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[Efecto citotóxico selectivo del 1-O-undecilglicerol en células de melanoma humano]

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Abstract

Context: 1-O-alkylglycerols are ether-linked glycerols derived from shark liver oil and found in small amounts in human milk. Previous studies showed antineoplastic activity for this family of compounds, structurally related to alkylphospholipids, but the activity of linear chain synthetic alkylglycerols in cancer cell lines is less documented. Melanoma is a high incidence cancer, highly resistant to potential treatments. Finding new anti-cancer compounds to improve melanoma prognosis is a relevant research issue.

Aims: To study the cytotoxic effect of 1-O-undecylglycerol in primary cultured normal fibroblasts and A375 human melanoma cell line.

Methods: Cells were treated with different concentrations of 1-O-undecylglycerol and viability assessed by MTT assay. Morphological changes were visualized by DAPI and acridine orange-ethidium bromide staining. Mitochondrial membrane potential was evaluated, and gene expression of P53 and BcL-2 was semi-quantified.

Results: 1-O-undecylglycerol decreased viability of A375 cells and exerted very low cytotoxicity on primary cultured normal fibroblasts. Necrosis appeared in A375 cells but not in fibroblasts, and no apoptotic changes were visualized in DAPI staining experiments. After 24 h fibroblasts and melanoma cells developed mitochondrial potential changes similar to valinomycin. The gene expression of P53 and BcL-2 decreased in treated cells.

Conclusions: 1-O-undecylglycerol exhibited selective cytotoxic activity in A375 melanoma cells when compared with primary cultured fibroblast. Its toxicity is mediated by necrosis that may be related with mitochondrial events and decrease in P53 and BcL-2 expression. The results suggest that UDG could be a useful strategy to combine with other chemotherapeutic agents in melanoma treatment.

Keywords: Alkylglycerols, cytotoxicity, melanoma, primary cultured fibroblasts.

Resumen

Contexto: Los 1-O-alquilgliceroles son éteres presentes en el aceite de hígado de tiburón y en la leche materna. Han mostrado actividad antineoplásica, pero está poco documentada para alquilgliceroles sintéticos de cadena lineal. La búsqueda de nuevos antitumorales para mejorar el pronóstico del melanoma resulta relevante, debido a su alta incidencia y resistencia.

Objetivos: Estudiar el efecto citotóxico del 1-O-undecilglicerol en cultivo primario de fibroblastos normales y la línea celular A375 de melanoma humano.

Métodos: En células tratadas con diferentes concentraciones de 1-O-undecilglicerol se monitoreó la viabilidad mediante ensayo con MTT. Los cambios morfológicos se visualizaron por tinción con DAPI y naranja acridina- bromuro de etidio. Se evaluó el potencial de membrana mitocondrial, y se semi-cuantificó la expresión de los genes P53 y BcL-2.

Resultados: El 1-O-undecilglicerol redujo la viabilidad de las células A375, y ejerció poca citotoxicidad en el cultivo primario de fibroblastos normales. Ocurrió necrosis en las células A375 pero no en los fibroblastos, y no se visualizaron cambios apoptóticos en la tinción con DAPI. Luego de 24h ambas células desarrollaron cambios en el potencial de membrana mitocondrial similar a la valinomicina, y disminuyó la expresión de los genes p53 y BcL-2.

Conclusiones: El 1-O-undecilglicerol mostró actividad citotóxica selectiva en células A375 al compararlo con cultivo primario de fibroblastos humanos. Su toxicidad es mediada por necrosis, probablemente por eventos mitocondriales y disminución en la expresión de P53 y BcL-2. Ello sugiere que el 1-O-undecilglicerol pudiera ser útil en combinación con otros quimioterapéuticos en el tratamiento del melanoma.

Palabras Clave: Alquilgliceroles, citotoxicidad, cultivo primario de fibroblastos melanoma, necrosis.

INTRODUCTION

Shark liver oil (SLO) is successfully used as therapy for several diseases, i.e. cancer, inflammation (Iannitti and Palmieri, 2010). Obtained from sharks that live in deep cold waters, it contains alkylglycerols (AKG) and squalene as major components, among other lipids as unsaturated fatty acids (Bordier et al., 1996, Qian et al., 2014). SLO exhibited antineoplastic activity in Walker 256 tumor-bearing rats (Iagher et al., 2013), and in human mammary, ovarian carcinoma and prostate cancer cell lines (Krotkiewski et al., 2003). This activity is related to AKG content, and SLO with low content of AKG showed no activity in transformed cells (Davidson et al., 2007).

AKG are alkyl ethers of glycerol that represent about 20% of SLO (Hallgren and Larson, 1962), usually as follows: 12:0 (1-O-dodecylglycerol), 1-2%; 14:0 (1-O-tetradecylglycerol), 1-3%; 16:0 (1-O-hexadecylglycerol, chimyl alcohol), 9-13%; 16:1 n-7 (1-O-hexadec-7-enylglycerol), 11-13%; 18:0 (1-O-octadecylglycerol, batyl alcohol), 1-5%; 18:1 n-9 (1-O-octadec-9-enylglycerol, selachyl alcohol), 54-68%; 18:1 n-7 (1-O-octadec-7-enylglycerol), 4-6%, and other (< 1%). This family of compounds acts as biological response modifiers. As anti-tumor agents, they have been related to inhibitory growth, antimetastatic activity, anti-neoangiogenic action, and induce differentiation and apoptosis in cancer cells (Deniau et al., 2011, Molina et al., 2013). Immune stimulation properties (Kantah et al., 2012; Qian et al., 2014) and brain drug delivery enhancing (Hülper et al., 2013) have also been attributed to these substances. The antineoplastic actions of linear chain synthetic AKG in cancer cell lines is less documented, but Sotomayor demonstrated cytotoxic activity in MCF-7 breast cancer cells (Sotomayor et al., 2006).

Non-selective cytotoxicity and drug resistance are recurrent issues in most of available cancer chemotherapy. In this context, natural products, derived from plants or animals, have drawn attentions of many scientists (Block et al., 2015).

Melanoma is a malignant skin tumor induced by transformation of melanocytes, whose incidence rate is rapidly increasing in the world. Metastatic melanoma has a very poor prognosis, a fact that is

in part due to its high resistance to cytotoxic agents (Cattaneo et al., 2015). Hence, finding new anti-cancer compounds, to improve melanoma prognosis is a relevant research issue. In melanoma, immune regulation at the level of the tumor microenvironment is a key factor in treatment (Galon et al., 2012), so immunomodulating agents as AKG could be a right choice. The combination of immunomodulatory action and antineoplastic potential could be a good strategy in melanoma treatment. It becomes relevant to study the cell death mechanisms associated with AKG cytotoxicity in melanoma cells.

In this context, the aim of the present study is to evaluate the cytotoxic properties of synthetic 1-O-undecylglycerol (UDG) in primary cultured normal fibroblasts and A375 human melanoma cells. It was further studied the cell death induced by UDG, its relationship with gene expression of death associated proteins, and changes in mitochondrial potential.

MATERIAL AND METHODS

Compound and reagents

All reagents were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA), unless otherwise stated. 1-O-undecylglycerol was synthesized in Food and Pharmacy Institute, with 95% of purity, by Williamson´s synthesis, as previously reported (León et al., 2002). A stock solution of UDG was prepared in absolute ethanol at 50 mM. In every experiment, it was diluted in complete culture media to 150 μM as work solution.

Primary culture of human fibroblasts (FF) and A375 human melanoma cells

Human fibroblasts were aseptically collected from a biopsy of a healthy donor of plastic surgery. It was consented and approved for ethical committee of Pediatric Hospital Juan Manuel Márquez, La Habana, Cuba. Donor sample was negative for HIV, hepatitis, and other possible infectious diseases. The culture was obtained as described previously (González et al., 2009). The trypsin/EDTA medium was replaced with fresh medium three times, and fi-

broblast were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin, in T-25 cm² tissue culture flask (Corning Costar Co., Lowell, MA, USA), and maintained in an incubator with a humid atmosphere at 37°C under 5% CO2.

A375 cells were obtained from the American Type Culture Collection (ATCC CRL-1619). The cell line was cultured in RPMI-1640 (Hyclone, UT, USA), supplemented with 10% fetal bovine serum (Gibco, Life Technologies, USA) and 50 µg/mL gentamycin, in T-25 cm² tissue culture flasks. Cells were passaged twice a week at the ratio of 1:5 to keep an exponentially growing state.

Cell viability assay

The viabilities of A375 cells or primary cultured fibroblasts were evaluated using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) colorimetric assay (Mossman, 1983). Both types of cells (6 x 10³) were incubated for 24 or 48 h in 96-well microtiter cell culture plates, in the absence (control cells) or presence of UDG, in a 200 µL final volume. After the treatments, cells were incubated for 4 h at 37°C with 20 µL of MTT 5 mg/mL. The blue MTT formazan precipitate was dissolved in 50 µL of isopropyl alcohol, and after 30 minutes of agitation at 37°C, the absorbance was measured at 570 nm on a multiwell plate reader SUMA PR-521 (TecnoSuma, Cuba). Cell viability is expressed as a percentage of these values in treated cells, compared to the non-treated (control) cells.

Morphological changes visualization by DAPI and acridine orange-ethidium bromide (AO/EtBr) staining

For visualization of morphological changes, A375 or FF cells (4 x 10⁴) were cultured in 24 wells plate, and treated with low concentrations of UDG for 24 or 48 h, or untreated. FF cells were treated with 18.75 µM UDG, correspondent to 1/8 of maximum concentration assayed. A375 cells were treated with 5 µM UDG, which represents 1/16 of concentration that inhibits 50% control viability after the incubation period (IC$_{50}$). Fixation solution was added, 500 µL of acetic acid/methanol (1:2) for 2 min, and then plates were washed twice. 6-

diamidino-2-phenylindole (DAPI) staining (1 mg/mL) was added for 20 minutes, at 20°C in the dark.

For AO/EtBr assay, cells were stained with a 1:1 mix of 100 µg/mL acridine orange and ethidium bromide in 1x PBS solution for 5 minutes. In both cases, cells were then observed under a fluorescence microscope (Olympus IX71, camera Olympus DP72, Japan) at 10x magnification.

Mitochondrial membrane potential

The mitochondrial membrane potential was visualized using Mitochondria Staining Kit (for mitochondrial potential changes detection; Sigma, St. Louis, MO, USA), which includes JC-1 dye. The JC-1 probe is a cationic dye that exhibits membrane potential-dependent accumulation in mitochondria, as indicated by a fluorescence emission shift from green to red. Mitochondrial depolarization is specifically indicated by a decrease in the red-to-green fluorescence intensity ratio (Cossarizza et al., 1993). The cells were incubated for 1, 12 or 24 h in the absence (control) or presence of UDG (18.75 µM in FF cells, and 5.25 µM for A375 cells) or valinomycin 1 µg/mL. After the JC-1 dying, fresh media was added, and observed under a fluorescence microscope (Olympus IX-71, Japan), with 480 nm and 520 nm filters. Fields were photographed with an Olympus DP-72 camera (Olympus, Japan).

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from treated and control cells, A375 and FF, at 24 and 48 h after treatment, using 1 mL Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction.

The extracted RNA was dissolved in RNase-free water. The RNA concentration was determined using spectrophotometer (Bio Photometer plus, Eppendorf). Equivalent volume for 2 µg of RNA was reverse transcribed using 2 µL of reverse transcriptase (RT). RT was carried out for 60 min at 42°C in a thermocycler (Nashita 4196, Spain). PCR was performed in a total reaction volume of 25 µL that contained 5 µL of cDNA solution and 1.25 µL of sense and antisense primers. The primers for GAPDH, p53 and Bcl-2 in forward and reverse were as follows: GAPDH (200 bp): 5'-

CCTTCCTGGGCATGGAGTCCTG-3' and 5'-GGAGCAATGAGCTTGATCTC-3'; p53 (100 bp): 5'-GGGTTAGTTTACAATCAGCCACATT-3' and 5'-GGCCTTGAAGTTAGAGAAAATTCA-3'; Bcl-2 (100 bp): 5'-CAGGTCCTCTCAGAGGCAGATAC-3' and 5'-CCTCTCCAGGGACCTTAACG-3'.

PCR was performed using the following procedure: 59^0C for 1 min followed 35 cycles of 94^0C for 1.5 min and 72^0C for 1 min, and completed with one cycle at 72^0C for 7 min. For visualization, PCR products were analyzed on a 2% agarose gel. The gel images and relative intensity of each band were obtained using Quantity one software (Bio-Rad, USA), after normalized to the corresponding GAPDH band.

Statistical analysis

The software program GraphPad Prism 5.0 (GraphPad Software Inc., USA) was used for statistical analysis. The data are expressed as mean ± standard error of mean (SEM). Comparisons between the different groups were performed by the one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test. Differences at $p < 0.05$ were considered statistically significant. The IC_{50} values for cell viability were estimated using a non-linear regression algorithm.

RESULTS

Cell viability assay

Incubation of A375 cells with UDG (Fig. 1A-B) promoted significant decrease in cell viability in concentration and time-dependent manner, as estimated by the MTT assay. The assessed IC_{50} value was 94.59 μM for 24 h and 82.06 μM for 48h. FF cells viability was slightly affected by UDG treatment for 24 h in a concentration-dependent manner. The IC_{50} for these cells was not reached for assayed concentrations. At 48 h (Fig. 1B), FF cells keep their viability, and A375 cells are more affected by UDG from 75 μM. Accordingly, these results reveal that UDG exhibited more selectively activity against melanoma cells, with low cytotoxicity to normal fibroblasts.

Morphological changes of FF and A375 cells by AO/EtBr and DAPI staining

Cells were stained with AO/EtBr to visualize cell death after 24 and 48 h of treatment. Morphological changes in A375 and FF cells were observed under the fluorescence microscope after exposure to UDG and shown in Fig. 2. After 24 h both cell lines were affected by UDG, and almost all cells exhibited orange-red fluorescence, indicating increased membrane disruption; and looked intact, with nuclear morphology resembling that of viable cells, which appears to be a necrotic event. At 48 h A375 cells showed clear growth inhibition, with an orange nucleus, determining necrotic events. FF cells exhibited yellow nucleus, as a signal of recovery of their viability. The absence of DNA fragmentation was confirmed by DAPI staining. Neither A375 nor FF cells showed apoptotic characteristics when stained with DAPI.

Changes in mitochondrial membrane potential

To explore the effects of UDG on the mitochondrial membrane potential, FF and A375 cells were cultured with UDG or valinomycin for 1, 12 and 24 h, and then processed for confocal microscopy analysis after being stained with fluorescent probe JC-1. The dye enters selectively into the mitochondria and reversibly changes its color from red to green as the membrane potential decreases (Cossarizza et al., 1993). After treatment with UDG, the cells showed increased green fluorescence similar to valinomycin as a positive control, in a time-dependent manner (Fig. 3). It suggests that UDG disrupts the mitochondrial membrane potential, resulting in the cytosolic accumulation of monomeric JC-1.

p53 and BcL-2 gene expression using RT-PCR

The expression levels of p53 and BcL-2 genes in FF and A375 cells were evaluated to explore the possible mechanism of UDG induced necrotic cell death by RT-PCR and agarose gels band semi-quantitation. In both cells p53 gene expression decreased in a time-dependent manner, and also, BcL-2 exhibited a similar pattern (Fig. 4).
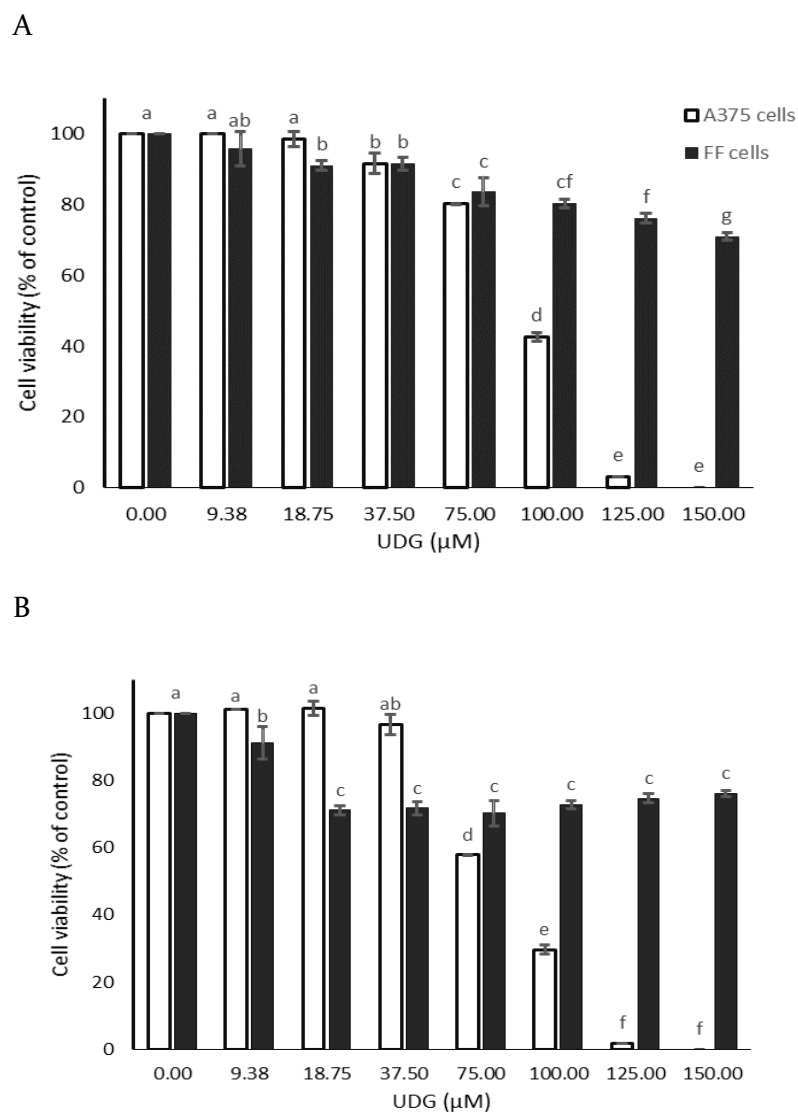
A

B

Figure 1. Effects of 1-O-undecylglycerol (UDG) on A375 cells and normal fibroblasts (FF) viabilities, estimated by the MTT assay. Panels **(A)** and **(B)** display the effects at 24 and 48 h, respectively.

The bars are mean ± SEM of three experiments. Different letters in the same series symbolize significant differences ($p < 0.05$) by ANOVA and post hoc Newman Keuls tests.
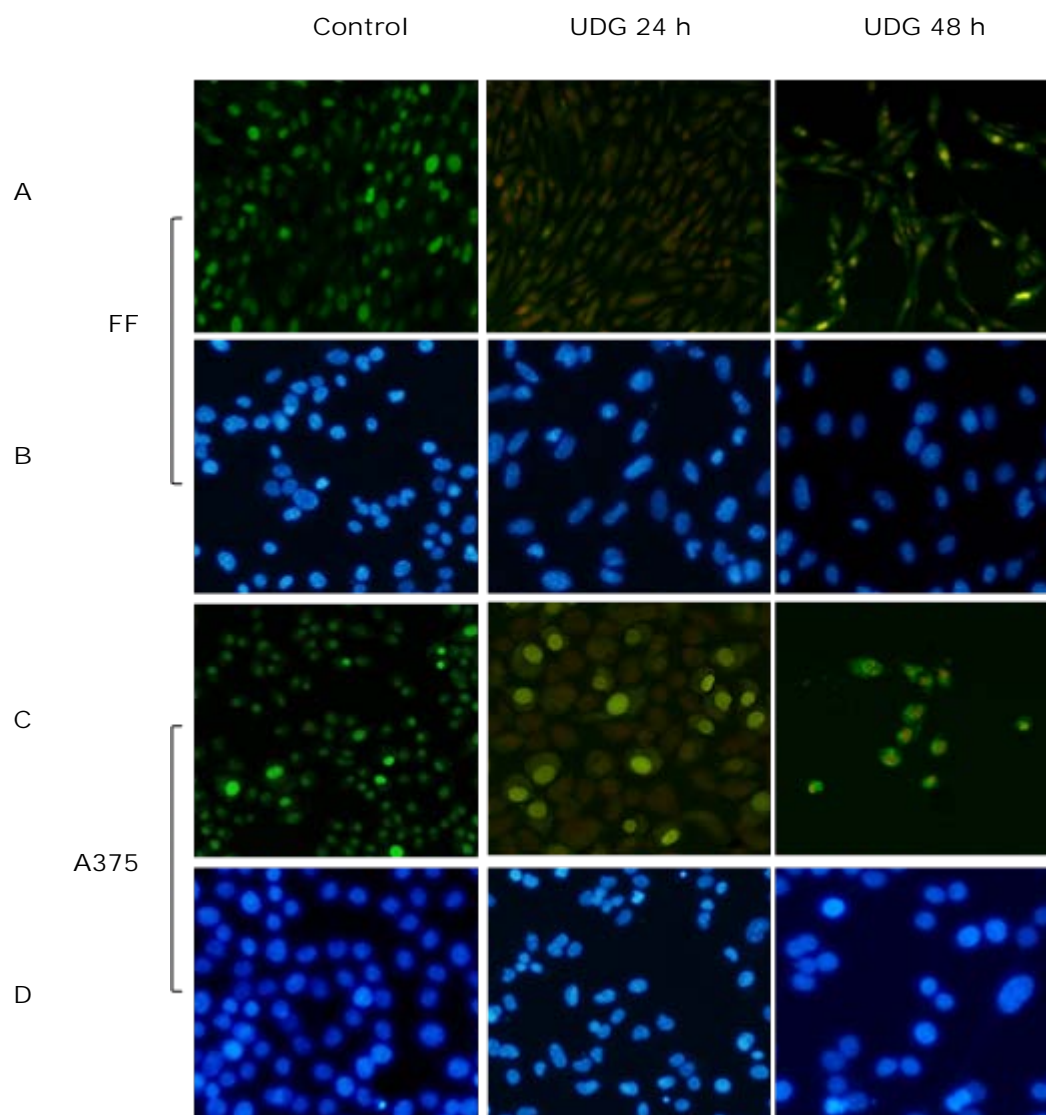
Figure 2. Effects of 1-O-undecylglycerol (UDG) on morphological changes in fibroblasts (FF) and A375 cells, detected by AO/EB double fluorescence (green-orange) and DAPI staining (blue).
FF and A375 cells were incubated with UDG 18.75 µM and 5 µM, respectively for 24 or 48 h. **(A)** FF cells stained with AO/EB and **(B)** with DAPI. **(C)** A375 cells stained with AO/EB and **(D)** with DAPI.
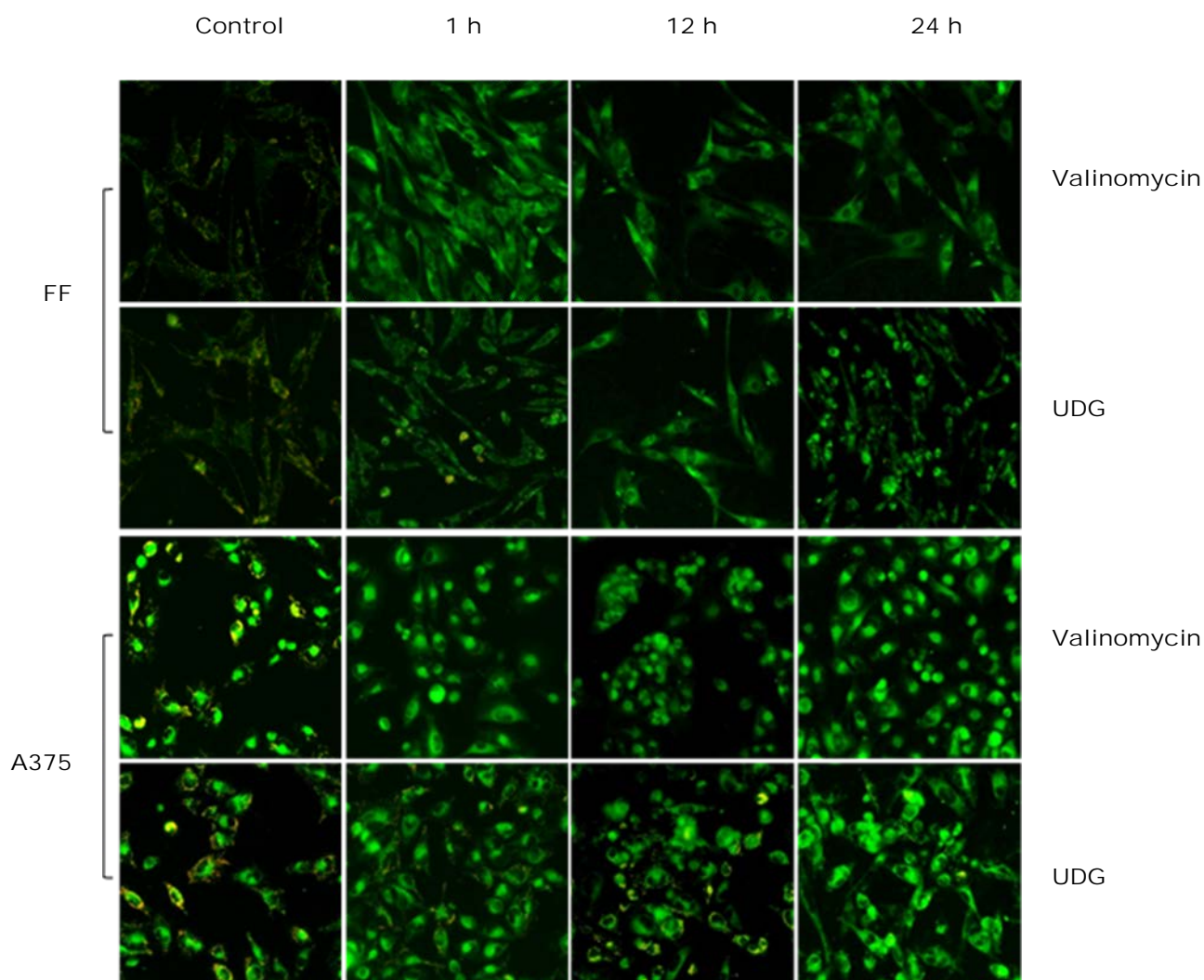Cells were observed using fluorescence microscopy (160X) after staining.

Figure 3. Effects of 1-O-undecylglycerol (UDG) and valinomycin on mitochondrial membrane potential changes in fibroblasts (FF) and A375 cells, detected by JC-1 staining.
FF and A375 cells were incubated with UDG 18.75 μM and 5 μM, respectively, or valinomycin 1 μg/mL for 1, 12 and 24 h.
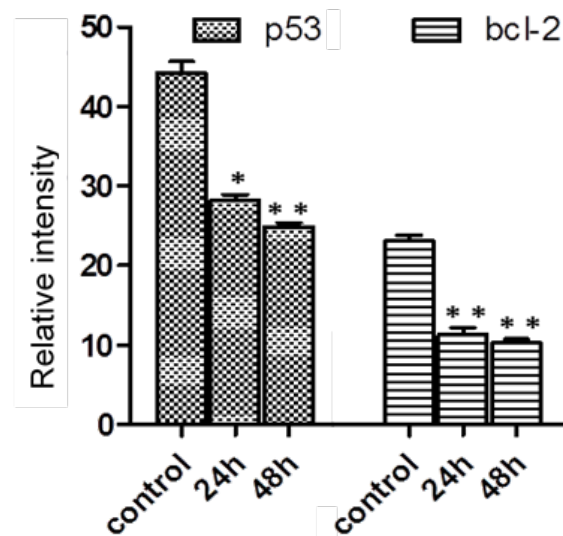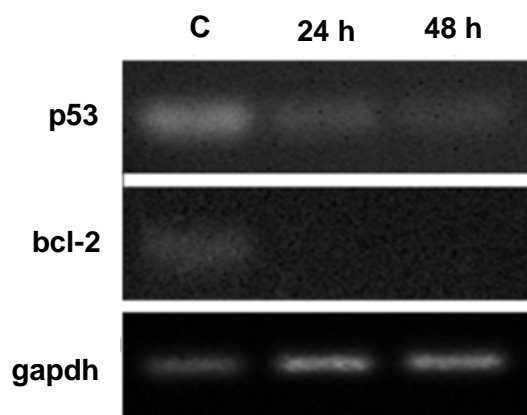Cells were observed using fluorescence microscopy (160X) after staining.
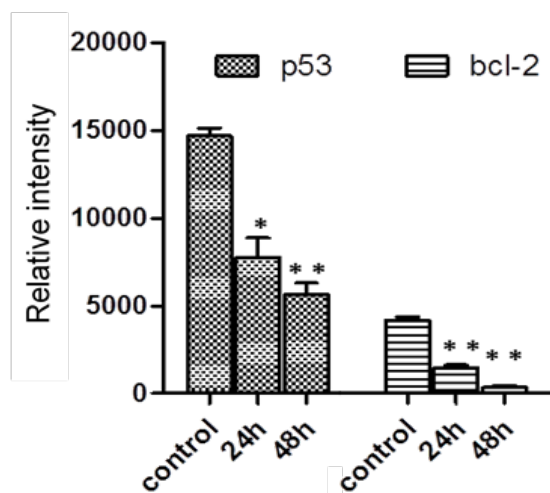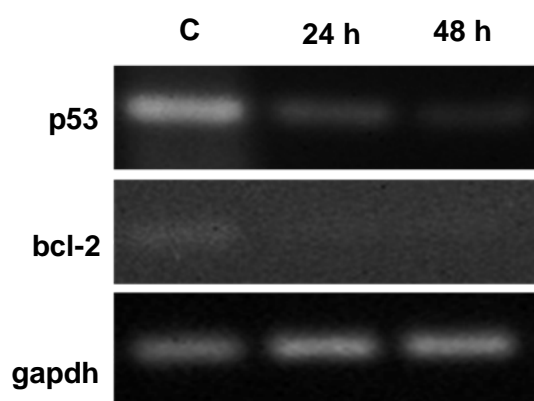
Figure 4. Semi-quantitative RT-PCR analysis of p53 and BcL-2 gene expression in fibroblasts **(A)** and A375 **(B)** cells, control (C) and treated with 1-O-undecylglycerol (UDG) for 24 or 48 h, with GAPDH as housekeeping gene. FF and A375 cells were incubated with UDG 18.75 μM and 5 μM, respectively.

The bars are mean ± SEM of three experiments. *p<0.05, ** p<0.01 respect to the control as measured by 1-way ANOVA.

DISCUSSION

Melanomas cause over 76% of skin cancer deaths annually. Their incidence continues to rise at an alarming rate (Siegel et al., 2015). They are among the most resistance cancers to chemotherapeutic agents. Thus, there is an urgent need to ex-plore other alternative and targeted therapies (Hutchinson, 2015). Alkyl-ether lipids from SLO have shown selective effects in inhibiting the proliferation of neoplastic cells, with practically no effects on normal cells (Krotkiewski et al., 2003), similar to

their alkylphospholipid synthetic analogues (van Blitterswijk and Verheij, 2013).

In the present study, the cytotoxic effects of UDG, a synthetic AKG, were analyzed in primary cultured human fibroblasts FF and A375 human melanoma cells. UDG exhibited selective antiproliferative activity on A375 cells, and FF cells were resistant to the treatment after 48 h, in a concentration-dependent manner (Fig. 1A-B).

Although AKG have been previously studied for anticancer properties (Ianniti and Palmieri, 2010; Deniau et al., 2011), such activity by UDG is here reported for the first time. The induction of apoptosis and/or necrosis by shark liver oil has been attributed to the presence of AKG on its composition (Krotkiewski et al., 2003), but UDG has not been reported as a usual component of that oil.

For a long time, necrosis has been considered as a merely accidental cell death mechanism and was defined by the absence of morphological traits of apoptosis or autophagy, but now is clear that necrotic cell death has a prominent role in multiple physiological and pathological settings (Galluzzi et al., 2012).

Multidrug-resistant tumors remain susceptible to necrotic death (Sasi et al., 2009). In this way, necrosis should become a focus of study in determining novel approaches to cancer prevention and treatment. To confirm if necrosis was present in UDG induced cell death, cells were stained with AO, which permeates all cells and the nuclei become green, and EtBr, which is only taken up when cytoplasmic membrane integrity is lost, and their nuclei are stained orange/red (Kasibhatla et al., 2006). UDG induced morphological changes consistent with necrotic events in both cell lines, but FF cells required higher concentration, and seems to get recovered, as shown in Fig. 2, where necrotic characteristics appeared in treated cells, and non-apoptotic features were detected by DAPI staining.

The primary molecular mechanism behind necrosis is believed to be the rapid depolarization of membranes leading to swelling and rupture of the plasma membrane (Lemasters et al., 1998), and AKG can incorporate into membrane phospholipids and modify lipid structure of membranes (Marigny et al., 2002) as part of their mechanism of cell death induction.

A hallmark of necrotic cell death is mitochondrial dysfunction itself, such that if mitochondrial structure function are preserved during a necrotic insult, the cells affected typically no longer die (Crompton, 1999). A rapid decrease in mitochondrial membrane potential ($\Delta\Psi$m) was detected after 1h of treatment with UDG, and at 24 h the effect was similar to valinomycin, used as positive control (Fig. 3). Edelfosine, an ether lipid structurally related with AKG, also affected $\Delta\Psi$m about cell death (Vrablic et al., 2001), so the glyceryl ether bond could be a factor in mitochondrial disruption induced by those compounds.

The mitochondrial loss of internal permeability is a primary factor for the loss of $\Delta\Psi$m and adenosine triphosphate (ATP) that promotes osmotic shock to the organelle. This is responsible of necrosis, due to loss of ion homeostasis and cell integrity (Bonora and Pinton, 2014). Mitochondria could be a potential target of selective activity of UDG because its membrane potential was affected in both fibroblasts and melanoma cells, but only melanoma cells exhibited significant cell death, so the relative number of mitochondria increased by Warburg effect (Kim and Dang, 2006) could be responsible for exposing these cells to necrosis induced by UDG.

Taking into account that A375 cells showed a significant decrease of cell viability in MTT assay (Fig. 1A-B), and a decrease in mitochondrial membrane potential, it should be extra-mitochondrial components sensitive to MTT dye. Recent research on the site of reduction of MTT has refuted the dogma that MTT is always reduced in the mitochondria (Bernas and Dobrucki, 2002). Studies indicate that NADH is responsible for most MTT reduction and is associated not only with the mitochondria but also with the cytoplasm and associated with membranes in the endosome/lysosome compartment as well as the plasma membrane. MTT reduction at the plasma membrane may account for observations of formazan crystals occurring outside of cells.

Necrosis was accompanied by a decrease of P53 and BcL-2 gene expression after treatment with UDG (Fig. 4). It is consistent with the fact that wild-type P53 in melanoma fails to function as a tumor suppressor, and the expression of its target genes is defective. So, knockdown of P53 in mela-

noma cells resulted in decreased proliferation (Avery-Kiejda et al., 2011), as was observed in A375 cells treated with UDG. The interference in the regulation of BcL-2 could be a factor in UDG necrosis induction although the role of BcL-2 in necrosis has not been extensively studied. It has been recognized as a protein that inhibits apoptosis, leading to cell survival (Tomek et al., 2012), which overexpression protected cancer cells from cell death induced by ether lipid edelfosine (Gajate and Mollinedo, 2014).

CONCLUSIONS

This study is the first to demonstrate cytotoxic activity of UDG. This activity is selective in A375 melanoma cells when compared with primary cultured fibroblast. A particular finding is that predominant cell death is necrosis, associated with mitochondrial events and decrease in P53 and BcL-2 expression. The results suggest that UDG could be a useful strategy to combine with other chemotherapeutic agents in melanoma treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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