

# Membrane Structure Modulation, Protein Kinase C $\alpha$ Activation, and Anticancer Activity of Minerval

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Received March 26, 2004; accepted November 5, 2004

## 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 nonlamellar (hexagonal H<sub>II</sub>) 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 PKC $\alpha$  expression (mRNA and protein levels) in A549 cells. This drug also induced PKC activation, which led to a p53-independent increase in p21<sup>CIP</sup> 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 (Escríbá et al., 1997). Indeed, certain antitumor drugs seem to act by regulating signal transduction in this way (Escríbá et al., 1995, 2002). Although it has been proposed that the cytotoxic effects of anthracyclines are based on a direct interaction with DNA, strong evidence has accumulated indicating that other interactions are also responsible for their pharmacological activ-

ity. First, anthracycline-agarose derivatives exert their antitumor activity by interacting with the plasma membrane without entering the cell (Triton and Yee, 1982). Second, anthracyclines that do not bind to DNA (e.g., *N*-trifluoroacetyladrriamycin 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 (Escríbá 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 (Escríbá et al., 1995). Moreover, this phenomenon is involved in the cellular relocation and activity of both G proteins and protein kinase C (PKC) (Escríbá et al., 1995; Giorgione et al., 1995). These proteins are crucial cell signaling components

This work was supported in part by grants FIS PI/031218 (to R.A.), FIS00/1029, SAF2001-0839, SAF2003-00232, SAF2004-05249, and CAO01-002 (to P.V.E.). 'Fundación Marathon', 'Conselleria de Sanitat', and 'Conselleria d'Innovació i Tecnologia (PRDIB-2002GC2-11) del Govern Balear' also provided funds for this work (to P.V.E.). J.C. and T.N. were supported by fellowships from the Spanish 'Ministerio de Educación, Cultura y Deportes'. J.P. was supported by a fellowship from the 'Conselleria d'Innovació i Tecnologia del Govern Balear'. R.A. and O.V. are "Ramón y Cajal" fellows.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
doi:10.1124/mol.104.000778.

**ABBREVIATIONS:** PKC, protein kinase C; HMBA, hexamethylene bisacetamide; PE, phosphatidylethanolamine; DEPE, dielaidoyl phosphatidylethanolamine; DOPC, dioleoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; PBS, phosphate-buffered saline; IOD, integrated optical density; QRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; RT, reverse transcription; PCR, polymerase chain reaction; cdk, cyclin-dependent kinase.

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 (Caponigro 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  $H_{II}$  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

**$^{31}\text{P}$  NMR.** Multilamellar lipid vesicles were prepared mixing 56 mg of bovine liver phosphatidylethanolamine (PE) with deuterated water [ $\text{PE}/\text{D}_2\text{O}$ , 15:85 (w/w)], in the absence or presence of Minerval [ $\text{PE}/\text{Minerval}$ , 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 ( $60^\circ\text{C}$ ) and freezing ( $-80^\circ\text{C}$ ) cycles, and equilibrated before data acquisition, as reported previously (Funari et al., 2003).  $^{31}\text{P}$  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  $55^\circ\text{C}$ , with increases of  $2.5^\circ\text{C}$ ) for 10 min before data were acquired.  $^{31}\text{P}$  NMR free induction decays were accumulated for 64 transients by using a 4.4- $\mu\text{s}$ ,  $90^\circ$  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  $\text{H}_2\text{O}$  (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  $70^\circ\text{C}$  and cooling to  $4^\circ\text{C}$ ). Then, they were immediately stored at  $-80^\circ\text{C}$  under argon until use. Before X-ray scattering experiments were performed, the samples were equilibrated at  $4^\circ\text{C}$  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  $57^\circ\text{C}$ . Interplanar distances,  $d_{hkl}$ , were calculated according to the equation  $s = 1/d_{hkl} = (2 \sin \theta)/\lambda$ , where  $s$  is the scattering vector,  $2\theta$  is the scattering angle,  $\lambda$  (0.15 nm) is the X-ray wavelength, and  $hkl$  are the Miller indexes of the scattering planes. For hexagonal ( $H_{II}$ ) phases, the unit-cell dimension,  $a$ , was calculated using the following relationship:  $a = 2d_{10}/3^{1/2}$ . The parameter  $a$  also corresponds to the diameter of the rods forming the hexagonal lattice.

**PKC $\alpha$  Binding to Liposomes.** For these experiments, vesicles containing 2% diacylglycerol, 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  $42^\circ\text{C}$  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, Alabaster, 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  $62,000g$  and  $25^\circ\text{C}$ . Finally, the pellet was resuspended in TK buffer to a final concentration of 0.6 mM lipid phosphorus. Model membranes (300  $\mu\text{l}$ ) were incubated in the presence of 10  $\mu\text{M}$   $\text{CaCl}_2$  and 25 ng of PKC $\alpha$  for 30 min at  $25^\circ\text{C}$  with agitation. PKC $\alpha$  bound to membranes was separated from the free enzyme by centrifugation as described above. The pellet containing bound PKC $\alpha$  was resuspended in 25  $\mu\text{l}$  of electrophoresis loading buffer (84 mM Tris-HCl buffer, pH 6.8, 4% SDS, 143 mM  $\beta$ -mercaptoethanol, 5% glycerol, and 0.01% bromophenol blue). Polyacrylamide 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  $\mu\text{g}/\text{ml}$  amphotericin B. When the cells reached approximately 70% of confluence, Minerval (100  $\mu\text{M}$ ) 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-dimethylthiazol-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.

**Electrophoresis (SDS-Polyacrylamide Gel Electrophoresis), Immunoblotting, and Protein Quantification.** For protein extraction from cultured cells, 300  $\mu\text{l}$  of 10 mM Tris-HCl buffer, pH

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  $\mu$ l were removed for total protein quantification. Then, 30  $\mu$ l of 10 $\times$  electrophoresis loading buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 50% glycerol, 0.1% bromophenol blue, and 10%  $\beta$ -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-PKC $\alpha$  (1:1000 dilution), anti-p21<sup>CIP</sup> (1:500), and anti-p53 (1:500) and anti- $\beta$ -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  $\mu$ m (400 dpi).

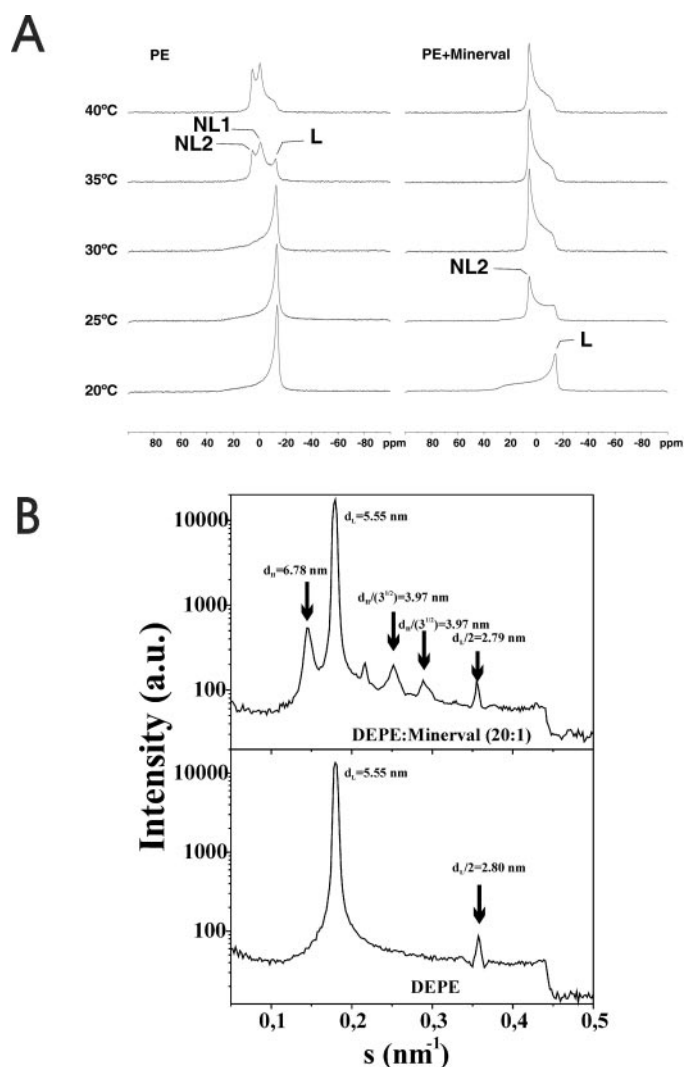
For protein quantification by immunoblotting, the samples to be tested were evaluated using standard curves [i.e., total protein loaded versus integrated optical density (IOD)] consisting of five points of different protein content from control cells (usually between 5 and 30  $\mu$ g of protein), all 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 ( $P_T$ ) 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 value ( $V$ ) 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 PKC $\alpha$  by Minerval (100  $\mu$ M, 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  $\mu$ l. For this purpose, 1  $\mu$ g of total RNA, 1  $\mu$ l of oligo(dT) (500  $\mu$ g/ml; Invitrogen, Carlsbad, CA), 1  $\mu$ l of 10 mM dNTPs (Invitrogen), and  $H_2O$  to 12  $\mu$ l were mixed in a tube and heated at 65°C for 5 min. The tubes were then chilled quickly on ice before 4  $\mu$ l of 5 $\times$  first-strand buffer (Invitrogen), 2  $\mu$ l of 0.1 M dithiothreitol, and 1  $\mu$ l of RNase OUT (recombinant ribonuclease inhibitor; Invitrogen) were

added to each tube, and they were incubated at 37°C for 2 min. Finally, 1  $\mu$ l (200 units) of Moloney murine leukemia virus reverse transcriptase (Invitrogen) was added to the mixture, which was incubated for 50 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 PKC $\alpha$  amplification were 5'-ATG GAT CAC ACT GAG AAG AGG-3' (forward) and 5'-TCA GCT CCG AAA CTC CAA AGG-3' (reverse). These primers were of identical length, GC content, and calculated  $T_m$ , and amplified a 342-base pair fragment. PKC $\alpha$  mRNA levels were corrected by  $\beta$ -actin mRNA levels to disregard possible experimental artifacts. For this purpose,  $\beta$ -actin was quantified in parallel experiments by QRT-PCR using the forward primer 5'-GCG GGA AAT CGT GCG TGA CAT T-3' and the reverse primer 5'-CTA CCT CAA CTT CCA TCA AAG CAC-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



**Fig. 1.** Effect of Minerval on the structure of model membranes. A,  $^{31}P$  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 ( $d_L$ ) and hexagonal phases ( $d_H$ ) are indicated.



Diagnostics). The DNA concentration was determined by SYBR Green I fluorescence. PCR reaction mixtures consisted of 1  $\mu$ l of DNA Master SYBR Green I mixture (containing 2.5 U of *Taq* polymerase), 0.8  $\mu$ l of  $MgCl_2$  (3 mM final concentration), 0.1  $\mu$ l of each forward and reverse primers (0.1  $\mu$ M final concentration), and 1  $\mu$ l of cDNA template (10-ng maximum) from the RT reaction, in a final volume of 10  $\mu$ l. These mixtures were denatured at 95°C for 4 min before cycling. For quantification of PKC $\alpha$  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  $\beta$ -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 85°C. The specificity of PCR products was determined by analysis of the amplified DNA melting curves and agarose gel electrophoresis.

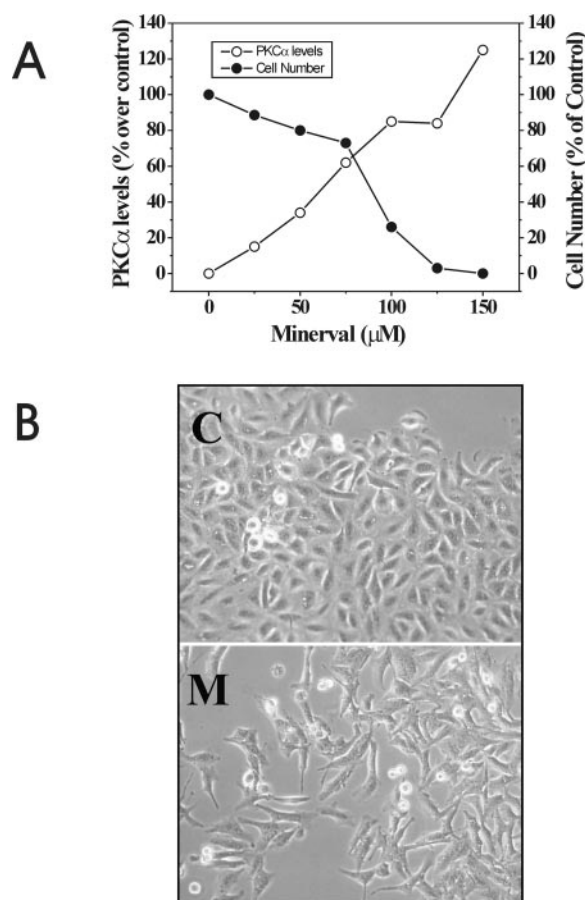
**Determination of PKC Activity.** Approximately  $3 \times 10^7$  A549 cells, cultured as described above, were collected in ice-cold PBS, washed twice, and resuspended in 1 ml of cold sample preparation buffer (50 mM Tris-HCl, pH 7.5, 50 mM  $\beta$ -mercaptoethanol, 10 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamide). The cell suspension was sonicated and then centrifuged at 100,000g 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  $MgCl_2$ , 2 mM  $CaCl_2$ , 0.1 mM ATP, 50  $\mu$ g/ml phosphatidylserine, 0.5 mM EDTA, 1 mM EGTA, and 5 mM  $\beta$ -mercaptoethanol, in a total volume of 108  $\mu$ l. 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(P)LRQKNV-C, where S(P) indicates the serine residue phosphorylated. In other series of experiments, activity of purified PKC $\alpha$  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 Minerval-treated cell fractions (soluble and particulate) was expressed as the percentage  $\pm$  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 ( $5 \times 10^6$  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  $(w^2 \times l)/2$ , 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 mm<sup>3</sup>. 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 Minerval (75 and 100 mg/kg twice daily). Tumor volumes of control (untreated) and Minerval-treated animals were measured after these treatments, as indicated above. In other series of treatments, DBA/2J  $\times$  C57BL/6 (F1) mice were infected with P388 murine leukemia cells ( $4 \times 10^5$  cells per animal i.p.). One week after infection, animals were treated with either 150 mg/kg Minerval (5 times weekly) or 3 mg/kg doxorubicin (twice monthly) or both (12 animals per group 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  $\pm$  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 Minerval on the lipid structure of model membranes using <sup>31</sup>P NMR and X-ray scattering. In bovine liver PE membranes, the lamellar-to-hexagonal ( $H_{II}$ ) 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  $H_{II}$  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 Minerval induced important changes in the membrane structure. On one hand, it lowered the lamellar-to-hexagonal transition temperature about 12.5°C ( $T_H \approx 22.5^\circ\text{C}$ ). On the other hand, this transition did not involve the occurrence of other nonlamellar structures (the isotropic phase peak was not ob-



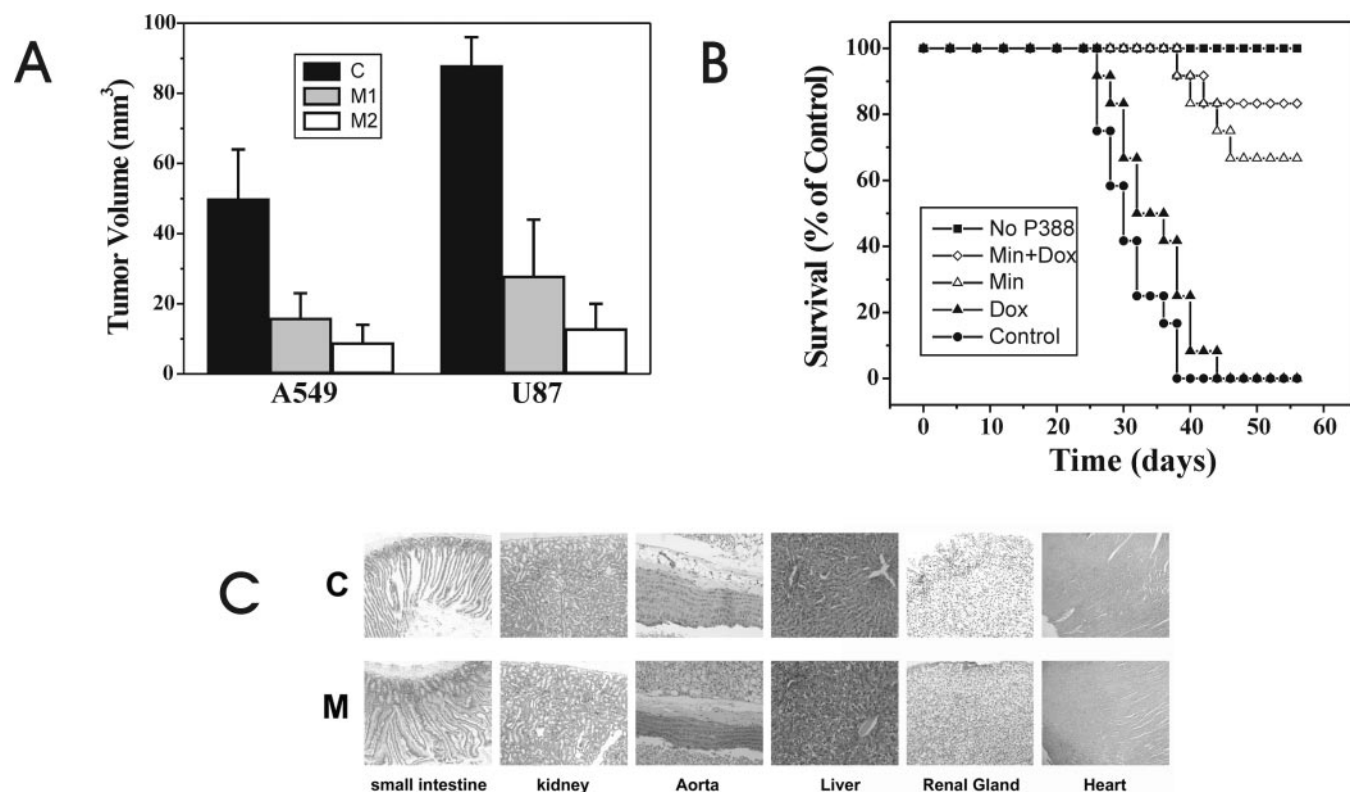
**Fig. 2.** Antiproliferative activity of Minerval. A, graph shows the effect of various concentrations of Minerval (24-h cultures) on the growth of A549 cells (solid circles). Control values correspond to untreated cells (100%,  $5 \times 10^5$  cells/ml). Open symbols correspond to the levels of PKC $\alpha$  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 Minerval-treated (M, 100  $\mu$ M) 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 HMBA, 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 PKC $\alpha$  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  $\times$  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



**Fig. 3.** Antitumor effects of Minerval. A, effect of Minerval on xenograft tumors from A549 (human lung adenocarcinoma) and U87 (human malignant glioma) cells in nude athymic mice. Tumor volumes (cubic millimeters) were determined before and after 5 days of treatment (days 10–14 after tumor cell implantation). Bars correspond to mean  $\pm$  S.E.M. of tumor volume increase in untreated (control) and Minerval-treated animals (75 mg/kg, M1 or 100 mg/kg, M2). B, Kaplan-Meier survival curves for mice infected with P388 murine leukemia cells. Mice (12 animals per group) were treated with saline ( $\bullet$ ), doxorubicin ( $\blacktriangle$ ), Minerval ( $\triangle$ ), or Minerval plus doxorubicin ( $\diamond$ ). Survival of noninfected animals is also shown ( $\blacksquare$ ). C, histological toxicity after Minerval treatments. Animals were treated with vehicle (control, C, top) or Minerval (M, bottom) (1200 mg/kg/day p.o., 1 week). Sections from paraformaldehyde-fixed and paraffin-embedded tissues were submitted to hematoxylin & eosin staining and photographed at a 50- to 100-fold magnification.

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  $\mu$ M), 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 paraptosis (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>CIP1/WAF1/Pic1</sup>, cdk-interacting protein (CIP), wild-type p53 activated fragment (WAF), and p53-regulated inhibitor of cdk (Pic)] were significantly increased in A549 cells exposed to Minerval (increases of  $255 \pm 22\%$ ,  $P < 0.001$ ) (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  $7 \pm 8\%$ ,  $P > 0.05$ ) (Fig. 4A), suggesting that this protein is not involved in the induction of p21<sup>CIP</sup>. In contrast, PKC $\alpha$  levels were also increased in the presence of Minerval ( $125 \pm 14\%$ ,  $P < 0.001$ ). 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 (Zezula 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 (Kashi-

wagi 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) (Zezula 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 PKC $\alpha$  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  $48 \pm 5\%$ ,  $P < 0.01$ ).

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 PKC $\alpha$  ( $387 \pm 56\%$ ,  $P < 0.001$ ) 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 PKC $\alpha$  binding to DOPE-containing membranes but not to membranes formed exclusively of DOPC ( $736 \pm 61\%$ ,  $P < 0.01$  and  $93 \pm 12\%$ ,  $P > 0.05$  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.

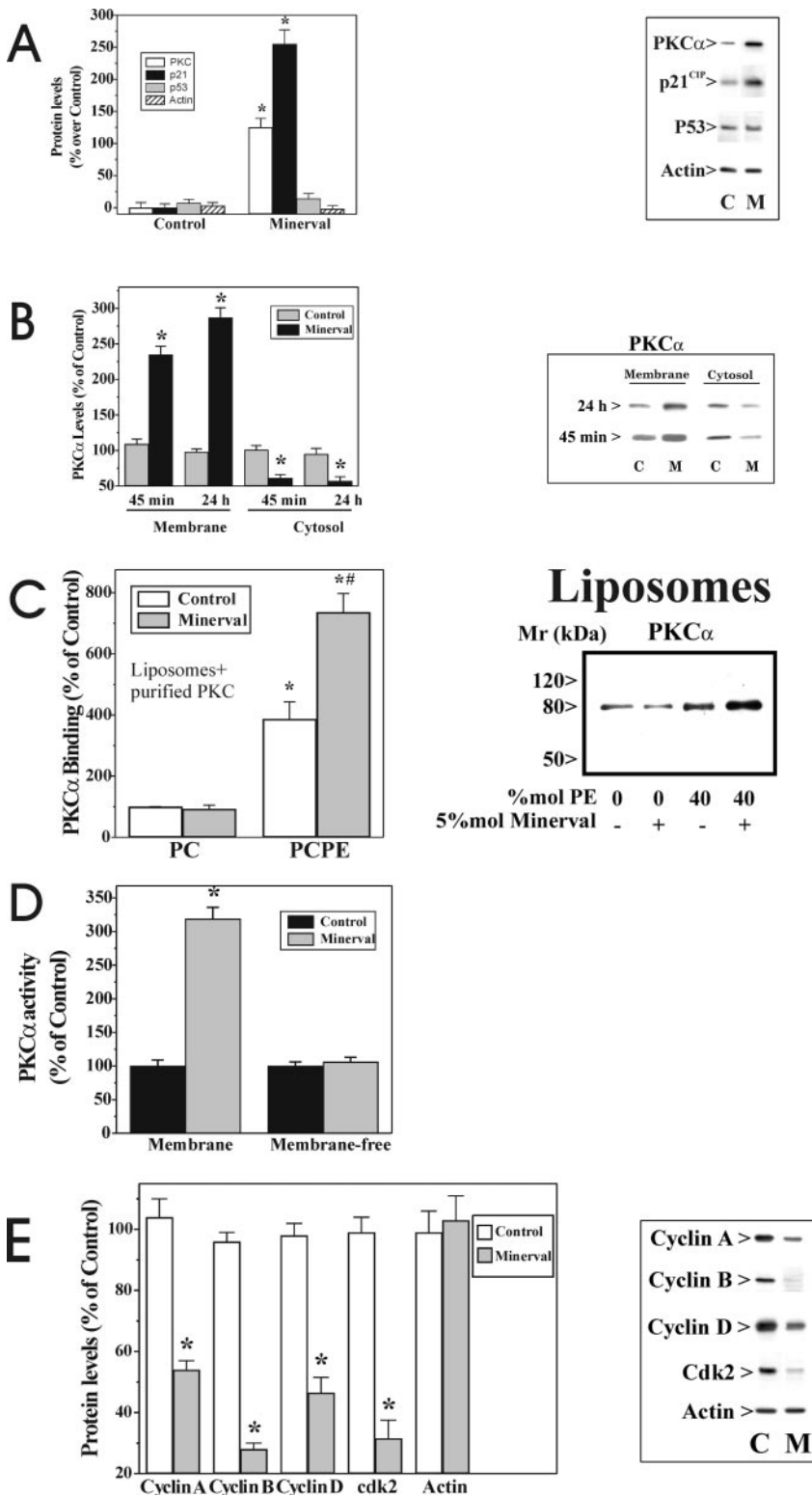
PKC translocation from cytosol to membranes is associated with activation of the enzyme (Pinton et al., 2002). Here, we found that besides PKC translocation to membranes, Minerval induced significant increases of PKC activity in membranes of Minerval-treated cells (increases of  $219 \pm 17\%$ ,  $P < 0.01$ ) (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 PKC $\alpha$  expression in A549 cells. In whole cells (not just membranes), PKC $\alpha$  protein levels were markedly and significantly higher after treatment with 100  $\mu$ M Minerval (increases of  $125 \pm 14\%$ ,  $P < 0.01$ , over untreated control cells, as measured by immunoblotting). In a similar manner, PKC $\alpha$  mRNA levels

were found elevated (increases of  $274 \pm 56\%$ ,  $P < 0.001$ , 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



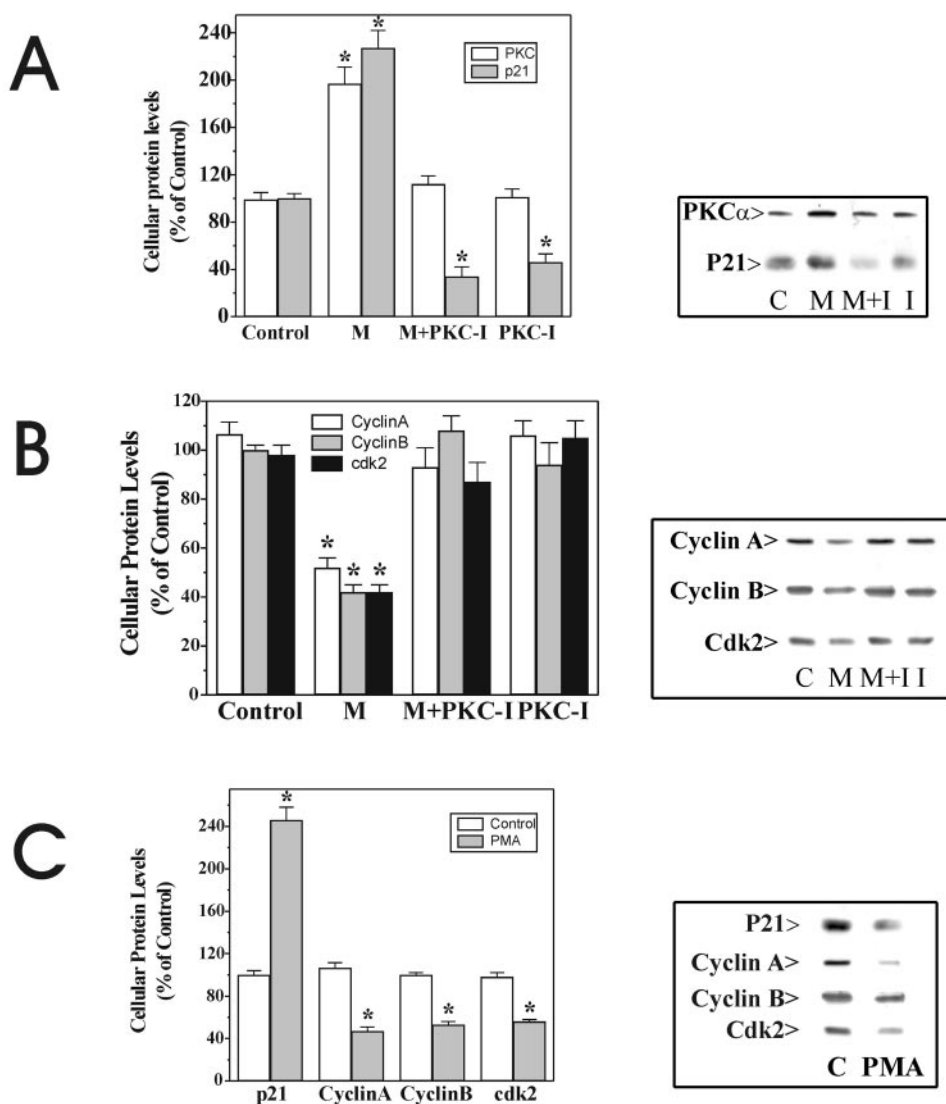
**Fig. 4.** Minerval mechanism of action. A, effects of Minerval treatments (100  $\mu$ M, 24 h) on the density of PKC $\alpha$ , p21<sup>CIP</sup>, and p53 in A549 cells, measured by immunoblotting analysis. Bars correspond to means of six independent experiments. Representative immunoblots are shown on the right. \*,  $P < 0.001$ . B, Minerval treatments on PKC $\alpha$  translocation to membranes. PKC $\alpha$  recruitment to A549 cell membranes after 45-min and 24-h treatments was measured by immunoblotting. The corresponding PKC $\alpha$  decreases in cytosol fractions were also measured from control (untreated) and Minerval-treated A549 cells. Bars are means of four independent experiments. \*,  $P < 0.01$ . C, binding of purified PKC $\alpha$  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, PKC $\alpha$  binding to membranes containing DOPE [nonlamellar-prone phospholipid, DOPC:DOPE 6:4 (mol:mol)] (PCPE) is shown. Bars are means of three independent experiments. \*,  $P < 0.001$  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  $\mu$ M), 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  $\beta$ -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 p21<sup>CIP</sup> regulates various cyclin/cdk2 complexes. The association of p21<sup>CIP</sup> 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  $\mu$ M Minerval) (Fig. 4E). It has been shown that p21<sup>CIP</sup> 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 cdks 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 PKC $\alpha$  and  $\beta$  (Ward and O'Brian, 1993). PKC inhibition prevented the overexpression of PKC $\alpha$  induced by Minerval and reversed its effect on p21<sup>CIP</sup> 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 p21<sup>CIP</sup> and down-regulation of cyclins and cdk2 (Fig. 5, A and



**Fig. 5.** Role of PKC activity in the molecular mechanism of Minerval. A, effect of the PKC inhibitor PKC-I on the expression of PKC $\alpha$  and p21<sup>CIP</sup>. Minerval (M, 100  $\mu$ M, 24 h) induced up-regulation of PKC $\alpha$  and p21<sup>CIP</sup> in A549 cells. PKC activity was inhibited by PKC-I, reversing PKC $\alpha$  overexpression and inducing a down-regulation of p21<sup>CIP</sup> either in the presence (M + PKC-I) or absence (PKC-I) of Minerval. B, effects of PKC inhibition on cyclin and cdk2 expression. PKC $\alpha$  inhibition reversed the effects of Minerval (M) on cyclin A and B and cdk2. C, PKC activation by the phorbol ester phorbol 12-myristate 13-acetate (PMA) induced similar effects to those induced by Minerval. \*,  $P < 0.01$ .



B) and in the antiproliferative effects mediated by the drug. Moreover, PKC activation by phorbol 12-myristate 13-acetate provoked the up-regulation of p21<sup>CIP</sup> and the down-regulation of cyclins and cdk2 (Fig. 5C). These data are in agreement with the involvement of PKC $\alpha$  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 p21<sup>CIP</sup> in endothelial and epithelial cells, respectively. Together, the present results show that PKC, p21<sup>CIP</sup>, 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; Vögler 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 $\alpha$  proteins and G $\beta\gamma$  dimers prefer non-lamellar-prone membranes, whereas the G $\alpha$ i monomer, formed upon agonist activation of G protein-coupled receptors, has a greater affinity for lamellar phases (Vögler et al., 2004). This differential interaction with membrane lipids drives in part the cellular localization and activity of resting (G $\alpha\beta\gamma$ ) and active (G $\alpha$  and G $\beta\gamma$ ) 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, nonimmobilizers, 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 unspecific 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-

ing such proteins is not questioned. A variety of cell membranes exist, containing different lipid species, lipid compositions, and lipid shapes. For example, the numerous organelles within a cell have different membrane types, each containing specific lipid species (Deshmukh et al., 1988). Indeed, even within an individual membrane, the exoplasmic and cytoplasmic leaflets can display very different lipid compositions (Verkley et al., 1973; Bloj and Zilversmit, 1976; Rothman and Lenard, 1977). Moreover, localized or mobile specialized membrane domains or membrane structures may also exist (e.g., membrane rafts, caveolae, synaptosomes, and brush borders) (Mañes et al., 1999; Vereb et al., 2003). This diversity and the exquisite regulation of the lipid levels argue in favor of the possibility of designing specific lipid therapies. In fact, treatments with drugs that modulate the structure of the membrane and diets rich in certain oils that regulate the membrane lipid properties also alter the expression of some genes (Escribá et al., 2002; Puskás et al., 2003).

Despite the variety of studies that we have carried out to reveal the molecular bases of Minerval action, many questions require further investigations. In this context, other molecular mechanisms could also be involved in the anticancer activity of Minerval. The presence of the hydroxyl moiety on its  $\alpha$  carbon may interfere with, for example, mitochondrial import of fatty acids,  $\beta$ -oxidation, and peroxisome proliferator-activated receptor activities. These molecular entities and/or mechanisms that Minerval may modulate are currently under investigation. However, the lack of relevant toxicology and side effects and the oral administration of Minerval, as well as its antiproliferative and antitumoral activities support its potential use in clinical therapies.

## References

- Akashi M, Osawa Y, Koeffler HP, and Hachiya M (1999) p21<sup>WAF1</sup> expression by an activator of protein kinase C is regulated mainly at the post-transcriptional level in cells lacking p53: important role of RNA stabilization. *Biochem J* **337**:607–616.
- Barceló F, Prades J, Funari SS, Frau J, Alemany R, and Escribá PV (2004) The hypotensive drug 2-hydroxyoleic acid modifies the structural properties of model membranes. *Mol Membr Biol* **21**:261–268.
- Belachew S, Aguirre AA, Wang H, Vautier F, Yuan X, Anderson S, Kirby M, and Gallo V (2002) Cyclin-dependent kinase-2 controls oligodendrocyte progenitor cell cycle progression and is downregulated in adult oligodendrocyte progenitors. *J Neurosci* **22**:8553–8562.
- Bloj B and Zilversmit DB (1976) Asymmetry and transposition rates of phosphatidylcholine in rat erythrocyte ghosts. *Biochemistry* **15**:1277–1283.
- Caponigro F, French RC, and Kaye SB (1997) Protein kinase C: a worthwhile target for anticancer drugs? *Anticancer Drugs* **8**:26–33.
- Cayrol C and Ducommun B (1998) Interaction with cyclin-dependent kinases and PCNA modulates proteasome-dependent degradation of p21. *Oncogene* **17**:2437–2444.
- Dawson RM, Irvine RF, Bray J, and Quinn PJ (1984) Long-chain unsaturated diacylglycerols cause a perturbation in the structure of phospholipid bilayers rendering them susceptible to phospholipase attack. *Biochem Biophys Res Commun* **125**:836–842.
- Deshmukh DS, Vorbrott AW, Lee PK, Bear WD, and Kuizon S (1988) Studies on the submicrosomal fractions of bovine oligodendroglia: lipid composition and glycolipid biosynthesis. *Neurochem Res* **13**:571–582.
- Detjen KM, Brembeck FH, Welzel M, Kaiser A, Haller H, Wiedenmann B, and Rosewicz S (2000) Activation of protein kinase Calpha inhibits growth of pancreatic cancer cells via p21<sup>CIP</sup>-mediated G $_1$  arrest. *J Cell Sci* **113**:3025–3035.
- Dobashi Y, Shoji M, Kitagawa M, Noguchi T, and Kameya T (2000) Simultaneous suppression of cdc2 and cdk2 activities induces neuronal differentiation of PC12 cells. *J Biol Chem* **275**:12572–12580.
- el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, and Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**:817–825.
- Epand RM (1985) Diacylglycerols, lysolipids or hydrocarbons markedly alter the bilayer to hexagonal phase transition temperature of phosphatidylethanolamines. *Biochemistry* **24**:7092–7095.
- Epand RM and Bottega R (1988) Determination of the phase behaviour of phosphatidylethanolamine admixed with other lipids and the effects of calcium chloride: implications for protein kinase C regulation. *Biochim Biophys Acta* **944**:144–154.
- Epand RM, Epand RF, Ahmed N, and Chen R (1991) Promotion of hexagonal phase formation and lipid mixing by fatty acids with varying degrees of unsaturation. *Chem Phys Lipids* **57**:75–80.

- Epand RM and Lester DS (1990) The role of membrane biophysical properties in the regulation of protein kinase C activity. *Trends Pharmacol Sci* **11**:317–320.
- Escribá PV, Ferrer-Montiel AV, Ferragut JA, and Gonzalez-Ros JM (1990) Role of membrane lipids in the interaction of daunomycin with plasma membranes from tumor cells: implications in drug-resistance phenomena. *Biochemistry* **29**:7275–7282.
- Escribá PV, Morales P, and Smith A (2002) Membrane phospholipid reorganization differentially regulates metallothionein and heme oxygenase by heme-hemopexin. *DNA Cell Biol* **21**:355–364.
- Escribá PV, Ozaita A, Ribas C, Miralles A, Fodor E, Farkas T, and Garcia-Sevilla JA (1997) Role of lipid polymorphism in G protein-membrane interactions: nonlamellar-prone phospholipids and peripheral protein binding to membranes. *Proc Natl Acad Sci USA* **94**:11375–11380.
- Escribá PV, Sánchez-Dominguez JM, Alemany R, Perona JS, and Ruiz-Gutierrez V (2003) Alteration of lipids, G proteins and PKC in cell membranes of elderly hypertensives. *Hypertension* **41**:176–182.
- Escribá PV, Sastre M, and Garcia-Sevilla JA (1995) Disruption of cellular signaling pathways by daunomycin through destabilization of nonlamellar membrane structures. *Proc Natl Acad Sci USA* **92**:7595–7599.
- Funari SS, Barcelo F, and Escrivá PV (2003) Effects of oleic acid and its congeners, elaidic and stearic acids, on the structural properties of phosphatidylethanolamine membranes. *J Lipid Res* **44**:567–575.
- Giorgione J, Epand RM, Buda C, and Farkas T (1995) Role of phospholipids containing docosahexaenoyl chains in modulating the activity of protein kinase C. *Proc Natl Acad Sci USA* **92**:9767–9770.
- Goldberg EM, Lester DS, Borchardt DB, and Zidovetzki R (1994) Effects of diacylglycerols and  $\text{Ca}^{2+}$  on structure of phosphatidylcholine/phosphatidyl-serine bilayers. *Biophys J* **66**:382–393.
- Goñi FM and Alonso A (1999) Structure and functional properties of diacylglycerols in membranes. *Prog Lipid Res* **38**:1–48.
- Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D, and Lassar AB (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science (Wash DC)* **267**:1018–1021.
- Han JW, Ahn SH, Kim YK, Bae GU, Yoon JW, Hong S, Lee HY, Lee YW, and Lee HW (2001) Activation of p21<sup>WAF1/Cip1</sup> transcription through Sp1 sites by histone deacetylase inhibitor apicidin: involvement of protein kinase C. *J Biol Chem* **276**:42084–42090.
- Israel M, Idriss JM, Koseki Y, and Khetarpal VK (1987) Comparative effects of adriamycin and DNA-non-binding analogues on DNA, RNA and protein synthesis in vitro. *Cancer Chemother Pharmacol* **20**:277–284.
- Janes N (1996) Curvature stress and polymorphism in membranes. *Chem Phys Lipids* **81**:133–150.
- Junoy B, Maccario H, Mas JL, Enjalbert A, and Drouva SV (2002) Proteasome implicated in phorbol ester- and GnRH-induced selective down-regulation of PKC ( $\alpha$ ,  $\epsilon$ ,  $\zeta$ ) in  $\alpha\text{T}_2$ -1 and L $\beta$ T<sub>2</sub> gonadotrope cell lines. *Endocrinology* **143**:1386–1403.
- Kashiwagi M, Ohba M, Watanabe H, Ishino K, Kasahara K, Sanai Y, Taya Y, and Kuroki T (2000) PKC $\epsilon$  associates with cyclin E/cdk2/p21 complex, phosphorylates p21 and inhibits cdk2 kinase in keratinocytes. *Oncogene* **19**:6334–6341.
- Kinnunen PKJ (1996) On the molecular-level mechanism of peripheral protein-membrane interactions induced by lipids forming inverted non-lamellar phases. *Chem Phys Lipids* **81**:151–166.
- Koubi L, Tarek M, Bandyopadhyay S, Klein ML, and Scharf D (2002) Membrane structural perturbations caused by anesthetics and nonimmobilizers: a molecular dynamics investigation. *Biophys J* **81**:3339–3345.
- Lester DS and Baumann D (1991) Action of organic solvents on protein kinase C. *Eur J Pharmacol* **206**:301–308.
- Lu G, Morinelli TA, Meier KE, Rosenzweig SA, and Egan BM (1996) Oleic acid-induced mitogenic signaling in vascular smooth muscle cells. A role for protein kinase C. *Circ Res* **79**:611–618.
- Mañes S, Mira E, Gomez-Mouton C, Lacalle RA, Keller P, Labrador JP, and Martínez AC (1999) Membrane raft microdomains mediate front-rear polarity in migrating cells. *EMBO (Eur Mol Biol Organ) J* **18**:6211–6220.
- Michaeli J, Busquets X, Orlow I, Younes A, Colomer D, Marks PA, Rifkind RA, and Kolesnick RN (1992) A rise and fall in 1,2-diacylglycerol content signal hexamethylene bisacetamide-induced erythropoiesis. *J Biol Chem* **267**:23463–23466.
- Mosior M, Golini ES, and Epand RM (1996) Chem specificity and physical properties of the bilayer in the regulation of protein kinase C by anionic phospholipids: evidence for the lack of a specific binding site for phosphatidylserine. *Proc Natl Acad Sci USA* **93**:1907–1912.
- Park JW, Jang MA, Lee YH, Passaniti A, and Kwon TK (2001) p53-independent elevation of p21 expression by PMA results from PKC-mediated mRNA stabilization. *Biochem Biophys Res Commun* **280**:244–248.
- Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, Olson EN, Harper JW, and Elledge SJ (1995) p53-independent expression of p21<sup>Cip1</sup> in muscle and other terminally differentiating cells. *Science (Wash DC)* **267**:1024–1027.
- Perkins WR, Dause RB, Parente RA, Minchey SR, Neuman KC, Gruner SM, Taraschi TF, and Janoff AS (1996) Role of lipid polymorphism in pulmonary surfactant. *Science (Wash DC)* **273**:330–332.
- Pinton P, Tsuboi T, Ainscow EK, Pozzan T, Rizzuto R, and Rutter GA (2002) Dynamics of glucose-induced membrane recruitment of protein kinase C  $\beta$ II in living pancreatic islet  $\beta$ -cells. *J Biol Chem* **277**:37702–37710.
- Pintou G, Tandolini B, Maioli M, Posadino AM, Gaspa L, and Ventura C (1999) Heparin down-regulates the phorbol ester-induced protein kinase C gene expression in human endothelial cells: enzyme-mediated autoregulation of protein kinase C- $\alpha$  and - $\delta$  genes. *FEBS Lett* **449**:135–140.
- Puskás LG, Kitajka K, Nyakas C, Barceló-Coblijn G, and Farkas T (2003) Short-term administration of omega 3 fatty acids from fish oil results in increased transthyretin transcription in old rat hippocampus. *Proc Natl Acad Sci USA* **100**:1580–1585.
- Rothman JE and Lenard J (1977) Membrane asymmetry. *Science (Wash DC)* **195**:743–753.
- Shen Y-MA, Chertihin OI, Biltonen RL, and Sando JJ (1999) Lipid-dependent activation of protein kinase C- $\alpha$  by normal alcohols. *J Biol Chem* **274**:34036–34044.
- Sieber FE, Traystman RJ, Brown PR, and Martin LJ (1998) Protein kinase C expression and activity after global incomplete cerebral ischemia in dogs. *Stroke* **29**:1445–1452.
- Spector AA and Burns CP (1987) Biological and therapeutic potential of membrane lipid modification in tumors. *Cancer Res* **47**:4529–4537.
- Sperandio S, de Belle I, and Bredesen DE (2000) An alternative, nonapoptotic form of programmed cell death. *Proc Natl Acad Sci USA* **97**:14376–14381.
- Strobeck MW, Fribourg AF, Puga A, and Knudsen ES (2000) Restoration of retinoblastoma mediated signaling to Cdk2 results in cell cycle arrest. *Oncogene* **19**:1857–1867.
- Sun XG and Rotenberg SA (1999) Overexpression of protein kinase C $\alpha$  in MCF-10A human breast cells engenders dramatic alterations in morphology, proliferation and motility. *Cell Growth Differ* **10**:343–352.
- Triton TR and Yee G (1982) The anticancer agent adriamycin can be actively cytotoxic without entering cells. *Science (Wash DC)* **217**:248–250.
- Vereb G, Szollosi J, Matko J, Nagy P, Farkas T, Vigh L, Matyus L, Waldmann TA, and Damjanovich S (2003) Dynamic, yet structured: the cell membrane three decades after the Singer-Nicolson model. *Proc Natl Acad Sci USA* **100**:8053–8058.
- Verkleij AJ, Zwaal RF, Roelofsens B, Comfurius P, Kastelijn D, and van Deenen LL (1973) The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim Biophys Acta* **323**:178–193.
- Vögler O, Casas J, Capó D, Nagy T, Borchert G, Martorell G, and Escrivá PV (2004) The G $\beta\gamma$  dimer drives the interaction of heterotrimeric Gi proteins with nonlamellar membrane structures. *J Biol Chem* **279**:36540–36545.
- Walker DH, Adami GR, Dold KM, and Babiss LE (1995) Misregulated expression of the cyclin dependent kinase 2 protein in human fibroblasts is accompanied by the inability to maintain a G2 arrest following DNA damage. *Cell Growth Differ* **6**:1053–1061.
- Ward NE and O'Brian CA (1993) Inhibition of protein kinase C by N-myristoylated peptide substrate analogs. *Biochemistry* **32**:11903–11909.
- Zezula J, Sexl V, Hutter C, Karel A, Schutz W, and Freissmuth M (1997) The cyclin-dependent kinase inhibitor p21<sup>Cip1</sup> mediates the growth inhibitory effect of phorbol esters in human venous endothelial cells. *J Biol Chem* **272**:29967–29974.

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