Pivotal role of dihydrofolate reductase knockdown in the anticancer activity of 2-hydroxyoleic acid

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α-Hydroxy-9-*cis*-octadecenoic acid, a synthetic fatty acid that modifies the composition and structure of lipid membranes. 2-Hydroxyoleic acid (HOA) generated interest due to its potent, yet nontoxic, anticancer activity. It induces cell cycle arrest in human lung cancer (A549) cells and apoptosis in human leukemia (Jurkat) cells. These two pathways may explain how HOA induces regression of a variety of cancers. We showed that HOA repressed the expression of dihydrofolate reductase (DHFR), the enzyme responsible for tetrahydrofolate (THF) synthesis. Folinic acid, which readily produces THF without the participation of DHFR, reverses the antitumor effects of HOA in A549 and Jurkat cells, as well as the inhibitory influence on cyclin D and cdk2 in A549 cells, and on DNA and PARP degradation in Jurkat cells. This effect was very specific, because either elaidic acid (an analog of HOA) or other lipids, failed to alter A549 or Jurkat cell growth. THF is a cofactor necessary for DNA synthesis. Thus, impairment of DNA synthesis appears to be a common mechanism involved in the different responses elicited by cancer cells following treatment with HOA, namely cell cycle arrest or apoptosis. Compared with other antifolates, such as methotrexate, HOA did not directly inhibit DHFR but rather, it repressed its expression, a mode of action that offers certain therapeutic advantages. These results not only demonstrate the effect of a fatty acid on the expression of DHFR, but also emphasize the potential of HOA to be used as a wide-spectrum drug against cancer.

anticancer | DNA | membrane-lipid therapy | neoplasia | nutrigenetics

2-Hydroxyoleic acid (HOA) is a potent anticancer drug whose molecular mechanism of action is still not fully understood. Although HOA induces cell cycle exit in human lung cancer (A549) cells (1), it induces apoptosis in human leukemia cells (2). The IC_{50} of this drug for most cancer cells studied is in the range of 30–150 μM, whereas its IC_{50} in normal cells is over 5,000 μM (2). Thus, this drug does not produce toxicity at therapeutic doses despite acting efficiently in cell and animal models of human cancers (2, 3). Hence, it is important to define the mode of action of this compound and whether common molecular events underlie its therapeutic effects and the regression of different types of cancer.

HOA was developed on the basis that the lipid composition and structure of the plasma membrane can be altered by certain antitumor drugs and that these modifications are involved in their action against cancer (4, 5). In this context, anthracyclines that are unable to enter cancer cells or bind to DNA still have strong antitumor activity (6). Indeed, those used in human therapy regulate plasma membrane structure, and they induce changes in the localization and activity of important peripheral signaling proteins, such as G proteins and PKC (4, 7, 8). This mode of action also appears to be responsible for the activity of hexamethylene bisacetamide against cancers (9, 10).

In the search for molecules capable of inducing similar regulatory effects on membrane lipid structure and peripheral protein function, oleic acid was found to be more potent than anthracyclines (8) and thus, a nonhydrolyzable analog of this molecule was designed and named Minerval (1, 11). This drug interacts with cell membranes, inducing changes in their composition and structure (1, 11, 12), and it triggers a series of events that ultimately result in the dramatic knockdown of the proliferation-inducing transcription factor E2F-1 (3).

Folates play a key role in one-carbon metabolism, which is essential for the biosynthesis of purines and hence, for DNA replication. Antifolates are classic antitumor agents that inhibit key enzymes in DNA synthesis, such as thymidylate synthase and DHFR, whose expression is regulated by E2F-1. One of the initial clinically useful blockers of DHFR was methotrexate (13, 14), which is frequently used in the treatment of acute leukemias and a number of solid tumors (15). Although this drug and other antifolate drugs are relevant in the treatment of cancer, drug resistance poses a major obstacle to their effectiveness. Because inhibition of DNA synthesis can induce cell cycle arrest or the induction of apoptosis, we studied how DHFR was involved in the mechanism by which two cancer cell lines respond differently to HOA.

Results

Having previously demonstrated that HOA produces potent cell cycle arrest of human lung cancer (A549) cells and apoptosis of lymphoblastic (T lymphocyte) leukemia (Jurkat cells) (1, 2), we investigated how a common molecular mechanism might explain these effects. Accordingly, we first studied the structure-function effects of HOA using its *trans* analog, elaidic acid (EA), and the membrane-binding lipids, phosphatidylcholine (PC) and cholesterol (CH) (Fig. 1). HOA, but not the other lipids, did regulate the lipid structure of membranes, facilitating the binding and activation of important membrane signaling proteins, such as PKC (1). A similar effect was previously shown by the natural *cis* but not the *trans* structures of elaidic acid and HOA (16).

In this context, HOA but not EA, PC, and CH, inhibited cancer cell growth (Fig. 1), arguing against any possible nonspecific effects mediated by the general disorganization of membrane lipid structure.

Among the events triggered by HOA, there is a marked knockdown of E2F-1, a key transcription factor that regulates the expression of a large number of cell cycle-related genes, including DHFR (17, 18). Due to the central role of this enzyme in regulating cell viability and proliferation, we investigated the effects of HOA on DHFR expression and as a consequence, on DNA synthesis and cell cycle progression. There was a significant decrease in DHFR expression (protein and mRNA) in both A549 and Jurkat cells exposed to HOA for 48 h (50–100 μM) (Fig. 2); evidence that HOA produces a common effect in different cancer cells. Because DNA

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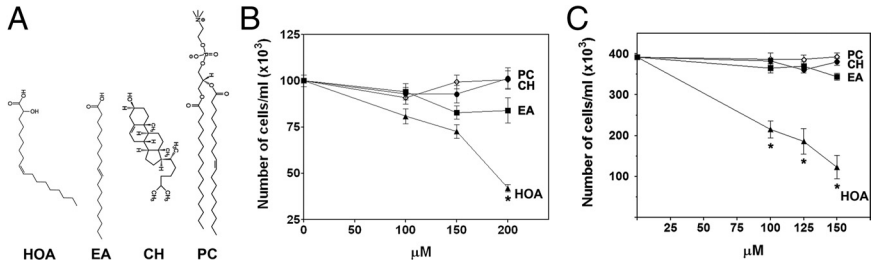

Fig. 1. Lipid structures and effects. (*A*) Structures of HOA (Minerval), phosphatydilcholine (PC), elaidic acid (EA), and cholesterol (CH). Effects of these lipids on A549 (*B*) and Jurkat (*C*) cell proliferation after 24 h at the concentrations indicated.

inhibition can induce either cell cycle arrest or apoptosis in A549 and Jurkat cells, this effect may explain the divergent anticancer effects of HOA (see *Discussion*).

Induction of the cell cycle arrest and apoptosis by HOA (100 μM) was further determined by flow cytometry, studying the proportion of cells in the various phases of the cell cycle (G_0/G_1, S, and G_2/M phases or the subG_1 peak for apoptosis) (Fig. 3*E* and Fig. 4*E*). HOA increased the proportion of A549 cells in the G_0/G_1 phase, consistent with cell cycle arrest (Fig. 3*E*). HOA also down-regulated the expression of cdk2 and cyclin D3 (Fig. 3 *B* and *C*).

To determine whether DHFR participated in the cell cycle arrest of A549 cells, these cells were maintained in the presence of folinic acid (200 μM), a molecule that is readily converted into THF without the participation of DHFR. A549 cell proliferation was restored in the presence of HOA (100 μM) and folinic acid (Fig. 3 *A* and *D*). Similarly, the levels of cdk2 and cyclin D3 as well as the number of cells in mitosis also increased significantly in the presence of folinic acid (Fig. 3 *B* and *C*).

Folinic acid also impaired the apoptosis induced by HOA in Jurkat cells (2), as witnessed by a decrease in the subG_1 peak (Fig. 4*E*) and PARP degradation (Fig. 4 *B* and *C*), as well as the concomitant recovery of Jurkat cell proliferation (Fig. 4 *A* and *D*).

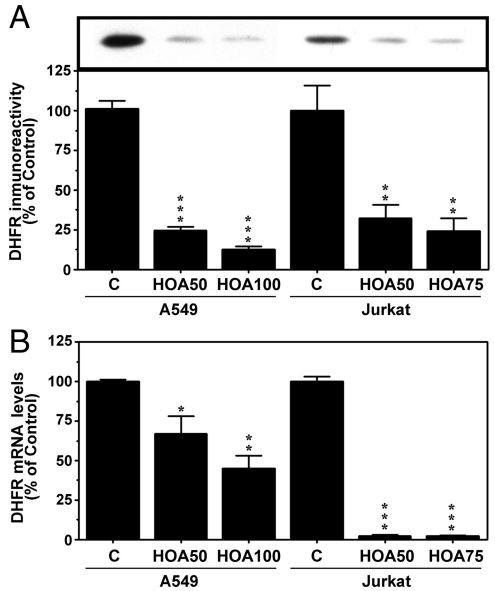

Fig. 2. Effect of HOA on DHFR in A549 and Jurkat cells. (*A*) Effect of HOA on DHFR protein levels as determined by quantitative immunoblotting (a representative immunoblot is also shown). A549 and Jurkat cells were incubated in the presence or absence (C, control) of HOA (50 μM, HOA50; 75 μM, H75; 100 μM, H100). (*B*) Effects of HOA on DHFR mRNA expression in A549 and Jurkat cells. Bars correspond to mean ± SEM values of five independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

These results clearly indicate the specificity of HOA and the relevance of DHFR in inducing cell cycle arrest in A549 cells and apoptosis in Jurkat cells. However, the fact that this recovery was not complete in both cell lines suggests that other pathways are also likely to be regulated by this drug.

Discussion

Pivotal Function of DHFR in the Action of HOA Against Cancer. HOA is a potent anticancer drug that inhibits tumor growth and induces tumor regression, markedly increasing the life span of

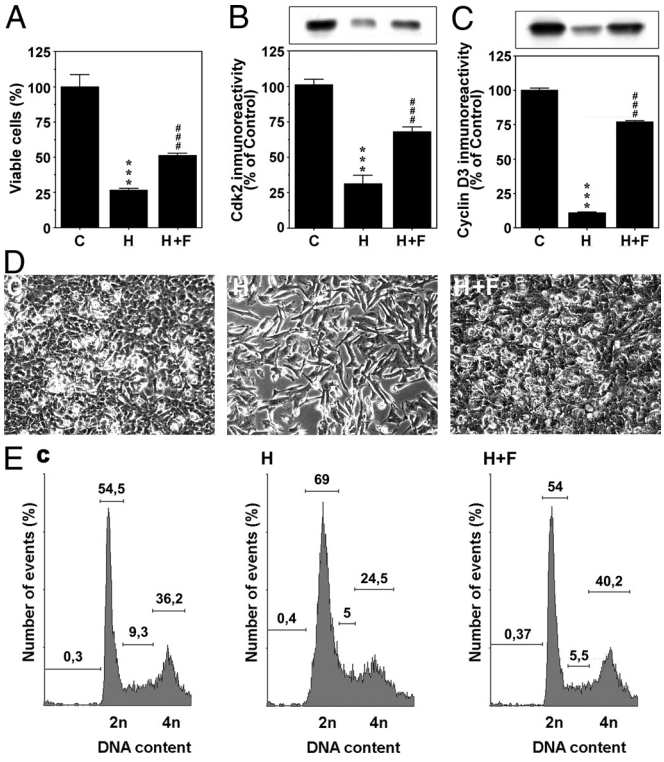

Fig. 3. Effects of HOA and folinic acid on A549 cells. (*A*) A549 cell proliferation in the presence or absence (C, control) of HOA or HOA plus folinic acid (H+F). (*B*) Effects of HOA on Cdk2 and (*C*) on cyclin D3 were determined by quantitative immunoblotting (a representative immunoblot is also shown). The bars correspond to mean ± SEM values of five independent experiments of A549 cells incubated for 48 h (100 μM HOA, 200 μM folinic acid; ***, $P < 0.001$ with respect to untreated cells; ###, $P < 0.001$ with respect to HOA-treated cells). (*D*) Representative phase-contrast micrographs (100× magnification) of A549 cells in the presence or absence (C, control) of HOA (H), or HOA plus folinic acid (H+F). (*E*) DNA content in A549 cells incubated in the presence or absence (C, control) of HOA (H, 100 μM) or HOA plus folinic acid (H+F, 100 μM HOA, 200 μM folinic acid). From left to right, the bars and indicated values correspond to the percentage of cells in the subG_1 (apoptosis), G_1, S, and G_2+M phases of the cell cycle (for further details, see *Materials and Methods*).

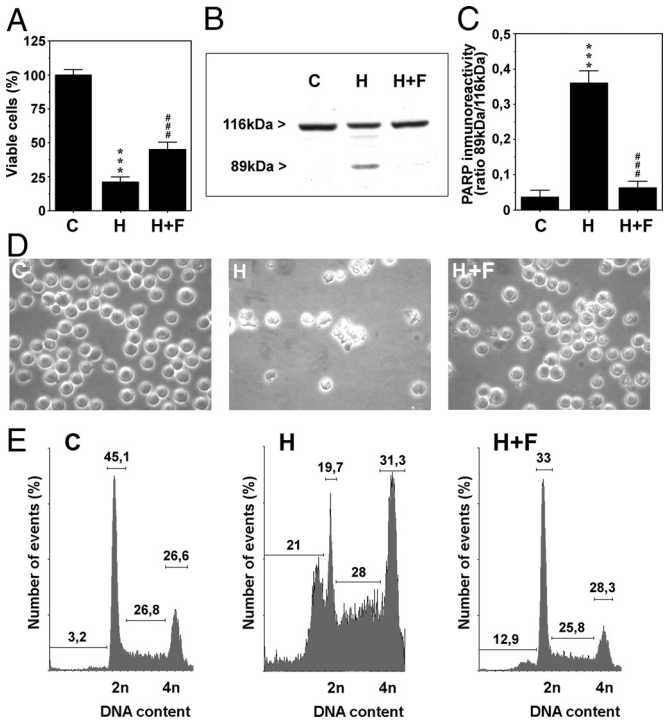

Fig. 4. Effects of HOA and folinic acid on Jurkat cells. (*A*) Jurkat cell proliferation in the presence or absence (C, control) of HOA (H), or HOA plus folinic acid (H+F). (*B*) Effects on PARP. (*C*) Representative immunoblot showing the effect of H and H+F on PARP. The bars correspond to the mean ± SEM of five independent experiments of Jurkat cells incubated for 48 h (75 μM HOA, 200 μM folinic acid; ***, $P < 0.001$ with respect to untreated cells; ###, $P < 0.001$ with respect to HOA-treated cells). (*D*) Representative phase-contrast micrographs (100× magnification) of Jurkat cells in the presence or absence (C, control) of HOA(H), or HOA plus folinic acid (H+F). (*E*) DNA content in Jurkat cells incubated in the presence or absence (C, control) of HOA (H, 75 μM), or HOA plus folinic acid (H+F, 75 μM HOA and 200 μM folinic acid). From left to right, the bars correspond the percentage of cells in the subG$_1$ (apoptosis), G$_1$, S, and G$_2$+M phases of the cell cycle (for further details, see *Materials and Methods*).

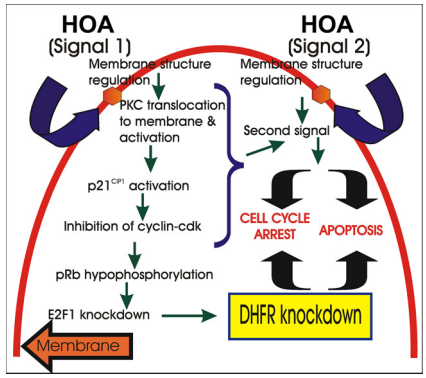

Fig. 5. Proposed mechanism for the anticancer effects of HOA. HOA modifies the membrane lipid structure (*Signal 1*), inducing PKC translocation to the membrane and its subsequent activation. This event is followed by activation of p21^{CIP1}, cyclin-cdk inhibition, pRb hypophosphorylation, and E2F1 knockdown (1, 3). E2F1 is a critical transcription factor that regulates several genes involved in cell proliferation, such as DHFR. Finally, DHFR down-regulation impairs DNA synthesis, which results in either the cell cycle arrest (A549 cells) or apoptosis (Jurkat cells). The particular molecular features of each cell line are responsible for the selection of one or another anticancer mechanism. Other molecular events may participate in the generation of a second signal, *Signal 2*, which could originate directly at the membrane or as a consequence of crosstalk with the *Signal 1* cascade. An example of the former is the clustering of the Fas receptor after altering membrane lipid structure (2), which might be involved in induction of apoptosis in Jurkat cells. By contrast, the latter may reflect the differential effect of cyclin-cdk inhibition, which induces the cell cycle arrest in A549 cells and apoptosis in Jurkat cells (2, 3, 22–24).

animals with cancer (1, 2). Two apparently divergent mechanisms appear to be associated with the therapeutic effects of this drug: (*i*) Inhibition of cancer cell proliferation and (*ii*) apoptosis. To date, these mechanisms have been studied in 12 different cancer cell lines, although A549 and Jurkat cells were used here as models to investigate the molecular bases underlying the induction of cell cycle arrest and apoptosis, respectively. We show that concentrations in the range of the IC_{50} for this drug induced marked and significant reductions of DHFR mRNA and protein expression, a pivotal enzyme necessary for DNA synthesis and hence, cell proliferation.

Analysis of the DNA content in A549 cells by flow cytometry showed that cells accumulate in the G_0/G_1 phase after exposure to HOA. Induction of cell accumulation by HOA in the G_0/G_1 phase was associated with the down-regulation of cdk2 and cyclin D3, both of which are involved in G_1 to S phase progression (19). However, although HOA does not induce apoptosis of A549 cells, it does promote the death of Jurkat cells, as witnessed by the degradation of PARP and the presence of a subG$_1$ flow cytometry peak. In this regard, the induction of apoptosis by a number of antifolate drugs in T cells is a well-characterized phenomenon (20). These results suggest that the HOA-induced impairment of DNA synthesis drives Jurkat cells into an apoptotic program.

DHFR knockdown in response to HOA was concentration-dependent in both A549 and Jurkat cells. Moreover, the pharmacological action of HOA was reversed in both cell lines in the presence of folinic acid (5-formyl-THF), a molecule that is readily converted to THF without the intervention of DHFR. These results indicate that DHFR knockdown (i.e., DNA synthesis inhibition) is a key event in the induction of the cell cycle arrest (A549 cells) or apoptosis (Jurkat cells). However, because two different anticancer mechanisms were triggered by HOA, and given that its anticancer action was not fully reversed, simultaneous or sequential mechanisms would appear to be involved in the pharmacological effect of this molecule (see Fig. 5). This second signal or pathway would define the final and complete anticancer effect of the drug (either inhibition of cell growth or apoptosis), and it might originate from the plasma membrane, from a downstream molecular event leading to DHFR reduction or from both. An example of such simultaneous membrane signals might be the induction of Fas receptor clustering in Jurkat cells (2). By contrast, the divergent effect of impairing cyclin and cdk activity that leads to cell cycle arrest in A549 or apoptosis in Jurkat cells, may also reflect the downstream crosstalk mechanisms (2, 3, 21–23).

A number of antineoplasic drugs alter the organization of the plasma membrane (4, 5, 8) and strongly influence the localization and activity of key signaling proteins. Such modifications can be used for therapeutic purposes, an approach termed "membrane-lipid therapy" (7, 24). Indeed, lipid molecules such as edelfosine and mitelfosine are potent antineoplastic drugs that target the membrane (25, 26), and they induce apoptosis in cancer cells through mechanisms that alter membrane lipid raft organization (26). By contrast with oleic acid, α-hydroxy derivatives of fatty acids (e.g., HOA) are less prone to be used by cells, and they are therefore likely to display better pharmacological properties (27).

Role of Lipid Structure in the Effects of HOA. The structural alterations to the cell membrane induced by HOA favor the translocation of PKC to the cell membrane and a series of events that

provoke the down-regulation of E2F1 (1, 3). E2F1 is an important transcription factor that regulates the expression of dozens of genes required for cell cycle progression (28). Among others, E2F1 controls the expression of DHFR (17, 18). We investigated the effect of different lipids on cancer cell growth and we found that, EA, the *trans* deoxylated isomer of HOA, did not significantly affect the growth rate of A549 and Jurkat cells. This result argues in favor of the structure-specific effects of HOA. In this context, the lamellar-prone lipid PC and the fluidity regulator CH did not change the growth rate of cancer cells either. On the whole, the data presented support and extend previous studies indicating that membrane lipid structure is crucial in the mode of action of HOA.

Specificity of HOA. In contrast to most drugs designed on a rational structural bases, HOA does not target proteins but rather membrane lipids. Fatty acids are mainly considered as cellular fuel, and their effects on membranes are mostly associated with changes in membrane fluidity.

Membranes have complex structural features, and subtle structural changes in lipids have a strong impact on the structural properties of membranes and on their pharmacological effects. For instance, *cis* (oleic acid, HOA) and *trans* (EA) isomers of octadecenoic acid (ω-9) have identical (oleic acid and EA) or similar (HOA has one extra oxygen with respect to the former) chemical compositions, but because their structure differs, so does their effect on membrane structure and on the localization and activity of peripheral membrane proteins (e.g., G proteins, PKC) (1, 8). In addition to the different structure of *cis*- and *trans*-monounsaturated fatty acids, we showed that these lipids differ in their potency against cancer cell growth. In fact, the biophysical, molecular, and cellular effects of EA are closer to those of stearic acid, which lacks double bonds but has a closer structure to that of EA, in accordance with the proposed structural basis of the effects of lipids on membranes. In addition, the presence of an oxygen atom on carbon 2 makes HOA far more effective against cancer growth than its precursor, oleic acid (2). These data demonstrate the structural basis underlying the cellular, physiological and pharmacological effects of fatty acids.

Although the above data clearly demonstrate the specificity of HOA, there are other arguments in support of this phenomenon, such as HOA's lack of general or cytological toxicity in animals. In addition to the absence of side effects in animals at a dose of up to 3 g/kg (3), this fatty acid appears to be about 100-fold less potent in killing normal cells when compared with cancer cells (2). From a therapeutic point of view, this result is the strongest evidence of specificity, and it establishes an important difference between HOA and most anticancer drugs.

The small number of genes modulated by this drug is further evidence of HOA's specificity; about 100 genes are up- or down-regulated (2). In addition, many of the proteins regulated by this lipid have been characterized, and those whose activity is regulated by protein-lipid interactions have been shown to display marked differences in their interactions with membranes that contain HOA (2).

Signals that inhibit DNA synthesis (e.g., knockdown of DHFR) or cell cycle progress (e.g., down-regulation of cyclins and cdks) should not markedly affect mature cells. Conversely, these types of signals will promote the death of cancer cells. The lack of toxicity and high efficacy of this drug indicates that HOA is more closely associated with the modulation of cell signaling than with cytotoxic phenomena.

DHFR Activity Modulation in Cancer Therapy. Methotrexate was one of the initial antimetabolites used to treat cancer. Developed in the 1940s, it was synthesized as a DHFR inhibitor to treat human leukemia, although it is also used to treat a wide variety of solid tumors (29). Like many other enzyme inhibitors, methotrexate induces an increase in DHFR expression as an adaptive mechanism to its binding and lowering of enzyme's activity (30), and its overexpression frequently leads to drug resistance (31). Methotrexate analogs, such as pemetrexed (32), do not fully overcome the phenomenon of the drug resistance developed by cancer cells exposed to methotrexate. By contrast, HOA reduces the activity of this pivotal enzyme by diminishing its expression. The way that HOA reduces DHFR activity may have a number of therapeutic advantages, including the evasion of drug resistance phenomena triggered by conventional antifolates. The evidence is indicative that this compound represses gene expression of one critical enzyme required for rapid cancer cell growth, but it is not repressed enough to prevent normal cell growth. The oral administration and lack of toxicity suggest that HOA (Minerval) could be used in first-line treatments and combinatory therapies.

Materials and Methods

Cell Culture. Human nonsmall lung adenocarcinoma (A549) and Jurkat T-lymphoblastic leukemia cells were obtained from the American Type Culture Collection. The cells were maintained at 37 °C in 5% CO_2 in air and in RPMI medium 1640 supplemented with 2 mM glutamine, containing 10% bovine calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Tissue culture medium and supplements were purchased from Sigma.

2-Hydroxyoleic Acid. HOA (GMP quality) was obtained from Avanti Polar Lipids. We analyzed the purity of the compound, by HPLC and gas chromatography, which was 99.7% (similar to that indicated in the technical data sheet provided by the manufacturer, 99.6%). Our analysis of impurities (amounting only 0.3%) also coincided with that of the manufacturer, being oleic acid (precursor reagent), hydroxystearic acid, the main byproducts found, with trace quantities of other fatty acids. The individual abundance of each of these species was under 0.1%, including those of transfatty acids, which were present only in trace amounts.

Cell Proliferation Assays. Cells were plated at a density of 1×10^5 (A549) cells or 1.5×10^5 (Jurkat) cells in 24-well plates with 0.5 mL culture medium with serum per well, and they were incubated overnight. To analyze the effect of lipids on cell proliferation, A549 cells were exposed for 24 h to between zero and 200 μM HOA, egg PC (Avanti Polar Lipids), CH (Sigma), or EA (Sigma). The fatty acid content of PC is 32.4% palmitic acid, 12.3% stearic acid, 32% oleic acid, 17.2% linoleic acid, 2.7% arachidonic acid, 1.1% palmitoleic acid, 0.4% docosahexaenoic acid, and less than 0.4% of other fatty acids. Jurkat cells were treated with the same lipids at concentrations between 0 and 150 μM for 24 h. The time and dose to which the cell lines were exposed was based on their different sensitivities to lipid treatments.

To determine how DHFR influences the effects of HOA, cells were incubated in the presence or absence of HOA (100 or 75 μM HOA for A549 and Jurkat cells, respectively), in the presence or absence of 200 μM folinic acid (Acros Organics) added every 12 h for 72 h. Unattached dividing cells were recovered by centrifugation for 5 min at room temperature (RT) and 600 $\times$ *g*, and they were combined with the adherent cells that were harvested by centrifugation after treatment for 5 min at 37 °C with 0.05% trypsin in sterile PBS (137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3). Viable cells were counted immediately using 0.2% trypan blue in PBS.

Cell Cycle Analysis. The cell cycle was analyzed by flow cytometry in cells maintained in the presence or absence of HOA for 72 h. After treatment, A549 cells were washed twice with PBS and detached using trypsin, whereas Jurkat cells were washed with PBS and collected by centrifugation. The cells were fixed with 100% methanol for 2 h at 4 °C, centrifuged for 5 min at RT and 600 $\times$ *g*, and then resuspended in PBS. Finally, cells were incubated for 30 min in the presence of 100 μg/mL ethidium bromide and 100 μg/mL RNase A and analyzed on a Beckman Coulter Epics XL flow cytometer. Cell populations in the different phases of cell cycle (subG$_1$, G$_0$/G$_1$, S, and G$_2$/M) were determined on the basis of their DNA content.

Electrophoresis (SDS/PAGE), Immunoblotting, and Protein Quantification. Cells were incubated in the presence or absence of HOA (100 μM for A549 and 75 μM for Jurkat cells), in six-well culture plates under the experimental conditions indicated above. The cells were then washed twice with PBS and har-

vested by using a rubber policeman in 300 μL 10 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1% SDS, 5 mM iodoacetamide, 1 mM phenylmethylsulfonylfluoride, 1 μM sodium orthovanadate, and 1 μM cantharidin. Jurkat cells were washed twice with PBS and collected by centrifugation at 600 $\times$ *g* for 5 min at room temperature. The cells were homogenized by ultrasound for 10 s at 50 W in a Braun Labsonic U sonicator (20% cycle), and 30-μL aliquots were removed for total protein quantification. Subsequently, 30 μL 10$\times$ electrophoresis loading buffer (120 mM Tris-HCl buffer, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 50% glycerol, and 0.1% bromophenol blue) were added to the samples, and they were boiled for 3 min. For immunoblotting, 25 μg total protein from HOA-treated samples or 5–60 μg from control samples (for standard curves) were resolved on the same SDS-polyacrylamide gel (9% polyacrylamide) and transferred to nitrocellulose membranes (Whatman, Schleicher, and Schuell). The membranes were incubated with blocking solution (PBS containing 5% nonfat dry milk, 0.5% BSA, and 0.1% Tween 20) for 1 h at RT and then overnight at 4 °C in fresh blocking solution containing the specific primary antibodies: mouse anti-cyclin D3 (diluted 1:1,000); mouse anti-cdk2 (diluted 1:1,000); mouse anti-DHFR (diluted 1:500) from BD Biosciences Transduction Laboratories; and a rabbit anti-poly ADP-ribose polymerase (anti-PARP, diluted 1:2,000) from Santa Cruz Biotechnology. Subsequently, the membranes were incubated with horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG (diluted 1:2,000 in blocking solution) for 1 h at room temperature. The immunoreactive protein bands were visualized using the ECL Western blotting detection system (Amersham Pharmacia) followed by exposure to ECL hyperfilm, and the films were scanned at a resolution of 600 dpi using the Foto 32 software (Agfa).

Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR). QRT-PCR was used to determine the regulatory effects of HOA on the levels of DHFR mRNA. Accordingly, cells were incubated in the presence or absence of HOA for 48 h and then, the total RNA was extracted from 3×10^6 A549 or Jurkat cells using the RNeasy Mini kit in combination with the RNase-free DNase kit (Qiagen) according to the manufacturer's instructions. Reverse transcription reactions were carried out using 1 μg total RNA in a final volume of 20 μL, containing the following reagents (from Invitrogen): First-Strand Buffer; oligo(dT) (2.5 μM); random hexamers (2.5 μM); dNTP mix (dGTP, dCTP, dATP, and dTTP; each at 500 μM); DTT (10 mM); ''RNase OUT'' (recombinant ribonuclease inhibitor, 0.4 U/μL); Moloney murine leukemia virus reverse transcriptase (10 U/μL), and RNase-free water. The reaction mixtures were then incubated at 65 °C (for 5 min), 37 °C (for 50 min), and 70 °C (for 15 min), and the cDNA samples obtained were stored at −20 °C before use.

For PCR amplification, the primers designed were based on the DHFR sequence in GenBank (accession no. 1719): 5′-TCACCCAGGCCATCTTAAAC-3′ (DHFR forward) and 5′-GAACACCTGGGTATTCTGGC-3′ (DHFR reverse). As endogenous control, the expression of 18S RNA (GenBank accession no. 100008588) was determined using the following primers: 5′-GAGGTGAAATTCTTGGACCGG-3′ (18S RNA forward) and 5′-CGAACCTCCGACTTTCGTTCT-3′ (18S RNA reverse). Real-time PCR amplifications were carried out in a LightCycler thermal cycler (Roche Diagnostics) using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics), which contained 0.5 μM each dNTP and 2.5 mM $MgCl_2$. An initial denaturation step at 95 °C for 5 min preceded thermal cycling. DNA amplification and fluorescence quantification was determined over 30 cycles with a denaturation step at 95 °C for 3 s, a 7-s annealing step at 60 °C for DHFR or 63 °C for 18S RNA, and an extension step at 72 °C for 12 s. Fluorescence quantification was carried out after each DNA extension step (72 °C), and the data were analyzed using the LightCycler software. The ratio between the expression of DHFR and 18S RNA (whose expression is not modulated by HOA) was determined by means of the equation described by Pfaffl et al. (33). This value was used to calculate the relative expression in HOA-treated cells with respect to untreated cells (control = 1). Melting curve analysis and agarose gel electrophoresis were used to further characterize the PCR products.

Statistics. The results were expressed as the mean ± SEM of at least three independent experiments, and the level of significance was set as $P < 0.05$ (Student's *t*-test).

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