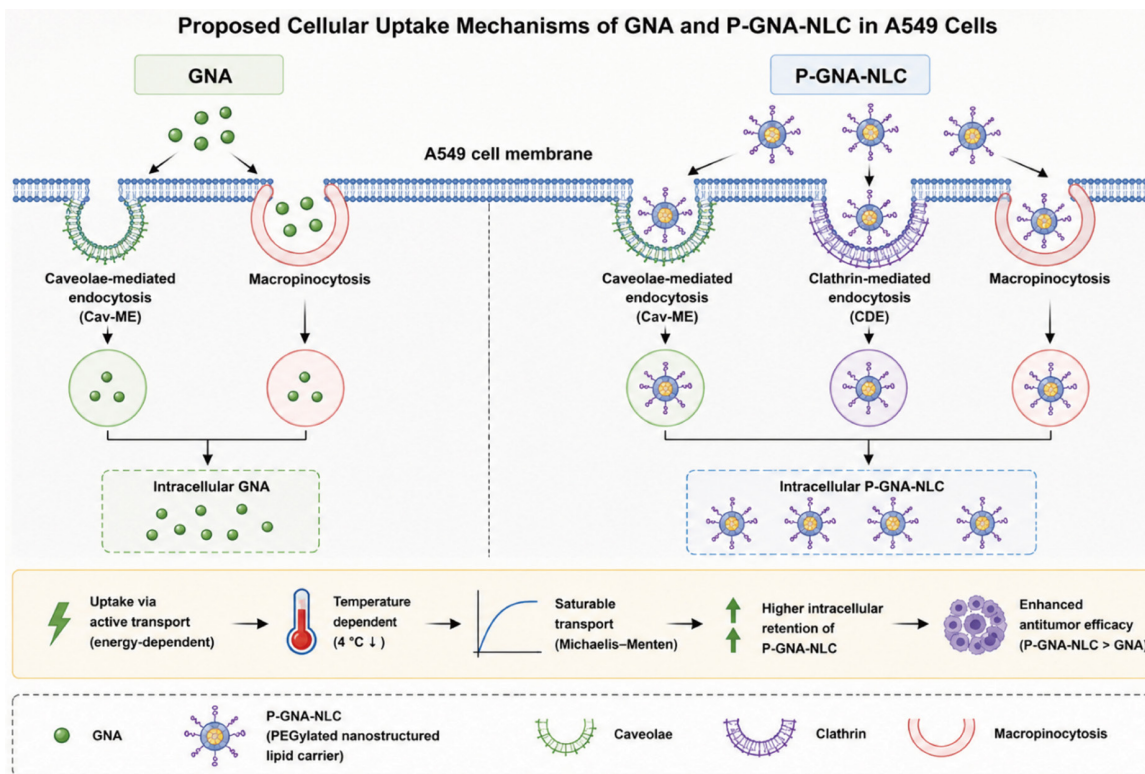


Figure 7. Schematic diagram of the cellular uptake mechanisms of GNA and P-GNA-NLC in A549 cells. Notes: GNA, gambogenic acid; P-GNA-NLC, PEGylated gambogenic acid nanostructured lipid carriers; Cav-ME, caveolae-mediated endocytosis; CDE, clathrin-mediated endocytosis.

encapsulation can enhance drug stability and improve cellular bioavailability [14, 15]. These findings further support the ability of nanocarrier systems to promote cellular uptake [16]. Carrier saturation experiments suggested that P-GNA-NLC is internalized predominantly as an intact nanoparticle complex rather than as free GNA [17]. This observation further supports the

role of nanolipid carriers in enhancing the efficiency of cellular uptake [18, 19] and suggests that P-GNA-NLC may utilize transport mechanisms distinct from those of free GNA [20, 21]. Based on the results of the endocytosis inhibition experiments, P-GNA-NLC appears to enter cells through multiple pathways, including Cav-ME, CDE, and macropinocytosis, whereas free

GNA is internalized primarily through Cav-ME and macropinocytosis [22-24]. The additional involvement of the CDE pathway in P-GNA-NLC uptake may result from the physicochemical characteristics of the nanolipid carrier. Clathrin-mediated endocytosis is a major pathway responsible for the internalization of extracellular macromolecules and nanoparticulate systems, and surface modification of nanocarriers may facilitate their entry through this pathway [25, 26]. Enhanced utilization of multiple endocytic pathways may contribute to the greater intracellular accumulation of P-GNA-NLC and could partially explain its superior anti-tumor activity compared with free GNA. These findings provide mechanistic insights into the cellular transport of GNA and P-GNA-NLC and may facilitate the rational design of future nanocarrier-based drug delivery systems.

In order to ascertain the effects of temperature and concentration on the efficiency of cellular uptake, the present study explored the relationship between these variables and the cellular uptake rate. The results demonstrated that the cellular uptake rate was significantly reduced under low-temperature conditions, thus suggesting that cellular uptake of GNA and P-GNA-NLC is an energy-dependent process. Furthermore, an increase in the cellular uptake rate was observed with an increase in drug concentration up to a certain level, which may be indicative of transporter saturation on the cell membrane. Notably, the inhibition rate of the energy inhibitor on GNA uptake (21.7%) was lower than that of low temperature (34.1%). Low temperature globally suppresses energy-dependent processes, membrane fluidity, and multiple transport pathways simultaneously, while the energy inhibitor only partially impairs ATP production. In addition, free GNA can enter cells via passive diffusion, which is less sensitive to ATP reduction but strongly inhibited at low temperature. This observation further supports the involvement of passive diffusion in GNA uptake. These findings underscore the necessity to take drug concentration, temperature, and other pertinent factors into account when aiming to optimize the uptake efficiency in practical applications [27]. In the endocytosis inhibitor assay, chlorpromazine was employed as an inhibitor of CDE [28]. The experimental results demonstrated that chlorpromazine significantly reduced the uptake of P-

GNA-NLC but had no significant effect on the uptake of GNA in A549 cells. These findings suggest that CDE contributes to the cellular internalization of P-GNA-NLC, whereas it is not a major pathway for the uptake of free GNA. Clathrin-mediated endocytosis is one of the best-characterized endocytic pathways and is responsible for the internalization of various nanoparticles and receptor-bound molecules. Clathrin proteins assemble into coated pits on the plasma membrane, which subsequently form intracellular vesicles [29, 30]. In the present study, GNA and P-GNA-NLC may be internalized through the formation of clathrin-coated vesicles following interactions with membrane-associated receptors or proteins. Chlorpromazine has been reported to disrupt the assembly of clathrin-coated pits, thereby inhibiting the CDE pathway [31]. In the present study, chlorpromazine significantly reduced the uptake of P-GNA-NLC but had no significant effect on the uptake of free GNA, consistent with the notion that CDE is specifically utilized by the nanoformulation. Furthermore, genistein was utilized as an inhibitor of Cav-ME [32]. The results demonstrated that genistein significantly inhibited the uptake of both GNA and P-GNA-NLC. These findings indicate that Cav-ME plays an important role in the cellular internalization of both formulations. Caveolae are flask-shaped invaginations of the plasma membrane enriched in cholesterol, sphingolipids, and caveolin proteins [33]. These specialized membrane domains mediate the uptake and intracellular trafficking of a variety of biomolecules and nanoscale carriers. In the present study, both GNA and P-GNA-NLC may enter A549 cells through caveolae-mediated transport. The stronger inhibitory effect observed for P-GNA-NLC suggests that nanoformulation may enhance interactions with caveolae-associated membrane structures, thereby promoting cellular uptake. It is noteworthy that, in addition to Cav-ME and CDE, we observed that P-GNA-NLC uptake was regulated to a certain extent by macropinocytosis. Macropinocytosis is a non-specific endocytic process that mediates the rapid internalization of extracellular fluid, macromolecules, and particulate matter. Therefore, P-GNA-NLC may also enter cells through macropinocytosis, although the underlying regulatory mechanisms require further investigation.

It is important to recognize certain limitations of this work. First, this study was conducted entirely *in vitro* using only A549 cells; therefore, the extent to which these findings apply to other tumour types or to the *in vivo* setting remains unknown. Each cell line is characterized by distinct cellular membranes, receptor expressions, and endocytic capacities. This could significantly influence the cellular uptake of GNA and P-GNA-NLC. Another limitation is that, while we identified the different endocytic pathways involved in this process, we did not investigate the exact molecular interactions or regulatory proteins that determine why one pathway is preferred above others. For instance, we did not perform immunofluorescence co-localization to visually confirm the association of P-GNA-NLC with clathrin, although the chlorpromazine inhibition data strongly support functional involvement of this pathway. Third, our analysis focused primarily on the internalization process; post-internalization events, such as endosomal escape and intracellular trafficking, were not investigated. These post-entry processes play a key role in determining the final pharmacological effect of the treatment. To address these limitations, several avenues for future research are suggested. Future studies should test the uptake mechanisms we identified across a range of different cancer cell lines, including those with varying drug resistance profiles, to confirm their reliability. Using siRNA knockdown or CRISPR-Cas9 knockout of essential proteins on pathways such as clathrin-mediated, caveolae-mediated, and macropinocytic would provide molecular evidence of its pathway. In addition, live-cell imaging with fluorescently labeled nanocarriers will enable us to observe the progression of the nanocarriers within the cells in real time to study how entry route affects its distribution in subcellular parts. *In vivo* studies with relevant tumor-bearing animal models are needed to assess whether the improved cellular uptake and altered pathways observed *in vitro* result in better tumor targeting, biodistribution and efficacy *in vivo*. Through this work the mechanistic understanding of cellular studies can be connected to the applicability of these nanocarriers in the clinic.

Conclusion

This study clarified the uptake mechanisms of GNA and its P-GNA-NLC formulation in A549

cells. Our results confirmed that P-GNA-NLC enters these cells with greater efficiency by leveraging multiple endocytic pathways, specifically CDE, Cav-ME, and macropinocytosis. Notably, when compared directly to GNA alone, P-GNA-NLC not only showed improved cellular uptake but also enhanced antitumor activity. This finding has considerable clinical relevance, as it offers a strategy to enhance both the bioavailability and therapeutic efficacy of GNA. Moreover, it lays out a new framework for the design and development of innovative antitumor drug delivery systems. Moving forward, further research should focus on investigating the *in vivo* pharmacokinetic characteristics of P-GNA-NLC, evaluating its antitumor efficacy across various tumor models, and verifying its clinical applicability, all critical steps toward unlocking its potential for use in clinical treatment.

Acknowledgements

This work was supported by Anhui Provincial Department of Education research major project (2024AH040232).

Disclosure of conflict of interest

None.

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