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Research paper

PEGylated conjugated linoleic acid stimulation of apoptosis via a p53-mediated signaling pathway in MCF-7 breast cancer cells

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ABSTRACT

The objective of this study was to investigate whether PEGylated conjugated linoleic acid (PCLA), as compared with conjugated linoleic acid (CLA) alone, displays anti-cancer properties in MCF-7 breast cancer cells. To generate PCLA, CLA was simply coupled to poly(ethylene glycol) (PEG) at the melting state of PEG without a solvent or a catalyst. The coupling reaction generated an ester linkage between the carboxyl group of CLA and hydroxyl one of PEG. The half-life of the generated PCLA was 52 h at pH 7.4 at 37 °C, indicating that PCLA potentially acts as a pro-drug. Apoptosis of MCF-7 breast cancer cells treated with PCLA showed a dose response to PCLA concentration during treatment. In addition, pro-apoptotic proteins such as Bax were up-regulated, whereas anti-apoptotic proteins, such as Bcl-2, were down-regulated by treatment with both CLA and PCLA. The tumor suppressor gene p53 was significantly up-regulated by treatment with increasing concentrations of PCLA, suggesting that PCLA-induced apoptosis is regulated by a p53-mediated signaling pathway. Overall, the anti-cancer effects of PCLA on MCF-7 breast cancer cells may have therapeutic significance.

1. Introduction

Conjugated linoleic acid (CLA) is a collective term referring to a mixture of positional geometric isomers of linoleic acid (*cis*-9, *cis*-12, octadecadienoic acid) in which the double bonds are conjugated in either the *cis* or *trans* configuration. CLA is converted by rumenal bacteria via an enzymatic isomerase reaction and naturally occurs at a high concentration in food produced from ruminant animals [1–2]. Most *in vitro* studies report that CLA mixtures are composed of two principal isomers: *cis*-9, *trans*-11 (*c*-9, *t*-11) CLA and *trans*-10, *cis*-12 (*t*-10, *c*-12) CLA [3–4].

CLA has received considerable attention because it has been associated with various health-related benefits in animals due to its anti-carcinogenic, anti-diabetic, anti-adipogenic, and anti-atherogenic properties as well as its immune modulation effects [4–7]. In particular, it has long been recognized that CLA inhibits chemically-induced carcinogenesis in tissues such as the mammary gland, skin, forestomach, and intestine. Furthermore, CLA has been shown to inhibit the proliferation of hepatoma, lung, colon, and breast cancer cells *in vitro* [3,8–9]. However, the molecular mechanisms of the effects of CLA on cancers have not been fully elucidated [10]. CLA is extremely insoluble in water, resulting in difficulty in examining its clinical applications. Therefore, chemical modification of CLA is necessary to overcome this limitation and allow CLA compounds to be evaluated in clinical trials.

Poly(ethylene glycol) (PEG) is a linear or branched neutral polyether with the chemical formula $HO–(CH_2CH_2O)_n–H$ [11], which has been found to form non-immunogenic, non-antigenic, non-toxic, and highly water-soluble polymers [12]. PEG is a widely available compound and is used in a wide range of biotechnological and pharmacological fields due to its unusual chemical and biological properties [13–14]. In addition, PEG is approved by the FDA for use in drugs [15], foods, and cosmetics [16]. PEG conjugation with biomolecules, called PEGylation, has several advantages for drug modification [17] and is a well-established system for the modification of drugs and proteins [18] because it results in an increased circulation time in the body [19] and promotes stability, improved biocompatibility, and enhanced solubility in water [12,18,20].

In the present study, chemical modification of CLA by PEG for the enhancement of water solubility and anti-cancer activity was performed. To evaluate the effects of PEGylation, the CLA was simply coupled to PEG without a solvent or a catalyst, and the anti-proliferative effects of PEGylated CLA (PCLA) on MCF-7 breast cancer cells were investigated.

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2. Materials and methods

2.1. Materials

The conjugated linoleic acid mixture (CLA; 45.98% *cis*-9, *trans*-11 and 49.88% *trans*-10, *cis*-12 isomers) was purchased from HK Biotech (Seoul, Korea). Poly (ethylene glycol) (PEG) was purchased from Aldrich (Milwaukee, USA).

2.2. Coupling of CLA to PEG

PEG (MW 2000, 10 g, 5 mmol) was reacted with CLA (MW 280.45, 20 g, 71.3 mmol) at 170 °C and for 5 h under nitrogen condition without a solvent or catalyst. Following the reaction period, un-reacted CLA was removed by ethyl ether and un-reacted PEG was removed using NANOCEP^{R} centrifugal devices (solute MW: 3 K) (PALL Corporation, Michigan, USA) following the manufacturer's protocol. The final reactant was dissolved in *d*-chloroform, and the degree of PEGylation in PCLA was estimated by ^{1}H NMR spectroscopy (Bruker, Avance 500).

2.3. Characterization of PCLA

2.3.1. Fourier transform infrared (FT-IR) spectroscopy

FT-IR was employed to characterize possible interactions between CLA and PEG in the solid state using an FT-IR multiscope spectrophotometer (Midac, Costa Mesa, USA).

2.3.2. Electrophoretic light scattering spectrophotometry (ELS)

The nanoparticle sizes and the surface charges of PCLA were evaluated using ELS (ELS 8000, Otsuka Electronics, Osaka, Japan) with 90° and 20° scattering angles at 25 °C.

2.3.3. Transmission electron microscopy (TEM)

The morphology of PCLA nanoparticles (NPs) was observed using TEM (JEM 1010, JEOL, Japan). A drop of PCLA dissolved in distilled water was placed on a copper grid and stained with a 2% uranyl acetate solution for 5 s. The grid was allowed to dry further for 15 min and was then observed with the electron microscope.

2.4. In vitro half-life of PCLA

To confirm PCLA as a pro-drug, DMEM was used for half-life analysis of PCLA *in vitro* at 37 °C and pH 7.4. PCLA (80 mg) was dissolved in 40 mL DMEM and incubated in a shaking incubator at 130 rpm. At specific time intervals, changes in the absorbance of the PCLA in aqueous solution were measured by UV absorbance spectroscopy (Optizen 2120 UV, Seoul, Korea) at 260 nm.

2.5. Cell culture and PCLA treatment

NIH 3T3 fibroblasts and MCF-7 human breast cancer cells were seeded in 6-, 12-, and 24-well tissue culture plates in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were grown at 37 °C in a 5% CO_2 incubator. After one day, serum was removed, and the cells were subsequently incubated with CLA and PCLA according to their various concentrations for three days.

2.6. Cell viability assay

The effects of different concentrations of CLA and PCLA on cell viability were determined using a 3-(4,5-dimethylthiazol-2yl)-2,5-dipyenyltetrazolium (MTT) proliferation assay kit (Sigma) as instructed by the manufacturer.

2.7. Indirect nuclear staining

Cells were cultured on circular glass slides in 12-well plates. Cells were fixed with 3.7% formaldehyde for 20 min and stained with 4,6-diamidino-2-phenylinodole (DAPI, 1 mg/mL in sterile dH_2O) for 30 min at room temperature. Slides were then washed in PBS and examined under a fluorescence microscope equipped with an UV light filter (Zeiss, Germany).

2.8. Flow cytometric analysis of cell cycle and apoptosis

MCF-7 cells in the presence or absence of CLA and PCLA were collected by centrifugation and fixed with 70% ethanol overnight at 4 °C. The 70% ethanol was then removed by centrifugation, and the DNA of the cells was stained with a PI staining solution (100 μg/mL PI, 0.1% Triton-X, 1 mM EDTA in PBS) for 45 min in the presence of an equal volume (200 μg/mL) of DNase-free RNase (Intron Co., Seoul, Korea). Stained cells were analyzed by Fluorescence Activated Cell Sorting (FACS).

2.9. Whole cell extracts

Plates were washed twice in PBS and cells were lysed in the plates by the addition of SDS sample buffer containing 2.5% SDS, 10% glycerol, 50 mM Tris–HCl (pH 6.8), 10 mM dithioerythritol, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor mixture (1/50 tablet per mL) (Boehringer Mannheim, Damstadt, Germany). Cell lysis was immediately followed by 3 min of boiling. Lysates were subsequently treated with benzon nuclease (Boehringer Mannheim, Damstadt, Germany). Whole cell extracts were stored at −80 °C. Protein concentrations were determined by the BCA method.

2.10. Western blot analysis

Equal amounts (50 μg/well) of protein were separated on SDS-polyacrylamide gels and proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech., Buckinghamshire, England). The membranes were blocked overnight in TBS containing 5% nonfat dry milk and 0.1% Tween 20 (Biosesang, Seoul, Korea). Incubation with primary and secondary antibodies was performed in TBS containing 5% nonfat dry milk for 2 h. After incubation with antibodies, membranes were washed in TBS containing 0.1% Tween 20. The primary antibodies used were mouse monoclonal p53, Bcl-2, Caspase-9, and rabbit polyclonal Bax. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Santa Cruz Biotech., San Francisco, USA). Enhanced chemiluminescence (ECL; Intron Biotech., Seoul, Korea) was used for detection.

2.11. Statistical analysis

Statistical analysis was performed using the Student's *t*-test. Data are expressed as means ± standard deviations (SD). Statistical significance is represented by * for *p*-values less than 0.05, by ** for *p*-values less than 0.01 and by *** for *p*-values less than 0.001.

3. Results and discussion

3.1. Synthesis of PCLA

CLA was coupled to PEG under dry nitrogen conditions at 170 °C and for 5 h. The synthetic scheme for PCLA is illustrated in Fig. 1. Double bonds were clearly detected at 5.2–6.2 ppm in synthesized PCLA (Fig. 2), an indication of the successful PEGyla-

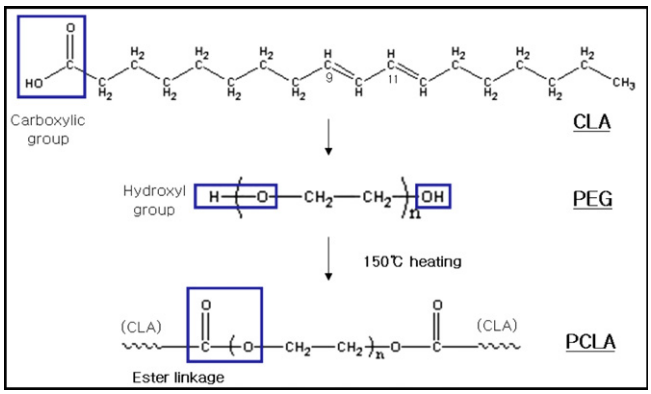

Fig. 1. The reaction scheme of PCLA. The CLA was simply coupled to PEG at the melting state without a solvent or a catalyst and handled under a dry nitrogen condition.

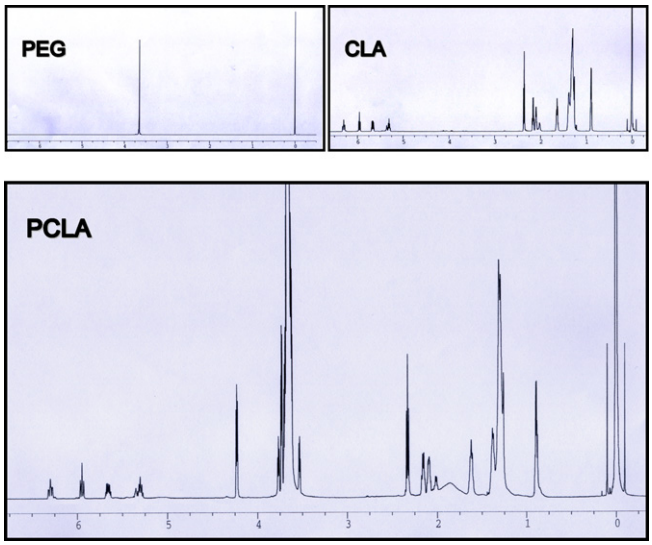

Fig. 2. ^{1}H NMR spectra of PCLA prepared at 170 °C for 5 h.

tion of CLA through ester linkage between the hydroxyl group of PEG and the carboxylic group of CLA, without reduction of CLA. The highest degree of PEGylation (81 mol%) was obtained when the coupling occurred at 170 °C for 5 h. In addition, oxidation of CLA did not occur under the nitrogen condition (data not shown), suggesting that PEGylation of CLA under nitrogen conditions was stable for CLA. FT-IR was used to evaluate how well CLA was coupled to PEG under reaction condition. The FT-IR spectra of PEG and PCLA are shown in Fig. 3. The C=O stretching band of ester bond in PCLA appeared at 1737.81 cm^{-1}, an indication of the occurrence of ester bonding [21] between the carboxylic group of CLA and hydroxyl one of PEG. Also, the C–O–C stretching band in PEG was assigned in the 1300–1000 cm^{-1} region [22,23]. Therefore, our data indicated that CLA was successfully coupled to PEG.

3.2. Characterization of PCLA

The particle sizes and size distributions of prepared PCLA nanoparticles in aqueous solution were determined by ELS, and the particle morphology was observed by TEM. The average PCLA particle sizes were around 25 nm with uniform size distributions (Fig. 4A), and the PCLA particles showed spherical morphology (Fig. 4B).

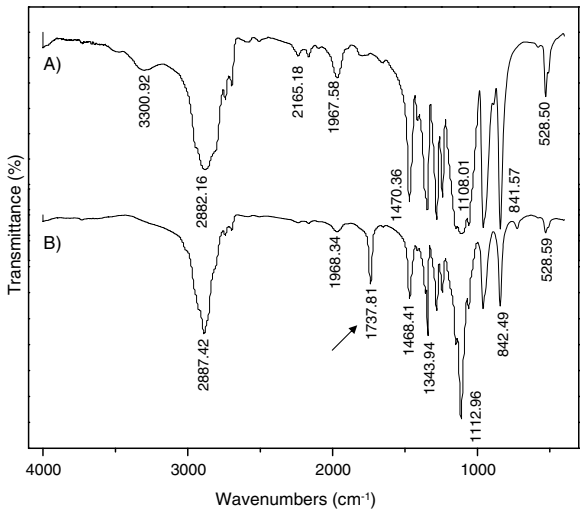

Fig. 3. FT-IR spectra of PEG (A) and PCLA (B).

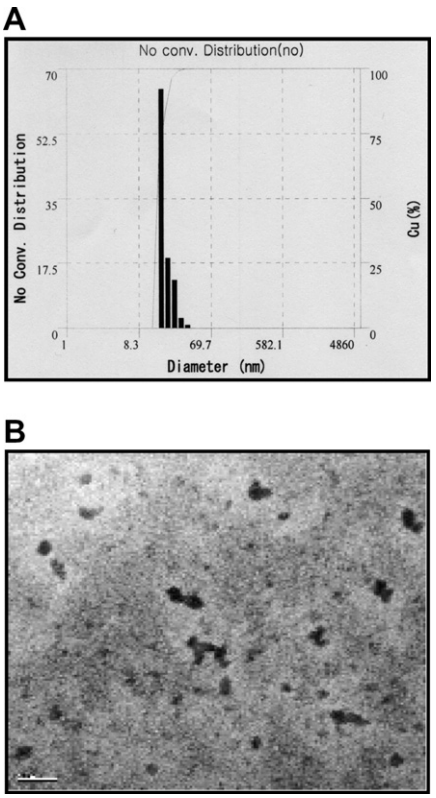

Fig. 4. ELS measurement (A) and TEM image (B).

Therefore, it can be speculated that PCLA may serve as an useful anti-cancer drug because of its small particle size. This is very important because carrier systems with a diameter from 10 to 200 nm are generally used for drug targeting to cancer cells [24–25]. In addition, some larger drug carriers could be easily and rapidly captured by the reticuloendothelial system (RES), suggesting that the diameter of an anti-cancer drug should be smaller than 400 nm [26–28].

3.3. Half-life of PCLA

The most often employed pro-drugs have been based on hydrolysable or enzymatically cleavable bonds, such as esters, carbon-

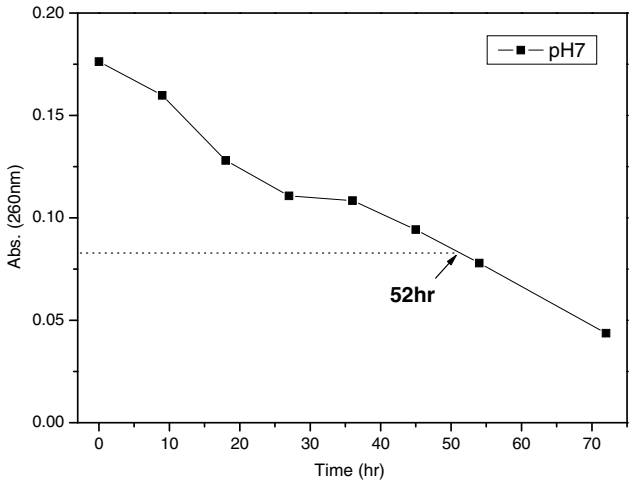

Fig. 5. *In vitro* half-life of PCLA.

ates, carbamates, and hydrazones [7,29–30]. On the basis of these early concepts, further investigation was made into whether PCLA can act as a pro-drug. The half-life of PCLA was 52 h under conditions similar to that found in the bloodstream (Fig. 5), indicating that PCLA acts as a pro-drug. In addition, it has been generally confirmed that body circulation times of drugs are increased after PEGylation when compared to the non-modified drug [11,17,20]. These results suggest that PCLA will likely have an increased body circulation time as compared with CLA alone.

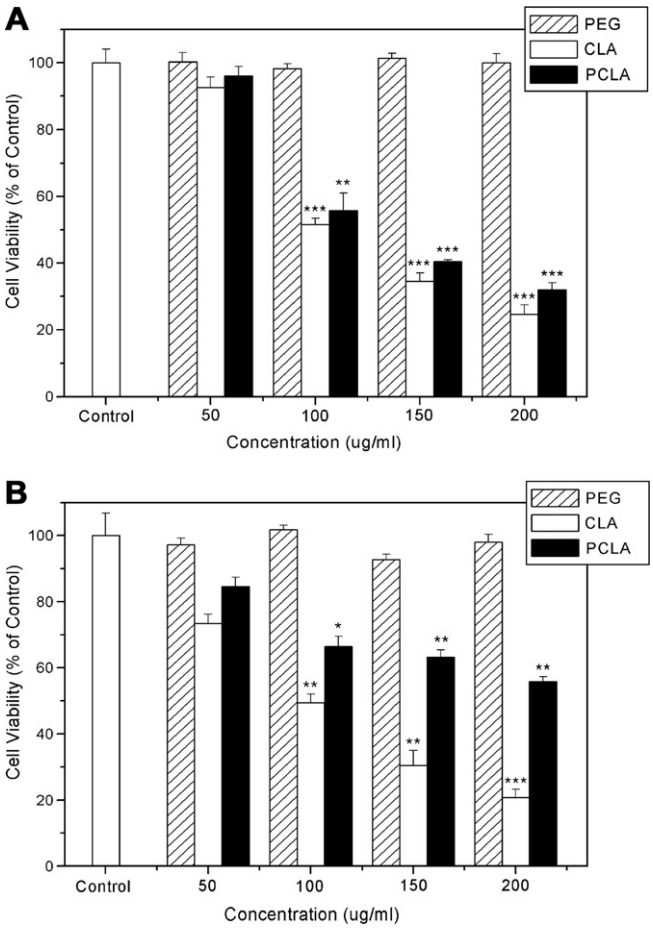

Fig. 6. Cell viability of PEG, CLA and PCLA. (A) MCF-7 breast cancer cells and (B) NIH 3T3 fibroblast cells.

3.4. Cell viability and nuclear population

To evaluate cell viability, the MTT assay was used, which provides a quantitative determination of metabolically active cells [2]. It has been reported that CLA has anti-cancer effects on hepatoma, lung, colon, and breast cancer cells *in vitro* [2–3,8–10]. To evaluate the effects of PCLA, MCF-7 breast cancer cells and NIH 3T3 fibroblasts were treated with PEG, CLA or PCLA in increasing concentrations. Both CLA and PCLA reduced cell viability in the MCF-7 cells after 72 h of treatment (Fig. 6). Although the reduced cell viability between the CLA and PCLA treatment groups with increasing concentrations in MCF-7 cell lines was not significantly different, PCLA-treated NIH 3T3 cells showed increased viability compared with CLA-treated cells, suggesting that PEGylation of CLA decreased cell viability in cancer cell. As further support for the cell viability data, the nuclear population of the MCF-7 breast cancer cells was examined using fluorescence microscopy. The results showed a reduced nuclear population following treatment with either CLA or PCLA, indicating that PCLA is a valuable anti-cancer pro-drug (Fig. 7).

3.5. Effects of PCLA on cell cycle and apoptosis

The effects of CLA and PCLA treatment on cell cycle were evaluated. The results showed that treatment with not only CLA but also PCLA in a range from 100 to 200 μM caused a delay of cell entry into the S phase and a reduction of cell accumulation in the G0/G1 phase (Fig. 8). In fact, only 8% of control cells were detected in the Sub G1 phase, whereas a 5-fold increase in the number of cells in the Sub G1 phase was observed following treatment with either 200 μM CLA or PCLA (Table 1).

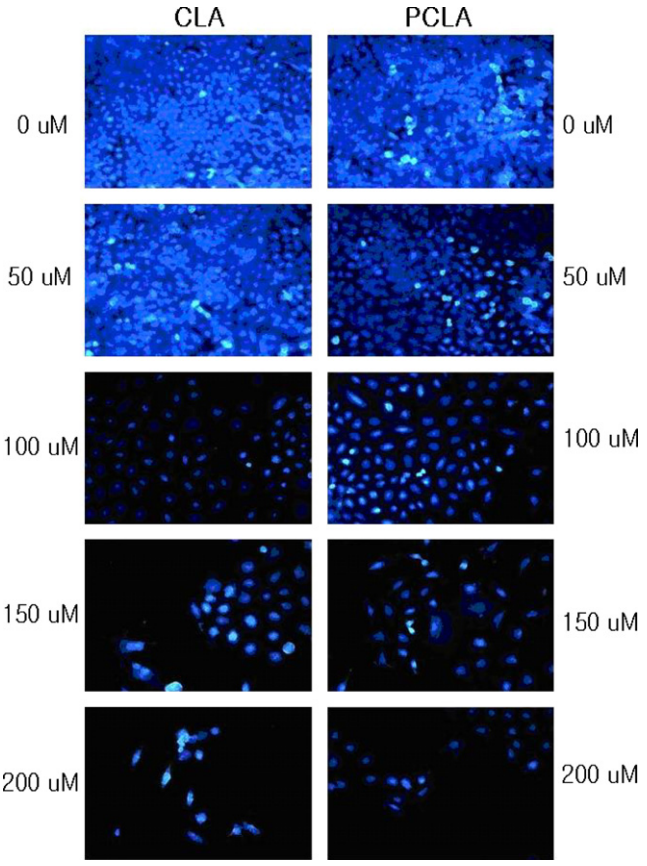

Fig. 7. DAPI staining of CLA and PCLA.

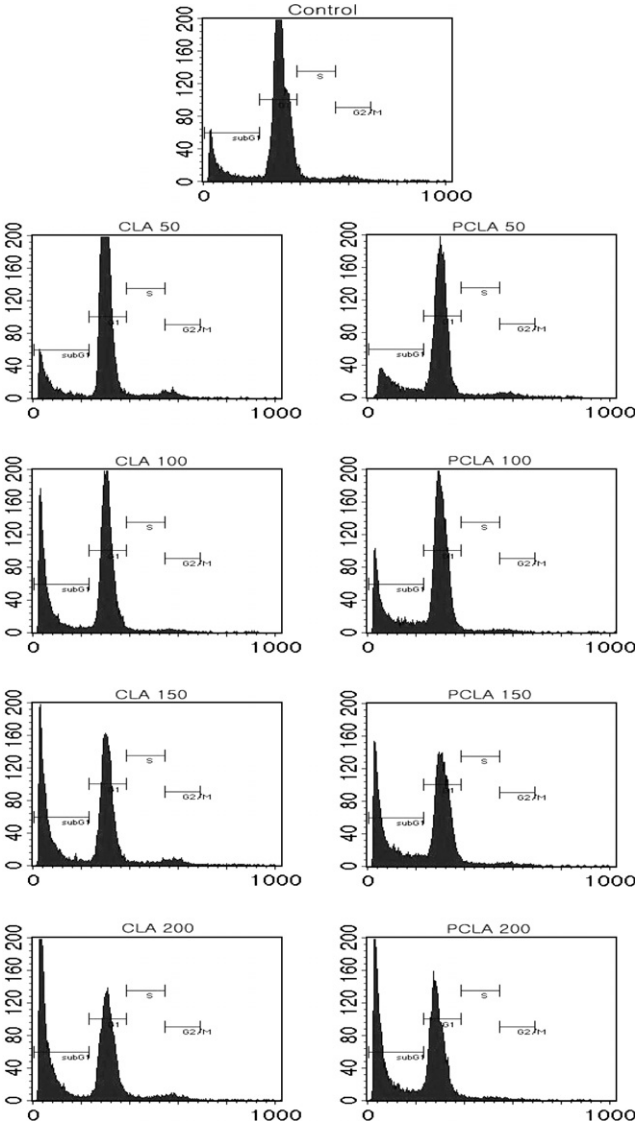

Fig. 8. Cell cycle and apoptosis analysis after treatment of CLA and PCLA in MCF-7 breast cancer cells.

Table 1
Effect of CLA and PCLA on different phases of the cell cycle of MCF-7 breast cancer cells

		Concentration of treatment (μM)				
		Control	50	100	150	200
Sub G1	CLA	8.26	12.35	32.53	38.57	47.08
	PCLA		16.09	26.95	37.51	46.95
G1	CLA	85.68	82.96	63.56	55.75	48.82
	PCLA		79.04	69.52	58.21	49.08
S	CLA	3.38	2.82	2.74	3.03	2.01
	PCLA		2.60	2.38	2.10	1.87
G2/M	CLA	2.68	1.87	1.09	2.65	2.09
	PCLA		2.27	1.91	2.18	2.1
All (%)	CLA	100	100	100	100	100
	PCLA		100	100	100	100

Previous study demonstrated that cell size increased in the G1 phase due to the need to produce RNA and synthesize proteins for DNA synthesis [31]. In addition, an increase in the apoptotic cell population accompanied the increase in the number of cells in the Sub G1 phase [32]. It has also been confirmed that CLA treatment

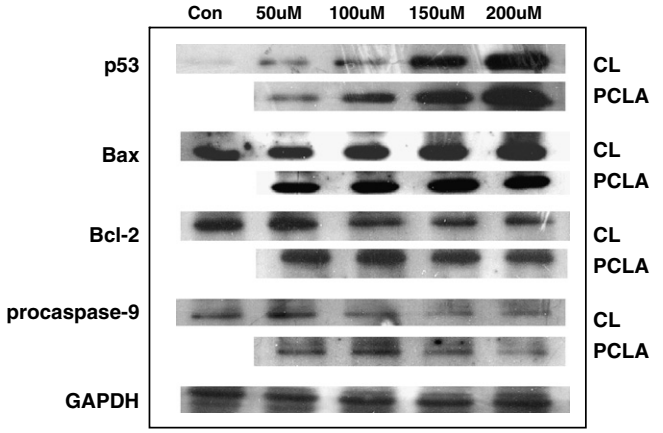

Fig. 9. Expression of p53, Bax, Bcl-2 and procaspase-9 after CLA and PCLA treatment of MCF-7 breast cancer cells in dose-dependent manners.

induces arrest of the cell cycle at the G0/G1 phase in MCF-7 breast cancer cells [7,33]. Results from the study described here are in agreement with these previous reports: CLA and PCLA treatment resulted in cell cycle arrest at the G0/G1 phase in MCF-7 cells, thereby leading to an induction of apoptosis and reduced cell growth and DNA synthesis.

To provide further support for PCLA as an inducer of apoptosis, the effects of PCLA treatment on the modulation of various anti- and pro-apoptotic proteins were examined. Generally, the mitochondrial pathway is thought to be important for both proliferation and apoptosis, mediated by the Bcl-2 family proteins [34]. Also, over-expression of Bcl-2 had been reported to protect tumor cells from apoptosis, whereas increased Bax expression promoted apoptosis [35–36]. This effect is of great importance because down-regulation of Bcl-2 is associated with better therapeutic outcome, specifically in breast cancer patients [37]. In the present study, Bax levels increased with increasing concentrations of CLA and PCLA treatments for 3 days (Fig. 9). On the contrary, Bcl-2 was decreased by CLA and PCLA treatments. These results suggested that PCLA-induced apoptosis was modulated by the up-regulation of Bax and down-regulation of Bcl-2. Procaspase-9 is an important factor in this process as the activation of caspases results in the induction of apoptosis [38–39], and further results from this study showed that levels of the procaspase-9 protein were decreased, indicating that treatment with PCLA may trigger apoptotic cell signaling downstream of caspase-9.

PCLA regulation of the tumor suppressor gene p53 was also examined. The Western blotting assay showed that both CLA and PCLA treatments increased the expression levels of p53, suggesting that PCLA-induced apoptosis is regulated by a p53-mediated signaling pathway. In fact, previous report showed that treatment with not only CLA-coupled poloxamer but also CLA itself had anti-cancer effects on MCF-7 cells via the up-regulation of a p53-dependent signaling pathway [38]. Importantly, p53 can limit cell proliferation by two mechanisms: one is by direct arrest of the cell cycle and the other is via the activation of apoptosis [40]. p53 may induce apoptotic cell death by up-regulating Bax and down-regulating Bcl-2 expression, in which it determines the survival or death of cells following an apoptotic stimulus [41]. Therefore, the inhibitory effects of PCLA on the expression of pro-apoptotic proteins and anti-apoptotic proteins in MCF-7 cells may have therapeutic significance.

4. Conclusions

This is the first report on the anti-proliferative effects of PCLA as a pro-drug on MCF-7 cells. PCLA-induced apoptosis was examined

as a tool to explore the molecular events that occur during the cancer proliferation process. Overall, the results from this study demonstrated that CLA was successfully coupled to PEG without oxidation, and water solubility was increased by PEGylation due to the hydrophilic properties of PEG. Also, PCLA was shown to have small particle sizes. PCLA treatment resulted in improved cell viability effects on NIH 3T3 cell lines over those seen with CLA treatment, suggesting that PCLA acts as a local anti-cancer drug. In addition, pro-apoptotic proteins were up-regulated and anti-apoptotic proteins were down-regulated by PCLA treatment. Importantly, the tumor suppressor gene p53 was significantly up-regulated by PCLA treatment with increasing concentrations, suggesting that PCLA-induced apoptosis is regulated by a p53-mediated signaling pathway. Although more studies are required to examine isomer-specific effects and mechanisms of PCLA action on animals and humans, the possibilities of using PCLA and other pro-drugs as anti-proliferative agents are encouraging, and their use in a variety of cancer situations is a distinct possibility.

Acknowledgements

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