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Research Article

Enhancement of Paclitaxel Therapeutic Effect by Aptamer Targeted Delivery in PLGA Nanoparticles

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Abstract

Objectives: Paclitaxel is a drug molecule used in the therapy of various cancer types, including breast cancer. It is one of the preferred chemotherapy agent due to its high efficacy. However, many side effects have been observed associated with paclitaxel use such as allergy, hair loss, diarrhea and pain.

Methods: We evaluated therapeutic efficacy of paclitaxel when it is actively targeted to breast cancer tumours inside a polymeric nanoparticle. Targeted delivery of paclitaxel to tumour sites has been reported as an improved cytotoxicity strategy with a variety of nanoparticles. In this study, poly Lactic-co-Glycolic Acid (PLGA) nanoparticles were used as drug carrier and nucleolin aptamers as affinity targeting agents.

Results: Paclitaxel molecules were entrapped during the synthesis of PLGA nanoparticles of 238 nm in diameter. The encapsulation and loading efficiencies of paclitaxel was 97% and 21% respectively. The paclitaxel loaded PLGA nanoparticles were functionalized with nucleolin aptamers and their targeting ability to cultured mouse cancer cells was determined for two cell lines (E0771 and 4T1). E0771 cell line was chosen for the preparation of allograph breast cancer mouse models. Evaluations of the targeted paclitaxel in PLGA nanoparticles showed 38% better performance in inhibiting tumour growth compared to free paclitaxel treatment groups of mouse models.

Conclusion: The chemotherapeutic effect of cancer drugs like paclitaxel can be increased by loading inside tumour targeted polymeric nanoparticles.

Keywords: DNA aptamers, nanoparticles, paclitaxel, drug delivery systems

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Breast cancer ranks first among cancer types, especially in terms of its incidence in women.[1] The side effects of the most common therapy options such as radiotherapy or chemotherapy, include reduced effectiveness because of dose losses.[2] In addition, non-targeted chemotherapy drugs interact with healthy cells and might exhibit various toxic effects. The specific transfer of paclitaxel (PTX) which is the antineoplastic drug we will use in our study, to the tumour tissue will be achieved with a targeted drug system. Thus, it will be possible to treat breast cancer more effectively and with fewer side effects associated with the paclitaxel therapy.

Aptamers are single stranded nucleic acids selected in vitro. They are synthetic oligonucleotides that can bind to a wide variety of target molecules (proteins, metal ions, monosaccharides, peptides, microorganisms, cells and tissues etc.) with high selectivity and affinity.[3, 4] Aptamers are frequently used in biosensor applications due to their similar affinity to antibodies, and they selectively recognize target molecules.[5, 6] They are easily produced, inexpensive, easily modified chemically, and can be easily integrated into different analytical designs. Due to such features, aptamer-based biosensors have advantages compared to antibodies.[7] Since aptamers can undergo conformational changes when bound to their targets, they are well suited to drug delivery system design. Aptamer targeted drug delivery enables efficient cancer therapy with reduced potential toxicity to healthy

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cells.[8, 9] Tumour size reduction has been demonstrated in a nude mouse glioma cancer model using PTX encapsulated in aptamer (AS1411 aptamer) coated PLGA.[10] In another study, cervical cancer was formed on nude mice and treated with AS1411 aptamer-coated docetaxel-encapsulated nanospheres, resulting in reduced tumour size.[11]

PLGA nanoparticles was prepared with synthetic polymers and approved for use in drug transport systems by the Federal Drug Administartion (FDA) for therapeutic use. It is a biocompatible and biodegradable polymer.[12] In this respect, it is among the most studied nanoparticles today. Synthetic polymers such as PLGA provide many advantages due to their chemical properties, being biodegradable with small particle size, thus easy production and controlled release.[13, 14]

In this study, targeted nanostructures will be synthesized by using AS1411 aptamer as targeting molecule, which exhibits high affinity for nucleolin protein as a specific target for breast cancer cells. Since the nucleolin protein is found more in the membrane of cancer cells, it is targeted to specifically release the antineoplastic drug to these areas. The cytotoxicity effects of the paclitaxel loaded inside the aptamer functionalized PLGA nanoparticles were determined for two mouse breast cancer cultured cells. The cell line with best targeting performance for nucleolin aptamer was used to obtain breast cancer cells allography mouse model. Finally, the allograph tumour sizes were evaluated for free paclitaxel or targeted paclitaxel in aptamer-PLGA nanoparticle (Apt-PLGA-PTX). A summary for the strategy used in this study is given in Figure 1.

Methods

Materials

PLGA, Resomer® RG 503 H), paclitaxel and all other chemicals were obtained from Sigma-Aldrich (Germany). E0771 (ATCC CRL-3461) and 4T1 (ATCC CRL-2539) cell lines were obtained from ATCC (USA).

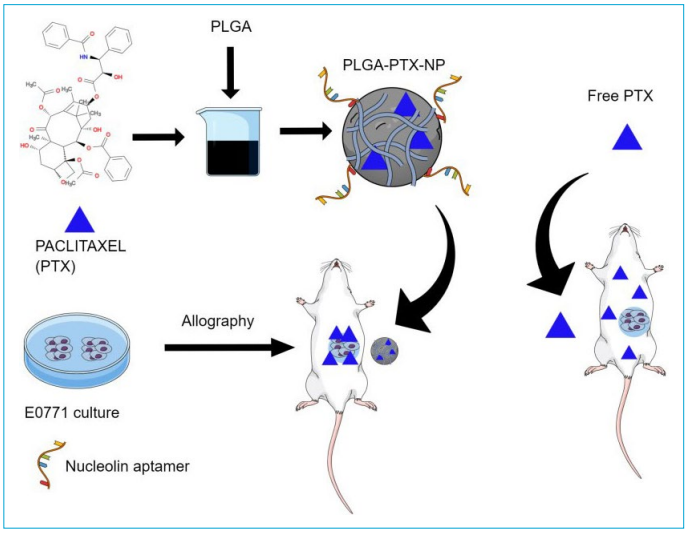

Figure 1. The summary of targeted paclitaxel experiments used in this study.

Methods

Allograph Breast Cancer Mouse Model

The breast cancer mouse model was prepared by injection of a mouse mammary tumour cell line into mice.[15] In this study, 6-8 weeks old female C57/BL6 mice were administered once by subcutaneous injection of 3-5 x 106 E0771 cell line. After the cells reached 60% confluency in culture, they were collected by trypsinization and washed with PBS and injected subcutaneously into the mouse. Tumour formation in the animal was observed in a short time, which is a characteristics for cancer cells in xenograph modelling studies (Fig. 2). Tumour development was observed by palpating breast tissue twice a week. The size of the tumor was measured with a caliper during palpation at its widest shape.[16] The mice were grown for 39 days (21 days for tumour formation and 18 days for tumour monitoring).

Three weeks after innoculation injections, the tumours reached 200-300 mm^3 and the treatments of paclitaxel were initiated by injections in PBS buffer. Treatment solutions were injected as intraperitoneal application with an insulin injector. The injections were according to weight of the Mouse at 0, 3^{th} and 6^{th} days. The control groups were injected with equal volume of PBS (1 ml) at the treatment days. The weight of the mice were monitored for every 5 days untill the end of the experiments (Table 1).

Synthesis of PLGA Nanoparticles

Oil-in-water emulsification-evaporation based synthesis of the PLGA nanoparticles and aptamer functionalization was according to previously published methods.[17, 18] Five mg poly-lactic acid (50:50) was mixed with dichloromethane to obtain the primary emulsion and five mg paclitaxel was added before sonication of the whole mixture by agitation. The solution was emulsified by adding polyvinyl alcohol (PVA) at 0.1 % (w/v) concentration with constant stirring, resulting in a double emulsion. Dichloromethane was evaporated by room temperature incubation for 24 hours. The nanoparticles were collected by centrifugation at 8.723 x g for 40 min. and washed with distilled water and kept at -20 °C until use.

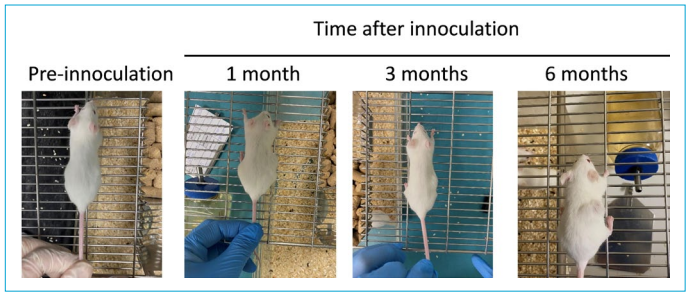

Figure 2. Typical picrtures of mouse used in this study. Female C57/BL6 mice were followed after innoculation of E0771 tumor forming cultured cells.

Table 1. The weights of the mice.

Days	Weight (Kg), No treatment Animals	Weight (Kg), Pactitaxel treatment Animals	Weight (Kg), Apt-PTX-PLGA treatment Animals
0	19.1±3.8	18.7±4.1	17.5±5.2
5	23.6±3.7	18.8±4.5	19.9±5.7
10	22.6±3.5	20.2±3.8	21.0±5.1
15	23.1±2.9	20.9±4.3	21.3±3.7
20	24.2±1.8	21.1±3.7	23.9±2.0
25	24.8±1.4	24.1±2.9	24.1±3.6
30	24.4±2.1	23.4±3.0	23.4±4.2
35	24.4±1.8	23.8±2.2	23.8±1.9
39	24.9±2.8	24.8±2.5	24.8±1.5

Encapsulation efficiency (EE) was calculated from total amount of paclitaxel used in loading experiments in PBS with equation 1.

$$EE\ (\%) = \frac{\text{Paclitaxel loaded } (\mu g \cdot ml^{-1})}{\text{Total Paclitaxel } (\mu g \cdot ml^{-1})} \times 100 \quad (1)$$

Loading efficiency (LE) was calculated from the same total amount value as used in EE% with equation 2.

$$LE\ (\%) = \frac{\text{Paclitaxel loaded } (\mu g \cdot ml^{-1})}{\text{Total nanoparticle weight } (\mu g \cdot ml^{-1})} \times 100 \quad (2)$$

Paclitaxel was quantified by fluorescence spectroscopy (excitation: 308 nm / emission: 551).[19] The samples were collected at various times up to 20 days during release experiments in PBS similar to a previous description.[17] The hydrodynamic average size of the nanoparticles were determined by dynamic light scattering (DLS) (NanoZS, Malvern Instruments Worcestershire, UK). The morphology of the particles was investigated by atomic force microscopy (AFM; Nanomagnetics inc., Ankara, Turkey).

The 5'-amino labelled nucleolin binding aptamer (AS1411) was synthesized according to published sequence (5'--CCACCACGGTGGTGGTGGTTGTGGTGC-3').[20] The aptamers were reacted with carboxyl functionalized PLGA nanoparticles by a EDC linker (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride). PLGA nanoparticles at 10 µg / µl concentration were mixed with 200 mM EDC and 50 mM N-hydroxysuccinimide (NHS), incubated for 15 min at room temperature by constant stirring.[21] Ten nanomol aptamer was added into the mixture and incubated for another 6 hours. The mixture was washed in PBS by centrifugation. The amount of aptamers conjugated to the surface of the nanoparticles were calculated from the difference of aptamer concentrations before and after the conjugation procedure.[22] Aptamer grafting details can be found in a previous publication.[17]

Cell Culture and Cytotoxicity Analysis

Mouse cancer cell lines (4T1 and E0771) was grown in RPMI medium supplemented with 10% (w/v) heat-inactivated fetal bovine serum (Sigma-Aldrich, Germany) and 0.1% penicillin/streptomycin (Sigma-Aldrich, Germany) by incubating in a humidified 5% CO^2 atmosphere at 37 °C and sub-cultured every 72 h when the cells reached about 80% confluency. The cytotoxic effect of paclitaxel or Aptamer-PLGA-PTX was determined with WST-1 cell proliferation assay was used (Cell Counting Kit-8, Sigma) for counting live cells. 104 cells were added to 96-well plate and incubated for 24 h with paclitaxel or nanoparticles. CCK-8 reagent was added to each well and the conversion of tetrazolium salts to formazan dye was evaluated on a microtiter plate reader for the absorbance at 440 nm (BMG Labtech, Omega).

Results

In this study, paclitaxel-loaded PLGA-aptamer (AS1411) conjugate was used in a mouse breast cancer model obtained by injection of the E0771 mouse cancer cell line to obtain palpable size tumor. Then, aptamer-conjugated PLGA nanoparticles with paclitaxel cargo were injected into model mice to investigate its effectiveness in mouse mammary tumor model.

Synthesis and Characterization of PLGA Nanoparticles

Paclitaxel was entrapped in PLGA nanoparticles by including equal amount of drug during the synthesis of the nanoparticles by two-phase evaporation method. The round morphology of the nanoparticles was observed with AFM analysis (Fig. 3A). The average diameter of the synthesized nanoparticles was measured to be 238±3.9 nm by DLS (Fig. 3B). The hydrodynamic size of the PLGA particles are slightly larger than the sizes of AFM images (Fig. 3). The encapsulation efficiency (EE%) of paclitaxel was 97% and loading efficiency was 21%. The morphology of the particles was investigated The amount of aptamer molecules immobilized on the surface of PLGA nanoaprticles were estimated as 22.4 nmol/mg.

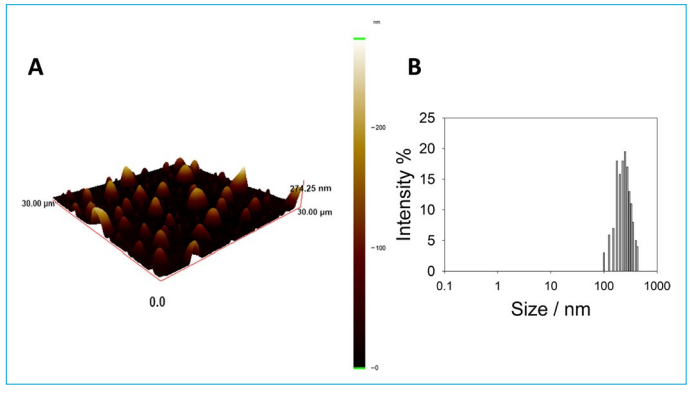

Figure 3. Characterization of PLGA nanoparticles. **(a)** AFM images for verticle morphologic analysis, **(b)** DLS particle size analysis.

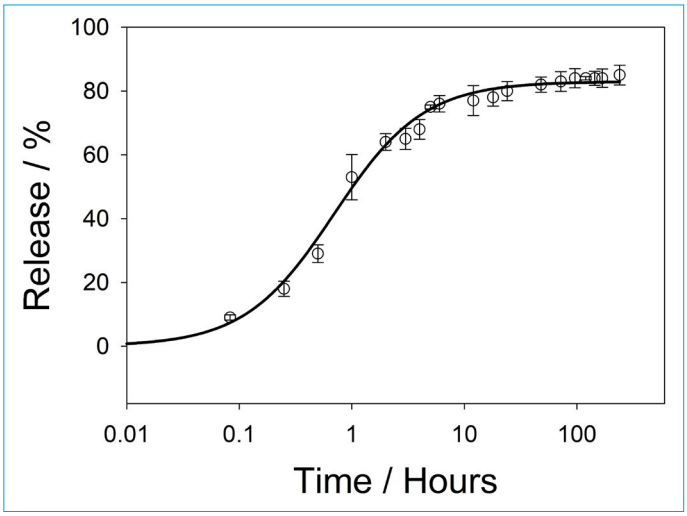

Figure 4. Paclitaxel release from Aptamer-PLGA-PTX nanoparticles. Paclitaxel were monitored in a time course. Error bars represent standard deviation of triplicate experiments.

We followed paclitaxel release from PLGA particles at 37 °C for 20 days after solubilization in PBS (pH=7.4). Paclitaxel leakage from particles were quite fast in the initial one hour of mixing in buffer solution up to 62%, then the release continued at a decreased rate (Fig. 4).

In Vitro Studies with Aptamer-PGLA-PTX Nanoparticles

The Aptamer-PLGA-PTX particles showed 3.3% and 8.3% more cytotoxic effect on E0771 cell lines for 5 ng/ml and 50 ng/ml paclitaxel treatments experiments (Fig. 5a). There was no significant difference for 0.05 and 0.5 ng/ml paclitaxel treatments. The control experiments with the growth medium RPMI or Aptamer-PLGA particles without any paclitaxel did not show any significant cytotoxic effect (Fig. 5a).

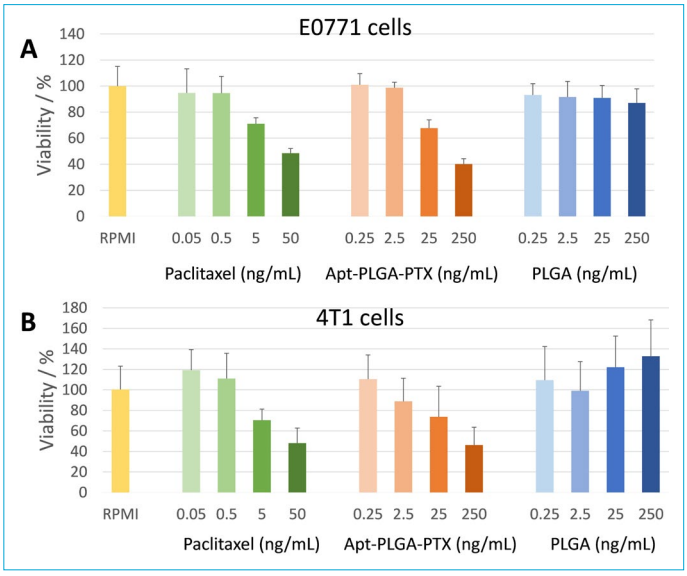

Figure 5. Cytotoxicity results with paclitaxel in free solutions or encapsulated in Aptamer-PLGA nanoparticles for cell lines **(a)** E0771 and **(b)** 4T1.

However, similar experiments with 4T1 cells resulted in only 2% cytotoxic difference betwee free paclitaxel and Aptamer-PLGA-PTX particles while there was no significant differences in cytotoxicity for 0.05, 0.5 or 5 ng/ml concnetrations (Fig. 5b).

In Vivo Studies with PGLA-PTX-Aptamer Nanoparticles

Equal amounts of paclitaxel were injected into allograph mice models in aqueous form (0.2 mg/Kg) or entrapped in PLGA-PTX-Aptamer particles (1 mg/Kg). The non-treated allograph mice group developed tumour up to 6800±282.7 mm^3 in 18 days (Fig. 6). The treatment group with free paclitaxel inhibited the tumour growth with 1604±9.2 mm^3 in 18 days, corresponding to 76.4 % smaller tumour lump (Fig. 6). The treatment with PLGA-PTX-Aptamer particles outperformed the free paclitaxel with 987±4.9 mm^3 at the end of 18 days (Fig. 6). Thus, targeted paclitaxel in PLGA nanoaprticles resulted in 38.4% better tumour inhibition compared to free paclitaxel treated mice.

Discussion

PLGA nanoparticles are one of the preferred drug carriers for targeted delivery studies due to its high biocompatibility and bioavailability. In this study, we synthesized paclitaxel entrapped PLGA nanoparticles by including the drug in the synthesis mixture. Subsequent grafting with nucleolin aptamers provided active targeting capability to the nanoparticle carrier to breast cancer tumour cells. The nanoparticle carrier was shown to have good targeting capacity in cultured mouse breast cancer cells. The size, loading and grafting characteristics of Aptamer-PLGA-PTX nanoparticles were similar to a previous study on PLGA nanoparticles loaded with antibiotics.[17]

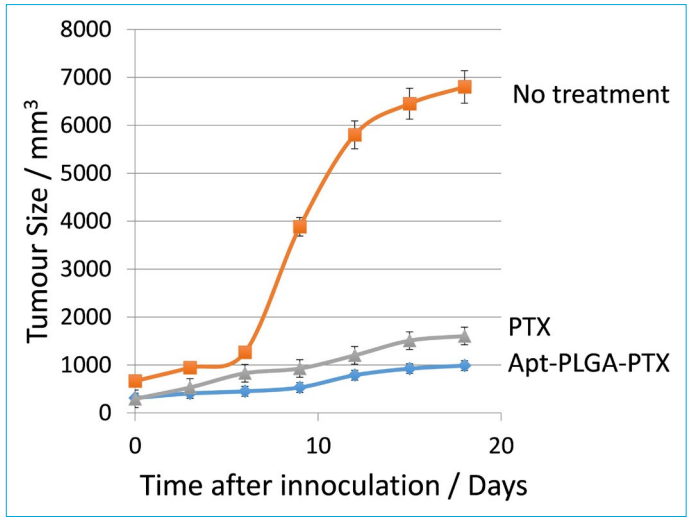

Figure 6. Time-course monitoring of tumor size after inoculation of E0771 cells in free paclitaxel treatment group or targeted paclitaxel in PLGA nanoparticles. Both treatments prevented the growth of the tumor compared to no treatment group. The error bars represents standard deviations of 9 independent experiments. The x-axis is the day after the treatment start.

Nucleolin binding aptamer was reported in numerous reports for its specific property to actively target tumour cells.[20, 23] 4T1 carcinoma cell line has been used in many studies due to its representation of invasive characteristics to simulate stage IV human breast cancer. In this study, we first tested the nucleolin aptamer ability to target mouse breast cancer cell lines. Aptamer-PLGA-PTX demonstrated better cytotoxicity impact on E0771 cells compared to 4T1 cells. There was 3.3% to 8.3% more cytotoxic effect on the viability of E0771 cells when paclitaxel was targeted in Aptamer-PLGA-PTX particles. Thus, E0771 cell line was chosen for preparation of allograph mouse model studies.

The breast cancer mouse model as prepared in this study has been reported in many successful tumour development and drug-treatment experiments.[15, 24] In addition, the use of cancer cell lines in allograph modeling has provided the most realistic imitation of tumours for research purposes, providing a realistic imitation of tissues and organs to be targeted, and tumour growth. Therefore, we tested the efficiency of our targeted paclitaxel nanoparticle carrier with E0771 mouse breast cancer cells allograph in female C57/BL6 mice. The results showed that paclitaxel effect increased the efficiency of chemotherapy when it is provided inside PLGA nanoparticles compared to free paclitaxel. In this manuscript, the effect of paclitaxel loaded PLGA was not included since many previous studies demonstrated that the effects of paclitaxel loaded PLGA and free paclitaxel almost were the same.[25, 26]

Conclusion

In conclusion, the chemotherapeutic effect of cancer drugs like paclitaxel can be increased by loading inside tumour targeted polymeric nanoparticles. PLGA is used to encapsulate paclitaxel and nucleolin aptamers were used for targeting breast cancer tumours in allograph mice models.

Disclosures

Ethics Committee Approval: This study was approved by the Yeditepe University, Animals Ethics Committee (Decree No: 738/ Date: 11.03.2019).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – A.D.D., V.C.O.; Design – A.D.D., B.M.; Supervision – B.M.; Materials – A.D.D., V.C.O.; Data collection &/or processing – S.U., F.P., E.Y.; Analysis and/or interpretation – A.D.D., S.U.; Literature search – S.U., F.P., E.Y.; Writing – A.D.D.; Critical review – B.M., V.C.O.

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