Docosahexaenoic Acid Sensitizes Leukemia Lymphocytes to Barasertib and Everolimus by ROS-dependent Mechanism Without Affecting the Level of ROS and Viability of Normal Lymphocytes

ZHIVKO ZHELEV[1,2], DONIKA IVANOVA[1], DESISLAVA LAZAROVA[3], ICHIO AOKI[4], RUMIANA BAKALOVA[3,4] and TSUNEO SAGA[4]

[1]*Medical Faculty, Trakia University, Stara Zagora, Bulgaria;*
[2]*Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria;*
[3]*Medical Faculty, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria;*
[4]*Molecular Imaging Center, National Institute of Radiological Sciences, Inage-ku, Chiba, Japan*

Abstract. *The aim of the present study was: (i) to investigate the possibility of sensitizing leukemia lymphocytes to anticancer drugs using docosahexaenoic acid (DHA); (ii) to find combinations with synergistic cytotoxic effect on leukemia lymphocytes, without or with only very low cytotoxicity towards normal lymphocytes; (iii) and to clarify the role of reactive oxygen species (ROS) in the induction of apoptosis and cytotoxicity by such combinations. The study covered 15 anticancer drugs, conventional and new-generation. Well-expressed synergistic cytotoxic effects were observed after treatment of leukemia lymphocytes (Jurkat) with DHA in combination with: barasertib, lonafarnib, everolimus, and palbociclib. We selected two synergistic combinations, DHA with everolimus or barasertib, and investigated their effects on viability of normal lymphocytes, as well as on the production of ROS and induction of apoptosis in both cell lines (leukemia and normal). At the selected concentrations, DHA, everolimus and barasertib (applied separately) were cytotoxic towards leukemia lymphocytes, but not normal lymphocytes. In leukemia cells, the cytotoxicity of combinations was accompanied by strong induction of apoptosis and production of ROS. In normal lymphocytes, drugs alone and in combination with DHA did not affect the level of ROS and did not induce apoptosis. To our knowledge, the present study is the first to report synergistic ROS-dependent cytotoxicity between DHA and new-generation anticancer drugs, such as everolimus and barasertib, that is cancer cell-specific (particularly for acute lymphoblastic leukemia cells Jurkat). These combinations are harmless to normal lymphocytes and do not induce abnormal production of ROS in these cells. The data suggest that DHA could be used as a supplementary component in anticancer chemotherapy, allowing therapeutic doses of everolimus and barasertib to be reduced, minimizing their side-effects.*

Correspondence to: Rumiana Bakalova, Ph.D., D.Sci., Molecular Imaging Center, National Institute of Radiological Science, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan. Tel: +81 8034307755, e-mail: bakalova@nirs.go.jp

Key Words: Docosahexaenoic acid, DHA, everolimus, barasertib, leukemia lymphocytes, reactive oxygen species.

Polyunsaturated fatty acids (PUFAs) are highly active molecules. They can act as transcription factors, ligands in signal transduction and membrane components that regulate the fluidity, permeability and dynamics of cell membranes. There is a growing body of evidence that long-chain *ω*-3 polyunsaturated fatty acids (*ω*-3PUFAs), particularly docosahexaenoic acid (DHA) – an essential fatty acid derived from fish and fish oils, has an important role in the prevention and treatment of coronary artery disease, hypertension, diabetes, arthritis, neurodegeneration and other inflammatory and autoimmune disorders (1-5). Many *in vitro* and *in vivo* studies suggest that DHA has anticancer activity and improves the efficiency of conventional cancer therapy: DHA suppressed tumor cell proliferation and reduced tumor growth in experimental animals (6-11), inhibited drug resistance in various cancer cell lines (12-14), and exerted cytotoxic effects on cancer cells, but it is significantly less cytotoxic towards normal cells (15-17). Moreover, new clinical trials and prospective studies on humans show that DHA is associated with a lower risk of cancer, as well as of Crohn's disease, in populations with high fish consumption (14, 18-21). The mechanisms behind these effects are still unclear and need to be elucidated.

Some authors suggest that reactive oxygen species (ROS) and lipid peroxidation have a crucial role in the anticancer activity of DHA (22-25). It is supposed that the susceptibility of DHA to free radical oxidation makes it capable of generating lipid peroxides, which can directly cause cytotoxicity or may influence intracellular signaling pathways, resulting in growth inhibition or death of tumor cells (22-25). It was also reported that DHA effectively killed cancer cells through down-regulation of the superoxide dismutase isoenzyme-1 (*SOD1*) gene (26, 27) and subsequent modulation of redox-sensitive transcription factors [*e.g.*, peroxisome proliferator-activated receptors (PPARs), hypoxia-inducible factors (HIFs), and nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB)] (11, 27-30). The human *SOD1* gene promoter has DNA-binding elements for these factors, which might be implicated in this process (11, 26-30). Other studies demonstrated that DHA can induce apoptosis of cancer cells by induction of stress in the endoplasmic reticulum, which is accompanied by: (i) disruption of Ca^{2+} homeostasis; (ii) cell growth arrest in G_1 phase; and (iii) phosphorylation of eukaryotic translation initiation factor 2a (eIF2a), which leads to attenuation of global protein synthesis (31-34). Clearly, DHA serves as a modulator of cellular redox homeostasis and is a key regulator of cell signaling.

It is widely accepted that acute oxidative stress triggers apoptosis or necrosis, but persistent oxidative stress (low or moderate level) induces genomic instability and has been implicated in malignant transformation, tumor progression and drug resistance (35). Usually, the therapeutic strategies in cancer (chemotherapy and radiation therapy) are directed to abnormal production of ROS in cancer cells. Many conventional anticancer drugs (*e.g.* doxorubicin, cisplatin, paclitaxel, and bleomycin) up-regulate the intracellular level of ROS, that enhances their cytostatic/cytotoxic efficiency (36). However, the harmful side-effects of these drugs are also usually due to ROS-mediated mechanisms, and disturbance of redox homeostasis of non-cancer cells and tissues. Using natural or synthetic redox-modulators in combination with chemotherapy (or radiation therapy) can protect normal cells against oxidative stress (37-39). However, it was found that conventional antioxidants may in fact reduce the therapeutic effect of anticancer drugs (37-39). In order to achieve therapeutic selectivity and efficiency in cancer using such combinations, it is necessary to take advantage of the fundamental differences in metabolism between cancer and normal cells. Targeting of unique biochemical alterations in cancer cells (as their redox homeostasis) might be a feasible approach to achieving therapeutic activity and selectivity, and perhaps in preventing the development of drug resistance and side-effects (35).

In this context, DHA seems to be a promising candidate due to its redox-modulating effects, which might be different in cancer and non-cancer cells. Many researchers have shown that DHA increases the efficacy of conventional anticancer drugs (12-14, 21, 40-50). Pre-enrichment of cancer cell lines with DHA enhances their sensitivity to a variety of anticancer drugs and more specifically to anthracyclines (44, 45, 47-50). For example, it was reported that ω-3 fatty acids (25-100 μM) increase chemosensitivity of EHEB and MEC-2 cells (derived from patients with B-cell chronic lymphoblastic leukemia and B-cell prolymphocytic leukemia, respectively) to doxorubicin, vincristine and fludarabine (13). DHA (30 μM) sensitizes breast cancer cells to doxorubicin, that is accompanied by increased levels of malondialdehyde and changes of glutathione-related enzymes (loss of cytosolic glutathione peroxidase activity) (47, 48). DHA (25-150 μM) sensitizes neuroblastoma cell lines (including multidrug-resistant cells) to doxorubicin, cisplatin, and irinotecan (49), which is accompanied by depolarization of the mitochondrial membrane, production of ROS (by mechanisms, involving intracellular peroxidation of DHA catalysed by 15-lipoxygenase or autoxidation) and accumulation of DNA in sub-G_1 phase of the cell cycle (49, 51). DHA delays and inhibits the growth of neuroblastoma xenograft in athymic rats (52). Most of these studies suggest a ROS-dependent mechanism of DHA-mediated cytotoxicity in cancer. However, there are publications demonstrating that DHA increases cytotoxicity of doxorubicin in glioblastoma cell lines (A-172, U-87 MG) and bronchial carcinoma cell line (A-427), without enhancement of lipid peroxidation products (50). Pettersen *et al.* also reported that oxidative stress response is not the cause of DHA-mediated cytotoxicity in colonic cancer cell lines (53). The combination of DHA and doxorubicin inhibited doxorubicin-induced ROS in non-cancer cells (*e.g.* renal tubular epithelial cells and cardiomyocytes) (16, 54).

These studies show that the mechanisms of enhancement of cytotoxicity of anticancer drugs by DHA are complex and cell-specific and do not always require an increased production of ROS.

The aim of the present study was: (i) to investigate the possibility of sensitizing leukemia lymphocytes to anticancer drugs using DHA; (ii) to find combinations with synergistic cytotoxic effect on leukemia lymphocytes and to investigate their cytotoxicity towards normal lymphocytes; (iii) and to clarify the role of ROS in the induction of apoptosis and cytotoxicity by these combinations. The study included 15 anticancer drugs, conventional and new-generation.

Materials and Methods

Cells and treatment protocol. The experiments were performed on Jurkat leukemia lymphocytes (Hayashibara Chem. Lab., Okayama, Japan), derived from patients with acute lymphoblastic leukemia, as well as on normal lymphocytes, isolated from healthy blood donors using Lymphosepar-I (Immuno-Biological Laboratories Co.,

Fujioka, Japan). The cells were cultured in RPMI-1640 medium (Sigma-Aldrich, Steinheim, Germany), supplemented with 10% heat-inactivated fetal bovine serum (FSB; Gibco, Auckland, New Zealand), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco), in a humidified atmosphere at 37˚C with 5% CO_2. All cells were collected by centrifugation (1000 × *g*, 10 min.) and placed in fresh medium without antibiotics before treatment with anticancer drugs.

The drugs were dissolved in dimethyl sulfoxide (DMSO; suitable for cell cultures; Sigma-Aldrich) or phosphate-buffered saline (PBS; 10 mM, pH 7.4). The final concentration of DMSO in the cell suspension did not exceed 1%. At this concentration, DMSO did not influence cell viability.

The drugs were applied to the cells (1×10^6 cells/ml) at the concentrations given below (single dose) and incubated for different times in a cell incubator. At each time point, aliquots of cells were used for cell viability assay.

The cells were incubated with DHA, drug, or drug plus DHA at the following concentrations: 12.5 μM DHA (Sigma-Aldrich), 0.25 μM palbociclib (Selleckchem, Huston, USA), 0.5 μM PI-103 (Selleckhem, Houston, TX, USA), 5 μM everolimus (Selleckchem), 0.5 μM lonafarnib (Selleckchem), 0.1 μM ABT-737 (Selleckchem), 0.1 μM doxorubicin (Sigma, Steinheim, Germany), 0.5 μM bleomycin (Noppon Kayaku Co., Tokyo, Japan), 0.1 μM AZD-7762 (Sigma), 0.01 μM MLN-2238 (Selleckchem), 0.025 μM MG-132 (Wako, Tokyo, Japan), 10 μM lomustine (Sigma), 2.5 μM cisplatin; Selleckchem), 0.025 μM BEZ-235 (Selleckchem), 0.01 μM bortezomib (Selleckchem), 0.05 μM or 0.01 μM barasertib (Selleckchem). The selected concentrations of drugs and DHA (applied separately) induced about 20% inhibition of cell growth.

Cell viability assay. Cell viability was analyzed using trypan blue staining and Countess™ Automated Cell Counter (Invitrogen, OR, USA) at very precise standardization of measurement. Three independent experiments (with two repetitive measurements) for each experiment were performed for each sample. Non-treated cells were used as controls. The data are presented as the mean±SD.

Intracellular ROS assay. The amount of ROS was analyzed using OxiSelect™ *In vitro* ROS/RNS Assay Kit – Green Flourescence (Cell Biolabs., Inc., San Diego, CA, USA). The method is based on the use of the fluorogenic probe, 2',7'-dichlorodihydrofluorescein (DCHF)–DiOxyQ. In the cytosol, the probe is de-acetylated to non-fluorescent DCHF. DCHF reacts with ROS and reactive nitrogen species (RNS) (predominantly H_2O_2, $ROO^{\bullet}$, NO, $ONOO^-$) with formation of the fluorescent product 2',7'-dichlorofluorescein (DCF). The intensity of DCF fluorescence (λ_{ex}=480 nm, λ_{em}=530 nm) is proportional to the amount of ROS/RNS in the biological sample. The amount of ROS/RNS was calculated by calibration curve based on DCF standard solution in PBS. The measurements were performed on a Tecan Infinite F200 PRO (Tecan Austria GmbH, Mannedorf, Austria) microplate reader.

Briefly, treated and untreated cells (1×10^6 cells/ml) were collected by centrifugation (1000 × *g*, 10 min) and lysed by using 300 μl of 0.1% sodium dodecylsulfate (SDS; dissolved in PBS) within 30 min on ice. The lysates were adjusted to have an equal protein concentration (in the range 1-10 mg/ml) using PBS. Protein concentration was analyzed using Bradford reagent (Sigma-Aldrich). Each sample was subjected to ROS/RNS assay, according to the manufacturer's instruction.

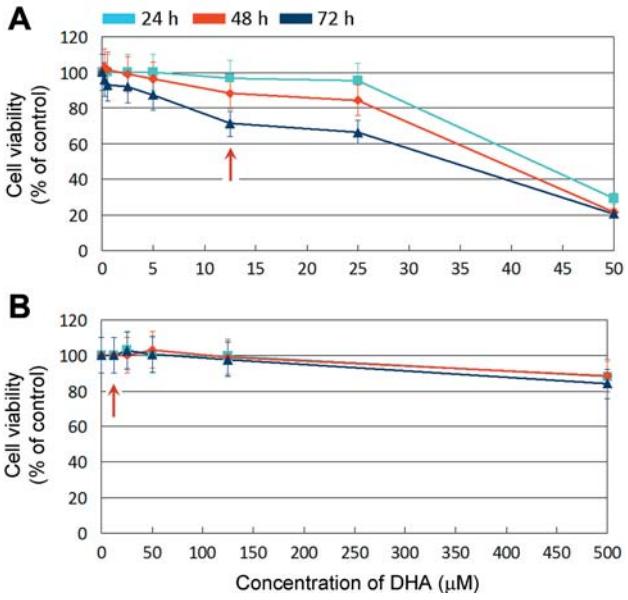

Figure 1. *Effect of docosahexaenoic adic (DHA) on viability of leukemia lymphocytes Jurkat (A) and normal lymphocytes (B) after different incubation times. The are the mean±SD of six independent experiments. The arrows indicate the concentration of DHA (12.5 μM), selected for further experiments in combination with anticancer drugs.*

Protein-carbonyl assay. The amount of protein-carbonyl products were analyzed using OxiSelect™ Protein Carbonyl Spectrophotometric Assay Kit (Cell Biolabs, Inc.). The most common products of protein oxidation in biological samples are the protein-carbonyl derivatives of proline, arginine, lysine and threonine. These derivatives are chemically stabile and serve as markers of oxidative stress. The analysis of these products is based on derivatization of the carbonyl groups with dinitrophenylhydrazine (DNFH) with formation of protein–hydrazone. The amount of protein–hydrazone was detected spectrophotometrically at 375 nm.

Briefly, treated and untreatedcells (1×10^6 cells/ml) were collected by centrifugation (1000 × *g*, 10 min) and lysed using 300 μl of 0.1% SDS (dissolved in PBS) within 30 min on ice. The lysates were adjusted to equal protein concentration (in the range 1-10 mg/ml) using PBS. Protein concentration was analyzed by Bradford assay. Each sample was subjected to the protein-carbonyl assay, according to the manufacturer's instruction. Oxidized bovine serum albumin was used as a standard.

Apoptosis assay. The induction of apoptosis was analyzed by the expression of phosphotidylserine (PSer) on the cell surface, using fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection Kit (BioVision, Milpitas, CA, USA).

Briefly, treated and untreated cells (1×10^6 cells/ml) were incubated with drug, DHA or their combination, under the conditions mentioned above. At each time-point, the cells were collected by centrifugation (1000×g, 10 min), washed twice with PBS, containing 2.5 mM $CaCl_2$ (annexin V-binding buffer) and re-suspended in the same buffer. One hundred microliters of the suspension were incubated with 5 μl of FITC-annexin V for 10 min

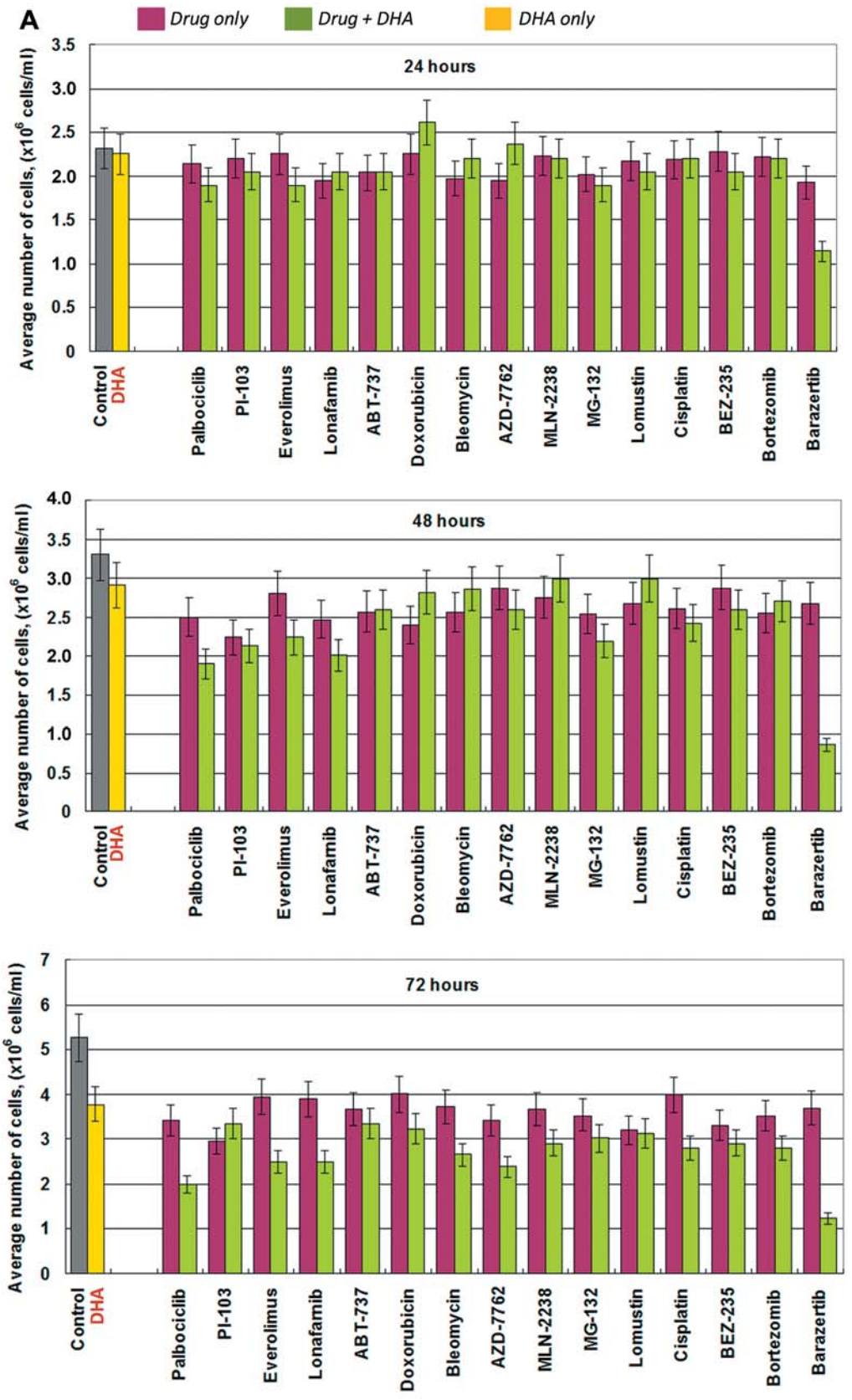

Figure 2. *continued*

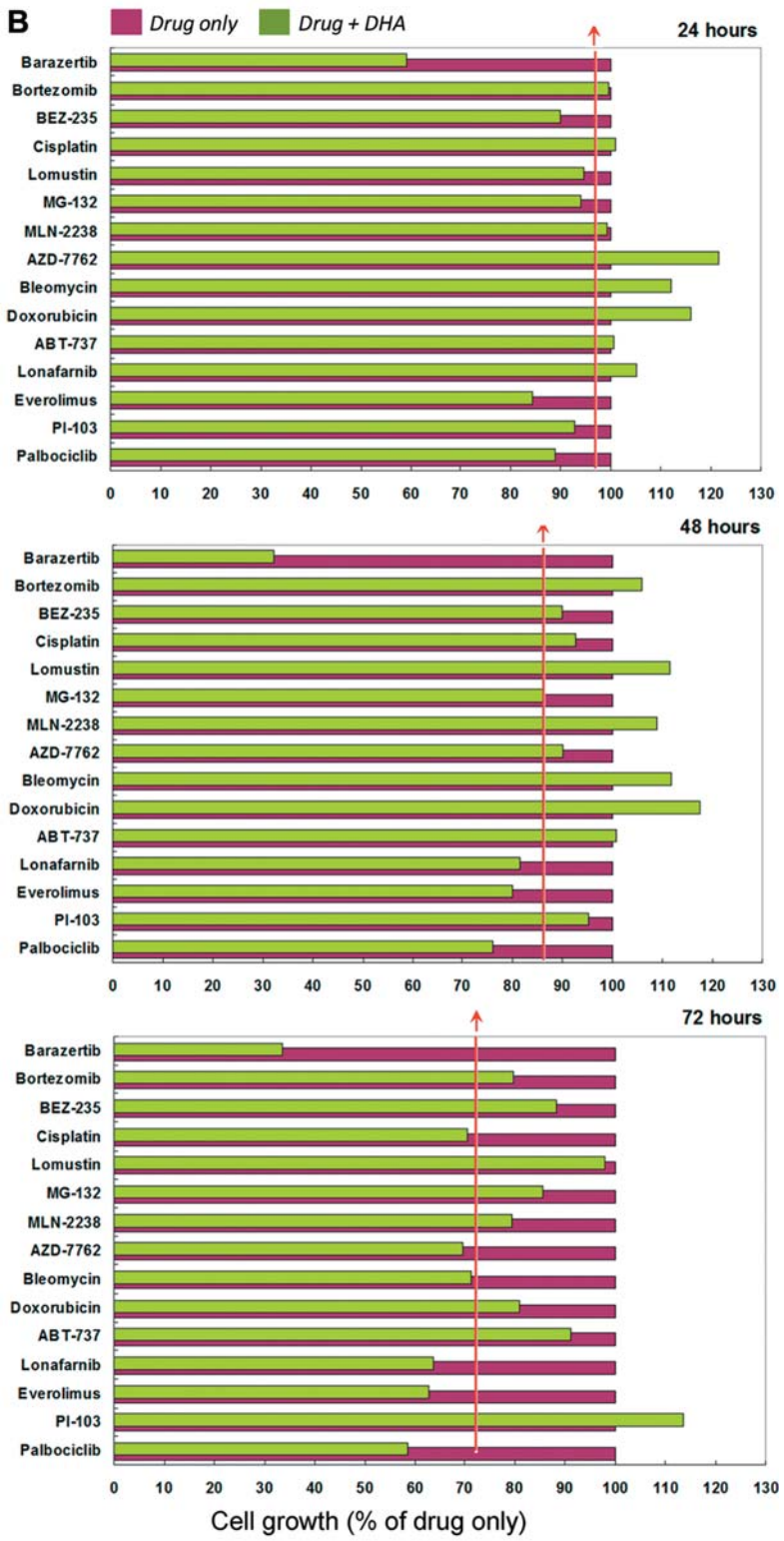

Figure 2. *A: Effect of docosahexaenoic acid (DHA) and anticancer drugs alone and in combination on cell proliferation of Jurkat cells after incubation for 24, 48 and 72 h. Incubation conditions: 1×10^6 cells/ml, 12.5 μM DHA/drug (at concentrations given in the Materials and Methods), at 37˚C in a humidified atmosphere. The data are the mean±SD of six independent experiments. B: Effect of each combination (drug plus DHA) on cell proliferation as a percentage of the effect of drug when applied alone. The arrow indicates the effect of DHA on cell proliferation activity of cancer cells as a percentage of the control (untreated cells). *In this experiment, the concentration of barasertib was 50 nM.*

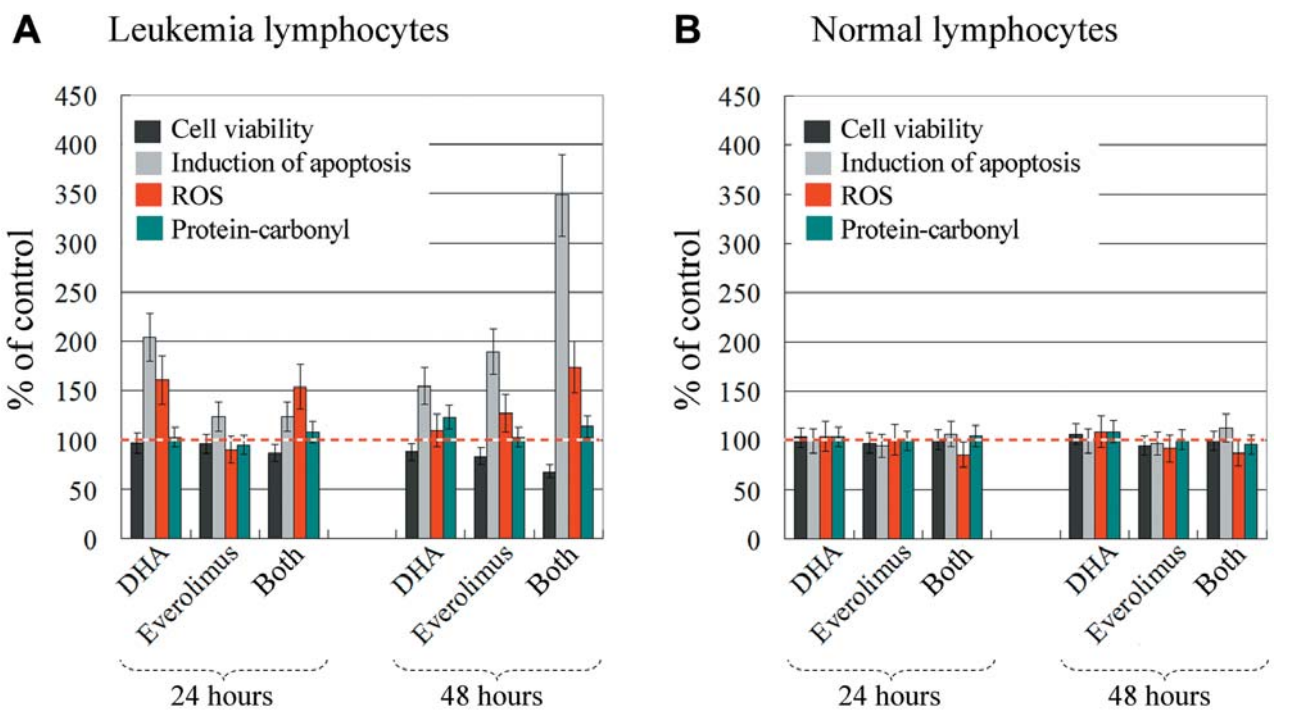

Figure 3. *Effects of docosahexaenoic acid (DHA; 12.5 μM), everolimus (5 μM) and their combination on cell viability, induction of apoptosis, level of reactive oxygen species (ROS) and level of protein-carbonyl products in Jurkat leukemia cells (A) and normal lymphocytes (B), after 24 and 48 h incubation at 37˚C in a humidified atmosphere. The data are the mean±SD of three independent experiments.*

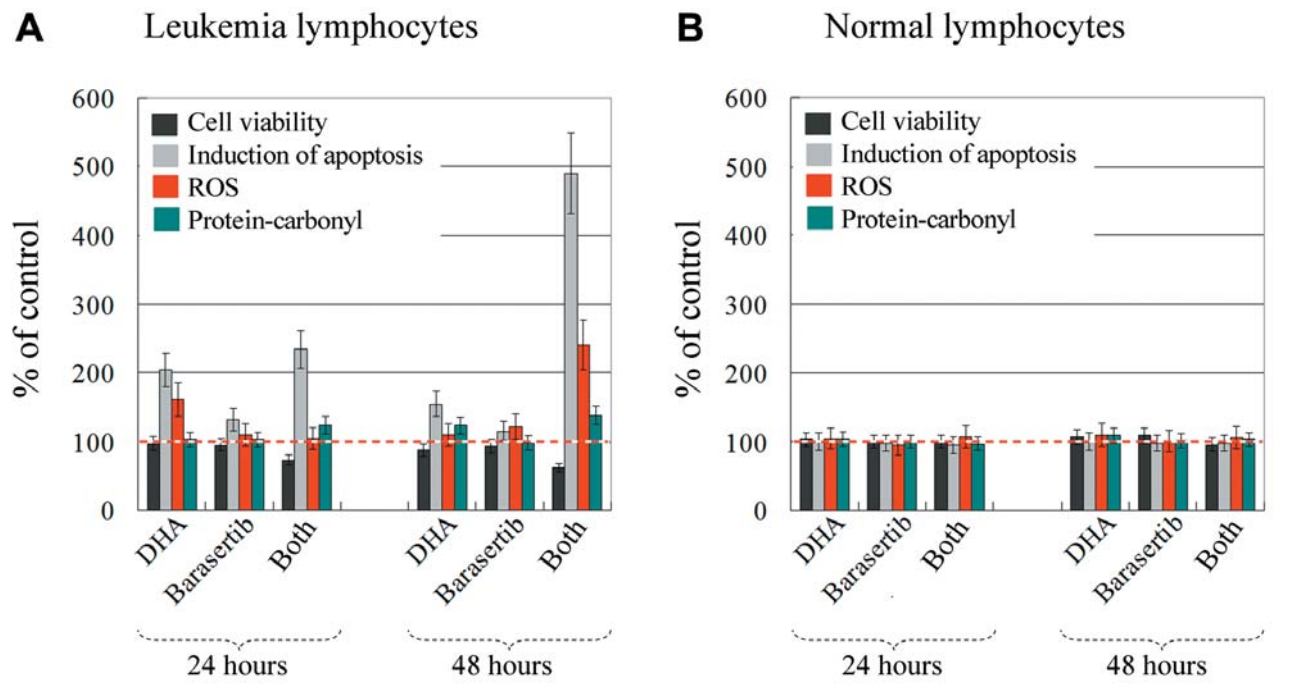

Figure 4. *Effects of docosahexaenoic acid (DHA; 12.5 μM), barasertib (0.01 μM) and their combination on cell viability, induction of apoptosis, level of reactive oxygen species (ROS) and level of protein-carbonyl products in Jurkat leukemia cells (A) and normal lymphocytes (B), after 24 and 48 h incubation at 37˚C in a humidified atmosphere. The data are the mean±SD from three independent experiments.*

at room temperature in the dark. The cells were then washed three times with annexin V-binding buffer and finally were re-suspended in 500 μl of the same buffer. FITC-annexin V, bound to PSer exposed on the cell surface, was detected spectrofluorimetrically at λ_{ex}=488 nm and λ_{em}=535 nm, using a Tecan Infinite F200 PRO (Tecan Austria GmbH) microplate reader.

Results and Discussion

The data in Figure 1 show the viability of leukemia and normal lymphocytes when treated by DHA at different concentrations and for different times. DHA (applied alone or 50 μM) exhibited a strong cytotoxicity (~80%) towards Jurkat cells, while it was completely non-cytotoxic to normal lymphocytes, even at higher concentrations. Other authors also reported similar results (15, 17). This experiment allowed the selection of the most appropriate concentration of DHA for application in combination with anticancer drugs – 12.5 μM, which is approximately the the concentration, which causes 20% inhibition of cell proliferation (IC_{20}) for leukemia lymphocytes at 48-hours of incubation (Figure 1A).

Jurkat cells were treated with: (i) drug only; (ii) DHA only; and (iii) combination of drug and DHA. The aim of this step of the study was to find combinations with synergistic cytotoxic effects on leukemia cells. The data in Figure 2 demonstrates the proliferation of leukemia lymphocytes, treated with DHA with/without drugs for 24-72 h. To distinguish the synergistic cytotoxic effect from antagonistic/additive effects in the combinations, we calculated the effect of each combination on cell proliferation as a percentage of the effect of the respective drug applied alone and compared it with the effect of DHA applied alone, as described previously (55). The red line in Figure 2B indicates the effect of DHA alone on cell proliferation. In the case of drug plus DHA, the data located to the left of the red line reflect synergistic cytotoxic effects, while the data located to the right of the red line represent antagonistic effects. All data matching the red line reflect an additive affect. The cytotoxicity of each drug (applied separately at the selected concentration) ranged from ~10-20% after 24-h incubation to ~20-30% after 48-h and 72-h incubation. The cytotoxicity of DHA ranged from 0 to ~25%, depending on the length of incubation. Most of combinations (drug plus DHA) were characterized by additive or antagonistic effects on cell proliferation of Jurkat cells compared with cells treated with drug only (Figure 2 B). Well-expressed synergistic cytotoxic effects were observed after treatment of Jurkat cells with DHA in combination with: barasertib, lonafarnib, everolimus, and palbociclib. This synergism increased with increasing incubation time.

Two synergistic combinations were selected for the next step of the study: (everolimus plus DHA) and (barasertib plus DHA). We investigated the effect of these combinations on viability of normal lymphocytes and clarified whether the cytotoxicity was accompanied by enhancement of ROS and induction of apoptosis in both cell types (leukemia and normal).

Everolimus (at 5 μM) was cytotoxic towards Jurkat cells, but not normal lymphocytes (Figure 3). The cytotoxic effect of the drug on Jurkat cells was ~15% after 48-h incubation (Figure 3A). DHA increased the cytotoxicity of everolimus by up to ~30%, but the combination did not affect the viability of normal lymphocytes. In Jurkat cells, the cytotoxicity of everolimus applied alone was accompanied by a strong induction of apoptosis (~twice that of the control level after 48-h incubation), but only relatively slight (insignificant) production of ROS. DHA increased the induction of apoptosis by everolimus, as well as the level of ROS, by up to 3.5-fold and ~75% of the control levels, respectively. The level of protein-carbonyl products did not change significantly in any of the samples. At the selected concentrations, neither everolimus nor its combination with DHA affected the level of ROS and protein-carbonyl products, nor did they induce apoptosis of normal lymphocytes (Figure 3B).

Similar tendencies were observed in cells treated with barasertib and its combination with DHA (Figure 4). The cells were treated with a very low concentration of barasertib, 10 nM. At this concentration, barasertib and DHA when applied alone did not affect the viability of either cell type. However, their combination induced a very strong cytotoxic effect on Jurkat cells (~40%), but not on normal lymphocytes. In Jurkat cells, the cytotoxicity of the combination of barasertib and DHA was accompanied by strong induction of apoptosis and production of ROS – about 5- and 2.5-fold the control levels, as detected after 48-h incubation (Figure 4A). The level of protein-carbonyl products increased slightly in leukemia cells, by about 25-30% compared to the control. Neither barasertib nor its combination with DHA significantly affected the level of ROS and protein-carbonyl products, not did they induce apoptosis of normal lymphocytes (Figure 4B).

In the literature, there are many data regarding sensitizing cancer cells to conventional anticancer drugs (such as doxorubicin, bleomycin, and cisplatin) by essential fatty acids, particularly DHA (12-14, 21, 40-50). Most of them suggest ROS-dependent mechanisms. A limited number of studies (only two) describe the effect of ω3-PUFAs on the cytotoxicity of new-generation anticancer drugs (recently approved for clinical use or still in clinical trials), such as proteasome inhibitors bortezomib and MG-132 (17, 56). There exist no data about the effect of ω3-PUFAs on the efficiency of new-generation anticancer drugs such as AZD-7762 (a selective inhibitor of checkpoint kinases), barasertib (a selective inhibitor of aurora B kinase), everolimus (mammalian target of rapamycin inhibitor), lonafarnib (a

farnesyltrasferase inhibitor), MLN-2238 (proteasome inhibitor), and palbociclib (a selective inhibitor of cyclin-dependent kinases).

Recently, Abdi *et al*. reported that eicosapentaenoic acid (EPA) and DHA induced apoptosis and increase sensitivity to bortezomib in myeloma cells, but did not affect viability of normal human peripheral mononuclear cells (17). They found that EPA and DHA inhibited NF-κB activity and induced apoptosis through mitochondrial perturbation and caspase-3 activation (17). This study suggests that EPA and DHA induce selective cytotoxic effects in myeloma cells and increase sensitivity to bortezomib.

Dimri *et al*. reported that DHA and EPA sensitized breast cancer cells to MG-132 through down-regulation of the polycomb group protein enhancer of zeste homologue 2 (EZH2), and up-regulation of E-cadherin and insulin-like growth factor-binding protein 3, which are known targets of EZH2 (56). Treatment with ω3-PUFAs, but not with ω6-PUFAs (linoleic acid and arachidonic acid), also led to a decrease in the invasiveness of breast cancer cells, an oncogenic phenotype that is known to be associated with EZH2. Theses studies suggest that EZH2 is an important target of ω3-PUFAs and that down-regulation of EZH2 may be involved in the mediation of anti-oncogenic and chemopreventive effects of ω3-PUFAs.

The molecular mechanisms of the synergism between new-generation anticancer drugs and ω3-PUFAs are still unknown. Currently, this is under intensive investigation. To our knowledge, our study is the first to report a synergistic cytotoxicity between DHA (as a redox modulator) and both everolimus and barasertib (as an anticancer drug) which is ROS-dependent, but specific for cancer cells (particularly for acute lymphoblastic leukemia cells Jurkat). These combinations are harmless to normal lymphocytes and do not induce abnormal production of ROS in those cells. The data suggest that DHA could be used as a supplementary component in anticancer chemotherapy, which allows for reduction of the therapeutic doses of everolimus and barasertib, minimizing their side-effects. This statement can also be made for lonafanib and palbociclib that also have synergistic cytotoxic effects in combination with DHA.

References

1 Fetterman JW Jr. and Zdanowicz MM: Therapeutic potential of n-3 polyunsaturated fatty acids in disease. Am J Health Syst Pharm *66*: 1169-1179, 2009.

2 Saravanan P, Davidson NC, Schmidt EB and Calder PC: Cardiovascular effects of marine omega-3 fatty acids. Lancet *376*: 540-550, 2010.

3 Swanson D, Block R and Mousa SA: Omega-3 fatty acids EPA and DHA: health benefits throughout life. Adv Nutr *3*: 1-7, 2012.

4 Julia V, Macia L and Dombrowicz D: The impact of diet on asthma and allergic diseases. Nat Rev Immunol *15*: 308-322, 2015.

5 Wysoczański T, Sokoła-Wysoczańska E, Pękala J, Lochyński S, Czyż K, Bodkowski R, Herbinger G, Patkowska-Sokoła B and Librowski T: Omega-3 fatty acids and their role in the central nervous system – a review. Curr Med Chem 2016 doi: 10.2174/0929867323666160122114439.

6 Karmali RA, Marsh J and Fuchs C: Effect of n-3 fatty acids on growth of a rat mammary tumor. J Natl Cancer Inst *73*: 457-461, 1984.

7 Gonzalez MJ, Schemmel RA, Gray JI, Dugan LJr, Sheffield LG and Welsch CW: Effect of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas in a thymic nude mice: relationship between carcinoma growth and lipid peroxidation product levels. Carcinogenesis *12*: 1231-1235, 1991.

8 Xia S, Lu Y, Wang J, He C, Hong S, Serhan CN and Kang JX: Melanoma growth is reduced in *Fat-1* transgenic mice: impact of omega-6/omega-3 essential fatty acids. Proc Nalt Acad Sci USA *103*: 12499-12504, 2006.

9 Jia Q, Lupton JR, Smith R, Weeks BR, Callaway E, Kang LA, McMurray DN and Chapkin RS: Reduced colitis-associated colon cancer in *Fat-1* (n-3 fatty acid desaturase) transgenic mice. Cancer Res *68*: 3985-3991, 2008.

10 Siddique RA, Harvey KA, Walker C, Altenburg J, Xu Z, Terry C, Camarillo I, Jones-Hall Y and Mariash C: Characterization of synergistic anticancer effects of docosahexaenoic acid and curcumin on DMBA-induced mammary tumorogenesis in mice. BMC Cancer *13*: 418, 2013.

11 Zou S, Meng X, Meng Y, Liu J, Liu B, Zhang S, Ding W, Wu J and Zhiu J: Microarray analysis of anticancer effects of docosahexaenoic acid on human colon cancer model in nude mice. Int J Clin Exp Med *8*: 5075-5084, 2015.

12 Gelsomino G, Corsetto PA, Campia I, Montorfano G, Kopecka J, Castella B, Gazzano E, Ghigo D, Rizzo AM and Riganti C: Omega 3 fatty acids chemosensitize multidrug/resistant colon cancer cells by down-regulation cholesterol synthesis and altering detergent-resistant membrane composition. Mol Cancer *12*: 137, 2013.

13 Fahrmann JF and Hardman WE: Omega 3 fatty acids increase chemosensitivity of B-CLL-derived cell lines EHEB and MEC-2 and of B-PLL-derived cell line JVM-2 to anticancer drugs doxorubicin, vincristine and fludarabine. Lipids Health Dis *12*: 36, 2013.

14 Fracasso PM, Picus J, Wildi JD, Goodner SA, Creekmore AN, Gao F, Govindan R, Ellis MJ, Tan BR, Linette GP, Fu CJ, Pentikis HS, Zumbrun SC, Egorin MJ and Bellet RE: Phase 1 and pharmacokinetic study of weekly docosahexaenoic acid–paclitaxel, taxoprexin, in resistant solid tumor malignancies. Cancer Chemother Pharmacol *63*: 451-458, 2009.

15 Anel A, Naval J, Desportes J, Gonzalez B, Uriel J and Pineiro A: Increased cytotoxicity of polyunsaturated fatty acids on human tumoral B and T-cell lines compared with normal lymphocytes. Leukemia *6*: 680-688, 1992.

16 Hsu HC, Chen CY and Chen MF: n-3 Polyunsaturated fatty acids decrease levels of doxorubicin-induced reactive oxygen species in cardiomyocytes – involvement of uncoupling protein UCP2. J Biomed Sci *21*: 101, 2014.

17 Abdi J, Garssen J, Faber J and Redegeld FA: Omega-3 fatty acids, EPA and DHA induce apoptosis and enhance drug sensitivity in multiple myeloma cells, but not in normal peripheral mononuclear cells. J Nutr Biochem *25*: 1254-1262, 2014.

18 Hidaka A, Shimazu T, Sawada N, Yamaji T, Iwasaki M, Sasazuki S, Inoue M, Tsugane S and Japan Public Health Center-based Prospective Study Group: Fish, n-3 PUFA consumption and pancreatic cancer risk in Japanese: a large, population-based, prospective cohort study. Am J Clin Nutr *102*: 1490-1497, 2015.
19 Sawada N, Inoue M, Iwasaki M, Sasazuki S, Shimazi T, Yamaji T, Takachi R, Tanaka Y, Mizokami M, Tsugane S and Japan Public Health Center-based Prospective Study Group: Consumption of n-3 fatty acids and fish reduces risk of hepatocellular carcinoma. Gastroenterology *142*: 1468-1475, 2012.
20 Chan SS, Luben R, Olsen A, Tjonneland A, Kaaks R, Lindgren S, Grip O, Bergmann MM, Boeing H, Hallmans G, Karling P, Overvad K, Venø SK, van Schaik F, Bueno-de-Mesquita B, Oldenburg B, Khaw KT, Riboli E and Hart AR: Association between high dietary intake of the n-3 polyunsaturated fatty acid docosahexaenoic acid and reduced risk of Crohn's disease. Aliment Pharmacol Ther *39*: 834-842, 2014.
21 Bougnoux P, Hajjaji N, Ferrasson MN, Giraudeau B, Couet C and Floch OL: Improving outcome of chemotherapy of metastatic breast cancer by docosahexaenoic acid: a phase II trial. Br J Cancer *101*: 1978-1985, 2009.
22 Merendino N, Costantini L, Manzi L, Molinari R, D'Eliseo D and Velotti F: Dietary ω-3 polyunsaturated fatty acid DHA: A potential adjuvant in the treatment of cancer. BioMed Res Int, Article ID 310186, 2013.
23 Gleissmann H, Johnsen I and Kogner P: Omega-3 fatty acids in cancer, the protectors of good and the killers of evil? Exp Cell Res *316*: 1365-1373, 2010.
24 Siddiqui RA, Harvey K and Stillwell W: Anticancer properties of oxidation products of docosahexaenoic acid. Chem Phys Lipids *153*: 47-56, 2008.
25 Trombetta A, Maggiora M, Martinasso G, Cotogni P, Canuto RA and Muzio G: Archidonic and docosahexaenoic acids reduce the growth of A549 human lung tumor cells increasing lipid peroxidation and PPARs. Chem Biol Interact *165*: 239-250, 2007.
26 Ding WQ, Vaught JL, Yamauchi H and Lind SE: Differential sensitivity of cancer cells to docosahexaenoic acid-induced cytotoxicity: the potential importance of down-regulation of superoxide dismutase 1 expression. Mol Cancer Ther *3*: 1109-1117, 2004.
27 Tuller ER, Beavers CT, Nitchel JR, Ihnat A, Benbriik MD and Ding WQ: Docosahexaenoic acid inhibits superoxide dismutase 1 gene transcription in human cencer cells: the involment of peroxisome proliferator-activated receptor α and hypoxia-inducible factor -2α signaling. Mol Pharmacol *76*: 588-595, 2009.
28 Diep QN, Touyz RM and Schiffrin EL: Docosahexaenoic acid, a peroxisome proliferator-activated receptor-alpha ligand, induces apoptosis in vascular smooth muscle cells by stimulation of p38 mitogen-activated protein kinase. Hypertension *36*: 851-855, 2000.
29 Manzi L, Costantini L, Molinari R and Merendino N: Effect of dietary w-3 polyunsaturated fatty acid DHA on glycolytic enzymes and Warburg phenotypes in cancer. Biomed Res Int, Article ID 137097, 2015.
30 Spencer L, Mann C, Metcalfe M, Webb M, Pollard C, Spencer D, Berry D, Steward W and Dennison A: The effect of omega-3 FAs on tumor angiogenesis and their therapeutic potential. Eur J Cancer *45*: 2077-2086, 2009.
31 Jakobsen CH, Storvold GL, Bremseth H, Follestad T, Sand K, Mack M, Olsen KS, Lundemo AG, Iversen JG, Krokan HE and Schonberg SA: DHA induced ER stress and growth arrest in human colon cancer cells: associations with cholesterol and calcium homeostasis. J Lipid Res *49*: 2089-2100, 2008.
32 Kolar SS, Barhoumi R, Callaway ES, Fan YY, Wang N, Lupton JR and Chapkin RS: Synergy between docosahexaenoic acid and butyrate elicits p53-independent apoptosis *via* mitochondrial Ca^{2+} accumulation in colonocytes. Am J Physiol Gastrointest Liver Physiol *293*: G935-G943, 2007.
33 Kolar SS, Barhoumi R, Lupton JR and Chapkin RS: Docosahexaenoic acid and butyrate synergistically induce colonocyte apoptosis by enhancing mitochondrial Ca^{2+} accumulation. Cancer Res *67*: 5561-5568, 2007.
34 Schroder M and Kaufman RJ: Endoplasmic reticulum stress and the unfolded protein response. Mutat Res *569*: 29-63, 2005.
35 Trachootham D, Alexandre J and Huang P: Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? Nat Rev Drug Discov *8*: 579-591, 2009.
36 Ozben T: Oxidative stress and apoptosis: impact of cancer therapy. J Pharm Sci *96*: 2181-2196, 2007.
37 De Larco J, Park CA, Dronava H and Furcht LT: Paradoxical roles for antioxidants in tumor prevention and eradication. Cancer Biol Ther *9*: 362-370, 2010.
38 Ivanova D, Bakalova R, Lazarova D, Gadjeva V and Zhelev Z: The impact of reactive oxygen species on anticancer therapeutic strategies. Adv Clin Exp Med *22*: 899-908, 2013.
39 Yasueda A, Urushima H and Ito T: Efficacy and interaction of antioxidant supplements as adjuvant therapy in cancer treatment: A systematic review. Integr Cancer Ther *15*: 17-39, 2016.
40 Larsson SC, Kumlin M, Ingelman-Sundberg M and Wolk A: Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. Am J Clin Nutr *79*: 935-945, 2004.
41 Pardini RS: Nutritional intervention with omega-3 fatty acids enhances tumor response to anti-neoplastic agents. Chem Biol Interact *162*: 89-105, 2006.
42 Chapkin RS, McMurray DN and Lupton JR: Colon cancer, fatty acids and anti-inflammatory compounds. Curr Opin Gastroenterol *23*: 48-54, 2007.
43 Berquin IM, Edwards IJ and Chen YQ: Multi-targeted therapy of cancer by omega-3 fatty acids. Cancer Letters *269*: 363-377, 2008.
44 Colas S, Maheo K, Denis F, Goupille C, Hoinard C, Champeroux P, Tranquart F and Bougnoux P: Sensitization by dietary docosahexaenoic acid of rat mammary carcinoma to anthracycline: a role for tumor vascularization. Clin Cancer Res *12*: 5879-5886, 2006.
45 Hardman WE, Avula CP, Fernandes G and Cameron IL: Three percent dietary fish oil concentrate increased efficacy of doxorubicin against MDA-MB 231 breast cancer xenografts. Clin Cancer Res *7*: 2041-2049, 2001.
46 Wang Z, Butt K, Wang L and Liu H: The effect of seal oil on paclitaxel-induced cytotoxicity and apoptosis in breast carcinoma MCF-7 and MDA-MB-231 cell lines. Nutr Cancer *58*: 230-238, 2007.
47 Maheo K, Vibet S, Steghensb JP, Dartigeasa C, Lehmana M, Bougnoux P and Gore J: Differential sensitization of cancer cells to doxorubicin by DHA: a role for lipoperoxidation. Free Radic Biol Med *39*: 742-751, 2005.

48 Vibet S, Goupille C, Bougnoux P, Steghens JP, Gore J and Maheo K. Sensitization by docosahexaenoic acid (DHA) of breast cancer cells to anthracyclines through loss of glutathione peroxidase (GPx1) response. Free Radic Biol Med *44*: 1483-1491, 2008.

49 Lindskog M, Gleissman H, Ponthan F, Castro J, Kogner P and Johnsen JI: Neuroblastoma cell death in response to docosahexaenoic acid: sensitization to chemotherapy and arsenic-induced oxidative stress. Int J Cancer *118*: 2584-2593, 2006.

50 Rudra PK and Krokan HE: Cell-specific enhancement of doxorubicin toxicity in human tumour cells by docosahexaenoic acid. Anticancer Res *21*: 29-38, 2001.

51 Gleissman H1, Yang R, Martinod K, Lindskog M, Serhan CN, Johnsen JI and Kogner P: Docosahexaenoic acid metabolome in neural tumors: identification of cytotoxic intermediates. FASEB J *24*: 906-915, 2010.

52 Gleissman H, Segerström L, Hamberg M, Ponthan F, Lindskog M, Johnsen JI and Kogner P: Omega-3 fatty acid supplementation delays the progression of neuroblastoma *in vivo*. Int J Cancer *128*: 1703-1711, 2011.

53 Pettersen K, Monsen VT, Hakvag-Pettersen CH, Overland HB, Pettersen G, Samdal H, Tesfahun AN, Lundemo AG, Bjorkoy G and Schonberg SA: DHA-induced stress response in human colon cancer cells – focus on oxidative stress and autophagy. Free Radic Biol Med *90*: 158-172, 2016.

54 Lin H, Hou CC, Cheng CF, Chiu TH, Hsu YH, Sue YM, Chen TH, Hou HH, Chao YC, Cheng TH and Chen CH: Peroxisomal proliferator-activated receptor-alpha protects renal tubular cells from doxorubicin-induced apoptosis. Mol Pharmacol *72*: 1238-1245, 2007.

55 Zhelev Z, Ivanova D, Aoki I, Saga T and Bakalova R: 2-Deoxy-D-glucose sensitizes cancer cells to Barasertib and Everolimus by ROS-independent mechanism(s). Anticancer Res *35*: 6623-6632, 2015.

56 Dimri M, Bommi PV, Sahasrabuddhe AA, Khandekar JD and Dimri GP: Dietary omega-3 polyunsaturated fatty acids suppress expression of EZH2 in breast cancer cells. Carcinogenesis *31*: 489-495, 2010.

Received January 28, 2016
Revised March 10, 2016
Accepted March 15, 2016