STUDIES ON THE MECHANISM OF SELECTIVE ANTIPROLIFERATIVE EFFECTS OF ALKYLLYSOPHOSPHOLIPIDS IN MAMMARY EPITHELIAL CELLS

By

Pranati Samadder

A thesis submitted to the faculty of Graduate Studies

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PRANATI SAMADDER

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Dedicated

To my late Parents

and

My beloved children, Urmi and Shubhashish

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LIST OF CONTENTS

LIST OF FIGURES AND TABLES

ABBREVIATIONS

Ab	antibody
ACGM	adapted cell growth medium
ACGM$^-$	adapted cell growth medium without prostaglandin $F_{2\alpha}$
ACGM$^+$	adapted cell growth medium with prostaglandin $F_{2\alpha}$
ADP	adenosine 5'-diphosphate
AEBSF	aminoethylbenzenesulfonyl fluoride
AEL	antitumor etherlipids
ALP	alkyllysophospholipids
APC	alkylphosphocholines
aPKCs	atypical PKCs (ζ, λ, ι)
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
β-GP	β-glycerophosphate
BM41•440	1-S-hexadecylthio-2-methoxymethyl-*sn*-glycero-3-phosphocholline (ilmofosine)
BPE	bovine pituitary extract
BSA	bovine serum albumin
°C	degree Celsius
cAMP	cyclic adenosine 3',5'-monophosphate
CAPK	ceramide-activated protein kinase

cGMP	cyclic guanosine 3';5'-monophosphate
Ci	Curie
cPKCs	conventional (or classical) PKCs (α, βI, βII, γ)
CR	conserved region
CRD	cysteine-rich domain
CREB	cAMP response element-binding protein
CT	cytidylyltransferase
C-terminus	carboxyl terminus or COOH-terminus
CTP	cytidine 5'-triphosphate
DAG	diacylglycerol
DDW	distilled deionized water
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diaminetetraacetic acid
EGF	epidermal growth factor
EGFr	epidermal growth factor receptor
EGTA	ethylenegylcol-bis-(β-amino-ethyl ether) N,N'-tetraaceticacid
ERK	extracellular signal-regulated protein kinase (1 or 2)
Est	estrogen

ET-18-OCH_3	1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (edelfosine)
ET-16-OCH_3	1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine
ET-16-OCH3-phosphatidate	1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphate
ET-16-OCH_3-phosphonate	4-*O*-hexadecyl-3(*S*)-*O*-methoxybutanephosphonate
ET-16S-OCH_3-	4-Thiohexadecyl-3(*S*)-*O*-methoxybutane-4-phosphonate
ET-16-phosphono-TDB	2'-(trimethylammonio)-ethyl-4-(hexadecyloxy)-3-methoxy-1-butenephosphonate
FBS	fetal bovine serum
GAEL	glycosylated antitumor ether lipid
GAP	GTPase-activating protein
GDP	guanosine 5'-diphosphate
GF	growth factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GNEF	guanine nucleotide exchange factor
Grb2	growth factor receptor-bound protein 2
GTP	guanosine 5'-triphosphate
GTPase	guanosine 5'-triphosphatase
h	hour
HBSS	Hank's balanced saline solution
HePC	hexadecylphosphocholine (also known as miltefosine)

HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
H+L	heavy and light chains of antibodies
HNME	human normal mammary epithelial cells
HRP-	horseradish peroxidase-conjugated
Hsp	heat shock protein
IgG	immunoglobulin G
IGF	insulin-like growth factor
IL-1	interleukin-1
IP_3	inositol triphosphate
IRS-1	insulin receptor substrate-1
JAK	Janus kinase
JNK	Jun N-terminus kinase
kDa	kilodalton
l	litre
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
M	molar
mAb	monoclonal antibody
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MAPKKK	MAPK kinase kinase
MBP	myelin basic protein

MEK	mitogen-activated protein kinase/extracellular signal regulated protein kinase kinase
MEBM	mammary epithelial basal medium
MEGM	mammary epithelial growth medium
MEM	minium essential medium
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mwt	molecular weight
NF-κB	nuclear factor-κB
nM	nanomolar
nmol	nanomole
nPKCs	novel PKCs (δ,ϵ,η,θ,μ)
N-terminus	amino- or NH_2-terminus
OD	optical density
PA	phosphatidic acid
PAF	platelet-activating factor
PAP	phosphatidic acid phosphohydrolase
PBS	phosphate buffer saline solution
PC	phosphatidylcholine
PC-PLC	phosphatidylcholine-specific phospholipase C

PC-PLD	phosphatidylcholine-specific phospholipase D
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PH	Pleckstrin-homology
PI	phosphatidylinositol
PIP	phosphatidylinositol (4) phosphate
PIP_2	posphatidylinositol (4,5) bisphosphate
PIP_3	phosphatidylinositol (3,4,5) triphosphate
PI-PLC	phosphoinositide-specific phospholipase C
PI 3-kinase	phosphoinositide 3-kinase
PI 4-kinase	phosphoinositide 4-kinase
PI 5-kinase	phosphoinositide 5-kinase
PI 3-P	phosphatidylinositol 3-phosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKI	protein kinase A inhibitor peptide
PLA	phospholipase A
cPLA	cytoplasmic phospholipase A
PLC	phospholipase C
PLD	phospholipase D

PMSF	phenylmethylsulfonyl fluoride
PTPα	protein tyrosine phosphatase α
RBD	Ras-binding domain
RK	reactivating kinase
RTK	receptor tyrosine kinase
SAPK	stress-activated protein kinase
SBM	supplemented basal medium
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s	second
ser	serine
SH2/3	Src-homology 2 or 3 domain
Shc	Src-homology 2/α-collagen-related
Sos	Son of sevenless (a guanine nucleotide exchange factor)
SRF	serum response factor
SRI 62-834	2-{hydroxy[tetrahydro-2-(octadecyloxy)methylfuran-2-1-methoxyl]phosphinyloxy}-*N,N,N*-trimethylethaniminium - hydroxide
Syp	SH2-containing protein tyrosine phosphatase (also known as SH-PTP2)
TCA	trichloroacetic acid
TDB-PC	ET-16-phosphono-TDB

TEMED	N,N,N',N'-tetramethyl ethylenediamide
Thr	threonine
TLC	thin-layer chromatography
TNF	tumor necrosis factor
TPA	12-*O*-tetradecanoyl phorbol-13-acetate (a phorbol ester)
Tyr	tyrosine
UV	ultraviolet
µ	micron
µCi	microcurie
µl	microlitre
µM	micromolar
v	volume
v/v	by volume
w	weight
w/v	weight/volume

ABSTRACT

The antiproliferative and cell-selective effects of alkyllysophospholipids (ALPs) have been well established by studies from numerous laboratories. A number of hypotheses have been proposed to explain the mechanisms underlying these effects. Previous studies with MCF-7 cells led to the proposal that ET-18-OCH_3, the prototypic ALP, perturbs the association of Raf-1 with cell membranes and thereby prevents its sustained activation. This in turn leads to inhibition of downstream kinases such as MAP kinase which leads to inhibition of cell proliferation. If inhibition of Raf-1 activation is responsible for the cytostatic activity of ALPs then the inability of ET-18-OCH_3 to inhibit the proliferation of some cells could be due to the utilization of Raf-1-independent pathways to activate MAP kinase or differences in the mechanism of activation of Raf-1 in ALP-sensitive and insensitive cells. Before exploring this hypothesis, studies were conducted to validate the suggestion that ALPs inhibit cell proliferation by inhibiting Raf-1 activation. Two enantiomers of a double-bond phosphonocholine ALP analog were identified which had differential effects on the proliferation of epithelial cancer cell lines. (*S*)-ET-16-phosphono-TDB inhibited cell proliferation whereas (*R*)-ET-16-phosphono-TDB was not able to inhibit cell proliferation. We demonstrated that while the (*S*) enantiomer of ET-16-phosphono-TDB was able to inhibit MEK, MAP kinase and S6 kinase activities in EGF-stimulated cells, the (*R*) enantiomer was not able to do so. Both enantiomers did not directly affect the kinase activity of catalytically active Raf-1, indicating that the inhibition of MEK activity is not due to a direct inhibition of Raf-1 by (*S*)-ET-16-phosphono-TDB. Unlike ET-18-OCH_3, both (*S*) and (*R*)-ET-16-phosphono-TDB did not inhibit the translocation of Raf-1 to the membrane but rather seemed

to promote it in the absence of growth factor stimulation. Taken together our results suggest that all these choline-containing ALPs interact with Raf-1 in the cytosol and while compounds such as (*S*)-ET-16-phosphono-TDB interfere in the events that lead to activation of Raf-1 once it is in the membrane, interaction of Raf-1 with ET-18-OCH_3 inhibits Raf-1 translocation to the membrane in response to cell stimulation.

After confirming the role of inhibition of Raf-1 activation in the mechanism of action of ALPs, studies were conducted to explore the mechanisms responsible for the observed selective effects. Serum-free media were developed and MCF-7 cells were adapted for growth in the media. The cells that were adapted for serum-free growth, MCF-7Ad$^+$, became insensitive to growth inhibition by ET-18-OCH_3. These cells, along with human normal mammary epithelial (HNME) cells that were also resistant to growth inhibition by ET-18-OCH_3, were used for our studies. We demonstrated that unlike in MCF-7 cells where inactive Raf is present in the cytosol, in HNME and MCF-7Ad$^+$ cells Raf-1 was found in both the membranes and the cytosol in unstimulated quiescent cells. Stimulation of both cells with growth factors resulted in the activation of Raf-1 without further translocation of cytosolic Raf-1. Preincubation of both cells with ET-18-OCH_3 had little effect on membrane Raf-1 levels and MAP kinase activity and phosphorylation. In contrast, MAP kinase activity in MCF-7 cells was severely inhibited by preincubation with ET-18-OCH_3. A progressive increase in membrane Raf-1 levels during the adaptation process which correlated with increasing resistance to ET-18-OCH_3 was demonstrated. Since ET-18-OCH_3 inhibits the translocation of Raf-1 to the membrane, the constitutive association of Raf-1 in the membrane by-passes the step that is inhibited by ET-18-OCH_3 and thereby confers resistance to the

growth-inhibitory effect of the compound. Our studies allow us to conclude that Raf-1 is a key intracellular target of choline-containing ALPs in epithelial cells and inhibition of Raf-1 activation by the compounds leads to inhibition of growth. Our results also strongly suggest that differences in the mechanism of activation of Raf-1 may explain why some cells are unaffected by concentrations of ET-18-OCH_3 that severely inhibit the proliferation of other cells.

1 BACKGROUND AND LITERATURE REVIEW

1.1 ANTITUMOR ETHER LIPIDS

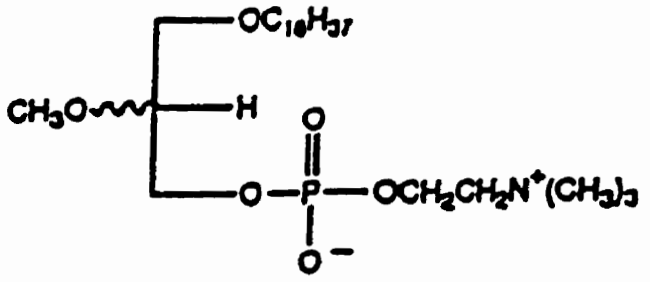
1.1.1 Alkyllysophospholipids - Synthesis and Development as Antitumor Agents:

The development of alkyllysophospholipids (ALP) originated from the discovery that lysophosphatidylcholine (LPC), which is formed by hydrolysis of phosphatidylcholine (PC), was a potentiator of macrophage activation (Burdzy *et al.*, 1964). This discovery led to the development of LPC analogs as potential immunomodulators. In cells, LPC is rapidly converted to PC or degraded by lysophospholipases, and consequently, it has a very short half-life. LPC analogs were therefore synthesized in which the metabolically active 2-hydroxy group was replaced by a metabolically stable small alkyl group, such as a methoxy group (Arnold *et al.*, 1967; Eibl *et al.*, 1967). At the *sn*-1 position, the acyl group was replaced by an alkyl group with 12-20 carbon atoms. These synthetic LPC-like compounds are known as alkyllysophospholipids (ALPs). Although the compounds were initially synthesized for use as potential immunomodulators, it was soon observed that they spontaneously accumulate in tumor tissue *in vitro* and were cytotoxic against a wide variety of tumor cell lines *in vitro*. In contrast, they were not toxic against normal or non-tumorigenic cells (Andreesen *et al.*, 1978; Arnold *et al.*, 1978; Hoffman *et al.*, 1984). In the last 30 years, many ether-linked lipids have been synthesized and examined for their antitumor activities (Daniel, 1993; Houlihan *et al.*, 1995; Lohmeyer and Bittman, 1994). Collectively these compounds are called antitumor ether lipids (AELs), since they do not all have the lysophospholipid structure like the ALPs.

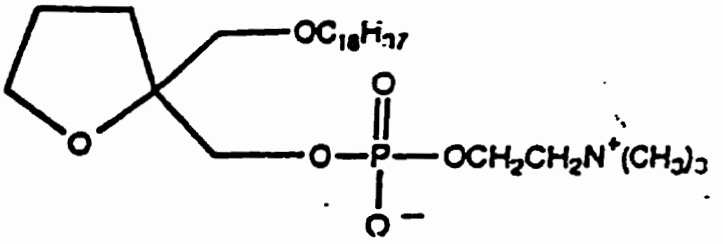
1.1.2 Types of AELs

There are two major well-established classes of AELs, the ALPs and the alkylphosphocholines (APC) **(Fig 1)**. The prototype ALP is the diether phospholipid, 1-*O*-octadecyl-2-*O*-methyl-glycero-phosphocholine (ET-18-OCH_3; edelfosine) while the prototype APC is hexadecylphosphocholine (miltefosine). APCs lack the glycerol backbone and are simple long chain alcohols esterified to a phosphobase. ET-18-OCH_3 and ET-16-OCH_3 are structurally analogous to the naturally occurring lipid mediator platelet-activating-factor (PAF, 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine), but are very dissimilar with respect to their biological properties. Other ALPs that have been developed include the thioether analog BM41.440 (ilmofosine) and the cyclic ether phospholipid SRI 62-834 **(Fig.1)**. Ilmofosine has a CH_2OCH_3 group at the 2- position of the glycerol backbone, whereas the *sn-2* *O*-alkyl group in the SRI 62-834 is incorporated into a tetrahydrofuran ring. A phosphonate analog of ET-16-OCH_3 was synthesized in which the oxygen linkage at *sn-3* position was replaced by a methylene group (Bittman *et al.*, 1993; 1994).

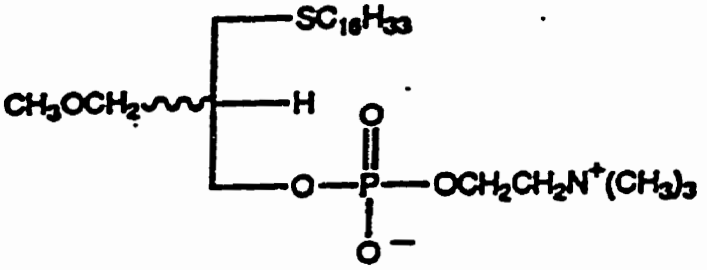
The glycosylated antitumor ether lipids (GAELs) represent a new generation of AELs. In these compounds, the phosphocholine has been substituted with a glycosyl moiety without compromising the antiproliferative properties (Guivisdalsky *et al.*, 1990). These compounds have proved to be very effective cytotoxic agents, especially the 2-glucosamino and 2-deoxyglucosyl derivatives (Lu *et al.*, 1994; Marino-Albernas *et al.*, 1996; Samadder *et al.*, 1998). They also reduce pro-aggregatory effects observed with some choline ALP analogs (Guivisdalsky *et al.*, 1990). AELs are distinguished by their very low rates of metabolism in cells.



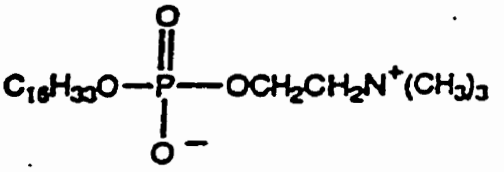
ET18-OCH3 (edelfosine)



SRI 62-834



BM 41.440 (ilmofosine)



HePC (miltefosine)

Figure 1. Structure of the antitumor ether lipids (AELs) ET-18-OCH_3 (edelfosine), BM 41.440 (ilmofosine), SRI 62-834 and hexadecylphosphocholine (HePC, miltefosine).

Most studies with AELs have used racemic mixture of the ALPs. Recently, the enantioselective synthesis of ET-18-OCH_3, SRI62-834 and BM41.440 analogs have been achieved (Bittman *et al.*, 1997; Lohmeyer and Bittman, 1994), which has allowed investigations on the effect of chirality on biological activity.

Clinical trials with AELs have produced encouraging results (Houlihan *et al.*, 1995; Lohmeyer and Bittman, 1994; Principe and Braquet, 1995) and have led to the approval of miltefosine for use against skin metastases in breast cancer. ET-18-OCH_3 is also being tested for use in purging bone marrow of leukemia cells prior to transplantation (Vogler *et al.*, 1990).

1.1.3 Antitumor activity of AELs

The antitumor activity of AELs has been demonstrated in a large variety of experimental tumors. The biological activity of AELs that contribute to their antitumor effects include activation of macrophages, inhibition of tumor metastases, shrinkage of tumours, differentiation of tumor cells, inhibition of tumor cell proliferation (Berdel *et al.*, 1985; Andreesen, 1988) and inhibition of angiogenesis (Candal *et al.*, 1994).

The antiproliferative effects of AELs have been demonstrated on a wide range of tumor cell lines and primary tumor cells including prostate carcinomas, urothelial carcinoma of bladder, hypernephroid carcinoma, terato-carcinomas (Berdel *et al.*, 1983a; 1983b; Neumann *et al.*, 1987); various human and murine leukemia (Hoffmann *et al.*, 1984; Salari *et al.*, 1992; Tidwell *et al.*, 1981; Unger *et al.*, 1987); human brain tumors (Ashagbley *et al.*, 1996; Houlihan *et al.*, 1987; Unger *et al.*, 1987); lung cancers (Herrmann and Neuman,

1987; Scholar, 1986), ovarian carcinoma (Fujiwara *et al.*, 1989; Neuman *et al.*, 1987), colon (Neuman *et al.*, 1987; Lu *et al.*, 1992a), breast (Herrmann and Neuman, 1987; Kosano and Takatani, 1988), gall bladder, cervix, uteri and corpus uteri (Neuman *et al.*, 1987) and fibrosarcomas (Houlihan *et al.*, 1987; Modolell *et al.*, 1979; Tarnowski *et al.*, 1978).

1.1.4 Mechanism of antiproliferation

The mechanism of antiproliferative action of the AELs is not yet fully understood, but unlike many other antitumor agents which exert their effect by interfering with DNA replication and transcription, AELs do not interact directly with DNA and are believed to mediate their effects by interacting with the plasma membrane. They are therefore not mutagenic. The mechanism of inhibition of cell proliferation by AELs has been considerably debated (Brachwitz and Vollgraf, 1995; Houlihan *et al.*, 1995; Lohmeyer and Bittman, 1994; Principe and Braquet, 1995). AELs have been proposed to inhibit proliferation via their effects on cellular transport systems (Berkovic *et al.*, 1992; Hoffman *et al.*, 1992; Junghahn *et al.* 1995; Vallari *et al.*, 1988), induction of apoptosis (Mollinedo *et al.*, 1997; Winkler *et al.*, 1996), inhibition of transacylation (Herrmann and Neuman, 1986; Winkler *et al.*, 1996), inhibition of phosphatidylcholine synthesis (Baburina and Jackowski, 1998; Boggs *et al.*, 1998; Geilen *et al.*, 1992; Haase *et al.*, 1991; Tronchere *et al.*, 1991), inhibition of signaling enzymes such as phosphatidylinositol 3-kinase (Berggren *et al.*, 1993), phosphatidylinositol specific phospholipase C (Powis *et al.*, 1992; Überall *et al.*, 1991), protein kinase C (Helfman *et al.*, 1983; Powis, 1991) and Raf-1 (Zhou *et al.*, 1996). It is worth bearing in mind that the mechanism of action of AELs may not be the same in different cell types; for example, AELs

do not induce apoptosis in all cell types (Houlihan *et al.*, 1995). Very few studies have correlated the effects of the drugs on specific molecules or metabolic processes with the inhibition of cell growth. Thus, it is unclear which are the primary sites of action and which ones are not. There is therefore a need to unequivocally establish the targets that are critical to the antineoplastic properties of AELs from those that are irrelevant.

1.1.5 Site of action of AELs

To establish the primary site of action of AELs in cells, knowledge of the cellular distribution of the compounds would be helpful. It is clear that AELs are directly absorbed and accumulate in cell membranes (Fleer *et al.*, 1992; Houlihan *et al.*, 1995; Kelly *et al.*, 1993). While the plasma membrane is widely believed to be the primary site of action of AELs (Berdel, 1991), there is strong indirect evidence that they are widely distributed intracellularly. There are suggestions that inhibition of lipid metabolism, particularly the synthesis of phosphatidylcholine (PC) is the mechanism via which AELs inhibit cell proliferation. For example, intracellular lipid-metabolising enzymes like CTP: phosphocholine cytidylyl transferase (CT) (Geilen *et al.*,1991; Haase *et al.*, 1991; Vogler *et al.*, 1996) are inhibited by AELs. This occurs by either direct inhibition of the enzyme or its translocation to membranes (Geilen *et al.*, 1992; Haase *et al.*, 1991; Tronchere *et al.*, 1991). However, for AELs to mediate their inhibitory effect on CT, the drugs have to be present in the nucleus, since CT is an intranuclear enzyme (Wang *et al.*, 1993; Watkins and Kent, 1992). ET-18-OCH_3 induced inhibition of phospholipid metabolism in MCF-7 and A549 cells seems to be due to the effect of ET-18-OCH_3 on enzymes that are present in cell compartments other than the

plasma membrane (Zhou and Arthur, 1995). The assessment of cellular distribution of AELs has been difficult because of the ability of the compounds to redistribute spontaneously within cellular compartments during the preparation of subcellular fractions (Vallari *et al.*, 1989). The resolution of this issue may lie in the development of fluorescent AEL analogs that would allow the subcellular distribution to be analyzed microscopically in intact cells.

1.2 AELs AND CELL SIGNAL TRANSDUCTION PATHWAYS

1.2.1 Introduction

Studies in our laboratory have demonstrated that inhibition of PC synthesis or reacylation of phospholipids by ET-18-OCH_3 is unlikely to be the cause of the cytotoxic effects of the compound in epithelial cancer cells (Lu and Arthur, 1992a; 1992b; Zhou and Arthur, 1995). Consequently, our focus in elucidating the mechanism of action of the compounds in epithelial cells has been on investigating their effects on signal transduction processes. In this section, I will therefore briefly review cell signaling pathways and the molecules participating in these pathways and discuss what is known about the impact of AELs on these molecules and their signaling pathways.

Cells have evolved many mechanisms that allow the binding of ligands to receptors on the outer surface of the plasma membrane to generate an intracellular signal. The activation of specific intracellular signal transduction pathways by extracellular signals depends on the mechanism by which the signal is transduced across the membrane. There are four basic mechanisms that transduce signals across the plasma membrane which are defined by the type of receptor interacting with the ligand. These include:

(1) Receptors with integral kinase activity: the most widely studied members of this group are those with tyrosine kinase activity. These receptor tyrosine kinases (RTKs) are single-pass transmembrane proteins with an extracellular N-terminal domain which serves as a ligand binding site, a transmembrane domain and a cytosolic C-terminal domain with the kinase activity. Upon ligand binding to the receptor the tyrosine kinase is activated resulting in the phosphorylation of specific Tyr residues on the receptors. This initiates events that transmit the signals intracellularly. Other than tyrosine kinase activities, receptors with associated tyrosine phosphatase (Hunter, 1996), guanylyl cyclase (Garbers and Lowe, 1994) and serine / threonine kinase activities (Kolodziejczyk and Hall, 1996) have also been described.

(2) G-protein coupled receptors: Binding of ligand to these receptors causes conformational changes that allow the association and activation of a G-protein. This in turn, activates an effector to generate a second messenger such as cyclic AMP (cAMP), cyclic GMP (cGMP), Ins 1, 4, 5-P_3, and diacylglycerol (DAG). The characteristic feature of these G-protein-coupled receptors is the presence of seven helical membrane-spanning domains (7-pass receptors).

(3) Single-pass receptors with no intrinsic enzymatic activities that associate with non-receptor tyrosine kinases: this family of receptors includes most of the cytokine receptors (Taniguchi, 1995). Dimerization of these receptors occurs upon ligand binding which leads to cross-phosphorylation and activation of the associated non-receptor tyrosine kinase. These phosphorylate the receptors at Tyr residues which allows the binding of signalling molecules to propagate the intracellular signal (Ihle, 1996; Taniguchi, 1995).

(4) Ligand-gated receptors: Binding of ligands to these receptors opens ion channels to allow selected ions to cross the membrane and generate intracellular signals.

Transduction of extracellular signals via the above mechanisms initiates intracellular signaling events that may result in cell division. Interruption or interference in the activation of the molecules that participate in these pathways would be expected to have a negative impact on cell proliferation. It is therefore not surprising that studies have been conducted to determine whether AELs interfere with the propagation of mitogenic signals. These studies will be reviewed in the following pages.

1.2.2 Effect of AELs on receptor activation

Studies on the effect of ET-18-OCH_3 on epidermal growth-factor (EGF) receptor interaction with its ligand were some of the first conducted to examine the effects of ET-18-OCH_3 on signaling molecules (Kosano and Takatani, 1988; 1989). Initial studies led to the suggestion that the ALP inhibits the interaction of EGF with its receptors (Kosano and Takatani, 1988), but a later study by the same authors which differentiated between internalized and surface bound ligands, led to the conclusion that ET-18-OCH_3 does not interfere with the binding of EGF to its receptor but rather it inhibits the internalization of these receptors in AEL-sensitive MCF-7 cells but not in AEL-insensitive BT-20 cell line (Kosano and Takatani, 1989). The suggestion that inhibition of EGF receptor internalization by ET-18-OCH_3 may play a role in the antiproliferative effect of the ALP (Kosano and Takatani, 1989) was not supported by a recent study which showed that even though ET-18-OCH_3 inhibits internalization of the EGF receptor, this occurred long after the inhibition of

the MAP kinase pathway by the ALP were observed (Zhou *et al.*, 1996). This suggests that the inhibition of internalization could not be responsible for growth inhibition. In Swiss 3T3 cells, ET-18-OCH_3 had little effect on the binding of PDGF to its receptor (Seewald *et al.*, 1990). Thus, it would appear that the antiproliferative effect of ET-18-OCH_3 is not due to its effect on the binding of ligands to RTKs or activation of these receptors (Zhou *et al.*, 1996).

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a cytokine that stimulates growth and differentiation of hemopoietic progenitor cells and some leukemic cells (Nicola, 1989). Incubation of leukemic cell lines with ET-18-OCH_3 for 3 h resulted in a dose dependent inhibition of GM-CSF binding to its receptor (Shoji *et al.*,1994). It was suggested that the inhibition of GM-CSF binding to its receptor could be due to membrane perturbations by the AEL that results in the masking of ligand binding sites on the receptors (Shoji *et al.*, 1994). What role, if any, these effects play in ALP-induced inhibition of proliferation of leukemic cells remains to be established, however, it is conceivable that a drastic reduction in levels of ligand binding could decrease the magnitude of the intracellular signal below that required for growth. As the GM-CSF receptor expression is limited to a few cell types, any growth-inhibiting effects of AELs that occur via this receptor will not account for the widespread growth-inhibitory effects of AELs.

Studies on the effect of AEL on the estrogen receptor-estradiol interaction showed that incubation of the estrogen positive breast adenocarcinoma MCF-7 cells with ET-18-OCH_3 for 12 hours decreased the estradiol uptake by 21% and inhibited cell growth, but ET-18-OCH_3 had no effect on the binding affinity of estradiol to its receptor in these cells

(Kosano *et al.*, 1990). As the estrogen receptors are intracellular, the reduced estrogen binding could be taken as evidence of a reduction in the receptor number by ET-18-OCH_3. In the same study, a 25% decrease of progesterone receptors was observed (Kosano *et al.*, 1990). Whether the moderate decrease in the number of estrogen and progesterone receptors leads to inhibition of growth is not known.

1.2.3 AELs and second messengers derived from phospholipids

The binding of growth factors and mitogens to their receptors leads to the activation of intracellular signaling pathways that regulate cell proliferation. It is now well established that a number of major intracellular signaling pathways are activated as a result of the production of lipid-derived second messengers. Second messengers produced from phospholipids include DAG, IP_3, phosphatidylinositol 3,4,5 triphosphate (PIP_3), lysophosphatidic acid (LPA), platelet - activating factor (PAF), fatty acids (eg. arachidonic acid), ceramide and sphingosine (Divecha and Irvine, 1995; Liscovitch and Cantley, 1994). The lipid-activating signaling pathways that have been implicated in the mechanism of action of AELs include protein kinase C (PKC), IP_3 (Ca^{2+}) and PIP_3 signaling pathways. Protein kinase C is activated in response to DAG production which may be derived from PIP_2 or PC, while IP_3 is produced from PIP_2 hydrolysis **(Fig 2)**. In the following sections, the production of these second messengers and the effects of AELs on the production and activation of the signaling pathways they activate is discussed.

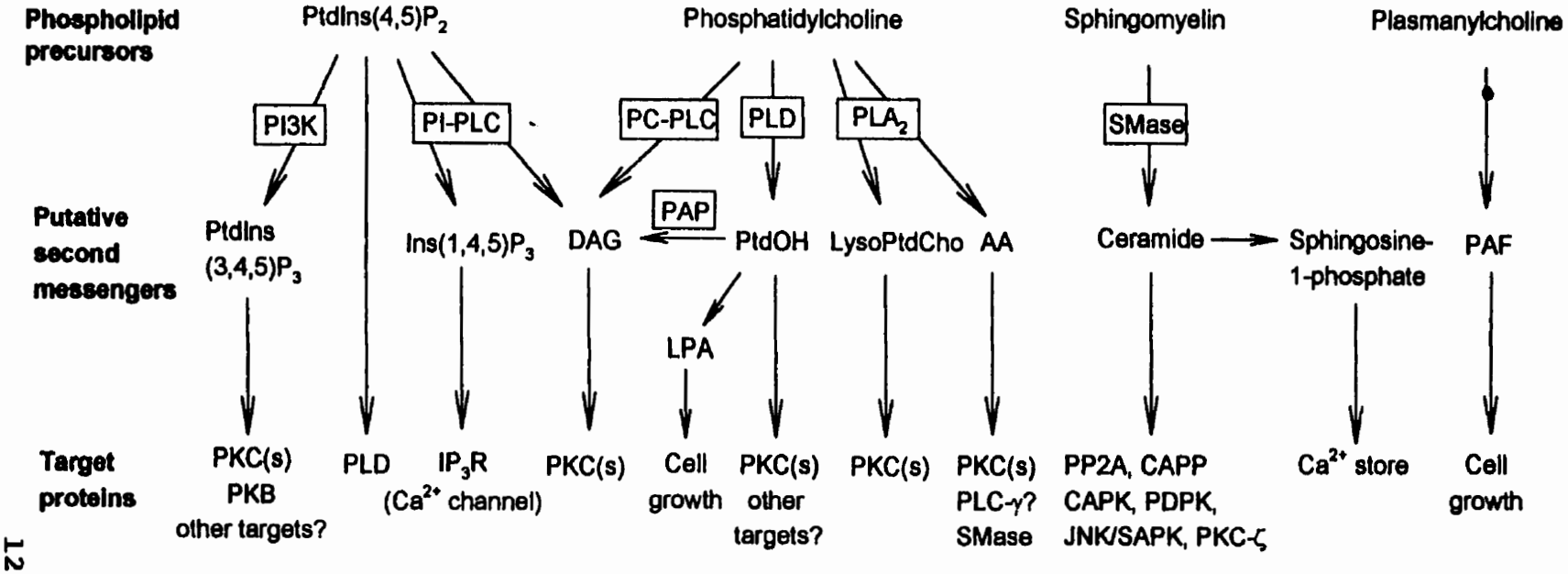


Figure 2. Lipid and lipid-derived signaling molecules and their signaling pathways. Phosphatidylinositol (4,5)bisphosphate (Ptd(4,5)P_2), phosphatidylcholine, sphingomyelin and plasmanylcholine function as major precursors of well-known and putative second messengers. These second messengers then induce diverse cellular responses by mediating functions of their target proteins. AA, arachidonic acid; CAPK, ceramide-activated protein kinase; CAPP, ceramide-activated protein phosphatase; DAG, diacylglycerol; Ins(1,4,5)P_3, inositol (1,4,5)triphosphate; IP_3R, the receptor of Ins(1,4,5)P_3; JNK/SAPK, c-Jun NH_2- terminal kinase/stress-activated protein kinase; lysoPtdCho, lysophosphatidylcholine; PAF, platelet-activating factor; PAP, phosphatidic acid phosphohydrolase ; PKB/C, protein kinase B or C; PC-PLC, phosphatidylcholine-specific PLC; PDPK, proline-directed protein kinase; PI3-kinase, phosphoinositide 3-kinase; PI-PLC, phosphoinositide-specific PLC; PLA_2, phospholipase A_2; PLCγ, phospholipase Cγ isoform; PLD, phospholipase D; PP2A, type-2A protein phosphatase; PtdIns(3,4,5)P_3, phosphatidylinositol (3,4,5)triphosphate; PtdOH, phosphatidic acid; SM, sphingomyelin; SMase, sphingomyelinase. (Modified from Liscovitch and Cantley, 1994).

1.2.3.1 AELS and IP_3 and DAG production from PIP_2

Although phosphoinositides make up only a small fraction of the total phospholipid mass, at least three second messengers, IP_3, DAG and PIP_3 , are generated from PIP_2 (Fig 2). A wide variety of agonists, such as neurotransmitters, hormones and growth factors that activate G-protein coupled receptors, receptor tyrosine kinases or non-receptor tyrosine kinases of the Src family (fyn, lck), cause the hydrolysis of PIP_2 by activating phosphoinositide-specific phospholipases C (PI-PLC) (Berridge, 1993; Liscovitch and Cantley, 1994). Both products of this reaction are second messengers; DAG activates PKC whereas IP_3 mobilizes intracellular Ca^{2+} (Berridge, 1993; Liscovitch and Cantley, 1994).

A number of cellular processes such as cell proliferation, cell transformation, fertilization, gametogenesis, secretion, smooth muscle contraction, sensory perception and neuronal signalling may be regulated by IP_3, DAG and other inositol lipids (Bansal and Majerus, 1990; Berridge, 1993; Divecha and Irvine, 1995; Downes and Macphee, 1990).

Evidence that inositol lipid-derived messengers play a role in cell proliferation includes the requirement of PIP_2 hydrolysis in the proliferation of some (Uno *et al.*, 1988), but not all cells (Cuadrado and Molley, 1990; Margolis *et al.*, 1990; Mohammadi *et al.*, 1992); the association of Xenopus embryonic cell mitosis with IP_3-triggered calcium oscillation (Berridge, 1993); the presence of the inositide cycle and the β isozyme of PI-PLC in the nucleus (Divecha *et al.*, 1993; Martelli *et al.*, 1992; Michell, 1992); the dramatic increase in the level of nuclear calcium upon mitogenic stimulation (Stricker *et al.*, 1992; Yamada *et al.*, 1991); the cell cycle related transient changes in the intracellular calcium level (Ciapa *et al.*, 1994; Hepler, 1992; Wahl and Gruenstein, 1993); and ability of the calcium to

activate mitogen activated protein (MAP) kinase (Chao *et al.*, 1992).

PI-PLC, the enzyme responsible for hydrolysis of PIP_2, can be classified into three main groups, β, γ and δ, with each group having more than one isoform (Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Rhee and Bae, 1997). So far, all the PI-PLC isoforms isolated from mammalian tissues are capable of hydrolysing PI, PIP and PIP_2, but not 3-phosphorylated inositol phospholipids (Nishizuka, 1995; Rhee and Choi, 1992). PLC-β is a membrane associated protein and activation of PLC-β isozymes (β_1, β_2 and β_3) is selectively mediated by βγ subunits of G_i or by the α subunit of G_q subsequent to receptor activation (Blank *et al.*, 1993; Camps *et al.*, 1992; Katz *et al.*, 1992; Noh *et al.*, 1995; Smrcka *et al.*, 1991; Taylor *et al.*, 1991). Activation of PLC-β by G_i or G_q is responsible for pertussis toxin-sensitive or -insensitive activation of PIP_2 hydrolysis, respectively. PLC-γ (1or 2) is found in the cytosol in resting cells but is translocated to the membrane by associating with activated RTK via their SH2 domains. PLC-γ is also activated by direct phosphorylation on tyrosine residues by the RTK or receptor associated tyrosine kinases (Cantley *et al.*, 1991; Meisenhelder *et al.*, 1989). Little is known about the mechanism of activation and regulation of PLC-δ, but they exhibit enzymatic activity in response to G-protein activation and Ca^{2+} mobilization (Banno *et al.*, 1994). A novel protein, p122 has been discovered that stimulates PLC-δ activity (Homma and Emori, 1995).

1.2.3.2 Effect of AELs on PI-PLC

The importance of PIP_2 hydrolysis in cell mitogenesis (Noh *et al.*, 1995; Smith *et al.*, 1989; 1990) has led to studies to determine whether AELs inhibit cell proliferation by

inhibiting PI-PLC. It was reported that ET-18-OCH_3 inhibited PDGF or AlF_4-induced IP_3 formation in Swiss 3T3 cells without interfering with the interaction between PDGF and its receptors (Seewald *et al.*, 1990). ET-18-OCH_3 was also able to inhibit PI-PLC activity when added directly to the cytosolic or membrane fraction prepared from Swiss 3T3 and BGI (ovarian carcinoma) cells (Powis *et al.*, 1992). HePC also inhibited the formation of IP_3 in bombesin-stimulated NIH 3T3 cells (Üherall *et al.*, 1991). The observed inhibition of PI-PLC by AELs in cells suggested that this could contribute to cell growth inhibition. However, in these studies no correlation was established between the decreased level of IP_3 formation and decreased cell growth. The role of AEL-induced inhibition of PI-PLC activity in decreased cell growth was questioned when it was observed that lyso-PAF, which is not a cytotoxic agent also inhibited PI-PLC as effectively as cytotoxic AELs in the same cells (Houlihan *et al.*, 1995; Powis *et al.*, 1992).

When the human mammary epithelial cell line H184 was transformed with the *v-erbB* oncogene, the transformed cells were more sensitive to HePC, equally sensitive to ET-18-OCH_3, and less sensitive to hexadecylphosphono (N-acetyl)-L-serine, relative to the parental line (Junghahn *et al.*, 1995). Preincubation of the cells with HePC for 24 hours followed by bombesin stimulation, resulted in a 90% inhibition of IP_3 formation in a transformed cells and a 60% inhibition in the parental cells. Similar studies with ET-18-OCH_3 resulted in 64% inhibition of IP_3 formation in both transformed and parental cell lines. On the other hand, hexadecylphosphono (N-acetyl)-L-serine inhibited IP_3 formation less than 10% in normal cells and by 65% in the transformed cells. These observations clearly dissociate the cell growth inhibitory effects of the AELs from their effect on IP_3 formation (Junghahn *et al.*, 1995).

As the role of PLC-δ in mitogenesis is not fully understood, the relevance of cell growth inhibition and the observed inhibition of purified PI-PLCδ by HePC is unclear (Pawelczyk and Lowenstein, 1993).

1.2.3.3 AELs and DAG production from PC

Hydrolysis of inositol phospholipids by activated PI-PLC results in a rapid and transient elevation in the level of DAG which is often followed by a slow, sustained DAG production in response to growth factors, cytokines and phorbol esters (Exton, 1994). Sometimes, sustained DAG production occurs alone in response to the mitogenic stimuli (Nishizuka, 1995). The sustained production of DAG is thought to be due to hydrolysis of PC by PC-PLC or the hydrolysis of PC by phospholipase D (PLD) followed by phosphatidic acid phosphohydrolase (PAP) action on the resultant phosphatidic acid (Exton, 1990; 1994; Nishizuka, 1995). The sustained DAG production maintains PKC activation and is believed to be important for cell growth and cell differentiation (Nishizuka, 1995). There are multiple mechanisms for the activation of receptor-coupled PLD (Exton, 1997). These include: 1) PKC-dependant activation; 2) ADP-ribosylation factor-dependent activation; 3) G protein-linked activation (heterotrimeric and small mwt G protein Rho-dependent activation and; 4) RTK or receptor-associated tyrosine kinase-dependent activation (Dennis *et al.*, 1991; Exton 1997; Thompson *et al.*, 1993).

AELs have little effect on the PC-PLC activity in NIH 3T3 cell membranes (Powis et al., 1992). With respect to PLD, concentrations of ET-18-OCH_3 below those cytotoxic to NIH 3T3 cells, had no effect on PLD activity in membranes prepared from these cells. In

intact cells, however, preincubation with high concentrations of ET-18-OCH_3 resulted in a slight activation of PLD activity (Powis *et al.*, 1992). Detergent solubilized rat brain PLD was not affected by ET-18-OCH_3 at concentrations of up to 400 μM (Gratas and Powis, 1993). It has also been reported that both HePC and ET-18-OCH_3 activated PLD in CCD-986-SK (normal mammary fibroblast cell line) when incubated with 30-40 μM of drug. Since AELs have either no effect, or activate PC-PLC or PC-PLD activity, it would appear that effects of AELs on these enzymes are unlikely to inhibit DAG production in cells for PKC activation or in the case of PLD, the production of PA which may itself be a second messenger (Berridge, 1993; Liscovitch, 1996).

1.2.3.4 AELs and protein kinase C activation

Protein kinase C (PKC) is a large family of Ser/Thr kinases involved in many diverse and critical cellular functions including the control of cell growth. Based on their sequence homology and cofactor requirements, at least twelve isozymes of PKC have been discovered and grouped into 3 classes: the classical (cPKC), novel (nPKC) and atypical (aPKC) isozymes (Dekker and Parker, 1994; Newton, 1995; Nishizuka, 1995). The cPKC are activated by Ca^{2+}, DAG, phorbol esters and PS or PE and include the α, βI / βII and γ isozymes. The nPKC are Ca^{2+}-independent but can be activated by DAG, phorbol esters and PS and include the δ, ϵ, η and θ isozymes. The aPKC isozymes, ζ, λ, and ι are not activated by Ca^{2+}, DAG or phorbol esters. Expression of different PKC isoforms varies between tissues and cell types (Asaoka *et al.*, 1992; Hug and Sarre, 1993; Nishizuka, 1995), indicating that different isoforms may have distinct cellular functions depending on the tissue and cell type. For

example, PKC-βII is required for human erythroleukemia (K562) cell proliferation (Murray *et al.*, 1993) whereas activation of PKC-ζ is necessary and sufficient for mitogenic activation in oocytes and NIH 3T3 cells (Berra *et al.*, 1993; Dominguez *et al.*, 1992).

Several different signalling pathways may be involved in PKC-mediated cell growth. There is evidence that activation of PKC leads to the activation of the MAP kinase pathway as a consequence of phosphorylation of Raf-1(Cai *et al.*, 1997; Carroll and May, 1994; Kolch *et al.*, 1993; Sozeri *et al.*, 1992; Ueda *et al.*, 1996). PKC may also be involved in the activation of the MAP kinase cascade by G-protein coupled receptors (Malarkey *et al.*, 1995; van Biesen *et al.*, 1996). Another signaling pathway leading to transcriptional activation that is modulated by PKC is the NFκB pathway. In quiescent cells NFκB is found in the cytosol in an inactive complex with the inhibitor IκB. Phosphorylation of IκB by PKC leads to its degradation and dissociation from NFκB which translocates to the nucleus to initiate transcription (Liou and Baltimore, 1993; Lozano *et al.*, 1994; Thanos and Maniatis, 1995).

Additional evidence of a role for PKC in cell proliferation comes from reports that activation of PKC α, δ, ϵ results in the activation of $P34^{cdc2}$ histone H1 kinase and mitogenesis in Swiss 3T3 cells (Takuwa *et al.*, 1992). PKC is also known to phosphorylate a host of nuclear proteins including nuclear cytoskeletal proteins, and proteins required for DNA repair and transcription (Buchner, 1995).

This involvement of PKC in cellular mitogenic signalling processes has led to the investigation of the effect of AELs on PKC and cell growth inhibition. Earlier studies demonstrated that several AELs directly inhibit PKC activity *in vitro* (Berkovic *et al.*, 1992; 1994; Chambers *et al.*, 1992; Geilen *et al.*, 1991; Helfman *et al.*, 1983; Shoji *et al.*, 1988;

Überall *et al.*, 1991; Zheng *et al.*, 1990). However, there is a report that ET-18-OCH_3 and HePC activate PKC instead of inhibiting it (Heesbeen *et al.*, 1991). These contradictory observations appear to be due to differences in assay protocol. Activation was observed when the AEL was incorporated into the membranes prior to their isolation and utilization as a source of enzyme, whereas inhibition was observed if the AEL was presented in mixed micelles with the substrate. It has been suggested that because of the amphiphilic detergent nature of AELs, they can activate or inhibit PKC activity depending on their effect on the lipid bilayer stability of the membrane which would depend on factors, such as AEL concentrations and other assay conditions (Houlihan *et al.*, 1995).

Any role of PKC in the mechanism of action of AELs has to be demonstrated in intact cells. Studies with intact NIH 3T3 cells demonstrated that HePC was able to inhibit the PKC-mediated Na^+/H^+ antiport (Überall *et al.*, 1991). In HL-60 cells, ET-18-OCH_3 inhibited tetradecanoyl phorbol 13-acetate (TPA)-induced cell differentiation in an additive manner with H-7, a PKC inhibitor (Shoji *et al.*, 1991). As H-7 is not a very specific inhibitor of PKC, the effects observed may not be due entirely to PKC-related events. ET-18-OCH_3 also inhibited-TPA induced activation of NF-kB but not TNFα or IL-1-induced NF-kB activation (Daniel *et al.*, 1995). These findings provided indirect evidence that AELs inhibit PKC activity in intact cells.

In contrast, there is indirect evidence that both HePC and ET-18-OCH_3 activate PKC in CCD-986-SK cells. This was based on the observation that the AELs activate PLD, whose activation had previously been established to be due to PKC activation (Wieder *et al.*, 1996). Other evidence against the involvement of PKC in the mechanism of action of AELs

include reports that a 20% - 30% inhibition of PKC was observed in both AEL-sensitive (HL - 60, WEH1-3B) cell lines and AEL-insensitive (K562, R6X - B15) cell lines (Berkovic *et al.*, 1994; Salari *et al.*, 1992). If confirmed, these observations would suggest that the ability of AELs to inhibit cell growth may not be related to their ability to inhibit the activation of PKC in cells.

The effect of AELs on PKC activation in cells has been examined by another approach that involved monitoring the phosphorylation of endogenous proteins by PKC in the presence and absence of the AEL. These studies revealed that phosphorylation of endogenous proteins by PKC in TPA- or DAG-stimulated cells was completely inhibited under conditions where preincubation with ET-18-OCH_3 inhibited MCF-7 cell growth (Zhou and Arthur, 1997). Thus inhibition of PKC by ET-18-OCH_3 might have some contribution in the inhibition of MCF-7 cell growth. In leukemic HL-60 cells, however, inhibition of PKC induced phosphorylation of endogenous proteins by ET-18-OCH_3 only occurred following stimulation with low concentrations (20 nM) of TPA but not with high concentrations (200 nM) of TPA (Kiss *et al.*, 1987). In the studies with MCF-7, cells inhibition of PKC-induced phosphorylation by ET-18-OCH_3 was observed in cells stimulated with 1 μM TPA (Zhou and Arthur, 1997). It is possible that these contrasting results in MCF-7 and HL-60 cells may be due to differences in the mechanisms of action of ET-18-OCH_3 in different cell types (Lu *et al.*, 1993; 1994; Zhou *et al.*, 1992).

1.2.3.5 Effect of AELs on intracellular calcium concentrations

Ca^{2+} plays an important role in cell signalling. An increase in the level of intracellular

Ca^{2+} has been related to various cell functions including the induction of cell death (Orrenius *et al.*, 1989). Mechanisms for the increase in the level of intracellular Ca^{2+} include the release of Ca^{2+} from intracellular stores, as a result of IP_3 production from PIP_2 hydrolysis (see above), or an influx from extracellular sources through Ca^{2+} channels.

In Swiss 3T3 cells, ET-18-OCH_3 inhibited the PDGF-, bradykynin- and vasopressin-induced increase in intracellular Ca^{2+}, partly by inhibiting PI-PLC activation and partly by direct inhibition of IP_3 induced Ca^{2+} release (Seewald *et al.*, 1990). Similar effects were observed with ET-16S-OC_2H_5, SRI62-834 and HePC (Überall *et al.*, 1991). On the other hand, others observed that AELs increase the intracellular Ca^{2+} levels independent of stimulation (Lazenby *et al.*, 1990; Lohmeyer and Workman, 1993; Palmblad *et al.*, 1990; Thompson and Hickman, 1988) with an initial transient increase followed by a sustained increase with increasing concentrations of drug. As high intracellular Ca^{2+} level is also known to induce cell death, it was suggested that the sustained increase in Ca^{2+} by AELs was the mechanism for the cytotoxic action of AELs (Lazenby *et al.*, 1990). Others argued that the sustained increase in intracellular Ca^{2+} by AELs is due to disruption of the cell membrane and is not the mechanism underlying the inhibitory effects of low concentrations of AELs (Lohmeyer and Workman, 1993). Recent studies confirm that the AEL induced cytotoxicity is independent of the level of intracellular Ca^{2+}. In the hybrid NH15-CA2 neuroblastoma x glioma cell line, experiments were conducted under conditions where membrane lysis by AEL could be ruled out. In these circumstances, when ET-18-OCH_3-induced Ca^{2+} influx was inhibited, the AEL-induced cell death was not prevented or inhibited. This suggested that the mechanism of cell death by AELs in the cells was independent of AEL-induced increase in

intracellular Ca^{2+} levels (Brinkmeier *et al.*, 1996).

1.2.3.6 Phosphoinositide 3-OH kinases

PI 3-Kinases are a family of enzymes that catalyse the phosphorylation of inositol lipids at the D-3 position of the inositol moiety (Stephens, 1995). There are three classes of PI 3-kinases. The class I PI 3-kinases are stimulated by extracellular ligands and have been implicated in cell proliferation. Not much is known about the regulation or physiological role of the class II enzymes (Vanhaesebroeck *et al.*, 1997) whereas the class III enzymes are agonist-insensitive and are responsible for the constitutive production of PI 3-P that may be involved in vesicular transport (Schu *et al.*, 1993; Vanhaesebroeck *et al.*, 1997). The most widely studied enzymes are the class I enzymes and of these, the best known is the heterodimer consisting of an 85 kDa subunit (p85) and 110 kDa subunit (p110). The p85 is the regulatory subunit and mediates the enzyme association with a variety of receptor tyrosine kinases or adaptor proteins (eg IRS-1) through its SH2 domain. The p110 is the catalytic subunit. Activation of the enzyme results in the phosphorylation of PIP_2 to produce PIP_3, a putative second messenger (Divecha and Irvine, 1995; Kapellar and Cantley, 1994; Stephens, 1995). An interesting feature of this enzyme is that it also possesses an intrinsic protein kinase activity and is able to phosphorylate the p85 regulatory subunit on Ser 608 (Dhand *et al.*, 1994) and IRS-1 on Ser residues, but the consequence of this is not yet established (Lam *et al.*, 1994). Recent studies indicate that PIP_3 produced by PI 3-kinase binds to the pleckstrin homology (PH) domain of protein kinase B (PKB, also known as Akt) and its activating kinase causing their translocation to the membrane and facilitating the

phosphorylation of PKB by the activating kinase (Burgering and Coffer, 1995; Didichenko *et al.*, 1996; Franke *et al.*, 1995). A signaling pathway from activated receptors to PI 3-kinase and sequential activation of PKB, $p70^{S6K}$ and cell proliferation is beginning to emerge (Cheatham *et al.*, 1994; Downward, 1995a,b). 3-Phosphorylated inositol lipids have also been observed to activate PKC δ, ϵ, ζ ,η isoforms (Nakanishi *et al.*, 1993). The role of this PKC phosphorylation in the regulation of cell proliferation is unclear.

ET-18-OCH_3, HePC and SRI-62 834 reportedly inhibited PI 3-kinase activity *in vitro* and ET-18-OCH_3 inhibition was non-competitive with ATP (Berggeren *et al.*, 1993). Preincubation of NIH 3T3 cells with ET-18-OCH_3 inhibited the cellular levels of PIP_3 subsequent to PDGF stimulation (Berggren *et al.*, 1993). Although observations from the above studies suggest that AELs inhibited the activity of PI 3-kinases in intact cells, a correlation between the observed decrease in activity of PI 3-kinase and inhibition of proliferation upon AEL treatment has yet to be established.

AELs do not appear to affect the activity of other phosphoinositide kinases such as PI 4- and 5-kinases whose activity results in the synthesis of PIP_2. Thus, ET-18-OCH_3 had no effect on the levels of PIP and PIP_2 in NIH 3T3 cells after the cells were treated with ET-18-OCH_3 for 2 h (Überall *et al.*, 1991). In Raji cells, D-*myo*-inositol transport was severely inhibited by HePC, but no effect on the incorporation of inositol into the membrane lipids was observed, suggesting that there was no AEL induced inhibition of PI 4- and 5-kinases (Berkovic *et al.*, 1992).

1.2.4 AEL and inhibition of signaling via the mitogen-activated protein (MAP) kinase cascade

The MAP kinase cascade is a major signaling pathway for mitogenesis in a wide variety of cell types (Blumer and Johnson, 1994; Nishida and Gotoh, 1993). The MAP kinase pathway is involved in transmitting signals from RTKs, non-receptor protein tyrosine kinases and G-protein coupled receptors (Inglese *et al.*, 1995; Lopez-Ilasaca *et al.*, 1997) through the cytosol into the nucleus (Davis, 1995; Hill and Treisman, 1995). In this pathway, signals travel stepwise from receptors -> Grb2 - Sos -> Ras -> Raf-1 (MAP kinase kinase kinase) -> MEK (MAP kinase kinase -> MAP kinase (ERK, extracellular signal - regulated protein kinase) **(Fig 3)**. Activated MAP kinase then phosphorylates various cytosolic and nuclear substrates, which eventually leads to cellular responses including cell proliferation (Pagès *et al.*, 1993; Troppmair *et al.*, 1994) or cell differentiation (Crompton and Gilmour, 1996; Ferrel, 1996). Recent studies in our lab have suggested that ET-18-OCH_3, the prototypic ALP may inhibit cell proliferation by inhibiting the transduction of growth signals via the MAP kinase pathway. Since my studies are an extension of this finding, I will discuss the molecules participating in the MAP kinase pathway and what is known about the effect of AELs on their activation in the following sections.

1.2.4.1 EGF receptor

Signaling via the MAP kinase pathway from RTKs begins with the binding of the ligand to the receptor. As most of the research that I conducted utilized EGF as the ligand to stimulate the cells, the focus of the background review will be on signaling from RTKs

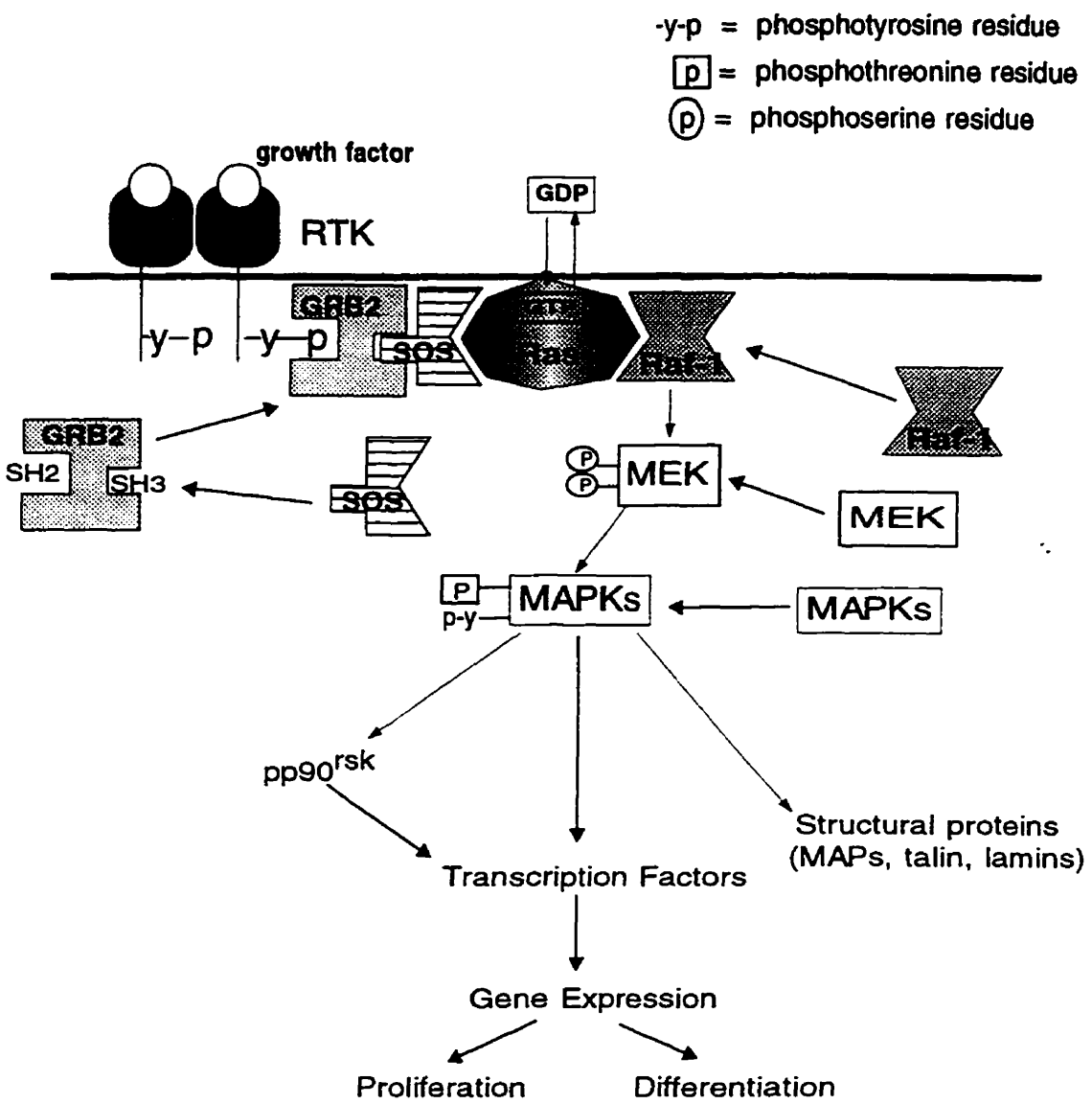


Figure 3. The Ras/Raf/MEK/ERK pathway. Upon EGF binding to receptors, the receptors undergo dimerization and tyrosine phosphorylation. An adaptor protein Grb2 binds to the phosphotyrosine residues via its SH2 domain. The SH3 domain of Grb2 binds to the C-terminal proline-rich sequence of Sos and recruites Sos to the plasma membrane. Subsequently, Sos activates Ras by unloading GDP from Ras followed by spontaneous association of GTP. The active GTP-bound Ras then interacts with Raf-1 and translocates Raf-1 to the membrane where it is activated by an unknown mechanism. After activation, Raf-1 phosphorylates and activates a dual specificity kinase MEK, which in turn activates MAPK (ERK) by phosphorylating its adjacent threonine and tyrosine residues. Activated ERK phosphorylates cytosolic substrates, cytoskeletal elements, and enters the nucleus to phosphorylate nuclear transcription factors, ultimately leading to cell proliferation or differentiation.

and from the EGF receptor in particular.

The EGF receptor is a 170 kDa polypeptide. It contains an extracellular EGF binding domain, a single hydrophobic transmembrane domain and a cytosolic tyrosine kinase domain (Cumming, *et al.* 1985). EGF binding to the extracellular domain of this receptor results in the dimerization of the receptor and activation of its tyrosine kinase activity. This results in transphosphorylation of specific Tyr residues on the EGF receptor (Panayotou and Waterfield, 1993) that provides binding sites for the downstream signaling molecules. For example, phosphorylation on Tyr 1068 and Tyr 1086 serve as binding sites for Grb2 (Buday and Downward, 1993; Li *et al.*, 1993; Okutani *et al.*, 1994; Songyang *et al.*, 1993); Tyr 1148 and Tyr 1173 for Shc (Okabayashi *et al.*, 1994) and Tyr 992 for PLC-γ (Rotin *et al.*, 1992; Songyang *et al.*, 1993).

After activation, EGF bound receptors are internalized by endocytosis. The endocytosis is mediated by receptor- associated adaptins and occurs in clathrin-coated pits (Nesterov *et al.*, 1995; Sorkin and Carpenter, 1993; Sorkin and Waters, 1993). This association of adaptin to receptors is dependent on the phosphorylation or activation of receptors (Nesterov *et al.*, 1995) and occurs in intact cells before the formation of fully assembled clathrin-coated pits (Sorkin and Carpenter, 1993). Clathrin-mediated endocytosis is not only important in attenuating EGF receptor signaling, but is also important in establishing and controlling EGF receptor specific signalling pathways (Vieira *et al.*, 1997).

As was discussed in **section 1.2.2**, AELs do not appear to inhibit either the affinity of EGF for its receptor or the activation of the receptor. Furthermore, the inhibition of internalization observed following incubation of cells with ET-18-OCH_3 is unlikely to be

responsible for inhibition of cell proliferation (Zhou *et al.*, 1996).

1.2.4.2 AEL and signal transduction from RTK to Ras

The activation of the MAP kinase pathway following the activation of the EGF receptor requires the activation of Ras. Signal transduction from the receptor to Ras is mediated by the Grb2 and Sos (Son of sevenless) complex.

Grb2 is a 25 kDa protein with one *src* homology 2 (SH2) domain and two SH3 domains. SH2 domains bind to specific phosphorylated tyrosines on proteins. Grb2 relays signals from the EGF receptor by either binding directly to the receptor or binding indirectly to other SH2 containing proteins (eg Shc, Syp and PTPα) that associate with the EGF receptor. The binding sites for Grb2 on EGF receptor are either Tyr-1068 or Tyr-1086; Tyr-1148 on Shc (Okutani *et al.*, 1994); Tyr-542 on Syp (Bennet *et al.*, 1994; Li *et al.*, 1994) and Tyr-789 on PTPα (den Hertog *et al.*, 1994). The SH3 domain of Grb2 binds to a guanine nucleotide exchange factor (GNEF), Sos, which is required to promote the release of guanosine diphosphate (GDP) from the membrane-bound inactive Ras-GDP complex. This promotion of GDP release facilitates binding of GTP, which is the predominant guanine nucleotide in the cytosol (Downward, 1992), to Ras to form an active Ras-GTP complex. There are two distinct mammalian forms of Sos, Sos 1 and Sos 2 (Bowtell *et al.*, 1992; Chardin *et al.*, 1993). A proline-rich sequence at the carboxy terminus is found on both Sos 1 and Sos 2, which binds to the SH3 domain of Grb2 (Yang *et al.*, 1995). Grb2 therefore acts to bring Sos to its target, the membrane-associated inactive Ras-GDP, where Sos exchanges the GDP on Ras for GTP, leading to the activation of Ras and Ras-mediated signaling pathways. Sos derivatives engineered to contain either a farnesylation or

myristoylation site are sufficient for Ras activation (Aronhiem *et al.*, 1994). This strongly suggests that the membrane recruitment of Sos upon binding to the SH3 domain of receptor-bound Grb 2 is the primary mechanism for Ras activation.

As far as I am aware, there have been no studies to directly examine the effects of AELs on the association of Grb2 with RTKs or Sos with Grb2. However, the report that preincubation of MCF-7 cells under conditions that inhibited cell proliferation had no effect on EGF-stimulated Ras activation suggests that ET-18-OCH_3 had no effect on the receptor/Grb2/Sos interactions required to activate Ras (Zhou *et al.*, 1996).

1.2.4.3 AELs and Ras activation

Ras proteins, $p21^{ras}$, are monomeric small molecular weight G-proteins with a high affinity for guanine nucleotides. When Ras is complexed with GTP, it acquires its active conformation whereas the complex with GDP produces the inactive form (Bourne *et al.*, 1990; 1991). Ras is synthesized as a pro-molecule that undergoes post-translational modifications at the C-terminus to form the final membrane-associated molecule. The most critical modification is the attachment of the isoprenoid farnesol to the Cys residue of the C-terminal CAAX box (C = Cys, A = Aliphatic amino acid, X = another amino acid) which targets Ras to the membrane. Other modifications occur following the attachment of the farnesol group; these include proteolytic cleavage of the AAX residues, carboxyl methylation of the farnesylated Cys and palmitoylation of Cys upstream of the CAAX motif. These increase the membrane affinity and biological activity of Ras (Gibbs *et al.*, 1994; Lowy and Willumsen , 1993).

Mutations in either the phosphoryl group or the purine ring-binding site regions on *ras* protooncogene results in *ras* oncogenes whose products are constitutively active and are found in a wide variety of human cancers (Barbacid, 1987). It is estimated that 30% of all human cancers contain mutated ras oncogenes (Marshall, 1993).

Ras has a very low intrinsic GTPase activity and in order to convert from the active to the inactive conformation it requires a GTPase-Activating Protein (GAP) to accelerate its intrinsic GTPase activity (Marshall, 1993). One of the highly Ras-specific GAP proteins is GAP1, a 120 kDa cytosolic protein that contains one SH3 and two SH2 domains (Trahey and McCormick, 1987). GAP1 forms a complex with activated receptors through its SH2 domain and is therefore recruited to the membrane where it interacts with Ras (Downward, 1992). It has been reported that membrane targeting of GAP1 by Ras-CAAX enabled GAP1 to act as a supressor of Ras function (Huang *et al.*, 1993).

Following its activation, Ras interacts with, and activates a number of effectors. The best characterised effector of Ras is the Ser/Thr protein kinase Raf-1 (Marshall, 1994). The N-terminal regulatory region of Raf - 1 binds to the effector domain of the activated Ras-GTP complex (Chuang *et al.*, 1994; Ghosh *et al.*, 1994; Hu C-D *et al.*, 1995; Zhang *et al.*, 1993). This binding to Ras translocates Raf-1 to the membrane, and is one of the key steps of Raf-1 activation. Other potential effectors of Ras include PI 3-kinases (Rodriguez-Viciana *et al.*, 1994), GAP1 (Boguski and McCormick, 1993; Polakis and McCormick, 1993) and Jun N-terminal kinase (JNK) (Adler *et al.*, 1995). Unfortunately little is known about the Ras mediated activation of these effectors.

As far as I am aware there has been only one study that examined the effect of AELs

on Ras activation (Zhou *et al.*, 1996). The results showed no effect of ET-18-OCH_3 on the levels of GTP-bound Ras relative to controls, indicating that there was no effect of AEL on Ras activation.

1.2.4.4 AELs and Raf activation

Raf proteins are members of the MAP kinase kinase kinase (MAPKKK) family and the initial kinases of the MAP kinase cascade. Raf proteins serve as a central intermediate in many of the developmental and proliferative signalling pathways, functioning to connect upstream tyrosine kinases and Ras with downstream Ser/Thr kinases (Avruch *et al.*, 1994). These Ser/Thr protein kinases are evolutionarily highly conserved and essential for growth and development in worms, flies, frogs and mammals (Daum *et al.*, 1994). A single functional Raf gene has been identified in Drosophila and in Caenorhabditis elegans, which encodes D-Raf and Ce-Raf (Brunner *et al.*, 1994; Han *et al.*, 1993) respectively. Three genes, namely *Raf-1, B-Raf and A-Raf* were described in vertebrates (Heidecker *et al.*, 1990). These Raf genes were mapped to three different chromosomes and located at sites that are frequently altered in human tumors (Sithanadam *et al.*, 1989; Storm *et al.*, 1990a; 1990b). The A-Raf protein is abundantly expressed in urogenital tissues, B-Raf in testes and cerebrum and c-Raf-1, a 74 KDA protein is ubiquitously expressed at variable levels (Storm, 1990b).

All Raf proteins share three highly conserved structurally homologous domains designated as conserved region 1(CR1), CR2 and CR3 **(Fig 4)**. Raf proteins can also be divided into two functional domains: 1) The N-terminal, regulatory domain, comprising the first 300 amino acids including CR1 and CR2 and; 2) the C-terminal kinase domain that

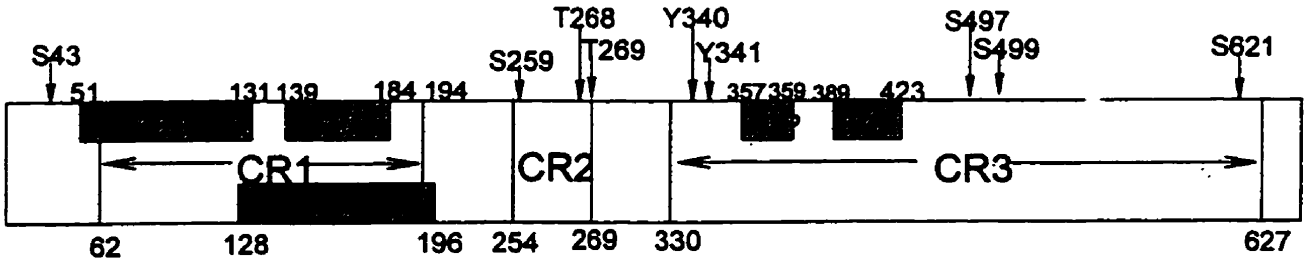


Figure 4. Structure of mammalian c-Raf-1. The figure shows the functional regions of Raf and amino acid residue numbers. CR1 (residues 62-196), CR2 (residues 254-269) and CR3 (residues 330-627). The conserved cysteine-rich domain (CRD) encompasses a zinc finger motif. Phosphatidylserine(PS)-binding site in CR1 (residues 128-196), phosphatidic acid (PA)-binding site is in CR3 (residues 389-423). ATP-binding site is in CR3 (residues 357-359). Sites of amino acid phosphorylation are shown using single-letter amino acid code. Substrate-binding domain in CR3 includes S497 and S499. (Modified from Morrison and Cutler, 1997).

contains CR3 (Daum *et al.*, 1994). The regulatory domain functions to suppress the catalytic activity of Raf-1. CR1 contains a Ras binding domain (RBD) (residues 51-131) and an adjacent zinc coordination site ($^{152}CX_2CX_9CX_2CX_7CX_7{}^{184}$) in the conserved cysteine-rich domain (CRD, residues 139-184), which has also been identified to be a second Ras binding site (Mott *et al.*, 1996). CR2 is rich in serine and threonine residues and is a site of regulatory phosphorylation and association with the 14-3-3 protein (Muslin *et al.*, 1996). CR3, the catalytic or kinase domain of Raf kinase, contains of both ATP and substrate binding sites and also associates with 14-3-3 protein (Freed *et al.*, 1994; Muslin *et al.*, 1996).

1.2.4.4.1 Interaction of proteins with Raf

Inactive Raf-1 is found in the cytosol constitutively associated with 14-3-3 and the molecular chaperones hsp90 and p50 in a multiprotein complex (Morrison and Cutler, 1997; Stancato *et al.*, 1993). 14-3-3 proteins are specific phosphoserine-binding proteins (Muslin *et al.*, 1996) that bind to the motif RxSxS*xP (where x represents any amino acid and S* represents phosphoserine). Two phosphorylation sites with 14-3-3 binding motifs have been identified on Raf-1, Ser 259 in the N-terminal regulatory domain and Ser 621 in the C-terminal kinase domain (Morrison *et al.*, 1993). Binding of 14-3-3 to phosphorylated Ser 259 in inactive Raf-1 may be required to maintain Raf-1 in an inactive conformation by obscuring the second Ras binding domain in the CRD. This idea came from observations that Raf-1 proteins that are unable to stably interact with 14-3-3 are activated (Michaud *et al.*, 1995) and a mutation that altered the consensus 14-3-3 binding motif surrounding Ser 259 of Raf-1, allowed the CRD to bind to Ras without also binding to the RBD (Drugan *et al.*, 1996).

This idea was further supported by fact that Ras binding interfered with the interaction between the Raf-1 N-terminal domain and 14-3-3 (Rommel *et al.*, 1996).

14-3-3 association with Raf-1 may also be indirectly involved in Raf-1 activation (Irie *et al.*, 1994; Fantl *et al.*, 1994) through stabilization of the active conformation of Raf-1 by protecting it from phosphatase action rather than direct stimulation (Dent *et al.*, 1995; Freed *et al.*, 1994; Muslin *et al.*, 1996). This function might be accomplished by the binding of 14-3-3 to Ser 621 in the kinase domain of activated Raf-1 (Morrison and Cutler, 1997).

The molecular chaperones hsp90 and p50 also associate with Raf-1 constitutively (Morrison and Cutler, 1997; Stancato *et al.*, 1993; Stepanova *et al.*, 1996). This complex of Raf-1 with hsp90 and p50 appears to be important for maintaining protein stability and for the proper localisation of Raf-1 within the cell. Disruption of the complex results in destabilization of Raf-1 and loss of Raf-Ras association (Schulte *et al.*, 1995; 1996). Raf-1 binds to hsp90 via its C-terminal catalytic domain (Stancato *et al.*, 1993) and remains complexed to hsp90 even when Raf-1 is bound to Ras at the plasma membrane (Wartmann and Davis, 1994).

1.2.4.4.2 Mechanism(s) of activation of Raf-1 kinase

Activation of Raf-kinase is a complex multistep process that is thought to involve protein-protein and protein-lipid interactions, and also phosphorylation (Heidecker *et al.*, 1990; Stanton *et al.*, 1989). At the simplest level, Raf-1 is regulated by reversibly removing the negative control effect of the N-terminus to allow the kinase domain to contact its effectors and substrates. This process of regulating Raf activity involves an intricate series

of events that may vary in different cell types and may differ with the stimulating event.

1.2.4.4.3 Ras/Raf-1 interaction

The current understanding of Raf-1 activation was aided by the discovery of the interaction between Raf-1 and Ras and the establishment of Raf kinase as a critical transformation effector of Ras (Avruch *et al.*, 1994; Bruder *et al.*, 1992; Hallberg *et al.*, 1994). In the RBD of Raf-1, Gln 66, Lys 84 and Arg 89 are primarily responsible for the association with the Ras effector domain (Block *et al.*, 1996). The interaction of Raf-1 through its RBD with activated Ras causes Raf-1 to translocate to the membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). The second Ras-binding domain comprising residues 139-184 in the CRD, associates with Ras-residues different from those that associate with the first RBD. Binding of the CRD to Ras may require a post-translational modification of Ras (Drugan *et al.*, 1996; Hu *et al.*, 1995). In full length Raf-1, the CRD is inaccessible for Ras binding (Drugan *et al.*, 1996) and it is only upon binding of Ras to the RBD that the CRD is unmasked (Brtva *et al.*, 1995; Drugan *et al.*, 1996; Hu *et al.*, 1995). For the full activation of Raf-1, binding of Ras to both the RBD and the CRD is required (Hu *et al.*, 1995; Mott *et al.*, 1996). The CRD also directly binds to the ζ form of the 14-3-3 protein (Clark *et al.*, 1997) and this binding may serve as a negative regulator of Raf-1 function. It was proposed that the Ras interaction with the RBD may displace the 14-3-3 from the CRD and initiate the subsequent events that lead to the full activation of Raf-1 (Clark *et al.*, 1997). The CRD has also been postulated to mediate the Raf-1 interaction with other molecules such as PS and ceramide (Ghosh *et al.*, 1996; Huwiler *et al.*, 1996).

1.2.4.4.4 Role of lipids in Raf-1 activation

The likelihood of lipids playing a role in Raf activation seems plausible based on the similarities between Raf and PKC. Evidence that this is indeed the case has been suggested by a number of studies (Cai *et al.*,1993; Dent *et al.*, 1995a). Other studies have, in fact, demonstrated the binding of lipids to Raf. Thus, the CRD of Raf-1 selectively binds to PS (Ghosh *et al.*, 1994) whereas residues in the C-terminal region of Raf-1 (residues 389-423) specifically and strongly interact with PA (Ghosh *et al.*, 1996). Ceramide has also been shown to specifically bind and activate Raf-1, suggesting that ceramide could be an endogenous lipid activator of Raf-1 (Huwiler *et al.*, 1996). The generation of PA via TPA-induced PLD activation in MDCK cells led to translocation and activation of Raf-1 which could be inhibited by the addition of ethanol (Ghosh *et al.*, 1996). It has in fact been suggested that PA and PS not only facilitate the association of Raf-1 with the membrane but may directly modulate Raf-1 kinase activity (Ghosh and Bell, 1997; Ghosh *et al.*, 1997).

1.2.4.4.5 Role of dimerization/oligomerization in Raf-1 activation

It has been reported that chemical-induced oligomerization of Raf-1 can cause its activation in either a Ras-independent (Farrar *et al.*, 1996) or Ras-dependant (Luo *et al.*, 1996) manner. In the case of Ras-independent activation, forced oligomerization activated Raf-1 by changing its conformation and reducing the suppressive effect of its N-terminal regulatory domain. On the other hand, this oligomerization could result in Raf-1 activation through increased transphosphorylation or autophosphorylation in the Ser/Thr-rich CR2 domain. The autophosphorylation sites are located in the CR2 domain and several mutations

in this region have been found to have an activating effect (Heidecker *et al.*,1990; Morrison *et al.*, 1993). In the case of Ras-dependent activation, oligomerization would result in more Raf-1 molecules being recruited to the membrane. However, both the Ras-independent and -dependent activation of oligomerized Raf-1 appear to be weak activating mechanisms in comparison to normal signalling events (Farrar *et al.*, 1996; Luo *et al.*, 1996). Finally, whether under normal conditions this oligomerization plays a role in Raf-1 activation remains unclear.

1.2.4.4.6 The role of phosphorylation in Raf activation

Evidence that phosphorylation plays a role in regulating Raf activity can be deduced from observations that purified or membrane-associated protein serine phosphatases (Dent *et al.*, 1995b) or a GTP-dependent protein tyrosine phosphatase can inactivate Raf kinase activity (Dent *et al.*, 1996). However, the precise role of phosphorylation in the physiological regulation of Raf-1 activity remains to be deciphered.

Raf-1 may be phosphorylated on several residues *in vivo* including: Ser 43, Ser 259, Ser 338, Ser 339, Ser 499, Ser 621, Thr 269, Tyr 340 and Tyr 341 (Diaz *et al.*, 1997; Duam *et al.*, 1994; Fabian *et al.*, 1993; Marais *et al.*, 1995; Morrison *et al.*, 1993; Wartmann *et al.*, 1997). The multiple nature of Raf-1 phosphorylation has made it difficult to decipher the role of phosphorylation at specific sites in the activation of the molecule. This is compounded by the fact that there appear to be phosphorylation-dependent and phosphorylation-independent means of activating Raf which may be differentially affected by further phosphorylation (Rommel *et al.*, 1996).

Phosphorylation at Tyr 340 and 341 reportedly activates Raf-1 activity (Marais *et al.*, 1995) however, these have been in systems that overexpress oncogenic non-receptor tyrosine kinases such as members of the Src kinase family (Marais *et al.*, 1995) and JAK-2 (Xia *et al.*, 1996). In other systems, activation occurs in the absence of Tyr phosphorylation and even when Tyr phosphorylation occurs, the majority of residues that are phosphorylated are Ser residues (Daum *et al.*, 1994). Thus the role of Tyr phosphorylation under physiological conditions is still unclear.

Some phosphorylation may be constitutive and required for the association of interacting proteins such as the association of 14-3-3 with Ser 259 of inactive Raf in the cytosol (Morrison *et al.*, 1993). Upon activation of Raf, 14-3-3 is thought to dissociate from Ser 259 and associate with a newly phosphorylated Ser 621 at the C-terminal domain and this interaction is thought to be crucial for maintaining Raf in the active conformation (Morrison and Cutler, 1997).

Phosphorylation of Ser 338 and 339 is thought to be essential for both biological and catalytic functions of Raf-1 (Diaz *et al.*, 1997) but the kinases that phosphorylate these sites have not been identified. Phosphorylation of Thr 269 by ceramide-activated protein kinase (CAPK) that is activated in response to cell stimulation by tumor necrosis factor(TNF), leads to Raf activation (Yao *et al.*, 1995). Phosphorylation on Ser 499 has been implicated in protein kinase C-mediated activation of Raf-1 (Kolch *et al.*, 1993; Morrison *et al.*, 1993; Sozeri *et al.*, 1992; Wartmann *et al.*, 1997). On the other hand, phosphorylation of Ser 43 by cAMP dependant PKA inhibits Raf activation but this has no effect if Raf is already active (Morrison *et al.*, 1993; Samuel *et al.*, 1993).

Recently, hyperphosphorylation of Raf-1 which causes a mobility shift on gels and has long been used as a measure of Raf activation was, in fact, demonstrated to represent a negative feedback mechanism that resulted in decreased affinity for the plasma membrane that contributes to the desensitization of the MAP kinase signalling cascade (Wartmann *et al.*, 1997).

Thus, the role of phosphorylation in Raf-1 activation is a complex one and may result in activation or inactivation of Raf-1 depending on the mode of activation and the sites of phosphorylation. This was clearly demonstrated in a recent study which showed that phosphorylation of Raf activated solely by interaction with Ras, resulted in decreased activity (Marais *et al.*, 1995).

1.2.4.4.7 A current model of Raf activation

A model for the activation of Raf-1 that brings together the individual events discussed above has been proposed **(Fig 5)**. In the unstimulated cell, Raf-1 exists as an inactive complex with hsp90, p50 and 14-3-3. The 14-3-3 is bound at Ser 259. Following activation of Ras and exposure of the Raf-binding sites, the RBD in Raf interacts with Ras which translocates it to the membrane and causes the displacement of 14-3-3 from Ser 259. This exposes the CRD site allowing it to bind to Ras to expose the kinase and substrate domains. The active conformation is stabilized by interaction with phospholipids (PA and PS) and binding of 14-3-3 to Ser 621. The ensuing events that increase the catalytic activity

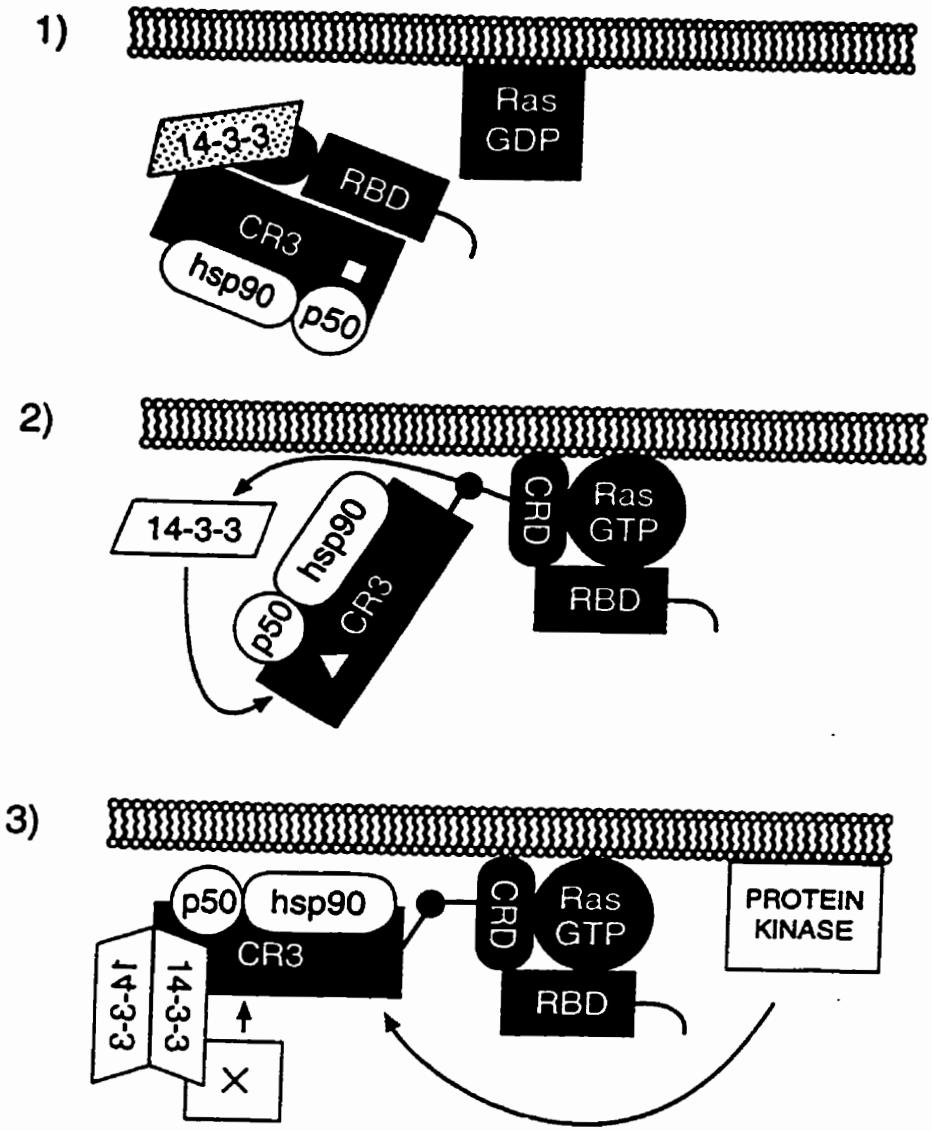


Figure 5. Putative model for Raf-1 activation. **1.** Inactive Raf-1 is in a closed conformation with 14-3-3 bound to phosphorylated Ser259 (●) and the CRD. hsp90 and p50 are constitutively associated with CR3. **2.** Upon stimulation, RBD of Raf-1 binds to Ras-GTP which results in the displacement of 14-3-3 from Ser259 (●) resulting in the assumption of an open conformation by Raf. This unmasks the CRD which becomes accessible for binding to Ras. The kinase domain (CR3) is exposed and Ser621 becomes phosphorylated (Δ). The free 14-3-3 now binds to this higher-affinity phosphorylated Ser621 site. **3.** The activity of Raf-1 may be further stimulated by other membrane-bound protein kinases or other 14-3-3-bound signalling molecules (X). (Adapted from Morrison and Cutler, 1997).

of the molecule are not very clear but could involve phosphorylation as discussed above.

Following its activation, Raf phosphorylates and activates its substrates. The best known substrates of Raf-1 are MEK-1 and MEK-2 (Daum *et al.*, 1994; Dent, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). Raf-1 binds to MEK via its COOH- terminus and the association may involve a proline-rich insert found in the C-terminal domains of MEK-1 and 2, because deletion of the insert impairs activation of MEK-1 by Raf-1 (Catling *et al.*, 1995). Raf phosphorylates Ser 218 and Ser 222 of MEK-1 and phosphorylation of either of these 2 sites is sufficient to activate of MEK 1 (Alessi *et al.*, 1994). The activation of MEK by Raf leads to the activation of MAP kinase.

1.2.4.4.8 Effect of AEL on Raf activation

There is evidence that Raf may be a key intracellular target of ET-18-OCH_3 (Zhou *et al.*, 1996). Under experimental conditions where MCF-7 cells had accumulated sufficient levels of ET-18-OCH_3 to completely inhibit the proliferation of the cells, diminished levels of membrane-bound Raf-1 were observed in cells incubated with the drug relative to controls. The ET-18-OCH_3-induced decrease in the quantities of Raf-1 associating with membranes resulted in an inability to sustain the phosphorylation of MEK, which consequently inhibited MAP kinase phosphorylation. ET-18-OCH_3 did not have a direct inhibitory effect on Raf-1 kinase activity. The ET-18-OCH_3-induced decrease in Raf-1 levels in the membrane could be due to interference with the translocation of Raf-1 or enhanced dissociation of Raf-1 from the membrane. These could be due to perturbation of the postulated interaction between Raf-1 and PA and PS that stabilizes the active conformation of Raf-1 (Ghosh *et al.*, 1996). We

can also not rule out an effect of the ALP on the unknown events, including phosphorylation, that lead to the increase in catalytic activity of the kinase.

Evidence that inhibition of Raf and consequently the MAP kinase cascade by ET-18-OCH_3 may play a major role in the ET-18-OCH_3-induced cell growth inhibition is supported by the demonstration of a direct correlation between the levels of ET-18-OCH_3 in the cells, the levels of Raf in the membrane, the degree of inhibition of MAP kinase and the extent of inhibition of cell proliferation (Zhou *et al.*, 1996). This notion was further supported by other studies that demonstrated that when cells were treated with ET-18-OCH_3 after transient stimulation of MAP kinase, the effect of the drug on cell cycle progression was blunted compared to cells treated with ET-18-OCH_3 prior to stimulation. Thus, Raf-1 appears to be a major site of AEL action for cell growth inhibition.

1.2.4.5 AELs and MEKs (MAP kinase / ERK kinases)

MEKs are members of the MAP kinase kinase (MAPKK) family of protein kinases that phosphorylate both Tyr and Ser/Thr residues (Hanks and Hunter, 1995). Six members of the MEK family, MEK1-6 have been identified and each MEK appears to act strongly in a single, or at most in two MAP kinase cascades (Brunet and Pouyssegur, 1996). MEK 1 and MEK 2 phosphorylate the TEY (Thr-Glu-Tyr) sequence on ERKs to activate the enzymes. MEK 3 (Dérijard *et al.*, 1995) and MEK 6 (Han *et al.*, 1996) probably activate the p38 subgroup of MAP kinases while MEK 4 acts on both JNK / SAPK and p38 subgroups of MAP kinases (Dérijard *et al.*, 1995; Lin *et al.*, 1995). No substrates have yet been found for MEK 5, although it binds to ERK 5 in yeast two-hybrid assay (Waskiewicz and Cooper,

1995; Zhou *et al.*, 1995). As far as I am aware there have been no studies to examine whether AELs have a direct effect on MEK activity, however, preincubation of MCF-7 cells with ET-18-OCH_3 led to decreased activation of MEK as a consequence of decreased phosphorylation by Raf (Zhou *et al.*, 1996).

1.2.4.6 AELs and MAP kinase activation

MAP kinases are proline-directed protein kinases that phosphorylate serine or threonine residues in the consensus sequence of PX (S or T) P where X is either a basic or neutral amino acid (Pelech and Sanghera, 1992). The MAP kinases are highly conserved throughout evolution (Marshall, 1994; Sprague, 1992) and are grouped into three main sub families based on the mode of their activation and substrate phosphorylation (Cano and Mahadevan, 1995).

The best studied and the archetypal members of the mammalian MAP kinases are the extracellular signal-regulated kinases ERK 1 (44 kDa) and ERK 2 (42 kDa). MEK 1 and MEK 2 activate ERKs by phosphorylating Thr 183 and Tyr 185 of ERK 2 or Thr 183 and Tyr 190 of ERK 1 within the TEY (Thr-Glu-Tyr) motif (Cano and Mahadevan, 1995; Posada and Cooper, 1992).

The c-Jun NH2-terminal kinases (JNK)/ stress-activated protein kinases (SAPK) sub family of MAP kinases have a TPY (Thr-Pro-Tyr) consensus sequence for tyrosine and threonine phosphorylation for activation of the subgroup (Cano and Mahadevan, 1995; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994). So far, five members of the JNK/SAPK sub family have been identified. JNK 1 (46 kDa) and JNK 2 (55kDa) phosphorylate c-Jun on Ser

63 and Ser 73 respectively upon the exposure of the cells to ultraviolet (UV) radiation (Dérijard *et al.*, 1994; Hibi *et al.*, 1993). The other three members of the JNK/SAPK sub family (SAPK α,β,γ) are potently activated by stress-inducing stimuli, such as translational inhibitors, heat shock or tumor necrosis factor (TNF).

The third subfamily of mammalian MAP kinases is p38 (Han *et al.*, 1994) and reactivating kinase (RK) (Rouse *et al.*, 1994). The defined dual phosphorylation sequence on this MAP kinase subfamily is TGY (Thr-Gly-Tyr). Both p38 and JNK are activated in response to osmotic stress in mammalian cells (Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994) and as described above, share common MEK activators (Dérijard *et al.*, 1995; Ichijo *et al.*, 1997), suggesting that they may be more closely related to each other than to the ERK subfamily.

1.2.4.6.1 The ERK subtype of MAP kinase

ERKs have many potential cytosolic, nuclear and membrane substrates. Activated ERKs activate ribosomal S-6 kinase ($p90^{rsk}$) in the cytosol (Ahn *et al.*, 1991; Blenis,1993; Chung *et al.*, 1991; Grove *et al.*, 1993; Lavoinne *et al.*, 1991). Both activated ERKs and $p90^{rsk}$ translocate from the cytosol to the nucleus where they phosphorylate Elk-1, a ternary complex factor (TCF), in its C-terminal domain (Hipskind *et al.*, 1994; Janknecht *et al.*, 1993; Marais , 1993; Rao and Reddy, 1994; Zinck *et al.*, 1993), and serum response factor (SRF) at Ser 103 (Rivera *et al.*, 1993), respectively. The phosphorylated Elk-1 and SRF together bind to the promoter region of many genes including *c-fos* to initiate gene expression. Another transcription factor, cAMP response element-binding protein (CREB) is also

phosphorylated and activated by $p90^{rsk}$ at Ser 133 (Xing *et al.*, 1996) to mediate the expression of many genes including c-fos (Bonni *et al.*, 1995; Ginty *et al.*, 1994; Robertson *et al.*, 1995). Thus ERKs and $p90^{rsk}$ relay the signals arising at the plasma membrane to gene expression in the nucleus.

Another well characterized ERK substrate is the cytoplasmic PLA_2 ($cPLA_2$). ERK mediated phosphorylation of $cPLA_2$ at Ser 505 activates the enzyme and increases the release of arachidonic acid and subsequent eicosanoid formation (Lin *et al.*, 1993). Thus ERKs are also responsible for triggering the generation of multiple secondary signaling molecules.

Activated ERK 1 and ERK 2 are essential elements in mitogenic signaling. Prolonged activation and nuclear retention of ERKs are required for the transcription of cyclin D1, indicating a connection between ERK activation and promotion of the cell to enter the cell cycle (Lavoie *et al.*, 1996). The magnitude and duration of activation of ERKs in cells may decide whether cells undergo proliferation or differentiation (Marshall, 1995). Activated ERKs are also found associated with the cytoskeleton, suggesting that they may play a role in the organization of the cytoskeleton in preparation for cell division (Reszka *et al.*, 1995).

1.2.4.6.2 Effect of AEL on MAP kinase activation

The effect of ET-18-OCH_3 on the activation of MAP kinase (ERKs) has been investigated (Zhou *et al.*, 1996). The results of these studies revealed that preincubation of quiescent MCF-7 cells with ET-18-OCH_3, under conditions that permitted the accumulation of the ALP in quantities that inhibited cell proliferation, resulted in a significant decrease in the magnitude and duration of MAP kinase activation in cells stimulated with EGF or serum

(Zhou *et al.*, 1996). Similar results have been observed with ilmofosine treated MCF-7 cells (Samadder and Arthur unpublished observations). ET-18-OCH_3 did not have a direct inhibitory effect on the catalytic activity of MAP kinases (Zhou *et al.*, 1996). It was determined that the diminished MAP kinase activity was due to the effect of the ALP on Raf-1 levels in the membrane, which in turn diminished the sustained phosphorylation of MEK leading to diminished phosphorylation and activation of MAP kinase (Zhou *et al.*, 1996).

1.3 HYPOTHESIS AND RESEARCH AIMS

1.3.1 Cell selective effects of AELs

One of the remarkable properties of AELs is their cell-selective effects. At low concentrations, they inhibit the growth of some cancer cells without affecting others, and even more remarkable with respect to therapeutic applications, AELs appear to selectively inhibit the proliferation of cancer cells at concentrations that have no effect on normal cells (Berdel *et al.*, 1985; Andreesen., 1978; 1979). This property has in fact been successfully exploited in the use of AELs in purging bone marrow for transplantation (Vogler *et al.*, 1992; Vogler, 1994). The mechanism underlying the selectivity displayed by AELs is not known, but has been variously attributed to differences in rates of uptake (Storch and Munder, 1987), extent of metabolism (Fleer *et al.*, 1987), cellular ether lipid content, cholesterol content, and perturbation of lipid metabolism (Chabot *et al.*, 1989; Diomede *et al.*, 1991). However, these hypotheses have not been supported by other studies (Lu and Arthur, 1992a; 1992b; Zhou *et al.*, 1992; Lu *et al.*, 1993; Chabot *et al.*, 1989; Hoffman *et al.*, 1986).

The overall goal of this work is to determine the reasons why some cells can

escape the growth-inhibitory effects of ALPs.

In order to achieve the above objective, a reasonable approach will be to establish the mechanism underlying the antiproliferative effects of AELs and then use the information as a basis to investigate why all cells do not respond in an identical manner to the compounds.

Studies in our lab suggest that the inhibition of the MAP kinase pathway by ET-18-OCH_3 may be the mechanism responsible for the antiproliferative effects of the ALP (Zhou et al 1996). This led to the working hypothesis that ET-18-OCH_3 inhibits cell growth by inhibiting the activation of Raf-1 which curtails the transduction of growth signals via the MAP kinase cascade. As Raf-1 is ubiquitously expressed, possible mechanisms to explain why some cells are not sensitive to the compound would have to explain why the activation of Raf-1 is either not affected in some cells, or if it is, why this has no effect on cell proliferation.

Since the above working hypothesis is fundamental to the proposed work on cell selectivity properties of AELs, it was essential to validate the postulated effect of ALPs on Raf activation using a different approach. The studies described in this thesis are divided into two sections, the first section deals with validation of the working hypothesis and the second section deals with studies on the mechanism of selective inhibition of cell proliferation by ALPs.

The specific goal of the first section was to determine whether inhibition of MCF-7 cell proliferation by ALPs is due to inhibition of Raf activation.

To answer this question my approach was to compare the effects of structurally related analogs of ALPs on cell proliferation in order to identify an active and inactive

pair of compounds and subsequently investigate if the activity correlates with their effects on Raf activation.

The specific goal of the second section, after confirming that perturbation of Raf activation is involved in the mechanism of action of ALPs was to determine whether differences in the mode of regulation of Raf-activation in ALP-sensitive and ALP-insensitive cells was responsible for the differential effects of the drugs on the cells, or whether differences in the signaling pathways that transduce growth signals (ie Raf-dependent versus Raf-independent) was the basis for the observed selective ALP effects.

2 MATERIALS

2.1 Cell Lines Media and Other Tissue Culture Materials

MCF-7, MDA-MB-468 (human breast adenocarcinoma), T-47D (human breast ductal carcinoma), A427, A549 (human lung carcinoma), DU145 (human prostate carcinoma), SK-N-SH and SK-N-MC (human neuroblastoma) cell lines were grown from frozen stocks originally obtained from the American Type Culture Collection (ATCC) (Rockville, Maryland).

Human normal mammary epithelial (HNME) cells, mammary epithelial cell basal medium (MEBM) and supplements required to formulate the growth media, mammary epithelial cell growth medium (MEGM), were obtained from Clonetics Inc (San Diego, CA).

DMEM, DMEM/F-12 (with 15 mM HEPES), F-12 and MEM media, bovine pituitary extract (BPE), trypsin/ETDA, insulin-transferrin-selenium-X supplement (insulin, 1 mg/ml, transferrin, 0.55 mg/ml, sodium selenite, 0.67 μg/ml and ethanolamine, 0.2 mg/ml) were obtained from Gibco BRL (Burlington, Ontario).

Insulin, $PGF_{2\alpha}$, estrogen, hydrocortisone, transferrin, fibronectin, soybean trypsin-inhibitor and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, Missouri).

Insulin-like growth factor I and II were obtained from Bachem California (Torrance, CA).

EGF, laminin, collagen I and collagen IV were obtained from Becton Dickinson (Mississauga, Ontario).

Fetal bovine serum (FBS) (Cellect Gold) was obtained from ICN Pharmaceuticals

(Montreal, Quebec).

Falcon tissue culture ware was obtained from Baxter Canlab Diagnostics Incorporation (Winnipeg, Manitoba).

2.2 Antibodies and related reagents

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) and HRP-goat anti-rabbit IgG (H + L) were obtained from BioRad Laboratories (Mississauga, Ontario).

HRP-rabbit anti-sheep IgG (H + L) antibody was purchased from Zymed laboratories Inc (South San Francisco, CA).

Rabbit polyclonal anti-ERK 1 [ERK 1(c-16)], rabbit polyclonal anti-ERK 2 [ERK 2 (c-14)], rabbit polyclonal anti-MEK [MEK (c-18)], rabbit polyclonal anti-Insulin receptor [Rβ (c-19)] antibodies were purchased from Santa Cruz Biotechnology Inc. (La Jolla, CA).

Anti-PKC-α, -β, -γ, and ϵ antibodies, Anti-c-Raf-mAb and anti-MEK-1 antibodies were purchased from Transduction Laboratories (Lexington, Kentucky).

Sheep polyclonal anti-EGF receptor antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, New York).

Rabbit polyclonal phospho-specific MAP kinase antibody raised against a synthetic peptide spanning residues 196-209 of p44 MAPK with a phosphorylated tyrosine residue (Tyr 204) in the TEY motif, and rabbit polyclonal phospho-specific MEK 1/2 antibody which recognizes phosphorylated Ser 217 and 221 in MEK whose phosphorylation results in the activation of MEK were obtained from New England Biolabs Inc. (Mississauga, Ontario).

2.3 Radiochemicals and related reagents

1-*O*-[^{3}H]Octadecyl-2-*O*-methyl-sn-glycero-3-phosphocholine ([^{3}H]ET-18-OCH_3) was obtained from Amersham International (Oakville, Ontario).

Adenosine 5'-[γ-^{32}P]triphosphate and Ecolite scintilation fluid were from ICN Biomedicals (St. Laurent, Quebec).

Kodak GBX developer and replenisher and Kodak rapid fixer with hardener were from Picker (Ontario).

BM Chemiluminescence Blotting Substrate (POP) was from Boehringer Mannheim (St. Laval, Quebec).

2.4 Proteins and peptides

GST-Ras fusion protein containing bound GTP-γ-S, or GDP were generously provided by Dr. R. Bhullar (Dept. of Oral Biology, University of Manitoba).

Full length MEK-1 [MEK(FL), a 55 kDa polyhistidine fusion protein] was obtained from Santa Cruz Biotechnology Inc. (La Jolla, CA).

Catalytically active c-Raf-1 was purchased from Upstate Biotechnology (Lake Placid NY)

Myelin basic protein (MBP) was purchased from Sigma Chemical Co. (St. Louis, Missouri).

S-6 phosphate acceptor peptide and PKI were purchased from Bachem (Torrance, CA).

2.5 Chemicals, reagents and chromatographic supplies

Acrylamide, N, N'-methylene bisacrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), N, N, N', N,-tetramethyl ethylenediamine (TEMED) were all made by Serva and were procured from Crescent Chemical Co., Inc (New York, NY).

Glacial acetic acid, o-phosphoric acid, glycine, methanol and chloroform were obtained from Fisher Scientific Company (Winnipeg, MB).

Glycerine, ethyleneglycol-bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), β-glycerophosphate (disodium salt) (β-GP), 1, 4-dithiothreitol (DTT), sodium orthovanadate (Na_3VO_4), ATP, calmidazolium, triton X-100, nonidet P-40 (NP-40), Tween 40, aprotinin, benzamidine, leupeptin, aminoethylbenzenesulfonyl fluoride (AEBSF), phenylmethylsulfonyl fluoride (PMSF), Trizma base, Trizma hydrochloride (Tris-HCl), magnesium chloride, 3-({N-morpholino}propanesulfonic acid) (MOPS) and trypan blue were purchased from Sigma Chemical Co. (St.Louis, Missouri).

N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) was purchased from Gibco BRL (Burlington, Ontario).

ET-18-OCH_3 was from Med Mark Pharmaceuticals (Gruenwald, Germany).

ET-16-OCH_3-phosphatidate, ET-16-OCH_3-phosphonate and ET-16S-OCH_3-phosphonate, (*S*)- and (*R*)-ET-16-phosphono-TDB and (*R*)- and (*S*)-oxo-Ilmofosine were synthesized and generously provided by Dr. R. Bittman (Queens College, City University of New York, Flushing, NY).

Ilmofosine was obtained from Dr. D. B. J. Herrmann (Boehringer Mannheim GmbH, Mannheim, Germany).

Coomassie protein assay solution was from Pierce (Rockford, Illinois).

Whatman P81 (cellulose acetate) filter paper was purchased from Mandel Scientific Company Ltd (Guelph, Ontario).

Supported nitrocellulose membranes (0.2 μm) were from Bio Rad Laboratories, Mississauga, Ontario.

Silica gel K6 thin-layer chromatography (TLC) plates were purchased from Baxter Canlab Diagnostics Inc. (Winnipeg, Manitoba).

2.6 Water and buffers

Distilled deionized water (DDW) (Conductance ≤ -18 μΩ) from a Milli Q Plus water purification system was used throughout these studies.

Buffer A: (preparation of cell lysates) 20 mM Tris-HCl (pH 7.4), 2mM EGTA, 100 mM β-glycerophosphate, 1 mM Na_3VO_4, 0.1 mM PMSF, 0.2 mM AEBSF, 0.2 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM dithiotreitol (DTT).

Buffer B: (solubilizing membranes) Buffer A plus 1% Triton X-100 and 0.5% NP-40.

Hanks balanced saline solution (HBSS): 0.04% KCl, 0.8% NaCl, 0.006% KH_2PO_4, 0.009% $Na_2HPO_4 \cdot 7H2O$ and 1% D-glucose (all w/v).

PBS: 0.8% NaCl, 0.02% KCl, 0.115% $Na_2HPO_4 \cdot 7H_2O$, 0.02% KH_2PO_4 (w/v)

2.7 Equipment

The following items of equipment were used in the course of this research: L-80 Ultracentrifuge, J2-HS centrifuge and LS3801 liquid scintillation counter (Beckman); Model

ZM Coulter counter (Coulter Electronics); Model TMS-F Microscope (Nikon); Model PDI 325oe High Resolution Color Scanner (Protein + DNA Imageware Systems, Huntington Station, NY); Microsonic XL ultrasonic cell disruptor; Hitachi U2000 spectrophotometer.

3 Experimental Methods

3.1 Cell culture

Cells were cultured in appropriate media (A427 and DU145 in MEM, A459 in F-12, MCF-7, T47D, MDA-MB-468, SK-N-SH and SK-N-MC in DMEM) supplemented with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and fungizone (0.3 μg/ml) at 37°C in 5% CO_2/95% humidified air atmosphere. Cells were subcultured by detaching with trypsin (0.25% Trypsin and 1 mM EDTA·4Na in HBSS) followed by addition of the serum-supplemented media to the detached cells. The cells were dispersed and passed through a 21-gauge needle to break up clumps, if necessary, prior to distribution into the tissue culture ware.

HNME cells were cultured in MEGM which consisted of MEBM supplemented with bovine pituitary extract (52 μg/ml), EGF (10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), gentamycin (50 μg/ml) and amphotericin (50 ng/ml).

MCF-7Ad$^+$ and MCF-7Ad$^-$ cells **(section 3.3)** were cultured in serum-free media designated adapted cell growth medium (ACGM). MCF-7Ad$^+$ cells were grown in DMEM/F12 (1:1) medium (2.0 g of $NaHCO_3$/liter), supplemented with EGF (20 ng/ml), insulin (5 μg/ml), hydrocortisone (1 μg/ml), fibronectin (7.5 μg/ml), transferrin (25 μg/ml), $PGF_{2\alpha}$ (100 ng/ml), BPE (52 μg/ml), insulin-transferrin-selenium-ethanolamine-supplement (1%, v/v), gentamycin (50 μg/ml) and amphotericin (50 ng/ml). This serum-free medium which contains $PGF_{2\alpha}$ was designated ACGM$^+$ to distinguish it from ACGM$^-$ which was identical but lacked $PGF_{2\alpha}$ and was used to grow MCF-7Ad$^-$ cells.

HNME, MCF-7Ad$^+$ and MCF-7Ad$^-$ cells were subcultured by detachment with

trypsin (0.025% trypsin/0.01% EDTA) followed by the addition of an equal volume of soybean trypsin inhibitor (0.2% w/v). HEPES buffered saline solution or the serum-free medium (10 vol) was added and the cells were centrifuged at 1000 x g for 5 min in a bench top centrifuge. The cell pellet was resuspended in serum-free growth medium for distribution into the appropriate tissue culture ware as dictated by the experiment. In experiments with the MCF-7Ad^{+} and MCF-7Ad^{-} cell lines, the cells were subcultured into tissue culture dishes that had been incubated with 10% FBS-supplemented DMEM for 24 h, followed by complete removal of the medium by suction.

3.2 Development of serum-free media for MCF-7 growth and attachment factors required for growth

Equal numbers of MCF-7 cells were seeded in 24-well plates in DMEM/F-12 media containing different combinations of growth factors. The cells were detached with trypsin and cell numbers were determined every 24 h using a Coulter counter.

To identify the adhesion and spreading factor(s) required for optimal growth of the cells in serum-free medium, 24-well plates were preincubated with collagen I, collagen IV, laminin or 10% serum containing media for 24 h before seeding the MCF-7 cells in serum-free media (ACGM). After 72 h, the morphology of the cells and spreading was examined under the microscope and compared with the cells grown in untreated 24-well plates.

3.3 Adaptation of MCF-7 cells for growth in serum-free medium

Confluent MCF-7 cells in 10% FBS-supplemented DMEM were subcultured into 10%

FBS-supplemented DMEM diluted 1:1 with ACGM. Subsequently at each passage, the FBS content was reduced by half by the addition of an equal volume of ACGM until after growing to 80% confluence in medium containing less than 0.1% FBS, the cells were subcultured directly into ACGM. Cells growing in media containing 0.625% FBS or less were subcultured into flasks preincubated for 24 h with 10% FBS-supplemented DMEM following removal of the medium. Using this strategy, two types of cell lines were obtained, MCF-7 cells adapted for growth in $ACGM^{+}$, $MCF\text{-}7Ad^{+}$, and MCF-7 cells adapted for growth in $ACGM^{-}$, $MCF\text{-}7Ad^{-}$.

3.4 Isolation of clones of MCF-7 cells

MCF-7 cells were detached by trypsin, as described above, and the cell suspension was passed through a 21-gauge needle to obtain a single cell suspension. The cell number was determined in aliquots of the cell suspension. Following calculation of the cell density, aliquots were taken and the cell density was diluted to 100 cells/ml. The cells were seeded in 150 mm plates at a density of 10 cells/dish. After colonies were observed, individual colonies were detached by trypsin and subcultured into 6-well dishes. Each colony was then expanded in T75 flasks and stocks were frozen in liquid N_2.

3.5 Determination of the effects of ALPs on the proliferation of epithelial cancer cell lines.

Thirty millimolar stock solutions of ALPs [ET-18-OCH_3, (*S*)- and (*R*)-ET-16-phosphono-TDB, ET-16-OCH_3-phosphatidate, ET-16-OCH_3-phosphonate, ET-16S-OCH_3-

phosphonate, ilmofosine, (*S*)- and (*R*)-oxo-ilmofosine] were prepared in 95% ethanol and stored at -20°C. Working concentrations of each ALP in growth medium required by each cell line under investigation was prepared fresh from the stock solution. The final concentration of ethanol in each medium was 0.1%. Equal numbers of cells growing in 10% FBS-supplemented medium were seeded in 24-well plates with an Eppendorf Repeater pipette and the cells were grown to log phase. On the day of the addition of the drugs (0 time), cells were detached from representative wells and the numbers were determined with a Model ZM Coulter counter. The media for the remainder of the plates were removed and replaced with the media containing 0-30 μM of the ALP under investigation. After 48 h incubation with the compounds, the cell numbers in the wells were determined after cells were detached with trypsin. Increases in cell numbers from the number at day 0 were determined for the different concentrations of the ALP and expressed as a percentage of the increase in numbers in control wells incubated in medium without ALP but with ethanol (0.1%).

In studies with cells growing in serum-free medium, the cells were detached with trypsin, followed by the addition of soybean trypsin inhibitor and medium as described above **(section 3.1)**. After centrifugation and resuspension in the appropriate serum-free medium, equal numbers were seeded in 24-well plates preincubated for 24 h with 10% FBS-supplemented DMEM/F12. When the cell growth was in log phase, the effect of the drugs on growth was determined as described above.

3.6 Determination of preincubation conditions with (*S*)-ET-16-phosphono-TDB that inhibit serum-induced proliferation of quiescent MCF-7 cells

MCF-7 cells were seeded in 6-well plates and allowed to attach and grow in the 10% serum-containing media. After 24 h, cells were washed and incubated in DMEM supplemented with BSA (0.5 mg/ml). The increase in cell numbers were monitored daily until the cells were quiescent, defined as an increase in the cell numbers of 10% or less in a 24 h period.

Working solutions of (*S*)- and (*R*)-ET-16-phosphono-TDB (30 μM) in 0.5 mg/ml BSA were freshly prepared from the 30 mM stock solutions. Medium containing 0.1% ethanol was also prepared. The medium was removed from the quiescent cells and replaced with one containing either (*S*)- or (*R*)-ET-16-phosphono-TDB (30 μM) or 0.1% (v/v) ethanol for 4 h. The concentration of ethanol in all wells was 0.1% (v/v). At the end of the incubation, the cells were washed with DMEM/BSA-containing media and incubated with FBS-supplemented medium. The cell number in representative wells in drug-treated and untreated groups, were counted at the end of the 4 h incubation to establish the cell number at day 0. The cell number was determined every 24 h up to 72 h.

In addition, at the end of the 4 h drug treatment of quiescent MCF-7 cells described above, cell viability was also determined in all three groups of cells using a trypan blue dye exclusion assay. The viability in drug treated groups were then compared with control group. For this assay, 0.5 ml of cell suspension (~ 2.5 x 10^6 cells/ml in HBSS) was throughly mixed with 0.5 ml of 0.08% trypan blue (diluted with HBSS) and was allowed to stand for 5-10 min. Cell suspension in trypan blue was then mixed with Pasteur pipette and a drop of this cell suspension was transferred into chambers of a hemocytometer and blue and non-blue staining cells were counted under the microscope in the appropriate quadrants of the hemocytometer.

The percentage of cell viability was calculated based on the following formula:

% of Cell Viability = Total viable cells (unstained cells) ÷ total cells (unstained and stained blue) x 100.

3.7 Determination of uptake and extent of metabolism of ET-18-OCH_3 in proliferating cells

[^{3}H]ET-18-OCH_3 (0.1 μCi/μg/ml) was prepared in the required medium and added to proliferating cells growing in 6-well plates for uptake studies, or 100 mm dishes (0-9 h) for an assessment of the extent of drug metabolism. At the selected times, the medium was removed and the cells were washed twice with medium containing BSA (0.5 mg/ml). The cells were detached with trypsin and a known volume of FBS-supplemented medium was added. The cells were transferred to tubes and dispersed with a 21-gauge needle. Aliquots were taken for determination of cell numbers using the Coulter counter and the remainder of the cells were pelleted by centrifugation. Pellets from cells obtained from the 6-well plates were dissolved in 1 ml of 1% SDS in 0.3 M NaOH, and the radioactivity was determined by scintillation counting after the addition of Ecolite to the samples.

To assess the extent of metabolism of ET-18-OCH_3, lipids were extracted from pellets obtained from the 100 mm plates. 5 ml of chloroform/methanol (1:1,v/v) was added to the pellets followed by mixing and centrifugation to separate the solvents from the pellet. After removal of the organic solvents, the pellets were extracted twice with chloroform/methanol (2:1,v/v) and the extracts were combined in silated glass tubes and the solvents were removed by evaporation under a stream of nitrogen. Known volumes of chloroform, methanol and

0.9% KCl were added to the dried lipid extracts. Each addition was followed by vigorous mixing resulting in a final biphasic system consisting of chloroform /methanol/0.9% KCl (4:2:3, v/v). The mixture was clarified by brief centrifugation and the lower phase with the lipids was stored in capped tubes at -20°C for lipid analysis. The lipids were analysed by thin-layer chromatography (TLC) on Whatman K6 plates. The plates were activated at 110°C for 1 h and the phospholipids were applied at marked origins on the plates. Aliquots of the stock [^{3}H]ET-18-OCH_3 were added to authentic phospholipid markers and applied to parallel lanes on the TLC plates. All the plates were developed in a solvent system of chloroform/methanol/water/acetic acid (50:30:8:6, v/v) (Lu *et al.*, 1993). The lipids were visualized by I_2 vapor and the ET-18-OCH_3 bands were marked and scraped into scintillation vials. The remainder of each lane was divided into manageable areas that were also scraped into scintillation vials. The associated radioactivity was determined by scintillation counting. The radioactivity in the ET-18-OCH_3 band was expressed as a percentage of the total label in each lane. The values were compared to those obtained for the stock [^{3}H]ET-18-OCH_3.

3.8 Determination of cellular phospholipid composition

Cells were grown in 150 mm dishes to 80% confluence. They were then detached by trypsin as described above **(section 3.1)**. The cells were pelleted by centrifugation and washed twice with HBSS. After the second wash the cells were resuspended in a known volume of HBSS, dispersed with a 21-gauge needle and aliquots were taken for determination of cell number.

Lipids were extracted from the remainder of the cells and phospholipids were

separated on TLC plates with a solvent system of chloroform/methanol/water/acetic acid (50:37.5:2:3.5, v/v) (Arthur and Page, 1991). The lipids were visualized with I_2 vapor and each lipid class was scraped into a test tube for quantitative determination of the lipid phosphorus following digestion with 1 ml of perchloric acid.

3.9 Quantitation of phospholipids

Phospholipids were quantitated by determination of inorganic phosphorus produced after perchloric acid digestion using the modified malachite green method (Zhou et al. 1992). To digest the phospholipids, 1 ml of perchloric acid was added to the silica gel scrapings (see above) and the tubes were placed in a block heater at 160°C until the digestion was complete. Silica gel from blank lanes on the TLC plates was also digested to correct for background. Aliquots of the digested phospholipids were transferred into tubes and distilled deionized water (DDW) was added to bring the volume to 0.4 ml, followed by mixing. Two ml of freshly-prepared 0.4% malachite green working solution was then added to each tube and mixed immediately by vortexing. The tubes were allowed to stand at room temperature for 20 min. The absorbance (OD) was read at 660 nm in a Hitachi U-2000 double-beam spectrophotometer with DDW as the reference. The quantities of phospholipids were calculated from standard curves plotted for each experiment.

3.10 Preparation of cytosolic and membrane fractions from cells

Quiescent cells were obtained by incubating proliferating cultures in DMEM or DMEM/F12 supplemented with 0.5 mg/ml fatty acid-free BSA until the 24 h increase in cell

number was 10% or less. The cells were incubated with or without the ALP (ET-18-OCH_3, (*S*)- or (*R*)-ET-16-phosphono-TDB) for the stipulated times, washed twice and stimulated with EGF or growth media for various periods. At the end of the incubation, the medium was aspirated, the cells were washed with ice-cold PBS, and scraped into ice cold Buffer A (20 mM tris-HCl (pH 7.4) 2 mM EGTA, 100 mM β-glycerophosphate, 1 mM Na_3VO_4, aprotinin (10 µg/ml), leupeptin (10 µg/ml), 0.2 mM aminoethylbenzylsulfonyl fluoride, 0.1 mM PMSF, 0.2 mM benzamidine and 1 mM dithiothreitol). The cells were disrupted by ultrasonication with a probe sonicator and centrifuged at 7000 x g for 10 min in a Beckman JA21 rotor in the J2-HS centrifuge at 4°C to pellet nuclei, cell debris and unbroken cells. The supernatant from this low-speed centrifugation was centrifuged at 200,000 X g for 30 min in a Beckman 70.1Ti rotor to separate the cytosol from the membrane fraction. The supernatant **(cytosol)** was removed, flash frozen and stored at -70°C. The pellets were dissolved in ice-cold buffer B **(section 2.6)** by ultrasonication. The soluble membrane proteins were obtained by re-centrifugation at 200,000 X g for 30 min as described above, and the samples were flash frozen and stored at -70°C until required.

3.11 Quantitation of cellular proteins

The protein content of the cellular fractions was determined by the Coomassie protein assay as described by the manufacturer (Pierce).

3.12 Assay of MAP kinase activity

MAP kinase activity was measured as the phosphorylation of MBP (Ahn *et al*, 1990;

Ahn and Krebs, 1990). The reaction mixture (30 µl) contained 2 µM protein kinase A inhibitor (PKI) peptide, 10 µM calmidazolium, 8 µg of MBP, 20mM $MgCl_2$, 0.1 mg/ml BSA, 40 mM β-glycerophosphate, 0.15 mM Na_3VO_4 and 0.15 mM ATP (3 x 10^6 cpm/nmol). The reaction was initiated by the addition of 1 µg of cytosolic protein and the mixture was incubated at 30°C for 10 min. Subsequently, 20 µl of the reaction mixture was transferred to P81 paper which was immediately placed in 150 mM phosphoric acid. After several changes of the 150 mM phosphoric acid solution, the paper was dried and the amount of bound ^{32}P on the paper was quantitated. The values were corrected for non-specific binding of ATP by subtracting counts of identical assays without MBP.

3.13 Assay of S6 kinase activity

S6 kinase assays were performed as described above for the P81 MAP kinase assay but with S6 peptide as substrate.

3.14 Western blot analysis of proteins

Western blot analysis was used to investigate the translocation of various molecules between the cytosol and membrane as well as the relative quantities of the molecules in the fractions. In addition, it was also used to measure the activation of protein kinases such as MAP kinase and MEK because of the availability of antibodies that only recognize the activated forms of the enzymes. Details of the quantities of proteins used for analysis are described in the legends to the individual Figures.

Proteins were separated by SDS-PAGE gel and the proteins were transferred to nitrocellulose membranes. Proteins of interest were probed with the appropriate antibodies

according to the instructions of the manufacturers and were detected using HRP-conjugated secondary antibodies and chemiluminescent detection.

3.15 Quantitation of immunoblots, and autoradiographs

Quantitation of immunoblots and autoradiographs was achieved by densitometric analysis with a high resolution color scanner model PDI 325oe using the ImageMaster scanning program (Pharmacia).

To ensure that changes in densitometric readings were quantitative for each protein of interest, Western blot analyses were initially conducted with different quantities of a selected cellular fraction followed by densitomeric analysis. The density was then plotted against the protein content in the fraction. Protein content within the linear portion of the plot was selected for the quantitative studies. In addition each blot was exposed for different periods and those that were not overexposed were used for quantitation.

3.16 Effect of (*S*)- or (*R*)-ET-16-phosphono-TDB on Raf-1 kinase activity

Raf kinase activity of human recombinant c-Raf was assayed according to the instructions of the manufacturer. Briefly, the assay mixture consisted of 10 µl of buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM DTT) 10 µl of inhibitor cocktail (20 µM PKC inhibitor peptide, 2 µM PKI and 20 µM calmidazolium in assay dilution buffer), 10 µl of [γ-^{32}P]ATP (20µCi/assay tube, freshly prepared 0.5 mM ATP / 75 mM $MgCl_2$) , 0.5 U Raf-1 and 0.5 µg MEK-1 in a total reaction volume of 35 µl. The reaction was started with the addition of the ATP. Assays were

conducted in the presence or absence of 40 μM (*S*)- or (*R*)-phosphono-TDB prepared in assay dilution buffer. The reaction mixture was then incubated at 30°C for 30 min. The reaction was stopped by the addition of 8.75 μl of 5x SDS sample buffer. After through mixing and boiling the proteins were separated by SDS-PAGE in 10% acrylamide. The gel was stained with Coomassie blue stain (to visualize the bands) followed by destaining overnight with acetic acid/methanol/DDW (1:1:8, v/v) mixture. After destaining, the gel was washed in DDW for at least 1 h, dried between cellophane and subjected to autoradiography and quantitation.

3.17 *In vitro* Ras/Raf-1 binding assay

To determine the effect of ALPs (ET-18-OCH_3, (*S*)- or (*R*)-phosphono-TDB) on Raf interaction with Ras, an *in vitro* assay was used. Quiescent cells were treated with the ALPs under conditions previously established to inhibit cell proliferation **(section 3.6)**. Cytosolic fractions were prepared from the cells **(section 3.10)** and used on the day of preparation. Freshly prepared cytosol (1mg protein) was incubated with 25μl of purified GST-Ras-fusion protein (GTP-γ-S or GDP loaded) in 100 mM NaCl and 20 mM Tris (pH 8.0) [Beads: buffer, 1:1, v/v] for 1.5 h at 4° C. The agarose beads were pelleted by centrifugation at 1,500 rpm for 1 min. After discarding the supernatant, the beads were washed three times with 100 mM MOPS/5mM $MgCl_2$. The agarose-bound proteins were dissolved in 1x SDS sample buffer, boiled for 5 min and centrifuged at 14, 000 rpm for 30 sec in a microfuge. The proteins were resolved by SDS-PAGE and subjected to Western blot analysis using anti Raf-1 antibodies. Blots were quantitated by densitometric analysis.

4 RESULTS

4.1 Role of inhibition of Raf-1 activation in the mechanism of action of ALPs

As discussed in the introduction, the mechanism of inhibition of cell proliferation by ALPs is still under debate, although there is increasing evidence that it may involve inhibition of mitogenic cell signals (Houlihan *et al.*, 1995; Kosano and Takatani, 1989; Lazenby *et al.*, 1990; Lohmeyer and Workman, 1993; Powis, 1991). Recent studies in our lab have indicated that ET-18-OCH_3 decreases the level of Raf-1 that associates with cell membranes in growth-factor stimulated MCF-7 cells (Zhou *et al.*, 1996). It was therefore postulated that decreasing the membrane-associated Raf-1, ET-18-OCH_3 effectively decreases the quantity of active Raf-1 available to activate MEK, which is necessary to sustain the activation of MAP kinase. This leads to a truncation in MAP kinase activity that results in decreased cell growth. In view of the large number of signaling molecules implicated in the mechanism of action of ALPs, it was important to confirm that this postulated role of Raf-1 in the mechanism of action of ET-18-OCH_3 was valid, since this formed the basis for my working hypothesis on the mechanism of selective antiproliferative effects of ALPs.

Our approach to establish the relevancy of ET-18-OCH_3-induced inhibition of Raf-1 in the mechanism of action of ALPs was to search for structurally related ALPs, one of which would be growth inhibitory while the other would not and therefore, could serve as a negative control. Thus, if Raf-1 is the target for ALPs we will expect the inhibitory compound to affect its activation whereas the inactive analogue would not.

To identify possible active/inactive pairs of ALPs we investigated the effects of lysophosphatidate (LPA) analogues of ET-16-OCH_3, enantiomers of an ilmofosine analogue,

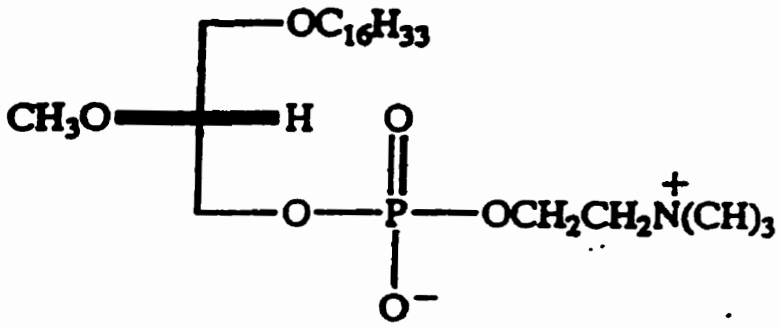
and enantiomers of a double bond phosphonocholine analogue of ET-16-OCH_3.

4.1.1 Effect of lysophosphatidate analogues of ET-16-OCH_3 on the proliferation of epithelial cancer cells

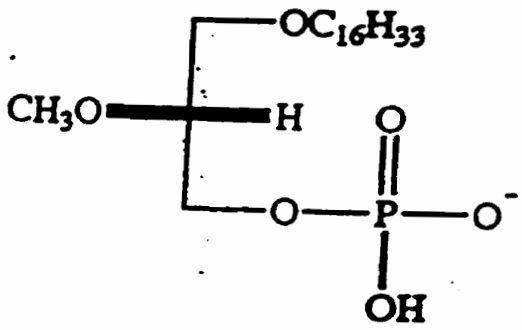
The lysophosphatidate analogues used in the studies were ET-16-OCH_3-phosphatidate, its phosphonate analogue, ET-16-OCH_3-phosphonate, and a thioether analogue, ET-16S-OCH_3-phosphonate. The structures of the compounds investigated are shown in **Fig 6**. The effect of these LPA analogues on the proliferation of epithelial cancer cells was investigated. The following cancer cells: MCF-7, T47D (breast), SK-N-MC, SK-N-SH (neuroblastoma), A549, A427 (lung), DU145 (prostate) and A498 (kidney) were used for these studies. To assess the relative potency of the compounds on cell proliferation, the effect of ET-18-OCH_3, the prototype ALP, on the proliferation of same cells was also investigated for comparison.

As shown in **Fig 7**, ET-18-OCH_3 inhibited the proliferation of all the cells tested. The cell lines tested showed a wide range of sensitivity to the compound. Some cell lines such as MCF-7 and SK-N-SH were very sensitive with IC_{50} values 1.6 and 2.9 μM respectively whereas others like A498 and DU145 were much less sensitive with IC_{50} values of 13.6 and 14.2 μM respectively.

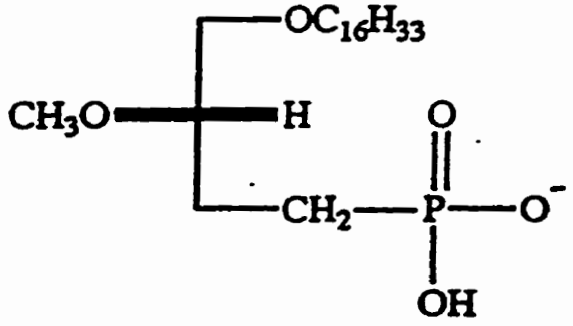
ET-16-OCH_3-phosphatidate had a weak inhibitory effect on the proliferation of the cancer cell lines **(Fig 8)**. At a concentration of 30 μM only 15 - 20% inhibition of growth was observed with most of the cell lines studied (SK-N-SH, SK-N-MC, T47D, MCF-7, A549



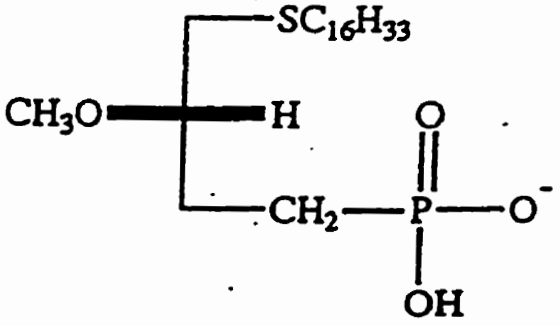
ET-16-OCH_3



ET-16-OCH_3-phosphatidate



ET-16-OCH_3-phosphonate



ET-16S-OCH_3-phosphonate

Figure 6. Structures of alkyllysophospholipid analogues of LPA.

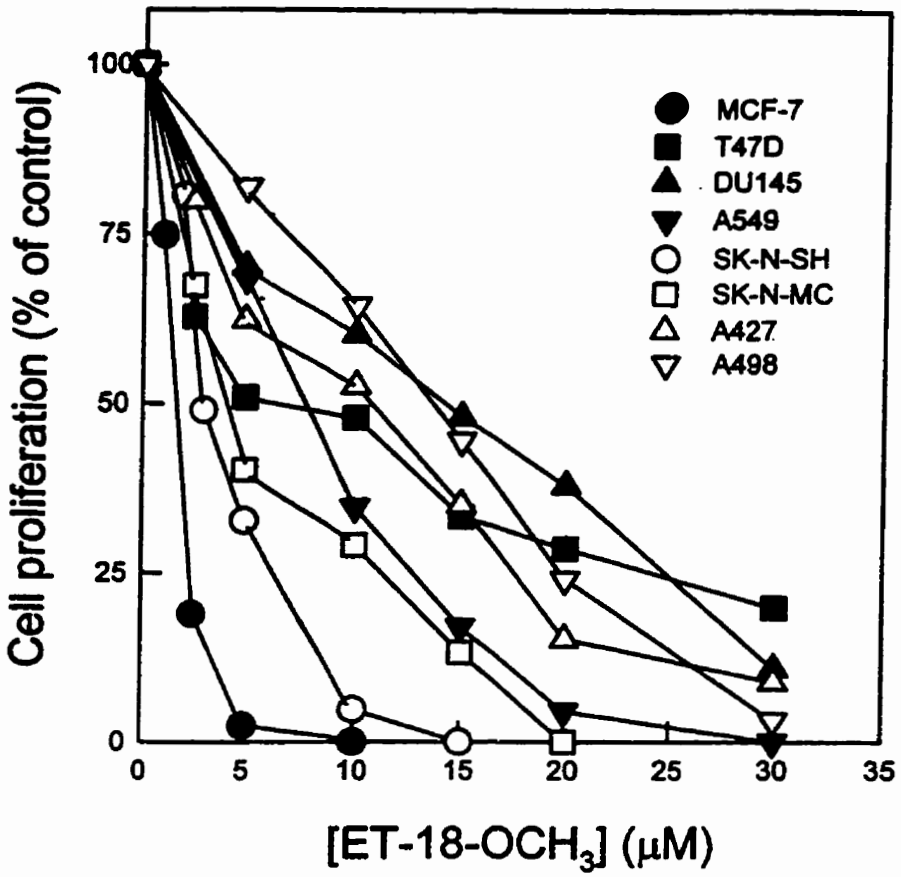


Figure 7. Effect of ET-18-OCH_3 on proliferation of epithelial cancer cell lines. Proliferating cells growing in 24-well plates were incubated with medium containing ET-18-OCH_3 (0-30 μM). Cells in representative wells were counted on day 0 prior to the addition of the compound. Forty-eight hours after the addition of the compound the numbers were determined and the increase over day 0 for each concentration was expressed as a % of that in control wells that did not receive the compound. The results are the means of 3 different studies with quadruplicate wells/experiments. The standard deviations were 14% or less of each mean value.

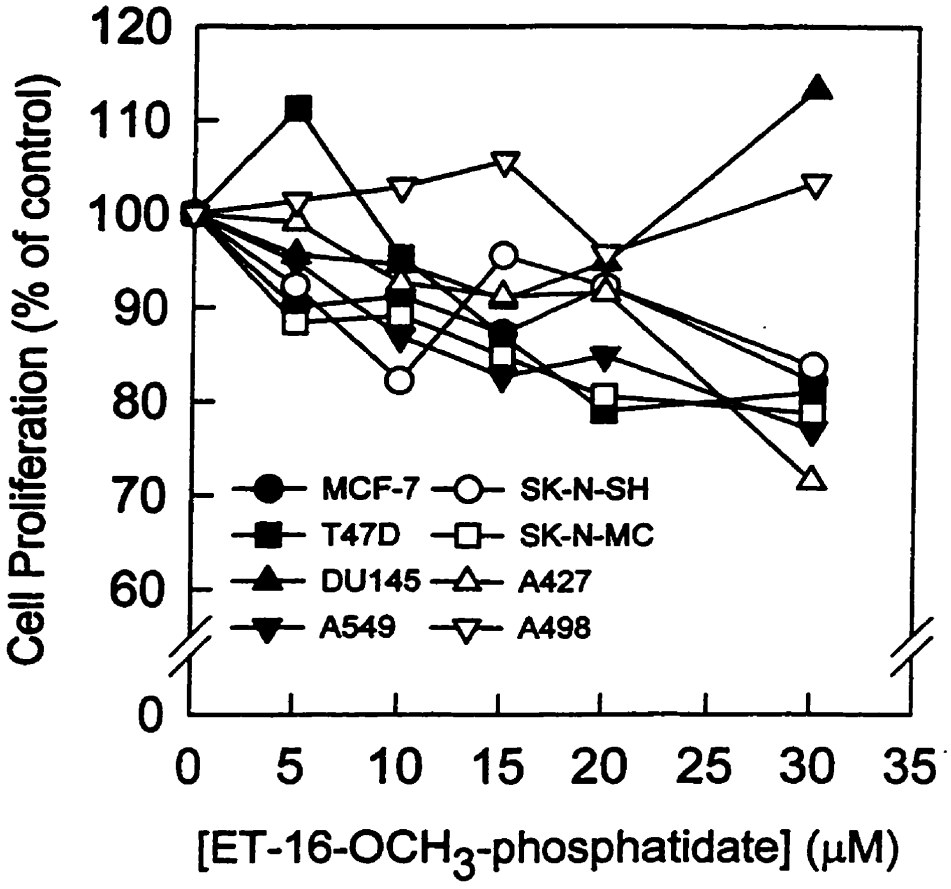


Figure 8. Effect of ET-16-OCH_3-phosphatidate on the proliferation of epithelial cancer cells. The experiments were conducted with varying concentrations of ET-16-OCH_3-phosphatidate as described in Figure 7. The results are the means of 3 different experiments with quadruplicate wells/experiment. The standard deviations were 10% or less of each mean value.

and A427). The growth of A498 was not decreased even with 30 μM of ET-16-OCH_3-phosphatidate whereas growth of DU145 was slightly enhanced after 48 hours of incubation with 30 μM of this ALP.

The results obtained from studies on the effects of ET-16-OCH_3-phosphonate on cell proliferation are shown in **Fig 9**. ET-16-OCH_3-phosphonate inhibited the proliferation of all the cell lines studied with T47D being the most resilient to the antiproliferative effect of this ALP. The IC_{50} values for A549 cells were similar to that obtained with ET-18-OCH_3. The ET-16-OCH_3-phosphonate IC_{50} values for A427, A498, and DU145 were even lower than those obtained with ET-18-OCH_3, indicating that ET-16-OCH_3-phosphonate was a potent antiproliferative compound.

ET-16S-OCH_3-phosphonate, the thioether analog of ET-16-OCH_3-phosphonate, was the least cytotoxic of all the compounds investigated **(Fig 10)**. At a concentration of 30 μM, proliferation of A427 and SK-N-SH were inhibited by 12% while the proliferation of SK-N-MC was inhibited by 25%. On the other hand, the proliferation of DU145 cells was enhanced with increasing concentrations of the compound reaching 145% of controls at a concentration of 30 μM.

The results of the above studies identified ET-16-OCH_3-phosphonate and ET-16S-OCH_3-phosphonate as a potential active/inactive pair of ALPs for use in the proposed studies.

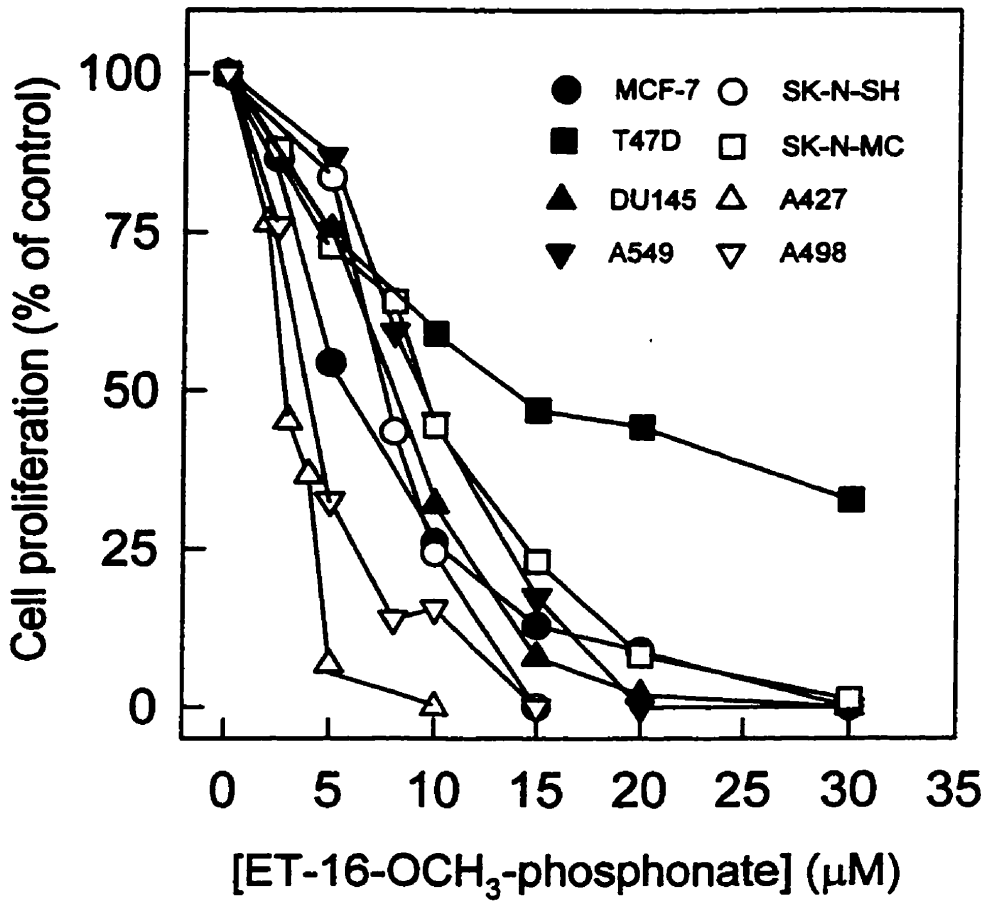


Figure 9. Effect of ET-16-OCH_3-phosphonate on the proliferation of epithelial cancer cells. The experiments were conducted with varying concentrations of ET-16-OCH_3-phosphonate as described in Figure 7. The results are the means of 3 different experiments with quadruplicate wells/experiment. The standard deviations were 8% or less of each mean value.

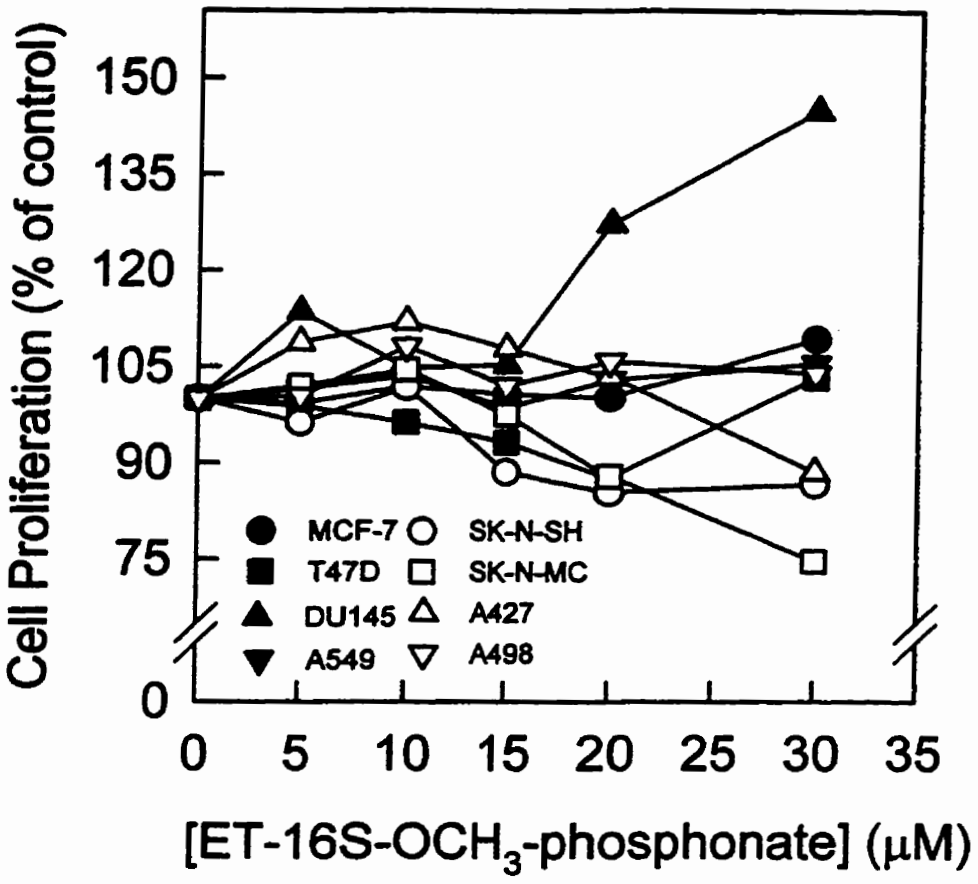
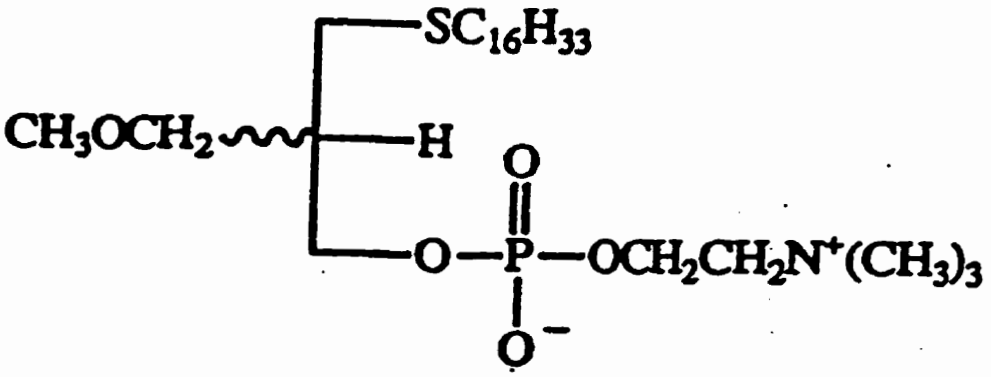


Figure 10. Effect of ET-16S-OCH_3-phosphonate on the proliferation of epithelial cancer cells. The experiments were conducted with varying concentrations of ET-16S-OCH_3-phosphonate as described in Figure 7. The results are the means of 3 different experiments with quadruplicate wells/experiment. The standard deviations were 10% or less of each mean value.

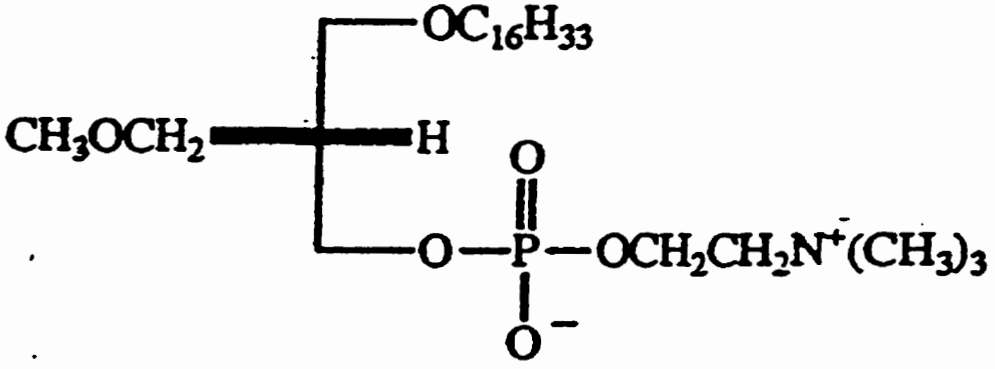
4.1.2 Effect of (*R*) and (*S*) enantiomers of 2'-(trimethylammonio)ethyl-3-(hexadecyloxy)-2-(methoxymethyl) propyl phosphate (oxo-ilmofosine) on the proliferation of epithelial cancer cell lines

(*Rac*)-Ilmofosine is an ALP with antiproliferative effects against malignant cell types of murine and human origin (Herrmann and Neumann, 1987; Neumann *et al.*, 1987 Winkelmann *et al.*, 1992). It differs from ET-18-OCH_3 in having a thioether linkage at the C-1 position and CH_3-O-CH_2 at the C-2 position. The oxygen analogues used for our studies, (*R*)- and (*S*)-oxo-ilmofosine **(Fig 11)** were synthesized and provided by Dr. Bittman (Bittman *et al*, 1997). The effects of (*R*)- and (*S*)-oxo-ilmofosine on the proliferation of three epithelial cancer cell lines, MCF-7, A549, and A427 were investigated. The order of decreasing sensitivity of the cells to (*S*)-oxo-ilmofosine was MCF-7 > A549 > A427 with IC_{50} values of 2.0, 7.5 and 20 μM respectively **(Fig 12)**. The antiproliferative profile of (*R*)-oxo-ilmofosine was very similar **(Fig 13)** to that of (*S*)-oxo-ilmofosine with IC_{50} values 2.0, 10 and ≥ 20 μM, against MCF-7, A549 and A427 cells, respectively. The relative potency of (*R*)- and (*S*)-oxo-ilmofosine on the proliferation of MCF-7 and A549 cells was assessed by comparing their antiproliferative effects against (*rac*)-ilmofosine. Both enantiomers of oxo-ilmofosine had similar growth inhibitory effects as (*rac*)-ilmofosine against MCF-7 **(Fig 14)** and A549 cells **(Fig 15)**.

The results of these studies revealed that (*S*)- and (*R*)-oxo-ilmofosine did not represent an active/inactive pair of ALPs and thus, would not be useful for our proposed studies.



rac-Ilmofosine (BM 41.440)



(R)-oxo-ilmofosine

Figure 11. Structure of ilmofosine (BM41.440) and oxo-ilmofosine.

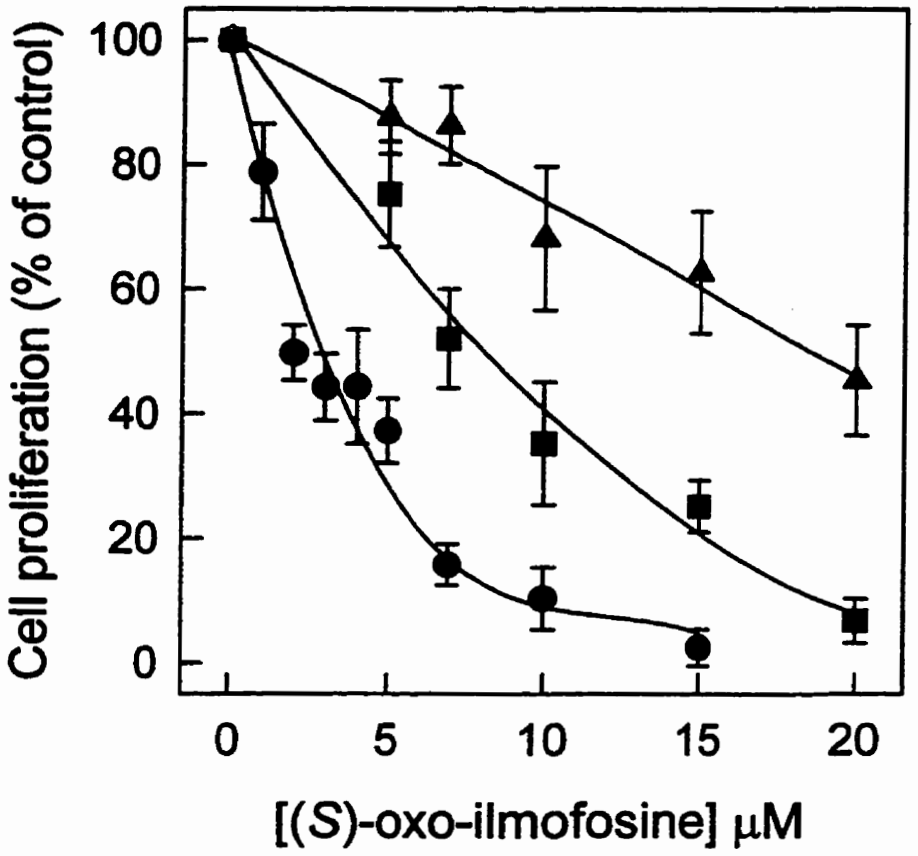


Figure 12. Effect of *(S)*-oxo-ilmofosine on the proliferation of epithelial cancer cells. The experiments were conducted with varying concentrations of *(S)*-oxo-ilmofosine (0-20 μM) on MCF-7 (●), A549 (■) and A427 (▲) cells using the experimental procedures described in Figure 7. The results are the means ± standard deviations of 3 different experiments with quadruplicate wells/experiment.

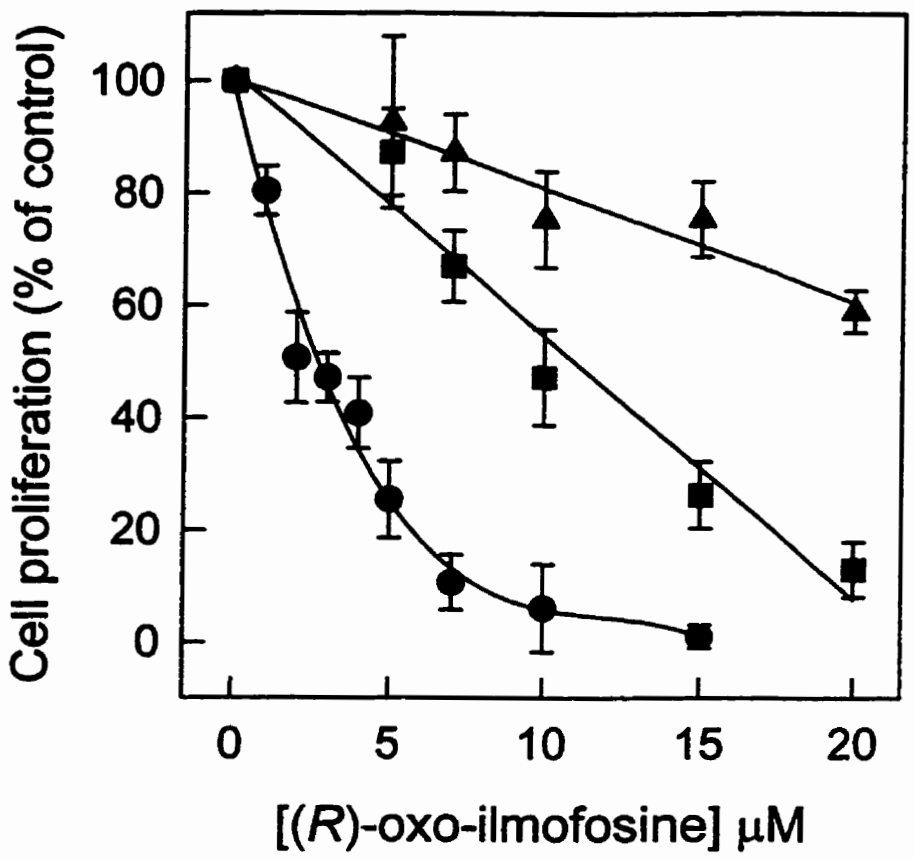


Figure 13. Effect of *(R)*-oxo-ilmofosine on the proliferation of epithelial cancer cells. The experiments were conducted with varying concentrations of *(R)*-oxo-ilmofosine (0-20 μM) on MCF-7 (●), A549 (■) and A427 (▲) cells using the experimental procedures described in Figure 7. The results are the means ± standard deviations of 3 different experiments with quadruplicate wells/experiment.

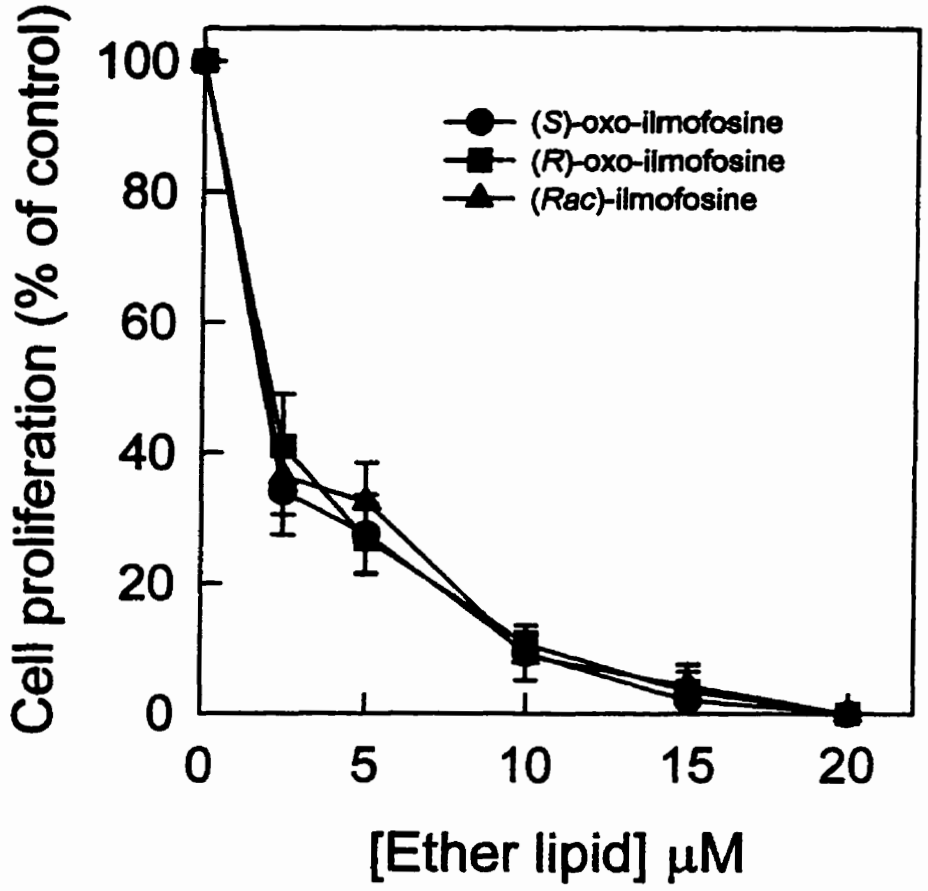


Figure 14. Effect of (*rac*)-ilmofosine, (*S*)-oxo- and (*R*)-oxo-ilmofosine on MCF-7 cell proliferation. The effect of the ilmofosine on proliferating MCF-7 cells were investigated with varying concentrations (0-20 μM) of (*rac*)-ilmofosine, (*S*)-oxo-ilmofosine or (*R*)-oxo-ilmofosine using the procedures described in Figure 7. The results are the means ± standard deviations of 2 different experiments.

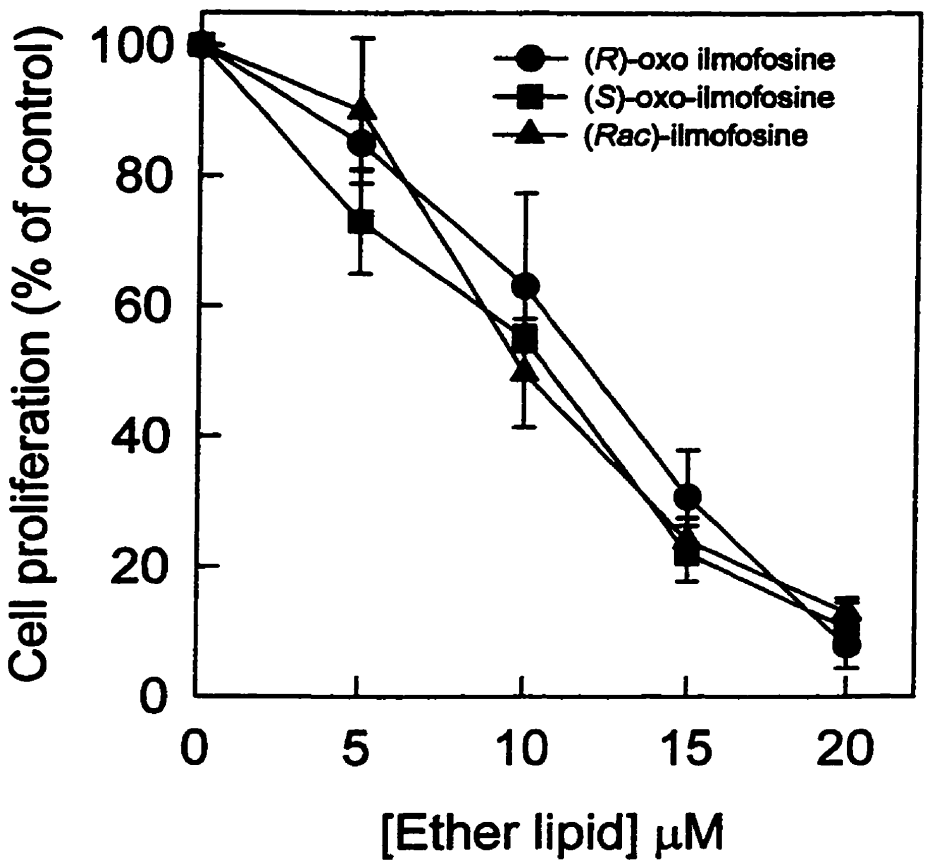
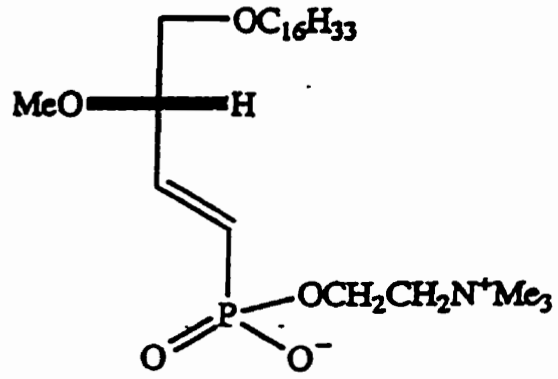


Figure 15. Effect of (*rac*)-ilmofosine, (*S*)-oxo- and (*R*)-oxo-ilmofosine on A549 cell proliferation. The effect of the ilmofosine on the proliferation of A549 were investigated with varying concentrations of (*rac*)-ilmofosine or (*S*)-oxo-ilmofosine or (*R*)-oxo-ilmofosine (0-20 µM) using the procedures described for MCF-7 cell lines (Fig 7). The results are the means ± standard deviations of 3 different experiments.

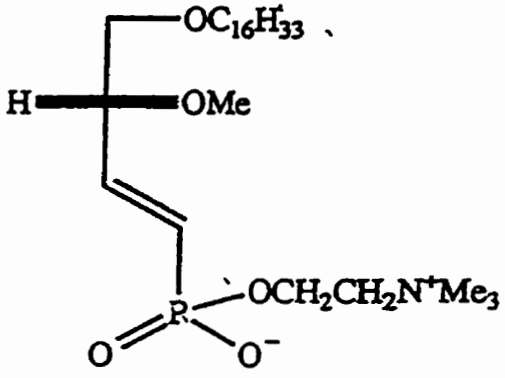
4.1.3 Effect of (*S*)- and (*R*)- enantiomers of 2'-(trimethylammonio)-ethyl-4-(hexadecyloxy)-3-methoxy-1-butenephosphonate, (ET-16-phosphono-TDB) on the proliferation of epithelial cancer cell lines

Phosphonocholine analogues of ET-16-OCH_3 were previously demonstrated to be potent antiproliferative agents (Bittmann *et al.*, 1993). These compounds are also expected to be more resilient to PLC hydrolysis due to the phosphono bond. In a previous study, no differences were observed in the potency of (*S*)- and (*R*)- enantiomers of ET-16-phosphonocholine in inhibiting the proliferation of cancer cell lines (Bittman *et al.*, 1993). In the next generation of phosphono-ALPs synthesized by Dr Bittman, a trans double bond was inserted between C-3 and C-4 to produce ET-16-phosphono-TDB **(Fig 16)**.

We investigated the effect of (*S*)- and (*R*)-ET-16-phosphono-TDB on the proliferation of MCF-7, T47D, MDA-MB-468, A427, SK-N-SH and SK-N-MC cell lines using the procedures described in **section 3.5**. The results of the study are displayed in **Fig 17 and 18.** (*R*)-ET-16-phosphono-TDB was unable to inhibit the proliferation of breast cancer cell lines, T47D and MDA-MB 468, neuroblastoma lines, SK-N-SH and SK-N-MC, and the lung cancer cell line A427 at concentrations of up to 30 μM **(Fig 17)**. Some inhibition of the proliferation of the breast cancer cell line MCF-7 was observed with increasing concentrations of (*R*)-ET-16-phosohono-TDB and at a concentration of 30 μM 50% inhibition of proliferation relative to the controls was observed. The results of the study with (*S*)-ET-16-phosphono-TDB on the proliferation of the epithelial cancer cells are shown in **Fig 18**. In contrast to the results obtained with (*R*)-ET-16-phosphono-TDB, (*S*)-ET-16-phosphono-TDB was able to inhibit the proliferation of all the cell lines studied.



(*R*)-ET-16-phosphono-TDB



(*S*)-ET-16-phosphono-TDB

Figure 16. Structures of (*S*)- and (*R*)- 2'-(Trimethylammonio)-ethyl-4-(hexadecyloxy)-3-methoxy-1-butenephosphonate, (ET-16-phosphono-TDB).

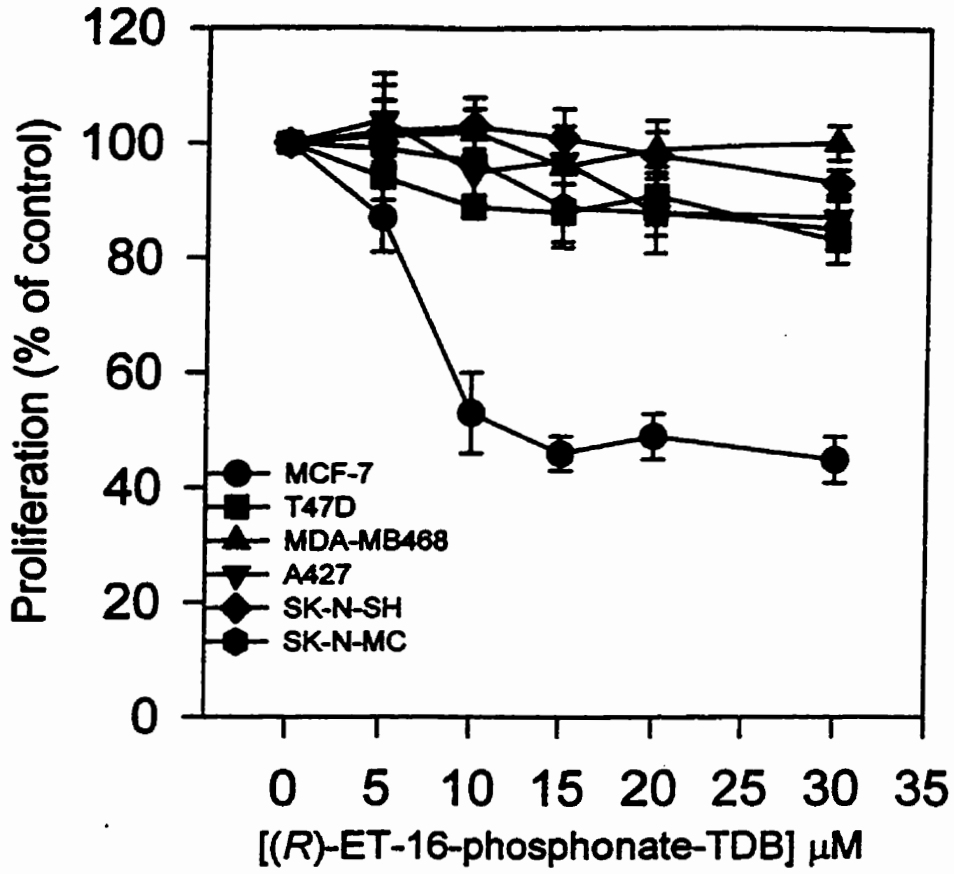


Figure 17. Effect of (*R*)-ET-16-phosphono-TDB on the proliferation of epithelial cancer cell lines. Log phase cells in 24-well plates were incubated with varying concentrations of (*R*)-ET-16-phosphono-TDB and the increase in cell numbers after 48 h were determined and expressed as a proportion of the increase in numbers of cells incubated without any drug. The results are the means ± standard deviations of quadruplicate wells from 3 different experiments.

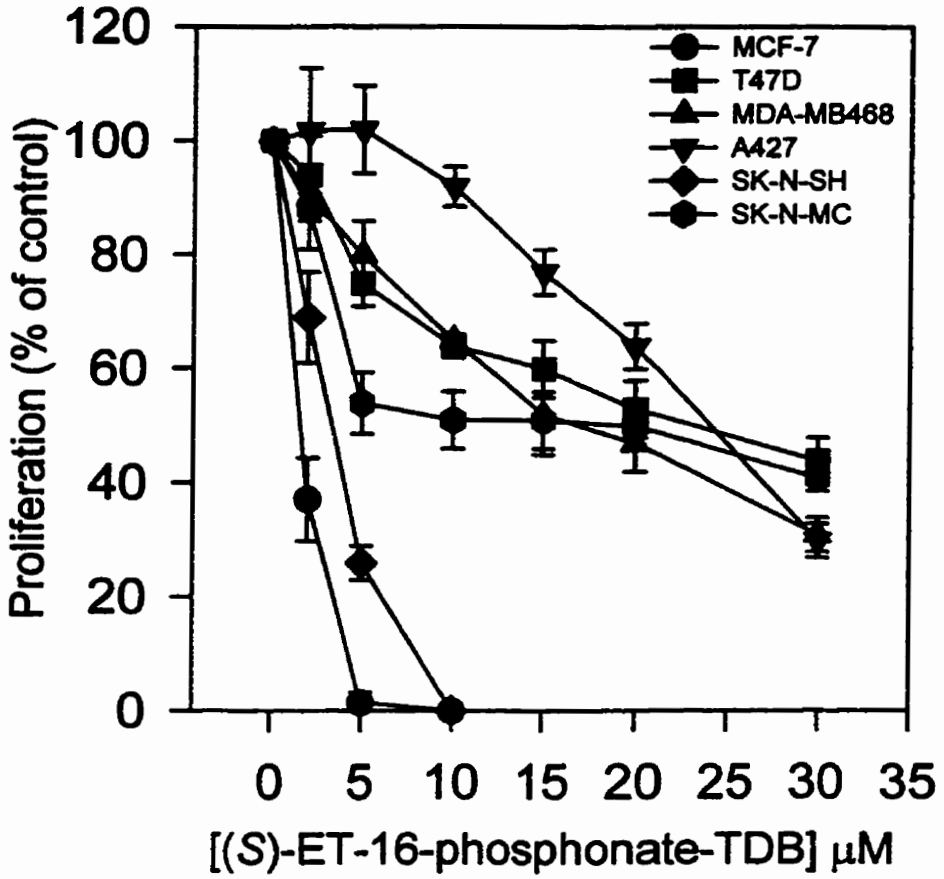


Figure 18. Effect of (*S*)-ET-16-phosphono-TDB on the proliferation of epithelial cancer cell lines. The experiments were conducted as described in Figure 17, but with *(S)*-ET-16-phosphono-TDB. The results are the means ± standard deviations of 3 different experiments.

Concentrations of 5 μM and higher completely inhibited the MCF-7 cell growth while the growth of SK-N-SH cells were completely inhibited with 10 μM of the (*S*) enantiomer. The growth of all other cell lines that were not affected by (*R*)-ET-16-phosphono-TDB were inhibited between 50-60% by (*S*)-ET-16-phosphono-TDB. Thus, the (*S*) enantiomer of ET-16-phosphono-TDB was significantly more active than *(R)* enantiomer for each cell line tested.

These results clearly identified (*S*) and (*R*)-ET-16-phosphono TDB as an active / inactive pair of compounds suitable for use in studies designed to identify cellular events whose perturbation are relevant to the mechanisms of growth inhibition by these ALPs. These compounds can therefore be used to investigate the postulated role of Raf-1 in the mechanism of action of ALPs.

4.2 Effects of (*S*) and (*R*)-ET-16-Phosphono-TDB on signal transduction via MAP kinase cascade in EGF-stimulated MCF-7 cells

Much is now known about the pathways that transmit mitogenic signals from receptor tyrosine kinases, such as the EGF receptor, to the nucleus to initiate processes that result in mitosis. Subsequent to the activation of the EGF receptor by EGF, a series of molecular events occur that culminate in the activation of Ras (Egan *et al.*, 1993; Panayotou and Waterfield, 1993). The transmission of signals from activated Ras to the nucleus involves the sequential activation of Raf, MEK and MAP kinase (Marshall, 1996). MAP kinase regulates the activity of a wide range of molecules including other kinases and nuclear transcription factors. Because the activation of MAP kinase is a key early event downstream of receptor

activation we have hypothesized that inhibition of its activation is involved in the mechanism of inhibition of cell growth by ALPs. If this hypothesis is valid we would expect the active (*S*)-ET-16-phosphono-TDB, which inhibits cell proliferation, to inhibit MAP kinase activation whereas the inactive (*R*) enantiomer, which does not inhibit cell proliferation, would not.

In view of the sensitivity of MCF-7 cells towards (*S*)-ET-16-phosphono-TDB **(Fig 18)**, and the use of these cells to establish our previous hypothesis on the role of Raf-1 in the mechanism of action of ALPs, we selected them for subsequent studies with (*S*)- and (*R*)-ET-16-phosphono-TDB to identify possible mechanisms by which the active compound achieves growth inhibition.

4.2.1 Establishment of incubation conditions with (*S*)-ET-16-phosphono-TDB to inhibit the proliferation of serum-stimulated quiescent MCF-7 cells

Before initiating studies on the effects of the double-bond phosphonocholines on the early cell signaling events, we established incubation conditions (concentration and incubation time) with (*S*)-ET-16-phosphono-TDB that inhibited the proliferation of quiescent MCF-7 cells following stimulation by 10% serum-supplemented DMEM. Quiescent MCF-7 cells were selected for these studies in order to allow a synchronised response by the cells subsequent to stimulation.

Quiescent MCF-7 cells in 6-well plates were obtained by serum-starvation using the procedures described in **section 3.6**. The cells were incubated with or without 30 μM of ET-16-phosphono-TDB in BSA-supplemented medium for various periods. The cells were then

washed and incubated with 10% FBS supplemented media and the cell number was counted every 24 h. As shown in **Fig 19**, stimulation of control quiescent cells with FBS supplemented media resulted in 2.6 and 4.4-fold increase in cell numbers after 48 and 72 h respectively. Preincubation of serum-starved quiescent MCF-7 cells with 30 μM (*S*)-ET-16-phosphono-TDB for 4 h followed by incubation with 10% FBS-supplemented medium resulted in almost complete inhibition of cell proliferation relative to control cells that had not been treated with any compound. In contrast, preincubation of the cells with 30 μM (*R*)-ET-16-phosphono-TDB for 4 hours had no effect on the cell growth following readdition of the growth medium. These results confirmed the lack of inhibitory activity of the (*R*) enantiomer, reinforcing its usefulness as a negative control compound. Assessment of cell viability by trypan blue dye exclusion assay revealed no differences between cells pretreated with (*R*)- or (*S*)-ET-16-phosphono-TDB and controls prior to readdition of the growth medium and at the end of the 72 h incubation.

Based on the above results incubation of the quiescent cells with 30 μM of (*S*)- and (*R*)-phosphono-TDB for 4 h was selected for the subsequent studies.

4.2.2 Effect of (*R*)- or (*S*)-ET-16-phosphono-TDB on MAP kinase activity in EGF-stimulated MCF-7 cells

Having established preincubation conditions with (*S*)-ET-16-phosphono-TDB which inhibited the growth of MCF-7 cells subsequent to cell stimulation, we investigated the activation of MAP kinase in control cells and in cells preincubated with the active (*S*) and inactive (*R*) enantiomers of ET-16-phosphono-TDB in response to EGF. MCF-7 cells were

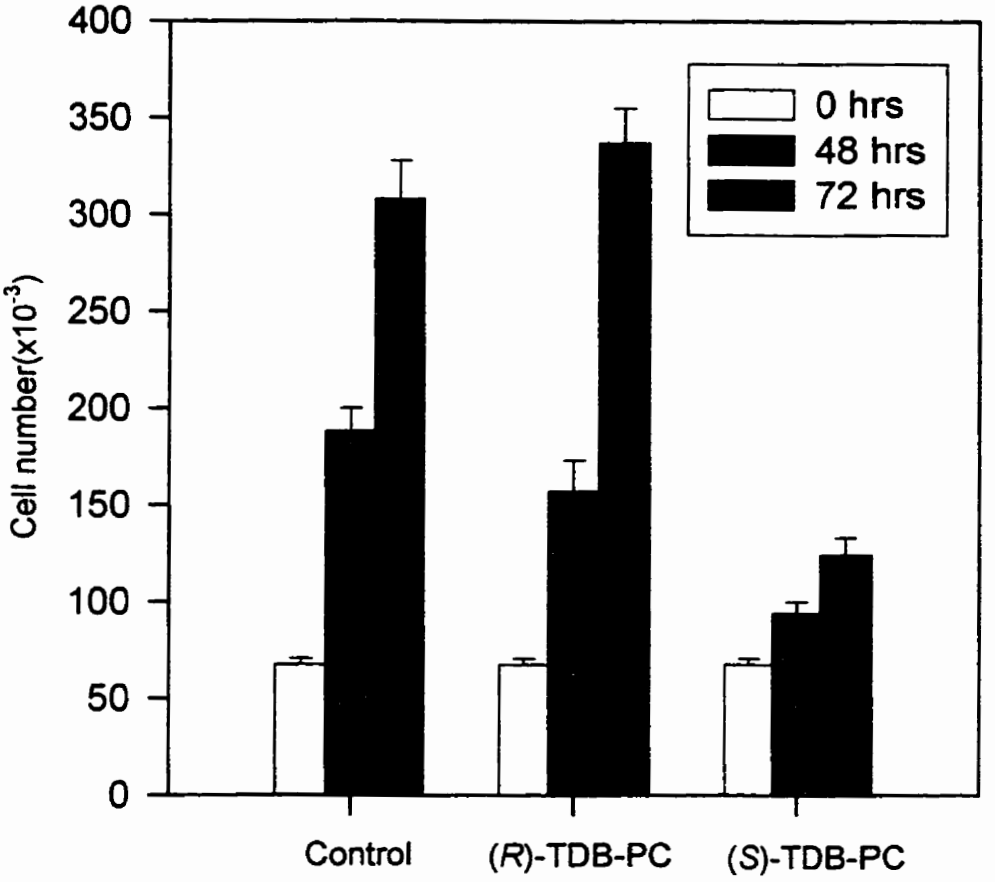


Figure 19. Effect of ET-16-phosphono-TDB preincubation on the growth of quiescent MCF-7 cells. Quiescent MCF-7 cells were incubated with 30 μM (*R*)- or (*S*)-ET-16-phosphono-TDB or no drug for 4 h. The cells were washed and incubated with 10% FBS supplemented growth medium and the increase in cell numbers were determined at the indicated times. The results are the average of 6 different determinations ±standard deviations.

made quiescent by serum starvation. The cells were then incubated with BSA-supplemented DMEM with ethanol (0.1%) or the 30 μM of each enantiomer of ET-16-phosphono-TDB for 4 h. The cells were washed, stimulated with EGF, harvested, and cell lysates were prepared as described in **section 3.10**. MAP kinase activity was measured by two different assays, *in vitro* phosphorylation of MBP and the extent of phosphorylation of MAP kinase proteins (ERK1 and ERK2). As shown in **Fig 20**, stimulation of quiescent MCF-7 cells with EGF resulted in activation of the MAP kinase activity that reached a peak between 4-10 min, followed by sustained activation at levels significantly greater than resting values for up to 30 min. EGF stimulation of cells preincubated with (*R*)-ET-16-phosphono-TDB yielded a profile of activation of MAP kinase that was relatively similar, with respect to the magnitude and duration, to that obtained in the controls. In contrast, MAP kinase activation in cells preincubated with the active (*S*)-ET-16-phosphono-TDB was prematurely truncated. The peak activity at 4 minute was significantly less than that observed in the control and cells pretreated with the (*R*) enantiomer; furthermore, the activity declined after 4 minutes, while in the controls, the activities were still increasing.

4.2.3 Direct effect of (*S*)-ET-16-phosphono-TDB on MAP kinase activity

In view of the key role that is attributed to MAP kinase in initiating cell proliferation (Marshall, 1995), our observations that (*S*)-ET-16-phosphono-TDB inhibited cell growth and was able to truncate the activation of MAP kinase, whereas (*R*)-ET-16-phosphono-TDB was unable to inhibit cell proliferation and did not affect the activation of MAP kinase suggested that inhibition of MAP kinase may be a key event in the mechanism of inhibition of cell

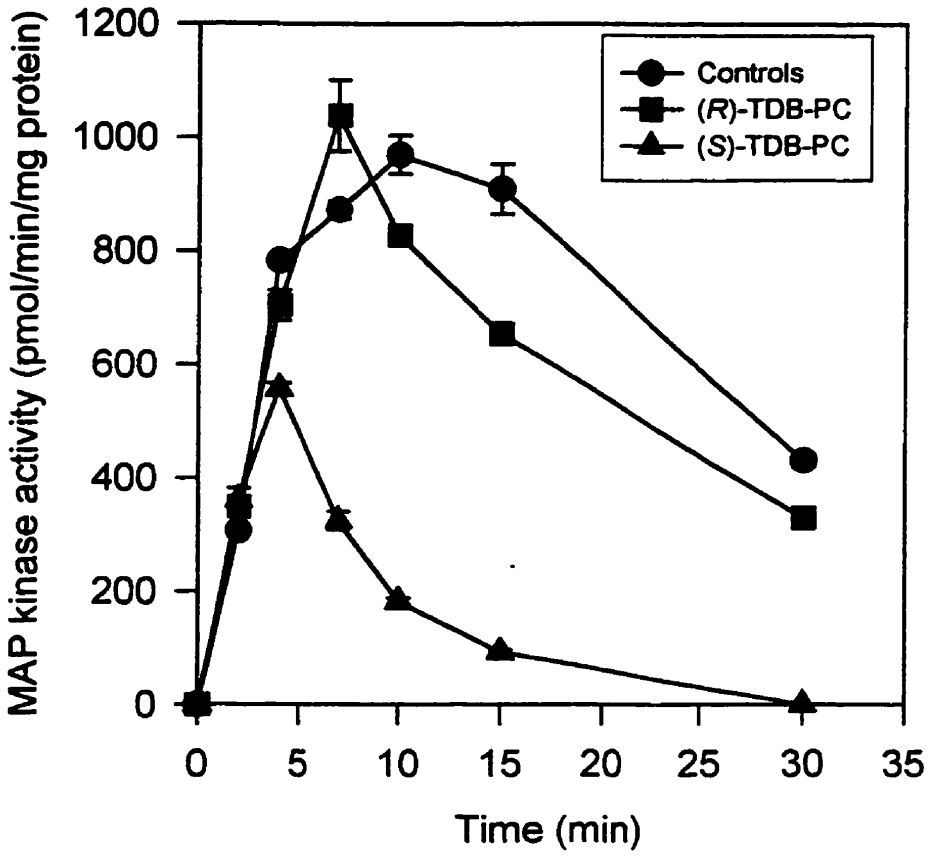


Figure 20. Effect of ET-16-phosphono-TDB preincubation on MAP kinase activity in EGF-stimulated MCF-7 cells. Quiescent MCF-7 cells were incubated without or with 30 μM (*R*)-ET-16-phosphono-TDB or (*S*)-ET-16-phosphono-TDB for 4 h. The cells were washed and stimulated with 10 ng/ml EGF for varying periods and cytosolic fractions were prepared as described in Methods section. MAP kinase activity in cytosolic fractions (1 μg protein) from cells incubated with (*R*)-ET-16-phosphono-TDB, (*S*)-ET-16-phosphono-TDB or controls without drug were measured as the phosphorylation of MBP using the *in vitro* kinase assay as described in Methods section. The results are the means ± standard deviations of triplicate incubations from a single experiment that is representative of results obtained with 3 different cell preparations.

proliferation by (*S*)-ET-16-phosphono-TDB. The activation of receptor tyrosine kinases such as the EGF receptor initiates a sequence of events that culminate in the activation of MAP kinase. Thus the perturbation of any of these events upstream of MAP kinase could conceivably result in the observed decrease in MAP kinase activation. Alternatively, the drug could have a direct effect on the activity of MAP kinase. To distinguish between these two possibilities, we investigated the effect of exogenous addition of (*S*)-ET-16-phosphono-TDB (0-50 μM) on the activity of MAP kinase using the *in vitro* MBP phosphorylation assay. Our results **(Fig 21)** showed that addition of (*S*)-ET-16-phosphono-TDB had no effect on the activity of MAP kinase in cytosolic fractions from cell lysates of EGF-stimulated control cells. These results suggest that the active compound was unlikely to interfere directly with MAP kinase enzyme to inhibit its activation, but most likely achieves this by perturbing a process (es) upstream, between the MAP kinase enzyme and the EGF receptor.

4.2.4 Effect of (*S*)-ET-16-phosphono-TDB on phosphorylation of MAP kinase

Phosphorylation of MAP kinase in cells was assessed by Western blot analysis using phospho-specific MAP kinase antibody. The results **(Fig 22)** revealed that at 4 and 10 min after EGF stimulation, phosphorylation of MAP kinase in control and (*R*)-ET-16-phosphono-TDB-pretreated cells was prominent and quantitatively similar, whereas, in cells treated with (*S*)-ET-16-phosphono-TDB the phosphorylation of MAP kinase was significantly lower than those in the control group. No differences were observed in the level of MAP kinase protein in cells treated with or without drugs **(Fig 22)**.

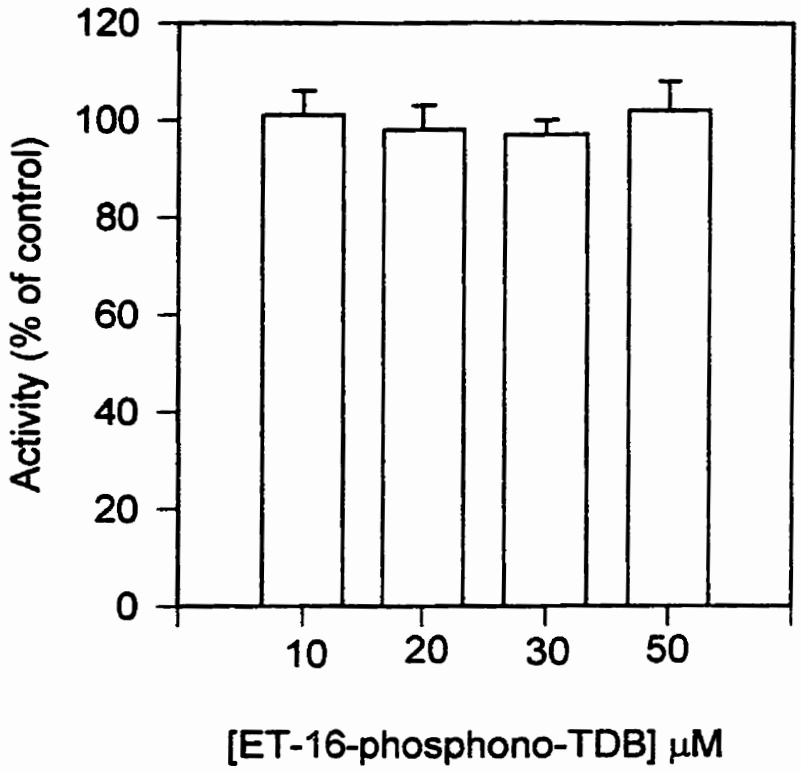


Figure 21. Effect of (*S*)-ET-16-phosphono-TDB on MAP kinase catalytic activity. MAP kinase activity in cytosolic fractions from control cells stimulated with EGF for 7 min (peak MAP kinase activity, Figure 20) was measured as the phosphorylation of MBP in the *in vitro* kinase assay in the presence of 0-50 μM (*S*)-ET-16-phosphono-TDB. The results are the means ± standard deviations of triplicate incubations from three separate experiments.

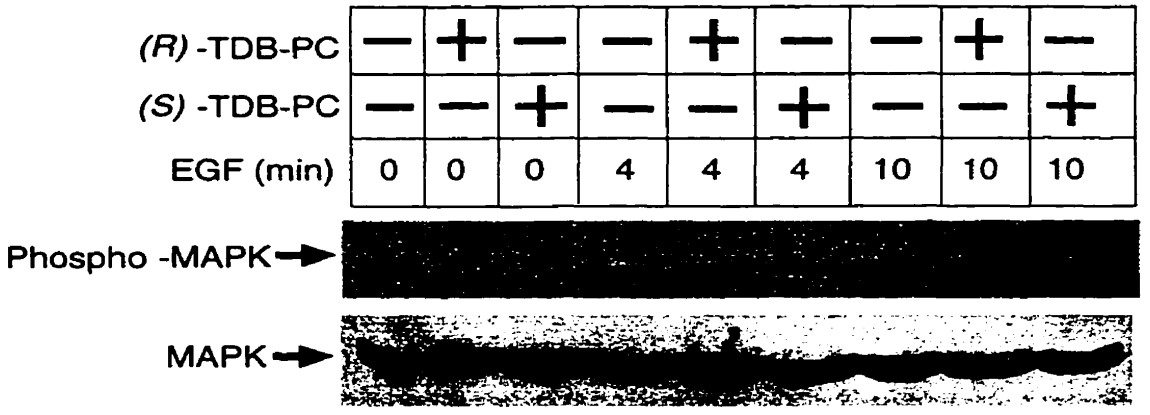


Figure 22. Effect of ET-16-phosphono-TDB preincubation on MAP kinase phosphorylation in MCF-7 cells. Quiescent MCF-7 cells were treated with or without (*R*)- or (*S*)-ET-16-phosphono-TDB for 4 h and cytosolic fractions were prepared as described in the legend to Figure 20. 30 μg or 15 μg of cytosolic protein were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and probed with either anti-phospho-MAP kinase antibody (gels loaded with 30 μg protein) or with the anti-MAP kinase antibody (gels loaded with 15 μg protein). The membranes were then incubated with HRP-conjugated secondary antibody and the bound antibody was visualised by chemiluminescence. The results are from a single experiment that is representative of those obtained with 3 different cell preparations.

Thus, preincubation of MCF-7 cells with (*S*)-ET-16-phosphono-TDB but not with (*R*)-ET-16-phosphono-TDB decreased MAP kinase activation as a consequence of decreased MAP kinase phosphorylation.

4.2.5 Effect of (*R*)- or (*S*)-ET-16-phosphono-TDB on S6 kinase activation in EGF-stimulated MCF-7 cells

There is ample evidence to support a role for MAP kinase in the activation of S6 kinase (Ahn and Krebs, 1990; Bogoyevitch *et al.*, 1994; Sturgill *et al.*, 1988). We therefore investigated whether the differential effects of (*S*)- and (*R*)-ET-16-phosphono-TDB on the activation of MAP kinase would translate to similar effects on S6 kinase activation. The kinetics of the phosphorylation of RRLSSLRA, a peptide that is specifically phosphorylated by S6 kinases, by lysates obtained from EGF-stimulated control cells and those obtained from cells preincubated with the phosphonocholines are shown in **Fig 23**. Very little differences were observed in the magnitude and duration of activation of S6 kinase by cytosol from controls and cells incubated with (*R*)-ET-16-phosphono-TDB. In cells incubated with (*S*)-ET-16-phosphono-TDB, a decrease in the magnitude and duration of activation of S6 kinase were observed in comparison to control groups. These results are what one would predict on the basis of the observed effects of the compounds on the activation of MAP kinase. Thus the extent of inhibition of MAP kinase activity by the (*S*)-ET-16-phosphono-TDB is sufficient to affect the transduction pathways downstream of MAP kinase.

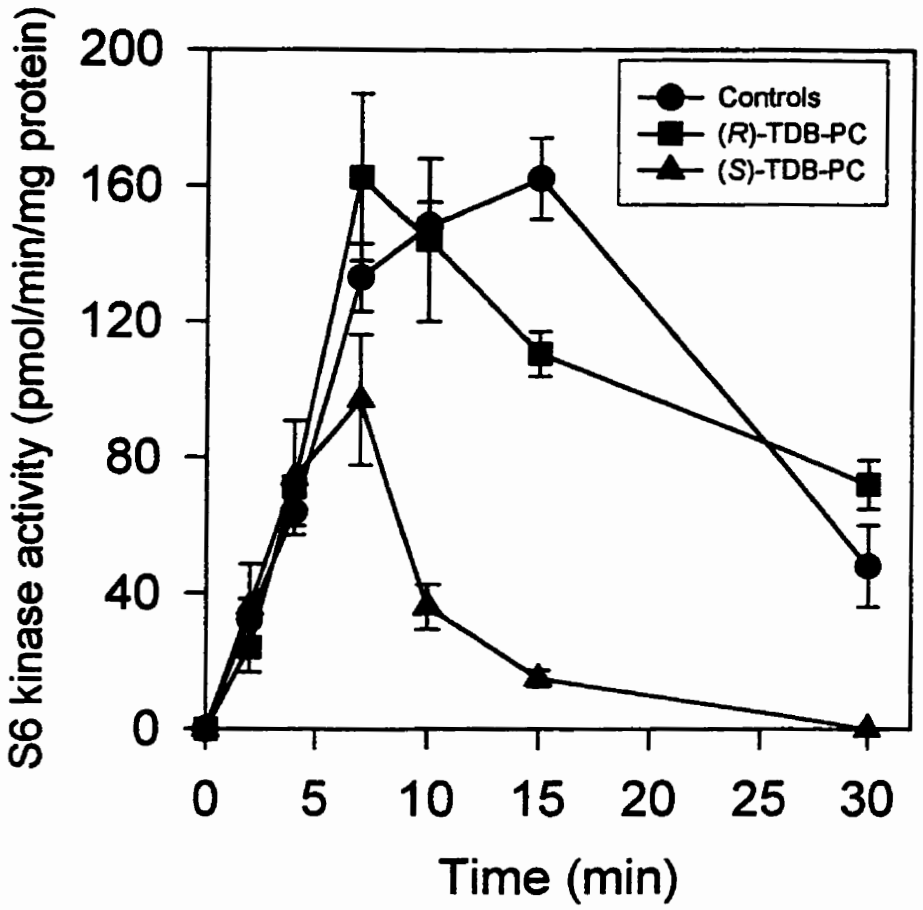


Figure 23. Effect of ET-16-phosphono-TDB on S6 kinase activity in EGF-stimulated MCF-7 cells. Cytosolic fractions from the experiment described in Figure 20 were used to assay S6 kinase activity. The kinase activity of fractions from cells incubated with (*R*)-ET-16-phosphono-TDB, (*S*)-ET-16-phosphono-TDB or without drug were measured as the phosphorylation of S6 peptide (RRLSSLRA) using the procedures described in the Methods section. The results are the means ± standard deviations of triplicate incubations from a single experiment that is representative of 3 with different cell preparations.

4.2.6 Effect of (*S*)- and (*R*)-ET-16-phosphono-TDB on the phosphorylation of MEK.

The activation of MAP kinase in EGF-stimulated cells requires the phosphorylation and activation of MEK by Raf (Posada *et al.*, 1993; Zhou *et al.*, 1996). The effects of (*R*)- or (*S*)-ET-16-phosphono-TDB on MEK phosphorylation was investigated as a measure of Raf activation. Quiescent MCF-7 cells were incubated with or without the double-bond phosphonocholines (30 μM) for 4 h, the cells were stimulated with EGF and subcellular fractions were prepared. Aliquots of the cytosolic fractions were subjected to Western blot analysis with an anti phospho-MEK-specific antibody. The results showed that in cells pretreated with (*S*)-ET-16-phosphono-TDB prior to stimulation with EGF, MEK phosphorylation was inhibited, whereas in cells treated with (*R*)-ET-16-phosphono-TDB, there was very little effect on the phosphorylation of MEK compared to control cells that were not treated with any ALP **(Fig 24)**. These results suggest that in cells preincubated with (*S*)-ET-16-phosphono-TDB, Raf-1 is unable to phosphorylate MEK to levels observed in control or (*R*)-ET-16-phosphono-TDB-treated cells.

4.2.7 Effect of (*R*)- or (*S*)-ET-16-phosphono-TDB on Raf-kinase activity

The inhibition of MEK phosphorylation by (*S*)-ET-16-phosphono-TDB observed in **section 4.2.6** indicated that the compound could have a direct effect on the catalytic activity of Raf-1. To investigate whether (*S*)-ET-16-phosphono-TDB was an inhibitor of Raf-1, (*R*)- or (*S*)-ET-16-phosphono-TDB were added to *in vitro* assays with recombinant human active Raf-1. As shown in **Fig 25**, the addition of 40 μM of either (*R*)- or (*S*)-ET-16-phosphono-TDB to the kinase assays had no effect on the phosphorylation of full length MEK by human

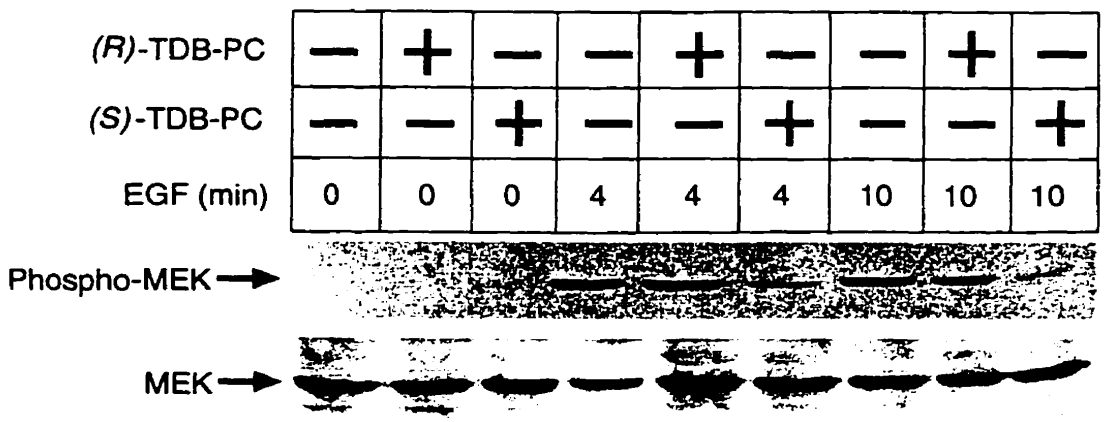


Figure 24. Effect of ET-16-phosphono-TDB preincubation on the phosphorylation of MEK. Quiescent MCF-7 cells were incubated with or without ET-16-phosphono-TDB enantiomers and the cytosolic fractions were prepared as described in Figure 20. Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes for Western blot analysis. 40 µg protein was used for Western blot analysis with the anti-phospho-MEK antibody and 20 µg for the anti-MEK antibody. The results are from a single experiment that is representative of results obtained with 3 different cell preparations.

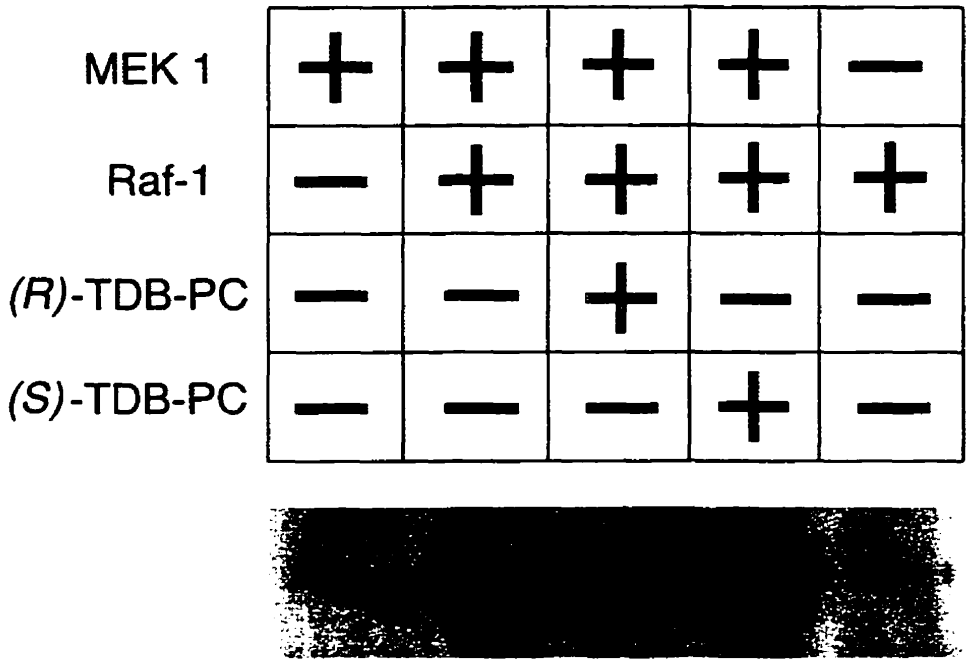


Figure 25. Effects of *(S)*- and *(R)*-ET-16-phosphono-TDB on Raf-1 kinase activity. The kinase activity of recombinant catalytically active Raf-1 was measured in the presence of *(R)*- or *(S)*-ET-16-phosphono-TDB by the *in vitro* kinase assay that measured the phosphorylation of full length MEK. The reaction was terminated by the addition of 1x SDS sample buffer and the sample were resolved by 10% SDS-PAGE and subjected to autoradiography. The results are from a single experiment that is representative of 2 different experiments.

active Raf-1 kinase. These results indicated that the decreased MEK phosphorylation observed in (*S*)-ET-16-phosphono-TDB treated cells is not due to a direct inhibition of Raf-1 kinase activity by the compound.

4.2.8 Effect of (*S*)- or (*R*)-ET-16-phosphono-TDB on membrane-associated Raf-1 levels in EGF-stimulated MCF-7 cells

As (*S*)-ET-16-phosphono-TDB does not inhibit Raf kinase activity directly but inhibited MEK phosphorylation, this indicated that the compound may be inhibiting the activation of Raf-1 as demonstrated for ET-18-OCH_3 (Zhou *et al* 1996). ET-18-OCH_3 achieved this by decreasing the quantity of Raf associated with cell membranes. We therefore investigated whether the levels of Raf-1 associating with the membranes were affected by pretreatment of the cells with (*S*)-ET-16-phosphono-TDB prior to EGF stimulation. Quiescent MCF-7 cells were incubated with or without (*S*) or (*R*)-ET-16-phosphono-TDB (30 μM) for 4 h. The cells were stimulated with EGF, followed by harvesting and isolation of the membrane fractions as described in **section 3.10**. The level of Raf associated with the membrane fraction was assessed by Western blot analysis with anti-Raf-1 antibodies. The results obtained revealed that in membranes isolated from unstimulated cells incubated with either (*R*) or (*S*)-ET-16-phosphono-TDB, the levels of Raf were higher than in membranes isolated from control cells that had not been treated with either compound **(Fig 26 and 27)**. Western blot analysis of identical drug-treated membrane extracts with PKC-specific antibodies revealed no translocation of PKC (α,γ or ϵ) to the membrane. Stimulation of control cells with EGF resulted in the expected translocation of Raf to the membrane (Fig

27). Densitometric analysis of the blots revealed that the level of Raf, in arbitrary units, increased from 0.068 in unstimulated control cells to 0.58 after 2 min stimulation with EGF. In cells treated with (*R*)-ET-16-phosphono-TDB, the corresponding values were 0.906 to 1.507 while the value for cells treated with (*S*)-ET16-phosphono-TDB went from 1.2 to 1.49 in the same period. Thus, in cells treated with either (*R*)- or (*S*)-ET-16-phosphocholine-TDB, stimulation with EGF also resulted in additional translocation of Raf-1 to the membrane. However, the changes relative to the resting levels in the phosphono-TDB-treated cells, were not as high as those observed in the control cells. These results indicate that although these compounds do not appear to affect the EGF-dependent translocation of Raf to the membrane, there may be a limit to the levels of Raf that can associate with the membrane.

4.2.9 Effect of (*R*)- or (*S*)-ET-16-phosphono-TDB on *in vitro* Raf-Ras interaction

The increase in Raf-1 translocation by (*S*)- and (*R*)-ET-16-phosphono-TDB in unstimulated cells was unexpected, as was the apparent inability of the (*S*) enantiomer to decrease the levels of membrane associated Raf in stimulated cells. Translocation of Raf to the membrane following cell stimulation is mediated by activated Ras (Ras-GTP) (Chang *et al.*, 1994; Ghosh and Bell, 1994; Hu et al., 1995; Zhang *et al.*, 1993). We therefore investigated whether the inability of the (*S*)-ET-16-phosphono-TDB to inhibit Raf translocation could be demonstrated in an *in vitro* assay.

Quiescent MCF-7 cells were treated with or without (*R*)- or (*S*)-ET-16-phosphono-TDB (30 μM) for 4 h. After washing, the cytosolic fractions were prepared from cells.

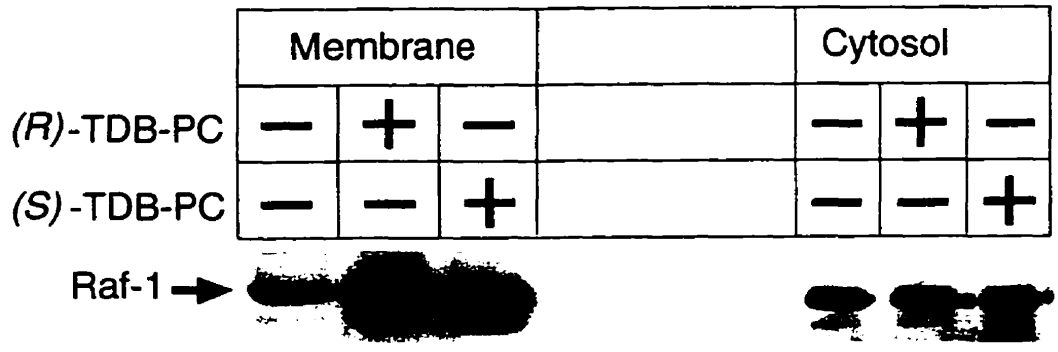


Figure 26. Effect of *(S)*- and *(R)*-ET-16-phosphono-TDB on levels of membrane-associated Raf-1 in unstimulated quiescent MCF-7 cells. MCF-7 cells were incubated without or with 30 μM of *(R)*- or *(S)*-ET-phosphono-TDB for 4 h. Membrane and cytosolic fractions were prepared by differential centrifugation as described in the methods. 15 μg of membrane and 5 μg of cytosolic protein were separated by 10% SDS-PAGE for Western blot analysis with the anti Raf-1 antibody. The results are from a single experiment that is representative of results from 4 different cell preparations.

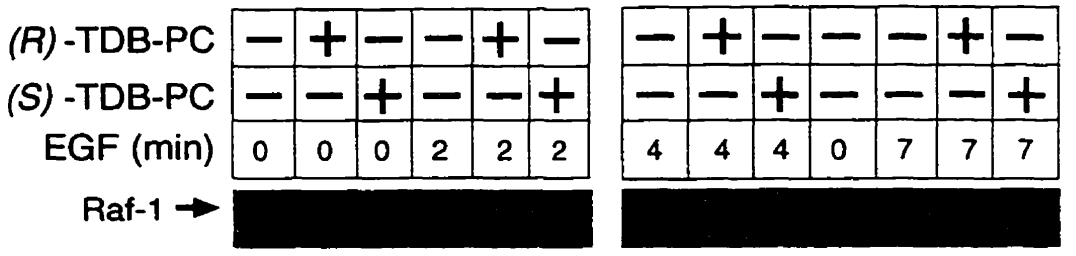


Figure 27. Effects of *(S)*- and *(R)*-ET-16-phosphono-TDB on the translocation of Raf-1 in EGF-stimulated in MCF-7 cells. Quiescent MCF-7 cells were treated with or without 30 μM *(S)*- or *(R)*-ET-16-phosphono-TDB for 4 h. The cells were washed and stimulated with EGF for selected periods and the membrane fractions were isolated and analysed for Raf content by Western blot analysis using the Raf-1-specific antibody. The results are from a single experiment that is representative of results obtained from 3 different experiments.

Purified GST-Ras fusion protein loaded with GTP-γ-S or GDP (Jilkina and Bhullar, 1996), were incubated with the cytosol (1 mg protein) for 1.5 h, at 4°C. The beads were sedimented by centrifugation and after washing, the proteins were dissolved in 1x SDS-sample buffer boiled and subjected to Western blot analysis with anti Raf-1 antibody. The blots were analysed by densitometry. The results which are displayed in **Fig 28,** showed that the affinity of Ras-GDP for Raf-1 in the cytosol of cells treated with either (*S*)- or (*R*)-ET-16-phosphono-TDB was not greater than that observed in experiments with cytosol from control cells. Preincubating the cells with ET-16-phosphono-TDB did not affect the levels of cytosolic Raf binding to Ras-GTP compared to controls. On the other hand, there was a 2-fold increase in the levels of Raf from cytosol isolated from control cells, (*S*)- or (*R*)-ET-16-phosphono-TDB- treated cells bound to Ras-GTP compared to Ras-GDP **(Fig 28)**. In similar experiments with cytosol from ET-18-OCH_3-treated cells (20 μM for 3 h, Zhou *et al.*, 1996), a 2-fold decrease in the binding of Raf-1 from ET-18-OCH_3-treated cells to Ras-GTP was observed **(Fig 29)**. These observations that preincubation of cells with ET-18-OCH_3 decreases the affinity of cytosolic Raf-1 for Ras-GTP, suggest that the decreased level of membrane-associated Raf-1 in ET-18-OCH_3 -treated cells may be due to inhibition of Ras-GTP-mediated translocation of Raf-1 by the ALP. On the other hand, both (*S*)- and (*R*)-ET-16-phosphono-TDB do not affect the affinity of Raf for Ras-GTP and do not inhibit Ras-GTP mediated Raf translocation.

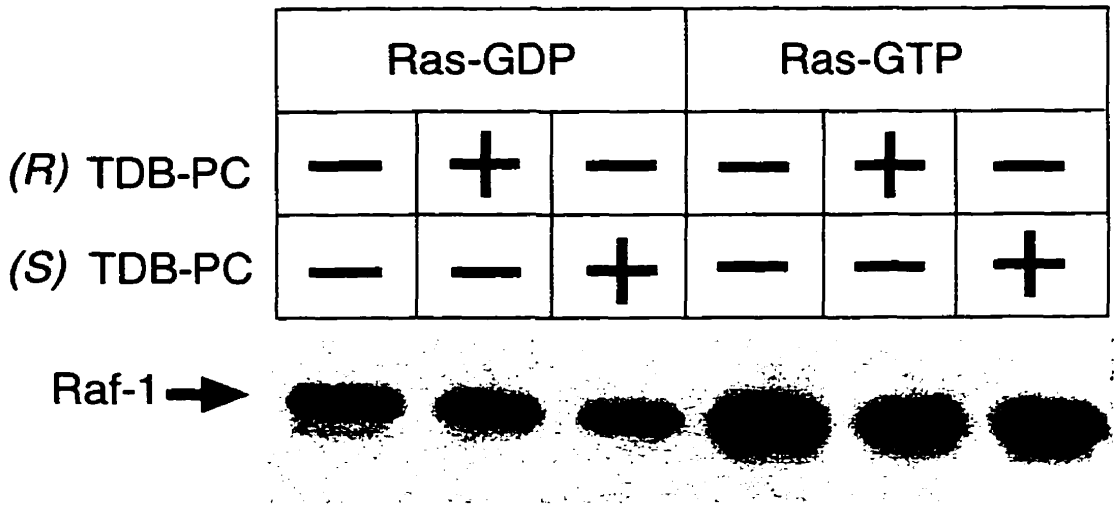


Figure 28. Effect of *(S)*- and *(R)*-ET-16-phosphono-TDB on the interaction of Raf with Ras-GDP and Ras-GTP. Quiescent MCF-7 cells were incubated without or with 30 μM (*S*)- or (*R*)-ET-16-phosphono-TDB for 4 h. Cytosolic fractions were prepared as described in the methods and 1 mg freshly prepared cytosolic protein was incubated with 25 μl of purified GST-Ras fusion protein loaded with GTP-γ-S or GDP for 1.5 h at 4^0C. The beads were obtained by brief centrifugation, washed 3 times and the proteins were dissolved in 1x SDS sample buffer. Samples were then subjected to Western blot analysis as described in Figure 22 using anti-Raf-1 antibody. The results are from a single experiment that is representative of that obtained with 3 different cell preparations.

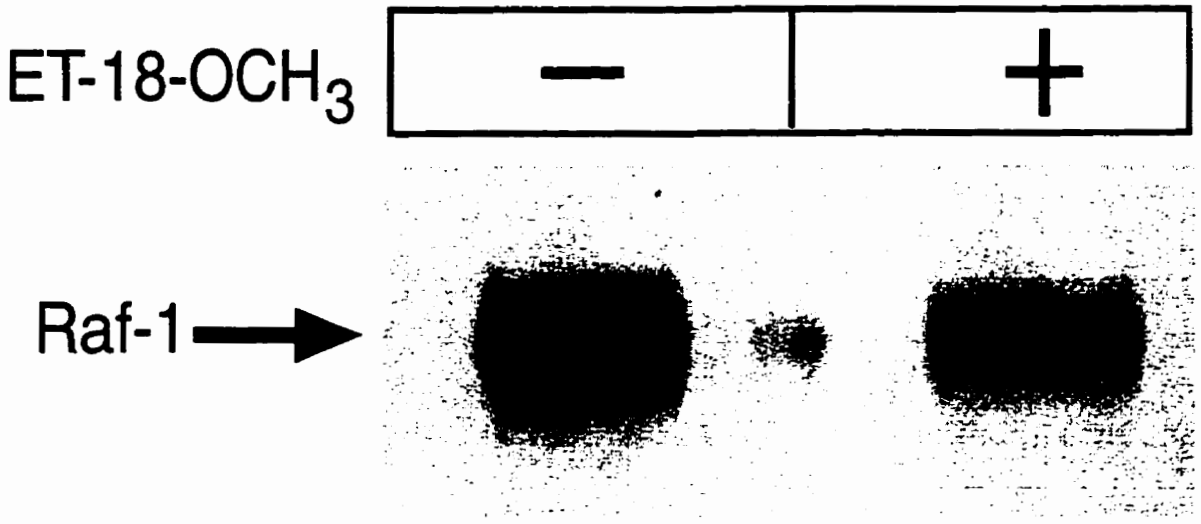


Figure 29. Effect of ET-18-OCH_3 on *in vitro* Raf-1/Ras-GTP interaction. Quiescent MCF-7 cells were preincubated without or with 20 μM ET-18-OCH_3 for 3 h. Cytosolic fractions were prepared and the Raf-1/Ras-GTP binding experiments were performed as described in Figure 28. The results are from a single experiment that is representative of that obtained with 3 different cell preparations .

4.3 Mechanism of selective inhibition of cell proliferation by ALPs

ALPs inhibit cell proliferation without interaction with cellular DNA (Berdel,1991; Lohmeyer and Bittman, 1994) and their antiproliferative effects are cancer cell-selective. More importantly, concentrations of ALPs that inhibit cancer cell proliferation do not appear to affect the growth of normal cells (Berdel *et al.*, 1985; Lohmeyer and Bittman, 1994). Although this selectivity of ALPs for cancer cells relative to normal cells is widely accepted, it is based primarily on comparative studies between leukemic cells and macrophages, myelocytes and normal bone marrow cells (Andreesen *et al.*, 1978; 1979; Berdel *et al.*, 1985). In spite of the fact that most cancers are epithelial in origin, few studies have compared the effects of ALPs between epithelial cancer cells and normal primary epithelial cells from the same tissue. The mechanism(s) underlying the selective cytotoxicity of these compounds has also not been established.

We have confirmed previous reports (Kosano *et al.*, 1990; Lu and Arthur, 1993) that the human mammary adenocarcinoma cell line, MCF-7, is very sensitive to the inhibitory effects of ET-18-OCH_3 (Fig 7). In the previous section, our studies with a pair of structurally related active/inactive ALPs have confirmed the suggestion that decreased Raf-1 activation which leads to inhibition of MAP kinase, is likely to be involved in the inhibition of cancer cell growth by ALPs. If ALPs inhibit Raf-1-dependent mitogenic signal transduction pathway as our studies suggest, then the cell-selective effects of ALPs might be either due to the use of Raf-1-independent mitogenic signaling pathways by insensitive cells, or fundamental differences in the processes of Raf-1 activation in sensitive and insensitive cells. To investigate which of these scenarios might be responsible for the selective effects of ALPs,

we conducted comparative studies between an ALP-sensitive cancer cell and its normal ALP-insensitive counterpart.

4.3.1 Effects of ET-18-OCH_3 on the proliferation of human normal mammary epithelial cells

The ALP-sensitive cancer cell selected for our studies was the MCF-7 cell line. As normal cells are apparently insensitive to growth inhibition by ALPs, we intended to use human normal mammary epithelial (HNME) cells, if they were ALP-insensitive, for the comparative studies with MCF-7 cells. We therefore needed to establish that HNME cells were indeed resistant to the growth-inhibitory effect of ET-18-OCH_3. Commercially available HNME cells are cultured in serum-free medium while MCF-7 cells are cultured in serum-supplemented medium. This precludes a direct comparison of the effects of ET-18-OCH_3 on the proliferation of the cells in their respective media since binding of ET-18-OCH_3 to proteins reduces the effective free concentration that interacts with the cell (Kotting *et al.*, 1992; Storch *et al.*, 1987). We therefore needed to equalize the protein content of the serum-free mammary epithelial growth medium (MEGM) and FBS-supplemented media. The protein content of 10% FBS-supplemented medium was determined to be 3 mg/ml. Consequently, before conducting studies on the effect of ET-18-OCH_3 on HNME cell proliferation, the media was supplemented with BSA to a value of 3 mg/ml. The proliferation rate of HNME cells growing in MEGM supplemented with BSA (3 mg/ml) is similar to that of MCF-7 cells growing in 10% FBS-supplemented medium (**Fig 30**).

The results obtained on the effect of increasing concentrations of ET-18-OCH_3 on

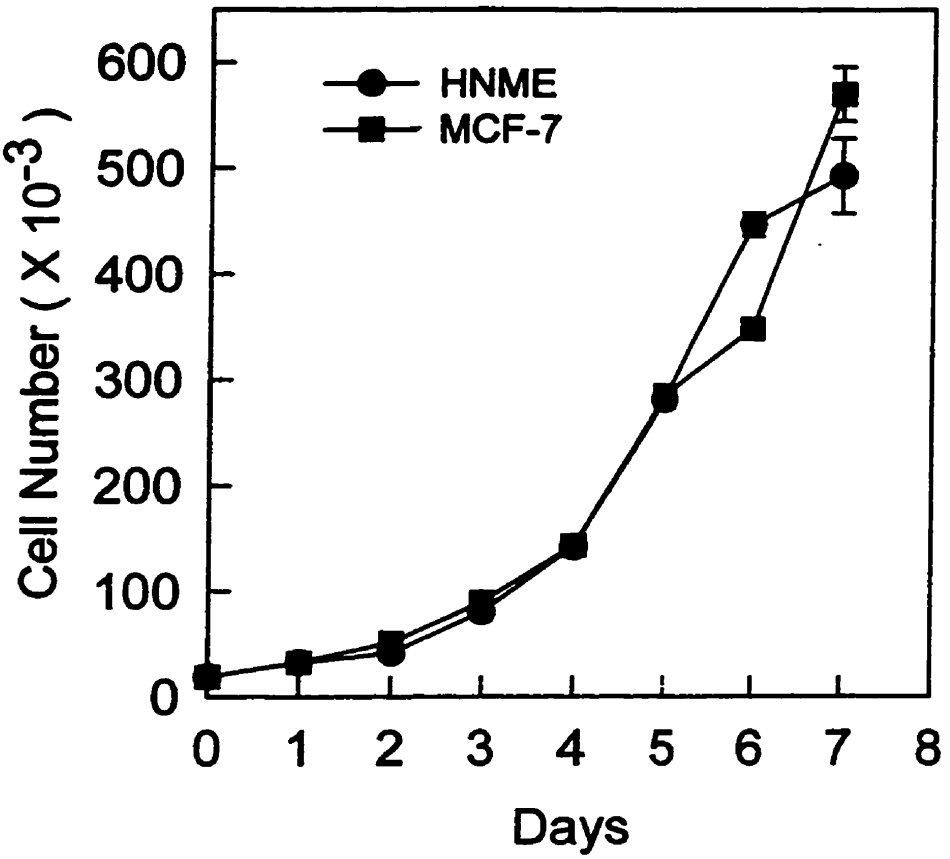


Figure 30. Comparison of growth rate between MCF-7 and HNME cells. MCF-7 cells were grown in 10% FBS-supplemented DMEM and HNME cells were grown in MEGM. The cell numbers were counted daily. The results are the means ± standard deviations of quadruplicate wells from 3 different studies.

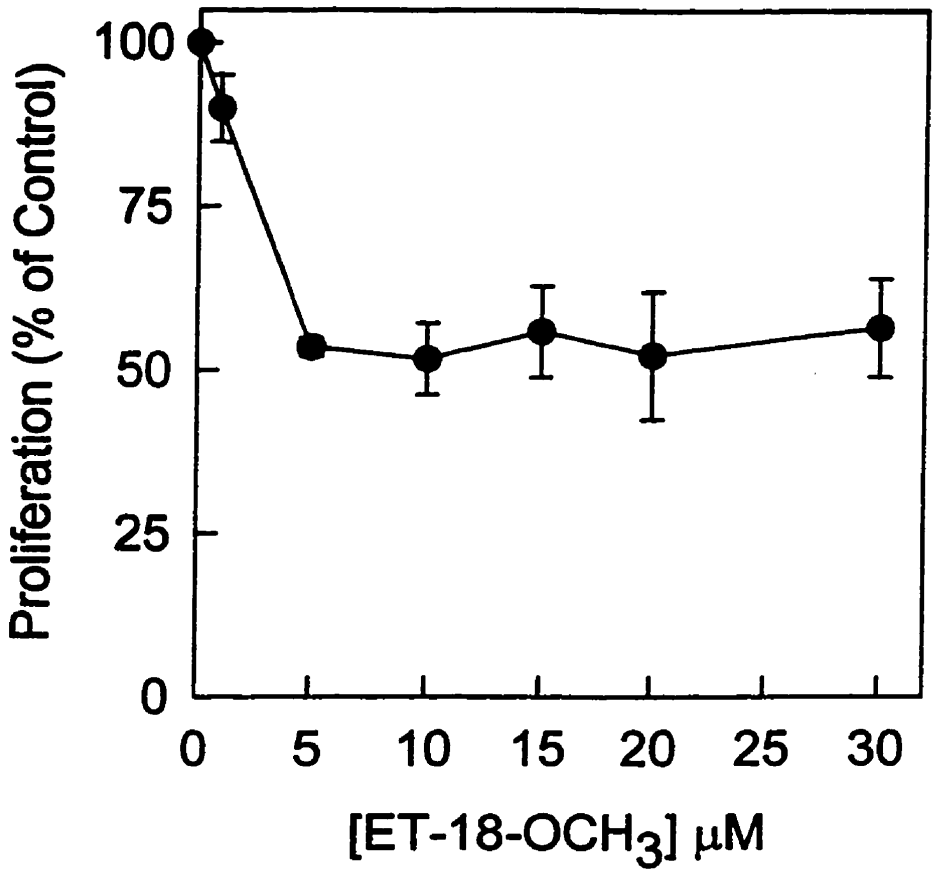


Figure 31. Effects of ET-18-OCH_3 on the proliferation of human normal mammary epithelial (HNME) cells. Proliferating log phase HNME cells growing in 24-well plates were incubated with mammary epithelial growth medium (MEGM) supplemented with 3 mg/ml BSA and different concentrations of ET-18-OCH_3 (0-30 μM). The cell numbers were determined 48 h after the addition of drug and the increase in cell number over 0 day was expressed as a % of the increase in controls. The results represent the means ± standard deviations of quadruplicate wells from 5 different studies. Each 24-well plate had its own controls with no ET-18-OCH_3.

the growth of HNME cells are shown in **Fig 31**. In contrast to MCF-7 cells, HNME cells were quite insensitive to the effect of the ALP. A 45% decrease in the rate of proliferation relative to controls was observed following incubation of HNME cells with 5 μM of ET-18-OCH_3, but no further decrease in proliferation occurred with concentrations of up to 30 μM ET-18-OCH_3. Hence this normal primary epithelial cell is relatively insensitive to growth inhibition by ET-18-OCH_3 indicating that the notion of differential effects of ET-18-OCH_3 on cancer and normal cells extends beyond hemopoietic cells.

Having demonstrated that HNME cells are relatively resistant to growth inhibition by ET-18-OCH_3, we decided to grow MCF-7 cells in serum-free medium because the HNME cells differentiate in serum-containing medium. This would then allow direct comparative studies to be conducted between both cell types in serum-free medium.

4.3.2 Formulation of two serum-free media for MCF-7 cell growth

Although the growth of MCF-7 cells in serum-free medium has been previously reported (Barnes, 1979), in our hands, we were unable to obtain any growth of MCF-7 cells in the formulation. The reasons for this are unclear. We therefore modified the medium, based on the above mentioned report (Barnes, 1979) and other reports in the literature (Band and Sager, 1989; Barnes and Sato, 1980) and examined their effect on the growth of MCF-7 cells. A supplemented basal medium (SBM) consisting of DMEM/F-12 (1:1) pH 7.4, supplemented with EGF (20 ng/ml), insulin (5 μg/ml), BPE (52 μg/ml), and insulin-transferrin-selenium-ethanolamine-supplemented (1%, v/v, added primarily to provide ethanolamine and selenium) was formulated. MCF-7 cells remained viable in this medium as

judged by the trypan blue dye exclusion assay and the increase in cell numbers over time. However, the rate of growth was much less than that of cells growing in FBS-supplemented medium. In an attempt to increase the growth rate, the effect of each of the following was investigated: hydrocortisone (1 μg/ml), 0.5 mg/ml fetuin, 7.5 μg/ml fibronectin, 100 ng/ml $PGF_{2\alpha}$ and 25 μg/ml transferrin. The highest rate of growth of MCF-7 cells was observed with SBM plus either $PGF_{2\alpha}$, transferrin or fibronectin **(Fig 32)**. Further studies with combinations of these factors led to the development of two types of serum-free growth medium designated as adapted cell growth media (ACGM). One was $ACGM^-$, which consisted of SBM with fibronectin (5 μg/ml) and transferrin (25 μg/ml) and the other $ACGM^+$, was made up of $ACGM^-$ with $PGF_{2\alpha}$ (100 ng/ml) **(section 3.1)**.

To further improve the growth rate of MCF-7 cells in the serum-free media, MCF-7 cells were adapted for growth in both $ACGM^-$ and $ACGM^+$. To achieve this, MCF-7 cells were grown to confluence in 10% FBS-supplemented DMEM and subcultured into medium diluted 1:1 with ACGM to give a final FBS content of 5%. Subsequently at every passage, the FBS content was sequentially reduced by 50% by diluting with the ACGM until when a concentration of 0.01% FBS was reached the cells were subcultured directly into the ACGM. The MCF-7 cells adapted in $ACGM^-$ were designated $MCF\text{-}7Ad^-$ and those adapted in $ACGM^+$ were designated $MCF\text{-}7Ad^+$. Although the cells proliferated, they did not spread or adhere as well as the parental MCF-7 cells. Comparative studies were therefore conducted on the effect of laminin (4 μg/ml), collagen I(20 μg/ml), collagen IV (20 μg/ml), FBS (10%) or BSA (3 mg/ml) on cell adhesion and spreading. Tissue culture-ware was incubated with the above factors for 24 h. The media was removed completely by suction prior to the

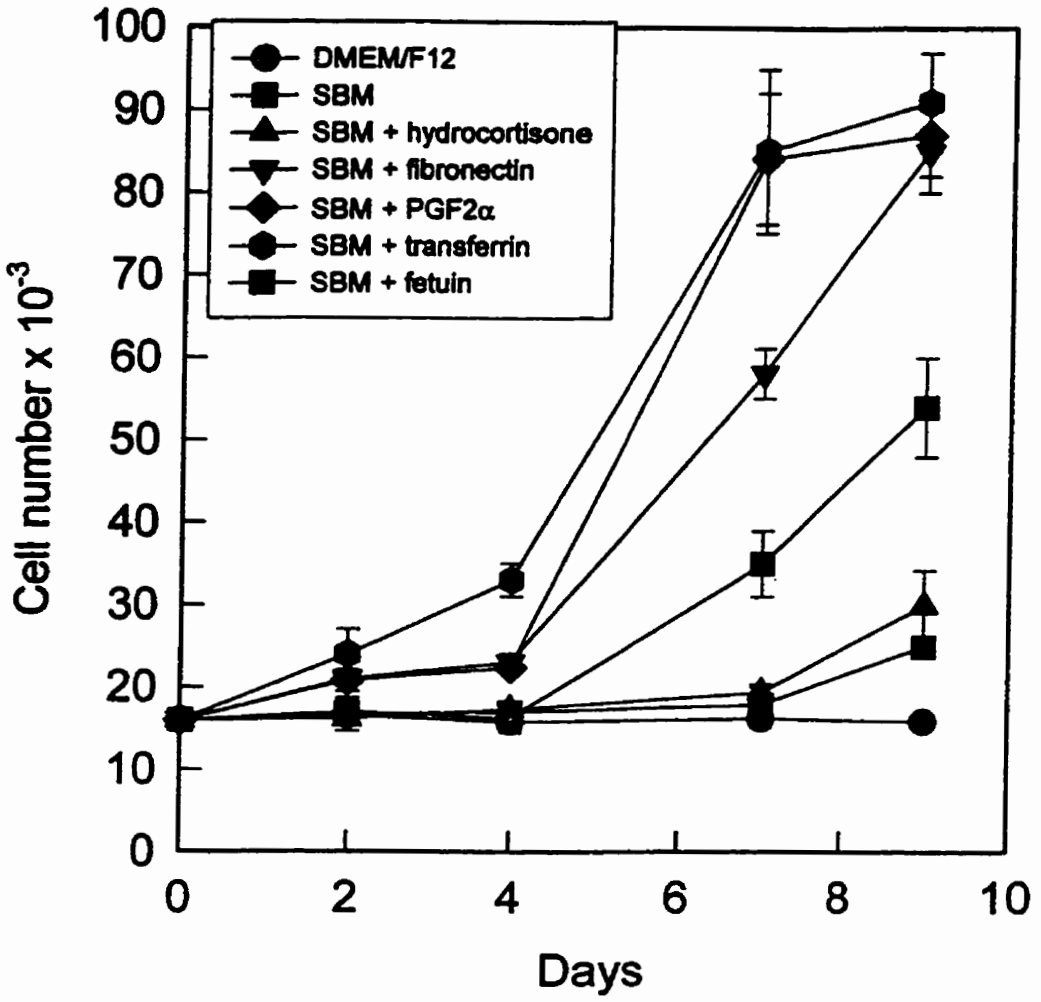


Figure 32. Growth of MCF-7 cells in supplemented basal medium (SBM) with different combinations of other serum-free cell growth promoting agents. MCF-7 cells were seeded in 24-well plates and incubated with DMEM/F12, SBM, and SBM supplemented with hydrocortisone, fetuin, fibronectin, $PGF_{2\alpha}$, or transferrin. The medium was changed every second day and cell numbers were also determined every two days. The results represent the means ± standard deviations of 3 different experiments.

addition of the adapted cells in the serum free media. After 72 h, examination of the cultures by a microscope revealed that the morphology of the cells in FBS preincubated dishes most closely resembled the parental cells. The morphology of MCF-7, MCF-7Ad$^+$ and MCF-7Ad$^-$ cells are shown in **Fig 33**. The adapted cells were more spindly and elongated relative to the parental MCF-7 cells. MCF-7Ad$^+$ and MCF-7Ad$^-$ cells had similar proliferation rates (**Fig 34**).

4.3.3 Effects of ET-18-OCH_3 on the proliferation of MCF-7Ad$^+$ and MCF-7Ad$^-$ cells

Having developed serum-free media and incubation conditions capable of supporting the growth of MCF-7 cells, we investigated the effect of the ET-18-OCH_3 on the proliferation of the parental MCF-7 and the adapted MCF-7Ad$^+$ cells using the procedures described in the methods (**section 3.5**). The protein content of the serum-free medium was supplemented with BSA to 3 mg/ml in order to bring the protein content to levels found in 10% FBS supplemented medium. The effect of increasing concentrations of ET-18-OCH_3 on the proliferation of MCF-7, MCF-7Ad$^+$ and MCF-7Ad$^-$ cells are shown in **Fig 35**. As expected, MCF-7 cells growing in FBS-supplemented medium were very sensitive to ET-18-OCH_3 with an IC_{50} value of 2 μM. Surprisingly, the MCF-7 cells adapted for growth in serum-free ACGM$^+$ and ACGM$^-$, MCF-7Ad$^+$ and MCF-7Ad$^-$, were less sensitive to growth inhibition by ET-18-OCH_3 compared to the parental MCF-7 cells. Furthermore, differences were observed in the effect of ET-18-OCH_3 on the proliferation of the two adapted cells (**Fig 35**). Proliferation of MCF-7Ad$^+$ cells was inhibited by a maximum of 40% compared to 85% inhibition of MCF-7Ad$^-$ by ET-18-OCH_3. MCF-7Ad$^-$ cells were therefore less sensitive than

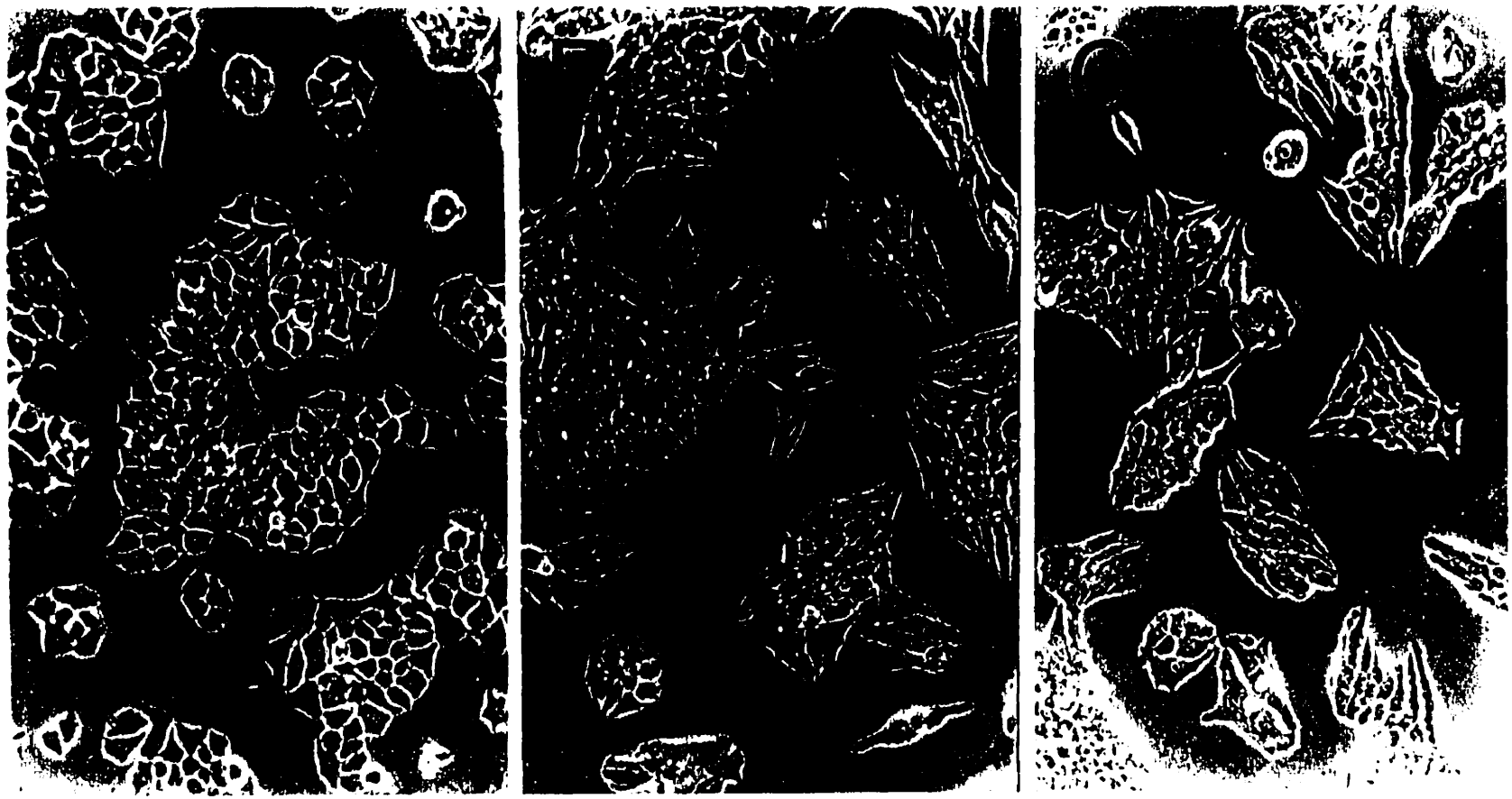


Figure 33. Morphology of the proliferating MCF-7 , MCF-7Ad$^-$ and MCF-7Ad$^+$ cells. The pictures of MCF-7 (A), MCF-7Ad$^-$ (B) and MCF-7Ad$^+$ (C) are shown at a magnification of x 200.

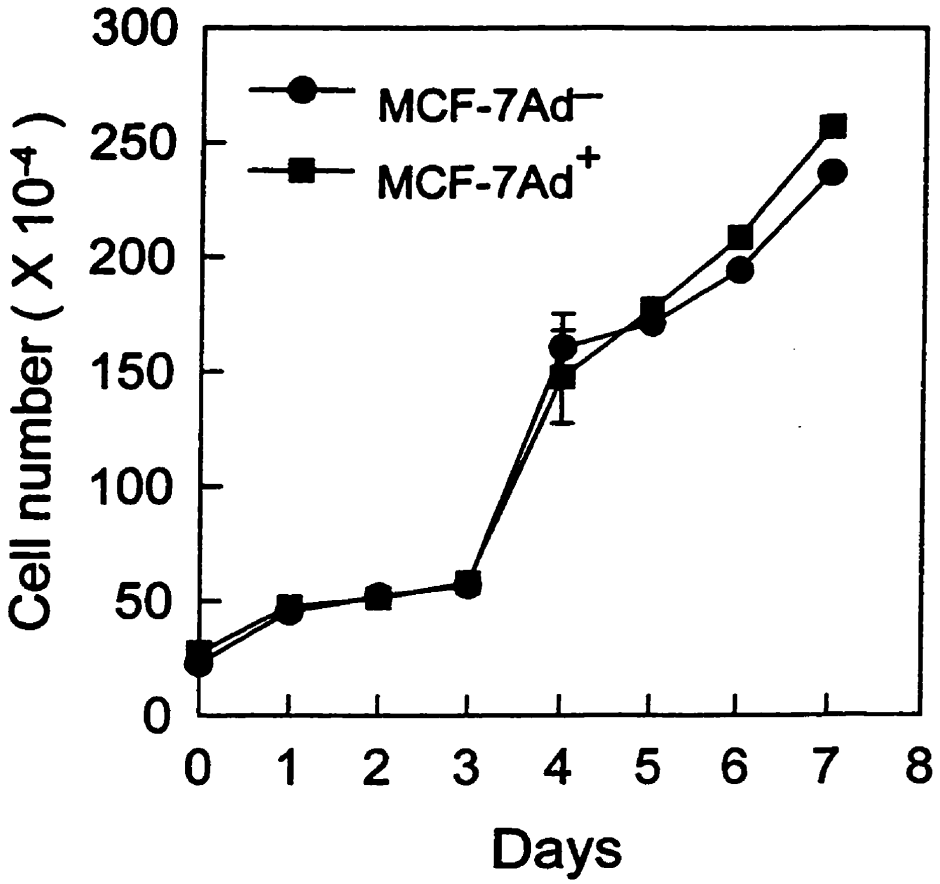


Figure 34. Comparison of growth rate between MCF-7Ad$^-$ and MCF-7Ad$^+$ cells. MCF-7Ad$^-$ and MCF-7Ad$^+$ cells were subcultured and grown in ACGM$^-$ and ACGM$^+$ respectively in 24-well plates. The cell numbers were counted daily. The results are the means ± standard deviations of quadruplicate determinations from 3 different experiments.

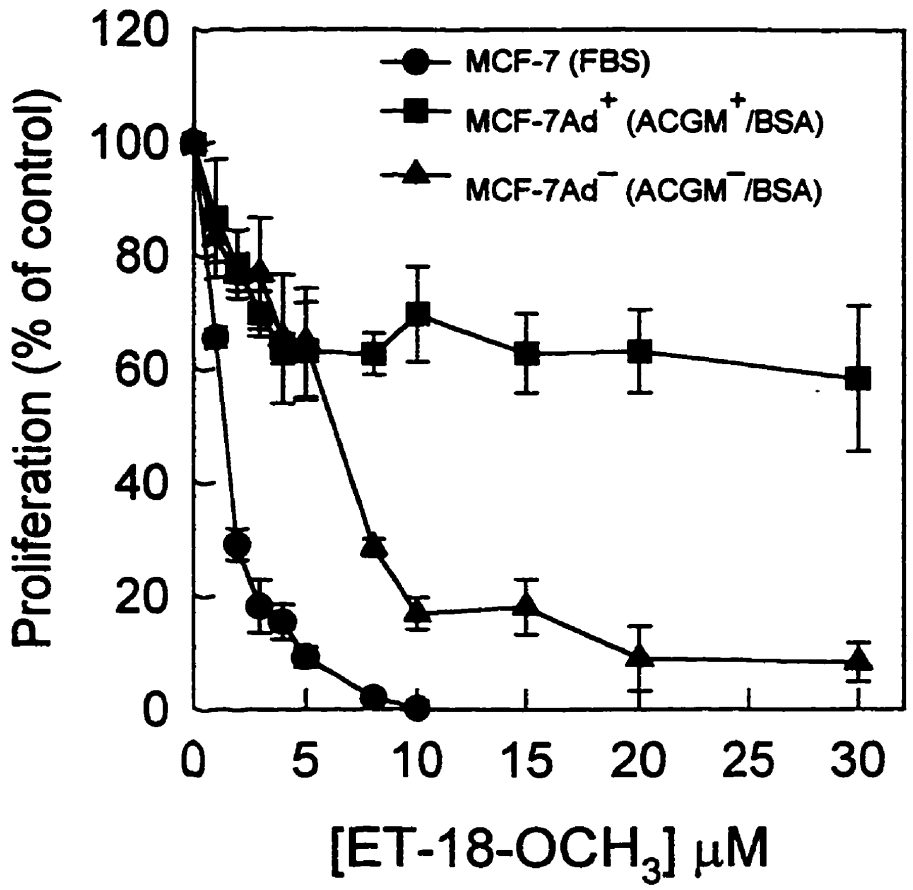


Figure 35. Effect of ET-18-OCH_3 on the proliferation of MCF-7, MCF-7Ad$^-$ and MCF-7Ad$^+$ cells. The effects of varying concentrations of ET-18-OCH_3 (0-30 μM) on the growth of log-phase MCF-7 cells growing in 10% FBS-containing medium, MCF-7Ad$^-$ cells and MCF-7Ad$^+$ cells growing in ACGM$^-$ and ACGM$^+$, respectively, were determined. The cells were incubated with the ALP (in the presence of 3 mg/ml BSA in ACGM$^-$ and ACGM$^+$) for 48 h. The results represent the means ± standard deviations of quadruplicate wells from 3-5 different experiments. Each 24-well plate had its own controls with no drug. The 48 h-increase in cell numbers of the controls were taken as the 100% value.

the parental MCF-7, cells but significantly more sensitive than MCF-7Ad$^+$ cells.

Our studies on the effect of ET-18-OCH_3 on the proliferation of adapted cells therefore revealed that both the adapted MCF-7 cells were now less sensitive to ET-18-OCH_3 in comparison to the parental MCF-7 cells. This suggests that MCF-7 cells had acquired ALP insensitivity to different degrees during the process of adaptation.

Since these MCF-7Ad$^-$ and MCF-7Ad$^+$ cells were derived from MCF-7 cells we elected to conduct our studies on the mechanisms of ALP-selectivity with these cells rather HNME cells. MCF-7Ad$^+$ cells were selected as the primary model for our investigations because they were more ET-18-OCH_3-insensitive than MCF-7Ad$^-$ cells.

4.3.4 Effect of experimental conditions on the ET-18-OCH_3-insensitivity of the adapted MCF-7 cells

The unexpected reduced sensitivity of MCF-7Ad$^+$ cells to ET-18-OCH_3 implied that during the adaptation process changes had occurred to the parental MCF-7 cells that made them somewhat resistant to the inhibitory effects of ET-18-OCH_3. We therefore conducted a series of studies to rule out the possibility that the insensitivity of the MCF-7 cells was a consequence of differences in the experimental conditions used for MCF-7 and MCF-7Ad$^+$ cells.

4.3.4.1 Effect of BSA or FBS on ALP-sensitivity

Since ET-18-OCH_3 was added to MCF-7Ad$^+$ cells in serum-free medium containing 3 mg/ml BSA, there was the possibility that the presence of BSA or absence of serum

components in the media could be responsible for the differences observed. To investigate this, ET-18-OCH_3 was added to proliferating MCF-7Ad$^+$ cells in 10% serum-containing ACGM$^+$ and the effect on growth after 48 h was monitored. The results which are displayed in **Fig 36**, showed that addition of the ALP in 10% FBS-supplemented medium had no effect on the previously observed insensitivity of MCF-7Ad$^+$ cells in medium with BSA. Conversely the incubation of MCF-7 cells for 48 h with ET-18-OCH_3 in BSA-supplemented medium did not decrease the sensitivity of the cells to the drug **(Fig 36)**.

4.3.4.2 Effect of growth rate on ALP-sensitivity

The growth rate of MCF-7Ad$^+$ cells in ACGM$^+$ medium is significantly lower than that of MCF-7 cells growing in FBS-supplemented medium or even MCF-7Ad$^+$ cells in medium supplemented with 10% serum **(Fig 37)**. We examined if the lower rate of proliferation of MCF-7Ad$^+$ cells was responsible for the insensitivity to ALP. To improve the growth rate we investigated the effect of estrogen, IGF-1 and IGF-2 on the proliferation of the cells, since breast cells have been reported to respond to these mitogens (Karey and Sirbasku, 1988; Rohlik *et al.*, 1987). IGF-1 and IGF-2 did not significantly improve the growth rate of the cells, but cells incubated with estrogen showed a significant increase in growth that approached the growth rate of MCF-7Ad$^+$ cells in 10% FBS-supplemented medium **(Fig 37)**. The effects of ET-18-OCH_3 on MCF-7Ad$^+$ cells grown in ACGM$^+$ with estrogen was investigated. The results, which are displayed in **Fig 36**, showed that the presence of estrogen in the growth medium had no effect on the insensitivity of the MCF-7Ad$^+$ cells to the inhibitory effects of the ALP. This suggested that the rate of growth was

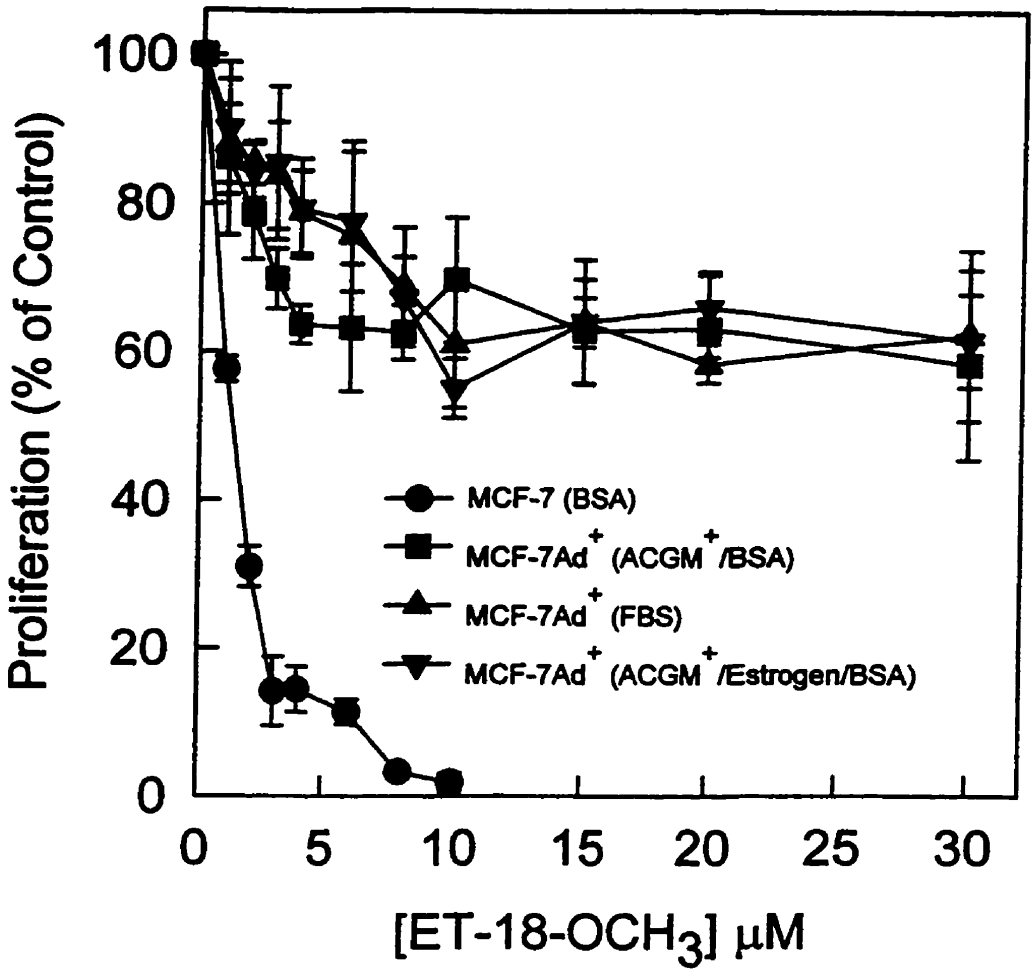


Figure 36. Effect of BSA, FBS and rate of cell proliferation on sensitivity of cells to ET-18-OCH_3. The medium of log-phase MCF-7 cells was replaced with BSA-supplemented DMEM with or without ET-18-OCH_3. MCF-7Ad^+ cells growing in $ACGM^+$ with 1 nM estrogen were treated with or without ET-18-OCH_3 in medium with BSA. In another group of MCF-7 Ad^+ cells growing in $ACGM^+$, the medium was replaced with one containing ET-18-OCH_3 in $ACGM^+$ supplemented with 10% FBS. The increase in cell numbers were determined after incubation of all cells with or without ET-18-OCH_3 for 48 h. The values are expressed as a % of controls without the drug. The values represent the means ± standard deviations of quadruplicate determinations from 4 different experiments.

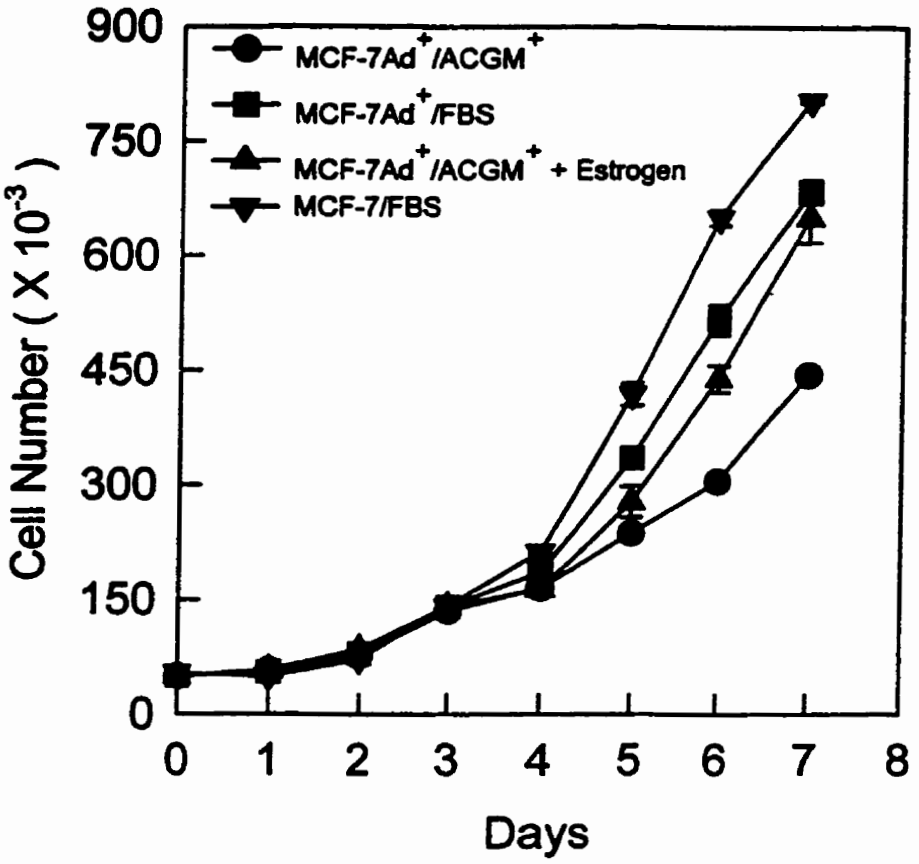


Figure 37. Comparison of growth rate between MCF-7 and MCF-7 Ad^{+} cells. MCF-7 cells were grown in 10% FBS supplemented DMEM, while MCF-7Ad^{+} cells were subcultured and grown in ACGM^{+}, 10% FBS-supplemented ACGM^{+} or in ACGM^{+} supplemented with 1 nM estrogen. The cell numbers were counted daily. The results are the means ± standard deviations of quadruplicate wells from 3 different studies.

not responsible for the different sensitivity of the cells to the ALP.

4.3.5 Comparison of the uptake and extent of breakdown of ET-18-OCH_3 in mammary cells

The basis for the relative insensitivity of HNME, MCF-7Ad$^-$ and MCF-7Ad$^+$ cells to growth inhibition by ET-18-OCH_3 was not known, however, decreased uptake or increased metabolism of ET-18-OCH_3 by these cells relative to MCF-7 cells could account for differential sensitivity to the ALP. To investigate this possibility, we determined the uptake of [^{3}H]ET-18-OCH_3 in proliferating MCF-7, MCF-7Ad$^-$, MCF-7Ad$^+$ and HNME cells. The results displayed in **Fig 38** revealed that the uptake of ET-18-OCH_3 was similar in proliferating MCF-7 and MCF-7Ad$^+$ cells, while the uptake in MCF-7Ad$^-$ cells was slightly higher than in MCF-7 cells. HNME cells, on the other hand, took up almost twice as much ET-18-OCH_3 as the sensitive MCF-7 cells.

To investigate whether there were differences in the extent of metabolism of ET-18-OCH_3 in the different cells that could account for the differential effects of the ALP on their proliferation, lipids were isolated from the cells following incubation with [^{3}H]ET-18-OCH_3 for different times. ET-18-OCH_3 was separated from the other lipids by TLC and the % distribution of label in the fraction was determined as described in **section 3.7**. The results revealed that between 96-98% of the label was associated with ET-18-OCH_3 extracted from the cells or from the stock solution. This indicates that there was very little hydrolysis of ET-18-OCH_3 in all the cells. The result of these uptake and metabolism studies lead us to conclude that the differential effect of ET-18-OCH_3 on the proliferation of cells is unrelated

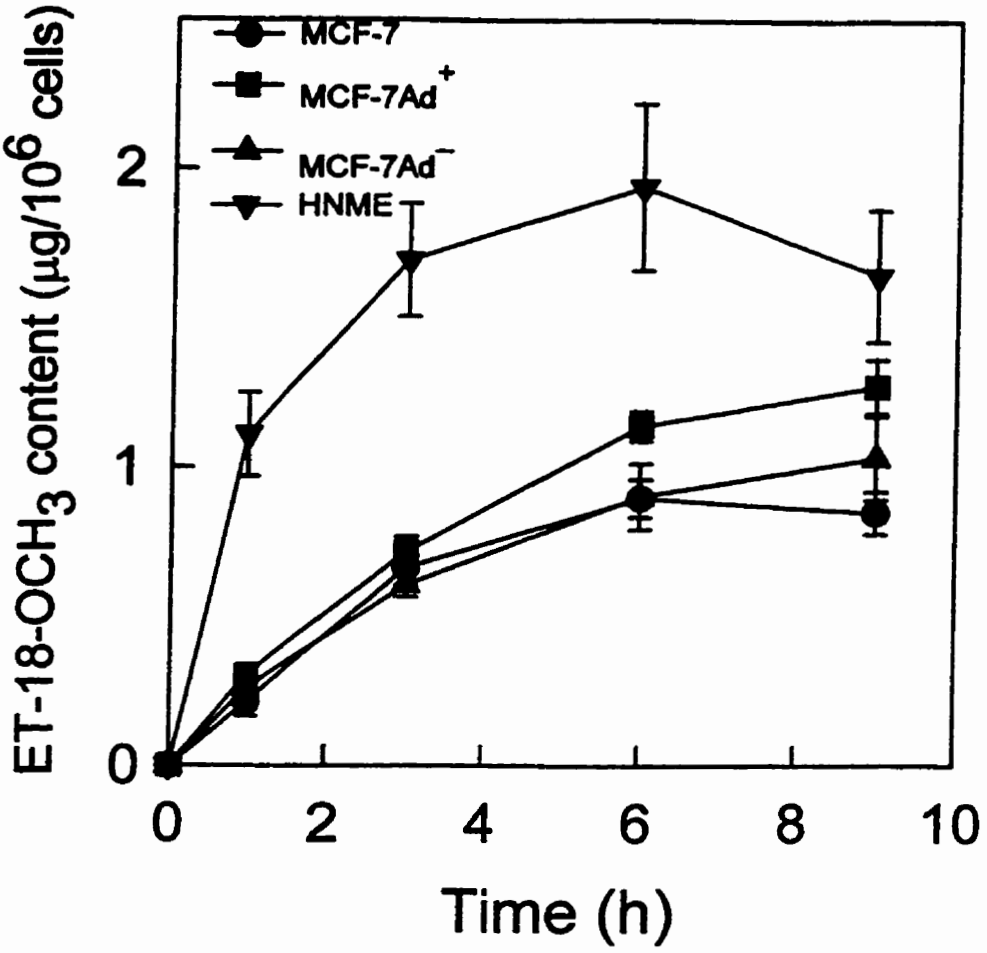


Figure 38. Uptake and incorporation of ET-18-OCH_3 into proliferating mammary epithelial cells. Proliferating MCF-7, MCF-7Ad$^-$, MCF-7Ad$^+$ and HNME cells were incubated with [^{3}H] ET-18-OCH_3 (10 µg/ml, 0.1 µCi/µg) for various times. The quantities of ET-18-OCH_3 incorporated into the cells were determined as described in the methods. The values represent the means ± standard deviations of 6 different experiments.

to the quantities present in the cell or differences in metabolism of the ALP.

4.3.6 Effect of ET-18-OCH_3 on the proliferation of $ACGM^+$-adapted MCF-7 clones

One possible explanation for the relative insensitivity of the adapted cells to ET-18-OCH_3 is that during the adaptation process there was a selection of ET-18-OCH_3-resistant cells from a mixed population of ET-18-OCH_3-resistant and ET-18-OCH_3-sensitive MCF-7 cells. To address this issue, our approach was to isolate clones of MCF-7 cells, adapt each clone independently for growth in $ACGM^+$ and compare the ET-18-OCH_3-sensitivity of the parental and adapted clones. Twenty clones of MCF-7 cells each derived from a single cell were obtained as described in the methods **(section 3.4)**. Five of the clones were arbitrarily selected for our investigations. The results on the effect of ET-18-OCH_3 on the proliferation of the parental clones are shown in **Fig 39**. All five clones were sensitive to ET-18-OCH_3 with IC_{50} values between 1.5- 2.5 μM **(Fig 39)**. Cells from each of the clones were then independently adapted for growth in $ACGM^+$ medium using the procedures described in the Methods **(section 3.3)**. The effect of ET-18-OCH_3 on each of the adapted clones was then investigated. The results showed that all the clones adapted for growth in serum-free media were ET-18-OCH_3-resistant compared to their parental counterparts **(Fig.40)**. The maximum inhibition observed in any clone resulted in proliferation rates that were 60% of that of the controls.

To investigate if the acquired insensitivity could be reversed, cells from each of the adapted clones were grown in 10% FBS-containing medium for 6 passages and subsequently their sensitivity to ET-18-OCH_3 was investigated. The results displayed in **Fig 41** showed

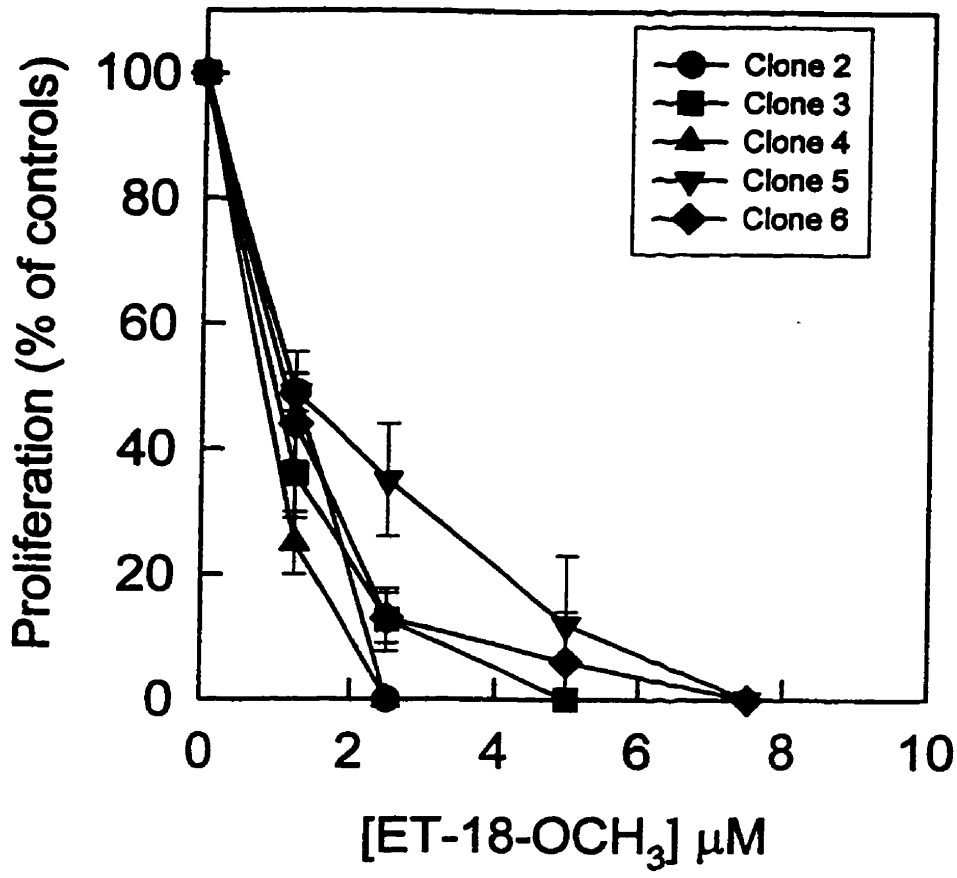


Figure 39. Effect of ET-18-OCH_3 on the proliferation of clones of MCF-7 cells. Clones of MCF-7 cells were isolated and grown as described in the methods. The effect of ET-18-OCH_3 on the proliferation of five arbitrarily selected clones, clones 2-6 was investigated using the procedures described in Fig 7. The results are the means ± standard deviations from quadruplicate wells from 4 different studies.

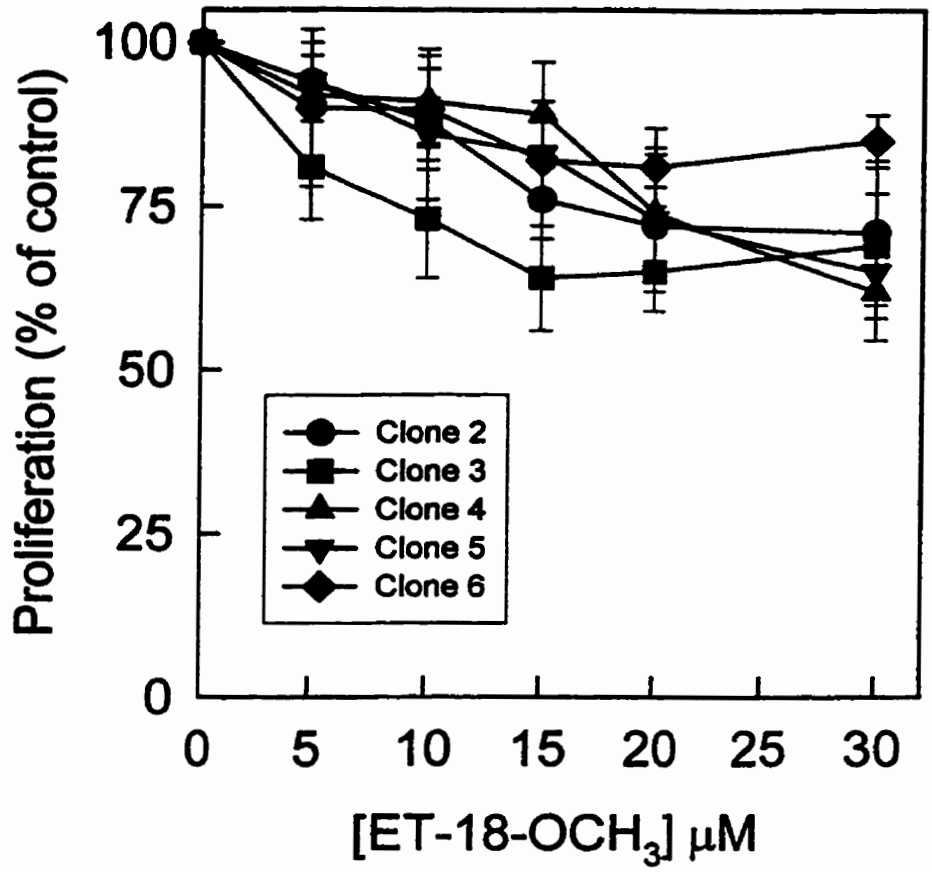


Figure 40. Effect of ET-18-OCH_3 on the proliferation of MCF-7 clones adapted for serum-free growth in $ACGM^+$. The five MCF-7 clones selected for our studies (clones 2-6) were each adapted for serum-free growth in $ACGM^+$ by the procedures described in the methods. The effect of ET-18-OCH_3 on proliferation was investigated with each adapted MCF-7 clone. The results are the means ± standard deviations from quadruplicate wells from 4 different studies.

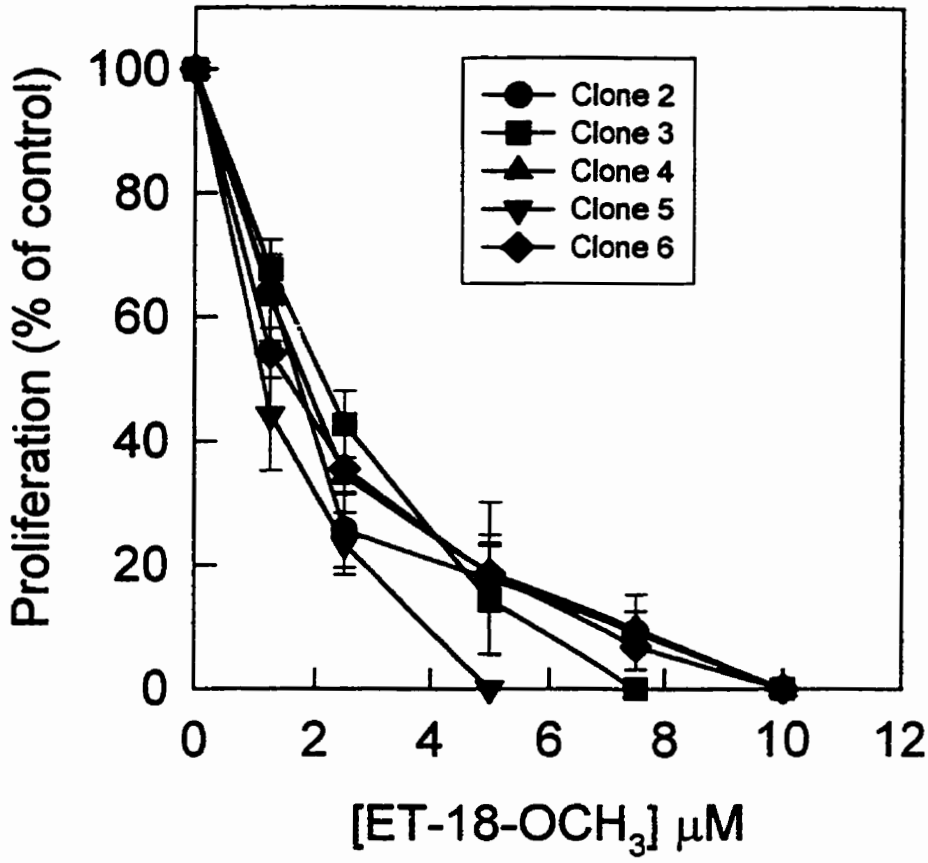


Figure 41. Effect of ET-18-OCH_3 on adapted MCF-7 clones grown in serum containing medium. The five selected MCF-7 clones (clones 2-6) adapted for serum-free growth were grown in 10% FBS-supplemented medium for six passages. The effect of various concentrations (0-10 μM) of ET-18-OCH_3 on the proliferation was then determined on these clones. Results are the means ± standard deviations from quadruplicate wells from 4 different studies.

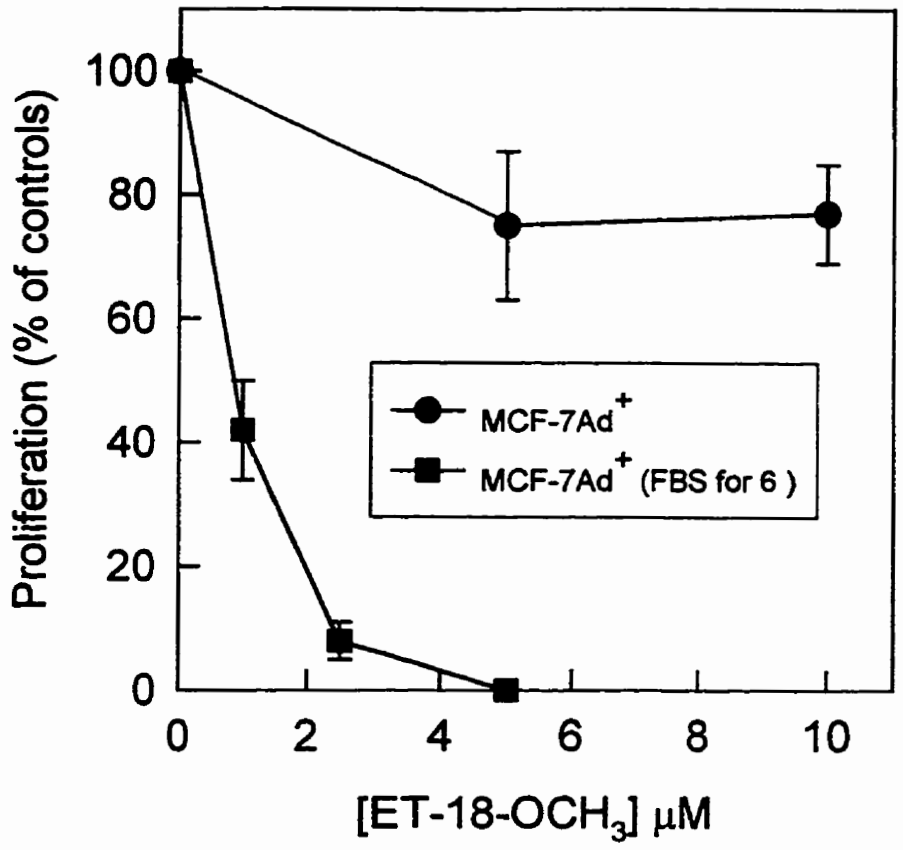


Figure 42. Effect of ET-18-OCH_3 on MCF-7 Ad^+ cells grown in serum containing medium. MCF-7 Ad^+ cells were grown in 10% serum containing medium for six passages. The effect of various concentrations (0-10 μM) of ET-18-OCH_3 on the proliferation was then determined as described in Figure 7. The results are the means ± standard deviations from quadruplicate wells from 4 different studies.

that all 5 adapted clones had become sensitive to ET-18-OCH_3 with IC_{50} of between 1.5 -2.2 μM. Similar results were obtained when MCF-7Ad$^+$cells adapted from the mixed population were grown in serum-containing medium for 6 passages (**Fig 42**).

These results clearly indicate that the ALP insensitivity/sensitivity of MCF-7 cells in serum-free/serum containing media respectively were not due to a selection process during the adaptation process, and furthermore, the process was reversible.

4.3.7 Prostaglandin $F_{2\alpha}$ and acquisition of ET-18-OCH_3-insensitivity in MCF-7 cells

The above results do not establish the basis of the differences in ALP-sensitivity between HNME, MCF-7, MCF-7Ad$^-$ and MCF-7Ad$^+$ cells but fortuitously, the results of our study provide clues on possible reasons for the differences in ET-18-OCH_3-sensitivity between MCF-7Ad$^-$ and MCF-7Ad$^+$. As shown in **Fig 35**, MCF-7Ad$^-$ cells were less sensitive to ET-18-OCH_3 relative to MCF-7 cells but were more sensitive than MCF-7Ad$^+$ cells. Since the only difference in the adaptation conditions was the presence of $PGF_{2\alpha}$ in ACGM$^+$, this indicates that the presence of $PGF_{2\alpha}$ in the medium is linked to the increased insensitivity of MCF-7Ad$^+$ cells. To explore this idea, we investigated whether serial passaging of MCF-7Ad$^-$ cells in ACGM$^+$ might increase the ET-18-OCH_3-resistance of the cells to the levels observed with MCF-7Ad$^+$ cells. MCF-7Ad$^-$ cells were continuously passaged in ACGM$^+$ and at selected passages the effect of ET-18-OCH_3 on cell proliferation was determined. The results displayed in **Fig 43** show that insensitivity of the cells to ET-18-OCH_3 increased progressively with growth in ACGM$^+$ such that after 16 passages, the resistance was similar to that obtained with MCF-7Ad$^+$ cells.

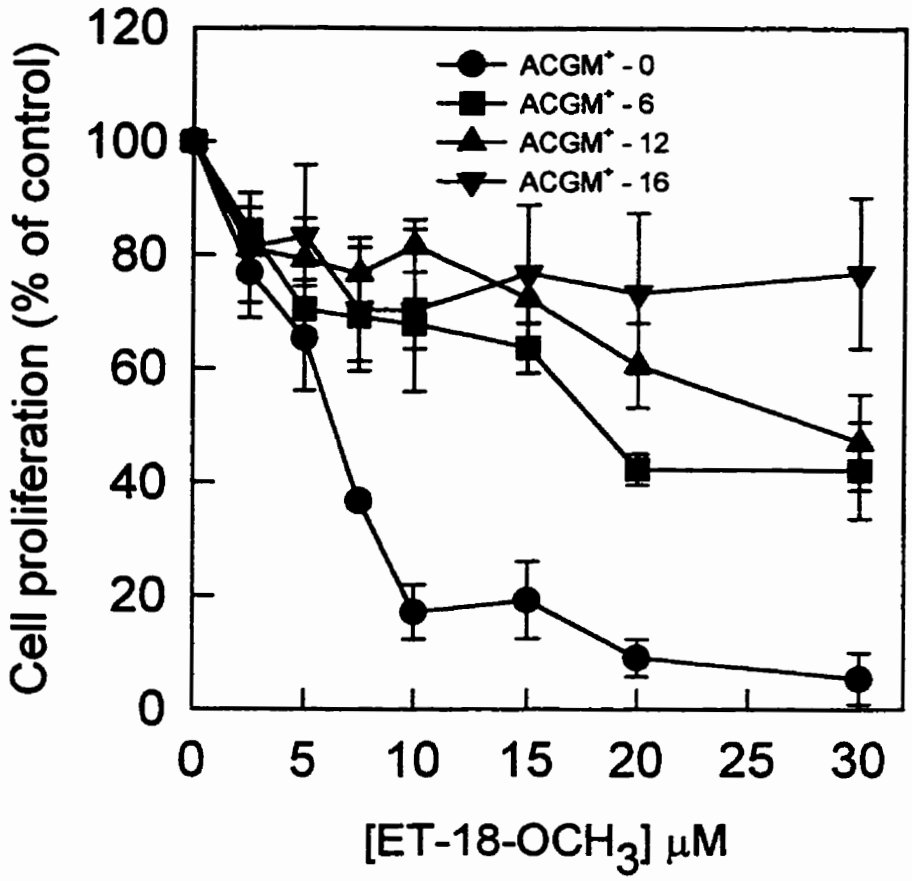


Figure 43. Effect of ET-18-OCH_3 on the proliferation of MCF-7Ad$^-$ cells passaged continuously in ACGM$^+$ medium. MCF-7Ad$^-$ cells passaged once in ACGM$^-$ were subsequently grown and passaged in ACGM$^+$ and the sensitivity of cells to ET-18-OCH_3 at selected passages in ACGM$^+$ (0, 6, 12 and 16) was determined. The results are the means ± standard deviations from quadruplicate wells from 3 different experiments.

4.3.8 Progressive acquisition of ET-18-OCH_3-insensitivity during the adaptation process

The above results prompted us to investigate whether a progressive decrease in the sensitivity of MCF-7 cells occurs during adaptation from growth in 10% FBS-supplemented medium to the serum-free $ACGM^+$. At different stages during the adaptation **(section 3.3)**, the effect of ET-18-OCH_3 on growth was assessed. In all experiments the final protein concentration of the medium was adjusted to 3 mg/ml with fatty acid-free BSA. The results observed **(Fig 44)** clearly showed that there was a progressive decrease in the sensitivity of the cells as the proportion of $ACGM^+$ increased with the coincidental decrease in the FBS content in the medium. A very large increase in insensitivity to ET-18-OCH_3 was observed between cells grown in medium containing 1.25% FBS and 0.625% FBS, and also between 0.31% and 0.156% FBS-containing medium. The IC_{50} values were 2.5 μM, 23 μM and >30 μM for the cells obtained from 1.25, 0.625 and 0.156% FBS-containing media respectively.

4.3.9 Mechanism of ET-18-OCH_3-insensitivity in adapted MCF-7 cells ($MCF\text{-}7Ad^+$)

Previous studies have demonstrated that in MCF-7 cells, inhibition of growth by ET-18-OCH_3 is related to the inhibition of the MAP kinase pathway as a consequence of the inhibition of Raf-1 association with the cell membranes (Zhou *et al.*, 1996). We therefore investigated whether uptake of ET-18-OCH_3 by $MCF\text{-}7Ad^+$ cells to levels similar to those that inhibit MAP kinase in MCF-7 cells, inhibits the activation of the MAP kinase in the adapted cells.

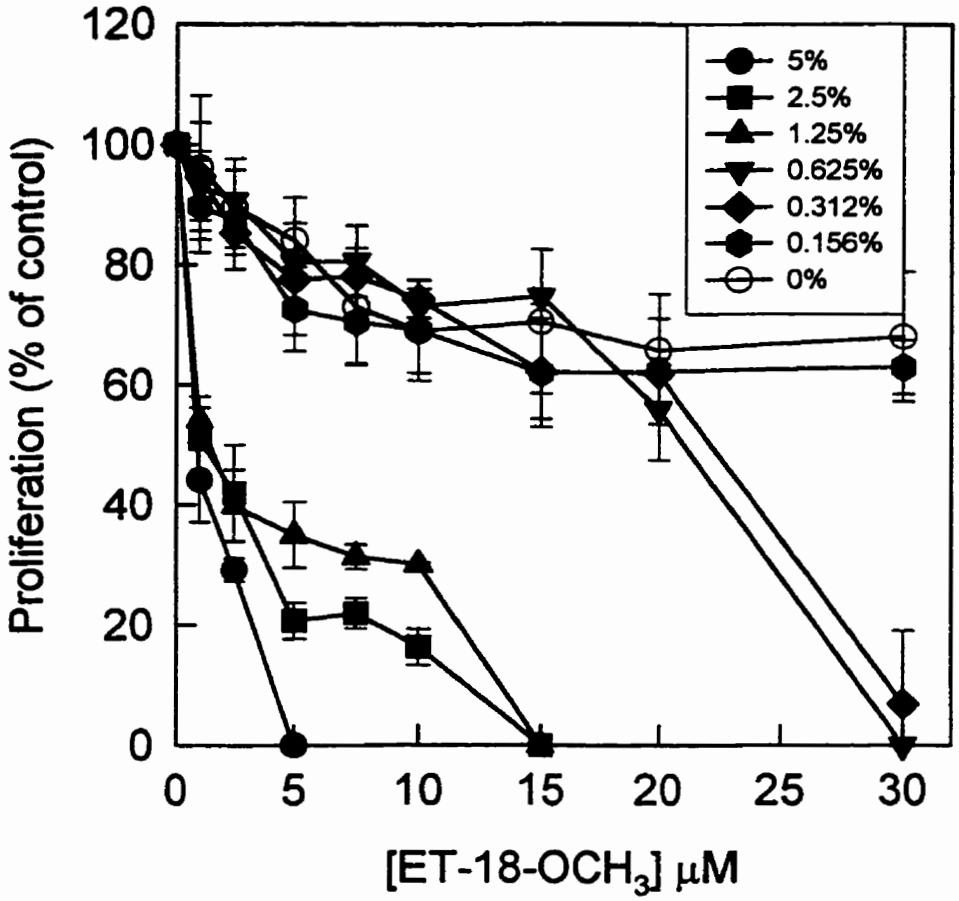


Figure 44. Changes in ET-18-OCH_3-sensitivity of MCF-7 cells during adaptation for growth in serum-free medium. MCF-7 cells were adapted for growth in $ACGM^+$ as described in the Methods section. During the adaptation process the effect of ET-18-OCH_3 on the proliferation of MCF-7 cells growing in medium containing 5%, 2.5% , 1.25% , 0.625%, 0.3125%, 0.156% and 0% FBS was determined. The protein content of the drug-containing medium was adjusted with the addition of BSA to give a protein concentration of 3 mg/ml. The effect of ET-18-OCH_3 on cell growth was determined after 48 h of incubation. The values represent the means ± standard deviations of quadruplicate determinations from 4 different studies.

To determine the incubation conditions with ET-18-OCH_3 that result in the accumulation of ET-18-OCH_3 in the cells to levels similar to the inhibitory levels observed in MCF-7 cells, quiescent MCF-7Ad$^+$, MCF-7Ad$^-$ and HNME cells were incubated with [^{3}H]ET-18-OCH_3 (20 μM) for varying periods (1-6 h). The quantity of the ALP taken up in the cells was determined as described in **section 3.7**. The results showed that after incubation for 1.5 h, quiescent HNME cells accumulated 0.83 μg ET-18-OCH_3/10^6 cells. Incubation of MCF-7Ad$^+$ and MCF-7Ad$^-$ cells for 5 h with 20 μM ET-18-OCH_3 resulted in the accumulation of 0.76 μg ET-18-OCH_3/10^6 cells. It has been previously established that incubation of MCF-7 cells with 20 μM ET-18-OCH_3 for 3 h results in the accumulation of 0.74 μg ET-18-OCH_3/10^6 cells (Zhou *et al.*, 1996). These levels of ET-18-OCH_3 were sufficient to inhibit MAP kinase activation and cell proliferation in response to growth factor or serum stimulation (Zhou *et al.*, 1996). Thus, the incubation conditions established above for the cell lines were used in the studies described below.

To examine the effect of ET-18-OCH_3 on the activation of MAP kinase in MCF-7Ad$^+$ cells, proliferating cells were made quiescent by incubation in BSA-supplemented DMEM/F12 (0.5 mg/ml) and the daily growth rate was monitored until growth dropped below 10%. On the day of the experiment the cells were incubated with 20 μM ET-18-OCH_3 for 5 h, the cells were washed twice and stimulated with either 10 ng/ml EGF or ACGM$^+$. After stimulation, cells were washed with ice-cold PBS, scraped in assay buffer **(section 3.10)** and cytosolic fractions were prepared for the MAP kinase assay. MAP kinase activity was assayed using the *in vitro* phosphorylation of MBP or quantitation of phosphorylated MAP kinase in the cytosol by Western blot analysis **(section 3.14)**. The results presented in **Fig 45**

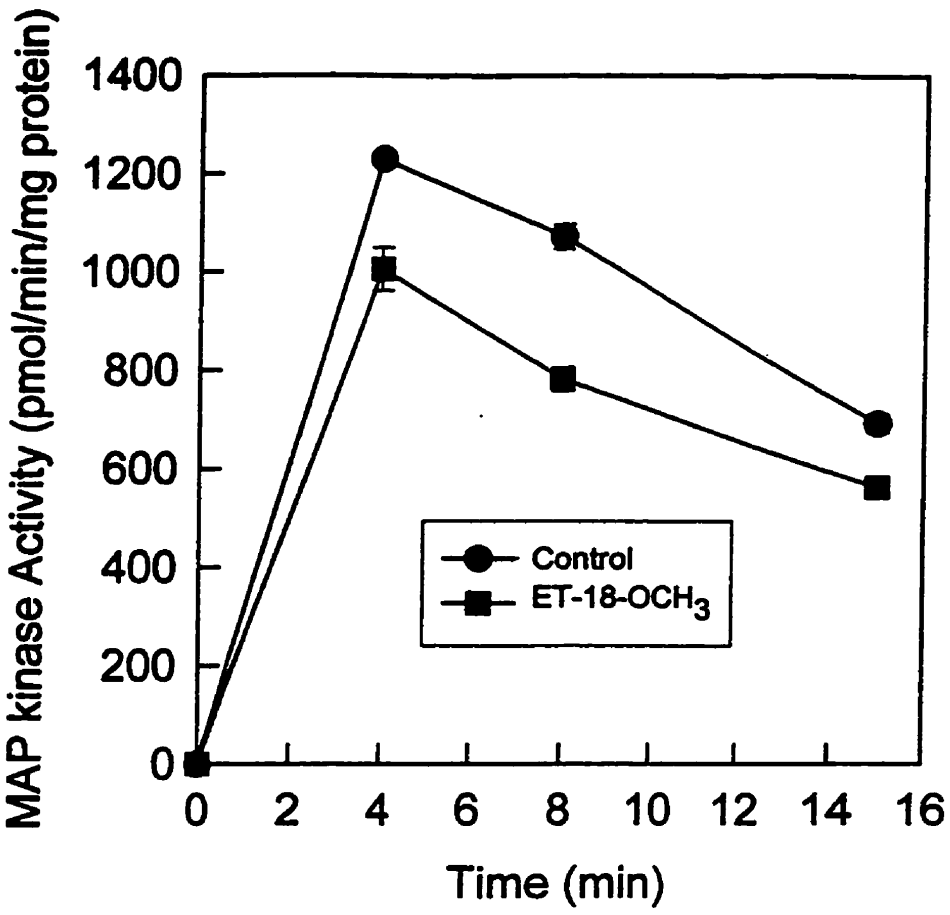


Figure 45. Effect of ET-18-OCH_3 on the MAP kinase activity in EGF-stimulated MCF-7Ad$^+$ cells. Quiescent MCF-7Ad$^+$ cells were preincubated without or with 20 μM ET-18-OCH_3 for 5 h. The cells were then washed and stimulated for varying periods with EGF. Cytosolic fractions were prepared and MAP kinase activity was measured using the *in vitro* MBP phosphorylation assay described in the methods. The results are the means ± standard deviations of triplicate incubations from a single experiment that is representative of results with 5 different cell preparations.

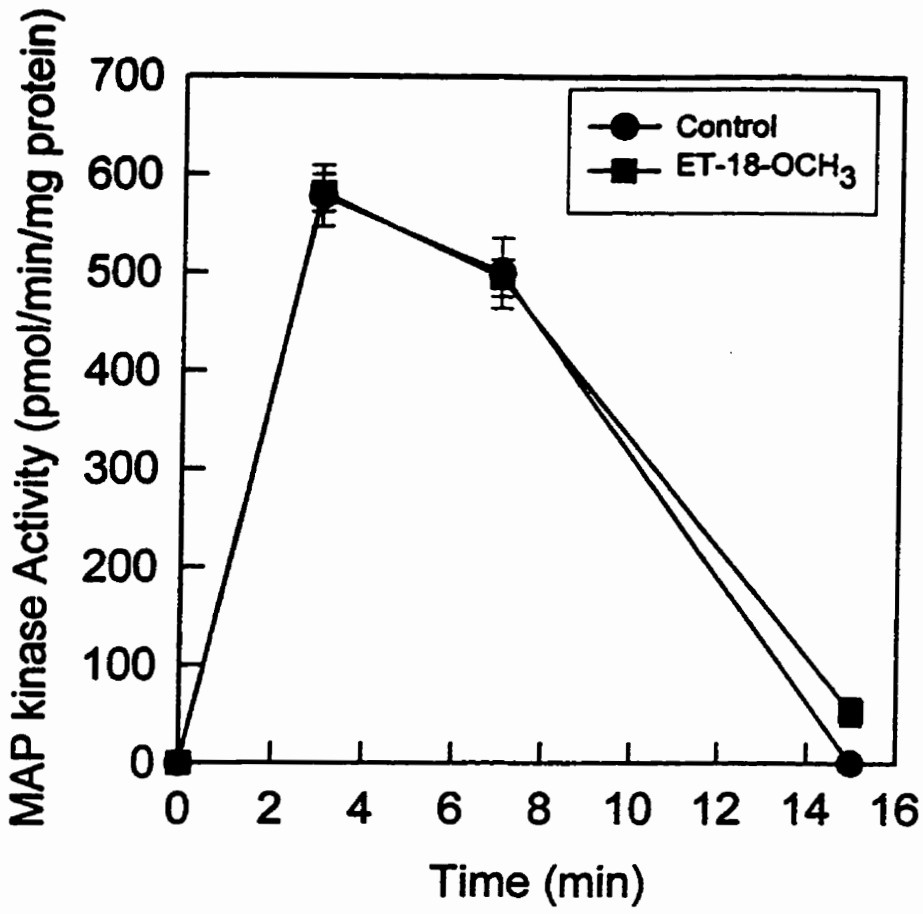


Figure 46. Effect of ET-18-OCH_3 on MAP kinase activity in $ACGM^+$ -stimulated $MCF\text{-}7Ad^+$ cells. Quiescent $MCF\text{-}7Ad^+$ cells were preincubated without or with 20 μM ET-18-OCH_3 for 5 h. The cells were then washed and stimulated for varying periods with $ACGM^+$. Cytosolic fractions were prepared and *in vitro* MAP kinase assay was conducted as described in the methods. The results are the means ± standard deviations of triplicate incubations from a single experiment that is representative of results from 3 different cell preparations.

showed that only a slight inhibition of MAP kinase activity occurred in the MCF-7Ad$^+$ cells preincubated with the drug prior to stimulation with EGF. In cells stimulated with ACGM$^+$, no inhibition of MAP kinase activity was observed **(Fig 46)**. In contrast, a severe inhibition of MAP kinase activity was observed in MCF-7 cells preincubated with ET-18-OCH_3 **(Fig 47)**.

These results were confirmed by assessing the levels of phosphorylated MAP kinase by Western blot analysis using anti- phospho-specific MAP kinase antibody **(Fig 48 and 49)**. There was little difference in MAP kinase phosphorylation between control and ET-18-OCH_3-treated and EGF or ACGM$^+$-stimulated MCF-7Ad$^+$ cells. Western blot analysis with anti MAP kinase Ab showed no differences in the amount of MAP kinase protein between control and drug treated group **(Fig 48, lower lane)**. We did observe that MAP kinase activity and phosphorylation in MCF-7Ad$^+$ cells was sustained for a longer period in response to EGF compared to ACGM$^+$.

4.3.9.1 Effect of ET-18-OCH_3 on MAP kinase activity in HNME cells

Studies were conducted with HNME cells to investigate whether the effect of ET-18-OCH_3 on MAP kinase activation was similar to that of the MCF-7Ad$^+$ cells. *In vitro* MAP kinase assays in control and ET-18-OCH_3-treated HNME cells showed about 25% inhibition in the maximum MAP kinase activity in drug treated cells **(Fig 50)** compared to controls. Western blot analysis with anti-phospho-MAP kinase antibody **(Fig 51)** and densitometric analysis of the blots showed that preincubation of the cells with ET-18-OCH_3 had very little effect on the phosphorylation of MAP kinase. In HNME cells, it was also apparent that the

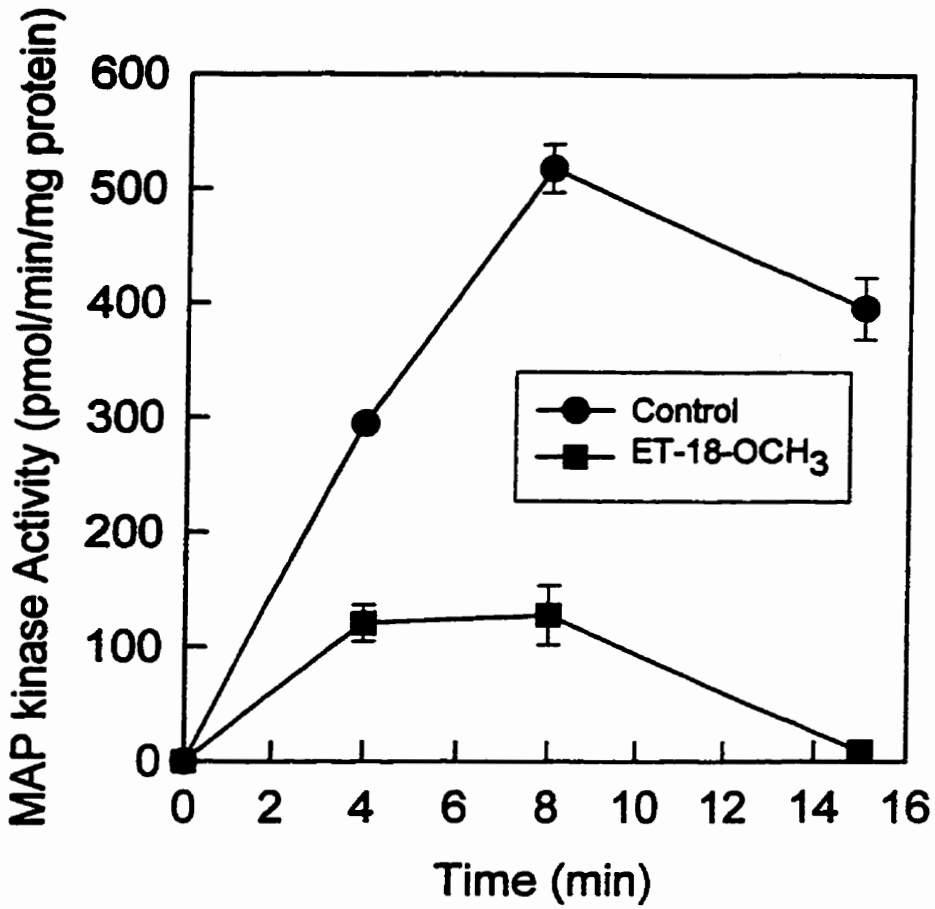


Figure 47. Effect of ET-18-OCH_3 on MAP kinase activity in EGF-stimulated MCF-7 cells. Quiescent MCF-7 cells were preincubated without or with 20 μM ET-18-OCH_3 for 3 h. The cells were then washed and stimulated for varying periods with EGF. Cytosolic fractions were prepared and *in vitro* MAP kinase assay was conducted as described in the methods. The results are the means ± standard deviations of triplicate assays from a single experiment that is representative of results obtained from 3 different cell preparations.

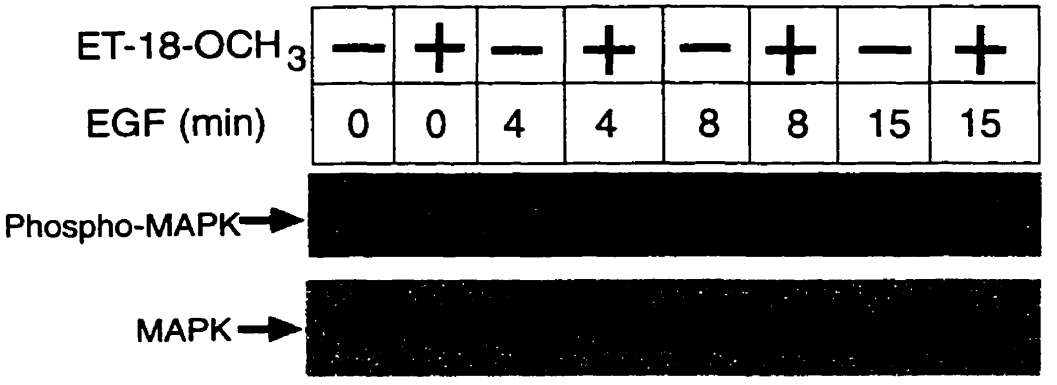


Figure 48. Effect of ET-18-OCH_3 on the phosphorylation of MAP kinase in EGF-stimulated MCF-7Ad$^+$ cells. Quiescent cells were treated with or without ET-18-OCH_3, stimulated with EGF, followed by isolation of cell cytosol as described in the legend to Figure 45. Cytosolic protein (30 μg/lane) were separated by SDS-PAGE on a 10% gel and subjected to Western blot analysis using the anti-phospho-MAP kinase antibody. Similar experiments with 15 μg protein were probed with anti-MAP kinase antibody. The results are from a single experiment that is representative of results obtained with 3 different cell preparations.

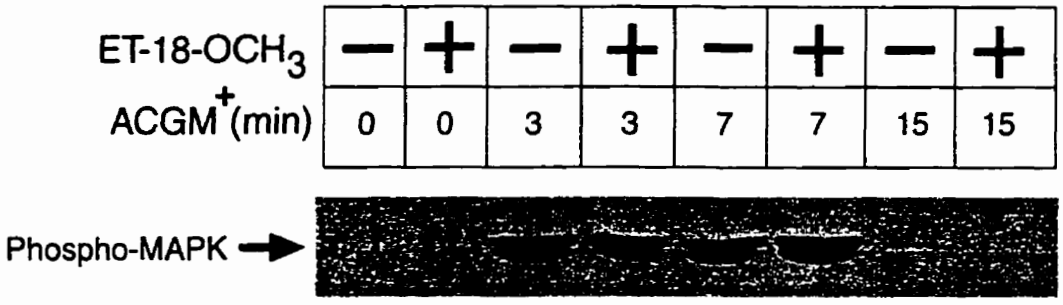


Figure 49. Effect of ET-18-OCH_3 on the phosphorylation of MAP kinase in $ACGM^+$- stimulated $MCF\text{-}7Ad^+$ cells. Quiescent $MCF\text{-}7Ad^+$ cells were treated with or without ET-18-OCH_3, stimulated with $ACGM^+$ and cell cytosol was isolated as described in the legend to Figure 45. Western blot analysis with the anti-phospho-MAP kinase antibody was conducted as described in Figure 48. The results are from a single experiment that is representative of results obtained with 3 different cell preparations.

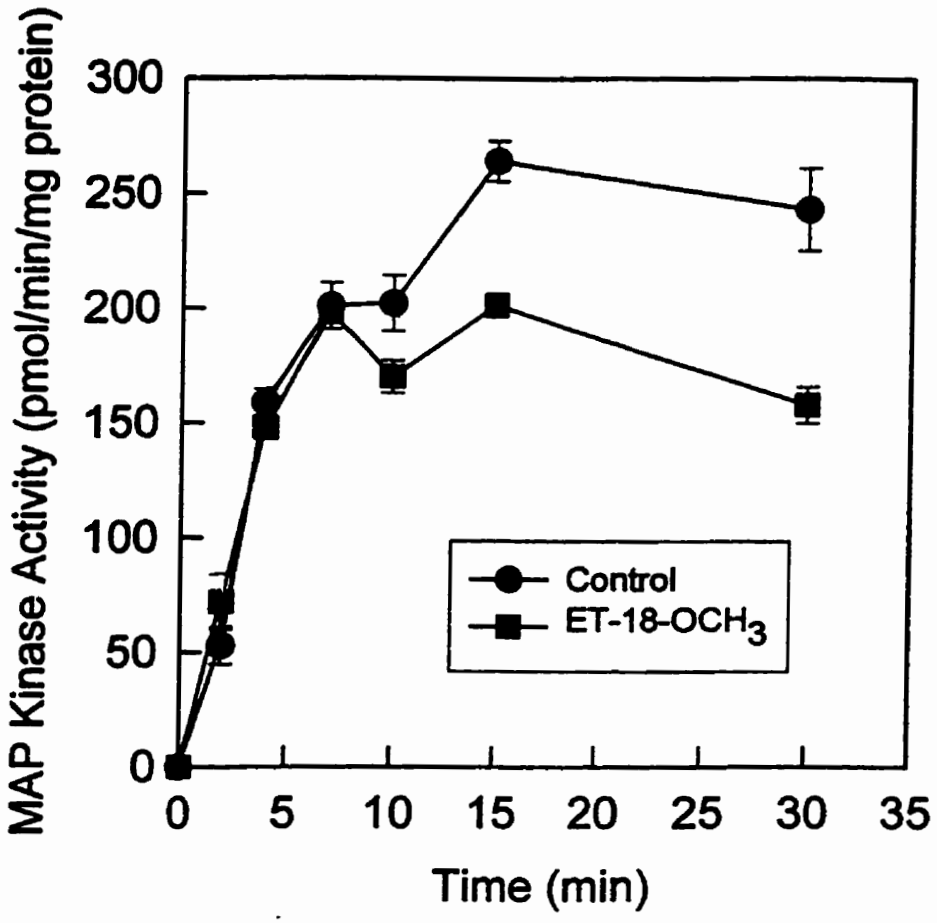


Figure 50. Effect of ET-18-OCH_3 on MAP kinase activity in human normal mammary epithelial cells (HNME). Quiescent HNME cells were incubated without or with 20 μM ET-18-OCH_3 for 1.5 h. The cells were stimulated with EGF for varying periods and cytosolic fractions were obtained by differential centrifugation. MAP kinase activity was determined using the *in vitro* assay as described in the methods. The results are the means ± standard deviations of triplicate incubations from a single experiment that is representative of results obtained with 2 different cell preparations.

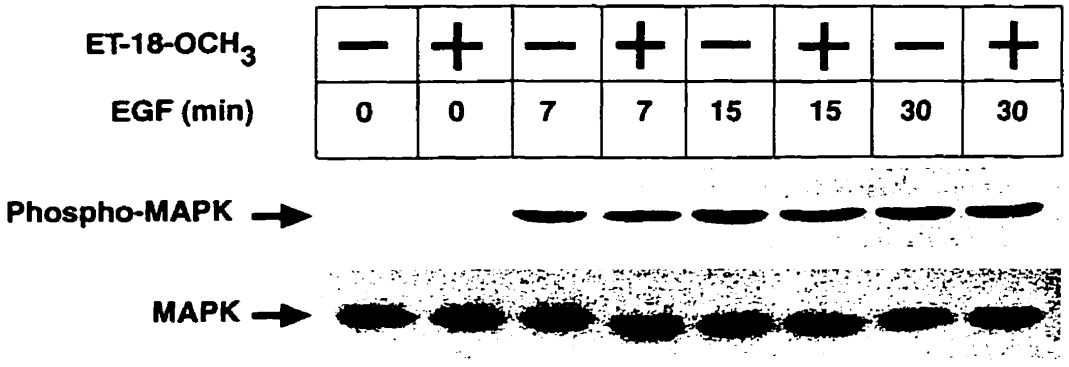


Figure 51. Effect of ET-18-OCH_3 on the phosphorylation of MAP kinase in HNME cells. The cytosolic fractions obtained from the experiments described in figure 50 were subjected to Western blot analysis with anti-phospho-MAP kinase or anti-MAP kinase antibodies. 30 μg of cytosolic protein was used for studies with anti-phospho-MAP kinase antibody and 15 μg protein for anti-MAP kinase antibody. The results are from a single experiment that is representative of results from 2 different cell preparations.

MAP kinase activity is sustained for prolonged periods compared to the MCF-7Ad$^+$ or MCF-7 cells following EGF stimulation, although the magnitude of activation is less.

4.3.10 Assessment of insulin and EGF receptor levels in MCF-7Ad$^+$ cells

One possible reason for the inability of ET-18-OCH_3 to inhibit MAP kinase activity in the adapted cells is that the incubation with insulin and EGF in the media during the adaptation process upregulates the insulin and EGF receptors, which would result in an amplification of the signal following cell stimulation. This could overwhelm any inhibitory effects caused by the ALP. We therefore compared the level of insulin and EGF receptors in membrane fractions of MCF-7 and MCF-7Ad$^+$ cells by Western blot analysis with receptor-specific antibodies and densitometric analysis of the blots. The results showed that there were no differences in the quantities of the insulin receptor proteins in these two cells (**Fig 52**). A slight increase of 15% was observed in the levels of the EGF receptors in MCF-7Ad$^+$ cells relative to MCF-7 cells.

4.3.11 Raf-1 membrane levels in MCF-7Ad$^+$ cells

Previous studies have reported that ET-18-OCH_3 inhibits the association of Raf-1 with membranes (Zhou *et al.*, 1996), an association that is required for the activation of its catalytic activity (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). We therefore examined the effect of ET-18-OCH_3 on Raf-1 translocation in the adapted cells. Membranes were isolated from MCF-7Ad$^+$ cells preincubated with ET-18-OCH_3 and the levels of Raf associated with the membranes were assessed by Western blot analysis. The results displayed in **Fig 53**

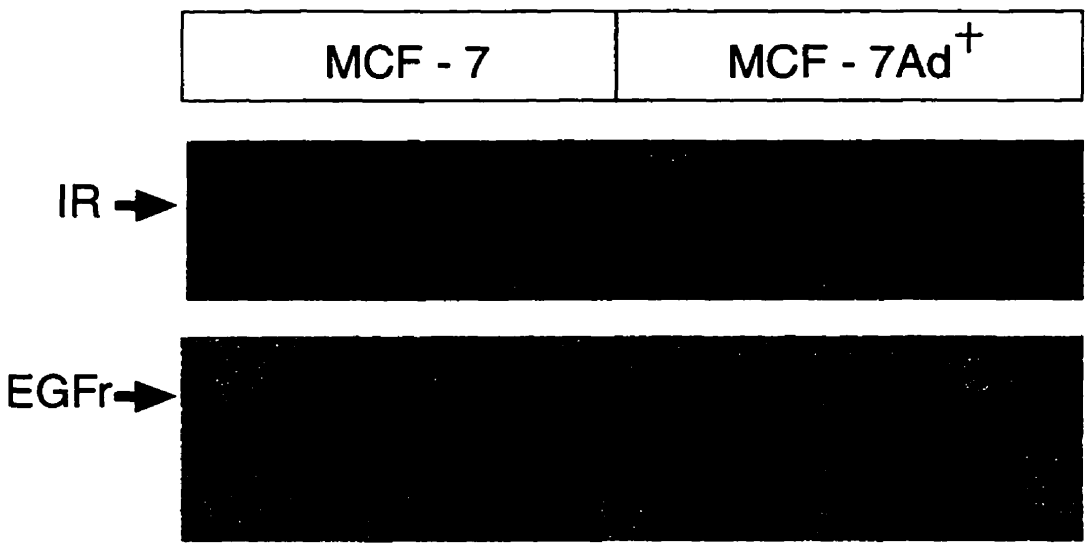


Figure 52. Comparison of the level of EGF receptors and insulin receptors between MCF-7 and MCF-7Ad$^+$ cells. Membrane fractions were prepared from quiescent unstimulated MCF-7 and MCF-7Ad$^+$ cells by differential centrifugation as described in the methods. Membrane protein (20 μg) was loaded in each lane and the proteins were separated by 10% SDS-PAGE. Western blot analysis was conducted using the anti-insulin receptor antibody and the anti-EGFr antibody. The results presented are from 2 different cell preparations. Similar results were obtained with preparations from 2 other cell preparations.

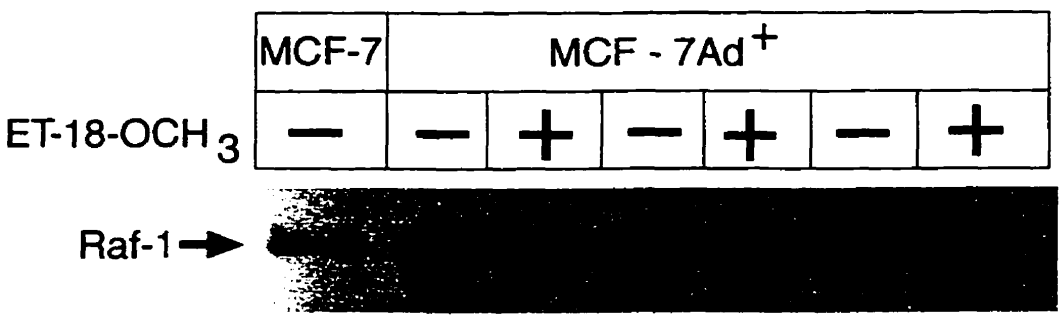


Figure 53. Raf-1 levels in membranes fron control and ET-18-OCH_3 treated MCF-7Ad$^+$ cells. Membrane fractions were prepared from quiescent MCF-7Ad$^+$ cells treated with or without ET-18-OCH_3 (20 μM) for 5 h. Western blot analysis was conducted with 15 μg membrane protein using the anti-Raf-1 antibody as described in Figure 22. Membrane fractions prepared from MCF-7 cells were also analysed for comparison. The results are from a single experiment that is representative of results obtained from 3 different cell preparations.

showed that in quiescent unstimulated MCF-7Ad$^+$ cells significant levels of Raf-1 were associated with the membranes compared to the levels in quiescent MCF-7 cells. Densitometric analysis revealed a 5-fold increase in the Raf-levels in MCF-7Ad$^+$ membranes relative to MCF-7 cells. Stimulation of the MCF-7Ad$^+$ cells with EGF or ACGM$^+$ did not significantly increase the levels of Raf-1 associated with the membrane **(Fig 54 and 55)**. Examination of Raf-1 levels in membranes isolated from proliferating cells showed that, as with the quiescent cells, the quantities of Raf-1 were greater in the MCF-7Ad$^+$ cells than in the proliferating MCF-7 cells **(Fig 56)**.

4.3.12 MEK activity in MCF-7Ad$^+$ cells

The artificial targeting of Raf-1 to cellular membranes by inserting the CAAX motif, resulted in Raf activation in the absence of growth factor stimulation (Stokoe *et al.*,1994). We therefore investigated whether the constitutive association of Raf-1 with membranes in MCF-7Ad$^+$ cells results in its activation. Raf activation was assessed by determining the level of phosphorylated MEK in the cells using Western blot analysis with phospho-specific MEK antibodies. The results which are displayed in **Fig 57** and **58** show that in unstimulated quiescent MCF-7Ad$^+$ cells, MEK was not phosphorylated, despite the large amounts of Raf associated with the membrane. Subsequent to stimulation with EGF or ACGM$^+$, MEK was phosphorylated in both control cells and those pretreated with ET-18-OCH_3 with little difference between the drug-treated and untreated groups **(Fig 57 and 58)**. Densitometric analysis of the blots showed that after 15 min, the relative increase in MEK phosphorylation in EGF-stimulated cells, as much greater than those in ACGM$^+$-treated cells. In control cells,

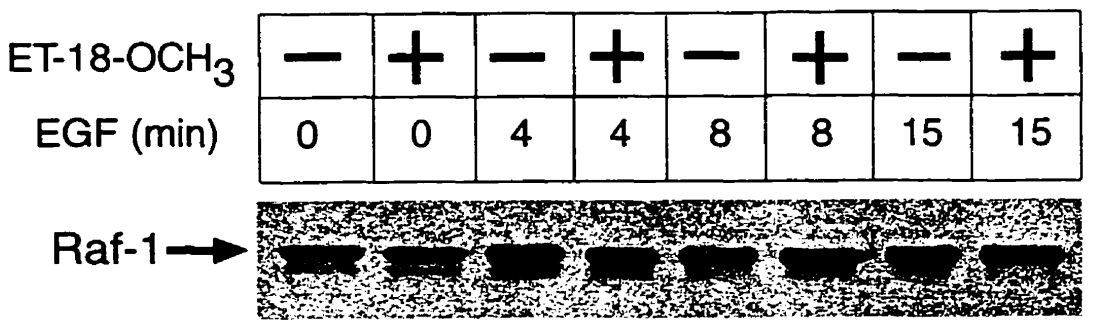


Figure 54. Effect of ET-18-OCH_3 on the association of Raf-1 to the membranes in EGF-stimulated MCF-7Ad$^+$ cells. Quiescent MCF-7Ad$^+$ cells were incubated with or without ET-18-OCH_3 (20 μM) for 5 h. The cells were washed and stimulated with EGF for various times. Membrane fractions were prepared and Western blot analysis was conducted using the anti-Raf-1 antibody as described in figure 26. The results are from a single experiment that is representative of results obtained from 4 different preparations.

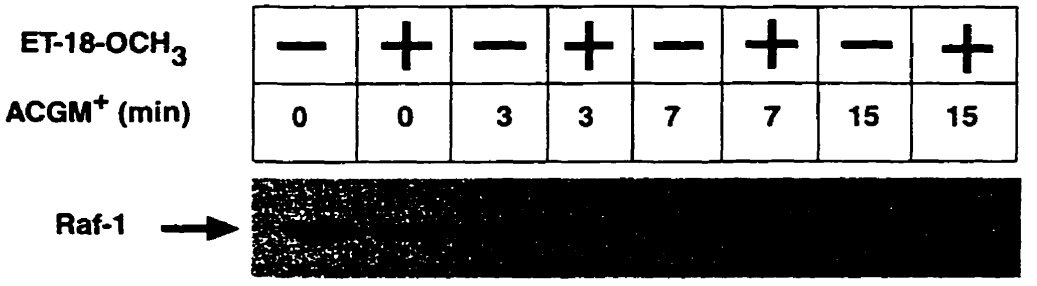


Figure 55. Effect of ET-18-OCH_3, on the association of Raf-1 to the membranes in $ACGM^+$-stimulated $MCF\text{-}7Ad^+$ cells. Quiescent $MCF\text{-}7Ad^+$ cells were incubated with or without ET-18-OCH_3 (20 μM) for 5 h. The cells were washed and stimulated with $ACGM^+$ for various times. Membrane fractions were preparped and Western blot analysis was conducted using the anti-Raf-1 antibody as described in Figure 26. The results are from a single experiment that is representative of results obtained from 4 different cell preparations.

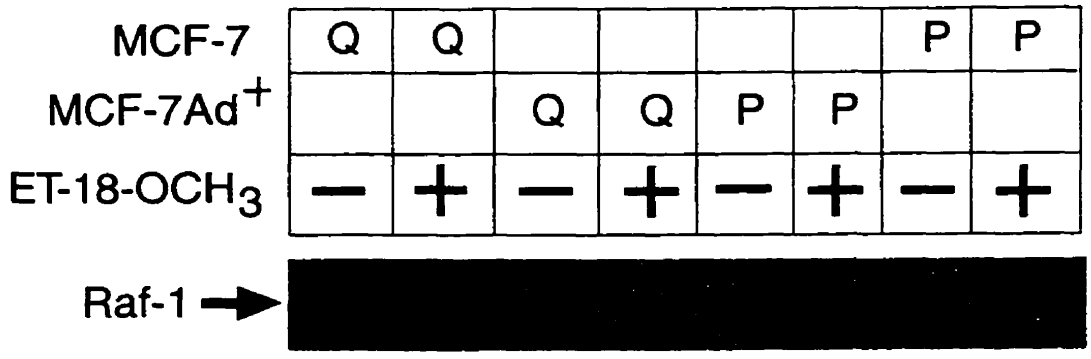


Figure 56. Comparison of the level of membrane bound Raf-1 in MCF-7 and MCF-7Ad$^+$ cells. Membrane fractions were prepared from unstimulated quiescent (Q) and proliferating (P) MCF-7 and MCF-7Ad$^+$ cells incubated with or without ET-18-OCH_3. Quiescent MCF-7 and MCF-7Ad$^+$ cells were incubated with ET-18-OCH_3 (20 μM) for 3 h and 5 h respectively. Proliferating MCF-7 and MCF-7Ad$^+$ cells were both incubated with ET-18-OCH_3 (20 μM) for 6 h. Western blot analysis was conducted with the anti-Raf-1 antibody as described in Figure 22 with 15 μg of membrane protein. The results are from a single experiment that is representative of results obtained with 3 different cell preparations.

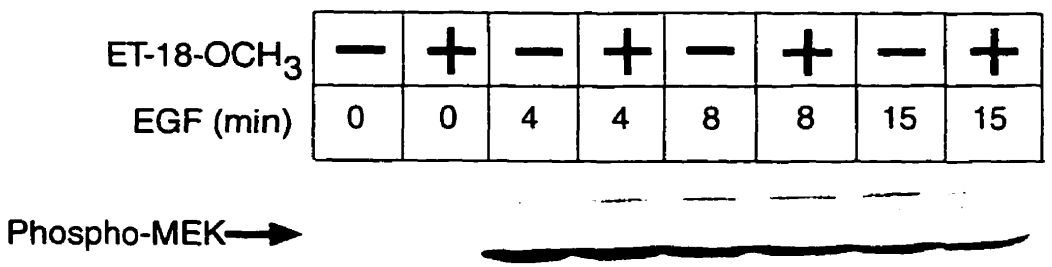


Figure 57. Effect of ET-18-OCH_3 on MEK phosphorylation in EGF-stimulated MCF-7Ad$^+$ cells. Quiescent MCF-7Ad$^+$ cells were incubated with ET-18-OCH_3 (20 μM) for 5 h. The cells were washed and stimulated with EGF for various times. Cytosolic fractions were prepared and Western blot analysis was conducted using the anti-phospho-MEK antibody after separating 50 μg protein by 10% SDS-PAGE. The results are from a single experiment that is representative of results obtained from 4 different cell preparations.

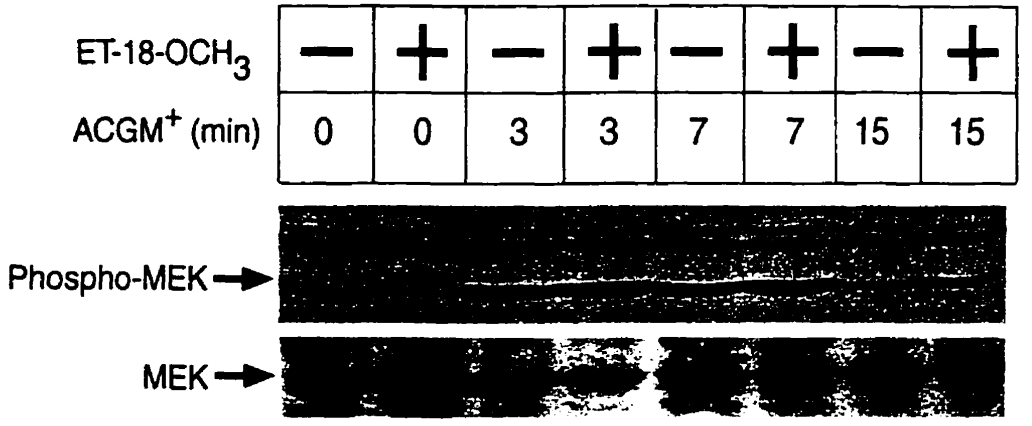


Figure 58. Effect of ET-18-OCH_3 on MEK phosphorylation in $ACGM^+$-stimulated $MCF\text{-}7Ad^+$ cells. Experiments were conducted as described in Figure 57, but the cells were stimulated with $ACGM^+$. Western blot analysis was performed with the anti-phospho-MEK and anti-MEK antibodies. The results are from a single experiment that is representative of results obtained from 4 different cell preparations.

for example, the densitometric values were 1.05 versus 0.004 in EGF-stimulated cells and 0.431 versus 0.051 in $ACGM^+$-stimulated cells. Thus, these results are consistent with the observation that MAP kinase activation is sustained for longer periods by EGF compared to $ACGM^+$.

4.3.13 Correlation between membrane Raf-1 levels and ET-18-OCH_3-insensitivity in adapted MCF-7 cells

The above results led us to investigate whether the association of Raf-1 to membranes correlates with acquisition of insensitivity to ET-18-OCH_3. MCF-7 cells were adapted for growth from FBS-supplemented medium into $ACGM^+$. At each subculturing stage during the adaptation process, some of the cells were used for studies on the effect of ET-18-OCH_3 on cell proliferation, while some were seeded into dishes for determination of the levels of Raf in the membrane of quiescent cells using the procedures described in **section 3.14**.

The results of the studies on the ET-18-OCH_3-sensitivity which are shown in **Fig 44** and were described in **section 4.3.8**, showed that increasing insensitivity results from increasing time spent in media with decreasing levels of FBS, or conversely increasing levels of $ACGM^+$. A significantly sharp decrease in ET-18-OCH_3-sensitivity was observed between cells grown in 1.25% serum-containing medium and 0.625% serum-containing medium implying that changes responsible for the insensitivity of the cells were optimally functional at this stage of the adaptation processes.

The results on the Raf-membrane levels showed that there was a progressive increase in the amount of Raf-1 associated with the membrane during the adaptation process **(Fig 59)**.

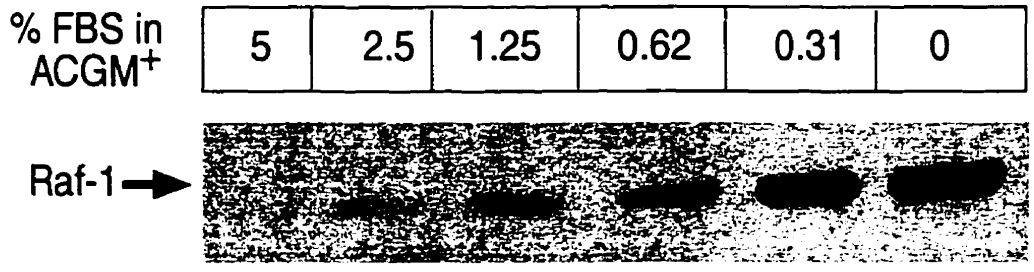


Figure 59. Raf-1 levels in membranes of MCF-7 cells during adaption for growth in serum-free medium. MCF-7 cells were adapted for growth in $ACGM^+$ using the procedures described in the methods. During the adaption process, cells growing in medium containing 5%. 2.5%, 1.25%, 0.62%, 0.31% and 0% FBS were made quiescent. Membrane fractions were prepared and the level of membrane-bound Raf-1 was determined by Western blot analysis using the anti-Raf-1 antibody. The results are from a single experiment that is representative of 4 different cell preparations.

Scanning of the blots yielded 0.004, 0.095, 0.367, 0.621, 1.675 and 2.124 (arbitrary units) for 5, 2.5, 1.25, 0.62, 0.31 and 0% (FBS content) respectively. Thus, there were very significant differences in the Raf levels at each stage in the adaptation process. The changes in membrane Raf-1 levels clearly correlate with the acquisition of ET-18-OCH_3-insensitivity.

As MCF-7Ad$^-$ cells are also less sensitive to ET-18-OCH_3 compared to MCF-7 cells, we investigated whether this could also be caused by increased level of Raf-1 in their membranes. Membranes were isolated from quiescent MCF-7Ad$^-$ cells and subjected to Western blot analysis with the Raf-1 antibody. The results revealed that there was increased Raf-1 in the membrane in the quiescent MCF-7Ad$^-$ cells compared to the quiescent MCF-7 cells **(Fig 60)**. When these MCF-7Ad$^-$ cells were grown in ACGM$^+$ medium, a progressive increase in the amount of Raf-1 associated with the membrane at different passages in ACGM$^+$ was observed **(Fig 60)**. Densitometric analysis revealed a progressive increase in the Raf-1 levels from 1.072 in MCF-7Ad$^-$ cells to 2.416 (arbitrary units) in MCF-7Ad$^-$ cells after 12 passages in ACGM$^+$. The values for MCF-7 and MCF-7Ad$^+$ which were run for comparison were 0.18 and 2.45, respectively. The increased levels of membrane Raf-1 as a consequence of growth of the cells in ACGM$^+$ correlates with increasing insensitivity of the cells to ET-18-OCH_3 **(Fig 43)**.

4.3.14 Raf-1 levels in membranes of HNME cells

HNME cells are insensitive to ET-18-OCH_3. In a preliminary study we investigated whether this insensitivity could be due to increased association of Raf-1 with cell membranes in these cells. Quiescent HNME cells were treated with or without ET-18-OCH_3 and

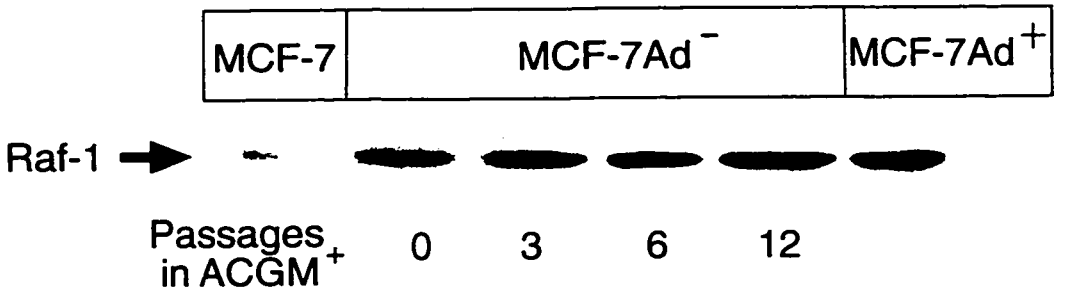


Figure 60. Raf-1 levels in membranes isolated from MCF-7Ad⁻ cells grown in ACGM⁺. Membrane fractions were prepared from quiescent MCF-7 cells, MCF-7Ad⁻ cells grown in ACGM+ for 0, 3, 6 and 12 passages and from MCF-7Ad⁺ cells. Western blot analysis was conducted using anti-Raf-1 antibodies as described in Figure 26. The results are from a single experiment that is representative of those obtained with 4 different cell preparations.

stimulated with EGF. Membranes were isolated from the cells and the levels of Raf were assessed by Western blot analysis with Raf-1 antibodies. Significant levels of Raf were found in membranes isolated from untreated and ET-18-OCH_3-treated quiescent HNME cells (**Fig 61**). A slight decrease in membrane associated Raf-1 was apparent in the drug-treated cells relative to controls. Stimulation with EGF did not increase the Raf levels but caused a slight decrease after prolonged stimulation.

4.3.15 Lipid composition of MCF-7 and MCF-7Ad$^+$ cells

There are suggestions that Raf associates specifically with membrane phospholipids (Ghosh *et al.*, 1994; Ghosh *et al.*, 1996). We therefore compared the lipid composition of MCF-7 and MCF-7Ad$^+$ to determine if there were any dramatic changes. MCF-7Ad$^+$ cells had 23% more phospholipids than the parental MCF-7 cells (**Table 1**). With respect to the phospholipid classes, the proportion of SM doubled from 3.4% in MCF-7 cells to 6.6% in MCF-7Ad$^+$ cells. The PE levels increased by 14% in MCF-7Ad$^+$ cells relative to MCF-7 cells. The PI and PA levels dropped by 24 and 43% in MCF-7Ad$^+$ compared to the levels in the parental MCF-7 cells. The proportion of the PC and PS were not significantly different.

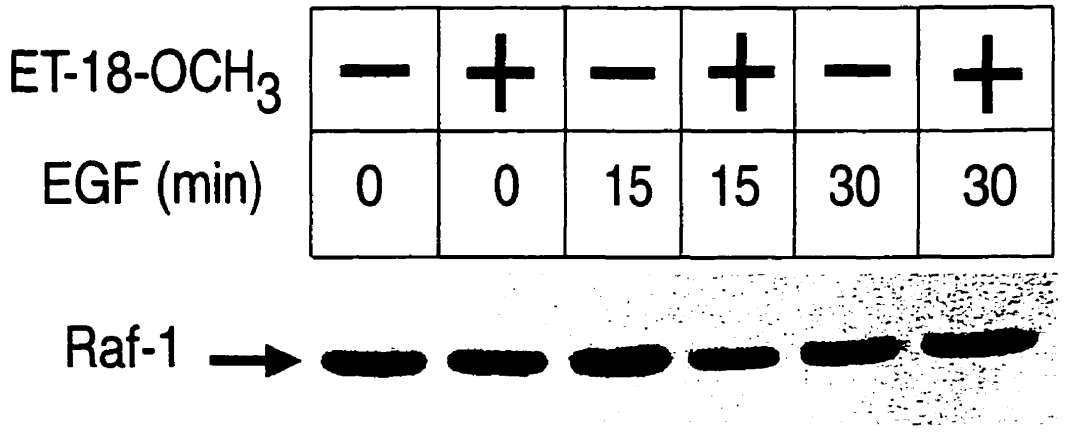


Figure 61. Effect of ET-18-OCH_3 on the association of Raf-1 in unstimulated and EGF stimulated HNME cell menbranes. Quiescent HNME cells were preincubated with 20 μM ET-18-OCH_3 for 1.5 h. The cells were stimulated with EGF for varying periods, washed and the cells were harvested as described in the methods. Membrane fractions were prepared and Western blot analysis was conducted using anti-Raf-1 antibody as described in Figure 26. The results are from a single experiment that is representative of those obtained with 2 different cell preparations.

Table 1. Phospholipid composition and content of MCF-7 and MCF-7Ad$^+$ cells. Log-phase MCF-7 or MCF-7Ad$^+$ cells grown in 150 mm dishes were harvested and washed in HBSS. Aliquots were taken for determination of cell number. Lipids were extracted and the phospholipid classes were separated by TLC. The phosphorus content in each phospholipid class was determined by the malachite green method described in **section 3.9** of the methods. The values represent the means ± S.D. of three experiments analyzed in duplicate. The asterisks indicate results of MCF-7Ad$^+$ cells that are significantly different from those of MCF-7 cells with a value of $p < 0.01$ or less by the Student's t test.

Phospholipid	MCF-7	MCF-7Ad$^+$
	% of total lipid phosphorus	
SM	3.4 ± 0.08	6.64 ± 0.26*
PC	56.64 ± 0.7	53.72 ± 0.89
PS	7.86 ± 0.4	7.31 ± 0.56
PI	7.4 ± 0.61	5.32 ± 0.28*
PE	22.83 ± 0.73	26.08 ± 0.77*
PA	1.85 ± 0.04	1.06 ± 0.05*
Phospholipid content[a]	45.66 ± 1.08	56.18 ± 0.93*

[a] nmol/10^6 cells

5 DISCUSSION

5.1 Introduction

The mechanism of inhibition of cell proliferation by ALPs has been ascribed to a variety of events including inhibition of nutrient uptake (Berkovic *et al.*, 1992; Hoffman *et al.*, 1992; Melchior *et al.*, 1990), increase in intracellular-free Ca^{2+} levels (Lazenby *et al.*, 1990; Lohmeyer and Workman, 1993), induction of apoptosis (Diomede, 1994; Fujiwara 1992; 1993; Vagnetti *et al.*, 1990), inhibition of phosphatidylcholine synthesis (Vogler *et al.*, 1985) and inhibition of cell signaling pathways including PKC, PI 3-kinase and the MAP kinase pathway (Berggren *et al.*, 1993; Grunicke *et al.*, 1989; Helfman *et al.*, 1983; Powis, 1991; Zhou *et al.*, 1996). The apparent involvement of such wide-ranging mechanisms is a testament of the ability of ALPs to affect the activity of a wide variety of molecules *in vitro*. It is also quite likely that the mechanism of inhibition of cell proliferation by ALPs may be different in different cell types. However, few studies have related the proposed changes to inhibition of cell proliferation. Studies in our laboratory on the mechanism of action of ET-18-OCH_3 in inhibiting the growth of MCF-7 cells and other epithelial cells, demonstrated that the cytotoxic effects of the drug on these cells were unlikely to be due to an effect of the compound in inhibiting lipid metabolism (Lu and Arthur 1992a; 1992b; Zhou and Arthur 1995). Subsequent studies demonstrated that ET-18-OCH_3 inhibited MAP kinase activity in MCF-7 cells (Zhou *et al.*, 1996). This inhibition appeared to be due to the ability of ET-18-OCH_3 to decrease the association of Raf-1 with the plasma membrane. As Raf-1 membrane association is required for its activation, the decreased Raf-1 levels prevent sustained signaling from Raf-1 and consequently, sustained activation of MAP kinase. In this study, a direct

correlation between decreased Raf-1 levels, decreased MAP kinase activation and inhibition of cell proliferation was demonstrated. These observations led to the hypothesis that the mechanism of inhibition of cell proliferation by ET-18-OCH_3 was due to its effect on Raf-1 activation (Zhou *et al.*, 1996).

If the above hypothesis is valid, then one could propose that the selective inhibitory effects of ET-18-OCH_3 are either due to differences in the mechanism of activation of Raf-1 between the ALP-sensitive and -insensitive cell lines or the transduction of mitogenic signals via Raf-independent mechanisms in the insensitive cell lines. The central role of Raf-1 in these hypotheses makes it essential to confirm the relevance of the inhibition of Raf-1 activation in the mechanism of action by ALPs, prior to studies to investigate these hypotheses. In this project studies were therefore conducted to validate the proposed role of inhibition of Raf-1 activation in the mechanism of action of ALPs. Subsequently, studies were conducted to examine whether the selective response of cells to ALPs is linked to Raf-1.

5.2 Mechanism of inhibition of cell proliferation by ALPs

To establish the relevancy of ET-18-OCH_3-induced inhibition of Raf-1 in the mechanism of action of ALP, our approach was to first identify a pair of structurally related analogues of ALP which had differential effects on the proliferation of cancer cells, and then investigate whether these effects correlated with the effect of the compounds on Raf-1 activation.

We initially examined the effects of lysophosphatidate analogs of ET-16-OCH_3 on proliferation of epithelial cells **(section 4.1.1)**. These studies showed that ET-16-OCH_3-

phosphatidate and ET-16S-OCH_3-phosphonate had little growth inhibitory effects on the epithelial cancer cells tested **(Fig 8 and 10)**, whereas ET-16-OCH_3-phosphonate very potently inhibited cell growth **(Fig 9)**. From these studies, we identified ET-16-OCH_3-phosphonate and ET-16S-OCH_3-phosphonate as closely related structural analogues that had differential effects on cell proliferation. The difference between the two compounds was the presence of a thioether linkage at the C-1 position in the inactive compound as opposed to an ether linkage in the active compound. It is unclear why the thioether bond should reduce the cytotoxicity of ET-16S-OCH_3, as ilmofosine also has a thioether bond at the C-1 position and is a potent antiproliferative compound (Neumann *et al.*, 1987; Winkelman *et al.*, 1992).

Enantiomers of ALPs would make an ideal active/inactive pair for probing the relevance of cellular events in their mechanism of action. The use of such related compounds would eliminate potential problems of differences in solubility that may arise by using non-enantiomers. This could result in significant differences in the quantities of the compounds that accumulate in the cell. Unfortunately, previous studies had demonstrated little differences in the potency of enantiomers or other chiral isomers of ALPs against tumor cells (Bittman *et al.*, 1993; Houlihan *et al.*, 1995). These included studies with *(R)* and *(S)*-enantiomers of ET-16-OCH_3 (Bittman *et al.*, 1994; Lohmeyer and Workman, 1992), as well as its phosphonocholine analogues (Bittman *et al.*, 1994). In another study, (*S*) and (*R*) enantiomers of SRI 62•834 showed no difference in their cytotoxicity against various cell lines (Houlihan *et al.*, 1992; Lohmeyer and Workman, 1992), but interestingly, in a mouse tumor model, the (*S*) enantiomer was 3-4 fold more effective in decreasing tumor size and

increasing host survival than the (*R*) enantiomer (Houlihan *et al.*, 1992). This led us to conduct studies with (*S*) and (*R*) enantiomers of oxo-ilmofosine **(Fig 11)** and ET-16-phosphono-TDB, a double bond phosphonocholine **(Fig 16)**.

The enantiomers of oxo-ilmofosine showed a similar antiproliferative profile against a number of epithelial cell lines **(Fig 12 and 13)** and both enantiomers had similar growth-inhibitory effects on MCF-7 **(Fig 14)** and A549 cells **(Fig 15)**, similar to that of (*rac*)-ilmofosine. Thus, (*S*)- and (*R*)-oxo-ilmofosine enantiomers did not represent an active/inactive pair of ALPs and were unsuitable for our study.

Studies with the enantiomers of ET-16-phosphono-TDB, in contrast, demonstrated differential effects on cell proliferation **(Fig 17 and 18)**. The (*S*) enantiomer inhibited the proliferation of all the cell lines examined relative to the (*R*) enantiomer. The potency of the *(S)*- ET-16-phosphono-TDB against MCF-7 cells can be appreciated by the fact that it completely inhibited the proliferation of MCF-7 cells at concentrations similar to those of (*rac*)-ET-18-OCH_3 **(Fig 7, 18)**. It was however, not as potent as ET-18-OCH_3 against the other cell lines. (*R*)-ET-16-phosphono-TDB had little effect on the proliferation of all the cell lines with the exception of the MCF-7 cell line which was inhibited by 50%, suggesting that MCF-7 cells are particularly sensitive to ALPs. The differential effects of the (*R*) and (*S*)-ET-16-phosphono-TDB allowed us to successfully establish incubation conditions with the (*S*)-enantiomer that inhibited the proliferation of quiescent MCF-7 cells subsequent to stimulation with growth medium. Under similar conditions, (*R*)-ET-16-phosphono-TDB had no effect on the growth of the cells **(Fig 19)**. The results of these studies clearly established that *(S)*- and *(R)*-ET-16-phosphono-TDB are an active/inactive pair of compounds **(Fig 17, 18)** that

will be extremely useful in identifying cellular events whose perturbation are relevant to the mechanism of growth-inhibition by ALPs in cells sensitive to the compounds. The phosphonocholine-TDB pair are more suitable for use than the pair of lysophosphatidate analogues because they would eliminate any solubility and uptake problems and their similarity to ET-18-OCH_3 makes it less likely that they would mediate their action via a completely different mechanism.

To assess the relevance of inhibition of MAP kinase activation in the mechanism of action of ALPs, we examined the activation of signaling molecules in the MAP kinase cascade in cells incubated with the active (*S*) and inactive (*R*) enantiomers of ET-16-phosphono-TDB. The results of our studies on the effects of *(S)*- and *(R)*-ET-16-phosphono-TDB on EGF-stimulated mitogenic signalling supports the hypothesis that ALPs inhibit cell growth as a consequence of their inhibition of the MAP kinase pathway **(Fig 20)**. The fact that the active enantiomer, incubated under conditions where it inhibited cell proliferation, was indeed able to inhibit MAP kinase phosphorylation and activation **(Fig 22)**, while the inactive enantiomer was unable to do so **(Fig 22)**, provides very strong evidence that the growth inhibitory effects are linked to the ability of the compounds to inhibit MAP kinase activation in these cells. Furthermore, the inhibition of MAP kinase by *(S)*-ET-16-phosphono-TDB resulted in an inhibition of S6 kinase activation, a signaling molecule downstream of MAP kinase **(Fig 23)**. Thus the inhibition of activation of MAP kinase by the *(S)*-ET-16-phosphono-TDB affects the activation of molecules downstream of this enzyme. In view of the key role attributed to MAP kinase in initiating cell proliferation (Marshall, 1995), our observations that *(S)*-ET-16-phosphono-TDB inhibited cell growth and

was able to truncate the activation of MAP kinase **(Fig 18 and 20)**, whereas *(R)*-ET-16-phosphono-TDB was weakly antiproliferative and did not affect the activation of MAP kinase **(Fig 17 and 20)**, suggests that inhibiting the activation of MAP kinase is a key event in the mechanism of inhibition of cell proliferation by *(S)*-ET-16-phosphono-TDB.

Our results also indicate that inhibition of MAP kinase activation by (*S*)-ET-16-phosphono-TDB is at the level of Raf activation. (*S*)-ET-16-phosphono-TDB did not directly inhibit the catalytic activity of MAP kinase **(Fig 21)**, but in cells incubated with the compound MAP kinase phosphorylation was drastically reduced **(Fig 22)**. MEK phosphorylation was also reduced in these cells, which would explain the reduced phosphorylation of MAP kinase. The reduced phosphorylation of MEK in EGF-stimulated cells treated with the *(S)* enantiomer **(Fig 24)**, implicates an effect of the compound on Raf activity. However, we were able to show that the compound is not an inhibitor of the catalytic activity of Raf-1 **(Fig 25)**. Since translocation of Raf to the membrane is required for its activation, and previous studies with ET-18-OCH_3 had shown that decreasing the levels of membrane-associated Raf led to decreased activation of MAP kinase (Zhou *et al.*, 1996), we investigated the effect of the double bond phosphonocholines on membrane Raf levels. Surprisingly, both (*R*) and (*S*)-ET-16-phosphono-TDB promoted the translocation of Raf to the membrane in quiescent cells in the absence of cell stimulation **(Fig 26)**. Neither (*S*)- nor (*R*)-ET-16-phosphono-TDB increased the membrane-translocation of PKC isozymes in unstimulated cells which indicates that (*S*)- and (*R*)-ET-16-phosphono-TDB-induced translocation of Raf-1 is not a nonspecific phenomenon. It is worth noting that the phosphonocholine-mediated translocation of Raf-1 to the membrane in quiescent cells did not result in Raf activation, since it did not lead to

MEK and MAP kinase phosporylation/activation. In contrast, in studies where attachment of a CAAX motif led to the membrane localisation of Raf, MEK and MAP kinase were activated in the absence of growth factors (Stokoe *et al.*, 1994). Our results showing that *(R)*-ET-16-phosphono-TDB is able to cause the translocation of Raf and permit its subsequent activation in response to EGF stimulation **(Fig 27)** provides direct evidence that distinct events mediate the activation of Raf once it is in the membrane.

To investigate the mechanism responsible for the translocation of Raf by the phosphonocholines, we examined the effect of the compounds on Raf-Ras interaction, as the interaction of Raf with Ras is required for its translocation (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). We were able to show that the increased translocation of Raf-1 to the membrane induced by both compounds is unlikely to be due to their ability to increase the affinity of Raf-1 for inactive Ras-GDP in the unstimulated cells. This is because *in vitro* studies with cytosolic Raf-1 from untreated, (*S*) or (*R*)-ET-16-phosphono-TDB-treated unstimulated cells, showed no differences in the affinity of Raf for Ras-GDP. In studies with Ras-GTP, the levels of associating Raf from cytosol from all three experimental groups were also similar and greater than the levels observed associating with Ras-GDP **(Fig 28)**. On the other hand, cytosolic Raf-1 from ET-18-OCH_3-treated unstimulated cells showed a much lower affinity for Ras-GTP than Raf from untreated cells **(Fig 29)**, suggesting that the reported decrease in membrane Raf levels in ET-18-OCH_3-treated cells (Zhou *et al.*, 1996) may be due to the effect of the compound on the translocation of cytosolic Raf-1 to membranes.

Preincubation of cells with the phosphonocholines did not affect the translocation of additional Raf-1 following EGF stimulation **(Fig 27)**. This indicates that neither *(R)*- or *(S)*-

ET-16-phosphono-TDB inhibit the association or translocation of Raf with the membrane as was observed for ET-18-OCH_3 (Zhou *et al.*, 1996). This observation is also consistent with the conclusions from the effect of the compounds on *in vitro* Ras/Raf interaction. However, it is worth pointing out that the levels of Raf translocated to membranes in response to EGF in cells pretreated with ET-16-phosphono-TDB were less than in untreated cells, suggesting that there may be a maximum limit to the quantity of Raf that can associate with the membrane.

The inability of EGF to activate Raf in cells preincubated with the (*S*)-ET-16-phosphono-TDB, even though Raf is present in the membranes, coupled with the lack of inhibition of Raf-kinase activity by the compound **(Fig 25)**, indicates that the inhibition of Raf activation by *(S)*-ET-16-phosphono-TDB is probably due to its effects on a process or processes that activate Raf. Alternately, it may prevent Raf-1 from assuming a favourable conformation that permits its activation. We think it is significant that in spite of the differences in their structure, both ET-18-OCH_3 and *(S)*-ET-16-phosphono-TDB inhibit the activation of the MAP kinase cascade in MCF-7 cells. Furthermore, this is achieved as a consequence of perturbation of Raf-1 activation even though the mechanism via which this is achieved is significantly different between the two compounds. Whereas ET-18-OCH_3 decreases the levels of Raf associating with the membrane, the ET-16-phosphono-TDB inhibits the activation process *per se*. The above finding leads us to suggest that Raf-1 is a key intracellular molecule targeted by choline-containing ALPs and that the compounds interact directly with Raf-1. Understanding the interaction between ALPs and Raf at the molecular level will provide a greater understanding of how these lipids are able to affect the

activation of this key signaling molecule. As indicated in the introduction, Raf is a lipid-binding protein. It has a PS binding domain in its CRD (128-196) and a PA binding site within 389-423 residues (Ghosh *et al.*, 1994; 1996). The binding of Raf to these lipids may be important in the translocation and activation events (Ghosh *et al.*, 1994; 1996). From our studies one can postulate that incubation of cells with ALPs results in accumulation of the compound not only in the membrane but also in the cytosol. Raf-1 which is present in the cytosol binds to these ALPs. Whether the binding occurs at the PA and/or PS sites or different sites, and whether the different ALPs bind at the same or different sites remains to be established. Depending on the ALP, binding to Raf-1 results in the acquisition of a configuration that can either be favourable or unfavourable for its translocation to the membrane and for its subsequent activation.

5.2.1 Model of the mechanism of inhibition of cell proliferation by choline-containing ALPs

Based on our results, we have proposed a model to explain the mechanism of inhibition of cell proliferation by choline-containing ALPs **(Fig 62)**. In quiescent MCF-7 cells, stimulation with growth factors leads to the association of cytosolic inactive Raf-1 with membrane-bound Ras-GTP. This initiates events (Morrison and Cutler, 1997) that lead to Raf translocation to the membrane, and changes that result in Raf-1 assuming a conformation favourable for its activation. This active conformation may be stabilized by its interaction with membrane lipids (Ghosh *et al.*, 1994; 1996). Activation of the kinase activity occurs via unknown events which may involve phosphorylation (Morrison *et al.*, 1993). Once activated

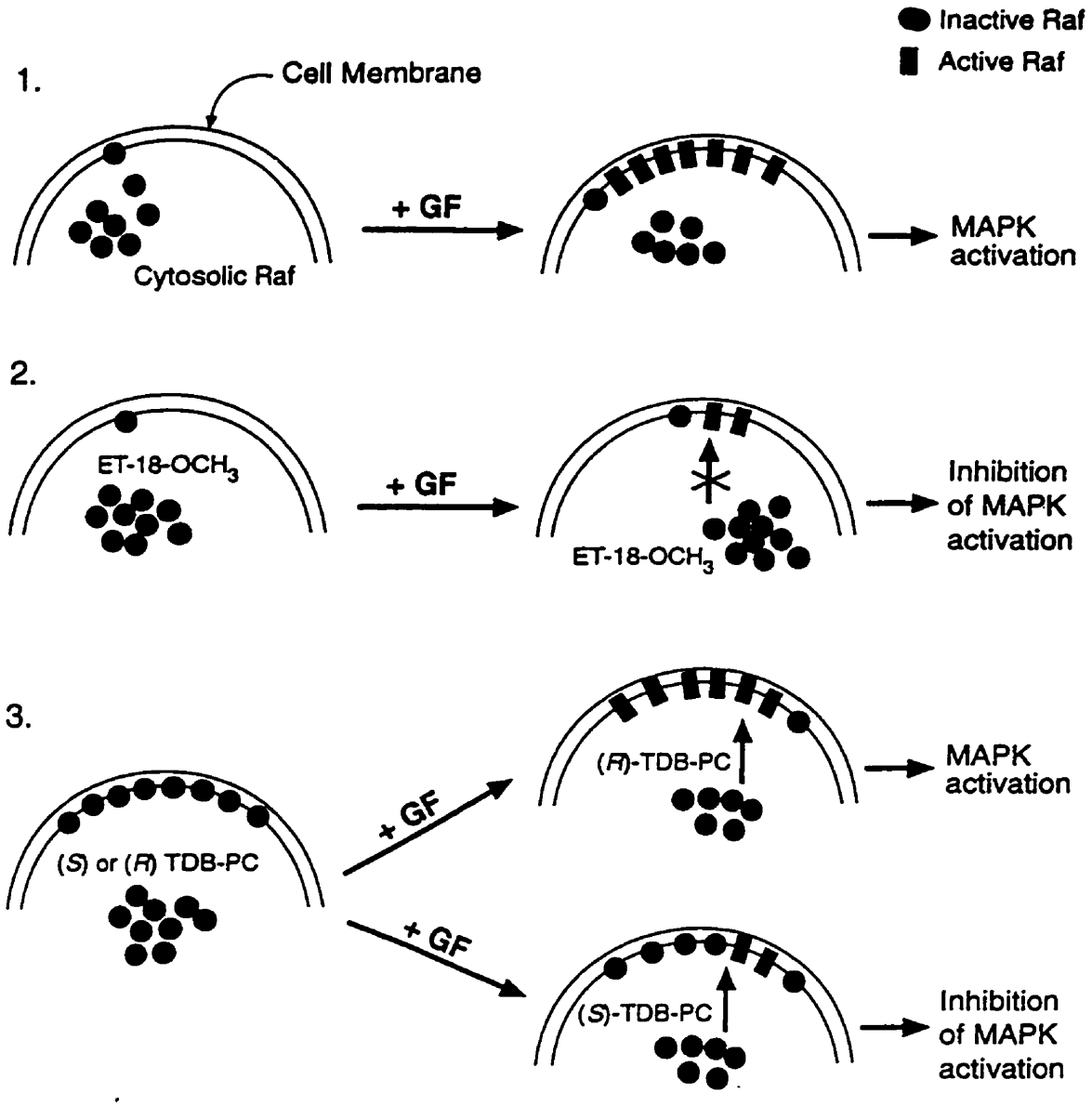


Figure 62. Postulated model of the mechanism of cell-growth inhibition by choline containing ALPs. 1. In quiescent MCF-7 cells Raf is mostly cytosolic and inactive (●). Upon stimulation, Raf translocates to the membrane followed by its activation (■) which leads to the activation of the MAPK cascade. 2. Treatment of the cells with ET-18-OCH_3 results in the inhibition of GF-stimulated Raf translocation and as a consequence, inhibition of sustained activation of Raf and MAPK cascade occurs. 3. Both (*S*)- or (*R*)- ET-16-phosphono-TDB induce Raf to translocate to the membrane in unstimulated cells treated with these drugs. Upon stimulation, only the membrane-bound Raf in (*R*)-ET-16-phosphono-TDB-treated cells becomes activated (■) whereas the membrane-bound Raf in (*S*)-ET-16-phosphono-TDB-treated cells is unable to be activated.

Raf phosphorylates and activates MEK, which then phosphorylates and activates MAP kinase. MAP kinase activates its downstream substrates to initiate a series of events that ultimately results in mitogenesis.

In ET-18-OCH_3-treated cells, cytosolic Raf-1 binds to the compound and acquires a conformation that is unfavourable for interaction with Ras. When ET-18-OCH_3-treated cells are stimulated with a growth factor, the small amount of Raf-1 which is not associated with ET-18-OCH_3 can interact with Ras and be translocated to the membrane and undergo the sequence of events described above for the untreated cells. As association and dissociation of Raf-1 from the membrane is expected to be a dynamic event, the loss of Raf from the membrane in these cells is not replaced by more cytosolic Raf since they are bound to the ET-18-OCH_3 and cannot interact with Ras. Consequently, the phosphorylation of MEK cannot be sustained and signaling via MAP kinase is prematurely terminated. Whether ET-18-OCH_3 promotes the dissociation of membrane-bound Raf-1(Zhou *et al.*, 1996), still remains to be explored.

In (*R*)- and (*S*)-ET-16-phosphono-TDB-treated cells, cytosolic Raf-1 binds to the compounds which results in the acquisition of a favourable conformation for Ras-independent membrane translocation. Upon stimulation, the membrane-bound Raf with associated (*R*)-ET-16-phosphono-TDB is able to be activated, which leads to the activation of MAP kinase. In contrast in the (*S*)-ET-16-phosphono-TDB-membrane associated Raf, the conformation is such that Raf cannot be activated and consequently there is no activation of MEK and MAP kinase. While the basis for the chiral effect of the double bond phosphonocholines is unclear, perhaps the introduction of the trans double bond in the phosphonocholine may restrict the

torsional movement (flexibility) of the molecule and thereby maintain the (*S*) enantiomer in a conformation that allows it to interact in such a way that it prevents Raf from assuming conformational changes essential for its activation. Alternatively the site of interaction of (*S*)-ET-16-phosphono-TDB with Raf may prevent the interaction of Raf with unknown molecules required for its activation. Elucidation of the site of interaction of the ALPs with Raf-1 in future studies will allow the molecular events to be fleshed out in greater detail and may lead to synthesis of even more potent ALPs that selectively interfere in the activation of Raf-1.

5.3 Mechanism of insensitivity of cells to ALPs

5.3.1 ET-18-OCH_3-insensitive cells

One of the characteristics of ALPs that makes them potentially valuable as anticancer drugs is the selectivity they display in inhibiting the growth of cells. Not only do they display selective effects among cancer cells, but they also appear to inhibit cancer cells at concentrations that do not affect the growth of normal cells (Berdel *et al.*, 1985; Lohmeyer and Bittman, 1994). To investigate the mechanisms responsible for the selective effects of ALPs we initially conducted studies to establish that the selectivity displayed by ALPs, which has primarily been established with hemopoietic cells, extended to epithelial cell lines. We therefore investigated the effect of ET-18-OCH_3 on the proliferation of HNME cells. The results revealed that these normal primary epithelial cells are relatively insensitive to growth inhibition by ET-18-OCH_3 **(Fig 31)**. Thus, the notion that ALPs have differential effects on cancer and normal cells extends beyond hemopoietic cells.

In order to minimize differences in experimental conditions between the MCF-7 and

HNME cells, the initial intention was to culture MCF-7 cells in serum-free medium, since HNME cells are cultured in serum-free media. We developed serum-free media that supported the growth of MCF-7 cells and adapted the cells for growth in the medium to improve the proliferation rate. Surprisingly, the MCF-7 cells obtained after adaptation, MCF-7Ad$^+$ and MCF-7Ad$^-$, were relatively insensitive to growth inhibition by ET-18-OCH_3 compared to the parental MCF-7 cells **(Fig 35)**. The response of the MCF-7Ad$^+$ cells to ET-18-OCH_3 was very similar to that obtained for the HNME cells **(Fig 31 and 35)**. This led to the decision to use MCF-7Ad$^+$ cells to elucidate the basis for the insensitivity to ET-18-OCH_3, because they were derived from MCF-7 cells and would more closely resemble the parental cell than the primary HNME cells. Since the MCF-7Ad$^+$ cells could be reversibly converted to MCF-7 cells, this provided an opportunity to discover reversible cellular changes that correlated with the transformation of MCF-7 to MCF-7Ad$^+$ cells and vice versa. Before embarking on the search for cellular changes, it was important to demonstrate that the ET-18-OCH_3-insensitivity of the adapted cells was real and not an artifact of the experimental system.

There were differences in the experimental systems used to investigate the effect of ET-18-OCH_3 on the proliferation of MCF-7 and MCF-7Ad$^+$ cells. Addition of the drug to MCF-7Ad$^+$ cells required supplementing the media with BSA to bring the protein concentration to the levels of that in FBS-supplemented medium. This is due to the fact that ALPs bind to proteins and thus the protein content determines the effective concentration available to the cells (Munder and Westphal, 1990). It was therefore important to show that the sensitivity of cells was independent of whether ET-18-OCH_3 was added to the cells in

medium containing BSA or FBS-supplemented medium. We showed that MCF-7Ad$^+$ cells were insensitive in the presence of 10% FBS or BSA (3 mg/ml) **(Fig 36)**. Conversely, the incubation of MCF-7 cells with ET-18-OCH_3 in BSA-supplemented medium did not decrease the sensitivity of the cells to the drug **(Fig 36)**. These results therefore discount any possibility that the altered sensitivity of MCF-7 cells was due to the presence of factors in the serum that were absent when BSA was used in the serum-free media to bring the protein level to that of medium containing FBS.

As mentioned earlier, the growth rate of MCF-7Ad$^+$ cells was about 50% lower than that of MCF-7 cells in 10% FBS medium **(Fig 37)**. We therefore investigated whether the sensitivity of cells to ET-18-OCH_3 was related to their growth rate. We demonstrated that the addition of estrogen to ACGM$^+$ which increased the growth rate of MCF-7Ad$^+$ cells to levels approaching that of cells grown in FBS-supplemented medium **(Fig 37)** had no effect on the insensitivity to ET-18-OCH_3 **(Fig 36)**. This suggested that the rate of growth was not a factor in determining the sensitivity of cells to ALPs. Further evidence in support of this comes from results of the study in which ET-18-OCH_3 was added in BSA-supplemented medium to proliferating MCF-7 cells. As a consequence of replacing FBS-supplemented medium with BSA, the cell numbers after 48 h in control cells without any ET-18-OCH_3 were 1.4 fold greater than the numbers at day 0 compared to a 3.7 fold increase in cells incubated with FBS-supplemented medium. In spite of the decrease in rate of cell proliferation, the MCF-7 cells were still as sensitive to the ALP as cells in FBS-supplemented medium **(Fig 36)**. In addition, HNME cells grow at similar rates in MEGM to MCF-7 cells in 10% FBS-supplemented DMEM **(Fig 30)** and yet the HNME cells were insensitive to ET-18-OCH_3

while the MCF-7 cells were not. Also, MCF-7Ad$^-$ cells grow at similar rates to MCF-7Ad$^+$ cells (**Fig 34**) and yet there are significant differences in their sensitivity to ET-18-OCH_3. Taken together the results indicate that the sensitivity of cells to ET-18-OCH_3 is unrelated to their growth rate.

We were also able to demonstrate that the insensitivity of cells that occurred after adaptation for growth in ACGM (**Fig 35**), and reacquiring of ET-18-OCH_3-sensitivity following growth of the adapted cells back to FBS-supplemented medium (**Fig 42**), was characteristic of all MCF-7 cells since similar results were obtained with five independent MCF-7 clones (**Figs 40 and 41**). These results suggest that it is unlikely that a population of ET-18-OCH_3-insensitive cells was selected during the adaptation process.

As a consequence of the above studies, we concluded that during the adaptation process, changes had occurred to the MCF-7 cells that had resulted in the cells becoming insensitive to ET-18-OCH_3. We therefore investigated the basis of the acquired ET-18-OCH_3-insensitivity.

5.3.2 Uptake and metabolism of ET-18-OCH_3 in sensitive and insensitive cells

A possible reason for the ET-18-OCH_3-insensitivity displayed by HNME, MCF-7Ad$^-$ and MCF-7Ad$^+$ could have been decreased uptake or increased metabolism of ET-18-OCH_3 by these cells relative to MCF-7 cells. However, when we determined the uptake of [^{3}H]ET-18-OCH_3 in proliferating MCF-7, MCF-7Ad$^-$, MCF-7Ad$^+$ and HNME cells, we observed that uptake of ET-18-OCH_3 was similar in proliferating MCF-7 and MCF-7Ad$^+$ cells, while the uptake in MCF-7Ad$^-$ cells was slightly higher than in MCF-7 cells. HNME cells took up

more ET-18-OCH_3 than the sensitive MCF-7 cells **(Fig 38)**. We were also unable to find any differences in the metabolism of the compound by the different cell lines **(section 4.3.5)**. The results of these uptake and metabolism studies led us to conclude that the differential effect of ET-18-OCH_3 on the proliferation of the cells was unrelated to the quantities present in the cell or differences in the metabolism of the ALP. This finding is consistent with that from other studies that have failed to observe a direct correlation between the accumulation of ALP and sensitivity of cells (Chabot *et al.*, 1989 Fleer *et al.*, 1987; Lu and Arthur, 1992a). We cannot, of course, discount the possibility of differential accumulation of the compound in specific organelles in sensitive and insensitive cells.

5.3.3 Raf-1 and ET-18-OCH_3-insensitivity

Having established that the ET-18-OCH_3-insensitivity was not due to differential uptake or metabolism of the drug we initiated a series of studies to examine the effects of ET-18-OCH_3 on the activation of Raf and MAP kinase in the insensitive cells. Studies with MCF-7 had indicated that accumulation of ET-18-OCH_3 in the cells led to diminished Raf levels in cell membranes that resulted in the inability to sustain MAP kinase phosphorylation and activation in the cells (Zhou *et al.*, 1996). Since accumulation of similar levels of ET-18-OCH_3 in MCF-7Ad$^+$ and HNME cells was expected to lead to a similar decrease in membrane Raf levels and Raf-dependent MAP kinase activation, the inability of the compound to inhibit cell proliferation suggested that (a) MAP kinase was activated by a Raf-1 independent mechanism in the ALP-insensitive cells, (b) the mechanism of Raf-1 activation was different between sensitive and insensitive cells or (c) in insensitive cells, mitogenesis was mediated by

signaling pathways that did not involve the activation of MAP kinase.

In developing $ACGM^+$ we established that EGF and insulin were the primary growth factors and since both act via the MAP kinase pathway this ruled out the possibility that the major mitogenic signaling pathway was a MAP kinase- independent one. We were able to establish that EGF or $ACGM^+$-induced activation of MAP kinase in ET-18-OCH_3-treated $MCF\text{-}7Ad^+$ cells, revealing that MAP kinase was only very slightly inhibited in EGF-stimulated cells **(Fig 45)** while in $ACGM^+$-stimulated cells there was no effect **(Fig 46)**. In contrast, preincubation of MCF-7 cells with ET-18-OCH_3 effectively inhibited MAP kinase activation **(Fig 47)** as had been reported (Zhou *et al.*, 1996). Interestingly, MAP kinase activity and phosphorylation in $MCF\text{-}7Ad^+$ cells was sustained for a longer period in EGF-stimulated cells compared to $ACGM^+$ stimulation **(Fig 48 and 49)**. We were able to demonstrate that preincubation of the cells with ET-18-OCH_3 had little effect on the phosphorylation of MEK subsequent to cell stimulation **(Fig 57 and 58)**.

The fact that accumulation of ET-18-OCH_3, which perturbs Raf activation, but was unable to inhibit the MAP kinase activity, now meant that either MAP kinase activation occurred independently of Raf or the mechanism of Raf-1 activation in these cells was different from that in MCF-7 cells.

Our examination of the levels of Raf associated with $MCF\text{-}7Ad^+$ cells revealed that in both ET-18-OCH_3-treated and untreated $MCF\text{-}7Ad^+$ cells a considerable amount of Raf-1 was associated with the membrane compared to unstimulated quiescent MCF-7 cells **(Fig 53)**. It is worth noting that in spite of the large levels of Raf in the quiescent $MCF\text{-}7Ad^+$ cells, neither MEK or MAP kinase were active **(Fig 48, 49, 57 and 58)**. We were also able to

show that proliferating MCF-7Ad$^+$ cells had more Raf in their membranes compared to MCF-7 cells. Thus it appears that a fundamental difference between the MCF-7 cells and the MCF-7Ad$^+$ cells is the constitutive association of Raf-1 in the membrane. The level of membrane bound Raf in the MCF-7Ad$^+$ cells was similar in both ALP treated and untreated cells. Stimulation of ET-18-OCH_3-treated and untreated quiescent MCF-7Ad$^+$ cells with EGF or ACGM$^+$ did not result in further translocation of Raf to the membrane **(Fig 54 and 55)** but MEK and MAP kinase were both phosphorylated.

The studies with HNME revealed that as with MCF-7Ad$^+$ cells, ET-18-OCH_3 had no effect on MAP kinase activation **(Fig 50 and 51)** and furthermore, Raf was associated with the membrane in unstimulated cells **(Fig 61)**. This strongly suggests that the mechanism of ET-18-OCH_3-insensitivity is similar in these two cells.

Constitutive association of Raf-1 with cell membranes would allow cells to escape the inhibitory effect of ET-18-OCH_3. This is because, if as postulated, ET-18-OCH_3 acts by preventing translocation of Raf to the membrane **(section 4.3.11)**, then it would have no effect if the activation of Raf does not depend on translocation, since it is already constitutively associated. Our studies suggest that insensitivity of MCF-7Ad$^+$, MCF-7Ad$^-$ and HNME cells is in fact due to the constitutive association of Raf with membranes in the cells. The evidence for this is as follows:

1. In quiescent MCF-7 cells which are very sensitive to ET-18-OCH_3, Raf is not found (or very small amount) in the membrane **(Fig 53)**. The levels of membrane-bound Raf in MCF-7Ad$^-$ cells, which are less sensitive than MCF-7 cells, was greater than in MCF-7 cells **(Fig 60)**. MCF-7Ad$^+$ and HNME cells which were the most

insensitive had much higher levels of Raf than MCF-7Ad$^-$ cells **(Fig 60 and 61)**. Thus, the levels of Raf in the cell membranes correlated with their relative insensitivity to ET-18-OCH_3.

2. We were also able to correlate the increasing ET-18-OCH_3-insensitivity of the MCF-7 cells during the adaptation process **(Fig 44)** with increasing levels of Raf-1 in the membranes at various stages of the adaptation process **(Fig 59)**. In addition, we have been able to show in a preliminary experiment that growth of MCF-7Ad$^+$ cells in serum-supplemented medium which results in restoration of ET-18-OCH_3-sensitivity **(Fig 42)** correlates with decreased levels of Raf-1 in the membranes.

3. We also showed that growth of MCF-7Ad$^-$ in ACGM$^+$ medium which results in increasing insensitivity to ET-18-OCH_3, eventually attaining the insensitivity of MCF-7Ad$^+$ cells **(Fig 43)**. This was also correlated with increasing levels of Raf-1 in the membrane of cells at various stages **(Fig 60)**.

4. It is highly unlikely that the slight increase in EGF-receptor levels (Fig 52) is responsible for the ET-18-OCH_3-insensitivity observed in MCF-7Ad$^+$ cells. If this was the case, one would not expect any difference in ET-18-OCH_3 sensitivity between MCF-7Ad$^+$ and MCF-7Ad$^-$ cells since they were both exposed to the same concentration of EGF for identical periods and hence the EGF receptors are expected to be upregulated in the MCF-7Ad$^-$ cells also.

5. The insensitivity of MCF-7Ad$^-$, MCF-7Ad$^+$ and HNME cells is not due to a lower uptake or enhanced metabolism of ET-18-OCH_3 **(Fig 38)**.

Taken together, we propose that differences in the mechanism of Raf activation in

MCF-7Ad$^+$ and HNME cells compared to MCF-7 cells is the basis for the insensitivity of the former cells to growth-inhibition by ET-18-OCH_3.

The reasons why Raf associates constitutively with the membrane in the cells is unknown. Clearly, this occurred during the adaptation of the MCF-7 cells for growth in the serum-free media. The fact that HNME cells, which are cultured in serum-free medium, exhibit the same phenomenon is an indication that serum-free growth and accumulation of Raf in the membrane are related. Our studies indicate that the composition of the media plays a significant role in the levels that accumulate. MCF-7Ad$^-$ cells had lower levels of Raf than MCF-7Ad$^+$ cells. The only difference in the adaptation conditions was the presence of $PGF_{2\alpha}$ in ACGM$^+$ but not in ACGM$^-$. This indicates that the effect of $PGF_{2\alpha}$ on the cells is involved in stimulating the processes that result in constitutive accumulation of Raf-1. $PGF_{2\alpha}$ was unable to activate MAP kinase activity in MCF-7 or MCF-7Ad$^+$ cells (data not shown). The ability to cause Raf accumulation is not restricted to $PGF_{2\alpha}$ since MEGM does not contain the prostaglandin but yet HNME cells had large levels of Raf in their membranes. It was not possible to examine whether growth in serum-containing medium would eliminate Raf from the membrane as the HNME cells differentiate in serum-containing medium.

The significance of the changes in lipid content of membranes to the constitutive association of Raf-1 in the membrane is hard to discern since there is no obvious link between the observed changes and Raf/membrane interaction. Further studies will clearly be needed to determine whether the changes in lipid composition, especially the dramatic increase in SM, contribute to the change in subcellular localisation of Raf-1.

It is also worth pointing out that even though our studies indicated that in both MCF-

$7Ad^+$ and HNME cells, ET-18-OCH_3-induced inhibition of MAP kinase activation is insignificant, we did observe a decrease in proliferation of cells incubated with the compound. Furthermore, the inhibition was maximal at relatively low concentrations. One explanation for this is that maximum cell proliferation in these cells depends not only on the MAP kinase pathway but also on mitogenic signals mediated from Raf-independent signaling pathways. Thus maximum stimulation of both pathways results in maximum rates of cell proliferation observed in untreated control cells. We suggest that this unidentified pathway(s) is inhibited by ET-18-OCH_3 in these cells and inhibition occurs at quite low concentrations. Thus, even though the Raf-dependent mitogenic signaling is not inhibited, the cells cannot grow at the maximum proliferation rates hence they proliferate at rates lower than the controls. We postulate that the degree of sensitivity cells display to ALPs depends on the relative contribution of ALP-sensitive signaling pathways to their maximum growth.

5.3.4 Model of mechanism of ET-18-OCH_3-insensitivity in cells

The following model is proposed to explain the mechanism of insensitivity of cells to ET-18-OCH_3 (Fig 63). In sensitive cells, Raf-1 is in the cytosol and addition of ET-18-OCH_3 results in association of the ALP to Raf-1 in the cytosol. This association inhibits the translocation of the Raf-1 to the membrane following stimulation by growth factors. Raf-1 cannot be activated and the MAP kinase activation cannot be sustained. In insensitive cells, we propose that Raf-1 is associated with the membrane in a very stable conformation. Stimulation of the cells by growth factors results in the activation of the membrane associated Raf-1. Translocation of Raf-1 to the membrane does not appear to be required. The

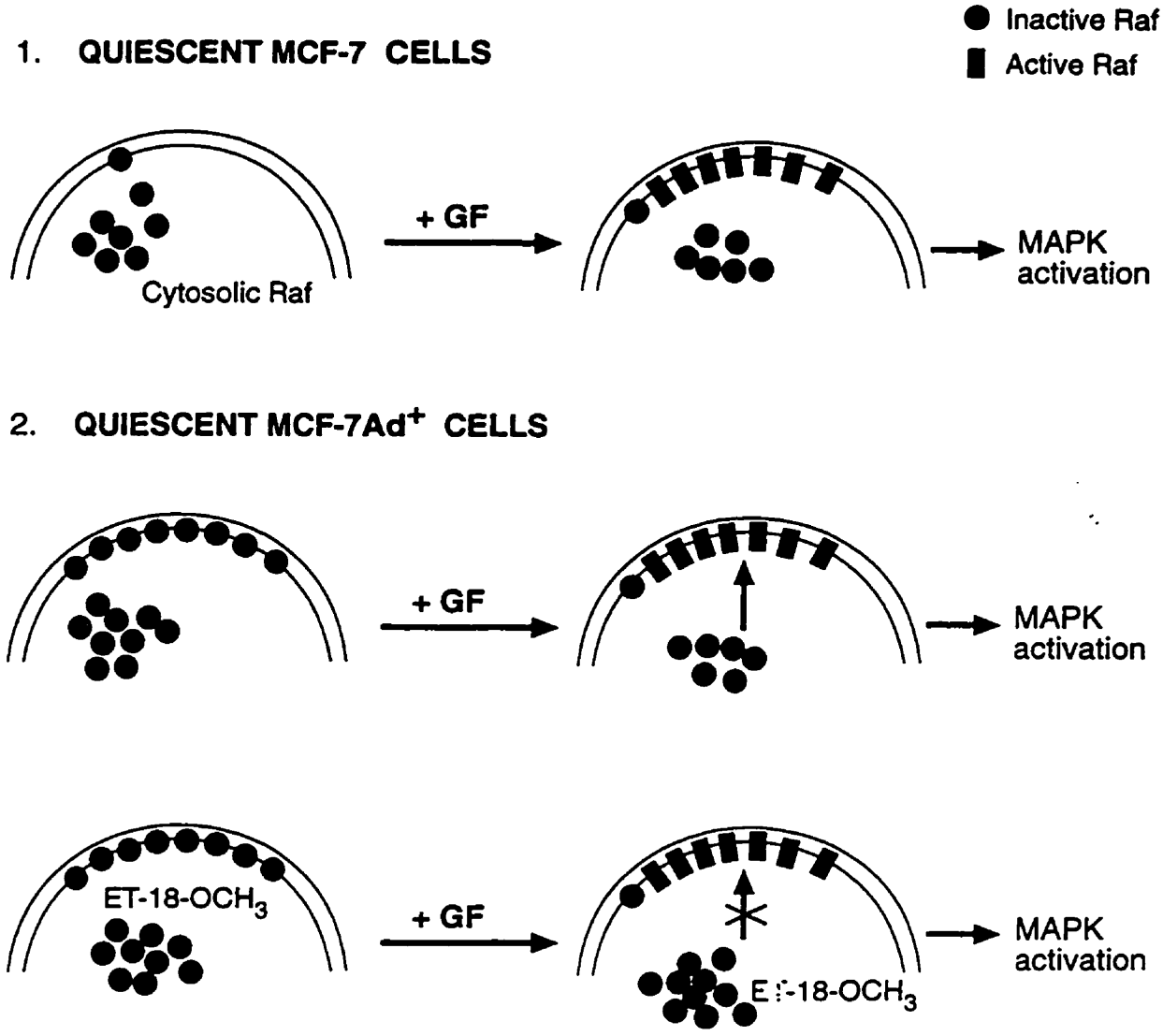


Figure 63. Postulated model of the mechanism of ET-18-OCH_3 insensitivity in cells. 1. In control quiescent MCF-7 cells, Raf is inactive and in the cytosol (●), upon stimulation, translocation and activation of Raf (■) occurs which leads to the activation of MAPK cascade. 2. In quiescent MCF-7Ad$^+$ cells, a substantial amount of Raf is constitutively bound to the membranes which is activated upon stimulation. ET-18-OCH_3-treatment interferes with Raf translocation in these cells, but as the Raf is already in the membrane prior to the drug treatment, it is able to become activated upon stimulation and thus the cells escape the inhibitory effect of the drug.

activation of Raf-1 leads to MEK phosphorylation and the activation of MAP kinase. In insensitive cells treated with ET-18-OCH_3, the compound binds to Raf in the cytosol. Following stimulation the ALP inhibits the translocation of additional Raf to the membrane, however, in this instance, there is sufficient Raf in the membrane which is activated to cause the activation of MEK and MAP kinase. Hence, the inhibition of translocation has very little effect on MAP kinase activation as the cells are activated independent of Raf-1 translocation. This difference in the mechanism of activation of Raf-1, translocation-dependent versus translocation-independent, is responsible for the observed ET-18-OCH_3-insensitivity.

5.3.5 Regulation of Raf-1 activation

Although the studies in the thesis were conducted to elucidate mechanisms for the selective inhibitory effects of ALPs, we have obtained novel information on the regulation of Raf-1 activity. The observation that Raf can constitutively associate with membranes in MCF-7Ad$^+$ and HNME cells without being activated, reinforces the results and conclusions obtained in the studies with ET-16-phosphono-TDB; translocation of Raf can be clearly dissociated from the events that activate the molecule. The fact that no further accumulation of Raf occurred in HNME and MCF-7Ad$^+$ cells subsequent to stimulation also supports our earlier suggestion that there is a limit to the levels of Raf that can associate with the membrane. These results also raise the question of the role of Ras in Raf activation. Ras was once thought to act solely to translocate Raf (Avruch et al., 1994; Bruder *et al.*, 1992; Hallberg *et al.*, 1994), but there is now some evidence that Ras interaction with Raf may serve an activating function that is independent of its translocation function (Morrison and

Cutler, 1997; Stokoe and McCormick, 1997; Tamada *et al.*, 1997). We believe these cells would be an excellent model to investigate these questions. We also think that the fact that the Raf levels did not decrease in the membrane when the signal from the growth factor was terminated, as assessed by the return of MEK and MAP kinase activities to resting levels, indicate that the mechanisms that inactivate Raf are in the membrane and do not necessarily involve dissociation of Raf from the membrane. Thus we postulate that the changes in Raf conformation/phosphorylation from the active to an inactive form can occur exclusively in the membrane. The MCF-7Ad^{+} cells would again be an excellent model to investigate the events that curtail Raf activity following growth factor stimulation. Very little is known about how this is achieved or regulated (Morrison and Cutler, 1997).

6 CONCLUSION

The antiproliferative activities and the cell selective effects of AELs are now firmly established, but the mechanisms responsible for these events are still under intense investigation. In our laboratory, studies on the mechanism of action of ET-18-OCH_3, the prototype ALP, on epithelial cancer cell lines led to the conclusion that ET-18-OCH_3 perturbs the association of Raf-1 with the membrane and this results in premature termination of signaling via the MAP kinase cascade. In this thesis we sought to confirm the role of inhibition of Raf-1 activity in the mechanism of inhibition of cell proliferation by ALPs and then proceeded to investigate the mechanism responsible for the insensitivity of some cells to growth-inhibition by ET-18-OCH_3.

The following are the conclusions from the studies in this thesis:

1. (*S*)-ET-16-phosphono-TDB completely inhibits the proliferation of MCF-7 cells at concentrations where (*R*)-ET-16-phosphono-TDB has only a slight effect on proliferation.

2. Under incubation conditions where (*S*)-ET-16-phosphono-TDB inhibits the proliferation of quiescent MCF-7 cells, Raf-1 activation is inhibited, which leads to inhibition of MEK and MAP kinase activation.

3. Unlike ET-18-OCH_3, (*S*)-ET-16-phosphono-TDB did not decrease Raf-1-membrane association; instead, both (*S*)- and (*R*)-ET-16-phosphono-TDB increased the levels of Raf-1 in the membrane of quiescent cells without causing its activation. Thus, the translocation of Raf-1 to the membrane does not automatically lead to its activation.

4. ALPs appears to interact with Raf in the cytosol. Interaction of (*S*) and (*R*)-ET-16-phosphono-TDB did not inhibit Ras/Raf interaction *in vitro* and neither did the compounds

inhibit Ras-dependent translocation of Raf. ET-18-OCH_3 on the other hand inhibited Raf/Ras interaction, suggesting that the ET-18-OCH_3-induced decrease in membrane Raf levels may be due to inhibition of Raf translocation.

5. (*S*)-ET-16-phosphono-TDB does not inhibit Raf-1 kinase activity, but interferes in processes that lead to the activation of the catalytic activity of membrane-associated Raf-1.

6. Adaptation of MCF-7 cells for growth in serum-free media resulted in decreased sensitivity of the adapted cells to ET-18-OCH_3.

7. The change in ET-18-OCH_3-sensitivity were not a function of the absence of serum components during incubation of cells with ET-18-OCH_3, slower growth rate, selection of a resistant population, or development of a new strain of MCF-7 cells.

8. ET-18-OCH_3-insensitivity of mammary epithelial cells was not due to lower uptake or increased breakdown of ET-18-OCH_3.

9. The constitutive association of Raf-1 with membrane in the MCF-7Ad$^-$, MCF-7Ad$^+$ and HNME cells is responsible for the inability of ET-18-OCH_3 to inhibit the proliferation of the cells.

10. In MCF-7Ad$^+$ cells, $PGF_{2\alpha}$ plays a key role in acquiring increased insensitivity to ET-18-OCH_3 beyond the levels developed in media without it, by facilitating increased constitutive Raf-1 binding to the membranes.

11. In the ET-18-OCH_3-insensitive MCF-7Ad$^+$ cells, the partial growth inhibition by ET-18-OCH_3, in spite of any apparent decrease in MAP kinase activity, might be due to the inhibitory effect of ET-18-OCH_3 on Raf-1-independent process(es) which together with signaling via the MAP kinase cascade is required for optimum growth of the cells.

In conclusion, the studies in this thesis have clearly implicated Raf-1 as a key intracellular target in the mechanism of action of choline-containing ALPs. Raf-1 was implicated in studies with ET-16-phosphono-TDBs and in the studies on the mechanism responsible for the selective effects of ALPs. Thus, if ALPs are able to inhibit Raf activation in cells whose growth is dependent on Raf-1-signaling pathways, cell growth is inhibited. Alternatively, if the cells are able to activate Raf via mechanisms that by-pass the step inhibited by the ALP, then the cells are insensitive to the compound. Hence the cell selective effect of ET-18-OCH_3 may be due to differences in the process of activation of Raf-1 between the ALP-sensitive and -insensitive cells and/or the importance of Raf-1 signaling pathways in cell proliferation.

The above postulate does not imply that differences in the mode of activation of Raf-1 is responsible for the insensitivity displayed by all cell lines. As indicated earlier, there is evidence that ALPs inhibit cell growth in different cell types by distinct mechanisms. One can also envisage that in cells with activating mutations downstream of Raf, ET-18-OCH_3 may not be an effective antiproliferative agent. There are now a large variety of AELs including different types of ALPs and evidence exists that they may not all act via a common mechanism. This indicates that against tumors of unknown pedigree, these compounds would be most effective if used as a combination of different compounds. Future studies to elucidate the mechanism of action of other ALPs and AELs including studies to understand the interaction of ALPs with Raf-1 and regulation of Raf-1 activity in epithelial cells, should hopefully lead to the rational use of ALPs and AELs in cancer therapy.

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IMAGE EVALUATION
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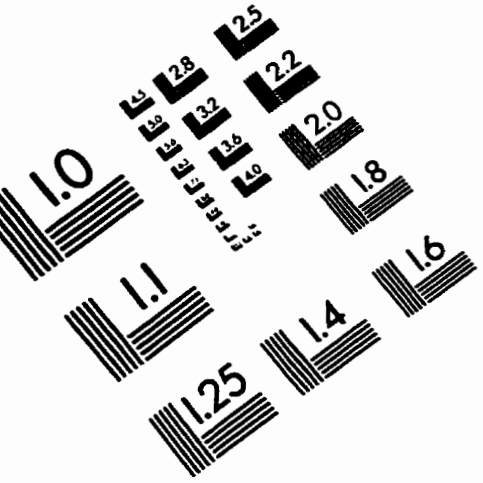

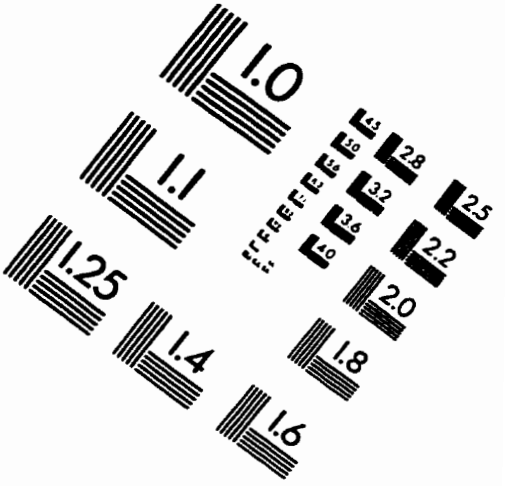

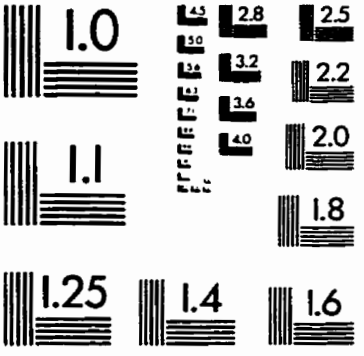

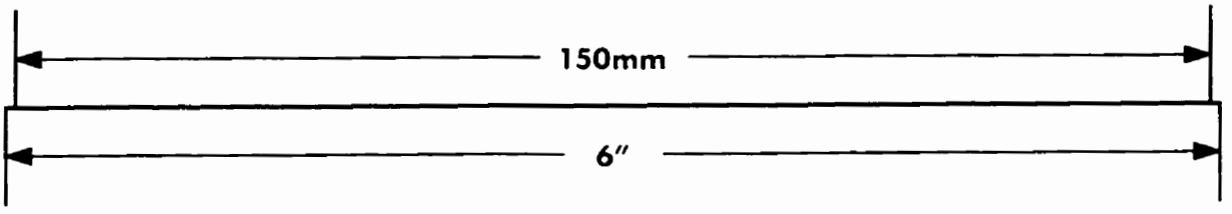

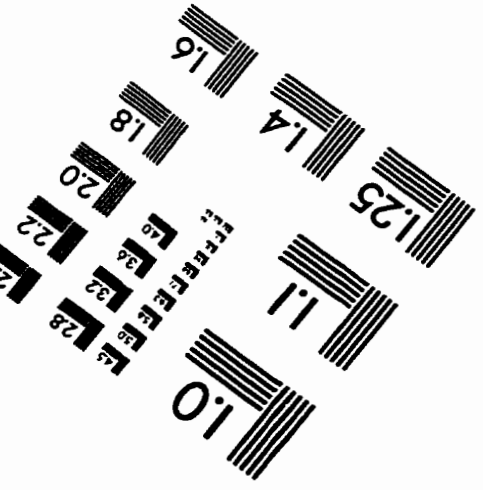

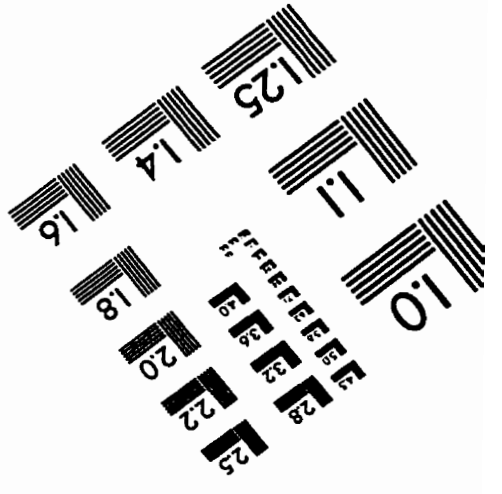

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