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POLYUNSATURATED FATTY ACIDS POTENTIATE CYTOTOXICITY OF CISPLATIN IN A549 CELLS

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Abstract: In normal and tumor cells, polyunsaturated fatty acids (PUFAs) act as intracellular second messengers, which play a role in signaling, proliferation and cell death. PUFAs have selective tumoricidal action and may alter sensitivity of tumor cells to cisplatin (CDDP), a commonly used anticancer agent. The aim of this study was to evaluate the influence of arachidonic acid (AA, 20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3), docosahexaenoic acid (DHA, 22:6 n-3) and CDDP on autophagy and apoptosis in A549 human lung adenocarcinoma cells. Viability of A549 cells treated with CDDP and PUFAs was measured using the XTT tetrazolium salt based assay. Caspase-3/7 activity was estimated using ApoTox-Glo kit (Promega). Autophagic vacuoles were detected by Cyto-ID Autophagy Detection Kit (Enzo). The results were compared to control cultures maintained in the absence of CDDP and PUFAs. PUFAs, in particular EPA and DHA, added to the cultivation medium, increased the antitumor activity of CDDP in A549 cells in a concentration dependent manner. In case of AA this effect was observed at the highest of the concentrations tested only (100 μM). Both, EPA and DHA, but not AA, significantly increased the amount of autophagic vacuoles and induced caspase-3/7 activity. The obtained results suggest that the antiproliferative effect of CDDP in A549 cells can be enhanced by AA and in particular by EPA and DHA through their influence on autophagic and apoptotic cell death. It is likely that EPA and DHA incorporated to the tumor cells may improve outcomes in lung cancer patients.

Keywords: polyunsaturated fatty acids, cisplatin, autophagy, apoptosis

Cisplatin [cis-diamminedichloroplatinum (II); CDDP] is one of the most potent and widely used anticancer drugs for the treatment of lung cancer. However, the treatment with CDDP 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 drugs limits their application (1–3). Administration of larger doses of CDDP to overcome the resistance may lead to severe organ toxicities.

It has been reported that acquired CDDP resistance in human lung adenocarcinoma cells is associated with enhanced autophagy (4). Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved catabolic process by which cells destruct their cytoplasmic content and organelles through the lysosomal machinery. Autophagy is initiated by the formation of the double-membrane bound short lived vacuoles (autophagosomes), which sequester cytosolic proteins and organelles such as mitochondria and endoplasmic reticulum. Autophagosomes fuse with acidic lysosomes to produce autolysosomes where the sequestered content is degraded by lysosomal enzymes, and amino acids and sugars are recycled into the cytosol for reuse (5). Emerging evidence shows that autophagy is important in the regulation of cancer development and progression. However, the role of autophagy is complicated and autophagy may cause divergent effects in cells. Autophagy may protect tumor cells from nutrient deficiency and hypoxia; but on the other hand, autophagy defects are associated with development of cancer (6, 7). The fact that autophagy can have both suppressive and promoting roles in carcinogenesis, makes it an attractive target in cancer research. As a tumor suppressing mechanism, autophagy serves as an alternative to apoptosis to eliminate transformed cells. Nevertheless, autophagy may facili-

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tate tumor cells resistance to certain anticancer drugs (6, 7).

It is well documented 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. Moreover, PUFAs can enhance their sensitivity to anticancer therapy and reverse the resistance (8, 9). This led us to investigate the effect of arachidonic acid (AA, 20 : 4, n-6), EPA, and DHA on autophagy and apoptosis in A549 human lung adenocarcinoma cells treated with CDDP.

EXPERIMENTAL

Cell culture

A549 human lung adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC) and cultured (25000 cells/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 CDDP and PUFAs

CDDP, AA, EPA, and DHA 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 CDDP were prepared freshly from stock solutions and diluted with the appropriate volumes of the growth medium. Twenty-four hours after cell seeding (96-well plates; 1×10^4 cells/well), the medium was replaced with the media supplemented with CDDP (5 µg/mL) and AA, EPA or DHA (25, 50, 100 µM). The CDDP concentration was chosen on the basis of the viability tests results obtained in our laboratory prior to the experiment. Control cells were cultured in the medium containing the same concentration of ethanol (v/v, 0.1%) as the experimental cultures for another 24 h. Previous observations showed, that ethanol at this concentration had not been toxic to the cells.

Viability assay

Survival of cells exposed to CDDP (5 µg/mL) or PUFAs (25, 50, 100 µM) and CDDP (5 µg/mL) together 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-carboxyanilide 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.

ApoTox-Glo Triplex assay

The ApoTox-Glo Triplex Assay (Promega) was used to measure A549 cells viability, cytotoxicity and apoptosis in the same sample following manufacturer's protocols. Briefly, viability and cytotoxicity are measured by fluorescent signals produced when either live-cell or dead-cell proteases cleave added substrates GF-AFC (viability) and bis-AAF-R110 (cytotoxicity). GF-AFC can enter cells and is therefore cleavable by live-cell protease only, which incidentally becomes inactive when cell membrane activity is lost; bis-AAF-R110 cannot enter the cell, and is cleaved only by dead-cell protease leaked from cells lacking membrane integrity. Both cleaved substrates have different excitation and emission spectra. Apoptosis is measured by the addition of a luminogenic caspase-3/7 substrate (Caspase-Glo 3/7) which is cleaved in apoptotic cells to produce a luminescent signal. Fluorescence at 365 Ex/500 Em (viability), 485 Ex/535 Em (cytotoxicity) and luminescence (apoptosis) were measured with a plate reader (Triad LT Multimode Detector, Dynex Technologies).

Cyto-ID autophagy detection assay

Cyto-ID kit (Enzo) was used according to the manufacturer's protocol for fluorescence microplate reader. Cyto-ID Autophagy Detection Kit measures autophagic vacuoles and monitors autophagic flux in live cells using a novel dye that selectively labels autophagic vacuoles. The dye has been optimized through the identification of titratable functional moieties that allow for minimal staining of lysosomes while exhibiting bright fluorescence upon incorporation into pre-autophagosomes, autophagosomes, and autolysosomes (autophagolysosomes). The assay offers a rapid and quantitative approach to monitoring autophagy in live cells without the need for cell transfection. Rapamycin and tamoxifen, typical inducers of autophagy, were used as a positive control and 3-methyladenine (3-MA) as inhibitor of autophagy (negative control).

Fluorescence was measured at 365 Ex/465 Em (Hoechst 33342 nuclear stain) and 485 Ex/535 Em (Cyto-ID green autophagy detection reagent) with a plate reader (Triad LT Multimode Detector, Dynex Technologies).

Statistical analysis

The data obtained from 4 independent series of experiments were expressed as the mean values ± standard deviations. Statistical comparisons were made by analysis of variance (ANOVA), followed

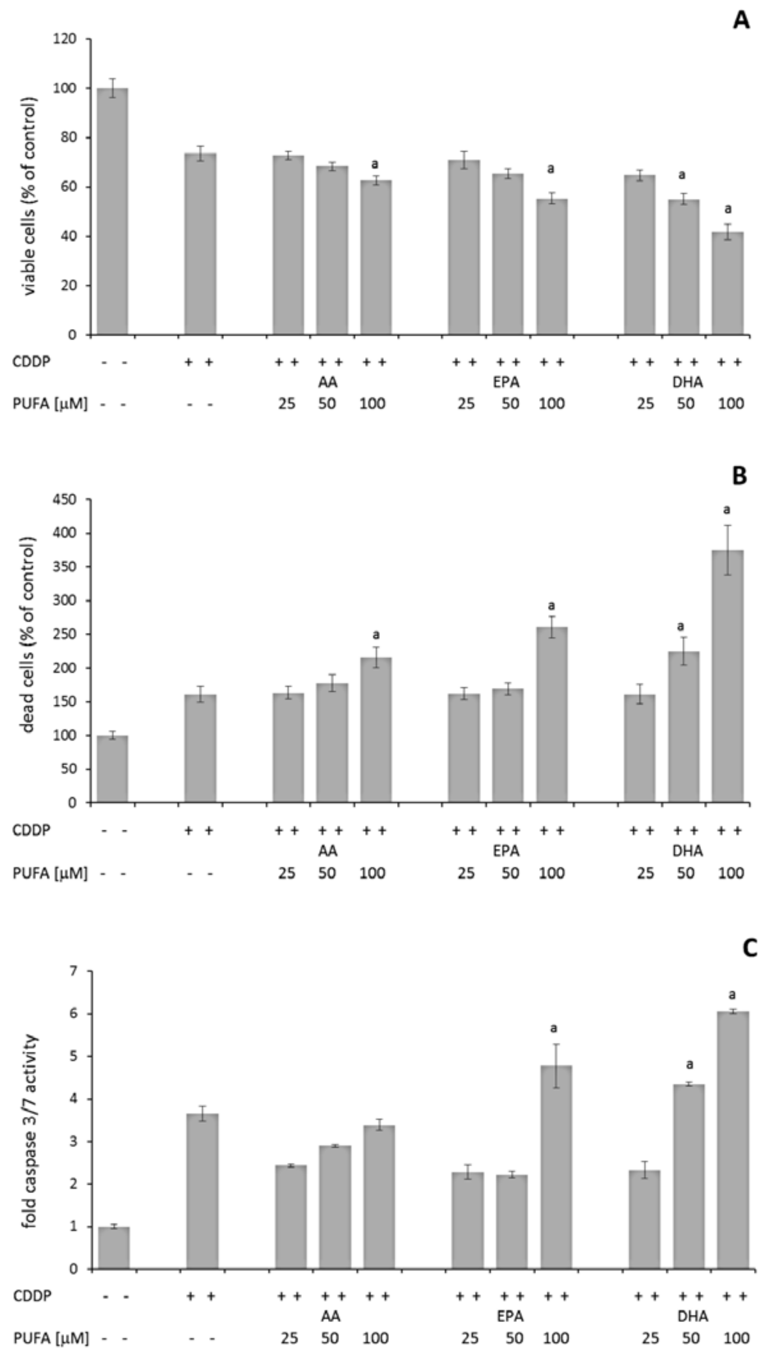

Figure 1. Concentration-dependent (25–100 µM) effect of arachidonic acid (AA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) on cisplatin (CDDP, 5 µg/mL) cytotoxicity in A549 cells. The viability (**A**), cytotoxicity (**B**) and apoptosis (**C**) of A549 cells exposed to cisplatin (CDDP) in the absence or presence PUFAs were detected by the ApoTox-Glo Triplex Assay. Data were calculated as the means ± SD from four independent experiments. [a] statistically significant differences ($p < 0.05$) *vs.* CDDP

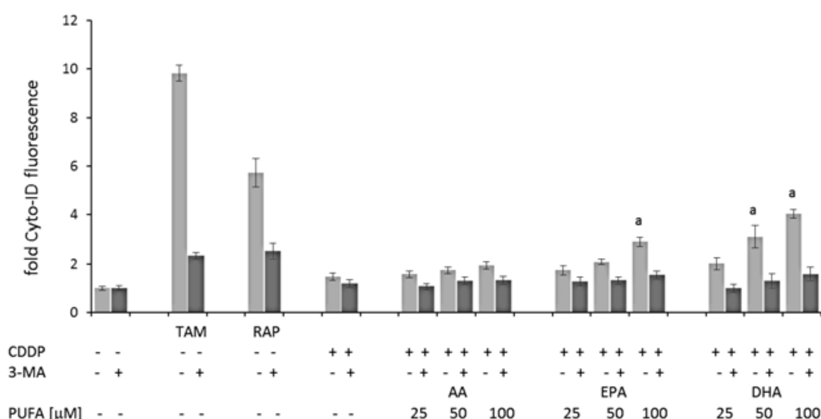

Figure 2. Cyto-ID fluorescence in A549 cells exposed to cisplatin (CDDP, 5 µg/mL) in the absence or presence of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) at various concentrations (25–100 µM) detected by the Cyto-ID autophagy detection assay. Rapamycin and tamoxifen, the typical inducers of autophagy, were used as a positive control and 3-methyladenine (3-MA; 5 mM) as inhibitor of autophagy (negative control). Data were calculated as the means ± SD from four independent experiments. [a] statistically significant differences ($p < 0.05$) *vs.* CDDP

by Tukey's HSD test. The p-value of less than 0.05 was considered significant. Statistical analysis was performed using Statistica 10 PL software for Windows (StatSoft, Poland).

RESULTS AND DISCUSSION

Cisplatin treatment is known to promote tumor cell resistance to apoptosis induction *via* multiple mechanisms involving gene expression, modulation of oncogenes, tumor suppressors and blockade of pro-apoptotic mitochondrial membrane permeabilization. Many cancers have disrupted intrinsic (mitochondrial dependent) and extrinsic (death receptor mediated) apoptosis signaling pathways. The consequential insufficient activation of caspases is thought to contribute to resistance to chemotherapy. Many papers have presented data suggesting that tumor cell autophagy induced by anticancer treatment inhibits tumor cell killing. However, it has also been proposed that autophagy is a cell death mechanism that could function as a backup when apoptosis is disabled. The form of cell death initiated by autophagy (autophagic cell death) is caspase independent. Moreover, an increasing number of studies have demonstrated that apoptosis and autophagy share some common signaling pathways and are mutually regulated (10, 11).

There is increasing interest in the new therapeutic approaches based on the knowledge of structure, function, and alteration of membrane lipids (12). Supplementation of tumors with long-chained ω-3 PUFAs results in enrichment of tumor phospholipid fractions with these PUFAs. As components of membrane phospholipids, PUFAs cause changes of membrane fluidity and membrane structure, which may influence receptor-ligand interactions, signal transduction properties and various membrane mediated cellular functions (13). Such cells have membranes with increased fluidity, an elevated unsaturation index, enhanced transport capabilities that result in accumulation of selective anticancer agents and alteration of signaling pathways important for cancer progression. (10).

Several *in vivo* and *in vitro* studies have reported the increased efficacy of CDDP against human tumors after DHA supplementation. It has been shown that both, EPA and DHA downregulated SOD1, SOD2, GPx-4 and GST-π genes expression in CDDP treated A549 cells, which may explain the increased efficacy of this drug against these cells (14). Moreover, incubation of CDDP-sensitive (GLC-4) and resistant (GLC-4-CP) small cell lung carcinoma cells with non-toxic levels of DHA resulted in an increased accumulation of DHA in the tumor membranes and a three-fold decrease in the resistance of GLC-4-CP cells toward CDDP, but had no influence on the cytotoxicity of CDDP toward the susceptible GLC-4 cells. DHA enhanced the sensitivity of drug resistant tumor cells to CDDP by increasing the intracellular platinum levels, total platinum bound to DNA and inter- strand cross-link-

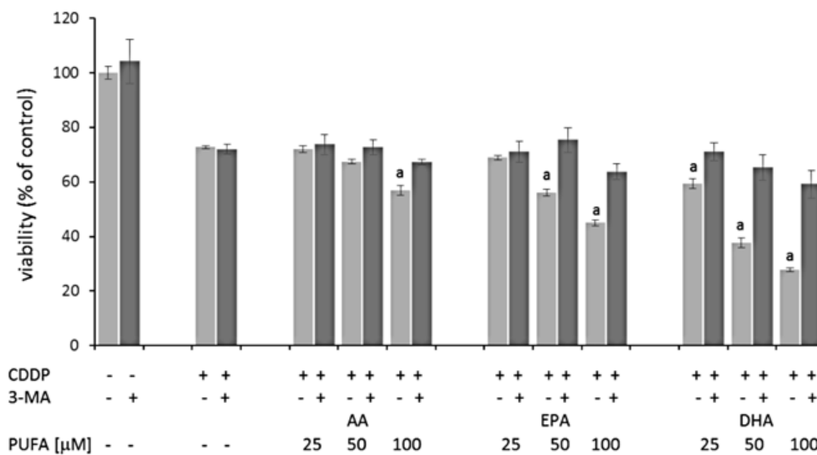

Figure 3. Viability (% of control) of A549 cells exposed to cisplatin (CDDP, 5 µg/mL) in the absence or presence of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) at various concentrations (25–100 µM) and the effect of 3-methyladenine (3-MA; 5 mM) on CDDP and PUFAs toxicity measured by XTT. Data were calculated as the means ± SD from four independent experiments. [a] statistically significant differences ($p < 0.05$) *vs.* CDDP

ing in the both cell lines (15). Similarly, the CDDP resistant ovarian cell line 2780- CP was sensitized to CDDP by pre-incubation with EPA, whereas, the parent ovarian line 2780 was not (16). Elmesary et al. evaluated the antitumor effects of DHA, alone or in combination with CDDP, in the EAC solid tumor mice model (17). The study found that DHA reduced the size of tumors, enhanced the positive effects of the CDDP chemotherapy, and limited its harmful side effects. In addition, DHA eradicated lethal CDDP-induced nephrotoxicity and renal tissue injury. In agreement with the *in vitro* and *in vivo* studies on human cancer cells, the presented results demonstrated that AA (100 µM), EPA (100 mM) and DHA (50, 100 µM), caused a significant decrease in cell viability and a significant increase in cytotoxicity of CDDP (5 µg/mL) in A549 cells (Figs. 1A, 1B). Several studies have documented that DHA simultaneously induced both autophagy and apoptosis in cancer cells by activating intrinsic and extrinsic apoptotic pathways (18, 19). Exposure of A549 cells to CDDP in the presence of EPA (100 µM) or DHA (50, 100 µM) significantly increased caspase activity and apoptosis (Fig. 1C).

To examine the possibility that PUFAs and CDDP induce autophagy in A549 cells, autophagy was monitored in live cells by measuring the fluorescence intensity using a novel dye (Cyto-ID) that exhibits bright green fluorescence when selectively labeling autophagic vacuoles including pre-autophagosomes, autophagosomes and autophagolysosomes. Recently, this assay has been recognized to be a rapid and quantitative approach to monitor autophagy in live cells (20). Figure 2 shows the Cyto-ID fluorescence in A549 cells exposed to CDDP in the presence or absence of PUFAs. 3-MA, as an inhibitor of autophagy, blocks the formation of autophagosomes, by controlling class I and class III phosphatidylinositol 3-kinases (PI3K) (21, 22). As expected, it caused a reduction in the cytoplasm vacuolization and therefore a decrease of Cyto-ID fluorescence intensity. As shown in Figure 2, the treatment of A549 cells with CDDP did not induce autophagy. These results are in agreement with some previous observations in A549 cells, indicating that 3-MA enhanced growth inhibition and apoptotic effect of CDDP in cells with acquired resistance (A549/DDP) and did not alter its cytotoxic effect to the parent A549 cell line (4, 23). After CDDP treatment in the presence of EPA (100 µM) or DHA (50, 100 µM) the Cyto-ID fluorescence intensity significantly increased (Fig. 2), what suggests that autophagic vacuolization in response to PUFAs treatment occurred. The effect was reverted when the autophagy inhibitor was applied (Fig. 2). Statistically significant differences were observed between CDDP and EPA or DHA treated cells and CDDP and EPA or DHA plus 3-MA treated cells ($p < 0.01$), confirming the reduction of autophagy (Fig. 2). Moreover, the inhibition of the autophagic process induced an increase in cell viability, what confirms the occurrence of cell death by autophagy (Fig. 3). 3-MA did not alter the viability of A549 cells. These results indicate that CDDP toxicity, at

least in part, is increased by EPA or DHA-induced autophagy. It is likely that EPA and DHA incorporated to the tumor cells may improve outcomes in lung cancer patients.

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