# 1-*O*-Octadecyl-2-*O*-methyl-glycerophosphocholine Inhibits the Transduction of Growth Signals via the MAPK Cascade in Cultured MCF-7 Cells

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## Abstract

**1-*O*-Octadecyl-2-*O*-methyl-glycerophosphocholine (ET18-$OCH_3$) is an ether lipid with selective antiproliferative properties whose mechanism of action is still unresolved. We hypothesized that since ET18-$OCH_3$ affects a wide variety of cells, its mechanism of action was likely to involve the inhibition of a common widely used pathway for transducing growth signals such as the mitogen-activated protein kinase (MAPK) cascade. To test this, we established conditions whereby quiescent MCF-7 cells took up ET18-$OCH_3$ in sufficient quantities that inhibited cell proliferation subsequent to the addition of growth medium and examined the activation of components of the MAPK cascade under these conditions. ET18-$OCH_3$ inhibited the sustained phosphorylation of MAPK resulting in a decrease in the magnitude and duration of activation of MAPK in cells stimulated with serum or EGF. ET18-$OCH_3$ had no effect on the binding of EGF to its receptors, their activation, or $p21^{ras}$ activation. However, an interference in the association of Raf-1 with membranes and a resultant decrease in Raf-1 kinase activity in membranes of ET18-$OCH_3$–treated cells was observed. ET18-$OCH_3$ had no direct effect on MAPK or Raf-1 kinase activity. A direct correlation between ET18-$OCH_3$ accumulation, inhibition of cell proliferation, Raf association with the membrane, and MAPK activation was also established. These results suggest that inhibition of the MAPK cascade by ET18-$OCH_3$ as a result of its effect on Raf-1 activation may be an important mechanism by which ET18-$OCH_3$ inhibits cell proliferation. (*J. Clin. Invest.* 1996. 98:934–944.)**

**Key words: phospholipid ethers • antineoplastic agents • cell division • signal transduction • protein kinases**

## Introduction

1-*O*-Octadecyl-2-*O*-methyl-glycerophosphocholine (ET18-$OCH_3$),[1] also known as edelfosine, is the prototype of a class of antitumor ether lipids, the alkyllysophospholipids (ALPs), with a wide range of antitumor activity that are undergoing clinical trials (1–3). Two characteristics of the antiproliferative effect exhibited by these compounds have generated considerable interest. Firstly, inhibition of cell proliferation appears to be achieved without any interaction with cellular DNA and secondly, these antiproliferative effects are cell selective (1). The mechanism of inhibition of cell proliferation by ALPs is still unresolved and although they affect a large number of cellular events including nutrient transport, TGF-α secretion, estradiol uptake, transferrin binding, and intracellular-free $Ca^{2+}$ levels (reviewed in 1–3), the role of these perturbations in inhibiting cell growth has yet to be established. ET18-$OCH_3$ also perturbs lipid metabolism but recent studies have suggested that this is unlikely to be the underlying cause for its antiproliferative effect (4–7).

There is a growing recognition that ALPs may inhibit cell growth by interfering in signal transduction processes. Inhibition of protein kinase C (PKC) has been proposed as the mechanism by which ET18-$OCH_3$ and related compounds inhibit cell proliferation (8), but this is contentious. ET18-$OCH_3$ may inhibit (9) or activate (10) PKC activity in in vitro assays depending on the mode of presentation of the ALP in the assay, but a number of studies have revealed no correlation between inhibition of cell growth and inhibition of PKC activity (2, 11, 12). Decreased inositol 1,4,5-trisphosphate ($InsP_3$) production in response to incubation of cells with antitumor ether lipids has been reported (13) and could be due to inhibition of phosphatidylinositol-specific phospholipase C (PI-PLC) (14). Observations that ET18-$OCH_3$ inhibited PI-3-OH-kinase (PI-3K) activity in purified preparations and cell lysates and reduced the production of 3-phosphorylated inositol lipids after PDGF stimulation of NIH 3T3 cells (15) led to the suggestion that the mechanism by which ALPs inhibit cell proliferation may involve the inhibition of PI-3K. PI-3K activation may not be sufficient to initiate cell proliferation and its contribution to proliferation is yet to be fully established (16). The wide range of cancer cells whose growth is inhibited by ET18-$OCH_3$ suggests that a common mechanism of action, if one existed, was likely to involve perturbation of a widely used signal transduction mechanism that initiates proliferation. One such pathway is the Ras-dependent activation of the mitogen-activated protein kinase (MAPK) cascade (Ras/Raf-1/MEK/ERK) which transduces signals from receptor tyrosine kinases, oncogenic tyrosine kinases, and G-protein-coupled receptors (17, 18).

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1. *Abbreviations used in this paper:* AEBSF, aminoethylbenzenesulfonyl fluoride; ALP, alkyllysophospholipid; DME/FBS (or/BSA), DME supplemented with 10% FBS (or with 0.5 mg/ml BSA); EGFr, EGF receptor; ERK, extracellular signal–regulated kinase; ET18-$OCH_3$, 1-*O*-octadecyl-2-*O*-methyl-glycerophosphocholine; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/extracellular signal-regulated kinase; PI-3K, phosphatidylinositol-3-OH kinase; PI-PLC, PI-specific phospholipase C; PKA, protein kinase A; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

We, therefore, investigated the effects of ET18-$OCH_3$ on signaling via the MAPK cascade in the human breast adenocarcinoma cell line, MCF-7, which is very sensitive to the antiproliferative effects of ET18-$OCH_3$ (19). Here we show that ET18-$OCH_3$ inhibits the transduction of growth signals via the MAPK cascade in MCF-7 cells by perturbing the association of Raf-1 with the membrane. This truncates Raf-1 kinase activity and prevents the sustained activation of the MAPK cascade.

## Methods

*Materials.* ET18-$OCH_3$ was obtained from Medmark (Gruenwald, Germany). EGF, myelin basic protein (MBP), all protease inhibitors, calmidazolium and $Na_3VO_4$, were purchased from Sigma Chemical Co. (St. Louis, MO). Coomassie protein assay reagent was a product of Pierce (Rockford, IL). FBS (Cellect Gold), [$^3$H]thymidine, 2 Ci/mmol [$\gamma^{32}$P]ATP, and [$^{32}$P]orthophosphate were obtained from ICN Pharmaceuticals (Montreal, Quebec). Cell culture medium and subculturing reagents were purchased from Gibco BRL (Burlington, Ontario). PEI cellulose TLC plates were purchased from E. Merck (Darmstedt, Germany). Anti-ERK1 (C-16), -ERK2 (C-14), -Ras (259), and -Raf-1 (C-12) antibodies and MEK-1 (FL) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sheep polyclonal anti-EGF receptor Ab and mouse antiphosphotyrosine mAb were procured from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-Raf-1 antiserum (SP63) was generously provided by Dr. U. Rapp (National Cancer Institute, NIH). cAMP[$^3$H] assay kit was from Amersham Canada (Oakville, Ontario). PKI and Kemptide were purchased from Bachem (Torrance, CA).

*Cell culture.* MCF-7 (human breast adenocarcinoma) cells from frozen stocks originally obtained from American Type Culture Collection (Rockville, MD) were cultured in DME/FBS (19). Quiescent cells were obtained by seeding cells in tissue culture dishes overnight in DME/FBS followed by incubation in DME/BSA for 4–6 d. The cells were considered quiescent when the 24 h increase in cell number of parallel cultures was 15% or less.

*ET18-$OCH_3$ preincubation and cell activation.* Working solutions of DME/BSA supplemented with ET18-$OCH_3$ were prepared fresh. Quiescent cells were incubated with or without ET18-$OCH_3$ (10 μg/ml) in fresh DME/BSA. At the end of the incubation, the medium was aspirated, the cells were washed and stimulated with DME/FBS or EGF (10 ng/ml) for selected times. The cells were then washed and scraped into the appropriate buffer and sonicated. Cytosol was obtained by centrifugation at 200,000 *g* for 30 min and frozen at −70°C until required. The protein content of fractions was determined by the Coomassie protein assay. The basic buffer for most of the studies, buffer A, consisted of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 100 mM β-glycerophosphate, 1 mM $Na_3VO_4$, aprotinin, leupeptin (10 μg/ml each), 0.2 mM aminoethylbenzenesulfonyl fluoride (AEBSF), 0.1 mM PMSF, and 0.2 mM benzamidine.

*Protein kinase assays.* Cells were scraped into buffer B (buffer A without NaCl plus 1 mM DTT), and disrupted by ultrasonication. Cell cytosol was obtained by centrifugation as described above. MAPK activity was measured as the phosphorylation of MBP in vitro (20) in the presence of 2 μM PKI peptide, 10 μM calmidazolium and 1 μM PKC inhibitor peptide or by the in-gel assay (21) after electrophoresis on 10% SDS gels. PKA activity was measured with Kemptide as substrate (22) and was taken as the difference in phosphorylation of the peptide in the absence and presence of PKI (2 μM).

*Phosphorylation of MAPK.* Quiescent cells were washed with Krebs buffer and incubated with phosphate-free DME/BSA containing 300 μCi/ml [$^{32}$P]orthophosphate for 3 h followed by washing and incubation with or without ET18-$OCH_3$ (10 μg/ml) for 3 h. At the end of the incubation the cells were washed and incubated with DME/BSA and stimulated for selected periods with EGF. The cells were then quickly washed three times with ice-cold Krebs buffer and scraped into buffer C (buffer A + 1% [wt/vol] Triton X-100, 0.5% [wt/vol] NP-40), vortexed immediately, and centrifuged at 200,000 *g* for 30 min. Cell cytosol was precleared followed by immunoprecipitation of $p42^{mapk}$ and $p44^{mapk}$ with agarose-conjugated polyclonal Abs to ERK1 (C-16) and ERK2 (C-14). Pellets were washed (eight times) with buffer C followed by boiling in SDS sample buffer. The proteins were separated on 10% SDS gels and phosphorylated bands were visualized by autoradiography.

*EGF receptor phosphorylation.* After the appropriate treatment and stimulation of cells with EGF, the cells were washed twice and lysed in 50 mM Hepes (pH 7.5), 150 mM NaCl, 1% (wt/vol) Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, protease inhibitors (aprotinin, leupeptin, benzamidine, AEBSF), 0.5 mM $Na_3VO_4$ and 100 mM β-glycerophosphate. The extracts were sonicated and centrifuged at 200,000 *g* for 30 min. The supernatant was used for immunoprecipitation with sheep polyclonal anti-EGFr Ab followed by Western blot analysis with the same Ab or mouse antiphosphotyrosine mAb.

*$p21^{ras}$ activation.* Ras was immunoprecipitated with a rat anti–v-H-Ras mAb (259) from [$^{32}$P]orthophosphate (300 μCi/ml, 4 h)–labeled quiescent cells stimulated for selected periods with EGF. The GTP/(GTP + GDP) content was determined as previously described (23).

*Translocation of Raf-1.* This was performed as described by Wartmann and Davis (24). Quiescent cells treated with or without ET18-$OCH_3$ were stimulated with EGF or serum. The cytosolic fraction and solubilized membrane fraction were prepared (24) and subjected to Western blot analysis with anti–Raf-1 (C-12) Ab after electrophoresis on 10% SDS-PAGE.

*Raf-1 kinase assays.* Membranes were obtained from control and ET18-$OCH_3$–treated cells, solubilized (24), and were precleared. Immunoprecipitation was achieved with anti–Raf-1 antiserum (SP63) (25). Raf-1 kinase activity was assayed (25) by using 10 μg of histone H1 or 2.5 μg of MEK (FL) as substrates. Radiolabeled histone H1 or MEK was separated on 12% SDS gels and visualized by autoradiography.

*[$^3$H]Thymidine incorporation in cells.* Quiescent cells in 6-well plates were preincubated with or without ET18-$OCH_3$ (10 μg/ml) for selected periods. After washing, the cells were incubated with DME/BSA or DME/FBS for 20 h followed by the addition of [$^3$H]thymidine 0.5 μCi/well for an additional 4 h. Radioactivity in TCA precipitable material was determined (26).

*Determination of cellular cAMP levels.* cAMP levels were measured with a kit from Amersham Corp. using the protocol enclosed with the kit without any modifications.

*Flow cytometry.* Cells were processed for flow cytometry analysis as previously described (27). Data were collected and analyzed with a cell sorter EPICS 753; Coulter Electronics Inc., Hialeah, FL and the PARA 1 analysis software (Coulter Electronics Inc.).

*Quantitation.* Quantitation of immunoblots and autoradiographs were obtained by densitometric analysis with a high resolution color scanner model PDI 325oe (Protein + DNA Imageware Systems, Huntington Station, NY) using the ImageMaster scanning program (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

## Results

*ET18-$OCH_3$ inhibits the serum-induced cell proliferation and [$^3$H]thymidine incorporation.* The approach adopted for this study was to investigate the effect of ET18-$OCH_3$ on the proliferation of quiescent cells. To correlate any observed cellular perturbations with the inhibition of cell growth, incubation conditions were established whereby quiescent MCF-7 cells accumulated sufficient ET18-$OCH_3$ to inhibit cell proliferation after the readdition of DME/FBS without loss of cell viability. The addition of DME/FBS to serum-deprived MCF-7 cells stimulated proliferation, while continued deprivation of serum did not (Fig. 1 *A*). Preincubation of the cells with ET18-$OCH_3$ (10 μg/ml) for 1 h and subsequent incubation with DME/FBS de-

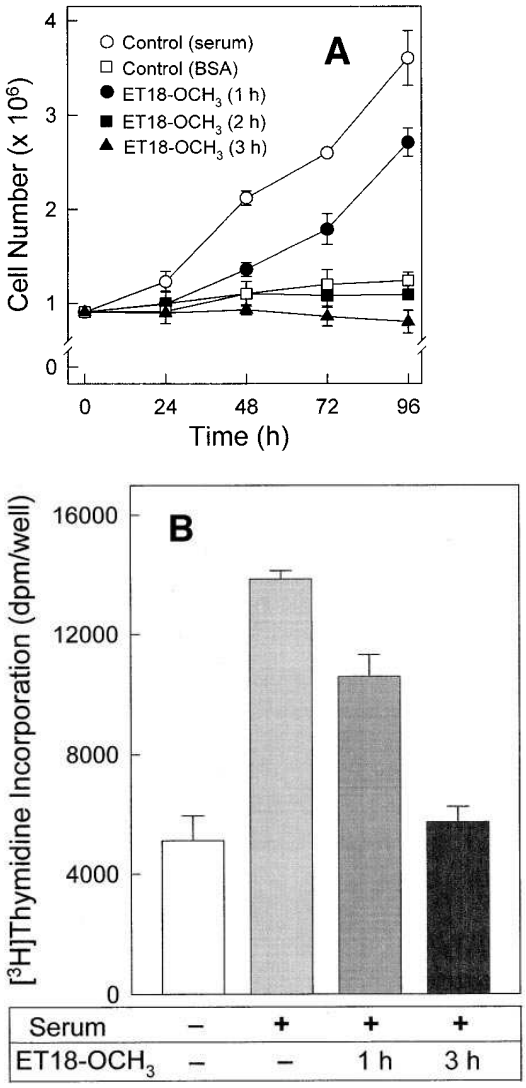
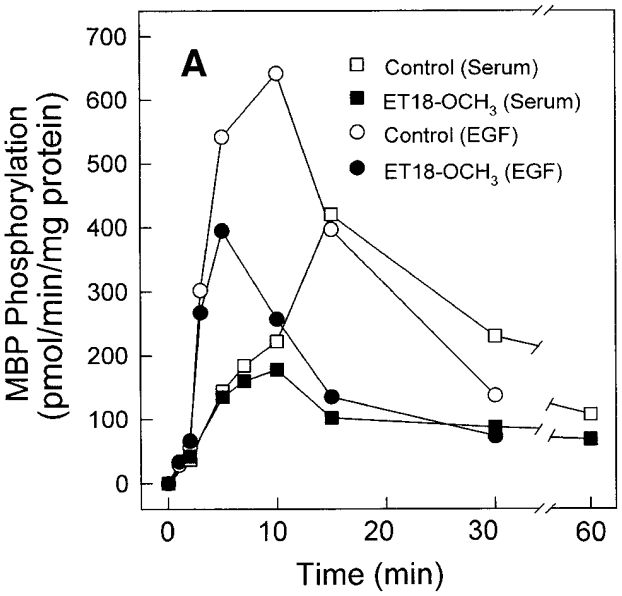



*Figure 1.* Preincubation of quiescent MCF-7 cells with ET18-$OCH_3$ inhibits cell proliferation and thymidine incorporation. (*A*) Quiescent MCF-7 cells were incubated with 10 μg/ml ET18-$OCH_3$ for 1 h (●), 2 h (■), and 3 h (▲). The cells were washed and incubated with DME/FBS and the cell numbers were determined at 24 h intervals. Control cells without ET18-$OCH_3$ pretreatment were incubated with DME/FBS (○) or DME/BSA (□). (*B*) [$^3$H]Thymidine incorporation in cells treated with or without ET18-$OCH_3$ (10 μg/ml) for the indicated periods followed by stimulation with serum was determined. The results of both studies are the mean of six separate determinations.

creased their rate of proliferation relative to controls while incubation for 2 h or more completely inhibited cell proliferation. In cells preincubated with ET18-$OCH_3$ for 3 h before incubation in DME/FBS for up to 4 d, the proportion of cells excluding trypan blue dye (> 90%) was similar to that of controls, indicating that the viability of the cells had not been compromised. Preincubation of quiescent MCF-7 cells with ET18-$OCH_3$ followed by stimulation with serum also inhibited the incorporation of [$^3$H]thymidine into the cells (Fig. 1 *B*) indicating that progression of the cells into S phase had been inhibited. The quantities of ET18-$OCH_3$ taken up by the cells after incubation for varying periods were determined with [$^3$H]ET18-$OCH_3$ (5). After incubation for 1, 2, and 3 h with 10 μg/ml ET18-$OCH_3$ the quantities of the ALP incorporated in the quiescent cells were 0.44, 0.63, and 0.74 μg/$10^6$ cells, respectively. The above results suggested that the accumulated ET18-$OCH_3$ could be blocking the transduction of the growth signals in serum to the appropriate cellular proliferation machinery.

*ET18-$OCH_3$ inhibits the activation of MAPK in MCF-7 cells.* The activation of MAPK (ERK) is crucial for cell proliferation after stimulation by extracellular ligands, such as EGF, acting on receptor tyrosine kinases (17). To determine if the inhibitory locus of ET18-$OCH_3$ was upstream or downstream of MAPK, cells were preincubated with or without ET18-$OCH_3$ (10 μg/ml) for 3 h, stimulated with EGF, and MAPK activity was measured in the cytosolic fractions. The results displayed in Fig. 2 *A* show that in control cells, MAPK activity peaked at 642 pmol/min per mg protein after 10 min stimulation whereas in cells preincubated with ET18-$OCH_3$ a peak activity of 395 pmol/min per mg protein was observed after 5 min. At 10 min, MAPK activity was 2.5 times higher in the controls compared with the cells preincubated with ET18-$OCH_3$. The in-gel MAPK assay revealed a similar inhibitory effect of ET18-$OCH_3$ preincubation on MBP phosphorylation in response to EGF (Fig. 2 *B*). There was little or no differences between the controls and experimental cells after 2 and 4 min but at 10 min, phosphorylation of MBP by $p42^{mapk}$ and $p44^{mapk}$ in the controls was still evident whereas that in the ET18-$OCH_3$–treated cells had declined considerably. ET18-$OCH_3$ had a similar effect on MAPK activation in cells stimulated with serum (Fig. 2 *A*). Peak phosphorylation of MBP in control cell cytosol occurred 15 min after serum stimulation. In contrast, preincubation of the cells with ET18-$OCH_3$ before serum stimulation yielded a peak activity at 10 min which was twofold lower than the peak activity in controls. 15 min after serum stimulation, MAPK activity in the ET18-$OCH_3$–loaded cell cytosol was fourfold lower than the activity in controls. Thus, the effect of preincubating MCF-7 cells with ET18-$OCH_3$ under conditions that inhibit their proliferation was a decrease in both the intensity and duration of MAPK activation in response to serum or EGF. The observed inhibition of MAPK activation in cells preincubated with ET18-$OCH_3$ is unlikely to be due to a direct inhibition of the enzyme activity by the compound because the activity of purified recombinant ERK2 and MAPK activity in the cytosolic fractions from stimulated cells were unaffected by the addition of up to 20 μg/ml exogenous ET18-$OCH_3$ (data not shown).

*ET18-$OCH_3$ decreases EGF-induced phosphorylation of $p42^{mapk}$ and $p44^{mapk}$.* As phosphorylation of MAPK is required for its activation (28) we investigated the effect of ET18-$OCH_3$ on this event. $p42^{mapk}$ and $p44^{mapk}$ were immunoprecipitated from [$^{32}$P]orthophosphate-labeled cells incubated with or without ET18-$OCH_3$ and stimulated with EGF. Phosphorylation was assessed by autoradiography (Fig. 2 *C*). 4 min after EGF stimulation there was little difference in the phosphorylation of the MAPK immunoprecipitates from the control and experimental cells. However, while phosphorylation had not decreased after 10 min in MAPK immunoprecipitated from the control, this had declined significantly in MAPK immunoprecipitates from cells incubated with the ALP (3.2- and 3.7-fold decrease in $p42^{mapk}$ and $p44^{mapk}$ by densitometric analysis, respectively). Western blot analysis of parallel gels revealed no differences in MAPK protein content (data not



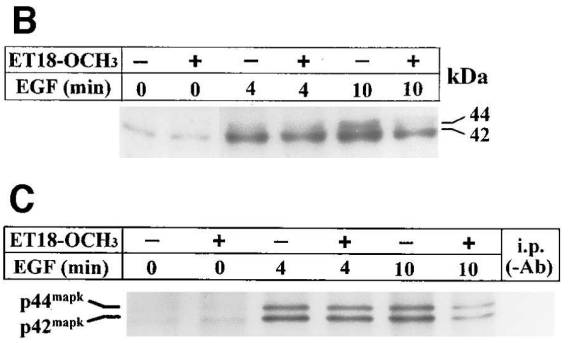




*Figure 2.* Preincubation of MCF-7 cells with ET18-$OCH_3$ attenuates activation of MAPK in response to stimulation by EGF or serum. (*A*) Quiescent cells were incubated without (*open symbols*) or with (*closed symbols*) ET18-$OCH_3$ (10 μg/ml) for 3 h, washed and stimulated with EGF (*circles*) or DME/FBS (*squares*). MAPK activity was measured in cell cytosol as the phosphorylation of MBP. These results are from a single experiment that is representative of four separate experiments and the values are the mean of triplicate incubations. (*B*) Assessment of MAPK activity by the in-gel assay. Results from control and ET18-$OCH_3$–treated cells stimulated with EGF are displayed. The results are from a single experiment that is representative of two identical experiments. (*C*) Assessement of $p42^{mapk}$ and $p44^{mapk}$ phosphorylation. Quiescent cells were prelabeled with [$^{32}$P]orthophosphate, washed, and subsequently incubated with or without ET18-$OCH_3$ (10 μg/ml) for 3 h and stimulated with EGF. MAPK was immunoprecipitated from 750 μg of cell cytosol protein with 3 μg each of anti-ERK1 and -ERK2 Abs and resolved on 10% SDS-PAGE. MAPK phosphorylation was visualized by autoradiography. The results are from a single experiment that was repeated with three different cell preparations with similar results.

shown). These results suggest that the truncated activation observed in cytosol from cells preincubated with ET18-$OCH_3$ was due to the decreased phosphorylation of MAPK, an indication that ET18-$OCH_3$ probably affected an event upstream of MAPK that was critical to its sustained phosphorylation and activation. Experiments were initiated to investigate the effect of preincubating cells with ET18-$OCH_3$ on the signaling events from EGF receptor to MAPK activation (17, 28–31).

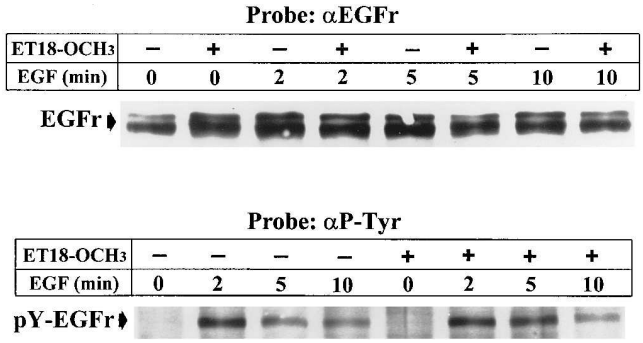



*Figure 3.* Preincubation with ET18-$OCH_3$ does not affect the phosphorylation of EGF receptors in MCF-7 cells. Western blot analysis of immunoprecipitates of EGF receptor from cells incubated with or without ET18-$OCH_3$ and subsequent simulation by EGF. Antibodies used for immunoblotting are indicated in the figures. The results are from a single experiment. Two independent experiments showed similar results.

*ET18-$OCH_3$ does not affect activation of the EGF receptor.* Studies on the binding and affinity of [$^{125}$I]EGF to its receptors in cells incubated with or without ET18-$OCH_3$ at 4 and 37°C revealed no significant differences in the quantity of EGF bound or affinity of EGF to the receptors (data not shown). Decreased internalization of EGF in cells preincubated with ET18-$OCH_3$ relative to controls was observed at 37°C, but this was only apparent after 20 min incubation with the ligand (data not shown). The effect of ET18-$OCH_3$ on EGF receptor (EGFr) activation was assessed by monitoring the tyrosine phosphorylation of EGFr (32). Immunoblotting of the immunoprecipitated EGFr with anti-EGF (αEGFr) Abs showed

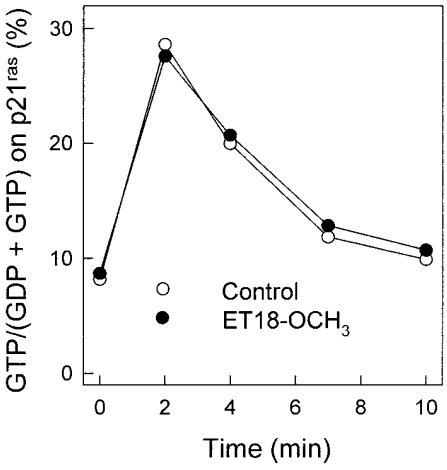



*Figure 4.* Pretreatment with ET18-$OCH_3$ does not affect $p21^{ras}$ activation. $p21^{ras}$ was immunoprecipitated from cell lysates (500 μg protein) obtained from [$^{32}$P]orthophosphate-labeled cells preincubated with or without ET18-$OCH_3$ (10 μg/ml) and subsequent stimulation with EGF. Guanine nucleotides were extracted from the immunoprecipitates and separated by TLC and the amount of associated radioactivity was visualized by autoradiography. Spots were scraped from the TLC plates and the radioactivity was quantitated. The results are from a single experiment that is representative of two identical experiments.

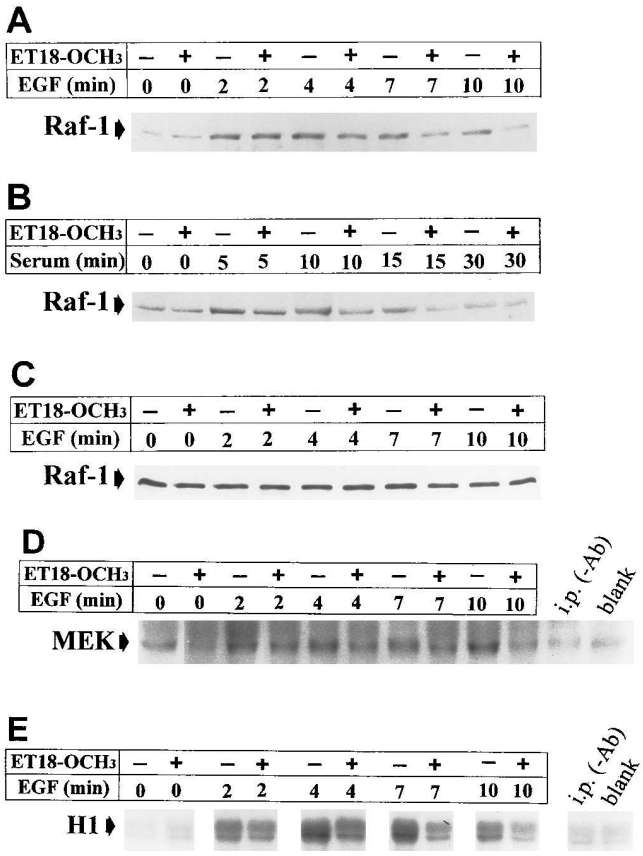



*Figure 5.* Preincubaton of MCF-7 cells with ET18-$OCH_3$ attenuates Raf-1 activation. (*A*) Effect of ET18-$OCH_3$ on Raf-1 membrane association in EGF-stimulated cells. Quiescent cells were incubated with or without ET18-$OCH_3$ (10 μg/ml) for 3 h and stimulated with EGF. Membranes were prepared, solubilized and 40 μg of protein were separated by 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti–Raf-1 Abs. The results are from a single experiment that is representative of those obtained with three different cell preparations. (*B*) Effect of ET18-$OCH_3$ on Raf-1 membrane association in serum-stimulated cells. Experiments and legends are similar to those in *A* except stimulation was with DME/FBS. The results are from a single experiment that is representative of two identical experiments. (*C*) Effect of ET18-$OCH_3$ on Raf-1 content in MCF-7 cells. Experiments were conducted as described in *A*, but after stimulation, the cells were lysed in detergent containing buffer. Each lane was loaded with 10 μg of total cell lysate protein. The legends are as described in *A*. The results are from a single experiment that is representative of two. (*D*) Raf-1 kinase activity in ET18-$OCH_3$–treated and control cells. Raf-1 was immunoprecipitated from membrane extracts of cells (300 μg protein) treated with or without ET18-$OCH_3$ (10 μg/ml) for 3 h followed by stimulation with EGF. The immunoprecipitates were washed and used in kinase assays to measure the phosphorylation of MEK-1 (*FL*). Controls without Abs in Raf-1 immunoprecipitates and nonspecific phosphorylation of MEK-1 in assays with just buffer and [$\gamma^{32}$P]ATP are also indicated. The results are from a single experiment. Two independent experiments showed similar results. (*E*) Experiments were conducted as in *D* but with histone H1 as substrate. Legends are the same as described in *D*. The results are from a single experiment that is representative of three independent experiments.

that similar amounts of the receptor were present in cells incubated with or without ET18-$OCH_3$ (Fig. 3). Immunoblotting with antiphosphotyrosine (αP-Tyr) Abs revealed that the extent and kinetics of EGFr phosphorylation were similar in both groups (Fig. 3). This was confirmed by densitometric analysis (data not shown).

*ET18-$OCH_3$ does not affect the activation of p21$^{ras}$.* The activation of EGFr leads to the formation of a complex with Grb2/mSos that leads to the activation of p21$^{ras}$ (29) which in turn leads to Raf-1 activation to initiate the kinase cascade. Inhibition of p21$^{ras}$ activity could lead to decreased activation of MAPK. Fig. 4 shows that preincubation of the cells with ET18-$OCH_3$ had no effect on the activation of p21$^{ras}$ which peaked between 2 and 4 min and declined to resting values by 8 min.

*ET18-$OCH_3$ perturbs Raf-1 association with cell membranes.* Activated p21$^{ras}$ mediates the translocation of Raf-1 from the cytosol to the membrane where it is activated by undefined events (17, 30, 31, 33). To investigate the effect of ET18-$OCH_3$ on Raf-1 activation, we examined its effect on the translocation of Raf from the cytosol to the membrane in control cells and those preincubated with ET18-$OCH_3$. In cells stimulated with EGF (Fig. 5 *A*), densitometric analysis of the blots revealed that the membrane-associated Raf-1 in controls and cells preincubated with ET18-$OCH_3$ increased similarly at 2 min while at 4 and 7 min, 1.8- and 3.4-fold more Raf-1, respectively, was associated with control membranes relative to membranes from ET18-$OCH_3$–loaded cells. Results from serum-stimulated cells (Fig. 5 *B*) also revealed that Raf-1 association with membranes from cells preincubated with ET18-$OCH_3$ never attained the levels of the controls and decreased more rapidly. After 5 and 10 min the levels in membranes from ET18-$OCH_3$–treated cells were 1.6- and 2.6-fold less than the levels in control membranes, respectively. Western blot analy-

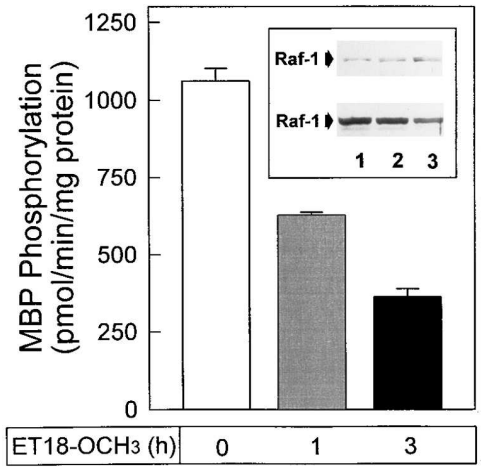



*Figure 6.* Effect of preincubation time with ET18-$OCH_3$ on Raf-1 membrane association and MAPK activity. Quiescent MCF-7 cells were incubated without (*open bar*; *inset* lane *1*) or with ET18-$OCH_3$ (10 μg/ml) for 1 h (*grey bar*; *inset* lane *2*), or 3 h (*black bar*; *inset* lane *3*). The cells were washed and stimulated with EGF for 7 min. MAPK activity in the cytosolic fraction (*bar graph*) and Raf-1 association with the membrane (*inset*) were determined as described in Methods. Raf-1 associated with membranes from unstimulated and stimulated cells are shown in the upper and lower panels of the inset, respectively. Two independent experiments showed similar results.

sis was performed on cell lysates to investigate whether the above differential association of Raf-1 with membranes was due to differences in the Raf-1 content in cells treated with or without ET18-$OCH_3$. The results revealed that there were no differences in the Raf-1 content between ET18-$OCH_3$–treated and control cells (Fig. 5 *C*).

*Preincubation of cells with ET18-$OCH_3$ inhibits Raf-1 kinase activity.* To investigate if the decreased Raf-1 association caused by ET18-$OCH_3$ preincubation translated into decreased Raf-1 kinase activity in the membrane, Raf-1 kinase activity was measured in Raf-1 immunoprecipitates from membranes of control and ET18-$OCH_3$–loaded cells. The results clearly showed that there was greater phosphorylation of MEK-1 and histone H1 in assays with immunoprecipitates from control cells compared to those from cells preincubated with ET18-$OCH_3$ (Fig. 5, *D* and *E*), and the time course of the activity paralleled that of Raf-1 membrane association. The ratio of the increase in phosphorylation of the substrates over blanks in controls relative to ET18-$OCH_3$–loaded cells as assessed densitometrically ranged from 1.5 at 2 min to 12 at 10 min for MEK-1 and 1.2 to 4.3 for histone H1 from 2 to 10 min, respectively. Addition of ET18-$OCH_3$ to the assays did not have any effect on the kinase activity of Raf-1 immunoprecipitates from EGF-stimulated and unstimulated cells (data not shown). Thus, ET18-$OCH_3$ has no direct effect on Raf-1 kinase activity.

*Correlation between ET18-$OCH_3$ accumulation, Raf-1 membrane association and MAPK activation.* As we observed a time-dependent accumulation of ET18-$OCH_3$ (see above), which could account for the differential effect on proliferation observed in Fig. 1, we investigated whether this could be correlated with differences in the extent of Raf-1/membrane association and MAPK activity. Quiescent MCF-7 cells were incubated with ET18-$OCH_3$ for 0, 1, and 3 h before stimulation with EGF for 7 min and cell fractions processed for examination of Raf-1/membrane association, and MAPK activity. The results (Fig. 6) show that Raf-1 association with the membrane decreased as a function of increasing incubation with ET18-$OCH_3$ and that the order of decreasing MAPK activity correlated with decreased membrane-bound Raf-1 and increasing ET18-$OCH_3$ accumulation.

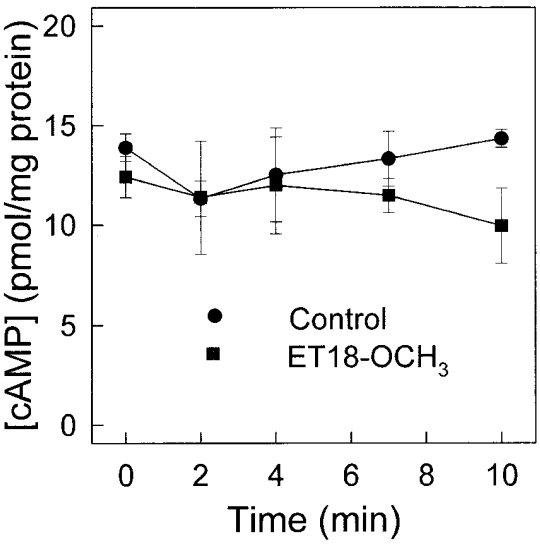



*Figure 7.* Effect of ET18-$OCH_3$ on cellular cAMP content. cAMP levels in MCF-7 cells preincubated with or without 10 μg/ml ET18-$OCH_3$ for 3 h and stimulated with EGF (10 ng/ml) were determined as described in Methods. The results are the mean of two separate experiments conducted in triplicates.

*Table I. Effect of Transient Stimulation of MCF-7 Cells on the Cytostatic Effects of ET18-$OCH_3$*

| Treatment | Cell cycle distribution (%±SD) | | |
|---|---|---|---|
| | $G_0/G_1$ | S | $G_2/M$ |
| BSA | 81.5±3.1 | 10.5±1.7 | 8.0±1.8 |
| ET18-$OCH_3$/FBS | 81.7±2.8 | 6.7±1.7 | 11.5±1.3 |
| FBS/BSA/FBS | 59.5±1.0 | 24.2±1.0 | 16.0±1.4 |
| FBS/ET18-$OCH_3$/FBS | 66.0±2.9 | 17.2±1.3 | 16.2±2.2 |

Quiescent MCF-7 cells were incubated with DME/FBS for 1 h. The medium was removed, the cells were washed and incubated with ET18-$OCH_3$ (10 μg/ml) in DME/BSA or DME/BSA alone for 3 h. After removal of medium and washing, the cells were incubated in DME/FBS. Another group of cells were incubated with ET18-$OCH_3$ (10 μg/ml) in DME/BSA for 3 h followed by washing and incubation with DME/FBS. All cells were harvested 25 h after initial treatment with DME/FBS and processed for flow cytometry analysis. The results are the mean of four independent experiments.

*Effect of ET18-$OCH_3$ on cellular cAMP levels.* Because PKA activation may lead to inhibition of Raf-1 activity (34), we determined the cAMP levels and PKA activity in ET18-$OCH_3$–pretreated and untreated MCF-7 cells to investigate whether the inhibitory effect of ET18-$OCH_3$ was mediated via activation of PKA. ET18-$OCH_3$ had no effect on unstimulated or EGF-stimulated cellular levels of cAMP (Fig. 7) or on PKA activity which remained unchanged from the basal level of 3.6±1.7 pmol/min per mg protein.

*Effect of transient MCF-7 cell stimulation on the cytostatic effect of ET18-$OCH_3$.* If the inhibition of the MAPK cascade by ET18-$OCH_3$ contributes significantly to the antiproliferative effects of the compound as the above studies suggest, these effects should be minimized if the ALP is added subsequent to the transient activation of the cells. Quiescent MCF-7 cells were therefore incubated with DME/FBS for 1 h to transiently activate MAPK (Fig. 2). The cells were washed, and incubated with ET18-$OCH_3$ for 3 h. At the end of this incubation the cells were washed and incubated with DME/FBS and subsequently processed for flow cytometric analysis. Table I shows that whereas preincubation with ET18-$OCH_3$ before the addition of FBS completely inhibited cell cycle progression into S phase, this inhibition was significantly blunted when the cells were transiently activated with FBS before addition of ET18-$OCH_3$. The above results demonstrate that inhibition of the early signaling events by ET18-$OCH_3$ contributes significantly to the antiproliferative action of the compound. However, the observation that there was a difference of 7% in the number of cells in S phase between control cells incubated without ET18-$OCH_3$ (FBS/BSA/FBS) and those incubated with ET18-$OCH_3$ after transient activation (FBS/ET18-$OCH_3$/FBS) suggests that the inhibition of cellular events by the ALP after the transient activation may also contribute to the inhibition of MCF-7 cell proliferation by ET18-$OCH_3$.

## Discussion

The results of the current study demonstrate that in an ET18-$OCH_3$–sensitive cell line, uptake of the drug inhibits the sustained phosphorylation and activation of MAPK as a consequence of its effect on Raf-1. This is based on the following: [*a*] ET18-$OCH_3$ had no effect on the binding of EGF to its receptors. Although EGFr internalization was inhibited as previously reported (8), this occurred after the decrease of MAPK activity to near-resting levels; [*b*] ET18-$OCH_3$ did not affect the extent and kinetics of tyrosine phosphorylation of the EGFr, suggesting that ET18-$OCH_3$ had no effect on EGFr kinase activity; [*c*] ET18-$OCH_3$ did not affect the activation of $p21^{ras}$ in the cells; [*d*] Preincubation of cells with ET18-$OCH_3$ did not reduce the Raf-1 content of the cells but significantly decreased the duration of the association of Raf-1 with the membrane within 4 min of stimulation. This reduced association correlated with a decrease in the membrane-associated Raf-1 kinase activity; [*e*] Since ET18-$OCH_3$ had no effect on the cellular cAMP levels and PKA activity, the inhibition of Raf-1 activity was not caused by a direct or indirect increase of PKA activity by ET18-$OCH_3$; and [*f*] The decrease in MAPK activity paralleled that of Raf-1 activity and resulted from decreased phosphorylation rather than a direct effect of ET18-$OCH_3$ on MAPK activity. The reduction in magnitude and duration of MAPK and the decreased association of Raf-1 with membranes in MCF-7 cells preincubated with ET18-$OCH_3$ after EGF or serum stimulation have also been observed in insulin-stimulated cells (Richard, C. and G. Arthur, personal observations).

The mechanism by which ET18-$OCH_3$ interferes in the association of Raf-1 with the membrane is not known. Since Ras activation, which localizes Raf-1 to the membrane (30, 31), is unaffected by ET18-$OCH_3$, it is not surprising that even though ET18-$OCH_3$ was already present in the membrane before stimulation of the cells with growth factors, it was unable to prevent the initial increase in Raf-1 association with membranes. We believe the initial activation of MAPK after cell stimulation is due to the Ras-induced translocation and subsequent activation of Raf-1, but the presence of ET18-$OCH_3$ in the membranes appears to activate or enhance a process that results in the dissociation of membrane-associated Raf-1 much earlier than in the control cells. However, we cannot yet categorically rule out an effect of ET18-$OCH_3$ on the translocation process. The enhanced dissociation of Raf-1 leads to the termination of the MAPK activity. Since growth inhibitory conditions with ET18-$OCH_3$ used in the study did not completely block MAPK activation, we can surmise that the magnitude and duration of activation of the enzyme were insufficient for the phosphorylation and activation of downstream molecules to levels required to initiate proliferation. This interpretation is consistent with the postulation that the magnitude and duration of activation of MAPK is crucial in determining cellular responses to stimulation (17).

We do not know whether ET18-$OCH_3$–induced inhibition of Raf-1 activation is due to a direct interaction of the compound with Raf-1, or whether this is achieved indirectly via its effects on unknown intermediary molecules required for the activation and/or attachment of Raf-1 to the membrane. It has been suggested that lipids may play a role in Raf-1 activation (35–37). Irrespective of the exact mode of action of ET18-$OCH_3$ on Raf-1, its ability to interfere in the activation of this key signaling molecule in a dose-dependent fashion should make it useful in assessing the effects of differential activation of the MAPK cascade on cellular responses.

The correlation between the accumulation of ET18-$OCH_3$, the extent of growth inhibition, Raf-1/membrane association and MAPK activation suggests that the inhibition of the MAPK pathway by ET18-$OCH_3$ is likely to contribute significantly to its antiproliferative effects. This view is supported by our recent studies demonstrating that *R* and *S* enantiomers of a double bond phosphonocholine ALP analog had differential effects on MCF-7 cell growth which correlated with their ability or inability to inhibit MAPK activation (Samadder, P., R. Bittman, H.-S. Byun, and G. Arthur, personal observations). The differential effects of the phosphonocholine enantiomers on cell proliferation also provides support for the widely held view that the inhibitory effects of ALPs are not due to nonspecific perturbation of membranes (reviewed in references 2, 3). The demonstration that inhibition of the early events in cell activation by ET18-$OCH_3$ significantly contributes to its antiproliferative effects clearly implicates the inhibition of the MAPK cascade in the mechanism of action of the compound. However, as other signaling molecules such as PI-3K, PLC, and PKC that may be activated within this period could be inhibited by ALPs, this cautions against attributing the inhibition of growth by ET18-$OCH_3$ solely to its ability to inhibit the MAPK cascade. An assessment of whether these molecules are inhibited under ET18-$OCH_3$ preincubation conditions that inhibit Raf-1 activation and whether their activation is essential for growth will be required before such a conclusion can be made. There is evidence that inhibition of PKC by ALPs does not correlate with inhibition of cell growth (2, 11, 12).

The localization of an inhibitory effect of ET18-$OCH_3$ downstream of Ras, implies that ET18-$OCH_3$ and related compounds should be effective against activating Ras mutations which have been observed in almost one-third of human cancers (38). Another implication of our study is that the apparent cell selective effects of ET18-$OCH_3$ may be due to fundamental differences in the regulation of Raf–membrane associations between the ALP-sensitive and -insensitive cells or differences in the signal transduction pathways (Raf-dependent or -independent pathways) that mediate cell proliferation in different cell types. Future studies to resolve the basis of the selective effects should lead to the rational use of these compounds in cancer therapy.

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