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RESEARCH PAPER

Multifunctional Tumor-Targeted Polymer-Peptide-Drug Delivery System for Treatment of Primary and Metastatic Cancers

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ABSTRACT

Purpose In order to improve drug delivery to drug-resistant ovarian tumors, we constructed a multifunctional polymer-peptide-drug conjugate (PPDC) system for effective treatment of primary and metastatic ovarian cancers.

Methods The PPDC consists of the poly(Ethylene Glycol) (PEG) polymeric carrier conjugated via citric acid spacers to anticancer drug (Camptothecin, CPT), tumor targeting moiety (LRHR, a synthetic analog of luteinizing hormone-releasing hormone) and a suppressor of cellular antiapoptotic defense (BH3 peptide). To test the conjugates *in vitro* and *in vivo*, cancer cells were isolated from tissue samples obtained from patients with ovarian primary tumor and metastatic malignant ascites.

Results It was found that cells isolated from malignant ascites were more aggressive in terms of tumor growth and more resistant to chemotherapy when compared with those isolated from primary tumors. PPDC containing two copies of drugs and peptides was most efficient in treatment of primary tumors and intraperitoneal metastases. Multiple treatments with this PPDC led to almost complete regression of primary tumor and prevented growth of malignant ascites.

Conclusion The proposed multifunctional polymeric delivery system which consists of multiple copies of the drug and peptides demonstrated significantly higher antitumor activity in primary and metastatic cancers when compared with drug alone and PEG-CPT conjugate.

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KEY WORDS BH3 peptide · camptothecin · LHRH peptide · PEG polymer · proapoptotic peptide

ABBREVIATIONS

APAF1	apoptotic protease activating factor 1
BCA	bicinchoninic acid
BH3 Peptide	CL2 Homology 3 Peptide
CA	citric acid
CASP3	Caspase 3
CASP9	Caspase 9
CPT	camptothecin
FITC	fluorescein isothiocyanate
LHRH	luteinizing hormone-releasing hormone
MDR	multidrug resistance
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PEG	poly(Ethylene Glycol)
PPDC	polymer-peptide-drug conjugate
RT-PCR	reverse transcriptase-polymerase chain reaction
β_2-m	β_2-microglobulin

INTRODUCTION

Ovarian cancer is the leading cause of human death from gynecologic malignances (1). A major challenge in the treatment of ovarian carcinoma is that this disease causes minimal and nonspecific symptoms during its initial stages. Consequently, most patients with ovarian carcinoma are

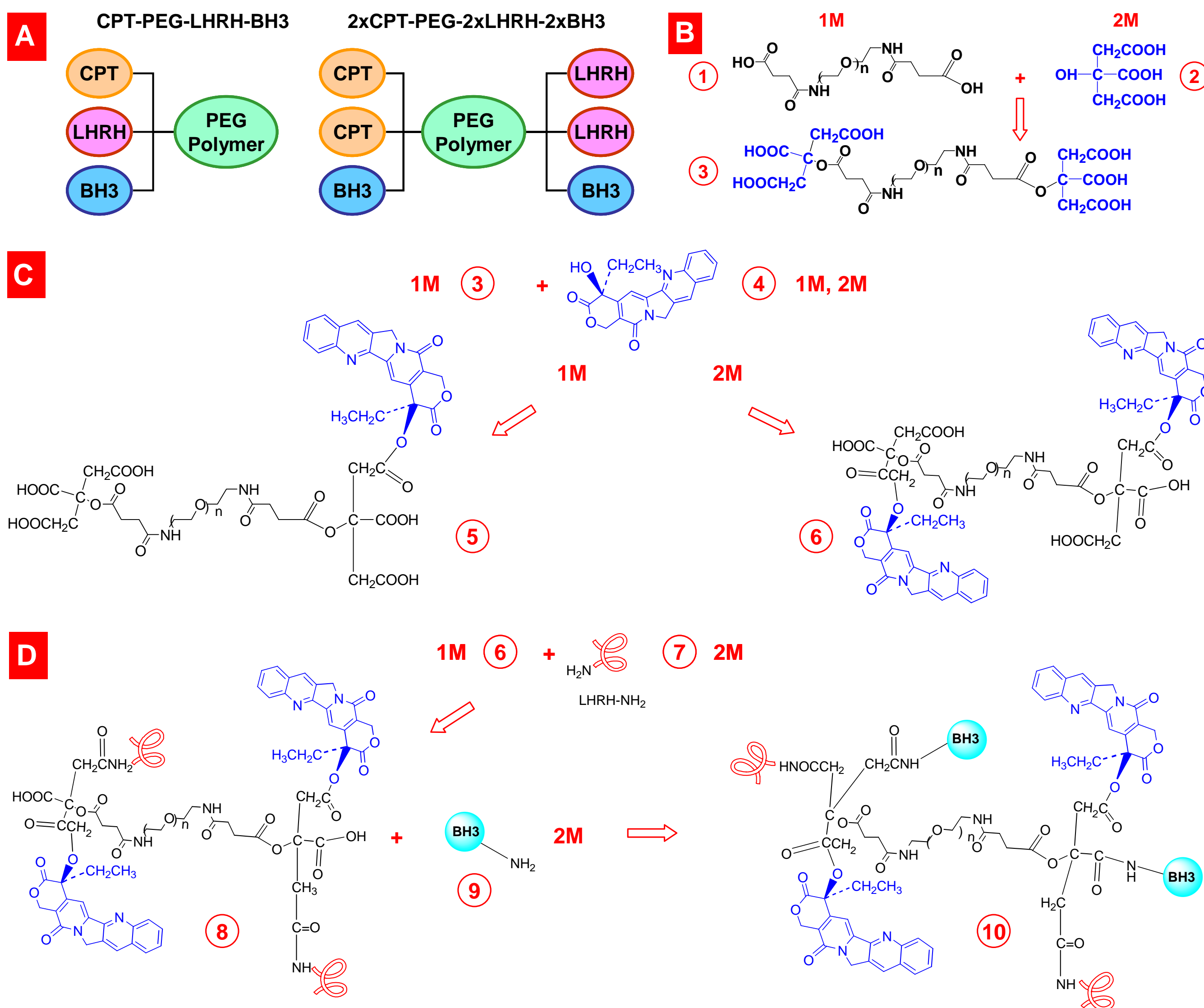
diagnosed in the advanced stage of the disease, which leads to an unfavorable prognosis. The main treatment choice for patients with ovarian cancer is aggressive “debulking” surgery followed by chemotherapy. However, intrinsic and acquired drug resistance of ovarian cancer substantially limits the efficiency of chemotherapeutic treatment (2). Several recent studies revealed that the major role in such resistance is played by an antiapoptotic defense of cancer cells that depends on the expression of BCL2 and BAX proteins (3–6). It was shown that overexpression of antiapoptotic BCL2 protein and a decrease in the expression of proapoptotic BAX proteins in cancer cells are the indicators of poor response to chemotherapy of ovarian carcinoma. In order to overcome such resistance, high doses of chemotherapy are generally used (7,8). However, the increase in the dose of chemotherapeutic drugs usually leads to the induction of high adverse side effects of the treatment on healthy organs. In order to suppress the resistance of cancer cells to chemotherapy and prevent systemic adverse side effects of chemotherapy, we recently proposed a multifunctional, multicomponent polymer-peptide-drug conjugate (PPDC) that include one or several copies of anticancer drug(s) conjugated to poly(Ethylene Glycol) (PEG) carrier via branched spacers together with one or several copies of luteinizing hormone-releasing hormone (LHRH) decapeptide and BCL2 Homology 3 domain (BH3) peptide for tumor targeting and suppression of cellular antiapoptotic defense, respectively (9). The proposed PPDCs were tested *in vivo* and *in vitro* using established human ovarian and lung cancer cells and showed promising results in these models (10–16). However, these cell lines were cultured for a relatively long period of time after the isolation from patient tumor tissues. Consequently, these cells probably do not exactly reflect real conditions in patients' tumor cells. Therefore, one of the aims of the present study is to test proposed PPDCs *in vivo* and *in vitro* using cancer models derived from the cells isolated from primary tumors obtained from patients with ovarian carcinoma.

Invasion and metastases of cancer cells along with the development of resistance to cancer therapies are the main causes of morbidity and mortality from ovarian cancer. Malignant ascites (the accumulation of fluid and cancer cells in the abdominal cavity caused by metastasizing of cancer cells into the abdominal cavity) represent the most abundant adverse effects of advanced ovarian carcinoma. Generally, patients suffering from malignant ascites have a poor prognosis (17). Consequently, the development of novel therapeutics for treatment of malignant ascites is an important task for an effective chemotherapy of advanced ovarian cancer. In the present study, for the first time, we plan to answer four questions: (1) Are the cells in malignant ascites more resistant to chemotherapy when compared to primary ovarian tumors, and are the mechanisms of drug resistance similar in malignant ascites and primary tumor isolates? (2) Can LHRH peptide be used for effective targeting of cancer cells in malignant ascites as well as primary tumor cells? (3) Will the proposed PPDC be equally effective in treatment of tumors derived from both primary tumor isolates and malignant ascites? (4) Are the mechanisms of apoptotic cell death induction by PPDC similar in both cases? The present paper is aimed at answering these questions and investigating novel multi-component polymer-peptide-drug conjugates for treatment of primary and metastatic cancer.

MATERIALS AND METHODS

Materials and Synthesis of Conjugates

Camptothecin (CPT), N,N-diisopropylethyl-amine, and 4-(methylamino)pyridine (DMAR) were obtained from Sigma Chemical Co. (Atlanta, GA); bis(2-carboxyethyl) PEG polymer and fluorescein isothiocyanate (FITC) were obtained from Fluka (Allentown, PA). LHRH analog, Lys6-des-Gly10-Pro9-ethylamide (Gln-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-NH-Et) and BH3-NH_2 (Ac-Met-Gly-Gln-Val-Gly-Arg-Gln-Leu-Ala-Ile-Ile-Asn-Arg-Arg-Tyr-Cys-NH_2) peptides were synthesized according to our design (11–13) by American Peptide (Sunnyvale, CA). The sequence of native LHRH peptide, which is similar in human, mouse, and rat, was modified to provide a reactive amino group only on the side chain of a lysine residue, which replaced Gly at position 6 to yield the superactive, degradation-resistant-Lys-6-des-Gly-10-Pro-9-ethylamide LHRH analog (18). The system (Scheme 1, A) contains bis-poly(ethylene glycol) (PEG) polymer as a carrier, one or two copies of LHRH peptide as a targeting moiety, one or two copies of Camptothecin (CPT) as an anticancer drug and one or two copies of BH3 peptide as a suppressor of cellular antiapoptotic defense. The conjugates were synthesized as previously described (10,12–14). Briefly, α,ω-bis-PEG3000-citric acid (CA) conjugate (Scheme 1, B, compound 3) was synthesized using one-step procedure by conjugation of 1 M of bis-(2-carboxyethyl) PEG (Scheme 1, B, compound 1) with 2 M of CA [CAS number: 77-92-9, 2-hydroxy-1,2,3-propanetricarboxylic acid] (Scheme 1, B, compound 2). The bis-PEG-CA conjugate (Scheme 1, C, compound 3) was coupled with one or two moles of CPT (Scheme 1, C compound 4) to obtain α,ω-bis-(2-carboxyethyl) PEG-CA-CPT conjugates (Scheme 1, C, compounds 5, 6). Further, one or two moles of LHRH (Scheme 1, D, compound 7) was conjugated with compounds 5 or 6 to obtain α, ω-bis-(2-carboxyethyl) PEG-citric acid-CPT-LHRH conjugates (Scheme 1, D, compound 8) having one or two copies of CPT and LHRH. Finally,



Scheme 1 Structure and synthesis of multicomponent polymer-peptide-drug conjugates. The system (**A**) contains bis-poly(ethylene glycol) (PEG) polymer as a carrier, one or two copies of LHRH peptide as a targeting moiety, one or two copies of camptothecin (CPT) as an anticancer drug and one or two copies of BH3 peptide as a suppressor of cellular antiapoptotic defense. (**B**) α,ω-bis-PEG3000-citric acid (CA) conjugate (3) was synthesized using one-step procedure by conjugation of 1 M of bis-(2-carboxyethyl) PEG (1) with 2 M of CA [CAS number: 77-92-9, 2-hydroxy-1,2,3-propanetricarboxylic acid] (2). The bis-PEG-CA conjugate (3) was coupled with one, or two moles of CPT (4) to obtain α,ω-bis-(2-carboxyethyl) PEG-CA-CPT conjugates (**C**, 5, 6). Further, one or two moles of LHRH (7) was conjugated with 5 or 6 to obtain α, ω-bis-(2-carboxyethyl) PEG-citric acid-CPT-LHRH conjugates (**D**, 8) having one or two copies of CPT and LHRH. Finally, CPT-PEG-LHRH conjugates with one or two copies of LHRH (8) were conjugated with one or two copies of BH3 peptide (9), respectively, to obtain CPT-PEG-LHRH-BH3 or 2xCPT-PEG-2xLHRH-2xBH3 (10) conjugates.

CPT-PEG-LHRH conjugates with one or two copies of LHRH (Scheme 1, D, compound 8) were conjugated with one or two copies of BH3 peptide (Scheme 1, D, compound 9), respectively, to obtain CPT-PEG-LHRH-BH3 or 2xCPT-PEG-2xLHRH-2xBH3 (Scheme 1, D, compound 10) conjugates.

Cancer Cells

Discarded anonymous pathological materials obtained from the Cancer Institute of New Jersey that do not allow identifying patient information were used to isolate cancer cells from tissues obtained from patients with ovarian carcinoma. Cancer cells were isolated from primary tumor tissue according to the standard protocol (19). The fluid with cancer cells was obtained from the peritoneum area of the patients with ovarian cancer. The samples were centrifuged for 20 min at 2,000 g; the supernatant was discarded, and cell pellets were consequently resuspended. The resuspended cells were cultured in RPMI media (Sigma, St. Louis, Mo) supplemented with fetal bovine serum (Fisher Chemicals, Fairlawn, NJ), 2.5 µg/mL insulin

and 1.2 mL/100 mL penicillin-streptomycin (Sigma, St. Louis, Mo). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (*v/v*) in air. All experiments were performed on cells in the exponential growth phase.

Animal Model and *In Vivo* Antitumor Activity

An animal model of human ovarian carcinoma xenografts was created as previously described (10,11,13,20–22). Briefly, human ascitic cells (2×10^6) were subcutaneously transplanted into the flanks of female athymic nu/nu mice. When the tumors reached a size of about 0.3 cm^3 (15–20 days after transplantation), mice were treated intraperitoneally with saline (control), CPT, CPT-PEG-BH3-LHRH and 2xCPT-PEG-2xBH3-2xLHRH. The doses of CPT in formulations (10 mg/kg) corresponded to the maximum tolerated dose of this drug. The maximum tolerated dose of CPT was estimated in separate experiments based on the animal weight change after the injection of increasing doses of CPT as previously described (10,11,13,14). The animals were treated 6 times within 3 weeks, and tumor size was measured by a caliper. Animal weight was evaluated every day during the treatment period. Changes in tumor size were used as an overall marker for antitumor activity.

Cellular Internalization of Polymers

To analyze cellular internalization of conjugates, PEG polymers were labeled by Fluorescein Isothiocyanate (FITC) as previously described (10,14). Cellular internalization of FITC-labeled PEG polymers was studied by confocal microscopy in living cells at 37°C within 4.5 h.

Cytotoxicity Assay

The cellular cytotoxicity of CPT and CPT conjugates was assessed using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (12,23–26).

Gene Expression

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used for the analysis of expression of genes encoding LHRH receptors (LHRHR), caspases 3 (CASP3) and 9 (CASP9), MDR1 and β_2-microglobulin (β_2-m). The pairs of primers used to amplify each type of gene were 5′-CCCATCATTGCAATAGCAGG-3′ and 5′-GTTCAAACTTCTGCTCCTGA-3′ (MDR1); 5′-GGA TTGTGGCCTTCTTTGAG-3′ and 5′-CCAAACTGAG CAGAGTCTTC-3′ (BCL2); 5′-TGACTGCCAA GAAAATGGTG-3′ and 5′-CAGCTGGTCCCATTGAA GAT-3′ (Caspase 9); 5′-ACCCCCACTGAAAAAGATGA-3′ and 5′-ATCTTCAAACCTCCATGATG-3′ (β_2-m). For the analysis of gene expression total cellular RNA was isolated using RNeasy kit (Qiagen, Valencia, CA) and QIA shredder micro spin homogenizer (Qiagen, Valencia, CA). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech., Piscataway, NJ) according to manufacturer instructions with 2 mg of total cellular RNA and 100 ng of random hexadeoxynucleotide primer (Amersham Pharmacia Biotech., Piscataway, NJ). β_2-microglobulin (β_2-m) was used as an internal standard. PCR was carried out using a Thermocycler (Parkin Elmer, Waltham, MA) with the diluted first-strand reaction mixture, 1 unit of Taq DNA Polymerase (Promega Corporation, Madison, NJ), and 0.5 mM of specific primers in a final volume of 50 mL. The PCR regimen used was 94°C/4 min, 55°C/1 min, 72°C/1 min for 1 cycle; 94°C/1 min, 55°C/50 s, 72°C/1 min for 28 cycles, 60°C for 10 min. Gel electrophoresis was used for the separation of PCR products by submarine electrophoresis using agarose gel (Lonza Rockland, ME) at 4% *w/v* concentration in 0.5 3 TBE buffer: 0.0445 M Tris/Borate, 0.001 M EDTA, pH 8.3 (Research Organics, Cleveland, OH). The gels were digitally scanned, and gene expression was calculated as a percentage of the internal standard (β_2-m).

Protein Expression

The expression of Caspase 9 and BCL2 proteins was assessed by Western immunoblotting analysis and processed using scanning densitometry to quantify the expressed protein as previously described (22,23). To this end, harvested cells were lysed in RIPA buffer (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) using a needle and syringe. Following incubation on ice for 45 min, the cells were centrifuged at 10,000 g for 10 min. Protein content in the supernatant was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL), and 50 µg of protein was run on a 15% sodium dodecyl sulphate (SDS) polyacrylamide gel immersed in Tris/Glycine/SDS buffer (BioRad, Hercules, CA) for 90 min at 70 V. Proteins were transferred to an Immobilon-P nitrocellulose membrane (Millipore, Bedford, MA) in a Tris/Glycine buffer (BioRad, Hercules, CA) for 90 min at 100 V. The membrane was blocked in non-fat milk for 30 min at room temperature on a rotating shaker to prevent non-specific binding, washed and incubated overnight with anti-BCL2 rabbit primary antibody (1:250 dilution, Stress Gene Biotechnologies, Victoria State, BC, Canada), anti-Caspase 9 rabbit primary antibody (1:500 dilution, Stress Gen Biotechnologies, Victoria State, BC Canada) or anti-β-actin mice primary antibody (1:2000 dilution, Oncogene Research, San Diego, CA) at 4°C. After further washing,

the membrane was immersed in goat anti-rabbit and goat anti-mouse IgG biotinylated antibody (1:3000 dilution and 1:1000 dilution, respectively, BioRad, Hercules, CA) at room temperature for 1.5 h on a rotating shaker. Bands were visualized using an alkaline phosphatase color development reagent (BioRad, Hercules, CA). The bands were digitally photographed and scanned using the Gel Documentation System 920 (NucleoTech, San Mateo, CA). Beta-actin was used as an internal standard to normalize protein expression. Band intensities of BCL2 protein and caspase 9 were expressed as the percentage of the β-actin band intensity, which was set at 100%. Similarly, the expression of BCL2, Caspase 9, P-gp and MRP2 was also measured in homogenates of tumor samples obtained from experimental animals.

Apoptosis

The apoptosis induction was analyzed by the measurement of the enrichment of histone-associated DNA fragments (mono- and oligo-nucleosomes) in homogenates of the tumor and other organs (liver, kidney, lung, heart and brain) using anti-histone and anti-DNA antibodies by a cell death detection ELISA Plus kit (Roche, Nutley, NJ) as previously described (13,23,25).

Statistical Analysis

Data obtained were analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as a mean value±standard deviation (SD) from five independent measurements. We analyzed data sets for significance with Student's test and considered P value of less than 0.05 as statistically significant.

RESULTS

Tumor-Targeted Proapoptotic Polymer-Anticancer Drug-Peptide Conjugates

We constructed complex multifunctional polymer-peptide-drug conjugates for effective treatment of primary and aggressive metastatic ovarian tumors (Scheme 1, A). The conjugates contain one or two copies of an anticancer drug, targeting moiety, and a suppressor of antiapoptotic cellular defense. In the present study, we used camptothecin as an anticancer drug, conjugating one or two CPT molecules to one polymer molecule. The detection of the expression of plasma receptors to LHRH peptide in tissues of a primary tumor and malignant ascites from patients with metastatic advanced ovarian carcinoma showed that these receptors are overexpressed in primary and metastatic (malignant ascites) cancers (Fig. 1, A, B). Moreover, the measurement of LHRH expression in cancerous and normal ovarian tissues obtained from the same patient showed that the expression of these receptors is substantially more pronounced in cancerous tissue when compared with normal ovarian tissue. Consequently, we used LHRH peptide as a targeting moiety to direct an entire conjugate specifically to cancer cells and enhance its penetration and intracellular uptake. The fluorescent microscopy analysis of cells incubated with FITC-labeled LHRH-polymer conjugates showed that, in fact, such conjugate effectively penetrated into cancer cells isolated from tumor tissues obtained from patients with ovarian carcinoma and distributed both in the cytoplasm and nuclei of the cells after 24 h long incubation period (Fig. 1, C). Consistently, based on our previous findings (10–12,14,23), in the present study, we used one or two copies of BH3 peptide as a suppressor of cellular antiapoptotic defense and one or two copies of LHRH peptide as a targeting moiety.

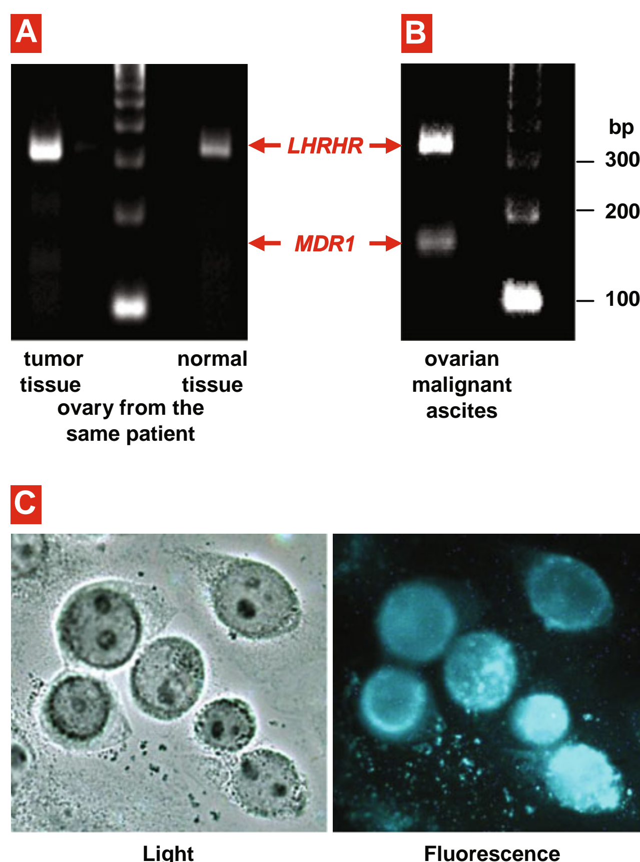


Fig. 1 Typical images of gel electrophoresis of RT-PCR products of gene encoding LHRH receptors (LHRHR) and P-glycoprotein (*MDR1*) in tissues obtained from patients with primary ovarian tumor (**A**) and intraperitoneal metastases (malignant ascites) (**B**); typical images of primary tumor isolates incubated 24 h with FITC-labeled LHRH-PEG polymer conjugates (**C**).

Apoptosis Induction *In Vitro*

The ability of different polymer-peptide-drug conjugates to induce apoptosis was studied in experiments using cells isolated from tissues obtained from patients with primary ovarian carcinoma (Fig. 2, A). The cells were separately incubated with different drug-peptide-conjugate formulations, and expression of two major caspases involved in apoptosis induction (caspase 9 and 3), as well as the rate of apoptotic cell death were studied (Fig. 2, B and C). It was found that incubation of cells isolated from primary tumor with free, non-conjugated CPT led to the activation of caspase 9 and slight but statistically significant ($P<0.05$) induction of apoptosis (compare bars 2 and 1 in Fig. 2, B and C). Conjugation of CPT to PEG polymer slightly increased expression of caspases 9 and 3 and apoptosis (bar 3 in Fig. 2, B and C). Further conjugation of CPT-PEG with LHRH to some extent enhanced the ability of CPT to induce apoptotic cell death by activating caspases 9 and 3 (bar 4 in Fig. 2, B and C). An addition of proapoptotic BH3 peptide to the non-targeted polymer-drug conjugate dramatically (more than three times when compared with control) augmented the expression of caspases and apoptosis induction (bar 5 in Fig. 2, B and C). Targeting of this proapoptotic conjugate to cancer cells by LHRH peptide significantly ($P<0.05$) increased the expression of caspases 9 and 3 (on 17% and 34%, respectively when compared with non-targeted conjugate; compare bars 6 and 5 in Fig. 2, B) and apoptosis induction (more than two times, compare bars 6 and 5 in Fig. 2, C). Finally, doubling the number of copies of CPT, LHRH and BH3 peptides substantially enhanced apoptosis-inducing ability of the conjugate. It led to a further significant ($P<0.05$) increase in the expression of caspases and more than two-fold increase in apoptosis induction (compare bars 7 an 6 in Fig. 2, C). As a result, the degree of apoptosis was augmented more than 15 times when compared with control (compare bars 7 and 1 in Fig. 2, C).

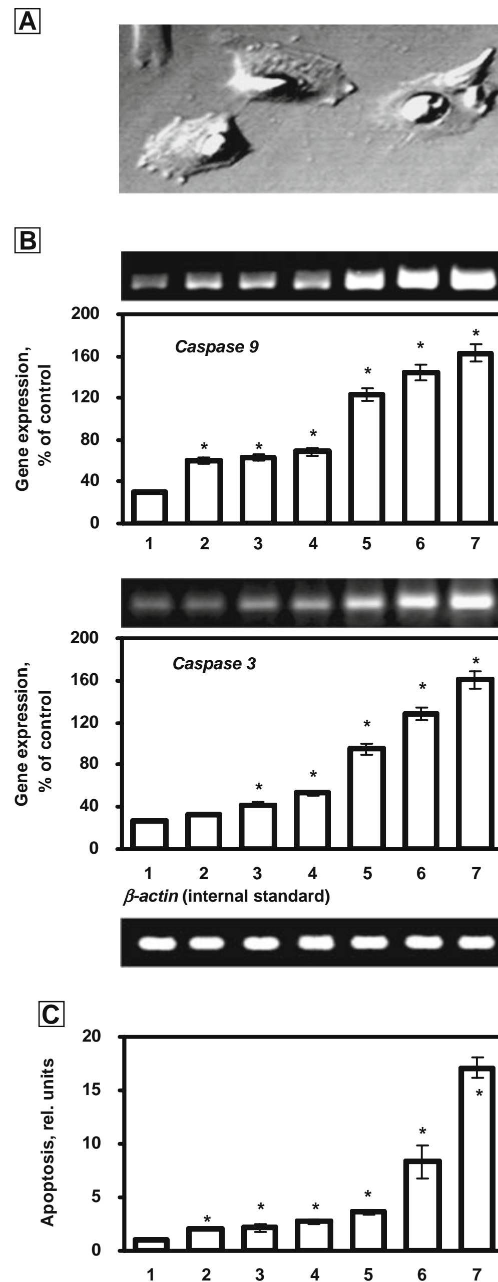


Fig. 2 Gene expression and apoptosis induction in primary tumor isolates. Typical microscopy image of cancer cells isolated from primary tumor tissue samples obtained from patients with ovarian carcinoma (**A**). Typical images of gel electrophoresis of the RT-PCR products and quantitative analysis of expression of *Caspases 9 and 3* genes (**B**) and apoptosis induction (**C**) in primary tumor isolates treated with the indicated formulations. Band intensities of studied genes are expressed as the percentage of the *β-actin* (internal standard) band intensity, which was set to 100%. The enrichment of histone-associated DNA fragments (mono and oligonucleotides) in control (1) was set to 1 unit, and the degree of apoptosis was expressed in relative units. 1—Untreated Control; 2—CPT; 3—CPT-PEG; 4—CPT-PEG-LHRH; 5—CPT-PEG-BH3; 6—CPT-PEG-LHRH-BH3; 7—2xCPT-PEG-2xLHRH-2xBH3. Means±SD are shown. * $P<0.05$ when compared with control.

Activation of Cell Death Signaling Pathways and Suppression of Cellular Antiapoptotic Defense *In Vivo*

The ability of developed conjugates to induce a cell death signal and suppress cellular antiapoptotic defense was studied on mice bearing xenografts of cells isolated from human malignant ascites. This tumor model represents a much more aggressive type of cancer when compared with tumor produced by inoculation of cells isolated from primary tumors of patients with ovarian carcinoma. The expression of *P53* (a central cell death signal), *BCL2* (an antiapoptotic defense), BAX (a major pro-apoptotic protein from the BCL2 family), *APAF1* (apoptotic protease activating factor 1), *caspase 9* (*CASP9*, a major apoptosis initiator),

caspase 3 (CASP3, a main apoptosis executor) was studied by RT-PCR using β_2-microglobulin (β_2-m) as an internal standard. In addition, a pro-apoptotic BAX/BCL2 ratio was calculated, and the cleavage of active form of caspase 9 from pro-caspase 9 was confirmed by Western blotting. The results of these series showed the following. Free, non-conjugated CPT led to the activation of both pro-apoptotic signal (P53, BAX) and antiapoptotic defense (BCL2). As a result, only a moderate activation of APAF1 and caspases were registered (compare bars 2 and 1 in Fig 3, A and B). Conjugation of CPT to tumor-targeted proapoptotic polymer-peptide complex (CTT-PEG-LHRH-BH3) substantially enhanced pro-apoptotic cell death signal and activated caspases more efficiently (bar 3 in Fig. 3, A and B). Doubling the number of active components in the conjugate (2xCPT-PEG-2xLHRH-2xBH3) further enhanced proapoptotic signal and suppressed antiapoptotic defense after a single (bar 4 in Fig. 3, A and B) and multiple (bar 5 in Fig. 3, A and B) treatments.

Apoptosis Induction and Adverse Side Effects *In Vivo*

Apoptosis induction was analyzed in the tumor and other organs of animals bearing xenografts of human cancer cells isolated from malignant ascites from patients with ovarian carcinoma (Fig. 4). The degree of apoptosis induction in the tumor was proportional to the antitumor effect of tested formulations, while that in healthy organs reflects adverse side effects. It was found that free CPT activates apoptosis not only in the tumor, but also in the heart, lung, liver, spleen, and kidney. This indicates wide unfavorable organ distribution of the free drug and its severe adverse side effects, inducing cell death in healthy organs. Incorporation of CPT into the targeted proapoptotic polymer conjugate containing one copy of each active component (CPT, LHRH and BH3) led to significant enhancement of apoptosis induction in the tumor. However, considerable side effects leading to a substantial induction of cell death in healthy organs were still preserved. Including two copies of each CPT, LHRH and BH3 in one polymeric conjugate (2xCPT-PEG-2xLHRH-2xBH3) not only augmented cell death in the tumor but substantially limited adverse side effects of the treatment on healthy organs. Repeating treatment with this conjugate further enhanced cell death induction in the tumor while preventing adverse side effects in normal healthy tissues (Fig. 4).

Antitumor Effect of the Conjugates

The efficiency of tested formulations to suppress tumor growth was investigated in two aggressive models of human tumor xenografts. In the first series of the experiments, cancer cells were isolated from solid tumor tissues of

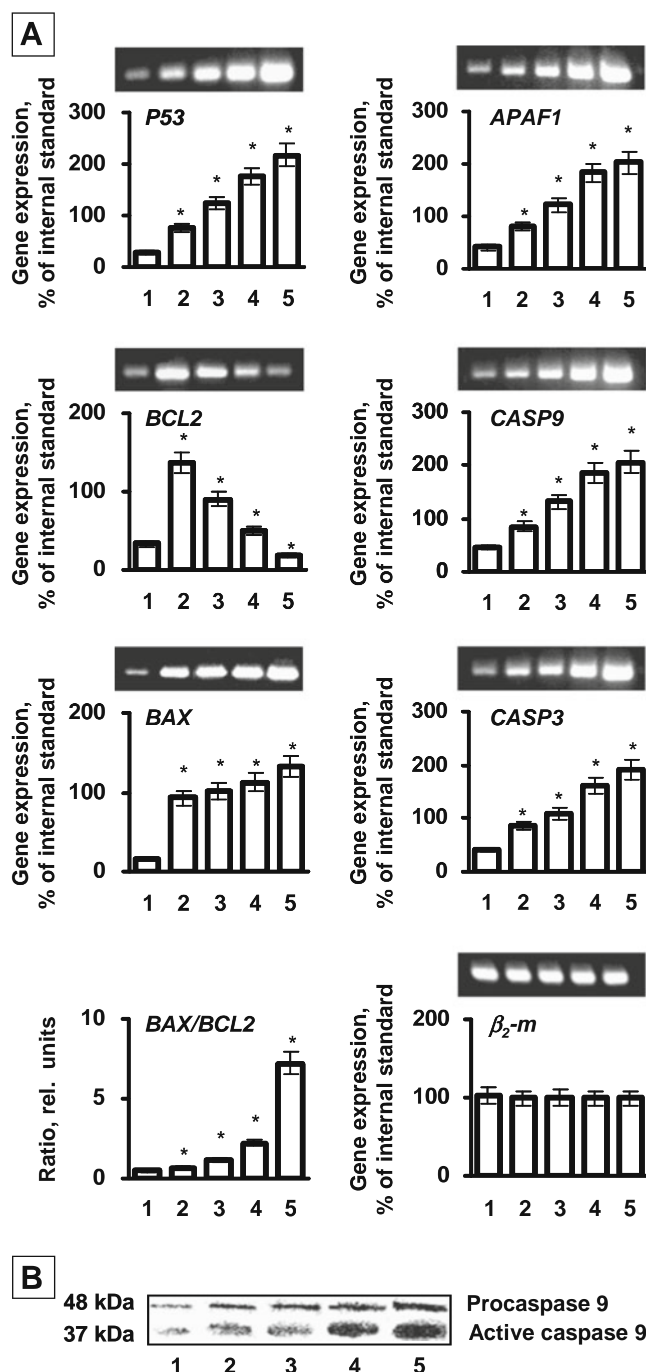


Fig. 3 Gene and protein expression in tumor samples from mice bearing xenografts of cancer cells isolated from malignant ascites obtained from patients with metastatic ovarian carcinoma. Mice were treated with the indicated formulations. Typical images of gel electrophoresis of the RT-PCR products and quantitative analysis of gene expression (**A**). Typical image of Western blot (**B**). Band intensities of studied genes are expressed as the percentage of the β_2-microglobulin (β_2-m, internal standard) band intensity, which was set to 100%. The BAX/BCL2 gene expression ratio is expressed in relative units. 1—Untreated control; 2—CPT (after a single treatment); 3—CPT-PEG-LHRH-BH3 (after a single treatment); 4—2xCPT-PEG-2xLHRH-2xBH3 (after a single treatment); 5—2xCPT-PEG-2xLHRH-2xBH3 (after 6 injections within 3 weeks). Means±SD are shown. * $P<0.05$ when compared with control.

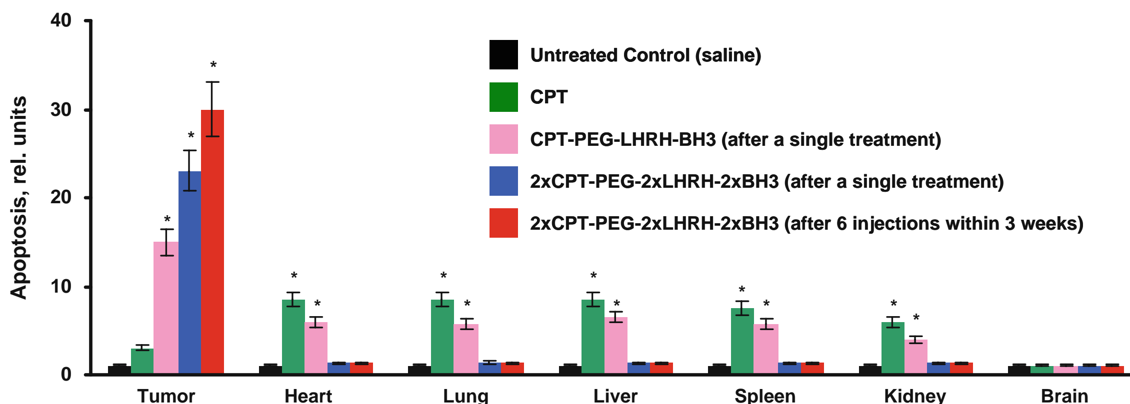


Fig. 4 Apoptosis induction in tumor samples from mice bearing xenografts of cancer cells isolated from malignant ascites obtained from patients with metastatic ovarian carcinoma. Mice were treated with the indicated formulations. The enrichment of histone-associated DNA fragments (mono and oligonucleotides) in control (1) was set to 1 unit, and the degree of apoptosis was expressed in relative units. Means±SD are shown. * $P<0.05$ when compared with control.

patients with primary ovarian carcinoma and were subcutaneously injected in the flanks of nude mice. In the second series of the experiments, the cells were isolated from malignant ascites of patients with ovarian carcinoma and were used for creation of subcutaneous mouse model. The size of the tumor was measured every day using a caliper, and tumor volume was calculated. It was found that cells isolated from metastatic tumor (malignant ascites) demonstrated more aggressive tumor growth when compared with cancer cells isolated from primary tumor. In fact, the volume of untreated tumors produced by primary tumor isolates increased within 14 days from 500 to 2,000 mm^3 (the maximal tumor size allowed for such experiments by the animal protocol approved by the Institutional Animal Care and Use Committee). In contrast, cells isolated from malignant ascites demonstrated more aggressive growth (approximately 1.4 times faster) reaching the same volume of subcutaneous tumor within 10 days. Consequently, proposed polymer-peptide-drug conjugates were substantially more effective in primary tumor isolates model when compared with model developed using malignant ascites. At the same time, the tendency in antitumor activity of tested formulations was preserved. Free non-conjugated CPT demonstrated lowest activity, PEGylated CPT was more efficient than free CPT, and tumor-targeted proapoptotic peptide-drug conjugate (CPT-PEG-LHRH-BH3) was more effective when compared with non-targeted conjugate. A doubling in the number of copies of all active components of delivery system led to significant increase in antitumor efficiency of the proposed multicomponent conjugate. It should be stressed, however, that even conjugates with two copies of each active component were substantially less effective in tumor models created using malignant ascites when compared with that produced by primary tumor isolates. In fact, while 2xCPT-PEG-2xLHRH-2xBH3 conjugate led to a substantial tumor regression in primary tumor model, a similar conjugate just stabilized the tumor volume and prevented its growth when the tumor was created using cells isolated from human malignant ascites (Fig. 5).

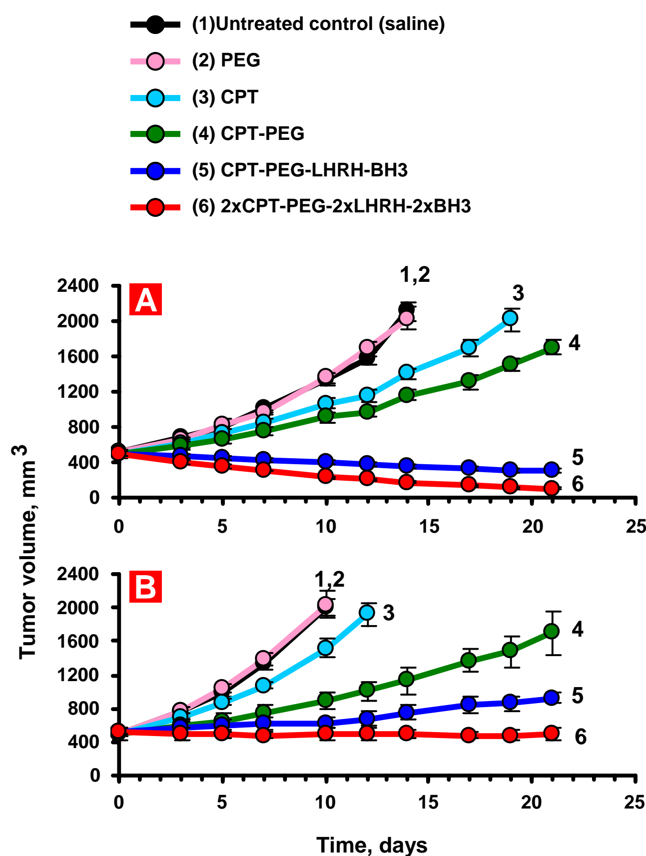


Fig. 5 Tumor volume in mice bearing xenografts of cancer cells isolated from primary tumor (**A**) or malignant ascites (**B**) obtained from patients with primary and metastatic ovarian carcinoma. Mice were treated six times twice per week within 3 weeks starting from the day 0 with the indicated formulations. Means±SD are shown.

DISCUSSION

Recently, we proposed complex multifunctional tumor-targeted proapoptotic polymer-peptide-drug conjugates (PPDC) (10). Such PPDC contains a carrier and one or several copies of anticancer drug, tumor-targeting peptide and proapoptotic peptide. A carrier combines all components together, providing an aqueous solubility of an entire complex. Several carriers have been tested for such conjugates, including linear polymers with branched spacers, liposomes, different modifications of dendrimers, *etc.* (13–15,27). An anticancer drug acts as a cell death inducer, while a proapoptotic peptide acts as a suppressor of cellular antiapoptotic defense. By suppressing such defense, a proapoptotic peptide enhances cell death induction by an anticancer drug. Tumor targeting moiety plays a dual role in this PPDC. First, it directs an entire complex specifically to tumor cells, thereby preventing the accumulation of such high toxic complex in normal tissues and consequently avoiding its severe adverse side effects on healthy organs. Second, targeting moiety enhances cellular uptake by cancer cells that expressed plasma membrane receptors specific to targeting peptide. We proposed and tested LHRH peptide as a tumor-targeting moiety (10,12–16,23,27). In the present study, we used a linear PEG polymer as a carrier, one or two copies of camptothecin, BH3 peptide and LHRH peptide as a cell-death inducer, tumor-targeting moiety and suppressor of cellular antiapoptotic defense, respectively. Preliminary data showed that further increase in the number of copies of BH3 peptide or CPT substantially decreases the solubility of an entire complex, limiting its bioavailability and toxicity. Previous detailed investigation of such conjugates showed that an entire complex acts as a prodrug, inactive and stable during its journey in the systemic circulation. This prodrug converts into a combination of active anticancer drugs and suppressors of cellular antiapoptotic defense after their cleavage from the conjugate via the enzymatic degradation inside cancer cells (14,28).

Previously, we tested such a system *in vitro* and *in vivo* against established A2780-sensitive and A2780/AD multidrug-resistant human ovarian cancer cell lines and their mice xenografts (10,12). In the present investigation, we used ovarian cancer cells isolated from primary tumor and metastatic malignant ascites from patients with ovarian carcinoma. In contrast to well-established cancer cells which were passed many times in standard *in vitro* conditions, losing their cancerogenous activity, these cells were processed only once after isolation in order to obtain a sufficient number of cancer cells to carry out *in vitro* and *in vivo* experiments. Therefore, these cells can be considered as those almost identical to patients' tumor or ascitic cells. In addition, such cells provide considerable variations in the expression of studied genes and proteins, ability to grow as xenografts of solid tumor in mice and resistance to chemotherapy, giving a more reliable experimental model of clinical ovarian cancer and its intraperitoneal metastases. Moreover, cells isolated from patients' malignant ascites demonstrated a substantially higher success rate in establishing mice tumor xenografts and faster tumor growth. All cells isolated from primary tumors and malignant ascites overexpressed plasma membrane receptors for targeting LHRH peptide and successfully internalized our PPDC containing both one and two molecules of each active component conjugated to one molecule of PEG polymer.

Both proposed variants of PPDC (CPT-PEG-LHRH-BH3 and 2xCPT-PEG-2xLHRH-2xBH3) demonstrated similar mechanisms of cell death induction by apoptosis in primary tumor isolates and cells from malignant ascites. These mechanisms included activation of central cell death signal by the anticancer drug. At the same time, BH3 peptide suppressed the activity of cellular antiapoptotic defense by interfering in the activity of its major player—BCL2 protein. It is known that the suppression of BCL2 leads to the leakage of cytochrome *c* from mitochondria and the formation of "apoptosome"—a combination of APAF1, procaspase 9 and cytochrome *c* (29,30). The activation of apoptosome by the cytochrome *c* cleaves procaspase into active caspase, which in turn activates a cascade of caspase-executors of apoptosis (31). The present study showed that all these steps are involved in apoptosis induction by all tested PPDCs in primary tumor isolates and malignant ascites. However, the degree of activation of cell death signal, caspase-dependent signaling pathways of apoptosis and, ultimately, antitumor effect of tested PPDC were significantly different. First, the antitumor activity of peptide-drug conjugates increased with doubling the number of copies of each active component conjugated to one molecule of the carrier. Second, the efficiency of the suppression of tumor growth by the same PPDC was significantly less pronounced in cells isolated from malignant ascites when compared with those originated from primary tumors. One possible explanation of this phenomenon is the overexpression of *MDR1* gene encoding P-glycoprotein in cancer cells isolated from malignant ascites (Fig. 1C). In contrast, cells isolated from primary tumor tissues do not demonstrate overexpression of *MDR1* gene (Fig. 1B). It is well known that expression of P-glycoprotein increases the resistance of cancer cells, often leading to multidrug resistance (32,33).

CONCLUSIONS

The present study clearly showed differences between cancer cells isolated from primary solid tumor and metastatic malignant ascites from patients with ovarian

carcinoma. First, the cells isolated from malignant ascites demonstrated higher tumorogeneric activity in mice bearing xenografts of human ovarian carcinoma. Second, malignant ascites were found to be more resistant to chemotherapy even if such chemotherapy was accompanied by the suppression of cellular antiapoptotic defense. Therefore, the proposed PPDC should be further modified in order to suppress multidrug resistance in metastatic cancer. Nevertheless, the proposed multifunctional polymeric delivery system which consists of multiple copies of the drug and peptides demonstrated significantly higher antitumor activity in primary and metastatic cancers when compared with drug alone and PEG-CPT conjugate.

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