Combined Therapy for Paclitaxel and Curcumin by Nonionic Surfactant Vesicles Leading to Enhanced Efficacy of Chemotherapy in Ovarian Cancer Cells Through Inhibiting the Serine/threonine Kinase Akt and Nuclear Factor KappaB Activity

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Abstract

The combination therapy of cytotoxic drugs and chemosensitizing agents encapsulated in nanoparticles has been highlighted as an effective treatment for various cancers. Combination therapy is promising to produce synergistic anticancer effects, to magnify the treatment effect and overcome multidrug resistance. In this investigation, we have studied augmentation of therapeutic efficacy upon a combinational treatment of paclitaxel (PCL) and curcumin (Cur), an inhibitor of nuclear factor kappa B (NF- κ B), in OVCAR-3 cell. PCL and Cur were encapsulated in nanoniosome formulations. Then, the effects of nanoniosome formulations on cytotoxicity, expression profile of *AKT-1* gene and NF- κ B activity were evaluated. The findings showed that nanoniosomes were highly efficient in delivering the PCL and Cur drugs to OVCAR-3 cell. A 3-fold and 3.6-fold reduction in Cur and PCL concentration were measured, respectively, when the Cur and PCL were administered in nanoniosomes compared to free Cur and free PCL solutions in OVCAR-3 cell. Moreover, curcumin could significantly increase cell growth inhibition of paclitaxel so that, in presence of NioCur, the IC_{50} of NioPCL was diminished to ~ 2.4 -fold. *AKT-1* gene expression studies showed that co-administration of curcumin/paclitaxel nanoniosome formulations caused 91.2% reduction in *AKT-1* gene expression compared to control group. On the other hand, this co-administration caused 79.42% reduction in the amount of NF- κ B activity and a 4-fold reduction in the activity of the MDR protein pumps in cancer cells compared to the control group. Our findings demonstrate that the combination therapy of PCL with Cur using the nanoniosomes delivery is a promising strategy for breast cancer more effective therapy

1. Introduction

There are some limitations for systematically administering single-drug chemotherapy, such as fast blood/renal clearance, multiple drug resistance, or poor bioavailability. Additionally, the drug is frequently at low levels at the tumor sites; thus, an effective drug dose cannot be reached. Consequently, higher doses of drug are required within administration, resulting in severe negative side effects (1–3). Combined chemotherapy for cancer treatment has been recently proposed that is a promising approach for generating synergistic antitumor impacts, reducing individual drug-associated toxicity, suppressing multiple drug resistance (MDR) through different action mechanisms, and reducing the required dosage of each drug. In combined chemotherapy, two or more active drugs are simultaneously administered for inducing cell cycle arrest in a different stage of the cell cycle, for modulating various signaling pathways in cancerous cells, for maximizing the therapeutic impact, and overcoming MDR. Combined treatment utilizes a pharmacologically safe and natural anticancer drug along with a very robust toxic commercial anticancer agent, which make it an attractive strategy for obviating these constraints. Toxicity in natural anticancer drug is significantly lower, it is easily available and safe (4–7). An example for these agents is Curcumin (Cur) (8). Cur (Diferuloylmethane) is a natural diphenol compound, purifying from the plant rhizome, *Curcuma longa* (which is a member of the ginger family) (9). Cur is popular for its anti-inflammatory, anti-amyloid, antibacterial and antioxidant properties, and anti-spasmodic activity. Moreover, it has also been reported as an anticancer agent with the ability of suppressing and treating

different types of malignancies. Cur also can indicate p-gp inhibitory activity independently by down-regulating the NF- κ B and PI3K/Akt pathways. Additionally, Paclitaxel (PCL) is a very powerful and anticancer, which is obtained from the bark of the Pacific yew tree, *Taxus brevifolia*. It is used for treating different types of cancers, like breast, lung, esophageal, and ovarian cancers. The PCL has an anticancer mechanism that blocks mitosis, resulting in arresting G2/M cell cycle and apoptosis cell death (7, 10). It has commonly been studied combined with other chemotherapeutic drugs for enhancing and increasing its therapeutic efficacy and reducing its toxicity. It is an attractive anticancer strategy to combine PCL and Cur. PCL is known as a potent microtubule-stabilizing drug, which causes cell cycle arrest, while Cur presents a biological attack through regulation of various signal transduction pathways (8). Although there are good therapeutic impacts, Cur and PCL have poor solubility in water. Hence, because of demands for effective PCL and Cur delivery approaches, attractive systems have been developed for PCL and Cur formulations, like polymeric micelles, polymeric NPs, liposomes, nanogels, niosomes, cyclodextrin, and biodegradable microspheres (11–14). Niosome is a non-ionic vesicle that is based on surfactant and developed by non-ionic surfactant and cholesterol incorporation as an excipient. Also, other excipients can be employed. Liposome-related drawbacks can be reduced by niosomes, which include chemical instability because of their tendency to oxidative degradation and varying purities of phospholipids. As drug carriers, niosomes present considerable advantages making them superior over other common vesicular delivery approaches. The main factors inspiring development of niosomal systems include chemical stability, biocompatibility, biodegradability, easy handling and storage, low toxicity, and low production cost (15–18). In the present work, the combined delivery of Cur and PCL, compressed in PEGylated niosomal formulations, is examined for augmenting their efficacy in OVCAR-3 human ovarian cancer cell. NF- κ B pathway inhibition and *Akt-1* gene down-expression were also investigated after single treatment and combined treatment in aqueous solution and nanoniosomal formulations.

2. Materials And Methods

2.1 Preparation of Cur/ PCL - loaded niosomes

The Cur/ PCL -loaded niosomes were developed using thin film hydration approach (19). We dissolved cholesterol (Sigma-Aldrich, USA), polyethylene glycol (Lipoid GmbH, Germany), and Tween 60 (Sigma-Aldrich, USA) (64.4: 27.6: 5 molar ratios) in chloroform. It was followed by dissolving Cur (Sigma-Aldrich, USA) and PCL (Stragen, Switzerland) in methanol, and it was mixed with the mix of lipids and surfactant. Fluorescent label DIL (Sigma, USA) was mixed with lipid phase at 0.1% mol for evaluating the cellular uptake. A dose of 0.5 mg.mL^{-1} was used for both drugs. Phosphate- Buffered Saline (PBS, pH = 7.4) was used for hydrating dry lipid film by rotary device (Heidolph, Germany). Then, we sonicated the vesicles were for decreasing the vesicles mean size. Afterward, free PCL and Cur (unloaded) were eliminated from niosomal PCL and Cur (NioPCL and NioCur) by a dialysis bag diffusion approach against PBS. It was accomplished at 4°C for 1 h (MW = 8–14 kDa). The dynamic light scattering (DLS) method was used for evaluating zeta potential and polydispersity index of Cur/ PCL -loaded niosomes and the particle size

distribution by Brookhaven Corp Instruments (Holtsville, NY). Spectroscopic measurements were performed at 236 nm and 429 nm (λ_{max} of drugs) for evaluating the entrapment efficiency of PCL and Cur in niosomal formulations. The dialysis of 1 mL of the niosome preparations was performed for assessing Niosomal Cur/PCL release. For this purpose, an 8-14-kDa cutoff dialysis tube was used in PBS pH 7.4 at 37°C under constant stirring. Spectrophotometry was used for detecting the release amount of Cur/PCL into vesicle at pre-specified time intervals by considering 1 mL of the dialysis medium and its replacement with the same volume of fresh PBS (20–23).

2.2 Cytotoxicity assays

The MTT (Sigma, USA) assay was used for calculating the IC_{50} values of Cur and PCL as single drugs or as combined treatment. In short, we seeded OVCAR-3 cell in 96-well plates at 1×10^4 cells per well. After 24 h, treatment of the cells with 200 μL fresh medium was done that contained serial dilutions of the various niosomes/ drugs formulations. The product was incubated for 48 h, and then, 20 μL MTT was mixed with well plates, followed by incubation at 37 °C for 3 h. Lastly, we added 180 μL of DMSO to the wells for dissolving the formed formazan crystals. EPOCH Microplate Spectrophotometer (BioTek, USA) was used for recording absorbance of the wells at 570 nm. GraphPad Prism 6 was used for calculating the IC_{50} values of Cur and PCL as single drugs or as combined treatment. The Combination Index (CI) value was calculated for appraising combined Cur and PCL. This index was obtained by Talalay and Chou Eq. and the CompuSyn software (20–22, 24–27).

2.3 Nanocarrier Uptake Surveys

Seeded OVCAR-3 cell in 6-well plates at a cell density of 1×10^4 cells/well were used for investigating the in-vitro cellular uptake of the Cur/ PCL-loaded niosomes. Cell growth was performed overnight so that 80% confluence is reached. Afterward, treatment with DIL-labeled PCL Niosome (DIL-NioPCL) and DIL-labeled Cur Niosome (DIL-NioCur) was done, and then incubation was done at 37°C for three hours. OVCAR-3 cell was then rinsed with PBS three times, and they were fixed with 4%, paraformaldehyde solution (Sigma, USA). Lastly, the cell incubation was performed with DAPI solution for 15 min, and a fluorescence microscope was employed for imaging the cells (20, 24, 28).

2.5 Evaluation of Annexin V-Fluorescein Isothiocyanate (FITC)/propidium iodide (PI)

Propidium iodide (PI) and Annexin V-FITC staining were used for determining apoptosis. We then seeded OVCAR-3 cells at a density of 100000 cell/well for 24 hours prior to experiment in 6-well plates. Afterward, cell treatment with PCL and Cur was performed, either as combined PCL + Cur or as single drugs. The drugs were administered in nano-niosome formulations or in aqueous solution. Following incubating for 24 h, cold PBS was used for trypsinizing and washing the cells. The PI and Annexin V-FITC were used for staining cells for 30 min. BD FACSCalibur instrument was employed for analyzing cells. Plots were classified into quadrants for assessing the percentages of apoptotic cells and living cells. FlowJo

software (FlowJo LLC, Ashland, OR, USA) was employed for data analysis. The control group included cells not receiving any drug treatment (29–31).

2.6 Real-time PCR analysis

real-time PCR analysis was performed for investigating the synergistic impacts of Cur and PCL on expression of Akt-1 gene. We seeded OVCAR-3 cell in 6-well plates at 1×10^6 cells in each well. After 24 h, the cell treatment with fresh medium was conducted. The medium contained the various niosomes/drugs formulations: free-Cur solution, free-PCL solution, free-PCL + free Cur physical mix, niosomal PCL, niosomal Cur, and co-administered niosomal PCL + niosomal Cur. The control group included cells not receiving any drug treatment. Following a 48-hour incubation, Super RNA extraction Kit (yekta tajhiz, Iran) was used for extraction of total RNA. RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used for performing the RNA reverse transcription in accordance with the instructions of the manufacturer. We used cDNA as a template for subsequent qPCR amplification by primers specific for Glycer Aldehyde 3-Phosphate Dehydrogenase (GAPDH) and *Akt-1* genes. These primers were used for qPCR: forward 5'- CTTCTATGGCGCTGAGATTGTG -3', reverse 5'- CAGCATGAGGTTCTCCAGCTT -3'; *Akt-1*, *GAPDH*, forward 5'- GCAAGAGCACAAGAGGAAGA -3' and reverse 5'- ACTGTGAGGAGGGGAGATTC -3'. The qPCR reaction contained a primary activation phase for 5 minutes at 94°C, followed by 35 cycles for 30 s at 94°C, annealing for 30 s at 60°C, extension for 30 s at 72°C, and a final extension for 7 min at 72°C. Rotor-Gene Q Real-Time PCR System (qiagen, USA) and 5x HOT FIREPol®EvaGreen® qPCR Mix Plus the real-time PCR kit (Solis BioDyne, Estonia) were used for performing qPCR. Rotor-Gene Q Real-Time PCR System Software provided raw data, which was exported in rdml format and imported to LinRegPCR for calculating the PCR efficiency. In each experiment, Ct values were obtained that were employed an indicator for gaining fold change in the target genes' expression normalized to GAPDH. $2^{-\Delta\Delta Ct}$ technique was used for calculating the relative expression levels regarding fold change by Gene software (MultiD Analyses AB, Sweden) (32–35).

2.7 Determining p65-NF-κB by ELISA assay

The DNA binding activity of NF-κB was examined by ELISA for investigating the synergistic impacts of Cur and PCL on p65-NF-κB activity. Abcam (Cambridge, United Kingdom) prepared the NF-κB p65 transcription factor assay kit (catalog no. ab133112). We seeded OVCAR-3 cells in 75-cm² tissue culture flask. After 24 h, the cell treatment with fresh medium was performed. The medium contained various niosomes/drugs formulations: free-Cur solution, free-PCL solution, free-PCL + free Cur physical mix, niosomal PCL, niosomal Cur, and co-administered administered niosomal PCL + niosomal Cur. Control group contained cells not receiving any drug treatment. Following a 48-h incubation, a nuclear extraction kit (ab113474, Abcam) was used for obtaining nuclear extracts based on the manufacturer's protocol. A sensitive ELISA assay was used for determining levels of nuclear p65 concentrations. Shortly, a certain double stranded DNA (dsDNA) sequence with the p65-NF-κB response element was immobilized onto the well bottoms on a 96-well plate. p65-NF-κB included in a nuclear extract attached to the p65-NF-κB response elements. Specific primary antibody directed against p65-NF-κB was added to detect p65-NF-

κB. It was followed by addition of a secondary antibody conjugated to HRP, and spectrophotometer was used for reading the absorbance at 450 nm (36–38).

2.8 Statistical analysis

GraphPad Prism 6 software was utilized to analyze statistical data, and data were presented as mean $\pm$ SD. For comparison of two independent groups, the student t-test was employed. Multiple samples were compared by the ANOVA test. P-Values smaller than 0.05 were regarded significant.

3. Results

3.1 Characterization of Cur / PCL loaded nanoniosomes

The mean size, PDI, zeta potential, and encapsulation efficiency of the formulation of Curcumin-loaded nano-niosome were 88.7 ± 1.7 nm, 0.148 ± 0.011 , -19.08 ± 2.46 and $97.22\% \pm 2.4$. The mean size, PDI, zeta potential and encapsulation efficiency of the formulation of paclitaxel-loaded nano-niosome were 97.8 ± 1.3 nm, 0.153 ± 0.012 , -17.92 ± 0.15 and $96.41\% \pm 1.7$.

In vitro assessment of the drug release was conducted by dialysis technique. The profile of PCL and Cur release from niosomal formulations at 37°C is indicated in Fig. 1. After 72 h, 33.39 was released for Cur and 30.28 % was released for PCL of the loaded drugs (Fig. 1). The PCL and Cur showed a biphasic release profile with a fast primary release. A slower release phase was then appeared.

Figure 1 The in vitro release profile of curcumin and paclitaxel from niosome formula.

3.2 Cytotoxicity assays

MTT assay was used for specifying the inhibitory impact of individual Cur and PCL as drug niosomal form on OVCAR-3 cell and a free form. Figure 2 indicates that independent therapies with the nanoniosomal form and the free form led to inhibited growth of OVCAR-3 cell in a dose-dependent manner. In Table 1, the IC_{50} values of the mentioned agents are evaluated. Figure 2b demonstrates higher efficiency of nanoniosomes in transporting PCL and Cur drugs to OVCAR-3 cell. With administrating PCL and Cur in nanoniosomes, PCL and Cur concentration was reduced by 3.8-fold and 2.8 fold reduction in Cur and PCL concentration in comparison with free PCL and free Cur and solutions in OVCAR-3 (Table 1). Then, we used the IC_{50} concentrations for generating fixed ratios for the next combination experiments and calculating combination index (CI). A combination study was carried out for determining the synergistic antitumor impacts of PCL and Cur, results of which are given in Table 2. The dose–response curves for OVCAR-3 cell exposed to combined treatment with PCL and Cur are shown in Fig. 3a, b. Combined treatment with PCL and Cur decreased cell viability more in OVCAR-3 than either PCL or Cur alone. When delivering PCL and Cur in nanoniosome formulations, such combined treatment has a significantly efficacy (p-value < 0.05) in comparison with a free solution (Table 2). Therefore, the concurrent usage of PCL and Cur led to enhancement of therapeutic potential. Also, the CI analysis of the Cur and PCL interaction in OVCAR-3 cell is illustrated in Fig. 3c, d. The PCL and Cur combination in

niosomal forms and free forms showed values of CI < 1, which demonstrates synergistic interaction of the two drugs for cell growth inhibition (Table 2, Fig. 3c,d).

Table 1
The IC₅₀ values of Cur and PCL alone and in combined form on OVCAR-3 cell, administered in the free drug forms and drug niosomal form. Data is expressed as the mean ± SD

Treatment type	IC ₅₀ values
Free Curcumin Solution	50.12 ± 1.76
Free Paclitaxel Solution	18.56 ± 0.65
Free Paclitaxel + Free Curcumin Solution	13.25 ± 1.22
Curcumin Niosome	17.94 ± 2.81
Paclitaxel Niosome	5.16 ± 1.36
Paclitaxel Niosome + Curcumin Niosome	2.312 ± 0.27

Figure 2 Inhibition of cell growth by curcumin and paclitaxel individual as a drug free form and drug nanoniosomal form in OVCAR-3 cell. a Free Cur vs Free PCL; b; NioCur vs NioPCL

Table 2
Curcumin and Paclitaxel Combination Index (CI) against OVCAR-3

Combination type	CI	interaction type
Free paclitaxel + free curcumin solution	0.27	synergistic
Curcumin niosome + Paclitaxel niosome	0.35	synergistic
CI < 1, synergistic; CI = 1, additive; CI > 1, antagonistic.		

Figure 3 Analysis of synergy between curcumin and paclitaxel for OVCAR-3. a, Dose–response curve of free Cur + Free PCL ; b dose–response curve of NioCur + NioPCL. CI values at different levels of growth inhibition effect (fraction affected, FA; c Free Cur + Free PCL ; d NioCur + NioPCL in OVCAR-3 cell)

3.3 Nanocarrier Uptake Studies

Using the fluorescence microscopy, we examined the cellular uptake of OVCAR-3 cell, incubated with DIL-NioPCL and DIL-NioCur. Fig. 4a, b illustrates that the efficient entry of nanoniosome formulations into the cancerous cells, which is principally into the nucleus. It seems that the endocytosis has a vital role in cell perpetration of the drug-loaded niosomes.

Figure.4 Cellular uptake of drugs-loaded niosome on OVCAR-3 line. [A1: Niosomal Cur: Nucleus, A2: Niosomal Cur, A3: Niosomal Cur. Merged; B1: Niosomal PCL: Nucleus, B2: Niosomal PCL, B3: Niosomal PCL: Merged.

3.4 Combination of Cur and PCL caused an increase in apoptosis than individual treatments, particularly when administered in drug niosomal form

Investigation of apoptosis was done in OVCAR-3 cell incubated with PCL and Cur, as combined PCL + Cur or as single agents, administered in nano-niosome formulations or aqueous solution (Fig. 5). The early apoptosis rate was 8.96 and 6.78 % in cells treated with the free PCL and free Cur. Nevertheless, this rate showed an increase to 28.8 and 13.8% after cell treatment with NioPCL and NioCur. Moreover, there was an increase in apoptosis by combined PCL and Cur in comparison with individual treatments, particularly when used in drug niosomal form (that is, 17.7 % early apoptosis in free PCL + free Cur solution vs. 49.4 % early apoptosis in PCL niosome + Cur niosome, $p < 0.05$). There is consistency between these results and the results of cytotoxicity assay (MTT approach) of Cur combined with PCL.

Figure 5 Cell apoptosis analyzed with Annexin V-FITC kit following the treatment of cells with Cur and PCL, either as single agents or as a Cur + PCL combination, administered in aqueous solution or in nano-niosome formulations. A: Control; B: Free Curcumin; C: Free Paclitaxel; D: Free Curcumin + Free Paclitaxel; E: Niosomal Curcumin; F: Niosomal Paclitaxel; G: Niosomal Curcumin + Niosomal Paclitaxel.

3.5 Combined treatment of Cur and PCL caused a reduction in expression of Akt-1 gene more than individual treatments with either PCL or Cur, particularly when administered in drug niosomal form

With performing the real-time PCR analysis, the synergistic impact of PCL and Cur on expression of *Akt-1* gene was investigated. According to results of qPCR (Fig. 6), with treating OVCAR-3 cells with free PCL solution and free Cur solution, it was observed that there is downregulation of expression of *Akt-1* gene in comparison with untreated cells (A 51.89 and 48.07% reduction in comparison with control group). Besides, with treatment of cells with NioPCL and NioCur, an enhancement was noticed in the Akt-1 gene expression downregulation by 85.15 and 81.26%. Additionally, combined treatment with PCL and Cur in both niosomal forms and free forms showed higher efficacy in reduction of expression of *Akt-1* gene compared to individually administered drugs**** $p < 0.0001$).

Besides, it was observed that the combined treatment caused a significant efficacy (**p-value = 0.0003) in cases that PCL and Cur were administered in nanoniosome formulations than free drug solution (72.89% reduction in free PCL + free Cur solution compared to 91% reduction in PCL niosome + Cur niosome), which shows a consistency with cytotoxicity experiments.

Figure 6. qPCR analysis of Akt-1 gene expression in OVCAR-3 cells following treatment with paclitaxel and curcumin either as a single agent or in combination, when administered in aqueous solution and nanoniosome formulations. *P < 0.0001 vs control. a: free Cur vs Free Cur + Free PCL (P < 0.0001) b: Free Cur vs NioCur (P < 0.0001). c: Free PCL vs Free Cur + Free PCL (P < 0.0001). d: Free PCL vs NioPCL (P < 0.0001). e: Free Cur + Free PCL vs NioCur + NioPCL (P = 0.0003)

3.6 Combined therapy with Cur and PCL reduced activity of NF-κB more than single therapies with either PCL or Cur, particularly when used in drug niosomal form

ELISA assay was used for investigating the synergistic impact of Cur and PCL on activity of p65-NF- κ B with analysis of the DNA binding activity of NF- κ B. As indicated by the results (Fig. 7), NF- κ B activity in OVCAR-3 cell was decreased due to prevention of NF- κ B activation and its translocation to the nucleus by free PCL and free Cur compared with the control (a 20.20 and 36.86 % reduction compared with the control). Furthermore, with treating the cells with NioPCL and NioCur, the reduced activity of NF- κ B was improved to 52.55 and 58.82 percent. Moreover, combined treatment with PCL in both niosomal and free forms has shown higher efficacy in decreasing NF- κ B activities compared with any drug individually ($p < 0.05$). Additionally, the combined treatment approach showed a significant effectiveness ($p < 0.05$) in cases that PCL and Cur were transferred in nanoniosome formulations rather than as free drug solution (a 56.47% reduction in free PCL + free Cur solution in comparison to 79.61% reduction in PCL niosome + Cur niosome). These findings show consistency with cytotoxicity experiments and results of qPCR analysis for expression of Akt-1 gene.

Figure 7. p65-NF- κ B activity in OVCAR-3 cells following treatment with paclitaxel and curcumin either as a single agent or in combination, when administered in aqueous solution and nanoniosome formulations. * $P < 0.05$ vs control. a: free Cur vs Free Cur + Free PCL ($P < 0.05$). b: Free Cur vs NioCur ($P < 0.05$). c: Free PCL vs Free Cur + Free PCL ($P = 0.0002$). d: Free PCL vs Nio PCL ($P = 0.0005$). e: Free Cur + Free PCL vs NioCur + NioPCL ($P < 0.05$).

4. Discussion

Different types of cancer are commonly treated with Chemotherapy as a standard therapy. However, it is accompanied by low therapeutic efficacy and high systemic toxicity (39). Combined chemotherapy is a promising approach for generating synergistic antitumor impact. An attractive anticancer approach is combining PCL and Cur (8). Although Cur and PCL represent satisfactory therapeutic impacts, their water solubility is poor. Accordingly, demands for effective strategies for delivery of PCL and Cur have led to attractive systems for formulations of PCL and Cur, like polymeric micelles, polymeric nanoparticles, liposomes, nanogels, biodegradable microspheres, cyclodextrin, and niosomes (11–14). In the present work, Cur and PCL were loaded into nanoniosome formulations in order to enhance effectiveness in OVCAR-3 human ovarian cancer cell. Our results showed high efficiency of nanoniosomes in delivery of Cur and PCL drugs to OVCAR-3 cell (Table 1). Concentration of PCL and Cur was reduced by 3.8 and 2.8 fold, when administering the PCL and Cur in nanoniosomes in comparison with free PCL and free Cur and solutions in OVCAR-3 cell. A combination study was carried out for determining the synergistic antitumor impact of Cur and PCL. As a result of combined treatment with PCL and cur, cell viability reduction was more in OVCAR-3 compared to either Cur or PCL individually. In delivering the PCL and Cur in nanoniosome formulations compared to a free solution, such a combined treatment approach was effective (p value < 0.05) (Table 2). Therefore, when the PCL and Cur are used together, the therapeutic potential is enhanced. Our results showed the ability of Cur for increasing the cell growth inhibition of PCL so that when free Cur was present, the IC_{50} of free PCL was decreased to about 1.4 –fold. The IC_{50} of NioPCL was decreased to about 2.2 –fold with presence of NioCur. It was indicated that Cur, lonely or

combined with other compounds, has an anticancer function by regulation of multiple signaling pathways, such as p53 signaling pathway, the apoptotic-signaling cascade, the Wnt/ β -catenin signaling pathway, NF- κ B pathway, JAK/ STAT signaling pathway, the AKT pathway, and AMPK/COX-2 pathway (40–43). The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is significant for many cellular functions, including control of growth, cell survival, cell proliferation, and metabolism. There is a high expression of AKT in breast cancer carcinogenesis because of its capacity for regulating cell growth, cycle, apoptosis, and proliferation (41, 44). We evaluated relative expression of *AKT-1* gene in OVCAR-3 cells following treatment with free Cur, free PCL, free Cur + free PCL, NioCur, NioPCL and NioCur + NioPCL. As demonstrated by the results, expression of *AKT-1* gene was reduced in OVCAR-3 cancerous cells by both free Cur and free PCL. Furthermore, a 85.15 and 81.26% reduction was observed in expression of *AKT-1* gene when administration of free PCL and free Cur in niosomal formulations that was significantly lower than the free forms ($P < 0.05$). Also, it was indicated that co-administering Cur and PCL nanoniosome formulations reduced expression of *AKT-1* gene by 91%, which shows higher efficacy compared to combined treatment of free drugs. Cur has the capability of down-regulation of NF- κ B pathways. NF- κ B pathway has responsibility of tumor cell survival and metastasis. NF- κ B is a transcription factor that is available in the cytoplasm as an inactive heterotrimer composed of p65, p50, and I κ B α subunits. I κ B α subunit is phosphorylated and degraded by I κ B kinase (IKK) in response to specific stimuli. Thus, NF- κ B is translocated into the nucleus, and the transcription of some genes is initiated. These genes are responsible for survival and metastasis of tumor cell. IKK is inhibited by Cur, and hence, the I κ B α degradation is prevented. As long as I κ B α is not degraded, NF- κ B stays in the inactive state, resulting in improved apoptosis in tumor cells. Accordingly, it is possible to use Cur as an efficient sensitizer for increasing therapeutic reaction of cancerous cells to common chemotherapeutic medicines (9, 45). The present research findings showed significant reduction of NF- κ B activation by both Cur and PCL. Moreover, with administering free PCL and free Cur in nanoniosomal formulations, 52.55 and 58.82% reduction was observed in NF- κ B activation, which was significantly lower compared to the free forms ($P < 0.05$). Also, it seems that nanoniosomal formulations has a role in improving activity through drugs' effective internalization. In comparison with combined treatment with Cur and PCL in aqueous solution, combined treatment with Cur and PCL in nanoniosomes showed higher efficiency in reducing activation of NF- κ B (a 56.47 % reduction in free PCL + free Cur solution in comparison with 79.61% reduction in PCL niosome + Cur niosome). Cell apoptosis is described as an autonomous cell death process that can be caused by various chemical and physical factors and different drugs. It has been described that apoptosis induction is the best and an standard strategy in anti-cancer therapy. Apoptosis is promoted by Cur as a natural polyphenolic complex, which is accomplished through interference in different cell survival signaling pathways, like Akt and NF- κ B (46, 47).

In the present work, it was found that both Cur and PCL had a significant role in increasing apoptosis. Nevertheless, an increase was observed in the rate of early apoptosis to 28.8 and 13.8 % after treatment of cells with NioPCL and NioCur. Besides, the combined administration of PCL and Cur increased apoptosis in comparison with individual therapies, particularly when applied in drug niosomal form (that

is, 17.7 % early apoptosis in free PCL + free Cur solution vs. 49.4 % early apoptosis in PCL niosome + Cur niosome, $p < 0.05$).

5. Conclusion

A nonionic surfactant vesicle of Cur and PTX was successfully developed in the present work, and it was characterized regarding size, loading efficiency, and in vitro drug release. The encapsulation efficiency of the drugs was high. As demonstrated by the release profile of the drugs, burst release was followed by a constant drug release for both agents. When Cur (an efficient chemosensitizer) and PCL (a robust anticancer drug) are used in a combined way, especially in nanoniosome formulations, the therapeutic efficacy of Cur and PCL can be increased in reducing AKT-1 gene expression, reducing activation of NF- κ B, and enhancing apoptosis. Considerable potential can be achieved in the cancer clinical management using this dual therapeutic strategy, particularly based on nonionic surfactant vesicles.

Declarations

Ethics approval and consent to participate: The manuscript was approved by the Abadan University of Medical Sciences Internal Review Board. There are no human subjects or animals involved in the study.

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Figures

Figure 1

The in vitro release profile of curcumin and paclitaxel from niosome formula.

Figure 2

Inhibition of cell growth by curcumin and paclitaxel individual as a drug free form and drug nanoniosomal form in OVCAR-3 cell. a Free Cur vs Free PCL; b; NioCur vs NioPCL

Figure 3

Analysis of synergy between curcumin and paclitaxel for OVCAR-3. a, Dose–response curve of free Cur + Free PCL ; b dose–response curve of NioCur + NioPCL. CI values at different levels of growth inhibition effect (fraction affected, FA; c Free Cur + Free PCL ; d NioCur + NioPCL in OVCAR-3 cell)

Figure 4

Cellular uptake of drugs-loaded niosome on OVCAR-3 line. [A1: Niosomal Cur: Nucleus, A2: Niosomal Cur, A3: Niosomal Cur: Merged; B1: Niosomal PCL: Nucleus, B2: Niosomal PCL, B3: Niosomal PCL: Merged.

Figure 5

Cell apoptosis analyzed with Annexin V-FITC kit following the treatment of cells with Cur and PCL, either as single agents or as a Cur + PCL combination, administered in aqueous solution or in nano-niosome formulations. A: Control; B: Free Curcumin; C: Free Paclitaxel; D: Free Curcumin + Free Paclitaxel; E: Niosomal Curcumin; F: Niosomal Paclitaxel; G: Niosomal Curcumin + Niosomal Paclitaxel.

Figure 6

qPCR analysis of Akt-1 gene expression in OVCAR-3 cells following treatment with paclitaxel and curcumin either as a single agent or in combination, when administered in aqueous solution and nanoniosome formulations. * $P < 0.0001$ vs control. a: free Cur vs Free Cur+ Free PCL ($P < 0.0001$) b: Free Cur vs NioCur ($P < 0.0001$). c: Free PCL vs Free Cur+ Free PCL($P < 0.0001$). d: Free PCL vs NioPCL($P < 0.0001$). e: Free Cur+ Free PCL vs NioCur+ NioPCL ($P = 0.0003$)

Figure 7

p65-NF- κ B activity in OVCAR-3 cells following treatment with paclitaxel and curcumin either as a single agent or in combination, when administered in aqueous solution and nanoniosome formulations. * $P < 0.05$ vs control. a: free Cur vs Free Cur+ Free PCL($P < 0.05$). b: Free Cur vs NioCur ($P < 0.05$). c: Free PCL vs Free Cur+ Free PCL($P = 0.0002$). d: Free PCL vs Nio PCL ($P = 0.0005$). e: Free Cur+ Free PCL vs NioCur+ NioPCL($P < 0.05$).

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