Membrane Structure Modulation, Protein Kinase C Activation, and Anticancer Activity of Minerval

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ABSTRACT

Most drugs currently used for human therapy interact with proteins, altering their activity to modulate the pathological cell physiology. In contrast, 2-hydroxy-9-cis-octadecenoic acid (Minerval) was designed to modify the lipid organization of the membrane. Its structure was deduced following the guidelines of the mechanism of action previously proposed by us for certain antitumor drugs. The antiproliferative activity of Minerval supports the above-mentioned hypothesis. This molecule augments the propensity of membrane lipids to organize into non-lamellar (hexagonal H₃) phases, promoting the subsequent recruitment of protein kinase C (PKC) to the cell membrane. The binding of the enzyme to membranes was marked and significantly elevated by Minerval in model (liposomes) and cell (A549) membranes and in heart membranes from animals treated with this drug. In addition, Minerval induced increased PKCα expression (mRNA and protein levels) in A549 cells. This drug also induced PKC activation, which led to a p53-independent increase in p21CIP expression, followed by a decrease in the cellular concentrations of cyclins A, B, and D3 and cdk2. These molecular changes impaired the cell cycle progression of A549 cells. At the cellular and physiological level, administration of Minerval inhibited the growth of cancer cells and exerted antitumor effects in animal models of cancer without apparent histological toxicity. The present results support the potential use of Minerval and related compounds in the treatment of tumor pathologies.

Alterations in the lipid structure of the plasma membrane can modify cell signaling events (Escribá et al., 1997). Indeed, certain antitumor drugs seem to act by regulating signal transduction in this way (Escribá et al., 1995, 2002). Although it has been proposed that the cytotoxic effects of anthracyclines are based on a direct interaction with DNA (e.g., acetyladriamycin 14-valerate, AD 32) show even greater antitumor effects than their DNA binding precursors (e.g., Adriamycin) (Israel et al., 1987). Third, the plasma membrane is the first cellular target encountered by anthracyclines, and membrane phospholipids bind large amounts of these drugs, accounting for their cytotoxic properties (Escribá et al., 1990). On the other hand, we found that these drugs alter the membrane structure, affecting the bilayer propensity to organize into nonlamellar phases (Escribá et al., 1995). Moreover, this phenomenon is involved in the cellular relocalization and activity of both G proteins and protein kinase C (PKC) (Escribá et al., 1995; Giorgione et al., 1995). These proteins are crucial cell signaling components.
and participate in cell proliferation and differentiation, thus their modulation might be responsible for the antitumor activity of some drugs. In line with this hypothesis, the antineoplastic agent hexamethylene bisacetamide (HMBA) induces cell differentiation by activating PKC and also alters the membrane lipid structure (Michaeli et al., 1992; Escribá et al., 2002).

During recent years, efforts have been made to identify potential targets for antitumor drugs. Several alternatives have been proposed to improve the success of chemotherapy in treating cancer, such as the development of new molecular therapeutic strategies. Albeit cell membranes themselves have not been generally considered as targets for chemotherapy, it was pointed out that they could be used to develop antineoplastic agents (Spector and Burns, 1987). It has also been suggested that PKC might be an appropriate target for antitumor drugs (Caonigro et al., 1997), due to the role it plays in cell proliferation. With this in mind, we have designed a molecule that fulfills both these criteria (new mechanism of action based on the modulation of the plasma membrane structure and involvement of PKC in the antineoplastic activity), 2-hydroxy-9-cis-octadecenoic acid (Minerval). This is the first report on the molecular bases of the antitumoral activity of this drug. Minerval was designed to alter the plasma membrane structure, regulating the occurrence of nonlamellar or hexagonal HII phases. As observed for anthracyclines and HMBA (Escribá et al., 1995, 2002), Minerval modulated the membrane lipid structure, producing molecular and cellular alterations that influenced cell signaling. These alterations in membrane structure provoked by Minerval regulated PKC localization and activity. These molecular events correlated with the antiproliferative and antitumor activities of this compound. This lipid therapy with Minerval is a pharmacological approach that differs from conventional therapies, focused on cellular proteins, and whose potential use in the clinical treatment of cancer will be determined during the next few years.

Materials and Methods

31P NMR. Multilamellar lipid vesicles were prepared mixing 56 mg of bovine liver phosphatidylethanolamine (PE) with deuterated water [PE/D_{2}O, 15.85 (w/w)], in the absence or presence of Minerval [PE/Minerval, 20:1 (mol/mol)]. The lipid suspensions were hydrated and homogenized with a pestle-type minihomogenizer (Sigma-Aldrich, St. Louis, MO) and submitted to vortex shaking until homogeneity. The suspensions were then submitted to 10 heating (60°C) and freezing (−80°C) cycles, and equilibrated before data acquisition, as reported previously (Funari et al., 2003). 31P NMR measurements were made in 5-mm tubes on an Advance-300 multinuclear NMR spectrometer (Bruker Instruments, Madrid, Spain). Samples were equilibrated at the assay temperatures (from 5 to 55°C, with increases of 2.5°C) for 10 min before data were acquired. 31P NMR free induction decays were accumulated for 64 transients by using a 4.4-μs, 90° radio frequency pulse, a 24.3-kHz sweep width, and 65,000 data points. The delay between transients was 2 s, and the spectra were obtained by scanning from lower to higher temperatures.

X-Ray Scattering. Multilamellar lipid vesicles of dielaidoyl phosphatidylethanolamine (DEPE) [15 DEPE/85 H_{2}O (w/w)] were prepared in 20 mM HEPES, pH 7.4, in the absence or presence of Minerval [DEPE/Minerval, 20:1 (mol/mol)]. Samples were thoroughly homogenized with a pestle-type minihomogenizer (Sigma-Aldrich) and vortexed until a homogeneous mixture was obtained. Lipid suspensions were submitted to three temperature cycles (heating to 70°C and cooling to 4°C). Then, they were immediately stored at −80°C under argon until use. Before X-ray scattering experiments were performed, the samples were equilibrated at 4°C for 72 h. Small- and wide-angle synchrotron radiation X-ray scattering data were collected simultaneously, using the standard procedures of the soft condensed matter beamline A2 at the storage ring DORIS III of the Deutsches Elektronen-Synchrotron, Hasylab (Hamburg, Germany). The positions of the observed peaks were converted into distances, d, after calibration using standards with well defined scattering patterns. Silver behenate and polyethylene terephthalate were used to calibrate the small- and wide-angle synchrotron radiation X-ray scattering regions, respectively. Data collection was carried out at 57°C. Interplanar distances, d_{hkl}, were calculated according to the equation s = 1/(2d_{hkl}) = (2 sin θ)/λ, where s is the scattering vector, 2 θ is the scattering angle, λ (0.15 nm) is the X-ray wavelength, and hkl are the Miller indexes of the scattering planes. For hexagonal (HII) phases, the unit-cell dimension, a, was calculated using the following relationship: a = 2d_{110}^{3/2}. The parameter a also corresponds to the diameter of the rods forming the hexagonal lattice.

PKCa Binding to Liposomes. For these experiments, vesicles containing 2% diacetylphoglycerol, 5% phosphatidyl-serine, and 95% dioleoyl phosphatidylcholine (DOPC) or DOPC/dioleoyl phosphatidylethanolamine (DOPE) [6:4 (mol/mol)], in the presence or absence of 5% mol of Minerval, were prepared by dissolving the lipids in chloroform and mixing the appropriate volumes in glass vials. The solvent was removed under an argon stream, and the residue was maintained in a vacuum for at least 3 h. Thereafter, 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl (TK buffer) and 200 mM sucrose, was added to the lipid film and incubated at 42°C for 1 h with vigorous vortexing every 15 min. This lipid suspension (large multilamellar liposomes) was passed through a 400-nm polycarbonate membrane 11 times using a mini-extruder (Avanti Polar Lipids, Alaaster, AL) to obtain monolamellar vesicles of defined and homogeneous diameter. Liposome suspensions in TK buffer were combined with another 2.5 volumes of TK buffer without sucrose and centrifuged for 45 min at 62,000 g and 25°C. Finally, the pellet was resuspended in TK buffer to a final concentration of 0.6 mM lipid phosphorus. Model membranes (300 μl) were incubated in the presence of 10 μM CaCl2 and 25 ng of PKCa for 30 min at 25°C with agitation. PKCa bound to membranes was separated from the free enzyme by centrifugation as described above. The pellet containing bound PKCa was resuspended in 25 μl of electrophoresis loading buffer (84 mM Tris-HCl buffer, pH 6.8, 4% SDS, 143 mM β-mercaptoethanol, 5% glycerol, and 0.01% bromphenol blue). Polycyclamylide gel electrophoresis, immunoblotting, and protein quantification were performed as indicated below.

Cell Culture. Human lung adenocarcinoma (A549) cells were grown in six-well plates containing 2 ml of RPMI 1640 medium, supplemented with 10 mM HEPES, pH 7.4, 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. When the cells reached approximately 70% of confluence, Minerval (100 μM) was added to the medium for 24 h, except where indicated. Immediately after the incubation period, the cell monolayers were washed twice with PBS, cell attachment was disrupted with a rubber policeman, and the cells were resuspended in the appropriate buffer (see the corresponding sections). Finally, total RNA or total protein was extracted from the cells and quantified. The number of cells was determined by two methods. Live cells were counted using the trypan blue exclusion method, whereas cell proliferation/viability was determined by the 3-(4,5-dimethylthiazolol-2-yl)-2,5-diphenyl tetrazolium bromide method. Both methods gave similar results. Cell number was expressed as a percentage with respect to the control (untreated) cell cultures.
7.4, containing 50 mM NaCl, 1 mM MgCl$_2$, 2 mM EDTA, 1% SDS, 5 mM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride was added to each tissue culture well. Cell monolayers were then scraped free with a rubber policeman, and the cell suspensions were transferred to 1.5-ml Eppendorf tubes before being submitted to ultrasound treatment for 10 s at 50 W in a Braun Labsonic U sonicator. Aliquots of 30 µl were removed for total protein quantification. Then, 30 µl of 10× electrophoresis loading buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 50% glycerol, 0.1% bromphenol blue, and 10% β-mercaptoethanol) was added to the remaining suspension, which was then boiled for 3 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels (15-well and 1.5 mm in thickness) and then transferred to nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany). After protein transfer, the nitrocellulose membranes were incubated for 1 h at room temperature in a blocking solution [PBS (137 mM NaCl, 2.7 mM potassium chloride, 12 mM dibasic sodium phosphate, and 1.38 mM monobasic potassium phosphate, pH 7.4) containing 5% nonfat dry milk, 0.5% bovine serum albumin, and 0.1% Tween 20]. Then, the membranes were incubated overnight at 4°C in blocking solution containing the specific primary antibodies: monoclonal anti-PKCα (1:1000 dilution), anti-p21$^{	ext{CIP}}$ (1:1500), and anti-β-actin (1:2000), anti-cyclin A (1:1000), anti-cyclin B (1:1000), anti-cyclin D3 (1:1000), and anti-cdk2 (1:1000) (BD Biosciences Transduction Laboratories, Heidelberg, Germany). After removing the primary antibody, the membranes were washed three times for 10 min with PBS, and they were then incubated with the horseradish peroxidase-linked goat anti-mouse IgG secondary antibody (1:2000 dilution) (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) for 2 h at room temperature in fresh blocking solution. Immunoreactivity was detected with the enhanced chemiluminescence Western blot detection system (Amersham Biosciences UK, Ltd.) followed by exposure to ECL hyperfilm (Amersham Biosciences UK, Ltd.; Fig. 1). Films were scanned with a resolution of 63.5 µm (400 dpi).

For protein quantification by immunoblotting, the samples to be tested were evaluated using standard curves (i.e., total protein loaded versus integrated optical density (IOD)) consisting of five points of different protein content from control cells (usually between 5 and 30 µg of protein), all loaded on the same gel. This quantification procedure was repeated four times in different gels for each experiment, and experiments were replicated at least three times so that the mean value corresponds to at least 12 measurements. A linear correlation between the amount of protein loaded on the gel and IOD values was observed. The theoretical amount of protein (PT) loaded on the gel was obtained by interpolation of the IOD value from a test sample band in the standard curve. This value was compared and IOD values was observed. The theoretical amount of protein (PT) loaded on the gel was obtained by interpolation of the IOD value from a test sample band in the standard curve. This value was compared with the real amount of protein (P$_R$) loaded onto the gel. The percentage change (V) with respect to controls was calculated as $V = (P_T/P_R) \times 100$, where $V = 100$ for the control sample used as the standard. On the other hand, the percentage change (C) with respect to control was calculated as $C = V - 100$, where C = 0 for a control sample used as standard.

**mRNA Determination by Quantitative Reverse Transcriptase-Polymerase Chain Reaction.** QRT-PCR was used to determine the transcriptional modulation of PKCα by Minerval (100 µM, 24-h treatment). For this purpose, total RNA was extracted from $3 \times 10^6$ A549 cells (human lung adenocarcinoma cells) using the RNeasy midi kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The RNA concentration and purity was determined by optical density at 260 and 280 nm and by agarose gel electrophoresis.

Reverse transcription (RT) reactions were performed in a total volume of 20 µl. For this purpose, 1 µg of total RNA, 1 µl of oligo(dT) (500 µg/ml; Invitrogen, Carlsbad, CA), 1 µl of 10 mM dNTPs (Invitrogen), and H$_2$O to 12 µl were mixed in a tube and heated at 65°C for 5 min. The tubes were then chilled quickly on ice before 4 µl of 5× first-strand buffer (Invitrogen), 2 µl of 0.1 M dithiothreitol, and 1 µl of RNase OUT (recombinant ribonuclease inhibitor; Invitrogen) were added to each tube, and they were incubated at 37°C for 2 min. Finally, 1 µl (200 units) of Moloney murine leukemia virus reverse transcriptase (Invitrogen) was added to the mixture, which was incubated for 30 min at 37°C. The RT reaction was stopped by heating the tubes to 70°C for 15 min.

PCR conditions were established using a gradient thermal cycler (Eppendorf HQ, Hamburg, Germany) before quantitative PCR was carried out using the following method. Oligonucleotides for PKCα amplification were 5′-ATG GAT CAC ACT GAG AGG-3′ (forward) and 5′-TCA GCT CGG AAA CTC CAA AGG-3′ (reverse). These primers were of identical length, GC content, and calculated Tm, and amplified a 342-base pair fragment. PKCα mRNA levels were corrected by β-actin mRNA levels to disregard possible experimental artifacts. For this purpose, β-actin was quantified in parallel experiments by QRT-PCR using the forward primer 5′-GGG GAA ATT CAT GCT-3′ and the reverse primer 5′-CTA CTC CAA ACC-3′, which yielded a fragment of 231 base pairs. Real-time PCR amplifications were carried out in a LightCycler thermal cycler (Roche Diagnostics, Mannheim, Germany), using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche NMR of bovine liver PE membranes in the absence (left) or presence (right) of Minerval at different temperatures. The observed peaks corresponded to the lamellar (L), nonlamellar isotropic (NL1), and nonlamellar H$_{II}$ (NL2) phases. B, X-ray scattering of DEPE membranes showed that Minerval induced the occurrence of nonlamellar (hexagonal H$_{II}$) phases. The peaks corresponding to lattice distances for lamellar (dL) and hexagonal phases (dH) are indicated.
Diagnostics). The DNA concentration was determined by SYBR Green I fluorescence. PCR reaction mixtures consisted of 1 µl of DNA Master SYBR Green I mixture (containing 2.5 U of Taq polymerase), 0.8 µl of MgCl₂ (3 mM final concentration), 0.1 µl of each forward and reverse primers (0.1 µM final concentration), and 1 µl of cDNA template (10-ng maximum) from the RT reaction, in a final volume of 10 µl. These mixtures were denatured at 95°C for 4 min before cycling. For quantification of PKCα levels, samples were submitted to 50 thermal cycles consisting of four thermal segments: 10 s at 95°C (denaturation), 10 s at 58°C (annealing), 18 s at 72°C (extension), and 1 s at 81°C (fluorescence determination). For quantification of β-actin, samples were submitted to 40 thermal cycles of four segments: 5 s at 95°C, 5 s at 48°C, 18 s at 72°C, and 1 s at 95°C. The specificity of PCR products was determined by analysis of the amplified DNA melting curves and agarose gel electrophoresis.

**Determination of PKC Activity.** Approximately 3 × 10⁷ A549 cells, cultured as described above, were collected in ice-cold PBS, washed twice, and resuspended in 1 ml of cold sample preparation buffer (50 mM Tris-HCl, pH 7.5, 50 mM β-mercaptoethanol, 10 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamide). The cell suspension was sonicated and then centrifuged at 100,000 g for 60 min at 4°C. The pellets and supernatants were collected separately, and they were incubated at 25°C for 10 min in 25 mM Tris-HCl, pH 7.0, 3 mM MgCl₂, 2 mM CaCl₂, 0.1 mM ATP, 50 µg/ml phosphatidyserine, 0.5 mM EDTA, 1 mM EGTA, and 5 mM β-mercaptoethanol, in a total volume of 108 µl. PKC activity was determined using a protein kinase assay kit (enzyme-linked immunosorbent assay; Medical and Biological Laboratories Ltd., Nagoya, Japan) that measures the phosphorylation of the peptide N-RFARKGS(V/L)RQKNV-C, where S(P) indicates the serine residue phosphorylated. In other series of experiments, activity of purified PKCα was also measured. The experiments were carried out similarly as described above, except that 50 ng of purified enzyme was used instead of cell extracts. PKC-mediated phosphorylation was detected using a specific biotinylated anti-phosphopeptide antibody provided by the manufacturer, which was incubated in the assay sample for 1 h at 25°C. After washing the unbound antibody, phosphorylated-peptide/biotinylated-antibody complexes were quantified by addition of peroxidase-conjugated streptavidin (1 h at 25°C) followed by substrate addition (5-min incubation) and optical density reading at 492 nm. PKC-mediated phosphorylation in Mineval-treated cell fractions (soluble and particulate) was expressed as the percentage ± S.E.M. values of control values (control is 100%) of enzyme activities.

**Minerval Treatments.** The protocols used in this study were revised and approved by the Ethics Committee of the University of the Balearic Islands. A-549 and U87 xenograft tumor models were established by s.c. injection of tissue culture-derived tumor cells (5 × 10⁶ cells/animal on the left dorsal surface) in 6- to 8-week-old NCr nu/nu female athymic nude mice (National Cancer Institute, Frederick, MD). The tumor volume (cubic millimeters) was calculated as (w² × l)/2, where w is the width and l is the length of the tumor, as measured with calipers. Animals were monitored until day 10 after implantation, when tumors reached an average size of approximately 55 mm³. The animals were randomized into groups of six animals, so that the average tumor volumes were approximately the same for each group at the onset of treatment. Animals were treated for five consecutive days (i.p. or p.o. twice daily) with Mineval (75 and 100 mg/kg twice daily). Tumor volumes of control (untreated) and Mineval-treated animals were measured after these treatments, as indicated above. In other series of treatments, DBA/2J × C57BL/6 (F1) mice were infected with P388 murine leukemia cells (4 × 10⁶ cells per animal i.p.). One week after infection, animals were treated with either 150 mg/kg Mineval (5 times weekly) or 5 mg/kg doxorubicin (twice monthly) or both (12 animals weekly in two independent series of treatments). The survival of the animals was monitored during 8 weeks (Kaplan-Meier survival curves).

**Statistics.** Results are expressed as mean ± S.E.M. of at least three independent experiments or 12 animals. Student’s t test or one-way analysis of variance followed by Fisher’s tests was used for statistical evaluations. The level of significance was chosen as P = 0.05.

**Results and Discussion**

**Regulation of Lipid Structure by Minerval.** We studied the effects of Mineval on the lipid structure of model membranes using 31P NMR and X-ray scattering. In bovine liver PE membranes, the lamellar-to-hexagonal (HII) phase transition was 35°C (Fig. 1A). Below 30°C, the lipid was only organized into lamellar phases (peak L at −12.20 ppm) (Fig. 1A). At higher temperatures (>45°C), PE molecules organized only into nonlamellar H2 phases (peak NL2 at 5.94 ppm). The peak at 0.10 ppm (NL1) corresponded to an isotropic phase, most likely an intermediate between lamellar and hexagonal phases (Fig. 1A). The presence of Mineval induced important changes in the membrane structure. On one hand, it lowered the lamellar-to-hexagonal transition temperature about 12.5°C (TM ~ 22.5°C). On the other hand, this transition did not involve the occurrence of other nonlamellar structures (the isotropic phase peak was not ob-

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**Fig. 2.** Antiproliferative activity of Mineval. A, graph shows the effect of various concentrations of Mineval (24-h cultures) on the growth of A549 cells (solid circles). Control values correspond to untreated cells (100%, 5 × 10⁶ cells/ml). Open symbols correspond to the levels of PKCα protein over control (untreated cells, 0% of increase). Results are means of 12 measurements from four independent experiments. B, pictures show typical subconfluent cultures of control (C) and Mineval-treated (M, 100 µM) A549 cells.
served). These results clearly indicate that Minerval had important effects on lipid mesomorphism, favoring the formation of nonlamellar H_{II} lipid phases. In model DEPE membranes, Minerval also induced the formation of H_{II} phases, unequivocally defined by the X-ray scattering pattern (Fig. 1B), in agreement with the above-mentioned data. PE usually is the most abundant phospholipid in the cytoplasmic leaflet of mammalian plasma membranes. It is mainly organized into lamellar structures because of the presence of other phospholipid species. However, regions rich in PE have special physical properties, such as the nonlamellar-phase propensity, which may result in negative monolayer curvature or even in transient nonlamellar phases. In this context, numerous reports show that the nonlamellar phases, or the propensity to form them, increase the binding to membranes and activity of PKC (Dawson et al., 1984; Epand et al., 1991; Escribá et al., 1995; Giorgione et al., 1995; Mosior et al., 1996; Goñi and Alonso, 1999). In fact, diacylglycerols activate PKC not only by a direct binding to the Cys-rich C1 domain of the enzyme but also by inducing hexagonal (H_{II}) phases or increasing the nonlamellar phase propensity of membranes (Dawson et al., 1984; Epand, 1985; Epand and Bottega, 1988; Epand and Lester, 1990; Goldberg et al., 1994; Janes, 1996). In a similar manner, Minerval and structural analogs are able to lower the temperature of the lamellar-to-hexagonal (H_{II}) phase transition of model membranes. This effect is caused by the “molecular shape” of the drug: only structural analogs of Minerval (e.g., fatty acids with cis bonds) stabilize H_{II} phases, whereas chemical analogs with divergent structure do not (Epand et al., 1991; Goñi and Alonso, 1999; Funari et al., 2003; Barceló et al., 2004). These alterations can affect the functional properties of cells for example by influencing activity of membrane proteins.

Antiproliferative and Antitumor Effects of Minerval. Similarly as anthracyclines and HMBAs, Minerval inhibited cell proliferation of A549 (human lung adenocarcinoma) cells in a concentration-dependent manner (Fig. 2A). In addition, an inverse correlation between cell proliferation and the rise of PKCα levels at increasing concentrations of Minerval was observed (Fig. 2). To determine the antitumorigenic activity of Minerval, the inhibition of tumor-cell proliferation provoked by this drug was evaluated in xenograft tumor models. In athymic nude female mice, Minerval produced a significant inhibition of the growth of human A549 and U87 (human malignant glioma) cancers (Fig. 3A). Reductions of about 80% in the volume of subcutaneous xenograft tumors were observed after 5 days of treatment with Minerval. In mice (DBA/2J × C57BL/6, F1) infected with murine leukemia (P388) cells, Minerval had a greater antitumor activity than doxorubicin, an anthracycline currently used to treat leukemias and other types of cancer in human patients (Fig. 3B). It is interesting that Minerval plus doxorubicin seemed to display more potent therapeutic effects (83.3% survival at the end of the assay) than each drug alone (66.6 and 0% of survival).
survival rates for Minerval and doxorubicin, respectively). These results indicate that the antiproliferative activity of this drug is not restricted to cultured cells and that it is effective in whole organisms and could be used for treatment of human tumors. An important feature of the lipid therapy with Minerval is the low toxicity of the compound. In this context, we were unable to determine the maximally tolerated dose of this drug because after treatments of 3 g/kg per day for 2 weeks, the animals were still alive. Histological analyses of several rat organs showed that high doses of Minerval (1.2 g/kg/day p.o.) did not induce observable toxicity (Fig. 3C). Only in liver, intracellular vesicles were observed in hepatocytes, a typical phenomenon associated with high fat intake. However, this effect was not observed at lower doses used in this study.

**Minerval Mechanism of Action.** To determine the molecular mechanisms that underlie the antiproliferative effects of Minerval, we first studied the possible involvement of apoptosis in the alterations leading to inhibition of cancer cell proliferation. After exposure to Minerval for 24 h (0–150 μM), we did not observe poly(ADP-ribose) polymerase degradation in immunoblots, caspase-3 activation in enzyme immunoassays and immunoblots, or DNA breakage as determined by agarose gel electrophoresis and flow cytometry (data not shown). These results suggested that under the experimental conditions used, Minerval did not promote apoptosis in A549 cells. However, other conditions or mechanisms related to apoptosis, such as parapoptosis (Sperandio et al., 2000), may operate in these cells. Therefore, in other cell types or under different physiological/experimental conditions, apoptosis or cytotoxicity should not be discarded. On the other hand, cell differentiation may justify inhibition of tumor progression after treatment with Minerval. In fact, PKC activation has been associated with differentiation of various tumor cell types (Michaeli et al., 1992; Sun and Rotenberg, 1999). Moreover, the modulation of the membrane structure, PKC recruitment to membranes and subsequent enzyme activation, have been related to the antitumor effects of HMBA (Michaeli et al., 1992; Escribá et al., 1995, 2002; Giorgione et al., 1995).

An alternative to apoptosis to explain the impairment of tumor progression by Minerval is the activation of antiproliferative signals. In this context, the levels of the antiproliferative protein p21<sup>CIP</sup> [p21<sup>CIP</sup>/WAF1/p53<sup>−/−</sup>], cdk-interacting protein (CIP), wild-type p53 activated fragment (WAF), and p53-regulated inhibitor of cdk5 (Pic) were significantly increased in A549 cells exposed to Minerval (increases of 255 ± 22%, P < 0.001) (Fig. 4A). Induction of p53 often results in the activation of p21<sup>CIP</sup> transcription (el-Deiry et al., 1993) although the latter has been shown to induce exit from the cell cycle independently of p53 (Parker et al., 1995). Here, we showed that Minerval did not alter p53 levels (increase of 7 ± 8%, P > 0.05) (Fig. 4A), suggesting that this protein is not involved in the induction of p21<sup>CIP</sup>. In contrast, PKCα levels were also increased in the presence of Minerval (125 ± 14%, P < 0.001). It is interesting that activation of PKC can induce p21<sup>CIP</sup> expression in a p53-independent manner in cancer cells, leading to cell cycle arrest (Zeuzela et al., 1997; Akashi et al., 1999; Detjen et al., 2000; Han et al., 2001; Park et al., 2001). Moreover, it has been reported that PKC-mediated phosphorylation of p21<sup>CIP</sup> is associated with a reduction in the activity of certain cyclin/cdk2/p21<sup>CIP</sup> complexes (Kashiwagi et al., 2000). In this context, different PKC isoforms can act not only directly on p21<sup>CIP</sup> (Kashiwagi et al., 2000) but also through other messengers (e.g., Mek1) (Zeuzela et al., 1997) to activate p21<sup>CIP</sup>. These studies show that induction of PKC can lead to activation of p21<sup>CIP</sup>, thus initiating inactivation of cyclin/cdk complexes that finally results in exit of the cell cycle. In this work, we also showed that PKC activation by phorbol esters induced the expression of p21<sup>CIP</sup> and blockade of the enzyme reversed its overexpression. The present results, along with the above-mentioned studies, suggest that PKC is involved in exit of the cell cycle induced by Minerval (see below).

Short-term incubations with Minerval (15–60 min) also induced the translocation of PKCα to A549 cell membranes (Fig. 4B). This effect was maintained during long-term (24-h) exposure to the drug (Fig. 4B), being the increase in membrane-associated PKC accompanied by a decrease in cytosolic PKC levels (Fig. 4B). Moreover, induction of binding of PKC to membranes was observed not only in A549 cell membranes but also in cardiac cell membranes from animals treated with Minerval (increases of 48 ± 5%, P < 0.01). We also studied the effect of the membrane structure on membrane-PKC interaction and its modulation by Minerval using a model system consisting of pure membrane-forming phospholipids and the purified enzyme (Fig. 4C). Membranes containing phospholipid DOPE, which was organized into H<sub>II</sub> phases under the experimental conditions used, showed a greater capacity to bind PKCα (387 ± 56%, P < 0.001) than membranes containing only the lamellar-forming phospholipid DOPC (Fig. 4C). DOPE and DOPC are identical in their hydrophobic core, although their polar heads are not chemically similar; the choline moiety in DOPC is larger than ethanolamine in DOPE. For this reason, DOPC organizes into bilayers and DOPE into hexagonal (H<sub>II</sub>) phases, at room temperature (experimental conditions). Therefore, these results indicate that the membrane structure does indeed play a pivotal role in the membrane association of PKC and its subsequent activation. Furthermore, the presence of Minerval increased PKCα binding to DOPE-containing membranes but not to membranes formed exclusively of DOPC (736 ± 61%, P < 0.01 and 93 ± 12%, P > 0.05 in the presence or absence of DOPE, respectively) (Fig. 4C). The increase of PKC binding in vesicles containing DOPE was similar to that found in the binding of this enzyme to biological membranes (Fig. 4A). These results show the importance of the lipid structure in PKC-membrane interactions and may explain in part the anticancer activity of Minerval. Our results may also explain the molecular/structural basis underlying PKC activation by certain unsaturated fatty acids (e.g., oleic acid and related compounds) (Lu et al., 1996), a question that remained elusive thus far. Albeit a direct interaction between fatty acids and PKC has not been described yet, it should not be discarded the possibility that this type of molecules also interacted directly with the enzyme. However, this point requires further investigation.

PKC translocation from cytosol to membranes is associated with activation of the enzyme (Pinton et al., 2002). Here, we found that besides PKC translocation to membranes, Minerval induced significant increases of PKC activity in membranes of Minerval-treated cells (increases of 219 ± 17%, P < 0.01) (Fig. 4D). This drug did not alter significantly PKC
activity in the absence of membranes (purified protein) (Fig. 4D).

On the other hand, Minerval induced PKCα expression in A549 cells. In whole cells (not just membranes), PKCα protein levels were markedly and significantly higher after treatment with 100 μM Minerval (increases of 125 ± 14%, P < 0.01, over untreated control cells, as measured by immunoblotting). In a similar manner, PKCα mRNA levels were found elevated (increases of 274 ± 56%, P < 0.001, over untreated control cells, as measured by RT-PCR, which is described in the materials and methods section). Although PKC activation by phorbol esters is followed by protein down-regulation, several reports demonstrate that under different physiological situations, enzyme activation is paralleled by increases in the levels of the protein (Sieber et al., 1998). In this context, PKC down-regulation induced by phorbol esters

![Anticancer Activity of Minerval](molpharm.aspetjournals.org)

**Fig. 4.** Minerval mechanism of action. A, effects of Minerval treatments (100 μM, 24 h) on the density of PKCα, p21CIP1, and p53 in A549 cells, measured by immunoblotting analysis. Bars correspond to means of six independent experiments. Representative immunoblots are shown on the right. *, P < 0.001. B, Minerval treatments on PKCα translocation to membranes. PKCα recruitment to A549 cell membranes after 45-min and 24-h treatments was measured by immunoblotting. The corresponding PKCα decreases in cytosol fractions were also measured from control (untreated) and Minerval-treated A549 cells. Bars are means of four independent experiments. *, P < 0.001. C, binding of purified PKCα to model membranes (liposomes) of DOPC (PC) (lamellar-prone phospholipid) in the absence or presence of Minerval are shown on the left side of the graph. On the right side, PKCα binding to membranes containing DOPE [nonlamellar-prone phospholipid, DOPC:DOPE 6:4 (mol:mol)] (PCPE) is shown. Bars are means of three independent experiments. #, P < 0.01 versus the corresponding PC vesicles; *, P < 0.01 versus PCPE without Minerval. D, effect of Minerval on PKC activity. The bars correspond to enzyme activity in membrane fractions (membrane) from A549 cells treated with Minerval and from untreated control cells. Minerval had no significant effect on PKC activity in the absence of membranes (purified protein, membrane-free). Results are expressed as percentage values of the corresponding control (in the absence of Minerval). Basal PKC activity in the presence of membranes was about 5-fold greater than that in a membrane-free system. Bars are means of three independent experiments. *, P < 0.01. E, effect of Minerval on cyclins A, B, and D3 and cdk2. A549 cells were treated during 24 h with Minerval (100 μM), and the cellular content of these proteins was quantified by immunoblotting and compared with the levels in untreated control cells (100%). Minerval treatments did not induce significant changes in the levels of β-actin. Bars are means of at least four independent experiments. *, P < 0.01 versus the corresponding control.
is caused by proteolytic degradation of the strikingly activated enzyme (Junoy et al., 2002). In turn, moderate PKC activation would result in elevated protein levels (Pintus et al., 1999), which are not masked by proteolysis.

The binding of p21CIP regulates various cyclin/cdk2 complexes. This association of p21CIP to these complexes promotes their degradation impairing the cell cycle progression (Cayrol and Ducommun, 1998). Consistent with this sequence of events, exposure to Minerval caused the down-regulation of cyclins A, B, and D3 and cdk2 in A549 cells (50–70% reductions after 24-h incubations with 100 µM Minerval) (Fig. 4E). It has been shown that p21CIP is involved in cellular quiescence and cell cycle arrest/exit through inactivation and/or down-regulation of cyclins (Halevy et al., 1995; Detjen et al., 2000). In general, cell cycle progression and exit has been associated with cyclin expression and degradation, whereas cdk's are often maintained at constant levels within the cell. Nevertheless, cdk2 was also down-regulated upon exposure to Minerval. In agreement with our data, it has been reported that cell cycle arrest in C33A tumor cells is associated with a decrease of both cyclin A and cdk2 protein levels (Strobeck et al., 2000). Moreover, a similar down-regulation of cyclin A and cdk2 has been observed after cell cycle arrest of cells with damaged DNA (Walker et al., 1995). In addition, reduced cellular proliferation and increased cellular differentiation have also been associated with decreases in cdk2 expression (Dobashi et al., 2000; Belachew et al., 2002). The effects of Minerval on the levels of cyclins and cdk2 were similar to those induced by serum deprivation on these proteins (reductions between 42 and 61%, data not shown), in agreement with the suggested antiproliferative activity of this drug. Thus, it seems likely that the changes in the levels of cyclins and cdk2, provoked by exposure to Minerval, accounted for the inhibition of A549 cell proliferation observed.

The involvement of PKC in the mechanism of action of Minerval was further investigated by inhibiting the enzyme with PKC-I, a peptide corresponding to the N-terminal (amino acids 20–28) myristoylated pseudosubstrate sequence for PKCα and β (Ward and O’Brian, 1993). PKC inhibition prevented the overexpression of PKCα induced by Minerval and reversed its effect on p21CIP in A549 human lung adenocarcinoma cells (Fig. 5A). Likewise, PKC-I reversed the effect of Minerval on the levels of cyclins and cdk2 (Fig. 5B). These results suggest the involvement of PKC in activation of p21CIP and down-regulation of cyclins and cdk2 (Fig. 5, A and B).
B) and in the antiproliferative effects mediated by the drug. Moreover, PKC activation by phorbol 12-myristate 13-acetate provoked the up-regulation of p21cip and the down-regulation of cyclins and cdk2 (Fig. 5C). These data are in agreement with the involvement of PKCα in the pharmacological mechanism of action of Minerval leading to impairment of cell proliferation, as described for other antitumor drugs (Michaeli et al., 1992; Escribá et al., 2002). In addition, in line with the present results, it has been shown that PKC activation mediates growth inhibition of endothelial cells, which can be also reversed by PKC inhibitors (Zezula et al., 1997). In that study and in this work, PKC activation by phorbol esters induced p21cip in endothelial and epithelial cells, respectively. Together, the present results show that PKC, p21cip, cyclins, and cdk2 are involved in the pharmacological effects exerted by Minerval.

Lipid Therapy. The membrane lipid structure has been shown to influence cell signaling (Escribá et al., 1997; Vogler et al., 2004). The lipid composition of membranes has also been associated with the development of human pathologies (Escribá et al., 2003). The present data suggest that the antiproliferative activity of Minerval could be related to the regulation of the membrane structure and subsequent PKC recruitment to the membrane. The hexagonal phase propensity of membranes has been associated with the interaction of several amphitropic proteins (Kinnunen, 1996). In line with this, heterotrimeric G proteins and Gβγ dimers prefer non-lamellar-prone membranes, whereas the Gαi monomer, formed upon agonist activation of G protein-coupled receptors, has a greater affinity for lamellar phases (Vogler et al., 2004). This differential interaction with membrane lipids drives in part the cellular localization and activity of resting (Gαiγ) and active (Gαi and Gβγ) G proteins. Because G proteins and related systems are targets for a great number of marketed drugs, the modulation of these systems is of great clinical relevance. Moreover, many cell functions occur in or around membranes, so that the differential modulation of the membrane lipid structure and/or composition will probably result in the modulation of other proteins. Although the modulation of the membrane structure itself has not been considered for the development of clinical drugs, evidence has been gathered showing that compounds that alter the membrane structure regulate cellular functions. Agents such as alcohols, detergents, anesthetics, non-oxidizers, organic solvents, surfactants and other types of molecules modulate the membrane structure with their own characteristic features (Lester and Baumann, 1991; Perkins et al., 1996; Shen et al., 1999; Koubi et al., 2002). Lipid therapy differs with other conventional therapeutic strategies in that the first target of the drug is not a protein itself, but the membrane lipids. Indeed, the potential for this approach is supported by the fact that the antitumor agents daunomycin and HMBA, which alter the cellular distribution and activity of PKC, also modulate the plasma membrane structure (Michaeli et al., 1992; Escribá et al., 1995, 2002; Giorgione et al., 1995).

Because of the current view of the cell membrane as a mere support for membrane proteins, one might envisage that lipid therapy would either be an unspecified or a poorly effective treatment. Nevertheless, the expression of certain proteins is often ubiquitous or widespread in cells, and the effectiveness and specificity of many drug treatments target-


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