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PEtOx-DOPE nanoliposomes functionalized with peptide 563 in targeted *BikDDA* delivery to prostate cancer

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Background: Nanocarrier-based systems have cultivated significant improvements in prostate cancer therapy. However, the efforts are still limited in clinical applicability, and more research is required for the development of effective strategies. Here, we describe a novel nanoliposomal system for targeted apoptotic gene delivery to prostate cancer.
Methods: Poly (2-ethyl-2-oxazoline) (PEtOx) dioleoyl phosphatidylethanolamine (DOPE) nanoliposomes were conjugated with the prostate-specific membrane antigen (PSMA)-targeting peptide GRFLTGGTGRLLRIS (P563) and loaded with *BikDDA*, a mutant form of the proapoptotic Bik. We selected 22Rv1 cells with moderate upregulation of PSMA to test the in vitro uptake, cell death, and in vivo anticancer activity of our formulation, P563-PEtOx-DOPE-BikDDA.
Results: *BikDDA* was upregulated in 22Rv1 cells, inducing cell death, and CD-1 nude mice xenografts administered with the formulation showed significant tumor regression.
Conclusion: We suggest that P563-PEtOx-DOPE-BikDDA nanoliposomes can serve as prominent gene carriers against prostate cancer.

Key words: Prostate cancer, poly(2-ethyl-2-oxazoline), liposome, *BikDDA*, peptide 563, targeted gene therapy

1. Introduction

Prostate cancer is one of the most frequently diagnosed malignancies among men worldwide (Siegel et al., 2022). Despite the notable advance in the experimental therapy of prostate cancer enabled by nanomaterial-based systems, in vivo precision-targeting, biocompatibility, and safety issues continue to pose major limitations in actual clinical application (Pranav et al., 2023). Therefore, profound research is required for developing safe and efficient nanodelivery systems for prostate cancer therapy.

While nanoparticles (NPs) can reach tumors through the enhanced permeability and retention (EPR) effect, achieving passive targeting due to the leaky tumor vasculature, active targeting is commonly utilized to further advance in vivo specificity (Batool et al., 2023). Prostate-specific membrane antigen (PSMA) is a prominent target given that it is upregulated on prostate cancer cell surfaces as well as the neovasculature of all major solid tumors (Chang et al., 1999). Our previous work showed that prostate cancer cells with moderate to high levels of PSMA expression could be effectively targeted by peptide 563 (P563; GRFLTGGTGRLLRIS), a highly selective PSMA-targeting peptide (Shen et al., 2013). Building on these findings, our group synthesized P563-conjugated PEtOx-DOPE (dioleoyl phosphatidylethanolamine) lipopolymers for targeted gene delivery to prostate cancer (Gulyuz et al., 2021). As for the cargo molecule, we selected *BikDDA*, a mutant form of the proapoptotic gene Bik, which has a prolonged half-life and possesses increased apoptotic activity (Jiao et al., 2014).

Here, we tested the novel P563-PEtOx-DOPE-BikDDA nanoliposome formulation for antitumor activity against

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prostate cancer in vitro, using 22Rv1 cells exhibiting PSMA expression, and in vivo, on 22Rv1 xenograft models. To the best of our knowledge, this is the first study that employs the use of the *BikDDA* gene as a treatment strategy for prostate cancer.

2. Materials and methods

2.1. Site-directed mutagenesis

The *BikDDA* cargo gene used in this study was obtained previously, as described in Oz et al. (2020). Briefly, the QuikChange Lightning Site-Directed Mutagenesis Kit was used to modify the Bik gene: Thr33 and Ser35 residues were mutated to aspartic acid, and the Ser124 residue was replaced with an alanine to obtain *BikDDA*.

2.2. Cell culture

PNT1A human prostate epithelial cells (95012614; Sigma-Aldrich, USA) and 22Rv1 prostate carcinoma cells (CRL-2505; American Type Culture Collection [ATCC]) were routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco) and 100 units/mL penicillin/streptomycin (Gibco). The cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

2.3. Quantitative real-time PCR

Total RNA was isolated from 22Rv1 cells (3×10^5 cells/well in 6-well plates) using a TRIzol reagent (Invitrogen), and cDNA was synthesized using Sensiscript reverse transcription kit (QIAGEN), following the respective instructions. Both 18S rRNA (QuantiTect Primer Assay, QIAGEN, Germany) and Bik forward (5'-GAGACATCTTGATGGAGACC-3') and reverse (5'-TCTAAGAACATCCCTGATGT-3') primers were used to amplify the respective PCR products (Bio-Rad, CFX96 Real-Time PCR System). The data was analyzed using CFX Manager.

2.4. Cell viability

P563-PEtOx-DOPE polymers were successfully synthesized (Gulyuz et al., 2021), and P563-PEtOx-DOPE nanoliposomes were prepared using the lipid hydration method, as previously demonstrated (Saka and Bozkır, 2018).

The effect of P563-PEtOx-DOPE-BikDDA nanoliposomes on 22Rv1 prostate cancer cell viability was assessed using a WST-1 cell proliferation reagent (Roche, USA). The 22Rv1 cells seeded into 96-well plates at a density of 5×10^4 cells/well were treated with 10 µg/mL of BikDDA plasmid (naked *BikDDA*) or nanoliposomes carrying an equal concentration of the gene P563-PEtOx-DOPE-BikDDA. The cells were also treated with an equivalent dose of the empty nanoliposome formulation (P563-PEtOx-DOPE). Following 72 h of treatment, WST-1 reagent was added at a dilution of 1:10 (v/v) in RPMI. Absorbance was measured at 450 nm using SpectraMax Paradigm Multimode Microplate Reader (Molecular Devices, San Jose, CA, USA). Untreated (control) cells were considered 100% viable, and the relative viability of cells under different treatment conditions was compared accordingly.

2.5. Cell death assay

At a density of 3×10^4 cells/well, 22Rv1 cells were seeded into 8-well chambers. Subsequently, they underwent treatment with P563-PEtOx-DOPE-BikDDA, P563-PEtOx-DOPE, or 1 µM staurosporine (positive control). The cells were fixed with paraformaldehyde (4% w/v) and permeabilized with a Triton X-100 (0.1% v/v) solution in sodium citrate (0.1% w/v). In between each step, the cells were washed three times with PBS. In Situ Cell Death Detection Kit (Roche, USA) was used to analyze cell death, following the manufacturer's protocol: the TUNEL reaction mixture was prepared, and the samples were labeled at 37 °C for 75 min. The cells were visualized using a Zeiss Axioscope fluorescent microscope, and average fluorescent intensity was calculated from five independent images for each condition using ImageJ.

2.6. Serum stability

P563-PEtOx-DOPE-BikDDA nanoliposomes in PBS were stored at 4 °C in amber tubes for two weeks. Nanoliposomes were mixed with Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at a 1:50 (v/v) ratio and incubated at 37 °C for 24 h. Serum stability was assessed by the size distribution and zeta potential of the nanoliposomes (Chan et al., 2009; Jones and Nicholas, 1991). The pH was approximately 7.4 for all media utilized during the characterization tests, including water, buffers, and serum-containing DMEM.

2.7. Assessment of in vivo antitumor activity

Athymic male CD-1 nu/nu mice were obtained from Charles River Laboratories (Germany) and allowed to rest in the animal facility of Yeditepe University (Türkiye) for two weeks before tumor inoculation. The animals were maintained under a 12 h light/12 h dark cycle and had ad libitum access to food and water. On the day of inoculation, the mice were injected with 22Rv1 cells in a Matrigel/PBS mixture as described in our previous work (Nezir et al., 2023). The mice were randomly assigned into P563-PEtOx-DOPE-BikDDA, P563-PEtOx-DOPE, naked *BikDDA*, and control (PBS) groups on the first day of injection and received a total of 8 injections over the course of 28 days. *BikDDA* administered in the naked or encapsulated form was 7 µg/injection/mice, while P563-PEtOx-DOPE was administered at a dose equivalent to the administered *BikDDA*-loaded nanoliposomes. Tumor tissues were collected following cervical dislocation, and tumor weight and volume data were collected. All tissues were stored in a 10% formalin solution until pathological

analyses. The following formula was used to calculate tumor volumes: tumor volume (mm^3) = 1/2 × length × $width^2$—length, long radius; width, short radius (Zhao et al., 2022). The study protocol was approved by the Animal Care and Welfare Committee of Yeditepe University (Türkiye, decision number: 644), and all experiments were performed in accordance with the ARRIVE guidelines.

2.8. Histopathological evaluation

All samples were transferred to Ümraniye Training and Research Hospital, Department of Pathology, for histopathological evaluations. Paraffin-embedded tissue sections were prepared from formalin-fixed tumor tissues and subjected to hematoxylin and eosin (H&E) staining as described previously (Gulyuz et al., 2021; Nezir et al., 2023). A score of 0 to 3 (0, absent; 1, mild; 2, moderate; 3, severe) was assigned to each sample in terms of necrosis, inflammation, and fibrosis.

2.9. Statistical analysis

Differences between group means were analyzed in GraphPad Prism 8 (GraphPad Software, USA) using one-way analysis of variance followed by Tukey posthoc test, and the data are expressed as mean ± standard deviation (SD). A two-sided p value of <0.05 was considered statistically significant.

3. Results

3.1. Serum stability of P563-PEtOx-DOPE-BikDDA

The particle size and zeta potential values of P563-PEtOx-DOPE-BikDDA are shown in Table 1. A 30-nm difference was observed in particle size after incubation of the nanoliposomes in serum, indicating a slight interaction between the nanoliposomes and serum proteins. While the zeta potential of the formulation increased significantly following serum incubation, no significant change was observed in charge distribution in the medium. No aggregation of the liposomes was observed at any phase of characterization, indicating the suitability of the materials to be tested under in vivo conditions.

3.2. BikDDA transfection efficiency of P563-PEtOx-*DOPE* nanoliposomes

BikDDA mRNA expression level in 22Rv1 prostate cancer cells transfected with P563-PEtOx-DOPE-BikDDA nanoliposomes was increased by 6-fold compared with untreated (control) cells and those treated with empty nanoliposomes (P563-PEtOx-DOPE) after 72 h of treatment, while no change in mRNA levels was observed at 48 h (Figure 1). These results imply that P563-PEtOx-DOPE-BikDDA could effectively increase the expression of *BikDDA* in 22Rv1 cells, given an appropriate treatment duration.

3.3. The effect of P563-PEtOx-DOPE-BikDDA on cell viability

A significant decrease to 60% viability was observed when 22Rv1 cells were treated with P563-PEtOx-DOPE-BikDDA for 72 h (Figure 2). An equal dose of naked *BikDDA* or the empty carrier (P563-PEtOx-DOPE) did not promote a significant change in cell viability compared with untreated (control) cells.

3.4. The effect of P563-PEtOx-DOPE-BikDDA on cell death

A TUNEL assay was performed to analyze cell death. Average fluorescent intensities calculated from five independent areas revealed a 6.2-fold increased signal intensity for P563-PEtOx-DOPE-BikDDA and 4.3-fold for staurosporine (positive control), compared with P563-PEtOx-DOPE (empty nanoliposomes). The change in cell morphology induced by the treatment conditions and the corresponding fluorescent images are shown in Figure 3.

3.5. In vivo antitumor efficacy of P563-PEtOx-DOPE-BikDDA

Mice administered with P563-PEtOx-DOPE-BikDDA had a significantly lower average tumor volume compared with both the control group that received PBS (107.2 vs. 503.4 mm^3, $p < 0.5$) and P563-PEtOx-DOPE (107.2 vs. 451.5 mm^3, $p < 0.5$) (Figure 4a). Average tumor weight in P563-PEtOx-DOPE-BikDDA, P563-PEtOx-DOPE, and control groups were 275.8, 772.8, and 946.4 mg, respectively, confirming the significant reduction in tumors in the P563-PEtOx-DOPE-BikDDA group ($p < 0.5$; Figure 4b). Tumors collected from all animals are shown in Figure 4c. These results indicated that *BikDDA* transfected in our nanoliposome formulation could effectively promote tumor regression in vivo.

All tumor samples were histopathologically confirmed to have a Gleason score (GS) of 5 + 5, indicating the

Table 1. Particle size and zeta potential values of P563-PEtOx-DOPE-BikDDA.

	Particle size (nm)		Zeta potential (mV)	
	Before incubation	**After incubation**	**Before incubation**	**After incubation**
P563-PEtOx-DOPE-BikDDA	146.0 ± 1.557	117.3 ± 3.936	–30.9 ± 0.794	–11.8 ± 0.153
DMEM with 10% FBS	-	148.0 ± 0.2082	-	–13.6 ± 1.0000

The values represent the mean ± SD of three independent experiments.

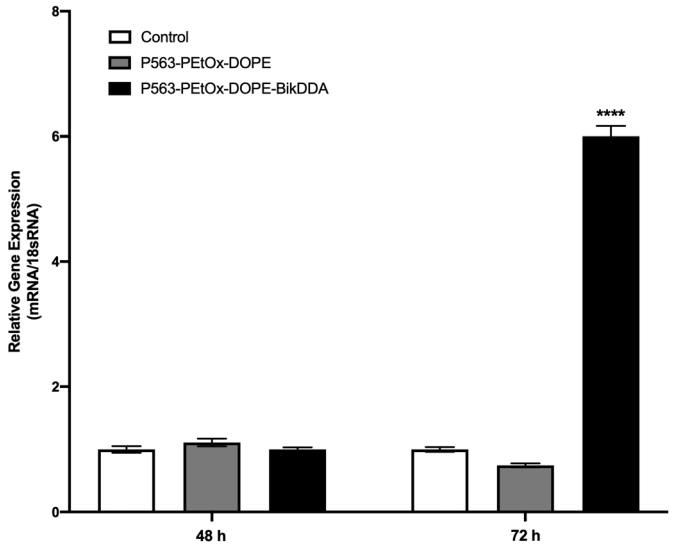

Figure 1. Relative *BikDDA* expression in 22Rv1 cell lines transfected with P563-PEtOx-DOPE-BikDDA nanoliposomes. The results were compared with those of untreated (control) and empty nanoliposome (P563-PEtOx-DOPE)-treated cells. The reference gene used was 18s rRNA. **** $p \leq 0.001$.

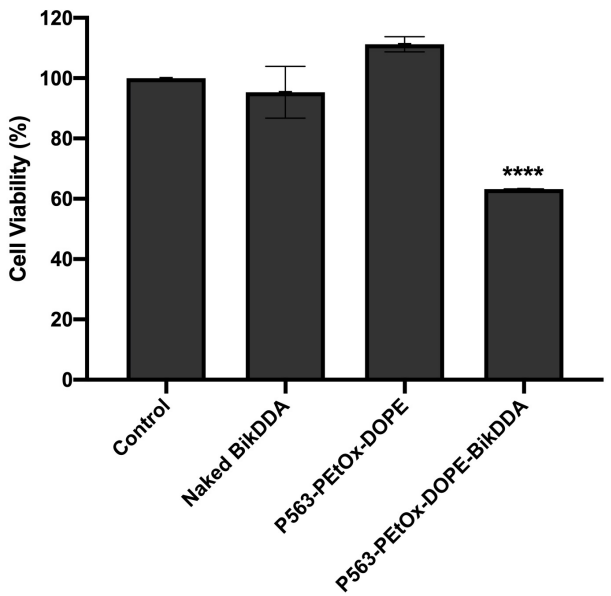

Figure 2. The effect of P563-PEtOx-DOPE-BikDDA on 22Rv1 cell viability. The cells were treated with P563-PEtOx-DOPE-BikDDA, an equal dose of naked *BikDDA*, or an equal dose of the empty nanoliposomes (P563-PEtOx-DOPE). Percentage cell viability was assessed by assuming the viability of untreated (control) cells to be 100%. **** $p \leq 0.001$.

successful establishment of the xenograft models. The average necrosis score was 1.3, inflammation was 1.0, and fibrosis was 1.0 in the control group mice that received PBS injections (Table 2). Similarly, these scores were 1.2, 0.8, and 1.0, respectively, in the P563-PEtOx-DOPE (empty nanoliposome) group. The lowest scores were assigned to the P563-PEtOx-DOPE-BikDDA group, with a score of 0.5 for all three factors (Table 2).

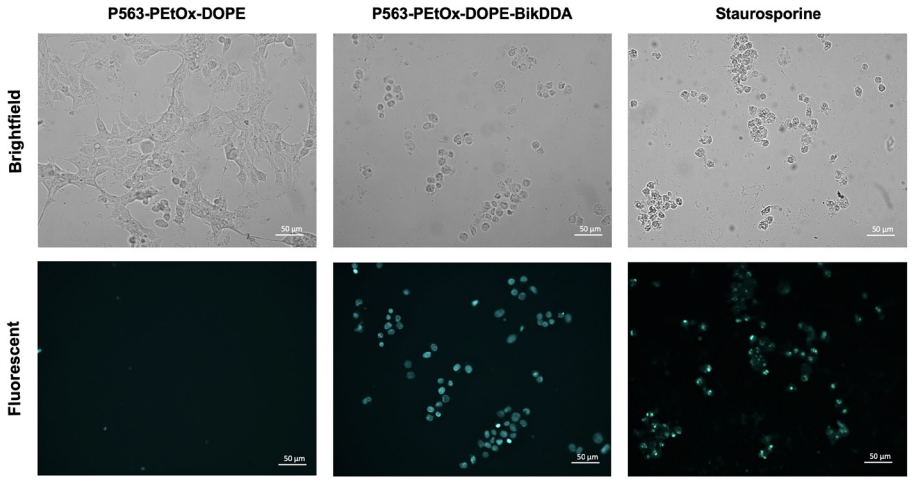

Figure 3. Representative fluorescent microscopy images showing 22Rv1 cell death detected by the TUNEL assay. DNA double-strand breaks were observed upon treatment with P563-PEtOx-DOPE-BikDDA and staurosporine (positive control). Objective: 20×; scale bars: 50 µm.

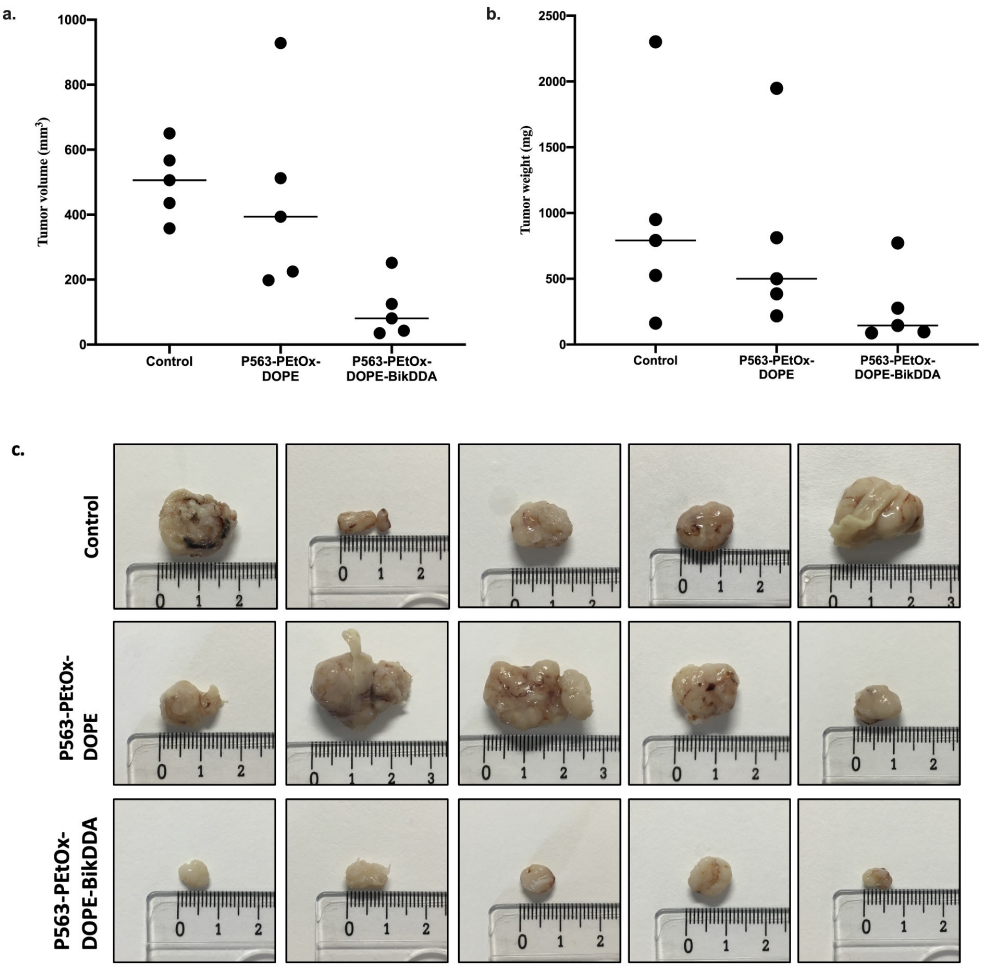

Figure 4. Changes in (a) tumor volume and (b) weight of CD-1 nu/nu male 22Rv1 tumor xenografts (n = 5) upon treatment with the vehicle control (PBS), P563-PEtOx-DOPE, or P563-PEtOx-DOPE-BikDDA. (c) Images of tumors in all groups (n = 5).

Table 2. Pathological evaluation of the tumors in all groups.

Group (n = 5)	Necrosis	Inflammation	Fibrosis
Control	1.3	1.0	1.0
P563-PEtOx-DOPE	1.2	0.8	1.0
P563-PEtOx-DOPE-BikDDA	0.5	0.5	0.5

The values represent the average of five pathological scores assigned to each group.

4. Discussion

Nanocarrier-based cancer therapies have yielded promising results in recent years. A rather successful example is BIND-014 (Hrkach et al., 2012), a polymeric nanoparticle (NP) formulation comprising poly(D,L-lactide) (PLA) and poly(ethylene glycol) (PEG), proven to be safe for human use (Avgoustakis, 2004). The NP encapsulated docetaxel, a chemotherapeutic drug efficient against prostate cancer, and was functionalized with a small molecule ligand targeting the extracellular domain of prostate-specific membrane antigen (PSMA) that is upregulated on prostate cancer cell surfaces as well as the neovasculature of all major solid tumors (Von Hoff et al., 2016). The formulation showed prolonged circulation in the vascular system, suppressed tumor growth in more than one animal model, and enabled tumor regression in patients at a dose lower than that used in clinical practice for the soluble form of docetaxel (Hrkach et al., 2012; Von Hoff et al., 2016). Most recently, in a phase 2 study, the number of PSMA-positive circulating tumor cells was reduced in patients treated with BIND-014. However, the study lacked comparison with soluble docetaxel, and a high ratio of the patients experienced adverse effects, including neuropathy (Autio et al., 2018).

Yet, only a few of the numerous nanodelivery systems developed to this day are in clinical trials. Therefore, extensive research has been focused on developing novel nanocarrier-based delivery systems. Recently, we developed a polymeric micelle formulation similar to BIND-014 in that it was loaded with a low dose of docetaxel and achieved active targeting through the tumor-homing peptide P563, a peptide with high affinity to PSMA (Nezir et al., 2023). Our formulation induced tumor regression in 22Rv1 xenografts and displayed a good safety profile.

In the present study, we tested a nanoliposome formulation employing the same PSMA-targeting approach for in vivo targeting of prostate cancer using 22Rv1 xenografts. This setting was designed to evaluate the potential of our formulation in targeted gene therapy. Therefore, instead of docetaxel, the cargo molecule was the apoptotic *BikDDA* gene. During apoptosis, p53 induces the transcription of several genes that produce BCL-2 homology domain 3-only proteins, including the proapoptotic Bik gene (Hao et al., 2023). The action of the protein product Bik, which is localized in the endoplasmic reticulum, mobilizes calcium ions to the mitochondria, resulting in the remodeling of the mitochondrial cristae and the induction of apoptosis through the mitochondrial pathway (Chinnadurai et al., 2008). Previously, *BikDDA* was shown to enhance apoptotic activity in triple-negative breast cancer cells, compared to another mutant, *BikDD* (Jiao et al., 2014). Here, for the first time in the literature, we have tested the apoptotic effect of the *BikDDA* gene in prostate cancer by targeting the androgen responsive 22Rv1 cell line and their in vivo xenograft model. In vitro assays showed that 22rv1 cells subjected to P563-PEtOx-DOPE-BikDDA were efficiently transfected with the gene, and apoptotic cell death was effectively induced by this longer half-life mutant form of Bik. In vivo, tumor volume and tumor weight were significantly reduced in mice administered with P563-PEtOx-DOPE-BikDDA. Moreover, tumor necrosis was reduced in the treatment group compared with the PBS and empty carrier controls, indicating a less aggressive tumor profile (Liu and Jiao, 2020). Overall, these results suggest that P563-PEtOx-DOPE nanoliposomes can serve as a promising gene delivery system to induce tumor regression.

5. Conclusion

In summary, this study highlights several important findings in prostate cancer therapy: (i) delivery of *BikDDA*, a mutated form of the proapoptotic Bik, can be considered a promising strategy in the field of gene therapy; (i) PEtOx-DOPE nanoliposomes can serve as safe and effective gene delivery platforms; and (iii) targeting of prostate cancer cells in vitro and in vivo can be achieved by employing the PSMA-targeting peptide P563, even for cells with only moderate upregulation of PSMA on the cell surface. Therefore, the findings presented here are expected to have a guiding significance for future advances in nanocarrier-based prostate cancer therapy.

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Conflict of interest
The authors declare that they have no conflict of interest.

Contribution of authors
Conceptualization, A.E.N., O.M.S., and D.T.; Development of methodology, A.E.N., O.M.S., S.G., U.U.O., O.Y., A.B., and D.T.; Investigation, A.E.N., Z.B.B., I.E.Z., and D.T.; Writing-Original Draft, A.E.N., O.M.S., and D.T.; Writing-Review and Editing, D.T. and F.S.; Supervision, D.T.

Ethics approval
Animal experiments were performed in accordance with the ARRIVE guidelines, and the study protocol was approved by Animal Care and Welfare Committee of Yeditepe University (Türkiye, decision number: 644).

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