The Novel Anticancer Drug Hydroxytriolein Inhibits Lung Cancer Cell Proliferation via a Protein Kinase Ca– and Extracellular Signal-Regulated Kinase 1/2–Dependent Mechanism

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ABSTRACT

Membrane lipid therapy is a novel approach to rationally design or discover therapeutic molecules that target membrane lipids. This strategy has been used to design synthetic fatty acid analogs that are currently under study in clinical trials for the treatment of cancer. In this context, and with the aim of controlling tumor cell growth, we have designed and synthesized a hydroxylated analog of triolein, hydroxytriolein (HTO). Both triolein and HTO regulate the biophysical properties of model membranes, and they inhibit the growth of non–small-cell lung cancer (NSCLC) cell lines in vitro. The molecular mechanism underlying the antiproliferative effect of HTO involves regulation of the lipid membrane structure, protein kinase C–α and extracellular signal-regulated kinase activation, the production of reactive oxygen species, and autophagy. In vivo studies on a mouse model of NSCLC showed that HTO, but not triolein, impairs tumor growth, which could be associated with the relative resistance of HTO to enzymatic degradation. The data presented explain in part why olive oil (whose main component is the tracyglycerol triolein) is preventive but not therapeutic, and they demonstrate a potent effect of HTO against cancer. HTO shows a good safety profile, it can be administered orally, and it does not induce nontumor cell (fibroblast) death in vitro or side effects in mice, reflecting its specificity for cancer cells. For these reasons, HTO is a good candidate as a drug to combat cancer that acts by regulating lipid structure and function in the cancer cell membrane.

Introduction

Non–small-cell lung cancer (NSCLC) accounts for about 80% of all lung cancers, and it is one of the leading causes of cancer deaths (Reck et al., 2013). The hypothesis that proliferation in cancer depends on the activity of oncogenic pathways has served to focus the design and discovery of anticancer drugs targeted against specific proteins (Lynch et al., 2004; Paez et al., 2004). However, lung cancer has a high 5-year mortality rate due to drug resistance and other problems (Sharma et al., 2007), highlighting an unmet clinical need and the fact that innovative approaches are required to develop new therapies against this devastating condition. We previously defined a new therapeutic approach called membrane-lipid therapy, whereby drugs interact with membrane lipids rather than proteins or nucleic acids (Escribá, 2006). Membrane-lipid therapy targets cell membranes, regulating their structure, the activity of relevant signaling proteins that associate with them, and their downstream events (Escribá et al., 2008). Drugs acting on the membrane are both efficacious and specific, in part because they regulate upstream events that affect several proteins. Despite the relatively recent description of this concept (Escribá, 2006), there are already two molecules, whose mechanism of action is based on regulating membrane composition and structure, that have shown safety and efficacy against cancer in clinical trials (Clinicaltrials.gov identifiers: NCT01792310 and NCT02201823, respectively) (Martin et al., 2013; Ibarguren et al., 2014).

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ABBREVIATIONS: DCFH-DA, 2′,7′-dichlorofluorescein diacetate; ERK, extracellular signal-regulated kinase; GF109203X/GF, 2-[1-(3-dimethylamino propyl)-5-(phenylaminocarbonyl)-3,4-\text{-}dichloro fluorescene diacetate; MEK, mitogen-activated protein/extracellular signal-regulated kinase; NSCLC, non–small-cell lung cancer; LC3B-I/II, light chain 3 B types I/II; LDH, lactate dehydrogenase; LPL, lipoprotein lipase; OA, oleic acid; PBS, phosphate-buffered saline; PKCα, protein kinase Ca; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; ROS, reactive oxygen species; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; XTT, sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4 methoxy-6 nitro)benzene sulfonic acid hydrate) 5-dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromide.
Lipids play several roles in the cell, serving as a source of energy and a supply of building blocks for membrane biosynthesis but also acting as signaling molecules. Moreover, membrane lipid composition may reflect different stages of a cell, and, indeed, lipid synthesis and localization is actively regulated by cells during cytokinesis (Attila-Gokcumen et al., 2014). In this context, it has been shown that the lipid composition of cancer cells differs from that of their normal counterparts (Meng et al., 2004).

There is now considerable evidence that the membrane lipid composition can be modified through diet. The protective effect of the Mediterranean diet against the development of cancer is well known; more specifically, high olive oil consumption is associated with a lower incidence of lung cancer (Fortes et al., 2003), breast cancer, and colon cancer (Stark and Madar, 2002). The main constituent of extra virgin olive oil is the triacylglycerol triolein, which contains three oleic acid (OA) molecules (70%–80% in olive oil). In vitro studies have shown that OA possesses antitumor activity that depends on the type of cancer, and it is linked to the inhibition of angiogenesis (Lamy et al., 2014), metastasis (Suzuki et al., 1997), and proliferation (Hughes-Fulford et al., 2001) as well as the induction of apoptosis (Menendez et al., 2005). OA has a protective effect against chemically-induced lung tumorigenesis (Yamaki et al., 2002), and it inhibits metastasis of Lewis lung carcinoma, although it has no significant effect on the primary solid tumor (Kimura, 2002).

The antitumor potential of triolein is limited due to its use as a cell fuel. Triolein is degraded by lipoprotein lipases (LPL), and the fatty acids released are used to provide energy via β-oxidation. We designed hydroxytriolein (HTO) as a more stable triolein analog (Fig. 1A) that is metabolized by LPL at a markedly lower rate than triolein. Moreover, the resulting hydroxoylate molecule cannot be degraded by β-oxidation but by α-oxidation, which is a slower catabolic pathway. Because both synthetic lipids (the hydroxylated triglyceride and the hydroxylated fatty acid) have antitumor activity, their efficacy is in part due to their enhanced metabolic stability.

In this study, we have used several human NSCLC lines as a model to evaluate the antitumor potential of HTO and its natural analog from olive oil, triolein. Accordingly, we found that substitution of three hydrogen atoms by hydroxyl moieties increased the antitumorigenic and therapeutic potential of triolein.

**Materials and Methods**

**Reagents and Antibodies.** We purchased 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) from Avanti Polar Lipids (Alabaster, AL), and HTO from Lipopharma (Palma de Mallorca, Spain). Triolein, oleic acid, rhodamine B, and the fluorescent probe HTO from Lipopharma (Palma de Mallorca, Spain). We obtained 1,2-diolein from Larodan (Malmö, Sweden), and HTO from Lipopharma (Palma de Mallorca, Spain). We purchased 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) from Avanti Polar Lipids (Alabaster, AL), and HTO from Lipopharma (Palma de Mallorca, Spain). Triolein, oleic acid, rhodamine B, and the fluorescent probe HTO from Lipopharma (Palma de Mallorca, Spain).

**Cell Lines and Culture.** Human NSCLC cells (A549, Calu-1, HOP-62, and NCI-H838) and normal lung fibroblasts (MRC-5) were obtained from the American Type Culture Collection (Manassas, VA). The cells were incubated at 37°C in a humidified atmosphere of 5%
CO₂ in RPMI 1640 (A549, Calu-1, HOP-62, and NCI-H838) or Dulbecco’s modified Eagle's medium (MRC-5) supplemented with 10% fetal bovine serum (v/v), 100 U/ml penicillin, and 0.1 µg/ml streptomycin. MRC-5 medium was also supplemented with 1% of nonessential amino acids. Media and nonessential amino acids were obtained from Sigma-Aldrich, penicillin-streptomycin from Biowest (Nuaillé, France), and fetal bovine serum from Biosera (Boussens, France).

HTO Incorporation into Cells. HTO emulsion containing 1 mM rhodamine B was stirred for 24 hours and then sonicated for 2 minutes. Cells were seeded at a density of 5 × 10⁵ cells/well in six-well plates, and the next day they were treated with the HTO emulsion containing rhodamine B. The cells were then fixed with 4% paraformaldehyde, and their nuclei were stained with Hoechst 33342 (Thermo Scientific, Waltham, MA) before visualizing them on a Nikon Eclipse TG 2000-S fluorescence microscope (Nikon, Tokyo, Japan).

Cell Proliferation Assays. Cell number was measured by trypan blue exclusion, whereby the cells were stained with trypan blue and the viable cells were counted in a Bürker chamber. Metabolically active cells were measured using the cell proliferation kit II from Roche (Basel, Switzerland). Briefly, cells were cultured in 96-well plates at a density of 3 × 10⁴ cells per well in the presence or absence of HTO or triolein and at the concentrations indicated in the figures. After different periods, the viable cells in the plate were measured, mixing XTT (sodium 3-[1-(phenylamino)carbonyl]-3,4-tetrazolium-bis (4 methoxy-6-nitro) benzene sulfonic acid hydrate) 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) with N-methyl dibenzopryrazine methyl sulfate and adding it to the cell culture medium according to manufacturer’s recommendations (Roche). The cells were incubated at 37°C and in 5% CO₂ until the color developed, and the absorbance was determined at 495 nm using a microplate reader with a reference wavelength of 650 nm (FLUOstar Omega; BMG Labtech, Ortenberg, Germany).

Lactate Dehydrogenase Release. Lactate dehydrogenase (LDH) released from cells after drug treatment was measured with the Cytotoxicity Detection KitPLUS (Roche) according to manufacturer’s recommendations. Briefly, the cells were cultured in 96-well plates at a density of 3 × 10⁴ cells per well in the presence or absence of HTO and at the concentrations indicated in the figures. After 24 hours, 2.5% lysis buffer was added to some cells to assess the maximum release of LDH. Next, the cell culture medium was collected, and the LDH reagent was added to the medium and incubated at room temperature at dark until color developed; the absorbance was determined at 492 nm using a microplate reader (FLUOstar Omega). A test medium was used as a background control. The cytotoxicity was analyzed in comparison with control cells by use of the following equation: [(HTO treated – Untreated)/ (Maximum LDH – Untreated)] × 100.

LPL Activity. LPL activity was determined by measuring the levels of glycerol using a kit to test the triacylglycerol concentration according to the manufacturer’s instructions (BioSystems, Barcelona, Spain). For this purpose, triolein or HTO were dissolved in ether from Roche and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). The membranes were then blocked for 1 hour at room temperature in phosphate-buffered saline (PBS) containing 5% nonfat dry milk and 0.1% Tween 20 (blocking solution), and they were then incubated overnight at 4°C with a primary antibody diluted in blocking solution. The membranes were then washed 3 times for 5 minutes with PBS, and the antibody was detected with a donkey anti-mouse or donkey anti-rabbit IgG labeled with IRDye800CW (1:5000). Proteins were quantified by image analysis of the membranes scanned at 800 nm using the infrared imaging system Odyssey (Li-COR, Lincoln, NE). The α-tubulin content in the samples was determined by the same procedure and used as a loading control. The intensity of the signal was analyzed using the program Total Laboratory (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK).

Generation of Reactive Oxygen Species. A549 cells were seeded in 6 cm² plates at a density of 1 × 10⁵ cells/well, and cultured at 37°C in the presence or absence of HTO for 24 hours or 48 hours in 5% CO₂, as described earlier. The cells were then harvested, washed with PBS, and resuspended in 300 µl of 10 mM 2’;7’-dichlorofluorescein diacetate for 30 minutes at 37°C. Samples were then analyzed immediately on a flow cytometer EPICS XL-MCL (Beckman Coulter, Brea, CA) with a 488-nm excitation wavelength.

Lipid Separation. A549 cells were seeded in 10 cm² culture plates at a density of 4 × 10⁵ cells/well, and cultured in the presence or absence of HTO for 24 hours. Membrane lipids were extracted directly from a monolayer of approximately 1 × 10⁶ cells by chloroform/methanol extraction (Bligh and Dyer, 1959). Protein levels were measured using a modified Lowry assay according to the manufacturer’s instructions (Bio-Rad Laboratories). Individual neutral lipids classes were separated by thin-layer chromatography on silica gel-60 plates (20 x 20 cm; Whatman/GE Healthcare, Maidstone, Kent, UK) using petroleum ether/diethyl ether/acetic acid (75:25:1.3 by volume). After thin-layer chromatography separation, the plates were air-dried, sprayed with 8% (wt/vol) H₃PO₄ containing 10% (by volume). After thin-layer chromatography separation, the plates were air-dried, sprayed with 8% (wt/vol) H₃PO₄ containing 10% (wt/vol) CuSO₄, and charred at 180°C for 10 minutes before the lipids were quantified by photodensitometry. The lipid fractions were identified using 1,2-diolene, oleic acid, and triolein as standards.

Cell Fractionation. A549 cells were seeded in 25 cm² culture plates at a density of 2 × 10⁶ cells/well, and cultured in the presence or absence of HTO. Approximately, 4 × 10⁶ cells were used for cell fractionation, first homogenizing the cells with a 25G needle in 600 µl of lysis buffer (20 mM HEPES, 2 mM EDTA, 0.5 mM EGTA, 1.5 mM MgCl₂, 1 mM EDTA, 0.5 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 1% SDS, 5 mM iodoacetamide and 1 mM phenylmethylsulfonyl fluoride. The cell suspensions were disrupted by ultrasound (70% cycle) for 10 seconds at 50 W using a Braun Labsonic U probe (type probe) sonicator (Braun, Kronberg, Germany), and 30 µl aliquots were removed for protein quantification using a modified Lowry assay according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA). Subsequently, 30 µl of 10 x electrophoresis loading buffer (120 mM Tris-HCl [pH 6.8], 4% SDS, 50% glycerol, 0.1% bromophenol blue, and 10% β-mercaptoethanol) was added, and the samples were boiled for 5 minutes.

For immunoblotting, 30 to 50 µg of total protein from the cell lyses was resolved by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). The membranes were then blocked for 1 hour at room temperature in phosphate-buffered saline (PBS) containing 5% nonfat dry milk and 0.1% Tween 20 (blocking solution), and they were then incubated overnight at 4°C with a primary antibody diluted in blocking solution. The membranes were then washed 3 times for 5 minutes with PBS, and the antibody was detected with a donkey anti-mouse or donkey anti-rabbit IgG labeled with IRDye800CW (1:5000). Proteins were quantified by image analysis of the membranes scanned at 800 nm using the infrared imaging system Odyssey (Li-COR, Lincoln, NE). The α-tubulin content in the samples was determined by the same procedure and used as a loading control. The intensity of the signal was analyzed using the program Total Laboratory (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK).

Animals, Tumor Xenografts, and Treatments. NUDE (Swiss) Crl:NIH (Ico)-Fox1tm1 mice (5 weeks old, 30–35 g; Charles River Laboratories, Paris, France) were maintained in a thermostated cabinet with a sterile airflow (28°C) (Eluet GmbH Y Co. KG Labor- und Pharmatechnik, Tulln an der Donau, Austria) at a relative humidity of 40%–60%, on a 12-hour dark/light cycle and with autoclaved food and water supplied ad libitum.

To induce subcutaneous xenograft tumors, 7.5 × 10⁵ NSCLC A549 cells were inoculated into the dorsal area on one side of the animal, and after 1 week the tumors were visible. Animals were then

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randomly divided into groups with a similar mean tumor volume, and for 29 days they received daily oral treatment with HTO or triolein (400 mg/kg), or the vehicle alone (water). Tumor volumes (v) were calculated as v = W × L/2, where W is the tumor width and L is its length. All experiments were performed in accordance with the animal welfare guidelines of the European Union and the Institutional Committee for Animal Research of the University of the Balearic Islands.

**Data Analysis.** Statistical analyses were performed using GraphPad Prism 4.01 (GraphPad Software, San Diego, CA). Unless otherwise indicated, the data are expressed as the mean ± S.E.M. of at least three independent experiments with duplicate samples. The experimental groups were compared using one-way analysis of variance followed by the Bonferroni’s multiple-comparison test or Student’s t test. P < 0.05 was considered a statistically significant difference: *P < 0.05, **P < 0.01, and ***P < 0.001.

**Results**

**Triolein and HTO Regulate Membrane Lipid Structure.** We studied the effect of triolein and HTO on membrane lipid structure using differential scanning microcalorimetry and small-angle X-ray diffraction. The microcalorimetry showed no differences in the gel-to-fluid (Lβ-to-Lα) transition temperature of POPE model membranes in the presence or absence of either hydroxylated or nonhydroxylated triglyceride. By contrast, a drop in the lamellar-to-inverted hexagonal (Lα-to-HII) phase-transition temperature was observed in the presence of 1 mol% triolein or HTO (from 69°C to approximately 61°C and approximately 66°C, respectively; Table 1). Moreover, the presence of 5 mol% HTO dramatically reduced the phase-transition temperature, and it increased the temperature range in which the Lα and HII phases coexisted, which is associated with a reduction in the bilayer’s lateral surface pressure (from approximately 24°C; Supplemental Methods and Supplemental Fig. 1). This property of the bilayer influences the type and abundance of peripheral membrane proteins bound to the membrane.

**HTO Impairs the Proliferation and Viability of Human NSCLC Cell Lines.** To treat cells in culture we formulated HTO as a lipid emulsion in which the average dispersity when measured with the nanosizer (Supplemental Methods and Supplemental Fig. 1). This property of the membrane lamellar-to-hexagonal phase (HII)

<table>
<thead>
<tr>
<th>Model Membrane</th>
<th>Tm, °C</th>
<th>ΔH</th>
<th>TH, °C</th>
<th>ΔH</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPE</td>
<td>23.6 ± 0.2</td>
<td>4.42 ± 0.03</td>
<td>69.1 ± 0.5</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>Triolein</td>
<td>23.3 ± 0.3</td>
<td>4.55 ± 0.09</td>
<td>60.8 ± 3.3</td>
<td>0.24 ± 0.11</td>
</tr>
<tr>
<td>+ HTO</td>
<td>23.8 ± 0.3</td>
<td>5.78 ± 0.08</td>
<td>66.0 ± 0.9</td>
<td>0.55 ± 0.06</td>
</tr>
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ΔH, transition enthalpy; Tm, gel-to-liquid crystalline (lamellar) phase-transition temperature. *P < 0.01. +POPE versus triolein or HTO, P < 0.01.

When the proliferation of NSCLC cells was evaluated, exposure to HTO reduced the number of viable cells in a dose- and time-dependent manner (Fig. 2, A–F). Cells showed standard growth dynamics in the absence of HTO, but exposure to 150–300 μM HTO induced NSCLC cell death (Fig. 2, G–J; Supplemental Fig. 2B). To determine whether the drop in the number of viable A549 cells was due to cell death or to cell cycle arrest, we examined the extent of apoptosis by flow cytometry of propidium iodide and annexin V-labeled cells. In this context, the presence of HTO significantly increased the number of early and late apoptotic cells, concomitant with a decrease in the number of viable cells (Supplemental Methods and Supplemental Fig. 2C).

We also analyzed the cytotoxicity of HTO in nontumor fibroblast (MRC-5) cells, and the IC50 value proved to be higher than that for A549 cells (>500 and 36.3 μM, respectively; Fig. 2K), which reflects the lack of signs of HTO toxicity observed in animals. These results further suggest that HTO acts specifically against tumors.

Triolein and HTO provoked a reduction in NSCLC cell number at all studied times; short incubation times (24 hours) showed no significant difference between HTO and triolein IC50 (Fig. 2, C–F). After 72 hours, the IC50 values obtained for HTO and triolein in A549 (32 ± 3.9 μM versus 60.85 ± 8.5 μM) and NCI-H838 (49.8 ± 16.7 μM versus 301.5 ± 61.6 μM) were statistically significantly different (P < 0.05). Moreover, in NCI-H838 the difference in the IC50 values was already statistically significant (P < 0.01) after 48 hours (117.74 ± 15.4 μM versus 401.9 ± 55.2 μM).

**LPL Differentially Degrades Triolein and HTO.** When we measured LPL activity in vitro, degradation was 4-fold slower for HTO than for triolein (Fig. 2L), which indicates that the metabolism of HTO in vivo could be impaired by the hydroxyl groups presence in the acyl moieties.

**HTO Activates the Mitogen-Activated Protein Kinase (ERK) Pathway.** Sphingomyelin levels increased significantly in the membranes of cells exposed to HTO (300 μM) for 48 hours (Supplemental Methods and Supplemental Fig. 2, D and E). Changes in membrane lipid composition of tumor cells have been related to altered cell signaling that might impair tumor growth (Pardini, 2006). We analyzed the phosphorylation status (i.e., activity) of the mitogen-activated protein kinase pathway signaling proteins ERK and MEK in human A549 cells, and we found a significant increase in phosphorylated ERK and MEK after 24 hours and 48 hours of exposure to HTO (Fig. 3A; Supplemental Fig. 3, A and B). Increases in ERK phosphorylation (activation) were statistically significant in NCI-H838 cells, but did not reach statistical significance in Calu-1 and HOP-62 cells (Fig. 3, B and C).

**HTO Induces Reactive Oxygen Species Production.** Signaling mediated by reactive oxygen species (ROS) has been associated with sustained ERK activation (Cagnol and Chambard, 2010), and a 24- or 48-hour exposure to HTO increased ROS levels in a dose-dependent manner (Fig. 3D). To investigate how ROS production influences the activity of HTO against cancer, we evaluated the effects of HTO on A549 cells in the presence or absence of the ROS scavengers α-tocopherol (vitamin E) or N-acetylcysteine. Of these, α-tocopherol alone abolished the increase in ROS levels induced by HTO (Fig. 3E) and blocked the HTO-induced death of A549 cells (Fig. 3F).
In addition to its antioxidant activity, α-tocopherol activates the diacylglycerol kinase that induces the conversion of diacylglycerol to phosphatidic acid (Lee et al., 1999). Diacylglycerol increased significantly in cells exposed to HTO (Fig. 4C), activating PKCa, such that this kinase may be inhibited by α-tocopherol. To confirm whether PKCa is involved in the antitumor mechanism of HTO, we investigated the cytosolic-to-membrane translocation of this enzyme. HTO impaired...
PKCα translocation to cytosol, and PKCα clearly accumulated in the membrane fraction of HTO-treated cells, which is associated with its enzymatic activation (Fig. 4, A and B).

**Antiproliferative Effects of HTO and Triolein Are Mediated by PKCα.** To determine whether PKCα is involved in the inhibition of A549 cell growth provoked by HTO, A549 cells were exposed to HTO or triolein in the presence or absence of general or specific PKC inhibitors. The antiproliferative effect of both triolein and HTO was reverted in the presence of α-tocopherol or the PKC inhibitor GF, further demonstrating the involvement of PKC in the effect of HTO against tumors (Fig. 5A). Because PKCα activation is associated with cancer cell differentiation, we analyzed gelsolin as a marker of differentiation. Both HTO and triolein enhanced the levels of gelsolin protein, an increase that was impeded by α-tocopherol and GF (Fig. 5, B and C).

**ERK Activation through PKC Is Responsible for the Effect of HTO on A549 Cell Growth.** ERK phosphorylation (i.e., activation) increased markedly and significantly in the presence of HTO and to a lesser extent in the presence of triolein (Fig. 5, B–D). By contrast, ERK was not activated in the presence of the PKC inhibitors GF or α-tocopherol, indicating that PKC was responsible for the rise in ERK phosphorylation. ERK is directly phosphorylated by MEK; indeed, HTO and triolein failed to inhibit A549 cell growth in the presence of the MEK inhibitor U0126 (Fig. 5E). General or specific PKC and MEK inhibitors reverted the HTO cytotoxicity in all studied cell lines, although they only reverted the HTO antiproliferative effect in Calu-1 cells (Fig. 5, F and G).

**HTO Induces Autophagy.** Autophagy serves as a pathway of recycling intracellular components; it occurs at a basal level in all cells and increases in response to multiple stressors such as ROS accumulation and PKCα activation (Kroemer et al., 2010; Sridharan et al., 2011). Moreover, it has been reported that excessive levels of autophagy can induce a form of cell death called autophagic cell death (Liu and Levine, 2015). To investigate the effect of HTO on autophagy, we analyzed the levels of the autophagy marker LC3B-II.
membrane fraction. Na⁺K⁺-ATPase was used as a membrane marker.

10 seconds, 1 hour, and 24 hours in the presence or absence of HTO
membranes. (A and B) PKCα recruitment to A549 cell membranes after
PKCα translocation to cell membranes. (A and B) PKCα recruitment to A549 cell membranes after 10 seconds, 1 hour, and 24 hours in the presence or absence of HTO (150 μM). Representative immunoblots are shown. C, cytosolic fraction. M, membrane fraction. Na⁺K⁺-ATPase was used as a membrane marker. Bars correspond to the mean ± S.E.M. values of one (HTO 10 seconds) to two independent experiments. *P < 0.05, **P < 0.01 (Student’s t test).

(A) A549 cells were maintained in the presence or absence of HTO (150 μM, Vit.E) for 24 hours before lipids were extracted and separated by thin-layer chromatography to measure the diacylglycerol (DAG), triacylglycerides (TG), and free fatty acids (FFA).

Upon induction of autophagy, LC3B-I is converted to LC3B-II by an ubiquitin-like conjugation system that conjugates a phosphatidylethanolamine moiety to LC3B-I. LC3B-II is more hydrophobic than LC3B-I and thus migrates more rapidly in SDS-PAGE compared with LC3B-I. HTO induced a marked increase in LC3B-II levels in all studied cell lines (Fig. 6A–C).

Higher levels of LC3B-II could be associated with either enhanced autophagosome synthesis or reduced autophagosome turnover. To distinguish between these two options, we treated the cells with bafilomycin A1, a known inhibitor of the late phase of autophagy that acts by inhibiting vacuolar H⁺-ATPase, thereby inhibiting lysosome acidification and increasing the levels of LC3B-II. Our results showed that bafilomycin A1 increased the levels of the autophagy marker LC3B-II both in control and HTO-treated cells (Fig. 6D), which indicates efficient autophagic signal propagation in HTO-treated cells.

HTO Inhibits Tumor Progression in a Xenograft Model of Human Lung Cancer. The Irwin test was performed in NUDE (Swiss) Crl:NU (Ico)-Foxn1nu mice, and no abnormalities were detected in the animal behavior at concentrations as high as 1500 mg/kg (Supplemental Tables 2 and 3). The efficacy of HTO and triolein against NSCL was tested in an A549 cell xenograft model of human lung cancer. HTO treatment (400 mg/kg per day) induced a marked and significant reduction in A549 tumors, whereas triolein appeared to only modestly reduce the volume of human lung cancer–derived tumors in a manner that failed to reach statistical significance (Fig. 7A). During the treatment, no changes in mice weight (Fig. 7B), mortality, or side effects were observed in association with HTO or triolein treatment, indicating that their oral administration was well tolerated. Moreover, euthanasia at the end of the treatment and autopsy to some animals revealed no macroscopic signs of toxicity on the lung, kidney, heart, or stomach; no fat accumulation was observed in the liver, and normal feces were found in the intestine.

Discussion

We have designed and synthesized a hydroxy-triglyceride (HTO) analogous to triolein that shows antitumor activity in vitro and that prevents tumor progression in vivo (Fig. 8). Lipid intake has been shown to modify the composition of cell membranes, regulating their biophysical properties and influencing protein-membrane interactions, thereby affecting cell signaling, metabolism, and viability (Spector and Burns, 1987; Barceló-Coblijn et al., 2011; Teres et al., 2012). HTO and triolein induce changes in membrane lipid composition and structure that might be associated with their antitumor activity, as shown previously for other anticaner drugs (Martínez et al., 2005). The triglyceride content of the cell membrane is lower than that of other lipid species (Lerique et al., 1994). However, exposing cells to HTO and triolein not only increases triacylglycerol content but also free fatty acids and diacylglycerol, which in turn increase the nonlamellar (HII) phase propensity of membranes (our study and Ibarguren et al., 2014). An increase in the nonlamellar phase propensity of lipid bilayers causes a reduction in the lateral surface pressure that controls the type and abundance of the signaling proteins that regulate cell proliferation, differentiation, and survival (Escribá et al., 1995; Martin et al., 2013).

In this scenario, nonlamellar prone lipids promote the cytoplasmic-to-membrane translocation of PKC (Goldberg et al., 1994; Escribá et al., 1995) and therefore its activity (Epand et al., 1991; Goldberg and Zidovetzki, 1998). We show here that HTO induces PKCα cytosol to membrane translocation and that PKCα is in part responsible for the antitumor effects of HTO. Through the stimulation of the protein phosphatase PP2A, α-tocopherol specifically inactivates PKCα, and in turn it dephosphorylates PKCα (Ricciarelli et al., 1998) and blocks HTO-induced cell death.

PKCα activation has been formerly related to cell proliferation and also to differentiation. Due to its effects on proliferation, PKC inhibition has been considered as a potential cancer therapy. However, PKCα levels are low in NSCLC cells, and it was recently demonstrated that PKCα functions as a tumor suppressor in the LSL-Kras in vivo murine lung adenocarcinoma model. In these mice the loss of PKCα leads to self-renewal and proliferation of broncho-alveolar stem cells. In lung cells, PKCα activates p38, regulating transforming growth factor-β signaling, which may be oncogenic in the presence of K-Ras (Hill et al., 2014).

In addition, PKCα has been seen to negatively regulate the Wnt/β-catenin signaling, which is upregulated in some cancers like NSCLC (Stewart, 2014). Most NSCLC cell lines...
Fig. 5. PKCα and the molecular mechanism of HTO and triolein activity. A549 cells were preincubated in the presence or absence of 100 μM α-tocopherol (Vit.E) or 10 μM of the PKC inhibitor GF109203X (PKCi) for 1 hour before exposure to 150 μM triolein or HTO. (A) Cell number was measured using the XTT assay. **P < 0.01, ***P < 0.001 versus control cells; #P < 0.001 versus HTO- or triolein-treated cells (analysis of variance followed by Bonferroni’s test). (B–D) Effect of vitamin E (Vit.E) and the PKC inhibitor GF109203X (PKCi) on gelsolin expression (C) and on ERK phosphorylation (D) after exposure to HTO or triolein. *P < 0.05, **P < 0.01, ***P < 0.001 versus control cells. (E) A549 cell number (percentage of control) after 24 hours of treatment with HTO and triolein after preincubation with 10 μM of the MEK inhibitor U0126 for 2 hours. Bars correspond to the mean ± S.E.M. of three independent experiments. **P < 0.01, ***P < 0.001 versus control untreated cells. (F) Cell proliferation and cytotoxicity of NSCLC (Calu-1, HOP-62, and NCI-H838) after HTO cotreatment with either Vit.E or PKC inhibitor GF109203X (PKCi). Bars correspond to the mean ± S.E.M. of three independent experiments. Proliferation analysis *P < 0.05, **P < 0.01, ***P < 0.001 versus control cells. (G) Cell proliferation and cytotoxicity of HTO after MEK inhibition on NSCLC (Calu-1, HOP-62, and NCI-H838) after HTO cotreatment with either Vit.E or PKC inhibitor GF109203X (PKCi). Cells were preincubated with 10 μM of the MEK inhibitor U0126 for 2 hours and then incubated for 24 hours with HTO (HTO) or left untreated (control). Bars correspond to the mean ± S.E.M. of three independent experiments. Proliferation analysis *P < 0.05, **P < 0.01, ***P < 0.001 versus control (analysis of variance followed by Bonferroni’s test).
have active Wnt signaling, and in the A549 cell-line activation of the Wnt pathway has been associated to cisplatin resistance (Gao et al., 2013).

Active PKCα phosphorylates β-catenin, inducing degradation of this proliferation-promoting protein through the proteasome system. Indeed, antisense oligonucleotides against PKCα were not efficient in a phase III trial in patients with NSCLC (Paz-Ares et al., 2006). Our results with HTO suggest that the activation of PKCα could be beneficial for NSCLC treatment, although further studies will be necessary to determine to what extent HTO can affect the transforming growth factor-β and Wnt/β-catenin signaling pathways.

We found that HTO activation of PKCα promoted, on the one hand, sustained ERK activation and ROS production and, on the other, increased levels of the actin-binding protein and cell-differentiation marker gelsolin. The correlation between gelsolin expression and tumorigenesis is controversial. Reduced gelsolin expression appears to be characteristic of a variety of transformed cells (Mullauer et al., 1993), yet high gelsolin levels are associated with poor survival in some subpopulations of NSCLC patients (Yang et al., 2004). This effect is possibly due to the fact that gelsolin may facilitate tumor dissemination and metastasis by promoting locomotion. However, there is a loss of gelsolin protein in late-stage NSCLCs, and it is weakly expressed or undetectable in most NSCLC cell lines (Dosaka-Akita et al., 1998). Gelsolin protein levels increase when lung cancer cells receive differentiation stimuli (Jarrard et al., 1998), and we previously detected cancer (glioma) cell differentiation after membrane lipid remodeling as an intermediate step before programmed cell death (Terés et al., 2012).

Fig. 6. HTO induces autophagy. Effect of HTO on LC3B-II levels and on the LC3B-I/II ratio. NSCLC were treated for 24 hours in the presence or absence of HTO. (A) A549 cells were treated with 0–300 μM HTO. Bars correspond to mean ± S.E.M. values of three independent experiments. Representative immunoblots are shown: *P < 0.05, **P < 0.01, ***P < 0.001 (analysis of variance followed by Bonferroni’s test). (B and C) Calu-1, HOP-62, and NCI-H838 cells were treated for 24 hours or 72 hours with 150 μM HTO. Bars correspond to the mean ± S.E.M. values of two independent experiments with duplicate samples. **P < 0.01, ***P < 0.001 (Student’s t test). (D) Effect of bafilomycin A1 on LC3B-II levels of HTO-treated cells. A549, HOP-62, and NCI-H838 cells were pretreated with 50 nM bafilomycin A (Baf.A1) for 2 hours before exposure to 150 μM of HTO.
Fatty acids can induce ROS production through mitochondrial events (Schonfeld and Wojtczak, 2008) and by enhanced activity of the NAD(P)H-oxidase stimulated by PKC (Chen et al., 2014). ROS are known to produce apoptosis and to act as mediators in signal transduction pathways, such as the mitogen-activated protein kinase pathway (Cagnol and Chambard, 2010). HTO triggered ERK activation, and its role in the antitumor effects of HTO was evident by the recovery of A549-cell growth upon inhibition of ERK phosphorylation. Nevertheless, the effects of sustained ERK activation depend on the cell type, and it varies from cell cycle arrest in hepatoma cells (HepG2) (Wen-Sheng, 2006) to apoptosis in leukemia cells (Stadheim and Kucera, 1998). In this context, exposing A549 cells to HTO and the ROS scavenger N-acetylcysteine does not inhibit cell death, indicating that the main stimulus activating ERK is PKC. Thus, PKCα phosphorylation of the Raf-kinase inhibitory protein RKIP-1 will impair Raf inhibition and therefore trigger ERK activation (Corbit et al., 2003).

To investigate the potential toxicity of HTO and triolein, we measured their effects on the growth of tumor (NSCLC cells) and nontumor (MRC-5 fibroblast) cells. HTO and triolein impaired the growth of NSCLC cells but not that of normal lung fibroblasts. These differences could in part be attributed to the fact that HTO can rapidly alter the membrane composition of cancer cells, given the high turnover of the tumor cell phospholipid fraction (Spector and Burns, 1987). HTO induces rapid and marked changes in the levels of membrane phospholipids, including an approximately 2-fold increase in the sphingomyelin/phosphatidylethanolamine ratio (Supplemental Fig. 2E). These changes have been associated with the translocation of inactive peripheral membrane proteins to the cytosol (e.g., Ras) and the loss of proliferation potential (Terés et al., 2012). Accordingly, PKC phosphorylates K-Ras in A549 cells, promoting its translocation from the plasma membrane to intracellular membranes within the cell, including those of the mitochondria (Bivona et al., 2006). The translocation of mutated active K-Ras from the plasma membrane to the cytoplasm reduces its oncogenic potential. Moreover, active K-Ras interacts with mitochondrial mBcl-XL and can induce apoptosis.

One reason to design triolein analogs was to augment the activity of olive oil (i.e., triolein) against cancer, introducing radicals that would impair their metabolism. We measured HTO and triolein metabolization by LPL, and we found that the latter was degraded approximately 4-fold faster than HTO in vitro. In addition, increased LPL activity has been reported in cancer tumors compared with normal lung tissue, most likely due to the energy requirements of tumor cells (Cerne et al., 2007), which potentially explains the lack of efficacy of olive oil despite its protective anticancer effects. LPL would hydrolyze triglycerides to fatty acids that the tumor could use as energy source. Indeed, higher LPL activity is inversely correlated with NSCLC patient survival (Trost et al., 2009). Further studies will be necessary to clarify whether the activity of this enzyme is inhibited by the presence of HTO or whether its activity is low due to lower affinity for HTO.
HTO induced an autophagy response in the various studied cell lines, the origins of which could be traced to the known inducers of the autophagy response: an increase in ROS, ERK, or PKCo activation (Kroemer et al., 2010; Sridharan et al., 2011). Moreover, HTO treatment induced lipid droplets (data not shown), a major depot of neutral lipids such as triglycerides, which have been reported to increase autophagy (Dupont et al., 2014). Oleate has recently been reported to trigger a noncanonical autophagy response that relies on an intact Golgi apparatus (Niso-Santano et al., 2015). Given the similarity between HTO and oleate (both containing the fatty acyl moieties hydroxylate and oleate, respectively), we hypothesized that HTO-induced autophagy would also depend on an intact Golgi apparatus.

In summary, HTO has a stronger antimutagenic effect than oleate, the major triglyceride of olive oil. Epidemiologic studies have shown that olive oil markedly prevents the occurrence of several types of cancer but that it lacks therapeutic activity (Martin-Moreno, 2000; Stark and Madar, 2002; Fortes et al., 2003). Moreover, OA-rich oils have protective effects against tumor growth and metastasis in animal models (Kimura, 2002; Yamaki et al., 2002). Consistent with our data, they indicate that oleate, and most likely OA, could have protective effects against tumorigenesis but no therapeutic activity against lung cancer. By contrast, HTO displays a high efficacy against NSCLC both in vitro and in vivo. Both oleate and HTO induce similar changes in membrane structure, and they have similar effects in vitro. The differences observed in vivo might be due to the lower rate of HTO metabolism, as described for OA and its hydroxylated analog (Lladó et al., 2010). In addition, HTO processing by LPL yields hydroxylated OA that, in contrast to OA, increases sphingomyelin synthase activity. The result is an increase in membrane sphingomyelin levels, which has been associated with the induction of cancer cell arrest, differentiation, and death (Barceló-Clobijín et al., 2011; Terés et al., 2012; Martin et al., 2013). Indeed, increased sphingomyelin synthase expression and gene copy number have been associated with longer cancer patient survival. Therefore, HTO constitutes a new anticancer drug class with great potential.

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Authorship Contributions

Participated in research design: Escribá, Guardiola-Serrano.
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