Lipid-Based Nanocarriers Provide Prolonged Anticancer Activity for Palbociclib: *In Vitro* and *in Vivo* Evaluations

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**Abstract** - Breast cancer therapy has remained one of the major healthcare challenges. Based on the critical role of cyclin-dependent kinase 4/6 (CDK 4/6) in cell cycle progression, targeting this signaling appears promising for cancer therapy. Palbociclib, a selective CDKs 4/6 inhibitor, is the first-line treatment for estrogen receptor-positive breast cancer. However, poor absorption or side effects may negatively affect its efficiency. This prompted us to incorporate palbociclib into the nanostructured lipid carriers (NLCs) and evaluate the anticancer effect of the nanoformulation (Pa-NLCs) in *in vitro* and *in vivo* models of breast cancer. Pa-NLCs were developed by high-pressure homogenization followed by assessment of the physicochemical characteristics and bioactivities in MCF-7 breast cancer cells and female Wistar rats exposed to the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA). The prepared Pa-NLCs demonstrated suitable physicochemical characteristics, including the controlled release pattern, efficient cellular uptake, and cytotoxicity, while free palbociclib failed to show significant effects. Rats treated with Pa-NLCs exhibited significantly reduced tumor volumes, increased survival rates, and histopathological improvement. Free palbociclib was significantly less efficient than Pa-NLCs. Pa-NLCs, by improving the pharmacological profile of palbociclib and providing longer-lasting effects, can be considered as a promising nanoformulation against breast cancer.

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**Keywords**: Palbociclib; Cyclin-dependent kinase 4/6 (CDK 4/6) inhibitor; Nanostructured lipid carriers; Breast cancer; Michigan cancer foundation-7 (MCF-7) cells; Rat

**Introduction**

Breast cancer is one of the most common causes of mortality in females (1). Since cell cycle dysregulation and activation of cyclin-dependent kinases (CDKs) are hallmarks of breast cancer, CDKs have been considered attractive targets in cancer treatment (2-5). The limited activity or serious side effects of the first-generation CDK inhibitors led to the development of the next-generation of drugs capable of inhibiting the activity of specific CDK subtypes, including the CDKs 4 and 6 (CDK 4/6), which are critically involved in the initiation and progression of various types of cancers. Following identification of the role of cyclin D1 and CDK4/6 in the pathogenesis of breast cancer (6), the selective CDK 4/6 inhibitors, including abemaciclib, ribociclib, and palbociclib (PD 0332991), were developed against advanced breast cancer (3,7). Palbociclib, a potent and highly selective CDK 4/6 inhibitor, has shown promising growth inhibitory activity against various types of tumors such as hepatocellular, renal cell, and non-small cell lung carcinoma neuroblastoma, multiple myeloma, esophageal adenocarcinoma, and melanoma (8-13). Palbociclib has been most extensively investigated in the field of breast cancer. About 80% of breast tumors express estrogen receptor (ER) and rely on ER signaling for their growth and survival (14). ER inhibition may result in cell cycle arrest in the G1 phase...
and reduction of tumor cell viability (15). In a panel of 47 breast cancer cell lines exposed to palbociclib, ER+ cells were the most sensitive ones to growth inhibition (16,17). Regarding the significance of targeting transcription factors for cancer therapy, palbociclib has been shown to inactivate the transcription factor, forkhead box M1 (FOXM1), leading to the anti-proliferation effects (18-20). As monotherapy, palbociclib blocks cell cycle progression via inhibiting hyperphosphorylation of RB protein in breast cancer cells (16). In combination therapy, palbociclib may sensitize cancer cells to ionizing radiation or chemotherapeutic agents (16,17). Palbociclib, in combination with non-steroidal aromatase inhibitor letrozole, has prolonged the progression-free survival in ER+ advanced breast cancer (21,22). The synergistic growth inhibitory activity of palbociclib with trastuzumab, paclitaxel, tamoxifen, or fulvestrant has also been shown (16,22-24). Palbociclib and letrozole have been represented as first-line treatment of ER+ and epidermal growth factor receptor 2-negative breast cancer (16,22,25,26). Meanwhile, palbociclib may be associated with poor absorption and a variety of side effects (7,16,22,25-31) that may negatively affect the efficiency of the drug. This prompted us to incorporate palbociclib into the nanostructured lipid carriers (NLCs), the colloidal drug carriers with high stability, biocompatibility, and drug loading extent, which are suitable for controlled drug release or targeted delivery and protect the loaded therapeutics against degradation prolong the residence of drugs in target organs and prevent their expulsion during the storage period (32-35). Afterward, we assessed the anticancer activity of nanoformulation in the in vitro and in vivo models of breast cancer which are relevant to human biology.

Materials and Methods

Materials

Materials for cell culture were obtained from GIBCO/Invitrogen, Germany. Tween 80 and cetyl palmitate were purchased from Merck (Darmstadt, Germany), and other chemicals were purchased from Sigma Aldrich, Germany.

Preparation of palbociclib-loaded NLCs (Pa-NLCs)

The lipid phase containing the oleic acid and cetyl palmitate (15:85 or 30:70) was prepared at 75 °C followed by the addition of palbociclib (5, 10, 20, 40, and 100 % w/w). The aqueous phase was provided at the same temperature and added to the lipid phase. Drug-loaded or blank NLCs were developed as previously described in detail (32-35).

Characterization of Pa-NLCs

Determination of the particle size, zeta potential (ZP), and polydispersity index (PDI)

Following re-dispersion, particle size, ZP, and PDI of NLCs were evaluated by photon correlation spectroscopy (Zetasizer Nano, Malvern Instruments, UK), (n=6).

Morphological evaluation of NLCs

Using a scanning electron microscope (KYKY-EM3200, KYKY Technology Development Ltd., China), the shape of lyophilized NLCs was evaluated.

Assessment of drug loading (DL) and entrapment efficiency (EE)

0.5 ml of Pa-NLCs dispersion was centrifuged at 10000 rpm for 40 min, and the amount of palbociclib in the supernatant was determined by HPLC [Hitachi Model D-7000, Merck-Hitachi, Darmstadt, Germany, equipped with a UV-Vis detector (Merck-Hitachi, L-4250, Germany) with C18 column]. The mobile phase included 0.1% triethylamine and acetonitrile (30:70, v/v), and peak absorption wavelength was recorded at 263 nm. Dilutions of palbociclib (10-300 µg/ml) were provided to construct the calibration curve. The limits of detection and quantification were 1.59 and 4.18 µg/ml, respectively. DL% and EE% were determined as follows:

\[
\text{EE}\% = \frac{\text{Total amount of palbociclib} - \text{the amount of free palbociclib x 100}}{\text{The total amount of palbociclib}}
\]

\[
\text{DL}\% = \frac{\text{The amount of palbociclib encapsulated in NLCs} \times 100}{\text{The total amount of NLCs}}
\]

Differential scanning calorimetry (DSC)

DSC apparatus (Mettler-Toledo, Switzerland) was applied for thermal analysis of cetyl palmitate, palbociclib, lyophilized Pa-NLCs, and blank NLCs. An aluminum pan was used for sample placement followed by heating at the range of 10-280 °C (10 °C/min).

Assessment of the in vitro release profile

The release pattern of palbociclib from NLCs was assessed using dialysis membrane technique (32-34,36).
Dose released percentage was plotted against time and palbociclib solution was considered as control. Experiments were performed in triplicate.

**Stability of nanoparticles during storage at 4°C**

Lyophilized samples were re-suspended at 0, 1, 3, and 6 months followed by evaluating the particle size, ZP, PDI, DL%, and EE%. The results were demonstrated as mean±SEM (n=6).

**Assessment of the bioactivity of Pa-NLCs in vitro**

**Cell culture**

MCF-7 breast cancer cells were cultured in RPMI-1640 medium supplemented with fetal bovine serum (10%), amphotericin B (0.25 μg/ml), penicillin G sodium (100 U/ml), and streptomycin sulfate (100 μg/ml) in 5% CO2 incubator (Tuttlingen, Germany) at 37°C.

**Evaluating the cellular uptake of NLCs**

Following loading red fluorescent probe (DiI) into the NLCs, Amicon® filter (Millipore, USA) with molecular weight cut-off of 100 kDa was used for removing the un-encapsulated DiI by centrifugation at 13000 g for 30 min. Fluorescence detector (W2475) was applied for quantification of DiI and chromatography was carried out on C18 column at flow rate of 1 ml/min using the mobile phase including the methanol and 0.05 M dimethyl sulfate (98.2 v/v). Emission and excitation wavelengths were 565 and 549 nm, respectively. Standard curve was linear in the range of 0.005-10 μg/ml with R² of 0.99. Limits of detection and quantification were 0.001 and 0.0028 μg/ml, respectively. For evaluation of the cellular uptake, mean fluorescence intensity was determined by flow cytometer (FACSCalibur, USA) using CellQuest Pro software. Experiments were carried out in triplicate and untreated cells served as control.

**Assessment of the cytotoxicity**

24 and 48 after treatment with palbociclib (100, 150, 200, and 300 nM), Pa-NLCs (containing 100, 150, 200, and 300 nM palbociclib), or blank NLCs, the viability of MCF-7 cells was assessed by MTT (32-34). Cell viability was expressed as mean±SEM (n=6).

**Morphological analysis of MCF-7 cells following exposure to palbociclib or Pa-NLCs**

Cells in 6-well plates (10⁵ cells/well) were treated with 100, 150, 200, or 300 nM palbociclib or Pa-NLCs (containing 100, 150, 200, or 300 nM palbociclib) for six days followed by fixation in 4% paraformaldehyde for 30 min, washing with PBS (0.02 M), and staining with Hoechst 33258 (32,34). Images were captured by fluorescence microscope (Leica, Germany). In each group, the morphologically altered cells were counted in six visual fields and the percentage over total number of cells was determined as previously described (37).

**In vivo experiments**

**Animals**

Female Wistar rats weighing 140-160 g from the experimental animal center of Pasteur institute (Tehran, Iran) were randomly assigned and housed 3 per cage under the standard laboratory conditions; temperature (24±2°C), humidity (55±10%), with a 12-h light/dark cycle and ad libitum access to water and food pellets. Animal experiments were carried out in accordance with the European Committee guidelines for the use of experimental animal and were approved by the local ethics committee.

**Inducing breast cancer and treatment groups**

Breast cancer was induced by subcutaneous injection of 7,12-dimethylbenz (a) anthracene (DMBA) at dose of 100 mg/kg (38,39), (group 1). Other groups received intravenous injections three times a week as follows; groups 2-5 received 10, 30, or 75 mg/kg of palbociclib (dissolved in sodium lactate (50 mM, pH 4) (40-43) or vehicle, groups 6-9 received Pa-NLCs (containing 10, 30, or 75 mg/kg of palbociclib) or blank NLCs. Injections were initiated two weeks before DMBA administration and continued for 12 weeks. Animals were checked twice a week for body weight alterations, abnormal mass, and mortality. According to the relevant animal studies, n=6/group was selected for statistical analysis.

**Determining the tumor volume**

Tumor volumes were determined by a caliper and following formula:

\[ V (\text{mm}^3) = \frac{(ab^2\pi)}{6} \]

(a: the largest diameter of tumor, b: the largest diameter of tumor at 90 degrees to a).

**Histological evaluation**

Animals were anesthetized by intramuscular injection of 80 mg/kg of ketamine-HCl and 10 mg/kg of xylazine followed by mammary gland isolation,
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cleaning, drying, weighing, immersion of mammary glands in Bouin’s solution overnight, dehydration, paraffin embedment, providing 5-μm thick sections by rotary microtome (Leica, Germany), mounting on the glass slides, and hematoxylin-eosin staining (38,44,45). Samples were visualized using the microscope (Wolfe, S9-0982, Germany) and photos were captured by digital camera (Canon, Power-Shot SX500).

Data analysis
Shapiro-Wilk test was used to verify normal data distribution. Analysis of variance (ANOVA) followed by Tukey’s test was used for analysis of data related to the physicochemical properties of NLCs and their stability, drug release profile in vitro, cytotoxicity, alterations of body weights, and tumor volumes. Mann-Whitney U test was used for evaluating the morphological alterations of cells. Statistical significance in the survival evaluation (plotted as Kaplan-Meier curves) was evaluated by Log-rank test. Data have been demonstrated as mean±SEM with significance level of P<0.05.

Results
Characterizing of Pa-NLCs
Using various ratios of liquid and solid lipids, palbociclib and lipid, and sonication time or surfactants, several formulations of Pa-NLCs were developed (Table 1). Using higher amounts of surfactant and oil lipid led to higher EE% and smaller size of particles. Considering DL% and EE%, Pa-NLCs-8 was determined as the optimum formulation (Table 1). As shown in Figure 1, spherical shapes of Pa-NLCs were preserved even three months after lyophilization. In DSC, palbociclib showed an endothermic peak at 269.43° C (Figure 2b) and the melting peak of Pa-NLCs was observed at 74.83° C (Figure 2a). Cetyl palmitate showed a melting peak at 54.73° C (Figure 2c) and blank NLCs demonstrated a melting peak at 52.17° C (Figure 2d). Palbociclib release from solution was faster than Pa-NLCs formulation which demonstrated controlled drug release profile (Figure 3).

The stability of lyophilized nanoparticles were preserved without remarkable changes of the physicochemical properties (Table 2, P>0.05).

Table 1. Physicochemical characteristics of various Pa-NLCs formulations

| Formulation code | Particle size (nm) | PDI | ZP (mV) | EE (%) | DL (%) | DR after 48 h (%) |
|------------------|--------------------|-----|---------|--------|--------|------------------|
| Pa-NLCs-1        | 134.6 ± 7.9        | 0.33 ± 0.07 | -23.7 ± 0.39 | 43.5 ± 2.7 | 1.43 ± 0.14 | 52.7 ± 4.5    |
| Pa-NLCs-2        | 121.8 ± 7.2        | 0.37 ± 0.05 | -24.6 ± 0.37 | 47.8 ± 4.2 | 1.38 ± 0.05 | 53.4 ± 3.9    |
| Pa-NLCs-3        | 109.3 ± 8.5        | 0.25 ± 0.03 | -23.9 ± 0.43 | 63.4 ± 3.8 | 1.57 ± 0.13 | 62.9 ± 3.7    |
| Pa-NLCs-4        | 65.3 ± 3.2         | 0.17 ± 0.05 | -25.3 ± 0.36 | 90.3 ± 5.7 | 3.56± 0.07 | 82.3 ± 3.3    |
| Pa-NLCs-5        | 76.5 ± 3.9         | 0.19 ± 0.05 | -22.5 ± 0.47 | 94.6 ± 6.2 | 5.93 ± 0.15 | 77.8 ± 5.2    |
| Pa-NLCs-6        | 89.7 ± 3.4         | 0.26 ± 0.07 | -19.2 ± 0.33 | 85.3 ± 4.5 | 11.86 ± 2.3 | 74.3 ± 3.5    |
| Pa-NLCs-7        | 93.9 ± 2.7         | 0.29 ± 0.03 | -28.9 ± 0.55 | 94.3 ± 7.3 | 28.32 ±2.5 | 65.7± 2.7     |
| Pa-NLCs-8        | 132.8 ± 5.4        | 0.23 ± 0.03 | -25.7± 0.45  | 87.9 ± 5.5 | 45.92 ± 3.7 | 63.9 ± 3.8    |
| Blank NLCs       | 52.9 ± 4.6         | 0.17 ± 0.03 | -18.8 ± 0.37 | -     |        |      |

Data are expressed as mean±SEM (n=6)
(ZP: zeta potential, PDI: polydispersity index, DL: drug loading, EE: entrapment efficiency, DR: drug release, Pa-NLCs: palbociclib-loaded nanostructured lipid carriers)

Figure 1. Scanning electron micrographs of Pa-NLCs. Well-dispersed nanoparticles have preserved their spherical shapes even three months after lyophilization (Pa-NLCs: Palbociclib-loaded nanostructured lipid carriers)
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Figure 2. DSC thermograms. a: Pa-NLCs, b: palbociclib, c: cetyl palmitate, d: blank NLCs

(DSC: differential scanning calorimetry, Pa-NLCs: Palbociclib-loaded nanostructured lipid carriers)

Figure 3. The release pattern of palbociclib in vitro. Palbociclib demonstrated a controlled release profile from Pa-NLCs. Data are demonstrated as mean±SEM (n=3). (Pa-NLCs: Palbociclib-loaded nanostructured lipid carriers)

Table 2. Stability profile of Pa-NLCs

|                | Initial          | 1st Month        | 3rd Month        | 6th Month       |
|----------------|------------------|------------------|------------------|-----------------|
|                | Size  | PDI    | ZP    | EE%  | DL%  | Size  | PDI    | ZP    | EE%  | DL%  | Size  | PDI    | ZP    | EE%  | DL%  |
| Size           | 132.8±5.4        | 0.23±0.05        | -25.7±0.4       | 87.9±5.5        | 45.9±3.7        | 135.5±4.9 | 0.22±0.03 | -25.4±0.7 | 85.5±4.7 | 42.7±2.5 |
| PDI            | 0.23±0.05        | 0.23±0.05        | 0.23±0.05       | 0.23±0.05       | 0.23±0.05       | 0.23±0.05 | 0.23±0.05 | 0.23±0.05 | 0.23±0.05 | 0.23±0.05 |
| ZP             | -25.7±0.4        | -25.7±0.4        | -25.7±0.4       | -25.7±0.4       | -25.7±0.4       | -25.7±0.4 | -25.7±0.4 | -25.7±0.4 | -25.7±0.4 | -25.7±0.4 |
| EE%            | 87.9±5.5         | 87.9±5.5         | 87.9±5.5        | 87.9±5.5        | 87.9±5.5        | 87.9±5.5 | 87.9±5.5 | 87.9±5.5 | 87.9±5.5 | 87.9±5.5 |
| DL%            | 45.9±3.7         | 45.9±3.7         | 45.9±3.7        | 45.9±3.7        | 45.9±3.7        | 45.9±3.7 | 45.9±3.7 | 45.9±3.7 | 45.9±3.7 | 45.9±3.7 |
| 3rd Month       | Size  | PDI    | ZP    | EE%  | DL%  | Size  | PDI    | ZP    | EE%  | DL%  | Size  | PDI    | ZP    | EE%  | DL%  |
| Size           | 139.5±3.5        | 0.25±0.07        | -26.5±0.9       | 80.7±5.3        | 39.8±0.4        | 141.7±3.9 | 0.28±0.09 | -25.6±0.3 | 77.36±2.9 | 37.3±1.5 |
| PDI            | 0.25±0.07        | 0.25±0.07        | 0.25±0.07       | 0.25±0.07       | 0.25±0.07       | 0.25±0.07 | 0.25±0.07 | 0.25±0.07 | 0.25±0.07 | 0.25±0.07 |
| ZP             | -26.5±0.9        | -26.5±0.9        | -26.5±0.9       | -26.5±0.9       | -26.5±0.9       | -26.5±0.9 | -26.5±0.9 | -26.5±0.9 | -26.5±0.9 | -26.5±0.9 |
| EE%            | 80.7±5.3         | 80.7±5.3         | 80.7±5.3        | 80.7±5.3        | 80.7±5.3        | 80.7±5.3 | 80.7±5.3 | 80.7±5.3 | 80.7±5.3 | 80.7±5.3 |
| DL%            | 39.8±0.4         | 39.8±0.4         | 39.8±0.4        | 39.8±0.4        | 39.8±0.4        | 39.8±0.4 | 39.8±0.4 | 39.8±0.4 | 39.8±0.4 | 39.8±0.4 |
| 6th Month       | Size  | PDI    | ZP    | EE%  | DL%  | Size  | PDI    | ZP    | EE%  | DL%  | Size  | PDI    | ZP    | EE%  | DL%  |
| Size           | 141.7±3.9        | 0.28±0.09        | -25.6±0.3       | 77.36±2.9       | 37.3±1.5        | 145.4±3.5 | 0.29±0.10 | -25.4±0.5 | 77.1±3.2 | 36.9±1.5 |
| PDI            | 0.28±0.09        | 0.28±0.09        | 0.28±0.09       | 0.28±0.09       | 0.28±0.09       | 0.28±0.09 | 0.28±0.09 | 0.28±0.09 | 0.28±0.09 | 0.28±0.09 |
| ZP             | -25.6±0.3        | -25.6±0.3        | -25.6±0.3       | -25.6±0.3       | -25.6±0.3       | -25.6±0.3 | -25.6±0.3 | -25.6±0.3 | -25.6±0.3 | -25.6±0.3 |
| EE%            | 77.36±2.9        | 77.36±2.9        | 77.36±2.9       | 77.36±2.9       | 77.36±2.9       | 77.36±2.9 | 77.36±2.9 | 77.36±2.9 | 77.36±2.9 | 77.36±2.9 |
| DL%            | 37.3±1.5         | 37.3±1.5         | 37.3±1.5        | 37.3±1.5        | 37.3±1.5        | 37.3±1.5 | 37.3±1.5 | 37.3±1.5 | 37.3±1.5 | 37.3±1.5 |

Data are demonstrated as mean ± SEM (n=6).

(Pa-NLCs: palbociclib-loaded nanostructured lipid carriers, ZP: zeta potential, PDI: polydispersity index, DL: drug loading, EE: entrapment efficiency)

Evaluation of the cellular uptake

Using the confocal microscopy, cellular uptake of DiI-loaded NLCs was visualized (Figure 4A–4C). 4 and 6 h after exposure, remarkable enhancement of fluorescence intensity was observed (Figure 4D, P<0.05 and P<0.01).
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Figure 4. Cellular uptake of DiI-loaded NLCs. A-C: Confocal microscopy images regarding the cellular uptake of DiI-loaded NLCs after 1, 4, and 6 h of incubation, respectively (scale bars = 20 μm), D: mean fluorescence intensity values following 1, 4, and 6 h (a-c, respectively) of incubation with NLCs. Data are expressed as mean±SE (n = 3). b P<0.05 vs. a and c, c P<0.05 vs. b and P<0.01 vs. a.

Assessment of the cell viability

24 and 48 h after treatment with Pa-NLCs (containing 200 or 300 nM palbociclib), cell viability was significantly reduced (Figure 5A and 5B, P<0.05, P<0.01, and P<0.001 vs. control), while free palbociclib did not significantly affect cell viability at any dose tested (Figure 5A and 5B, P>0.05 vs. control).

Figure 5. Assessment of cell viability in MCF-7 cell culture exposed to palbociclib or Pa-NLCs. 24 and 48 h after treatment (A and B, respectively), the viability of MCF-7 cells was evaluated by MTT assay. Pa-NLCs dose-dependently reduced the cell viability, while free palbociclib failed to exert a significant effect at equivalent doses. Data are presented as mean ± SEM of 6 experiments. (Pa-100, 150, 200, and 300: treatment with palbociclib (100, 150, 200, and 300 nM), Pa-NLCs-100, 150, 200, and 300: treatment with Pa-NLCs (containing 100, 150, 200, and 300 nM palbociclib). Pa-NLCs: palbociclib-loaded nanostructured lipid carriers. *P<0.05, **P<0.01, and ***P<0.001 vs. control
Morphological evaluation of MCF-7 cells

As compared to the control (Figure 6A), six-day treatment with 200 or 300 nM palbociclib did not significantly affect cellular morphology (Figure 6B and 6C, respectively), while altered cellular morphology (condensed nuclei) was observed following six-day exposure to Pa-NLCs (containing 200 or 300 nM palbociclib, Figure 6D, and 6E, respectively). Damaged cells were significantly increased in Pa-NLCs groups (Figure 6F, *P*<0.01 and *P*<0.001 vs. palbociclib-treated groups and *P*<0.001 vs. control). In palbociclib-treated groups, the number of the morphologically altered cells did not significantly differ from that in the control group (Figure 6F, *P*>0.05).

![Figure 6](image_url)

**Figure 6.** Morphological evaluation of MCF-7 cells. Hoechst stain was used for assessment of the cellular damage after six-day exposure to free palbociclib or Pa-NLCs

A: control cells, B and C: treatment with palbociclib (200 and 300 nM, respectively) did not significantly affect cellular morphology, D and E: alterations of the cellular morphology (increased condensed nuclei) after treatment with Pa-NLCs (containing 200 and 300 nM palbociclib, respectively). (Scale bars = 20 μm), F: Morphologically-altered cells presented as the percentage of total cells counted. Data are expressed as mean±SEM (*n* = 6). *a* *P*< 0.01 vs. c and *P*< 0.001 vs. a and b, *d* *P*< 0.001 vs. a, b, and c. (Pa-NLCs: palbociclib-loaded nanostructured lipid carriers)

**In vivo studies**

Effects of DMBA, palbociclib, and Pa-NLCs on the bodyweight

A significant reduction of body weight was observed in DMBA-treated rats (Table 3, *P*<0.01 vs. the vehicle- and blank NLCs-treated groups). Furthermore, palbociclib and (75 mg/kg) and Pa-NLCs (containing 75 mg/kg of palbociclib) significantly reduced body weight (*P*<0.05 vs. vehicle- and blank NLCs-treated groups), however, a significant difference was observed between palbociclib- and Pa-NLCs-treated groups (*P*<0.05).
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Table 3. Effects of DMBA, palbociclib, and Pa-NLCs on the bodyweight

| Experimental groups | Initial body weight (g) | Final body weight (g) |
|---------------------|-------------------------|-----------------------|
| Vehicle             | 146.3 ± 6.2             | 398.9 ± 10.7          |
| Blank NLCs          | 148.7 ± 5.4             | 383.7 ± 12.3          |
| DMBA                | 151.5 ± 5.9             | 193.5 ± 7.5           |
| Palbociclib+DMBA    | 154.8 ± 3.7             | 204.3 ± 3.2           |
| Pa-NLCs+DMBA        | 146.3 ± 9.3             | 297.7 ± 5.9           |
| Blank NLCs+DMBA     | 153.6 ± 6.7             | 199.6 ± 9.7           |

Data are expressed as mean ± SEM (n=6). a P<0.01 vs. the vehicle- and blank NLCs-treated groups, b P<0.05 vs. vehicle- and blank NLCs-treated groups, c P<0.05 vs. vehicle-, blank NLCs, and palbociclib+DMBA-treated groups. (Pa-NLCs: palbociclib-loaded nanostructured lipid carriers), c vs. b: P<0.05.

Effects of palbociclib and Pa-NLCs on the survival rate following treatment with DMBA

Treatment with Pa-NLCs formulation (containing 75 mg/kg of palbociclib) provided a significantly higher survival rate (P<0.01) as compared to free palbociclib (75 mg/kg), blank NLCs-, or DMBA-treated group (Figure 7).

![Figure 7](image)

Figure 7. Kaplan-Meier curves demonstrating the survival data in DMBA-treated rats exposed to palbociclib, Pa-NLCs, or blank NLCs. The difference between the survival curves was evaluated by a log-rank test. (Pa-NLCs: palbociclib-loaded nanostructured carriers)

Effects of DMBA, palbociclib, and Pa-NLCs on tumor growth

DMBA induced significant tumor growth (P<0.05, P<0.01, and P<0.001 vs. the group treated with Pa-NLCs). Palbociclib even at the highest dose tested (75 mg/kg) did not significantly affect tumor growth as compared to the vehicle-, blank NLCs, and DMBA-treated groups (Figure 8, P>0.05), while, Pa-NLCs (containing 75 mg/kg of palbociclib) significantly reduced tumor volume (Figure 8, P<0.05, P<0.01, and P<0.001 vs. other groups as mentioned in the figure legend).

![Figure 8](image)

Figure 8. Effects of palbociclib and Pa-NLCs on tumor volume in DMBA-treated animals. a and b: P<0.05 vs. all groups receiving DMBA, c: P<0.05 vs. the vehicle and blank NLCs groups and P<0.01 vs. the DMBA-treated groups, d: P<0.01 vs. the vehicle and blank NLCs groups and P<0.001 vs. other groups as mentioned in the figure legend, e: P<0.001 vs. the vehicle and blank NLCs groups and DMBA-treated groups

Effects of palbociclib and Pa-NLCs on DMBA-induced histopathological alterations

DMBA-induced histopathological alterations, including the dilated ducts and hyper-chromatic nuclei and eosinophilic cytoplasm in the luminal cells (Figure 9B), were improved following treatment with Pa-NLCs.
(containing 75 mg/kg palbociclib) (Figure 9D). Reduced amounts of connective tissues and dilated ducts were observed in animals treated with free palbociclib (75 mg/kg) (Figure 9C).

**Figure 9.** Effects of palbociclib and Pa-NLCs on DMBA-induced histopathological alterations. A: mammary glands in the control animals demonstrated normal histological patterns, B: morphological alterations including the hyper-chromatic nuclei and eosinophilic cytoplasms in the luminal cells and dilated ducts in carcinogen-treated animals, C: reduced amounts of connective tissues and dilated ducts in animals treated with free palbociclib (75 mg/kg), D: moderate lymphocytic infiltration following treatment with Pa-NLCs (containing 75 mg/kg palbociclib), (magnifications; A-C: 400 x, D: 10 x)

**Discussion**

Treatment of breast cancer has remained one of the major healthcare challenges. In recent years, targeting the cell cycle and inhibition of CDKs have gained considerable attention. Selective CDK4/6 inhibitors have shown therapeutic potentials against various types of tumors. Palbociclib, which is a highly selective inhibitor of CDKs 4/6, has been approved as the first-line treatment of ER⁺ breast cancer. However, poor solubility and frequency- or dose-related side effects (7,16,22,25,26) may negatively affect its efficiency. Over the last decades, increasing research efforts in nanotechnology have resulted in the development of biocompatible nanostructures and optimized nanocarriers in order to protect the encapsulated drugs or biomolecules against excretion or metabolism and overcome the potential chemo-resistance. High-resolution imaging, real-time detection of a variety of biomarkers, controlled drug delivery, and targeted therapy may improve the efficiency of therapeutics and reduce their side effects. In this context, several nanoformulations with improved bioavailability and half-lives have proved to be useful against a variety of disorders (46-49). The development of nanotechnology-based imaging techniques facilitates detection of early-stage tumors or identification of the molecular expressions of neoplasms (50). Application of the nanobiosensors or nanowires for early detection of malignant lesions (51,52) might be of critical significance in cancer treatment. Using paramagnetic nanoparticles or polymeric dendrimers for detection of lymph node metastases (53,54), bimodal nanoparticles for intra-operative visualization of lesions (55), nanoparticle probes targeted with recognition agents for detecting tumor markers (56), or metal-based nanovectors for thermal ablation of tumors (57) indicate the significance of nanotechnology-based approaches in designing the novel generations of anticancer therapeutics.

In order to overcome the problems associated with older nanomaterials, advanced nanocarriers, including lipid-based colloidal drug delivery systems, have been designed. This type of nanoparticle facilitates targeted drug delivery, protects the encapsulated therapeutic agents against degradation, and improves their bioavailability and efficiency. Because of the limitations of solid lipid nanoparticles, such as the limited capacity of drug loading or drug expulsion, NLCs are preferred for the incorporation of poorly water-soluble drugs (35). Application of NLCs with suitable biocompatibility, long-term stability, high capacity of drug loading, and controlled drug release pattern may lead to improved pharmacological profiles, reduced dose frequency, and side effects of drugs (32-35,58). Besides its usefulness against fungal infections or ischemic neural injuries, the application of NLCs may improve the therapeutic index of encapsulated anticancer drugs (59,60). Loading of tyrphostin AG-1478, an inhibitor of epidermal growth factor receptor, into NLCs has provided promising therapeutic effects in human hepatocarcinoma cells (61). Based on this background, we have encapsulated palbociclib into the NLCs for obtaining longer-lasting therapeutic effects. During six months of storage, Pa-NLCs demonstrated high stability profile (Table 2) that may be related to the binary lipid mixture, increased imperfection, and reduced expulsion of palbociclib. The spherical shapes of Pa-NLCs with the suitable distribution of particle size and ZP were maintained during the storage period (Table 1, Figure 1) that may be
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due to the application of appropriate materials and preparation methods.

Using DSC, thermal behaviors of Pa-NLCs, bulk materials, and blank NLCs were evaluated (Figure 2). The disappearance of the endothermic peak of palbociclib in the DSC thermogram of Pa-NLCs indicates drug distribution in the amorphous state. Shifting of the melting curve peaks of Pa-NLCs and blank NLCs to the lower temperatures suggests the transformation of bulk materials into the nanoparticulate formation, increased surface area of particles, lower-ordered positioning of NLCs, and small size of particles. The melting point of Pa-NLCs was lower than that of blank NLCs (Figure 2), which may be due to the encapsulation of palbociclib into the lipid matrix and defect elevation in the crystal lattice.

Palbociclib showed a controlled-release profile from the NLCs (Figure 3) that might be due to the interactions between the lipid-surfactant or lipid-palbociclib, drug partitioning between the aqueous and lipid phases, or drug diffusion from the lipid core. This type of release profile which provides a sustained drug concentration for longer periods of time, might be of key therapeutic importance.

Following the efficient cellular uptake of NLCs (Figure 4), the MTT assay demonstrated that Pa-NLCs, but not free palbociclib, exhibit cytotoxic effects in a dose-dependent fashion (Figure 5). Unlike free palbociclib, six-day exposure to Pa-NLCs induced cellular damage in a dose-dependent manner (Figure 6). These findings represent NLCs as promising nano reservoirs capable of providing sustained drug concentrations that may result in the increased therapeutic efficiency.

Alterations of body weights were routinely monitored as a sign of in vivo toxicity. A significant loss of body weight was observed in animals treated with DMBA, free palbociclib, or Pa-NLCs (Table 3). Meanwhile, weight loss in Pa-NLCs-treated rats was statistically lower than those receiving free palbociclib (Table 3), indicating the reduced toxicity of the drug due to the application of NLCs.

As shown in Figure 8, Pa-NLCs significantly inhibited tumor growth. This might be of therapeutic significance because of the key role of cell proliferation in the process of carcinogenesis, including the initiation and progression (62,63). Higher survival rate and improved DMBA-induced histopathological alterations by Pa-NLCs (Figures 7 and 9) demonstrated higher efficiency of nanoformulation as compared to the free drug.

Nanotechnology, by which a variety of biomaterials or advanced devices may be designed for targeted therapy or early diagnosis of disorders, has opened up new frontiers in biomedical research. Using nanotechnology-based tools provides possibilities to acquire a better understanding of the pathophysiology of various diseases and develop efficient drug delivery systems that might improve treatment outcomes. The development of nanoformulations with increased bioavailability and reduced toxicity which interact with the biological systems may be of key therapeutic importance. In the present study, NLCs have been shown as promising nano reservoirs for encapsulation and delivery of palbociclib with therapeutic potentials against a variety of malignancies. Pa-NLCs via improvement of the pharmacological profile of palbociclib appears as a suitable nanoformulation that provides prolonged effects leading to the reduced dose frequency and side effects of palbociclib.

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