Carrier-Free Multifunctional Nanomedicine For Intraperitoneal Disseminated Ovarian Cancer Therapy

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Research Article

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Abstract

Background: Ovarian cancer is the most lethal gynecological cancer which is characterized by extensive peritoneal implantation metastasis and malignant ascites. Despite advances in diagnosis and treatment in recent years, the five-year survival rate is only 25 - 30%. Therefore, developing multifunctional nanomedicine with abilities of promoting apoptosis and inhibiting migration on tumor cells would be a promising strategy to improve the antitumor effect.

Methods and results: In this study, we developed a novel ACaT nanomedicine composed of alendronate, calcium ions and cyclin-dependent kinase 7 (CDK7) inhibitor THZ1. With the average size of 164 nm and zeta potential of 12.4 mV, the spherical ACaT nanoparticles were selectively internalized by tumor cells and effectively accumulated in the tumor site. Results of RNA-sequencing and in vitro experiments showed that ACaT promoted tumor cell apoptosis and inhibited tumor cell migration by arresting the cell cycle, increasing ROS and affecting calcium homeostasis. Weekly intraperitoneally administered of ACaT for 8 cycles significantly inhibited the growth of tumor and prolonged the survival of intraperitoneal xenograft mice.

Conclusion: In summary, this study presents a new self-assembly nanomedicine with favorable tumor targeting, antitumor activity and good biocompatibility, providing a novel therapeutic strategy for advanced ovarian cancer.

Background

Ovarian cancer (OC) is a common malignant tumor of female reproductive organs [1], which is often diagnosed in advanced stage [2], and easy to be widely implanted and transferred to the pelvic and abdominal cavity, forming malignant ascites [3]. Despite advances in diagnosis and treatment in recent years, the five-year survival rate is only 25 - 30%, which is the lowest among all gynecological malignancies [4]. Most notably, malignant ascites of ovarian cancer is the result of peritoneal infiltration and metastasis of tumor cells, which seriously affects the quality of life of patients and is one of the main causes of death of patients [5]. The treatment of advanced ovarian cancer largely depends on surgical and platinum-based chemotherapy. Over the past decades, intraperitoneal chemotherapy in advanced ovarian cancer has the potential to improve cytotoxicity and inhibit ascites production by increasing tumor exposure to antineoplastic agents [6]. Cisplatin and other platinum drugs, paclitaxel and mitomycin are commonly used in clinical and experimental intraperitoneal infusion [7, 8]. However, the small molecule drugs enter the circulatory system through the peritoneum-vascular barrier, resulting in systemic side effects and less tumor accumulation. In addition, drug resistance is the most common problem for recurrence of advanced ovarian cancer [9].

Recently, suppression of tumor cell proliferation by inhibiting super-enhancers attracts a huge attention of the clinical society. Cyclin-dependent kinase 7 (CDK7) is a component of transcription factor II H (TFIIH) and it plays an essential role in transcription initiation by phosphorylating the serine S5 of C-terminal
domain of RNAPII [10]. CDK7 inhibitor THZ1, a novel transcription-targeting compound, has been reported to significantly reduce the activity of super-enhancers and associated oncogene transcription factors through binding with CDK7 [11]. THZ1 has been demonstrated to have extensive cytotoxicity against ovarian tumors [12]. However, short half-life, poor bioavailability, and potential toxicity of THZ1 are drawbacks to be overcome. The half-life of THZ1 is only 45 min in mouse plasma, which limits its clinical application [13]. Therefore, developing a strategy that could improve bioavailability, prolong half-life time, and reduce potential toxicity, may increase the opportunity for the therapeutic application of THZ1.

Accompanying with the development of nanotechnology, nanomaterial has been used for cancer diagnosis and treatment in clinical trials [14–17]. An intraperitoneal chemotherapy study using xenograft mice demonstrated that the paclitaxel-loaded nanoparticles, compared to free paclitaxel, exhibited a 3.2-fold increase residence time in peritoneal cavity [18]. Injecting cisplatin-loaded nanoparticles showed enhanced antitumor activity in a rat of peritoneal carcinomatosis (PC), compared to free cisplatin [19]. The combination of nanostructured protein inhibitor and cisplatin by intraperitoneal injection could effectively downregulate tumor-promoting protein in metastatic ovarian cancer and ascites, and improve the survival rate of mice with metastatic ovarian cancer [20]. These results suggested that nanomedicine had stronger antitumor effects and a long-time accumulation in the abdominal cavity. Carrier-free nanomedicines have drawn great attentions due to the high load rate and non-toxic side effects of exogenous carriers [21, 22]. *In vivo* studies have shown that nanomedicine exhibited longer blood half-life, better tumor selectivity, enhanced tumor accumulation, and significantly improved antitumor efficacy compared with free drugs [23, 24].

The current carrier-free nanomedicines mostly suppress tumor growth by inhibiting tumor cell proliferation, and few can inhibit the metastasis and invasion of tumor. Therefore, a multifunctional nanomedicine which can simultaneously inhibit tumor cell proliferation and migration is highly demanded. Alendronate, a nitrogen-containing bisphosphonate, is clinically used for the treatment of osteoporosis and bone metastasis [25]. It has been reported that alendronate could inhibit the mevalonic acid pathway by reducing Rho activation and has an effect on reducing tumor burden and ascites, thus inhibiting ovarian cancer cell migration [26].

Herein, an ACaT carrier-free nanomedicine, which was composed of alendronate, THZ1 and Ca\(^{2+}\), was designed for intraperitoneal disseminated ovarian cancer therapy. Alendronate and Ca\(^{2+}\) formed network structure through coordination interaction, and THZ1 was self-assembled into the structure by hydrophobic attraction. This carrier-free nanomedicine could specifically target tumor and simultaneously achieve anti-migration and pro-apoptosis in an intraperitoneal xenograft model with human ovarian cancer. Moreover, ACaT could be degraded in low pH and showed good biocompatibility. Overall, this novel carrier-free nanomedicine ACaT provides new enlightenment for the clinical treatment of ovarian cancer.

**Materials And Methods**
Materials

Calcium chloride dihydrate (CaCl$_2$·2H$_2$O), alendronate sodium trihydrate (NaALN·3H$_2$O) were purchased from Shanghai Aladdin Bio-Chem Technology Co., LTD (Shanghai, China). CDK7 inhibitor THZ1 and the cell counting kit-8 (CCK8) assay kit were purchased from MedChemExpress (MCE, Shanghai). Dimethyl sulfoxide (DMSO) and penicillin-streptomycin (pen/strep) solution (100 X) were purchased from Sigma Aldrich (MO, USA). Cell culture medium, trypsin-EDTA and fetal bovine serum (FBS) were provided by Gibco (Guangzhou, China). Annexin V-FITC/PI apoptosis detection kits and mitochondrial membrane potential detection kit were purchased from Beyotime (Shanghai, China). 6-carboxy fluorescein (6-FAM), DAPI dihydrochloride and DiR iodide were purchased from Invitrogen (Carlsbad, CA, USA). D-Luciferin potassium salt and Fluo-4, AM were acquired from Yeasen Biotech Co., Ltd (Shanghai, China). Acridine orange-ethidium bromide (AO/EB) staining kit was purchased from Leagene Biotechnology (Beijing, China). Bcl-2, Bax and cleaved caspase-3 antibodies were bought from Absin Bioscience (Shanghai, China). β-actin and secondary antibodies were purchased from Proteintech (Wuhan, China). BCA protein quantitation kit and enhanced chemiluminescence (ECL) kit were purchased from Perkin-Elmer (Waltham, USA). All other reagents were consistent with previous report [27].

Cell Culture and Animal

SKOV3 cells (human ovarian cancer cell line), human renal proximal convoluted tubule epithelial cells HK2 and human peritoneal mesenchymal cells HMrSVS were purchased from American Type Culture Collection (ATCC, USA) in May 2016. All cells were routinely tested for mycoplasmas contamination using a mycoplasma detection kit (Beyotime, China) before used. SKOV3, HK2 and HMrSVS cultured in McCoy’s 5A, DMEM/F12 and RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1% pen/strep solution in a humidified atmosphere of 5% CO$_2$ and 95% air at 37°C. Firefly luciferase labeled SKOV3 cells (SKOV3-Luc) was generated as previously described [16]. Five-week-old to six-week-old female BALB/c nude mice (18-22 g) were purchased from GemPharmatech Co., Ltd (Guangzhou, China). All animal experiments were approved by the Animal Care and Use Committee (SYSU-IACUC-2021-B0815) of Sun Yat-sen University.

Preparation and Characterization of ACaT

ACaT were prepared through the self-assembly of THZ1, NaALN and Ca$^{2+}$. Firstly, 0.66 g of NaALN·3H$_2$O and 0.2 g of CaCl$_2$·2H$_2$O were dissolved in 75 mL of water, respectively, and mixed them up. Then, 50 mg of THZ1 (4 mg/mL) was added to the above solution. The pH of this mixture was adjusted to 7.0 by NaOH and stirred for 30 min at 4°C. ACaT nanomedicine was obtained by washing and centrifugation. The morphologies of obtained formulations were observed by TEM and SEM. Particle size and zeta potential were measured by Zetasizer Nano ZS90 (Malvern, Britain). X-ray powder diffraction (XRD) patterns were recorded using an X-ray diffractometer (Rigaku D/max 2550 V, Japan). UV-vis and fluorescence spectra were recorded by Evolution 300 UV-vis (Thermo Scientific, USA) and LS55 luminescence spectrometer (Perkin-Elmer, USA).
In Vitro Cellular Uptake of ACaT

ACaT nanoparticles were labeled with 6-FAM for 24 h at room temperature. In brief, SKOV3 cells were seeded in confocal dish and cultured until 60% density. Cells were then incubated with ACaT/6-FAM at 37°C for 1, 2, 4 and 24 h, respectively. After incubation, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 8 min, then stained with 5 μg/mL of DAPI, and photographed by confocal laser scanning microscopy (CLSM, Zeiss LSM880, Germany).

In Vitro Cytotoxicity Study

The in vitro cytotoxicity of THZ1, NaALN and ACaT were tested by CCK8 cell proliferation assay kit. According to the manufacturer's instructions, 5000 SKOV3 cells were seeded into a 96-well plate per well and cultured overnight. Then cells were incubated with various concentrations of THZ1, NaALN and ACaT for 24, 48 and 72 h, respectively. At each time point, 10 μL of CCK-8 reagent was added into each well and incubated for another 3 h. Optical density (OD) of 450 nm was measured by a microplate reader (BioTek, SynergyH1, USA). Cell viabilities were calculated using the formula: cell viability % = (OD\text{sample} - OD\text{blank}) / (OD\text{control} - OD\text{blank}) \times 100%.

Analysis of Cell Apoptosis

Cell apoptosis was assessed via AO/EB staining, flow cytometry and western blot analysis. Briefly, SKOV3 cells were pre-seeded in 6 well plates (5 \times 10^4 cells per well) overnight. Then ACaT with varied concentrations were added into each well and incubated for 24, 48 or 72 h, respectively. The cells were stained by AO/EB assay kit at different time points. Then image acquisition and analysis were carried out by a fluorescent microscopy (Leica, DMi8, Germany). Apoptosis was also investigated by flow cytometry using annexin V/PI co-staining as described previously [27]. For western blot analysis, the SKOV3 cells were washed and lysed in 150 μL of lysis buffer containing phenylmethylsulfonyl fluoride (PMSF, 1 mM) to collect total proteins. Total protein concentrations were measured using BCA protein quantitation kit (Waltham, USA). The protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 30 min followed by 120 V for 60 min, and then electrotransferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% defatted milk for 2 h and incubated with primary antibody overnight at 4°C, followed by incubation with secondary antibody for 2 h at room temperature. After that, the membranes were visualized in ECL reagent for 1-5 min and western blot images were collected on ChemiDoc Imaging System (Bio-Rad, America).

Analysis of Migration

Briefly, 5 \times 10^5 cells were seeded per well overnight in 6-well culture plates until 80% confluency was achieved. On the second day, a vertical scratch was performed using a 10 μL pipette tip to draw horizontal four lines (0.5 cm apart) and one vertical line evenly with ruler, and the cells were washed twice using fresh PBS to remove the dead or floating cells. Subsequently, the cells were incubated with various concentrations of ACaT (6.25 mg/L), NaALN (200 μM), THZ1 (0.01 μM). The control group was incubated...
with culture medium. Scratches were imaged by Nikon camera at 0 and 24 h. Data were analyzed using Image J software.

**Measurement of Intracellular Calcium Ion**

Intracellular calcium ion accumulation was detected by using CLSM and Fluo-4 AM dye as fluorescent probe. SKOV3 cells were incubated with media containing ACaT, NaALN, and THZ1 for 48 h at concentration of 1.56 mg/L, 200 μM and 0.01 μM, respectively. The control group was incubated with media containing the same volume of PBS. After 48 h of treatment, media was removed and cells were stained with 1 μM of Fluo-4 AM for 60 min. The cells were washed with HBSS for 3 times, and then incubated in a 37°C incubator for 30 min to ensure that Fluo-4 AM were completely transformed into Fluo-4 in the cells. And the fluorescence was recorded by CLSM at an excitation of 488 nm laser.

**Detection of Mitochondrial Membrane Potential**

The mitochondrial membrane potential was detected by a JC-1 based mitochondrial membrane potential kit. Cell culture in the same manner as described for migration assay above. The original media was removed, and 1 mL of JC-1 working solution was added. The cells were maintained at 37°C/5% CO₂ in an incubator for 20 min, washed twice with JC-1 washing buffer. Then 2 mL of cell medium was added. The samples were observed and photographed under a Leica DMI8 fluorescent microscope (Leica).

**mRNA Extraction and Sequencing**

Cells were treated with THZ1 (0.5 μM), NaALN (850 μM), and ACaT (100 mg/L) for 24 h. After treatments, cells were washed with ice-cold PBS three times and total RNA was extracted using Qiagen RNeasy Mini kit (Germany) according to the manufacturer protocol. RNA samples were quantified using the Qubit 2.0 (Thermo Fisher Scientific) and treated with DNase I to remove residual DNA. The quality control of mRNA samples were conducted using the Bioanalyzer 2100 (Agilent Technologies). RNA-seq libraries were generated and sequenced following the standard mRNA protocols on a Hiseq 2500 (Illumina, PE150). Clean reads were obtained from the raw reads by removing the adaptor sequences, low quality sequences and reads containing poly-N with Trimmomatic software. All the downstream analyses were based on the clean data with high quality. DESeq2 was selected to identify differentially expressed genes (DEGs). Genes with an adjusted P value < 0.05 and abs (log₂ (fold change)) > 1 were assigned as DEGs. Gene set enrichment analysis (GSEA) was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

**ACaT Biodistributions In Vivo**

SKOV3-Luc cells were trypsinized, washed, resuspended with ice-cold PBS, and kept on ice prior to implantation in mice. Peritoneal tumor model was initiated in mice by intraperitoneal injecting 5 × 10⁶ SKOV3 cells per mouse intraperitoneal. ACaT nanomedicine was prelabeled with DiR dye. To further evaluate the biodistribution of ACaT, SKOV3 tumor-bearing BALB/c nude mice were intraperitoneally
injected with free DiR and ACaT-DiR, and then monitored by real time in vivo imaging system (IVIS). Mice were imaged with an IVIS at 1, 4, 24, 72 and 168 h post injection. At 72 h and 168 h post injection, mice were sacrificed, and tumors as well as major organs were collected and fluorescently imaged immediately. The quantitative analyses of fluorescence signals were performed using AnitView100.

Antitumor Effect of ACaT In Vivo

When peritoneal metastasis model was established after 5 days, the tumor-bearing mice were randomly divided into four groups (n = 8) and intraperitoneally administered with PBS, NaALN (8 mg/kg), THZ1 (1 mg/kg) and ACaT (10 mg/kg) in 400 μL of PBS solution for 8 cycles. The body weights and abdomen circumference in mice were measured once a week. An AnitView100 IVIS imaging system was used for live mice imaging at one week interval. Five mice were fixed and subjected to weekly bioluminescence imaging. The other three mice in each group were killed under deep anesthetized with an overdose of sodium pentobarbital and dissected on day 35. Ascites volumes were measured and the excised tumors were photographed and weighed. Tumors and organs such as heart, liver, spleen, lung and kidney were collected for H&E and TUNEL staining.

Biocompatibility Assessment In Vitro and In Vivo

HK2 and HMrSVS cells were incubated on 96-well plates overnight and ACaT with different concentrations were added and incubated at 37°C for 24, 48 and 72 h, respectively. In addition, hemolytic activity test was also performed at the same time. The fresh red cells was constituted with normal saline (0.9%) into 2% red blood cell suspension, and mixed with different concentrations of ACaT solutions at 37°C for 4 h. The negative control was incubated with normal saline (0.9%), while the positive control was incubated with sterilized water. After incubation, all groups were centrifuged at 1500 rpm for 5 min. For each concentration, 100 μL of supernatant was added to a 96-well plate and absorbance at 570 nm were detected by microplate reader. HR% = (A_{sample} - A_{negative}) / (A_{positive} - A_{negative}) \times 100\% [27]. To evaluate the biocompatibility of ACaT in vivo, BALB/c mice were sacrificed at 72 h after injected with ACaT nanomedicine. Organs such as heart, liver, spleen, lung and kidney were collected for H&E staining.

Statistical Analysis

Data are shown as the average (± SD) taken from at least three independent experiments. Statistical analysis was performed using Prism 8.0 (GraphPad). Statistical differences between the values were evaluated using one-way ANOVA analysis of variance

Results And Discussion

Characterizations of ACaT

Alendronate-calcium-THZ1 (ACaT) nanomedicine was prepared by the self-assembly of alendronate, Ca^{2+}, and THZ1. As shown in Figure 1A, alendronate and Ca^{2+} formed infinite coordination structure
through coordination interaction, and THZ1 were self-assembled into the structure by hydrophobic attraction. The obtained ACaT exhibited amorphous structure and spherical morphology with particle size of 164 nm (Figure 1B-D). Zeta potential of ACaT was 12.4 mV, which could be easily uptaken by cancer cells. Fourier transform Infrared (FTIR) analysis was employed to verify the existence of alendronate and THZ1. The phosphate group bands related to alendronate were shown in the 960-1143 cm\(^{-1}\) (red asterisks) wavenumber range (Figure 1E) [28, 29]. Absorption peaks centered at 3179.97 cm\(^{-1}\) and 1639.07 cm\(^{-1}\) (benzene ring, black asterisks) were related to THZ1 [30, 31]. The degradation of nanoparticles were detected by incubating ACaT with PBS at different pH values, indicating that the degradation rate of ACaT in acidic (pH = 4.5, 6.0) environment was faster than that in neutral (pH = 7.0) condition (Figure 1F). It was suggested that in the acidic lysosomal environment (pH = 4.5), ACaT could be almost completely degraded, accompanied by the release of alendronate and THZ1 to cytoplasm. In physiological environment (pH = 7.0~7.4), the degradation of nanoparticles was slow, and the size of nanoparticles remained relatively stable for over 72 h (Figure 1G). Collectively, a novel pH-sensitive carrier-free nanomedicine ACaT with mean size of 164 nm and positive surface charge was successfully prepared.

**Therapeutic Effects of Alendronate and THZ1 In Vitro**

THZ1 have been reported to downregulate the expression of super-enhancer associated genes by inhibiting CDK7, which in turn block cell cycle and transcription of cancer cells [32]. Transcriptome sequencing was performed on THZ1 treated SKOV3 cells (Figure S1). The gene set enrichment analysis (GSEA) showed that genes related to cell cycle were concentrated, and differential genes were enriched in down-regulated regions (Figure 2A), indicating that THZ1 might perturb cell cycle progression. Flow cytometry was used to evaluate cell cycle distribution after treatment with different concentrations of THZ1. The results showed that the proportion of SKOV3 cells in G2/M phase was elevated with increased THZ1 concentration, suggesting the G2/M phase cell-cycle was arrested, which was consistent with the results of previous studies (Figure 2B-C) [33, 34]. Disruption of cancer cell cycle is known to inhibit cell growth and induce apoptosis. As shown in Figure 2D-E, THZ1 could significantly inhibit cancer cell proliferation and induce apoptosis at low concentration (1 µM).

Alendronate, an FDA-approved drug for the treatment of osteoporosis, has been reported to inhibit tumor metastasis [35]. Figure 2F-G showed that cancer cell migration ability was significantly suppressed after treatment with different concentrations of alendronate. The KEGG Orthology Based Annotation System (KOBAS) database was utilized to identify KEGG pathway enrichment of DEGs. Notably, focal adhesion pathway, which was associated with migration, was significantly downregulated (Figure S2). This observation was further confirmed in KEGG pathway map 04510 (Figure S3). In addition, treatment with alendronate for 48 h also showed obvious growth inhibition effect on SKOV3 cells (Figure 2H). The exact mechanism of apoptosis induced by alendronate still remains elusive. The previously reported alendronate treatment induced apoptosis in osteosarcoma cell through inhibiting PI3K-Akt-NFκB cell survival pathway [36].
Antitumor Effect and Mechanism of ACaT In Vitro

Antitumor drugs based on nanotechnology have been widely reported to change the pharmacokinetic characteristics and reduce system toxicity of small molecule drugs. In this study, ACaT nanomedicine was precisely designed for intraperitoneal disseminated cancer treatment. To investigate the uptake efficiency of ACaT by SKOV3 cells, ACaT was labelled with fluorescent probe 6-FAM (ACaT/6-FAM, green) to track the internalization in SKOV3 cells. The results showed that the intracellular uptake of ACaT by SKOV3 cells was time-dependent and the ACaT fluorescence intensity in the cytoplasm of SKOV3 was strong when the incubation time at 24 h (Figure 3A), indicating that ACaT nanomedicine could be quickly and efficiently internalized, which was conducive to further evaluation of anticancer effect in vitro and in vivo.

In order to explore the antitumor effect and mechanism of ACaT in vitro, the cell proliferation and migration abilities were evaluated. Firstly, RNA transcriptome sequencing and flow cytometry were applied for assessing the cell cycle distribution. As shown in Figure 3B and Figure S4, THZ1 and ACaT significantly inhibited the cell cycle process, making it arrested in G2/M phase. In addition to cell cycle, KEGG enrichment assay showed that cell apoptosis pathway in ACaT group was significantly affected as well. And cell apoptosis and cell cycle-related pathways were significantly enriched in bubble diagram (Figure 3C), indicating that the THZ1 component in ACaT had significant effects on apoptosis and cell cycle. In addition, cell apoptosis was detected by AO/EB co-staining. As shown in Figure 3D-E, ACaT exhibited time- and concentration-dependent killing against SKOV3 cells. The higher the ACaT concentration leads to the better killing effect. In addition to blocking cell cycle and inducing apoptosis, THZ1 was also demonstrated to induce intracellular ROS elevation (Figure S5) [37]. The increased ROS may lead to the disruption of mitochondrial membrane system function, calcium homeostasis disequilibrium, and eventually cell apoptosis [38, 39]. In our study, calcium ion accumulation and mitochondrial membrane potential loss were observed in the ACaT group (Figure S6-7). Calcium alone has little direct killing effect on SKOV3 cells (Figure S8). Therefore, we inferred that the increased ROS production and calcium accumulation facilitated each other in cell apoptosis.

Overall, ACaT induced cancer cell apoptosis through variety of mechanisms. Western blot was used to confirm the apoptosis of SKOV3 cells treated with different drugs for 24 h. As shown in Figure 3F, the cells treated with both THZ1 and ACaT exhibited the increased pro-apoptotic protein Bax and the downregulated anti-apoptotic protein Bcl-2 to induce apoptosis. However, alendronate treatment had no effect on the expressions of Bax and Bcl-2, illustrating that alendronate contributed little to cell apoptosis. Cleaved-caspase-3, classical apoptosis marker, was detected in SKOV3 cell lysates after treatment with THZ1 and ACaT. We reasoned therefore that THZ1 was the primary effector of ACaT-induced apoptosis in SKOV3 cells. On the other hand, inhibiting migration of cancer cells by ACaT was verified by cell scratch assay (Figure 3G and S9). The results showed that both NaALN and ACaT significantly reduced the migration rate of SKOV3 cells. Generally, each component of ACaT retained its biological activity and exerted anticancer effects via multiple mechanisms.
Distribution of ACaT In Vivo

In order to explore the biodistribution and anticancer effect of ACaT in ovarian cancer bearing mouse, SKOV3-Luc cells expressing firefly luciferase were used to establish an ovarian cancer peritoneal tumor model in BALB/c nude mice. The tumor progression could be conveniently detected by the in vivo imaging system. After 5 days of tumor cell transplantation, the mice were sacrificed, dissected and photographed. A solid tumor with a diameter of about 4 mm could be found in the lower right corner of the liver (Figure S10). Liver and spleen metastases began to appear around day 30 (Figure S11). The biological distribution of ACaT was studied on the 15 days after tumor transplantation. Free DiR and DiR labeled ACaT were intraperitoneally injected into mouse respectively. As shown in Figure 4A, free DiR was distributed in the abdominal cavity at 1 h after injection, enriched in liver and tumor 72 h after injection (Figure 4E and S12). No obvious fluorescent signal was detected at 168 h, implying most of free DiR excreted out of the body 168 h after injection (Figure 4B and F). ACaT-DiR was distributed throughout the abdominal cavity 1 h after injection (Figure 4C), followed by preferentially accumulated in the tumor site even at 168 h post-injection (Figure 4D-F).

Antitumor Effect of ACaT on Human Ovarian Cancer Bearing Mice

Encouraged by the strong antitumor activity and tumor cell uptake characters in vitro, the anticancer effect of ACaT nanomedicine in intraperitoneal xenograft mouse model was evaluated. The therapeutic schedule of animal experiment was shown in Figure 5A. After the successful establishment of abdominal tumor model, the mice were randomly divided into four groups with eight mice in each group. Treatment was performed every week for a total of 8 weeks. On day 35, three mice in each group were sacrificed, and the tumor weight and ascites weight were recorded. The experiment was ended on day 60 and all mice were euthanized. According to the quantitative results of fluorescence intensity, ACaT group had the best inhibitory effect on tumor growth, while NaALN had a modest inhibitory effect on tumor growth compared with the control group (Figure 5B-C). Nevertheless, the bioluminescence value of THZ1 group was similar to that of the control group, implying that THZ1 could not inhibit tumor growth (Figure 5B-D). It was probably because THZ1 had low bioavailability and short half-life in vivo.

At 35 d post administration, ACaT group exhibited a strong antitumor effect by inhibiting tumor growth and ascites formation (Figure 5E-F). It could also be seen from abdominal circumference data (Figure S13) wherein abdominal circumference of the mice in PBS and THZ1 groups increased significantly at the later stage, which was consistent with the volumes of ascites. The average survival time of mice was 36 days in PBS group but prominently prolonged in ACaT treatment group (Figure 5G). 4/5 mice survived at the end of the experiment. H&E and TUNEL staining were used to further confirm the cell necrosis and apoptosis in tumor tissue of ACaT group (Figure 5H). These results demonstrated that ACaT nanomedicine showed higher antitumor activity than THZ1 and NaALN in an intraperitoneal xenograft model with human ovarian cancer. All treated mice behaved normally, and their weights were well maintained, suggesting good biocompatibility of ACaT (Figure S14).

Biocompatibility of ACaT Nanomedicine
The biocompatibility of ACaT was systematically evaluated. The toxicities of ACaT on HK2 and HMrSVS were determined by CCK8 assay. As shown in Figure 6A-B, ACaT had no significant cytotoxic effect on human normal cells HK2 and HMrSVS. *In vitro* hemolysis test was a general method to evaluate the blood compatibility of nanomedicine. Hemolysis is defined as the hemolysis rate of human erythrocytes exceeding 5%. As shown in Figure 6C-D, the hemolysis rates of each ACaT concentration were less than 5%, and no signs of hemolysis were observed even at the high concentration (100 mg/ L). Finally, the histocompatibility of ACaT were evaluated by H&E staining. After 8 cycles of treatments, the tumor bearing mice were sacrificed and the major organs were extracted. As can be seen from Figure 6E, the heart, liver, spleen, lung and kidney of mice in NaALN, THZ1 and ACaT groups had no obvious histological damage, indicating the excellent biocompatibility of as-synthesized ACaT nanomedicine both *in vitro* and *in vivo*.

**Conclusions**

In summary, a novel ACaT nanomedicine was designed and successfully synthesized by self-assembly of alendronate, calcium and small molecule inhibitor THZ1 via coordination polymerization and hydrophobic forces. It had high drug loading rate and good stability in physiological environment. After being internalized by tumor cells, ACaT induced cell cycle arrest, apoptosis and inhibited cell migration by releasing anticancer drugs. Its antitumor properties and biocompatibility had been systematically evaluated by *in vitro* screening assays. In an intraperitoneal xenograft model with human ovarian cancer, ACaT could accurately target tumors, effectively suppress tumor growth, and significantly inhibit ascites production. Our work provides a versatile carrier-free nanomedicine platform for the treatment of advanced ovarian cancer.

**Declarations**

**Ethics approval and consent to participate**

All animal experiments were approved by the Animal Care and Use Committee (SYSU-IACUC-2021-B0815) of Sun Yat-sen University.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing financial interest.

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**Author’s Contributions**

XH and MQ contributed equally to this work. XH: investigation, formal analysis, data curation, software, writing original draft. MQ: formal analysis, data curation, resources, software, visualization. BL, SZ: investigation, writing - review & editing. TZ: resources, software. PL, QW, ZQ: resources, software. CZ,MW: supervision, validation. JZ: conceptualization, methodology, funding acquisition, project administration, writing – review. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Figures
Figure 1
Characterizations of ACaT nanomedicine. (A) Schematic representation, (B) TEM images, (C) size distribution, (D) XRD pattern, and (E) FTIR spectrum of ACaT. (F) Degradation of ACaT nanoparticles in PBS at different pH values. (G) Stability of ACaT nanomedicine in PBS with pH of 7.4.

Figure 2
Therapeutic effect and mechanism of ACaT nanomedicine. (A) GESA plot for genes of cell cycle after THZ1 treatment. (B) Flow cytometric analysis of SKOV3 cells treated with THZ1 for 24 h at different concentrations. (C) Quantitative cell cycle analysis of different phases in THZ1 treatment groups with different concentrations. (D) Cell viability of SKOV3 treated with THZ1 at different concentrations for 24 and 48 h. (E) Detection of apoptotic SKOV3 cells after treatment with THZ1 at concentration of 0, 0.25, 0.5 and 1 μM for 24 h by flow cytometry. (F) Scratch assay for cell migration. Scratch edges were recorded at 24 h after scratching and treating with alendronate at concentration of 75, 150 and 300 μM (yellow dotted boxes marked the scratch edges). (G) Quantification of the percentage of the wound area
in scratch migration assay. (H) Cell viability of SKOV3 treated with different concentrations of NaALN for 24 and 48 h.

**Figure 3**

Evaluation of cellular uptake, antitumor activity and mechanism of ACaT *in vitro*. (A) CLSM images of SKOV3 cells after treatment with ACaT/6-FAM for 1, 2, 4 and 24 h. Scale bar, 30 μm for the three columns on the left and 10 μm for the enlarge column. (B) Quantitative cell cycle analysis of different phases in different treatment groups. (C) Top 10 KEGG pathways enrichment analysis bubble-plot. Cells were treated with 100 mg/L of ACaT for 24 h before mRNA sequencing. Black arrow indicated cell apoptosis and cell cycle pathway. (D) Dual AO/EB fluorescent staining of SKOV3 cells after treatment with different concentrations of ACaT for 48 h (green for living cells and red for dead cells). Scale bar, 150 μm. (E) Cell viability of SKOV3 treated with ACaT at different concentrations for 24, 48 and 72 h. (F) Western blots for Bax, Bcl-2, and cleaved caspase-3 after NaALN (850 μM), THZ1 (0.5 μM), ACaT (100 mg/L) treatment for 24 h in SKOV3 cells. (G) Scratch assay for cell migration. Cell migration was recorded at 0 and 24 h after scratching. Cells were treated with NaALN (200 μM), THZ1 (0.01 μM), ACaT (6.25 mg/L) at the beginning of the experiment (yellow dotted boxes marked the scratch edges).

**Figure 4**

The *in vivo* biodistribution of free DiR and ACaT-DiR in female SKOV3-Luc tumor-bearing mice. The *in vivo* bioluminescence images (Luc) and fluorescent images (Fluo) of mice after intraperitoneal injection of (A) Free DiR and (C) ACaT-DiR at the indicated time points. The ex vivo bioluminescence images (Luc) and fluorescent images (Fluo) for tumor tissues and major organs of (B) free DiR and (D) ACaT-DiR at 168 h intraperitoneal injection. The quantitative analyses of pixel fluorescence intensity from tumor tissues and major organs after intraperitoneal injection 72 h (E) and 168 h (F). *p < 0.05 and ***p < 0.005.

**Figure 5**

*In vivo* antitumor effect of ACaT on SKOV3 ovarian tumor-bearing mice. (A) The timeline of animal experiment. (B) *In vivo* bioluminescence images of mice after different treatments (*n* = 5). (C) Individual fluorescence intensity of mice treated with PBS, NaALN, THZ1 and ACaT calculated from the *in vivo* bioluminescence image. (D) Average fluorescence intensity of mice treated with PBS, NaALN, THZ1 and ACaT (means ± SD, *n* = 5). (E) Tumor weight and representative images of tumor tissues harvested on day 35 (means ± SD, *n* = 3). (F) Ascites weight and representative images of ascites harvested on day 35.
(means ± SD, n = 3). (G) Kaplan-Meier survival curves of mice in the different treatment groups. (H) H&E and TUNEL staining images of tumor tissues for each group after 35 days treatment. Scale bar: 100 μm.

Figure 6

The biosafety evaluation of ACaT in vitro and in vivo. Cell viability of (A) HK2 cells and (B) HMrSVS cells cultured with various concentrations of ACaT for 24 and 48 h, respectively. (C) The photographs of human erythrocytes treated with ACaT at different concentrations. Samples 1-10: negative control (NS),
eight different concentrations of ACaT, positive control (dd H₂O). (D) Hemolysis rate of ACaT, NS and dd H₂O. (E) H&E staining of tissue slice of main organs in PBS, NaALN, THZ1 and ACaT groups \((n = 3)\). Scale bar, 50 μm.

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