

**PRO- AND ANTI-APOPTOTIC FUNCTIONS OF ETOPOSIDE AND THE
MECHANISM OF CARDIOLIPIN OXIDATION BY CYTOCHROME C.**

by

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Cytochrome *c* (cyt *c*) binds with high affinity with anionic phospholipids such as phosphatidylserine (PS) and cardiolipin (CL) to form a complex with peroxidase activity capable of oxidizing polyunsaturated phospholipids, including CL. Release of cyt *c* from mitochondria plays a pivotal role in cytosolic triggering of apoptotic caspase cascades whereby CL oxidation is involved in mitochondrial membrane permeabilization. Thus control of CL oxidation is critical to regulation of early stages of apoptosis. Given the lipid antioxidant potency of etoposide as well as its ability to induce apoptosis, we hypothesized that cyt *c* catalyzed CL oxidation during apoptosis can be sensitive to etoposide, hence affect execution of the apoptotic program. We analyzed how the apparent inability of etoposide to prevent apoptosis is related to the mechanism of mitochondrial cardiolipin oxidation by hydrogen peroxide catalyzed by cyt *c* molecules which has public health relevance since etoposide is a commonly used anti-tumor drug. In a model biochemical system, we showed that 160 pmol lipid hydroperoxides / nmol CL were generated when 100 μ M liposomes containing a mixture tetralinoleoyl-CL (TLCL) with dioleoyl phosphatidylcholine (DOPC) [1:1] were incubated with 4 μ M cyt *c* and 100 μ M H₂O₂ or 250 μ M AMVN. Etoposide inhibited CL hydroperoxide production in a concentration dependent manner but with different sensitivity to the different oxidizing systems. Etoposide was more effective in AMVN system (I_{50} =3 μ M) compared to cyt *c*/H₂O₂ system (I_{50} =15 μ M), suggesting CL is not oxidized randomly but via a definite pathway. Next we tried to outline the pathway of cardiolipin

oxidation using EPR techniques and PAGE studies. Characterizing the protein derived (tyrosyl) radical and etoposide-phenoxy radical we noted that cardiolipin oxidation occurs via the heme of the cyt *c* peroxidase and also partly by the protein derived (tyrosyl) radical generated as result of cyt *c* peroxidase reaction. Etoposide, in the model system inhibits cardiolipin oxidation by preventing formation of protein derived (tyrosyl) radical. However, in HL-60 cells, etoposide enhanced CL oxidation while suppressing AMVN-induced oxidation of other phospholipids. Thus etoposide-dependent inhibition of CL oxidation is not likely to interfere with the execution of apoptotic program via prevention of mitochondrial membrane permeabilization.

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PREFACE

I would like to express my sincere gratitude and respect to Dr. Valerian E. Kagan, my research advisor and chairperson of the Thesis Committee, for the opportunity to conduct this research project under his direction, for the valuable advice and support, and for allowing me to work independently for the three years I spent in his laboratory. Thanks to Dr. Jack Yalowich, Dr. James Fabisiak, Dr. Michael Epperly and Dr. Rhobert Evans for serving on my committee.

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1. INTRODUCTION

Mitochondria play a central role in the execution of apoptotic programs realized through intrinsic mechanisms and extrinsic pathways in type II cells [1-4]. It is well documented that one of the early mitochondrial responses to pro-apoptotic stimuli is the generation of reactive oxygen species (ROS) [5]. While the overall significance of ROS production in apoptosis has been established by its inhibition by different antioxidant enzymes and free radical scavengers [6-10], specific ROS-dependent mechanisms of apoptosis are still elusive. We have recently demonstrated that cytochrome *c* acts as a redox catalyst in oxidizing a mitochondria-specific phospholipid, cardiolipin (CL), thus facilitating accumulation of CL hydroperoxides (CL-OOH) required for the release of pro-apoptotic factors from mitochondria into the cytosol [11].

Etoposide (VP-16), a popular anti-tumor drug and a topoisomerase II inhibitor induces apoptosis accompanied by mitochondrial ROS production [12-16]. At the same time, others and we have reported that etoposide is an effective scavenger of lipid radicals participating in propagation of lipid peroxidation [17-19] and as such should prevent apoptosis. In this contribution we analyzed dual pro- and anti- apoptotic role of etoposide to resolve this apparent contradiction and relate to the mechanism of CL oxidation by *cyt c*.

The CL oxidation can proceed enzymatically whereby *cyt c*/CL complex acts as a CL-specific oxygenase inserting hydroxy and hydroperoxide groups in one or more of the four polyunsaturated fatty acid residues of CL, similar to cyclooxygenase-catalyzed oxygenation of arachidonic acid [20]. Alternatively, *cyt c*/CL complex can be involved in catalytic initiation of CL oxidation followed by a non-enzymatic propagation of free radical CL oxidation, similar to

well known mechanisms of peroxidation of polyunsaturated lipids in biomembranes [21]. Given an important role of CL oxidation in apoptosis [22-26], characterization of these different pathways is important for the regulation of apoptosis and development of new-targeted therapeutic interventions. We hypothesize that the details of the mechanism of CL oxidation in cyt *c*/CL complex is an important factor in the inability of antioxidant properties of etoposide to prevent the apoptosis. Therefore, in the current work, we attempted to discriminate between the two mechanisms of CL oxidation monitoring the development of apoptosis and generation of lipid hydroperoxide and free radicals at different etoposide concentration and reaction conditions *in vivo* and *in vitro* experiment. Etoposide phenoxyl radicals have very low reactivity towards lipids but relatively high reactivity towards ascorbate, GSH, and protein cysteines; therefore it is an excellent and specific lipid antioxidant that does not protect other important biomolecules against oxidation [27,28]. Thus, probing lipid oxidation pathways of apoptosis with etoposide is specific and is not likely to interfere with the other redox-sensitive mechanisms [17]. Using this approach, we now demonstrate that CL oxidation during apoptosis is not likely realized via a random lipid peroxidation mechanism but rather proceeds as a result of peroxidase reaction in a tight cyt *c*/CL complex that inhibit interactions of antioxidant etoposide with lipid radicals generated in the course of the reaction. We further show that at high concentrations, etoposide can inhibit CL oxidation by competing with it as a peroxidase substrate. These inhibitory effects of etoposide, however, are realized at far higher concentrations than those at which it induces apoptotic cell death. Thus, essential for apoptosis oxidation of CL by cyt *c*/ CL peroxidase complex is not inhibited by pro-apoptotic concentrations of the drug.

2. MATERIALS AND METHODS

2.1. REAGENTS

Etoposide (VP16, demethylepipodophyllotoxin-ethylenediene-glucofuranoside), horse heart cytochrome *c* (cyt *c*), diethylenetriaminopentaacetic acid (DTPA), EGTA, SDS, BHT, catalase, and porcine pancreas-derived phospholipase A₂ (PLA₂) were purchased from Sigma Aldrich (St. Louis, MO). Amplex Red (N-acetyl-3, 7-dihydroxyphenoxazine) reagent was obtained from Molecular Probes (Eugene, OR). HPLC grade solvents, fetal bovine serum, RPMI 1640 with phenol red, RPMI 1640 without phenol red, and phosphate buffered saline (PBS) from Invitrogen (Carlsbad, CA). Tetraoleoyl cardiolipin (TOCL), tetralinoleoyl cardiolipin (TLCL) and dioleoyl phosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids, Inc. (Albaster, AL). The azo-initiator, 2,2'-azo-bis(2,4-dimethyl-valeronitrile)(AMVN) was from Wako Chemicals U.S.A (Richmond, VA).

2.2. EXPERIMENTAL PROCEDURE

2.2.1. Cell culture

HL-60 human promyelocytic leukemia cells (American Type Culture Collection) were grown in RPMI 1640 with phenol red supplemented with 12.5% heat inactivated fetal bovine serum at 37°C in a humidified atmosphere (5% CO₂ plus 95% air). Cells from passages 45-50 were used for the experiments. The density of the cells at collection time was 0.5 X 10⁶ cell/ml. HL-60 cells

were incubated with either AMVN (500 μ M) or etoposide (25 μ M) or both together in fetal bovine serum-free RPMI 1640 medium without phenol red for 2h at 37°C.

2.2.2. Preparation of TOCL- and TLCL-containing liposomes

Small unilamellar liposomes containing 50% PC and 50 % TOCL or TLCL were produced as described by Fadok et al [29]. Individual phospholipids, stored in chloroform were dried under nitrogen. PBS containing 100 μ M DTPA was added to obtain the phospholipid concentration of 200 μ M, and the lipid mixture was vortexed and sonicated for 3 mins on ice. All liposomes were used immediately after preparation.

2.2.3. Oxidation of phospholipids

Liposomes were incubated with etoposide (1, 3, 5, 10, 25, 50 and 100 μ M) for 15min on ice. Samples were treated with 250 μ M AMVN or with cytochrome *c* (4 μ M) and H₂O₂ (100 μ M) for 1h at 37°C in a water bath. H₂O₂ was added four times (every 15mins) during the incubation period. Catalase (2units) was added to neutralize H₂O₂. Lipids were then extracted in Chloroform: Methanol (2:1) solvent. The extracted lipids were used to determine phosphorus content and measure the amount of lipid hydroperoxide generated as documented below.

2.2.4. Assay for phospholipid peroxidation

2.2.4.1. HPTLC

The extracted lipids (either from cells or the liposomes) were dried under nitrogen and separated by two-dimensional thin layer chromatography (HPTLC) on silica G plates. The plates were first developed with a solvent system consisting of chloroform: methanol: 28% ammonium hydroxide

(65:25:5, by vol.) After drying the plates with a forced air blower to remove the solvent, the plates were developed in the second dimension with a solvent system consisting of chloroform: acetone: methanol: glacial acetic acid: water (50:20:10:10:5, by vol.) The phospholipids were visualized by exposure to iodine vapor and identified by comparison with migration of authentic phospholipid standards. The spots identified by iodine staining were scraped, and the silicic acid was transferred to tubes. Lipid phosphorus was determined by the sub-micro method as described by Bottcher *et al.* [30]. The identity of each phospholipid was established by comparison with the *R_f* values measured for authentic standards.

2.2.4.2. Amplex Red assay

Lipid extracts were dried under nitrogen and then incubated with 100µl buffer containing 25mM NaH₂PO₄, 0.5mM EGTA and 1mM Calcium (pH 8), 10µl of 5mM SDS and 1µl PLA₂ for 30mins at room temperature. Two µL 1mM HCl and 1mM EGTA were added to adjust the pH of the samples. 1.5µl of Amplex red (50µM) and microperoxidase (MP-11, 1µg/µl) solution was added and incubated on ice for 40mins. Reaction was stopped using 100µl of stop reagent (solution of 10mM HCl, 4mM BHT in ethanol). The samples were centrifuged at 15,000x g for 5min and the supernatant was used for HPLC analysis. Aliquots (5µl) were injected into a C-18 reverse phase column (Eclipse XDB-C18, 5µm, 150 x 4.6 mm). The column was eluted by mobile phase composed of 25mM NaH₂PO₄ (pH 7.0)/ methanol (60:40 v/v) with 1ml/min flow rate. Lipid phosphorus was determined by a micro-method. The resorufin (an Amplex Red oxidation product) fluorescence was measured at 590 nm after excitation at 560 nm using a Shimadzu LC-100AT *vp* HPLC system equipped with fluorescence detector (model RF-10Ax1) and auto sampler (model SIL-10AD *vp*).

2.2.5. EPR measurement of Etoposide phenoxyl radical

Small unilamellar liposomes (200 μ M) containing 100 μ M DOPC and 100 μ M TOCL or TLCL were incubated with etoposide (5,10, 25, 50 and 100 μ M) for 15 min on ice followed by 1min incubation at room temperature with 4 μ M *cyt c*. Time course of etoposide phenoxyl radical EPR signal was recorded 1min after H₂O₂ (100 μ M) addition, using a JEOL-REIX spectrometer at 25°C in gas-permeable Teflon tubing (0.8mm i.d., 0.013mm thickness) obtained from Alpha Wire Corp. (Elizabeth, NJ). The tube was filled with 60 μ l of mixed sample, folded doubly and placed in an open 3.0mm internal diameter EPR quartz tube. Etoposide phenoxyl radical spectra were recorded under N₂ at 335 mT, center field; 10mW, microwave power; 0.05 mT, field modulation; 5 mT, sweep width; 10³, receiver gain; 0.03 sec, time constant; 4 min, scan time. The time course of etoposide radical EPR signals was obtained by repeated scanning of the field (5 mT, sweep width; 335mM, centered field; other instrumental conditions were the same).

2.2.6. EPR measurement of Tyrosyl radical

Liposomes (10mM) (DOPC: CL ratio 1:1) were incubated with 200 μ M etoposide for 15min on ice, followed by addition of 40 μ M *cyt c* and 1mM H₂O₂. Reactions were stopped after 20s by freezing the samples in liquid nitrogen. EPR spectra of the frozen samples were detected at 77K under the conditions: 325 mT – centered field; 10 mT – sweep width; 0.05 mT, field modulation; 0.2mW, microwave power; 0.1 s, time constant; 4 min, time scan.

2.2.7. Fluorescence measurement of dityrosine formation

After incubating 10 μ M cyt *c* with 250 μ M TOCL or TLCL liposomes in the presence of 100 μ M H₂O₂ in presence or absence of etoposide for 1h at 37°C, fluorescence was measured at $\lambda_{\text{excitation}}$ 284 nm and $\lambda_{\text{emission}}$ 420 nm.

2.2.8. PAGE electrophoresis of cyt *c*/CL complexes

Liposomes (200 μ M, at DOPC: CL ratio 1:1) were incubated with cyt *c* (4 μ M) in the presence of 100 μ M H₂O₂. Electrophoresis was performed in 12.5% PAGE. Gels were stained by silver using GelCode SilverSNAP Stain Kit II (Pierce).

2.2.9. Statistics

The results are presented as mean \pm S.E. values from at least three experiments, and statistical analysis were performed by either paired or unpaired Student's *t* test of one-way ANOVA. The statistical significance of difference was set at $p < 0.05$.

3. RESULTS

3.1. Effect of etoposide on phospholipid oxidation in HL-60 cells

Etoposide, a commonly used antitumor agent, initiates apoptotic signaling via inhibition of DNA topoisomerase II and consequent DNA cleavage [12-14]. It is well documented that induction of apoptosis by etoposide takes place via the intrinsic pathway, where mitochondria play a pivotal role [16,17]. CL oxidation is suggested to be an early apoptotic event, required for the release of cyt *c* from the mitochondria and activation of the apoptotic cascade [26]. Our previous work has shown that cyt *c* interaction with CL and the formation of a peroxidase complex capable of oxidizing CL is essential for the execution of apoptosis [11]. Clearly, inhibition of CL oxidation by etoposide would interfere with its pro-apoptotic properties. To this end we incubated HL-60 cells with lipid peroxidation initiator, an azo-generator of peroxy radicals, AMVN (2,2'-Azo-bis(2,4-dimethylvaleronitrile)) (500 μ M) or 25 μ M etoposide or a combination of both for 2h at 37°C, extracted phospholipids and separated them by 2-dimensional HPTLC. A typical HPTLC separation is illustrated in Fig.1A. Phosphatidylcholine (PC) represented about one half of the total phospholipids with phosphatidylethanolamine (PE) being the next most abundant phospholipid class (about 20%). Additionally, the phospholipids in the order of their abundance - phosphatidylinositol (PI) > sphingomyelin (SP) > phosphatidylserine (PS) > CL - were detectable on the HPTLC plate. There were no significant differences in the pattern of distribution of phospholipid classes between treated and untreated HL-60 cells. Spots corresponding to different phospholipids were scraped and the amounts of phospholipid hydroperoxides generated in etoposide treated and untreated HL-60 cells were determined using

our newly developed fluorescence HPLC/Amplex Red-based assay [31]. We found that AMVN induced apoptosis [32] (data not shown) and oxidation of various phospholipids, including PC, PE, PS and CL (Fig. 1B). In presence of etoposide, AMVN-induced oxidation of PC, PE and PS was suppressed. However, oxidation of CL was enhanced under similar conditions (estimated as 85.39 ± 3.73 (vs. 59.2 ± 3.17 in etoposide alone) pmol CL hydroperoxide/nmol CL). Etoposide alone caused apoptosis (data not shown) and selective oxidation of CL (estimated as 59.2 ± 3.17 (vs. 7.63 ± 1.06 in control) pmol CL hydroperoxide/nmol CL). In contrast more abundant phospholipids such as PC, PE, PI and PS did not undergo oxidation in etoposide-treated cells (in line with our previously published results [19]). Thus, the ability of relatively high concentrations of etoposide to inhibit oxidation of CL catalyzed by cyt *c* /CL complexes in apoptotic cells does interfere with the pro-apoptotic effects of low concentrations of etoposide.

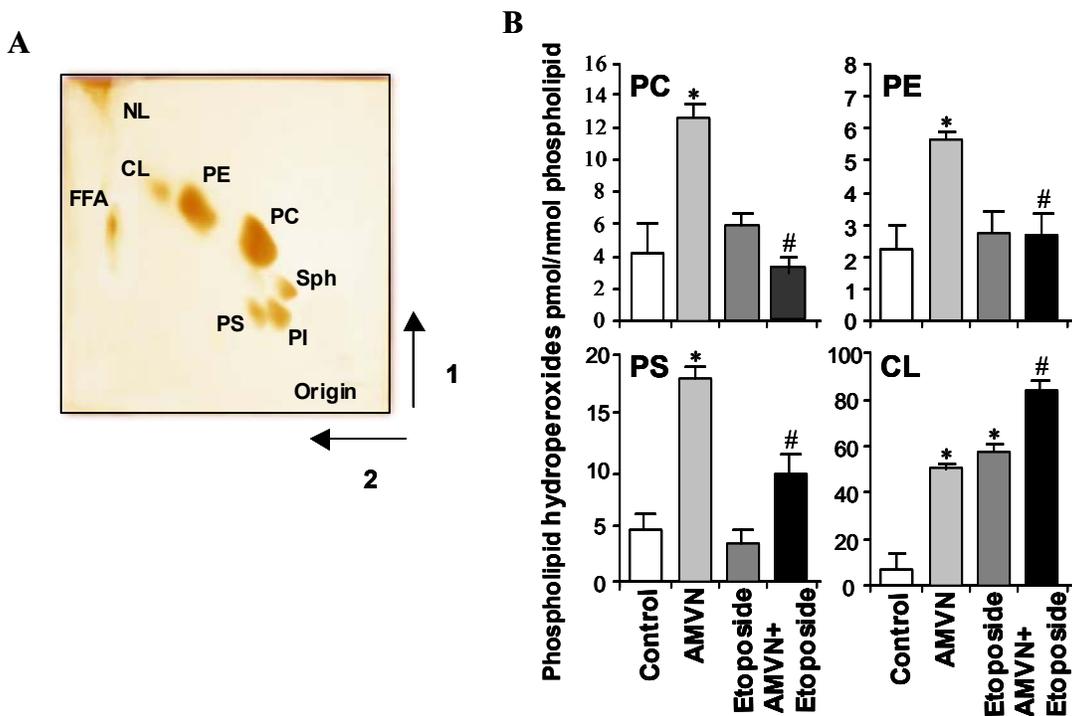


Figure 1: Effect of etoposide and AMVN on peroxidation of phospholipids in HL-60 cells.

HL-60 cells (2×10^6 /ml) were incubated with either AMVN ($500 \mu\text{M}$) or Etoposide ($25 \mu\text{M}$) or both together for 2h at 37°C . At the end of incubation lipids were extracted and resolved by 2D-HPTLC. 50 mg of lipids were used for analysis. (A) Typical 2D-HPTLC of total lipids extracted from HL-60 cells shows spots for NL, neutral lipids; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; FFA, free fatty acids identified by exposure to iodine crystals. Phospholipids spots were scraped from the plate and phospholipids were extracted. (B) Phospholipid hydroperoxides were determined by fluorescence HPLC using Amplex Red protocol. Data presented as Mean \pm S.E. ($n=3$); *, $p < 0.001$ versus control; #, $p < 0.001$ versus AMVN.

3.2. Effect of etoposide on CL oxidation in model system

However, our previous work has established that phenolic moiety of etoposide confers a potent lipid radical scavenging activity on the molecule [17,19]. We were eager to determine the extent to which etoposide was effective in inhibiting CL oxidation induced by AMVN, comparatively to its ability to suppress *c*/CL oxidation. To this end, we used liposomes containing dioleoylphosphotidylcholine (DOPC): tetralinoleoyl (TLCL) at a ratio of 1:1. TLCL was a natural choice since it is the most abundant CL species found in the animal mitochondria. Liposomes were incubated in PBS (containing 100 μ M DTPA) for 1h at 37°C with 250 μ M AMVN, which generates peroxy radicals at constant rate at a given temperature [33]. At the end of the incubation phospholipids were extracted, resolved by 1-dimentional HPTLC to separate etoposide from the phospholipids and the content of CL hydroperoxides was determined using our newly developed Amplex Red fluorescence HPLC-based assay [31]. We found that incubation of liposomes with AMVN resulted in accumulation of up to 155.8 \pm 13.0pmol CL hydroperoxides/nmol CL (~1.55 nmol/ml). This corresponds well with the total amount of radicals generated by AMVN (1.6 nmol/ml) during this period of time given that its decomposition rate is $1.36 \times 10^{-6} [\text{AMVN}] \text{ M}^{-1} \text{ s}^{-1}$ [34]. Addition of etoposide to the incubation system resulted in significant decrease of CL oxidation the magnitude of which was proportional to etoposide concentration. Half-maximal inhibition (I_{50}) was achieved at ~3 μ M etoposide, whereas nearly complete inhibition of hydroperoxide formation was obtained at 25 μ M etoposide. (Fig.2A). These results demonstrate that etoposide is an effective radical scavenger as has been demonstrated previously by us in other systems [9,19].

We next evaluated the potency of etoposide as an inhibitor of enzymatic *cyt c*/CL catalyzed oxidation. We chose the conditions (incubation of TLCL liposomes in PBS for 1h at 37°C with *cyt c* (4μM) and H₂O₂ (100μM, added every 15mins) yielding approximately the same CL oxidation level (162±38 pmol CL hydroperoxide/ nmol CL) as the one generated by AMVN system (compare Fig.2A and 2B). Etoposide protected TLCL against enzymatic *cyt c* catalyzed oxidation. However, in this system the potency of etoposide was approximately 5-fold lower with I₅₀ = 15μM (Fig.2B). The lower antioxidant activity of etoposide in the *cyt c*/H₂O₂ system compared to AMVN induced CL oxidation is not entirely unexpected and may be related to its hindered interactions with reactive intermediates of *cyt c*/CL peroxidase complex (compound I, compound II, protein-derived (tyrosyl) amino acid radicals) as compared to stochastic interaction with peroxy radicals during AMVN induced TLCL oxidation. To get better understanding of the etoposide participation in *cyt c*/CL peroxidase reaction, we applied EPR spectroscopy to detect etoposide phenoxyl radicals as well as protein-derived (tyrosyl) radicals generated by the *cyt c*/CL complexes [35].

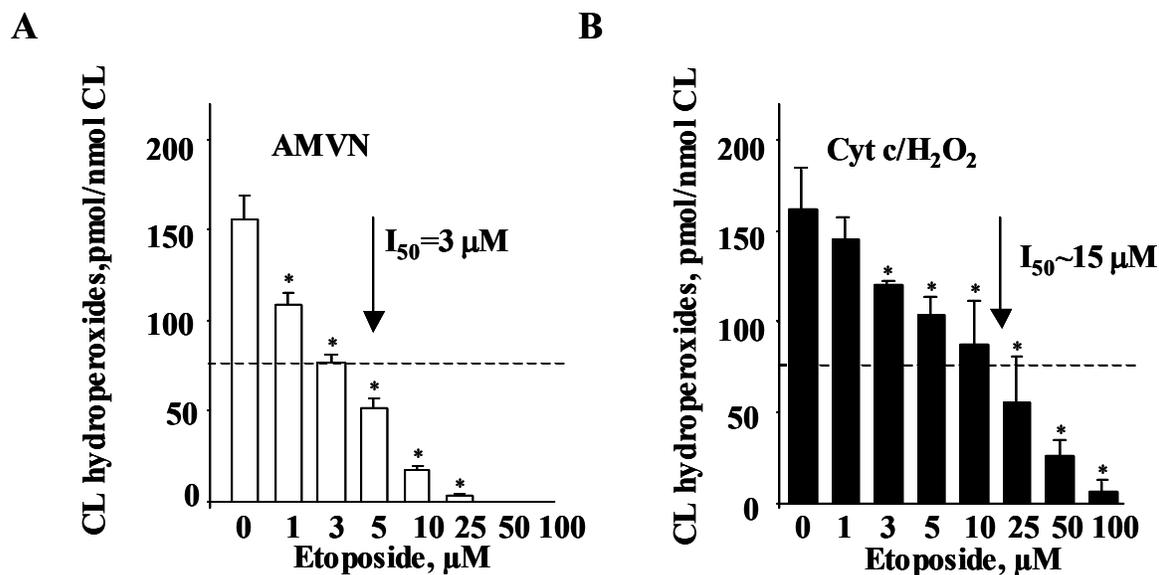


Figure 2: Effect of etoposide on CL oxidation induced by CL/cyt *c* complex in the presence of H_2O_2 or a lipid-soluble azo-initiator of peroxy radicals, AMVN.

PC/ TLCL (200 μM , at ratio 1:1) liposomes were incubated in PBS pH 7.4, containing 100 μM DTPA with (A) AMVN (250 μM) or in the presence of (B) cyt *c* (4 μM) and H_2O_2 (100 μM , 4times every 15mins) for 60 mins at 37°C. At the end of the incubation, lipids were extracted and hydroperoxides of CL were determined by fluorescence HPLC using Amplex Red protocol. Amount of hydroperoxides generated are presented as pmol CL hydroperoxide/nmol CL. Control group represents liposomes without etoposide treatment. Data are presented as Mean \pm S.E. (n=3);

*, $p < 0.001$ versus control.

3.3. EPR measurements of etoposide phenoxyl radical formation.

One-electron oxidation of etoposide yields its phenoxyl radical with characteristic signals in the EPR spectra [36]. Indeed, incubation of TLCL liposomes with *cyt c*, etoposide and H₂O₂ (under nitrogen) produced the characteristic EPR signal of etoposide phenoxyl radical (Fig.3A). This signal was not observed in the absence of H₂O₂ (data not shown). The magnitude of the signal was dependent on etoposide concentration with half-maximal response at approximately 15 μM, i.e., similar to I₅₀ for etoposide inhibition of TLCL peroxidation by *cyt c*/H₂O₂ (compare Figs. 2B and 3B). This suggests that one-electron oxidation of etoposide by the peroxidase activity of *cyt c*/TLCL complex may be responsible for its inhibitory effects on TLCL oxidation. However, in the presence of highly oxidizable TLCL, etoposide phenoxyl radicals can be generated by *cyt c*/H₂O₂ either directly through interactions with reactive intermediates of the enzyme - compounds I, II as well as *cyt c* protein radicals [21,35,36] - or indirectly in reactions with lipid alkyl and peroxy radicals scavenged by etoposide. To eliminate the contribution of lipid radicals into the etoposide phenoxyl radical formation, we used tetraoleoylcardiolipin (TOCL) a mono-unsaturated CL species that is not readily oxidized by *cyt c* to form peroxy radicals (Fig.2B, insert). Similarly, well-resolved etoposide phenoxyl radical EPR signals were detectable upon incubation of *cyt c* (4 μM) /H₂O₂ (100 μM) in the presence of TOCL liposomes and low concentrations of etoposide (Fig.3B). The magnitude of EPR signal increased in etoposide concentration dependent manner and reached a plateau at 50 μM with half-maximal response at approximately 15 μM etoposide. This suggests that peroxidase metabolism of etoposide by *cyt c*/CL complexes is likely a significant contributor to etoposide inhibition of TLCL peroxidation. In line with this, anaerobic (under nitrogen) incubation of *cyt c*/TOCL with

H₂O₂ (100μM) and etoposide also produced etoposide phenoxyl radicals by peroxidase activity of cyt *c* /CL complex (data not shown). Interestingly, the magnitude of EPR signal of etoposide phenoxyl radical in the presence of TOCL was significantly higher than with TLCL (Fig.3B). The difference in the magnitude of the observed etoposide phenoxyl radical signal between the two phospholipids was observed only at low etoposide concentrations. Since, TOCL does not undergo peroxidation (see Fig.3, insert), oxidizing equivalents generated by the peroxidase reaction are spent exclusively on etoposide oxidation resulting in the generation of its phenoxyl radicals. In the case of TLCL, a readily peroxidizable lipid substrate, the peroxidase reaction can be directed towards TLCL peroxidation and etoposide oxidation resulting in lower levels of generation of etoposide radical. To further characterize involvement of peroxidase metabolism of etoposide in its inhibition of TLCL oxidation we performed low temperature EPR studies of protein (tyrosyl) radical intermediates formed by cyt *c*/CL complexes in the presence of H₂O₂ and etoposide.

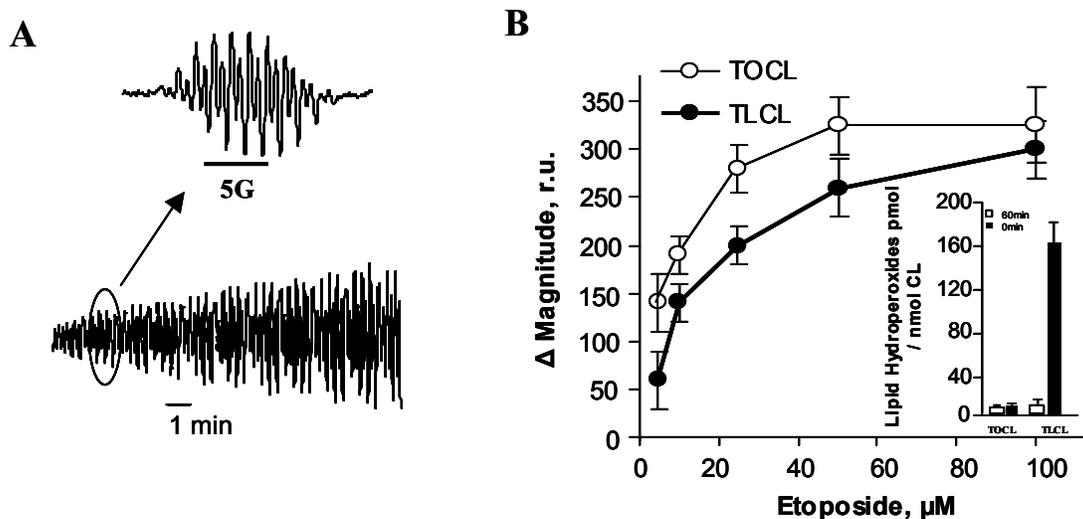


Figure 3: Changes in etoposide-phenoxyl radical in presence of TOCL and TLCL.

Liposomes (100μM DOPC and 100μM TOCL or TLCL) were incubated with etoposide for 15 min on ice. Cyt *c* (4μM) was incubated with liposomes for 1min at room temperature and (A) time course of 4min of EPR signal of etoposide was recorded within 1min after 100μM H₂O₂ addition (a part of the upper spectrum is presented repeatedly to demonstrate kinetics). (B,insert) Oxidation of TOCL and TLCL at (□) 0min and (■) 60 min after incubation with cyt *c* and H₂O₂. (B) Change of magnitude of etoposide radical EPR signal with increase in etoposide concentration. TOCL (■) or TLCL (□). The data illustrated are from one representative experiment of a total of four experiments (with duplicate measurements for each etoposide concentration). Etoposide phenoxyl radical spectra were recorded under the following conditions: 335 mT, center field; 5 mM sweep width; 10 mW, microwave power; 0.05 mT, field modulation; 10³, receiver gain; 0.03 sec, time constant; 4 min, scan time. The time course of etoposide radical EPR signals was obtained by repeated scanning of the field (0.25 mT, sweep width; other instrumental conditions were the same) corresponding to a part of the EPR signal.

3.4. EPR measurements of protein-derived (tyrosyl) radical formation

Catalytic activation of peroxidases is known to form highly reactive oxidizing intermediates – compounds I and II [37]. The production of the latter intermediate is also associated with the generation of another oxidizing species, amino acid centered (often tyrosyl) radical detectable by EPR [38-41]. Thermodynamically, any of these reactive intermediates of cyt *c*/CL peroxidase complex can oxidize etoposide to generate its phenoxyl radical [42,43, see also Discussion]. To better understand etoposide's inhibitory effects of cyt *c*/H₂O₂ induced oxidation of CL, we performed EPR measurements of protein-derived (tyrosyl) radicals of cyt *c* at 77 K [44,45]. As shown in Fig.4A, a characteristic low temperature EPR signal of (tyrosyl) radical with peak-trough width of 1.6mT and $g = 2.005$ was readily detectable in cyt *c* /CL system immediately upon addition of H₂O₂. These signals were only barely detectable from cyt *c* plus H₂O₂ in the absence of CL or from the mixture of cyt *c* with PC liposomes plus H₂O₂ (data not shown). The magnitude of the (tyrosyl) radical signal from cyt *c* /CL complex (Fig.4Ab) was about 30 ± 7 % lower for readily peroxidizable TLCL than for non-oxidizable TOCL (Fig.4Aa) – in line with the above results on etoposide phenoxyl radicals (see Fig.3B). It is likely that the difference is due to partial quenching of the radical by TLCL undergoing oxidation. Addition of etoposide to either TOCL- or TLCL- complexes of cyt *c* (Fig.4Ac) resulted (in the presence of H₂O₂) in approximately two-fold increase of the magnitude of the EPR signal and change of its shape ($g = 2.005$ and peak and trough width of 1.15mT). This seemingly “unexpected” growth of the signal may be explained by superposition of the protein-derived (tyrosyl) radical signal with the etoposide phenoxyl radical signal. In fact, as both of them have similar g -factors (2.005 and 2.005, respectively), and half-widths (1.6mT and 1.15mT, respectively), their simultaneous

formation is likely to cause an increase of the overall EPR signal. If oxidation of etoposide to its phenoxyl radical occurred on the protein-derived (tyrosyl) radical of cyt *c*/CL complex, one would expect to detect a decrease of its magnitude (due to its reduction by etoposide). Given very similar parameters for both of the signals - the protein-derived (tyrosyl) radical and etoposide phenoxyl radical – their interaction is likely to mutually compensate the changes in the signal magnitude. The observed large increase of the signal indicates that oxidizing intermediates distinct from the protein-derived (tyrosyl) radical (likely compound I or II) was involved in etoposide oxidation. This is also supported by our results on power saturation of etoposide phenoxyl radical and protein-derived (tyrosyl) radical EPR signals (Figure.4B). The etoposide phenoxyl radical EPR signal saturated at a significantly lower power of magnetic field than protein-derived (tyrosyl) radical signal. Power saturation of radical EPR signals depends very strongly on the distance between a radical and metal ion [45]. Thus, the results are indicative of spatial separation of the sites where these two radicals were formed, provided that relatively long-lived etoposide phenoxyl radicals did not significantly diffuse from the immediate site of their generation.

3.5. Formation of dityrosines in cyt *c* peroxidase reaction

A protein-derived tyrosyl radical formed during peroxidase reaction of cyt *c*-CL complex is relatively long-lived and can isomerize and combine with another tyrosyl radical with subsequent enolization [46]. As a result a stable, covalent, carbon–carbon bond is generated yielding 1,3-dityrosine. The latter is a highly fluorescent molecule that is distinguished by the intense 420nm fluorescence, measurable upon excitation within either 315nm (alkaline solutions) or 284nm

(acidic solutions) absorption bands [46]. Several oxidizing systems were found to produce dityrosines during oxidant exposure of both purified proteins in vitro and intact cells. We found a markedly increased fluorescence characteristic of dityrosines from cyt *c* /TOCL complex incubated in the presence of H₂O₂. The fluorescence response was quenched by etoposide in a concentration-dependent manner (Fig.4C). Notably, no characteristic dityrosine fluorescence was observed from cyt *c* /TLCL complexes treated with H₂O₂ in presence or absence of etoposide.

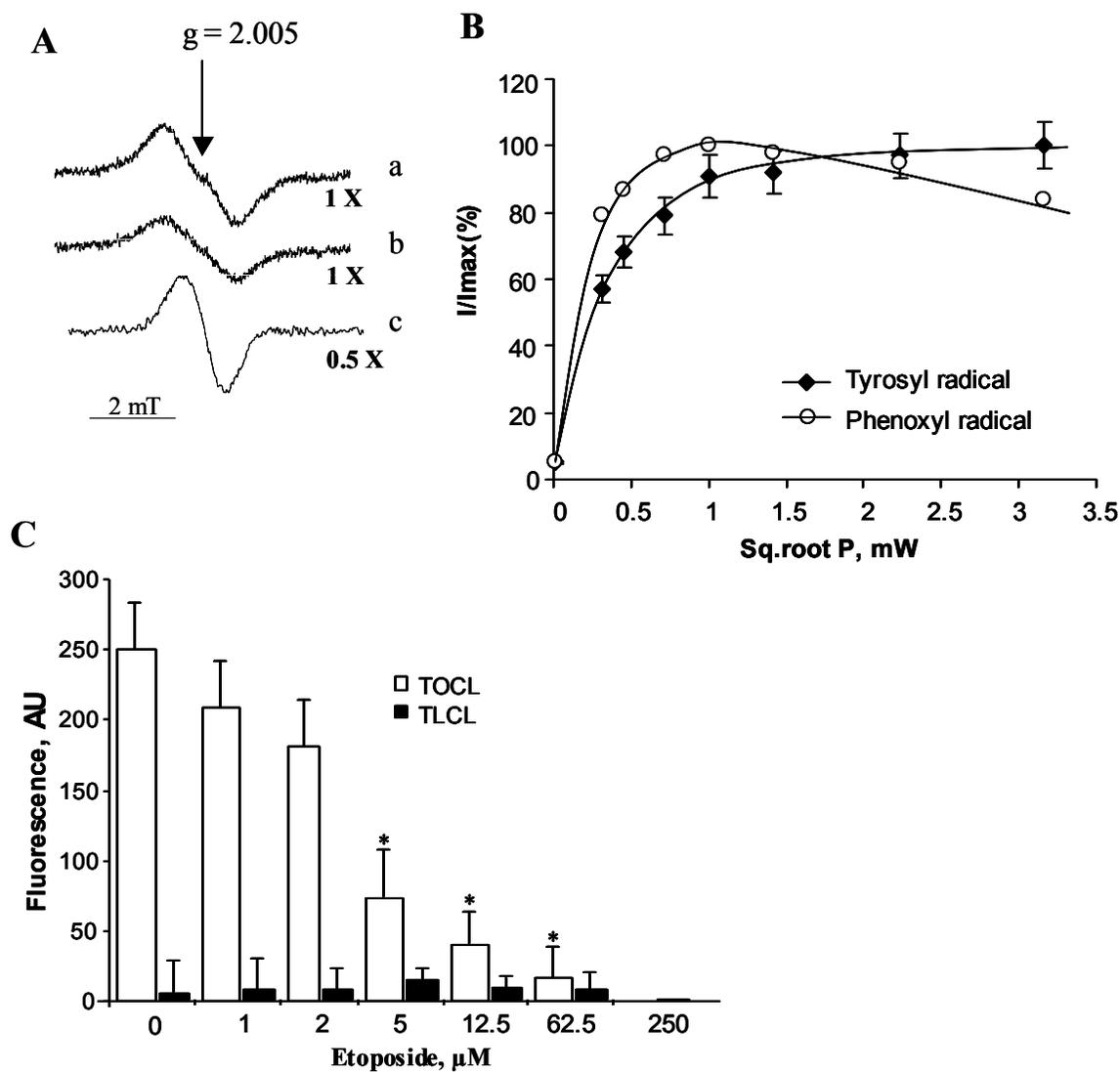


Figure 4: Low temperature EPR spectra of compound II associated tyrosyl radicals of *cyt c*.

Liposomes (10 mM, DOPC: TOCL or TLCL at ratio 1:1) were incubated with *cyt c* (200 μM) for 1 min at room temperature followed by addition of H₂O₂ (1 mM). Reaction was stopped after 20 s by freezing the samples in liquid nitrogen. (A) EPR-spectra of H₂O₂-induced protein-derived radical from *cyt c* / TOCL (a), TLCL (b) or in presence of 500 μM etoposide (c) complex was recorded at 77K under the conditions: 323 mT – centered field; 10 mT – sweep width; 0.5 mT, field modulation; 1 mW, microwave power; gain 4 × 10³, with relative gain indicated as x times; 0.1 s, time constant; 4 min, time scan. (B) Saturation curve, etoposide phenoxyl radical (○) and protein-

derived tyrosyl radical (●). Saturation curves are the same with TOCL/ cyt *c* and TLCL/cyt *c* complex. Dependence of relative magnitude (% from maximal one) of EPR signals of phenoxyl radicals on square root from microwave power (mW) was determined and presented as (C) Fluorescence was measured at 420nm in presence of varying concentrations of etoposide (0-250μM), TOCL (□) and TLCL (■). Data are presented as Mean ± S.E. (n=3); *, $p < 0.005$ versus in absence of etoposide.

3.6. Detection of *cyt c* / CL oligomerization products by PAGE electrophoresis

Formation of dityrosine between *cyt c* molecules should be associated with its cross-linking and accumulation of different protein oligomers detectable on PAGE gels under denaturing conditions [48]. Indeed, when *cyt c* /TOCL complexes were incubated with H₂O₂, dimers, trimers, tetramers of *cyt c* and larger aggregates, which did not enter the gel due to their very high molecular weight, were observed along with the monomer species after silver staining of the gels (Fig.5A). Etoposide effectively inhibited oligomerization of *cyt c*/TOCL complexes; particularly very high molecular weight aggregates, thus preserving monomeric form of *cyt c* (Fig. 5Ba). The *cyt c* /TLCL complexes did not display di-, tri-, or tetra-oligomers of *cyt c* upon exposure to H₂O₂. Instead only very high molecular weight aggregates that did not enter the gel accumulated (Fig. 5A). These very high molecular weight aggregates are typical of cross-linking by the secondary bifunctional lipid peroxidation products (e.g., dialdehydes) [49]. Etoposide was able to significantly prevent accumulation of these cross-links (Fig.5Bb). Thus two different types of oligomerization products were formed from complexes of *cyt c* with non-peroxidizable TOCL and readily peroxidizable TLCL. The former resulted from the production of dityrosine cross-links and exerted characteristic fluorescence while the latter were cross-linked by secondary lipid peroxidation products that did not exert characteristic di-tyrosine fluorescence. Etoposide was able to substantially inhibit aggregation of *cyt c* complexes with both TOCL and TLCL at concentrations exceeding 100μM. These results show that at higher concentrations etoposide prevents CL oxidation by competing with it as a peroxidase substrate.

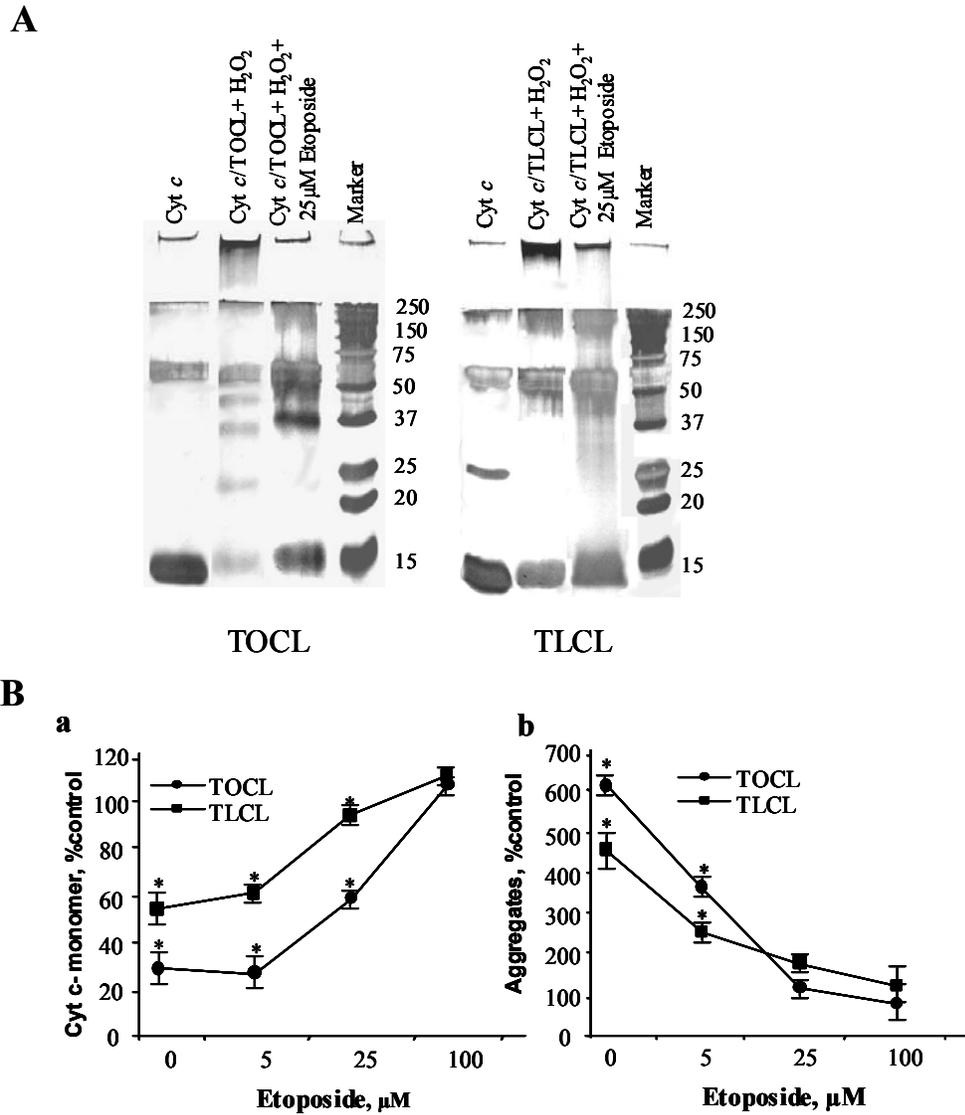


Figure 5: Electrophoresis of cyt c / CL complexes

Liposomes (PC: TOCL/ TLCL) were incubated with cyt c in PBS at 37°C for 1h with addition of 100 μM H₂O₂ (every 15 min). (A) Electrophoresis was performed in 12.5% PAGE. Silver stain was used to identify the different molecular weight bands on the gels. The bands were densitometrically analyzed (Ba)- Cyt c monomer (Bb) Aggregates at the base of the stacking gel. Data presented as % of control, Mean \pm S.E. (n=3); *, $p < 0.005$ versus control, where control is cyt c alone.

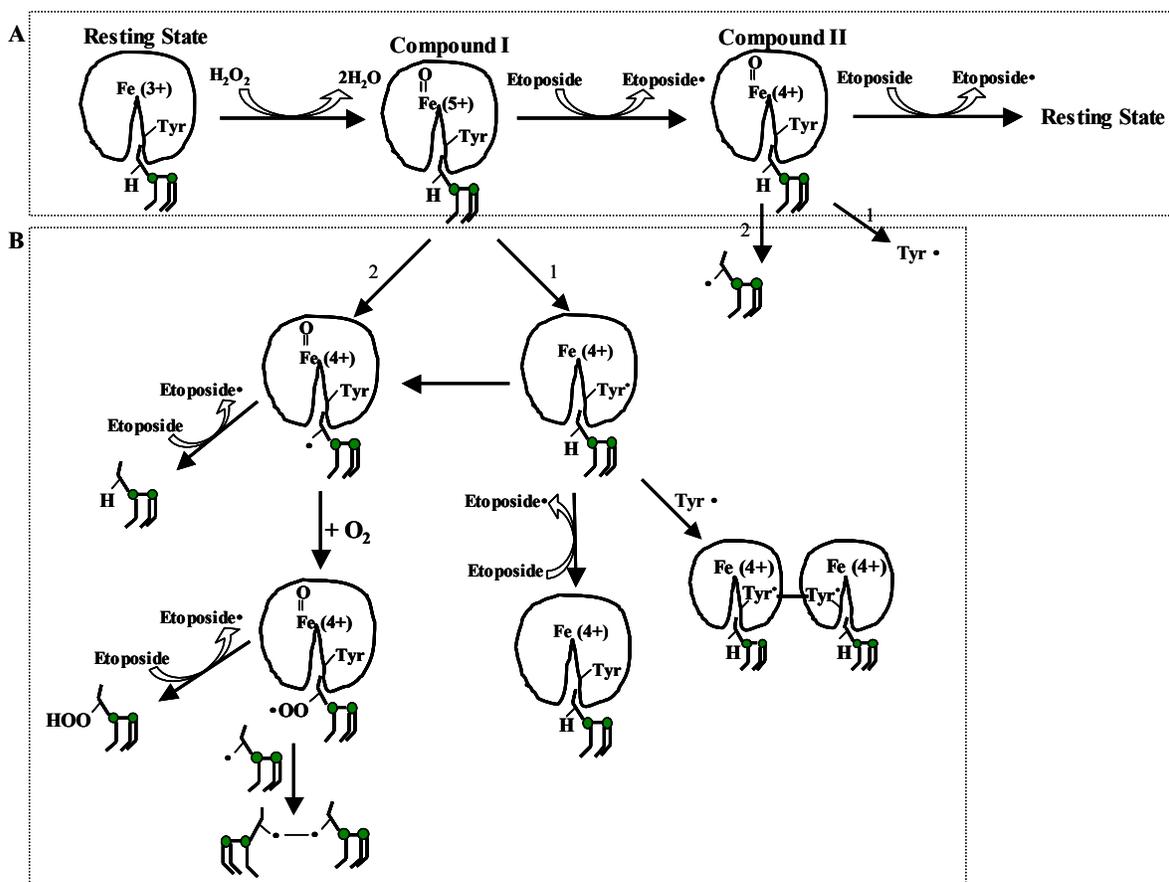
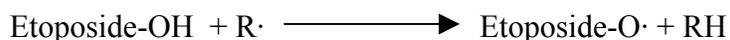


Figure 6: Schema

Cyt *c* on binding to anionic phospholipid CL forms a peroxidase, which in presence of H₂O₂ undergoes a typical peroxidase reaction, which leads to oxidation of CL. The reaction forms intermediates like compound I and compound II, which are thermodynamically equivalent and can undergo similar reactions. In the present schema, in presence of high concentrations of etoposide (A), the reaction proceeds as shown on the first line and the enzyme recycles. However in absence or at lower etoposide concentrations (B), the reaction may proceed in one of the two ways. It can oxidize protein amino acid (1), tyrosine, which can dimerize and form oligomers. The other pathway is through direct oxidation of the lipid by the heme of the peroxidase (2). The lipid radical formed can form peroxyl radical under aerobic conditions where it can combine with another lipid radical forming lipid aggregates. For non-oxidizable lipids the first pathway would be dominant where oxidizable lipids may undergo oxidation via the second pathway.

4. DISCUSSION

Etoposide (VP-16), a semisynthetic epipodophyllotoxin, was introduced in 1971 and since then has become one of the most widely used anticancer drugs. It is a DNA topoisomerase II poison containing a critical hindered phenolic ring necessary for its antitumor activity. It induces apoptosis via the intrinsic pathway accompanied by generation of reactive oxygen species [12-16]. Paradoxically, it has also been established as a powerful lipid antioxidant over the past few years. Etoposide with its hindered ring, can act as an effective donor of protons or electrons for scavenging reactive radicals



As indicated by the above reaction, etoposide acts as an antioxidant and its protective effect against lipid peroxidation have been demonstrated in model biochemical and cellular systems [17-19]. The main purpose of our study was to elucidate why etoposide readily induces apoptosis notwithstanding its known antioxidant properties

Others and we have shown that negatively charged unsaturated phospholipids like PS and CL are selectively oxidized during apoptosis [17]. The selective oxidation of PS and CL are considered to occur at different times in the apoptotic process, wherein, CL oxidation is shown to be an early apoptotic event [22,23] and PS oxidation a late apoptotic event [17]. Thus, regulation of oxidation of these phospholipids could influence the execution of apoptosis. Indeed, etoposide, a lipid radical scavenger, blocks PS oxidation and its subsequent externalization on the surface of apoptotic cells and their clearance by macrophages [17]. Taking into account the antioxidant ability of etoposide against PS oxidation, we asked ourselves whether it could prevent CL oxidation in a similar manner. Consistent with our expectations, etoposide blocked CL oxidation in model systems via scavenging of peroxy radicals, generated by AMVN

(Fig.2A). Using a more physiologically relevant system, *cyt c* / CL /H₂O₂, we observed blocking of CL oxidation at a much higher etoposide concentration (Fig.2B). The decreased antioxidant potency of etoposide in the *cyt c* / CL /H₂O₂ system can be attributed to a tight complex formed by *cyt c* and CL that in turn leads to close interactions of peroxidase reaction intermediates like compound I, compound II and amino-acid radicals with CL and prevent their inactivation by etoposide. Schema 1 presents possible pathways and substrates in the *cyt c*-catalyzed peroxidase reaction. Reactive heme species formed as a result of *cyt c* peroxidase reaction may oxidize CL, etoposide or aromatic amino acids in *cyt c* protein itself. Conversely, CL may be oxidized at 2 different sites, by the heme-centered compound I and compound II species or by amino-acid radicals. The latter may be the dominant pathway, because very active oxidative species compounds I or II should be able to oxidize both TOCL (monounsaturated CL species that are not easily oxidized) (Schema1), and TLCL (polyunsaturated CL species that are easily oxidized) while aromatic amino-acid radicals would be able to oxidize only TLCL and only TLCL oxidation was observed as seen in Fig.3 (insert).

As a substrate of peroxidase reaction, etoposide forms a phenoxy radical, which can be detected by EPR [36]. We observed a greater magnitude of etoposide phenoxy radical EPR signal in the case of TOCL than that of TLCL at low etoposide concentration. These results suggest that at low etoposide concentrations, it acts as a preferred substrate in presence of TOCL (and hence higher magnitude of the radical signal), whereas easily oxidized TLCL is preferred giving a lower magnitude of etoposide phenoxy radical signal. However, at higher concentrations, etoposide oxidation out competes CL (TOCL or TLCL) oxidation, thus exhibiting antioxidant effect. As mentioned earlier, the peroxidase reaction also generates amino-acid centered radicals that can be observed using EPR technique at low temperatures (77K). Our

results show that protein-derived radicals in the case of TLCL were quenched as compared to TOCL by 30 ± 7 % (Fig.4A). This quenching may be the result of oxidation of TLCL by amino-acid radicals.

It should be noted that a significant oxidation of cyt *c* in the presence of TLCL liposomes occurs even without addition of H_2O_2 as evidenced by cyt *c* crosslinking evident in from results presented in Fig.4A. Lipid radicals and peroxides are generated during TLCL liposomes preparation ($>1\%$ of lipids). Although the degree of lipid oxidation is small, for large concentration of CL (5 mM) it may result in considerable concentration of lipid peroxides that participate in cyt *c* catalyzed peroxidase reaction resulting in additional EPR signals [22,23,25]. These additional EPR signals may be either from alkoxy radical (signal centered at $g=2.012$ with line width about 1.4 mT) [50] or protein-derived radicals. Also, in the presence of etoposide, the amplitude of the EPR signal observed was twice greater than that of the tyrosyl radical signal in the absence of etoposide quenching. These results suggest that etoposide is largely oxidized at a different site, probably by heme-associated compound I and II species.

The specificity of oxidation process can also be explained considering the redox potentials of redox species involved. Compound I and II with redox potentials of $\sim 0.95 - 1.0$ V [42] are strong oxidants capable of oxidizing a wide range of substrates. Tyrosyl radical with $E^\circ = 0.7 - 0.9$ V [42] can oxidize TLCL ($E^\circ = 0.6$ V) [43] and etoposide ($E^\circ = 0.56$ V) [51]. Mono-unsaturated TOCL has higher redox potential ($E^\circ = 0.9$ V) [44], and tyrosyl radicals cannot oxidize it.

In the case of TOCL, peroxidase reaction is directed towards the protein amino acids oxidation and hence we see greater formation tyrosyl radicals. These stable radicals can dimerize forming dityrosine (Schema: pathway B1) that we observe measuring fluorescence at 470nm. This fluorescence was decreased upon addition of etoposide. No dityrosine formation was seen

in TLCL liposomes in the absence of etoposide, indicating tyrosyl radicals formed may be spontaneously recombining with lipid radicals or is utilized in oxidizing the lipid rather than participating in dityrosine formation. A similar phenomenon was observed in our PAGE experiment, where TOCL or TLCL in absence of etoposide formed high molecular weight aggregates at the base of the well along with formation of polymeric forms of *cyt c* only in case of TOCL. The formation of aggregates was reduced in presence of etoposide suggesting that etoposide prevents formation of tyrosyl radicals. With TLCL, however there was no formation of *cyt c* polymeric forms because the oxidizing potential of the peroxidase reaction was directed towards oxidation of the lipid (Schema: pathway B2).

We have shown that etoposide can suppress CL oxidation in model systems. We also examined the mechanism of etoposide antioxidant action. Etoposide exerts its protective effect by acting as a preferential substrate for the *cyt c* peroxidase activity. This diverts the peroxidase reaction from oxidation of CL to oxidizing etoposide. However, antioxidant protection of CL was observed only at rather high ($>15\mu\text{M}$) concentrations of etoposide in our HL-60 cellular model. Though, etoposide protected other phospholipids against oxidation, CL was still oxidized. These results indicate that oxidation of CL proceeds in tight *cyt c*/CL complexes that provide high rate and specificity of CL oxidation therefore rather high concentrations of etoposide are required to block CL oxidation. Apoptosis is induced by lower concentrations of etoposide ($<10\mu\text{M}$). Thus etoposide-dependent inhibition of CL oxidation is not likely to interfere with the execution of apoptotic program and mitochondrial membrane permeabilization that were shown to depend on CL oxidation

BIBLIOGRAPHY

1. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, and Peter ME. (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* **17**, 1675-1687
2. VanLoo G, Saelens X, VanGrup M, MacFarlane M, Martin SJ, Vandenabeele P. (2002) The role of mitochondrial factors in apoptosis: a Russian roulette with more than a bullet. *Cell. Death Differ.* **9**:1031-1042
3. Martinou JC, Green DR. (2001) Breaking the mitochondrial barrier. *Nature Reviews Mol. Cell Biol.* **2**: 63-67
4. Leblanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D, Ashkenzi A. (2002) Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic homolog Bax. *Nat Med.* **8**: 274-281
5. Raha, S and Robinson, BH. (2001) Mitochondria, oxygen free radicals and apoptosis. *Am. J. Med. Genet.* **106**:62-70
6. Zamzani N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G. (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med.* **182**(2):367-77.
7. Genova ML, Pich MM, Biondi A, Bernacchia A, Falasca A, Bovina C, Formiggini G, Parenti Castelli G, Lenaz G. Mitochondrial production of oxygen radical species and the role of Coenzyme Q as an antioxidant. (2003) *Exp Biol Med* (Maywood) **228**(5):506-13. Review.
8. Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell.* **75**(2):241-51.
9. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB. (1998) Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J Biol Chem.* **273**(21):13245-54.
10. Nomura K, Imai H, Koumura T, Arai M, Nakagawa Y. (1999) Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. *J Biol Chem.* **274**(41):29294-302.

11. Kagan VE, Borisenko GG, Tyurina YY, Tyurin VA, Jiang J, Potapovich AI, Kini V, Amoscato AA, Fujii Y. Oxidative lipidomics of apoptosis: redox catalytic interactions of cytochrome c with cardiolipin and phosphatidylserine. (2004) *Free Radic Biol Med.* **37**(12):1963-85
12. Gorman A, McGowan A, and Cotter T. (1997) Role of peroxide and superoxide anion during tumour cell apoptosis. *FEBS Lett.* **404**, 27-33
13. Pham NA, and Hedley DW. (2001) Respiratory chain-generated oxidative stress following treatment of leukemic blasts with DNA-damaging agents. *Exp. Cell Res.* **264**, 345-352
14. Corbett AH and Osheroff N. (1993) When good enzymes go bad: Conversion of topoisomerase II to a cellular toxin by antineoplastic drugs. *Chem. Res. Toxicol.* **6**:585-597
15. Loike JD and Horwitz SB. (1976) Effect of VP-16-213 on the intracellular degradation of DNA in HeLa cells. *Biochemistry* **15**: 5443-5448
16. Slevin ML (1991) The clinical pharmacology of etoposide. *Cancer* **67**:319-329
17. Tyurina YY, Serikan FB, Tyurin VA, Kini V, Yalowich JC, Schroit AJ, Fadeel B, and Kagan VE. (2004) Lipid antioxidant, etoposide, inhibits phosphatidylserine externalization and macrophage clearance of apoptotic cells by preventing phosphatidylserine oxidation. *J Biol Chem.* **279**(7), 6056-6064
18. Kalyanaraman B, Nemeč J, Sinha BK. (1989) Characterization of free radicals produced during oxidation of etoposide (VP-16) and its catechol and quinone derivatives. An ESR Study. *Biochemistry.* **28**(11):4839-46.
19. Kagan VE, Kuzmenko AI, Tyurina YY, Shvedova AA, Matsura T, and Yalowich JC. (2001) Pro-oxidant and antioxidant mechanisms of etoposide in HL-60 cells: role of myeloperoxidase, *Cancer Res.* **61**, 7777-7784
20. Egan RW, Paxton J, Kuehl FA Jr. (1976) Mechanism for irreversible self-deactivation of prostaglandin synthetase. *J Biol Chem.* **251**(23):7329-35.
21. Kagan VE. (1988) Lipid peroxidation in biomembranes. *CDC Press, Boca Raton, Florida.* 1-181.
22. Petrosillo G, Ruggiero FM, Paradies G. (2003) Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. *FASEB J.* **17**(15):2202-2208.
23. Garcia Fernandez M, Troiano L, Moretti L, Nasi M, Pinti M, Salvioli S, Dobrucki J, Cossarizza A. (2002) Early changes in intramitochondrial cardiolipin distribution during apoptosis. *Cell Growth Differ.* **13**(9):449-55.

24. Zamzami N, Kroemer G. (2003) Apoptosis: mitochondrial membrane permeabilization--the (w)hole story? *Curr Biol.* **13**(2):R71-3.
25. Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. (2002) Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage. *Gene.* **286**(1):135-41.
26. Petrosillo G, Ruggiero FM, Pistolese M, Paradies G. (2001) Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis. *FEBS Lett.* **509**(3):435-8.
27. Kagan VE, Yalowich JC, Day BW, Goldman R, Gantchev TG, Stoyanovsky DA. (1994) Ascorbate is the primary reductant of the phenoxyl radical of etoposide in the presence of thiols both in cell homogenates and in model systems. *Biochemistry.* **33**(32):9651-60.
28. Yalowich JC, Tyurina YY, Tyurin VA, Allan WP, and Kagan VE. (1996) Reduction of phenoxyl radicals of the antitumor agent etoposide (VP-16) by glutathione and protein sulfhydryls in human leukemia cells: implications for cytotoxicity. *Toxicol. In Vitro.* **10**:59-68
29. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL and Henson PM. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immuno.* **148**, 2207-2216
30. Bottcher CJF, Van Gent CM and Pries C. (1961) A rapid and sensitive sub-micro phosphorus determination. *Anal Chim Acta* **24**: 203–204.
31. Ritov VB, Tyurin VA, Tyurina YY, and Kagan VE. Quantitative analysis of phospholipids peroxidation: appearance of oxidized phosphatidylserine on the surface of HL-60 cells during intrinsic apoptosis. Abstract of 43rd Annual Meeting for Society of Toxicology, Baltimore, March 21-25, 2004.
32. Grune T, Davies KJ. (2003) The proteosomal system and HNE-modified proteins. *Mol. Aspects Med.* **24**(4-5):195-204
33. Niki E. (1987) Antioxidants in relation to lipid peroxidation. *Chem. Phys. Lipids* **44**, 227-253
34. Niki E. (1990) Free radical initiators as source of water- or lipid- soluble peroxy radicals. *Methods Enzymol.* **186**:100-108.
35. Kalyanaraman B, Nemeč J and Sinha BK. (1989) Characterization of free radicals produced during oxidation of etoposide (VP-16) and its catechol and quinine derivatives. *Biochemistry* **28**, 4839-4846

36. Kagan VE, Yalowich JC, Borisenko GG, Tyurina YY, Tyurin VA, Thampatty P, Fabisiak JP. (1999) Mechanism-based chemopreventive strategies against etoposide-induced acute myeloid leukemia: free radical/antioxidant approach. *Mol Pharmacol.* **56**(3):494-506.
37. Goldman R, Michei WBC, Day BW and Kagan VE. (1997) Environmental and nutritional phenols: Bioactivation to phenoxyl radicals and their cytotoxic and/or protective interactions with intracellular reductants. *Envir. Nutr. Inter.* **1**:97-118
38. Marnett LJ. (2000) Cyclooxygenase mechanism. *Curr. Opin. Chem. Biol.* **4**, 545-552
39. Tsai AL, Kulmacz RJ, Wang JS, Wang Y, Van Wart HE, Palmer G. (1993) Heme coordination of prostaglandin H synthase. *J. Biol. Chem.* **268** (12), 8554-8563.
40. Barr DP, Gunther MR, Deterding LJ, Tomer KB, Mason RP. (1996) ESR spin trapping of a protein-derived tyrosyl radical from the reaction of cytochrome c with hydrogen peroxide. *J Biol Chem.* **271**(26):15498-503.
41. Smith WL, DeWitt DL, Garavito RM. (2000) Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem.* **69**:145-82.
42. Buettner GR. (1993) The pecking order of free radicals and antioxidants: Lipid peroxidation, α -tocopherol and ascorbate. *Arch Biochem Biophys* **300**(2); 535-543
43. Koppenol WH. (1990) Oxyradical reactions: from bond-dissociation energies to reduction potentials. *FEBS.* **264**(2); 165-167
44. Chen YR, Chen CL, Chen W, Zweier JL, Augusto O, Radi R, Mason RP. (2004) Formation of protein tyrosine ortho-semiquinone radical and nitrotyrosine from cytochrome c-derived tyrosyl radical. *J Biol Chem.* **279**(17): 18054-62.
45. Lassmann G, Odenwaller R, Curtis JF, DeGray JA, Mason RP, Marnett LJ and Eling TE (1991) Electron spin resonance investigation of tyrosyl radicals of prostaglandin H synthase. Relation to enzyme catalysis. *J Biol Chem.* **266**(30): 20045-55
46. Giulivi C, Traaseth NJ, and Davies KJA. (2003) Tyrosine oxidation products: analysis and biological relevance. *Amino Acids.* **25**: 227-232
47. Malencik DA, Sprouse JF, Swanson CA., and Anderson SR. (1996) Dityrosine: Preparation, Isolation, and Analysis. *Anal. Biochem.* **242**, 202-213
48. Chen YR, Chen CL, Liu X, Li H, Zweier JL, Mason RP. (2004) Involvement of protein radical, protein aggregation, and effects on NO metabolism in the hypochlorite-mediated oxidation of mitochondrial cytochrome c. *Free Radic Biol Med.* **37**(10):1591-603.

49. Fabisiak JP, Tyurina YY, Tyurin VA, Lazo JS, Kagan VE. (1998) Random versus selective membrane phospholipid oxidation in apoptosis: role of phosphatidylserine. *Biochemistry*. **37**(39):13781-90.
50. Chen YR, Deterding LJ, Sturgeon BE, Tomer KB, Mason R.P. (2002) Protein oxidation of cytochrome C by reactive halogen species enhances its peroxidase activity. *J Biol Chem*. **277**(33):29781-91.
51. Wardman P. (1989) *J. Phys. Chem. Ref. Data* **18**, 1637-1755