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Eicosatetraynoic and arachidonic acid-induced changes in cell membrane fluidity consonant with differences in computer-aided design-structures

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ETYA (5,8,11,14-eicosatetraynoic acid), a competitive analogue of arachidonic acid (AA), inhibits the proliferation of U937 (human monoblastoid) and PC3 (human prostate) cancer cells, without the overt cytotoxicity associated with AA at similar concentrations. The mechanism of inhibition is not established. ETYA at 100 μM acutely increased whole cell and isolated microsomal membrane fluidity of both cell lines to a greater extent than arachidonic acid. PC3 cells incubated with ETYA for 72 h evidenced increased membrane fluidity. This was measured by the fluorescence polarization parameter, R, using the probes TMA-DPH and DPH for whole cell and isolated membrane fractions, respectively. Compared with whole cells, isolated membranes yielded a 10–20-fold increase in fluorescence intensity. The intramolecular conformational profiles of both ETYA and AA were explored using a combination of molecular mechanics energy minimization and molecular dynamics simulation. While it is possible that not all of the low energy conformational states of either molecule were sampled, the large number of low-energy conformers determined for ETYA correspond to kink deformed conformers relative to the family of AA conformers. These kinks make the molecular cross sections of ETYA larger than AA and arise from the four alkyne bond geometries. This structural finding is consistent with ETYA's greater effect on membrane fluidity. Dissociation between the extent of change in membrane fluidity due to ETYA or AA and inhibition of DNA synthesis can suggest that either (A) increased fluidity and inhibition of DNA synthesis are independent, or as we believe more likely, (B) greater membrane fluidity evoked by ETYA is important for inhibiting DNA synthesis, while changes induced by AA are insufficient or differ qualitatively from those required to initiate and sustain these nonlethal events.

Introduction

ETYA (5,8,11,14-eicosatetraynoic acid), a competitive inhibitor of arachidonic acid (AA), induces remarkably pleotropic effects in three different types of transformed human cancer cell lines [1–6]. They include reversible inhibition of DNA synthesis in all three cell lines, that did not depend upon cytotoxicity, as judged by several criteria, and the induction of a limited differentiation in the U937 monoblstoid and A172 glioblastoma lines but not in prostate PC3 cells [3,5]. In order to examine the events responsible for, or at least associated with these changes, we measured the effect of ETYA on membrane fluidity [7]. This was assessed by fluorescence polarization of TMA-DPH with whole cells and of DPH with isolated microsomal membranes from U937 and PC3 cells. While higher concentrations of arachidonic acid (AA) inhibit cellular replication, this is accompanied by an increased uptake of Trypan blue [8] and evidence of damage from free radicals [9]. Concentrations of AA from 30–40 μM that were not overtly cytotoxic did not inhibit PC3 DNA synthesis to the same extent as equimolar ETYA [10].

ETYA is a structural isomorph of arachidonic acid in which the four alkene bonds of the parent compound are replaced by four alkyne bonds [11]. The differences in fluidity due to ETYA and AA suggested a comparison of their minimum energy structures, employing programs for molecular modeling. The imputed differences in structure were consonent with the greater change in membrane fluidity induced by ETYA, as

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described subsequently. These results are consistent with a role for ETYA-induced perturbation of membrane fluidity as an early event in downregulating DNA synthesis of the two cell lines.

Methods

Cell culture. PC3 cells, originally derived from a patient with prostate cancer [12], were cultured in 75 cm 2 plastic flasks with 5% CO_2 at 37°C. The culture medium included RPMI 1640 with 5 or 10% fetal bovine serum, 2 mM L-glutamine, 50 u/ml penicillin and 50 μg/ml streptomycin (all from Gibco Lab., Grand Island, NY). U937 cells, originally from a patient with a lymphoma [13], were cultured under similar conditions, except 5% CPSR3 replacement serum from Sigma Biochemical, St. Louis, MO was substituted for FBS. Microsomes were isolated after disruption of cells by vigorous homogenization in a Potter-Elvejhem homogenizer, and centrifugation at 105 K at 4°C for 1 h of the supernatant remaining after preliminary centrifugation at $1000 \times g$ and $9000 \times g$ for 10 and 20 min, respectively.

Electron microscopy. Purified membrane fractions were fixed in glutaraldehyde, post-fixed with osmium tetroxide, stained with uranyl acetate, dehydrated with ethanol and proplylene oxide and embedded in epon/araldite [3]. Grids were counterstained with Reynold's lead and uranyl acetate and examined in a 100 CX electron microscope.

Steady-state fluorescence spectroscopy. Approx. $1 \cdot 10^6$ cells suspended in $1 \cdot 10^{-6}$ M TMA-DPH (trimethylammoniumdipenylhexatiene in KRP buffer, from Molecular Probes, Eugene, OR) were incubated with the dye at 37°C for 1 min, fluorescence anisotropy read at 425 nm on a Perkin-Elmer 650-40 spectrofluorometer (Perkin-Elwood, Norwalk, CT) with sample illumination at 340 nm. Samples were treated with 100 μM ETYA, AA or NDGA (nordihydroguaiaretic acid) or an equivalent quantity of the DMSO vehicle, and changes in polarization measured after 1 min at 37°C. Corrections for parasitic light scattering were made. Microsomes (400 μg protein/ml) were incubated with 1 μl of a stock solution of DPH (diphenylhexatriene) in tetrahydrofuran (0.5 mM DHP from Molecular Probes) for 2 h at 37°C and fluorescence anisotropy measured with an excitation frequency of 365 nm and emission at 430 nm before and after the aforementioned additions. The fluorescence anisotropy was calculated as

$$r_s = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2 \cdot I_{\perp})$$

This parameter reflects the steady-state anisotropy, range or probe motion component of fluidity, to which it is inversely related.

Molecular modelling. The ETYA and AA molecules were built and their structures optimised using the MMFF option of the CHEMLAB-II molecular modelling package [14]. An extended Allinger-MMII molecular mechanics force field employing monopole–monopole electrostatic interactions were used in the structure optimisation [15]. Monopole charges were determined using the CNDO/2 method [16]. The optimised structures were then subjected to molecular dynamics simulations at 10, 100, 200 and 300 K for 20 ps, using the same force field as employed in MMFF. The MOLSIM program [17] was used to perform the molecular dynamics. The resulting conformational trajectories of ETYA and AA were compared at common temperature and conformational differences established. Low-energy families of common families of conformations were displayed using the Molecular Viewer Program [18].

Results

Membrane ultrastructure

Electron microscopy of U937 and PC3 microsomes, prepared by a standard technique revealed predominant membrane components in the U937 fraction, and a somewhat lesser amount of membrane material with some admixture of ribosomes and amorphous cellular debris in the PC3 fractions (Fig. 1).

Fluidity measurements

The changes in r, the steady-state fluorescence anisotropy, of U937 whole cells and microscomal membranes acutely exposed to 100 μM ETYA or AA are presented in Table I. ETYA produced a 9.5% decline in r, from 0.287 to 0.260 with a $P < 0.01$ for $n = 13$. Arachidonic acid reduced r_s by 2.8%, $P < 0.05$. The effect of ETYA on U937 microsomal membrane fluidity was more marked, with a change from 0.170 to

TABLE I

The effect of ETYA or arachidonic acid on U937 whole cell and microsomal fluorescence anisotropy

100 μM final concentrations of either agent in 0.1% DMSO in Krebs Ringer phosphate buffer was used. TMA-DPH or DPH were employed with whole cells or micorsomal membrane fractions, as discussed in Methods. * $P < 0.01$; ** $0.01 < P < 0.05$.

	r_s
U937 whole cells	
Control ($n = 13$)	0.287 ± 0.02
ETYA ($n = 13$)	0.260 ± 0.009 *
AA ($n = 13$)	0.279 ± 0.02 **
U937 microsomes	
Control ($n = 13$)	0.170 ± 0.005
ETYA ($n = 13$)	0.149 ± 0.005 *
AA ($n = 13$)	0.161 ± 0.004 **

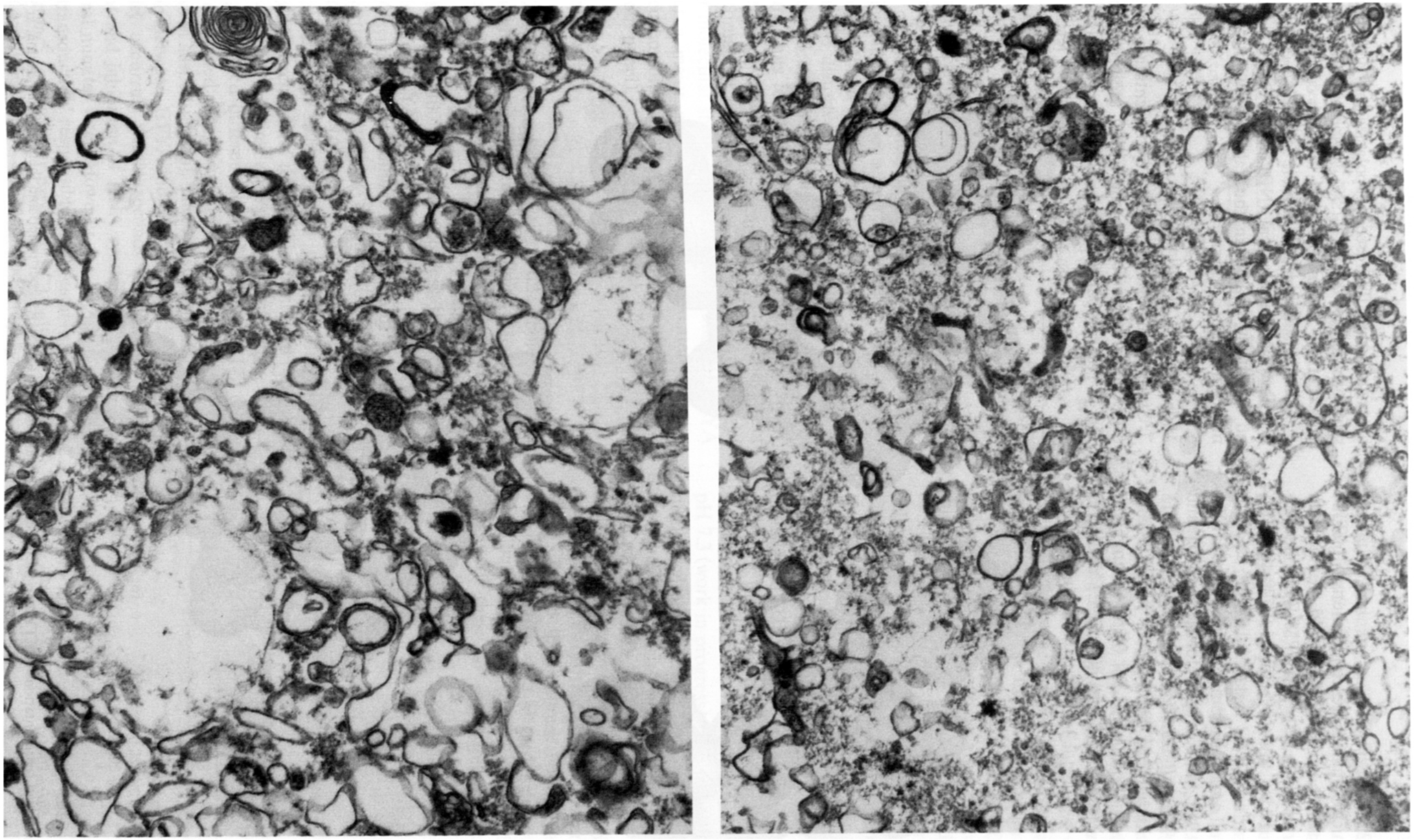

Fig. 1. Electron photomicrographs of representative membrane fractions from U937 (left-hand panel) and PC3 (right-hand panel) cells, prepared as described in Methods. Both ×57400.

0.149 for a 12.4% decline in r_s, at $n = 13$ and a $P < 0.01$. The microsomal fluorescence intensity of TPA was characteristically from 10- to 20-fold greater than that obtained with whole cells and TPM-DPH. Arachidonic acid reduced r_s by 6.3%, from 0.170 to 0.161, with $P < 0.05$.

PC3 whole cell and microsomal r_s values for ETYA declined by 10.9%, with $n = 16$, for a $P < 0.01$ and 28.7% at $n = 19$ and $P < 0.01$, respectively (Table II). Arachidonic acid reduced these values by 2.7% and 6.9%, respectively, both with $P < 0.05$. When U937 and PC3 cells were cultured with 40 μM ETYA for 72 h, washed and fluorescence polarization determined, decreases of 6.6% and 12.5%, respectively, were obtained at $n = 16$, and $P < 0.01$ for both cell lines.

Dimethylsulfoxide, the vehicle for ETYA or 100 μM nordihydroguaiaretic acid (NDGA) in DMSO caused no significant change in the values of whole cell or microsomal r_s (not shown).

Molecular modelling

Stereo structural models of representative low-energy conformations of AA and ETYA are presented in Fig. 2. The comparisons are between the two molecules

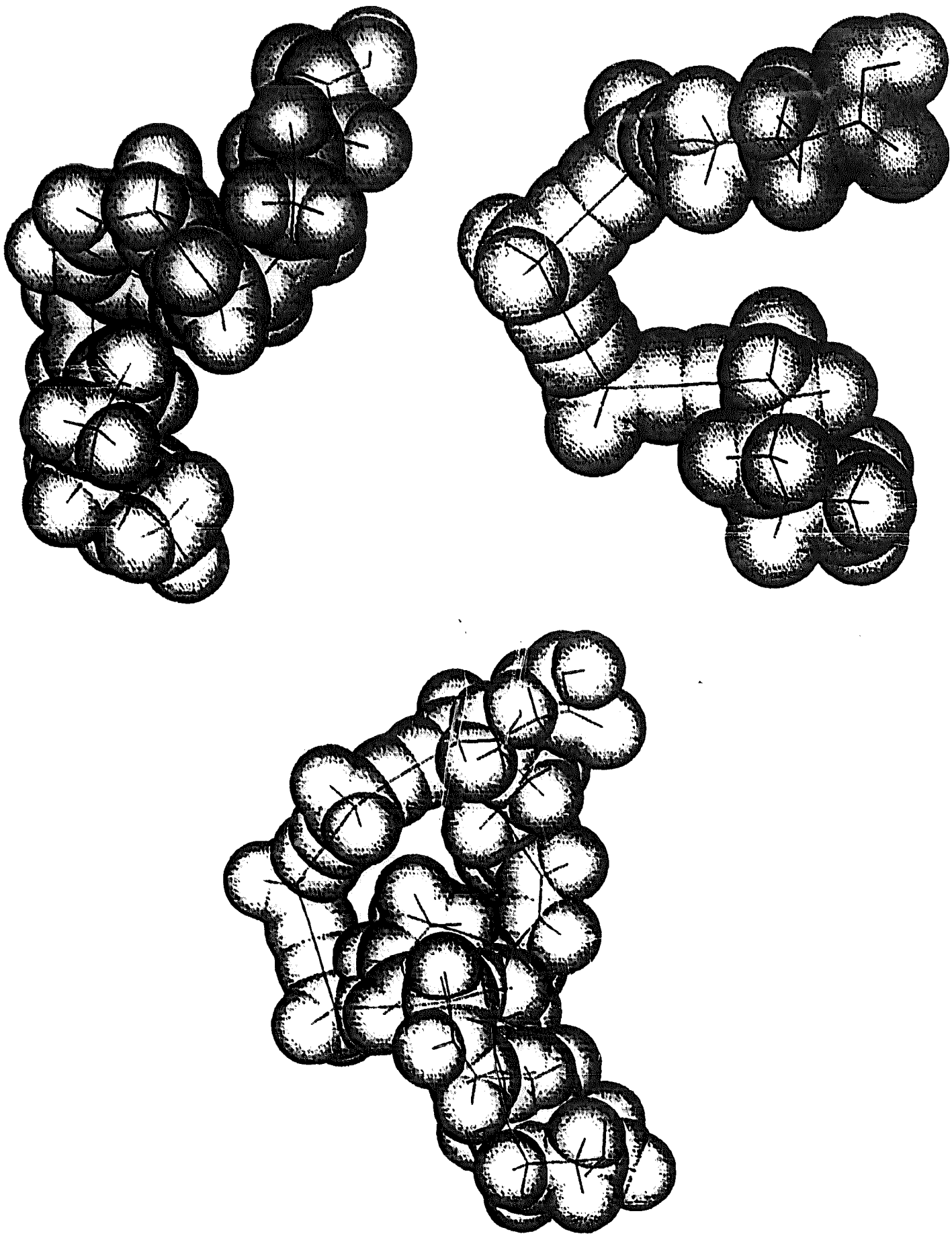

Fig. 2. Chemlab II-derived representations of ETYA (right upper panel), arachidonic acid (left upper panel) and their superimposition. at 300 K. Carboxyl groups at the top of each figure.

TABLE II

Membrane fluidity, as assessed by r_s values of PC3 whole cells and microsomal membranes

Concentrations as in Table I, with * $P < 0.01$; ** $0.01 < P < 0.05$.

	r_s
PC3 whole cells	
Control ($n = 16$)	0.258 ± 0.01
ETYA ($n = 16$)	0.230 ± 0.02 *
AA ($n = 16$)	0.251 ± 0.02 **
PC3 microsomes	
Control ($n = 19$)	0.202 ± 0.003
ETYA ($n = 19$)	0.144 ± 0.003 *
AA ($n = 19$)	0.188 ± 0.003 **

in which carbonyl groups and two additional carbon atoms, one in the middle and the other on the CH3 end of the molecules, are aligned. Although arachidonic acid undergoes a partial helical twist, it remains largely within a single major plane. On the other hand, ETYA is distorted out of its major plane. The calculated molecular surface areas are 460.448 and 419.844 square units for AA and ETYA, respectively; the 9% difference would imply that the molecular volume of the former exceeds the latter. However, inspection of their molecular shapes suggests that rotation of ETYA about a major axis encompasses a greater effective molecular volume than a similar rotation of AA (Fig. 2, bottom panel).

Discussion

ETYA increased U937 and PC3 whole cell and isolated microsomal membrane fluidity to a greater extent than arachidonic acid. Although AA is a fluidizing 'class A' fatty acid [19], the extent of change produced by 100 μM AA was less than that from equimolar ETYA. In addition, while AA can inhibit DNA synthesis and cellular replication of transformed cells, the concentrations required are cytotoxic [9]. ETYA inhibits DNA synthesis and cellular replication in transformed cells without overt cytotoxicity, as judged by exclusion of trypan blue, unaltered release of ^{51}Cr-labeled proteins, reversibility of inhibition, induction of limited differentiation (U937 and A172 cells), and continued attachment of PC3 and A172 cells (U937 grows in suspension) [6]. These results provide a correlation between ETYA-induced changes in membrane fludity occurring within seconds of exposure to the agent and inhibition of DNA synthesis, first detected several minutes later. However the nature of any 'signal transduction' responsible is not established.

From 5 to 20 s after exposing PC3 cells to 100 μM ETYA, intracellular Ca^{2+} increased from 2- to 5-fold, gradually declining toward the initial value during the succeeding 5 or more minutes [10,20]. A change in the activity of a membrane-bound enzyme such as phospholipase C, possibly due to increased membrane fluidity, with release of inositol 1,4,5-trisphosphate, or a direct physical effect of ETYA on intracellular storage sites for ionized calcium or on a membrane ion channel are possible explanations for the recruitment of Ca^{2+} from intra or extracellular sites.

The probe TMA-DPH is considered specific for plasma membrane fluidity measurements [21], at least prior to any diffusion into the cells. The relative lack of altered fluidity in cells first incubated with ETYA for 72 h may be due to homeoviscous adaptation The much more intense fluorescence from isolated membranes and DPH, compared to TMA-DPH and whole cells may be due in part to a greater concentration of microsomal membrane with which the dye can interact.

ETYA also rapidly inhibits about one third of PC3 cellular respiration (manuscript submitted) and about 45% of U937 respiration (work in progress). Inhibition by ETYA of oxygen uptake by gunea pig alveolar macrophages has been reported [22], while polyunsaturated fatty acids inhibit mitochondrial oxidative phosphorylation [23]. In addition, we have observed acidification of the medium from ETYA-treated PC3 cells, e.g. pH 6.95 at a time when control cell medium was at pH 7.3 (work in progress). This may suggest an ETYA-induced alteration in ion transport, and release of H^+ into the medium in exchange for a cation, probably Na^+ with alkalinization of the cytosol [24]. Since the cells remain attached and exclude Trypan blue after trypsinization, cytotoxicity does not seem to be responsible. Changes in membrane-bound Na^+/K^+ ATPase activity occurring with altered membrane fluidity are described [25]; possibly ETYA-induced change in the activity of these or other ion channel or transport

TABLE III

r_s values of PC3 and U937 whole cells incubated for 72 h with 40 μM ETYA

The cells were washed and analyzed as described in the Methods, and Tables I and II. * $P < 0.01$; ** $0.01 < P < 0.05$.

	r_s
PC3 cells	
Control ($n = 16$)	0.301 ± 0.02
ETYA ($n = 16$)	0.281 ± 0.01 *
AA ($n = 16$)	0.295 ± 0.01 **
U937 cells	
Control ($n = 16$)	0.344 ± 0.03
ETYA ($n = 16$)	0.301 ± 0.02 *
AA ($n = 16$)	0.337 ± 0.03 **

proteins for low molecular weight substances can be demonstrated in this experimental system.

Molecular modelling studies reveal significant differences between the low energy molecular structures of ETYA and AA. While AA can be represented as adopting a somewhat 'C'-shaped 'pseudocycloid' configuration, ETYA is severely twisted up and out of its 'C' plane, presumably due to the configurational strain from the four alkyne bonds on the intervening carbon-carbon single bonds. The calculated molecular surface area of AA exceeds that of ETYA by 9%. However, it appears that the effective volume occupied by rotating ETYA 360 degrees would exceed that of AA. The physical insertion of ETYA into phospholipid bilayers should reduce opportunities for the alignment of fatty acyl molecules due to van der Waals forces. It has been reported that ETYA can covalently replace AA in phospholipids of at least one cell line [26]. This physical picture is certainly oversimplified, since unidentified effects of solvent and of adjacent molecules on the structure of ETYA or AA cannot be taken into account. However, the amount of intramolecular energy needed to 'compress' ETYA into a compact shape more native to AA is so large as to eliminate such an event from intermolecular packing considerations. Thus, the free space kinked conformation of ETYA is considered representative of the class of conformations this molecule adopts in any environment.

In summary, the correlation between increased membrane fluidity and inhibited DNA synthesis in cells incubated with ETYA is consistent with their functional relation. Imputed differences in the structures of ETYA and AA, obtained with a computer aided design program, are believed to be related to their differing effects on membrane fluidity, inhibition of DNA synthesis and cytotoxicity. The relationship between potential ETYA-induced molecular 'signals' for rapidly downregulating DNA synthesis, which may include an acute rise in intracellular Ca^{2+}, rapidly inhibited oxygen consumption or effects on Na^{+}/H^{+} exchange with an increase in intracellular pH, as any of them may be altered by increased membrane fluidity, remain to be defined.

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