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RESEARCH ARTICLE

Folate-Targeted Curcumin Nanoliposomes for Receptor-Mediated Delivery in Triple-Negative Breast Cancer Cells

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Abstract

Introduction: Curcumin has reported anticancer activity, but its poor aqueous solubility, limited bioavailability, and relatively low intrinsic potency restrict its therapeutic translation. This study developed folate-conjugated PEGylated curcumin nanoliposomes (FA-Lipo-Cur) to improve curcumin delivery to folate receptor–positive MDA-MB-231 triple-negative breast cancer cells. **Methods:** Curcumin-loaded liposomes were prepared by thin-film hydration followed by post-insertion of DSPE-PEG(2000)-folate. The formulations were characterized for particle size, polydispersity index, ζ-potential, morphology, encapsulation efficiency, and in vitro release. Cytotoxicity was evaluated by MTT assay after 48 h exposure to free curcumin, non-targeted Lipo-Cur, and FA-Lipo-Cur. Blank liposomes and a concentration-dependent folate receptor-blocking assay were included to assess carrier-related toxicity and targeting specificity. **Results:** Both liposomal formulations showed predominantly spherical morphology, acceptable size distribution, negative ζ-potential, and high curcumin encapsulation. FA-Lipo-Cur had a mean size of 218 ± 8.6 nm, PDI of 0.17 ± 0.03, ζ-potential of −28.4 ± 2.3 mV, and encapsulation efficiency of 81.6 ± 2.8%. Both formulations showed sustained curcumin release over 50 h, with FA-Lipo-Cur exhibiting slower release than Lipo-Cur. FA-Lipo-Cur produced the lowest IC_{50} value (33 ± 3.3 μg/mL), followed by Lipo-Cur (48 ± 3.9 μg/mL) and free curcumin (60 ± 4.1 μg/mL). Folate receptor blocking increased the IC_{50} of FA-Lipo-Cur to 46.5 ± 3.2 μg/mL and progressively increased cell viability in the concentration-dependent blocking assay. **Conclusion:** FA-Lipo-Cur improved curcumin delivery and in vitro cytotoxic activity in MDA-MB-231 cells, with evidence supporting partial folate receptor-mediated uptake. These findings support further investigation of FA-Lipo-Cur as an adjunctive or combination-oriented nanocarrier platform rather than as a replacement for standard chemotherapy.

Keywords: folate-targeted liposomes; curcumin; triple-negative breast cancer; MDA-MB-231; receptor-mediated delivery

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Introduction

Breast cancer remains one of the leading causes of cancer-related morbidity and mortality among women worldwide [1-3]. Despite major progress in surgery, chemotherapy, radiotherapy, endocrine therapy, immunotherapy, and targeted treatment, therapeutic outcomes remain limited by systemic toxicity, drug resistance, tumor heterogeneity, and insufficient drug accumulation at the tumor site [2-5]. These limitations are particularly relevant in triple-negative breast cancer (TNBC), an aggressive subtype defined by the absence of estrogen receptor, progesterone receptor, and HER2 expression, which restricts the use of established receptor-directed therapies [2, 5-7]. Curcumin, a polyphenolic compound derived from Curcuma longa, has attracted attention as a multifunctional anticancer agent because of its reported antiproliferative, pro-apoptotic, anti-inflammatory, antioxidant-modulating, and anti-metastatic activities [8, 9]. In breast cancer models, including TNBC cell lines such as MDA-MB-231, curcumin has been reported to modulate several oncogenic signaling pathways, including NF-κB, STAT3, PI3K/AKT, MAPK, apoptosis-related pathways, and oxidative stress-associated mechanisms [8, 9]. However, the therapeutic translation of curcumin is limited by poor aqueous solubility, low stability, rapid metabolism, short systemic half-life, low bioavailability, and relatively

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high effective cytotoxic concentrations compared with conventional chemotherapeutic agents [4, 9]. Therefore, curcumin is more appropriately considered a candidate for improved delivery, adjunctive therapy, or combination-based strategies rather than a direct replacement for established chemotherapy [4, 9, 10]. Nanocarrier-based drug delivery offers a rational strategy to address some of these pharmaceutical limitations by improving dispersion, protecting the payload, modifying release behavior, and enhancing cellular uptake [4, 11, 12]. Liposomes are especially attractive nanocarriers because they are biocompatible vesicular systems capable of incorporating hydrophobic compounds within the lipid bilayer and supporting sustained or controlled release [11-15]. Surface modification of liposomes with targeting ligands can further enhance cellular uptake through receptor-mediated internalization and may improve selectivity toward receptor-expressing tumor cells [11, 14-16]. Folic acid is a small, stable, and widely used targeting ligand that binds folate receptors, which are overexpressed in several malignancies and in subsets of breast cancer [14, 16, 17]. However, folate receptor targeting has important limitations that must be considered before interpreting folate-decorated liposomes as uniformly selective tumor-targeted systems [5, 6, 16-18]. Folate receptor α expression is heterogeneous among breast cancer subtypes and even within TNBC; therefore, folate-mediated targeting may be most relevant for folate receptor-positive tumors and may be reduced or absent in receptor-negative disease [5, 6, 17]. Moreover, folate receptor β is expressed on activated macrophages and inflammatory cells, raising the possibility of non-tumor uptake in vivo, particularly in macrophage-rich tumor microenvironments [18]. Folate-decorated liposomes may also undergo receptor-independent uptake through non-specific endocytosis or other cellular internalization mechanisms, so receptor specificity should be supported by blocking or uptake-control experiments [14, 16, 19]. These considerations highlight the need to interpret folate-targeted delivery data cautiously and to validate targeting specificity using appropriate blank carrier controls, receptor-blocking assays, and comparative non-targeted formulations [16, 19]. In this study, folic acid-conjugated curcumin-loaded nanoliposomes were developed to improve curcumin delivery to folate receptor-positive MDA-MB-231 TNBC cells, based on previous evidence supporting curcumin nanodelivery and folate-targeted liposomal strategies [4, 10, 16, 20]. The formulation was prepared using thin-film hydration followed by folate-lipid post-insertion, a common approach for constructing folate-functionalized liposomal systems [14, 16, 21]. The resulting formulations were characterized for particle size, polydispersity index, ζ-potential, morphology, encapsulation efficiency, and in vitro release behavior to determine whether folate functionalization preserved acceptable physicochemical properties [11-16]. Cytotoxicity was evaluated using the MTT assay, and a concentration-dependent folate receptor-blocking experiment was included to assess the contribution of receptor-mediated uptake while recognizing the limitations of curcumin potency and folate receptor heterogeneity.

Materials and Methods

Materials

DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), DSPE-mPEG(2000), and DSPE-PEG(2000)-Folate were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Cholesterol, MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], sodium pentanesulphonate, sodium edetate, folic acid, doxorubicin hydrochloride, Tween-80, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, DMEM medium, fetal bovine serum, penicillin/streptomycin, and other cell culture reagents were obtained from Gibco (Grand Island, NY, USA). The MDA-MB-231 human breast cancer cell line was obtained from the National Cell Bank of Iran/ Pasteur Institute of Iran (Tehran, Iran).

Preparation of nanoliposomes

Curcumin-loaded and curcumin-free blank nanoliposomes were prepared using the thin-film hydration method. Briefly, DSPC, cholesterol, and DSPE-mPEG(2000) were mixed at a predetermined lipid ratio and dissolved in chloroform:methanol (2:1, v/v). For the non-targeted formulation, 160 mg DSPC, 15 mg cholesterol, and 40 mg DSPE-mPEG (2000) were dissolved in 40 mL of the organic solvent mixture. The lipid solution was transferred to a round-bottom flask and evaporated under reduced pressure using a rotary evaporator at 50 °C and 100 rpm until a thin, uniform lipid film formed on the flask wall. Residual organic solvent was removed by nitrogen purging for 5 min. The dried lipid film was hydrated with phosphate-buffered saline (PBS, pH 7.2) containing 9 mg curcumin for drug-loaded liposomes. For blank liposome controls, the same procedure was performed using plain PBS without curcumin. The hydrated lipid film was gently agitated at 50 °C for 30 min to promote lipid swelling and vesicle formation. The resulting multilamellar vesicles were then sonicated under controlled conditions to reduce vesicle size and obtain nanosized liposomes. For the folate-targeted formulation, DSPE-PEG(2000)-Folate was incorporated into preformed PEGylated liposomes by post-insertion at 50 °C. In this formulation, part of the DSPE-mPEG (2000) was replaced with DSPE-PEG (2000)-Folate, while the total PEG-lipid content was kept equivalent to that of the non-targeted liposomes. Non-targeted Lipo-Cur and blank non-targeted liposomes were prepared in parallel using DSPE-mPEG(2000) without folate. Blank folate-conjugated liposomes were also prepared using the same lipid composition as FA-Lipo-Cur but without curcumin, and were included as carrier controls to evaluate whether the lipid, PEGylated lipid, or folate-conjugated components contributed to cytotoxicity independently of curcumin. After preparation, curcumin-

loaded liposomes were purified by Sephadex G-50 gel filtration pre-equilibrated with PBS (pH 7.2) to remove unencapsulated curcumin. The purified vesicles were collected and stored at 4 °C until further physicochemical characterization and biological evaluation.

Characterization of nanoliposomes

The physicochemical properties of curcumin-loaded and blank liposomal formulations were evaluated after purification. The mean hydrodynamic diameter, polydispersity index (PDI), and ζ-potential were measured by dynamic light scattering using a Zetasizer Nano instrument. Before analysis, liposomal dispersions were gently resuspended, briefly sonicated to minimize reversible aggregation, and diluted 1:50 in PBS (pH 7.4). The morphology of Lipo-Cur and FA-Lipo-Cur was examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM analysis, diluted liposomal suspensions were placed on silicon stubs, air-dried, and sputter-coated with a thin Au/Pd layer before imaging. For TEM analysis, a drop of each diluted sample was placed on a carbon-coated copper grid, excess liquid was removed, and the grids were air-dried before examination. To reduce non-specific adsorption of curcumin during handling and analysis, tubes and pipette tips were pre-rinsed with 0.1% Tween-80 solution when required. Blank non-targeted and blank folate-conjugated liposomes were used as formulation controls during spectrophotometric measurements to correct for background absorbance and possible light scattering from lipid vesicles. Encapsulation efficiency (EE%) was determined by separating unencapsulated curcumin from liposome-associated curcumin using ultracentrifugation. Briefly, 1 mL of each curcumin-loaded liposomal suspension was centrifuged at 120,000 × g for 60 min at 4 °C. The supernatant containing unencapsulated curcumin was carefully collected and quantified by UV–Vis spectrophotometry at 425 nm using an appropriate curcumin calibration curve. Blank liposome suspensions were used as background controls. Curcumin standards were prepared under the same solvent and buffer conditions used for sample analysis to minimize matrix-related variation. Encapsulation efficiency was calculated using the following equation:

EE (%) = [(Initial amount of curcumin − Free curcumin in the supernatant) / Initial amount of curcumin] × 100

where the initial amount of curcumin refers to the total curcumin added during formulation preparation, and free curcumin refers to the unencapsulated curcumin detected in the supernatant after separation. All measurements were performed using three independent formulation batches, and results were reported as mean ± SD.

In vitro drug-release

The in vitro release of curcumin from non-targeted liposomes (Lipo-Cur) and folate-conjugated liposomes (FA-Lipo-Cur) was evaluated using a dialysis diffusion method. Briefly, 1 mL of each purified formulation, containing approximately 300 µg of encapsulated curcumin, was placed into a pre-soaked dialysis bag with a molecular weight cut-off of 10 kDa and immersed in 50 mL of phosphate-buffered saline (PBS, pH 7.4) containing 0.5% (w/v) Tween-80. Tween-80 was added to improve curcumin dispersion in the release medium and to reduce non-specific adsorption to the dialysis membrane and glassware. The release system was maintained at 37 ± 0.5 °C under gentle shaking at 120 rpm. Based on the cumulative release profile, the maximum calculated curcumin concentration in the external medium at 50 h was approximately 3.54 µg/mL for Lipo-Cur and 2.88 µg/mL for FA-Lipo-Cur. These concentrations remained well below 20% of the apparent solubility threshold, indicating that sink conditions were maintained throughout the release experiment. At predetermined time points of 1, 4, 8, 11, 15, 21, 26, 32, 42, and 50 h, 2 mL of release medium was withdrawn and immediately replaced with an equal volume of fresh pre-warmed PBS/Tween-80 medium. Curcumin concentration in the collected samples was quantified by UV–Vis spectrophotometry at 425 nm using a standard calibration curve prepared under the same medium conditions. Blank liposome samples were used as background controls to correct for possible light scattering or formulation-related absorbance.

Cumulative release values were corrected for sample withdrawal and medium replacement using the following equation:

Cumulative release (%) = $[(C_nV_0 + V_s\Sigma C_i) / Qtotal] \times 100$

where C_n is the curcumin concentration measured in the release medium at sampling time n, V_0 is the total volume of the release medium, V_s is the withdrawn sample volume, ΣC_i is the sum of curcumin concentrations measured in all previously collected samples, and Qtotal is the total amount of curcumin initially encapsulated in the liposomal formulation placed inside the dialysis bag.

In vitro cytotoxicity studies

The cytotoxic effects of free curcumin and curcumin-loaded liposomal formulations were evaluated in MDA-MB-231 human breast cancer cells using the MTT assay. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in complete culture medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and amphotericin B. For the assay, cells were seeded in 96-well plates at a density of 1.0×10^4 cells/well in 200 µL of medium and allowed to attach for 24 h. After cell attachment, the medium was replaced with treatment medium containing free curcumin, non-targeted curcumin-loaded liposomes (Lipo-Cur), or folate-conjugated curcumin-loaded liposomes (FA-Lipo-Cur) at curcumin-equivalent concentrations of 4, 8, 16, 32, 48, 72, and 96 µg/mL. The curcumin-equivalent concentration of each liposomal formulation was calculated according to its encapsulation efficiency and drug-loading content. Free curcumin was first dissolved in DMSO and then diluted with culture medium, with the final DMSO concentration

maintained below 0.1% (v/v). Vehicle-treated cells were used as solvent controls. To assess possible carrier-related cytotoxicity, blank non-targeted liposomes and blank folate-conjugated liposomes were evaluated under the same experimental conditions at lipid-equivalent concentrations corresponding to those used for the curcumin-loaded formulations. These blank controls were included to distinguish curcumin-mediated cytotoxicity from any potential effects of the lipid, PEGylated lipid, or folate-conjugated carrier components. Cells were treated for 48 h in medium containing 2% FBS. After treatment, the medium was removed and replaced with 180 µL of fresh medium and 20 µL of MTT solution (5 mg/mL in PBS). Plates were incubated for 3 h at 37 °C to allow formazan formation. The medium was then discarded, and the formazan crystals were dissolved in 200 µL of DMSO. Absorbance was measured at 570 nm using a microplate reader, and cell viability was expressed as a percentage of untreated control cells. To further evaluate the role of folate receptor-mediated uptake, a concentration-dependent receptor-blocking assay was performed. MDA-MB-231 cells were pre-incubated with increasing concentrations of free folic acid (0, 10, 50, 100, 500, and 1000 µM) for 2 h at 37 °C before treatment with FA-Lipo-Cur. After pre-incubation, cells were exposed to FA-Lipo-Cur at a fixed curcumin-equivalent concentration of 48 µg/mL for 48 h, followed by the MTT assay as described above. This experiment was designed to determine whether increasing concentrations of free folic acid could progressively reduce the cytotoxic advantage of FA-Lipo-Cur through competitive inhibition of folate receptor-mediated uptake. For IC_{50} determination of the blocked group, cells were pre-incubated with 1000 µM free folic acid for 2 h and then treated with the same concentration range of FA-Lipo-Cur. Unblocked FA-Lipo-Cur-treated cells served as the matched comparison group. Doxorubicin was included as a positive cytotoxicity control using a concentration range of 0.1–5 µg/mL under the same 48 h exposure and serum conditions. This control was used only as a potency benchmark and was not interpreted as a direct therapeutic equivalence comparison with curcumin formulations. IC_{50} values were calculated from concentration–response curves using nonlinear regression analysis. Statistical comparisons among free curcumin, Lipo-Cur, FA-Lipo-Cur, blocked FA-Lipo-Cur, and doxorubicin were performed as described in the statistical analysis section. Blank liposomes were evaluated separately as carrier controls at lipid-equivalent concentrations. Data are reported as mean ± SD from independent experiments.

Statistical Analysis

All experiments were performed using three independent formulation batches, and each measurement was conducted in triplicate. Data are presented as mean ± SD. Statistical analyses were performed using SPSS version 19. IC_{50} values were calculated from concentration–response curves by nonlinear regression using GraphPad Prism version 6.0. One-way ANOVA followed by Tukey's post hoc test was used to compare IC_{50} values among free curcumin, Lipo-Cur, FA-Lipo-Cur, blocked FA-Lipo-Cur, and doxorubicin where applicable. Blank liposomes were analyzed as carrier controls using lipid-equivalent concentrations; because IC_{50} was not reached within the tested range, they were not interpreted as a curcumin-equivalent IC_{50} group. Release profiles were analyzed using two-way ANOVA followed by Tukey's post hoc test to compare formulation type and time-dependent release behavior. Receptor-blocking results were analyzed by comparing the concentration-dependent folic acid pre-incubation groups and by comparing blocked FA-Lipo-Cur with unblocked FA-Lipo-Cur and non-targeted Lipo-Cur. A p-value < 0.05 was considered statistically significant. Significance thresholds are reported in the Results section and figure captions as ns, $*p < 0.05$, and $**p < 0.01$.

Results

Physicochemical characterization

Both curcumin-loaded liposomal formulations exhibited predominantly spherical vesicular morphology with relatively uniform particle distribution, as observed in SEM and TEM micrographs (Figure 1). The SEM images showed smooth, rounded particles with limited surface irregularity, while TEM micrographs confirmed nanoscale vesicles with mild clustering, which is commonly observed after drying during sample preparation. The folate-conjugated formulation showed a slightly smaller mean hydrodynamic diameter than the non-targeted liposomes. The mean particle size of FA-Lipo-Cur was 218 ± 8.6 nm, whereas Lipo-Cur showed a mean size of 236 ± 10.4 nm. Both formulations displayed acceptable size homogeneity, with PDI values below 0.25. The PDI of FA-Lipo-Cur was 0.17 ± 0.03, compared with 0.21 ± 0.04 for Lipo-Cur, indicating that folate functionalization did not increase particle heterogeneity. FA-Lipo-Cur showed a ζ-potential of −28.4 ± 2.3 mV, while Lipo-Cur showed −23.7 ± 2.0 mV. The slightly higher negative surface charge of FA-Lipo-Cur may be related to the presence of folate groups on the liposomal surface. Curcumin encapsulation efficiency was also high in both systems, reaching 81.6 ± 2.8% for FA-Lipo-Cur and 76.9 ± 3.2% for Lipo-Cur. These findings suggest that folate modification preserved the spherical vesicular structure, maintained acceptable particle uniformity, and supported efficient curcumin encapsulation. The physicochemical profile of FA-Lipo-Cur was therefore suitable for subsequent in vitro release and cytotoxicity evaluation.

In vitro drug-release

As shown in Figure 2, both liposomal formulations exhibited sustained curcumin release over the 50 h study period. In both groups, release increased during the early phase and then continued more gradually at later time points, suggesting a controlled diffusion-dominated release pattern. Throughout the experiment, Lipo-Cur released curcumin more rapidly than FA-Lipo-Cur, resulting in consistently higher cumulative release values. The difference between the two formulations became

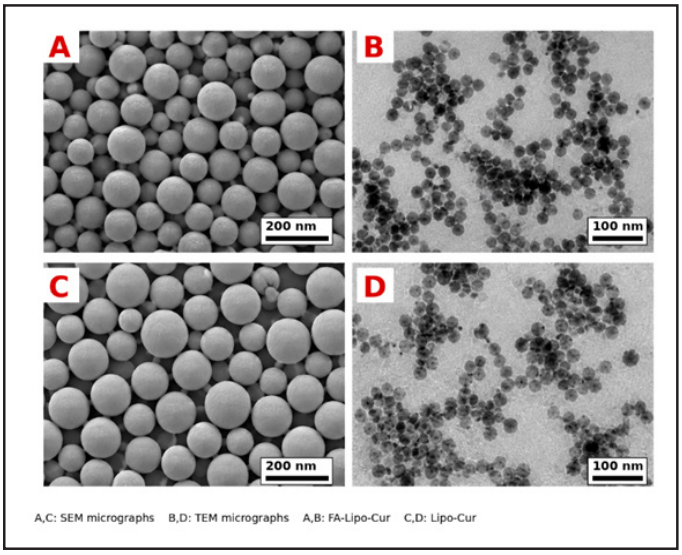

Figure 1. Representative SEM and TEM Micrographs of Curcumin-Loaded Liposomal Formulations. Panels A and B show FA-Lipo-Cur, while panels C and D show non-targeted Lipo-Cur. SEM images are shown in panels A and C, and TEM images are shown in panels B and D. Scale bars: A and C = 200 nm; B and D = 100 nm.

more evident during the intermediate and late phases of the study. Specifically, Lipo-Cur showed significantly higher cumulative release than FA-Lipo-Cur at 21, 26, 32, 42, and 50 h ($p < 0.05$, two-way ANOVA followed by Tukey's post hoc test). At 50 h, cumulative release from Lipo-Cur reached approximately 59%, whereas FA-Lipo-Cur reached approximately 48%. The slower release from FA-Lipo-Cur may be related to the PEG–folate surface layer, which can increase steric stabilization and reduce membrane permeability, thereby moderating curcumin diffusion without preventing sustained release. Overall, these results indicate that folate conjugation altered the release behavior of curcumin-loaded liposomes and produced a slower, more controlled release profile compared with the non-targeted formulation. Therefore, the release findings should be interpreted as a formulation-to-formulation comparison of release behavior rather than as a direct prediction of in vivo curcumin availability.

In vitro cytotoxicity studies

The cytotoxic effects of free curcumin, Lipo-Cur, and FA-Lipo-Cur were evaluated in MDA-MB-231 cells after 48 h exposure. As shown in Figure 3B, all curcumin-containing formulations reduced cell viability in a concentration-dependent manner. Free curcumin showed the weakest cytotoxic effect, whereas Lipo-Cur and FA-Lipo-Cur produced stronger reductions in cell viability. Among the tested formulations, FA-Lipo-Cur showed the greatest cytotoxic effect, suggesting improved intracellular delivery by the folate-conjugated liposomal system. The concentration-dependent receptor-blocking assay supported the involvement of folate receptor-mediated

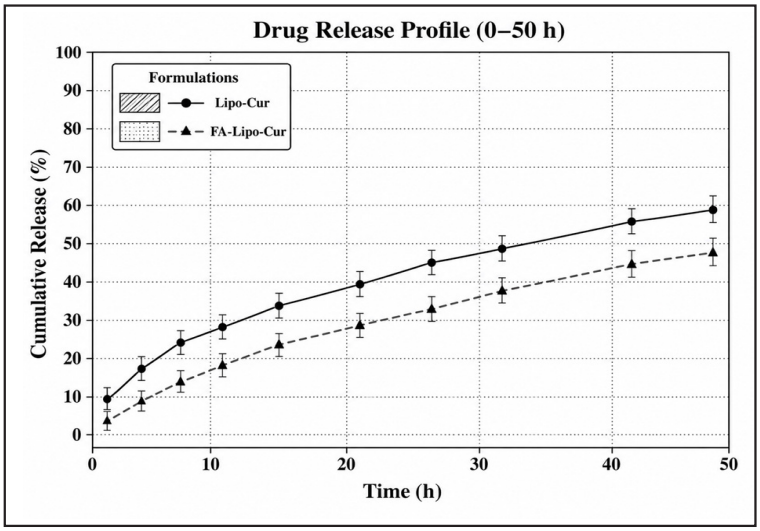

Figure 2. Cumulative Curcumin Release Profiles of Lipo-Cur and FA-Lipo-Cur in PBS (pH 7.4) Containing 0.5% Tween-80 at 37 °C over 0–50 h (mean ± SD, n = 3). Samples were collected at 1, 4, 8, 11, 15, 21, 26, 32, 42, and 50 h. Both formulations showed sustained release behavior, while FA-Lipo-Cur exhibited a slower and more controlled release profile than Lipo-Cur. Release profiles were analyzed using two-way ANOVA followed by Tukey's post hoc test, with significant differences observed at 21, 26, 32, 42, and 50 h ($p < 0.05$).

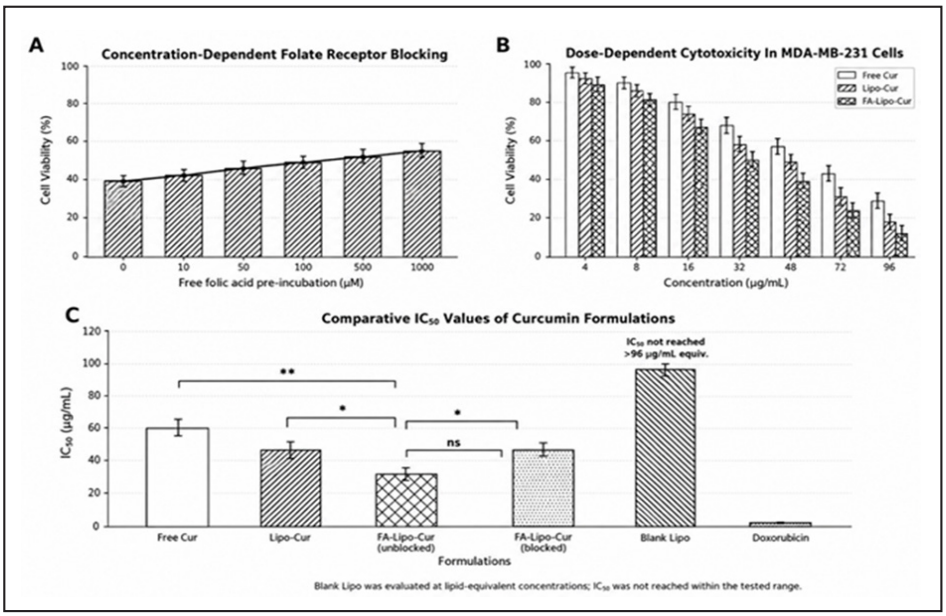

Figure 3. *In vitro* Cytotoxicity and Folate Receptor-Blocking Analysis of Curcumin Formulations in MDA-MB-231 Cells. (A) Concentration-dependent folate receptor-blocking assay. Cells were pre-incubated with increasing concentrations of free folic acid before treatment with FA-Lipo-Cur at a fixed curcumin-equivalent concentration of 48 μg/mL. The gradual increase in cell viability supports competitive inhibition of folate receptor-mediated uptake. (B) Dose-dependent cytotoxicity of free curcumin, Lipo-Cur, and FA-Lipo-Cur after 48 h exposure. FA-Lipo-Cur produced the strongest reduction in cell viability among the curcumin-containing groups. (C) Comparative IC_{50} values of free curcumin, Lipo-Cur, FA-Lipo-Cur, folate-blocked FA-Lipo-Cur, blank liposome controls, and doxorubicin. FA-Lipo-Cur showed significantly lower IC_{50} values than free curcumin ($p < 0.01$) and Lipo-Cur ($p < 0.05$). Folate receptor blocking increased the IC_{50} of FA-Lipo-Cur compared with unblocked FA-Lipo-Cur ($p < 0.05$), making it statistically comparable to Lipo-Cur ($p > 0.05$). Blank liposome controls were evaluated at lipid-equivalent concentrations, and IC_{50} was not reached within the tested range (>96 μg/mL lipid-equivalent); therefore, they were interpreted as carrier-control groups rather than curcumin-equivalent IC_{50} groups. Doxorubicin was included only as a positive cytotoxicity benchmark. Data are presented as mean ± SD. ns, not significant; *$p < 0.05$; **$p < 0.01$.

uptake. As shown in Figure 3A, pre-incubation with increasing concentrations of free folic acid before treatment with FA-Lipo-Cur at a fixed curcumin-equivalent concentration of 48 μg/mL gradually increased cell viability from approximately 39% in the unblocked condition to approximately 55% at 1000 μM folic acid. This partial reversal indicates competitive inhibition of folate receptor-mediated uptake, while also suggesting that receptor-independent uptake and the intrinsic cytotoxicity of curcumin may contribute to the remaining effect. IC_{50} analysis is summarized in Figure 3C and Table 1. FA-Lipo-Cur showed the lowest IC_{50} value among the curcumin-containing formulations (33 ± 3.3 μg/mL), followed by Lipo-Cur (48 ± 3.9 μg/mL) and free curcumin (60 ± 4.1 μg/mL). The IC_{50} of FA-Lipo-Cur was significantly lower than that of free curcumin ($p < 0.01$) and Lipo-Cur ($p < 0.05$). After folate receptor blocking, the IC_{50} of FA-Lipo-Cur increased to 46.5 ± 3.2 μg/mL, which was significantly higher than unblocked FA-Lipo-Cur ($p < 0.05$) and statistically comparable to non-targeted Lipo-Cur ($p > 0.05$). These findings indicate that the enhanced cytotoxicity of FA-Lipo-Cur was at least partly dependent on folate receptor-mediated internalization. Blank liposomes showed minimal cytotoxicity within the tested lipid-equivalent concentration range, and IC_{50} was not reached within this range (>96 μg/mL lipid-equivalent). Therefore, the blank liposome group was interpreted only as a carrier-control reference and not as a curcumin-equivalent IC_{50} group. This result indicates that the cytotoxicity of Lipo-Cur and FA-Lipo-Cur was mainly attributable to curcumin delivery rather than to the lipid, PEGylated lipid, or folate-conjugated carrier components alone. Doxorubicin showed a markedly lower IC_{50} value (1.2 ± 0.08 μg/mL) and was included only as a positive cytotoxicity benchmark, not as a direct therapeutic comparator. Overall, these results support FA-Lipo-Cur as an improved folate-targeted curcumin delivery system for MDA-MB-231 cells, while its potential application should be interpreted as adjunctive or combination-oriented rather than as a replacement for standard chemotherapy.

Discussion

This study evaluated folate-conjugated curcumin-loaded nanoliposomes as an in vitro delivery platform for improving curcumin activity in MDA-MB-231 breast cancer cells. The results showed that liposomal encapsulation improved the physicochemical behavior and cytotoxic performance of curcumin. Folate conjugation further enhanced this effect, at least partly through folate receptor-associated uptake [4,9,10,16,20]. Both Lipo-Cur and FA-Lipo-Cur showed favorable physicochemical characteristics. These included predominantly spherical morphology, acceptable particle-size distribution, negative ζ-potential, and high encapsulation efficiency. Such properties are consistent with liposomal systems

Table 1. Comparative IC_{50} Values, Blank-Carrier Control Interpretation, and Statistical Comparisons for the Cytotoxicity Analysis

Treatment group	IC_{50} or control result	Key statistical comparison	Interpretation
Free Cur	60 ± 4.1 µg/mL	Reference curcumin group	Weakest cytotoxic activity among curcumin-containing groups
Lipo-Cur	48 ± 3.9 µg/mL	$p < 0.05$ vs Free Cur; $p > 0.05$ vs blocked FA-Lipo-Cur	Non-targeted liposomal delivery improved curcumin cytotoxicity
FA-Lipo-Cur	33 ± 3.3 µg/mL	$p < 0.01$ vs Free Cur; $p < 0.05$ vs Lipo-Cur	Folate conjugation provided the strongest in vitro cytotoxic effect
FA-Lipo-Cur + folate block	46.5 ± 3.2 µg/mL	$p < 0.05$ vs unblocked FA-Lipo-Cur; $p > 0.05$ vs Lipo-Cur	Blocking reduced the targeting advantage and supported partial receptor-mediated uptake
Blank liposomes	IC_{50} not reached (>96 µg/mL lipid-equivalent)	Not compared as a curcumin-equivalent IC_{50} group	Minimal carrier-related cytotoxicity within the tested lipid-equivalent range
Doxorubicin	1.2 ± 0.08 µg/mL	Positive benchmark under the same 48 h and 2% FBS conditions	Not interpreted as therapeutic equivalence with curcumin formulations

Note: Values are reported as mean ± SD. Statistical comparisons were based on one-way ANOVA followed by Tukey's post hoc test. Blank liposomes were analyzed as lipid-equivalent carrier controls rather than curcumin-equivalent drug groups

designed to improve dispersion, protect hydrophobic payloads, and support controlled release behavior [11-16]. FA-Lipo-Cur showed a slightly smaller particle size and lower PDI than Lipo-Cur. This suggests that folate modification did not disrupt liposome formation or increase formulation heterogeneity. This point is important because excessive heterogeneity can affect reproducibility, release behavior, and cellular interaction [11-13, 16]. The in vitro release study showed sustained curcumin release from both liposomal formulations over 50 h. Lipo-Cur released curcumin more rapidly than FA-Lipo-Cur. In contrast, FA-Lipo-Cur showed a slower and more controlled release profile. This difference may be related to the PEG-folate surface layer. This surface layer can provide steric stabilization and may reduce membrane permeability, thereby moderating curcumin diffusion from the liposomal bilayer [11, 13-16, 21]. The slower release profile of FA-Lipo-Cur may help maintain prolonged drug availability. However, its biological relevance should be confirmed in more complex experimental models [13, 20-23]. The cytotoxicity results were consistent with the delivery advantage of the liposomal formulations. Free curcumin showed the weakest reduction in cell viability. Lipo-Cur and FA-Lipo-Cur produced stronger dose-dependent cytotoxic effects. This pattern agrees with previous studies showing that liposomal and nanosystem-based curcumin delivery can improve cellular uptake and cytotoxic activity compared with free curcumin [9, 10, 20]. Among the curcumin-containing groups, FA-Lipo-Cur produced the greatest reduction in MDA-MB-231 cell viability and showed the lowest IC_{50} value. These findings suggest that folate conjugation improved the in vitro activity of curcumin-loaded liposomes by increasing interaction with folate receptor-expressing cells [14, 16, 19, 21]. The concentration-dependent folate receptor-blocking assay provided additional mechanistic support. Pre-incubation with increasing concentrations of free folic acid progressively reduced the cytotoxic effect of FA-Lipo-Cur. This was reflected by a gradual increase in cell viability. Such competitive inhibition is consistent with receptor-associated uptake of folate-decorated liposomes. However, the reversal was only partial. Therefore, receptor-independent endocytosis, non-specific uptake, and the intrinsic cytotoxicity of curcumin may also have contributed to the observed effect [14, 16, 19]. In addition, folate receptor blocking increased the IC_{50} of FA-Lipo-Cur toward that of non-targeted Lipo-Cur. This supports the interpretation that the cytotoxic advantage of FA-Lipo-Cur was at least partly dependent on folate receptor-mediated internalization [14, 16, 19, 21-23]. The inclusion of blank liposome controls strengthened the interpretation of the cytotoxicity data. Blank liposomes showed minimal cytotoxicity within the tested lipid-equivalent concentration range. This suggests that the reduction in cell viability was mainly associated with curcumin delivery rather than toxicity from the lipid carrier, PEGylated lipid, or folate-conjugated components alone. This interpretation is consistent with the established use of liposomes as generally biocompatible drug carriers. It also highlights the importance of carrier controls in nanomedicine studies [11, 12, 15, 16]. Despite these encouraging findings, several limitations should be acknowledged. First, although FA-Lipo-Cur improved curcumin activity compared with free curcumin and non-targeted liposomes, the IC_{50} values remained in the tens of µg/mL range. This reflects the limited intrinsic potency and pharmaceutical limitations of curcumin compared with standard cytotoxic drugs [8, 9]. Doxorubicin showed a much lower IC_{50} and was included only as a positive cytotoxicity benchmark. It should not be interpreted as a direct therapeutic comparator. Therefore, FA-Lipo-Cur should not be presented as a replacement for conventional chemotherapy. A more appropriate interpretation is that this formulation may have potential as an adjunctive

or combination-oriented curcumin delivery platform, especially in settings where curcumin may complement conventional agents [5-7, 9, 10]. A second limitation is the heterogeneity of folate receptor expression in TNBC. MDA-MB-231 cells were used as a folate receptor-positive model in this study. However, not all TNBC tumors express sufficient levels of folate receptors. Therefore, the targeting advantage of FA-Lipo-Cur may be reduced or absent in FR-negative or low-FR-expressing TNBC subtypes [5 ,6, 17]. In addition, folate receptor β is expressed on activated macrophages, including tumor-associated macrophages. This may lead to non-tumor uptake of folate-decorated liposomes in vivo [18]. For this reason, the selectivity of FA-Lipo-Cur should be interpreted cautiously until it is validated in additional FR-positive and FR-negative breast cancer models, macrophage-containing systems, and in vivo biodistribution studies [5, 6, 17, 18]. Another limitation is that the present study was restricted to a single in vitro breast cancer cell model. Future studies should include additional breast cancer cell lines with different folate receptor expression profiles. Normal cell controls, direct cellular uptake assays, and mechanistic analyses using fluorescence microscopy or flow cytometry are also needed. Moreover, pharmacokinetic, biodistribution, safety, and tumor-inhibition studies are required to determine whether the improved in vitro performance of FA-Lipo-Cur translates into meaningful in vivo benefit [4, 14-16, 19]. Overall, the findings indicate that folate conjugation improved the in vitro performance of curcumin-loaded liposomes. This improvement was associated with enhanced cytotoxic activity, partial receptor-associated uptake, and controlled release behavior. However, the current study remains limited to in vitro conditions. Therefore, the translational relevance of FA-Lipo-Cur should be evaluated in future preclinical studies, particularly in adjunctive or combination-based therapeutic settings.

In conclusion, folate-conjugated curcumin-loaded nanoliposomes improved the in vitro delivery and cytotoxic activity of curcumin in MDA-MB-231 breast cancer cells. FA-Lipo-Cur showed favorable physicochemical properties, sustained release behavior, and a lower IC_{50} than both free curcumin and non-targeted Lipo-Cur. The concentration-dependent receptor-blocking assay, together with the increase in IC_{50} after folic acid blocking, supports the involvement of folate receptor-mediated uptake. However, this effect should be interpreted as partial rather than exclusive receptor-mediated targeting. Blank liposome controls showed minimal cytotoxicity within the tested lipid-equivalent concentration range. This indicates that the observed effects were mainly associated with curcumin delivery rather than carrier-related toxicity. Nevertheless, the relatively high IC_{50} values of the curcumin formulations, folate receptor heterogeneity in TNBC, and the in vitro nature of the study limit direct therapeutic interpretation. Therefore, FA-Lipo-Cur should be considered an adjunctive or combination-oriented nanocarrier platform rather than a substitute for standard chemotherapy. Further studies using additional FR-positive and FR-negative cell models, direct uptake assays, macrophage-containing systems, 3D tumor models, and in vivo evaluation are required. The comparative IC_{50} summary and carrier-control interpretation in Table 1 further support that the observed effect was formulation-associated, but not sufficient to claim replacement of standard chemotherapy.

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Consent for publication

All authors have given consent for publication.

Conflict of interest

The authors declare no potential conflict of interest.

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