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# Codelivery of a miR-124 Mimic and Obatoclax by Cholesterol-Penetratin Micelles Simultaneously Induces Apoptosis and Inhibits Autophagic Flux in Breast Cancer in Vitro and in Vivo

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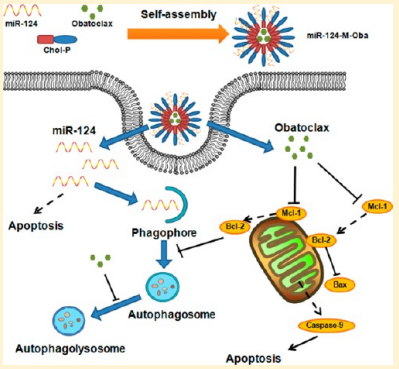
Supporting Information

**ABSTRACT:** Penetratin is a classical cell-penetrating peptide with the potential to assist in the transmembrane delivery of proteins or drugs. However, the synthesis and application of cholesterol-penetratin (Chol-P) conjugates as nonviral delivery systems for microRNAs or drugs have not previously been reported. In this study, the amphiphilic Chol-P was shown to self-assemble into micelles and efficiently deliver miR-124 and obatoclax. The codelivered miR-124-M-Oba had a homogeneous particle size and a positive zeta potential. Treatment with miR-124 mincreased cytotoxicity, and cell proliferation, was promoted by miR-124 inhibitor-loaded micelles in MCF-7 human breast cancer cells. Moreover, the inhibitory effects on cell proliferation, colony formation, and cell migration were increased in the miR-124-M-Oba group compared to the miR-124-M group. miR-124-M-Oba induced higher levels of mitochondrial apoptosis via Bax and caspase-9 activation. In addition, we found that the cationic Chol-P and miR-124-M could potently induce autophagy, and miR-124 was degraded in the corresponding autophagolysosomes. The obatoclax encapsulated in miR-124-M-Oba could inhibit the degradation of miR-124 and p62 in autophagolysosomes, which consequently maintained the concentration of miR-124 in breast cancer cells. Furthermore, miR-124-M-Oba potently inhibited tumor growth in subcutaneous xenograft breast cancer models. In summary, the miR-124-M-Oba prepared in this work showed improved apoptosis induction and autophagic flux inhibitory effects in MCF-7 cells, and miR-124-M-Oba may have potential applications in breast cancer therapy.




**KEYWORDS:** *penetratin, miR-124, obatoclax, apoptosis, autophagic flux, breast cancer*

## 1. INTRODUCTION

Breast cancer is one of the leading causes of mortality for adult females worldwide.[1−3] Advances in systemic therapy and screening, including surgery, radiation, and chemotherapy, have resulted in a reduced mortality rate; however, as with most solid tumors, distant metastases caused more than 90% of breast cancer-related deaths.[4−6] Currently, the multiple sequential steps of breast cancer metastasis are not fully understood, and further investigation of the underlying mechanisms is urgently needed.[6,7]

As one of the most important types of noncoding RNAs, microRNAs (miR) have many functions including regulation of mRNA degradation or translation inhibition of target genes via imperfect base pairing to the 3′-UTR (untranslated region).[8−10] In general, microRNAs regulate diverse biological processes including cell migration, proliferation, programmed cell death, and cell differentiation.[11−13] Moreover, microRNAs can also function as oncogenes or tumor suppressor genes in carcinogenesis, cancer progression, and metastasis.[14,15] Therefore, microRNAs are attractive targets in clinical prognostic analysis, cancer diagnosis, and targeted therapy.[16,17] A microRNA profile analysis of breast cancer and normal tissues identified several differentially expressed microRNAs including miR-124, which is a brain-enriched microRNA.[18] The function of miR-124 was initially reported as regulation of neuron development and regeneration.[19−22] Subsequent functional studies suggested that miR-124 is epigenetically silenced in breast cancer and suppresses cancer cell proliferation, metastasis, and endothelial−mesenchymal transition by targeting Sphk1, RAC1, EZH2, ROCK1, CDK4, and CD151.[23−32] Although many studies have reported that miR-124 acts as cancer suppressor in breast cancer, miR-124 can also play multifaceted roles in different diseases; for example, miR-124 is overexpressed in bone marrow-derived mesenchymal stem cells and promotes their transformation into neurons.[33−36] Considering the low expression of miR-124 in breast cancer cells and its anticancer effects, selective delivery of miR-124 mimics into breast cancer cells may be an attractive therapeutic strategy.[37−43]

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In addition to the differentially expressed microRNAs, the B-cell lymphoma 2 (Bcl-2) family, including many antiapoptotic (Bcl-2, Bcl-xL, and Mcl-1) and pro-apoptotic proteins (Bax, Bak, and BH3-only proteins), was also altered during the progression of breast cancers. Bcl-2 is often overexpressed in breast cancer and is a typical favorable prognostic indicator; it also interacts with Beclin-1, a key regulator of autophagy in the autophagosome formation.[44,45] Obatoclax (also called GX15−070, see Figure 1A), an indole derivative, is a pan-Bcl-2 inhibitor, which is being tested in clinical trials for leukemia, lymphoma, and other cancers.[46−54] However, application of obatoclax is restricted due to its highly hydrophobic properties.[55−57] Currently, self-assembled micelles are widely used to prepare aqueous formulations of hydrophobic drugs or gene therapeutics.[58−62] However, cationic nonviral gene vectors may rapidly activate autophagy and generate tubulovesicular autophagosomes and are then captured into autophagosomes, finally fusing slowly into lysosomes.[63−65] Recently, Liu et al. reported that the activation of autophagy effectively eliminated specific microRNAs for therapeutic usages. Moreover, obatoclax could inhibit autophagy independent of the Beclin-1 complex, and accumulation of both LC3 and p62 was simultaneously observed.[66−68] It has been hypothesized that following obatoclax treatment, autophagosomes are initiated and formed but do not fuse into autophagolysosomes for degradation.[69,70]

In this work, cholesterol conjugates of penetratin (Chol-P, see Figure 1B), a typical cell-penetrating peptide (CPP), and their self-assembling micelles were synthesized for simultaneous delivery of miR-124 and obatoclax to breast cancer cells. As a well-studied CPP, penetratin can increase the cell membrane translocation efficiency of the attached nanovectors. On the other hand, cholesterol is one of the most commonly used hydrophobic molecules, with a rigid, planar tetracyclic unit and a flexible aliphatic chain. The hydrophobic obatoclax could enter the Chol-P micelles by hydrophobic interactions, and the anionic miR-124 mimics could be adsorbed on the outer portion of the micelles via electrostatic interactions. Many nanoscale drug-gene codelivery systems have shown enhanced transfection efficacy with CPPs. The goals of the current study are to (1) design, synthesize, and characterize Chol-P micelles loaded with both obatoclax and miR-124 mimics; (2) comparatively evaluate delivery efficacy after enhancing cell uptake by CPPs; (3) determine the therapeutic effect of the codelivery of Chol-P micelles and the synergistic effect of obatoclax and miR-124 mimics; and (4) assess the potential mechanisms of crosstalk between the inhibition of autophagy by obatoclax and the regulation of apoptosis by miR-124 mimics.

## 2. MATERIALS AND METHODS

**2.1. Regents and Materials.** The penetratin peptide and its cholesterol conjugate Chol-P were synthesized by standard solid-phase peptide synthesis (SPPS) protocols on a CSBio 136XT peptide synthesis instrument.[71−74] The peptides were purified by high-performance liquid chromatography (HPLC) to 95% purity. The molecular weights were confirmed by mass spectrometry. MCF-10A cells (human breast epithelial cells), MCF-7 cells, and MD-MBA-231 cells (human breast cancer cells) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% (v/v) fetal bovine serum (FBS, Gibco, USA). MCF-10A cells and MD-MBA-231 cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Gibco) containing 10% FBS (Gibco, USA). All the above cell lines were cultivated at 37 °C in a 5% $CO_2$ incubator.

**2.2. Preparation, Characterization, and Drug Release Profiles of Chol-P Micelles.** The obatoclax-loaded Chol-P micelles (M-Oba), miR-124-loaded Chol-P micelles (miR-124-M), and the miR-124/obatoclax codelivery Chol-P micelles (miR-124-M-Oba) were prepared as follows: obatoclax and Chol-P (1:49, w/w) were fully dissolved in ethanol, and the solution was then evaporated in a rotary evaporator under vacuum. The residues were redissolved in normal saline to form M-Oba. For the preparation of miR-124-M, Chol-P was fully dissolved in ethanol, and after evaporation and hydration by saline, miR-124 mimics or miR-Ctrl were added. The obtained miR-124-M were filtered and lyophilized into powder form. The obtained M-Oba were incubated with the miR-124 mimics or miR-Ctrl to prepare miR-124-M-Oba, filtered using a 0.22 mm Millex-LG filter (Millipore Co., USA), and then lyophilized into a power before use. The particle size and zeta potential distribution of miR-124-M-Oba were determined using a Malvern Nano-ZS 90 laser particle size analyzer. The morphological characteristics of miR-124-M-Oba were examined using atomic force microscopy (AFM) (NSK Ltd., Tokyo, Japan). The drug loading and encapsulation efficiency of the Chol-P micelles were determined using an HPLC (Shimadzu LC-20) instrument. Detection was achieved on a UV detector, and chromatographic separations were performed on a reversed-phase $C_{18}$ column (4.6 × 150 $mm^2$, 5 $\mu$m, Inertsil/WondaSil, Shimadzu-GL, Kyoto, Japan). The drug loading and encapsulation efficiency of Chol-P micelles were calculated according to the following equations:

$$\text{DL}\ (\%) = \text{Drug}/(\text{Drug} + \text{Polymer}) \times 100 \quad (1)$$

$$\text{EE}\ (\%) = \text{Drug in micelles}/\text{drug in feed} \times 100 \quad (2)$$

The obatoclax released from Chol-P micelles was analyzed using a modified dialysis method. In general, samples of 1 mL of M-Oba or miR-124-M-Oba (with a fixed obatoclax concentration of 1 mg/mL) was placed in dialysis bags (molecular weight cutoff = 3500 Da) after incubation in 10 mL of thermostated neutral PBS buffers containing 0.5 wt % of Tween80, and the dialysis bags were gently shaken at 37 °C. After specific time intervals, the released media were collected, stored at −20 °C until analysis, and replaced by thermostated fresh release media. The concentration of released obatoclax in each sample was quantified with an HPLC method similar to drug loading experiment.

**2.3. Cell Proliferation Assay.** The effects of Chol-P micelles on cellular proliferation were evaluated in MCF-7 breast cancer cells. MTT assays were used to evaluate the potential cytotoxicity of Chol-P micelles with or without miR-124 mimics, inhibitors, and free obatoclax; the final concentrations of miR-124 mimics, inhibitors, and obatoclax were set to 10 nmol/L, 20 nmol/L, and 10 nmol/L, respectively. Cancer cells seeded in 96-well plates were treated with different concentrations for 48 h. The mean percentage of cell survival was determined from three individual experiments, and all the data are expressed as the mean ± SD. Cellular viability was evaluated by adding MTT solution (5 mg/mL) to the cells and incubating the cells for 3 h. Then the MTT solution was removed, and the formazan crystals were dissolved


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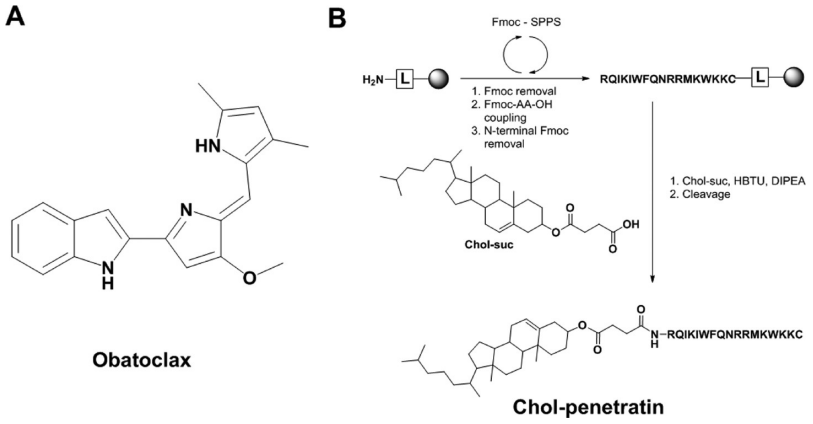


**Figure 1.** Chemical structure of obatoclax (A); the synthesis of Chol-penetratin (B).

in DMSO. The absorbance of the solution was measured at 570 nm. The control group consisted of untreated cells.

**2.4. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR).** Total RNA from cells was extracted by TRIzol Reagent (Invitrogen, CA, USA). RNA extraction and quantitative real-time PCR were carried out as previously reported. The stem−loop real-time RT-PCR was performed with a specific stem−loop real-time PCR miRNA kit (RiboBio, Guangzhou, China). Real-time PCR was performed as described previously. The relative expression of 5S rRNA in each sample was calculated and compared. The experiments were performed in triplicate. All procedures were performed according to the manufacturer's instructions.

**2.5. Wound-Healing Assay.** To determine the impacts of Chol-P micelles on cell migration, MCF7 breast cancer cells were cultured in six-well plates for 24 h and treated with Chol-P micelles with or without miR-124 mimics or free obatoclax. Then the confluent MDA-MB-231 and MCF7 cells cultivated in six-well plates were scraped in a straight line to create a scratch in the middle of the well using a 200 $\mu$L pipet tip, and the debris was rinsed with PBS. The morphological changes over a designated time period (0, 24, 48 h) were evaluated using a phase-contrast microscope (Olympus IX81, Tokyo, Japan).

**2.6. Colony Formation Assays.** Cells were seeded in six-well plates at $5 \times 10^2$ cells per well and treated with M-Oba, miR-124-M, and miR-124-M-Oba. After incubation for 2 weeks for the colony formation assay, the cells were then washed twice with cold PBS, fixed with methanol/acetic acid (3:1, v/v), and stained with 0.5% crystal violet (Sigma, St Louis, MO, USA). An optical microscope (Olympus IX81, Tokyo, Japan) was used to count the colonies.

**2.7. Apoptosis and Autophagy Assay.** Apoptosis assays with M-Oba, miR-124-M, and miR-124-M-Oba were carried out with MCF7 cells. Flow cytometric (FCM) analysis was used to confirm the apoptotic induction effect of different micelles. Apoptosis of MCF7 cells treated with M-Oba, miR-124-M, miR-124-M-Oba, or blank micelles was determined after cells were gently trypsinized without EDTA and centrifuged at 2000*g* for 5 min. Then the harvested cells were washed with 1.0 mL of cold PBS by centrifugation at 2000*g* for 5 min, resuspended in 500 $\mu$L of binding buffer (1×) containing 5 $\mu$L of Annexin V-FITC and 5 $\mu$L of propidium iodide (PI), and incubated for 15 min at room temperature. The samples were then measured by FCM (BD FACS Calibur, BD, USA) using the FL1 channel for Annexin V-FITC and the FL2 channel for PI. Both early apoptotic (Annexin V+/PI−) and late apoptotic (Annexin V+/PI+) cells were included in cell apoptosis determinations. The autophagy assay was performed on GFP-LC3-transfected MCF-7 cells. In brief, the GFP-LC3-transfected MCF-7 cells were treated with the Chol-P micelles or miR-124-M-Oba for specific times, and the cells were then fixed and observed under a fluorescence microscope. The occurrence of autophagy was confirmed by the aggregation of GFP-LC3 in the MCF-7 cells.

**2.8. Western Blot Analysis.** All primary antibodies were purchased from Cell Signaling Technology or Santa Cruz Biotechnology. After various treatments as indicated in the figure legends, MCF7 cells were harvested by trypsinization and then washed with cold PBS. The cells were lysed in RIPA buffer (Invitrogen, CA, USA) on ice for 30 min followed by sonication denaturation. Cell lysates were then centrifuged at 13 000*g* for 30 min at 4 °C. Supernatants were collected, and the protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo, USA). The protein was applied to a 10−15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and then detected with the appropriate primary and secondary antibodies before visualization by a chemical luminescence kit (Millipore, USA).

**2.9. Xenograft Breast Cancer Models and Histological Analysis.** The in vivo therapy experiments performed in this study were approved by the Institutional Animal Care and Use Committee of the West China Hospital, Sichuan University. Specific pathogen-free female nude mice, aged 6−8 weeks old, were obtained from Huafukang Biotechnology Co., Ltd. All animals were weighed and coded and randomly assigned to experimental groups of $n = 6-8$. After approximately 1 week of adaptation, $2 \times 10^6$ MCF-7 cells per 100 $\mu$L of phosphate buffer were injected s.c. into their right flank. Treatments were initiated when tumors were of measurable size (approximate leg diameter of 6 mm, the volume of tumor tissue was calculated according to formula: $V = ab^2 0.5$). Animals were intravenously injected either miR-124-M-Oba, miR-124-M, or M-Oba or saline on days 1−14 and were monitored on a daily basis during treatment (tumor measurements, body weights). All drugs were administrated every day at a rate of 2.0 mg/kg of miR-124 mimic and 1.5 mg/kg of obatoclax for animal body weight. On day 15, the animals were sacrificed, and the tumors and main organs were isolated and weighed. The tumor tissue was processed with 4% paraformaldehyde for IHC (immunohistochemistry) analysis. Sections were stained with TUNEL, $K_i$-67, and caspase-9 antibodies as described previously.[22]


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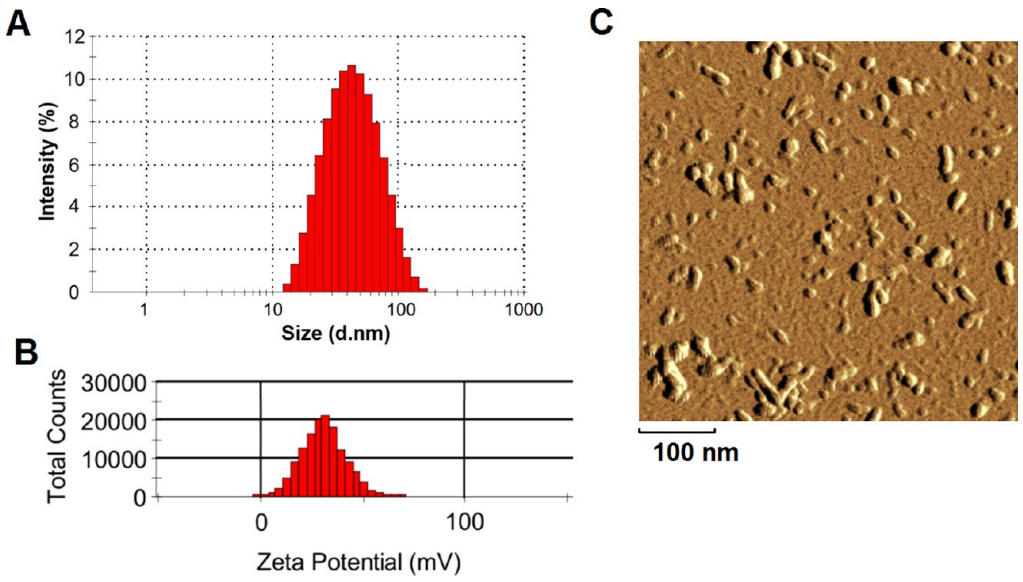


**Figure 2.** Size distribution (A), zeta potential (B), and AFM image (C) of miR-124-M-Oba.

## 3. RESULTS

**3.1. Preparation and Characterization of Chol-P and Obatoclax/miR-124 Codelivery Micelles.** A standard solid-phase peptide synthesis procedure was initiated with Rink-MBHA resin (Figure 1) that was loaded with lysine. After Fmoc removal and coupling of the subsequent amino acids, the monocholesterol ester of succinate was coupled with a Rink-MBHA resin-linked penetratin peptide. The Chol-P conjugate was cleaved from the resin using the Fmoc peptide synthesis method with trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/$H_2O$ to yield a crude product.

After purification by preparative HPLC and lyophilization, the Chol-P was successfully synthesized, and its purity and structure were characterized using HPLC analysis and the mass spectrum (see Figure S1). In general, miR-124-M-Oba was prepared by a convenient self-assembly method. The average particle size of the prepared miR-124-M-Oba was 42.5 ± 2.8 nm with a polydispersity index (PDI) of 0.29, and the zeta potential was 31.7 ± 1.2 mV (Figure 2A,B). According to the particle size distribution spectrum shown in Figure 2, panel A, miR-124-M-Oba had a narrow particle size distribution. The AFM image of miR-124-M-Oba and Chol-P micelles is presented in Figures 2, panel C, and S2, respectively, which show that miR-124-M-Oba is almost spherical in shape. The diameter of the miR-124-M-Oba observed by AFM was in good agreement with the results of the particle size analysis, which indicates that the prepared miR-124-M-Oba is stable and could be well-dispersed in aqueous solution. The drug loading and entrapment efficiency of miR-124-M-Oba were 1.82% and 90.7%, respectively. The drug release profiles were determined via dialysis methods. The obatoclax released from free drug, M-Oba, and miR-124-M-Oba was continuously monitored for 144 h (Figure 3). Compared with the fast release of the free drug, the cumulative release rate of drug-loaded micelles was much slower. In the first 24 h, approximately 93.1 ± 3.6% of obatoclax was released from the free drug, whereas the cumulative release rate of M-Oba or miR-124-M-Oba was 72.0 ± 2.5% or 64.8 ± 1.9%, respectively, which was much lower than that of free drug group. Moreover, the release of obatoclax from miR-124-M-Oba was slightly slower than that

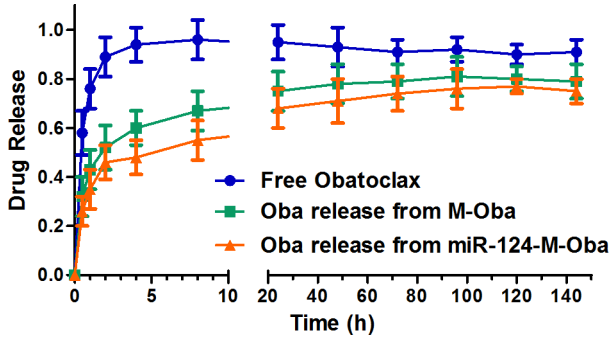


**Figure 3.** In vitro release profiles of obatoclax from free obatoclax, M-Oba, and miR-124-M-Oba.

from M-Oba, which indicated that the miR-124 might retard the drug release from codelivery micelles. In brief, the sustained release property of miR-124-M-Oba was suitable for antitumor drug delivery.

**3.2. miR-124 Is Down-Regulated in Breast Cancer Cells and Is Involved in Cell Proliferation, Colony Formation, and Migration.** We compared the levels of miR-124 in breast cancer cells (MCF-7 and MD-MB231) and normal breast epithelial cells (MCF-10A) using real-time PCR and found that miR-124 was significantly down-regulated in the breast cancer cell lines compared to breast epithelial cells (Figure 4A). To determine whether the down-regulated miR-124 affected the function of breast cancer cells, we treated breast cancer cells with a miR-124 control, miR-124 mimic, and miR-124 inhibitor. There was a significant decrease in cell proliferation in the miR-124 mimic-treated group compared to the miR-124 control group (Figure 4B,C), and cell proliferation was substantially increased in the miR-124 inhibitor-treated group compared to the miR-124 control group (Figure 4D,E).

Next, a colony formation assay was performed to confirm the antiproliferative effects of miR-124 on MCF-7 cells. The results showed that miR-124-M treatment strongly reduced the colony number compared to the miR-124 control-loaded Chol-P micelles group (Figure 5). Moreover, the miR-124 control-loaded Chol-P micelles also slightly reduced the number of colonies. The effects of miR-124 on cell migration were tested


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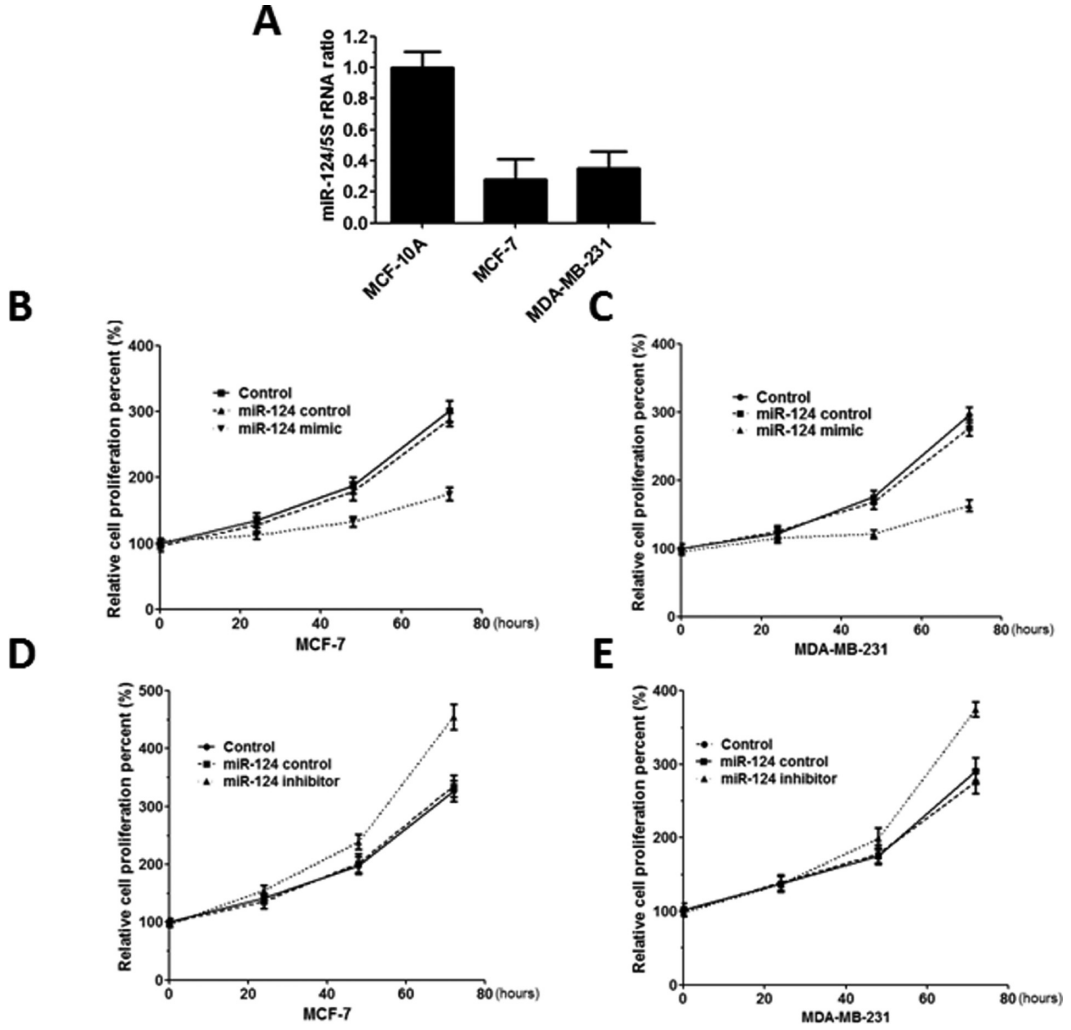


**Figure 4.** miR-124 is decreased in MDA-MB-231 and MCF-7 cells (A); miR-124 mimic-loaded Chol-P micelles potently inhibited MCF-7 cell proliferation (B); miR-124 mimic-loaded Chol-P micelles potently inhibited MDA-MB-231 cell proliferation (C); miR-124 inhibitor-loaded Chol-P micelles promoted MCF-7 cell proliferation (D); miR-124 inhibitor-loaded Chol-P micelles promoted MDA-MB-231 cell proliferation (E).

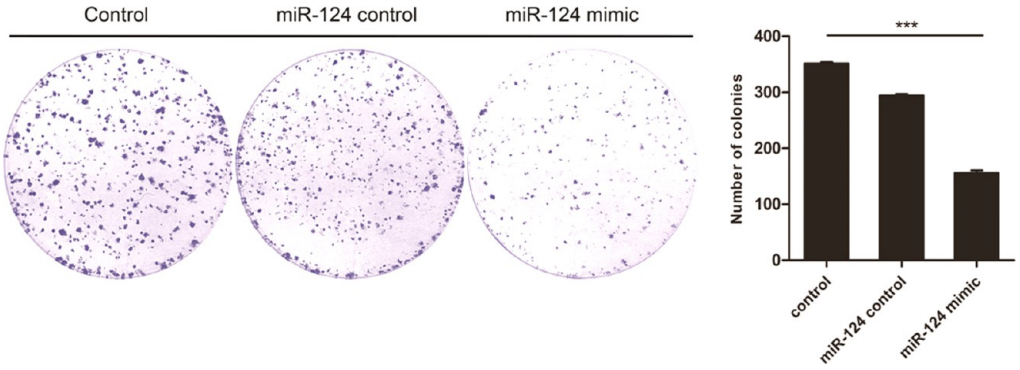


**Figure 5.** miR-124 mimic-loaded Chol-P micelles potently inhibited MCF-7 colony formation.

using a wound-healing assay. MCF-7 cells treated with the miR-124 mimic migrated significantly slower than cells from the miR-124 control and the control groups (Figure 6). In addition, the synergistic cytotoxicity of miR-124 and obatoclaxco-delivered by Chol-P micelles in MCF-7 cells was determined with cell proliferation assays. As shown in Figure 7, panel A, the miR-124 mimic and Chol-P alone did not affect the proliferation of MCF-7 cells. The miR-124-M slightly suppressed MCF-7 cell proliferation, and the free obatoclax and M-Oba moderately inhibited MCF-7 cell proliferation. The inhibitory effects of M-Oba were appreciably stronger than those of the free Oba, but these differences were not statistically significant. The miR-124-M-Oba demonstrated the most potent inhibitory activity, in a time-dependent manner, which suggests that the miR-124 and obatoclax may have a synergistic effect on MCF-7 cell proliferation.



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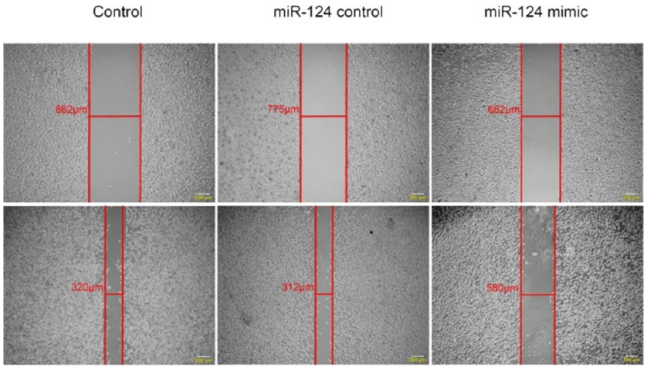


**Figure 6.** miR-124 mimic-loaded Chol-P micelles potently inhibited MCF-7 cell migration.

**3.3. Synergistic Cytotoxicity of Obatoclax and miR-124 on Breast Cancer Cells via Induction of Apoptosis.** Flow cytometry was used to assess the synergistic effect of different obatoclax or miR-124 mimic treatments on apoptotic cell death in MCF-7 cells. The early and late stages of apoptosis were differentiated by detecting phosphatidylserine on the outer leaflet of the apoptotic cells and DNA fragmentation using an Annexin V-FITC/PI staining kit. As shown in Figures 7, panel B, and S3, in the control group, only 1% of early apoptotic cells and 1.4% of late apoptotic cells were observed. After the miR-124 mimic administration, only a small number of apoptotic cells were identified. Moreover, the Chol-P micelles induced approximately 3.1% of early stage and 9.8% of late-stage apoptotic cells. In the free obatoclax group, we observed 46.5% of early stage apoptotic cells, and 26.8% of early stage and 18.8% of late-stage apoptotic cells were observed with M-Oba treatment. Furthermore, 11.9% of early stage and 24.8% of late-stage apoptotic cells were found after miR-124-M administration. Treatment with miR-124-M-Oba resulted in a significantly greater increase in apoptotic cells compared with miR-124-M and an increase in the percentage of late apoptotic cells compared with M-Oba as well as free obatoclax, suggesting that the synergistic cytotoxicity of miR-124 and obatoclax may be due to their capacity to induce apoptosis.

**3.4. Autophagy Influx Triggered by Chol-P Micelles May Enhance miR-124 Degradation.** After administration of Chol-P micelles or miR-124-M, autophagy in GFP-LC3-transfected MCF-7 cells was measured by GFP-LC3 mediated fluorescence aggregation (Figure 8A). Then we determined the protein levels of LC3 in cells treated with Chol-P micelles and

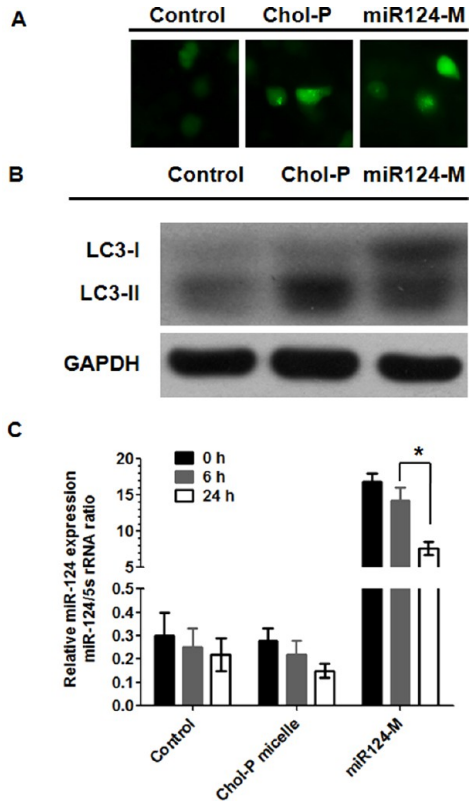


**Figure 8.** miR-124 mimic-loaded Chol-P micelles induce autophagy; fluorescent microscopic image (A); Western blot analysis of LC-3 (B); RT-PCR of relative miR-124 expression levels (C).

miR-124-M. The expression level of LC3 was increased after treatment with Chol-P micelles or miR-124-M, and these results confirmed that autophagy was induced by these micelles (Figure 8B). According to the literature, microRNAs are degraded in autophagolysosomes; thus, we hypothesized that Chol-P micelles may induce miR-124 degradation. Using real-time PCR, we measured the expression of miR-124. Degradation of miR-124 was observed after administration of Chol-P micelles or miR-124-M in a time-dependent manner. After 24 h, approximately half of the miR-124 molecules had

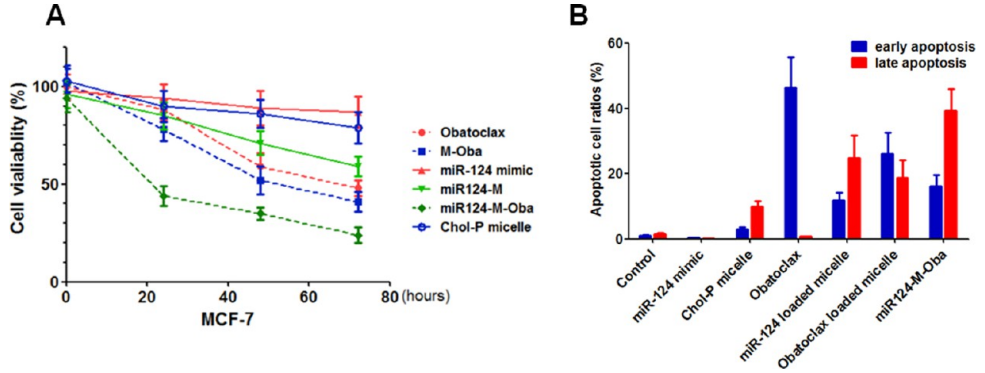


**Figure 7.** Cytotoxicity of the miR-124 mimic (red line), Chol-P micelles (blue line), miR-124-M (green line), free obatoclax (red dashed line), M-Oba (blue dashed line), and miR-124-M-Oba (green dashed line) in MCF-7 cells (A); apoptosis assays by FCM, the early apoptotic (blue) and late apoptotic (red) cell ratios in different groups (B).



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been degraded in the miR-124-M group compared to the 6 h treatment groups (Figure 8C).

**3.5. Obatoclax Loaded into miR-124-M-Oba Inhibited the Autophagolysosomal Degradation of the miR-124 Mimic.** To further study the complicated interactions between miR-124, obatoclax, and autophagy, the expression levels of LC3, p62, and miR-124 were examined following administration of M-Oba or miR-124-M-Oba with or without autophagy regulators. The results demonstrated that the addition of obatoclax could inhibit accumulation of both LC3 and p62 (Figure 9A). In the miR-124-M-Oba group, the activation of LC3 was slightly stronger than that of M-Oba, and the expression level of p62 was lower than that of the M-Oba group, which suggested that the induction of autophagy in the miR-124-M-Oba group was stronger than that of the M-Oba group. Using RT-PCR, as shown in Figures 8, panel C, and 9, panel A, we found that the addition of obatoclax strongly suppressed the degradation of miR-124 after treatment for 24 h. As shown in Figure 9, panel B, the addition of the autophagy agonist rapamycin clearly increased the LC3 levels and decreased the p62 levels. These results suggest that autophagosomes, as well as autophagolysosomes, were formed, and as a result, the degradation of miR-124 was also enhanced. On the other hand, the addition of the autophagy inhibitor chloroquine decreased the LC3 levels and the resulting p62 accumulation, but there were no apparent changes observed in the miR-124 level, which indicated that there were competitive effects, rather than synergistic effects, in the inhibition of autophagy by chloroquine and obatoclax (Figure 9C).

**3.6. Codelivered miR-124 and Obatoclax Synergistically Induced Mitochondrial Apoptosis.** To determine the potential molecular mechanisms underlying the induction of apoptosis by miR-124-M-Oba, the expression levels of apoptosis-related proteins were determined by Western blot analysis. The results show that the levels of Fas, FADD, and pro-caspase-8 were not changed, indicating that miR-124-M and miR-124-M-Oba may not induce apoptosis via the death receptor pathway (Figure 10). The expression level of pro-caspase-3 was low in all groups because the MCF-7 cells were deficient in caspase-3; therefore, apoptosis was not dependent on the caspase-3-mediated pathway. After the administration of miR-124-M, an increase in Bax and activation of caspase-9 was observed. Moreover, the administration of miR-124-M-Oba also increased the accumulation of Bax and the cleavage of caspase-9. In addition, miR-124-M-Oba eliminated the expression of Mcl-1. These results suggest that the apoptosis induced by miR-124-M-Oba was predominantly dependent on the mitochondrial pathway, and the codelivered miR-124 and obatoclax could synergisticly induce mitochondrial apoptosis.

**3.7. Codelivered miR-124 and Obatoclax Inhibited Breast Cancer Growth in Vivo.** We subsequently assessed the antitumor effects of the codelivery micelles in vivo. A MCF-7 human breast cancer model has been established and was used to evaluate the efficacy of miR-124-M-Oba. After 14 days of treatment with miR-124-M-Oba, changes in the tumors were noted, as shown in Figure 11. miR-124-M-Oba showed stronger antitumor activity than miR-124 and M-Oba. The miR-124-M-Oba showed the lowest rate of tumor inhibition, which was over 70%. Moreover, the miR-124 and obatoclax encapsulated in micelles caused a synergistic decrease in tumor volume and weight compared with the control group ($p < 0.01$), but there were no clear changes in daily body weight (Figure 11). Therefore, this study demonstrates that miR-124-

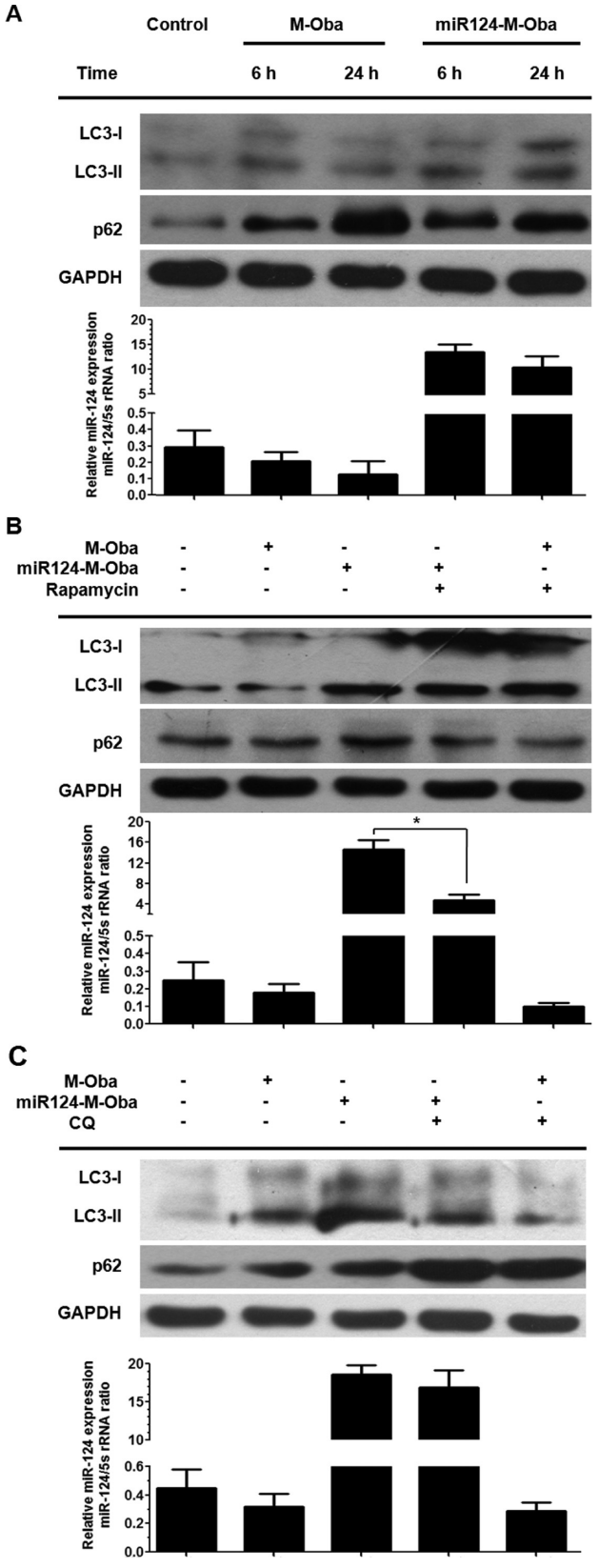


**Figure 9.** Both M-Oba and miR-124-M-Oba could induce the accumulation of LC3-II and p62 (A); the autophagy inducer rapamycin could increase the LC3 levels and enhance the degradation of miR-124 and p62 after treatment for 24 h (B); the autophagy inhibitor CQ could reduce the degradation of miR-124 and p62, and no synergistic effects were observed with obatoclax (C).



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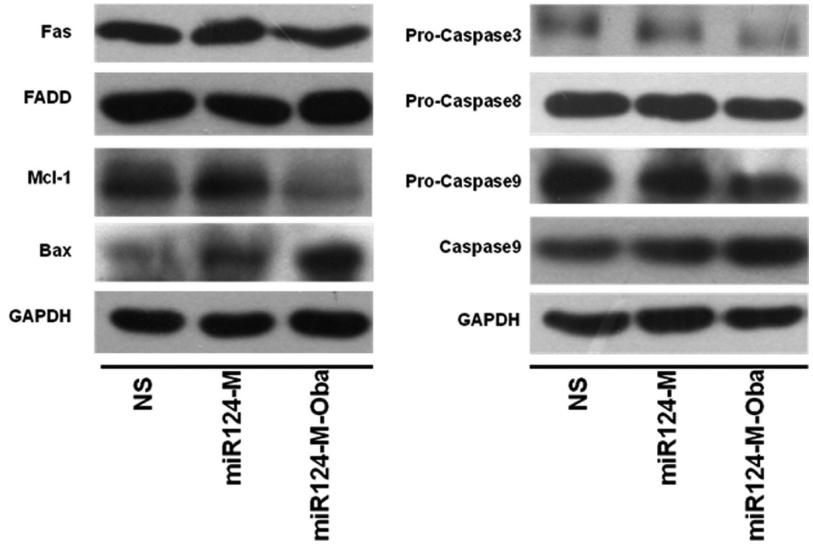


**Figure 10.** Western blot analysis of apoptosis-related proteins.

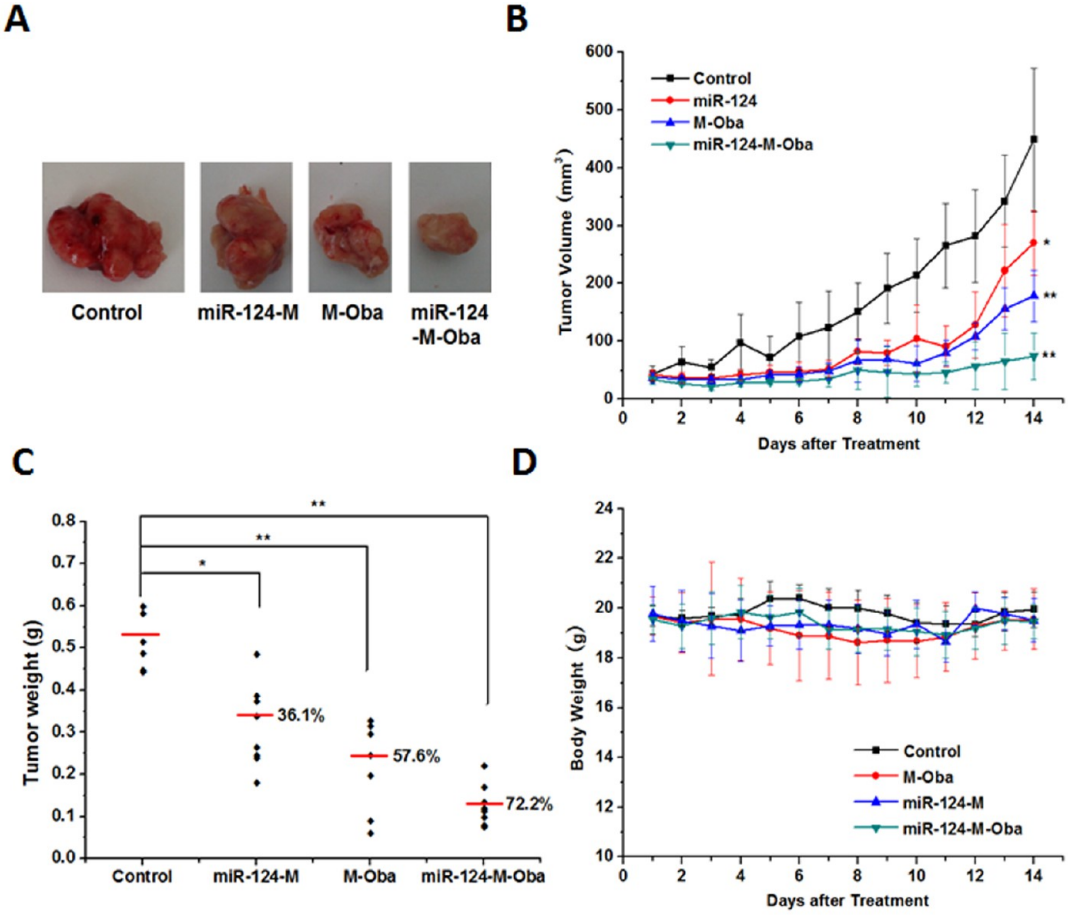


**Figure 11.** miR-124-M-Oba inhibited tumor growth in MCF-7 xenograft models. Representative photographs of subcutaneous tumors in each group (A); the change in daily tumor volume in each group (B); the mean tumor weight in each group (C); the change in daily body weight in each group (D); ∗, significantly different from the tumor volume of the control group, $P < 0.05$; ∗∗, significantly different from the tumor volume of the control group, $P < 0.01$.

M-Oba effectively inhibited the growth of breast cancer cell-derived tumors in a xenograft model in mice.

Furthermore, to confirm the potential antitumor mechanisms of miR-124-M-Oba, changes in apoptosis-related proteins were measured using IHC. $K_i$-67 is a proliferation indicator and is also used as prognostic and predictive marker in breast cancer. The results of the immunohistochemical experiments were analyzed using semiquantitative statistical methods and are presented in Figures 12−14. Apoptosis was assessed histological by TUNEL staining, and the TUNEL-positive apoptotic



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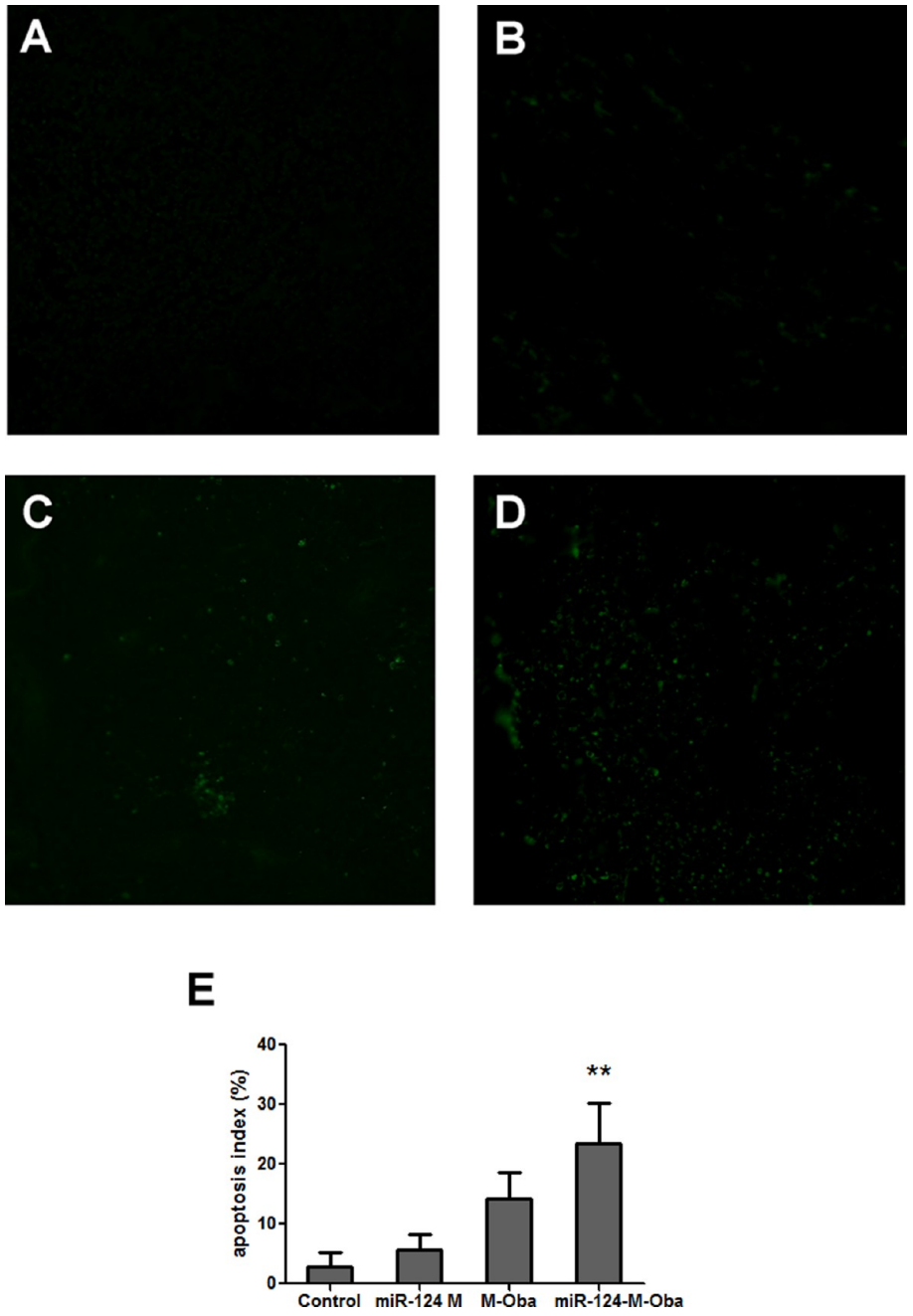


**Figure 12.** TUNEL immunofluorescence analysis of the tumors in each group. Representative images of the control (A), miR-124-M (B), M-Oba (C), and miR-124-M-Oba (D) groups, and the mean apoptotic index in each group (E); **, significantly different from the tumor volume of the control group, $P < 0.01$.

nuclei were significantly increased by treatment with miR-124-M-Oba compared to miR-124-M and M-Oba (Figure 12). A significant reduction in the expression of $K_i$-67 was observed following treatment with miR-124-M-Oba (Figure 13). In addition, higher cleaved caspase-9 levels were observed in the miR-124-M-Oba-treated group compared to the miR-124-M and M-Oba groups (Figure 14).

## 4. DISCUSSIONS

MicroRNAs are small noncoding RNA molecules that play diverse roles in various cancers.[8] miRNAs can target multiple mRNA transcripts and consequently regulate the expression of target genes.[10,12,15] The changes in miRNA expression modulate important biological processes involved in carcinogenesis, tumor growth, invasion, and metastasis.[9,11] The differential expression profiles of miRNAs can provide novel insights into the regulatory mechanisms of tumor growth and metastasis.[15] In recent years, more studies have indicated that miRNAs have important roles in the regulation of both biological processes and chemo-sensitivity in cancers.[13] Therefore, miRNAs are attractive diagnostic markers and therapeutic targets for cancers. Previous reports and our experimental results showed that up-regulation of miR-124 can significantly inhibit the proliferation of various cancer cells.[18,34,40,75,76] In breast cancer, miR-124 has been shown to be down-regulated. Additionally, miR-124 targets EZH2, flotillin-1, CDK4, and CD151, among others. Although the important roles of miR-


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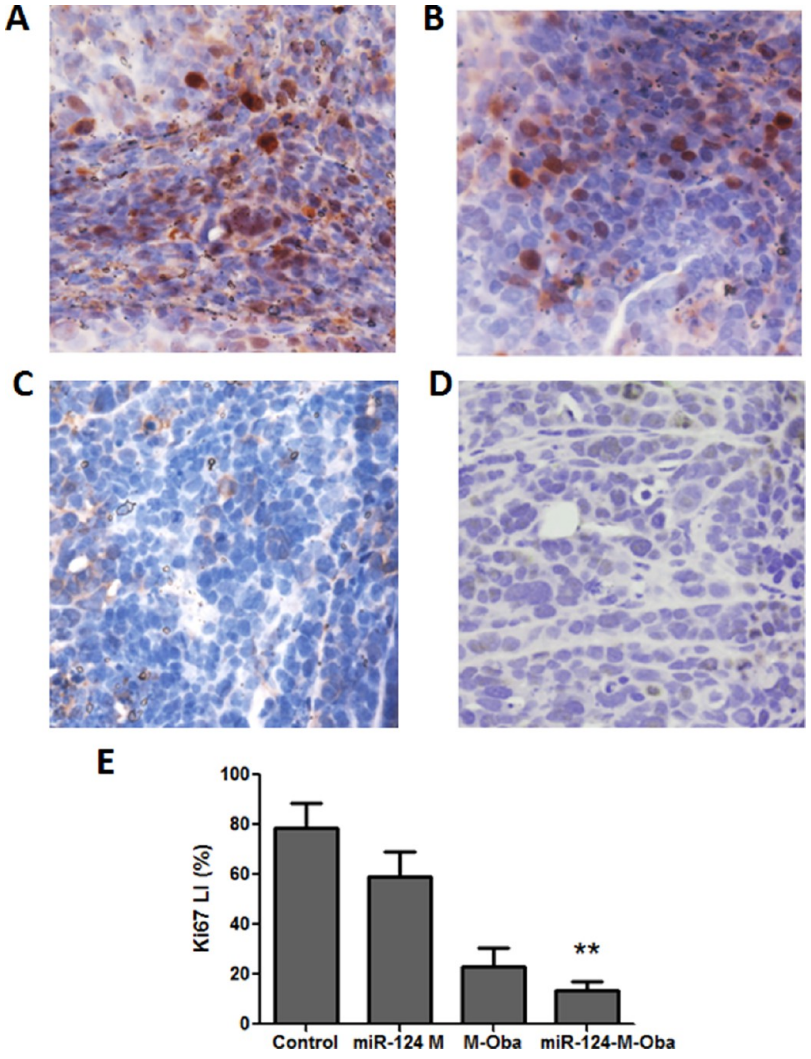


**Figure 13.** $K_i$-67 IHC analysis of the tumors for each group. Representative images of the control (A), miR-124-M (B), M-Oba (C), and miR-124-M-Oba (D) groups, and the mean apoptotic index in each group (E); **, significantly different from the tumor volume of the control group, $P < 0.01$.

124 in proliferation and migration in breast cancer have been elucidated, and miR-124 has been shown to be an independent unfavorable prognostic factor for breast cancer, the therapeutic efficiency of miR-124 in breast cancer has not been fully investigated.[26,28,39,41,43]

Currently, nonviral carriers have been used for miRNA delivery and have been shown to be an efficient and safe method to target a broad range of cancer cells. There are growing reports demonstrating that microRNAs and drugs can be codelivered in nanocarriers that simultaneously encapsulate and deliver multiple therapeutic agents against cancer and other syndromes.[77−82] In our previous study, we explored the viability of delivering plasmids together with docetaxel with a cationic polymeric nanoparticle for cancer therapy and carried out targeted delivery of claudin-3 shRNA by a folic acid-modified cationic liposome.[83−86] Although these studies have shown the feasibility of delivering RNA molecules and drugs by cationic nanocarriers, the efficacy of microRNA/drug codelivery systems is still unknown. In the current study, we selected miR-124 and obatoclax as a novel combination for the treatment of breast cancer. The rationale was based on the fact that the cationic nanocarriers may trigger autophagy or necrosis of target cells and subsequently lead to cell lysis. It was reported that obatoclax could inhibit autophagosomal lysis in breast cancer cells, and we then reasoned that the codelivery of miR-124 together with obatoclax using a cholesterol-penetratin micelle may potentially produce synergetic effects to induce apoptotic cell death and improve therapeutic outcomes in breast cancer cells.

In this study, the Chol-P conjugates were successfully synthesized and characterized using the standard solid-phase peptide synthesis method reported previously. The novel miR-124-M-Oba was also prepared by a self-assembly method. We took the advantage of the cationic and cell-penetrating features of the penetratin peptide, which adsorbed miR-124 and encapsulated miR-124 into micelles, thereby codelivering miR-124 and obatoclax. The obatoclax monodelivery micelles showed similar cytotoxicity and enhanced apoptosis compared to free obatoclax. Moreover, the miR-124-M could also induce apoptotic cell death in MCF-7 cells. However, despite the improved antitumor efficacy of the monodelivering M-Oba or miR-124-M, they show insufficient therapeutic efficiency. Thus, our efforts focused on combining obatoclax with a miR-124 mimic in the Chol-P micelles. We hypothesized that obatoclax would inhibit autophagosomal degradation of miR-124, which is induced by cationic nanocarriers, and would synergistically


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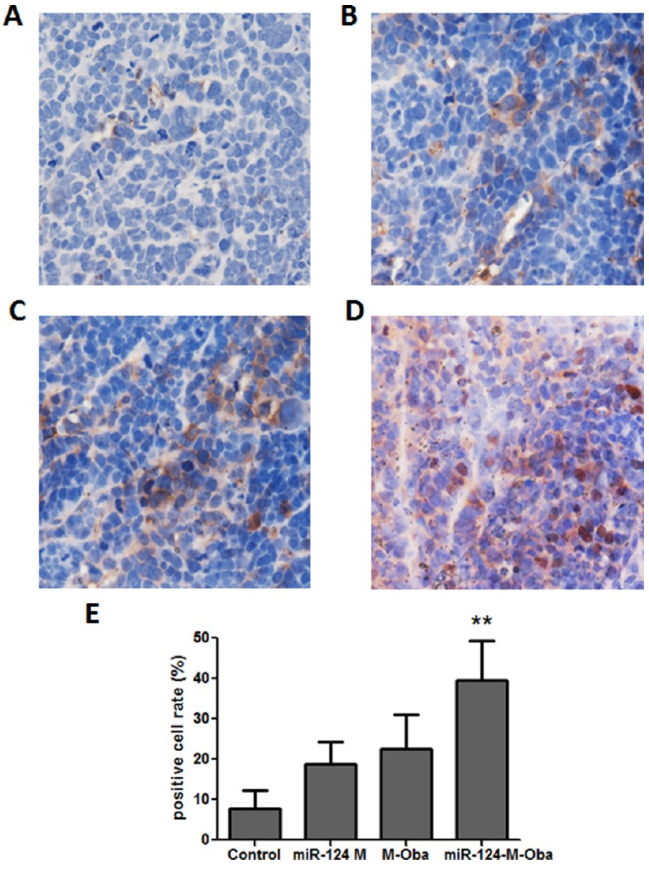


**Figure 14.** IHC analysis of cleaved caspase-9 in tumors for each group. Representative images of the control (A), miR-124-M (B), M-Oba (C), and miR-124-M-Oba (D) groups, and the mean apoptotic index in each group (E); **, significantly different from the tumor volume of the control group, $P < 0.01$.

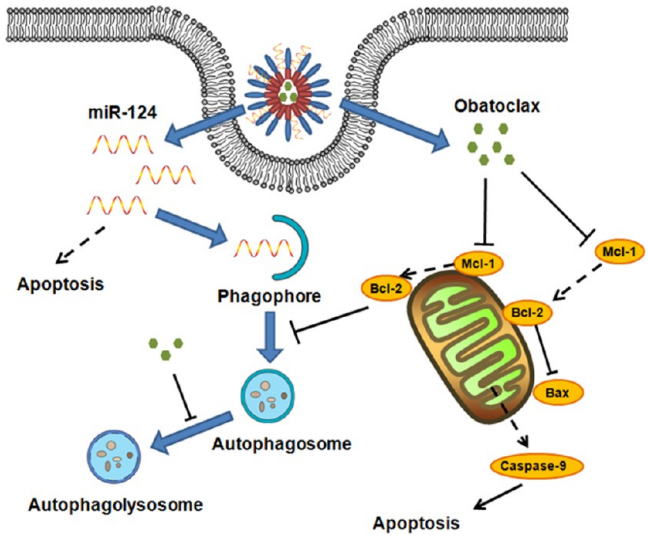


**Figure 15.** Potential mechanism of miR-124-M-Oba is dependent on the regulation of mitochondrial apoptosis and autophagy.

increase apoptosis in MCF-7 cells. The role of miR-124 in breast cancer has been previously reported; many studies have shown that miR-124 was significantly down-regulated in both breast cancer patients and cell lines. Overexpression of miR-124 in breast cancer cells suppressed cell proliferation, colony formation, and cell migration/invasion simultaneously. It is well-known that the benefits of monotherapy of a gene or drug alone are limited in breast cancer. Therefore, we developed Chol-P micelles as a cationic carrier for codelivery of miR-124 and obatoclax. The results demonstrate that miR-124-M-Oba efficiently delivered miR-124 and obatoclax to MCF-7 cells and ensured efficient cytoplasmic delivery of miR-124 via inhibition of the autophagosomal degradation of microRNAs (Figure 15). Our results also suggest that miR-124 and obatoclax delivered by miR-124-M-Oba produced a synergetic effect on cell proliferation via induction of apoptosis both in vitro and in vivo. Both miR-124-M and miR-124-M-Oba activated of Bax and caspase-9, which implied that the mitochondrial pathway was involved in the apoptotic cell death. Moreover, the expression level of miR-124-M-Oba was apparently decreased, which suggested that the Mcl-1/Bcl-2/Bax/caspase-9 axis may play a key role in the apoptosis induced by the codelivery micelles. In addition, obatoclax encapsulated in miR-124-M-Oba abolished the formation of autophagolysosomes and thus reduced the degradation of miR-124 after micelle administration. Our findings suggest that the obatoclax mediated autophagic flux inhibition may help to sensitize miR-124 induced apoptosis in breast cancer cells. On another hand, apoptosis via autophagy inhibition is not individually responsible for Obatoclax-induced apoptosis. In brief, it could be regarded that the inducing of mitochondrial apoptosis and inhibition of autophagic flux is a two-faced mechanism of obatoclax action and cooperated with miR-124 mimic. To the best of our knowledge, this is the first report on the therapeutic value of miR-124 in breast cancer cells when it is codelivered with obatoclax in penetratin-based self-assembly micelles. We show that codelivery nanocarriers with a drug and microRNA could synergisticly modulate programmed cell death of breast cancer cells, which represent an attractive therapeutic method for cancer combination therapy.

## 5. CONCLUSIONS

In conclusion, we synthesized a novel cholesterol conjugate of a classical cell-penetrating peptide, penetratin. The novel Chol-P conjugate could self-assemble into homogeneous micelles to codeliver a miR-124 mimic and obatoclax. The miR-124-M-Oba was monodisperse in nanoscale size and zeta potential distribution. The obatoclax was efficiently encapsulated in the micelles and showed sustained release within 144 h. Primary colony formation, cell proliferation, and migration assay results suggested that the miR-124-M could potently suppress the proliferation and motility of the MCF-7 and MD-MBA231 breast cancer cells. Moreover, the codelivery miR-124-M-Oba micelles induced more apoptotic cells than the corresponding single drug/microRNA-loaded micelles. Furthermore, we also found that both the Chol-P micelles and miR-124-M could initiate autophagy accompanied by the degradation of miR-124. However, the codelivered obatoclax could inhibit the autophagic flux by preventing the transformation of autophagosomes to autophagolysosomes, maintaining the intracellular level of miR-124. Studies of the molecular mechanisms of miR-124-M-Oba revealed that mitochondrial apoptosis was predominantly involved in the synergistic effects of these codelivery systems. In short, the miR-124-M-Oba showed improved apoptosis-inducing and autophagic flux inhibitory effects in vitro, and the miR-124-M-Oba may have potential applications in breast cancer therapy.


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## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00211.

HPLC and mass spectra of Chol-P; AFM image of Chol-P micelles; FCM results (PDF)

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### Notes

The authors declare no competing financial interest.

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