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ORIGINAL ARTICLE

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Growth suppression of a tumorigenic rat liver cell line by the anticancer agent, ET-18-O-CH_3, is mediated by inhibition of cytokinesis

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Abstract *Purpose*: This study was undertaken to elucidate the potential mechanism of the antitumor activity of ET-18-O-CH_3, a synthetic analogue of lysophosphatidyl choline, and a known antitumor agent and specific inhibitor of phosphoinositide phospholipase C (PI-PLC). *Methods*: A normal rat liver epithelial "oval" cell line (WB-F344) was neoplastically transformed by the H-ras oncogene (WB-ras2) and treated with a series of ET-18-O-CH_3 concentrates for a number of days. Cell growth, morphological "differentiation", cell cycle regulation, karyotypic changes, growth in soft agar (anchorage-independent growth) and the expression of cdk2, cdc2 and ERK genes were studied to determine the effect of ET-18-O-CH_3 on these neoplastic cells. *Results*: ET-18-O-CH_3 at 5 and 10 μg/ml was found to cause an increase in cell size, suppress cell growth, reduce the colony-forming efficiency and inhibit the anchorage-independent growth of the WB-ras2 cells. Significantly, flow-cytometric analysis revealed that while control cells and cells treated with concentrations of ET-18-O-CH_3 below 5 μg/ml were diploid, cell populations treated with 5 and 10 μg/ml ET-18-O-CH_3 comprised 33–37% diploid cells and over 60% tetraploid cells (4n–8n cycle cells). ET-18-O-CH_3 was found to induce aberrant cytokinesis as evidenced by the presence of a high frequency of enlarged cells, which were binucleated or multinucleated and mitotic cells with 4n and 8n numbers of chromosomes. ET-18-O-CH_3 was also capable of inhibiting both the expression of cdk2 and cdc2 and the activation of ERK1/2, while no effect was found on the expression of p21 ras. *Conclusions*: The effect of ET-18-O-CH_3 on neoplastically transformed H-ras rat liver cells has been interpreted as the result of an altered phenotype characterized by an enlarged and flattened cell morphology with ploidy changes caused by inhibition of cytokinesis.

Keywords Tetraploidization · Cytokinesis · Anticancer, ras-transformation

Abbreviations *AIG* Anchorage independent growth · *CdK* Cyclin-dependent kinase · *ERK* Extracellular signal-regulated kinase · *MAPK* Mitogen-activated protein kinase · *PI3-kinase* Phosphatidylinositol 3-kinase · *PI-PLC* Phosphoinositide phospholipase · *PKC* Protein kinase C

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Introduction

The ether lipid, 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosophocholine (ET-18-O-CH_3, edelfosine), has been found to show antitumor activity in vivo and in vitro [1]. It has been shown to inhibit the invasive growth of tumor cells in normal tissue [2, 3], to induce apoptosis in various cancer [4, 5] and transformed cells, to induce differentiation [6, 7] and to cause cell cycle arrest [8, 9]. ET-18-O-CH_3 has been shown to selectively inhibit the growth of cancer cells compared with that of noncancer cells [10]. Therefore, this drug is undergoing clinical trials in the treatment of lung, skin and breast cancers, as well as lymphomas. The precise molecular mechanism of the ET-18-O-CH_3-induced antiproliferative effect is not yet completely defined. Apoptosis induction by ET-18-O-CH_3 is mediated by c-jun NH_2-terminal kinase (JNK) activation and c-Jun via Fas (Apo-1/CD 95) receptor/ligand system [11, 12]. Recent studies have indicated that ET-18-O-CH_3 primarily acts on the membrane phospholipids. This suggests that ET-18-O-CH_3 could interfere with the function of enzymes involved in intracellular signal transduction processes. It has been proposed that inhibition of PI-PLC [13, 14, 15], PKC [6], and PI3-kinase [16] by ET-18-O-CH_3 affects the regulation of downstream signaling cascades, and this might subsequently lead to inhibition of cell

proliferation. The proteins mentioned above play a crucial role in oncogenic Ras-induced cellular proliferation and differentiation [17, 18].

Ras proteins are small monomeric GTP-binding proteins that relay signals from the receptor tyrosine kinases to the nucleus via activation of a number of intracellular molecules [17, 19]. Ras mutations are found in a wide variety of human malignancies, with the highest incidences observed in adenocarcinomas of the pancreas (90%), the colon (50%), and the lung (30%) [20]. However, mutations of the *Ras* gene are not sufficient to bring about an oncogenic response. Activation of a downstream signaling protein is necessary to modulate the expression of a specific subset of genes related to oncogenic proliferation, differentiation and transformation [21]. The Ras signaling pathway begins with upstream activation at the cell surface via tyrosine kinase or cytokine receptors, or $\beta\gamma$ subunits of heterotrimeric G proteins. Multiple Ras effectors have been identified including Raf, an integral member of the MAPK family, and PI3-kinase [18, 21, 22]. These effectors play a unique role in Ras-induced cellular transformation. Targeting the components of the Ras signaling pathways has been proposed as an approach to the development of anti-Ras drugs for cancer treatment [23, 24].

An increase in the level of inositol phosphates has been detected in cells expressing activated Ras, and Ras might regulate mammalian PLC activity [25, 26, 27, 28]. Recently, it has been shown that PLC is one of the effector proteins of Ras [29, 30]. Therefore, we considered that ET-18-O-CH_3, a direct inhibitor of PI-PLC, might show a chemotherapeutic effect in oncogenic *ras*-transformed cells. This study was undertaken to elucidate the mechanism involved in the antitumor activity of ET-18-O-CH_3 in a rat liver cell line (WB-F344) neoplastically transformed by the H-*ras* oncogene (WB-ras2). Specifically, we investigated the effect of ET-18-O-CH_3 on cell growth, colony-forming ability, anchorage-independent growth, the expression of ras and cell cycle regulation genes and karyotypic changes.

Material and methods

Chemicals

ET-18-O-CH_3 was from Biomol Research Laboratories (Plymouth Meeting, Pa.). Anti-cdk2, anti-cdc2, anti-ERK and anti-pERK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-pan-ras (Ab-2) monoclonal antibody was from Calbiochem (La Jolla, Calif.). ECL chemiluminescent detection reagent was purchased from Amersham Corporation (Arlington Heights, Ill.). Giemsa was purchased from Searle (High Wycombe, UK). Propidium Iodide was from Molecular Probes (Eugene, Ore.). Colcemid was purchased from GIBCO (Grand Island, N.Y.).

Cell culture

WB-ras2 cells, derived from the WB-F344 cell line, after infection with a virus carrying the H-*ras* oncogene and the neomycin-resistance gene, were cultured as described previously [31]. ET-18-O-CH_3 was dissolved in 50% ethanol and then applied to the cells at 1–10 μg/ml in the medium. Cells treated with an equal amount of ethanol were used as a solvent control.

Cell growth assay

The cells were inoculated into 35-mm dishes for 5 h and then exposed to the medium with the chemical after 5 h. After 3 days of incubation, the cells were harvested with 0.25% trypsin. Viable cells were determined by trypan blue exclusion and counted by a hemocytometer. Each treatment used triplicate plates.

Flow cytometry

For DNA content analysis, 2×10^5 cells in a 25-cm^2 flask were treated with various concentrations of ET-18-O-CH_3 and harvested by trypsinization at specific time-points. The cells were fixed with 1 ml 70% cold ethanol and stored at −20°C until use. Cells were centrifuged at 2100 rpm for 10 min and stained with propidium iodide (50 μg/ml) in phosphate-buffered saline (PBS) containing 0.1% Triton X-100, 0.1 m*M* EDTA (pH 7.4), 0.05 mg/ml RNase A, and stored at 4°C overnight in the dark. Cell cycle analysis was done at the Michigan State University FACS facility following a procedure described previously [32].

Karyotype analysis

The cells were inoculated on 22×22-mm coverslips (no. 2, Clay Adams, Gold Seal) in 35-mm dishes and treated with ET-18-O-CH_3 for 3 days. After colcemid treatment (0.1 μg/ml) for 3 h, the cells were washed with PBS, treated with hypotonic solution (0.7% sodium citrate) for 20 min and fixed with methanol/acetic acid (3:1) for 2 min. After the coverslips had been air-dried, the cells were stained with 5% Giemsa in Sorensen's buffer (pH 6.8, 34 m*M* KH_2PO4, 33 m*M* $Na_2HPO_4.2H_2O$) for 10 min and then rinsed with water. The coverslips were again air-dried, soaked in xylene and mounted with Permount on slide glasses. The karyotypes of cells were observed and photographed using a Nikon microscope.

Anchorage-independent growth

AIG was determined by a method described previously [33]. To assay for AIG, 1000 cells were mixed into 2.5 ml top agarose containing 0.33% agarose in the medium and seeded onto 2.5 ml solidified bottom agarose containing 0.5% agarose in the medium. After overnight incubation, 2.5 ml medium containing ET-18-O-CH_3 was added on top of the agar. The medium was renewed once every 3 days. Plates were observed daily for colony development for 3 weeks and photographs were taken.

Colony-forming efficiency

After 3 days of treatment with 10 μg /ml of ET-18-O-CH_3, cells were trypsinized and 1000 were replated on 100-mm plates in quadruplicate. The cells were grown for 5 days without chemical for determination of colony-forming efficiency. The colonies developed were washed once with PBS, stained with 0.5% crystal violet, and counted.

Western blot analysis

The cells were treated with ET-18-O-CH_3 (1, 5 or 10 μg/ml) for 3 days. The proteins were extracted with 20% SDS solution containing 1 m*M* phenylmethylsulfonylfluoride (PMSF) buffer. Equal amounts of proteins (20 μg/lane) were separated by 12.5% SDS-PAGE and transferred from the gel to PVDF membranes.

Immunoblotting was carried out with appropriate antibodies, and immunoreactive protein complexes were detected by ECL detection reagent.

Statistical analyses

All data are expressed as the mean group value ± SEM. For statistical analysis, the data were first analyzed using one-way analysis of variance (ANOVA). Significant differences between solvent control and ET-18-O-CH_3 treatment were evaluated using Dunnett's method. Statistical analyses were performed using a commercial statistical analysis protocol (SigmaStat 2.0; Jandel Scientific Software, San Rafael, Calif.). The level of statistical significance was set at $P < 0.05$.

Results

Effect of ET-18-O-CH_3 on cell morphology and cell growth

ET-18-O-CH_3 inhibited the growth of WB-ras2 cells in a dose-dependent manner (Fig. 1). Treatment of cells with 5 and 10 μg/ml of ET-18-O-CH_3 was found to reduce cell growth dramatically as compared to the control. Concomitantly, morphological changes were also found to be induced in cells treated with both concentrations of the chemical (Fig. 2). The WB-ras2 cells showed no contact inhibition at confluency with spindle-like morphology. Following 3 days of treatment with 5 and 10 μg/ml of ET-18-O-CH_3, the cells showed contact inhibition and became enlarged, flattened and more epithelial-like. Multiple nuclei were observed in most of the enlarged cells (Fig. 2).

Effect of ET-18-O-CH_3 on DNA content and cell cycle progression in the WB-ras2 cells

To determine if the antiproliferative effect of ET-18-O-CH_3 on WB-ras2 cells was associated with cell cycle arrest or abnormal cell division, we analyzed the DNA content and cell cycle progression of WB-ras2 cells treated with ET-18-O-CH_3 by flow cytometry (Fig. 3). In control cells and those treated with the low dose of ET-18-O-CH_3 (1 μg/ml) for 3 days, a typical higher peak of 2n DNA content cells (G_0/G_1 cells) and a low peak of 4n DNA content cells (G_2/M) were observed. This distribution of cells was dramatically changed in cells treated with 5 or 10 μg/ml ET-18-O-CH_3 for 3 days. In these cell populations, the 4n DNA content cells became the dominant peak. In addition to the 2n and 4n peaks, a new peak of 8n DNA content cells emerged. Clearly, the diploid cells had given rise to tetraploid cells as a consequence of the drug treatment.

Flow cytometry showed that the cell populations of the control and low-dose treatment entirely comprised diploid cells undergoing normal cell division (i.e. 2n–4n cycle cells), whereas the cell populations treated with 5 or 10 μg/ml of ET-18-O-CH_3 were 37–33% diploid and over 60% tetraploid (4n–8n cycle cells; Table 1). To determine if the effect of ET-18-O-CH_3 on the emergence of the 8n DNA pattern was reversible, ET-18-O-CH_3 was removed after 3 days of treatment for 2 days and the cells were reanalyzed for DNA content by flow cytometry. There was no change in the distribution of DNA content of cells 2 days after the removal of ET-18-O-CH_3 (data not shown), indicating that the karyotypic changes were irreversible.

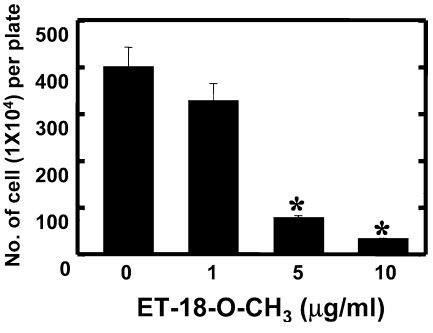

Fig. 1 Effect of ET-18-O-CH_3 on growth of WB-ras2 cells. WB-ras2 cells were inoculated into 35-mm dishes in triplicate at 10,000 cells per dish and exposed to medium with various concentrations of ET-18-O-CH_3. After a 3-day incubation, the cells were removed by trypsinization. Viable cells were determined by trypan blue exclusion and counted by a hematocytometer. Bars represent means ± SEM of triplicate plates. $*P < 0.05$

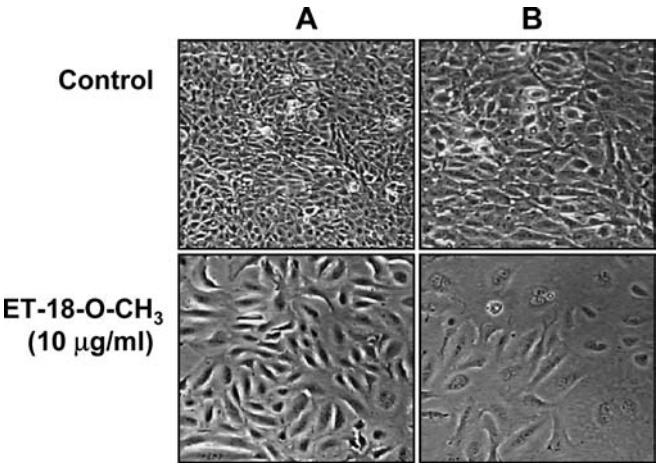

Fig. 2A, B Phase-contrast photographs of WB-ras2 cells treated with ET-18-O-CH_3. WB-ras2 cells were treated with 10 μg/ml of ET-18-O-CH_3 or ethanol (solvent control) for 3 days. The photographs of the cells were taken with a Nikon microscope at different magnification (**A** ×10 objective, **B** ×20 objective)

ET-18-O-CH_3 induced binucleated cells by inhibiting cytokinesis

Although ET-18-O-CH_3 at 5 and 10 μg/ml showed an antiproliferative effect on WB-ras2 cells (Fig. 1), it did not inhibit DNA synthesis or chromosome replication of the initial cell population since mitotic cells were

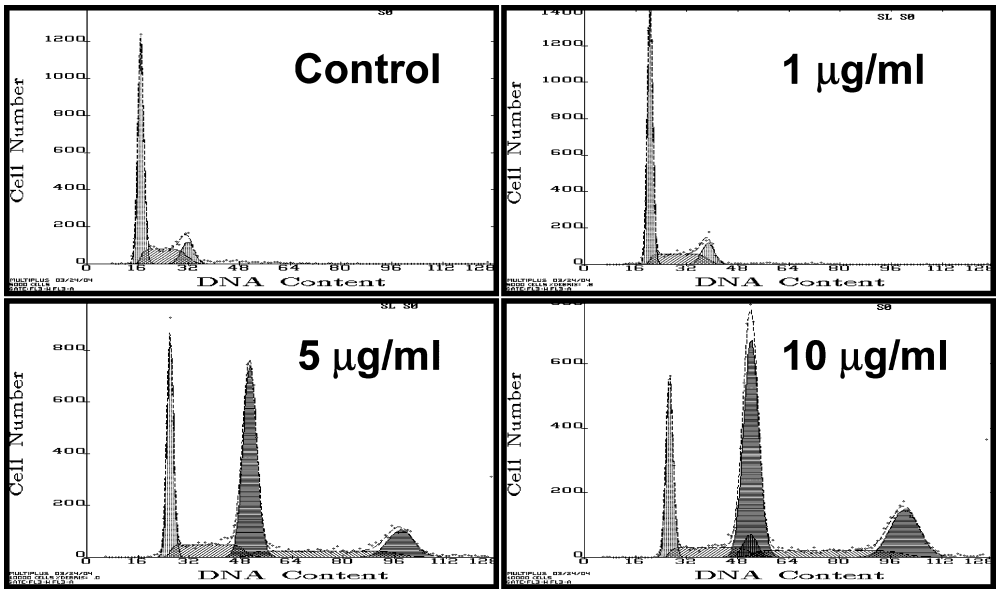

Fig. 3 Cell cycle analysis of WB-ras2 cells treated with various concentrations of ET-18-O-CH_3 by flow cytometry. WB-ras2 cells were treated with various concentrations of ET-18-O-CH_3 for 3 days. The cells were fixed with 70% cold ethanol at −20°C, stained with propidium iodide overnight at 4°C, and analyzed by flow cytometry

Table 1 Cell cycle phase distribution of WB-ras2 cells treated with various concentrations of ET-18-O-CH_3 for 3 days. The data presented are the means ± SEM of triplicate experiments

ET-18-O-CH_3 (μg/ml)	Cells in 2n → 4n cycle (%)	G_1	S	G_2/M	Cells in 4n → 8n cycle (%)	G_1	S	G_2/M
0	100	62.0 ± 1.5	25.4 ± 1.2	12.6 ± 0.5	0	–	–	–
1	100	66.1 ± 0.3	21.3 ± 0.6	12.6 ± 0.2	0	–	–	–
5	37.6 ± 2.3	69.4 ± 2.0	27.0 ± 3.5	3.6 ± 2.1	62.4 ± 2.3	66.5 ± 1.2	17.6 ± 0.5	15.9 ± 0.8
10	33.7 ± 4.0	67.9 ± 2.4	26.4 ± 2.3	5.7 ± 0.2	66.3 ± 4.0	66.7 ± 2.8	13.4 ± 0.4	19.9 ± 2.3

readily observable in cells treated with the drug for 3 days. The karyotypic analysis of Giemsa-stained chromosomes and nuclei of these cells, however, revealed that the drug did affect normal cell division (Fig. 4). In control cells and those treated with the low dose of ET-18-O-CH_3 (1 μg/ml), the 2n number of mitotic chromosomes (40) (Fig. 4A) and resting stage mononucleated cells were observed in almost all of the cells. In cells treated with 5 or 10 μg/ml of ET-18-O-CH_3 for 3 days, more than 50% of the cells had 4n (Fig. 4B) or 8n (Fig. 4C, D) numbers of mitotic chromosomes. At resting stage, similar proportions of cells were binucleated or multinucleated with a larger cell size (Fig. 4E, F). This observation indicates that ET-18-O-CH_3 was able to inhibit cytokinesis. The resulting multinucleated polyploid cells with enlarged cell size may become terminally differentiated.

Effect of ET-18-O-CH3 on the colony-forming efficiency of WB-ras2 cells

We used a clonogenic assay to measure the viability of cells. From the population containing multinucleated cells after treatment with 10 μg/ml ET-18-O-CH_3 for 3 days, 1000 cells were replated. The colony-forming efficiency of these WB-ras2 cells was reduced by 50% compared to control cells (Fig. 5), indicating that multinucleated cells may be terminally differentiated and lose colony-forming ability.

Effect of ET-18-O-CH_3 on anchorage-independent growth

To determine whether ET-18-O-CH_3-induced karyotypic/morphological changes and whether antiproliferative effects would lead to attenuation of the oncogenic activity of *ras*, AIG was investigated. The parental WB-F344 cells did not grow on soft agar (Fig. 6A), whereas the WB-ras2 cells were capable of AIG (Fig. 6B, C), confirming the neoplastic transformation of the WB-ras2 cells. ET-18-O-CH_3 inhibited AIG of WB-ras2 cells in a dose-dependent manner (Fig. 6D–F). ET-18-O-CH_3 at 10 μg/ml completely abolished AIG (Fig. 6F) and the treated WB-ras2 cells behaved similarly to wild-type WB-F344 cells (Fig. 6A).

Effect of ET-18-O-CH_3 on the expression of p21ras, Erk1/2 and cdk2

Oncogenes such as *ras, src, raf* and *mos* have been shown to transform cells by activating MAPK kinase and

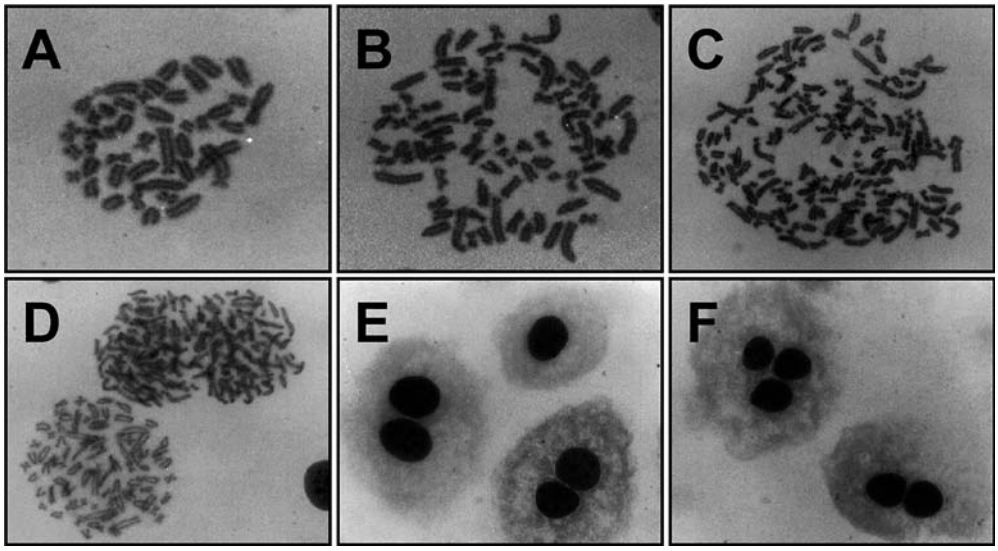

Fig. 4A–F Representative karyotypes of WB-ras2 cells treated with 5 or 10 μg/ml ET-18-O-CH_3 showing 2n, 4n and 8n number of chromosomes. WB-ras2 cells were grown on glass coverslips. After a 3-day treatment with 10 μg/ml of ET-18-O-CH_3, the cells were treated with colcemid for 3 h and then exposed to hypotonic solution and fixed with methanol/acetic acid (3:1). After air-drying, the coverslips were stained with 5% Giemsa in Sorensen's buffer for 10 min. After air-drying again, the coverslips were soaked in xylene and mounted on slide-glasses. Photographs were taken using a Nikon microscope. **A** karyotype with 2n chromosomes; **B** karyotype with 4n chromosomes; **C** karyotype with 8n chromosomes; **D** 8n chromosomes in each nucleus of two contiguous cells; **E, F** binucleated and trinucleated cells with larger cytoplasm

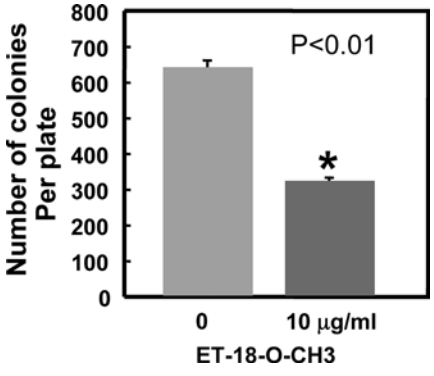

Fig. 5 Effect of ET-18-O-CH_3 on colony-forming ability of WB-ras2 cells. After treatment with ET-18-O-CH_3 at 10 μg/ml, 1000 cells were replated in each of four 9-cm plates. The average number of colonies was 327 ± 8 (329, 303, 341, 334) and 645 ± 19 (592, 651, 655, 682) for the treated plates and the control plates, respectively. The difference is highly significant

downstream components in the signaling pathway [34]. Activation of Ras leads to activation of the MAPK cascade pathway [21, 22] and eventual cell proliferation. To determine if the antiproliferative effect of ET-18-O-CH_3 on WB-ras2 cells was due to inhibition of the expression of p21ras and downstream signal components of Ras, especially ERK1/2, Western blot analyses were carried out (Fig. 7). At 5 and 10 μg/ml, ET-18-O-CH_3 inhibited the activation of ERK1/2 but had no effect on p21ras. In addition, the effects of ET-18-O-CH_3 on cell cycle-regulation proteins such as cdc2 and cdk were investigated. At 10 μg/ml, ET-18-O-CH_3 decreased protein expression of cdk2 and cdc2 which regulate cell cycle progression at the G_0/G_1 and G_2/M phases, respectively. At 5 μg/ml, ET-18-O-CH_3 also decreased the expression of cdk2 but not that of cdc2.

Discussion

Chemotherapeutic agents stop cell proliferation and induce cell death by various mechanisms. The classical targets of conventional anticancer drugs are DNA (e.g. doxorubicin hydrochloride), DNA polymerase (e.g. cytarabine), topoisomerase (e.g. etoposide), antimetabolites of nucleic acids (e.g. methotrexate), tubulin (paclitaxel) and transcription of specific growth promoting gene (e.g. tamoxifen). More recently, many drugs have been developed to target specific oncogenes, tumor-suppressor or cell cycle-regulating genes, cell growth signaling pathways, telomerase, angiogenesis factors and metastasis enzymes. ET-18-O-CH_3 has been shown to be an inhibitor of PLC [13, 14, 15], a pivotal enzyme controlling cell proliferation mediated by the action of some growth factors and oncogenes. Therefore, the chemical may be considered as affecting cell growth signaling pathways (i.e. PKC, MAPK and PI3-kinase). However, our studies showed that the chemical has a major effect on karyotypic changes and cell differentiation.

The mechanism of growth inhibition by ET-18-O-CH_3 is not well understood. Besides the induction of apoptosis reported in the literature [11, 12], our studies indicated that it could induce enlarged and flattened morphology of cells and increase DNA ploidy. The reduction in colony-forming ability and the abrogation of AIG after drug treatment could be part of the manifestation of this phenotype. Furthermore, we found that this phenotype may be initiated by the absence of

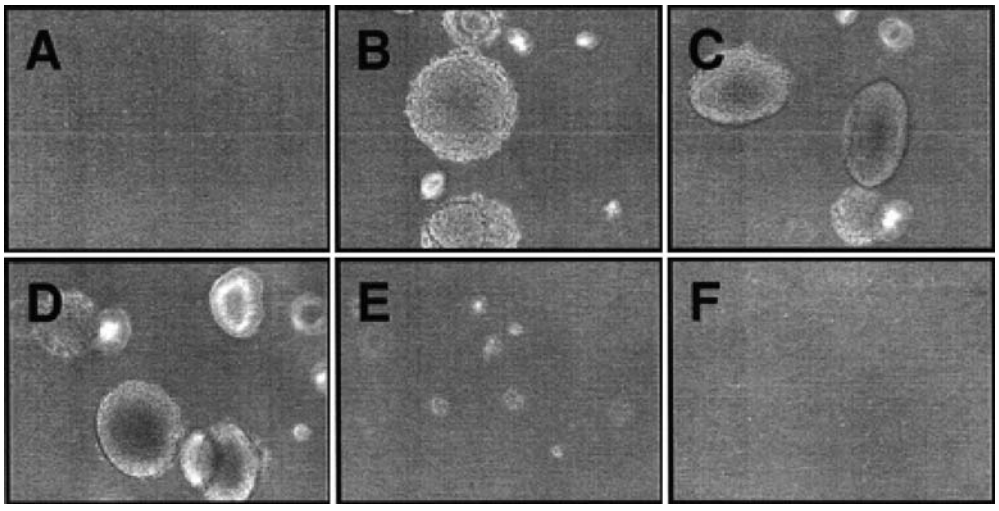

Fig. 6A–F Effect of ET-18-O-CH_3 on anchorage-independent growth of WB-ras2 cells. Cells (10,000 in 2.5 ml 0.33% agarose medium) were plated on top of 2.5 ml prehardened 0.5% agarose medium. The cells were cultured with the medium containing various concentrations of ET-18-O-CH_3 for 3 weeks. Photographs were taken 3 weeks after inoculation. **A** WB cells, the non-*ras* transformed parental cells, are anchorage-dependent (AIG^-); **B, C** WB-ras2 cells are anchorage-independent (AIG^+) with (**C**) or without (**B**) solvent (ethanol). ET-18-O-CH_3 inhibited AIG in a dose-dependent manner: **D** 1 μg/ml of ET-18-O-CH_3, **E** 5 μg/ml of ET-18-O-CH_3, **F** 10 μg/ml of ET-18-O-CH_3

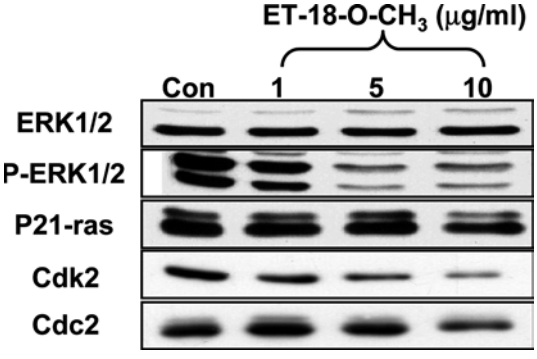

Fig. 7 Effect of ET-18-O-CH_3 on expression of cdk2, cdc2 and activation of ERK in WB-ras2 cells. WB-ras2 cells were treated with various concentrations of ET-18-O-CH_3 for 3 days. Protein extracts from the cells were immunoblotted with the indicated antibodies

cytokinesis induced by ET-18-O-CH_3. This is evidenced by the karyotypic analyses that showed the presence of 4n and 8n numbers of chromosomes in mitotic cells and the presence of enlarged cells that were binucleated or multinucleated. The increased DNA ploidy (4n and 8n DNA content) was confirmed by flow cytometric analysis. Cells with 8n number of chromosomes with duplicated chromatids should contain 16n DNA content. This peak was not seen in flow cytometric analysis. It is possible that these cells with excessive numbers of chromosomes might be insignificant in number or too fragile to remain intact after harvesting for analysis.

The increase in ploidy and DNA content of cells may be accomplished by different mechanisms, e.g. endoreduplication and absence of cytokinesis. The latter appears to be the mechanism for ET-18-O-CH_3. Since ET-18-O-CH_3 is known to be an inhibitor of PLC as well as PKC, the results of this study appear to indicate a role of PLC and/or PKC in normal cytokinesis. Sun et al. have demonstrated that diacylglycerol, generated by nuclear PI-PLC activity, is the driving force for entry into mitosis and decreased nuclear PI-PLC activity, caused by PI-PLC specific inhibitor, leads to cell cycle blockade in the G_2/M phase [35]. Neri et al. have demonstrated that nuclear diacylglycerol produced by PI-PLC is responsible for nuclear translocation of PKC-α, and PI-PLC activity appears to be essential for the G_0/G_1 to S phase transition [36]. Although these reports are consistent with our observation that cdc2 and cdk2 were downregulated by ET-18-O-CH_3, the specific roles of PLC and PKC in cytokinesis remain to be determined.

This study showed a new effect of antiproliferation (i.e. the induction of large, contact-inhibited cells) and a new mechanism of action (i.e. the inhibition of cytokinesis and tetraploidization) of ET-18-O-CH_3.

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