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RESEARCH PAPER

The Cytotoxic Action of Cytochrome C/Cardiolipin Nanocomplex (Cyt-CL) on Cancer Cells in Culture

Yury A. Vladimirov[1,3] • Can Sarisozen[2] • Georgy K. Vladimirov[1,3] • Nina Filipczak[2] • Anastasia M. Polimova[3] • Vladimir P. Torchilin[2]

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ABSTRACT

Purpose The effect of existing anti-cancer therapies is based mainly on the stimulation of apoptosis in cancer cells. Here, we have demonstrated the ability of a catalytically-reactive nanoparticle-based complex of cytochrome c with cardiolipin (Cyt-CL) to induce the apoptosis and killing of cancer cells in a monolayer cell culture.

Methods Cyt-CL nanoparticles were prepared by complexing CytC with different molar excesses of CL. Following characterization, cytotoxicity and apoptosis inducing effects of nanoparticles were investigated. In an attempt to identify the anticancer activity mechanism of Cyt-CL, pseudo-lipoxygenase and lipoperoxidase reaction kinetics were measured by chemiluminescence.

Results Using chemiluminescence, we have demonstrated that the Cyt-CL complex produces lipoperoxide radicals in two reactions: by decomposition of lipid hydroperoxides, and by lipid peroxidation under the action of H_2O_2. Antioxidants inhibited the formation of lipid radicals. Cyt-CL nanoparticles, but not the CytC alone, dramatically enhanced the level of apoptosis and cell death in two cell lines: drug-sensitive (A2780) and doxorubicin-resistant (A2780-Adr). The proposed mechanism of the cytotoxic action of Cyt-CL involves either penetration through the cytoplasm and outer mitochondrial membrane and catalysis of lipid peroxidation reactions at the inner mitochondrial membrane, or/and activation of lipid peroxidation within the cytoplasmic membrane.

Conclusions Here we propose a new type of anticancer nanoformulation, with an action based on the catalytic action of Cyt-CL nanoparticles on the cell membrane and and/or mitochondrial membranes that results in lipid peroxidation reactions, which give rise to activation of apoptosis in cancer cells, including multidrug resistant cells.

KEY WORDS apoptosis · cytotoxicity · cytochrome c-cardiolipin complex · lipid peroxidation · lipid peroxyl radicals

ABBREVIATIONS

Bcl-2	B-cell/lymphoma 2
BCL	Bovine heart cardiolipin
CytC	Cytochrome c
Cyt-CL	Complex of cytochrome c with cardiolipin
IMM and OMM	Outer and inner mitochondrial membranes
MDR	Multidrug resistance
PDI	Polydispersity index
TOCL	1,1′,2,2′-Tetraoleoyl cardiolipin
PBS	10 mM NaH_2PO_4-Na_2HPO_4 (pH = 7.4)

INTRODUCTION

Cell death mechanism called apoptosis (1) is the major way to maintain the homeostasis in the vertebrates (2). Decoding the principles of apoptosis revealed a tightly controlled genetic process that usually proceeds via the intrinsic, so-called mitochondrial pathway (3). Upon the activation of the mitochondrial apoptosis pathway by cell stress and DNA damage-inducing reagents and/or conditions, several key proteins are released from the mitochondria into the cytosol including cytochrome c (Cyt*C*) and the apoptosis-promoting factor Apaf-1. The release of CytC to the cytosol is controlled by

✉ Yury A. Vladimirov
yuvlad@mail.ru; info@fbm.msu.ru

[1] Faculty of Basic Medicine M.V. Lomonosov Moscow State University 27/1 Lomonosovsky Prospekt Moscow 119192, Russian Federation

[2] Center for Pharmaceutical Biotechnology and Nanomedicine Northeastern University 360 Huntington Avenue Boston, Massachusetts 02115, USA

[3] Federal Research Center: Crystallography and Photonics Russian Academy of Sciences Leninsky Prospekt 59 Moscow 119333, Russian Federation

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the outer mitochondrial membrane permeabilization. Paradoxically, the permeability of the outer mitochondrial membrane is caused by events in the inner mitochondrial membrane, primarily the complex formation of CytC with specific mitochondrial highly anionic phospholipid, cardiolipin (CL), and lipid peroxidation in the inner membrane catalyzed by the complex. In the inner mitochondrial membrane, CL accounts for 25% of all phospholipids it consists of (4–6). When CytC assumes its electron shuttle role, the functional positions in its heme iron are occupied. However, when CytC is bound to CL, its confirmation changes and it becomes partially unfolded (7). This change allows an opening for hydrogen peroxide to bind with the heme and imparts CytC a peroxidase activity (8). This change in activity is followed by CL oxidation, mitochondrial inner membrane permeability transition, outer membrane permeabilization, and CytC release into the cytosol (9).

One of the major characteristics of the cancer cells is their ability to evade or down-regulate the apoptosis. The oncogenic mutations that disrupt apoptosis cause tumor initiation, progression, and metastasis. That is why many agents used for cancer chemotherapy kill tumor cells *in vitro* and *in vivo* through launching/restoring the mechanisms of apoptosis (10). Anticancer agents directed at this target are listed in the review (11), where they are classified as those acting on Apoptotic Proteins in the Extrinsic, Intrinsic, and Common Pathways (see also (12)). In their turn, cancer cells develop resistance to drugs, based on the elimination of the drugs from the cell or overexpression of cellular antiapoptotic proteins. The majority of these are the mitochondrial membrane proteins called B-cell/lymphoma 2 (Bcl-2) family, which prevent permeabilization of outer mitochondrial membrane, and thus the release of CytC (13). Bcl-2 and Bcl-xL are the most commonly overexpressed proteins in cancer cells that act as antiapoptotic factors and prevent CytC release into the cytoplasm (14). The overexpression of Bcl-2 is also associated with multidrug resistance (MDR) phenotype of the cancer cells, which results in the decreased sensitivity to chemically unrelated anticancer agents and diminishes the clinical success of chemotherapy.

Thus, one can assume that the intracellular delivery of CytC from outside of the cell could initiate the apoptosis in the cancer cells by bypassing the factors that prevent the "own" CytC release from the cell mitochondria. However, most proteins, including CytC, are cellular membrane impermeable. To overcome this obstacle, several different nanosized CytC delivery systems including mesoporous silica nanoparticles (15), polymer-based nanoparticles (16) or lipoprotein-based nanoparticles (17) have been recently studied. Most of the mentioned systems have used CytC carriers formulated with synthetic compounds, in other words, have tried to mimic a naturally occurring event using non-naturally occurring compounds.

In our study, in the light of the current information, we have developed a biomaterial-based nanoparticle formulation containing CytC and a natural lipid (cardiolipin). Due to the great affinity of CytC for cardiolipins compared to other lipids, such as phosphatidylcholine or phosphatidylserine, we have used tetraoleoyl cardiolipin (TOCL), which is not susceptible to lipid peroxidation, inevitable in the system of "CytC + oxidizable lipid". Cyt-CL complexes that contain TOCL have been studied extensively by different methods such as NMR, mass spectrometry and X-ray structural analysis (see review (18)). According to the small-angle X-Ray scattering data, the Cyt-CL complexes represent closely packed nanospheres of 11.1 ± 1 nm in diameter (19). Along with the spectrophotometric titration (19) and protein fluorescence measurements, the data show that each nanosphere represents a melted globe of CytC with the diameter of about 5.5 nm (18), surrounded by cardiolipin monolayer, with hydrophobic tails turned to outside. Such hydrophobic nanoparticles may penetrate lipid bilayer (20–22), (see also discussion in (23)) and hence – cell and mitochondrial membranes.

It should be stressed that Cyt-CL nanosphere is not merely the CytC carrier across the cell membrane, but mostly important the enzymatic complex performing lipoperoxidase action inside the cell. The complex of CytC with anionic lipids, including CL in the inner and outer membrane of mitochondria (9,24–29) and phosphatidylserine in the cytoplasmic membrane (30–33), catalyzes the lipid peroxidation that results in permeability transition in mitochondria, the efflux of CytC, and apoptosis initiation on one hand, and provokes phosphatidyl serine externalization on the other hand to send out 'eat-me signal' for phagocytes.

Based on all the above considerations, we have investigated the cytotoxic and pro-apoptotic action of the Cyt-CL complex in cancer cells, both drug-sensitive and -resistant, and studied the mechanism of reactions of free radical formation by the Cyt-CL complex, since these reactions could mediate the cytotoxic and pro-apoptotic effects observed. Our results allow us to propose a new type of anticancer agent, the action of which is based on introducing Cyt-CL into the cancer cell, catalysis of lipid peroxidation reactions in mitochondrial and cytoplasmic membranes, and activation of apoptosis in cancer cells, including multidrug resistant cells.

EXPERIMENTAL PROCEDURES

Materials

1,1′,2,2′-Tetraoleoyl cardiolipin (TOCL, CAS 115404–77-8) in chloroform (10 mg/ml) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cytochrome c from bovine heart (CytC, CAS 9007–43-6) was purchased from Sigma-Aldrich (St. Louise, MO, USA). The phosphate buffer pH 7.4 (10 mM

NaH_2PO4-Na_2HPO_4) was prepared in ultrapure water. Streptomycin (25 μg/ml)/Penicillin (10,000 U/ml) solution, RPMI-1640 media and Trypsin/EDTA were purchased from Corning/Mediatech (Manassas, VA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Flowery Branch, GA). A2780 human ovarian carcinoma cell line and its doxorubicin-resistant derivative (A2780-Adr) were from ECACC and purchased from Sigma-Aldrich (USA). All media were supplemented with 10% (*v*/v) FBS and 1% (*v*/v) streptomycin/penicillin, unless otherwise indicated. Cells were all grown at 37°C with 5% CO_2. CellTiter-Blue® cell viability assay was purchased from Promega Corporation (Fitchburg, WI). Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit with Alexa Fluor 488 Annexin V and PI was from Molecular Probes (Eugene, OR, USA).

Cyt-CL Complex Preparation

To prepare the nanospheres of CytC and CL (Cyt-CL), first Cyt*C* was dissolved in the phosphate buffer. Different molar excesses (25, 35, and 40) of TOCL over CytC were calculated and required volumes were taken from the TOCL chloroform stock solution into separate tubes. Chloroform was evaporated under nitrogen to form a thin film and the tubes were further freeze-dried to remove the trace solvent. Resulted TOCL film was dissolved in methanol. Concentrated TOCL solution was then added to the CytC solution dropwise while mixing. The methanol concentration was kept at not more than 5% (*v*/v). Then the dispersion was subjected to probe sonication on ice for 20 s at 10 W for 6 times to obtain a clear nanoparticle complex solution. The empty CL particles consisted of only TOCL were prepared in the same way but without CytC in the buffer.

Particle Size and Polydispersity Index Measurements

Following the preparation of the Cyt-CL nanoparticles, the particle sizes of the formulations were measured using Malvern Zetasizer ZS90 (Malvern Instruments, UK). The formulations were diluted in ultrapure water, and particle size distribution of all samples was measured in triplicate at 90-degree scattering angle. Same instrument was used to determine the zeta potential of the formulations.

Chemiluminescence Kinetic Measurements

The chemiluminescence kinetics was measured using Lum-100 luminometer (DiSoft, Russia). The operation of the device, the analysis of the results of photon counting, and plotting were performed using the computer program Power Graph (34). The method described in this paper, involves the sequential measurements of lipoxygenase activity and lipoperoxidase activity in one experiment.

The measurements began with the recording of *background luminescence*: 100 μl of 100 μM CytC in PBS, 25 μl of 1mM coumarin C-334 in methanol and 775 μl of PBS were placed in the cuvette of luminometer, and the registration was started, which lasted for 25 s.

To start the record of *lipoxygenase reaction* kinetics, the luminometer lid was opened, 100 μl of 6 mM BCL methanol solution were rapidly injected into the cuvette, the lid was closed, and the recording of the kinetics of lipoxygenase reaction was immediately started, so that the non-registered interval between reagent mixing and measurement was as small as 5–7 s. In the absence of coumarin C-334, the chemiluminescence curve was about five times as low as with C-334, due to the sensitizing effect of the dye, typical of quinolizin (9a,9,l-gh)-substituted coumarins (35). Because the luminescence was oxygen-dependent (data not shown), but observed in the absence of H_2O_2, it could be accounted for quasi-lipoxygenase activity of CytC (36), in particular in complex with cardiolipin (9). After adding BCL methanol solution, the chemiluminescence intensity was measured for 3.5 min, and then the cuvette was removed from a chemiluminometer, covered with filter paper, and allowed to stand.

To record the kinetics of *lipoperoxidase reaction*, hydrogen peroxide was added to the mixture of reagents in the first cuvette. The lid of the luminometer was opened and second cuvette was placed there, which contained 100 μl of 1.5 mM H_2O_2 solution, then the luminometer lid was closed, and the record of the chemiluminescence was continued for 10 s. Then, the lid was opened, and 900 μL of the first cuvette content were added in the second cuvette, the lid closed, and the recording continued for 6 min. By adding a larger volume into a smaller, we provided a good stirring of the sample.

The influence of the hydrogen peroxide concentration on the lipoperoxidase function of Cyt-CL was studied by varying the concentration of hydrogen peroxide added before the registration of the lipoperoxidase reaction kinetics.

The effect of antioxidants on the lipoxygenase and lipoperoxidase functions of Cyt-CL was studied by adding 50 μL of an antioxidant solution of a certain concentration before the background luminescence registration started.

Cell Toxicity

A2780 cells were cultured in RPMI-1640 complete medium in cell culture flasks and passaged two times a week for routine culture. A2780-Adr cells were cultured in RPMI-1640 complete medium containing 100 nM doxorubicin HCl. For cytotoxicity experiments, 3000 A2780 and A2780-Adr cells were seeded in each well of 96-well flat-bottom cell culture treated plates and allowed to grow 24 h prior to the treatments. The cells were treated with empty CL particles, Cyt-CL particles, or free CytC in phosphate buffer in serum-containing complete medium for 24 h and 48 h continuously.

5% (*v*/v) methanol-containing buffer was used as the control and cell viability was measured by the CellTiter-Blue® assay according to the manufacturer's protocol. All treatments were carried out in triplicate.

Apoptosis Enhancement by Cyt-CL

The externalization of phosphatidylserine at the cell surface as an indicator of apoptosis was investigated by flow cytometry using annexin V and propidium iodide (PI). A2780 and A2780-Adr cells were seeded into the wells of 12-well plate at a density of 80,000 cells/well. The cells were allowed to attach and grow for 24 h and then treated with empty CL particles, Cyt-CL particles, free CytC in phosphate buffer, and buffer control for 48 h in serum-containing complete medium at the CytC concentration of 62.5 μg/ml. Following the treatments, the cells were harvested, washed with the PBS three times and resuspended in the annexin-binding buffer. The cells were analyzed by flow cytometer (FACSCalibur, Beckton Dickinson, Franklin Lakes, NJ) using Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit according to the manufacturer's protocol. The fluorescent signal from the cells was measured using 488 nm blue laser for excitation and FL1 (for Alexa Fluor® 488, 530/30 bandpass filter) and FL3 (for PI, 670 longpass filter) channels for recording. A total of 10,000 live cells were acquired after gating the population.

RESULTS

Cyt-CL Complex Preparation and Characterization

Upon the mixing of CL with CytC solution, a red-brown colored dense dispersion was immediately formed, the particles of which are the microcrystals composed of microspheres of Cyt-CL (19). The centrifuged sediment was first resuspended in the PBS, pH 7.4, and then subjected to the probe sonication, which resulted in the clear red-colored solution containing the nanoparticles with the size of 93.8 ± 1.35 nm, PDI of 0.340, and zeta potential of −61.11 ± 1.21 mV (for TOCL:CytC molar ratio of 40:1). The detailed particle size information of the formulations is given in Table 1. The sonication of the formulations with different molar excess of TOCL yielded similar sized nanoparticle dispersions (data not shown). For the further cell culture experiments, the probe-sonicated Cyt-CL formulation with 40 mol excess of TOCL was used, due to complete coverage of CytC molecules by TOCL, its small particle size distribution and high Cyt-CL peroxidase activity.

Cyt-CL Nanoparticles Demonstrate Efficient Cancer Cell Killing

The A2780 and doxorubicin-resistant A2780-Adr cancer cells were treated with Cyt-CL nanoparticle complexes. At the end of the 48 h treatment time, the wells were washed twice with the cell culture medium prior to the addition of the CellTiter-Blue® reagent into the wells. The cell viability results were normalized compared to the non-treated control cells and graphed against the CytC concentration. The weight ratio between the TOCL and CytC in the nanoparticles was 4.9 (the molar ratio was 40/1) and the TOCL concentration in the empty CL group was the same as in the Cyt-CL complexes. 5% methanol in the PBS, pH 7.4, was used as the buffer control to investigate the effect of the dispersion medium. Free CytC was applied in only buffer. The A2780 sensitive cell cytotoxicity results are given in Fig. 1. As can be seen from the results, the buffer cytotoxicity was negligible in all concentrations. Moreover, free CytC did not exert cytotoxic effect even at the highest concentration; only ~15% cell death was obtained at 1 mg/ml CytC. The empty CL formulations caused higher cytotoxicity compared to buffer control or free CytC at the studied concentrations. However, the Cyt-CL cytotoxicity was still significantly higher than that of the empty CL formulations, in particular, at low Cyt-CL (and empty CL) concentrations. The IC_{50} of the Cyt-CL complex have been found to be 0.27 ± 0.06 mg/ml. The results suggest that the Cyt-CL complex could induce apoptosis, while CytC is ineffective when administered as its free form. This change in the activity could be explained by the appearance of the peroxidase activity of CytC when complexed with TOCL as reported previously (9,37).

Then, the cytotoxic effect of the Cyt-CL complexes against multidrug resistant (MDR) cells was investigated using doxorubicin HCl (Adriamycin®)-resistant A2780-Adr cells. The treatment results are presented in Fig. 2. Free CytC and the buffer treatments were found to be non-cytotoxic in a wide range of concentrations, similar to the A2780 sensitive cell line results. Cyt-CL nanoparticle complexes were able to kill the MDR cells in a dose-dependent manner, and the cytotoxicity of the Cyt-CL complexes was significantly higher than the empty CL formulations at all studied concentrations. The IC_{50} of the CytC was found to be 0.46 ± 0.03 mg/ml, when it is formulated in Cyt-CL nanoparticles. The results clearly show that the Cyt-CL nanoparticles were effective against even the Pgp (38,39) and Bcl-2 (40) overexpressing MDR cells, despite their elevated resistance mechanisms that allow them to bypass apoptosis by preventing endogenous CytC release into the cytoplasm.

When the empty CL results were compared between the sensitive and MDR cells, it was found that the cytotoxicity values of the only TOCL-containing formulation were significantly different only at the concentration that corresponds to

Table I Summary of particle size, polydispersity index and zeta potential values of the complexes

TOCL:CytC (mole:mole)	Time after preparation (hours)	Mean particle size (nm)	PDI*	Zeta Potential (mV)
25:1	$t = 0$	217.1 ± 77	0.254	−54.62 ± 2.16
	After resuspension	225.1 ± 79	0.242	
35:1	$t = 0$	248.6 ± 86	0.235	−64.77 ± 1.94
	After resuspension	252.5 ± 90	0.264	
40:1	$t = 0$	218.7 ± 73	0.206	−63.94 ± 1.64
	After resuspension	218.0 ± 75	0.225	
	After probe sonication	93.8 ± 1.35	0.340	−61.11 ± 1.21
Only TOCL	After probe sonication	154.3 ± 3.73	0.358	-58.66 ± 6.21

*PDI polydispersity index

62.5 μg/ml CytC (Fig. 3A). This suggests that the empty CL cytotoxicity is not cell type- and drug resistance-dependent. On the other hand, the Cyt-CL nanoparticle cytotoxicity was slightly lower in the A2780-Adr cells compared to their sensitive derivative, which is also evident from the IC_{50} values, due to elevated resistance mechanisms (Fig. 3B).

A comparison of the diagrams in Fig. 3A and B shows that, although the complex Cyt-CL exhibited a pronounced cytotoxic effect, the latter was obscured by the fact that Empty CL also had a cytotoxic effect, especially at high concentrations (Fig. 3A). This action was nonspecific and almost the same in A2780 and A2780-Adr cells. At low concentrations of Cyt-CL and CL (31 and 62 mg CytC/l), cytotoxic effect of Cyt-CL exceeded that of Empty CL by 8 and 5 times. The nature of nonspecific cytotoxic effects of CL remains unclear. In the next series of experiments, we used a low concentration of Cyt-CL (62,5 mg CytC/l), in which the effect of the CL was absent or very small (Fig. 4).

Cyt-CL Nanoparticle Treatment Enhances the Apoptosis

To investigate the factors that are resulted in the effective cell killing of sensitive and resistant ovarian cancer cells, we have analyzed the apoptosis enhancement following the formulation treatment. The results are given in Fig. 4. Annexin V was used as the early apoptosis marker of the cells that preserve the membrane integrity. The translocation of the phosphatidylserine from the inner cell membrane to the outer surface allows annexin V to recognize the apoptotic cells and stain them. PI was used as the second dye to distinguish the cells with damaged membranes, i.e. necrotic and dead cells. It should be noted that using this double stain method does not allow to distinguish the necrotic cells from the late apoptotic cells due to the diminished membrane integrity.

To better investigate the apoptosis enhancement properties of the formulations, instead of their cell killing action, we have treated the cells at 62.5 μg/ml CytC concentration. As can be

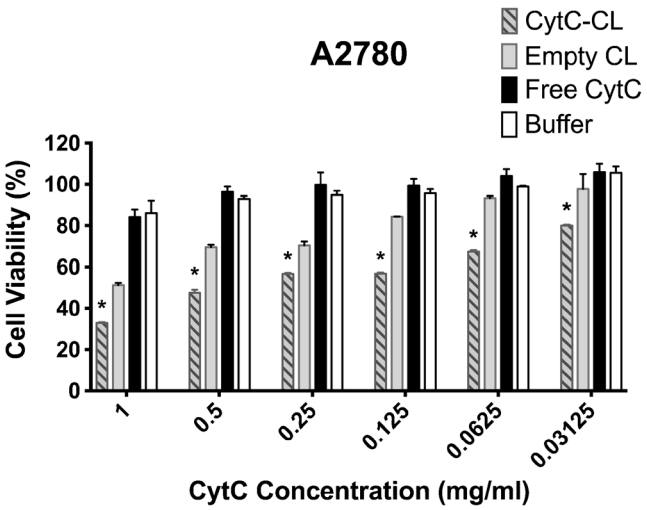

Fig. 1 Cell viability of A2780 cells after 48 h continuous incubation with different formulations at various concentrations of CytC. Data shown indicate the triplicate mean ± SD, **P < 0.0001*, two-way ANOVA with Tukey's multiple comparison test.

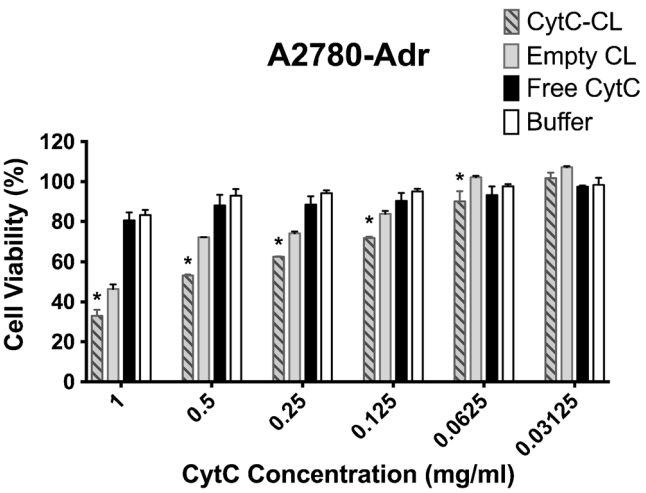

Fig. 2 Cell viability of MDR A2780-Adr cells after 48 h continuous incubation with different formulations at various concentrations of CytC. Data shown indicate the triplicate mean ± SD, **P < 0.0001*, two-way ANOVA with Tukey's multiple comparison test.

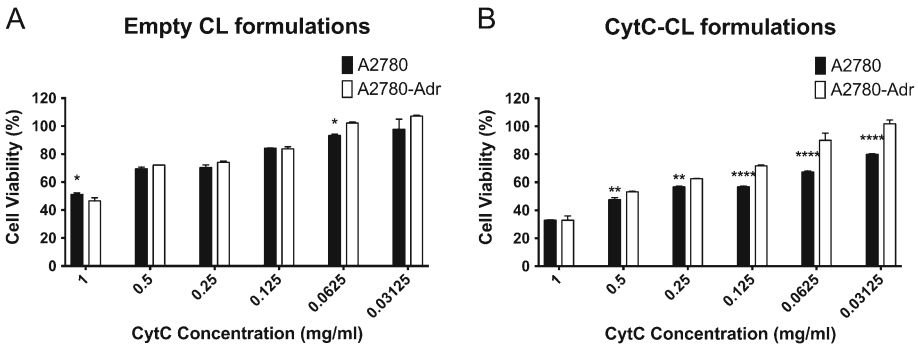

Fig. 3 Comparison of the cell viability values of empty CL and Cyt-CL formulations between the A2780 sensitive and A2780-Adr cell lines. Data shown indicate triplicate mean ± SD, $^{**}P < 0.02$, $^{***}P < 0.0002$, $^{****}P < 0.0001$, two-way ANOVA with Sidak multiple comparison test.

seen from the Fig. 4, even at this low concentration of CytC, the Cyt-CL complexes caused significantly increased apoptotic cell populations in both sensitive and MDR cell lines. The viable cell population was somewhat higher in the A2780-Adr cells, which supports the cytotoxicity results. At this low concentration, empty CL formulations did not cause any significant cell death or apoptosis when compared to free CytC or the control. The same concentration of CytC in nanoparticle complexes resulted in more apoptosis enhancement in the A2780-Adr cells compared to A2780 cells, in which they show mainly cell killing effect. The results suggest that the Cyt-CL complexes successfully enhance the apoptotic mechanisms in the both studied sensitive and resistant cell lines, which in turn causes significant cytotoxic efficacy against them.

Lipoperoxyl Radical Formation in Pseudo-Lipoxygenase and Lipoperoxidase Reactions Catalyzed by the Complex Cyt-CL Detected by C-334 Enhanced Chemiluminescence

In an attempt to identify the mechanisms underlying the anticancer activity of Cyt-CL nanocomplexes, we have investigated some aspects of the enzymatic activity of these complexes. Lipid peroxidation is a chain reaction mediated by the reactions

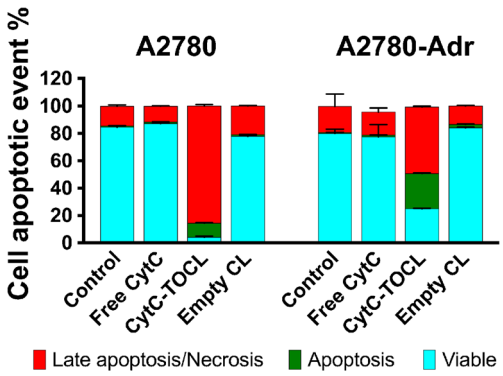

Fig. 4 Different cell populations after the 48 h continuous treatment with the formulations at low dose of CytC. Values represent the percentage of gated 10,000 events after annexin V and PI dual staining (Error bars represent mean ± SD, $n = 3$).

of lipid free radicals $L^{\bullet}$ and $LOO^{\bullet}$, forming and disappearing at each coin of the chain. The interaction of two $LOO^{\bullet}$ radicals is accompanied by the "ultra-weak" luminescence (41,42), which can be increased by several orders of magnitude with special CL enhancers, quinolizin-(9a,9,l-gh)-substituted coumarins, such as C-525, C-338, etc. (35). They typically do not influence the kinetics of the chemical reaction (35) and the biological status of cells (43), but strongly increase the quantum yield of the chemiluminescence due to the electronic energy transfer from the CL-reaction product to the enhancer (35). In this paper, we have used the C-334 as the enhancer in order to determine the steady state concentration of $LOO^{\bullet}$ in reactions, catalyzed by Cyt-CL in poly-unsaturated fatty acid (PUFA)-containing systems, hoping to model the reactions occurring in biological membranes in the presence of Cyt-CL complex.

The addition of the natural cardiolipin (BCL) to CytC brings about (1) the formation of Cyt-CL complex and (2) the reaction of Cyt-CL with preexisting in the system lipid hydroperoxides (LOOH). The latter represents the heterolytic cleavage of LOOH (44) in the one-electron reduction reaction:

$$LOOH \rightarrow LO^{\bullet} + OH$$

In the presence of oxygen, $LOO^{\bullet}$ radicals would form, and C-334-dependend chemiluminescence is expected:

$$LO^{\cdot} + LH + O_2 \rightarrow LOH + LOO^{\cdot} \rightarrow C-334-\text{dependent luminescence}$$

This hypothesis was supported by the experimental results shown in Fig. 5.

The curves shown on the left panel in the Fig. 5 represent the pseudo-lipoxygenase process induced by Cyt-CL and show also the comparison of the chemiluminescence kinetics in presence of Cyt-CL with the kinetics provided by the stand-alone components of Cyt-CL system. The addition of the free CytC in PBS did not show any chemiluminescence (the beginning of the CL-curves in both diagrams in Fig. 5). In contrast, the bovine heart cardiolipin mediated the C-334-dependent chemiluminescence even in the absence of the CytC (Left diagram, curve BCL). Whatever the mechanism is, the chemiluminescence shows the current concentration of

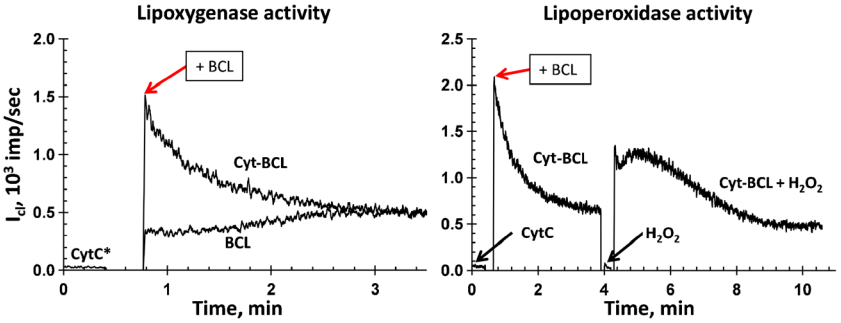

Fig. 5 Kinetics of coumarine-induced chemiluminescence in the presence of CytC-BCL complex. **Left:** CL kinetics in solutions of CytC, BCL and CytC + BCL (CytC-BCL complex) in PBS. *Curves*: *CytC-BCL: 25 μM C-334, 10 μM CytC, 600 μM BCL*; *BCL: 25 μM C-334, 600 μM BCL*; *CytC: 25 μM C-334, 10 μM CytC (* chemiluminescence of that mixture was similar to that of 25 μM C-334 methanol solution)*. **Right:** Lipoperoxidase reaction in presence of CytC-BCL complex. The reaction starts after the addition of H_2O_2 to CytC-BCL pre-incubated during 3.5 min (left curves). The concentration of the added H_2O_2 was 150 μM.

lipid peroxyl radical ($LOO^{\bullet}$) appearing in the system as a result of the autoxidation of PUFA residues, spontaneous or catalyzed by the transient metal contaminations in the system (35,45).

This "background" luminescence should be subtracted from that associated with the formation of $LOO^{\bullet}$ radicals during the decomposition of the hydroperoxides catalyzed by Cyt-CL. Subtracting the BCL curve from the CytC-BCL curve, we obtain the kinetics of the lipoxygenase processes catalyzed by the Cyt-CL complex, which, as expected, has an exponential form (calculation data not shown).

Since the formation of lipid peroxyl radicals occurs in the absence of H_2O_2, but in the presence of oxygen, the reaction may be called pseudo-oxygenase.

After adding hydrogen peroxide to the same probe, we see the formation of a new portion of lipid radicals, which provide a rise of chemiluminescence. Observed chemiluminescence kinetics is different from the kinetics observed during the decomposition of lipid hydroperoxides. This suggests that in this case, a new reaction proceeds, PUFA (LH) oxidation by hydrogen peroxide, and $LOO^{\bullet}$ radicals are formed:

$$LH + H_2O_2 \rightarrow LOO^{\cdot} \rightarrow C{-}334{-}\text{dependent luminescence}$$

The mechanism of this reaction is similar to the mechanism of the luminol radical formation when luminol is oxidized by the horseradish peroxidase or Cyt-CL, and therefore such a reaction should be called a lipoperoxidase reaction.

We hypothesize that the quasi-lipoxygenase and lipoperoxidase reactions occur in mitochondria under the conditions of the oxidative stress and lead to the apoptosis, which is consistent with the literature data (7,9,27).

The Effect of Antioxidants on Cyt-CL Lipoperoxidase Function

The luminescence kinetics found in the study on the effect of antioxidants on the formation of radicals in the reaction catalyzed by Cyt-CL complex in the presence of hydrogen peroxide, confirms the hypothesis that in presence of hydrogen peroxide, the Cyt-CL complex acquires the properties of the peroxidase.

We have shown earlier that (1) antioxidants that have a high rate constant of coupling with radicals ("strong" antioxidants), such as Trolox, increase the latent period of the chemiluminescence in the peroxidase reaction, catalyzed by the horseradish peroxidase, (2) antioxidants with low rate constant ("weak antioxidants"), e.g. Mexidol, decrease the intensity of the luminescence throughout the reaction, and antioxidants of medium strength change the initial rate of the luminescence development (46). Figure 6 shows that at the given concentrations, Trolox (left diagram) had no effect on the kinetics of the lipoxygenase reaction, while the lipoperoxidase reaction was temporarily suppressed in the presence of the same concentrations of Trolox (the latent period of chemiluminescence was observed).

Taxifolin (Fig. 6, right diagram) in given concentrations insignificantly reduced the intensity of the luminescence in the lipoxygenase reaction. In the same concentrations, taxifolin depressed free radical formation in lipoperoxidase reaction showing the significant decrease in the light intensity over the entire time interval. The fact that two different antioxidants demonstrated the same effect on the kinetics of the formation of radicals in the "classic" peroxidase reaction (horseradish peroxidase with luminol as a substrate) and on the kinetics of the lipid radical production by the Cyt-CL complex in presence of H_2O_2, one can consider as an evidence that the mechanism of the lipoperoxidase reaction catalyzed by the Cyt-CL is similar to that in typical peroxidase reactions.

DISCUSSION

The apoptosis is the main physiological process that cleans and safely eliminates the damaged cells. Mitochondria serve as the arena of the cascade of events that lead the cell to its death. CytC release from the mitochondria into the cytoplasm due to mitochondrial membrane permeabilization and

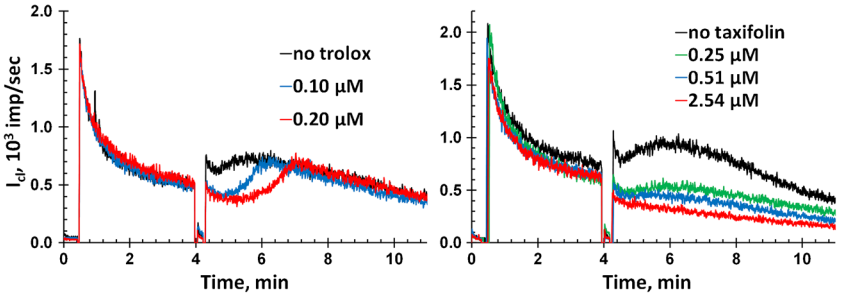

Fig. 6 Effect of antioxidants on CytC-BCL-induced lipoperoxidase reaction. **Left** – effect of Trolox. **Right** – effect of taxifolin. Reaction system: ***Antioxidant*** + 25 μM C-334, 10 μM CytC, 600 μM BCL, 100 μM H_2O_2. Numbers at the curves are concentrations of antioxidant, μM. I_{cl} – Intensity of chemiluminescence, 10^3 imp/s.

subsequent activation of caspases is considered as the starting point of this pathway (47). In cancers, this pathway of programmed cell death is inhibited via many different mechanisms (48) to allow cancer cells to proliferate, metastasize and differentiate. When CytC is released into the cytosol, it directly activates Apaf-1, which leads to the cascade resulting in the activation of the effector caspase-3. Bcl-2 or Bcl-xL expression inhibits the CytC release from the mitochondria in cells as well as from the isolated mitochondria (49,50). These genes are significantly overexpressed in many MDR cancers.

In the current study, our aim was to introduce the apoptosis in cancer cells by adding Cyt-CL nanoparticle complexes from outside. While the formation of the reverse-micelle like structure of the Cyt-CL complex formation has been explained earlier, briefly, the addition of the concentrated methanolic CL to the CytC solutions results in the coating of the CytC surface by the CL molecules, and the resulting nanospheres form microcrystals having low solubility in water (19,21). We have used TOCL as the lipid of choice, which is not susceptible to the lipid peroxidation by itself and by CytC (51).

When the particle size data of the formulations were evaluated (Table 1), it can be seen that the size of the particles does not change with the increasing ratios of TOCL over CytC, which supports the previously published hypothesis that there is no intermediate phase of the complex formation. The positive charge of the CytC was shielded by coverage the protein surface by the TOCL molecules and the surface area of the CytC was completely covered at and above 35-fold mol excess of the highly negatively charged lipid. When the equilibrium of complete surface coating of the CytC is reached, the negative zeta potential of the particles increases. Probe sonication of the complexes with 40x mol excess of TOCL yielded a clear, red-pink colored solution without any visible precipitate. The micelle-looking dispersion gave smaller particle size but same zeta potential as its non-sonicated counterpart. Further centrifugation at 13000 rpm for 30 min did not result any precipitation due to small particle size of the microcrystals. Short sonication pulse time (20 s) and long delays between the pulses prevented both the heat generation which can cause significant degradation of the protein and also the formation of excessive air cavities during the process. In the light of the mentioned results, we believe that the sonication energy did not destruct the Cyt-CL nanoparticles but rather dispersed the dense aggregates of the microcrystals.

The cytotoxicity evaluation of the formulations revealed that the Cyt-CL nanoparticles can successfully kill the cancer cells, while the free CytC does not exert a cytotoxic effect. This can be explained by the impermeability of cell membrane for the free CytC and also means that Cyt-CL is unable to destroy cell membrane despite of its lipoperoxidase property (44). Importantly, the cytotoxic effect was shown both for drug-sensitive cells (Fig. 1) and MDR cells (Fig. 2). The CytC IC_{50} values of 0.27 mg/ml and 0.46 mg/ml for A2780 and A2780-Adr cells, respectively, corresponds to ca. 0.022 and 0.037 μM. As a comparison, the IC_{50} values of the most commonly used anticancer drugs for ovarian cancer were extracted from The Genomics of Drug Sensitivity in Cancer (GDSC) database (52) and were summarized in Table 2. The data indicates that the Cyt-CL is significantly more effective than majority of the drugs used for this ovarian cancer cell. Moreover, our results become even more intriguing when the A2780-Adr IC_{50} value was taken into consideration. It has been reported that MDR A2780 cell lines are resistant to anticancer drugs 18 to 65 times more compared to their parental cell line A2780 (53–55). However, the IC_{50} of the CytC in our Cyt-CL nanoparticle formulation was only 1.7-fold higher, which indicates a successful overcoming of

Table 2 Most commonly used anticancer drugs and their IC_{50} values for A2780 human ovarian cancer cell line

Drug	IC_{50} (μM)
Cisplatin	4.930
5-Fluorouracil	1.970
Doxorubicin	0.069
Methotrexate	0.047
Cyt-CL	0.022
Gemcitabine	0.007
Docetaxel	0.005

resistance in A2780-Adr ovarian cancer cells with our delivery strategy as discussed in further detail in the following sections.

It has been shown that the Bcl-2, which can prevent the CytC release from the mitochondria, is significantly overexpressed in the A2780-Adr cells as a result of their MDR phenotype (40). The common resistance mechanisms the cancer cells deploy to prevent undergoing apoptosis, will interfere with the mitochondrial apoptosis pathway and result in the inhibited apoptosis. In particular, Bcl-2 overexpression gives rise to the inactivation of BH-123 pro-apoptotic proteins, Bax and Bak, that are responsible for the mitochondrial outer membrane permeabilization (MOMP) (See review (3)). We were able to bypass these mechanisms by directly introducing Cyt-CL into the cells and, probably, also in mitochondria (Figs. 7, *1–2*). The subsequent events may be illustrated by the scheme in Fig. 7. One way is the interaction of CytC, released from Cyt-CL nanoparticles inside the cytoplasm (Fig. 7-*3*), with other apoptotic proteins that induces the caspase cascade activation, ending with the cell death. Despite its simplicity, this mechanism still seems questionable, since CytC has a very high affinity to cardiolipin, so that it would rather pull the cardiolipin phospholipid out of the bilayer membrane, than release it into such a highly polar medium like water. The second way of Cyt-CL action is its penetration through the outer mitochondrial membrane (OMM) and incorporation into IMM (Figs. 7-*4*). We may hypothesize (Cf. (18, 23)) that Cyt-CL nanosphere would diffuse in IMM along the membrane lipid layer until rich VDAC-ANT complex, with continuous peroxidation of lipids by mechanisms described in this paper (see the Results section on pseudo-lipoxygenase and lipoperoxidase reactions and Figs. 7, *5–6*). The lipid peroxidation brings about the mitochondrial matrix swelling (56–58) and opening megapores in OMM (59). In cell cultures, the peroxidation of cardiolipin immediately preceded CytC efflux from the mitochondria (9). Hence, the way of the apoptosis development via the route 1–2, 4–7 in Fig. 7 is in a good agreement both with our and literature data.

The third possible way of cell death, consistent with our experiments, is shown in Figs. 7, *9*. According to this hypothesis, Cyt-CL incorporates directly into the cell membrane and catalyzes the lipid peroxidation via quasi-lipoxygenase and lipoperoxidase mechanisms described earlier in the results.

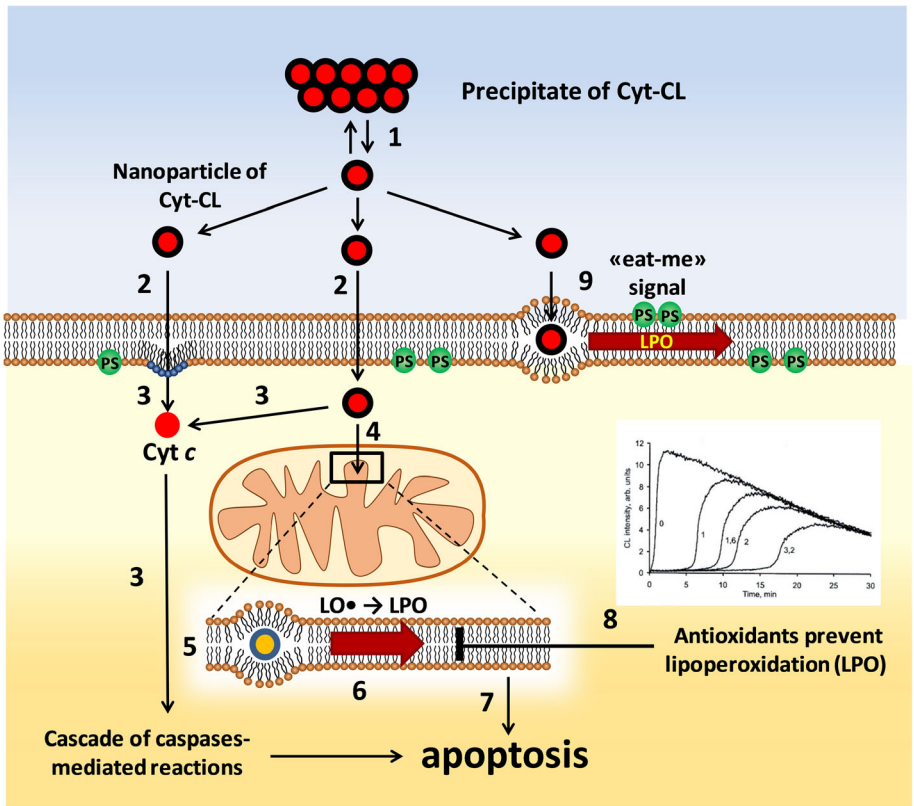

Fig. 7 The possible mechanisms of pro-apoptotic and cytotoxic effects of Cyt-CL on cancer cells. 1. Cyt-CL microcrystals are in equilibrium with hydrophobic nanospheres of Cyt-CL complex. 2. Complex Cyt-CL can pass through the cell membrane. 3. The nanospheres gets rid of its lipid envelope, and "naked" cytochrome *c* enters the cytosol. This event could activate the cascade of caspase-mediated reactions terminated by the cell death, apoptosis. 4. A more likely event is the passage of cytochrome CL in the mitochondria and its inclusion in the IMM. 5. The hydrophobic nanosphere Cyt-CL diffuse inside lipid bilayer and (6) initiate lipid peroxidation anywhere along its way. 7 –In the end, the above processes lead to apoptosis. 8 – Antioxidants block the peroxidation. 9 – Alternatively to step 2, Cyt-CL can be embedded in the outer membrane and catalyze lipid peroxidation. This will lead to the externalization of phosphatidylserine and the appearance on the cell surface of the signal "eat me" to phagocytes.

This is followed by the lipid peroxidation and phosphatidyl serine (PS) appearance on the cell surface. The PS is a "eat me" signal for macrophages, which recognize the damaged cell and remove it. The mechanisms of the lipid peroxidation in the cell membrane, catalyzed by CytC and followed by scramblase inactivation, PS oxidation, and its externalization, are now well documented (60,61). In our experiments, these phenomena may explain the data obtained using the annexin V to recognize the apoptotic cells (Fig. 4). The more durable treatment of cells with Cyt-CL could destroy the membrane lipid layer integrity and increase the membrane permeability for PI (late apoptosis/necrosis in Fig. 4). As a result, the cells became not viable (that is they lose the ability to convert a redox dye (resazurin) into the fluorescent end product, Figs. 1, 2 and 3). Note that the mechanism based on the assumption that the target for Cyt-CL peroxidase activity is the cell membrane (*1*, *9* in Fig. 7), explains the equal response of sensitive A2780 cells and doxorubicin-resistant A2780-Adr cells (as well as the mechanism based on the assumption that the inner mitochondrial membrane is the target, Figs. 7, *1–3*, *4–8*). To differentiate between these two possibilities, further investigations will be performed. It should be noted that our study outlines the proof of principle of an alternative approach and reports that the Cyt-CL nanoparticles acts as a complete system with all of its components. Thus, in the future studies cancer cell targeting would be required, as for any other neoplastic agents, not only to minimize the toxicity against noncancerous cells *in vivo* but also to overcome the off-site empty CL cytotoxicity.

Conclusion

Whichever the precise mechanism of the Cyt-CL cytotoxic and pro-apoptotic action, our results indicate that the extra/intracellularly delivered Cyt-CL nanoparticle complexes could successfully start the apoptosis cascade in both the sensitive and drug resistant cancer cells (ovarian cancer cells in this particular case). Delivering these complexes into the cells can push the cells to enter the apoptosis even when they overexpress the factors that normally prevent them from doing so. The completely biomaterial-based Cyt-CL formulation is able to act like a nano-sized machinery in different parts of the cancer cells to start different cascades that cumulatively result in effective anticancer activity. Our finding could be of interest for developing novel approaches to cancer therapy.

Acknowledgments and Disclosures

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