ET-18-OCH_3 (Edelfosine): A Selective Antitumour Lipid Targeting Apoptosis Through Intracellular Activation of Fas/CD95 Death Receptor

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Abstract: Synthetic ether-linked analogues of phosphatidylcholine and lysophosphatidylcholine, collectively named as antitumour lipids (ATLs), were initially synthesized in the late 60s, but have attracted a renewed interest since the finding that the ether lipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3, edelfosine), a synthetic analogue of 2-lysophosphatidylcholine considered the ATL prototype, induces a selective apoptotic response in tumour cells, sparing normal cells. Unlike most chemotherapeutic agents currently used, ET-18-OCH_3 does not interact with DNA, but act at the cell membrane, and thereby its effects seem to be independent of the proliferative state of target cells. Each part of the molecular structure of ET-18-OCH_3 is important for its optimal proapoptotic activity. Recent progress has unveiled the molecular mechanism underlying the apoptotic action of ET-18-OCH_3, involving membrane rafts and Fas/CD95 death receptor, and has led to the proposal of a two-step model for the ET-18-OCH_3 selective action on cancer cells, namely: a) ET-18-OCH_3 uptake into the tumour cell, but not in normal cells; b) intracellular activation of Fas/CD95 through its translocation and capping into membrane rafts. ET-18-OCH_3 constitutes the first antitumour drug acting through the intracellular activation of the Fas/CD95 death receptor. Computational docking studies have allowed us to propose a molecular model for the putative interaction of ET-18-OCH_3 with the intracellular Fas/CD95 death domain. This novel mechanism of action represents a new way to target tumour cells in cancer chemotherapy and can be of interest as a new framework in designing novel and more selective proapoptotic antitumour drugs.

Keywords: ET-18-OCH_3; edelfosine; antitumour ether lipid; apoptosis; signal transduction; Fas/CD95; molecular modelling; cancer.

INTRODUCTION

Synthetic antitumour lipids (ATLs) include two major subtypes [1]: a) the alkyl ether phospholipids (AEPs), widely referred collectively as antitumour ether lipids (AELs) or alkyl-lysophospholipid analogues (ALPs), containing ether bonds in the glycerol backbone of the phospholipid, with the prototypical 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3; edelfosine) (Fig. **1**), b) and the alkylphosphocholines (APCs), lacking the glycerol backbone and formed by a simple long-chain alcohol esterified into a phosphobase, with the prototypical hexadecylphosphocholine (HPC; miltefosine) (Fig. **1**). Additional AEP and APC analogues have been synthesized that show promising antitumour activities, including 1-hexadecylthio-2-methoxymethyl-*rac*-glycero-3-phosphocholine (BM 41.440, ilmofosine) [2-5], the cyclic analogue SRI 62-834 [6], octadecyl-(N,N-dimethyl-piperidinio-4-yl)-phosphate (D-21266, perifosine) [7-9] and erucylphosphocholine [10-13] (Fig. **1**). Two remarkable features of AEPs and APCs are their low metabolism rates *in vitro* and *in vivo*, and the fact that they do not target the DNA, but act at the cell membrane. Although the first ATLs were synthesized in the late 60s as metabolically stable analogues of lysophosphocholine [14], the finding in 1997 of the selective apoptosis in tumour cells treated with the ATL prototype ET-18-OCH_3 [15] has rekindled interest in these compounds, and especially in ET-18-OCH_3 as a promising and selective antitumour drug. Apoptosis or cell suicide is an intrinsic cell death programme that occurs in various physiological and pathological situations [16-18]. Apoptosis is characterised by typical biochemical and morphological hallmarks, including caspase activation, membrane blebbing, nuclear DNA fragmentation and cell shrinkage [18], and is mainly initiated through either a death receptor- or mitochondrial-mediated pathway, known also as extrinsic or intrinsic pathways, respectively [19-22]. Defects in apoptotic cell-death regulation contribute to several diseases, including disorders associated with cell accumulation such as cancer, autoimmunity and inflammation. Failure to mount an apoptotic response in tumour cells leads to cancer development and drug resistance. Cancer treatment by chemotherapy, γ-irradiation or immunotherapy kills ultimately the target cells by inducing apoptosis [1,23-29] although the triggering mechanisms for this apoptotic

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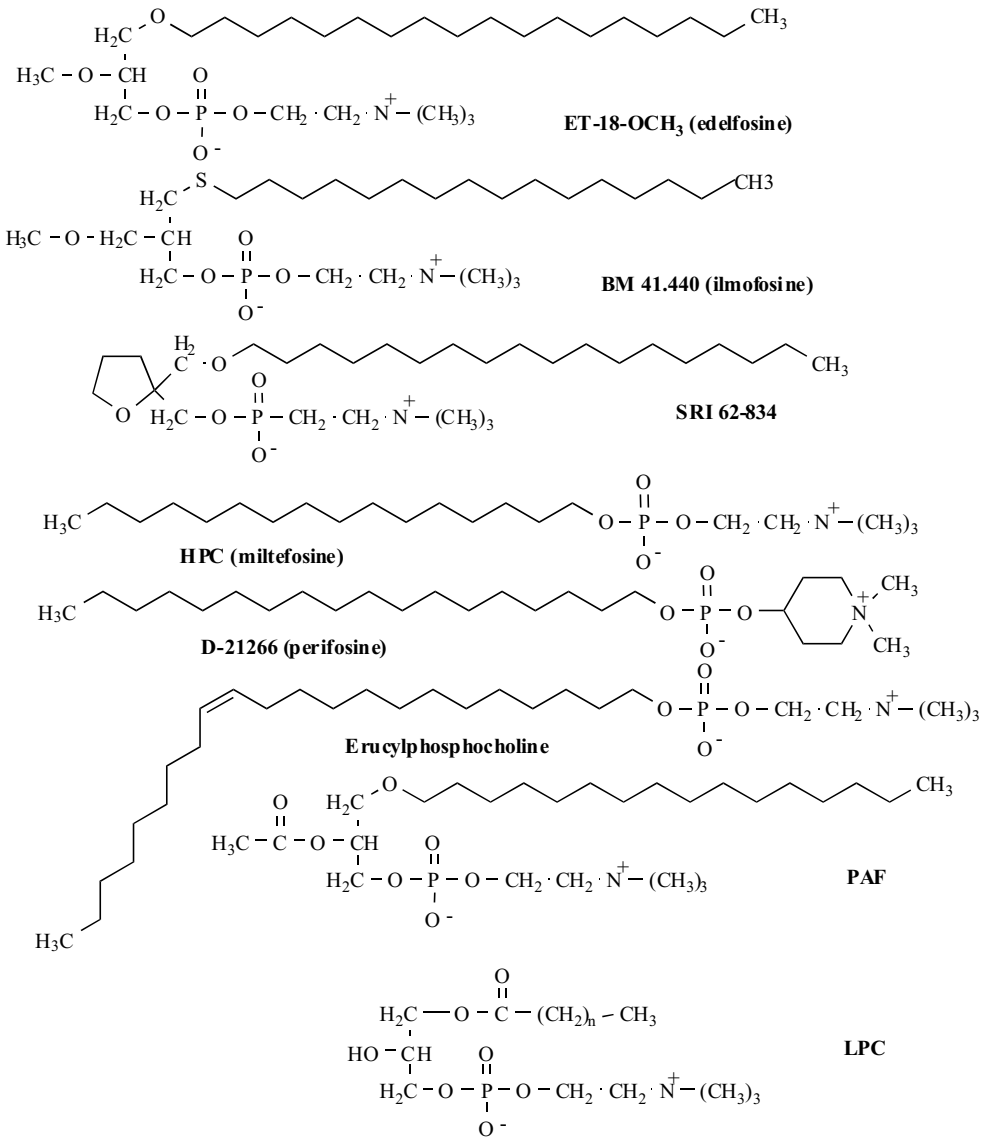

Fig. (1). Chemical structures of synthetic ether lipids. ET-18-OCH_3 (edelfosine), BM 41.440 (ilmofosine), SRI 62-834, HPC (miltefosine), octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate (perifosine) and erucylphosphocholine. The chemical structures of the natural lipids LPC and PAF are also included.

induction are stimulus dependent, involve multiple steps and have not been well defined. Cells sense damage to DNA or to other critical molecules or biochemical processes and trigger a cellular stress response that eventually leads to apoptosis in a rather indirect way. The current high interest for ET-18-OCH_3 underlies in its capacity to resensitise tumour cells to apoptosis, enhancing the activity of the apoptotic machinery. Recent evidence indicates that ET-18-OCH_3 is the first antitumour drug acting through intracellular activation of the Fas/CD95 death receptor [15,30,31], involving a raft-mediated process [31,32]. This represents a novel and unique mechanism of action in cancer chemotherapy. The present review covers the state of the art in the underlying mechanisms of the antitumour effect of ET-18-OCH_3, emphasising the effect of ET-18-OCH_3 in triggering apoptosis in tumour cells through activation of the apoptotic machinery that was dormant in cancer cells. We also propose a tentative molecular model for the intracellular activation of the Fas/CD95 death receptor by ET-18-OCH_3 as a novel way to target cancer cells.

HISTORICAL NOTES

The first ATLs were synthesised as 2-lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phosphocholine, LPC) (Fig. **1**) analogues in a search for immune modulators. The underlying basis for this synthetic effort goes back to the early 1960s when Herbert Fischer and Paul Gerhard Munder, working at the Max-Planck-Institut für Immunbiologie in Freiburg (Germany), found phospholipase A_2-mediated formation of LPC in macrophages during phagocytosis of silicogenic quartz particles and in response to substances with adjuvant activity, and that exogenous LPC strongly enhanced the phagocytic activity of peritoneal macrophages both *in vitro* and *in vivo* [33-35]. This suggested an immunomodulatory role for LPC in the

defense mechanisms of the immune system, but the naturally occurring LPC was rapidly metabolised by an acyltransferase to phosphatidylcholine or by a lysophospholipase to glycerophosphocholine. Thus, LPC analogues with longer *in vivo* half-life times were synthesized in the following years following a joint effort of different groups led by Herbert Fisher, Otto Westphal, Hans Ulrich Weltzien and Paul Gerhard Munder in Freiburg [6,14]. Particular emphasis was placed on changes in the positions C1 and C2 of the glycerol backbone in the LPC molecule, replacing ester bonds for ether linkages in order to render analogues unable to be metabolised by either acyltransferases or lysophospholipases. A number of these synthesised ether analogues of LPC turned out to be potent immune modulators, but surprisingly Munder and co-workers found that some these ether lipids exerted strong antitumour activities *in vitro* and *in vivo* in a rather selective way [36-38]. Among the synthesized LPC ether lipid analogues, ET-18-OCH$_3$ turned out to be the prototype and the most effective antitumoural compound, and rapidly became the effective standard in trials on antitumoural activities of such ether lipids [14]. The first synthesis of ET-18-OCH$_3$ was described by Guenter Kny in 1969 [39], a chemical diploma student in O. Westphal's laboratory, making use of the previous experience of Bernd Arnold and Hans Ulrich Weltzien with the synthesis of 1-*O*-alkyl- and 2-*O*-methyl derivatives of glycerol. Subsequent single crystal x-ray structure of D-ET-18-OCH$_3$ monohydrate has shown the ether lipid molecules arranged in a "bilayer" structure with interdigitating and strongly tilting hydrocarbon chains [40]. A three-dimensional view of ET-18-OCH$_3$ is shown in Fig **2**).

APOPTOSIS, CANCER CHEMOTHERAPY AND ET-18-OCH$_3$

Apoptosis (from the Greek words απο -apo-, "away from, off, detached", and πτωσιζ -ptosis-, "fall, corpse", meaning "falling off" as leaves from a tree) is a physiological cell death that was initially described by its morphological characteristics, including membrane blebbing, chromatin condensation, nuclear fragmentation, cell shrinkage, and breakdown of the cells into small, membrane-surrounded fragments (apoptotic bodies) which were cleared by phagocytosis without prompting an inflammatory response [41-43]. Critical for the absence of an inflammatory response during apoptosis is the preservation of an intact plasma membrane surrounding the apoptotic cell that prevents the leakage of intracellular contents. This is a pivotal feature of apoptosis and a major difference with a necrotic cell death, where membranes are disrupted and cell contents are spilled to the extracellular medium causing a potentially damaging inflammatory response. It is now well established that cellular suicide (apoptosis) is central to a number of physiological cellular processes and is essential in the maintenance of homeostasis and survival of multicellular organisms. In adult tissues, cell death rate exactly balances cell division rate to keep homeostasis. A massive flux of cell birth and death occurs in the self-renewing tissues of the body (skin, gut, bone marrow, sex organs), and it is estimated that an average adult produces and in parallel eradicates 50-70 billion cells per day. Apoptosis malfunctioning is critical for the pathogenesis of many human diseases, leading to cancer and autoimmune diseases when there is too little apoptosis, and stroke damage and neurodegenerative diseases, such as Alzheimer, when there is too much.

Apoptosis is usually the final common mechanism of many cytotoxic agents that are used in cancer chemotherapy. Thus, the effectiveness of anticancer drugs reflects the cell's ability to detect and respond to the perturbation induced by the drug [1]. Agents with diverse primary targets, including microtubule-active agents, topoisomerase II inhibitors, DNA-alkylating agents, antibiotics and folate antagonists, trigger signalling pathways leading ultimately to apoptosis. Conversely, because drugs with different targets induce apoptosis through similar apoptotic mechanisms, mutations in the apoptotic program lead to multidrug resistance and treatment failure.

The majority of anticancer drugs target DNA synthesis, whereas others affect cellular metabolism and cell division. In this regard, induction of apoptosis by these drugs is rather indirect because effects on cell cycle or cell metabolism must

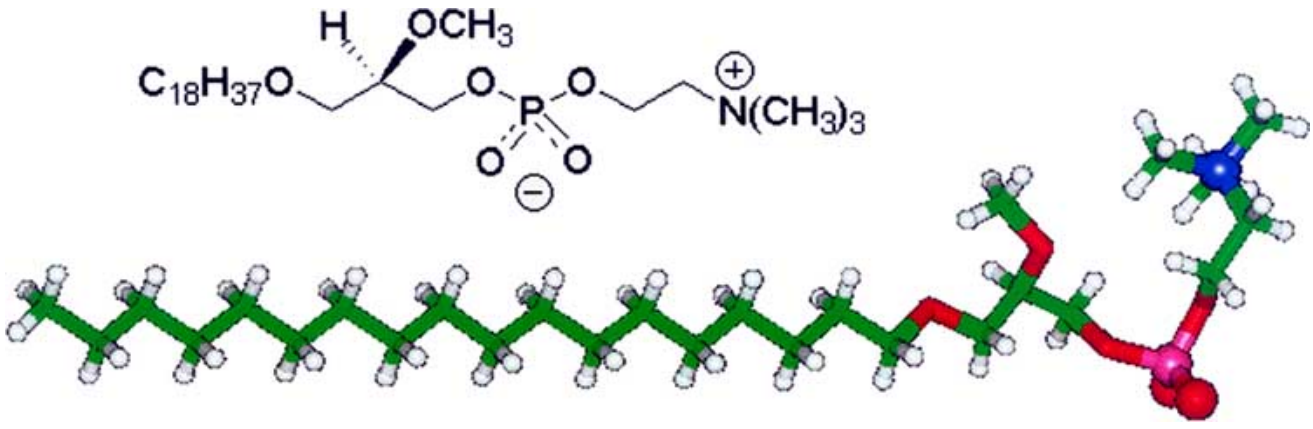

Fig. (2). Chemical composition and three-dimensional structure based on X-ray crystallography data [40] of ET-18-OCH$_3$. The corresponding atoms are colored as follows, C (green), O (red), N (blue), P (magenta), H (grey).

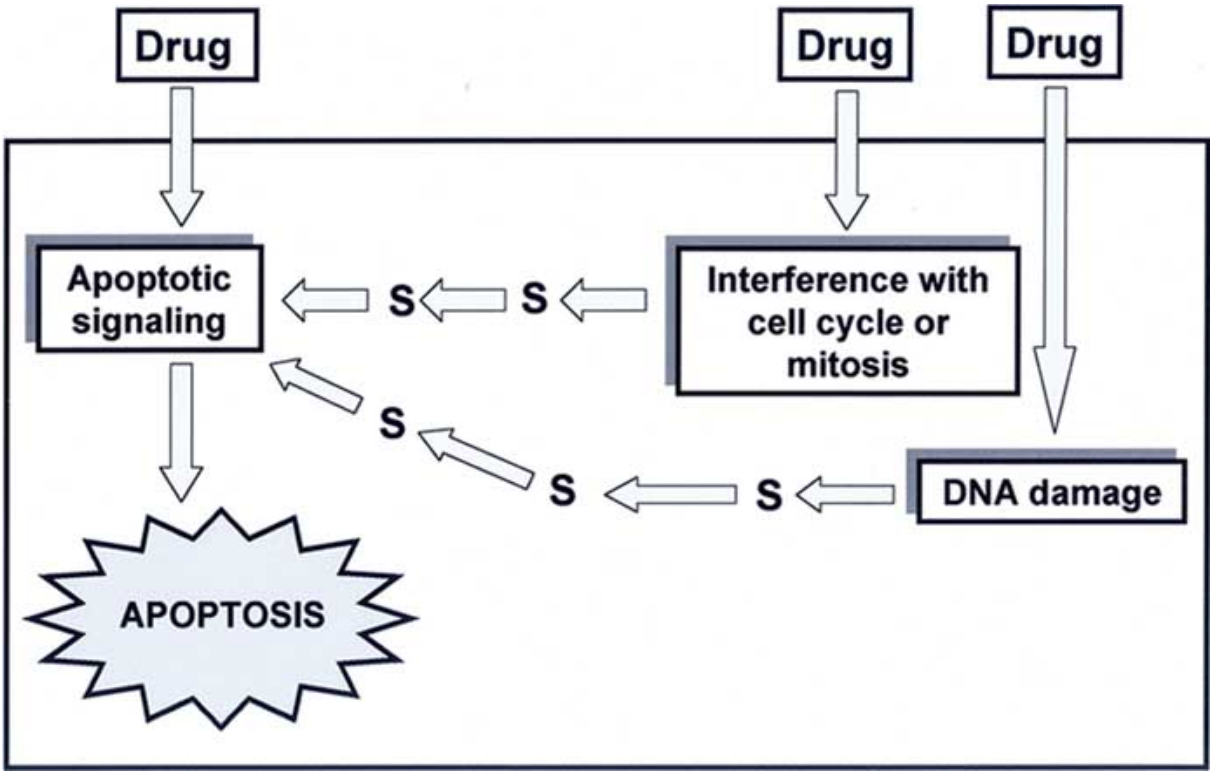

Fig. (3). Induction of apoptosis in cancer chemotherapy. Cells undergo drug-induced damages on cell cycle and DNA, and through the presence of "sensors" (S) calibrate these lesions and trigger an apoptotic response by a rather indirect and long way involving successive sensors. Cancer cells often get rid of these sensors, either through mutation or inactivation, to avoid their own cell death by blocking or hindering the signalling events connecting drug-induced damage with the triggering of apoptosis. A direct activation of the apoptotic signalling could hypothetically circumvent these obstacles and lead to rapid demise of cancer cells.

be sensed by the cells as signals that, after reaching a certain threshold, trigger an apoptotic response [1] (Fig. **3**). Thus, cells sense this drug-induced damage or cellular perturbation through the presence of "sensors", calibrate these lesions and mount a response to these aggressions, reacting according to their phenotype [23]. When this drug-induced metabolic, biochemical or DNA damage is excessive or difficult to be repaired, the cell decides to self-destruct by apoptosis avoiding in this way to threaten adjacent cells and tissues. The failure of some tumour cells to die following a chemotherapeutic treatment may be due to either their inability to sense the drug-promoted harm or their resistance to engage apoptosis. This implies the existence of an "*apoptosis threshold*", in response to damage that is set differently in distinct cell types [1]. Differences in apoptosis thresholds between different cell types may reflect differences in the expression or sensitivity of cellular sensors or in down-stream signalling events set in motion by the sensors. Tumour cells may either inhibit the molecular processes that lead to their own death, and/or increase remarkably the apoptosis threshold required to realise that they are receiving a signal driving them to commit suicide [1]. Tumour cells with an enhanced apoptosis threshold tolerate a high number of external insults or damages before mounting an apoptotic response. Thus, mutations in sensors involved in triggering an apoptotic response hamper the onset of apoptosis, and it is well-known that cancer cells try to get rid of sensors that can lead to cell death [1]. In this regard, the tumour suppressor gene p53, involved in cell-cycle control, apoptosis, and in the maintenance of genetic stability, is mutated in about half of all human cancers.

In order to evade putative mutations in the sensors linking cell damage to a cell death response that could hinder or frustrate the use of anti-cancer therapies, a direct activation of the apoptotic machinery should be more appropriate to kill rapidly and efficiently the tumour cell (Fig. **3**). The idea of developing drugs able to target and induce apoptosis in tumour cells constitutes an attractive and promising approach in cancer treatment. Direct activation of the cell death machinery could circumvent the action of cellular sensors and checkpoints, which are frequently mutated or altered in cancer, and therefore their mutant state should be largely irrelevant to this therapeutic approach (Fig. **3**). This new avenue in cancer therapy can fit the need to overcome the dead-lock in the present chemotherapeutical state of cancer treatment with further developments of new chemotherapeutic drugs different from the classical compounds used in clinical practice.

SELECTIVE INDUCTION OF APOPTOSIS AS A MAJOR EFFECT IN ET-18-OCH_3 ANTITUMOUR ACTION

In 1993, Diomede and co-workers [44] in Milan (Italy) and Mollinedo and co-workers [45] in Madrid (Spain)

independently demonstrated that ET-18-OCH$_3$ exerted a major proapoptotic effect on cancer cells. This apoptotic action proved to be a key finding in the elucidation of the processes involved in the antineoplastic effect of ET-18-OCH$_3$ and accounted for the major antitumour effect of the ether lipid [15,44-46]. The apoptotic response was optimally induced after treatment with 3-5 μg/ml (about 6-10 μM) of ET-18-OCH$_3$ (molecular weight, 524) [47], when tumour cells were cultured in the presence of 10% fetal calf serum. Subsequent reports have shown that other ATLs are also able to induce an apoptotic response in tumour cells [10,13,48,49]. Because ET-18-OCH$_3$ binds strongly to serum proteins, primarily albumin and high density lipoprotein (HDL) [50], the action of ET-18-OCH$_3$ is very dependent on the amount of serum present in the assay. Increasing amounts of serum inhibit the cytotoxic effects of ET-18-OCH$_3$ in a concentration-dependent manner [50-52](Gajate, C. and Mollinedo, F., unpublished data). The specific action of ET-18-OCH$_3$ and other ATLs on apoptosis should be separated from their non-specific lytic effect on membranes (detergent-like action) shown by ATLs at elevated doses. When used at high concentrations, ATLs act as membrane-active detergent molecules disrupting the orderly bilayer structure of the plasma membrane and forming micellar clusters, which give rise to pores and holes in cell membranes [53,54]. Nevertheless, the antitumour effect of ET-18-OCH$_3$ and other ATLs is not related to their detergent-like effects [1,6,14,55-58]. ET-18-OCH$_3$ is selective in its ability to induce apoptosis, encountering considerable resistance in many normal human cells, including fibroblasts, polymorphonuclear neutrophils, resting lymphocytes, and bone marrow progenitor cells [15,30,59]. Normal endothelial cells from human umbilical vein (HUVEC) or bovine aortic (BAEC) are also resistant to the action of the ether lipid [30,60]. This selectivity of ET-18-OCH$_3$ in achieving an apoptotic response in tumour cells, sparing normal cells, is an outstanding hallmark in its antitumour action. However, not all tumour cells are sensitive to ET-18-OCH$_3$, and a number of cancer cells, such as the chronic myelogenous leukemia K562 and the T lymphoid leukemia HPB-ALL, show resistance to the ET-18-OCH$_3$ action [30,47]. Structurally related analogues of ET-18-OCH$_3$ that show similar physicochemical properties lack proapoptotic activity [15]. Cell and compound selectivity would not be expected if the detergent properties of the ether lipid were responsible for its antitumour effect. Furthermore, the ET-18-OCH$_3$ concentrations required for cell lysis are far higher than those at which potent antiproliferative and apoptotic effects are observed. Thus, cell lysis is not an important cytotoxic mechanism at pharmacologically relevant concentrations. However, caution must be taken when ATLs are used in serum-free conditions, as the lytic concentrations for different types of ATLs generally correspond well to their respective critical micellar concentrations which are in the low μM range in water [61,62].

STRUCTURE-ACTIVITY RELATIONSHIP STUDIES IN ET-18-OCH$_3$-INDUCED APOPTOSIS

A large number of compounds structurally related to ET-18-OCH$_3$ have been synthesised and tested for its cytotoxic activity [6,56]. Compounds having aliphatic chains either shorter or longer than 16-18 C atoms at *sn*-1 showed lower activity. However, although a large number of modifications in the ET-18-OCH$_3$ structure have been explored, leading to a great amount of structurally related compounds, the lack of reliable biochemical parameters to monitor their cytotoxic activity by the time of their synthesis prevented to define a pharmacophoric group. Since the therapeutic concentrations of these compounds are close to the critical micelle concentration, it has been difficult in the past to distinguish between specific and non-specific cytotoxic effects, such as the lytic disruption of cell membranes. Once induction of apoptosis has been unveiled as the major effect induced by ET-18-OCH$_3$ that accounted for its antitumour activity, assessment of apoptosis can be used as a quantitative and specific parameter directly related to its antitumour activity. Thus, the measurement of apoptosis provides a reliable marker for the putative antitumour activity of different, structurally related analogues of ET-18-OCH$_3$. The available information on structure-apoptotic activity relationship studies on ET-18-OCH$_3$ is rather scarce. We have tested the relative capacity of 22 different structurally related ET-18-OCH$_3$ analogues to induce apoptosis in the human myeloid leukaemic HL-60 cell line [15]. Some hints can be drawn for the molecular structure requirements in the ET-18-OCH$_3$ molecule to promote apoptosis (Fig. **4**). Substitution of the methoxy group for a OH, H or acetyl group in C2, and the change of the phosphocholine group at C3 for a phosphoserine group abolished the capacity to induce apoptosis. In addition, the lack of the phosphocholine group blocked apoptosis. However, the *O*-octadecyl tail at C1 could be substituted for other similar long *O*-alkyl tails, such as *O*-hexadecyl or an *O*-octadecenyl[9-10] chain, without affecting the apoptotic capacity of the ether lipid. These structure-activity relationship studies indicate the key importance of the molecular structure of ET-18-OCH$_3$ for the induction of apoptosis, as small variations in the chemical structure can abrogate its proapoptotic activity. Thus, these data emphasise the specificity of the ET-18-OCH$_3$ effect and suggest that the presence of a short nonhydrolyzable *O*-methyl group at C2 and a polar phosphocholine head group at C3 are critical for its apoptotic actions, whereas the *O*-octadecyl chain at C1 could be substituted for other long *O*-alkyl tails without affecting the ability of the ether lipid to induce apoptosis. ET-18-OCH$_3$ is usually used as a racemic mixture, and we have not found significant differences between the respective apoptotic activities of both D- and L-enantiomers in leukaemic human HL-60 cells [15]. Although the D-isomer has been reported to be more cytotoxic than the L-isomer against Raji tumour cells [63], most of the data so far reported show that both D- and L- isomers of ET-18-OCH$_3$ have similar cytotoxic effects in different human leukemia cell lines [6,64]. Thus, it seems that there is not a marked enantiomer specificity in the ET-18-OCH$_3$ action that could suggest a lack of stereospecific interactions with a macromolecular target. It has been reported that ET-18-OCH$_3$ can be a substrate for a phospholipase C-like enzyme, able to split off or exchange the phosphocholine moiety, leading to the notion that the generation of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerol could be responsible for the cytotoxic properties of ET-18-OCH$_3$ [65,66]. However, we found no apoptotic effect when HL-60 cells were treated with a number ET-18-OCH$_3$- or ET-16-OCH$_3$-derived diglycerides

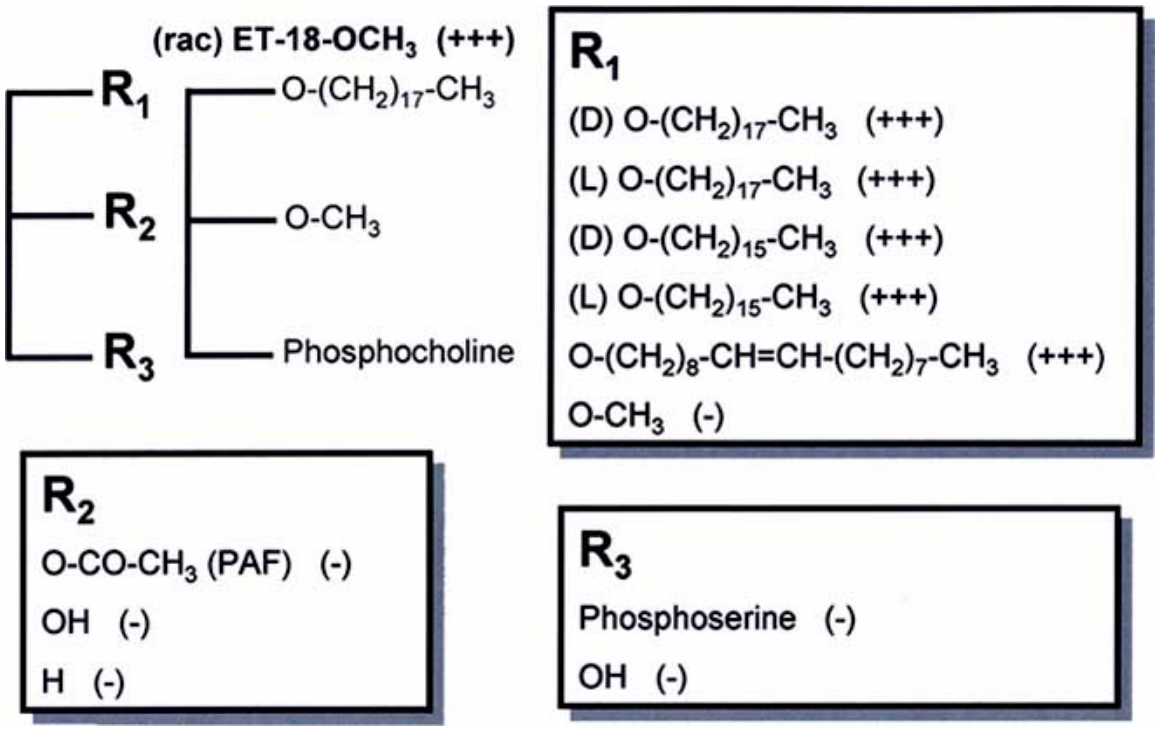

Fig. (4). Effect of several ET-18-OCH_3 structurally related compounds on apoptosis induction. Apoptosis was assessed after incubation of leukaemic HL-60 cells with 10 μM of the indicated compounds [15]. (+++), strong apoptotic response; (-) no apoptotic response.

and analogues [15], indicating that the products generated by a phospholipase C-mediated degradation of ET-18-OCH_3 are unable to induce an apoptotic response. This is in agreement with previous reports showing no cytotoxic activity by phospholipase C-mediated metabolites of ET-18-OCH_3 [67,68]. Thus, our data suggest that ET-18-OCH_3 is not metabolised to exert its proapoptotic activity. ET-18-OCH_3 is not a substrate for phospholipase A_2 due to the ether alkyl bond in C2, but could be theoretically metabolized through three major pathways [1]: a) phospholipase C hydrolysis, with formation of 1-*O*-alkyl-2-*O*-methyl-glycerol and phosphocholine; b) phospholipase D hydrolysis, with formation of 1-*O*-alkyl-2-*O*-methyl-phosphatidic acid and choline; c) an intriguing cleavage of the alkyl group through a glycerol-ether monooxygenase, with the formation of 1-lyso-2-*O*-methyl-phosphocholine. However, ET-18-OCH_3 is metabolically a very stable compound, in comparison with its natural counterparts alkyl-lysophosphatidylcholine, LPC and platelet-activating factor (PAF) (Fig. **1**). More than 98% of ET-18-OCH_3 remained unmodified in tumour cells after 24-h incubation [69,70], and the small ET-18-OCH_3 metabolism was basically due to the action of phospholipase C and phospholipase D activities [70]. These data indicate that ET-18-OCH_3 is active *per se* and not a prodrug.

ET-18-OCH_3-INDUCED APOPTOSIS IS NOT MEDIATED BY THE PAF RECEPTOR

Interestingly, the physiological product PAF, which plays an important role in inflammation [71-74], was identified in 1979, that is a decade after the synthesis of ET-18-OCH_3, as a 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine [75,76], in which the alkyl group is mainly a C16 or C18 aliphatic chain (Fig. **1**). Therefore, only by chance ET-18-OCH_3 can be considered as a 2-*O*-methyl synthetic analogue of PAF, differing in their respective molecular structures only in the position *sn*-2 of the glycerol backbone, *i.e.*, a methoxy group in ET-18-OCH_3 and an acetyl group in PAF (Fig. **1**). However, PAF, in tolerable doses, has no antitumour activity *in vitro* or *in vivo*, and ET-18-OCH_3 does not exert significant PAF or anti-PAF activity *in vivo*. PAF is an extremely potent biological mediator and its effects, achieved at concentrations as low as 10^{-9}-10^{-12} M, are mediated through a cell surface receptor [77-79]. A number of evidence indicate that ET-18-OCH_3 uptake and cytotoxicity proceed via a mechanism independent of the interaction of ET-18-OCH_3 with the PAF receptor. There is a lack of correlation between the presence of PAF receptor and ET-18-OCH_3 uptake and cytotoxicity [15,67,80,81]. Tumour cells devoid of PAF receptors, such as HL-60 cells, take up the ether lipid and undergo ET-18-OCH_3-mediated apoptosis [15] (Fig. **5**). Interestingly, PAF receptors develop following granulocytic differentiation of HL-60 cells induced by dimethyl sulfoxide (DMSO) treatment [82,83], when these cells become resistant to the ether lipid and do not incorporate the drug [15,67,81,84] (Fig. **5**). Neither PAF nor PAF antagonists prevent ET-18-OCH_3 uptake and cytotoxicity [15,80,81,85], and PAF does not induce apoptosis in ET-18-OCH_3 sensitive cells [15,81].

ET-18-OCH_3 is able to induce an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) through the PAF receptor as evidenced by cross-desensitization assays and by

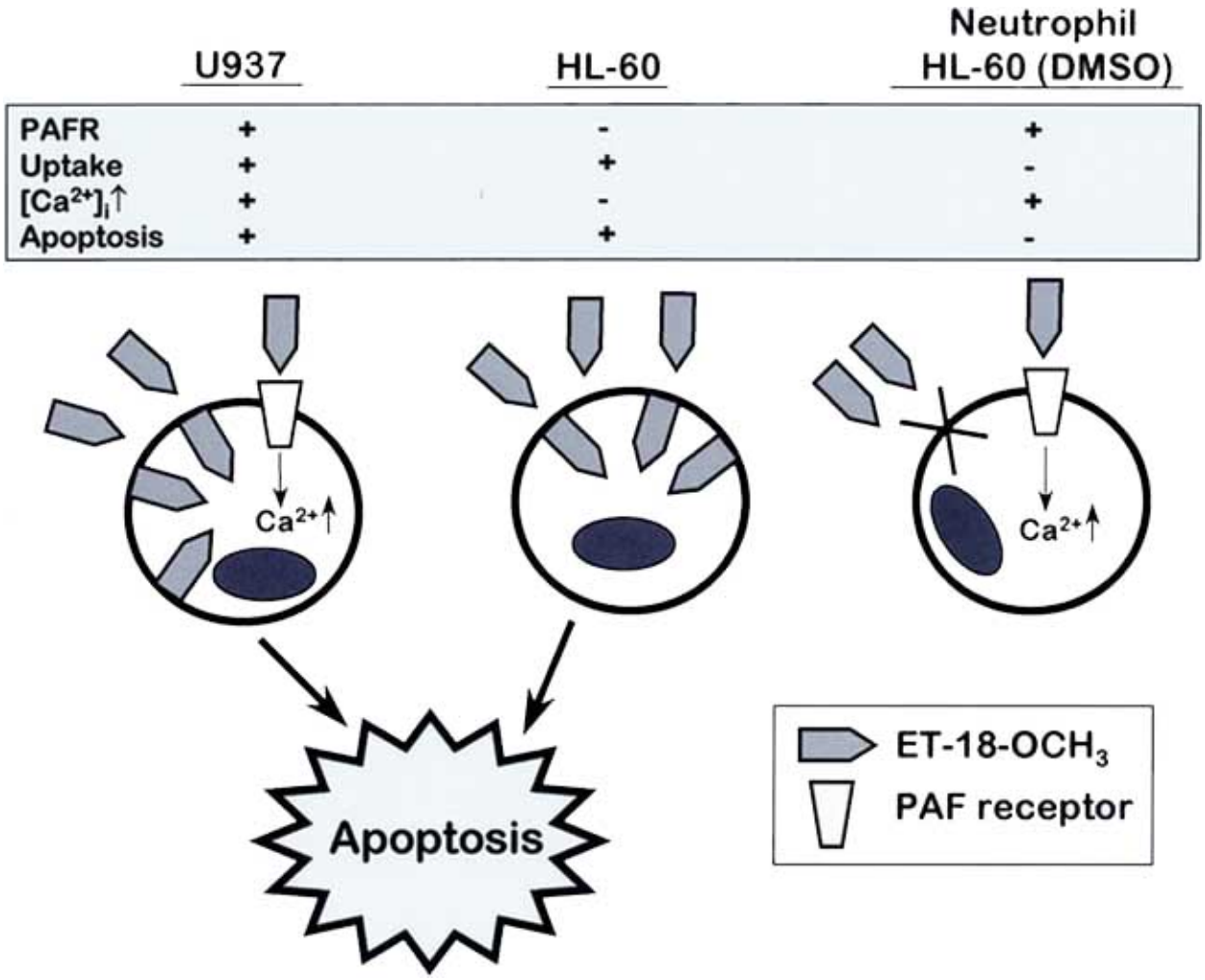

Fig. (5). ET-18-OCH$_3$-induced apoptosis is mediated by its cellular uptake but not by the presence of PAF receptors or changes in cytosolic free calcium concentration in the target cell. This is a schematic view showing that the presence (+) or absence (-) of PAF receptor (PAFR) or ET-18-OCH$_3$-induced increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i\uparrow$) do not affect either cellular uptake of the ether lipid or ET-18-OCH$_3$-induced apoptosis. Leukaemic U937 and HL-60 cells, HL-60 cells differentiated towards neutrophil-like cells (HL-60 (DMSO)) and mature normal neutrophils were analyzed [15,81]. ET-18-OCH$_3$ is thought to be incorporated into the inner part of the plasma membrane.

prevention of the $[Ca^{2+}]_i$ changes by PAF antagonists [81,86]. However, comparison of the dose-response curves for the effects of PAF and ET-18-OCH$_3$ on the increase in $[Ca^{2+}]_i$ in human neutrophils led to EC_{50} values of 5 x 10^{-11} and 2.5 x 10^{-7} M, respectively [81]. Thus, synthetic ET-18-OCH$_3$ seems to activate PAF receptors with a much smaller affinity than the natural lipid, in spite of their similarity in molecular structure (Fig. **1**). The affinity of the PAF receptor for ET-18-OCH$_3$ was about 5000 times smaller that for the natural PAF lipid [81]. In addition, no correlations were found between the effects of ET-18-OCH$_3$ on the increase in $[Ca^{2+}]_i$ and apoptosis. Thus, ET-18-OCH$_3$ is unable to promote apoptosis in HL-60 cells differentiated towards granulocytes and in peripheral blood mature neutrophils where the effects on $[Ca^{2+}]_i$ are bigger, whereas ET-18-OCH$_3$ induces apoptosis in undifferentiated leukaemic HL-60 cells, where it hardly affected $[Ca^{2+}]_i$ [81] (Fig. **5**). Unlike undifferentiated HL-60 cells, neutrophil-differentiated HL-60 cells as well as peripheral blood neutrophils express PAF receptors at their cell surface (Fig. **5**). PAF antagonists prevented the effects of ET-18-OCH$_3$ on $[Ca^{2+}]_i$ in neutrophil-differentiated HL-60 cells and mature neutrophils but had no effect on ET-18-OCH$_3$-induced apoptosis in tumour cells, and PAF, which reproduces the effects of ET-18-OCH$_3$ on $[Ca^{2+}]_i$, was not able to promote apoptosis [15,81]. Thus, the apoptotic effect of ET-18-OCH$_3$ is not related to its interaction with plasma membrane PAF receptors or to rapid changes in cytosolic free calcium concentration (Fig. **5**) [15,81].

INCORPORATION OF ET-18-OCH$_3$ INTO THE TARGET CELL IS CRUCIAL FOR ITS SELECTIVE ANTITUMOUR ACTION

AEPs and APCs do not directly interact with DNA, but due to their phospholipid nature they are readily incorporated into cellular membranes [87-89]. Uptake of ET-18-OCH$_3$

into tumour cells is a critical event for its selective anticancer effect, and is essential for the triggering of an apoptotic response [15,30]. There is a strong correlation between the cellular uptake of ET-18-OCH_3 and the cytotoxic ability of the drug [15,30,59,84,90-92]. Normal cells are unable to incorporate significant amounts of the ether lipid and are spared, whereas most tumour cells incorporate ET-18-OCH_3 undergoing the subsequently apoptosis [1,15,30]. Non-transformed 3T3 cells were resistant to the apoptotic action of ET-18-OCH_3 and incorporated only small amounts of the ether lipid, while upon transformation with SV40, these cells took up high amounts of the lipid and became sensitive to ET-18-OCH_3 [15]. Conversely, human leukaemic HL-60 cells incorporated high amounts of ET-18-OCH_3 and were sensitive to its apoptotic action, whereas following DMSO treatment HL-60 cells were differentiated towards cells with features of normal non-transformed mature neutrophils [93], and in this differentiated state the uptake of the ether lipid was dramatically decreased as well as the sensitivity to the drug [15,67,81,84]. These data indicate that the action of the ether lipid is specific for tumour cells and that both cellular uptake and ET-18-OCH_3-induced apoptosis are dependent on the malignant state of the cells. In addition, combining microinjection and *in situ* DNA fragmentation assays, we have further demonstrated that incorporation of ET-18-OCH_3 into the cell is a necessary requirement for ET-18-OCH_3-induced apoptosis [30]. Normal human fibroblasts are resistant to the exogenous addition of ET-18-OCH_3 because they do not take up significant amounts of the ether lipid. Nevertheless, these cells undergo rapidly apoptosis following microinjection of the ether lipid [30], indicating that the cell surface acts as a barrier to the ether lipid in normal cells. Interestingly, apoptosis was not detected when these cells were microinjected with two structurally related ET-18-OCH_3 analogues [30], 1-*O*-octadecyl-*rac*-glycero-3-phosphocholine (ET-18-OH) or 1-*O*-octadecyl-propanediol-3-phosphocholine (ET-18-H) that were also unable to induce apoptosis when added exogenously to ET-18-OCH_3-sensitive cancer cells [15,47]. These data conclusively demonstrate that incorporation of ET-18-OCH_3 into the cell is a crucial and necessary event in the selective apoptotic action of the ether lipid, and this response is highly specific for its molecular structure. In addition, we found that microinjected ET-18-OCH_3 induced apoptosis in a dose-dependent way [30], suggesting that a threshold for intracellular ET-18-OCH_3 concentration must be reached in order to trigger apoptosis. However, the mechanism by which ET-18-OCH_3 is incorporated into the tumour cell remains to be established and has been a matter of debate [1].

ET-18-OCH_3 has been reported to be preferentially accumulated in the plasma membrane of transformed cells to the extent that accounted for up to 17% of the purified membrane phospholipids [87,94,95]. [^{3}H]ET-18-OCH_3 was found to bind in similar amounts to both resistant and sensitive cells [92] at low temperatures (4ºC) when cellular processes, such as membrane internalisation or possible protein-facilitated transbilayer movement are greatly slowed or blocked, suggesting that ether lipids tend to interact unspecifically with membranes. However, when cells were warmed at room temperature to allow internalisation processes to become active, about 80% of the pre-bound ET-18-OCH_3 was internalised in sensitive cells, whereas only 10-20% of pre-bound ether lipid was taken up by resistant cells [92]. On these grounds, cellular uptake of ET-18-OCH_3 seems to be dependent on an internalisation process. The internalised lipid pool can be readily evaluated through extensive washing of the cells in either serum- or bovine serum albumin (BSA)-containing medium [96]. Internalised ET-18-OCH_3 is unavailable to BSA and therefore cannot be removed, but loosely membrane-bound ether lipid can be washed off, due to its high affinity binding to serum albumin [90,97].

We hypothesise that a yet unidentified cell surface protein present or appropriately modified in cancer cells, but not in normal cells, could be responsible for the selective incorporation of the ether lipid into the malignant cell [15,30], but solid evidence supporting this notion is still lacking. Disruption of ligand-receptor interactions by suramin treatment, a broad-specificity membrane impermeable inhibitor of ligand-receptor interactions [98-103], inhibits both ET-18-OCH_3 uptake and cytotoxicity [15], suggesting that ET-18-OCH_3 can interact with a cell surface protein involved in the transport of the ether lipid inside the cell. A tight association of the drug with cellular structures inside the cell can be suggested from the lack of ET-18-OCH_3 efflux from preloaded cells [50]. In this regard, a photoactivatable analogue of ET-18-OCH_3 is able to interact with two cytosolic proteins of MCF-7 human breast cancer cells of molecular masses of 47 and 170 kDa [104]. A plausible uptake mechanism related to a hypothetic protein-directed internalisation of ET-18-OCH_3 could involve unspecific binding of the ether lipid to the plasma membrane followed by flip-flop internalisation to the cytoplasmic face of the lipid bilayer [105]. Because flip-flop is inherently slow and ET-18-OCH_3 uptake follows a rapid kinetics, the assistance of a transmembrane transporter should be required. In this regard, the transporters Lem3p (ligand-effect modulator 3 protein) [106] and LdMT (*Leishmania donovani* putative Miltefosine Transporter) [107] have been recently found to be required for the uptake of alkylphosphocholine drugs, including ET-18-OCH_3, into *Saccharomyces cerevisiae* yeast and *Leishmania donovani* parasite, respectively. The putative participation of transporters, which play a role in the maintenance or dissipation of transbilayer lipid asymmetry, in the ET-18-OCH_3 uptake in tumour cells remains to be analysed. These transporters can be divided into three classes, based on the direction of transport of the lipid substrate [108-112]: a) "scramblases", which facilitate the bi-directional movement of lipids across the bilayer; b) "flippases", which catalyze the cytofacially directed transport of lipids; and c) "floppases", which promote the movement of lipids from the cytoplasmic to the external face of the membrane.

Different mechanisms for ether lipid uptake have been suggested, including direct adsorption onto plasma membrane, lipid flip-flop and endocytosis [62,113,114]. Despite controversial experimental support [15,50,92,114,115], endocytosis or passive diffusion do not explain the general observation that ET-18-OCH_3-sensitive cells take up significant amounts of the ether lipid while resistant cells do not. In this regard, flow cytometry analysis with fluorescein-labelled albumin showed no correlation

between endocytosis and ET-18-OCH$_3$ uptake [15]. Furthermore, Zoeller and co-workers [92], using ethyl methanesulfonate as mutagen [116], isolated mutant strains from the murine macrophage-like cell line RAW 264.7 that were resistant to the cytotoxic effect of ET-18-OCH$_3$, and found no differences both in receptor-mediated and fluid-phase endocytosis between ET-18-OCH$_3$-resistant mutants and the ET-18-OCH$_3$-sensitive parent cells. However, whereas the parent cells took up significant amounts of ET-18-OCH$_3$, the mutant cells did not. Thus, ET-18-OCH$_3$ appears to be taken up through a more specific process than general endocytosis.

ET-18-OCH$_3$ has been recently reported to accumulate into lipid rafts and to be internalised via endocytosis, mediated by the GTPase dynamin and intact rafts, in both mouse S49.1 lymphoma cells and human epitheloid cervix carcinoma HeLa cells [32,117]. Following this internalisation route, ET-18-OCH$_3$ would gain access to the intracellular location of CTP:phosphocholine cytidylyltransferase (CCT), likely the endoplasmic reticulum [118], as a putative target of ET-18-OCH$_3$ leading to inhibition of phosphatidylcholine (PC) biosynthesis and subsequent mitotic arrest and cell death [32,117]. In order to be activated, CCT translocates from the cytosol to the endoplasmic reticulum [118], and therefore ET-18-OCH$_3$ would need to be internalised to inhibit CCT at this location [117,119]. Raft-mediated endocytosis does not target the lysosomes, but is directed towards a rapid cycling pathway, via the Golgi or the endoplasmic reticulum, back to the plasma membrane [120-123]. Interestingly, the uptake of LPC, which shows a high structural similarity to ET-18-OCH$_3$ (Fig. **1**) and overrides its cytotoxic affects [124], follows a different route independent of rafts and dynamin, very likely not by endocytosis but by flipping over the plasma membrane lipid bilayer [117]. The fact that ET-18-OCH$_3$, but not its natural analogue LPC, is preferentially located into lipid rafts highlights the importance of ether-bonds for partitioning in membrane rafts [117].

EFFECTS OF ET-18-OCH$_3$ ON CELL CYCLE AND MITOSIS

In addition to the apoptotic effect, ET-18-OCH$_3$ has been reported to inhibit cell division without concurrent inhibition of nuclear division, leading to accumulation of cells in G$_2$/M, multinucleate cell formation, and subsequent cell death through apoptosis [124-131] (Mollinedo, F., del Canto-Jañez, E., Verhaegen, S. and Gajate, C., unpublished data). Inhibition of cell growth by ET-18-OCH$_3$ resulted from inhibition of cytokinesis [131,132] (Mollinedo, F., del Canto-Jañez, E., Verhaegen, S. and Gajate, C., unpublished data) by an unknown mechanism. ET-18-OCH$_3$-treated cells

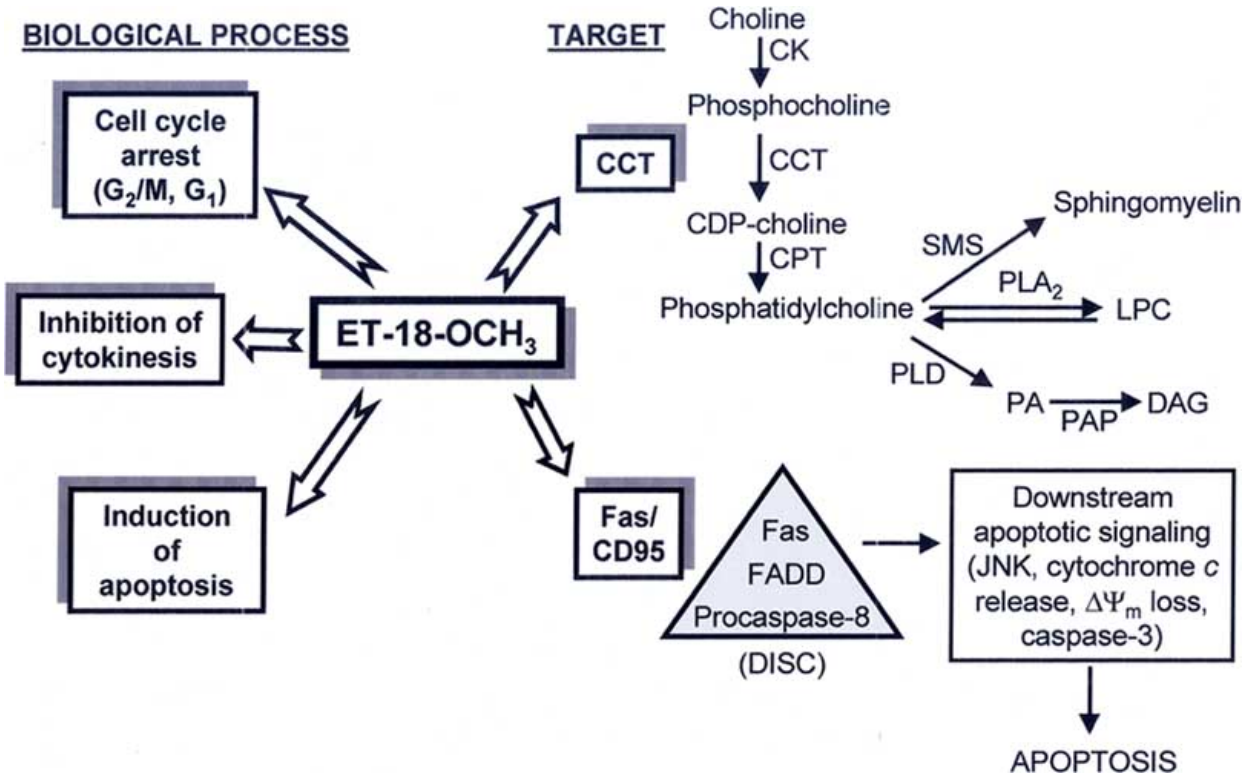

Fig. (6). Major biological processes and putative targets involved in the antitumour action of ET-18-OCH$_3$. CCT is involved in *de novo* synthesis of phosphatidylcholine biosynthesis, and Fas/CD95 induces apoptosis through the formation of the DISC, containing trimerized Fas/CD95, FADD and procaspase-8, and the ensuing downstream apoptotic signalling, including JNK activation, release of cytochrome *c* from mitochondria, loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and caspase-3 activation. CK, choline kinase. CCT, CTP:phosphocholine cytidylyltransferase. CPT, choline phosphotransferase. SMS, sphingomyelin synthase. PLA$_2$, phospholipase A$_2$. PLD, phospholipase D. LPC, lysophosphatidylcholine. PA, phosphatidic acid. PAP, phosphatidic acid phosphatase. DAG, diacylglycerol.

proceeded through the full cell cycle, but failed to divide, and instead accumulated as tetraploid or octaploid cells at G_0/G_1 phase of the cell cycle [131]. Microtubule assembly appears to be unaffected by exposure to the ether lipid [133], although F-actin filaments could be collapsed [6,131]. Also, a number of reports show that ET-18-OCH_3-treated cells are arrested in the G_0/G_1 and G_2/M phases of the cell cycle [124,127,128,131]. ET-18-OCH_3 is able to promote both a direct apoptotic effect or a cytostatic effect by inhibition of cytokinesis in the same cell type (Fig **6**), and the relative predominance of each effect is cell type-specific and drug dose-dependent (Mollinedo, F., de la Iglesia-Vicente, J. and Gajate, C., unpublished data). Interestingly, ET-18-OCH_3 is one of the few potential chemotherapeutic agents having a greater cytotoxic effect against non-small cell lung cancer (NSCLC) cells than small cell lung cancer (SCLC) cells [134]. In an attempt to unravel differences in cell signalling between NSCLC and SCLC cells that could account for their distinct ET-18-OCH_3 sensitivity, SCLC cells were found to exhibit greater phospholipase C-β1 expression and ET-18-OCH_3 resistance compared to NSCLC cells [135]. Lung cancer is currently among the most commonly occurring malignancies in the world and one of the leading causes of cancer mortality [136-138]. Malignant lung tumours are generally divided into two major subtypes, SCLC and NSLC. SCLC accounts for 20-25% of all bronchogenic malignancies, follows the most aggressive clinical course; although initially highly responsive to radiation and chemotherapeutic regimes, less than 4% of SLCL patients survive 5 years past the initial diagnosis. NSCLC patients are unresponsive to chemotherapy and about 13% of them survive 5 years after diagnosis. Shafer and Williams [134] found that three ET-18-OCH_3-sensitive NSCLC cell lines (NCI-H157, NCI-H520, NCI-H522) exhibited G_2/M arrest and significant apoptosis in response to drug treatment, whereas none of the SCLC cell lines used in their study showed ET-18-OCH_3-induced G_2/M arrest or significant apoptosis. These authors found little correlation between ET-18-OCH_3-induced cytotoxicity and altered activities of either the apoptotic c-Jun NH_2-terminal kinase (JNK) signalling pathway and the mitogenic/survival mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling pathway [134]. This could suggest that apoptotic and cell cycle arrest processes induced by ET-18-OCH_3 could be mediated through rather different signalling mechanisms, and their respective predominance would depend on the cell signalling pathways prevalent in a particular cell (cell type specificity), and the extent to which each signalling is affected (drug dose dependency). On these grounds, some cells would undergo apoptosis following ET-18-OCH_3 treatment if the signalling involved in this response were predominant over the one involved in cell cycle arrest, and others would undergo cell cycle arrest if the corresponding underlying signalling route prevailed over the apoptotic one. Subsequently, a prolonged cell cycle arrest will eventually lead to apoptosis but through a different and indirect process mediated by signalling events triggered as a result of a blockade in cell cycle progression [139].

Inhibition of PC biosynthesis might be envisaged as a putative mechanism involved in the ET-18-OCH_3-mediated inhibition of mitosis [38,140], as PC is the major membrane phospholipid of animal cells (about 50% of the phospholipid mass) and therefore its biosynthesis is required to provide increased quantities of cellular membranes during mitosis. ET-18-OCH_3 has been shown to inhibit PC biosynthesis [38] through inhibition of CCT [119] (Fig. **6**), which has long been recognized as the key enzyme controlling the *de novo* PC biosynthesis pathway [141-143], and this effect correlated with inhibition of cell growth in a number of cancer cell lines. Overriding this PC inhibition by the addition of LPC, which provides an exogenous source of cellular phospholipid through its acylation to PC, an alternate route for PC production [119,124], or by overexpressing CCT [144] prevented ET-18-OCH_3-dependent accumulation of cells in G_2/M and the subsequent apoptotic response, but did not overcome the ET-18-OCH_3-dependent G_0/G_1 block. This indicates that restoring PC biosynthesis overrides the cytotoxic but not the cytostatic activity of ET-18-OCH_3. Thus, neither CCT overexpression nor LPC supplementation allowed the tumour cells to proliferate in the presence of ET-18-OCH_3, suggesting that the cytostatic action of ET-18-OCH_3 was independent of its effect on membrane phospholipid synthesis activity [144]. CCTα is a soluble enzyme mainly localized in the nucleus, whose activity is regulated by phosphorylation, and becomes activated when induced to associate with the nuclear membrane [145-148]. Additional subcellular localisations have been reported, such as Golgi, endoplasmic reticulum and transport vesicles [145,149,150]. A new isoform named CCTβ lacks the nuclear targeting sequence and the phosphorylation domain of CCTα, suggesting that the new isoform differs from CCTα with regard to its subcellular localisation and regulation [151]. Whether the reported effect of the ether lipid on decreasing the active membrane-form of CCT [124] is mediated by direct CCT-drug interaction or through other indirect processes is not yet established. On the other hand, some results question a primary association of an alteration in the PC metabolism with the antitumour action of ET-18-OCH_3 [1,152-154]: a) induction of apoptosis by ET-18-OCH_3 is very rapid in certain leukaemic cells, occurring before any detectable change in PC metabolism; b) lack of correlation between interference with phospholipid metabolism and the sensitivity of different cell lines to the blockade of proliferation by ET-18-OCH_3. Arthur's group [152-154] found a dissociation between the inhibition of PC biosynthesis and the inhibition of cell growth by ET-18-OCH_3 in a number of epithelial tumour cells, and suggested the presence of some compensatory mechanisms that overcome initial lipid metabolism perturbations. Thus, it has yet to be conclusively established that the interference in lipid metabolism is a primary and general cause for the inhibition of cell growth and cytotoxic action of ET-18-OCH_3.

APOPTOTIC AND SURVIVAL SIGNALLING IN ET-18-OCH_3 ANTITUMOUR ACTION

Although treatment with ET-18-OCH_3 leads both to cytostatic and cytotoxic effects on tumour cells (Fig. **6**), apoptosis has been shown to be the major effect of the antitumour action of the ether lipid [15]. ET-18-OCH_3-induced apoptosis involves Fas/CD95 death receptor [30,31], JNK [47,60], mitochondria [155-157], reactive

oxygen species [156,157], c-Jun [47,60] and caspase-3 [156]. In addition, apoptotic concentrations (5-25 μM) of ET-18-OCH$_3$ have been reported to inhibit the MAPK/ERK mitogenic pathway and the Akt/protein kinase B (PKB) survival pathway [9,60,158]. It has been suggested that inhibition of the MAPK/ERK cascade by ET-18-OCH$_3$ is not due to any direct effect on ERK activity but as a result of interfering in the association of Raf-1 with membranes and subsequent decrease in Raf-1 kinase activity [159]. Thus, ET-18-OCH$_3$ can activate proapoptotic signalling pathways (JNK) and inhibit mitogenic/survival signalling (ERK, Akt). A dramatic and persistent activation of JNK signalling is required for ET-18-OCH$_3$-mediated apoptosis [47,60]. It could be envisaged that this sustained JNK signalling activation ultimately drives cells to apoptosis due to an accumulation of JNK signalling-derived signals until reaching a certain threshold. On the other hand, the balance between proapoptotic JNK pathway and the antiapoptotic MAPK cascade may be critical in the cell's fate to die or to survive [160,161].

Current evidence also indicates that ET-18-OCH$_3$-induced apoptosis involves mitochondria. ET-18-OCH$_3$-induced apoptosis is prevented by Bcl-2 or Bcl-x$_L$ overexpression by gene transfer [15,156]. ET-18-OCH$_3$ induces disruption of the mitochondrial transmembrane potential followed by the production of reactive oxygen species [156,157] and release of mitochondrial cytochrome *c* [155]. In addition ET-18-OCH$_3$ activates caspase-3 and its inactivation prevents the apoptotic response elicited by the ether lipid [156].

Interestingly, submicromolar concentrations (10-500 nM) of ET-18-OCH$_3$ have been shown to activate the MAPK/ERK pathway in A431 human epidermoid carcinoma cells [162]. In addition, 500 nM ET-18-OCH$_3$ trigger rapid clustering and internalisation of epidermal growth factor receptor (EGFR) in A431 cells, which overexpress EGFR (2.6-3.7 x 10^6 EGFRs/cell) [163], concomitant with MAPK/ERK activation [162]. In this regard, ET-18-OCH$_3$ has been reported to reduce the number of EGFR sites without affecting the affinity of the receptors in human breast cancer cell lines [164], and this reduction in the epidermal growth factor binding capacity might be related to the ET-18-OCH$_3$-induced inhibition of the growth of hormone-dependent breast cancer cells. Although the mechanism by which ET-18-OCH$_3$ induces EGFR internalisation and MAPK/ERK activation remains to be established, this effect seems to be somewhat cell-type specific as it is not observed in other cell types, including the breast carcinoma cell line MDA-MB-468 [162] that also overexpresses EGFR (1.3 x 10^6 EGFRs/cell) [165].

ET-18-OCH$_3$ has been reported to inhibit additional signalling pathways related to survival and mitogenesis [1,58], such as phosphoinositide-specific phospholipase C [135,166], phospholipase D [167], phosphatidylinositol-3-kinase [168], and protein kinase C (PKC) [169,170]. However, a major role for these signalling pathways in the antitumour action of ET-18-OCH$_3$ can be questioned, as some inactive analogues show similar actions on these signalling pathways, there is a lack of correlation between the ether lipid doses required to show the above inhibitory effects and those needed for antitumour activity, and inhibitory actions on the above signalling pathways are observed in both ether lipid sensitive and resistant cells. In addition, contradictory results have been reported showing either activation, inhibition or no effect on PKC by ET-18-OCH$_3$ [65,169,171-177], and cells depleted of PKC activity show similar sensitivity or resistance to ET-18-OCH$_3$ as cells expressing PKC activity [172].

ET-18-OCH$_3$ AFFECTS GENE EXPRESSION

Although ET-18-OCH$_3$-induced apoptosis does not require protein synthesis [156], this ether lipid is able to induce the expression of a number of transcription factors in tumour cells. In this regard, it is worthwhile to note the dramatic up-regulation of c-*myc* and c-*jun* by ET-18-OCH$_3$ [15,47,178]. Thus, despite ET-18-OCH$_3$ exerts its major effects at the plasma membrane level, it also affects gene expression by modulating the expression and activity of transcription factors.

Overexpression of c-*myc* has been correlated with induction of apoptosis in several systems [179-183], and it has been reported that c-Myc acts downstream of the Fas/CD95 receptor by sensitising cells to the Fas/CD95 death signal [184]. Interestingly, ET-18-OCH$_3$ increased dramatically the steady-state mRNA levels of c-*myc* in a number of leukaemic cells [15]. We observed this ET-18-OCH$_3$-mediated c-*myc* upregulation even in human leukaemic HL-60 cells [15], which constitutively overexpress c-*myc* [93] due to an amplification of DNA sequences encompassing the c-*myc* gene [185]. In addition, enforced expression of *bcl-2* has been shown to suppress both c-*myc*- and ET-18-OCH$_3$-mediated apoptosis [15,156,186,187]. These data suggest that ET-18-OCH$_3$-mediated upregulation of c-*myc* can facilitate, sensitize and potentiate a Fas-mediated cell killing mechanism induced by the ether lipid (see below). This could be relevant to therapy, given the ubiquitous activation and overexpression of c-Myc in human cancers. Thus, it could be envisaged that overexpression of this gene in tumour cells could even facilitate their killing by ET-18-OCH$_3$. However, the role of c-*myc* in ET-18-OCH$_3$-mediated apoptosis remains to be elucidated.

ET-18-OCH$_3$ is also able to induce the expression of *fos* and *jun* proto-oncogenes and to activate the transcription factor AP-1 in human leukaemic cells [47,178]. We have observed that the steady state mRNA level of *c-ju*n was dramatically enhanced following ET-18-OCH$_3$ treatment [178]. This increase in the c-*jun* mRNA levels was persistent along the incubation time and could be a result of the sustained activation of JNK by the ether lipid [47]. Antisense oligonucleotides against c-*jun* or overexpression of a dominant-negative mutant of c-Jun blocked ET-18-OCH$_3$-induced apoptosis, indicating a role for c-Jun in this apoptotic response [47,60]. The role of c-Jun in the cascade of events triggered by ET-18-OCH$_3$ remains to be elucidated, but because ET-18-OCH$_3$-induced apoptosis does not require protein synthesis [156], it is tempting to envisage the involvement of c-Jun in transcription-independent events leading to cell death. On the other hand, c-Jun is a major component of the transcription factor AP-1, and therefore ET-18-OCH$_3$ could regulate transcription. In fact, we found that ET-18-OCH$_3$ treatment led to activation

of the AP-1 transcription factor, as assessed by an enhanced binding activity of transcription factor AP-1 to its cognate DNA sequence as well as by stimulation of transcription from an AP-1 enhancer element [47,178]. The regulation of gene expression by ET-18-OCH_3 may explain some previously observed effects of this ether lipid. ET-18-OCH_3 is able to activate macrophages acquiring cytotoxicity against tumour cells [36]. As the transcription factor AP-1 regulates the expression of a number of cytokines that play a crucial role in macrophage function, it could be suggested that the enhancement of the macrophage tumouricidal activity after incubation with ET-18-OCH_3 might be, at least in part, mediated by the action of this ether lipid on the activity of transcription factor AP-1 and other putative additional transcription factors.

The weak induction of c-*fos* by ET-18-OCH_3 [47,178,188] was not related to the induction of apoptosis, as antisense oligonucleotides against c-*fos* did not prevent ET-18-OCH_3-induced apoptosis [47]. This is in agreement with the evidence from c-Fos-deficient mice demonstrating that c-*fos* is not essential for the induction of apoptosis [189]. We have also found that ectopic overexpression of *bcl-2* or *bcl-*x_L prevents apoptosis induced by ET-18-OCH_3, but not its cellular uptake [15], suggesting that high levels of Bcl-2 and Bcl-x_L can block the intracellular signalling route leading to cell death triggered by ET-18-OCH_3. However, p53 is dispensable for ET-18-OCH_3-induced apoptosis as HL-60 cells are very sensitive to the apoptotic action of ET-18-OCH_3 [15,45] and do not express p53 [190].

In addition, ET-18-OCH_3 has been shown to inhibit the activation of the nuclear factor-kappa B (NF-κB) transcription factor by 12-O-tetradecanoylphorbol-13-acetate, a PKC activator, but not by tumour necrosis factor-α (TNFα) or interleukin 1α [191], suggesting that this effect is mediated by the inhibitory action of ET-18-OCH_3 on PKC activity [169,170].

Although ET-18-OCH_3-induced apoptosis is prevented by caspase inhibitors and overexpression of Bcl-2, this ether lipid does not affect the expression of a number of caspases (caspase-2, caspase-3, caspase-4, caspase-7, caspase-8) and Bcl-2-related genes (*bcl-2*, *bcl-*x_L, *bik*, *bak*, *bax*, *A1/bfl-1*) [15,156], and therefore its proapoptotic action is not mediated by modulating the mRNA expression levels of these genes.

FAS/CD95 INVOLVEMENT IN ET-18-OCH_3-MEDIATED APOPTOSIS

The Fas/CD95 death receptor (also called APO-1), a major member of the tumour necrosis factor receptor (TNF-R) family, transmits apoptotic signals through the presence of a "death domain" (DD) within the cytoplasmic portion of the receptor [192-194]. Stimulation of Fas/CD95 with its specific ligand FasL/CD95L results in receptor aggregation [195,196], previously assembled in trimers [197,198], and recruitment of the adaptor molecule Fas-associated death domain protein (FADD) [199] through interaction between its own DD and the clustered receptor DDs. FADD also contains a "death effector domain" (DED) that binds to an analogous DED domain repeated in tandem within the zymogen form of procaspase-8 [200]. Upon recruitment by FADD, procaspase-8 oligomerisation drives its activation through self-cleavage, activating downstream effector caspases [19]. Thus, activation of Fas/CD95 results in receptor aggregation and formation of the so-called "death-inducing signalling complex" (DISC) [201], containing trimerized Fas/CD95, FADD and procaspase-8.

We have recently found that ET-18-OCH_3 stimulates from inside the cell a Fas/CD95-mediated apoptotic signalling pathway (Fig. **6**), independently of the natural ligand FasL/CD95L [30,31,59], representing a novel and unique way of killing cells in cancer chemotherapy. ET-18-OCH_3 only induces apoptosis in Fas/CD95-containing cells [30]. Fas/CD95-deficient cells are spared from the apoptotic effect of ET-18-OCH_3, but they become sensitive to the ether lipid after transfection with Fas, provided cells are able to incorporate the ether lipid [30]. Fas/CD95-expressing cells that do not take up ET-18-OCH_3 from the culture medium are unaffected by the ether lipid when this latter is added exogenously, but they undergo rapid apoptosis following microinjection of ET-18-OCH_3 [30]. This demonstrates that putative interactions between ET-18-OCH_3 and extracellular portions of Fas/CD95, if any, are irrelevant to the apoptotic effect of the ether lipid, and that ET-18-OCH_3 must be intracellularly located to trigger the Fas/CD95-mediated apoptotic response. The mechanism of action of additional ATLs also seem to involve a Fas/CD95-FADD-mediated signalling pathway [202]. Interestingly, an increasing number of diverse agents and experimental conditions have been recently reported to induce Fas/CD95-mediated apoptosis independently of FasL/CD95L [203], including ultraviolet light [204,205] and deoxycholic acid [206], suggesting that FasL/CD95L-independent activation of Fas/CD95 is a more general process of death receptor activation than initially believed [203].

Bringing together current evidences from different investigators, we can conclude that ET-18-OCH_3 elicits three major biological processes related to its antitumour action, namely cell cycle arrest at G_2/M and G_1, inhibition of cytokinesis and induction of apoptosis (Fig. **6**). These actions seem to be due to the effect of ET-18-OCH_3 on two major putative targets in the tumour cell, CCT and Fas/CD95 (Fig. **6**).

INVOLVEMENT OF MEMBRANE RAFTS IN ET-18-OCH_3-MEDIATED APOPTOSIS

The plasma membrane contains microdomains named membrane rafts, consisting of dynamic assemblies of cholesterol and sphingolipids [207-209]. The presence of saturated hydrocarbon chains in sphingolipids allows for cholesterol to be tightly intercalated, leading to the presence of distinct liquid-ordered phases, membrane rafts, dispersed in the liquid-disordered matrix, and thereby more fluid, lipid bilayer [209]. One key property of membrane rafts is that they can include or exclude proteins to varying degrees. Membrane rafts may serve as foci for recruitment and concentration of signalling molecules at the plasma membrane, and thus they have been implicated in signal transduction from cell surface receptors [207-209].

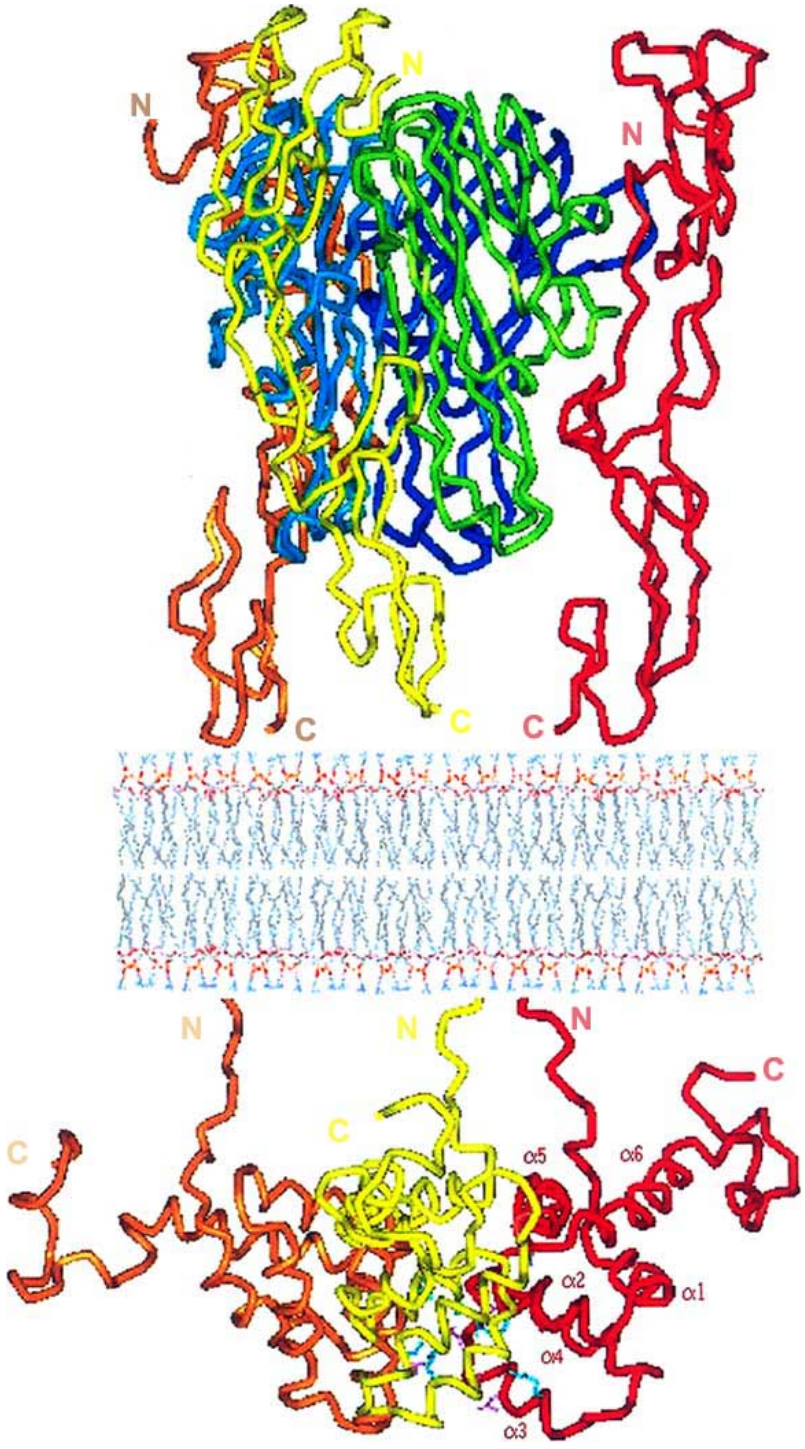

Fig. (7). Schematic representation of a tentative model of activated Fas/CD95 receptor. Coordinates for the extracellular domain (upper part) correspond to those of the sTNF-R1, highly homologous to the extracellular domain of Fas/CD95, in complex with TNFβ [220]. The three subunits of TNFβ are colored in cyan, blue and green, respectively. The three sTNF-R1 molecules are colored orange, yellow and red, respectively. The intracellular region of Fas/CD95 (lower part) was built from that of monomeric soluble Fas death domain (FasDD) [217], and automatically docked to provide the trimer. The three intracellular FasDDs are colored orange, yellow and red, respectively. The locations of the N and C termini of both the extracellular and intracellular domains of the receptors are indicated. Note the good orientation of the N termini of the three FasDDs upon oligomerization pointing to the corresponding C termini of the extracellular region. The six amphipathic α-helices of the FasDD are shown. The membrane-spanning region of the receptor is not shown. Membrane is not to scale. Key amino acid side chains relevant to the proposed model are shown as pink (negatively charged amino acids) and cyan (positively charged amino acids) sticks (see Fig. **8**).

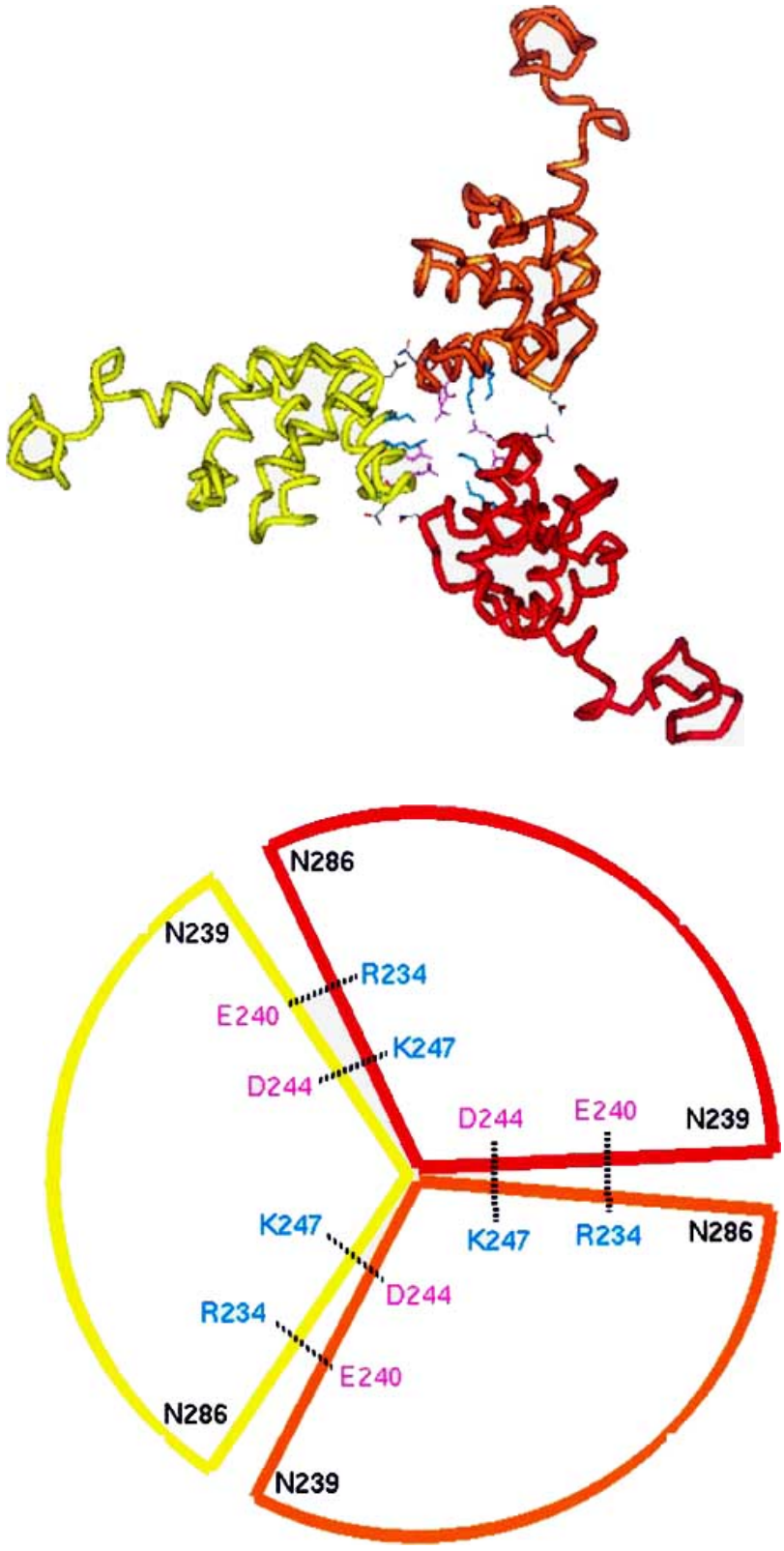

Fig. (8). Model for putative interactions involved in intracellular FasDD trimerization of activated Fas/CD95 receptor. (**A**) Top view (perpendicular to the plane of the membrane) of the intracellular death domain in activated Fas/CD95 showing putative interactions between different amino acid side chains. Cyan and pink colors are used to highlight positively and negatively charged amino acids, respectively. Side chains at the turns, colored according to the atom type, correspond to asparagine residues, which might also be involved in the association. (**B**) Simplified representation of (A) showing the labels of the amino acids and their putative interactions.

Interestingly, we have found that activation of Fas/CD95 by ET-18-OCH$_3$ was mediated by the translocation and capping of Fas/CD95 into membrane rafts [31], what, in turn, was the first evidence for the translocation of Fas/CD95 into rafts. Subsequent studies also found that Fas/CD95 was accumulated into rafts following activation by its ligand FasL/CD95L [210,211]. In addition, disruption of membrane rafts by pretreatment of cells with methyl-β-cyclodextrin or filipin, which extract cholesterol from the membranes, inhibited both ET-18-OCH$_3$-induced Fas/CD95 capping and apoptosis [31]. Because membrane rafts function as recruitment cores for signalling molecules at the plasma membrane, the ET-18-OCH$_3$-mediated translocation of Fas/CD95 into membrane rafts may provide a mechanism for amplifying Fas/CD95 signalling by the reorganisation of membrane microdomains [31]. In addition, ET-18-OCH$_3$ has been found to accumulate in membrane rafts [32] (Gajate, C., Acuña, A.U., Amat-Guerri, F. and Mollinedo, F., unpublished data), and thereby could affect a number of different signalling molecules that localise into these membrane domains. This could be an explanation for the manifold effects assigned to ET-18-OCH$_3$ on distinct signalling processes. ET-18-OCH$_3$, with the ether bonds and its saturated, long alkyl chain, is predicted to accommodate to the rigid, liquid-ordered, cholesterol- and sphingolipid-enriched microdomains [209]. The involvement of lipid rafts in ET-18-OCH$_3$ action could also be related to the previous observations concerning the temperature-dependency of the ET-18-OCH$_3$ cytotoxic activity [113,129,212,213].

MOLECULAR MODEL OF FAS DEATH DOMAIN TRIMERIZATION

Because ET-18-OCH$_3$-mediated apoptosis requires intracellular activation of Fas/CD95 and its translocation into membrane rafts, we analysed by molecular modelling a putative interaction between ET-18-OCH$_3$ and the intracellular portion of the death receptor. To this aim we have carried out a computational study to elucidate the structure of the intracellular portion of Fas/CD95 and the putative binding of ET-18-OCH$_3$ to this region.

Fas/CD95 is a 48-kDa type I transmembrane receptor of 319 amino acids with a single transmembrane domain of 17 amino acids, an N-terminal, cysteine-rich extracellular domain and a C-terminal cytoplasmic DD of about 80 amino acids, which plays a crucial role in transmitting the death signal from the cell's surface to intracellular pathways [192,193]. The fifteen carboxy-terminal residues of Fas/CD95 play a negative role [193] and interact with the protein tyrosine phosphatase FAP-1 (Fas-associated phosphatase 1) [214-216]. The structure of the intracellular Fas death domain (FasDD) has been reported only as an isolated monomer in solution at pH 4.0 [217], consisting of six amphipathic α-helices arranged antiparallel to one another [217,218] (Fig. **7**). Since trimer formation is widely believed to be responsible for recruitment of the adaptor protein FADD through its own DD to the clustered receptor DDs, and subsequent triggering of the apoptosis execution machinery [194], we searched for a plausible model of trimer formation using a computational method that could take into account the available evidence about the oligomerisation interface. Ten thousand possible dimer solutions were automatically generated by the FTDock programme [219], which permits an exhaustive search of translational and rotational space, for interaction of two FasDD monomers. After an automatic filtering process consisting of accepting only those solutions in which residues Arg-234, Asp-244, Glu-240, Glu-245, and Lys-247 in one of the monomers were in close proximity to the other monomers (i.e. at a distance equal to or less than 4.5 Å), 20 complexes were selected for visual inspection. Only one complex was found that simultaneously fulfilled the criteria of suitable interfacing residues, proper orientation of the N-terminal region arising from the membrane and provision of a suitable docking site for a third monomer. When this dimer and an additional monomer had been put into the same programme, the unique solution shown in Fig. (**7**) was produced. According to this model, three FasDDs associate mainly through interactions between exposed charged residues in their α2 and α3 helices. A network of crossed interactions involving ion pairs between residues from different monomers is therefore feasible (e.g. Glu-240:Arg-234 and Lys-247:Asp244, or reciprocally, Glu-240:Lys-247 and Arg-234:Asp-244). Alternatively, positively charged Arg-234 and Lys-235 could interact with the carbonyl oxygens at the C-terminus of helix α2 (Fig. **8**). In comparison, nonpolar interactions are less likely and could only involve the side chains of Ala-241 and Val-251. According to this tentative model, Fas/CD95 receptor trimerization would lead to a particular disposition of the intracellular C-terminal DDs such that acidic and basic residues from one Fas/CD95 molecule would interact with their basic and acidic counterparts on the other two monomers thus facilitating trimer formation. Interestingly, as shown in Fig. (**7**), when the intracellular domains are organized as described, the charged side chains on one side of helices α5 and α6 (*i.e.* Lys-272, Glu-273, Asp-276, Lys-280, Lys-283, and Lys-284) point towards the membrane (*i.e.* the polar heads of the inner layer's phospholipids). Also, the N-terminal segments of the death domains set aligned with the C-terminal segments of the extracellular domains of the Fas/CD95-related death receptor tumour necrosis factor receptor 1 (sTNF-R1) in complex with TNFβ [220] (Fig. **7**). Since Fas/CD95 and TNF-R1 receptors are expected to adopt similar three-dimensional structures because of their high homology, this structural alignment supports the feasibility of the model presented here.

IDENTIFICATION OF A PUTATIVE BINDING SITE FOR ET-18-OCH$_3$ ON FASDD

We envision two alternative explanations to account for the molecular mechanism of intracellular Fas/CD95 activation by ET-18-OCH$_3$: (i) direct interaction of the drug with the oligomerization interface, thereby facilitating trimer formation; (ii) binding to a region distinct from the interface and promoting a conformational change in the monomer that mimics the conformational change induced by the natural ligand (FasL/CD95L). In this respect, we note that ET-18-OCH$_3$ is an amphipathic molecule with a long hydrophobic hydrocarbon chain and a charged phosphocholine head, which includes a negatively charged phosphate and a positively charged ammonium group (Fig. **2**). The FasDD

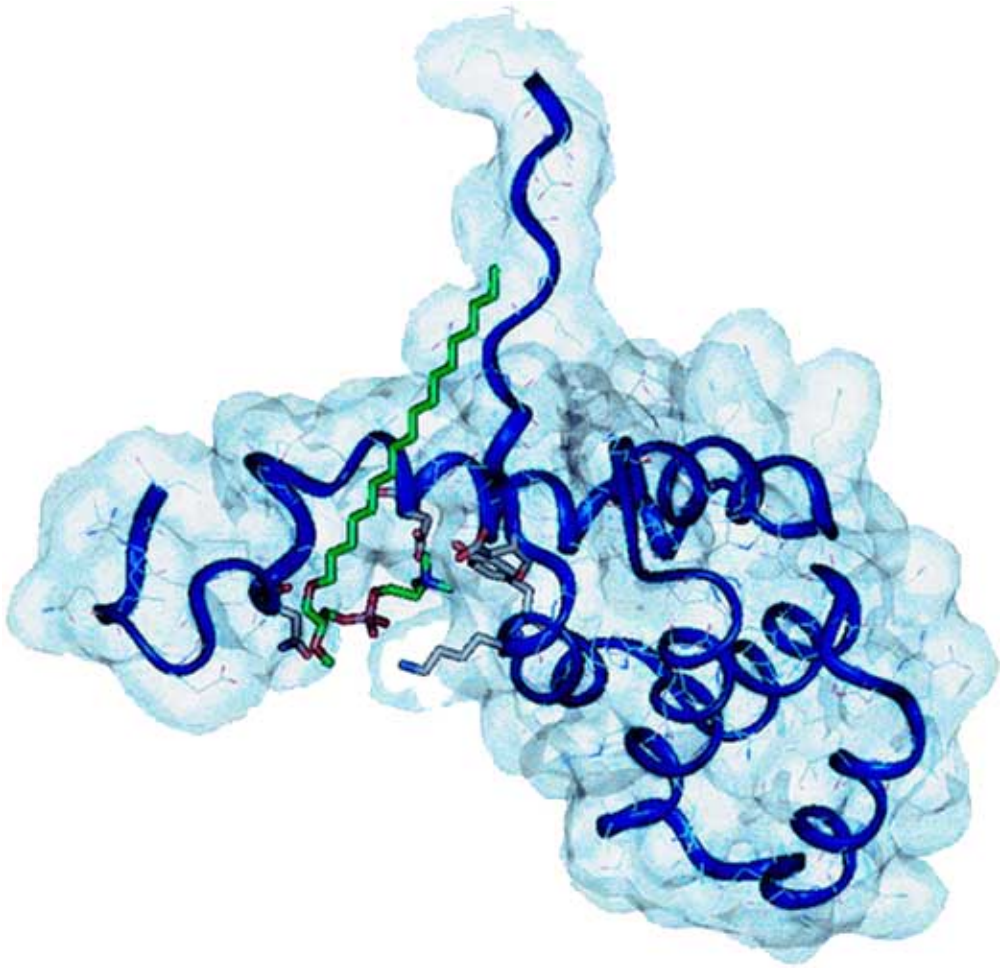

Fig. (9). Tentative model showing the putative site of interaction between a FasDD monomer (blue ribbon Cα trace and relevant residues as sticks with carbon atoms in gray) and ET-18-OCH_3 (sticks with C atoms in green, O in red, N in blue, and P in magenta). The corresponding atoms of ET-18-OCH_3 are colored as in Fig. (**2**). A semitransparent solvent-accessible surface surrounds the whole of the FasDD. Note that both the aliphatic hydrocarbon tail of ET-18-OCH_3 and the N-terminal region of FasDD point in a similar direction towards the membrane.

consists of a bundle of three pairs of antiparallel helices (α1/α2, α3/α4, and α5/α6) with the loops connecting α1/α2 and α4/α5, crossing over each other [217,218] (Fig. **7**). This helix arrangement gives rise to several hydrophobic clefts with positively and negatively charged residues, which could make up potential binding sites for ET-18-OCH_3. A positively charged residue (either a lysine or an arginine) is likely to interact with the phosphate group of the drug, and these residues are numerous over the surface of the protein. On the other hand, in addition to negatively charged residues (i.e. Asp or Glu) for binding to biological macromolecules, ligand choline groups usually make use of what is called cation-π interactions [221], involving the π electrons of up to three aromatic rings such as those present in tryptophan, phenylalanine and tyrosine. In this regard, aromatic residues of FasDD that are relatively close to each other in space and could provide electron density for establishing cation-π interactions with the positively charged head of ET-18-OCH_3 are restricted to only two pairs: Phe-232 and Trp-265, and Tyr-216 and Tyr-275. When the programme GRID [222] had been used to determine energetically the favourable binding sites on FasDD for an N^+ probe representing the trimethylammonium group present in the ET-18-OCH_3 molecule, a very distinct region was highlighted at the interface between helices α1, α5, and α6 (Fig. **9**). The interaction maps for the methyl carbon, the phosphate and ether oxygen probes, on the other hand, were less marked, and appeared scattered over relatively large areas of the protein. Of these, the most interesting ones were those observed in the vicinity of the site reported above for the choline head and, particularly, a positive region close to the side chain of Lys-215 that could lead to ion-pair formation with the phosphate group present in ET-18-OCH_3. Taken together, these interaction maps are strongly suggestive of a preferred location for binding of ET-18-OCH_3 polar head on FasDD in the vicinity of the aromatic rings of Tyr-216 and Tyr-275, the side chain carboxylate of Asp-301 and the backbone carbonyl oxygen of Val-211. At the same time, in this orientation, the hydrophobic tail could be partially inserted in the cytoplasmic membrane.

A complementary unbiased exploration of this region as a suitable binding site for ET-18-OCH$_3$ was carried out with the automated docking programme AutoDock [223]. Rigid-body docking with ET-18-OCH$_3$ yielded one main cluster with 62 hits (root-mean-square deviation from one another less than 1 Å) over 12 multi-member conformational clusters predicted. This cluster was ranked as the fourth by lowest docked energy over a total of 25 distinct possibilities. When flexible docking was used, i.e. changes in torsional angles involving rotatable bonds were allowed, 10 multi-member conformational clusters were found of which two presented the highest occurrence frequency (11 and 10 hits). The two clusters were ranked as 13th and 14th over 70 distinct possible solutions and both closely reproduced the conformation and orientation for ET-18-OCH$_3$ found when rigid docking was used. The putative binding mode, which is shown in Fig. (**9**), is in excellent agreement with the GRID results and predicts the following paired interactions: the phosphate group with Lys-215, the ammonium group with the aromatic side chain of Tyr-216 and the negatively charged carboxylate of Asp-301, and the methoxy group in a suitable position for additional interactions with Asn-310 and Asn-313. In this orientation, the long alkyl chain of the ether lipid would be pointing toward the membrane possibly interacting with the transmembrane region of the receptor. It is interesting that this side of FasDD is different from both the proposed trimerisation interface (helices α2 and α3) and the face responsible for association with the adaptor protein FADD (helices α1 and α4). It is also noteworthy that the putative binding site for ET-18-OCH$_3$ is spatially very close to the 15 C-terminal residues of FasDD, which have been shown to play a negative regulatory role in Fas/CD95 antibody-induced killing activity [193]. Thus, the conformational changes induced by ET-18-OCH$_3$ binding to FasDD could trigger Fas/CD95 receptor activation through removal of the inhibitory role of this C-terminal domain.

Although the details of the proposed Fas/CD95-ET-18-OCH$_3$ interaction must await currently ongoing structural

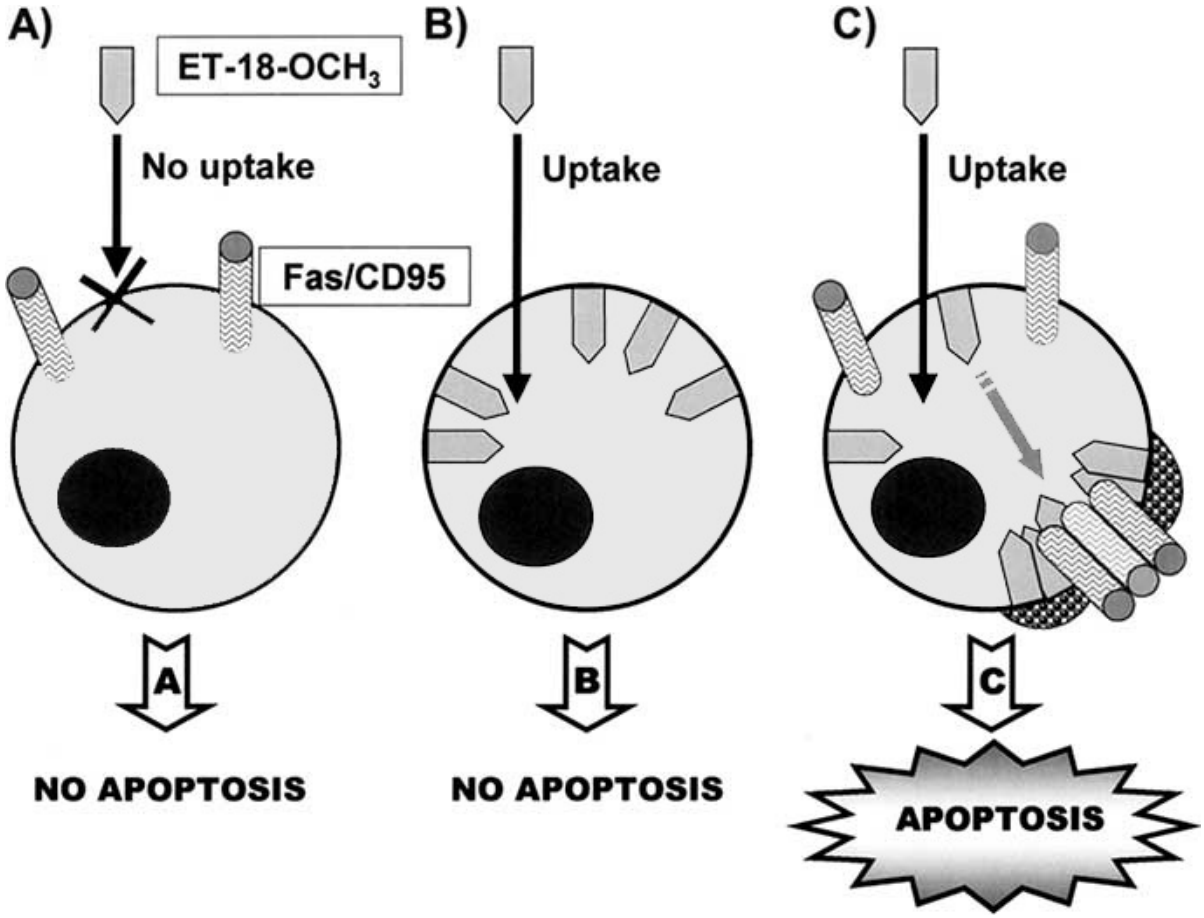

Fig. (10). Schematic model for the mechanism of action of ET-18-OCH$_3$. This mechanism involves two critical steps: a) selective incorporation of ET-18-OCH$_3$ within the cell, likely to the inner leaflet of the plasma membrane, and accumulation in lipid rafts; b) induction of apoptosis following intracellular triggering of Fas/CD95 oligomerization and capping in membrane rafts, independently of FasL/CD95L, and activation of a Fas/CD95-mediated signalling route. Three major scenarios can be found following incubation of ET-18-OCH$_3$ with distinct cell types. (**A**) Cells are unable to take up ET-18-OCH$_3$, and thereby they are spared after ET-18-OCH$_3$ treatment, despite of expressing Fas/CD95 (non-transformed, normal cells and resting T cells). (**B**) Cells are able to incorporate ET-18-OCH$_3$, but they do not undergo apoptosis following ET-18-OCH$_3$ treatment because of Fas/CD95 absence (some resistant tumour cells). (**C**) Fas/CD95-expressing cells are able to take up ET-18-OCH$_3$ and, once inside the cell, ET-18-OCH$_3$ triggers Fas/CD95 oligomerization and capping into membrane rafts (black dots) leading to apoptosis (cancer cells and activated T cells).

studies, the present model provides a fertile ground for further theoretical and experimental work.

SELECTIVE UPTAKE OF ET-18-OCH_3 AND INTRACELLULAR FAS/CD95 ACTIVATION AS A NOVEL APPROACH IN THE TREATMENT OF CANCER AND AUTOIMMUNE DISEASES

Our data suggest that the proapoptotic action of ET-18-OCH_3 appears to take advantage of some plasma membrane components uniquely expressed or modified in the cell surface of transformed cells as well as of the efficient Fas/CD95 cell death signalling route present in both cancer and normal cells. This latter Fas/CD95 activation is not selective for cancer cells, as ET-18-OCH_3-microinjected normal human fibroblasts undergo apoptosis [30], and therefore the selective action of the ether lipid depends on its uptake only in tumour cells.

ET-18-OCH_3 is the first small molecule that has been shown to induce tumour cell death through Fas/CD95 activation independently of FasL [30]. ET-18-OCH_3 promotes apoptosis in human leukaemic cells and other solid tumours [1,15,30], and has been shown to be effective in purging leukaemic bone marrow prior to autologous bone marrow transplantation [224]. In addition, ET-18-OCH_3 has been shown *in vivo* to be effective in inhibiting the development of a model of autoimmune disease, the Lewis rat model of experimental allergic encephalomyelitis (EAE), a widespread animal model for multiple sclerosis in humans. ET-18-OCH_3 delayed the onset, reduced the duration, and limited the severity of EAE [225-228]. This latter protective effect can be due to the reported induction of Fas/CD95-mediated apoptosis in activated T cells by ET-18-OCH_3, while resting T cells were spared [59]. Pilot studies with a limited number of patients have shown that ET-18-OCH_3 can be a promising drug in the treatment of multiple sclerosis and in purging leukaemic cells from bone marrow prior to autologous transplantation [14,224]. Recent evidence strongly suggests that the beneficial actions of ET-18-OCH_3 on both autoimmune disease and leukaemia are promoted by Fas/CD95-mediated apoptosis of activated T cells and leukaemic cells [30,31,59]. Furthermore, ET-18-OCH_3-induced apoptosis and triggering of Fas/CD95 is rather specific for the molecular structure of ET-18-OCH_3, as structurally related analogues are inactive [15,30,47]. Non-transformed cells and resting T cells are spared by ET-18-OCH_3 due to their inability to take up the drug in significant amounts [15,30,31,59]. This accounts for the low incidence of haematological or systemic side effects in the clinical studies performed with ET-18-OCH_3, even after prolonged therapy [1]. Nonetheless, normal cells can be rendered susceptible to ET-18-OCH_3-induced apoptosis by microinjecting the drug, provided they express Fas/CD95 [30]. This novel action is accomplished by the specific intracellular triggering of Fas/CD95, involving the cytoplasmic portion of Fas/CD95, and the ensuing direct activation of the apoptotic machinery through a mechanism that bypasses processes regulated by sensors and checkpoint genes that are frequently affected in tumour cells (e.g. p53). It also avoids the need to use non-specific immunosuppressive drugs in the treatment of autoimmune disorders and the deleterious and toxic actions on liver of extracellular Fas/CD95 activation [229]. Since ET-18-OCH_3 is metabolically stable, the long-lasting, drug-induced activation of Fas/CD95 could be related to the persistent activation of JNK [47,60] and sustained induction of c-*jun* [47,178] observed upon ET-18-OCH_3 treatment.

Thus, our findings have led us to postulate a novel mechanism of action for the proapoptotic drug ET-18-OCH_3 (Fig. **10**), which constitutes an attractive model for the selective induction of apoptosis in eukaryotic cells as well as a novel approach to treat autoimmune disease and cancer. The experimental evidences supporting the above mechanism can be summarised as follows [15,30-32,47,59,94,156]: a) ET-18-OCH_3 induces apoptosis from the interior of Fas/CD95-bearing cells, involving Fas/CD95 capping into membrane rafts; b) ET-18-OCH_3 accumulates at the cell surface in membrane rafts; c) Fas/CD95 activation requires trimerisation which involves the self-association of the cytoplasmic death domains; d) some steric and electronic requirements in the ET-18-OCH_3 molecule must exist for its apoptotic activity since structurally related analogues are unable to induce Fas/CD95 capping and apoptosis.

CONCLUSION

A great effort is being made by pharmaceutical companies in searching and designing small-molecule drugs acting as inhibitors of crucial processes for cell survival [230]. Recent developments indicate that small-molecule inhibitors of several signalling proteins, mostly protein kinases, show remarkable selectivity and potency [230-233]. ET-18-OCH_3 is the first small-molecule drug that acts as a direct activator of cellular signalling, in particular activating the apoptotic machinery of the cell through intracellular Fas/CD95 activation, and thereby it can constitute the leading compound of a new class of synthetic drugs targeting apoptosis. A liposomal formulation of the ether lipid ET-18-OCH_3 named TLC ELL-12 (Elan Biopharmaceuticals, USA) is currently in phase I clinical trials, and so far the drug has been well tolerated and no myelosuppression has been observed [234]. Previous phase I studies with ET-18-OCH_3, orally given, have also shown low toxicity and no myelosuppression [1]. Importantly, hematological or systemic side effects such as myelosuppression, nephro-, neuro- or hepatotoxicity have been rarely observed in the ongoing clinical studies with ET-18-OCH_3, even after prolonged therapy [1]. This apparent lack of toxicity distinguishes this antitumour agent from the anticancer agents currently available in the clinic.

The novel and unique mechanism of action of ET-18-OCH_3, involving its selective uptake by tumour cells and subsequent intracellular activation of Fas/CD95 cell death receptor through its translocation and capping into membrane rafts, also represents the first evidence for a raft-dependent killing of tumour cells, and indicate that rafts can represent a potential target for therapeutic intervention. Because Fas/CD95 is translocated into membrane rafts by ET-18-OCH_3 and this ether lipid incorporates into membranes and accumulates into lipid rafts, it is tempting to suggest a putative interaction between ET-18-OCH_3 and Fas/CD95 as the one described here, which could trigger the

apoptotic response in tumour cells. On these grounds, ET-18-OCH$_3$ should putatively concentrate in the inner leaflet of the tumour cell plasma membrane to interact with Fas/CD95. Elucidation of this selective uptake is a major challenge for future studies, and could lead to a new framework in the design of selective chemotherapeutic drugs and drug transporters in cancer therapy. A tentative model for the putative ether lipid uptake mechanism could consist of two steps: a) ET-18-OCH$_3$ binds first in a rather unspecific way to the outer leaflet of the plasma membrane from which can be washed off by repeated washings or by BSA extraction; b) the ether lipid is flipped across the plasma membrane, being translocated into the cytoplasmic leaflet of the lipid bilayer, through still unidentified inwardly directed transporters or flippases.

Molecular understanding of how ET-18-OCH$_3$ enters the tumour cell and triggers Fas/CD95 activation from inside the cell will constitute major subjects of research in the following years. Elucidation of these processes will open a new way to target tumour cells in cancer chemotherapy. Furthermore, the fact that ET-18-OCH$_3$ acts through its effects on apoptotic signalling indicates that it can be active against a wide array of tumour cells independently of their proliferative capacity. This is important as many tumours have a low proliferation rate, but instead show a deficient apoptotic response, and thereby a high apoptotic threshold [1]. In addition, the mechanism of action of ET-18-OCH$_3$ could also be of use for the treatment of additional maladies, such as autoimmune diseases. On the other hand, the selectivity of ET-18-OCH$_3$ for cancer cells as well as its accumulation in tumours turns this drug into an extremely attractive compound to identify tumour localisation and metastasis. Altogether the above data indicate that ET-18-OCH$_3$, beyond its putative clinical importance, is an interesting model compound for the development of more selective drugs for cancer therapy, and further investigation to fully unravel its mechanism of action will lead to novel strategies for cancer treatment.

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ABBREVIATIONS

AEL	=	Antitumour ether lipid
AEP	=	Alkyl ether phospholipid
ALP	=	Alkyl-lysophospholipid analogue
APC	=	Alkylphosphocholine
ATL	=	Antitumour lipid
BAE	=	Bovine aortic endothelial cells
BSA	=	Bovine serum albumin
[Ca^{2+}]	=	Cytosolic free Ca^{2+} concentration
CCT	=	CTP:phosphocholine cytidylyltransferase
DED	=	Death effector domain
DD	=	Death domain
DISC	=	Death-inducing signalling complex
DMSO	=	Dimethyl sulfoxide
EAE	=	Experimental allergic encephalomyelitis
EGFR	=	Epidermal growth factor receptor
ERK	=	Extracellular signal-regulated kinase
ET-18-OCH$_3$	=	1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (edelfosine)
FADD	=	Fas-associated death domain protein
FasDD	=	Fas death domain
FasL	=	Fas ligand
HDL	=	High density lipoprotein
HPC	=	Hexadecylphosphocholine (miltefosine)
HUVEC	=	Human umbilical vein endothelial cells
JNK	=	c-Jun NH_2-terminal kinase
kDa	=	Kilodalton
LPC	=	2-Lysophosphatidylcholine
MAPK	=	Mitogen-activated protein kinase
NSCLC	=	Non-small cell lung cancer
PAF	=	Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine)
PC	=	Phosphatidylcholine
PKB	=	Protein kinase B
PKC	=	Protein kinase C
SCLC	=	Small cell lung cancer
sTNF-R1	=	Extracellular domain of tumour necrosis factor receptor 1
TNF	=	Tumour necrosis factor
TNF-R	=	Tumour necrosis factor receptor

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