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EFFECTS OF POLYUNSATURATED FATTY ACIDS ON MULTIDRUG RESISTANCE AND DNA METHYLATION IN HUMAN CANCER CELL LINES

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ABSTRACT

Accumulating evidences indicate that dietary intake of long-chain polyunsaturated fatty acids (PUFAs) can affect various cellular processes and improve response of cancer cells to chemotherapy. The mechanisms by which PUFAs affect this response are not well understood. P-glycoprotein (P-gp), encoded by the multidrug resistance gene MDR1, is a drug efflux transporter that plays an important role in the bioavailability of anti-cancer drugs. Effects of long-chain polyunsaturated fatty acids on MDR1 gene expression and functional activity in the human colon cancer cell line Caco-2 were studied in this research. Caco-2 cells were treated with different concentrations of three PUFAs: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA). All three PUFAs down-regulated the expression of the MDR1 gene (EPA, 34%, DHA, 32% and AA, 27%). The inhibition of gene expression by these PUFAs was accompanied by reduction in protein levels of P-gp. The calcein-AM efflux assay indicated that EPA, DHA, and AA can increase intracellular accumulation (hence decrease the efflux) of calcein-AM (a P-gp substrate) by 25% to 31%. In addition, incubation of cells with PUFAs greatly enhanced the cytotoxicity of the anti-cancer drug paclitaxel. All three PUFAs also induced apoptosis and enhanced paclitaxel-induced apoptosis in Caco-2 cells. Together, these results suggest that inhibition of the multidrug resistance MDR1/P-gp is one mechanism through which dietary polyunsaturated fatty acids exert a positive effect on the response of tumor cells to anti-cancer drugs. In addition,

transcriptional promotion of the nuclear receptors CAR and PXR by PUFAs was also observed in this study.

Moreover, to determine whether the eicosapentaenoic acid affects BRCA1 expression through promoter methylation, BRCA1 promoter methylation patterns and gene expression in U937 cells were examined. The methylation status of the BRCA1 promoter was evaluated by methylation-specific PCR (MSP) of bisulfite conversion products. The results indicate that methylation of BRCA1 promoter DNA is reduced in EPA-treated cells. The reduction of methylation in the BRCA1 promoter was accompanied by an increase in mRNA levels obtained by real-time quantitative PCR (qPCR), suggesting that DNA methylation is a possible mechanism by which the dietary ω-3 polyunsaturated fatty acids mediate gene expression in human cells.

Because of these characteristics, use of PUFAs as adjuvants presents a promising strategy in cancer prevention and therapeutics.

DEDICATION

This dissertation is dedicated to those in my life who have provided so much to me: my parents, for your unlimited love, support, encouragement, and caring for me; my husband, Chih-Chao, for your love, thoughtfulness, patience, and encouragement to accompany me the pursuit of success and happiness; my sister and her husband, for your trust and patience; the rest of my family, for your support; my friends in Taiwan and in United States, for the friendship between us. Finally, this dissertation is especially dedicated to my father Yu-Lan Kuan, a remarkable person who enjoyed learning in his lifetime and always inspired me to do my best. Although he is not able to be with me as I finally earn my Ph.D., my memories of him are a blessing to me each day in my life.

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CHAPTER I

INTRODUCTION

Dietary polyunsaturated fatty acids (PUFAs) play important roles in cell physiology including control of cell division and cell growth, and they have been implicated in response to cancer therapeutics and in diseases of the cardiovascular system [1], obesity [2], diabetes [3], arthritis [4], bipolar disorder [5], as well as in cancer [6-8]. Studies demonstrate that ω-3 PUFAs can increase the sensitivity of tumor cells to anti-cancer drugs and suggest that there may be several means by which PUFAs are able to modulate this response [6, 9-12].

The active efflux of a broad range of anticancer drugs through the cellular membrane is one mechanism by which a cell achieves multiple drug resistance (MDR) [13]. P-gp (phospho-glycoprotein), encoded by the MDR1 gene, is a membrane efflux transporter which facilitates the movement of cytotoxic drugs out of the cell and is overexpressed in MDR cells [14-16]. Variations in P-gp expression or activity may contribute to the therapeutic efficacy of chemotherapy [17-19]. The ability to inhibit the activity of MDR1 has been proposed as one mechanism by which ω-3 PUFAs affect cancer therapeutics [20], however, the mechanisms by which ω-3 fatty acids exhibit these effects are not fully established.

Another mechanism by which components may affect response to anti-cancer drugs is through transcriptional inactivation (by DNA hypermethylation) of

genes associated with cell cycle control [21, 22]. DNA methylation refers to covalent addition of a methyl group at the 5' carbon of the cytosine ring by DNA methyltransferases (DNMTs) to generate 5-methylcytosine [23]. DNA methylation inhibits binding of transcription factors to gene promoters leading to suppression of gene transcription [24]. BRCA1, a tumor suppressor gene, is repressed due to promoter hypermethylation in 11–31% of breast cancers, 5–15% of ovarian cancers, 60% of pancreatic ductal carcinomas, and 38% of acute myeloid leukemia [25-27].

Although numerous studies link PUFAs to sensitizing cancer cells to anti-cancer drugs, there is no research on whether PUFAs exert this ability by regulating the expression and function of MDR1/P-gp. Examinations of the effect of PUFAs on the expression/activity of MDR1 and on methylation/expression of a tumor suppressor gene are among the studies undertaken to clarify the effects of PUFAs in cancer chemotherapeutics.

Objectives: The results of my studies related to the following specific objectives are presented in manuscript form in Chapters III and IV.

1. Evaluate possible mechanisms involved in modulation of MDR1/P-gp expression by PUFAs in Caco-2 cells.
    a. Determine the effect of the ω-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the ω-6 PUFA arachidonic acid (AA) on the expression of the MDR1 gene, levels of its encoded P-gp

protein and its efflux function in the human colon cancer cell line Caco-2.

b. Determine the effects of PUFAs on the sensitivity of Caco-2 cells to the anti-cancer drug paclitaxel and paclitaxel-induced apoptosis.

c. Determine the effect of PUFAs on the expression of nuclear receptors CAR and PXR.

2. Evaluate the effect of PUFAs on gene methylation

Determine the effect of EPA on the methylation status and expression of the tumor suppressor gene BRCA1 in human leukemic U937 cells.

CHAPTER II

LITERATURE REVIEW

2.1 Polyunsaturated fatty acids (PUFAs)

2.1.1. Structure and metabolic pathways

Polyunsaturated fatty acids (PUFAs) are carboxylic acids containing double bonds. There are two categories of PUFAs, ω-6 (omega-6) and ω-3 (omega-3), distinguished by the position of the first double bond from the methyl terminus of the hydrocarbon chain. Longer ω-6 and ω-3 PUFAs are metabolized from shorter chain precursors by a series of elongation and desaturation reactions (Figures 2.1 and 2.2) [6, 28, 29]. Linoleic acid (LA; 18:2ω-6) is converted to arachidonic acid (AA; 20:4ω-6), and α-linolenic acid (α-LNA; 18:3ω-3) is converted to eicosapentaenoic acid (EPA; 20:5ω-3) and docosahexaenoic acid (DHA; 22:6ω-3).

Twenty-carbon fatty acids of both families (e.g. dihomo-gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid) can derive to different eicosanoids including leukotrienes (LT), prostaglandins (PG) and thromboxanes (TX) [28], metabolites known to be involved in different and conflicting aspects of tumor cell metabolism (Figure 2.2).

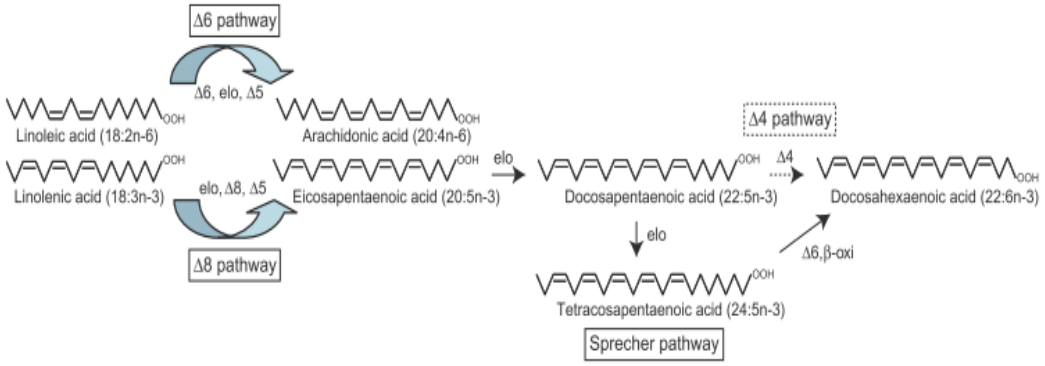

Figure 2.1. Synthesis pathways of long-chain polyunsaturated fatty acids [6]. The fatty acids linoleic acid (LA) and α-linolenic acid (α-LNA) compete for metabolism through a series of enzymes (desaturases and elongases). LA and α-LNA are converted to arachidonic acid (AA) and eicosapentaenoic acid (EPA), respectively, through a sequential action of delta 6 fatty acid desaturase (Δ6), elongase (elo), and delta 5 fatty acid desaturase (Δ5). EPA is further elongated to docosapentaenoic acid (DPA), desaturated by delta 4 fatty acid desaturase (Δ4) to form docosahexaenoic acid (DHA). DPA can also be elongated to tetracosapentaenoic acid (TPA), desaturated to tetracosahexaenoic acid (THA) by Δ6 desaturase, translocated to peroxisomes, and β-oxidized (β-oxi) to docosahexaenoic acid (DHA) (Sprecher pathway).

2.1.2. Sources

LA and α-LNA cannot be synthesized by mammalian organisms due to the lack of required desaturase enzymes (Δ12 and Δ15). Therefore, these two PUFAs are essential and must be obtained from diet to maintain physical

functions. Sources of LA are vegetable seeds and oils (soybean, coconut, corn and sunflower), while sources of α-LNA are dark green leafy plants and perilla, linseed, rapeseed, walnut, canola oil, flaxseed and blackcurrant seed oils [28]. The longer-chain EPA and DHA are found in cold-water fatty fish such as tuna, salmon, herring, sardines and mackerel [30, 31]. EPA and DHA can be produced from α-LNA by human beings; however, the extent of this conversion is inefficient (< 5–10% for EPA and 2-5% for DHA) [30, 31], such that EPA and DHA are acquired mainly through consumption of fish.

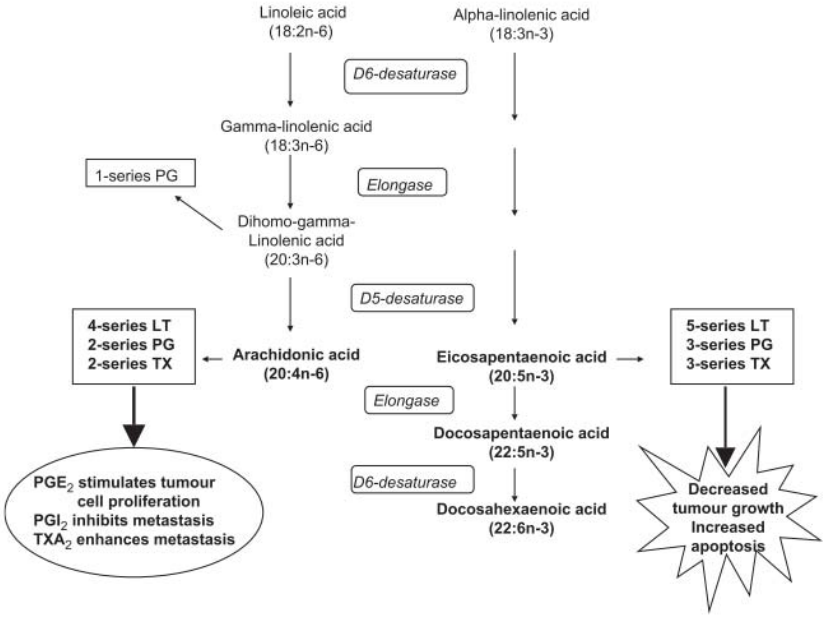

Figure 2.2. Pathways of ω-6 and ω-3 PUFAs metabolism and effects on tumor biology [28]. ω-6 and ω-3 fatty acids are metabolized through a series of enzymes (desaturases and elongases). Twenty-carbon fatty acids of both families (dihomo-gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid)

derive to different series of eicosanoids which include leukotrienes (LT), prostaglandins (PG) and thromboxanes (TX).

Intake of PUFAs leads to their incorporation into membrane phospholipids [32, 33], and diet significantly influences the lipid composition of cell membranes. The relative amounts and types of dietary PUFAs consumed are believed to be of critical importance in determining the effects of PUFAs on cell function.

2.1.3. Effects of PUFAs on human health

Over the past 40 years, increasing evidence indicates that ω-6 and ω-3 PUFAs impact numerous processes in the body (Figure 2.3) [34, 35]. These dietary polyunsaturated fatty acids (PUFAs) have been implicated in diseases of the cardiovascular system [1], obesity [2], diabetes [3], arthritis [4], bipolar disorder [5], as well as in cancer [6-8].

The mechanisms by which PUFAs are thought to affect cellular function are (1) modulation of eicosanoid production [36, 37]; (2) alteration of membrane fluidity or permeability [38, 39]; (3) changes in the production of lipid second messengers [40, 41]; (4) incorporation into lipid rafts, leading to changes in the distribution and/or activity of raft-related signaling proteins [35, 42]; (5) modulation of gene expression [43]; (6) modulation of gene expression via activation of peroxisome proliferator-activated receptors [44]; and (7) modulation of transcription factor activity [45, 46].

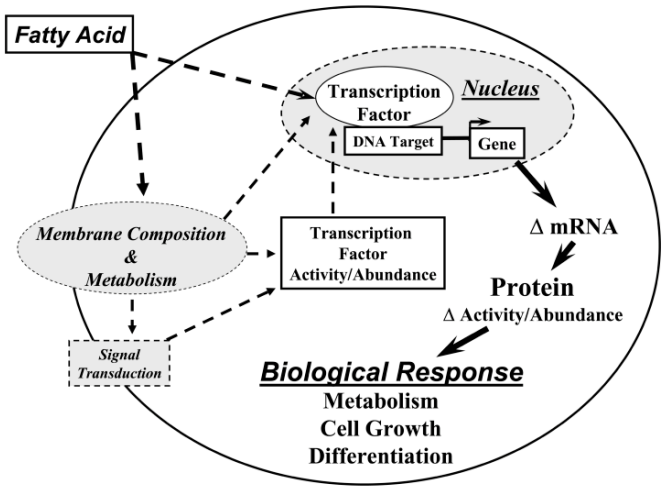

Figure 2.3. Effects of PUFAs on cell functions [35]. Fatty acids regulate some transcription factors by direct binding or by controlling mechanisms that affect nuclear abundance or activity of the transcription factors.

Of particular interest, studies have demonstrated that ω-3 PUFAs can increase the sensitivity of tumor cells to anti-cancer drugs in cell culture and in tumor-bearing animals. For example, DHA and/or EPA enhance the cytotoxic effect of several anti-cancer drugs including doxorubicin [47], epirubicin [48], taxane [49], 5-fluorouracil [50], mitomycin [51], arsenic trioxide [52], and tamoxifen [53] in cell lines derived from human neoplasms (e.g. breast, colon, bladder cancers). In animal studies, treatment with combinations of anti-cancer agents and ω-3 PUFA resulted in decrease of tumor size [48, 54, 55], reduction of side effects [56-59] and increased survival time [60]. In clinical studies, administration of the anti-cancer agents with PUFAs also resulted in the

improvement of nutritional status and quality of life assessments [61] and significant increase of body weight and energy level [62].

2.1.4. Mechanisms by which ω-3 PUFAs may enhance efficacy of chemo-therapeutic drugs

The mechanisms by which ω-3 PUFAs exert a beneficial role as supplements before or during chemotherapy are still not clearly established. Elucidation of these mechanisms is essential to ensure optimal efficacy of chemotherapy drugs and optimal influence of target levels within patients receiving supplements of ω-3 PUFA. Potential mechanisms examined include [10] (1) enhancement of drug uptake or intracellular accumulation [14, 63]; (2) alteration of membrane-associated signal transduction factors [53, 64]; (3) decreasing NF-κB activity [45, 64-66]; (4) lipid peroxidation [47]; (5) modulating expression or function of apoptotic proteins [48, 67, 68] and (6) enhancement of nucleoside analogue drug activity [69]. These potential pathways are discussed below. Of note, unlike many chemotherapy agents used, ω-3 PUFA administration does not appear to have toxic effects on normal cells [70].

2.1.4.1. Enhancement of drug uptake or intracellular accumulation

Studies have demonstrated that incorporation of ω-3 PUFAs into cell membranes modifies membrane permeability and fluidity *in vivo* [71] and *in vitro* [38]. A consequence is modification of the influx and efflux of drugs (particularly

hydrophobic drugs that pass through the membrane by diffusion) into and/or out of tumor cells [72]. (See discussion in MDR section) Increased drug uptake is also related to an effect of ω-3 PUFAs on transport proteins within the membrane [10].

2.1.4.2. Alteration of membrane-associated signal transduction factors

Among the membrane associated signaling factors that may be affected by PUFAs are Ras proteins, Akt activity and Her-2/Leu protein levels. Ras proteins are guanine-nucleotide binding proteins that cycle between inactive GDP-bound and active GTP-bound forms. They promote tumor cell growth, differentiation, survival and resistance to apoptosis [73], and association with lipid membranes is necessary for their function [74]. In colon tumor bearing rats fed a high fat fish oil diet, there are decreased levels of membrane bound Ras, increased levels of cytosolic Ras and decreased activity of farnesyl transferase compared to rats fed a high fat corn oil diet [75, 76]. Further, *in vitro* studies indicate that mouse colon cells treated with DHA have decreased Ras GTP binding/localization to the plasma membrane, compared to cells treated with linoleic acid [77]. These results suggest that ω-3 PUFAs may affect Ras activation by changing its membrane localization.

The PI3K/Akt signaling pathway plays an important role in cell cycle progression, affecting cellular functions including proliferation, apoptosis, differentiation and chemotaxis. Cells are protected from apoptosis by Akt, which

inactivates components needed for cell apoptosis pathways (e.g., caspase-9 and BAD). In addition, Akt can indirectly promote cell survival by activating NF-κB, a pro-survival transcription factor [78]. EPA and/or DHA decrease both Akt phosphorylation [64] and activity [53] *in vitro,* and EPA renders breast cancer cells with high Akt activity more responsive to the anti-cancer drug, tamoxifen [53].

Overexpression of Her-2/Leu, a transmembrane tyrosine kinase receptor belonging to the epidermal growth factor family, leads to taxane-based resistance. Therefore, tumor cells are more sensitive to taxanes when Her-2/Leu is down-regulated [79, 80]. For example, treating Her-2/Leu-overexpressing breast cancer cells with DHA reduced Her-2/Leu protein expression and enhanced the cytotoxic effects of paclitaxel and docetaxel [49].

2.1.4.3. Activity/expression of NF-κB

Nuclear factor-κB (NF-κB) is a transcription factor that plays an important role in cellular survival, growth, differentiation, adhesion and inflammation [81]. NF-κB acts as a promoter of tumorigenesis because it regulates genes (e.g., cyclin D1, bcl-2, bcl-x, matrix metalloproteinases) related to cell survival and inhibition of apoptosis. Patients with breast tumors that express activated NF-κB prior to chemotherapy treatment have a lower clinical response than patients with undetectable NF-κB staining [82]. Other studies have shown that inhibition of NF-κB expression can enhance sensitivity of tumor cells to chemotherapeutic drugs

[81, 83-86]. Long-chain ω-3 PUFAs decrease NF-κB activity or expression in human breast cancer cells [64, 66], macrophages [45] and T cells [87]. These studies suggest that ω-3 PUFA may be able to sensitize tumor cells to chemotherapy by decreasing NF-κB activity or expression.

2.1.4.4. Lipid peroxidation

Long-chain ω-3 PUFAs are susceptible to free radical attack, leading to formation of lipid hydroperoxides. The major effects of lipid peroxidation are inhibition of DNA synthesis, cell division and tumor growth, and induction of tumor cell death [88-90].

Drugs belonging to the anthracycline family of chemotherapeutic drugs (e.g., doxorubicin, epirubicin and daunorubicin) are thought to induce tumor cell death by stimulating formation of oxygen free radicals, which causes irreversible cell damage [91]. Addition of an oxidant such as DHA was reported to enhance cytotoxicity of doxorubicin in human breast cancer cells. This effect was decreased by addition of an antioxidant such as α-tocopherol [92]. Further, the efficacy of doxorubicin in treatment of mammary tumors is increased in mice fed fish oil (3%, w/w) 5 weeks prior to chemotherapy [55]. DHA is also reported to enhance the efficacy of arsenic trioxide, an antineoplastic agent used in treatment of acute promyelocytic leukemia, by inducing apoptosis through a reactive oxygen species-dependent pathway [52].

2.1.4.5. Modulation of expression or function of apoptotic proteins

Most chemotherapeutic drugs are believed to kill tumor cells by inducing or increasing apoptosis [93, 94]. Resistance to apoptosis can result in a decrease in tumor cell sensitivity to many chemotherapeutic agents. Therefore, agents that can increase expression of pro-apoptotic proteins and down-regulate anti-apoptotic proteins may be able to increase the efficacy of standard chemotherapies. Recent findings also demonstrate that most anti-cancer agents induce apoptosis through modification in tumor cell membrane fluidity (reviewed in [95]).

There is extensive evidence that ω-3 PUFAs can regulate expression or activity of apoptotic proteins in tumor cells (reviewed in [96]). DHA or EPA decrease the expression of the anti-apoptosis proteins in many cancer cells [67, 68, 97-99] and the combination of DHA and 5-fluorouracil decreases expression of bcl-2 and bcl-xL more than either agent alone [100].

2.1.5. The roles of ω-6 PUFAs in tumor biology

Whereas long chain ω-3 PUFAs show inhibitory effects on cancer, ω-6 PUFAs are widely considered as factors promoting the development of tumors [101, 102]. Linoleic acid (LA) has been identified as an agent in dietary fat responsible for an up-regulation of tumor growth *in vivo* [103]. However, the ω-6 PUFA arachidonic acid (AA) has been shown to exert a pro-apoptotic effect in many cell lines including HCT116 and U937 [104, 105]. Among the mechanisms

suggested to account for this effect are the ability of AA to (1) activate caspase-3 dependent and shingomielyn-ceramide apoptosis pathways [104, 106, 107]; (2) induce mitochondrial permeability transition and cytochrome c release [108]; (3) up-regulate transcription factors such as AP-1 involved in apoptosis [104, 109, 110]; (4) induce cellular oxidative stress [111].

2.2. Multidrug Resistance (MDR)

A focus of this study was to determine the effect of PUFAs on tumor biology through modulation of multidrug resistance (MDR) (see Chapter III). A tumor is defined as anti-cancer drug resistant if the drug dosage in the tumor is not effective in achieving a clinically ascertained response [112]. Anti-cancer multidrug resistance is defined as the ability of cancer cells to survive in the presence of chemotherapeutic drugs of dissimilar structure and function [19, 113, 114]. Despite remarkable progress in the last decades in understanding the molecular mechanisms leading to cellular resistance to anti-cancer drugs and the clinical introduction of new agents, MDR is still a major reason of therapy failure in the clinical treatment of cancer patients [112, 115-118].

Various cellular pathways and molecular mechanisms may occur simultaneously and/or sequentially lead to the multi-drug resistance (Figure 2.4) [16]. Mechanisms implicated in MDR include (1) decreased drug uptake and increased drug efflux [119]; (2) induction of repair of drug-induced DNA damage,

e.g. induction of MGMT (O^6-methylguanine-DNA methyltransferase) expression [120]; (3) activation of detoxifying systems, e.g. cytochrome P450 oxidases [121, 122]; (4) blocked apoptosis, e.g. disruptions in p53 or ceramide apoptotic signaling pathways [123, 124]. Other factors that may contribute to MDR are the tumor microenvironment, e.g. vascularization, diffusion, hypoxia [125] and the architecture of barriers such as the blood brain barrier [126, 127].

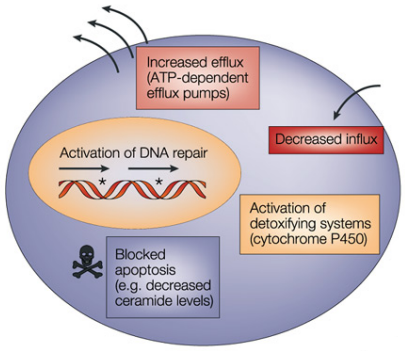

Figure 2.4 Mechanisms of cellular multidrug resistance [16].

Drug influx/intracellular retention is important in achieving sufficient targeting of an anti-cancer drug to the tumor. Membrane transporters/carriers or hydrophilic channels are required for delivery of hydrophilic drugs such as cisplatin, nucleoside analogues and antifolates [128, 129]. Decreased intracellular accumulation for this kind of anti-cancer drug is due to defective transporter/carrier systems [130]. For hydrophobic drugs, such as the natural products vinblastine, vincristine, doxorubicin, daunorubicin, actinomycin D, etoposide, teniposide, and paclitaxel, drug entry occurs by diffusion across the plasma membrane, without any specific drug carriers. Activation of energy-

dependent transport systems, such as ATP-binding cassette (ABC) transporters, is able to keep these drugs out of cells [131, 132].

The ATP-binding cassette transporters share similar sequence and structure. So far, 48 human ABC genes have been identified. They can be divided into seven distinct subfamilies (ABC-A–>ABC-G) on the basis of their sequence homology and domain organization [133]. The first efflux transporter that was identified as mediating a multidrug-resistant phenotype in an *in vitro* model was the P-glycoprotein (P-gp) [134-136] (see below).

2.3. P-glycoprotein (P-gp)

P-gp, encoded by the MDR1 (ABC-B1) gene, is a broad-spectrum transporter expressed in the enterocytes at the villus tip of the small intestine, hepatocytes, renal proximal tubular cells and capillary endothelial cells comprising the blood-brain barrier [137]. P-gp acts as a barrier to uptake of xenobiotics by promoting expulsion of different classes of cytotoxic agents from cells (Table 2.1[16] and Table 2.2 [132]).

Table 2.1. Tissue localization and possible functions of P-gp (modified from [16])

Common Name	Systematic name	Tissue	Non-Chemotherapy substrates	Chemotherapy substrates (known and suspected)
MDR1/ P-gp	ABCB1	Intestine Liver Kidney Placenta Blood-brain barrier	Neutral and cationic organic compounds many commonly used drugs	Doxorubicin Daunorubicin Vincristine Vinblastine Actinomycin-D Paclitaxel Docetaxel Etoposide Teniposide Bisantrene Homoharringtonine (STI-571)

P-gp has 12 transmembrane regions and two ATP binding domains (Figure 2.5). Substrates bind to the transmembrane domains and are exported to extracellular space by the energy of ATP hydrolysis [138]. Two ATP hydrolysis events, which do not occur simultaneously, are needed to transport one drug

molecule [139]. Binding of substrate to the transmembrane domains stimulates the ATPase activity of P-gp, causing a conformational change that releases substrate from membrane [140]. Hydrolysis triggered at the second ATP site seems to be able to reboot the transporter so that it can bind substrate again, completing one catalytic cycle [141].

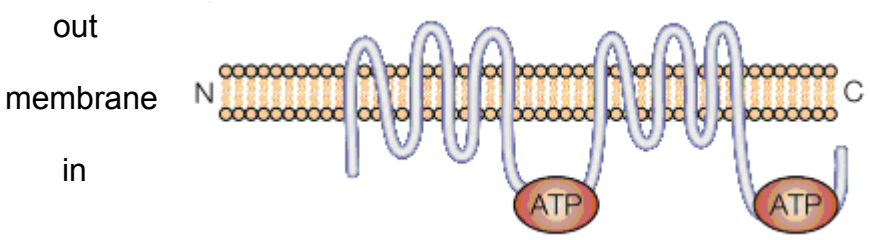

Figure 2.5. Structure of P-gp (modified from [16]). P-gp has 12 transmembrane domains and two ATP binding sites. The line attached to ATPs is protein strand. N: amino-terminal; C: carboxy-terminal.

Overexpression of P-gp results in lower levels of anti-cancer drugs in the blood and reduced diffusion of drugs from the blood into the tumor mass [142]. There is increased tissue concentrations of P-gp substrates in Mdr1a/Mdr1b-knockout mice [16, 143], and when a P-gp inhibitor is administered with P-gp substrates, there is increased absorption of the substrates into tissue [144, 145].

Ample evidences show that expression levels of MDRI impact cancer recurrence [15, 146]. MDR1 is expressed at high levels in many cancers, including kidney, liver, and colon cancer. For some cancers (leukemias

lymphomas, and multiple myeloma), P-gp levels are low at beginning, however, the levels elevate when recurrence occurs after chemotherapy. In other cancers, including chronic myelogenous leukemia in blast crisis and neuroblastoma, the development of the tumor appears to be associated with the turning on of P-gp expression [15].

These observations support the use of inhibitors for P-gp as a strategy to increase the bioavailability of a chemotherapeutic drugs [142]. A number of inhibitors/mediators of ABC-transporters have been identified/developed over the past years (reviewed in [147]). The hope was that these “drug resistance factor-targeting” compounds would reverse/inhibit the ability of drug resistance in cancer cells, thereby enhancing the efficacy of the anti-cancer drugs. Unfortunately, there have been unsatisfactory results in clinical trials, with unwanted side effects [112, 148]. For example, the necessary concentration of verapamil for blocking P-gp in tumors resulted in cardiac toxicity [149], and a Phase III trial with tariquidar, a third-generation inhibitor, was abandoned due to problems with toxicity [150]. Other approaches to modify efflux pump function have included the development of monoclonal antibodies directed against an extracellular epitope of MDR1/P-gp [151] and RNA interference (RNAi) approaches [152]. However, co-administration of an anti-cancer drug with a chemosensitizer that impairs P-gp function is still the basic strategy to circumvent MDR. Selected substrates and modulators of P-gp are listed in Table 2.2 [132].

Table 2.2 Substrates and modulators of P-gp [132]

Substrates	Modulators
Vinca alkaloids	Calcium channel blockers
Vinblastine	Verapamil
Vincristine	Dihydropyridines
Anthracyclines	Antiarrhythmics
Daunorubicin	Quinine
Doxorubicin	Antihypertensives
Antibiotics	Reserpine
Dactinomycin	Antibiotics
Actinomycin D	Cephalosporins
Other cytotoxic agents	Immunosuppressants
Mitomycin	Cyclosporin A
Taxol	Steroid hormones
Colchicine	Progesterone
Puromycin	HIV protease inhibitors
Digoxin	Sequinavir
	Alcoholism treatment drug
	Disulfiram
	Phytochemical
	Curcumin

2.3.1. Transcription of P-gp

Expression of the ABC transporters is highly regulated, particularly at the level of transcription [153]. Some studies indicate that the expression of drug transporter genes can be enhanced in response to chemotherapeutics [154, 155]. Although it was believed that only the MDR-drugs can induce the genes, recent studies indicate that non-MDR drugs , antifolates and hydoxyurea , may also induce MDR1 transcription [156].

We have observed that exposure to PUFAs reduces the expression of MDR1 in Caco-2 cells (see Chapter III). Caco-2 cells, derived from a human colonic adenocarcinoma, express P-gp and other multiple drug resistance proteins at levels similar to those in normal small intestinal epithelial cells and are an appropriate model for studying the effect of PUFAs on P-gp [157, 158]. Our results also indicate that in cells where MDR1 expression was reduced, the efflux function of MDR1/P-gp was decreased and paclitaxel toxicity increased. These observations are consistent with the thesis that inhibition of MDR1 gene expression accounts, at least in part, for the ability of PUFAs to increase the bioavailability of anti-cancer drugs, i.e. counteracts MDR1-mediated drug resistance, in cancer cells.

2.3.2. Other factors affecting P-gp expression and activity

2.3.2.1. Nuclear receptors

Binding of agonist ligands to a heterodimers of nuclear proteins such as the retinoid X receptor (RXR) results in dissociation of a repressor protein from the complex and recruitment of a co-activator protein that in turn promotes transcription of the downstream target gene into mRNA. Nuclear receptor proteins that have been shown to be involved in transcription of drug transporters include the retinoic acid receptor (RAR), farnesoid receptor (FXR), the steroid-activated receptor (SXR), the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) [159-161].

2.3.2.2. Methylation status of local chromatin

Chromatin is transcriptionally inactive in its basal state because DNA in a nucleosomal complex is generally inaccessible to transcription factors. However, signals from the environment can trigger conformation changes in chromatin that alter transcription potential. Promoter-hypomethylated DNA is transcriptionally active, whereas promoter- hypermethylated DNA is transcriptionally inactive (see below). Chromatin-modifying enzymes, i.e. histone acetylases, histone deacetylases and DNA methylases are involved in the regulation of the MDR1 gene [162, 163].

2.3.2.3. The Ras signaling pathway

The MDR1 gene is also a target of the ras/raf signaling pathway [164, 165]. The activation of the MDR1 promoter occurs in the presence of an activated H-

Ras oncogene [166]. Of note, mutations of Ras oncogenes result in down-regulation of MDR1 expression in acute myeloid leukemia [164].

2.3.2.4. Inflammation

The MDR1 gene responds to stress signals such as inflammation [167]. Under inflammatory conditions, the MDR1 gene is induced in the liver [168]. Cyclooxygenase-2 (COX-2) and its product, prostaglandin E2 (PGE2), are involved in inflammatory responses [169] and overexpression of COX-2 results in upregulation of MDR1 expression [170, 171] .

2.3.2.5. P-gp and apoptosis

Apoptosis is one of the main types of programmed cell death, and is commonly associated with activation of caspases. Apoptosis may also be caspase-independent [172]. A link between expression of MDR1/P-gp and cellular apoptotic pathways has been shown [173, 174]. For example, cancer cells expressing MDR1/P-gp exhibit resistance to anti-cancer drugs that trigger apoptosis. This response is associated with the blocking of mitochondrial cytochrome c release into the cytosol [175, 176]. This anti-apoptotic feature of MDR1/P-gp-expressing cancer cells appears to be dependent on the over-expression of Bcl-XL [112].

2.3.2.6. Effect of membrane composition on P-gp activity

Membrane composition and fluidity plays an important role in modulating activity of P-gp [177]. P-gp is located in cholesterol and sphingolipid-enriched domains of the plasma membrane called lipid rafts [178]. Removal of cholesterol from membranes changes P-gp localization and results in loss of P-gp function [179], and the inhibition of P-gp activity by excipients such as PEG are reported to be related to changes in membrane fluidity [180].

2.4. DNA methylation in mammalian cells

2.4.1. Epigenetic modifications

Epigenetic change is defined as a change in gene expression maintained through cell divisions, which is not due to a change in DNA sequence [21, 181]. Within cells, histone proteins bind to DNA and organize chromatin into a compact form to fit into nucleosomes [182]. Epigenetic modifications, including DNA methylation and histone post-translational modifications, affect chromatin structure and gene transcription (Figure 2.6) [183].

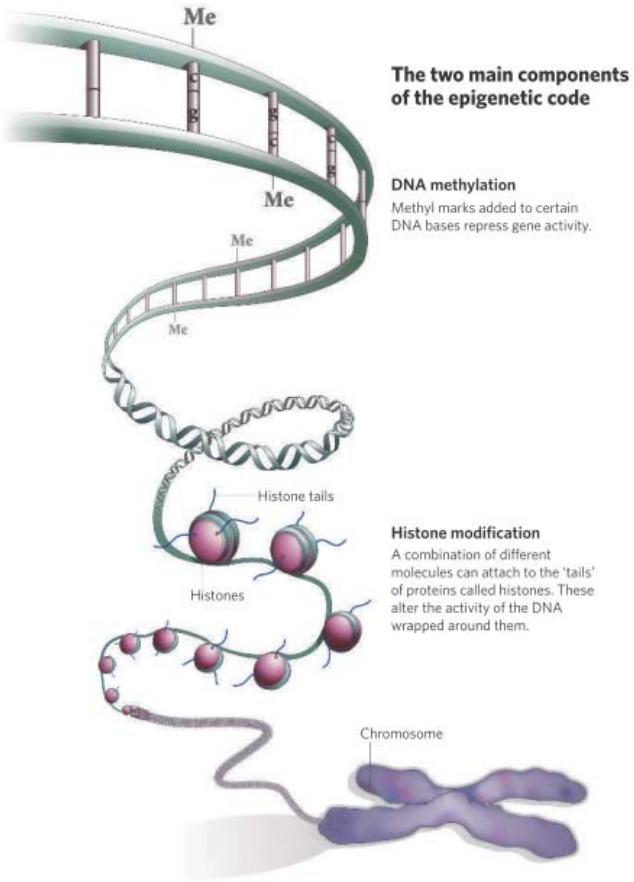

Figure 2.6. Epigenetic modification: DNA methylation and histone modification [184].

In DNA methylation there is covalent addition of a methyl group at the 5' carbon of the cytosine ring to produce 5-methylcytosine (Figure 2.7) [23]. There are two components involved in mammalian DNA methylation: the DNA methyl-transferases (DNMTs) that establish and maintain DNA methylation patterns, and the methyl-CpG binding proteins (MBDs) that recognize methylation sites (Figure 2.8) [185]. In mammalian DNA, cytosines in cytosine-phosphate-guanine dinucleotides (CpGs) are highly (70-80%) methylated [186]. These methyl groups

protrude into the major groove of DNA and can effectively inhibit transcription [187].

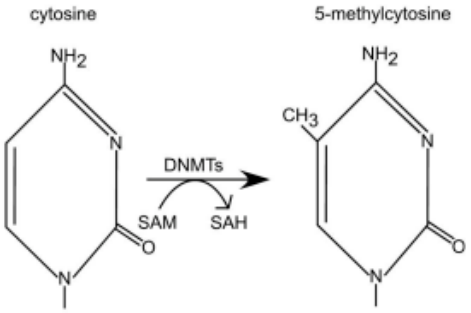

Figure 2.7. Structures of cytosine and 5-methylcytosine [183]. The DNMT enzymes use S-adenylmethionine (SAM) as the methyl donor to add a methyl group to the 5' carbon of the cytosine ring.

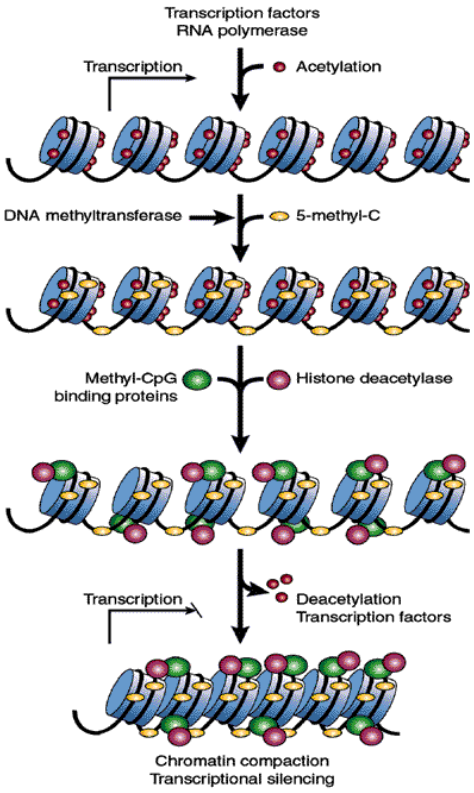

Figure 2.8. DNA methylation and histone deacetylation cooperate to repress transcription [185]. Hyperacetylated and hypomethylated chromatin regions result in a transcriptionally active state. A DNA or a gene destined for silencing recruits DNA methyltransferases. The resulting methylated DNA recruits methyl-CpG binding proteins and histone deacetylases. The DNA will then be heritably maintained in an inactive state.

2.4.2. DNA methylation and cancer

DNA methylation plays an important role affecting cellular function, and alternation in methylation patterns may lead to tumorigenesis [188-191]. The changes in DNA methylation patterns that are commonly observed in cancer cells are outlined in Figure 2.9 [189]. In cancer cells there is often hypo-methylation in repetitive regions, which leads to genomic instability [192-194], as well as activation of normally silenced repetitive DNA elements [195].

De novo methylation of CpG rich regions of DNA (CpG islands), often associated with the promoters of genes, can also occur during tumor development. An average of 600 CpG islands aberrantly methylated in tumors [196]. This number can vary widely between tumor types [196]. Studies indicate that DNA methylation inhibits binding of transcription factors to gene promoters leading to suppression of gene transcription [24]. Transcriptional inactivation of genes associated with cell cycle control, apoptotic signaling and DNA repair by DNA hypermethylation contributes directly to carcinogenesis and tumor

development [21, 22]. Of note, silencing of a specific tumor suppressor gene through DNA methylation affects the growth rate of cancer cells [197].

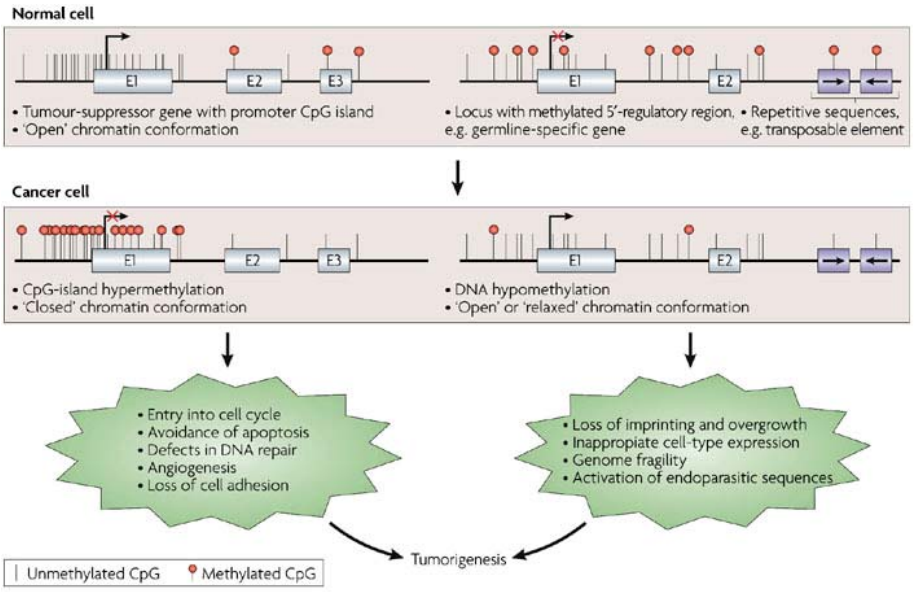

Figure 2.9. Altered DNA-methylation patterns in tumorigenesis [189].

2.4.2.1. DNA methylation as a biomarker for cancer

Improved clinical outcome of most cancers can be achieved by early detection. Therefore, development of early detection is important [198]. Because of their frequency and early occurrence in carcinogenesis, changes in DNA methylation, particularly CpG island hypermethylation, have been extensively examined as a method for early detection of cancers [199]. Methylation specific PCR (MSP) are sufficient and sensitive to detect promoter hypermethylation in serum, plasma, urine and fecal material [200]. Studying epigenetic profiles, DNA

methylation patterns and histone analysis, is expected to provide guidance for optimization of the epigenetic therapies with conventional chemotherapy and tools of diagnosis and prognosis [21, 184]. Several cellular pathways [201] targeted for aberrant DNA methylation-mediated gene silencing are listed in Table 2.3.

Table 2.3. Cellular pathways affected by DNA promoter CpG island hyper-methylation of tumor genes (modified from [201])

Pathways	Representative hypermethylated genes
DNA repair	hMLH1, MGMT, BRCA1
Vitamin response	RARB2, CRBP1
Ras signaling	RASSF1A, NORE1A
Cell cycle	p16INK4a, p15INK4b, Rb
P53 network	p14ARF, p73, HIC-1
Cell adherence and invasion	E-cadherin, H-cadherin, EXT-1
Apoptosis	TMS1, DAPK1, WIF-1, SFRP1
Wnt signaling	APC, DKK-1, IGFBP-3
Transcription factors	GATA-4, GATA-5, ID4
Other pathways	GSTP-1, COX-2, TPEF/HPP1

2.4.2.2. DNA methylation as a target for chemoprevention

Genes silenced by DNA methylation are intact and can be reactivated by inhibitors of the DNMTs [199]. Targeting DNA methylation and DNA methyl-transferases thus provides a promising strategy for cancer treatment [22]. DNMT inhibitors dramatically reduce the incidence of colon cancer [202]. Further, changes in DNA methylation can be readily detected with non-invasive assays. Thus drugs that target DNA methylation or DNMTs are now at the forefront of cancer treatments [184].

Methylation inhibitors (or hypomethylating agents) belong to three classes: (a) nucleoside inhibitors; (b) non-nucleoside inhibitors; and (c) rationally-designed inhibitors [203]. These inhibitors operate by reversing aberrant promoter hypermethylation, resulting in gene reactivation and restoration of cell growth control, apoptosis and DNA repair capacity [204-206]. Two nucleoside inhibitors, 5-azacitidine and Decitabine (Figure 2.10), have been approved by the FDA and are now in clinical use for myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [183, 207].

The cytosine analogues are incorporated into newly synthesized DNA during replication. When DNMTs catalyst the analogues, a covalent complex is formed [208]. This results in trapped DNMT at these sites. Trapped DNMT enzymes cause enzyme degradation, lower DNMT levels, and ultimately, hypomethylation [183].

Unfortunately, when used in high doses, these azanucleosides can produce serious toxicities in patients [203, 209]. As a single agent therapy for MDS, the most success for these drugs has been obtained using low doses and more cycles of therapy [210]. However, the effect of nucleoside inhibitors on gene activation is non-specific and usually involved in multiple cellular pathways [187, 203, 211, 212]. Thus, these demethylating agents may not suitable for application in cancer prevention (requiring long-term administration) due to safety concerns. Other agents such as procainamide [213], zebularine [214] and valproic acid [215] have shown the capacity to reverse DNA methylation and may

be safer for long-term use.

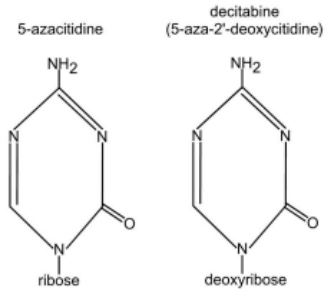

Figure 2.10. Structures of the two FDA-approved hypomethylating agents, 5-azacitidine and Decitabine [183].

2.4.3. BRCA1/Cancer/DNA methylation

BRCA1 is known to be involved in repair of DNA oxidative lesions [216, 217] and in induction of cell cycle arrest and apoptosis [218]. The BRCA1 gene plays a major role in the genesis of breast and ovarian tumors [219-221]. Germline mutations of BRCA1 appear in about 50% of patients with inherited breast cancer [222, 223]. Frequent loss of the wild-type allele in tumors of BRCA1 mutation carriers suggests that BRCA1 acts as a tumor suppressor gene [224].

A decrease in BRCA1 expression [225], associated with inactivation of the promoter by methylation, has been found in some cases of sporadic breast cancer [226-228]. In addition, reduced BRCA1 expression is frequently observed in therapy-related AML (t-AML) and contributes to secondary leukaemogenesis [27].

Of especial interest, DNA methylation patterns are highly sensitive to the chemical environment [191, 229] and studies have suggested that bioactive components of food can modify DNA methylation patterns [230]. For example, tea catechins are effective inhibitors of human DNA methyltransferase-mediated DNA methylation *in vitro* [202, 231]. A number of dietary compounds have also been shown to impact methylation, raising the possibility that an individual's diet may also influence DNA methylation [232]. A better understanding of the relationship between dietary components and cellular epigenetics could suggest ways of suppressing DNA methylation changes associated with aging and cancer [233].

Dietary ω-3 polyunsaturated fatty acids have already been reported to impact to prevent cancer (reviewed in [28, 234, 235]) and to increase the sensitivity to anti-cancer drugs [48, 52, 100]. Moreover, research shows that ω-3 polyunsaturated fatty acids can increase BRCA1 transcription and protein levels in human breast cancer cell lines [236] and *in vivo* [237]. In our study, we examined whether ω-3 polyunsaturated fatty acids can increase BRCA1 gene expression through decreasing promoter DNA methylation.

2.4.4. Analysis of gene methylation by bisulfite conversion and methylation-specific PCR (MSP)

The methylation status of DNA can be assessed using bisulfite conversion and methylation specific PCR (MSP), first described by Australian researchers in 1992 [238]. In this protocol, sodium bisulfite converts unmethylated cytosines (C)

to uracil (U) while methylated cytosines remain unchanged (Figure 2.11). After conversion, PCR is performed using specific primer sets for unmethylated and methylated DNA [239, 240]. The MSP products can be sequenced, visualized by agarose gel electrophoresis or analyzed by qPCR.

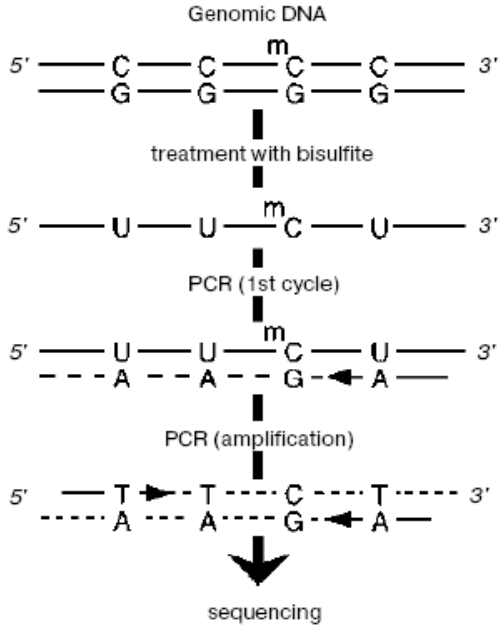

Figure 2.11 Bisulfite conversion (modified from [240]). DNA is denatured and treated with sodium bisulfite. PCR is performed with primers specific to one of two modified chains.

Of note, bisulfite treatment of DNA has some limitations and shortcomings, e.g., a decrease in the sensitivity may occur because of incomplete conversion from cytosine to uracil or partial degradation of DNA caused during bisulfite treatment [241, 242]. A number of approaches have been developed to improve these drawbacks [243].

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CHAPTER III

LONG-CHAIN POLYUNSATURATED FATTY ACIDS INHIBIT THE TRANSCRIPTION AND ACTIVITY OF MDR1/P-GP IN THE HUMAN COLON CANCER CELL LINE CACO-2

ABSTRACT

Accumulating evidence in both humans and animal models indicate that dietary intake of long-chain polyunsaturated fatty acids (PUFAs) can improve response to chemotherapy. The mechanisms by which PUFAs affect this response are not well understood. P-glycoprotein (P-gp), encoded by the multidrug resistance gene MDR1, is a drug efflux transporter that plays an important role in the bioavailability of anti-cancer drugs. Effects of long-chain polyunsaturated fatty acids on MDR1 gene expression and functional activity in the human colon cancer cell line Caco-2 were studied in this research. Caco-2 cells were treated with different concentrations of three PUFAs; eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA). All three PUFAs down-regulated the expression of the MDR1 gene (EPA, 34%, DHA, 32% and AA, 27%). The inhibition of gene expression by these PUFAs was accompanied by reduction in protein levels of P-gp. The calcein-AM efflux assay indicated that EPA, DHA, and AA can increase intracellular accumulation (hence decrease the efflux) of calcein-AM (a P-gp substrate) by 25% to 31%. In addition,

incubation of cells with PUFAs greatly enhanced the cytotoxicity of the anti-cancer drug paclitaxel. All three PUFAs also induced apoptosis and enhanced paclitaxel-induced apoptosis in Caco-2 cells. Together, these results suggest that inhibition of the multidrug resistance MDR1/P-glycoprotein is one mechanism through which dietary polyunsaturated fatty acids exert a positive effect on the response of tumor cells to anti-cancer drugs. In addition, we have shown that PUFAs promote transcription of the nuclear receptors CAR and PXR. Our results support the notion for using PUFAs as promising adjuvants in cancer therapeutics.

3.1. Introduction

Dietary long-chain polyunsaturated fatty acids (PUFAs) are known to mediate numerous cellular processes [1, 2]. There are two main classes of PUFAs, omega-3 (ω-3) and omega-6 (ω-6), distinguished by the position of the first double bond at the methyl terminus of the hydrocarbon chain. Of particular interest, several studies have demonstrated that ω-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) sensitize tumor-bearing animals and tumor cells in culture to cytotoxicity of anti-cancer drugs [3-6]. The mechanisms by which ω-3 fatty acids induce these effects are not fully established.

There are conflicting reports concerning the effect of ω-6 PUFAs on tumor

cells. For example, over-expression of the ω-6 PUFA, arachidonic acid (AA), has been linked to induction of apoptosis in the human colon cancer cell line HCT116 [7] and the human leukemic cell line U937 [8], but others have demonstrated that, unlike the ω-3 PUFAs, AA stimulates the growth of pancreatic and prostate cancer cells in vitro [9, 10].

Treatment of cancer is complicated by the fact that cancer cells have the ability to become resistant to different anticancer drugs. The active efflux of a broad range of anticancer drugs through the cellular membrane is one mechanism by which a cell achieves multiple drug resistance [11]. The human multidrug-resistance MDR1 gene encodes the P-glycoprotein (P-gp), which functions as an energy-dependent membrane efflux pump for a wide variety of lipophilic compounds [12]. The P-gp protein plays an important role in multidrug resistance by decreasing the intracellular retention of many anticancer drugs [13-15], thus preventing the interaction of these agents with their target molecules. Studies indicate that the MDR response is inversely related to the level of P-gp expression in various human cancers [16-18], suggesting that the ability to inhibit the activity of MDR1 has been proposed as one mechanism by which ω-3 PUFAs affect cancer therapeutics [19].

The ability of PUFAs to modulate expression of nuclear receptor genes involved in drug metabolism [20] is another mechanism by which PUFAs may impact MDR1. The constitutive androstane receptor (CAR) [21] and the pregnane X receptor (PXR) [22] function by heterodimerizing with the retinoic acid x

receptor (RXR) and binding to response elements in the promoter regions of target genes [23]. They are reported to be involved in transcription of multiple drug transporters [21, 22, 24, 25], in particular to mediate MDR1 gene expression [25], and have been identified as candidates for mediating gene regulation by fatty acids [26, 27].

Several pathways by which PUFAs may impact MDR were examined in this research. Effects of the ω-3 PUFAs, EPA and DHA, and the ω-6 PUFA AA on the expression of the MDR1/P-gp gene and on the levels and function of its protein product in cells of the human colon cancer cell line Caco-2 were also investigated. To determine whether PUFAs can increase the efficacy of the anti-cancer drug, paclitaxel, effects of PUFAs on paclitaxel-induced cytotoxicity were studied. Since cancer cells expressing MDR1/P-gp are more resistant to caspase-dependent apoptosis-triggering agents than normal cells [28, 29], whether PUFAs can enhance apoptosis in paclitaxel-treated Caco-2 cells was investigated. In addition, we evaluated whether PUFAs modulate MDR1/P-gp via regulation of two nuclear receptors, CAR and PXR, which are reported to be involved in modulation of the expression of MDR1.

3.2. Materials and Methods

3.2.1. Materials and reagents

The sodium salts of arachidonic and eicosapentaenoic acids were purchased from Nu-Chek Prep, Inc. (Elysian, MN). Paclitaxel and the sodium salt of docosahexaenoic acid were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.2. Cell culture

Cultures of Caco-2 cells (human colorectal adenocarcinoma) at passage 12 were obtained from the American Type Culture Collection (Manassas, VA, USA). For sub-culture, phosphate buffered saline (PBS) washed cells at 60-70% confluence were detached from the flask surface with 0.25% (w/v) trypsin-EDTA (0.022%, w/v) and centrifuged at 1,600 rpm for 5 min. Cell pellets were re-suspended in complete medium at a ratio of 1:3 in T75 flasks and maintained at 37°C in 5% CO_2 and 95% humidity. Complete medium contains 10% bovine growth serum (BGS) in minimum essential medium (MEM) with the following supplements: 1% MEM nonessential amino acids, 1% sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. All reagents were purchased from Hyclone, Inc. (Logan, UT). Plastic ware for cell culture was obtained from Fisher Scientific (Pittsburgh, PA) and VWR (West Chester, PA). Caco-2 cells at passage 30 to 40 were used for the experiments in this study.

3.2.3. Preparation of the BSA/fatty acid complex

Serum albumin serves as carrier for free fatty acids in blood [30]. Bovine serum albumin, a well-studied fatty acid carrier protein *in vitro* [30, 31], was used

as the PUFA carrier for these studies. To prepare BSA/PUFA complexes for application to cells, a modification of the method of Ho and Storch [32] was used. Briefly, BSA was coupled to PUFAs by adding the sodium salt of the fatty acid (0.01 g) to MEM medium (9mL) containing 825μM fatty acid-free BSA. The mixture was incubated at 37°C for 12 hr to obtain an optically clear solution, and aliquots of the BSA/fatty acid complex solution were stored under N_2 gas at -20°C. The aliquots were filter-sterilized before addition to the experimental medium. The molar ratio of fatty acid to BSA was 4:1 [33].

3.2.4. Cell viability

Cell viability was measured every 24h for a 72h time period, using the CellTiter-Blue® reagent (CTB, Promega, Madison, WI). The viability assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal.

Caco-2 cells were seeded into flat-bottomed 96-well plates at a density of 10^3 cells/well in a volume of 200 μl of complete medium. After a 24 hr incubation period to complete attachment, cells were exposed to 0–100 μM PUFA. As controls, cells were exposed to complete medium alone. After removal of 100 μl of the cell medium, 20 μl CTB reagent was added to the remaining 100 μl of well medium and cells were incubated at 37°C for 4 hr before quantization of fluorescence on a Synergy HT multi-mode plate reader (Bio-tek Instruments,

Winooski, VT) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The fluorescence values were normalized to the controls and expressed as percent viability.

3.2.5. RNA extraction and reverse transcription (RT)

Caco-2 cells were seeded into T25 flasks at a density of 1.5 x 10^6 cells/flask. After 24h to assure attachment, cells were exposed to 0–100 µM PUFAs. As controls, cells were exposed to complete medium alone. After incubation, cells were washed with PBS and trypsinized with 0.25% (w/v) trypsin-EDTA (0.022%, w/v). Cells pelleted at 1,600 rpm for 5 min were resuspended in PBS. After DNAse I treatment to remove genomic DNA, total RNA was isolated using the SV Total RNA isolation kit (Promega, Madison, WI) according to the manufacturer's instructions. The concentrations and purity of the RNA samples were assessed spectrophotometrically using the DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). RT was performed using the reagents provided in the Verso cDNA kit (Abgene, Rochester, New York). The RT reaction mixture (20 µl) contained 1 µg total RNA, 1 µl anchored oligo-dT primer, 500 µM dNTP mix, 4 ml 5× cDNA synthesis buffer, 1 µl RT enhancer and 1 µl Verso enzyme mix. RT was performed at 42°C for 30 min and terminated by heating at 95°C for 2 min. cDNAs were stored at -20°C.

3.2.6. Real-time quantitative PCR (qPCR)

Real-time quantitative PCR for MDR1, CAR, and PXR was conducted using an Mx3000P real-time PCR system (Stratagene, La Jolla, CA). The gene-specific primers (Fisher-Operon, Huntsville, AL) used for qPCR are shown in Table 3.1 and after initial heating at 95°C for 2 min, amplification proceeded according to the PCR cycling information provided in Table 3.1.

Table 3.1. Primers and cycling information

Gene	Forward sequence	Reverse sequence	Annealing temp (°C)	Annealing time (s)	Cycle	Amplicon (bp)
MDR1	GCTCCTGACTATGCCAAAGC	TCTTCACCTCCAGGCTCAGT	60	40	40	202
CAR	GGGGTTCCAGGTAGAGTTT	GTCGGTCAGGAGAGAAGAG	58	40	40	122
PXR	CGCTTCCTGAGTCTTTTCA	CGCCTGCCGATGAGTACA	58	60	40	115
β-Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	60	40	40	250

All reactions were performed in a singleplex mode. The total reaction mix (20 µl) contained 10 µl 2x SYBR Green PCR master mix (Bio-Rad Laboratories, Hercules, CA), 200 nmol/L of each primer, and 0.8-1 µl cDNA as template. Dissociation curve analyses were done for all samples and only sharp melting points were observed, indicating a specific signal and no primer dimers or mispriming. A negative template control (H_2O) was always included in each run to check for the presence of exogenous contaminant DNA. After the threshold cycle values in real-time PCR were obtained, the amounts of MDR1 were determined from a standard curve generated by serial dilutions of cDNA. After normalization to β-actin values, the expression levels of MDR1 for each condition

were expressed as % of control cells. Each sample was analyzed in triplicate, and data were analyzed using Mx3000P software (Stratagene, La Jolla, CA).

3.2.7. Western blotting

To prepare whole cell lysates, the cells were collected in lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml of pepstatin A, 1 μg/ml of leupeptin, 1 mM NaF, 1 mM Na_3VO_4), and centrifuged at 13000xg for 30 min at 4 °C. Total protein was determined by the BCA (bicinchoninic acid) assay (Pierce, Rockford, IL) and samples were prepared for electrophoresis by boiling for 5 min in 30 μl SDS-sample buffer (Boston Bioproducducts, Worcester, MA). For each condition, 30 μg of total protein was separated on 10% SDS-polyacrylamide gels and electro-transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with blocking reagents of WesternBreeze® chromogenic kit (Invitrogen, Carlsbad, CA) and then incubated with anti-P-gp mAb (Abcam Cambridge, MA) or anti-β-actin mAb (Santa Cruz Biotechnology Inc. Santa Cruz, CA). The bands were detected using reagents provided in the WesternBreeze® chromogenic kit (Invitrogen, Carlsbad, CA). The relative intensity of each protein band was measured using the Quantity One software (Bio-Rad Laboratories, Hercules, CA).

3.2.8. Measurement of drug efflux

The efflux function of P-gp was measured using reagents supplied in the Vybrant^{TM} Multidrug Resistance (MDR) Assay kit (Molecular Probes, Eugene, OR) [34]. This assay uses calcein acetoxymethyl ester (calcein-AM), a highly lipid soluble, non-fluorescent dye that penetrates the plasma membrane of cells, to measure the efflux function of P-gp. Once calcein-AM is inside the cell, the ester bond is cleaved, resulting in formation of the hydrophilic and fluorescent calcein, which is bound and pumped out of the cells by P-gp.

For the assay, cells were seeded at 1.2 x 10^5 cells/well in 12-well plates. After 24 hr, the cells were treated with 100 μM PUFA for 24 hr and then harvested by trypsinization. Cell pellets were washed with PBS and resuspended in PBS containing calcein-AM (final concentration 0.25 μM). Cells treated with DMSO or 20 μM verapamil served as negative and positive controls, respectively. Cells were incubated at 37°C for 20 min, centrifuged at 1,600 rpm for 5 min, washed (3 times) and resuspended in cold PBS. Calcein retention was measured by florescence at 494 nm for excitation and 517 nm for emission using a flow cytometer equipped with the CellQuest software (BD Biosciences, San Jose, CA).

3.2.9. Effect of PUFAs on cell toxicity of paclitaxel

Caco-2 cells were seeded into flat-bottomed 96-well plates at a density of 10^3 cells/well in a volume of 200 μl of complete medium. After a 24 hr incubation period to complete attachment, cells were treated with 0-100 nM of the

anticancer drug paclitaxel (Sigma-Aldrich, St. Louis, MO), with or without addition of 100 μM PUFAs, for 72 hr. The medium and drugs were replenished at 24 hr to avoid inactivation. The results are presented as percent of the solvent control.

3.2.10. Apoptosis

A characteristic of apoptosis is the translocation of phosphatidylserine (PS) to the outer surface of the plasma membrane. Apoptosis of Caco-2 cells was assayed using The Vybrant® apoptosis assay kit #2 (Molecular Probes, Eugene, OR). The kit supplies green-fluorescent Alexa Fluor 488 annexin V and red-fluorescent propidium iodide nucleic acid stain. The externalization of phosphatidylserine in apoptotic cells is detected via annexin V, a phospholipid-binding protein that has a high affinity for PS. Propidium iodide stains necrotic cells with red fluorescence. After treatment with both probes, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence.

For the assay, cells seeded in 12-well plates at a cell density of $5x10^5$ cells/well for 24 hr were treated with 2.5 nM paclitaxel with or without 100 μM PUFA for 72 hr. Paclitaxel and PUFAs were replenished at 24 hr to avoid inactivation of reagents. Cells washed with cold PBS were resuspended in annexin-binding buffer and incubated with annexinV-AlexaFluor 488 conjugate/ propidium iodide according to the supplier's directions for 20 min at room temperature. Cells were then washed with PBS and the fluorescent images were

captured using an Axiovert 135 inverted microscope (Carl Zeiss, Thornwood, NY) with 485nm filter, Image-Pro 5.1 software (Media Cybernetics, Silver Spring, MD), and a ProRes^{TM} C10^{Plus} digital camera (Chori Imaging Corporation, Yokohama, Japan).

3.2.11. Evaluation of mRNA expression of nuclear receptors, CAR and PXR

The effects of PUFAs (24h treatment) on the mRNA levels of the nuclear receptors, CAR and PXR, were evaluated using qPCR (Section 3.2.6). The primer sequences and PCR conditions are listed in Table 3.1.

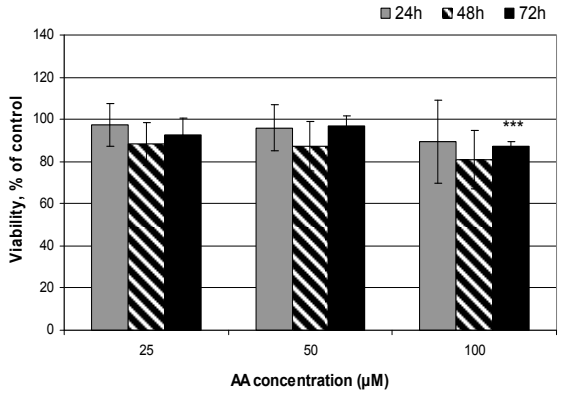
3.2.12. Statistical Analysis

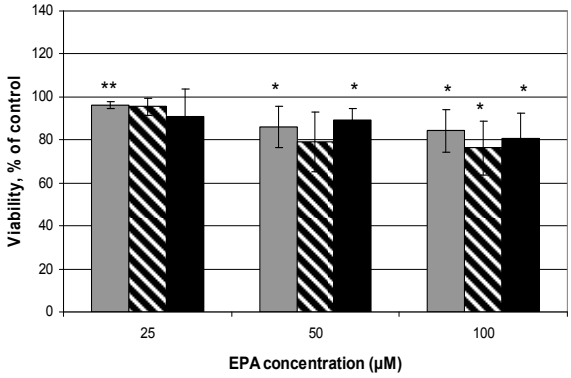
Experimental results were analyzed by a pairwise *t*-test using EXCEL package (Microsoft, Redmount, WA).

3.3. Results

3.3.1. Effect of Polyunsaturated Fatty Acids (PUFAs) on the Viability of Caco-2 Cells.

Three polyunsaturated fatty acids, AA, EPA and DHA were selected for our studies of the mechanisms by which PUFAs contribute to the therapeutic efficacy of chemotherapy. Because polyunsaturated fatty acids can be toxic to human cell growth [35-37], cell viability studies were conducted to determine concentrations of PUFAs appropriate for subsequent experiments. Human colon cancer cell line Caco-2 was incubated with different concentrations of the three PUFAs for 24, 48, and 72 hr. Although inhibition generally increased somewhat when cells were treated with higher concentrations of PUFAs, all three PUFAs, even at the highest concentration of 100 μM, had a modest inhibitory effect (~ 20% of solvent controls) on the growth of Caco-2 cells (Figure 3.1). After 24 hr incubation at concentrations of 50 and 100 μM, EPA appeared to have the most potential for inhibition of cell growth.


24h
48h
72h
Viability, % of control
140
120
100
80
60
40
20
0
***
25
50
100
AA concentration (µM)


Viability, % of control
140
120
100
80
60
40
20
0
**
*
*
*
*
*
25
50
100
EPA concentration (µM)

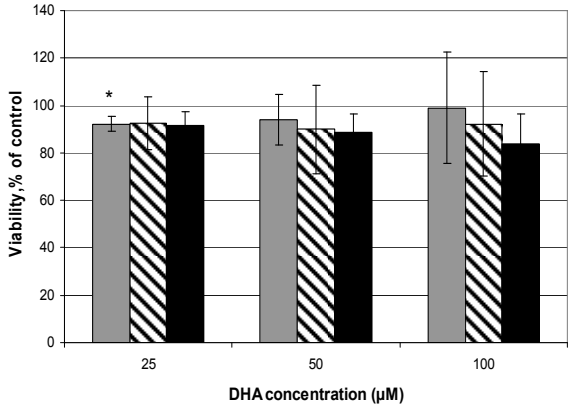

Viability,% of control
140
120
100
80
60
40
20
0
*
25
50
100
DHA concentration (µM)

Figure 3.1. The effect of PUFAs on viability of Caco-2 cells. Cells were incubated for 24, 48, and 72 hours with 25, 50 and 100 μM of the following PUFAs: AA, EPA, and DHA. The viability of treated cells is presented as the means of cell number (± SD) compared to control (untreated) cells. For each experiment, n=3. * (P < 0.05); ** (P < 0.01); *** (P < 0.001) indicate significant differences from control values.

3.3.2. Effect of PUFAs on MDR1 gene expression in the Caco-2 cells

Whether PUFAs modulate the expression of the MDR1 gene in Caco-2 cells was next addressed. Analysis by real-time quantitative PCR (qPCR) suggested that all three PUFAs down-regulated MDR1 expression (Figure 3.2). The degree of down-regulation increased with incubation time, from 4 hrs to 24 hrs. For the 24 hr incubation period, EPA treatment reduced the total mRNA level by ~34%, DHA treatment by 32% and AA treatment by 27%, compared to the solvent controls.

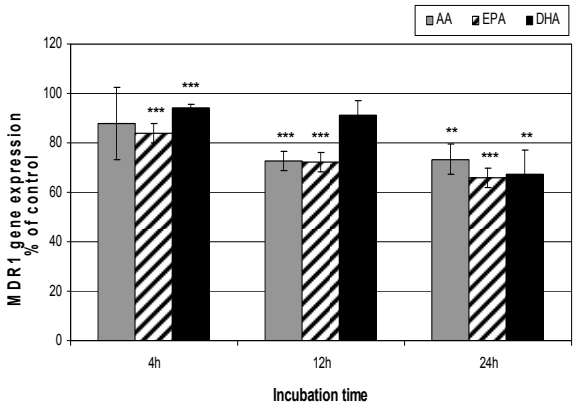


Figure 3.2. The effect of PUFAs on MDR1 gene expression. Caco-2 cells were incubated with 100 μM PUFAs for 4, 12, and 24 hours. mRNA levels were determined by qPCR (see **Materials and Methods**), from samples obtained from three independent experiments. * (P< 0.05); ** (P< 0.01); *** (P< 0.001) indicate significant differences from control values.

To determine whether the inhibition of MDR1 expression by PUFAs occurs in a dose-dependent manner, effects of a second concentration (50 μM) of PUFAs on MDR1 gene expression were examined. Incubation of Caco-2 cells with 100 μM EPA produced greater inhibition of MDR1 expression than did 50 μM EPA (Figure 3.3) at incubation times from 4 hr to 24 hr, indicating that inhibition of MDR1 expression by EPA is dose dependant. Similar results were observed for DHA and AA (data not shown).

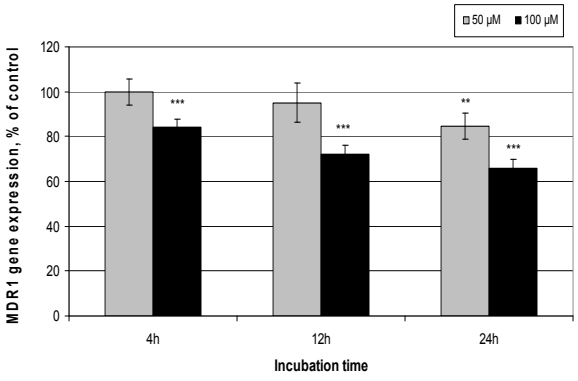


Figure 3.3. The effect of EPA on MDR1 gene expression at concentrations of 50 μM and 100 μM. MDR1 gene expression was determined by real-time

quantitative PCR (qPCR) using RNA samples obtained from three independent experiments. * (P< 0.05); ** (P< 0.01); *** (P< 0.001) indicate significant differences from control values.

3.3.3. PUFAs reduce protein levels of MDR1 in Caco-2 cells

As expected, decrease in levels of the MDR1 gene product P-gp was a consequence of PUFA-induced inhibition of MDR1 gene expression. Western-blot analysis indicated that all three PUFAs reduced levels of P-gp (Figure 3.4). Compared to the control, the level of P-gp protein was reduced by 37% for EPA, by 27% for DHA, and by 48% for AA.

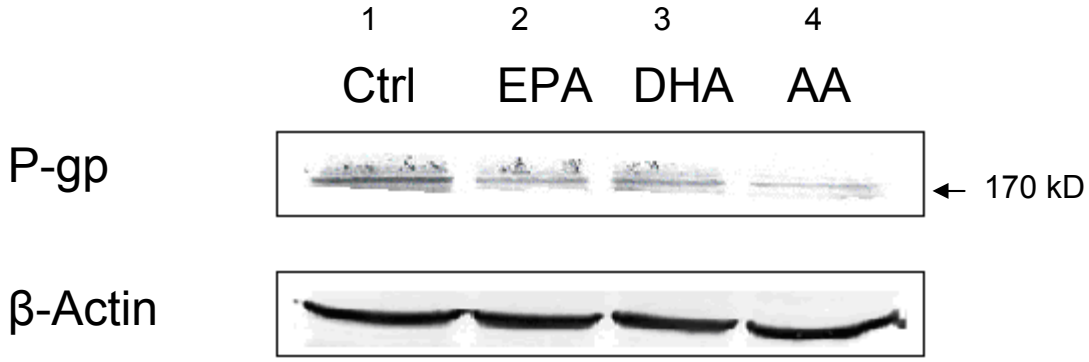


Figure 3.4. The effect of PUFAs on cellular levels of the MDR1 gene product P-gp. Western blot analysis of P-gp (top), β-actin (bottom). Caco-2 cells were treated with 100 μM PUFAs for 24 hours. Lane 1, control; Lane 2, EPA; Lane 3, DHA; Lane 4, AA). These images are representative of three independent experiments.

3.3.4. PUFAs inhibit the efflux function of the MDR1 gene product P-gp

To address whether PUFAs can affect the efflux function of MDR1, we examined whether PUFA-treatment affects drug accumulation in Caco-2 cells. Calcein-AM was used as a probe to monitor the ability of MDR1/P-gp to retain the fluorescent metabolite within the cells. The inhibition of activity was quantified by the change of intracellular fluorescence intensity of calcein.

Exposure of the cells to PUFAs increased intracellular fluorescence by 20-30% compared to controls (Figure 3.5). The increase was similar for all three PUFAs. This increase in intensity indicates that less calcein was exported from cells via P-gp in cells treated with PUFAs than in control cells.

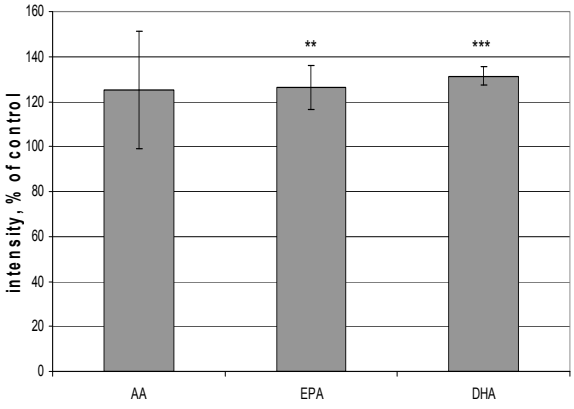


Figure 3.5. The effect of PUFAs on calcein-AM efflux in Caco-2 cells: intracellular fluorescence intensity. Caco-2 cells were incubated with 100 μM of PUFA for 24 hours followed by addition of 0.25 μM of calcein-AM. The results are averages of three independent experiments.

3.3.5. Effect of PUFAs on cytotoxicity of the anticancer drug paclitaxel

To address whether the PUFA-induced reduction in the expression and function of MDR1 is associated with enhanced drug toxicity in cancer cells, the effect of the three PUFAs on the cytotoxicity of paclitaxel were studied. The results suggest that all three PUFAs enhance the cell toxicity of paclitaxel in a dose-dependant manner in Caco-2 cells (Figure 3.6). AA and EPA were in general more effective than DHA in increasing cell toxicity (pairwise *t*-test, $P < 0.01$ and $P < 0.05$, respectively).

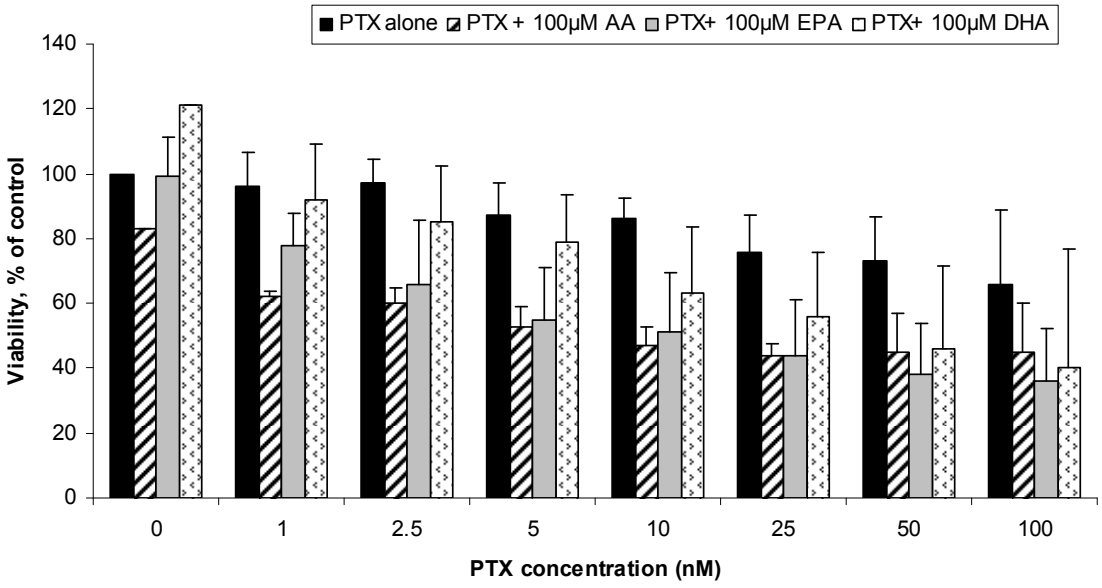


Figure 3.6. The effect of PUFAs on cytotoxicity of paclitaxel (PTX). Results are averages from three independent experiments.

3.3.6. Effect of PUFAs on apoptosis of Caco-2 cells

Tumor growth is often associated with defective apoptosis. Whether enhancement of apoptosis accompanies the effect of PUFAs on MDR1

expression and drug efflux was investigated. Compared to control cells, apoptosis was dramatically increased in cells exposed to PUFAs (Figure 3.7). Because induction of apoptosis is thought to be the main mechanism for the anti-tumor effect of paclitaxel [38, 39], whether PUFAs affect the ability of paclitaxel to induce apoptosis was examined. Whereas exposure to paclitaxel alone induced only modest numbers of apoptotic cells, exposure of cells to both paclitaxel and PUFAs resulted in a dramatic increase in apoptosis. Further, for all three PUFAs, apoptosis of Caco-2 cells exposed to both paclitaxel and PUFA exceeded that of cells exposed to PUFA alone, suggesting that these PUFAs have the ability to enhance drug-induced apoptosis.

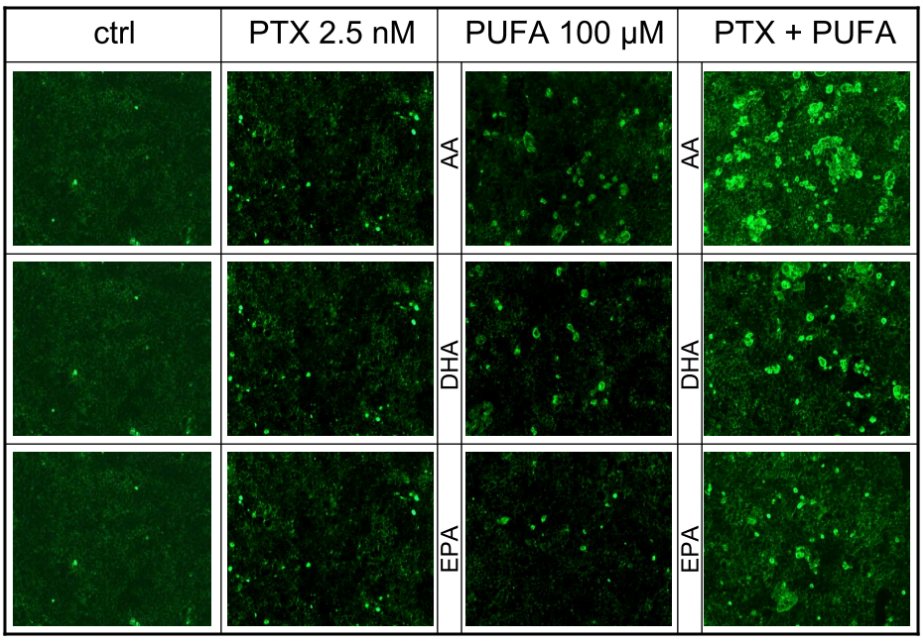


Figure 3.7. The effect of PUFAs on apoptosis. Exposure to PUFAs dramatically increases apoptosis of Caco-2 cells and enhances paclitaxel-induced apoptosis in these cells. Images in Column 1 are cells incubated with solvent controls;

Column 2, cells treated with paclitaxel (PTX); Column 3, cells treated with PUFAs (from top to bottom, AA, DHA, and EPA, respectively); Column 4, cells treated with paclitaxel plus PUFAs (from top to bottom, AA, DHA, and EPA, respectively).

3.3.7. Effect of PUFAs on the expression of the nuclear receptors CAR and PXR

Nuclear receptor proteins that have been shown to be involved in transcription of drug transporters include the pregnane x receptor (PXR) and the constitutive androstane receptor (CAR) [21, 25]. To determine whether the effects of PUFAs on MDR1 are related to these known modulators of MDR1, the relationship between PUFA treatment and expression of CAR and PXR at the mRNA level in Caco-2 cells was evaluated. The results suggest that the expression of the CAR and PXR genes is elevated by treatment with AA, EPA, and DHA (Figure 3.8).

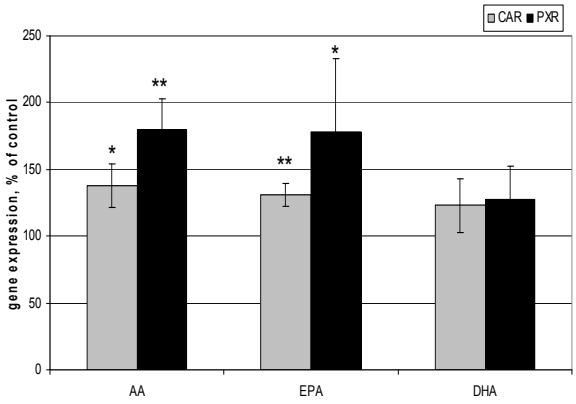


Figure 3.8. The effect of PUFAs on the expression of the CAR and PXR genes. Caco-2 cells were incubated with 100 μM of PUFA for 24 hours. Gene

expression was determined by real time quantitative PCR (qPCR) using RNA samples obtained from three independent experiments.

3.4. Discussion

We report here that exposure to PUFAs reduces the expression of MDR1 in Caco-2 cells (Figure 3.3). The results also indicate that in cells with reduced MDR1 expression, the efflux function of MDR1/P-gp was decreased and paclitaxel toxicity increased (Figures 3.5 and 3.6). These observations are consistent with the thesis that inhibition of MDR1 gene expression accounts, at least in part, for the ability of PUFAs to increase the bioavailability of anticancer drugs, i.e. counteract MDR1-mediated drug resistance, in cancer cells.

Since over-expression of P-gp results in lower blood drug levels and reduced drug diffusion into the tumor mass, one strategy suggested for increasing drug bioavailability involves the use of P-gp inhibitors [40]. A number of natural or synthetic compounds have been discovered that target P-gp, but these reagents produced severe side effects or low activity in vivo [41, 42]. Our data are consistent with previous observations that attribute the potency of ω-3 PUFAs in drug potentiation to the ability to modulate P-gp efflux pump activity [43] and provide further evidence that PUFAs are valuable as potential tools to combat multidrug resistance.

Treatment with PUFAs also dramatically increased apoptosis of Caco-2

cells (Figure 3.7), suggesting a link between decrease in P-gp expression and triggering of apoptotic pathways. These results are consistent with previous observations linking the activity of ABC-transporters, e.g. MDR1, to protection from apoptosis [16]. It has been reported that ω-3 PUFAs induce apoptosis by enhancing phospholipid oxidation and mitochondrial Ca^{++} accumulation [44]; however, the precise mechanism by which PUFAs increase apoptosis in these cells is unclear.

Exposure to PUFAs also enhanced paclitaxel-induced apoptosis in Caco-2 cells (Figure 3.7). Paclitaxel, a member of the taxane drug family, is a commonly used drug for the treatment of solid tumors, including ovarian, breast, head and neck, lung, and prostate cancer [45-48]. Paclitaxel is thought to exert a cytotoxic effect by binding to and inhibiting the function of mitotic microtubules [38, 39, 49, 50], thus triggering apoptosis [51]. Cancer cells expressing MDR1/P-gp are more resistant to caspase-dependent apoptosis-triggering agents than normal cells [28, 29, 52] and the known ability of MDR1/P-gp to export paclitaxel from cancer cells [53-55] may account for some of the paclitaxel resistance phenotypes in cancer patients [56]. Our results support the thesis that exposure of cells to PUFAs contributes to paclitaxel-induced apoptosis by increasing retention of the drug.

Our data also suggest that ω-6 PUFAs can contribute to modulation of MDR. Numerous reports have indicated that ω-3 polyunsaturated fatty acids are beneficial in cancer treatment [3, 5, 6, 57, 58] and that differences exist between the effects of ω-3 and ω-6 PUFAs in cellular function and tumor progression [59].

Notably, the ω-6 PUFA AA has been shown to stimulate rather than inhibit the growth of certain cancer cells, including pancreatic cancer cell growth in vitro [10]. Our data indicate that AA decreases expression of the MDR1 gene (Figures 3.2 and 3.4) and that this effect is accompanied by enhancement of paclitaxel drug efficacy as well as increased apoptosis in Caco-2 cells (Figures 3.6 and 3.7). Interestingly, the extent of these effects is similar to what was observed for the ω-3 PUFAs EPA and DHA (Figures 3.2, 3.4-3.7).

Pregnane X receptor (PXR) and the constitutive X receptor (CAR) are widely recognized as determinants in the regulation of various genes involved in the metabolism and disposition of xeno- and endo-biotics, including multidrug resistance-associated transporters such as MDR1 (P-gp) [25, 60]. The induction of MDR1 gene expression requires PXR binding to the nuclear receptor response elements in the proximal promoter and/or enhancer regions [22]. CAR appears to be another regulator of MDR1 for expression of endogenous MDR1 is high in cells stably expressing CAR [24]. Current models favor the view that PXR and CAR affect transcription of target genes through a process involving heterodimerization with the retinoid x receptor (RXR) and binding to response elements in the promoter region of the target genes [61]. Our studies demonstrate that all three PUFAs up-regulate the mRNA expression of PXR and CAR (Figure 3.8), while reducing MDR1 gene expression (Figure 3.2). Although MDR1 is a potential target gene of PXR and CAR [21, 22, 24], our results suggest that the relationship between the mRNA level of PXR and CAR with

their target gene MDR1 is not linear. PUFAs are not ligands of PXR and CAR [62, 63], we thus hypothesize that without appropriate ligands, higher expression of PXR and CAR might not induce the target genes. Further examination will be required to test the applicability of the hypothesis in other systems.

One difference between our study and those of others is the effect of PUFAs on cell viability. We observed only moderate inhibition of cell viability (Figure 3.1) while others have reported that PUFAs dramatically inhibit the growth of Caco-2 cells [35-37]. Unlike others who used a serum-poor system [35-37], cells were cultivated with medium containing serum in this study so as to more closely model physiological conditions. Since serum contains polypeptide growth factors and other cell protective substances [64-66], differences in data may be due to the use of serum in our cell culture medium. The choice of PUFA carrier and/or the preparation of the PUFA/carrier complex may also contribute to differences in results among different laboratories.

3.5. Conclusions

Despite the extensive evidence linking dietary fat intake to certain cancers (e.g., breast and colon cancer), the precise molecular mechanisms by which the dietary ω-3 and ω-6 PUFA classes impact tumor biology have not been fully established. We have demonstrated that both ω-3 and ω-6 PUFAs modulate expression of MDR1 and increase apoptosis but the effects are CAR-, PXR-

independent. Both ω-6 and ω-3 fatty acids can influence expression of a gene important in multidrug resistance is of especial current interest because the typical Western diet contains approximately 10 times more ω-6 than ω-3 PUFA [67].

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CHAPTER IV

DIETARY ω-3 POLYUNSATURATED FATTY ACID EPA INHIBITS PROMOTER DNA METHYLATION AND INCREASES GENE EXPRESSION OF BRCA1 IN THE HUMAN LEUKEMIC U937 CELLS

ABSTRACT

Despite numerous studies, the precise mechanisms by which dietary ω-3 polyunsaturated fatty acids mediate gene expression in human cells are not clear. To determine whether eicosapentaenoic acid (EPA) affects BRCA1 expression through promoter methylation, BRCA1 DNA promoter methylation and gene expression in U937 cells were examined. The methylation status of the BRCA1 promoter was evaluated by methylation-specific PCR (MSP) of bisulfite conversion products. The results indicate that methylation of BRCA promoter DNA is reduced in EPA-treated cells. The reduction of methylation in the BRCA1 promoter was accompanied by an increase in mRNA levels obtained by real-time quantitative PCR (qPCR), suggesting that DNA methylation is a possible mechanism by which the dietary ω-3 polyunsaturated fatty acids mediate gene expression in human cells.

4.1. Introduction

It is well known that ω-3 fatty acids affect various cellular processes including membrane composition, lipid metabolism, signal transduction and gene expression [1-3]. Of particular interest, ω-3 polyunsaturated fatty acids interact with multiple transcription factors and function as ligands for several nuclear receptors [4]. However, the precise mechanisms by which ω-3 polyunsaturated fatty acids act on gene regulation in cells are still elusive.

DNA methylation refers to covalent addition of a methyl group at the 5' carbon of the cytosine ring to produce 5-methylcytosine [5]. In mammalian DNA, 5-methylcytosine concentrates primarily at cytosine–guanine dinucleotides (CpGs). These methyl groups protrude into the major groove of DNA thereby inhibiting transcription [6]. DNA methylation plays a crucial role in development [7], germ-cell development [8], and tumorigenesis [9-11]. Hypermethylation of CpG islands within the promoters of tumor suppressor genes can cause silencing of the tumor suppressor genes, thereby enabling malignant growth [12].

BRCA1 is often highly expressed in actively dividing cells and is involved in cell proliferation, DNA repair, genomic integrity and apoptosis [13]. Of note, due to promoter hypermethylation, BRCA1 expression is suppressed in 11–31% of breast cancers, 5–15% of ovarian cancers, 60% of pancreatic ductal carcinomas, and 38% of acute myeloid leukemias [14-16].

Dietary ω-3 polyunsaturated fatty acids can be beneficial in cancer prevention [17-19]. Further, studies indicate that exposure to ω-3 poly-unsaturated fatty acids can increase BRCA1 transcription and protein levels in

several human breast cancer cell lines [20] and in vivo [21]. Therefore, clarifying how ω-3 polyunsaturated fatty acids modulate BRCA1 is important.

In this study, treating U937 cells with EPA was examined to understand whether methylation is decreased in the promoter region of BRCA1 and whether decrease in promoter methylation increases the expression of BRCA1.

4.2. Materials and Methods

4.2.1. Materials and reagents

5-Aza-2′-deoxycytidine (decitabine) and the sodium salt of EPA were obtained from Sigma-Aldrich (St. Louis, MO) and Nu-Chek Prep Inc. (Elysian, MN), respectively. PCR primer oligos were obtained from Operon Biotechnologies (Huntsville, AL). Taq polymerase was obtained from New England Biolabs (Ipswich, MA). Human leukemic U937 cells (ATCC# CRL-1593.2) were obtained from the ATCC (Manassas, VA, USA) and cultivated according to protocols provided by ATCC.

4.2.2. Preparation of the BSA/EPA complex solution

Serum albumin serves as carrier for free fatty acids in blood [22]. Bovine serum albumin, a well-studied fatty acid carrier protein in vitro [22, 23], was used as the EPA carrier for these studies. To prepare BSA/EPA complexes for delivery to cells, we used a modification of the method of Ho and Storch [24]. Briefly,

complexes of BSA and EPA were formed by adding the sodium salt of the EPA (0.01 g) to MEM medium (9mL) containing 825μM fatty acid-free BSA. The mixture was incubated at 37°C for 12 hr to obtain an optically clear solution, and aliquots of the BSA/EPA complex solution were stored under N_2 gas at -20°C. The aliquots were filter-sterilized before addition to the experimental medium. The molar ratio of EPA to BSA was 4:1 [25].

4.2.3. Cell viability

Cell viability was assayed using the CellTiter-Blue® reagent (CTB, Promega, Madison, WI). The viability assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal. U937 cells were seeded into flat-bottomed 96-well plates at a density of 10^3 cells/well in a volume of 200 μl of complete medium and were exposed to 10 μM Decitabine and 100 μM EPA. As controls, cells were exposed to complete medium with the same volume of DMSO (solvent control). After 36h incubation, 20μl CTB reagent was added to the wells and cells were incubated at 37^0C for 4 hr before quantization of fluorescence on a Synergy HT multi-mode plate reader (Bio-tek Instruments, Winooski, VT) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The fluorescence values were normalized to the controls and expressed as percent viability.

4.2.4. DNA isolation, bisulfite conversion and methyl-specific PCR (MSP)

Cells were seeded at the density of 5 x10^5 cell/mL in T25 culture flasks and incubated with DMSO (solvent control), Decitabine (10 μM), or EPA (100 μM), at 37°C in a humidified atmosphere with 5% CO_2 for 24 hours. Cells were harvested by centrifuging at 3,000 rpm for 5 min and rinsed with PBS once before isolating DNA (QIAamp DNA mini kit, Qiagen, Valencia, CA) from the cell pellets.

DNA was isolated from cell pellets using QIAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentrations and purity of the DNA samples were assessed spectrophotometrically using the DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). The methylation status of the promoter region for the two tumor suppressor genes, BRCA1 and p16, was determined by bisulfite conversion and methylation-specific PCR (MSP). Sodium bisulfite converts unmethylated cytosines to uracil and methylated cytosines remain unchanged. For preparation of methylated DNA, approximately 0.6μg of DNA was treated with sodium bisulfite using EZ DNA methylation kit (Zymo Research, Orange, CA), followed the instruction by the manufacturer.

An adaptation of the nested two-step approach of MSP, a modification that increases the sensitivity for detecting targeted sequences [26], was used here. The bisulfite induced sequence differences were detected using specific primer sets for unmethylated and methylated DNA. The primers in stage 1 were designed to amplify a region that is devoid of CpG nucleotides. This allowed

amplification independent of its methylation status. The PCR products of stage 1 were diluted 1:300 and subjected to the second stage of MSP. In the second stage, two PCR reactions were performed, with one primer pair specific for methylated DNA (M) and the other for unmethylated DNA (U). Successful amplification from the M pair and the U pair indicate methylation and unmethylation, respectively. MSP primers for BRCA1 [26] and p16 [27], as well as PCR cycling information, are shown in Table 4.1. The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and visualized under UV illumination.

This assay requires only 24 hours for incubating cells with reagents and takes an additional day to extract DNA, conduct the bisulfite reaction and PCR analysis. Overall, the complete assay takes less than three days.

Table 4.1. MSP primer sequences and the PCR conditions

Gene	Type	Forward sequence	Reverse sequence	Annealing temp (°C)	Annealing time (s)	Cycle	Amplicon (bp)
BRCA1	Stage1	GAGAGGTTGTTGTTTAGYGGTAGTTTT	TCTAAAAAACCCCACAACCTATCC	60	40	30	143
	M	TCGTGGTAACGGAAAAGCGC	AAATCTCAACGAACTCACGCCG	59	20	27	75
	U	TTGGTTTTTGTGGTAATGGAAAAGTGT	CAAAAAATCTCAACAAACTCACACCA	59	20	27	86
	mRNA	GGTGGTACATGCACAGTTGC	TGACTCTGGGGCTCTGTCTT	55	40	40	245
p16	Stage1	GAGGAAGAAAGAGGAGGGGTT	GCTACAAACCCTCTACCCACCTA	62	10	30	277
	M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	62	10	30	150
	U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	62	10	30	151
β-Actin	mRNA	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	55	40	40	250

4.2.5. RNA extraction, reverse transcription (RT) and quantitative real-time PCR (qPCR)

U937 cells were seeded into T25 flasks at a density of 5 x 10^5 cells/mL and exposed to 10 μM Decitabine and 100 μM EPA. As controls, cells were exposed to complete medium with same volume DMSO. After 36h incubation, cells were harvested by centrifuging at 3,000 rpm for 5 min and rinsed with PBS once. After DNAse I treatment to remove genomic DNA, total RNA was isolated using the SV Total RNA isolation kit (Promega, Madison, WI) according to the manufacturer's instructions. The concentrations and purity of the RNA samples were assessed spectro-photometrically using the DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). RT was performed using the reagents provided in the Verso cDNA kit (Abgene, Rochester, New York). The RT reaction mixture (20 μl) contained 1 μg total RNA, 1 μl anchored oligo-dT primer, 500 μM dNTP mix, 4 μl 5× cDNA synthesis buffer, 1 μl RT enhancer and 1 μl Verso enzyme mix. RT was performed at 42°C for 30 min and terminated by heating at 95°C for 2 min. cDNAs were stored at -20°C.

Real-time quantitative PCR (qPCR) for mRNA level of BRCA1 was conducted using an Mx3000P real-time PCR system (Stratagene, La Jolla, CA). The gene-specific primers (Fisher-Operon, Huntsville, AL) used for our qPCR reactions are listed in Table 4.1. All reactions were performed in a singleplex mode. The total reaction mix (20 μl) contained 10 μl 2x SYBR Green PCR master mix (Bio-Rad Laboratories, Hercules, CA), 200 nmol/L of each primer, and 0.8-1 μl cDNA as template. After initial heating to 95°C for 2 min, 40 cycles were run at 95°C for 20s, 55°C for 40s, and 70°C for 30s. Dissociation curve analyses were

done for all samples and only sharp melting points were observed, indicating a specific signal and no primer dimers or mispriming. A negative template control (H_2O) was always included in each run to check for the presence of exogenous contaminant DNA. After the threshold cycle values in real-time PCR were obtained, the amounts of BRCA1 were determined from a standard curve generated by serial dilutions of cDNA. After normalization to β-actin values, the expression levels of BRCA1 were expressed as % of control cells. Each sample was analyzed in triplicate, and data were analyzed using Mx3000P software (Stratagene, La Jolla, CA).

4.3. Results

4.3.1. Effect EPA and Decitabine on the Viability of U937 Cells.

Eicosapentaenoic acid (EPA, a -3 PUFA) was selected as our model for studying the effect of PUFAs on CpG methylation. Decitabine, a nucleoside inhibitor of gene activation, served as positive control. Because both of them can be toxic to human cell growth [28-30], cell viability studies was conducted to determine concentrations of EPA and Decitabine appropriate for subsequent experiments. U937 cells were incubated with 100μM EPA and 10μM Decitabine for 36 hours. Both EPA and Decitabine had a modest inhibitory effect (~ 20% of solvent controls) on the growth of U937 cells (Figure 4.1).

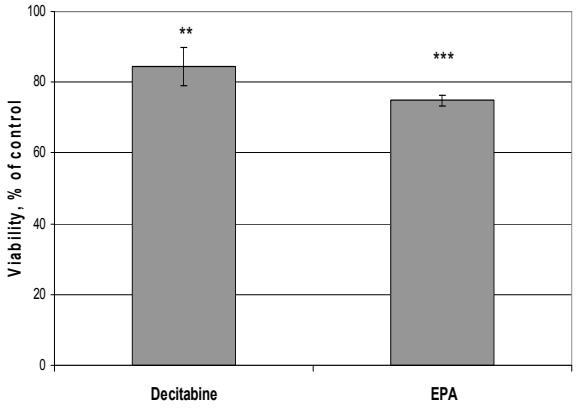

Figure 4.1. The effect of EPA and Decitabine on viability of U937 cells. Cells were incubated for 36 hours with 100 μM EPA and 10 μM Decitabine. The viability of treated cells is presented as the means of cell number (± SD) compared to control (solvent treated) cells. For each experiment, n=3. ** ($P< 0.01$); *** ($P< 0.001$) indicate significant differences from control values.

4.3.2 Effects of EPA on the promoter methylation status of the tumor suppressor genes BRCA1 and p16

The methylation status of the promoter region for BRCA1 and p16 was determined by methylation-specific PCR (MSP) using a two-step protocol (See Section 4.2.4). In the solvent control cells, there was intense amplification of unmethylated sequences in the promoter region of BRCA1 and less but still ample amplification of methylated sequences (Figure 4.2). In U397 cells treated with Decitabine or EPA for 24h, there was a dramatic reduction in BRCA1 methylation compared to control cells (Figure 4.2), and the degree of methylation appeared similar for Decitabine and EPA-treated cells.

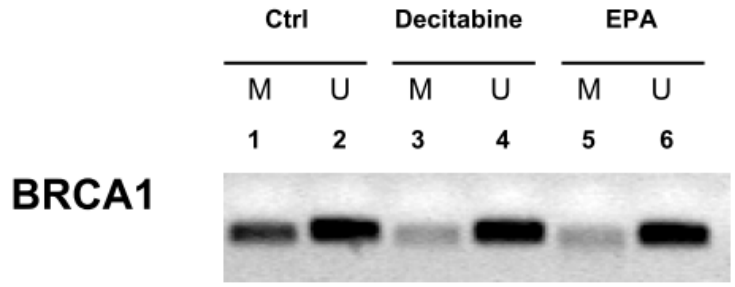

Figure 4.2. EPA and Decitabine inhibit DNA methylation of the BRCA1 gene promoter: Analysis of MSP products by agarose gel electrophoresis. DNA from U937 cells treated with 100μM EPA or 10 μM Decitabine was subjected to MSP. Ctrl: solvent control; U: unmethylated PCR product; M: methylated PCR product.

MSP is a powerful approach for evaluating DNA methylation [31] but does not provide quantitative information for assessment of methylation. To obtain quantification of the changes in BRCA1 methylation induced by EPA and Decitabine, a modified protocol based on existing quantitative MSP (qMSP) [32, 33] was conducted. For this protocol, the products from stage 1 MSP, diluted 1:300, served as DNA templates. Stage 2 results obtained from qPCR were normalized to Stage 1 products and expressed as fraction of control. qMSP results indicate that BRCA1 promoter methylation level decreased to 34% of control after treatment of U937 cells with EPA for 24h (Table 4.2). The methylation ratio is defined as the ratio of the fluorescence emission intensity values for the methylation-specific PCR products of the gene promoters to their stage 1-products.

Table 4.2. Degree of methylation of BRCA1 promoter

Treatment	ctrl	Decitabine	EPA
Ratio (M /stage 1)	1	0.18***	0.34**

To determine whether the effect of EPA on BRCA1 is specific, we performed a similar assay for the p16 gene, another well-known tumor suppressor [34]. Both methylated and unmethylated sequence amplification was detected in cells treated with Decitabine (Figure 4.3), but only methylated sequence was detected for p16 in EPA treated cells in a pattern similar to control cells. The absence of amplification of unmethylated sequence for the p16 promoter suggests that EPA modulation of gene methylation is not a global mechanism.

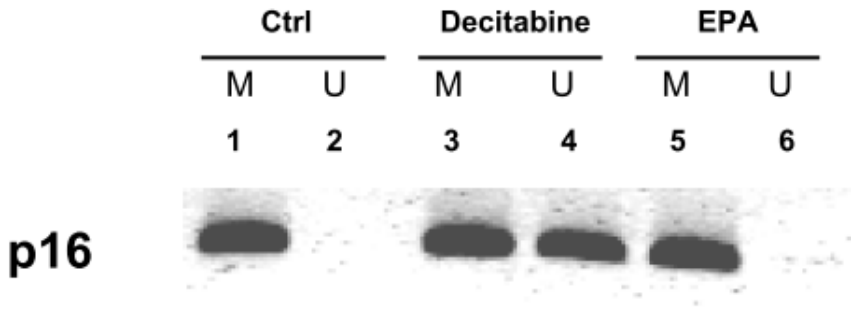

Figure 4.3. The effect of EPA and Decitabine on DNA methylation of the p16 gene promoter. Analysis of MSP products by agarose gel electrophoresis. DNA from U937 cells treated with 100μM EPA or 10 μM decitabine was subjected to MSP. Ctrl: solvent control; U: unmethylated PCR product; M: methylated PCR product.

4.3.3. Effects of EPA on BRCA1 gene expression in the U937 cells

Whether EPA modulates the expression of the BRCA1 gene in U937 cells was addressed next. Analysis by real time PCR suggests that both EPA and Decitabine up-regulate BRC1 expression (Figure 4.4). For the 36 hr incubation period, EPA treatment increased the total mRNA level by ~50% compared to the solvent controls. The degree of increase is similar to that induced by Decitabine treatment.

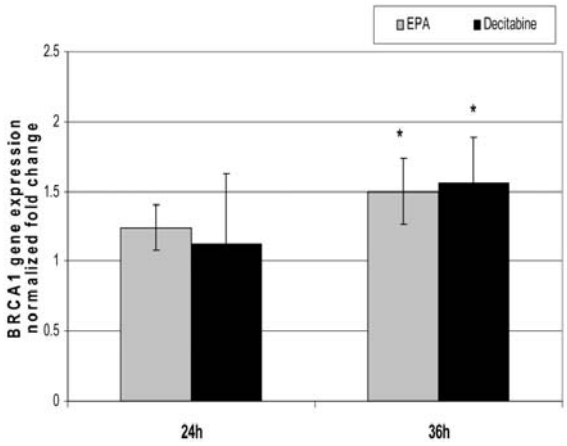

Figure 4.4. The effect of EPA on BRCA1 gene expression. U937 cells were incubated with 100 μM EPA or 10 μM Decitabine for 24 and 36 hours. mRNA levels were determined by qPCR (see **Materials and Methods**) using samples obtained from three independent experiments. * ($P < 0.05$) indicates significant differences from control values.

4.4. Discussion

Certain genes, such as BRCA1, involved in tumor suppression, have been shown to be hypermethylated in many tumor types. The potential of dietary polyunsaturated fatty acid EPA to decrease methylation in the promoter regions of these genes was studied. Treatment of cells with EPA decreased promoter methylation of BRCA1 and increased expression of BRCA1 (Figures 4.2 and 4.4). The inhibition of promoter methylation by EPA and the effect of EPA on BRCA1 gene expression approached levels observed for the nucleoside inhibitor of methylation Decitabine (Figures 4.2 and 4.4; Table 4.2). We did not detect an effect of EPA on the methylation status of the p16 promoter (Figure 4.3). These observations indicate a specificity in the ability of EPA to modulate gene silencing.

In order to ensure that the hypomethylation effect is not due to cell toxicity, cell viability assays were conducted. Both Decitabine and EPA have only a moderate cytotoxic effect on U937 cells within the assay time period (Figure 4.1). Of note, the concentration of Decitabine used in our study (10μM) is higher than that commonly used in other studies [35, 36]. We used the higher concentration of Decitabine to ensure a consistent hypomethlation effect in our screening model.

The mechanism(s) by which EPA affects DNA methylation is not clear. ω-3 polyunsaturated fatty acids are readily incorporated into cell membranes and lipid rafts, and their incorporation may affect membrane-associated signaling proteins. One hypothesis is that EPA modulates DNA methylation by interfering with Ras signaling for DNA methylation. Constitutive activation of Ras induces

expression of DNA methyl-transferase 1 (DNMT1) at the transcriptional level [37-39] and excess of DNMT levels may target certain genes for hypermethylation. A high fat fish oil diet fed to rats decreased protein levels of total and membrane bound Ras and increased proteins levels of cytosolic Ras in colon tumors compared to rats fed a high fat corn oil diet [40, 41]. This result suggests that ω-3 PUFAs might interfere with Ras activation by decreasing its membrane localization. Of note, recent data implicate lipid rafts as coordinators of signals generating from the cell membrane and mechanisms linking DNA methylation and chromatin dynamics [42].

DNA demethylating agents such as 5-azacitidine and Decitabine have been approved by FDA in the clinical treatment of several hematologic malignancies, including myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) [43, 44]. However, the effect of these nucleoside inhibitors on gene activation is non-specific and usually involved in multiple cellular pathways [6, 45-47]. In addition, when used in high doses, these azanucleosides can produce serious toxicities in patients [45, 48]. Recently, a number of dietary compounds have been shown to impact methylation, raising the possibility that an individual's diet may also influence DNA methylation [49]. Although the contribution of diet to the risk of cancer is now widely acknowledged, the multiple possible mechanisms are only starting to be resolved [50, 51].

4.5. Conclusions

In summary, our study clearly demonstrates that EPA inhibits promoter methylation and increases gene expression of BRCA1. To our knowledge, this is the first demonstration of such an activity for a commonly consumed dietary polyunsaturated fatty acid. By linking a major tumor suppressor gene associated with epigenetic modification to key nutritional factors involved in the incidence rate of the cancer, our study opens up further perspectives for the use of dietary interventions in cancer.

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CHAPTER V

SUMMARY AND RECOMMENDATIONS

Accumulating evidence in both humans and animal models indicate that dietary intake of long-chain polyunsaturated fatty acids (PUFAs) can improve response to chemotherapy. The mechanisms by which PUFAs affect this response are not well understood. P-glycoprotein (P-gp), encoded by the multidrug resistance gene MDR1, is a drug efflux transporter that plays an important role in the bioavailability of anti-cancer drugs. We studied the effects of three long-chain polyunsaturated fatty acids, the ω-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the ω-6 PUFA arachidonic acid (AA) on MDR1 gene expression and functional activity in the human colon cancer cell line Caco-2. All three PUFAs down-regulated the expression of the MDR1 gene. The inhibition of gene expression by these PUFAs was accompanied by reduction in protein levels of P-gp. EPA, DHA and AA also increased the intracellular accumulation (i.e. decreased the efflux) of a P-gp substrate. In addition, incubation of cells with PUFAs greatly enhanced the cytotoxicity of the anti-cancer drug paclitaxel. All three PUFAs also induced apoptosis and enhanced paclitaxel-induced apoptosis in Caco-2 cells. Of particular interest, our studies indicate that a ω-6 PUFA AA, as well as the ω-3 PUFAs EPA and DHA, can inhibit MDR1/P-gp expression and function.

Hypermethylation of promoter CpG islands is related to inhibition of gene expression. A thesis is that methylation (silencing) of tumor suppressor genes enables malignant growth. Using our protocol based on bisulfite conversion and methylation-specific PCR, we demonstrated that treatment of U937 cells with EPA decreased promoter methylation of the tumor suppressor gene BRCA1. The reduction of methylation in the BRCA1 promoter was accompanied by an increase in the mRNA levels of BRCA1, suggesting that DNA methylation is a possible mechanism by which the dietary ω-3 polyunsaturated fatty acids mediate gene expression in human cells. Treatment with EPA did not, however, significantly affect methylation in the p16 gene promoter, suggesting specificity for EPA modulation of gene methylation.

Together these studies suggest that use of PUFAs as adjuvants presents a promising strategy to improve cancer therapeutics.

Recommendations for Future Study

One major findings of the study is that PUFAs inhibit MDR1/P-gp expression and enhance paclitaxel-induced apoptosis. However, PUFAs have no significant effect on modulators of MDR1 inlcuding COX-2, CAR and PXR, expression. This study also reveals that EPA increases the BRCA1 expression while at the same time decreasing promoter methylated level. Based on these findings, I suggest the following future studies:

1. To study the effects of PUFAs on other mediators which are known to affect MDR1/P-gp gene expression under cetain conditions. Previous studies report that PUFAs can affect the activity of NF-κB. In addition, NF-κB is one of the modulators involved in MDR1 expression. Moreover, NF-κB is also related to apoptosis pathway. Therefore, it is important to examine the linkage between PUFAs, NF-κB, MDR1, and apoptosis.

2. To explore the strategy of co-administration PUFAs with anti-cancer drugs in cancer treatment. In consideration of the interactions between PUFAs and anti-cancer drugs, it is important to optimize the timing for PUFA administrations.

3. To study the effects of membrane composition changes by PUFA incorporation on P-gp expression. Several laboratories show that PUFAs can modulate cellular membrane composition and membrane proteins/transporters. Hence, the relationship between PUFAs and membrane composition is important to understand.

4. To further study the relationship between promoter DNA methylation and MDR1 expression. Because promoter DNA methylation is one of the

mechanisms for gene expression, it is interesting to understand whether PUFAs can regulate MDR1 expression through DNA methylation.

5. To study the mechanisms which are involved in changes of BRCA1 promoter methylation pattern results from EPA treatment. For example, DNMTs are the major enzymes in DNA methylation. A critical question to address is whether EPA modulates the DNA methylation through affecting the DNMTs .

6. To study the ability of EPA on modulating promoter DNA methylation in other genes or cell models. DNA methylation is highly gene- and tissue-specific. Whether EPA affects DNA methylation in other genes and cells remains to be elucidated.

APPENDICES

APPENDIX A

Table A.1 Viability of Caco-2 cells treated with PUFAs

Viability				
AA				
24h	conc (μM)	25	50	100
%	expt1	108.10	106.70	108.50
	expt2	87.48	84.78	69.08
	expt3	96.76	96.27	90.95
48h				
%	expt1	97.50	96.20	92.40
	expt2	77.30	73.90	65.60
	expt3	90.00	91.60	84.50
72h				
%	expt1	83.01	102.20	84.70
	expt2	98.10	94.20	87.60
	expt3	96.20	93.80	89.50
EPA				
24h	conc (μM)	25	50	100
%	expt1	94.00	90.00	91.00
	expt2	97.00	75.00	87.00
	expt3	96.00	76.00	67.00
	expt4	95.00	92.00	85.00
	expt5	98.67	96.01	91.26
48h				
%	expt1	99.00	81.00	78.00
	expt2	91.00	64.00	63.00
	expt3	97.00	92.00	88.00
72h				
%	expt1	99.00	83.00	67.00
	expt2	97.31	94.22	88.78
	expt3	75.60	89.70	86.10
DHA				
24h	conc (μM)	25	50	100
%	expt1	92.07	99.41	121.44
	expt2	89.10	81.90	74.32
	expt3	95.64	100.66	101.13
48h				
%	expt1	105.36	110.61	116.2
	expt2	85.95	74.66	72.94
	expt3	86.00	84.67	87.41
72h				
%	expt1	92.32	91.6	92.44
	expt2	85.52	80.23	68.66
	expt3	97.22	94.82	89.69

Table A.2 Gene expression of MDR1 in Caco-2 cells treated with PUFAs

MDR1				
100μM	**AA**	4h	12h	24h
fold change	expt1	1.04	0.71	0.79
	expt2	0.83	0.7	0.74
	expt3	0.76	0.77	0.67
100μM	**EPA**	4h	12h	24h
fold change	expt1	0.89	0.72	0.62
	expt2	0.83	0.68	0.67
	expt3	0.8	0.77	0.7
	expt4	0.83	0.7	
100μM	**DHA**	4h	12h	24h
fold change	expt1	0.95	0.85	0.56
	expt2	0.95	0.92	0.75
	expt3	0.93	0.97	0.71
50μM	**EPA**	4h	12h	24h
fold change	expt1	0.97	0.9	0.91
	expt2	0.97	1.05	0.8
	expt3	1.07	0.9	0.83

Table A.3 Intensity (%) of Calcein-AM efflux in Caco-2 cells treated with PUFAs

Calcein-AM efflux				
24h				
		expt1	expt2	expt3
%	AA	149.06	129.37	97.06
	EPA	122.25	118.81	137.68
	DHA	133.41	134.19	126.48

Table A.4 Viability of Caco-2 cells treated with paclitaxel (PTX) with/without PUFAs for 72h

72h, %			
PTX alone			
0	100.00%	100.00%	100.00%
1	103.19%	83.93%	101.68%
2.5	104.15%	88.87%	96.99%
5	98.26%	82.97%	78.41%
10	91.56%	87.98%	79.37%
25	86.20%	77.80%	63.84%
50	83.27%	78.18%	57.51%
100	77.33%	81.60%	40.48%
PTX+ 100μM AA			
0	83.24%		83.60%
1	61.18%		63.69%
2.5	57.71%	56.23%	65.27%
5	50.63%	48.08%	59.15%
10	44.28%	43.09%	53.57%
25	40.83%	43.12%	47.34%
50	58.35%	35.11%	41.13%
100	60.49%	30.54%	45.27%
PTX+ 100μM EPA			
0	90.53%		107.77%
1	70.79%		84.71%
2.5	66.33%	45.42%	85.01%
5	64.59%	36.66%	65.23%
10	62.86%	29.56%	61.03%
25	52.57%	24.44%	55.82%
50	48.02%	19.91%	46.51%
100	49.26%	17.78%	41.00%
PTX+ 100μM DHA			
0	121.16%	120.90%	118.71%
1	96.07%	88.31%	93.84%
2.5	83.81%	86.00%	89.63%
5	77.26%	81.70%	76.21%
10	64.09%	62.45%	68.12%
25	57.20%	54.76%	60.87%
50	44.18%	47.27%	52.23%
100	38.75%	40.31%	42.52%

Table A.5 Gene expression of CAR and PXR in Caco-2 treated with PUFAs for 24h

	CAR			**PXR**		
100μM	AA	EPA	DHA	AA	EPA	DHA
fold change	1.528	1.296	1.077	2.022	1.190	1.541
	1.209	1.233	1.459	1.804	1.582	1.041
	1.400	1.402	1.156	1.560	2.503	1.241
					1.848	

APPENDIX B

Table B.1 Viability of U937 cells treated with Decitabine and EPA for 36h

Viability of U937 (%)			
ctrl	100	100	100
Decitabine, 10μM	90.5	81.9	80.6
EPA, 100μM	73	75.8	75.8

Table B.2 qMSP of BRCA1 promoter in U937 cells treated with Decitabine and EPA for 24h

qMSP, 24h		
	Decitabine	EPA
expt1	0.22	0.56
expt2	0.31	0.12
expt3	0	0.35

Table B.3 Gene expression of BRCA1 in U937 cells treated with Decitabine and EPA for 24h and 36h

BRCA1, 24h		
	Decitabine	EPA
expt1	0.83	1.28
expt2	0.85	1.06
expt3	1.7	1.38
BRCA1, 36h		
	Decitabine	EPA
expt1	1.98	1.59
expt2	1.59	1.24
expt3	1.47	1.69
expt4	1.21	