

Aptamer-Mediated Dual-Loaded Liposomal Nanosystem for Synergistic Therapy in Hepatocellular Carcinoma via mTOR/HIF-1 α /VEGF Pathway

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Purpose: Cantharidin (CTD) is a natural anticancer compound whose clinical application is limited by poor water solubility, low bioavailability, and significant toxicity. To develop a more effective and safer therapeutic strategy, we proposed a synergistic combination therapy by integrating CTD with staurosporine (STS), a protein kinase inhibitor that shares complementary mechanisms of action targeting the mTOR/HIF-1 α /VEGF pathway. We further developed an aptamer-guided liposomal nanosystem for the co-delivery of CTD and STS (Apt/CTD-STS/NL), aiming to enhance tumor targeting, improve bioavailability, and reduce the systemic toxicity of both drugs.

Methods: We constructed aptamer-guided co-delivery nanoliposomes encapsulating CTD and STS (Apt/CTD-STS/NL). The targeted delivery efficiency, synergistic antitumor efficacy, and biocompatibility of this nanosystem were comprehensively evaluated through both in vitro and in vivo experiments.

Results: Apt/CTD-STS/NL achieved approximately 1.3-fold higher cellular uptake in HCC cells compared to non-targeted liposomes in vitro. In vivo, it demonstrated superior tumor accumulation. In a murine HCC model, Apt/CTD-STS/NL exhibited the strongest tumor growth inhibition ($79.50 \pm 4.39\%$), significantly outperforming free CTD, STS, or single-drug loaded liposomes. Mechanistic studies revealed that the core synergy between CTD and STS enabled simultaneous inhibition of the key oncoproteins mTOR, HIF-1 α , and VEGF, thereby disrupting tumor proliferation, survival, and angiogenesis.

Conclusion: This study demonstrates a novel targeted nanoplateform that combines an active ingredient from traditional Chinese medicine with a modern kinase inhibitor. This strategy achieves a synergistic anti-HCC effect through multi-pathway inhibition and offers a promising approach for cancer therapy with enhanced efficacy and reduced toxicity.

Keywords: cantharidin, staurosporine, aptamer, drug co-delivery, hepatocellular carcinoma

Introduction

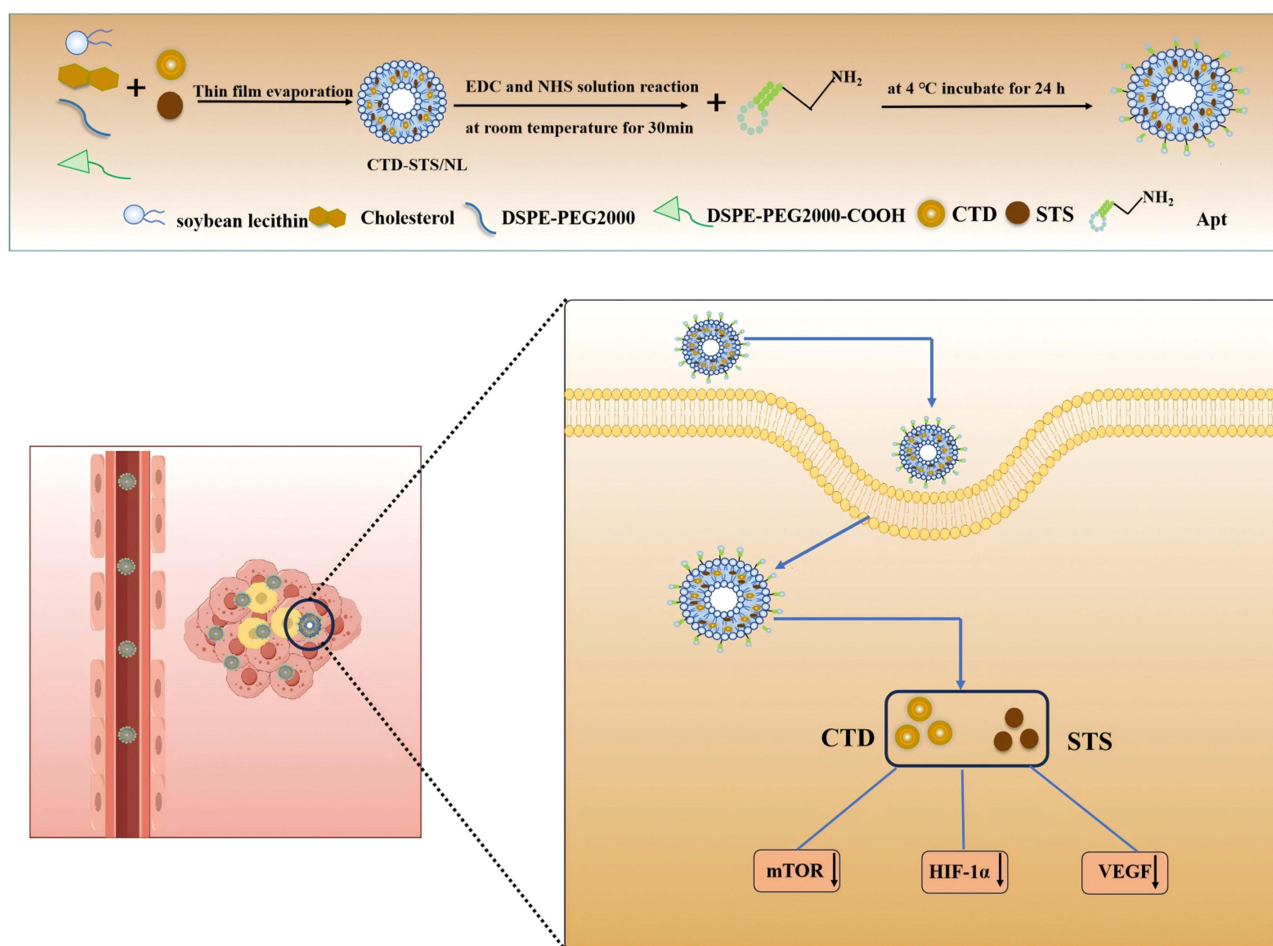
Hepatocellular carcinoma has become the sixth most common cancer and the third leading cause of cancer death worldwide, posing a serious threat to human health.¹ The hepatocellular carcinoma is insidious and progresses rapidly, with most patients progressing to advanced stages at diagnosis and thus missing opportunities for hepatic resection or liver transplantation.² Currently, traditional cytotoxic chemotherapeutic drugs have little efficacy in the treatment of hepatocellular carcinoma. Current domestic and international guidelines recommend targeted drugs as standard treatment option for advanced hepatocellular carcinoma, including sorafenib, lenvatinib and regorafenib, these are pan-targeted protein kinase inhibitors targeting the vascular endothelial growth factor receptors.³ However, all these drugs develop

resistance after a period of treatment, extending survival in hepatocellular carcinoma patients by weeks or months with limited effect. The rise of immunotherapy has brought new hope to liver cancer patients, but the current problems of dose-limiting toxicity and low patient response rate limit the use of this method.⁴ In recent years, traditional Chinese medicine (TCM) has played an important role in oncology with unique and precise efficacy advantages. The multi-target, multi-pathway, multi-link and multi-pathway synergistic effects of Chinese medicines can inhibit the growth of tumour angiogenesis, inhibit the proliferation and metastasis of tumour cells, induce cancer cell demise and neo-angiogenesis and effectively reverse the multidrug resistance of tumour cells to anticancer drugs to play an antitumour role.⁵ However, there are problems of no targeting and high toxicity and side effects, which seriously affect the clinical application.⁶ Therefore, the research and development of multi-targeted, non-drug-resistant Chinese medicine composite anti-hepatocellular carcinoma targeted nano-preparations, to achieve the effect of precise targeting, efficiency and toxicity reduction has become an important issue in the field of health.

The mammalian target of rapamycin (mTOR) is a central serine/threonine kinase that integrates signals from growth factors and nutrients to regulate cell growth, proliferation, and survival, playing a pivotal role in HCC pathogenesis.⁷ The mTOR signaling network exhibits close functional interactions with the PI3K/Akt pathway and serves as a key regulatory hub for hypoxia-inducible factor-1 α (HIF-1 α) and its critical downstream effector - vascular endothelial growth factor (VEGF).^{8,9} Within the tumor microenvironment, HIF-1 α functions as a master transcriptional regulator of cellular response to hypoxic stress, directly activating VEGF expression to drive tumor angiogenesis and malignant progression.⁹ A major challenge in current HCC targeted therapy lies in the high adaptability of signaling pathways: specific mTOR inhibitors trigger feedback activation of PI3K/Akt signaling, ultimately leading to drug resistance;^{10–13} similarly, although anti-VEGF therapies effectively inhibit tumor neovascularization, activation of compensatory pro-angiogenic pathways frequently results in primary or secondary resistance.^{14,15} These mechanisms collectively highlight the necessity of developing multi-target therapeutic strategies, suggesting that simultaneous inhibition of multiple key nodes within the oncogenic signaling axis may yield more substantial and durable antitumor efficacy.

The TCM Mylabris is primarily used to treat scrofula and gonorrhea. It has the effects of eroding dead tissue, promoting diuresis, and alleviating furuncle toxicity. Modern studies have shown that its active ingredient, Cantharidin (CTD), has obvious inhibitory effects on cancers such as liver, lung, prostate, and bladder cancers, and has a definite therapeutic effect on primary hepatocellular carcinoma.^{16–20} CTD can down-regulate EphB4 in HepG2 cells, inhibit the PI3K/Akt signalling pathway, thereby blocking the mTOR pathway, down-regulate VEGF, inhibit liver cancer, and inhibit the mTOR pathway, downregulates VEGF, inhibits hepatocellular carcinoma cell growth and promotes apoptosis.^{21–23} However, its poor water solubility, low targeting and significant irritation of the urinary system severely limit its clinical use.^{24,25} Staurosporine (STS), an alkaloid extracted from *Streptomyces*, is a typical ATP competitive kinase inhibitor and protein kinase C (PKC) inhibitor that blocks the transfer of the phosphodiester bond from DNA to tyrosine residues in the active site of topoisomerase II, thereby inhibiting its enzymatic activity.²⁶ STS inhibits the proliferation of HepG2 cells through Omi/HtrA2-mediated PDK1 degradation to inhibit the PI3K/Akt signalling pathway.²⁷ STS can directly inhibit mTOR kinase activity, interfere with the mTOR signalling pathway, and inhibit the expression of VEGF through multiple signalling pathways, with the potential to inhibit tumour growth and angiogenesis.^{27–34} However, poor solubility and low targeting lead to obvious toxic effects, making STS severely hindered in clinical application. In summary, in this study, CTD and STS were co-loaded into a nano-delivery system to reduce their off-target toxicity, improve their solubility and bioavailability, and achieve synergistic anti-hepatocellular carcinoma effects by inhibiting multiple target proteins of mTOR, HIF-1 α and VEGF through multiple pathways and reversing the resistance to single-targeted therapies.

With the advancement of nanomedicine, liposomes have emerged as one of the most extensively utilized drug delivery vehicles due to their excellent biocompatibility and capacity for co-encapsulating both hydrophilic and hydrophobic agents.³⁵ They enable passive drug targeting to tumor sites, thereby enhancing drug bioavailability, reducing systemic toxicity, and achieving controlled and targeted drug release.^{36–38} However, surface functionalization of liposomes is often necessary to achieve active targeting and improve therapeutic outcomes. Aptamers (Apt), short single-stranded oligonucleotides selected via the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technique, bind to targets with high affinity and specificity. We selected aptamers over conventional monoclonal



Scheme 1 Schematic Diagram of Preparation and anti-tumor therapy of aptamer-modified cantharidin/staurosporine co-loaded targeted nanoliposomes.

Note: This figure was drawn using figdraw <https://www.figdraw.com>, accessed on 25 September 2024), export ID: wS5==82008.

antibodies based on several key advantages: (1) smaller size, which may facilitate superior tumor tissue penetration; (2) ease of chemical synthesis and modification, offering excellent batch-to-batch consistency and lower production costs; (3) reduced immunogenicity; and (4) higher stability.^{39–41} These characteristics make aptamers particularly suitable for constructing robust and efficient targeted nanocarriers. In this study, we proposed a strategy to construct an Apt-modified targeted nanoliposome delivery system co-loaded with CTD and STS (Apt/CTD-STS/NL) by a thin film dispersion-binding post-linkage method using an aptamer that specifically recognises H22 as the targeting molecule (Scheme 1). This system can provide a therapeutic solution to improve the sensitivity of hepatocellular carcinoma cells to drugs, reduce the toxicity and side effects of drugs, and achieve multi-target synergism and potent treatment by synergistically inhibiting multiple key proteins such as mTOR, HIF-1α and VEGF.

Materials and Methods

Materials

Cantharidin (CTD, 98%) was purchased from Wuhan Xinxinjiali Biotechnology Co. Ltd. (Wuhan, China). Staurosporine (STS, 98%) was purchased Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethyleneglycol)-2000] (DSPE-PEG2000-COOH) was purchased Pongsure Bi-ological. (Shanghai, China). H22 Aptamer (CGTCGCTGCACATTCGAATAGTCTGAGCGGAATCAAGTGGTGC GGTGAGTTAGAGAGATCAACGCACAGCTGGGAGTAC-3, NH₂) was purchased from Sangon Biotech. (Shanghai, China). 1, 2-distearoyl-sn-

glycero-3-phosphoethanolamine-N-[methoxy(poly-ethylene glycol)-2000] (DSPE-mPEG2000, Cat.20220501), and soybean lecithin were obtained from Lipoid (Germany). Cholesterol was purchased from Sinopharm Chemical Reagent Co.Ltd. (Shanghai, China). N-hydroxysuccinimide, Coumarin-6, and 1-ethyl- (3-dimethylaminopropyl) carbamyl diimide hydrochloride (EDC) were Shanghai Aladdin Biochemical Technology Co.Ltd. (Shanghai, China). Fetal bovine serum (FBS), and penicillin-streptomycin (P/S) were purchased from Wuhan Pricella Biotechnology Co.Ltd. (Wuhan, China). Cell Counting Kit-8 (CCK-8) assay kit, Annexin V-FITC/PI double staining Apoptosis Detection Kit, Aspartate aminotransferase (AST/GOT) activity assay kit, Alanine aminotransferase (ALT/GPT) activity assay kit, Urea (BUN) Colorimetric assay kit, and Creatinine (CRE) colorimetric assay kit were obtained from Elabscience Biotechnology Co. Ltd. (Wuhan, China). The antibodies of mTOR, and VEGF were from Proteintech. (Wuhan, China). Anti-HIF-1 α antibody (HIF-1 α) was from Abcam (England).

Cells Culture

Mouse H22 hepatocellular carcinoma cells were cultured in RPMI1640 complete medium containing 10% (v/v) FBS, 1% (v/v) P/S solution. Mouse normal hepatocytes AML-12 were cultured in DMEM/F12 complete medium containing 10% (v/v) FBS, 1% (v/v) P/S solution. The cells were grown at 37 °C in a humidified incubator in 5% CO₂.

Synergistic Effect of CTD and STS

Chou-Talalay method was used to determine the interaction between CTD and STS. Log-grown H22 cells (6×10^3 cells/well) were collected and inoculated in 96-well plates at 37 °C in 5% CO₂ for 15 h. CTD (0.7, 0.5, 1.4 μ g/mL) and STS (0.45, 0.4, 0.6, 0.85, 0.3 ng/mL) were pipetted to prepare a mixture of a series of proportions, followed by incubation with the cells for 48 h, and then CCK8 solution was then added for 1 h. The absorbance of each group was measured at 450 nm with microplate reader. Combined index (CI) values were determined using CompuSyn software according to the Chou-Talalay method. The cell viability was calculated by the following equation:

$$\text{Cell viability (\%)} = (A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100\% \quad (1)$$

Preparation of Apt/CTD-STS/NL

Apt/CTD-STS/NL was prepared by thin film dispersion method. Briefly, soy lecithin, cholesterol, DSPE-PEG2000, CTD, DSPE-PEG2000-COOH, and STS (13:1:2.4:0.8:0.4:0.00068, mass ratio) was weighed precisely and placed in a cigar shaped bottle, ultrasonically dissolved in methylene chloride, rotary evaporation at 55 °C, and the inner wall of the cigar shaped bottle was formed into a homogeneous film. PBS buffer (pH 7.4) was added, hydrated at 55 °C for 1 h, and the probe was sonicated for 10 min (total power 100 W, ultrasonication for 2 s with 2 s interval), and filtered through 0.22 μ m microporous membrane to obtain CTD-STS/NL. 25 μ L of 0.16 mM NHS and 25 μ L of 0.64 mM EDC were added, respectively, and the sample was incubated for 30 min at room temperature with activated COOH, followed by the addition of 5 μ L 100 μ M H22 aptamer, mix thoroughly and incubate at 4 °C for 24 h. The Apt/CTD-STS/NL was obtained.

Characterization of Aptamer Modification

Agarose gel electrophoresis was used to verify whether aptamers were successfully modified. A 2% agarose gel was prepared by mixing 0.5 g agarose powder with 25 mL Tris acetate-EDTA (TAE), heating in microwave oven, cooling to 50 ~ 60 °C, and adding 2 μ L of GelRed nucleic acid stain to mix with it. Using 1 mol mL⁻¹ TAE as electrophoresis solution. Then mixed 5 μ L of Apt and Apt/CTD-STS/NL with 15 μ L of 6 $\times$ loading buffer respectively, used a 50–500 bp marker as a control, and then spotted sequentially. The samples were detected by agarose gel electrophoresis (135 V, 25 min), and images were observed by gel imager (BioRad, USA).

Characterization of Apt/CTD-STS/NL

Dynamic laser particle size analyzer (DLS, Zetasizer Nano-ZS90, Malvern Instruments, UK) was used to determine the particle size, polydispersity index (PDI), and zeta potential of Apt/CTD-STS/NL. The morphology of nano-liposomes was observed by transmission electron microscopy (TEM, JEM-F200, JEOL, Japan).

The encapsulation efficiency (EE) and drug-loading capacity (LC%) of CTD and STS was determined by centrifugation of dextran gel microcolumn combined with high-performance liquid chromatography (HPLC, Agilent, USA). The free CTD and STS were removed by centrifugation of 500 μ L of the sample on a 5 mL dextran gel microcolumn and eluted with PBS for 4 times. The centrifuge solution was collected in a 5 mL volumetric flask, and the solution was fixed by adding methanol-acetonitrile (1:1, v/v), ultrasonicated for 30 min, and then allowed to stand for 24 h at room temperature to break the emulsification. The EE of CTD and STS were determined by HPLC. The chromatographic conditions were acetonitrile and 0.1% phosphoric acid solution (45:55, v/v) for CTD, and acetonitrile and 0.1% phosphoric acid solution (32:68, v/v) for STS. The EE was calculated using the following equation (2). The LC was calculated by the following formula (3).

$$EE (\%) = W_{\text{encapsulated drug}} / W_{\text{total drug}} \times 100\% \quad (2)$$

$$LC(\%) = W_{\text{encapsulated drug}} / W_{\text{total Apt/CTD-STS/NL}} \times 100\% \quad (3)$$

$W_{\text{total drug}}$: the weight of CTD,STS in liposome solution

$W_{\text{encapsulated drug}}$: the weight of encapsulated CTD,STS

$W_{\text{total Apt/CTD-STS/NL}}$: total weight of Apt/CTD-STS/NL

Stability

Apt/CTD-STS/NL was mixed with RPMI-1640 complete medium (containing 1% penicillin-streptomycin and 10% fetal bovine serum) in equal volumes and incubated in a shaker at 37 °C for 48 h. The particle size, PDI, and zeta potential were determined by DLS every 8 h. In addition, we investigated the stability of Apt/CTD-STS/NL in phosphate-buffered saline (PBS, pH 7.4). The formulation was mixed with PBS and stored at 4 °C for 40 days, with the particle size, PDI, and zeta potential measured at 10-day intervals.

In vitro Hemolysis and Cytotoxicity Study

The haematological safety of the nanosystems was investigated. Apt/CTD-STS/NL was added to 2% erythrocyte suspension at different concentrations, and equal amounts of saline and deionized water were added as negative and positive controls, respectively. The samples were incubated at 37 °C for 3 h and then centrifuged at 4500 rpm for 5 min. The absorbance of the supernatant at 576 nm was determined, photographed and the hemolysis rate of the sample was calculated according to the following equation:

$$\text{Hemolysis rate } (\%) = (A_{\text{sample}} - A_{\text{negative control}}) / (A_{\text{positive control}} - A_{\text{negative control}}) \times 100\% \quad (4)$$

The toxicity of blank nanocarriers and Apt/CTD-STS/NL in normal hepatic AML-12 cells was determined by CCK8 assay. Briefly, AML-12 cells were seeded in 96-well dishes for 15 h at densities of 5×10^3 cells/well. After the cells were wall-adhered, different concentrations of blank liposomes (NL) and aptamer-modified blank liposome (Apt-NL) were added, and after a total of 48 h of incubation. Afterward, 20 μ L CCK-8 was added to each well, followed by incubation for another 1 h. Finally, the absorbance was measured at 450 nm by a microplate reader (Agilent 800TS-SN, Agilent, USA), and the cell survival rate was calculated.

Cellular Uptake of Apt/CTD-STS/NL

H22 cells were inoculated in 6-well plates (2×10^6 cells/well) and cultured for 24 h. RPMI-1640 basal culture containing H22 cells was used as negative control, and free coumarin 6 (C6), C6-labeled C6/NL, and Apt/C6/NL (final concentration of C6 was 0.5 μ g/mL) were incubated with the cells for 4 h. Subsequently, the cells were washed with pre-cooled PBS and fixed with 500 μ L of 4% (w/v) paraformaldehyde at room temperature for 30 minutes. After washing with PBS, the cells were stained with Hoechst 33342 staining solution (10 μ g/mL) and incubated at room temperature in the dark for 15 min. Following another PBS wash, the cells were resuspended in 500 μ L of PBS. Cellular uptake was analyzed using a confocal laser scanning microscope (CLSM, LSM780 NLO, Zeiss, Germany). Meanwhile, for fluorescence quantification, after 4 h of incubation in each group, the cells were washed with PBS and resuspended in 500 μ L of PBS.

Flow cytometry (BD LSRFortessa647794L6, BD, America) was used for analysis, and fluorescence quantification was performed using FlowJo v10.6.2.

Cell Inhibitory Proliferation

The CCK-8 assay was used to evaluate the *in vitro* anticancer effects of Apt/CTD-STS/NL in H22 cells. Briefly, H22 cells (6×10^3 cells/well) were inoculated in 96-well plates and cultured for 15 h. Different concentrations of CTD, STS, Apt/CTD/NL, CTD-STS, CTD-STS/NL, and Apt/CTD-STS/NL were added to incubate the cells for 48 h, and then added with CCK-8 solution for another 1 h. Finally, the absorbance was measured at 450 nm by microplate reader and cell viability was calculated.

Cell Apoptosis Analysis

H22 cells were inoculated in 6-well plates (1×10^6 cells/mL) and cultured for 15 h. CTD, STS, CTD-STS, CTD-STS/NL, and Apt/CTD-STS/NL were added and incubated for 48 h. Finally, cells were collected and washed twice with PBS, stained with AnnexinV-FITC/PI apoptosis kit, and flow cytometry was performed for detection of apoptotic cells, and apoptosis in each group was analyzed by FlowJo v10.6.2.

Western Blot Analysis

H22 cells were inoculated in 6-well plates and incubated with CTD, STS, CTD-STS, CTD-STS/NL, Apt/CTD-STS/NL, and Apt/CTD/NL, respectively, for 48 h. The cells were collected and lysed by adding RIPA buffer for 30 min under ice, then cell lysate was collected and centrifuged (12000 rpm for 15 min) to obtain the supernatant. After the protein level was determined using the BCA kit, the samples were mixed with SDS sampling buffer and boiled at 100 °C for 5 min to denature the proteins. The proteins were separated by SDS-PAGE gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes, and the PVDF membranes were sealed using 5% skimmed milk. Primary antibodies for VEGF, HIF-1 α , mTOR and α -Tubulin were added and incubated overnight at 4 °C. After sufficient washing using TBST, the membranes were incubated using secondary antibodies at room temperature for 1 h. Finally, the visualised proteins were detected by chemiluminescence imaging (BioSpectrum 300, UVP). The information of the primary and secondary antibodies and their working concentrations used in our experiments were detailed in [Table S1](#).

In vivo Tumor Models

BALB/C mice (4–6 weeks old, female) were obtained from Hunan Slake Kingda Laboratory Animal Co. (Changsha, China). The mice were kept under SPF conditions at the Animal Laboratory Center of Hunan University of Chinese Medicine. All animal experiments adhered to ethical standards and received ethical certification (LL2022071302). H22 cells (2×10^7 cells/mL) were injected subcutaneously into the axillary subcutis of BALB/C mice to establish a mouse tumour bearing model.

In vivo Biodistribution

When the tumour volume reached 150 mm³, DIR was used as a fluorescent probe instead of CTD and STS to prepare DIR liposome nanosystems. Free DIR, DIR/NL, and Apt/DIR/NL (DIR: 0.25 mg/kg) were injected by tail vein, respectively. Fluorescence intensity was detected using an IVIS Lumina XRMS series instrument (PerkinElmer, Waltham, MA) 24 h after administration, and the tumor bearing mice were executed, and the tumours and tissues of major organs (heart, liver, spleen, lung, and kidney) were taken for *ex vivo* imaging.

In vivo Antitumor Efficacy

When the tumour volume grew to about 100 mm³, the mice were randomly divided into eight groups (n = 6). PBS, CTD (0.6 mg/kg), STS (0.6 mg/kg), CTD-STS (CTD: 0.4 mg/kg), Apt/CTD/NL (CTD: 0.4 mg/kg), CTD-STS/NL (CTD: 0.4 mg/kg), Apt/CTD-STS/NL (CTD: 0.4 mg/kg) were injected via tail vein on days 0, 2, 4, 6 and 8, respectively, whereas Sorafenib was injected intraperitoneally using 10 mg/kg. The tumors were measured once in 2 days to construct a tumor volume curve, and tumor volume was calculated according to the following formula (5). On the 10th day, the

tumour tissue was peeled off and immersed in 4% paraformaldehyde tissue fixative and subjected to Hematoxylin eosin (H&E) staining in order to observe the damage to the tumour tissues after administration of each group. The tumor inhibition rate (TIR) for each treatment group was calculated relative to the PBS control group according to Formula (6).

$$\text{Tumour Volume (mm}^3\text{)} = \text{Length} \times \text{Width}^2/2 \quad (5)$$

$$\text{TIR} = (1 - \text{tumor volume of each treatment group/tumor volume of PBS group}) \times 100\% \quad (6)$$

Safety Evaluation

During the treatment period, the body weights of tumor-bearing mice were recorded every 2 days, and the body weight change curve was plotted. H&E staining was performed on major organs. The levels of serum alanine aminotransferase (ALT), creatinine (CRE), aspartate aminotransferase (AST), and urea nitrogen (BUN) were detected in each group of tumour bearing mice using standard kits. The organ index and blood routine were measured. The organ indices were calculated according to the following formula:

$$\text{OI(\%)} = \text{organ mass(g)/body weight(g)} \times 100\% \quad (7)$$

Statistical Analysis

All data were presented as (mean $\pm$ SD) and were performed 3 times in parallel. GraphPad prism 6.0 was used for statistical analysis. A *t*-test was employed to compare two independent samples. For comparisons with more than two groups, analysis of variance (ANOVA) with Tukey's multiple comparisons test. A *p* value of less than 0.05 was considered statistically significant. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, **** *p* < 0.0001.

Results and Discussion

Synergistic and Characterization of Apt/CTD-STS/NL

The effect of CTD combined with STS on H22 cells was detected by CCK8 assay. The interaction of the combination was evaluated by CI. Where CI < 1 is synergistic, CI = 1 is additive, and CI > 1 is antagonistic.⁴² In [Figure 1A and B](#), the CI values calculated by Compusyn are all less than 1, indicating a strong synergistic effect in the combination of CTD and STS under our investigation. The closer the CI value is to 0, the stronger the synergistic effect. One of the optimal combinations is CTD:STS at a ratio of 0.5:0.85 (concentration ratio, CTD: $\mu\text{g/mL}$, STS: ng/mL), with a CI value of 0.22. This compelling in vitro synergy provided a robust rationale for their subsequent co-encapsulation into a single nanocarrier, ensuring simultaneous delivery for optimal combinatorial activity. Meanwhile, the synergistic effect of the ratio was verified by using the ratio of the optimal mass concentration, and the results showed that all of them had strong synergistic effect on the H22 cells at this concentration ratio ([Figure S1](#)).

The conjugation of the H22 aptamer to CTD-STS/NL was primarily achieved through a chemical method. Specifically, the 3' end of the H22 aptamer was modified with $-\text{NH}_2$, while the surface of the liposomes was functionalized with DSPE-PEG₂₀₀₀-COOH. The $-\text{NH}_2$ and $-\text{COOH}$ were linked via a condensation reaction to conjugate the aptamer to the liposomes. After the aptamer is linked to the liposome, the overall size and configuration of the complex change. This can be verified by agarose gel electrophoresis to confirm whether the aptamer has been successfully modified on the surface of the liposome. As shown in [Figure 1C](#), compared to the Apt group, a distinct band shift was observed in the lane of the Apt/CTD-STS/NL group. In contrast, unmodified aptamer and the Apt group showed bands at lower positions, primarily because CTD-STS/NL was conjugated to the aptamer, resulting in reduced migration speed. These results demonstrate that the H22 aptamer was successfully modified onto CTD-STS/NL, confirming the successful construction of Apt/CTD-STS/NL.

Apt/CTD-STS/NL had an average particle size of 90.70 ± 0.79 nm, a PDI of (0.19 ± 0.15) , and an average zeta potential of -2.42 ± 0.15 mV, whereas CTD-STS/NL had an average particle size of 82.44 ± 1.13 nm, a PDI of (0.20 ± 0.15) , and an average zeta potential of -1.12 ± 0.15 mV. As shown in [Figure 1D and E](#), the aptamer modification resulted in slightly larger particle size and lower zeta potential. This may be because the nucleic acid aptamer is a negatively

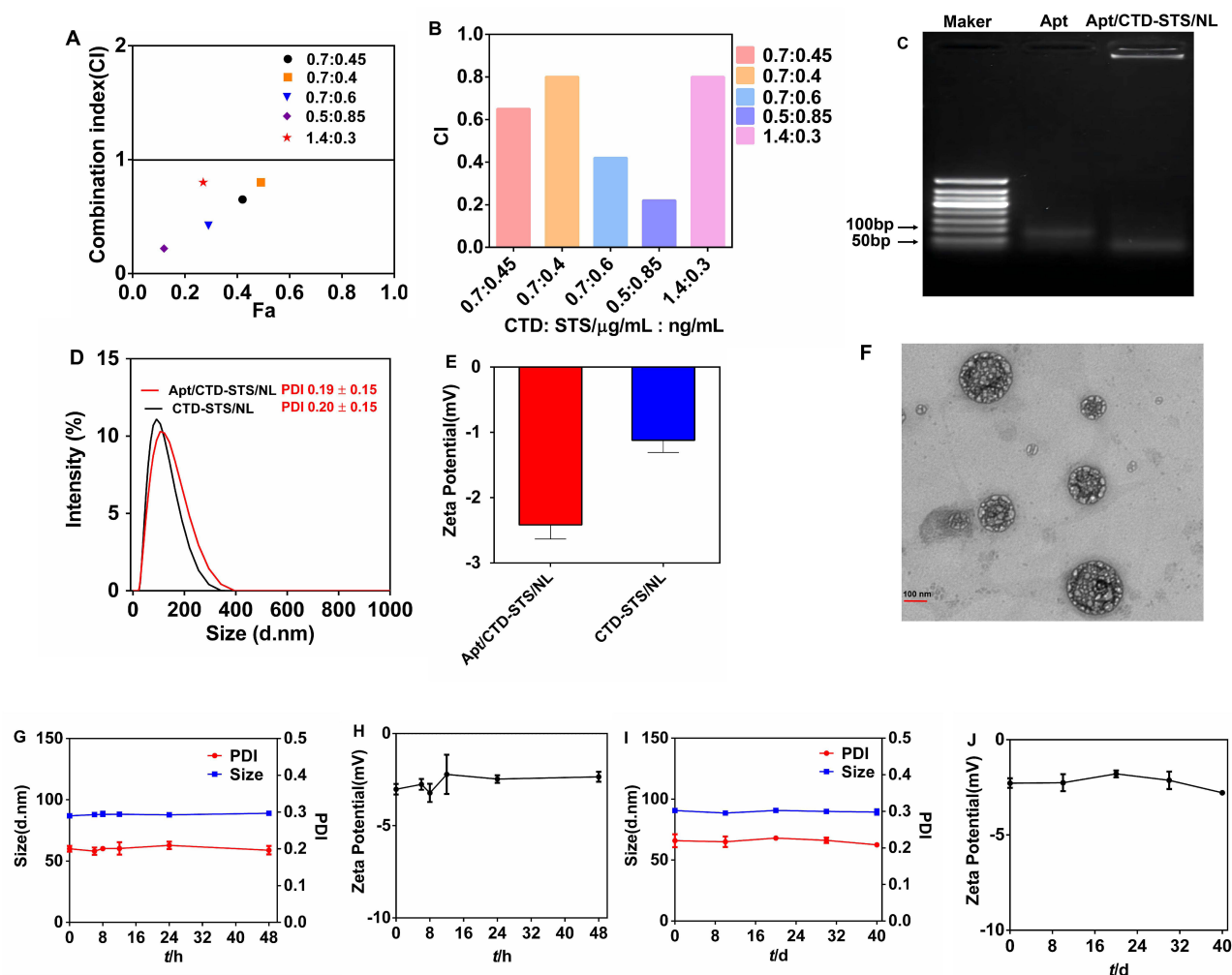


Figure 1 Physicochemical properties of Apt/CTD-STS/NL. (A) The curve of the CI-Fa of CTD-STS at different combined ratios analyzed on H22 cells. (B) CI of CTD-STS at different combined ratios. (C) Agarose electrophoresis gel. (D) Size distribution, PDI of CTD-STS/NL and Apt/CTD-STS/NL. (E) Zeta potential of CTD-STS/NL and Apt/CTD-STS/NL. (F) TEM image of Apt/CTD-STS/NL. Scale bar: 100 nm. (G) The particle size change of Apt/CTD-STS/NL after being placed in RPMI-1640 complete medium (containing 10% FBS) for 48 h. (H) The zeta potential change of Apt/CTD-STS/NL after being placed in RPMI-1640 complete medium (containing 10% FBS) for 48 h. (I) The particle size changes of Apt/CTD-STS/NL after 40-day storage in PBS at 4°C. (J) The zeta potential changes of Apt/CTD-STS/NL after 40-day storage in PBS at 4°C.

charged small molecule. Its modification on the surface of Apt/CTD-STS/NL has little effect on particle size but can lower the zeta potential compared to CTD-STS/NL, thereby further verifying that the H22 aptamer has been successfully modified on the surface of CTD-STS/NL. The morphology of Apt/CTD-STS/NL was observed by TEM, as shown in Figure 1F, all the nanoparticles were distributed in the form of single particles with clear spherical structure and uniform distribution. The experimental results showed that the prepared Apt/CTD-STS/NL had uniform particle size and good dispersion, which was conducive to the aggregation of liposomes at the tumour site and met the requirements of subsequent experiments. The EE of Apt/CTD-STS/NL were $(89.62 \pm 0.67)\%$ for CTD and $(96.01 \pm 1.02)\%$ for STS, and the LC of Apt/CTD-STS/NL were $(3.84 \pm 0.093)\%$ for CTD and $(0.0071 \pm 0.00021)\%$ for STS (Table S2). These physicochemical characteristics are ideal for systemic administration and passive tumor targeting via the EPR effect.

After 48 h of incubation in RPMI-1640 medium (containing 10% FBS), no significant changes were observed in the particle size, PDI, or zeta potential of Apt/CTD-STS/NL (Figure 1G and H), indicating its stability in the RPMI-1640 medium (with 10% FBS). In Figures 1I and J, the particle size, zeta potential, and PDI values of Apt/CTD-STS/NL showed no significant changes, indicating that Apt/CTD-STS/NL can maintain stability for 40 days in PBS medium at 4°C. Although the zeta potential of our Apt/CTD-STS/NL is close to neutral, it remains stable for 40 days. This observed stability is likely attributable to the steric hindrance provided by the surface-bound hydrophilic DSPE-PEG2000 chains

and nucleic acid aptamer, which effectively prevents nanoparticle aggregation. However, the exact underlying mechanism requires further experimental validation.

Hemolysis Qssay and Cytotoxicity Evaluation

Low toxicity and good biocompatibility of biological nanomaterials and drug carriers are essential for in vivo applications. To further investigate the biocompatibility of the nanocarriers, the haemolysis of the Apt/CTD-STS/NL was examined. Apt/CTD-STS/NL at 20 ~ 150 $\mu\text{g/mL}$ were all non-haemolysed and the haemolysis rates were all less than 5% (Figure 2A and B). The results indicated that Apt/CTD-STS/NL had good haemocompatibility and aptamer modification did not affect the haemocompatibility of serum albumin.

The results of the CCK8 assay to detect the toxicity of nanocarriers in normal hepatocytes AML-12 showed that at 48 h of administration, the cell survival rate of both NL and Apt-NL groups was greater than 95% in the concentration of 0 ~ 4 $\mu\text{g/mL}$ (Figure 2C), which indicated that the blank nanocarriers were non-toxic to the AML-12 cells. This high biocompatibility of the blank carrier is a critical prerequisite for its use as a safe drug delivery platform.

In vitro Cellular Uptake

The in vitro cellular uptake of nanoliposome-targeted delivery system was determined against H22 cells by confocal laser scanning microscopy (CLSM). C 6 (green dye) was used instead of CTD and STS encapsulated in liposomes to track the distribution of liposomes, and Hoechst 33342 (blue dye) was chosen to stain the nucleus. At 4 h, all groups showed a distinct Hoechst 33342 excited blue fluorescence, indicating that the cells in all groups were in good condition (Figure 3A). Compared with the free C6 and C6/NL groups, the Apt/C6/NL group superimposed the strongest fluorescence intensity and green fluorescence filled the whole cells, probably due to the specific recognition of H22

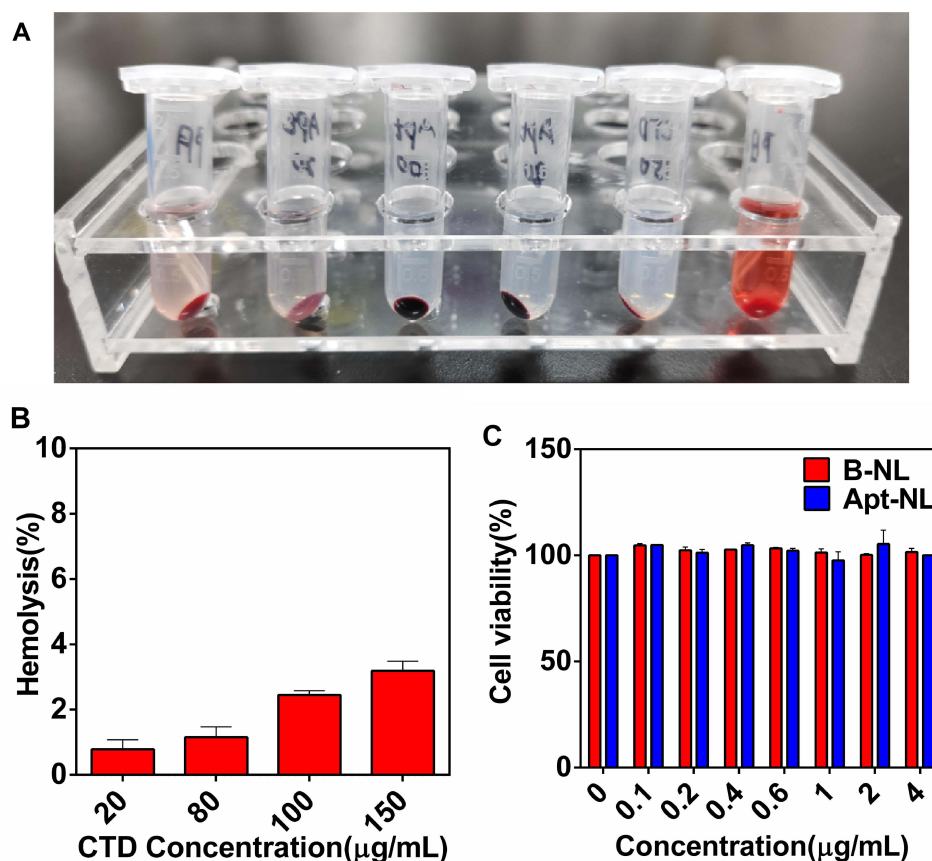


Figure 2 Biocompatibility of Apt/CTD-STS/NL. (A) Hemolysis test of fabricated nanoparticles ($n=3$). (B) Hemolysis ratio of fabricated nanoparticles ($n=3$). (C) Cell viabilities of blank nanoparticles in AML-12 cells ($n=6$). All data are expressed as the mean $\pm$ SD.

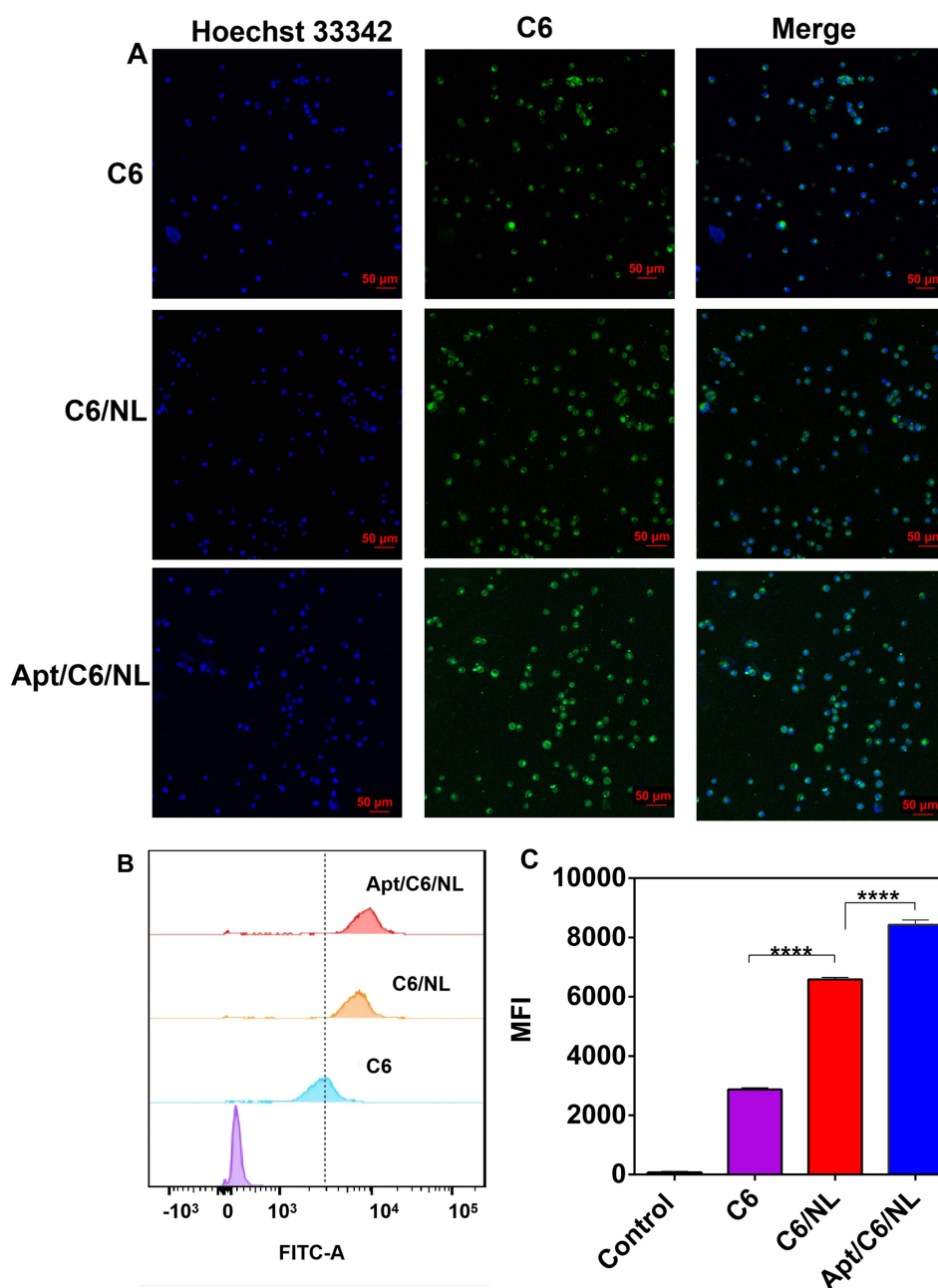


Figure 3 In vitro cellular uptake in H22 cells. (A) Fluorescence images of H22 cells incubated with different nanoparticles for 4 h. (B) FACS analysis of cellular uptake of the nanoparticles. (C) Quantification of cellular uptake of C6/NL and Apt/C6/NL. All data are expressed as the mean $\pm$ SD. (n = 3, **** $p < 0.0001$). Scale bar: 50 μ m.

cells by the aptamer against H22 cells, which enhanced the uptake of Apt/C6/NL by H22 cells. Quantitative fluorescence analysis of cellular uptake was performed by flow cytometry. The fluorescence intensity of the C6/NL and Apt/C6/NL groups was higher than free C6 group, with the strongest fluorescence intensity in the Apt/C6/NL group, which was 1.3-fold higher than that of the C6/NL group ($p < 0.001$) (Figure 3B and C). The results of flow cytometry and confocal fluorescence microscopy were consistent, both indicating that the aptamer-modified nanoliposome-targeted delivery system enhanced liposomes uptake by H22 cells. This enhanced cellular internalization is a direct consequence of aptamer-mediated active targeting and is pivotal for improving the subsequent therapeutic efficacy.

In vitro Anticancer Efficacy

The inhibitory effect of Apt/CTD-STS/NL on the proliferation of H22 cells was investigated using the CCK8 assay. CTD-STS exhibited higher cytotoxicity compared to CTD and STS at CTD mass concentrations of 0.05 ~ 0.5 $\mu\text{g/mL}$, suggesting that the combination of CTD and STS has a synergistic inhibitory effect on proliferation. Interestingly, The Apt/CTD-STS/NL and CTD-STS/NL showed stronger inhibition of cell proliferation than CTD-STS, whereas Apt/CTD-STS/NL showed stronger inhibition of cell proliferation than CTD-STS/NL. The greater inhibition of proliferation of H22 cells in the CTD-STS group at a concentration of 1 $\mu\text{g/mL}$ may be due to the fact that Apt/CTD-STS/NL and CTD-STS/NL failed to release all the drug, while free CTD-STS rapidly entered the tumour cells through passive diffusion and exerted synergistic killing effects. The IC_{50} of Apt/CTD-STS/NL was the smallest compared with the other groups (Figure 4A and B). This may be

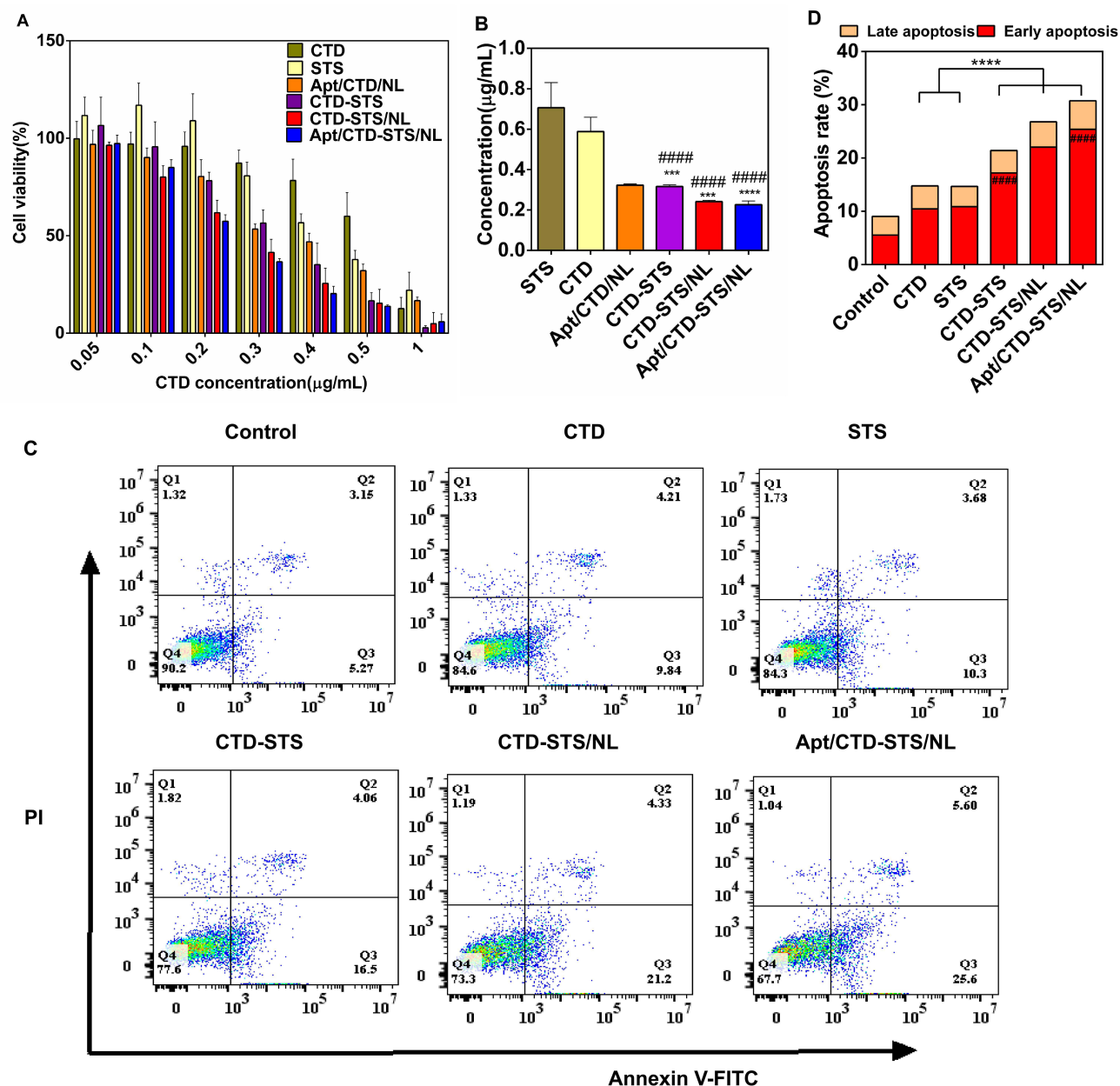


Figure 4 In vitro anticancer activity of Apt/CTD-STS/NL. (A) Cell viability of H22 cells treated with different preparations for 48 h. #: represents comparison with the STS group, *represents comparison with CTD, n = 6. (B) IC_{50} of each group (n = 3). (C) Representative scatter plots of Annexin V/PI analysis after H22 cells were treated with various CTD preparations. (D) Quantitative data on the apoptosis rate of H22 cells. #: represents comparison with the CTD-STS/NL group (n = 3). All data are expressed as mean $\pm$ SD. *** $p < 0.001$, ##### $p < 0.0001$, **** $p < 0.0001$.

attributed to the fact that modified aptamers specifically recognise H22 cells, enhancing the uptake of liposomes by tumour cells and improving the drug delivery efficiency, resulting in a better synergistic effect of CTD and STS inside the cells.

The apoptosis of H22 cells was detected by flow cytometry with Annexin V-FITC/PI double staining. The results of flow cytometry and fluorescence staining were consistent, as shown in [Figure 4C](#) and [D](#). The apoptosis rates of free CTD and free STS groups were lower than that of CTD-STS group, which indicated that the apoptosis rate of H22 cells could be significantly increased by the combination of CTD-STS. The apoptosis rate ($30.75 \pm 1.19\%$) of H22 cells in Apt/CTD-STS/NL group was the highest compared with other groups. This result indicated that the synergistic effect of CTD and STS can significantly enhance the induction of apoptosis in hepatocellular carcinoma cells, and the aptamer modification enhanced the enrichment of CTD-STS in H22 cells and enhanced apoptosis.

Study on Synergistic Mechanism

The mechanism of action of Apt/CTD-STS/NL synergistic anti-hepatocellular carcinoma was detected by Western blot. The results as shown in [Figure 5A–D](#), showed that the expression of VEGF, HIF-1 α and mTOR was significantly reduced compared with that of control group. Notably, HIF-1 α is a well-established upstream regulator of VEGF, acting as a key transcription factor that promotes VEGF expression under hypoxic conditions.⁴³ The concurrent downregulation of HIF-1 α and VEGF observed here suggests an effective disruption of this canonical signaling axis. Compared with the STS and CTD groups, the Apt/CTD-STS/NL group significantly down-regulated the expression of VEGF, HIF-1 α and mTOR, indicating that Apt/CTD-STS/NL could synergistically act on multiple targets of VEGF, HIF-1 α and mTOR to enhance the anti-hepatocellular carcinoma effect and improve the therapeutic effect of hepatocellular carcinoma. We propose that the core synergy stems from complementary mechanisms: CTD acts upstream by inhibiting PI3K/Akt to indirectly suppress mTOR, while STS directly targets mTOR kinase and independently destabilizes HIF-1 α . This multi-layered attack on the mTOR/HIF-1 α /VEGF axis comprehensively disrupts tumor proliferation, survival, and angiogenesis, providing a compelling rationale for the observed potent synergy.

In vivo Biodistribution Study

To study the biodistribution of liposomes in vivo, liposomes were labeled with DIR. In [Figure 6A](#), it can be observed that after 24 h, the fluorescence intensity of the liposome group was significantly stronger than that of the free DIR group, while the Apt/DIR/NL group exhibited the strongest fluorescence signal. This may be attributed to the enhanced circulation retention capacity of DIR in vivo facilitated by the liposomes. Additionally, we extracted major organs and tumor tissues from tumor-bearing mice for ex vivo fluorescence imaging and performed quantitative analysis ([Figure 6B](#) and [C](#)). The results revealed that fluorescence in each group was primarily distributed in the tumors and liver, with stronger fluorescence observed in the liposome group, and the highest fluorescence intensity in the Apt/DIR/NL group ($p < 0.05$). These findings suggest that liposomal encapsulation enhances drug accumulation at the tumor site, and aptamer modification further strengthens active targeting capability in vivo. This provides a foundation for the ability of Apt/CTD-STS/NL to target tumor sites, thereby improving drug delivery efficiency and reducing toxic side effects.

In vivo Antitumor Efficacy

To evaluate the anti-tumor effect of tumor-bearing mice in vivo, the mice were randomly divided into eight groups and given tail vein injections of different formulations. Normal mice from the normal control (NC) group were used as controls. There was no significant change of body weight in Apt/CTD-STS/NL Group, which indicated that nano-preparation had no acute toxicity and had good biological safety ([Figure 7A](#)). The results of tumour volume measurement in mice ([Figure 7B](#)), the tumour volume of mice in the PBS group grew rapidly, indicating the successful establishment of the mouse model. The tumour volume in the Apt/CTD-STS/NL group was the smallest compared with the other control groups, and the tumour growth was significantly inhibited. The results of the tumour inhibitory effect of each dosing group were shown in [Figure 7C–E](#). Apt/CTD-STS/NL had the strongest

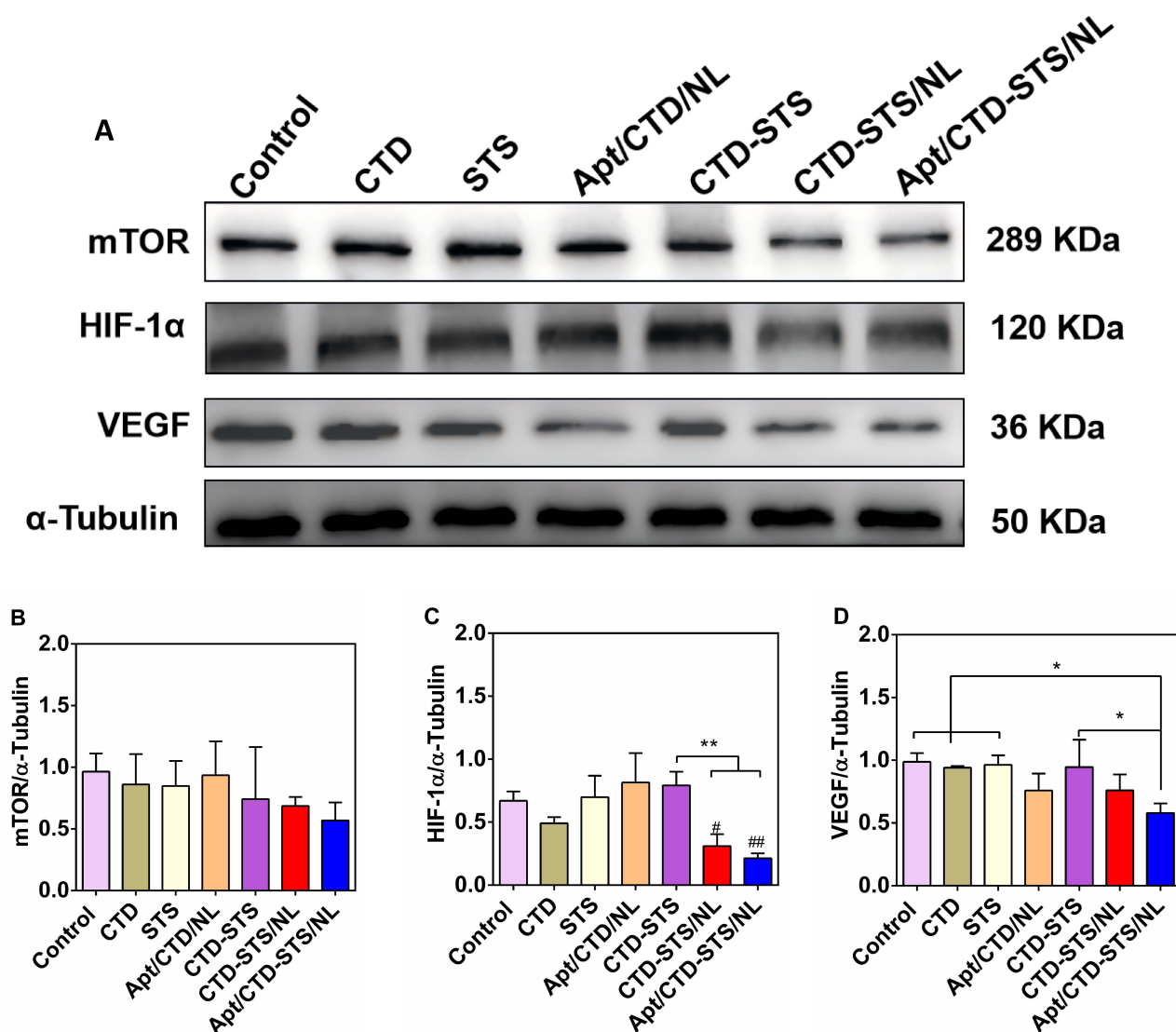


Figure 5 (A) Western blot assay of mTOR, HIF-1α and VEGF in H22 cells treated with different preparations respectively. The Western blot bands shown in the figure are representative of three independent experiments. **(B-D)** Quantitative results of mTOR, HIF-1α, and VEGF. All data are expressed as the mean ± SD. #: represents comparison with the control group (n = 3, *p < 0.05, **p < 0.01, #p < 0.05, ###p < 0.01).

tumour inhibitory effect, with a tumour inhibition rate of $(79.50 \pm 4.39)\%$. It was better than the 58.50% tumour inhibition in sorafenib group (Figure 7D and Table S3). The inhibitory rates of CTD and STS were $(70.67 \pm 9.83)\%$ and $(55.67 \pm 15.17)\%$ respectively. In contrast, the dose of Apt/CTD-STS/NL was lower than that of the free group, but had the strongest anti-tumor effect, indicating that the tumor inhibition rate of Apt/CTD-STS/NL was still better than that of the free group after the dose reduction, the synergistic effect of synergistic enhancement and attenuation can be achieved.

Tumour tissue was examined by H&E staining to evaluate the antitumour therapeutic efficacy of Apt/CTD-STS/NL. The degree of cell necrosis and nuclear consolidation was significantly higher in the Apt/CTD-STS/NL group, the CTD-STS/NL group and the sorafenib group than in the other groups (Figure 7F), suggesting that the Apt/CTD-STS/NL achieved optimal antitumor effects.

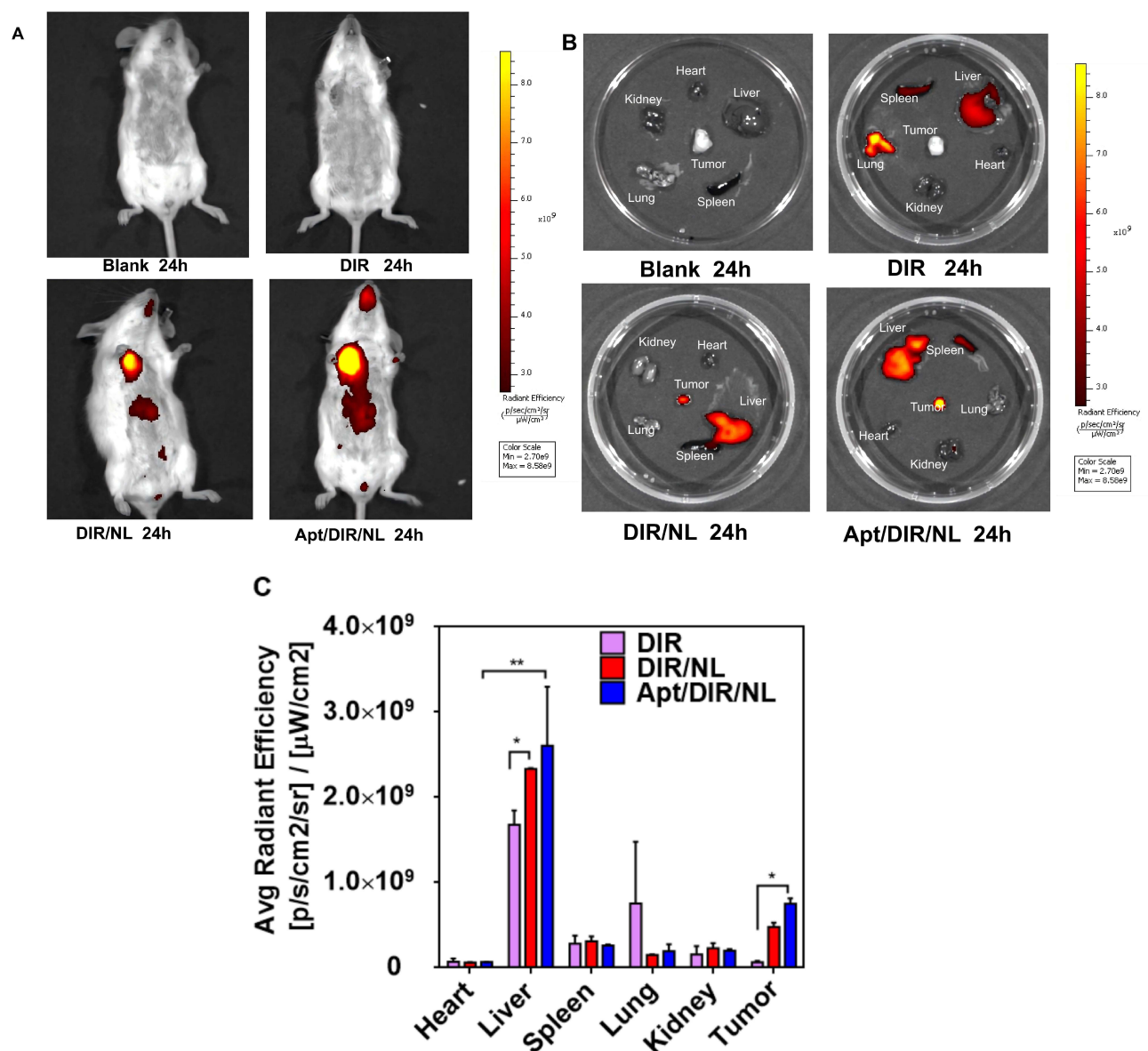


Figure 6 In vivo biodistribution. (A) Fluorescence images of tumor-bearing mice in vivo and (B) in vitro after different treatments. (C) The fluorescent intensity of tumors and organs after 24 h of injection. All data are expressed as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

In vivo Safety Evaluation

In order to evaluate the biosafety of Apt/CTD-STS/NL, blood routine, blood biochemical indexes, organ indices, and organ pathological sections were tested. The H&E results showed that the organs in the NC and PBS groups exhibited intact and well-arranged cellular structures, along with clear nuclear staining (Figure 8). Compared with the blank group, no pathological changes were observed in the heart, liver, spleen, lungs, or kidneys of mice in the CTD, STS, CTD-STS, Apt/CTD/NL, CTD-STS/NL, and Apt/CTD-STS/NL groups, indicating no significant damage or lesions in the major organs of the mice during the administration period. There was no significant change in the major organ indices of Apt/CTD-STS/NL compared to the PBS and NC groups (Figure S2), indicating that no systemic toxicity was induced. Compared with the NC group, the spleen index and thymus index of Apt/CTD-STS/NL were significantly higher, and the spleen and thymus were the largest immune organs in the body, which play an important role in regulating the immune function. The haematological parameters (Figure S3) in the Apt/CTD-STS/NL group were

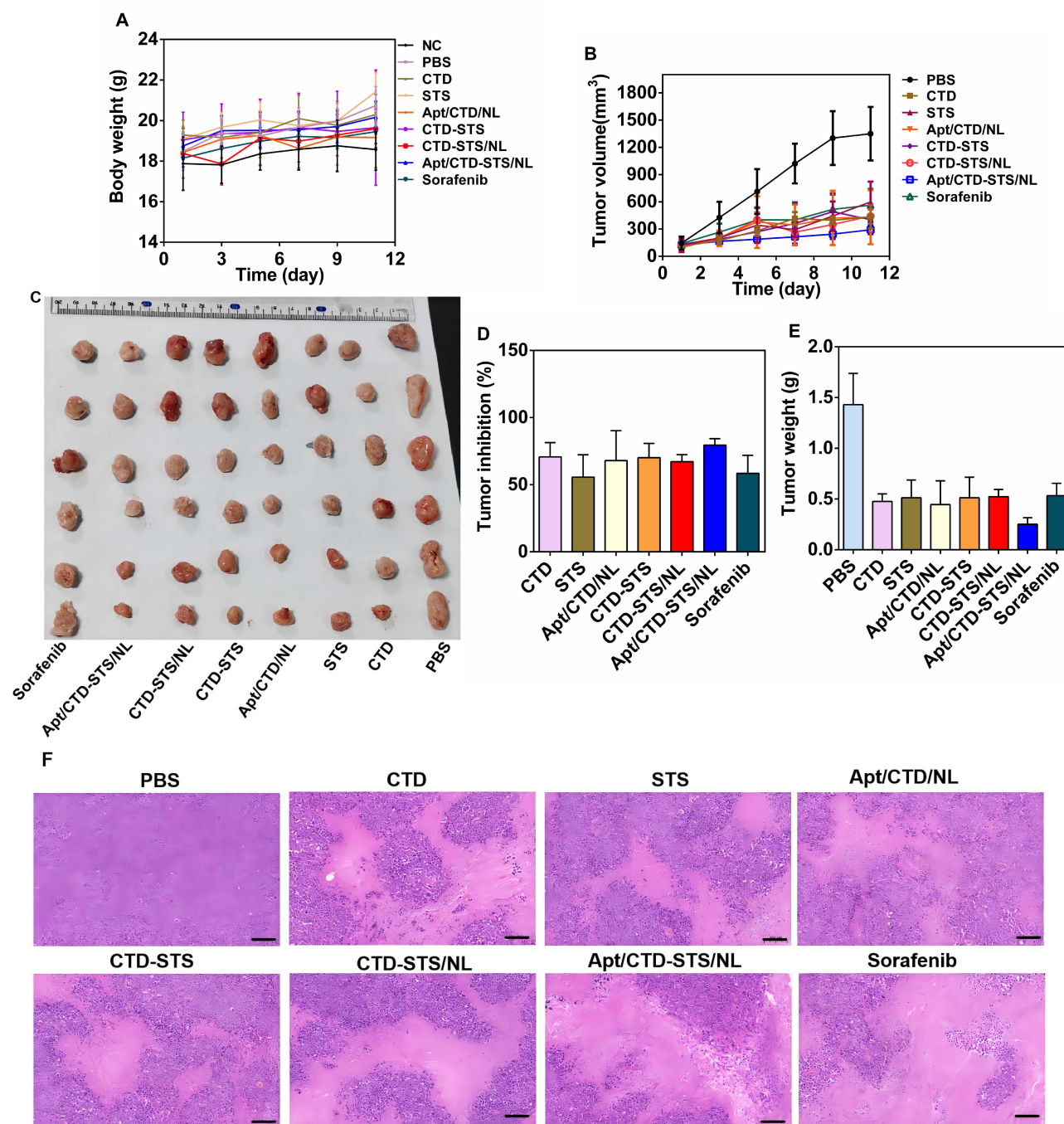


Figure 7 In vivo antitumor effects of Apt/CTD-STs/NL. (A) Body weight changes of mice (n = 6). (B) Tumor volume of each group (n = 6). (C) Tumor images of each group (n = 6). (D) Tumor inhibition of each group (n = 6). (E) Tumor weight of each group (n = 6). (F) H&E staining of tumors in each group at the end of treatment (Scale bar: 100 μ m). Data were presented as mean $\pm$ SD.

maintained at normal levels compared to the NC group, indicating no haematotoxicity. The analysis of serum biochemical indexes after treatment showed that the liver and kidney functions were within the normal range, indicating that there was no hepatorenal toxicity in Apt/CTD-STs/NL (Figure S4). In conclusion, Apt/CTD-STs/NL is a drug delivery system with high biosafety.

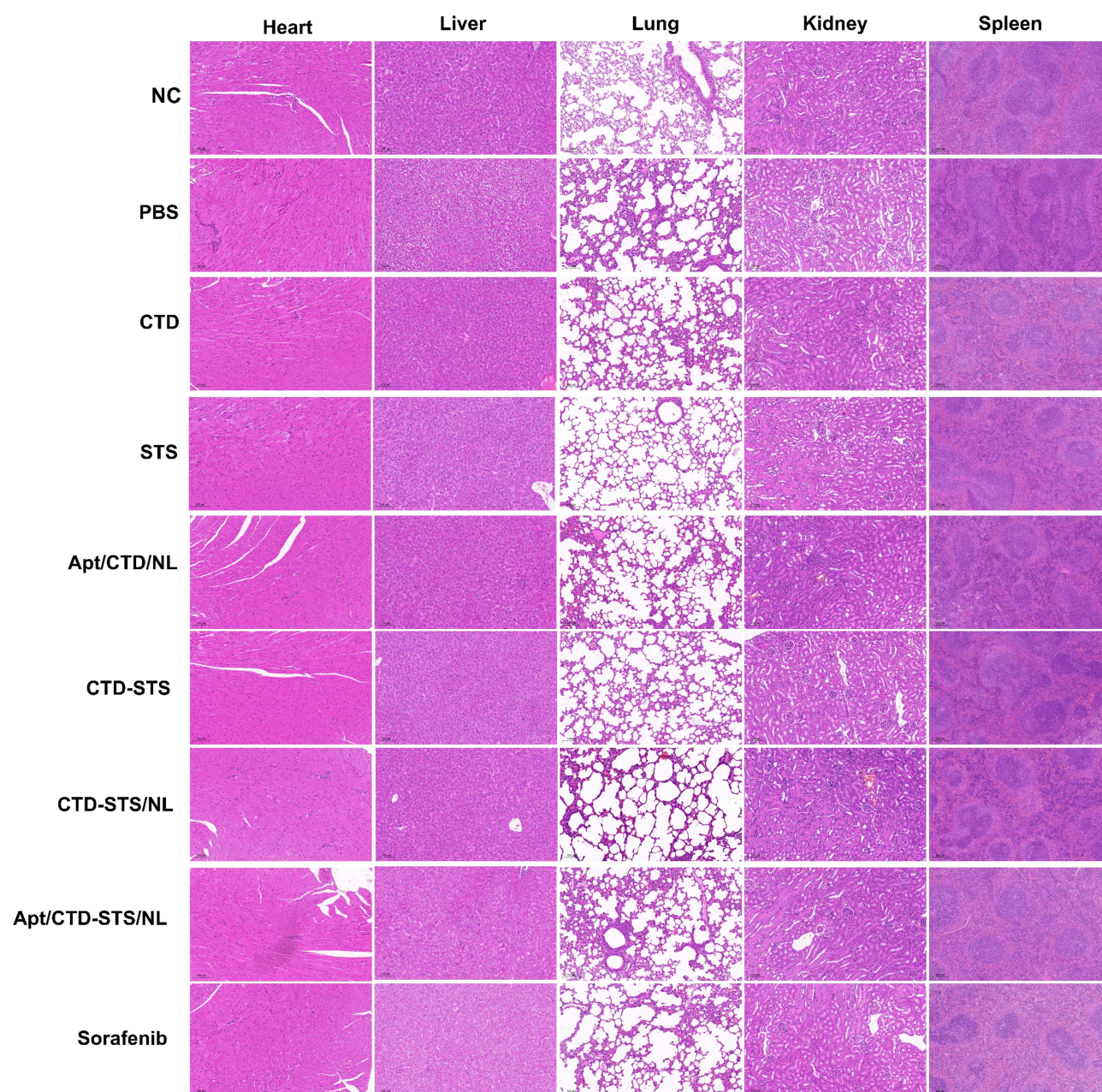


Figure 8 Histological evaluation for tissue damage of the major organs. Representative views for H&E of heart, liver, lung and kidney slices (scale bar: 100 μ m, The scale bar of spleen: 200 μ m).

Conclusion

In summary, we successfully designed and evaluated aptamer-modified nanoliposomes for the co-delivery of cantharidin (CTD) and staurosporine (STS) (Apt/CTD-STS/NL), which demonstrated effective tumor targeting and favorable biosafety. At an optimal synergistic mass ratio of CTD to STS was 0.5:0.85 (CTD: μ g/mL, STS:ng/mL), Apt/CTD-STS/NL significantly enhanced anti-HCC efficacy while reducing systemic toxicity at a lower administered dose. Mechanistic studies revealed that the combination synergistically suppressed the expression of key oncoproteins—mTOR, HIF-1 α , and VEGF—suggesting effective disruption of the mTOR/HIF-1 α /VEGF signaling cascade, in which HIF-1 α acts as a critical transcriptional regulator of VEGF.⁴³ This multi-targeting strategy not only strengthens therapeutic synergy but may also help overcome tumor drug resistance.

Notwithstanding these promising results, several limitations of this study should be noted. The specificity of the H22 aptamer, though supported by in vitro and in vivo targeting data, warrants further validation using a scrambled-sequence control. In addition, stability was assessed primarily under physiological pH; evaluation across a broader pH range simulating the tumor microenvironment would better predict in vivo performance. Lastly, while the multi-target mechanism implies potential to reverse drug resistance, direct evidence using established resistant HCC models remains to be established.

Future work will prioritize these aspects, including the use of resistant tumor models to systematically investigate the reversal of drug resistance. Collectively, this study establishes a foundational strategy for the development of targeted combination nanotherapies against hepatocellular carcinoma, with significant potential for translational advancement.

Ethics Approval and Consent to Participate

All animal experiments were conducted in strict accordance with the guide of the Laboratory Animal Welfare and Ethics Committee of Hunan University of Chinese Medicine (No. LL2022071302).

Acknowledgments

This work was supported by the Natural Science Foundation of Hunan Province (NO. 2023JJ50286), a Project Supported by Scientific Research Fund of Hunan Provincial Education Department (NO. 21A0255), the Hunan University of Chinese Medicine Research Grant (NO. 2020XJJ009), the Key Discipline Project on Chinese Pharmacology of Hunan University of Chinese Medicine [202302].

Disclosure

The authors declare no conflicts of interest.

References

- Li Z-Y, Shen Q-M, Wang J, et al. Prediagnostic plasma metabolite concentrations and liver cancer risk: a population-based study of Chinese men. *EBioMedicine*. 2024;100:104990. doi:10.1016/j.ebiom.2024.104990
- World Health Organization. The global cancer observatory: cancer today. 2022. Available from: <https://gco.iarc.fr/today/fact-sheets-cancers>. Accessed January 22, 2026.
- Finn RS, Zhu AX. Evolution of systemic therapy for hepatocellular carcinoma. *Hepatology*. 2021;73(Suppl 1):150–157. doi:10.1002/hep.31306
- Chai Y, Xu T, Chen X, et al. Immunotherapy targeting liver cancer tumor-initiating cells: challenges, mechanisms, and emerging therapeutic horizons. *Front Immunol*. 2025;16:1621243. doi:10.3389/fimmu.2025.1621243
- Li S, Chen X, Shi H, et al. Tailoring traditional Chinese medicine in cancer therapy. *Mol Cancer*. 2025;24(1):27. doi:10.1186/s12943-024-02213-6
- Kang C, Wang J, Li R, et al. Smart targeted delivery systems for enhancing antitumor therapy of active ingredients in traditional Chinese Medicine. *Molecules*. 2023;28(16):5955. doi:10.3390/molecules28165955
- Xie Y, Lei X, Zhao G, et al. mTOR in programmed cell death and its therapeutic implications. *Cytokine Growth Factor Rev*. 2023;71-72:66–81. doi:10.1016/j.cytogfr.2023.06.002
- Chen G, Tian TT, Wang FQ, et al. Chanling Gao suppresses colorectal cancer via PI3K/Akt/mTOR pathway modulation and enhances quality of survival. *Environ Toxicol: Int J*. 2024;39(3):1107–1118. doi:10.1002/tox.23994
- Liu LP, Ho RL, Chen GG, et al. Sorafenib inhibits hypoxia-inducible factor-1 alpha synthesis: implications for antiangiogenic activity in hepatocellular carcinoma. *Clin Cancer Res*. 2012;18(20):5662–5671. doi:10.1158/1078-0432.CCR-12-0552
- Yoon MS. The role of mammalian target of Rapamycin (mTOR) in insulin signaling. *Nutrients*. 2017;9(11):1176. doi:10.3390/nu9111176
- Harrington LS, Findlay GM, Gray A, et al. The TSC1-2 tumor suppressor control insulin-PI3K signaling via regulation of IRS proteins. *J Cell Biol*. 2004;166(2):213–223. doi:10.1083/jcb.200403069
- Wick KR, Werner ED, Langlais P, et al. Grb10 inhibits insulin-stimulated insulin receptor substrate(IRS)-phosphatidylinositol 3-kinase/Akt Signaling pathway by disrupting the association of IRS-1/IRS-2 with the insulin receptor. *J Biol Chem*. 2003;278(10):8460–8467. doi:10.1074/jbc.M208518200
- Julien LA, Carriere A, Moreau J, et al. mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. *Mol Cell Biol*. 2010;30(4):908–921. doi:10.1128/MCB.00601-09
- Cao S, Zhang W, Pan H, et al. Bioactive lipid-nanoparticles with inherent self-therapeutic and anti-angiogenic properties for cancer therapy. *Acta Biomater*. 2023;157:500–510. doi:10.1016/j.actbio.2022.12.022
- Zhu YJ, Zheng B, Wang HY, et al. New knowledge of the mechanisms of sorafenib resistance in liver cancer. *Acta Pharmacol Sin*. 2017;38(5):614–622. doi:10.1038/aps.2017.5
- Swagatika S, Tomar RS. Cantharidin downregulates PSD1 expression and inhibits autophagic flux in yeast cells. *FEBS Open Bio*. 2022;12(5):1017–1035. doi:10.1002/2211-5463.13196
- Song M, Wang X, Luo Y, et al. Cantharidin suppresses gastric cancer cell migration/invasion by inhibiting the PI3K/Akt signaling pathway via CCAT1. *Chem Biol Interact*. 2020;317:108939. doi:10.1016/j.cbi.2020.108939

18. Wang CQ, Zheng XT, Chen XF, et al. The optimal adjuvant strategy of Aidi injection with gemcitabine and cisplatin in advanced non-small cell lung cancer: a meta-analysis of 70 randomized controlled trials. *Front Pharmacol.* **2021**;12:582447. doi:10.3389/fphar.2021.582447
19. Shen M, Wu MY, Chen LP, et al. Cantharidin represses invasion of pancreatic cancer cells through accelerated degradation of MMP2 mRNA. *Sci Rep.* **2015**;5:11836. doi:10.1038/srep11836
20. Zhu K, Zhou L, Zou M, et al. Xia.18-GA-Suc modified liposome loading cantharidin for augmenting hepatic specificity: preparation, characterization, antitumor effects, and liver-targeting efficiency. *J Pharm Sci.* **2020**;109(6):2038–2047. doi:10.1016/j.xphs.2020.03.001
21. Zhu M, Shi X, Gong Z, et al. Cantharidin treatment inhibits hepatocellular carcinoma development by regulating the JAK2/STAT3 and PI3K/Akt pathways in an EphB4-dependent manner. *Pharmacol Res.* **2020**;158:104868. doi:10.1016/j.phrs.2020.104868
22. Ren Y, Kinghorn ADK. Antitumor potential of the protein phosphatase inhibitor, cantharidin, and selected derivatives. *Bioorg Med Chem.* **2021**;32:116012. doi:10.1016/j.bmc.2021.116012
23. Yang T, Yu R, Cheng C, et al. Cantharidin induces apoptosis of human triple negative breast cancer cells through mir-607-mediated downregulation of EGFR. *J Transl Med.* **2023**;21(1):597. doi:10.1186/s12967-023-04483-y
24. Yao H, Zhao J, Wang Z, et al. Enhanced anticancer efficacy of cantharidin by mPEG-PLGA micellar encapsulation: an effective strategy for application of a poisonous traditional Chinese medicine. *Colloids Surf B Biointerfaces.* **2020**;196:111285. doi:10.1016/j.colsurfb.2020.111285
25. Shao H, Dong L, Feng Y, et al. The protective effect of L-glutamine against acute Cantharidin-induced Cardiotoxicity in the mice. *BMC Pharmacol Toxicol.* **2020**;21(1):71. doi:10.1186/s40360-020-00449-8
26. Yadav SS, Prasad CB, Prasad SB, et al. Narayan. Anti-tumor activity of staurosporine in the tumor microenvironment of cervical cancer: an in vitro study. *Life Sci.* **2015**;133:21–28. doi:10.1016/j.lfs.2015.04.019
27. Ding Y, Wang B, Chen X, et al. Staurosporine suppresses survival of HepG2 cancer cells through Omi/HtrA2-mediated inhibition of PI3K/Akt signaling pathway. *Tumour Biol.* **2017**;39(3):1010428317694317. doi:10.1177/1010428317694317
28. Hua H, Kong Q, Zhang H, et al. Targeting mTOR for cancer therapy. *J Hematol Oncol.* **2019**;12(1):71. doi:10.1186/s13045-019-0754-1
29. Patel R, Islam SA, Bommareddy RR, et al. Simultaneous inhibition of atypical protein kinase-C and mTOR impedes bladder cancer cell progression. *Int J Oncol.* **2020**;56(6):1373–1386. doi:10.3892/ijo.2020.5021
30. Tee AR, Proud CG. Staurosporine inhibits phosphorylation of translational regulators linked to mTOR. *Cell Death Differ.* **2001**;8(8):841–849. doi:10.1038/sj.cdd.4400876
31. Schnier JB, Nishi K, Goodrich DW, et al. G1 arrest and down-regulation of cyclin E/cyclin-dependent kinase 2 by the protein kinase inhibitor staurosporine are dependent on the retinoblastoma protein in the bladder carcinoma cell line 5637. *Proc Natl Acad Sci USA.* **1996**;93(12):5941–5946. doi:10.1073/pnas.93.12.5941
32. Chabot-Fletcher M, Breton JJ. Effect of staurosporine on transcription factor nf-kb in human keratinocytes. *Biochem Pharmacol.* **1998**;56(1):71–78. doi:10.1016/s0006-2952(98)00117-8
33. Schnitzer SE, Schmid T, Zhou J, et al. Hypoxia and HIF-1 alpha protect A549 cells from drug-induced apoptosis. *Cell Death Differ.* **2006**;13(9):1611–1613. doi:10.1038/sj.cdd.4401864
34. Bohensky J, Shapiro IM, Leshinsky S, et al. HIF-1 regulation of chondrocyte apoptosis: induction of the autophagic pathway. *Autophagy.* **2007**;3(3):207–214. doi:10.4161/auto.3708
35. Wang CJ, Sun XQ, Feng LZ, et al. Rational design of a nonclassical liposomal nanoscale drug delivery system for innovative cancer therapy. *BME Mat.* **2024**;2(3):e12083. doi:10.1002/bmm2.12083
36. Fu Z, Xiang J. Aptamer-functionalized nanoparticles in targeted delivery and cancer therapy. *Int J Mol Sci.* **2020**;21(23):9123. doi:10.3390/ijms21239123
37. Soloman R, Gabizon AA. Clinical pharmacology of liposomal anthracyclines: focus on pegylated liposomal doxorubicin. *Clin Lymphoma Myeloma.* **2008**;8(1):21–32. doi:10.3816/clm.2008.n.001
38. Magar KT, Boafu GF, Xiaotong L, et al. Liposome-based delivery of biological drugs. *Chin. Chem. Lett.* **2022**;33(2):587–596. doi:10.1016/j.cclet.2021.08.020
39. Wu L, Wang Y, Xu X, et al. Aptamer-based detection of circulating targets for precision medicine. *Chem Rev.* **2021**;121(19):12035–12105. doi:10.1021/acs.chemrev.0c01140
40. Zhu G, Chen X. Aptamer-based targeted therapy. *Adv Drug Deliv Rev.* **2018**;134:65–78. doi:10.1016/j.addr.2018.08.005
41. Sameiyan E, Bagheri E, Dehghani S, et al. Aptamer-based ATP-responsive delivery systems for cancer diagnosis and treatment. *Acta Biomater.* **2021**;123:110–122. doi:10.1016/j.actbio.2020.12.057
42. Luo K, Yang L, Yan C, et al. A Dual-targeting liposome enhances triple-negative breast cancer chemoimmunotherapy through inducing immunogenic cell death and inhibiting STAT3 activation. *Small.* **2023**;19(40):e2302834. doi:10.1002/sml.202302834
43. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer.* **2003**;3(10):721–732. doi:10.1038/nrc1187

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