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*CORRESPONDENCE Yaling Li, Ilylapothecary@swmu.edu.cn Dan Zhang, Ilylapothecary@swmu.edu.cn

¹These authors have contributed equally to this work

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Efficient pH-sensitive nanosystem containing Atractylenolide I for improving tumor hypoxic microenvironment and photothermal conversion capability for synergistic treatment of colorectal cancer

Yuqing Zhu^{1†}, Qiang Ye^{2†}, Shi Feng¹, Xiujuan Fu¹, Siwei Chen¹, Hao Liu¹, Jialin Ning¹, Yaling Li^{1*} and Dan Zhang^{1*}

¹Department of Pharmacy of Traditional Chinese Medicine, School of Pharmacy, Southwest Medical University, Luzhou, Sichuan, China, ²State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources, Chengdu University of Traditional Chinese Medicine, Chengdu, China

Background: Photodynamic therapy (PDT) displays several beneficial characteristics, but it is still limited by the poor water solubility of photosensitizers, the insufficient targetability of the system, and the hypoxic microenvironment of tumors. It has been demonstrated that Atractylenolide I(ATL-1) not only alleviates the tumor hypoxic microenvironment but also has considerable potential in antitumor therapy. Hence the combination of the above two is anticipated to achieve more efficacious antitumor effects.

Methods: Nanosystems of pH-sensitive materials coated with Atractylenolide I and Ce6 were prepared by thin-film hydration method for targeted delivery to the acidic microenvironment of colorectal cancer. The morphology and stability of the nanosystems were evaluated by particle size, zeta potential, and phenology. The *in vitro* anti-tumor activity and mechanism of the nanosystems were evaluated using CCK-8 assay, live/dead cell double staining assay, ROS level, WB, and flow-through. Establishment of mouse ectopic colorectal cancer model. The safety, *in vivo* anti-tumor activity, and mechanism of the nanosystems were evaluated by mouse tumor volume change curves, biochemical indexes, and H&E staining.

Results: The results demonstrated that the prepared ATL-1/Ce6-pH Lip had desirable physicochemical properties, drug release behaviors, cellular uptake and cytotoxicity, satisfactory biocompatibility and *in vivo* antitumor effect.

Conclusion: This paper described the process of co-loading ATL-1 and Ce6 into pH-sensitive liposomes (ATL-1/Ce6-pH Lip), which utilized the tumor-specific acidic microenvironment to facilitate the release of drugs for the synergistic treatment of CRC. Our findings demonstrated that ATL-1 enhanced the oxygen content of the microenvironment, providing the essential raw material for the generation of ROS. At the same time, PDT was observed to obstruct blood vessels in tumor tissues, thereby damaging the blood supply to the tumor tissues.

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Additionally, PDT was demonstrated to destroy blood vessels in tumor tissues, leading to more exposure of the tumor cells to the drug environment. When it comes to the ATL-1/Ce6-pH Lip, which successfully combine the above two therapeutic approaches, chemotherapy and PDT treatment, a synergistic effect can be expected. In conclusion, the ATL-1/Ce6-pH Lip has considerable potential in the treatment of CRC.

KEYWORDS

CRC, ATL-1, Ce6, pH sensitive liposome, PDT

1 Introduction

Cancer is a significant cause of mortality that poses a significant threat to human health. Colorectal cancer (CRC) is the third most common malignant tumor in the world. In 2022, CRC became the second most deadly cancer (Sung et al., 2021). Treatments for CRC patients are currently conventional modalities, including surgery, chemotherapy, radiotherapy, and immunotherapy, *etc.* However, these approaches often induce severe side effects, invasiveness, and increased resistance to clinical chemotherapeutic agents, as well as nonspecific toxicity (Vodenkova et al., 2020). As a result, the treatment effect against CRC can hardly meet the expectation, and a novel therapeutic approach is urgently needed (Shinji et al., 2022).

Among the innovative therapies developed in recent years, photodynamic therapy (PDT) shows several advantages in the treatment of CRC, including minimal invasiveness, reduced systemic toxicity and restrained drug resistance. PDT primarily damages tumor cells by activating photosensitizers (PSs) to generate reactive oxygen species (ROS). However, ROS have a short half-life of less than 40 nanoseconds and are only effective for a limited distance of less than 20 nm after they are generated (van Straten et al., 2017). Therefore, it is preferable to deliver PSs to the key subcellular organelles that are more sensitive to ROS rather than just to tumor cells. It has been shown that Chlorin e6 (Ce6) is primarily localized on the endoplasmic reticulum and is activated by light to generate hydroxyl radicals that affect the synthesis of endoplasmic reticulum proteins and other proteins, resulting in over 90% mortality of tumor cells. In addition, Ce6 has low toxicity and no drug resistance (Hak et al., 2023; Gilson et al., 2019).

Despite the numerous advantages of Ce6-mediated PDT for the treatment of CRC, Ce6 exhibits poor water solubility, low targeting, and difficulty in treating deep tumors due to the low luminescent tissue penetration (Gunaydin et al., 2021; Dinakaran and Wilson, 2023). However, there is a significant limitation on the efficacy of PDT since the cytotoxic mechanism of PDT is the conversion of nontoxic oxygen into toxic ROS, which demonds substantial oxygen that is lack in the tumor microenvironment (Li et al., 2021). Consequently, there is an increasing interest in combining PDT with other therapeutic modalities, such as chemotherapy, with the aim to improve the tumor hypoxic microenvironment and enhancing the targeting behavior in order to achieve synergistic effects, thereby ameliorating the therapeutic efficacy of PDT (Choi et al., 2022).

Recently, researches on traditional Chinese medicines (TCM), particularly with regard to their potential anticancer properties, have yielded encouraging results (Zhou et al., 2023; Ge et al., 2022). Atractylenolide I (ATL-1), the primary active ingredient of the traditional Chinese medicine *Rhizoma Atractylodis* Macrocephalae, has shown promising results against cancer (Bailly, 2021). Studies have demonstrated that ATL-1 inhibits mitochondrial fission and induces mitochondrial dysfunction and apoptosis, which to a certain extent increases the oxygen content for ROS generation and improves the efficiency of PDT (Wang et al., 2009; Sun et al., 2021; Jia et al., 2023; Fukai and Ushio-Fukai, 2020). In addition to this, ATL-1 inhibits tumor cell proliferation through multiple pathways and is effective in inhibiting CRC (Ren et al., 2021; Wang et al., 2020; Qin et al., 2021; Tang et al., 2020). Concurrently, PDT can destroy blood vessels in tumor tissues (by damaging the blood supply to tumor tissues) and expose more tumor cells to the drug environment, thereby enhancing the efficacy of the treatment. However, the therapeutic efficacy of these agents is often limited by their poor water solubility and targeting ability. The rapid development of oncology and nanomaterials calls for the design of a variety of responsive nanocarrier drug delivery systems (NDDS) to provide new strategies for oncology therapy. These systems can efficiently carry and specifically deliver anticancer drugs to the tumor site and increase drug bioavailability (Da Silva et al., 2020).

The tumor microenvironment is distinguished from the normal physiological environment on account of a relatively low oxygen content and pH value (Wu and Dai, 2017). Of the above two characteristics, the acidic micro-environment is prevalent in various tumors and plays a pivotal role in the generation and development of tumors, and also contributes a lot in drug resistance restraining. Therefore, tumor microenvironment-responsive nanodrug carriers have demonstrated remarkable efficacy in controlling drug release at tumor sites, removal of carrier protective shells, and tumor targeting (Xu et al., 2023b; Tian et al., 2022). Based on the acidic microenvironment, the pH-responsive drug delivery system is designed. That is, the system is stable under normal physiological conditions, while after the carrier reaches the tumor site, the acidic microenvironment causes the carrier to undergo a series of variations, such as dissolution, surface charge flipping, and chemical bond rupture, which increases the accumulation of the carrier in the tumor, promotes the cellular uptake, resulting in the specific release of the drug, and eventually enhance the therapeutic effect of the chemotherapeutic drug and reduce the toxic side-effects (Gyanani and Goswami, 2023; Leng et al., 2023; Kansız and Elçin, 2023). According to this, we took advantage of the acidic nature of the tumor microenvironment to construct pH-sensitive drug-delivery liposomes for a better utilization and targeting of Ce6 and ATL-1 (Plana et al., 2022).

This paper described the construction of ATL-1 and Ce6 cocoated pH-sensitive liposomes (ATL-1/Ce6-pH Lip) for the inhibition of CRC. The ATL-1/Ce6-pH Lip can precisely release Ce6 and ATL-1 in an acidic tumor environment. Upon irradiation with a low-dose laser (808 nm), the PDT was found to damage the blood vessels in the tumor tissues, which disrupted the blood supply to the tumor tissue, exposing more tumor cells to the drug environment. Meanwhile, ATL-1 provides raw materials for ROS generation to interfere the function of mitochondria. This allows for the safe and efficient treatment of combining antitumor drugs with PDT, inhibiting the growth and development of CRC, which has a broad prospect of application in the precision treatment of tumors.

2 Materials and methods

2.1 Materials

Atractylenolide I (ATL-1, MW 230.30). Soybean Lecithin were purchased from Beijing Xinshengke Technology Co. LTD. (CAS). Cholesterol (CHOL, MW 386.65) was from J&K Scientific (Beijing, China). PhospHatidyl ethanolamine (PE, MW 744.03), and Cholesteryl hemisuccinate (CHEMS, MW 486.73) were purchased from Sigma-Aldrich. Soybean Lecithin (MW 758.06) was purchased from Qingdao Rishui Bio-technologies Co., Ltd. Chlorin e6 (Ce6, MW 596.67) were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. 0.01 M PBS (powder, pH 7.2–7.4) were purchased from Shanghai Acmec Biochemical Co., Ltd. Fetal bovine serum (FBS), and Dulbecco's modified Eagle medium (DMEM) were purchased from GIBCO (Thermo Fisher Scientific, Carlsbad, California, United States).

The Cell Counting Kit-8 (CCK-8) was obtained from APExBIO Technology (Houston, TX, United States). Antibodies were obtained from Abcam (Cambridge, United Kingdom) against Bcl-2 (ab196495). Penicillin-streptomycin Solution (Biosharp). Methylene chloride and methanol were purchased from Shanghai Macklin Biochemical Co., Ltd.

2.2 Cells and animals

The HCT116 cell line was obtained from the Pharmacology Laboratory at the School of Pharmacy, Southwest Medical University in China. Male Balb/c nude mice, aged 4–6 weeks and weighing 20 \pm 2 g, were purchased from Gempharmatech Co., Ltd. Certificate of Conformity No. SYXK (Chuan) 2023–034. The animals were reared and tested in accordance with the Ethical Guidelines for the Welfare of Experimental Animals of Southwest Medical University and approved by the Institutional Animal Care and Ethics Committee of Southwest Medical University (License No. 20220624–009). To establish a xenograft colorectal cancer mouse model, 15 × 10⁵ HCT116 cells were injected into the right axillary subcutis of nude mice.

2.3 Preparation and characterization of ATL-1/Ce6-pH Lip

To prepare ATL-1/Ce6-pH Lip, we mixed the lipid material, Ce6, and ATL-1 in dichloromethane (5 mL) at a mass ratio of 7:1:3:1. The mixed solution was evaporated on a rotary evaporator at 45°C to form a lipid film. The lipid film was then rehydrated in PBS (10 mL) for 10 min and sonicated for 2 min with a probe of 65 W , All preparations were filtered through a 220 nm membrane prior to the experiment to remove unencapsulated drug.

Particle size and zeta potential were measured using dynamic light scattering (Nano ZS 90, Malvern, Malvern city, United Kingdom). The samples were diluted to 2 mg/mL with PBS, dispersed onto a copper grid, stained with 10 μ L of 1% (v/v) phosphotungstic acid in PBS, and then dried in air before being evaluated for surface topography, size, and morphology using a transmission electron microscope (JEM-200CX, JEOL, Tokyo, Japan).

In order to obtain the irradiation conditions for the administration of ATL-1/Ce6-pH Lip, we placed different concentrations of ATL-1/Ce6-pH Lip under an 808 nm laser with a power of 1.0 W/cm³ and determined the optimal experimental conditions by comparing the heating of the preparation after irradiation for different times.

2.4 Drug loading efficiency (DLE)

The drug encapsulation efficiency (DEE) is the percentage of drug encapsulated in the lipid bilayer of liposomes, relative to the total drug delivery. This rate is used to evaluate the quality of liposomes and to screen for the optimal formulations and processes. It is a key indicator of the effectiveness of drug encapsulation in liposomes. On the other hand, drug loading efficiency (DLE) is used to assess the quality of liposomes, which also facilitates the formulation and the processing optimization. The DLE and DEE of ATL-1/Ce6-pH Lip were determined using the centrifugation method (1×10^4 r/min, 20 min). The 1 mL of the supernatant was precisely extracted and adjusted to 10 mL. The peak area was obtained by injecting and measuring the samples, and the concentration of free ATL-1 was calculated as c. The volume of ATL-1/Ce6-pH Lip was recorded as V. Then, the mass of free ATL-1 was calculated as $W = c \times V \times dilution$, and the mass of ATL-1 was known to be M. The concentration of ATL-1 was determined using high-performance liquid chromatography with a mobile phase of acetonitrile and 0.2% phosphate buffer (80:20, v/v) and a measurement wavelength of 274 nm. DLE and DEE were calculated as follows:

$$\label{eq:def} \begin{split} DEE\,\% &=\,(M-W)\big/M\times100\,\%\\ DLE\,\% &=\,(M-W)\big/\,(M+Amount~of~material)\times100\,\% \end{split}$$

2.5 In vitro drug release investigation

The dialysis method was used to assay the release of ATL-1 from ATL-1/Ce6-pH Lip *in vitro*. ATL-1/Ce6-pH Lip (2 mL, 0.5 mg/mL of ATL-1) in PBS was placed in a dialysis bag with a molecular weight cut-off of 10 kDa (Shanghai Titan Technology), which was immersed in 20 mL of PBS (pH adjusted to 7.4, 6.5, 5.0) containing 0.5% Tween 80 with stirring (100 rpm). At the indicated time points, an aliquot (100 μ L) was withdrawn and replaced with the same volume of fresh PBS. The aliquot was

assayed for ATL-1 using high-performance liquid chromatography, and the cumulative amount of drug released was calculated.

2.6 Stability evaluation of liposome

The stability of freshly prepared ATL-1/Ce6-pH Lip was investigated by dynamic light scattering respectively in PBS, distilled water, FBS and after storage at 4 $^{\circ}$ C for 1 week in the untreated state.

2.7 Cellular uptake study

Confocal Laser Scanning Microscope (CLSM, TCS SP2, Leica) and flow cytometer (FCM, Becton Dickinson, Franklin Lakes, NJ, United States) were used to observe the uptake of ATL-1/Ce6pH Lip by HCT116 cells. HCT116 cells were first cultured overnight in 24-well plates (5×10^4 cells per well) placed with coverslips. Subsequently, cells were exposed to ATL-1/Ce6-pH Lip (808 nm), free Ce6 (808 nm) and incubated for 2 h. In all case, the Ce6 concentration was 0.1 mg/mL. After being washed 2–3 times with PBS, the cells were fixed with 4% paraformaldehyde for 30 min and stained with DAPI (blue) for 5 min. The intracellular localization and cell internalization were visualized using CLSM. For flow cytometry, the cells were collected and resuspended after being washed with PBS, and the uptake of cells was measured by FCM.

2.8 In vitro cytotoxicity efficacy

The cytotoxicity of ATL-1 and PDT was investigated with CCK-8 assay on HCT116 cells, which were cultured in 96-well plates (8,000 cells per well) and incubated in different concentrations of ATL-1, ATL-1 Lip, Ce6 (light/no light), Ce6 Lip (light/no light), or ATL-1/Ce6-pH Lip (light/no light). Next, cells were irradiated with laser at 808 nm at 1.0 W/cm³ for 5 min. The IC₅₀ value was obtained to evaluate the synergistic effect of the ATL-1/Ce6-pH Lip treatment.

2.9 Assessment of the ROS generation ability

HCT116 cells were seeded in 12-well plates (10×10^4 cells per well) and placed in an incubator overnight to acquire complete adhesion of the cells to the wall. Respectively, different groups were set up, that is the ATL-1 group, ATL-1 Lip group, Ce6 (light/no light) group, Ce6 Lip (light/no light) group and ATL-1/Ce6-pH Lip (light/no light) group. The light group was exposed to light (808 nm, 5 min, 1.0 W/cm³) after 4 h of incubation. Subsequently, the cells were incubated in a constant temperature incubator in the dark for 12 h. The 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) working solution (2μ M) was prepared before use. The 12-well plates were removed, 1 mL of working solution was added to each well after cleaning and incubated in a constant temperature cell incubator at 37°C in the dark for 30 min. The working solution was removed by suction, the cells were washed 2–3 times with PBS, and the

intracellular fluorescence was observed under an inverted microscope.

2.10 Live/dead cell staining assay, and apoptosis evaluation by flow cytometry

To observe the anti-tumor effect of ATL-1/Ce6-pH Lip, HCT116 cells were cultured respectively in 12-well plates (10 \times 10⁴ cells per well) with ATL-1, ATL-1 Lip, Ce6 (light/no light), Ce6 Lip (light/no light), ATL-1/Ce6-pH Lip (light/no light), respectively. After illumination and culture according to the method under "2.9", 1 mL of working solution containing Calcein-AM (1 μ M) and propidium iodide (PI, 5 μ M) was added respectively. After incubation, the cell survival rates in each group were compared by fluorescence microscopy. The *in vitro* properties of ATL-1/Ce6-pH Lip were further investigated.

2.11 Western blotting analysis

HCT116 cells were seeded into 6-well plates $(10 \times 10^4$ cells per well), incubated for 24 h, then treated for 24 h with various drug formulations. As a control, HCT116 cells were cultured alone and treated the same way. Cells were harvested, lysed with radio-immunoprecipitation assay (RIPA) buffer, microcentrifuged to pellet debris, and the supernatant was fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were incubated 12 h with primary rabbit antibodies against Bcl-2 or caspase 3, followed by incubation for 1 h at 25°C with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. Antibody binding was detected using enhanced chemiluminescence (Image Quant LAS 4000 Mini; Fuji, Tokyo, Japan) and quantified using Gel Image System 4.00 (Tanon, Shanghai, China).

2.12 In vivo dynamic biodistribution

HCT116 cells were subcutaneously injected into the right armpit of nude mice, and the tumor volumes (length \times width²/2) were measured by caliper. When the tumor volume reached about 50 cm³, free Ce6 and ATL-1/Ce6-pH Lip were injected into the tail vein of the mice. *In vivo* imaging (IVIS Spectrum, Perkin Elmer, United States) was used to observe the biodistribution and tumor targeting of the latter two in nude mice at different time points.

2.13 In vivo antitumor effect

Given the excellent chemical activity of ATL-1/Ce6-pH Lip *in vitro* and its good pharmacokinetics and biodistribution *in vivo*, we further tested its anti-tumor activity in an ectopic tumor bearing mouse model. After subcutaneous injection of HCT116 cells into the right armpit of nude mice, the tumor volumes (length × width $^{2}/_{2}$) were measured using calipers. When the tumor volume reached approximately 50 cm³, the mice were randomly divided into eight groups (blank group, ATL-1 group, ATL-1 Lip group, Ce6 (808 nm) group, Ce6 Lip (808 nm) group,



ATL-1/Ce6-pH Lip (light/no light) group) and injected into the tail vein every other day. Six consecutive doses were administered. At the end of the 12th h, the intra-tumor ATL-1/Ce6-pH Lip accumulation peaked, so the ATL-1/Ce6-pH Lip illumination group was laser irradiated at the subcutaneous tumor site 12 h after intravenous injection. After the last administration, the mice were executed, and the tumor tissues were removed, weighed and photographed. Five tissues were collected, and H&E staining was used to examine the histopathological characteristics of the tissues and tumor sections. The cell apoptosis and tumor proliferation were observed by TUNEL and Ki67 immunochemical staining.

2.14 Statistical analysis

The data were recorded as mean \pm standard deviation (SD) and analyzed using GraphPadPrism 8.0.1. The pairwise differences were assessed using the two-tailed Student's t-test, while the differences among the three or more groups were assessed using one-way analysis of variance.

3 Results

3.1 Preparation and characterization of ATL-1/Ce6-pH Lip

Prolonging blood circulation is an important way to make the intravenous nanomedicine more effective (Li et al., 2020). Researches have indicated that nanoparticles (NPs) with a diameter over 200 nm are susceptible to clearance by monocytes and the reticuloendothelial system. Conversely, NPs with smaller particle sizes (<200 nm) exhibit enhanced drug accumulation in tumor cells through augmented permeability and prolonged retention (Xu et al., 2023a; Blanco et al., 2015). DLS showed that the diameter of ATL-1/Ce6-pH Lip was 159.53 ± 19.55 nm (n = 3) (Figure 1A). The zeta potential of ATL-1/Ce6-pH Lip was $-40.79 \pm$ 3.06 mV (n = 3), and PDI was 0.213 ± 0.013 (n = 3). This suggests that ATL-1/Ce6-pH Lip is monodispersed and has a suitable diameter for tissue extravasation. As confirmed by TEM images, the morphology of ATL-1/Ce6-pH Lip was homogeneous and spherical, with diameters ranging from 50 to 100 nm, and showed aggregation or slight enlargement due to drug encapsulation compared to naked liposomes. This was in good agreement with the DLS results (Figures 1B,C), indicating that ATL-1/Ce6-pH Lip has a good morphological size. The DEE of ATL-1 in ATL-1/Ce6-pH Lip was 94.37% \pm 0.58% (n = 3) as determined by centrifugation, and the DLE was 23.2% \pm 0.1%.

It has been shown that a spherical supramolecular structure with a uniform size distribution contributes to the stability of ATL-1/Ce6pH Lip. The changes in size and Zeta potential of ATL-1/Ce6-pH Lip were assessed through dynamic light scattering studies after 1 week under various solvent conditions, as depicted in Figure (Figures 2A,B). It was observed that there were no significant alterations in the size and Zeta potential of ATL-1/Ce6-pH Lip, indicating its excellent stability. The release of ATL-1 in vitro by ATL-1/Ce6-pH Lip (light/no light) in the pH releasing medium that mimicked the normal physiological environment, tumor environment, and lysosomal environment is shown (Figure 2C). By comparing the amount of drug released at the same time point, it can be seen that the preparation released steadily under the condition of pH = 5.0, which prove that the nano-preparation has sensitive pH responsiveness. Under the light condition, ATL-1/Ce6pH Lip had a larger initial rate and a faster release rate. After 12 h, the release of ATL-1 tended to be stable, and at the last 72 h, the maximum release of ATL-1/Ce6-pH Lip was 95.17% ± 4.40% (n = 3) at pH 5.0 after light exposure.

After irradiating the drug formulations using 808 nm laser, the warming of the different groups is shown in Figure 3, where the PBS group only warmed up to 10.1°C, whereas ATL-1/Ce6-pH Lip at a concentration of 20 μ g/mL Ce6 reached a temperature of 49.2°C after irradiation for 5 min. Considering the acclimatization of the mice, the final light condition was determined to be 1.0 W/cm³ for 5 min (Figures 3A,B).

3.2 Cellular uptake

Since Ce6 itself is fluorescent, its fluorescence was used to follow the internalization of nanoparticles by CLSM and FCM in HCT116 cells ($\lambda_{ex} = 633$ nm, $\lambda_{em} = 650-710$ nm) (Gilson et al., 2019; Tan et al., 2016). First, we examined the cellular uptake capacity of ATL-1/Ce6-pH Lip using CLSM, as shown in Figure 4A, where a clear red fluorescence was detected in the cytoplasm after 2 h of incubation. FCM results showed that ATL-1/Ce6-pH Lip was efficiently taken up by HCT116 cells,



and the uptake of ATL-1/Ce6-pH Lip was significantly higher than that of the free Ce6 (Figure 4B).

groups, indicating that ATL-1/Ce6-pH Lip exhibited excellent anticancer activity.

3.3 Effect of NPs combined with PDT on HCT116

The chemo-PDT cytotoxicity of ATL-1/Ce6-pH Lip was determined by CCK-8 assay. The IC_{50} of blank group, ATL-1 group, ATL-1 Lip group, Ce6 (808 nm) group, Ce6 Lip (808 nm) group, ATL-1/Ce6-pH Lip (light/no light) group at 24 h were 51.05, 27.91, 36.84, 32.41, 17.46, 15.84 (µg/mL), respectively (Figures 5A,B). It can be seen from the figure that after 24 h of incubation, the anticancer activity of ATL-1/Ce6-pH Lip+808 nm laser irradiation group was the strongest followed by the ATL-1/Ce6-pH Lip (no light) group, ATL-1 Lip group, Ce6 Lip (808 nm) group, Ce6 (808 nm) group and ATL-1 group. In addition, a concentration-dependent manner was observed in the preparation

3.4 Assessment of the ROS generation ability

The extent of ROS generated after 808 nm laser irradiation induced by ATL-1/Ce6-pH Lip was assessed using the DCFH-DA in PBS solution. The results demonstrated that ATL-1 Lip and ATL-1/ Ce6-pH Lip under 808 nm laser irradiation produced strong green fluorescence, suggesting a greater ROS production. However, the control group, ATL-1 group, Ce6 (808 m) group, Ce6 Lip (808 nm) group, and ATL-1/Ce6-pH Lip (no light) group failed to exhibit a significant green fluorescence. The results indicated that ATL-1 was capable of inducing the generation of ROS, and under 808 nm laser irradiation, ATL-1/Ce6-pH Lip was able to produce a higher amount of ROS, thus exerting an inhibitory effect (Figure 6A). Figure 6B shows the results of semi-quantitative analysis of the mean





Uptake of drug formulations by HC1116 tumor cells. (A) Confocal micrographs showing uptake of drug formulations by HC1116 cells cultured a Scale bar, 50 µm. (B) FCM image of Ce6 fluorescence intensity.

fluorescence intensity of the different drug formulation groups with the following formula:

Mean fluorescence intensity (Mean)

= Sum of fluorescence intensity of the area (IntDen) / Area of the area (Area)

3.5 Live/dead cell staining assay

As shown in Figure 7, the ATL-1/Ce6-pH Lip +808 nm group had more red fluorescence (that is, dead cells) and less green

fluorescence (that is, surviving cells). The intensity of red fluorescence in each treatment group was in the order of: ATL-1/Ce6-pH Lip (808) group > ATL-1/Ce6-pH Lip group > Ce6 Lip (808 nm) group > ATL-1 Lip group > Ce6 (808 nm) group > Ce6 Lip group > Ce6 group > ATL-1 group > blank group. In the control group, more obvious green fluorescence was observed, without red fluorescence. The results showed that ATL-1/Ce6-pH Lip had a significant anticancer effect when irradiated with 808 nm laser. The anti-tumor activity of the groups treated with ATL-1 and Ce6 alone was remarkably less than that of ATL-1/Ce6-pH Lip after illumination, indicating that the drug of this preparation has a strong synergistic effect with PDT.



FIGURE 5

Cytotoxicity of different drug formulations against HCT116 tumor cells. (A,B) Cytotoxicity was assessed using the Cell Counting Kit-8 assay with HCT116 tumor cells cultured with (A) ATL-1, (B) ATL-1 Lip, Ce6 (808 nm), Ce6 Lip (808 nm), Lip or Lip (808 nm). Data are mean \pm SD (n = 5). ****p < 0.0001, ***p < 0.001, *p < 0.05.



3.6 Western blotting analysis

Bcl-2 is one of the key proteins in the regulation of cell apoptosis, whose high expression inhibits apoptosis and accelerates cell growth, which in turn leads to the development of malignant tumors, and thus is closely related to the generation, development and treatment of tumors. In contrast, caspase 3, a apoptosis indicator of cellular function, belongs to the critical execution proteins downstream of apoptosis. Both the cellular Bcl-2 and Caspase 3 protein expression levels were detected by Western blot method after treatment of HCT116 cells with different preparations (Figure 8A). Semi-quantitative results showed that ATL-1/Ce6-pH Lip group could effectively inhibit Bcl-2 protein expression and upregulate Caspase 3 protein



FIGURE 7

Cytotoxicity in tumor cells *in vitro*. Cytotoxicity against HCT116 monocultures was assessed through staining with calcein-AM and propidium iodide. Live cells appear green; dead cells, red. Scale bar, 100 μ m, n = 3.



expression, thus displaying anti-tumor effects, further verifying the cytotoxicity results of CCK-8 (Figures 8B,C). The results demonstrated that ATL-1/Ce6-pH Lip was able to reduce the expression of anti-apoptotic protein Bcl-2 and increase the expression of pro-apoptotic protein Caspase 3.

3.7 In vivo dynamic biodistribution

The accumulation of nanoparticles in the tumor will affect the final therapeutic effect. The biodistribution of ATL-1/Ce6-pH Lip and free Ce6 in Babl/c nude mice carrying ectopic colorectal cancer



Antitumor efficacy of drug formulations *in vivo*. HCT116 cells were injected into the right axilla of nude mice; after the tumors had reached the appropriate size, the animals were given systemic injections of the drug formulations described in Figure 5. "Control" animals were injected with PBS. **(A)** Photographs of tumors excised on day 12 after injection. **(B)** Tumor growth curve. **(C)** Mouse body weight curve. n = 5.



can be directly determined by the *in vivo* imaging technology (shown in Figure 8D) ATL-1/Ce6-pH Lip peaked at 12 h in mice, and ATL-1/Ce6-pH Lip sticked in tumors after 48 h. Meanwhile, *ex vivo* imaging and semi-quantitative analysis of

major organs also showed that ATL-1/Ce6-pH Lip fluorescence was significantly stronger than that of the Ce6, and these results indicated that ATL-1/Ce6-pH Lip had a significant tumor accumulation and retention effect with a wide therapeutic window.



3.8 In vivo anti-tumor effect

The results above demonstrated that ATL-1/Ce6-pH Lip showed good accumulation and permeability in tumor, and therefore, we performed experiments to evaluate the combined treatment effect of ATL-1/Ce6-pH Lip in HCT116 mice. The result of anti-tumor experiments in vivo showed that compared with the blank group, the other groups all had anti-tumor effects, with the ATL-1/Ce6-pH Lip+808 group displaying the strongest anti-tumor effect (Figure 9A). After drug administration and light irradiation, the tumor volume was inhibited to 174.63 mm³ (n = 6), and the treatment effect of ATL-1@pH Lip and Ce6@ pH Lip groups was better than that of the free ATL-1 and the free Ce6 groups. This finding suggested that the formulation had significant tumor targeting ability. In addition, the tumor volume of the ATL-1/Ce6-pH Lip+808 group was 0.18 times that of the blank group, showing a notable tumor inhibition effect (Figure 9B). During the administration period, no significant weight loss was observed in each experimental group compared with the blank group, indicating that ATL-1/Ce6-pH Lip had better biological safety (Figure 9C). These data suggested that the combination of chemotherapy and phototherapy of ATL-1/Ce6pH Lip had prominent anti-tumor ability and presented a greater therapeutic advantage than free drugs with individual treatment approaches.

Biological safety was evaluated by blood biochemical analysis of Balb/c nude mice and H&E examination of normal organs of mice in different experimental groups. The results showed that there was no necrosis, apoptosis or inflammation of the cells in the major organs after treatment with the nanopreparations, and the nuclei were largely fusiform or spherical without apparent changes, exibiting favorable biocompatibility (Figure 10). Additionally, the damage in the tumor showed that the ATL-1/Ce6-pH Lip+808 group was the most serious. Secondly, the expression of Ki-67 was in the following order: control, ATL-1/Ce6-pH Lip (808 nm), ATL-1/Ce6-pH Lip, ATL-1 Lip, Ce6 Lip, ATL-1, and Ce6, which were consistent with the



anti-tumor effects described above *in vivo* (Figure 10A). TUNEL results further confirmed the anti-tumor effect of ATL-1/Ce6pH Lip (808 nm), which showed the highest degree of apoptosis (Figure 11B).

Finally, the results of blood physiological and biochemical parameters showed that alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), UREA and creatine kinase (CK) of the mice treated with ATL-1/Ce6pH Lip+808 were not significantly different from those of the PBS-treated mice (Figure 1B).

4 Discussion

CRC is one of the most common human malignant tumors in the digestive tract with high mortality. Current therapies for cancer, such as transplantation, minimally invasive, local excision, and molecularly targeted therapies, are unable to completely inhibit the growth and metastasis of tumor cells, so there is an urgent need for research, development, and innovation of new agents to counteract it (Dey et al., 2025).

In recent years, it has been found that natural products of traditional Chinese medicines are highly effective and low toxicity, thus have been included in the development of novel antitumor drugs. ATL -1 is one of the compound variants extracted from the traditional Chinese medicine *Atractylodes macrocephala*. In previous studies, ATL-1 was found to have various anti-tumor activities, such as downregulation of CTGF expression in fibroblasts, activation of the MAPK-JNK/c-Jun signaling pathway, and inhibition of the EPAS1 pathway (Ren et al., 2021; Wang et al., 2021; Li et al., 2024). In addition, it was

also found that ATL-1 can disrupt tumor cell glucose metabolism and produce excess oxygen through the regulation of AKT/mTOR pathway, which shows that ATL-1 has the potential to be used for molecularly targeted cancer therapy (Wang et al., 2020; Xie et al., 2024). At the same time, this reminds us of PDT, a photodynamic therapy that has received much attention in recent years (Modi et al., 2025). PDT has been approved for the treatment of certain cancers and precancerous lesions due to its high spatiotemporal selectivity, low invasiveness and low systemic toxicity (Huis In 't Veld et al., 2023). However, the therapeutic efficacy of PDT itself is limited by the fact that it exacerbates hypoxia in the tumor microenvironment by consuming oxygen to generate highly reactive singlet oxygen and thereby producing cytotoxicity (Shabnum et al., 2024). Therefore, we hypothesized that coadministration of ATL-1 and PDT might improve the therapeutic effect of PDT on the basis of the original tumor growth inhibition of ATL-1. At the same time, PDT is able to destroy tumor tissue, exposing more of the drug to the tumor environment and helping to address challenges such as deepseated tumors, metastasis or recurrence.

In addition, we also considered that both ATL-1 and PS have poor water solubility, low permeability, and low bioavailability for direct drug delivery, so we chose the commonly used nanoformulations as drug carriers. It is well known that the acidic microenvironment induced by massive anaerobic glycolysis is one of the important features of malignant tumors, and with the in-depth study of acidic TEM, it becomes a new target for tumortargeted drug delivery nano-formulations, which is of great significance for the design of pH-responsive nano-formulations (Yang et al., 2021).

In summary, the ATL-1/Ce6-pH Lip formulation exhibited encouraging antitumor activity, thereby establishing a novel, viable approach for the treatment of additional tumors. Nevertheless, the study is not without its limitations. First of all, in the initial phase of large-scale liposome production, there is a necessity to optimize the parameters to ensure stability between batches. This optimization should address the issues of particle size growth and drug precipitation during storage (Yu et al., 2021). Furthermore, there is a need to investigate the potential chronic toxicity of long-term repeated use of the drug. Following these investigations, there is ample opportunity for research in subsequent clinical translation. Furthermore, the antitumor activity of ATL-1/Ce6-pH Lip was investigated in this study through a mouse model of ectopic colon cancer, but its efficacy in situ colon cancer needs to be further investigated due to the problems of light source penetration depth limitation, immunosuppressive microenvironment, high pressure in colon cancer mesenchyme, and dense extracellular matrix (ECM) that significantly limits drug penetration. In conclusion, we found that the combination of ATL-1 and PDT has good anti-tumor activity, but this is only the result of the comprehensive comparison, in the subsequent more in-depth study, we will use network pharmacology to analyze the interaction between the two, to further clarify the key targets and core pathways, and ultimately to verify the synergistic mechanism through experiments.

5 Conclusion

In summary, we have successfully developed an efficient and multifunctional nano-carrier that integrates drug therapy with photodynamic therapy. The ATL-1/Ce6-pH Lip nanosystem had optimal particle size, zeta potential, PDI, encapsulation efficiency, and anti-tumor activity. Specifically released in an acidic environment, ATL-1/Ce6-pH Lip demonstrated excellent cytotoxicity *in vitro*. Significant cellular uptake and ROS generation were also observed by direct *in vitro* assays, which were able to significantly enhance the inhibitory effect of PDT (Figure 12).

In addition, the synergistic therapeutic effect and tumor suppression of ATL-1/Ce6-pH Lip was also evident despite the more complex biodistribution or clearance and tumor hypoxic microenvironment in the *in vivo* studies. These properties rendered the ATL-1/Ce6-pH Lip a promising carrier for combined drug therapy and photodynamic therapy of cancer cells, offering novel insights into the treatment modality of colorectal cancer.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Experimental Animal Ethics Committee of Southwest Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YZ: Conceptualization, Formal Analysis, Investigation, Methodology, Software, Writing original draft. _ OY: Conceptualization, Formal Analysis, Investigation, Methodology, Software, Writing - original draft, Writing - review and editing. SF: Data curation, Writing - original draft. XF: Investigation, Visualization, Writing - review and editing. SC: Resources, Supervision, Writing - review and editing. HL: Software, Writing - review and editing. JN: Supervision, Validation, Writing - original draft. YL: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review and editing. DZ: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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