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Effect of Tin and Lead Chlorotriphenyl Analogues on Selected Living Cells

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ABSTRACT: Three kinds of living cells, *human embryonic kidney cells*, *Saccharomyces cerevisiae*, and *Escherichia coli*, were tested for their sensitivity to chlorotriphenyltin and chlorotriphenyllead. The tin compound proved definitely more toxic than the lead derivative, particularly in the case of the human embryonic kidney cells devoid of any protective cell wall. Electron paramagnetic resonance (EPR) comparative studies carried out by using a natural model liposome system (egg yolk lecithin) confirmed considerable changes within the lipid bilayer upon doping by the aforementioned additives, which may be crucial to the mechanism of the observed cell cleavage. The individual dopants revealed diverse impact upon the membrane's condition, chlorotriphenyltin distinctly fluidized the lipid system, whereas chlorotriphenyllead stiffened the medium within the membrane. A theoretical approach concerning such different behaviors of studied tin and lead analogues because of their high toxicity in living cells has been presented. © 2010 Wiley Periodicals, Inc. J Biochem Mol Toxicol 25:231–237, 2011; View this article online at wileyonlinelibrary.com. DOI 10:1002/jbt.20380

KEYWORDS: **Chlorotriphenyllead; Chlorotriphenyltin; *Escherichia coli*; Electron Paramagnetic Resonance; Human Embryonic Kidney Cells; Yeast**

INTRODUCTION

Metalloorganic tin and lead derivatives have been considered as the most common toxicants for living organisms. Because of their widespread use in various chemicals, especially tin- and/or lead-based biocides [1], such compounds have entered into the environment and consequently accumulated in plants, animals, and microorganisms, thus poisoning them [2–4]. Organotin and organolead compounds readily dissolve in the lipid fraction of cells' membranes [5–7]. The degree of cell damage generally depends on the toxicant's chemistry, that is, molecular geometry, length of the alkyl chain, the presence of aromatic rings, and the metal involved, as well as its hydrophobicity. Tin- and lead-containing organic species usually cause cell lysis, which has been often related to the overproduction of reactive oxygen species (ROS) within the cellular system; however, the molecular basis of this process has not been resolved so far [8]. It is well known that these compounds affect a number of important biochemical processes. Organoleads are believed to produce arachidonic acid from biological membranes [9], whereas organotins are proved to mediate the chloride–hydroxide exchange across membranes [10] and were found to inhibit the ATP-ase complex in mitochondria [11] and chloroplasts [12]. In the literature, the lead compounds have been claimed as much more cell-destructive than those containing tin. Obviously, the final effect manifested in a cell is to be related to the peculiar chemical activity the metal derivative might have under specific conditions within the system. Hence, in some cases the results may not follow the commonly observed trend, as for Sn and Pb analogues of chlorotriphenylmethane (CTP-C) (Figure 1) explored in tests carried out on yeasts as well as model membranes. Interestingly, the Sn compound proved considerably more toxic than the Pb compound [13].

This work refers to a recent study on the influence of chlorotriphenyltin (CTP-Sn), chlorotriphenyllead (CTP-Pb), and CTP-C, as a nonmetallic reference, on eukaryotic human embryonic kidney cells (HEK 293) and selected microorganisms (*Saccharomyces cerevisiae* and *Escherichia coli*). Our study focused on a probable relationship between the cell's degradation and changes within a cell membrane due to exposure to stannous and lead lipophilic species. The electron spin

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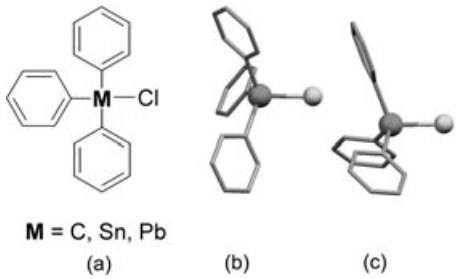

FIGURE 1. General chemical structure (a) and possible arrangement of phenyl rings in molecules of chlorotriphenyl-analogues of C, Sn, and Pb: propeller type (b) and edge-to-face type (c).

resonance (ESR) spin label technique was used to identify fluctuations in fluidity within the lipid bilayer due to doping. Essentially this method has been applied in model liposome studies, although it also proved successful in exploring the plasma membrane of the simple erythrocyte cellular system [14,15]. In our article, chemical and structural calculations based on density functional theory (DFT) were included to support the experimental work and to put forward a plausible explanation. It must be emphasized, however, that the hypothesis presented later to highlight the effect of the chlorotriphenyl analogues on the functionality of living organisms and to further initiate discussion on this subject. We are hopeful that any advancement in the understanding of the mechanism of organic Sn and Pb compounds' impact on liposome membranes and consequently on living cells may be relevant to their potential application to pharmacy (e.g., tumor treatment), toxicology, and ecology (poisoning prevention).

MATERIALS AND METHODS

Chemicals and Spin Labels

For doping of the biological material, pure grade chemicals: CTP-Sn, CTP-Pb, and CTP-C were purchased from Sigma-Aldrich (Poland). Analytical-grade dimethylformamide (DMF) was used to prepare solutions of the above-mentioned compounds. Two different spin labels (purchased from Sigma-Aldrich) were used in ESR tests: (1) 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and (2) 2-ethyl-2-(15-methoxy-15-oxopentadecyl)-4,4-dimethyl-3-oxazolidinyloxy (16-DOXYL).

Cells and Growth Conditions

Two different microorganisms and kidney tumor cells were characterized and were used in the study. To perform the cell growth, Yeast Extract Peptone Dextrose (YPD) (BioShop, Canada), LB, and Dulbecco Park Memorial Institute—Liquid Tissue Culture Medium (DPMI) (Difco, USA) media were applied.

(1) Human embryonic kidney cells (HEK 293) were maintained in a DPMI medium supplemented with 10% fetal bovine serum, 1% vitamins, and 1% penicillin–streptomycin–neomycin antibiotics (Invitrogen, USA). The cells were cured at 37°C with 5% CO_2 and maximum humidity. (2) *Saccharomyces cerevisiae* wild strain W303 *MATa ade 2–1 leu 2–3 112 his 3–11, 15 trp 1–1, ura3–1* [16] (W303) was used. The yeast was grown in the YPD medium (1% yeast extract, 1% bactopeptone, 2% glucose) at 30°C. For planting, the medium was supplemented with 2% bactoagar. (3) *Escherichia coli* strain *DH5α* supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA1 thi1 relA1[17] (DH5α) was cultivated in the LB medium (1% yeast extract, 1% bactotryptone, 0,1% glucose, 0,5% NaCl) at 30°C.

The cells were subjected to survival tests carried out according to a standard procedure [18] in a liquid medium supplemented with a definite amount of the particular dopant dissolved in DMF. The quantities of CTP-Sn and CTP-Pb fed into the investigated system are presented in Table 1. Exactly the same concentrations were used for CTP-C. The dopants' concentration ranged from 0.1 to 20 μM (μmol·dm^{-3}), which corresponds approximately to 0.05–10 μg·mL^{-1}. The content of DMF was 0.5% vol relative to the sample's volume. Similar tests were performed on two control groups: In the first one the pure medium was used for the cell growth and for the another group 0.5% vol of pure DMF was used.

ESR Method and Liposome Testing

Liposomes were obtained from hen egg yolk lecithin (EYL) and/or synthetic dipalmitoilphosphatidylcholine (DPPC) lecithin in distilled water, in a sonication process (ultrasonic disintegrator TECHPAN UD-20). The procedure included alternating cycles of 30 s of sonication followed by 40 s of cooling, and the total preparation time was 5 min for each 1.5 mL sample. The concentration of EYL and/or DPPC in each sample was fixed at 0.04 M, whereas that of the spin label was 0.5% relative to the lecithin (i.e., molar ratio to lipid is 0.005).

An electron paramagnetic resonance (EPR) spectrometer MX-201R (TU Wroclaw, Poland) was used in the tests under the following operating conditions: modulation amplitude 0.032 mT and microwave power 600–620 mW (damping 1 dB). In all experiments, the scanning range and the sweep speed of the EPR spectrometer were 5 mT (50 G) and 256 s, respectively, and the results are an average of three to five tests for an individual sample.

ESR studies were carried out by using the same spin probe technique reported in our previous work [19]. The tested dopants (dissolved in DMF) were

TABLE 1. Results of Survival Tests for Living Cells HEK 293, W 303, and DH5α Exposed to CTP-Sn and CTP-Pb

		Cell Growth[a] vs. Dopant Concentration (μM)									
Cell Strain	*Dopant*	*0.1*	*0.2*	*0.4*	*0.6*	*1.0*	*2.0*	*5.0*	*10*	*15*	*20*
HEK 293	CTP-Sn	–	–	–	–	–	–	–	–	–	–
	CTP-Pb	+	+	+	±	–	–	–	–	–	–
W 303	CTP-Sn	+	+	+	+	±	–	–	–	–	–
	CTP-Pb	+	+	+	+	+	±	–	–	–	–
DH5α	CTP-Sn	+	+	+	+	+	±	–	–	–	–
	CTP-Pb	+	+	+	+	+	+	+	±	–	–

[a] (+) Intensive growth, (±) weak growth, (–) without growth.

added to a suspension of previously formed liposomes, and their concentration was gradually increased from 0% to 10% (molar) relative to the lecithin. The amount of DMF introduced into the system was in proportion to that used for the living cells (0.5% vol). Measurements were performed at 22°C, and the total time of a single test did not exceed 120 min. Two diverse spin probes sensing different parts of the membrane were used. The TEMPO spin label penetrates to the interface region of the lipid bilayer and dissolves in both the hydrophobic part of the membrane and in the water medium. From the ESR spectra of this spin probe, the spectroscopic partition parameter (F), reflecting the probe distribution between the membrane and its ambient, was determined. The value of F is expressed as a ratio of amplitudes corresponding to the low-field line (H) related to the lipid medium and the high-field line (P) referred to the water ambient (Figure 2a). It is also related to the fluidity of the membrane's surface layer [20]. The 16-DOXYL spin probe locates itself in the central region of the lipid bilayer. From the spectra of this probe, the spectroscopic parameter τ (rotational correlation time) was determined. The value of τ depends among other factors on the membrane's fluidity degree and the greater it is the more rigid (better organized) is the ambient in which the spin label has been placed [21] (Figure 2b). A relative error determined in the measurements of F and τ parameters was 5% and 3%, respectively.

Molecular Modeling by the DFT Method

Theoretical calculations were performed to evaluate the potential impact of molecular structure related factors on the diverse behavior of the applied dopants in the lipid medium and consequently in the cell. A similar procedure was used in our previous work to assess structural details in some other lipophilic metalloorganic systems [22]. All structures of the CTP-M molecules (M = C, Sn or Pb) studied in this work were fully optimized starting from the two possible geometries with different arrangements of the phenyl rings (see Figures 1b and 1c). All calculations were carried out with Gaussian 09 [23] via DFT, using the hybrid functional B3LYP [24]. Two kinds of basis sets corresponding to different atoms were used: 6-31G** for C, H and Cl and LANL2DZ for Sn and Pb (with the keyword genecp). The bond energies of the M-Cl bonds were estimated by single-point potential energy scans and an M to Cl distance was used as a variable in the scanning. Results are presented in Table 2.

RESULTS

Survival Tests

All investigated cell strains exhibited typical and stable growth while cured in the respective media only,

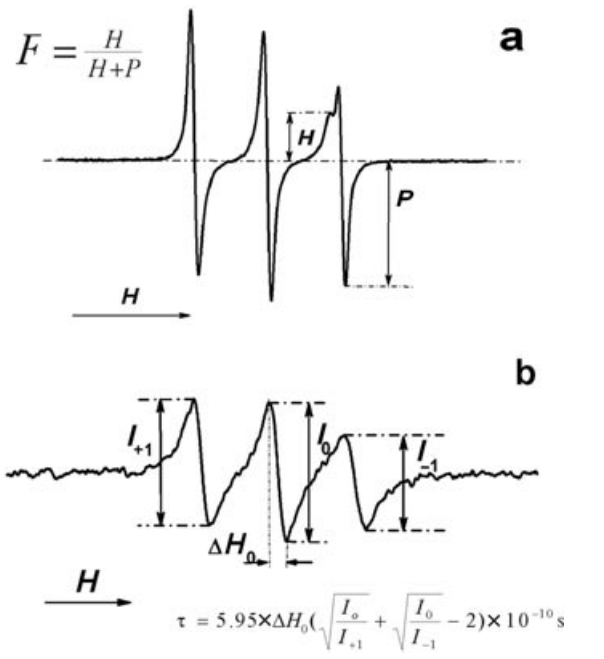

FIGURE 2. ESR spectra provided by the TEMPO (a) and 16-DOXYL spin labels (b).

TABLE 2. Structural Details of the Explored Chlorotriphenyl-Derivatives (CTP) Provided by DFT Calculations

			Partial Electric Charge per Atom				*Distance, (Å)*	
Compound	*Structure Type*[a]	E^{b}_{M-Cl} *(kcal/mol)*	C^{c}_{ph}	*M*	*Cl*	$\mu_D(D)$	*M–Cl*	C_{ph}–*M*
CTP-C	Propeller	82.3	+0.15	−0.32	−0.11	2.35	1.906	1.534
CTP-Sn	Propeller	103.6	−0.32	+1.15	−0.44	3.86	2.381	2.127
	Edge-to-face		−0.32	+1.16	−0.45	3.70	2.382	2.131
CTP-Pb	Edge-to-face	97.0	−0.25	+0.96	−0.47	4.28	2.459	2.182

M = C, Sn, Pb.
[a] Ref. Figure 1.
[b] Bond energy M–Cl.
[c] Phenyl C atom linked to M.

and addition of 0.5% of DMF did not affect the growth at all. Hence, the phenomenon observed for the doped cell-systems may definitely be attributed to the activity of the admixture applied.

The base-compound, CTP-C, proved completely passive in the tests and exhibited no destructive action against the cells. Thus, the lethal effect revealed by the other compounds is to be because of the metal they contained, that is, Sn or Pb. In fact, the result of cell doping depends on both the cell type and physicochemical properties of the metalloorganic compound applied. In all cases tested, the tin compound, CTP-Sn, appeared distinctly more toxic to the cells than the lead derivative (Table 1). The impact of CTP-Sn was more pronounced in the case of HEK 293 cells in which the dopant produced 100% cell-mortality within the concentration range explored (Figure 3). In the case of CTP-Pb, a comparable lethal effect was triggered off only when a dose of at least 1 μM was fed into the system. The other cell strains proved definitely more resistant, in particular the DH5α, which still exhibited a weak growth after doping by 2 and/or 10 μM of the Sn and Pb compounds, respectively. The yeast sample, W 303, showed similar susceptibility to both dopants; however, it seems to be somewhat more sensitive to the Sn derivative.

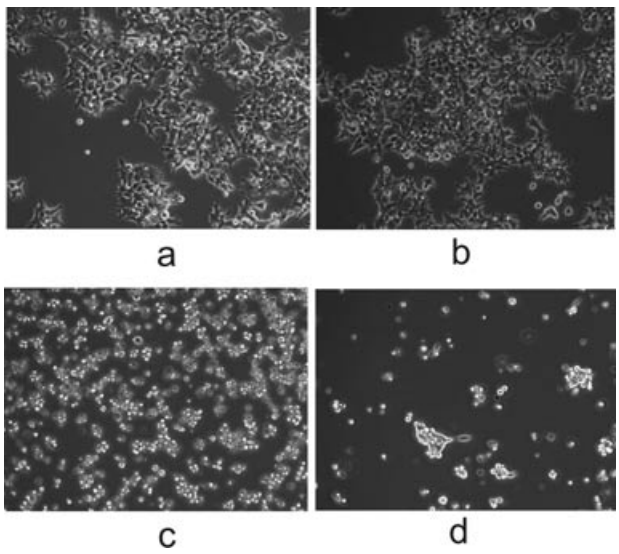

FIGURE 3. Micrographs of the HEK 293 cells cultivated on pure and doped DPMI media: (a) living cells on pure medium, (b) living cells on DPMI medium containing 0.5% vol. DMF, (c) dead cells on medium doped by 0.1 μM of CTP-Sn, and (d) dead and living cells on medium doped by 0.6 μM of CTP-Pb.

EPR Studies of Liposome Membrane

Liquid-Crystalline Lipid System

The EPR studies allowed to estimate the distribution of the CTP analogues inside the lipid double layer as well as their effect on the membrane's condition. The ESR spectra of the TEMPO spin label (penetrating to the edge of the membrane) show that only at concentrations of the dopants up to 2% different activities within the interface layer were recognized (Figure 4a). The most distinct effect was demonstrated by the Pb derivative, whereas the Sn compound did not affect the spin probe in a particular way. Higher dopant quantities only slightly influenced the partition parameter F, which practically became independent of the compound, presumably due to saturation of the membrane by particular additives used in the tests. It must be noted that DMF used to dissolve the organic dopants does affect the TEMPO–ESR spectra; the F parameter tends to increase with the increase in the DMF concentration in the liposome samples. Although the quantity of DMF in all samples was kept constant, time resolved changes in the concentration of the hydrophobic dopants are possible, which could have resulted in fluctuations in the fluidity of the lipid system within the interface layer. This fact may be attributed to the scattering of the data shown in Figure 4a, presumably reflecting the formation of specific dynamic lipid structures because of random variations in the dopant concentration. Such phenomenon may be crucial to the distribution of the studied additives throughout the liposome

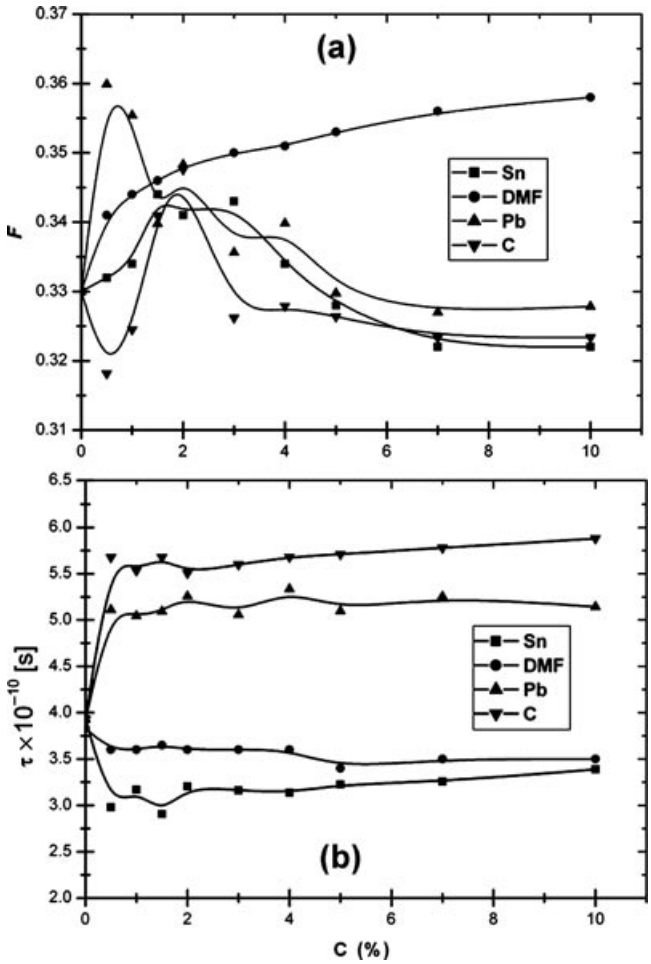

FIGURE 4. Effect of the dopant concentration (with respect to the lecithin) on the behavior of the spin labels within the liquid-crystalline EYL liposome membrane, based on the measured ESR spectra: (a) changes in the partition parameter (F) of the TEMPO spin label, and (b) changes in the 16-DOXYL spin probe relaxation rate (characterized by the τ parameter) reflecting the fluidity of the lipid system in the center of the membrane.

membrane. Similar changes in fluidity within the interface layer were reported in a previous work [25].

ESR spectra of the 16-DOXYL spin probe revealed significant dopant-induced changes in fluidity within the central part of the membrane. In this case, DMF does not affect the relaxation rate of the 16-DOXYL spin label, which means that the fluidity profile of the membrane's interior may not be related to the solvent. Either of the metal CTP analogue exhibited completely different impact on the lipid system inside the membrane, as shown in Figure 4b. The lead compound, CTP-Pb, produced a more rigid lipid structure within the bilayer relative to the undoped liposome sample, whereas addition of the tin derivative, CTP-Sn, definitely increased the fluidity of the lipid system as shown by a decrease in the τ value (rotational correlation time). Note that for concentrations of the Sn-dopant $\geq$2%, the fluidity tends to decrease (an increase in τ) presumably due to the saturation effect. However, the bilayer remains evidently more fluidized compared to its initial condition (while undoped). These results are perfectly similar to those reported in a previous work [13]. The nonmetal reference, CTP-C, has acted similarly to the CTP-Pb dopant but the membrane becomes even more rigid. By comparing the results provided by the TEMPO and 16-DOXYL spin labels, it is to be noted that the extent of changes in τ values was considerably greater than in the case of the F parameter. Maximum differences in τ for the doped liposomes relative to the control sample (undoped) amounted to 44% (CTP-C), 30% (CTP-Pb), and 24% (CTP-Sn), whereas for the F parameter they were only ca. 5% (CTP-C), 6% (CTP-Pb), and 3% (CTP-Sn). This indicates that the tested dopants display a definitely higher activity in the central part of the membrane than within the interface layer. Obviously, the interior of the lipid bilayer offers a more friendly environment for the hydrophobic and lipophilic chlorotriphenyl analogues than the region close to the membrane's surface. Hence, the studied dopants are concentrated in the bilayer's center rather than within the interface layers.

Gel-Phase Lipid System

The DPPC membranes proved considerably less permeable to the chlorotriphenyl dopants than the EYL ones. Penetration of the individual compounds to the membrane was found rather restricted. Furthermore, changes in the τ parameter characterizing the bilayer's interior (16-DOXYL) indicated the stiffening of the membrane's lipid structure due to doping, no matter what the dopant was. The most rigid structure was again revealed by liposomes containing CTP-C than CTP-Pb and the least effect was produced by CTP-Sn. However, in this case the raw experimental results turned out to be quite unsatisfactory (due to an unfavorable signal to noise ratio during the measurements) and hence did not allow us to precise quantitative conclusions.

Using Spin Labels in Living Cell System

In a parallel study, both the DOXYL and TEMPO spin labels were applied to the cell system of W303 yeast following the procedure used for the EYL liposomes. Our attempts failed because we were unable to measure any sensible ESR signals from the probes. This could be expected because only very simple cellular

systems (e.g., erythrocyte) seem suitable for ESR studies [14]. The cells investigated in our work are much more complex and thus the behavior of a radical spin probe anchored somewhere inside of such a membrane is practically unpredictable; hence, it is rather difficult to expect a clear-cut and reasonable result.

Dopant–Structure Effect on the Membrane Fluidity: Theoretical Considerations

Structural properties of the particular additives were analyzed for their possible influence on the fluidity of the studied lipid system. Both the metalloorganic species display a similar molecular size and are significantly larger than the typical organic CTP-C compound. Higher dipole moment (μ_D), different electronic density distribution, and hence definitely a stronger bonding of the Cl atom also distinguish the Sn and Pb derivatives from the basic C molecule. These factors may control the chemical behavior of the individual dopants within the liposome membrane. DFT calculations revealed that two stable and almost isoenergetic structures are possible for CTP-Sn (molecular energy difference is only 0.04 kcal/mol) and only the "edge-to-face" structure for CTP-Pb (Figure 1). Attempts to generate a stable "propeller" form of the lead derivative failed, and hence one may assume that most probably only one molecular modification is in favor of this case. However, the CTP-C was proved to adopt exclusively the "propeller" structure. The results presented in Table 2 indicate that due to inherent structure apparently similar (chemically) species, CTP-Sn, and CTP-Pb, display so extremely different membrane effects (Figure 4b). On the other hand, small molecule volume and distribution of the electronic charge as determined in CTP-C may explain its specific interaction with the phospholipid chains, which to some extent should restrict the lipid's movements, resulting in membrane's lower fluidity.

DISCUSSION

Results suggest that the impact of CTP-Sn and CTP-Pb on the studied living cells is due to the modification of properties of the cell membrane and substantial changes in fluidity in the lipid bilayer. Any fluctuations that increase or decrease the fluidity of the lipid bilayer may appear to be harmful to the cell itself. Both organometallic dopants produce such effects; however, they may be opposite in nature. Lipophilic and hydrophobic toxic species much easily penetrate to a more fluidized membrane, which could finally result in the disruption of the lipid bilayer system and consequently to the cell's death. The Sn compound, due to its strong fluidizing action, presumably produces cell lysis, which may account for the higher lethal factor in cells exposed to CTP-Sn than to those doped by the lead derivative (Table 1, Figure 3). On the other hand, stiffening of the lipid system within the membrane reduces its capacity to permeate vital nutrients to the cells. This might have explained the "delayed" effect of CTP-Pb particularly in the case of the HEK 293 cells (Table 1).

Evidently, the diverse susceptibility to the applied dopants demonstrated by the particular living cells follows from the structure of the cell wall and cell membrane. Eukaryotic cells HEK 293, which proved to be the least resistant, do not have a protective cell wall; hence, they may be rather sensitive to any alterations induced within the cell membrane. Definitely more protection is offered by the cell wall of yeast W 303, which consisted of chitin, manno protein, β1,3 glucan, and β1,6 glucan [26]. In this case, dysfunction of the membrane due to doping does not cause such a dramatic response to the weakened cellular system, as featured by the HEK 293 cells. The cell wall in *E. coli* (DH5α) is characterized by a multilayer structure including a large quantity of lipids, which offers evidently better conditions for the cells to survive a potential membrane cleavage. Most probably, the CTP dopants have been transferred to the cells by means of active carriers and membrane pumps, the lack and/or increased activity of which may have considerably affected the cell growth. Moreover, chemical modifications related to possible oxidation of cell membrane proteins could be relevant to the observed toxic effects, although it is not obvious whether CTP-Sn or CTP-Pb do somehow promote generation of ROS and in consequence protein peroxidation that may have destroyed the cell.

Diverse impact of the both metalloorganic compounds on the cell membrane may be related to their molecular structure. According to the DFT study, the tin compound (two isoenergetic structures possible) should be significantly more flexible and mobile than the rigid structure of CTP-Pb and therefore better adaptable to the dynamic conditions inside the lipid bilayer. This fact may highlight the peculiar ability of CTP-Sn to fluidize the membrane, as distinct from CTP-Pb. The less elastic and heavier lead species presumably creates only a kind of a static arrangement within the lipid system, which is supposed to stiffen the bilayer's setup. Despite the reduced fluidity, membranes doped by CTP-C seem to be easily permeable to supplementary ingredients important to the cells' survival, than in the case of the bulky CTP-Pb and CTP-Sn additives. Hence, CTP-C proved practically nontoxic for the studied living cells.

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