Combination of Etoposide and Docosahexaenoic Acid via Targeting PI3K/MAPK Pathway May Be A New Therapeutic Strategy for Medulloblastoma

Shuang Zhou
Department of Pharmaceutical Sciences,
North Dakota State University, Fargo, ND, USA

Fengfei Wang
Department of Pharmaceutical Sciences,
North Dakota State University, Fargo, ND, USA

Erxi Wu*
Department of Pharmaceutical Sciences,
North Dakota State University, Fargo, ND, USA

*Corresponding author
1 Introduction

Medulloblastoma (MB), an embryonal neuroepithelial tumor of the cerebellum, is the most common malignant brain tumor occurring in children (Gilbertson & Ellison, 2008). Current therapy includes surgical resection, craniospinal irradiation, and aggressive chemotherapy supplemented by bone marrow transplant (Gajjar et al., 2006; Gottardo et al., 2013; Northcott et al., 2012a). Although the outcome for children with MB has been improved over the past several decades, approximately one-third of patients with metastatic MB tumors at diagnosis remain incurable. These metastatic tumors are impossible to be removed completely by surgery, and the remaining chemo-resistant tumor cells are capable to spread to the spinal cord and other organs (Gottardo et al., 2013; Smoll, 2011). There is no effective therapy currently available for the metastatic MBs, and the long-term survivors suffer from significant disabilities caused by the concomitant toxicities of current treatments.

Docosahexaenoic Acid (DHA) is an omega-3 fatty acid which can be synthesized from alpha-linolenic acid (ALA) or obtained from maternal milk or fish oil. Despite its uncertain role in cancer prevention, recent studies suggest that DHA has therapeutic value in cancer treatment in several types of cancers using in vitro and in vivo model systems (Albino et al., 2000; Calviello et al., 1998; Denkins et al., 2005; Iigo et al., 1997; Kimura, 2002; Merendino et al., 2005; Merendino et al., 2003; Calviello et al., 2005, Schley et al., 2005). The studies on the combination of DHA with different anticancer regimens show synergistic effects on various types of tumor models (Duncan et al., 2005; Menendez et al., 2005; Payne et al., 2006; Ding et al., 2006, Wang et al., 2011). Because of the promising anti-cancer effects of DHA in vitro and in vivo models, a few clinical trials using DHA alone or as a supplement are being conducted in breast cancer survivors (clinical trial phase 2, NCT01849250), metastatic breast cancer patients (clinical trial phase 3, NCT01548534), and patients with advanced lung cancer (clinical trial phase2/3, NCT01048970). However, as a primary structural component of the human brain and an important modulator for brain development and function, whether DHA possesses therapeutic value for brain tumors, especially MB, is not clear. In a goal of elucidating the possible therapeutic value of DHA in MB therapy, we have herein summarized the latest view regarding MB, reviewed the major signaling pathways involved in MB formation and progression, analyzed the data obtained in our studies of DHA and its combination with etoposide in MB cells and discussed the pathways of cell growth and apoptosis that differentially influenced by DHA and the combined DHA-etoposide treatments.
2 MB Subtypes

MB is thought to arise from primitive pluripotent precursor cells of the ventricular zone and cerebellar external germinal layer (Northcott et al., 2012b). Five histological variants of MB have been recognized by the World Health Organization (WHO), including: (1) the classic MB, (2) desmoplastic/nodular MB, (3) large-cell anaplastic (LCA) MB, (4) melanotic MB, and (5) medulloblastoma variants (Giangaspero, 2003; Gilbertson, 2004). However, we do not have a clear understanding regarding the

<table>
<thead>
<tr>
<th>MB classification</th>
<th>WNT</th>
<th>SHH</th>
<th>Group 3</th>
<th>Group 4</th>
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<td>MYCN+</td>
<td>MYC++</td>
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<td>Rarely LCA</td>
<td>Medulloblastoma with extensive nodularity (MBEN)</td>
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<td>Rare</td>
<td>Variable</td>
<td>Frequent</td>
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<tr>
<td>5 Year Survival Rate</td>
<td>94%</td>
<td>87%</td>
<td>32%</td>
<td>76%</td>
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Table 1. MB subgroup classification. Based on the studies performed by several research groups, MB subgroups can be classified into four main subgroups with distinct molecular signatures (Cho et al., 2011; Clifford et al., 2006; Eberhart, 2012; Ellison et al., 2011; Ellison et al., 2005; Gajjar et al., 2006; Gibson et al., 2012; Kool et al., 2008; Northcott et al., 2012c;
between the histological properties and MB development so far. For example, despite many MB present identical histologic features, they show distinct responses to MB treatment (de Bont et al., 2008b; Kool et al., 2014). These differential responses may be ascribed to the involvement of genetic alterations and aberrant signaling pathways in MBs. Indeed, integrated analysis of MB tumors using genomic technologies, such as next-generation sequencing and complementary high-density genomic technologies (Shendure and Ji, 2008), demonstrated that MBs contain multiple alterations at the genomic level and can be sub-grouped in terms of the demographic, transcriptional, genetic, and clinical differences (Northcott et al., 2012a; Northcott et al., 2012b; Northcott et al., 2012d; Parsons et al., 2011; Ramaswamy et al., 2013; Taylor et al., 2011). So far, at least four distinct subgroups of MB have been identified with significantly dysfunction in their specific signaling pathways: SHH, Wnt, Group 3, and Group 4 (Table 1) (Northcott et al., 2012a; Northcott et al., 2012b; Northcott et al., 2012d; Ramaswamy et al., 2013; Taylor et al., 2011). Among the 4 subgroups of MBs, Wnt MB has a much better outcome; while Group 3 MB has a lowest 5-year survival rate. Notably, MYC, a pro-oncogene and frequently altered gene, is expressed in most of subgroups, especially amplified in Group 3 MBs.

3 Major Signaling Pathways Associated with MB Formation and Progression

Molecular dysfunctions in several key developmental signaling pathways, such as SHH, Wnt, and Notch signaling, have been linked to MB development and progression (Fig. 1). Mutations in the SHH signaling pathway have been linked to the desmoplastic/nodule subtype of human MB (Ellison et al., 2011; Schabert et al., 1997). The activating mutation of Ptc1, encoding a seven-pass transmembrane receptor in SHH, was identified in up to 20% of sporadic MB (Dong et al., 2000; Zurawel et al., 2000), the predominant desmoplastic MB. Activating mutations of smoothened (Smo) (Zurawel et al., 2000) and somatic mutations in suppressor of fused (SuFu) (Taylor et al., 2002) were also identified, though they occur less frequently. Over-expression of SHH targets Gli1 (Thompson et al., 2006) and Bmi1 (Leung et al., 2004) were detected in up to 30% and 67% of MB, respectively. In addition, deletion of tumor suppressor KCTD11 in SHH tumors has been identified in up to 39% of sporadic human MBs (Di Marcotullio et al., 2004). A significant reduction of KCTD11 expression compared to adult normal cerebellum was detected
in 14 of 20 (70%) of MB samples (Zawlik et al., 2006). The notion of abnormal activation resulting in MB progression was further supported by that a small molecule, CDG-449 targeting SHH signaling suppressed refractory smoothened mutants in a mouse model (Dijkgraaf et al., 2011). In Wnt signaling pathway, the mostly described mutations have been found to lie in the β-catenin gene since its first discovery in 1998 (Clifford et al., 2006; Zurawel et al., 1998); other mutations including mutations in APC (Huang et al., 2000) and AXIN2 genes (Koch et al., 2007), deletions of the AXIN1 gene (Dahmen et al., 2001), and over-expression of the SOX gene family (de Bont et al., 2008a). Notch1 and Notch2

**Figure 1. Key signaling pathways associated with MB development.** From left to right: Wnt binds to the frizzled and LRP receptors which allows for the accumulation of β-catenin, which, in turn, binds to Wnt effector TCF/LEF to activate expression of c-Myc and other target genes. The interaction of Notch/its ligands, Delta/Jagged activates the enzymes associated with Notch signaling to release Notch intracellular domain (NICD). NICD then forms a complex with CSL transcription factors and co-activators (CoA) to activate its downstream target genes, such as HES1. Hedgehog protein binds to Patched transmembrane receptor which re-
lieves its inhibition on Smoothened and leads to the activation of Gli complex. Gli1/2 will then activate the transcription of Wnt and other target genes (de Bont et al., 2008b; Guessous et al., 2008; Mendoza et al., 2011).

were found to have opposite effects on MB growth and cell proliferation, soft agar colony formation, and xenograft growth (Notch2 has promotion functions while Notch1 bears inhibition functions during these processes) (Fan et al., 2004). Notch2 and the Notch target gene, HES5, were also significantly elevated in Smoothened-induced MB tumors in human and mouse models (Dakubo et al., 2006; Hallahan et al., 2004). As the critical role of Notch signaling in neuron differentiation, the dysfunction of Notch signaling has been linked with all subgroups of MB (Bernard et al., 2010; Manoranjan et al., 2012; Palomero and Ferrando, 2008). Although rational targeted therapy based on genetics is not currently being used in MB treatment, the above mentioned findings provided the basis for future personalized MB treatment.

4 PI3K/MAPK Signaling Pathways:

Besides the dysfunctions of above major pathways, deregulations of several receptor tyrosine kinases (RTK), such as ERBB2, c-Met, IGFR, PDGFR and VEGFR, or the related signaling pathways, such as IGF/phosphatidylinositol 3-kinase (PI3K) and PDGFR-RAS/mitogen-activated protein kinase (MAPK), have also been associated with MB progression (Abouantoun et al., 2010; Abouantoun and Macdonald, 2009; Gilbertson et al., 2006; Guerreiro et al., 2008; Johnson et al., 2013; Li et al., 2008; MacDonald et al., 2001; Wang et al., 2001; Wojtalla et al., 2012; Yuan et al., 2010; Zhou et al., 2014). The following section focuses on the importance of PI3K and MAPK pathways in MB progression, and use the combination of DHA and etoposide to regulate these signaling pathways in human MB cells as an example to provide insight on development of novel anti-MB therapeutics.

4.1 PI3K Pathway in MB

PI3K signaling pathway regulates a broad range of cellular events such as cell growth, proliferation, survival, motility, and metabolism in both normal and pathological conditions. This pathway can be stimulated by G protein-coupled receptors (GPCR), RTK, cytokine receptors, B cell receptors or integrins (Fresno Vara et al., 2004; Zhang et al., 2007; Zhang et al., 2003). Activated PI3Ks, which belong to a lipid kinase family that is capable of phosphorylating the 3’-OH group in inositol phospholipides, convert phosphatidylinositol (4,5)-bisphosphate (PIP-2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP-3). The
interaction of the pleckstrin homology (PH) domain of a serine/threonine-specific protein kinase called Akt, also known as Protein Kinase B (PKB), with these phospholipids is thought to provoke conformational changes in Akt and leads to the exposure of its phosphorylation sites. Consequently, Akt can be phosphorylated and activated by phosphoinositide dependent kinase 1 (PDK1) and mammalian targets of rapamycin complex 2 (mTORC2) (Zhang et al., 2007). The activated Akt can then act on its various substrates via its kinase activity and promotes cell growth, proliferation, survival, motility, angiogenesis, and other effects (Fig.2) (Hartmann et al., 2006; Pei et al., 2012).

**Figure 2. PI3K/MAPK Pathways in MB.** PI3K pathway: Signaling through the RTKs, such as c-Met, ERBB2, PDGFR, TrKC, IGF, results in the activation of PI3K which converts PIP-2 to PIP-3. PTEN opposes the activity of PI3K. The activated Akt by PIP-3 phosphorylates Forkhead/Foxo (FKH) to prevent FKH from entering the nucleus to stimulate the transcription of its target genes, such as p27. Akt also inhibits the GSK3 and induces NFκB to activate the transcription of its target genes, e.g., c-Myc. MAPK pathway: MAPK signaling pathway could also be activated by RTKs through activation of RAS GTPase and RAF. The activated MEK subsequently phosphorylates ERK, which further activates its downstream targets, such as ETS domain-containing protein (ELK) and Ribosomal s6 kinase (RSK), to modulate target
gene transcription. The aberrant deregulation of these pathways in MB leads to alterations in cell growth, proliferation, survival, cell cycle progression, migration, and differentiation (de Bont et al., 2008b; Guessous et al., 2008; Hartmann et al., 2006; Mendoza et al., 2011; Pei et al., 2012).

It is well recognized that effective cancer therapies targeting RTKs leads to concomitant downregulation of the PI3K signaling (Berns et al., 2007; Bianco et al., 2003; Engelman et al., 2005; Moulder et al., 2001). Activation or over-expression of RTKs, e.g., insulin-like growth factor 1 receptor (IGF-1R), c-Met, ERBB receptor, and neurotrophin-3 receptor (TrkC), which leads to activation of PI3K pathway, is frequently observed in MB primary tumor and mouse models (Gilbertson et al., 2001; Gilbertson et al., 1998; Grotzer et al., 2000; Hernan et al., 2003; Kim et al., 1999; Northcott et al., 2012a; Rao et al., 2004; Segal et al., 1994; Wang et al., 2001). IGF signaling is essential for neuronal development and is involved in brain tumor development. Wang and colleagues have revealed that the activation of the IGF-1R contributes to malignant MB growth in human and mice (Wang et al., 2001). In addition, the incidence of SHH-induced tumor formation (15%) in mice was found to be enhanced by coexpression with IGF2 (39%) and Akt (48%) (Rao et al., 2004). The induced tumors showed upregulated expression of insulin receptor substrate 1 and phosphorylated forms of IGF-1R and Akt, which mimicks the activated IGF signaling found in human MBs (Rao et al., 2004). Activated c-Met signaling by its ligand hepatocyte growth factor (HGF) in MB, which is evidenced by the phosphorylation of Akt and MAPK, resulted in the promotion of tumor cell proliferation, cell cycle progression, anchorage independent growth, and the prevention of MB cells from apoptosis (Li et al., 2008; Li et al., 2005). ERBB2 is expressed in up to 86% of MBs, and the expression of ERBB2 has been associated with MB metastasis and poor prognosis in MB patients (Gilbertson et al., 2001; Gilbertson et al., 1998). Hernan et al. found that ERBB2 signaling up-regulates the expression of prometastatic genes such as S100A4 via a pathway involving PI3K and Akt1 in MB in vitro as well as in vivo (Hernan et al., 2003). Contrary to other RTKs, elevated level of TrkC mRNA expression in MB is a valuable predictor of favorable clinical outcome (Eberhart et al., 2004; Grotzer et al., 2000; Segal et al., 1994) and activation of TrkC induces MB cell apoptosis and inhibits tumor growth in mice (Kim et al., 1999).

Besides the activation of PI3K pathway by RTKs in MBs, a small subset of MBs (~ 5%) contains mutations in the α catalytic subunit of PI3K (PIK3CA) and the mutation of this molecule can also activate PI3K signaling and induce MB formation (Broderick et al., 2004; Guerreiro et al., 2008; Northcott et al., 2012b). In addition, PTEN, a phosphatase with dual activity on lipids and proteins, negatively regulates
PI3K pathway through its PIP-3 dephosphorylation activation. Inactivation or hypoactivation of PTEN can result in permanent activation of PI3K signaling. Although it remains unclear whether PTEN loss alone is sufficient to activate PI3K pathway, decreased expression of PTEN mRNA and protein in human MBs may contribute to the activation of PI3K/Akt pathways (Hartmann et al., 2006). Allelic loss of PTEN has been observed in 16% of MB (Hartmann et al., 2006). In addition, the PTEN promoter has been found to be hypermethylated in 5 of 10 MB human cases (Hartmann et al., 2006). Hambardzumyan et al. reported that activation of Akt signaling via PTEN loss could transform MB cells to a nonproliferating extensive nodularity morphology (Hambardzumyan et al., 2008). Similarly, using the SmoA1 transgenic mouse model of MB, Castellino and colleagues showed PTEN deficient tumors exhibited extensive nodularity with neuronal differentiation separated by focal areas of intense staining for proliferation and virtually absent apoptosis (Castellino et al., 2010). Recently, PTEN loss was also found to mitigate the response of MB to Hedgehog pathway inhibition in mouse model (Metcalfe et al., 2013).

Taken together, the inappropriate activation of PI3K pathway in MB has been linked with MB progression and therapy resistance. In addition, PI3K signaling has been found to regulate survival of cancer stem cells following radiation in MB in vivo (Hambardzumyan et al., 2008). Recently, it has also been shown that the inhibition of components in the PI3K pathway suppresses MB progression in some preclinical studies (Baryawno et al., 2010; Buonamici et al., 2010; Guerreiro et al., 2008). Targeting PIK3CA by RNA interference impaired the growth of MB cells and inhibits chemoresistance and migration (Guerreiro et al., 2008). The PDK1 inhibitor OSU03012 suppressed the growth of established MB xenograft tumors and augmented the antitumor effects of mTOR inhibitor CCI-779 (Baryawno et al., 2010). A combination of the PI3K class I inhibitor NVP-BKM120 or the dual PI3K/mTOR inhibitor NVP-BEZ235 with the Smo antagonist NVP-LDE225 markedly delayed the development of treatment resistance in mice with MB tumors (Buonamici et al., 2010). Thus, targeting the components of this pathway may provide novel therapeutic strategies to treat MB tumors.

### 4.2 MAPK Pathway in MB

MAPK cascades are evolutionarily conserved signal transduction pathways relaying signals from a diverse range of stimuli, such as cytokines, hormones, cellular stress, heat shock, mitogen, growth factors. They regulate a wide array of cellular responses including mitosis, metabolism, motility, survival, apoptosis, differentiation, and inflammatory responses. Each MAPK cascade consists of a core of three components: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK. MAPK cascades begin with the phosphorylation and activation of the protein Ser/Thr kinases MAP3Ks by small GTPases, such as RAS, Rac, and Rap1, or other enzymes in response to extracellular stimuli. The activated
MAP3Ks then phosphorylate the Ser/Thr/Tyr kinases MAP2Ks, which in turn stimulates the activity of the Ser/Thr kinases MAPKs through phosphorylation on their threonine and tyrosine residues. The activated MAPKs consequently phosphorylate their various downstream substrate proteins, leading to different cellular responses (Cossa et al., 2013; Guldal et al., 2012; Santarpia et al., 2012) (Fig.2). Fourteen MAPKs have been identified in mammals, including extracellular signal-regulated kinase (ERK)1/2, ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase (JNK)1/2/3 and the p38 isoforms α/β/γ/δ. The most intensely studied MAPKs are ERK1/2, JNKs, and the p38 family, which are classified as conventional MAPKs (Pearson et al., 2001).

Given the pivotal role of MAPK pathways in various cellular events, several studies have provided evidence for the possible involvement of MAPK cascades in MB progression (Anne et al., 2013; Gilbertson et al., 2006; Hallahan et al., 2003; Kool et al., 2008; MacDonald et al., 2014; MacDonald et al., 2001). Fults and colleagues reported that the N-RAS oncogene is activated in the human MB cell line TE 671 by a mutation at the third position of codon 61. A point mutation at this location corresponds to a substitution of histidine for glutamine in the N-RAS gene product, p21 (Fults et al., 1989). A low frequency mutation (3 out of 32 examined MB tissues) of N-RAS oncogene was later found in MBs (Iolascon et al., 1991). However, a recent report from Gilbertson et al. showed that, when analyzing 29 primary MB tumors, no oncogenic mutation was observed affecting NRAS, KRAS, HRAS, BRAF or PDGFRB in any tested case (Gilbertson et al., 2006). Consistent with this study, Kool and colleagues sequenced the KRAS, NRAS, and HRAS genes for codon 12, 13, and 61 but detected no mutation in 52 MB tumors tested (Kool et al., 2008). Studies with larger sample sizes are required to draw a conclusion on this debate.

Over-expression or activating mutations of RTKs also frequently contribute to the abnormalities of MAPK pathway in MB. For instance, PDGFR and members of the downstream RAS/MAPK signal transduction pathway are up-regulated in metastatic MBs compared with non-metastasis ones (MacDonald et al., 2014; MacDonald et al., 2001) implicating an essential role of MAPK pathway in MB metastasis. Yuan and colleagues also show that Rac1/Pak1(p21-activated kinase 1) signaling is critical to MB cell migration and is functionally dependent on PDGFRβ/ERK activity using MB cells and patient tissues (Yuan et al., 2010). In addition, up-regulation of mTOR, Akt, and ERK was observed in MBs with extensive nodularity (Jozwiak et al., 2011). Very recently, high levels of p38α and elevated levels of members of the p38 pathway, such as ASK1, MKK3, and ATF2, were found in CGNPs, the cells of origin for SHH-driven MB, in mouse and human MB, indicating the potentially critical role of p38 MAPK signaling in SHH-driven MB (Guldal et al., 2012).
With regard to MB treatment, Hallahan et al. found that bone morphogenetic protein-2 (BMP-2) induced p38 MAPK signaling is necessary for retinoids, and caused extensive apoptosis of MB cells (Hallahan et al., 2003). Moreover, over-expression of either MEK5 plus ERK5 or its target myocyte enhancer factor 2 (MEF2) has been found to be sufficient to induce MB cell death (Sturla et al., 2005). Recently, WNT3 has been shown to inhibit cerebellar granule neuron progenitor proliferation and MB formation via MAPK activation in vitro and in vivo (Anne et al., 2013).

5 Targeting PI3K and MAPK Pathways as Novel Therapeutic Strategies

5.1 DHA Sensitizes Medulloblastoma Cells to Etoposide-Induced Apoptosis

The interest in the beneficial effects of n-3 long chain fatty acids has been continuously increased during recent years. Among the three major dietary n-3 fatty acids: ALA (C18:3), eicosapentaenoic acid (EPA) (C20:5), and DHA (c22:6), DHA is the most abundant omega-3 fatty acid in the brain (40% of the polyunsaturated fatty acids (PUFAs) in the brain) and retina (60% of the PUFAs in the retina) (Innis, 2007). Because of the marked benefits of DHA in the maintenance of normal brain function (Innis, 2007) and DHA has been considered as an essential component of nutrition for people at all ages, researchers have recently given their attention to the effects of DHA on brain tumor therapy. For example, DHA has been found to regulate COX-2-mediated invasion in brain-metastatic melanoma (Denkins et al., 2005). With the encouraging results from the combinations of DHA with some well-studied anticancer drugs, including 5-fluorouracil, carboplatin, cyclophosphamide, doxorubicin, taxane, and celecoxib toward other types of human cancers (Harries et al., 2004; Merendino et al., 2013; Narayanan et al., 2006; Narayanan et al., 2005; Swamy et al., 2004; Wynter et al., 2004; Zhou et al., 2009), we investigated the effects and potential underlying mechanism of DHA alone and in combination with etoposide, an anticancer drug currently used in treating MB. Our results suggest that the addition of DHA or etoposide individually can inhibit the proliferation of Daoy and D283 human MB cells in a dose-dependent manner. The combination of these two agents results in an additive inhibitory effect on MB cell proliferation. The cytotoxicity of DHA, etoposide, and their combination was further validated for cell death by using Hoechst 33342/PI staining. The results showed increased apoptosis in Daoy and D283 cells treated with combined treatment compared to the single treatment. Noteworthy, no significant cytotoxic effect of DHA combined with etoposide was observed in U87 and U138 glioblastoma cell lines which implies that DHA modulates the cytotoxic effect of etoposide in a cell type dependent manner (Wang et al., 2011).
Based on the investigations of DHA’s mechanism of action, several pathways have been proposed to be involved. Studies suggest that DHA inhibits cancer cell growth and increases the therapeutic efficiency of chemotherapeutic agents at least in part by modulating the redox status in tumor cells since one important feature of DHA is its high susceptibility to oxidation (Lyberg et al., 2005; Madsen et al., 1999). Because of the higher levels of reactive species in cancer cells than in normal cells, DHA is able to exert high levels of lipid peroxidation. The lipid peroxidation results in toxic products, such as malondialdehyde, which can, in turn, interact with nucleic acid bases and induce cell apoptosis (Del Rio et al., 2005). Since reactive oxygen species (ROS) affect multiple target genes, such as NFκB, p53, HIF-1α, Akt, and p38 MAPK phosphatase (Horak et al., 2010; Liu et al., 2008; McCubrey et al., 2006; Silva et al., 2011), studies have been performed to analyse the effects of DHA on these molecules or related signaling pathways in cancer. DHA has been shown to activate p53 in prostate and colon cancer cells (Kato et al., 2007; Narayanan et al., 2006). The combination of DHA with 5-fluorouracil up-regulates the expression of Bcl2-associated X protein (BAX) in gastric carcinoma cells (Zhuo et al., 2009). Co-treatment with docetaxel and DHA in prostate cancer cells suppresses the levels of genes in NFκB pathway (Shaikh et al., 2008). Moreover, DHA induces apoptosis in colorectal carcinoma and neuroblastoma cells by targeting PI3K/MAPK or ERK signaling pathways (Akbar & Kim, 2002; Toit-Kohn et al., 2009; Wu et al., 2009). Although many studies have been performed to define the action mechanism for DHA’s anti-tumor activity, the exact mechanism(s) for DHA and the combination therapy of DHA with other drugs has not been elucidated yet.

To elucidate the mechanism by which DHA potentiated the effects of etoposide in MB cells, we performed Human Cancer Pathway Finder OligoGEArray to analyze the gene profile in Daoy and D283 cells treated with single or combined treatment of DHA and etoposide. Fourteen genes, including Akt, were observed to be up-regulated (Table 2). In contrast, thirteen genes were down regulated by DHA including those involved in growth signaling (e.g., MAPK14), cell cycle control, DNA damage repair, and anti-apoptotic pathway in both cells (Table 2).

<table>
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<th>Regulation of Gene Expressions in MB Cells Treated with DHA</th>
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<tr>
<td><strong>Up-regulated</strong></td>
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<td>BCL2L1, CDKN1B, CCNE1, BCL2, ERBB2, AKT1, BAXTIMP3, CDK4, ATM, TWIST1, ANGPT1, CCND1, BAD, BAI1</td>
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<tr>
<td><strong>Down-regulated</strong></td>
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<tr>
<td>MCAM, MAPK14, ITGA4, GZMA, MDM2, CHEK2, MTA2, RAF1, IL8, PNN, PLAU, PLAUR, PDGFB</td>
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<td>Differentially regulated (different effects in the two MB cell lines)</td>
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Table 2. The expression of genes in MB cells regulated by DHA.

In etoposide treated cells, ten genes, including Akt1, were up regulated (Table 3), while ten genes were down regulated including MAPK14 which is pivotal for MAPK signalling (Table 3). Additional genes including PTEN and Tp53 were found to be differentially regulated in Daoy and D283 cells by DHA or etoposide (Table 2&3).

<table>
<thead>
<tr>
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<td><strong>Up-regulated</strong></td>
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<tr>
<td><strong>Down-regulated</strong></td>
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<tr>
<td><strong>Differentially regulated (different effects in the MB cell lines)</strong></td>
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Table 3. The expression of genes in MB cells regulated by etoposide.

Compared to exposure to etoposide alone, over sixty genes were significantly down regulated by DHA and etoposide combined treatment in both Daoy and D283 cells. Among these genes, key components of PI3K pathway (e.g., Akt, PTEN, PIK3R1 and PIK3CB) and MAPK pathway (e.g., JUN, MAP2K1, MAPK14) are markedly down-regulated. Notably, PDGFA and PDGFB which usually associate with the activation of the PI3K/MAPK pathway, and have been implicated in the MAPK signaling pathway mediated MB metastasis, were also down-regulated (Table 4). This was further validated by using real-time PCR analysis for the gene expression of PDGFA and PDGFB using cDNA from MB cells treated with 30mM DHA and additional exposure to 0.8 µM of etoposide for 24 hours (Wang et al., 2011).

| Regulation of Gene Expressions in MB Cells Treated with DHA and Etoposide |
| Down-regulated | PRKDC, PDGFA, NME4, JUN, RASA1, CDC25A, ITGB1, E2F1, PIK3CB, BIRC5, BRCA2, CDKN1A, CDK4,CCNE1, PDGFB, MAP2K1, MCAM, AKT1, CDKN1B, MTA1, MTA2, BCL2, BCL2L1, PTEN, PNN, IFNA1, FGF2, RAF1, ETS2, CASP8, MMP1, MDM2, BRCA1, CD44, CHEK2, MAPK14, EGF, KISS1, ITGAV, ATM, CASP9, ITGA4, CDH1, APAF1, SYK, IFNB1, CFLAR, COL18A1, HTATIP2, BAD, PIK3RA, RB1, ITGA6, NFKB1, CD82, SNCG, FOS, TWIST1, FLT1, MYC, ITGA1 |

Table 4. The gene expressions in MB cells regulated by the combination of DHA and etoposide.

Taken together, the combination of DHA and etoposide exhibits profound inhibitory effects on human MB cells and its underlying mechanism of action at least in part relies on the down regulation of PI3K/MAPK signaling.

5.2 Benefits of Using DHA to Target PI3K and MAPK Pathways in MB

Loss of PTEN is associated in MB patients with a poor outcome (Castellino et al., 2010), and activation of PI3K/AKT signalling results in reduced expression of PTEN (Hartmann et al., 2006). Schley et al. discovered that DHA inhibits breast cancer cell proliferation and induces apoptosis by decreasing the Akt/NFκB signaling (Schley et al., 2005). A similar phenomenon was observed by Friedrichs et al. in which the inhibition of cancer progression by DHA correlated with a decrease in androgen receptor expression and suppression of the Akt/mTOR signaling pathway in human prostate cancer cells in vitro (Friedrichs et al., 2011). In addition, feeding PTEN(−/−) mice with a DHA enriched diet results in decreased phosphorylation of PDK1, Akt, and Bcl-2-associated death promoter (Bad) in prostates (Hu et al., 2010). In our study, DHA alone sensitized the expression of Akt in MB cells by stimulating its expression, while the combination of DHA and etoposide markedly reduced Akt expression (Wang et al., 2011). These studies suggest that MB patients may benefit from DHA suppression of the PI3K/AKT pathway.

DHA supplementation resulted in a reduction in phosphorylated MAPK in human breast cancer cells (Lu et al., 2010) and mouse mammary tissue (Sun et al., 2011). Reduction of VEGF expression and inhibited ERK-1 and-2 phosphorylation were observed in DHA treated human colon cancer cells (Calvillo et al., 2004). In addition, DHA induced cell death and DNA fragmentation occurs in parallel with the activation of ERK and c-Jun N-terminal kinases (JNK) in gastric cancer cells (Lee et al., 2009). These
results suggest that DHA’s anti-cancer activity is associated with suppression of MAPK. Given MAPK pathway plays an essential role in MB progression, MB patients are likely to benefit from regimens suppressing MAPK pathway. Noteworthy, in contrast with the evidence for DHA’s cytotoxic effects in cancer, Wu et al. showed that human neuroblastoma SH-SY5Y cells exposed to DHA exhibit significant increases in the percentage of cells with longer neuritis with up-regulated levels of MEK and ERK1/2 phosphorylation. This substantiates the important role of DHA in enhancing neurite out growth (Wu et al., 2009).

Although DHA alone or combined with etoposide modulates multiple signaling pathways in MB, DHA single treatment was shown to down-regulate MAPK14 and PDGFB, indicating its inhibitory effects on PI3K/MAPK pathways. When treated with the combination of DHA and etoposide, MB cells showed marked down regulation of key component genes PI3K/MAPK pathways, including Akt1, PTEN, PIK3R1, PIK3CB, JUN, MAP2K1, and MAPK14. Moreover, the down-stream targets of these pathways, such as NFkB and MYC were also suppressed by the treatment. These results systematically revealed the modulation of signaling pathways by the treatment of DHA and etoposide in MB.

Previous research revealed that PDGF/PDGFR signaling plays an essential role in MB cell proliferation, cell death, and metastasis (Gilbertson & Clifford, 2003; Gilbertson et al., 2006). Recent reports further support that targeting of PDGF/PDGFR signalling is a rational approach for MB treatment (MacDonald et al., 2014; Tian et al., 2011; Zhou et al., 2014). This notion is further supported by the results from another recent study using a cancer stem cell inhibitor, salinomycin, to treat MB cells. Salinomycin induced significant MB cell death accompanied with sharply down regulated expression of PDGFRβ (Zhou et al., 2014). The expressions of PDGFα and PDGFβ in MB cells were found to be down-regulated in response to DHA and etoposide combined therapy, but not etoposide alone. When key downstream mediators of the PDGFR signaling, PI3K/MAPK signaling is inevitably down-regulated, the survival of MB cells are subsequently disrupted. This is evidenced by the down regulation of components of PI3K/MAPK pathways and the cytotoxic effects of DHA and etoposide combined treatment on MB (Wang et al., 2011).

It is well known that DHA is a popular dietary supplement with marked health benefits. In our previous study, we extended the knowledge of DHA’s function in the therapy of human MB. Based on our observation, DHA at higher than 30µM significantly increased cell death and inhibited cell proliferation in MB cells; and additive activities of DHA combined with etoposide were shown on MB cell proliferation and cell death (Wang et al., 2011). These results indicate that possibly MB patients may benefit from taking DHA as an adjuvant of chemotherapeutic agents, such as etoposide.
6 Conclusion

In conclusion, the combination of DHA and etoposide treatment possesses anti-cancer effects against MB in vitro, and such effects are associated with DHA’s activities in modulating PI3K/MAPK pathways. Given the pivotal role of PI3K/MAPK signaling in MB progression and DHA’s well recognized benefits in maintaining brain functions, better understanding of the regulatory mechanism of DHA, as well as the combination of DHA and etoposide, in regulating PI3K/MAPK signaling in MB might provide clues for the evaluation of whether this combination will bring benefit to patients with MB. In addition, given the observed inhibitory effects of the DHA-etoposide combination treatment on MYC, MB patients with high MYC expression are likely benefited from this combination therapy. Future studies for DHA supplementation with chemotherapeutic and radio-therapeutic anticancer regimens using animal models and in humans are largely needed to determine the benefit of treating MB using the combination of DHA with current therapies.

Abbreviations:

ADAM: A disintegrin and metalloproteinase; ALA: Alpha-linolenic acid; APC: Adenomatous polyposis coli; ASK1: Apoptosis signal-regulating kinase 1; ATF2: Activating transcription factor 2; AXIN1: Axis inhibition protein 1; AXIN2: Axis inhibition protein 2; Bad: Bcl-2-associated death promoter; BAX: Bcl2-associated X protein; Bel-2: B-cell lymphoma 2; Bmi1: B lymphoma Mo-MLV insertion region 1 homolog; BMP-2: Bone morphogenetic protein-2; BRAF: V-raf murine sarcoma viral oncogene homolog B; CDHA: Conjugated DHA; CGNP: Cerebellar granule neuron precursor; c-Met: Hepatocyte Growth Factor Receptor; CoA: Coenzyme A; Cos2: Costal2; CSL: CBF1, Suppressor of Hairless, Lag-1; DHA: Docosahexaenoic Acid; ELK: ETS domain-containing protein; EPA: Eicosapentaenoic acid; ERBB2: V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2; ERK: Extracellular-signal-regulated kinase; FKH: Forkhead/Foxo; FU: Fused; Glii: Glioma-associated oncogene homolog 1; GPCR: G protein-coupled receptors; GSK3: Glycogen synthase kinase 3; Hes1: Hairy and enhancer of split-1; HIF-1α: Hypoxia-inducible factor 1-alpha; HRAS: Harvey rat sarcoma viral oncogene homolog; IGF: Insulin-like growth factor; IGF-1R: Insulin-like growth factor 1 receptor; IGFR: Insulin-like growth factor receptor; ITCH: Itchy E3 Ubiquitin Protein Ligase; JNK: Jun N-terminal kinase; KCTD11: Potassium channel tetramerization domain containing 11; KRAS: Kirsten rat sarcoma viral oncogene homolog; LCA: Large-cell anaplastic; LEF: Lymphoid enhancer-binding factor; LRP: Low density lipoprotein receptor-related protein; MB: Medulloblastoma; MAPK:
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