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ARAŞTIRMA YAZISI / RESEARCH ARTICLE

İNSAN MEME KANSERİ HÜCRELERİNDE DOSETAKSEL VE KOMBRETASTATİN A4 YÜKLÜ PEG-PLGA NANOPARTİKÜLLERİNİN TEDAVİ ETKİNLİĞİ

THERAPEUTIC EFFICACY OF DOCETAXEL AND COMBRETASTATIN A4 LOADED PEG-PLGA NANOPARTICLES IN HUMAN BREAST CANCER CELLS

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ÖZET

AMAÇ: Kemoterapi kanser tedavisinde yaygın olarak kullanılmasına rağmen, kemoterapi ajanlarının nonspesifik biyodağılımları ve yan etkileri nedeniyle problemler mevcuttur. Bu çalışmada, hem ilaç direncinin oluşumunu önleyebilen hem de tedavinin etkinliğini artırabilen bir nanoformülasyon geliştirilmiştir.

GEREÇ VE YÖNTEM: Docetaxel (DTX) ve Combretastatin A4 (CA4) yüklü PEG-PLGA (Poli(etilen glikol)-blok-Poli(laktid-ko-glikolid) polimerik nanopartiküller mono ve ikili ilaç yüklemesi ile üretildi. Nanopartiküllerin boyutu ve morfolojisi Transmisyon Elektron Mikroskobu (TEM) kullanılarak karakterize edildi. Tekli ve ikili ilaç yüklü nanopartiküllerin kritik misel konsantrasyonu (CMC) değerleri, ilaç kapsülleme verimliliği ve ilaç salım profilleri UV-Vis spektrometresi ile belirlendi. DTX ve CA4 yüklü PEG-PLGA nanopartiküllerinin *in vitro* etkinliği, serbest ilaç formülasyonlarıyla karşılaştırılarak, insan meme kanseri hücre hattında (MCF-7) test edildi.

BULGULAR: PEG-PLGA nanopartiküllerine ait CMC değeri 0,0126 mM olarak bulunmuştur. Partikül boyutları yaklaşık 5–10 nm aralığında ölçülmüştür. Nanopartiküller, pH 5,5 koşullarında, 4 gün içinde neredeyse tüm yüklü içeriği serbest bırakırken, pH 7,4 ortamında bu oran %60 seviyesinde kalmıştır. DTX ve CA4 yüklü nanopartiküller, ikili serbest ilaç formülasyonuna kıyasla daha düşük bir EC50 değeri sergilemiştir.

SONUÇ: Tasarlanan ikili nano ilaç formülasyonu, kanser tedavisi için alternatif bir kemoterapi çözümü olabilir.

ANAHTAR KELİMELER: Docetaksel, Kombretastatin A4, Nanopartikül, İkili terapi, Kanser.

ABSTRACT

OBJECTIVE: Although chemotherapy is widely used in the treatment of cancer, it suffers due to the nonspecific biodistribution and side effects of chemotherapy agents. In this study, a nano formulation that can both prevent the formation of drug resistance and increase the effectiveness of treatment was developed.

MATERIAL AND METHODS: Docetaxel (DTX) and Combretastatin A4 (CA4) loaded PEG-PLGA (Poly(ethylene glycol)-block-Poly(lactide-co-glycolide) polymeric nanoparticles were fabricated with mono and binary drug loading. The size and morphology of the nanoparticles were characterized using Transmission Electron Microscopy (TEM). The UV-Vis spectrometer determined critical Micelle Concentration (CMC) values, drug encapsulation efficiency, and drug release profiles of the mono and binary drug-loaded nanoparticles. *In vitro* efficiency of the DTX and CA4 loaded PEG-PLGA nanoparticles was tested using a human breast cancer cell line (MCF-7) compared to free drug formulations.

RESULTS: PEG-PLGA nanoparticles had a CMC value of 0.0126 mM. The size of the particles was measured at around 5-10 nm. They released almost all loaded content in 4 days at pH 5.5, whereas this value remained 60% at pH 7.4. DTX and CA4 loaded nanoparticles showed a lower EC50 value than the dual free drug formulation.

CONCLUSIONS: The designed nano dual drug formulation can be an alternative chemotherapy solution for cancer treatment.

KEYWORDS: Docetaxel, Combretastatin A4, Nanoparticle, Dual Therapy, Cancer.

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INTRODUCTION

Cancer has a high morbidity and mortality rate worldwide. The International Agency for Research on Cancer has predicted that the number of cancer cases will increase to 27.5 million by 2040 (1). Chemotherapy is still one of the major treatments for cancer patients. According to the action mechanism, chemotherapy agents can be grouped into anti-mitotic agents, alkylating agents, antiangiogenic agents, tyrosine kinase inhibitors, and antimetabolites (2-4).

In general, chemotherapy targets proliferating abnormal cells non-selectively, causing toxic exposure of both healthy and cancerous cells. So, chemotherapy agents have significant disadvantages due to their advanced side effects and low bioavailability (5). Exposure of healthy cells to the toxic agent affects bone marrow cells. It significantly reduces their immune system, increasing susceptibility to host diseases and leading to physiologically serious side effects in the body (6). Also, poor pharmacokinetic profiles such as short half-life and high clearance rate, low drug ratio in the tumor region, poor water-solubility, and non-specific distribution limit their success in the application (7). Besides these drawbacks, one of the significant obstacles to chemotherapy is multidrug resistance (MDR). It is responsible for 90% of cancer deaths. Although various additive factors cause MDR, such as genetic factors, growth factors, DNA repair ability of cancer cells, and xenobiotic metabolism, the MDR is mostly related to the efflux of chemotherapy agents from the cancer cells (8). Several proteins, such as P-glycoprotein, control the efflux of the active agents. There are several attempts to inhibit P-glycoprotein in clinical trials to prevent MDR (9, 10). Increasing the drug dose would be another solution, but it would cause systemic toxicity. Combining two or more therapeutics that target cancer progression through multiple metabolic pathways, such as proliferation, apoptosis, and angiogenesis, is an essential approach for MDR (11). Combination therapy is often more effective than monotherapy (12). Since different metabolic pathways are targeted in combination therapy, the treatment pathways work synergistically or additively, resulting in effective treatment and reduced side effects due to dose regulation (13). However, for this codelivery approach, where different binary or multiple drug combinations are used, smart drug delivery platforms are needed that enable drug molecules to reach the target tissue (mostly tumors) at the desired high dose.

Developments in nanotechnology have brought us nano drug formulations that provide specific biodistribution and keep drugs inactive in systemic circulation until they reach their target. This nano approach also offers advantages for binary or multiple codelivery applications (14). Nanoparticles provide drug localization at tumor sites with enhanced permeability and retention (EPR) effect and enter cancer cells, increasing the carcinogenic effect with lower doses of drugs while reducing the exposure of healthy cells (15).

Drug-loaded nanoparticles increase drug bioavailability in tumor regions with the enhanced permeability and retention (EPR) effect. This allows for more effective treatment with lower drug doses (16). In addition, since they ensure the inactivity of drugs in circulation and show less accumulation in healthy tissues and organs, they can also allow higher doses that cannot be applied due to toxicity (17).

When these extraordinary properties of nanoparticles and the dual delivery approach provided by dual and multiple drugs are used together, more effective platforms can be developed for MDR that need to be tackled in chemotherapy (18). Multidrug-loaded nanoformulations increase the serum stability of active drugs, enhance their pharmacokinetic profile, and deliver drugs to target sites at higher doses. Thus, the therapeutic synergistic effect of anticarcinogenic drug combinations can be further improved (19).

Babos and coworkers (20) loaded Doxorubicin and Sorafenib into PLGA-based nanoparticles and compared therapeutic efficacy with free drugs in HT-29 cancer cells. Results showed that nanoformulation of doxorubicin and sorafenib showed higher cytotoxicity. Karimian-Shaddel and coworkers (21) utilized a codelivery platform for Metformin and Methotrexate using

chitosan nanoparticles for triple-negative breast cancer therapy. The platform showed significantly improved antitumor efficacy and reduced toxicity compared to free drug combinations in animal studies. Elyazat and coworkers (22) showed superior treatment efficacy of Gefitinib and Azacitidine loaded biodegradable lipid nanoparticles over free drug combinations for metastatic-resistant lung cancer with in vivo studies (22). Rong and coworkers (23) studied the codelivery of camptothecin and MiR-145 by lipid nanoparticles and showed the synergistic effect for carcinoma with both in vitro and in vivo studies. Yadav and coworkers (24) used a nanoemulsion formulation to load Paclitaxel and Baicalein for breast cancer. In vitro and in vivo results proved the efficiency of the codelivery platform over free drug formulations (24).

In this study, a dual drug delivery system obtained by loading the chemotherapy agents Docetaxel (DTX) and Combretastatin A4 (CA4) into PEG-PLGA based nanoparticles was designed. DTX as an antimitotic agent can induce tubulin polymerization and inhibit depolymerization, resulting in mitotic arrest in the G2/M phase (25). CA4 is an antiangiogenic chemotherapy agent (26). PEG-PLGA copolymer is an amphiphilic block copolymer with both PEG block and PLGA block approved by the FDA. The hydrophobic structure of PLGA enables the self-assembly of PEG-PLGA in an aqueous environment while ensuring hydrophobic molecules are encapsulated inside the formed micelle-type nanoparticle (27). DTX and CA4 loaded PEG-PLGA nanoparticles were characterized using a transmission electron microscope (TEM). Drug loading efficiency and release profiles were determined. Cytotoxicity of the nanoparticles was performed on human breast cancer cells.

Breast cancer treatment faces significant challenges due to MDR, which limits the effectiveness of conventional chemotherapy. Breast cancer treatment encounters significant challenges due to MDR, which restricts the effectiveness of conventional chemotherapy. Breast cancer treatment faces significant challenges due to MDR, which limits the effectiveness of conventional chemotherapy. To overcome this, novel strategies involving co-delivery of synergistic drugs are needed to enhance therapeutic efficacy and reduce systemic toxicity. This study hypothesizes that encapsulating Docetaxel (DTX) and Combretastatin A4 (CA4) within a biocompatible PEG-PLGA nanoparticle system will improve drug stability, enable controlled release, and synergistically inhibit breast cancer cell growth. To overcome this, novel strategies involving the co-delivery of synergistic drugs are needed to enhance therapeutic efficacy and reduce systemic toxicity. This study hypothesizes that encapsulating DTX and CA4 within a biocompatible PEG-PLGA nanoparticle system will improve drug stability, enable controlled release, and synergistically inhibit breast cancer cell growth.

MATERIALS AND METHODS

Poly(ethylene glycol) methyl ether-block-poly(lactide-co-glycolide) (PEG average Mn 5,000, PLGA Mn 7,000), Oil Red O, Nile Red, Combretastatin A4, Docetaxel, and Thiazolyl blue tetrazolium bromide were purchased from Sigma-Aldrich. RPMI 1640 Medium (Roswell Park Memorial Institute 1640 Medium) and Fetal Bovine Serum (FBS) were purchased from Biowest. All solvents were HPLC-grade and used as received without any further purification.

Determination of critical micelle concentration (CMC) of PEG-PLGA nanoparticles: The CMC value was determined to understand the concentration value above which the PEG-PLGA polymer forms micelle-type polymeric nanoparticles by self-assembly. For this purpose, an equal amount of Nile Red solution (0.1 mg/500 µL) was added to 250 µL solutions obtained by serial dilution from the stock solution obtained by dissolving the PEG-PLGA polymer in acetone. Then, 1.5 mL of ultrapure water was added to each vial. After the acetone was evaporated at 37 °C, PEG-PLGA solutions, each containing 0.1 mg Nile Red, were obtained in ultrapure water with concentration values ranging between 1.6×10^{-1} mM and 1.6×10^{-4} mM. In this experiment with 3 repetitions, the absorbance values of the solutions obtained at 532 nm were measured, and a graph was created against the concentration values. In the graph, the value at the intersection point of the line where the absorbance values did not increase significant-

ly despite the increase in concentration value, and the line showing the absorbance values rising in correlation with the concentration value was determined as the CMC value (28).

Release profile of PEG-PLGA nanoparticles: Oil red O loaded PEG-PLGA nanoparticles were used to understand the release profile. 12 mg PEG-PLGA polymer was dissolved in 1.5 mL of acetone. In another vial, 1.2 mg Oil red O was dissolved in 1.5 mL of acetone. 250 µL aliquots of PEG-PLGA and Oil red O solutions were combined in six vials. 1.5 mL of PBS (pH 7.4) was added to three of these six vials, and 1.5 mL of acetate buffer (pH 5.5) was added to the other three. Acetone was evaporated on an orbital shaker overnight. The calibration curve was obtained by plotting absorbance values of standard solutions of Oil Red O at 515 nm with a UV-VIS spectrophotometer. The absorbance of filtered samples was recorded at this wavelength to find the loaded amount (n=3 for PBS (pH 7.4) and acetate buffer (pH 5.5). The release study was conducted at 37°C in an incubator. The absorbance values of released Oil Red O for each sample were measured at 515 nm at 0h, 24h, 48h, and 96h, and the amounts of released Oil Red O were calculated using the calibration curve.

Preparation of Drug-Loaded Nanoparticles CA4 Loaded PEG-PLGA Nanoparticles (CA4-PEG-PLGA NP): CA4 loaded PEG-PLGA nanoparticles were prepared using the emulsion solvent evaporation method. 2 mg PEG-PLGA copolymer and 0.15 mg CA4 were dissolved in 500 µL of acetone. This solution was mixed with 500 µL of distilled water to form an emulsion solution. Acetone was evaporated using an orbital shaker overnight. After evaporation, the solution was centrifuged at 6000 RPM to precipitate the unloaded CA4. The aliquot was separated, and the unloaded CA4 precipitate was dissolved with DMSO. The amount of unloaded CA4 was measured using the calibration curve of CA4 at 259 nm. The percent loading efficiency of CA4 was calculated using the equation below.

$$Loading\ Efficiency\ (\%)\ of\ CA4 = \frac{Initial\ Amount\ of\ CA4 - Unloaded\ Amount\ of\ CA4}{Initial\ Amount\ of\ CA4} X100$$

DTX Loaded PEG-PLGA Nanoparticles (DTX-PEG-PLGA NP): DTX loaded PEG-PLGA nanoparticles were prepared similarly to the ones with CA4. 2 mg PEG-PLGA copolymer and 0.40 mg DTX were dissolved in 500 µL of acetone. This solution was mixed with 500 µL of distilled water to form an emulsion solution. Acetone was evaporated using an orbital shaker overnight. After evaporation, the solution was centrifuged at 6000 RPM to precipitate the unloaded DTX. The aliquot was separated, and the unloaded DTX precipitate was dissolved with DMSO. The amount of unloaded DTX was measured using the calibration curve of DTX at 273 nm. The percent loading efficiency of DTX was calculated using the equation below.

$$Loading\ Efficiency\ (\%)\ of\ DTX = \frac{Initial\ Amount\ of\ DTX - Unloaded\ Amount\ of\ DTX}{Initial\ Amount\ of\ DTX} X100$$

CA4 and DTX Loaded PEG-PLGA Nanoparticles (CA4-DTX-PEG-PLGA NP): 4 mg PEG-PLGA copolymer, 0.15 mg CA4, and 0.40 mg DTX were dissolved in 500 µL of acetone. This solution was added to 500 µL of distilled water. Acetone was evaporated, and the solution was centrifuged at 6000 rpm to precipitate the unloaded CA4 and DTX. The aliquot was separated, and the unloaded CA4 and DTX precipitate was dissolved with DMSO. The unloaded CA4 and DTX amounts were measured using the calibration curve of CA4 and DTX at 259 nm and 273 nm, respectively. The percent loading efficiencies of CA4 and DTX were calculated using the equations above. The percent loading capacities of PEG-PLGA nanoparticles were calculated using the equation below.

$$Loading\ Capacity\ (\%) = \frac{The\ total\ amount\ of\ loaded\ drug\ molecules}{The\ total\ amount\ of\ loaded\ drug\ molecules + PEG - PLGA\ amount} X100$$

Morphology of the Nanoparticles: Transmission electron microscopy (TEM) was used to determine the morphological properties of PEG-PLGA nanoparticles. 10 µL of aqueous nanoparticle solutions (CA4, DTX, and CA4+-DTX loaded nanoparticles) were dropped on the TEM grids. The nanoparticle solution

samples were dried for 24 hours and observed under the TEM (JEOL 1220 JEM) at 120 kV.

In Vitro Cytotoxicity: The human breast cancer cell line (MCF-7) was cultured in RPMI-1640 medium supplemented with 10% FBS and 100 U/mL penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded in 96-well plates at a density of 5,000 cells per well and allowed to adhere for 24 h. Subsequently, cells were treated with free CA4, free DTX, free CA4+DTX combination, and DTX-PEG-PLGA, CA4-PEG-PLGA, and DTX-CA4-PEG-PLGA nanoparticle formulations at various concentrations ranging from 10^{-4} M to 10^{-12} M for 48 h. Cell viability was assessed using the MTT assay as follows: the culture medium was replaced with fresh medium containing 0.05 mg/mL MTT solution, and the cells were incubated for an additional 4 h at 37 °C. After incubation, the medium was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formed formazan crystals. Absorbance was measured at 570 nm using a microplate reader.

Ethical Committee

A commercially purchased cell line was used in the study. Therefore, it does not require any ethics committee approval.

Statistical Analysis

Data are presented as mean ± SD. The independent experiments were performed in triplicate, and the statistical analyses were carried out by one-way ANOVA. Significant differences between the groups were discerned at (*) $p \leq 0.05$ and (**) $p \leq 0.01$. Statistical software GraphPad Prism 5.0 and Origin 8 Pro versions were used for all graphical and statistical illustrations.

RESULTS

CMC of PEG-PLGA Nanoparticles

The CMC of PEG-PLGA was determined using a UV-Vis Spectrophotometer. As shown in **Figure 1**, the CMC of PEG-PLGA nanoparticles was an average of 0.0126 mM. This result suggests that hydrophobic Nil Red molecules were loaded in the nanoparticles' hydrophobic core without disturbing the micellar structure above a PEG-PLGA concentration of 0,0126 mM.

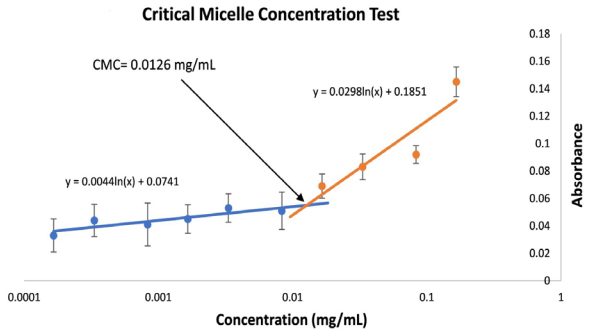

Figure 1: Critical micelle concentration test of PEG-PLGA nanoparticles

Nanoparticle Release Profile

The time-dependent release profile of nanoparticles was tested at different time intervals (0, 24, 48, 72, and 96 h) and pH conditions (pH 7.4 and 5.5). Oil red O, as a hydrophobic molecule, was loaded into the nanoparticles using the solvent evaporation technique. The prepared nanoparticle solutions were incubated at 37°C. The release profile was determined by measuring absorbance values at 0, 24, 48, 72, and 96 hours of incubation **(Figure 2)**.

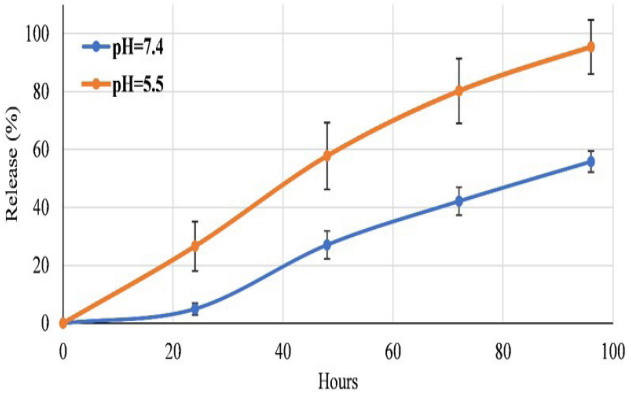

Figure 2: Release profile of PEG-PLGA nanoparticles. Data was obtained with absorbance values measured between 0-96 hours (n=3).

Results showed that Oil red O was gradually released until 96 h at pH 7.4 or pH 5.5. PEG-PLGA nanoparticles released ~60% of Oil red O in 48 hours in a pH 5.5 environment and ~60% of Oil red O in 96 hours in a pH 7.4 environment.

Drug Loading Efficiency of PEG-PLGA Nanoparticles

PEG-PLGA nanoparticles were loaded with anticarcinogenic agents DTX, CA4, and both (DTX and CA4). The percent drug loading content of the nanoparticles is shown in **Table 1**.

Table 1: Drug loading efficiencies and capacities of PEG-PLGA nanoparticles

PEG-PLGA Nanoparticles	Loading Efficiency (%)	Loading Capacity (%)
CA4-PEG-PLGA	92 (CA4)	6.45 (CA4)
DTX-PEG-PLGA	45 (DTX)	8.25 (DTX)
CA4-DTX-PEG-PLGA	85 (CA4) and 33 (DTX)	3.10 (CA4) + 3.15 (DTX)

Characterization of PEG-PLGA Nanoparticles

CA4-PEG-PLGA, DTX-PEG-PLGA, and CA4-DTX-PEG-PLGA nanoparticle samples were analyzed with TEM. Spherical and small (~ 5 to 10 nm in diameter) nanoparticles were observed for all tested nanodrug formulations. There was no significant difference between the PEG-PLGA nanoparticles loaded with CA4 and DTX, both in size and morphology. TEM images of the PEG-PLGA nanoparticles are shown in **Figure 3**.

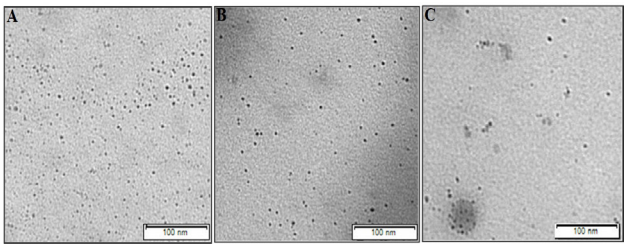

Figure 3: TEM images of PEG-PLGA nanoparticles. A; CA4-PEG-PLGA, B; DTX-PEG-PLGA, and C; CA4-DTX-PEG-PLGA.

Cytotoxicity of PEG-PLGA Nanoparticles

The cytotoxicity assay was performed for free CA4, free DTX, free DTX+CA4, CA4-PEG-PLGA nanoparticles, DTX-PEG-PLGA nanoparticles, and CA4-DTX-PEG-PLGA nanoparticles using MTT assay in MCF-7 cells **(Figure 4)**. The solutions of free drugs (CA4 and DTX) in the concentration range of 10^{-4} M and 10^{-12} M were prepared. Also, CA4-PEG-PLGA and DTX-PEG-PLGA formulations containing equivalent amounts of CA4 and DTX were prepared in the same range. Binary CA4+DTX combination solutions were prepared based on the mass ratio between CA4 and DTX in the CA4-DTX-PEG-PLGA formulation. Drug concentration values in CA4+DTX and CA4-DTX-PEG-PLGA formulations express the sum of CA4 and DTX concentration values.The results showed that the viability of human breast cancer cell lines was decreased in a dose-dependent manner in 48 h. The EC50 values of the free CA4 and CA4-PEG-PLGA nanoparticles were $1.27x10^{-8}$ and $1.83x10^{-9}$ M (Fig. 4A) while in free DTX and DTX-PEG-PLGA nanoparticles the EC_{50} values were $9.11x10^{-9}$ and $4.32x10^{-9}$ M in MCF-7 cells, respectively (Fig. 4B). Additionally, the EC_{50} values of the DTX+CA4 combination and CA4-DTX-PEG-PLGA nanoparticles were $1.28x10^{-7}$ and $1.87x10^{-8}$ M (Fig. 4C).

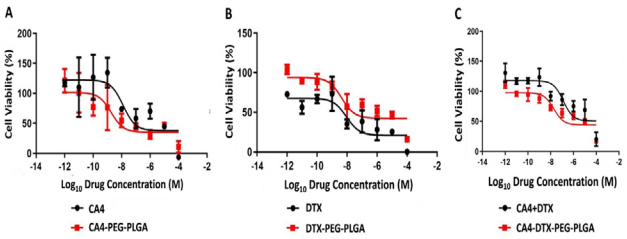

Figure 4: Cytotoxicity test results of CA4, CA4-PEG-PLGA (A), DTX, DTX-PEG-PLGA (B), DTX+CA4 combination, CA4-DTX-PEG-PLGA (C) in MCF-7 cells using MTT assay. Data represent mean ± SD (n=4).

DISCUSSION

In this study, CA4 and DTX drugs were loaded onto PEG-PLGA polymeric nanoparticles, and the suitability of PEG-PLGA for dual therapy was determined by *in vitro* tests. For this purpose, PEG-PLGA polymeric nanoparticles were evaluated by CMC, release profile, encapsulation efficiency, morphology, and cytotoxicity tests.

CMC test was performed to verify the ability of PEG-PLGA block copolymer to form micelles under specified conditions and to find the critical concentration value required for micelle formation. CMC value shows the micelle formation potential of amphiphilic copolymers for drug delivery systems (29). CMC can be measured by parameters such as osmotic pressure, molar conductivity, surface tension change (30), and by changes in the emission and absorption of organic dyes (31). In the study, Nile Red fluorescent dye was used to measure CMC. In the CMC test, the hydrophobic Nile Red molecule is trapped in the hydrophobic centers during the self-assembly of the hydrophobic PLGA segment by evaporation of the acetone used as an organic solvent. However, the self-assembly behavior exhibited by this amphiphilic block copolymer structure occurs above a certain concentration, depending on the hydrophilic-hydrophobic balance and strength. In cases where self-assembly does not occur, no significant absorbance is observed despite the presence of the same amount of Nile Red

in the medium. After the concentration at which self-assembly begins, significant increases in absorbance values are observed as more Nile Red molecules accumulate in the hydrophobic centers. Because the Nile Red molecule does not give color in water, but in hydrophobic environments. It has been shown that CMC can be determined by analyzing both the wavelength shift and the rapid increase in absorption depending on the polymer concentration (32). Within the scope of this study, CMC value for PEG-PLGA nanoparticles was determined by using the change in Nile Red absorption at 532 nm depending on the concentration of PEG-PLGA using a UV-Vis Spectrophotometer with the method used by Basu Ray et al. (33). A CMC graph was obtained from the results, and the CMC value for PEG-PLGA polymeric nanoparticles was determined as 0.0126 mM.

Release studies were conducted to investigate the drug release profiles of PEG-PLGA polymeric nanoparticles in acidic tumor (pH=5.5) and neutral physiological (pH=7.4) conditions. As a hydrophobic drug model, Oil red O was used in the release studies (34). Samples were incubated in PBS (pH=7.4) and acetate buffer (pH=5.5) solutions at 37 ºC. Samples in pH=7.4 PBS buffer showed a release of 5% in the first 24 hours. At the 96th hour, PEG-PLGA released 56% of Oil red O. On the other hand, samples in acidic acetate buffer (pH=5.5) released 26.58% of Oil red O in the first 24 hours, and 95% of Oil red O in 96 hours. Although release occurred in both environments, the release in the acidic environment was faster. Babos et al. (2018) loaded Sorafenib and Doxorubicin into PEG-PLGA polymeric nanoparticles and examined the release profile of PEG-PLGA at pH = 5.5 (20). They reported complete release for both drugs within 150 hours in acidic medium. In the study by Nahar and Jain (2009), amphotericin B drug was loaded into PEG-PLGA polymeric nanoparticles, and the release profiles were examined in PBS and acetate buffer solutions (35). Similarly, it was stated that the drug was released faster and with a higher ratio (91.7%) in acidic medium compared to the neutral one. The encapsulation efficiencies of DTX and CA4 drugs in PEG-PLGA polymeric nanoparticles were calculated separately and together. It was found that approximately 0.18 mg of 0.5 µmol (0.40 mg) DTX drug in 2 mg PEG-PLGA polymer solution was encapsulated by PEG-PLGA nanoparticles formed by evaporating the organic solvent. The PEG-PLGA encapsulation efficiency for docetaxel was 45%. In 2013, Wang and colleagues loaded DTX into PEG-PLGA and determined the encapsulation efficiency as 53.4% (36). In a study conducted by Rafiei and Haddadi in 2017, it was found that the encapsulation efficiency of DTX loaded into PEG-PLGA was 59.30% (37). It was found that the DTX encapsulation efficiency was similar to these results. Approximately 0.138 mg of 0.15 mg of CA4 was loaded in solution containing 2.0 mg of PEG-PLGA, which means 92% encapsulation efficiency. Although the molar numbers were kept equal, this difference between the encapsulation efficiencies of DTX and CA4 is due to the different molecular weights. This situation reveals the dependence of the hydrophobic mass ratio for loading hydrophobic molecules into amphiphilic block copolymer structures. In the encapsulation study where 4 mg PEG-PLGA block copolymer was used and DTX and CA4 were loaded together, it was determined that DTX and CA4 were loaded with 33% and 85% efficiency, respectively. The results showed that PEG-PLGA nanoparticles had a drug loading capacity of 6.45% and 8.25% for CA4, DTX, respectively, and 6.25% for the CA4+DTX combination. These values seem to be within an acceptable range compared to the drug loading capacity of PEGylated PLGA nanoparticles (38).

PEG-PLGA polymeric nanoparticles were imaged by TEM and found to have a regular spherical shape, an average diameter of 5-10 nm, and a homogeneous size distribution. No important changes were observed between the PEG-PLGA nanoparticles loaded with CA4, DTX, or both.

The cytotoxic behaviors of CA4 and DTX drugs, separately and in combination, were compared with the cytotoxicity behaviors of CA4-PEG-PLGA, DTX-PEG-PLGA, and CA4-DTX-PEG-PLGA nanoparticles, and the dual drug delivery potential of PEG-PLGA nanoparticles was investigated. For this purpose, formulations of free drugs (CA4 and DTX) and CA4-PEG-PLGA and DTX-PEG-PLGA nanoparticles having equivalent amounts of free drugs were com-

pared. The CA4+DTX formulation was compared with the CA4-DTX-PEG-PLGA formulation.

Cell internalization ability of the drugs is a parameter that should be considered when comparing drug-loaded nanoparticles with free drug formulations. It has been reported that drug-loaded polymeric nanoparticles are generally taken into cells via internalization routes such as clathrin or caveolae-mediated endocytosis, pinocytosis, and phagocytosis (39, 40). It has been shown that PEGylated nanoparticles show a stronger preference for clathrin and caveolae-mediated endocytosis compared to non-PEGylated ones (41). Such pathways generally result in drug-loaded nanoparticles taking up more drug than free drug in a shorter time (42).

The release rate of drugs is another important parameter to be considered when comparing drug-loaded nanoparticles with free drug formulations. Release studies reveal that PEG-PLGA nanoparticles are released faster under acidic conditions. This is due to the biodegradable structure of the PLGA. However, in addition to acidic conditions, lysosomal activities and other enzymatic conditions accelerate the degradation process of biodegradable structures *in vitro* conditions (43).

The results show that CA4-PEG-PLGA nanoparticles are more cytotoxic than free CA4. Considering the cell internalization and drug release kinetics mentioned above, this situation can be explained by the faster internalization of CA4-PEG-PLGA nanoparticles compared to free CA4 and rapid drug release under acidic *in vitro* conditions. In the study by Hassan et al. in 2020, CA4-loaded PLGA nanoparticles and free CA4 were compared. It was determined that CA4 was more cytotoxic when loaded into the nanoparticle (44). CA4 is a microtubule-targeting drug and accelerates microtubule depolymerization. When microtubule-targeting drugs enter the cell, they disrupt microtubule functions and trigger apoptosis. Cancer cells internalize polymeric nanoparticles via various internalization routes, which increase drug concentration. This explains why cytotoxicity was higher when CA4 was loaded into the nanoparticle (45).

When the EC_{50} values of free DTX and DTX-PEG-PLGA were compared, it was determined that DTX was slightly more cytotoxic than DTX-PEG-PLGA. In the study by Noori et al. (46), the cytotoxicity of DTX-loaded PEG-PDLA and DTX was compared, and it was determined that DTX was more toxic. Drug release profiles in vitro differ, and some drugs' release may vary depending on their environment. In cases where drug release is slow, nanocarrier systems may show fewer toxic effects than free drugs in short periods, such as 48 hours. However, although nanocarrier systems are less cytotoxic in various studies than free drugs in *in vitro* conditions, they are more successful in in vivo trials. This is explained as a situation that occurs due to both the enhanced permeability and retention (EPR) effect and the completion of drug release in in vivo anti-tumor studies over extended periods (47).

According to the MTT analysis results, the CA4-DTX-PEG-PLGA nanoparticle showed a lower EC_{50} value than the CA4+DTX Combination. According to the results, the PEG-PLGA nanoparticle formulation is more cytotoxic than the free CA4+DTX drug combination *in vitro*. Considering the anti-angiogenic effect of the CA4 drug in addition to its anti-mitotic effect (26) and the dual impact that will occur when used in the PEG-PLGA nano formulation together with DTX (25), which has a tubulin stabilizing effect, it is predicted that more successful results will be obtained in vivo.

In this study, PEG-PLGA polymeric nanoparticles were shown to be suitable drug delivery platforms in the combination drug therapy of cancer by *in vitro* tests. Controllable drug release profile is one of the advantageous aspects of the PEG-PLGA drug delivery system. *In vitro* cytotoxicity studies showed that loading different drugs into PEG-PLGA nanoparticles produced different results. Studies conducted with CA4 showed that CA4-PEG-PLGA was more cytotoxic than free CA4, while cytotoxicity studies conducted with DTX showed that the DTX group was more cytotoxic than the DTX-PEG-PLGA group. Both of these situations are encountered in the literature and may be due to differences such as the mechanisms of action of drugs, release profiles, and chemical structures of drugs. To evaluate the free combination with nano combination platform, free CA4 and DTX combination, and CA4-DTX-PEG-PLGA

nano formulation were compared. CA4-DTX-PEG-PLGA group (EC_{50}=1.866 x 10^{-8} M) showed more cytotoxic effect than the free CA4 and DTX combination group (EC_{50}=1.278 x 10^{-7} M). This supports the accuracy of the judgment forming the hypothesis of the study. When the data are considered together, it can be said that PEG-PLGA amphiphilic block copolymer is an ideal nanocarrier in combination therapy at the *in vitro* level. Especially when the *in vitro* conditions for cytotoxicity parameters, the anti-angiogenic property of CA4, and the known EPR effect of nanocarriers are considered together, it is predicted that more successful results will be obtained with this PEG-PLGA based nano drug delivery platform in systemic application compared to free dual drug applications.

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