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The Expression of Genes Encoding Antioxidant Enzymes in Lung Adenocarcinoma A549 Cells Exposed to Cisplatin Supplemented by Polyunsaturated Fatty Acids*

Ekspresja genów kodujących enzymy antyoksydacyjne w suplementowanych wielonienasyconymi kwasami tłuszczowymi komórkach gruczolakoraka płuca A549 eksponowanych na cisplatynę

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Abstract

Background. The effectiveness of cisplatin (CPT) in the treatment of tumors is often limited by primary or acquired resitance. Supplementation with polyunsaturated fatty acids, in particular eicosapentaenoic acid (EPA, 20 : 5, n-3) and docosahexaenoic acid (DHA, 22 : 6, n-3), can enhance drug sensitivity of tumor cells to anticancer therapy or reverse cell resistance. Antioxidant enzymes, highly expressed in lung tumors, are among the determinant factors in the sensitivity of lung tumors to chemotherapy.
Objectives. Investigation of the effect of EPA and DHA at different concentrations on gene expression of selected enzymes controlling cell redox status, such as superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), catalase (CAT), phospholipid hydroperoxide glutathione peroxidase (GPx-4), and glutathione S-transferase pi (GST-pi) in human lung adenocarcinoma cells (A549) exposed to CPT.
Material and Methods. Viability of A549 cells treated with CPT and EPA or DHA was measured using the XTT tetrazolium salt based assay. Expression of genes encoding the antioxidant enzymes was determined by quantitative reverse-transcription polymerase chain reaction (QRT-PCR) analysis after RNA isolation from A549 cells.
Results. EPA and DHA added to the culture medium, increased the antitumor activity of CPT in A549 cells in a concentration dependent manner. The SOD2 and GST-pi expression showed marked increase after CPT treatment, while supplementation with EPA and DHA down-regulated their expression in the CPT treated A549 cells. The observed changes in mRNA levels of CAT were not statistically significant.
Conclusions. The reduction of antioxidant potential in cancer cells may sensitize these cells to anticancer therapy. PUFAs supplementation during CPT-based anticancer therapy may enhance effectiveness of the treatment (**Adv Clin Exp Med 2010, 19, 5, 585–591**).

Key words: antioxidant enzymes, PUFAs, cisplatin, A549 cells.

Streszczenie

Wprowadzenie. Pierwotna lub nabyta oporność często ogranicza zastosowanie cisplatyny (CPT) w leczeniu nowotworów. Suplementacja wielonienasyconymi kwasami tłuszczowymi (w.n.k.t.), w szczególności eikozapentaenowym (EPA, 20 : 5, n-3) i dokozaheksaenowym (DHA, 22 : 6, n-3), może zwiększać wrażliwość komórek nowotworowych na leki albo znosić ich oporność. Zwiększona ekspresja enzymów antyoksydacyjnych w nowotworach płuca jest czynnikiem determinującym ich wrażliwość na chemioterapię.
Cel pracy. Zbadanie wpływu EPA i DHA, w różnych stężeniach, na ekspresję genów kodujących wybrane enzymy kontrolujące potencjał redox: dysmutazę ponatlenkową 1 (SOD1), dysmutazę ponatlenkową 2 (SOD2), katalazę (CAT), peroksydazę glutationowa wodoronadtlenków fosfolipidów (GPx-4) i S-transferazę glutationową pi (GST-pi) w ludzkich komórkach gruczolakoraka płuca (A549) eksponowanych na CPT.

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Materiał i metody. Przeżywalność komórek A549 eksponowanych na CPT i EPA lub DHA była mierzona za pomocą testu wykorzystującego sól tetrazoliową XTT. Ekspresja genów kodujących ww. enzymy antyoksydacyjne w ekstraktach RNA z komórek A549 została oznaczona metodą ilościowej reakcji łańcuchowej polimerazy poprzedzonej odwrotną transkrypcją (QRT-PCR).
Wyniki. EPA i DHA dodane do medium hodowlanego zwiększały, w sposób zależny od stężenia, przeciwnowotworową aktywność CPT. Suplementacja w.n.k.t. komórek A549 eksponowanych na CPT zmniejszała ekspresję SOD2 i GST-pi, która po ekspozycji tylko na CPT była znacznie podwyższona.
Wnioski. Ograniczenie potencjału antyoksydacyjnego w komórkach nowotworowych może zwiększać ich wrażliwość na terapię przeciwnowotworową. Suplementacja w.n.k.t. podczas terapii przeciwnowotworowej z udziałem CPT może poprawić skuteczność leczenia (**Adv Clin Exp Med 2010, 19, 5, 585–591**).

Słowa kluczowe: enzymy antyoksydacyjne, w.n.k.t., cisplatyna, komórki A549.

Cisplatin [cis-diamminedichloroplatinum (II); CPT] is one of the most potent and widely used anticancer drug for the treatment of lung cancer. However, the treatment with CPT and its analogs is often limited by their side-effects including nephrotoxicity, neurologic damage, and ototoxicity. Furthermore, both primary and acquired resistance to platinum-based agents limits their application [1–3]. CPT resistance evokes an important clinical problem because administration of large doses of drug to overcome the resistance may lead to severe organ toxicities. Some of the cytotoxic effects of CPT are closely associated with increased generation of reactive oxygen species (ROS). Therefore intracellular antioxidants may contribute to a drug resistance in cancer [4–6]. While acute oxidative stress, imbalance between the cellular redox state and defense systems, triggers cell apoptosis or necrosis, persistent oxidative stress induces genomic instability and has been implicated in tumor progression and drug resistance [5]. There is increasing evidence that antioxidant enzymes (AOEs), highly expressed in lung tumors, are determinant factors in the sensitivity of lung tumors to anticancer drugs and are associated with lymph node status and prognosis in non small cell lung cancer NSCLC [7, 8].

Several strategies have been proposed to reverse drug resistance. One of the strategies to overcome anticancer agent resistance is the application of chemosensitizers or drug-resistance modulators. There is also an increasing interest to enhance efficacy and minimize toxicity of drugs used to treat cancer by dietary supplements. It is well known that polyunsaturated fatty acids (PUFAs) found in fish oil, in particular eicosapentaenoic acid (EPA, 20 : 5, n-3) and docosahexaenoic acid (DHA, 22 : 6, n-3), exert selective cytotoxicity against various types of cancer cells [9, 10]. Moreover, PUFAs can enhance sensitivity of tumor cells to anticancer therapy and reverse cancer cell resistance [11]. Nevertheless, there is a paucity of information regarding their combined effects with CPT. However, molecular mechanism for the anticancer action of PUFAs have been intensively studied only a few of papers have suggested that anticancer activities of both EPA and DHA are associated with their ability to modulate the AOEs activity [12–14].

This led us to investigate the effect of different concentrations of EPA and DHA on gene expression of several enzymes controlling redox status, such as superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), catalase (CAT), phospholipid hydroperoxide glutathione peroxidase (GPx-4), and glutathione S-transferase pi (GST-pi) in human lung adenocarcinoma cells (A549) exposed to CPT.

Material and Methods

Cell Culture

A549 human lung adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC) and cultured (25000 cm^{-2}) in Modified Eagle's Medium (MEM) supplemented with 10% heat inactivated fetal bovine serum (FBS; PAA The Cell Culture Company), 10 mM buffer HEPES (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell Exposure to CPT and PUFAs

Cisplatin, eicosapentaenoic acid (EPA, 20 : 5, n-3), and docosahexaenoic acid (DHA, 22 : 6, n-3) were purchased from Sigma. The fatty acids were dissolved in 99% ethanol and stored as stock solutions (100 mM) under nitrogen at –20°C. To achieve experimental conditions, PUFAs and CPT were prepared freshly from stock solutions and diluted with the appropriate volumes of the growth medium.

Twenty-four hours after cell seeding, the medium was replaced with the medium supplemented with EPA or DHA (25 μM, 50 μM or 100 μM). After following 24 h, the culture medium was again

replaced with the medium containing additionally CPT (3 μg/ml) for 4 hours. The CPT concentration was selected on the basis of previous studies performed in our laboratory and preliminary viability tests conducted for this study. Control cells were cultured in the medium containing the same concentration of ethanol (v/v; 0.1%) as the experimental cultures. Previous observations showed, that ethanol at this concentration has not been toxic to the cells.

Cytotoxicity Assay

Survival of cells exposed to CPT (3 μg/ml) or PUFAs (25 μM, 50 μM, 100 μM) and CPT (3 μg/ml) was assessed by the XTT method (In Vitro Toxicology Assay Kit XTT Based, TOX-2, Sigma) with a commercial kit according to the manufacturer's instruction. The method based on the ability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxy-anilide inner salt), yielding orange formazan crystals, which are soluble in aqueous solutions. Absorbance of formazan was measured at 450 nm with the plate reader (Triad LT Multimode Detector, Dynex Technologies). Cell viability was expressed as a percentage of absorbance measured in the treated wells relative to that in the untreated control wells.

Extraction of mRNA and Analysis

Total RNA was extracted from cells using TRIZOL (Invitrogen) following the manufacturer's instruction and quantified by UV absorbance spectrophotometry using GeneQuant II (Pharmacia Biotech).

Quantitative RT-PCR was performed using QuantiTect Probe RT-PCR Kit (Qiagen) and the number of mRNA copies of SOD1, SOD2, CAT, GST-pi, GPx-4 genes was quantified with ABI PRISM 7000 Sequence Detection System (Applied Biosystems), according to the manufacturer's protocols. TaqMan gene expression assays for analyzing the mRNA expression of SOD1 (assay ID Hs00166575_m1), SOD2 (assay ID Hs00167309_m1), CAT (assay ID Hs00156308_m1), GST-pi (assay ID Hs00168310_m1) and GPx-4 (assay ID Hs00157812_m1) were obtained from Applied Biosystems.

mRNA expression levels of target genes were normalized to GAPDH mRNA (Applied Biosystems; assay ID Hs99999905_m1). The amount of target genes mRNA expression (target/reference ratio) in each sample was expressed as a percentage of control.

Statistical Analysis

The data obtained from three independent experiments were expressed as mean values ± standard deviations. Statistical significance analysis based on analysis of variance (ANOVA) followed by Tukey's HSD test. The P-value of less than 0.03 was considered significant. Statistical analysis was performed using Statistica 8 PL software for Windows (StatSoft, Poland).

Results

Effect of PUFAs on CPT Cytotoxicity

Figure 1 shows the cell survival of A549 cells exposed to CPT (3 μg/ml) in the presence or absence of PUFAs. Results are expressed as a percentage of viability of PUFAs+CPT-treated cells vs. control cells. CPT-treatment reduced growth of the A549 cells by about 35%. Both, DHA and EPA (25–100 μM) increased the antitumor activity of CPT in these cells in a concentration dependent manner. Treatment with CPT + EPA (100 μM) or CPT + DHA (100 μM) caused nearly 70 % reduction of cell viability of the A549 cells.

Effect of PUFAs on AOEs mRNA Level

The effects of PUFAs-induced alterations of AOEs mRNA expression in A549 cells exposed to CPT (3 μg/ml) are summarized in Table 1. The treatment of A549 cells with CPT caused a decrease of SOD1 and GPx-4 mRNA levels. The target/reference ratio for these genes, expressed as percentage of control, decreased to 83.4 and 40.3%, respectively. The relative quantification of mRNA/GAPDH ratio of the SOD2, and GST-pi showed marked increase after CPT treatment. The target/reference ratio for these genes, expressed as percentage of control, increased to 128.7 and 164.1 %, respectively. The observed changes in mRNA levels of CAT were not statistically significant.

Compared to CPT alone, EPA and DHA addition down-regulated SOD2, and GST-pi genes expression in CPT treated A549 cells in a concentration dependent manner. After supplementation with EPA and DHA at 50 and 100 μM of A549 cells exposed to CPT, the value of target/reference ratio of the SOD2 and GST-pi decreased below the control value. DHA at 25 μM up-regulated SOD1 gene expression in CPT treated A549 cells.

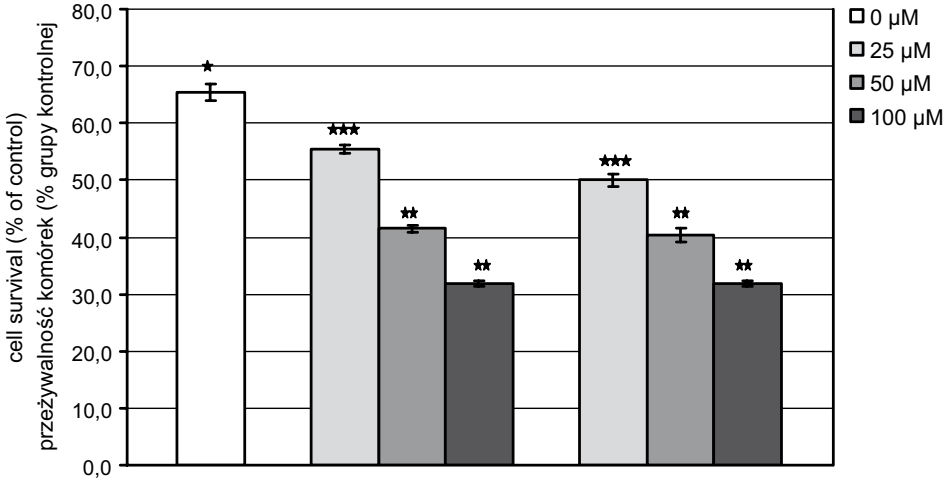

Fig. 1. Survival (% of control) of A549 cells exposed to cisplatin (CPT, 3 μg/ml) in the absence or presence of eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) at various concentrations (25–100 μM). Data were calculated as the means ± SD from three independent experiments. Statistically significant differences ($p < 0.02$): *vs. control, **vs. control and CPT, ***vs. control and CPT, EPA+CPT vs. DHA+CPT

Ryc. 1. Przeżywalność (% kontroli) komórek A549 eksponowanych na cisplatynę (CPT; 3 μg/ml) w nieobecności i obecności kwasu eikozapentaenowego (EPA) lub dokozaheksaenowego (DHA) w różnych stężeniach (25–100 μM). Dane są średnimi ± SD trzech niezależnych eksperymentów. Różnice istotne statystycznie ($p < 0.02$): *względem grupy kontrolnej, **względem grupy kontrolnej i CPT, ***względem grupy kontrolnej i CPT oraz EPA+CPT względem DHA+CPT

Table 1. PUFAs-induced alterations of AOEs mRNA expression in A549 cells exposed to cisplatin (CPT, 3 μg/ml). QRT-PCR data are expressed as AOEs/GAPDH mRNA ratio (% of control). Data were calculated as the means ± SD from three independent experiments

Tabela 1. Zmiany ekspresji mRNA enzymów antyoksydacyjnych (AOEs) indukowane przez w.n.k.t. w komórkach A549 eksponowanych na cisplatynę (CPT, 3 μg/ml). Wyniki QRT-PCR są wyrażone jako stosunek liczby kopii mRNA AOEs/GAPDH (% grupy kontrolnej). Dane są średnimi ± SD trzech niezależnych eksperymentów

PUFAs concentration (Stężenia w.n.k.t.)	AOEs/GAPDH mRNA ratio – % of control (Stosunek liczby kopii mRNA AOEs/GAPDH – % grupy kontrolnej)				
	SOD1	SOD2	CAT	GPx-4	GST-pi
CPT	83.4 ± 4.0[a]	128.7 ± 12.2[a]	99.9 ± 13.9	40.3 ± 3.0[a]	164.1 ± 7.0[a]
CPT + EPA (25 μM)	65.2 ± 4.8[a,b,c]	92.7 ± 3.9[b,c]	91.3 ± 2.5	39.5 ± 3.2[a,c]	105.8 ± 2.6[b]
CPT + EPA (50 μM)	24.6 ± 2.3[a,b,c]	56.3 ± 3.3[a,b,c]	75.9 ± 16.3	33.8 ± 2.1[a,b,c]	50.8 ± 1.3[a,b,c]
CPT + EPA (100 μM)	8.7 ± 0.7[a,b]	46.3 ± 3.3[a,b]	90.4 ± 3.7	26.0 ± 2.5[a,b,c]	32.2 ± 1.3[a,b]
CPT + DHA (25 μM)	148.4 ± 7.9[a,b,c]	103.2 ± 7.4[b,c]	101.8 ± 6.5	47.9 ± 1.4[a,c]	103.0 ± 2.0[b]
CPT + DHA (50 μM)	63.7 ± 4.5 [a,b,c]	73.2 ± 6.0[a,b,c]	92.1 ± 8.3	42.5 ± 2.3[a,c]	59.9 ± 2.0[a,b,c]
CPT + DHA (100 μM)	13.5 ± 1.2[a,b]	33.0 ± 0.7[a,b]	96.5 ± 7.6	9.2 ± 1.1[a,b,c]	33.4 ± 1.4[a,b]

[a] – significant difference in comparison with control.
[b] – significant difference in comparison with CPT.
[c] – significant difference EPA+CPT vs. DHA+CPT.

[a] – istotna statystycznie różnica w porównaniu z grupą kontrolną.
[b] – istotna statystycznie różnica w porównaniu z CPT.
[c] – istotna statystycznie różnica EPA+CPT *vs* DHA+CPT.

Discussion

Some tumor cells, including lung cancer cells, enhance total antioxidant capacity by up-regulation of the expression of genes that encode various cytoprotective enzymes, allowing them to survive damage from chemotherapy or radiotherapy [5, 8]. Many of these AOEs (e.g., heme oxygenase-1, superoxide dismutases SODs, glutathione reductase, glutathione peroxidases GPx, thioredoxin reductase, peroxiredoxins), phase II detoxification enzymes (e.g., NADP[H] quinone oxidoreductase 1, glutathione-S-transferases GSTs), several ATP-dependent drug efflux pumps (e.g., multidrug resistance protein MRP) and cysteine/glutamate transporter (SLC7A11), are under the control of

NF-E2-related factor 2 (Nrf2), which is associated with Kelch-like ECH associating protein 1 (Keap1) in the cytoplasm. Keap1 is the major regulator of Nrf2 activation. Upon exposure of cells to oxidative stress or chemopreventive compounds, after sequestration of Nrf2 by Keap1, Nrf2 translocates to the nucleus, forms a heterodimer with small Maf proteins or other leucine zipper proteins, and binds to antioxidant response element (ARE) in the promoters of its target genes. In a consequence, the production of ROS is decreased upon Nrf2 induction. The Nrf2–Keap1 signal pathway has been reported to be impaired in many lung cancer tissues and lung carcinoma cell lines [15, 16]. In lung cancer cells, low Keap1 activity (due to mutations or low-level expression) led to nuclear localization and constitutive activation of Nrf2. The latter resulted in constitutive expression of cytoprotective genes encoding AOEs, MRP and phase II detoxifying enzymes. Up- regulation of these target genes in lung cancer cells led to CPT resistance [15–18].

Nrf2 downstream genes such as AOEs are believed to have an important role in cellular proliferation, tumor infiltration, and in the development of chemo-resistance by malignant cells [5, 17]. SODs reduce superoxide anion into hydrogen peroxide. There are three SODs, all of which are expressed also in human lung. SOD2 (MnSOD) is mitochondrial, SOD1 (CuZnSOD) cytosolic and extracellular [19]. CAT and GPx, convert hydrogen peroxide to water. GPx, a selenoenzyme, detoxifies hydrogen and fatty acid peroxides by using glutathione (GSH) as a hydrogen donor [13]. GSTs catalyze the conjugation of electrophilic metabolites or drugs to GSH to facilitate their detoxication in the cells [8]. The cellular thiols such as GSH and metallothioneins have been shown to play an important role in detoxication of platinum-based anticancer agents. Increased thiol contents have been often observed in many types of CPT-resistant human cancer [3].

High levels of SODs, often found in malignant tumors including lung cancer, are also associated with resistance to anticancer drugs or radiation and with a poor prognosis [19]. The basal or induced level of SOD2 did not correlate with the development of drug resistance in A549 cells and human mesothelioma cells (M14K) during epirubicin treatment [20]. Very little is known about CAT in malignant cells or about its role in drug resistance. It has been shown that CAT is not important in the drug resistance of human mesothelioma cells and A549 cells and inhibition of CAT did not enhance epirubicin-related toxicity [20]. A number of studies demonstrate that the amount of GST isoenzymes is even higher in lung tumors than in the surrounding normal tissues [8]. GST-pi overexpression has been associated with increased resistance to various chemotherapeutic agents including a lack of response to CPT-based chemotherapy and with a poor overall survival. The high protein level of GST-pi contributes to this process either via its direct detoxifying effect towards the platinum-based drugs, or via the inhibitory effect on MAP kinase signal pathways [6, 21]. The reported findings on human mesothelioma cells and adenocarcinoma A549 cells suggest that glutathione-associated mechanisms play an important role in the resistance of these cells to antineoplastic agents [20]. Nuclear GST-pi accumulates in A549 cells in response to CPT and inhibition of its nuclear transport enhances the sensitivity of the cancer cells to CPT [21]. GPx-4 is a unique antioxidant enzyme that can directly reduce phospholipid hydroperoxides in membranes and lipoproteins [13]. Overexpression of mitochondrial GPx-4 in breast tumor epithelial cells protects photochemically generated cholesterol hydroperoxide-induced cell death [22]. Mitochondrial GPx-4 prevents cell death by reducing intracellular hydroperoxides [13]. In agreement with *in vitro* and *in vivo* studies on human cancer cells, the presented results demonstrate that up-regulation of the expression of SOD2, and GST-pi may protect A549 cells from CPT-dependent cytotoxicity.

PUFAs supplementation has been recognized as one of the agents involved in modulation of AOEs activity and response of cells to oxidative stress during anticancer treatment. It has been shown that DHA selectively down-regulates SOD1 expression in human lymphoma DHL-4 cells [12] and reduces the level of GPx-4 protein expression in various human cancer lines [13]. The present study showed that both, EPA and DHA down--regulated SOD1, SOD2, GPx-4, and GST-pi genes expression in CPT treated A549 cells. Exactly how DHA regulates expression of AOEs has not been established. PUFAs may enter the nucleus via fatty acid binding protein and affect gene transcription. Alternatively, lipid peroxidation products may destabilize the SOD1 mRNA, leading to lower expression of the enzyme [12]. In contrast, the induced by DHA decreased GPx-1 activity in breast cancer cells was accompanied by a decreased protein level but not a decreased mRNA, what suggests the effect of DHA at the post-transcriptional level [14]. GPx can also be damaged by lipid peroxidation products, which lead to a loss of GPx activity, probably by a modification of the selenocysteine residue at the active site of the enzyme [23].

It is known that DHA and EPA, exert selectively cytotoxic effects on cancer cells and are significantly less toxic toward normal cells [9, 11]. One major hypothesis explaining the antitumor effects of n-3

PUFAs is their susceptibility to peroxidation, which leads to generation of lipid peroxides, which may directly cause cytotoxicity or may influence intracellular signaling pathways leading to growth inhibition or death of tumor cells [10, 24]. Altering tumor lipid fatty acid composition may change drug-resistant phenotypes to drug-sensitive [14, 24].

Several *in vivo* and *in vitro* studies have reported the increased efficacy of CPT against human tumors after of DHA supplementation. Incubation of CPT-sensitive (GLC-4) and resistant (GLC-4--CP) small cell lung carcinoma cells with non-toxic levels of DHA resulted in increased accumulation of DHA in the tumor membranes and a three-fold decrease in the resistance of GLC-4-CP cells toward CPT, but had no influence on the cytotoxicity of CPT toward the susceptible GLC-4 cells. DHA enhanced the sensitivity of drug resistant tumor cells to CPT by increasing the intracellular platinum levels, total platinum bound to DNA, and inter-strand cross-linking in the both cell lines [25]. Similarly, the CPT resistant ovarian cell line 2780-CP was sensitized to CPT by pre-incubation with EPA, whereas, the parent ovarian line 2780 was not [26]. Elmesary et al. evaluated the antitumor effects of DHA, alone or in combination with CPT, in the EAC solid tumor mice model [27]. The study found that DHA reduced the size of tumors and enhanced the positive effects of the CPT chemotherapy, and limited its harmful side effects. In addition, DHA eradicated lethal CPT-induced nephrotoxicity and renal tissue injury. The present study showed that both, EPA and DHA, increased the antitumor activity of CPT in A549 cells in a concentration dependent manner. The concentrations of DHA and EPA used can be found in human plasma under physiological conditions or during supplementation [28].

About 40% of currently used anticancer drugs have been reported to induce oxidative stress [29]. Therefore, the reduction of AOEs potential in cancer cells may sensitize these cells to anticancer therapy. In conclusion, we suggest that PUFAs supplementation in conjunction with antineoplastic agents can enhance the positive effect of the CPT treatment.

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