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Dual Effects of Omega -6, and -9 Fatty Acids on Ovarian Cancer Cell Viability and Their Ability to Induce Apoptosis

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Abstract

It has been proposed that unsaturated fatty acids (UFAs) have cytotoxic effects on different cancer cell lines mostly colorectal, breast and prostate cancer cells. Unsaturated fatty acids with more than one double band are called polyunsaturated fatty acids and consist of omega-3 and omega-6 fatty acids. The omega-3 series of fatty acids seem to possess anti- cancer actions and have the ability to inhibit cell division and induce apoptosis, whereas the products of omega-6 fatty acids are believed to enhance cancer cell proliferation. The cytotoxic effects of some UFAs including Linoleic Acid (LA), Arachidonic Acid (AA), A-Linolenic Acid (ALA), and Oleic Acid (OA) was investigated in SKOV-3 human ovarian adenocarcinoma cells using MTT assay. The apoptosis induction of LA and OA was further characterized using an annexin-V-FLUOS staining kit. Linoleic acid (LA) and oleic acid (OA), as omega-6 and omega-9 fatty acids respectively, were abletoinhibitSKOV-3cell growth at concentrations above 500µmol, while at low concentrations (300–500 µmol) they promoted the proliferation of cells. However, ALA and AA, omega-3 and - 6 fatty acids respectively, showed no remarkable effect on viability of SKOV-3 cells. LA and OA had significant apoptotic effect on SKOV3 ovarian cancer cell line. It seems that there is a critical concentration for some UF as in confronting with some cancer cells and this critical concentration depends on the type of cell and also unsaturated fatty acid itself. UFAs put cytotoxic effect on cancer cells and this cytotoxicity is resulted from apoptosis induction.

Keywords: Apoptosis; Cytotoxicity; Unsaturated Fatty Acids

Introduction

Despite recent progress in cancer treatment, cancer still remains as a great challenge for medicine. In gynecologic oncology, ovarian cancer is a major problem because of its therapeutic resistance [1]. Epithelial ovarian cancer (EOC) is at the first place among other gynecologic malignancies to drive fatality [2] and in women suffering from cancer, is at the fourth place to cause morbidity and mortality [3,4]. Although there are moderate advances in chemotherapy for epithelial ovarian cancer, it still remains as one of the most aggressive cancer types which have a survival rate of 5years and an overall cure rate of approximately30% [5]. So, finding new drugs and approaches for epithelial ovarian cancer treatment seems to be very crucial.

There is considerable evidence that unsaturated fatty acids, despite their role as an energy source, might affect both cancer development and progression [6-8]. Unsaturated fatty acids are categorized into two groups of polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) based on the number of carbon-carbon double bonds in their carbon chain [9,10] which both have specific effects on cancer [11,12]. It should be noted that most investigations in this field have been focused on the effects of PUFAs and there are less studies about the effects of MUFAs, such as Oleic acid (OA; n-918:1), on cancer. PUFAs including omega-3 and omega-6 families are able to exert anti-cancer activities both *in vitro* and *in vivo* [13-16]. The omega-6 Linoleic acid (LA; n-618:2) andomega-3α-linolenic acid (ALA; n-318:3) are essential fatty acids (EFAs) and used to synthesis of long chain PUFAs of their family. The omega-6 long chain polyunsaturated fatty acids (LCPUFAs) include γ-linolenic acid (GLA; n-6 18:3), dihomo-

GLA (DGLA; n-6 20:3) and arachidonic acid (AA; n-6 20:4), and LCPUFAs of omega-3 family are eicosapentaenoic acid (EPA; n-3 20:5) and docosahexaenoic acid (DHA; n-3 22:6) [17]. These long chain PUFAs can give rise to the action of their precursors (EFAs), and hence areal so called functional EFAs [18]. It has been demonstrated that EFAs and their products can remarkably inhibit the cancer cell growth both *in vitro* and *in vivo* [15]. Previously, it has been clarified that some PUFAs exhibit selective cytotoxic effect on various cancer cells *in vitro* and *in vivo*, meaning that when the appropriate concentration is used, the tumor cells are killed without any harm or damage to the normal cells. But the mechanisms behind this tumoricidal function of PUFAs are not fully understood.

Oleic acid (OA; 18:1) is an omega-9 monounsaturated fatty acid abundantly found in olive oil. Traditionally it has been demonstrated that olive oil consumption links to an anti-cancer effect due to the existence of OA [19,20]. A broad range of epidemiological and animal researches have shown a protective effect for OA against several cancers such as breast, colorectal, prostate and ovarian [21,27]. OA can act selectively on cell growth as it has a promoting effect on growth of non-malignant cells and an inhibitory effect on malignant ones [28,29]. Several studies clarified that OA can inhibit proliferation of various cancer cell lines [11,30,31]. Yet, in this context some controversial results have been also reported, representing different effects (from non-promoting to completely promoting) on tumor cell growth [32,35]. But all these investigations have been focused on breast cancer cell lines.

In a large population-based case–control study, there was no evidence that the more consumption oftotalomega-3polyunsat uratedfattyacidsoreachomega-3fatty acid (ALA,EPA,DPAorDHA) individually helps to the lowered incidence of ovarian cancer. But consumption of higher a mounts of total omega-6 fatty acids inversely related to ovarian cancer development, and this appeared to be primarily driven by linoleic acid [36].

According to our knowledge, the cytotoxic and apoptotic effect of unsaturated fatty acids such as ALA(n-3;18:3),LA(n-6;18:2),AA(n-6;20:4),andOA(n-9;18:1)onSKOV-3cellshasnotyetbeen examined. Thus, we conducted present study to evaluate such effects on mentioned cell line.

Materials and Methods

The unsaturated fatty acids including Alpha-linolenic acid (n-3; 18:3), Linoleic acid (n-6; 18:2), Arachidonic acid (n-6;20:4),and Oleic acid (n-9;18:1)were purchased from Sigma-Aldrich. The unsaturated fatty acids were dissolved in pure ethanol, filter-sterilized and stored at -70˚C. The stock solutions were diluted with cell culture medium for use. The SKOV-3, a human epithelial ovarian cancer cell line, was obtained from NCBI (National Cell Bank of Iran). The MTT powder (Sigma-Aldrich, M2128) was kindly provided by Dr. Elham Tafsiri, Molecular Medicine Department, Biotechnology Research Center, Pasteur Institute of Iran. The annexin-V-FLUOS staining kit (Roche Life Science) was kindly provided by Dr.Mehdi Edalati Fathabad, Hematology Department, Tehran University of Medical Sciences.

Cell Culture

The SKOV-3 cell line was cultured in RPMI-1640 medium (Biosera, LM-R1637) supplemented with 10% heat-inactivated FBS (fetal bovine serum, Gibco, 26140-079) and 1% penicillin-streptomycin (Gibco, 15140-122). The cells were incubated at 37˚C in a humidified incubator containing 5% CO_2, and sub-cultured beyond 80% confluency.

Cell Viability Assessment

The cell viability was evaluated by usage of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5- di phenyl tetrazolium bromide] dye. The SKOV-3 cells were seeded in 96-well plates at 1.0×10^4 cells/well density and incubated overnight to attach. Then the medium was gently removed and fresh medium (RPMI-1640, 10% FBS) containing different concentrations of ALA, LA, AA and OA was added and incubated for 24, 48, and 72 hours without replacement of the medium. In all the experiments, untreated cells which didn't receive any concentration of unsaturated fatty acids but were treated with equal content of ethanol served as controls. The doses for LA and OA fatty acids ranged from 300 to 700μM, and from 300 to 1000μM for ALA and AA. After spending proper incubation time, the medium was gently discarded, and each well was treated with 10μl of MTT solution [5 mg/ml in phosphate buffered saline (PBS)] and 90μl medium at 37˚C for 2½ hours. The formed formazan crystals were then dissolved in 100 μl/ well dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm by a 96-well micro plate ELISA reader (Synergy4, nBioTek). The viability of cells in each well was presented as percentage of the control. Triplicate tests were set up for each treatment and for control. The IC50 was calculated from mean±SD values.

Quantification of Apoptosis Using Flow Cytometry

In order to detect the apoptotic and necrotic cells, the annexin-V-FLUOS staining kit (RocheLife Science, 11 858 777 001) was utilized. According to its dual-staining protocol, the Annexin-V- FLUOS (green fluorescence) was used to stain the apoptotic cells, and the propidium iodide (PI; red fluorescence) was used to stain the necrotic ones. The SKOV-3 cells were seeded in 6-well plates at 3×10^5cells/well density and incubated overnight. Then, the medium was discarded and freshRPMI-1640medium with LA and OA each at 600μM concentration was added and the cells were treated for 24,48, and 72h. After the treatment, the cells were trypsinized, washed with PBS, and labeled according to the manufacturer's instruction. The labeled cells were finally analyzed using a flow cytometry (Cyflow, Partec) and the percentages of apoptotic cells were determined using Cyflogic software.

Statistical Analysis

All the experiments were performed in triplicate. The statistical analysis was accomplished by repeated measures ANOVA and Tukey's post hoc tests (α = 0.05). Values are means (n=3) ± SD. (P-value <0.05). Data were analyzed using SPSS19.

Results

SKOV-3 Cell Viability After UFA Treatment

To evaluate the cytotoxicity of omega-3, -6 and -9 UFAs on SKOV-3 cells, the cells were treated with different concentrations (300, 400, 500, 600, and 700 µM) of LA and OA, and (300, 400,500, 600, 700, and 1000 µM) of ALA and AA for 24, 48, and 72 hours. Then, the viability of treated cells was measured by MTT assay. The LA (shortchainomega-6PUFA) and OA (omega- 9 MUFA) induced cytotoxic effects on human ovarian adenocarcinoma SKOV-3 cells. On the other hand, ALA (shortchainomega-3PUFA) and AA (longchainomega-6PUFA) were notable to affect the viability of SKOV-3 cells at used concentrations after 72 hours. The LA and OA performed dual effects on SKOV-3 cells at concentrations of 300-700 µM as they promoted the cell proliferation at concentrations below 500 µM, while reduced the cell viability at concentrations above 500 µM (Figure 1). The half maximal inhibitory concentrations (IC_{50}) of LA and OA were calculated and demonstrated in Table 1.

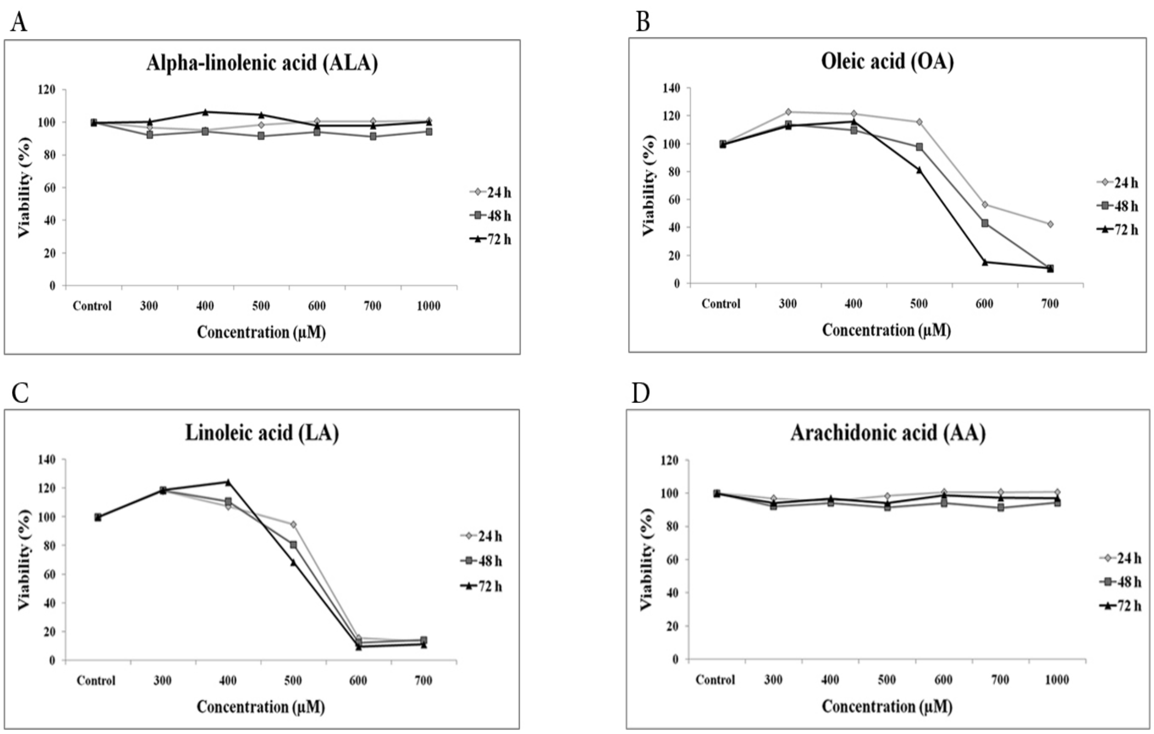

Figure 1: MTT assay results**:** The viability of SKOV-3 cells after 24, 48, and 72 hours of treatment with A) ALA; B) OA; C) LA; and D) AA. The dual effect of LA and OA on SKOV-3 cell viability is evident.

Cell line	**Unsaturated fatty acid**	**IC50 (µM)**		
		24 h	**48 h**	**72 h**
SKOV-3	Linoleic acid (LA)	541.92	522.5	513.21
	Oleic acid (OA)	669.52	589	532.3

Table 1: The IC50 of cytotoxic unsaturated fatty acids.

Flow Cytometric Analysis of Apoptosis

To determine whether the cytotoxicity of LA and OA was caused by apoptosis induction, the LA- and OA-treated SKOV-3 cells were stained with Annexin-V-FLUOS staining kit. According to the flow cytometry results, it was revealed that the cytotoxic action of LA and OA on SKOV-3 cells is caused by the ability of these FAs to induce apoptosis. It was also indicated that the apoptosis induction ability of mentioned fatty acids is promoted through time (Figure 2 and3).

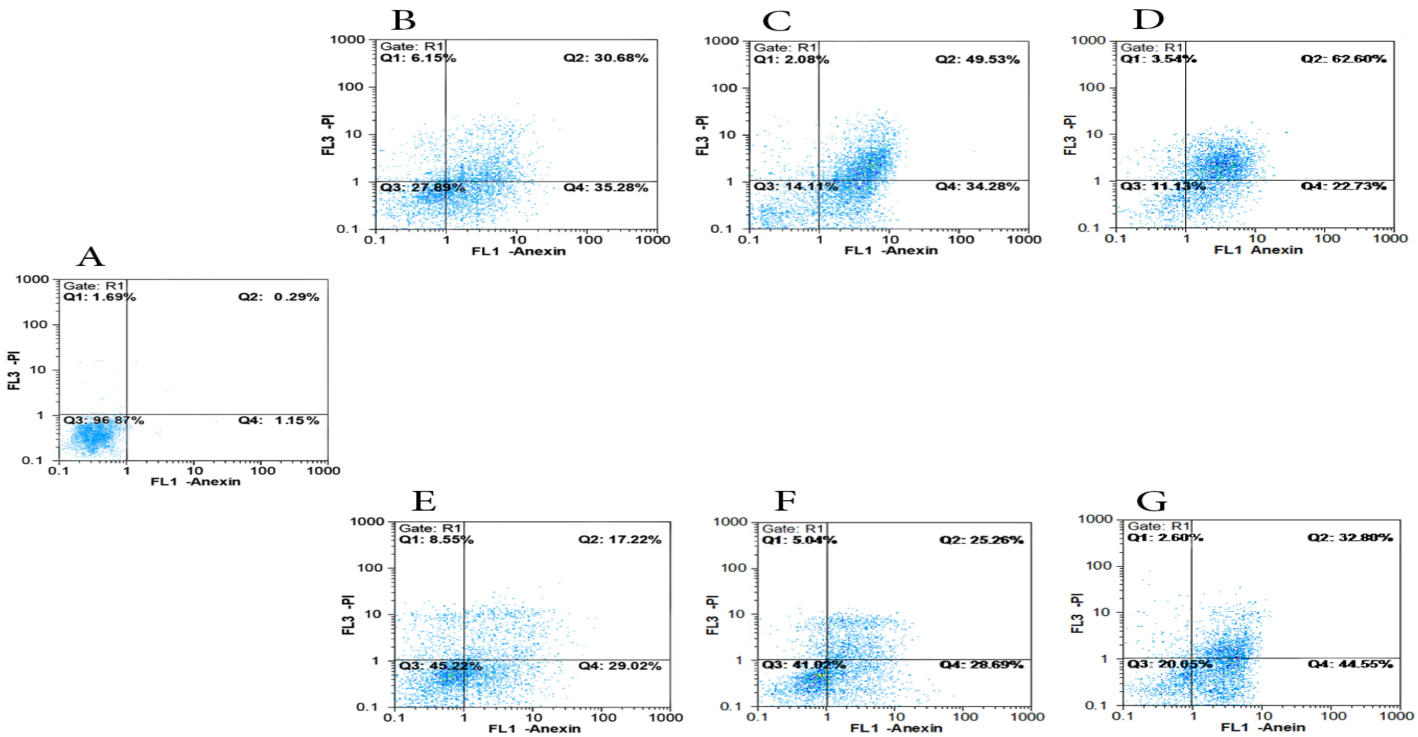

Figure 2: Flow cytometric assessment of apoptosis after treatment with LA and OA. A) Control; B) LA-treated cells after 24h; C) LA-treated cells after 48h; D) LA-treated cells after 72h; E) OA- treated cells after 24h; F) OA- treated cells after 48h; and G) OA- treated cells after 72h.

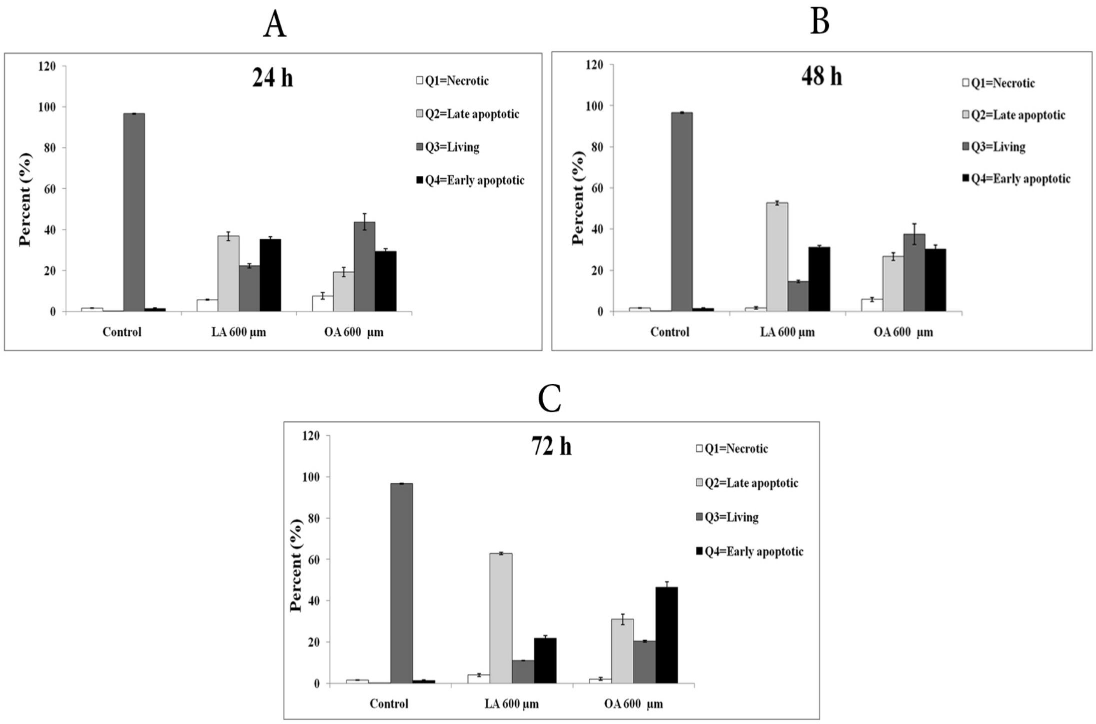

Figure 3: Apoptosis in SKOV-3 cells after treatment with LA and OA. A) 24 hours after the treatments, B)48 hours after the treatments, and C) 72 hours after the treatments with LA and OA. LA and OA are able to induce apoptosis in SKOV-3 cell line. The percentage of apoptotic cells after treatment with LA and OA is significant. Q1: Debries, Q2: Necrotic, Q3: Living, and Q4: Apoptotic cells.

Discussion

In this study, it has been demonstrated that LA and OA unsaturated fatty acids have cytotoxic and apoptotic effects onSKOV-3 cancer cell line. In contrast, we didn't observe significant changes in cell viability of SKOV-3 cells by ALA and AA. The LA and OA cytotoxic effects on SKOV-3 were obtained in concentrations higher than 500 µM which were enhanced in a time-dependent manner. On the other hand, there was a minor increase in cell proliferation through the concentrations under500µM.Previous studies have shown that unsaturated fatty acids can induce cell death in tumor cells [37] but the sensitivity of these cells seems to be diverse based on their nature, fatty acid type, and the used concentration [38,40]. Also, it was mentioned that the concentration at which unsaturated fatty acids can reduce cell viability is dependent on the cell density [41]. Similar to the current study, the dual effects of some types of unsaturated fatty acids have been referred in other studies i.e., they promote cell proliferation at low concentrations and inversely induce the cell death at higher concentrations. It was noted that when cancer cells at a density of 1×10^4 were exposed to LA at the concentration of 40 µg/ml, the growth was inhibited, whileatconcentrationsof5-10µg/ml,the cell proliferation was enhanced in some, if not all, types of cancer cells tested [42]. In another study performed on colorectal cancer cells, LA was growth- promoting at concentrations below 300 µM/L, but induced the inhibition at higher concentrations [43]. InMOLT-4leukemia cell line, the inhibitory and stimulatory effects of LA were obtained at 400µM and 200µM, respectively [44]. There is also evidence supporting such effect for OA [45]. The stimulatory effect LA and the inhibitory and/or cytotoxic effect of OA are reported in other studies [46,47]. In line with this investigation, it has been shown that AA is not able to influence the viability of some cell lines of breast cancer, but has cytotoxicity on others [39]. The *in vitro* studies have revealed that the cytotoxic potency is different among PUFAs and it seems that ALA and AA have the weakest [48]. In addition, according to some reports on tumor cells, ALA seems to need more time than other PUFAs to have an influence on cancer cells [49].

Also, it has been clarified that the cytotoxic effect of LA and OA on SKOV-3 cells is caused by apoptosis induction. Apoptosis maintains the balance between cell birth and cell death in our body and as a result, many biological circumstances are regulated [50]. Cancerous cells can block apoptotic pathways, so that they continue to growth and proliferation [51]. There are many efforts to find the elements for apoptosis induction in different cancer cells. The results of the present study are clearly compatible with recent reports that demonstrate the apoptosis induction of unsaturated fatty acids in various cell lines at high concentrations [15,52]. According to other studies it seems that fatty acids can cause cell death through apoptosis or necrosis (at higher concentrations) [53]. As observed in recent study and also previous studies, LA is more potent to induce apoptosis than OA. Unsaturated fatty acids induce apoptosis through the activation of caspases- 3, 6, 7, 8, and 9 [40,54,55]. They can disrupt redox state of cells by generating free radicals and lipid peroxides through lipid peroxidation, compelling cells to be a rapoptosis [38,56]. Lipid peroxidatin increases after treatment with PUFAs. The lipid derived metabolites may activate caspases and induce apoptosis [57]. Moreover, unsaturated fatty acids can cause loss of mitochondrial potential which may lead to the elevated levels of reactive oxygen species (ROS) [58]. In addition, PUFAs can cause the cleavage of Bid and the cytochrome *c* leakage from mitochondria [40,59].

Conclusions

OurresultsindicatedthatLAandOAhavedualeffectsonSKOV-3ovariancancercellline.These effects include cell death induction at high concentrations (≥500 µM) and the promotion of cell proliferation at low concentrations (<500µM). In general, different cells have different sensitivity against unsaturated fatty acids. Also, the fatty acid concentration and the exposure time of cells affect the degree of cell death and it seems that there is difference in the potency of unsaturated fatty acids to induce apoptosis.

Given our results and according to the previous studies in this content, a critical concentration seems to exist for some unsaturated fatty acids in confronting with some cancer cells and it relies on the cell type and also the unsaturated fatty acid itself. LA and OA have cytotoxic effect on SKOV-3 cells through their apoptosis induction ability. However, the distinct molecular mechanisms behind the apoptosis induction of LA and OA need more investigations.

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