High dose ω3 eicosapentaenoic acid and docosahexaenoic acid block, whereas ω6 arachidonic acid accelerates, *MYCN*-driven tumorigenesis *in vivo*.

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Abstract
Neuroblastoma (NB) is a genetically diverse, highly metastatic pediatric cancer that accounts for an estimated 8% of childhood cancer incidence but is responsible for 15% of childhood cancer deaths. High-risk and relapsed cases have less than 50% and near 5% survival, respectively. The current standard of care is highly genotoxic, resulting in life-long health issues and an increased risk of new cancer incidence. Effective and less toxic approaches to neuroblastoma remain elusive. We show here that ω3 and ω6 highly unsaturated fatty acids (HUFA) have differential effects on tumor formation in a syngeneic model of neuroblastoma. We observe that 20 gram/d doses of ω3 DHA and EPA have a robust antitumor effect, wherein DHA and high-dose EPA completely abrogate tumor formation. In contrast, ω6 arachidonic acid (ARA) has the opposite effect, driving higher tumor penetrance and shorter latency. When used together, ARA and EPA treatment results in a reduced tumor burden analogous to the control group, indicating that EPA may directly oppose the mechanism of ARA-mediated tumor formation. ROS-resistant deuterated DHA (D-DHA), which has deuterium atoms in place of ordinary ^{1}H (protium) in the oxidatively labile *bis* allylic positions, also completely abrogated tumor formation, strongly suggesting that the mechanism of action is not through oxidation as might have been expected. Interestingly, despite high levels of fatty acid delivery, very little tissue accumulation was observed. These results suggest that high doses of ω3 HUFA DHA and EPA may represent a viable, low-toxicity avenue for neuroblastoma therapy.

Introduction

Neuroblastoma (NB) is a highly metastatic cancer that accounts for 15% of childhood cancer deaths.[1, 2] We recently showed that unsaturated fatty acid synthesis (UFAS) genes have many predicted binding sites for let-7 and related NB-related miRNA. UFAS gene expression is strongly associated with NB outcomes.[3] We hypothesized that dietary exogenous HUFA would modulate tumorigenesis in a syngeneic mouse model of MYCN-driven NB. We used high levels of ω6 arachidonic acid (ARA), omega-3 eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and a deuterium (D) substituted ROS-resistant isotopologue of DHA (D-DHA)[4, 5] to saturate tissue before subcutaneous injection of MYCN over-expressing mouse cells into immunologically intact wild-type mice to investigate their effects on tumorigenesis.

Fatty acids are an important structural component of cellular membranes. Highly unsaturated fatty acid (HUFA) are released from membranes by PLA2s and as FFA are converted to eicosanoids and docosanoids, collectively known as oxylipins. They are fundamental to paracrine signaling, including immune-stimulating species such as prostaglandins, 20-HETE, and leukotrienes.[6] With respect to cancer, fatty acids contribute to tumor formation or inhibition.[7-9] As we recently showed for neuroblastoma, transformation to malignancy alters fatty acid synthesis and levels within cells; fatty acid inputs are known to influence tumorigenesis.[10]

In this study, we aimed to understand the effects of high-dose ω3 and ω6 HUFA DHA and EPA, and ARA, respectively. Prior reports on the effects of these HUFA suggest that all three can inhibit cancer cell growth, with a common mechanism of ROS reported. Earlier pre-clinical and clinical trials attempted to bring these observations forward but used only medium to low doses of relevant HUFA, and the outcomes were mixed. Other studies on humans have shown very high doses of ω3 can yield significant benefits for several human diseases. We reasoned that such high-level dosing might yield significant benefits for neuroblastoma in a continued effort to identify low-toxicity therapies.

Methods

Diets and oils.

Mice were fed on customary facility mouse chow with a composition approximating AIN-93G. ARA was from a single-cell triglyceride oil with about 44% ARA and the rest monounsaturated and saturated fatty acids. EPA and DHA were from dietary supplement triglyceride oils of about 88% EPA and 82% DHA, respectively. Oils were analyzed in-house prior to use, as outlined below. Doses are presented in Table 1.

Mouse gavage and injection procedure

Experiment 1.

A total of 8 mice per group were orally administered ARA and EPA for seven days before the cell injection, respectively; on day 7, 2.5×10^6 Neruro-2a MYCN transfected cells were injected into the mice by subcutaneous route, followed by alternative day orally administered the ARA and EPA to the mice for ten days post cell injection. At the end of the study, the mice were euthanized, tumors were collected from the mice, and tumor weight was measured.

Experiment 2.

Tumor study methodology: A total of 10 mice per group were orally administered Arachidonic acid (ARA) (250microliters) and EPA (250 microliters) and a combination of ARA(150 microliters) and EPA(150 microliters) for seven days before the cell injection, respectively; on day 7, 2*10^6

Neruro-2a MYCN transfected cells were injected into the mice by S.C. route, followed by alternative day orally administration the ARA (250 microliters)and EPA (250 microliters) and combination of ARA(150 microliters) and EPA(150 microliters) to the mice for 20 days post cell injection. At the end of the study, the mice were euthanized, tumors were collected for the mice, and tumor weight was measured. Tumor incidence: The incidence of the tumor was monitored post-injection in the mice till the end of the study to assess the growth of the tumor. By using vernier calipers, the growth of the tumor was measured every alternative day till the end of the study.

Table 1

	Dose (uL/d)	HUFA Dose (mg)	HUFA dose (mg/kg bw)	Cal	%Cal	Human dose (cal)*	Human Dose (g)
Control	0	0	0	0	0%	0	0
ARA (Exp1)	75	30	746	0.27	1.7%	41	4.6
ARA	125	50	1,243	0.45	2.7%	54	6.0
DHA	125	103	2,578	0.93	5.5%	110	12.2
D-DHA	125	125	3,125	1.13	6.6%	131	14.6
EPA (Exp1)	75	66	1,643	0.59	0.36%	71	7.9
EPA	125	110	2,739	0.99	5.8%	116	12.9
ARA+EPA	150	95	2,363	0.85	5.0%	101	11.2

All doses from Experiment 2 except as noted ("Exp 1").
*Human dose in calories assuming 2,000 calories per day total intake.

Fatty Acid Analysis

Doses and tissues were analyzed according to routine methods in our laboratory.[11] Briefly, tissue samples are minced to apparent homogeneity, or oils used as is, and treated with an aqueous phase to liberate fatty acids and convert to fatty acid methyl esters (FAME), and simultaneously extracted into an organic phase.[12] FAME mixtures are evaporated, resuspended in heptane and injected into a gas chromatograph (GC) equipped with a BPX-70 capillary column. An equal weight standard is used to verify response factors. Data are expressed as % weight-for-weight (%,w/w) of total fatty acids.

Ethics. Studies were approved by the University of Texas IACUC.

Results

Experiment 1.

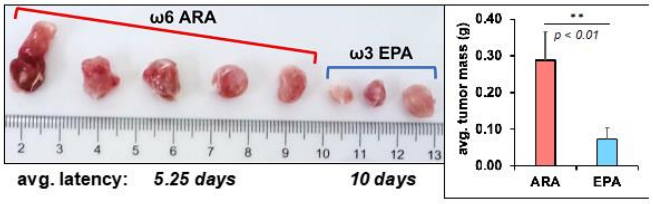
Mice gavaged with ARA produced tumors sooner and larger and, in more mice, than those gavaged with EPA (Figure 1). The mean latency to palpable tumor was 5.25 d for the EPA mice and 10 d for ARA mice, or about double the time. At 14 d post-injection, the mean weight of ARA mice tumors was about 3-fold greater than that of the EPA mice. Control mice with no gavage were a third group in this experiment. Four of eight control mice developed tumors at intermediate latency and of intermediate size compared to ARA (earlier, larger) and EPA (later, smaller) (data not shown).

Experiment 2.

Ten mice were in each group. Compared to Experiment 1, doses were adjusted upward by about 60% for ARA and EPA. Similar doses were used for DHA and D-DHA. An additional group with a combined dose of ARA+EPA at levels similar to Experiment 1 was the final group (Table 1).

The ARA group again had earlier, more, and larger tumors than the control, EPA, and ARA+EPA groups ($p<0.05$) (Figure 2). Remarkably, zero of the mice provided with DHA or D-DHA had any tumors at 14 d post-injection.

a.



b.

Neuro 2a		
Groups	Mice Injected	Tumor Incidence
ARA	8	5
EPA	8	3

c.

Neuro 2a			
Groups	Tumor Length (mm)	Tumor width (mm)	Tumor weight (gm)
ARA	22	11	0.39
	9	10	0.29
	12	12	0.33
	11	10	0.24
	9	8	0.19
EPA	9	7	0.05
	4	7	0.06
	7	8	0.11

Figure 1. Experiment 1 results. a, b) ARA-treated mice. 5 of the 8 mice produced at a mean latency (time to appearance) of 5.25 d, and a mean weight of about 0.28 g. EPA-treated mice. 3 of 8 produced tumors at mean latency of 10 d and eight of 0.08 g. c) Detailed tumor data. Control (no gavage) mice, 4 of 8 mice produced tumors of mean size greater than the EPA mice but smaller than the ARA mice.

We also observed in ARA mice pronounced subcutaneous blood vessels leading to the tumor (Figure 2C).

Fatty acid data on selected tissue showed modulation of ARA but not DHA or EPA due to dosing (Figure 3). Compared to controls, liver ARA was lower in EPA mice, higher in ARA mice, and lower in ARA+EPA mice. Liver ARA was about twice that in the ARA mice than in the EPA mice, indicating enhancement and suppression of ARA by doses of ARA and EPA, respectively. The EPA and ARA+EPA levels were similar, indicating that equal amounts of the two HUFA effectively balanced one another. Means for skin ARA followed a trend similar to liver but was not significant. Neither DHA nor EPA changed significantly in liver, muscle, or skin due to dosing despite the major suppression of tumorigenesis. Remarkably, despite the effect of EPA on tumorigenesis, it did not rise above a mean of 0.4% of fatty acids even in EPA mice.

Discussion

The mechanism of action of EPA and DHA in moderating or eliminating tumors will likely be via multiple underlying processes. Both ω3 HUFA compete with ARA for incorporation into some but

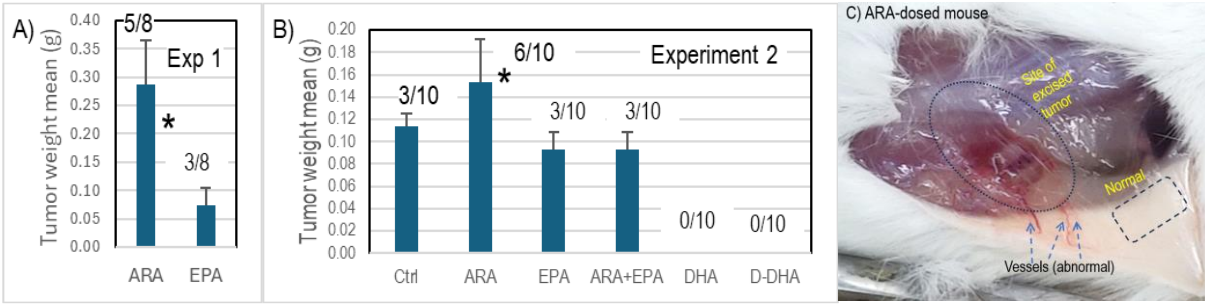


Figure 2. Experiment 2 results. A) Repeat of Experiment 1 tumor weights for comparison. B) Experiment 2 tumor results show 6 of 10 ARA mice had high tumor weights ($p<0.05$). Control, EPA, and ARA+EPA had similar tumor numbers (3 of 10) and mean weights. <u>DHA and D-DHA had zero tumors</u>. C) Photograph of subcutaneous region around the tumor of an ARA mouse. Blood vessels are prominent near the tumor but not adjacent to it.

not all membranes, from which they are liberated before conversion to signaling molecules, thus reducing the amount of ARA available for this purpose. Once liberated, typically by a phospholipase A2, they compete with ARA for access to the COXs, LOXs, and other enzymes for conversion to prostaglandins, leukotrienes, thromboxanes, and related molecules, which signal for a wide range of functions, including inflammation, thrombosis, vessel tone, and chemotaxis. Many studies also report their conversion to specialized pro-resolving lipid mediators.[6] The capillary endothelium is normally rich in ARA, suggesting that angiogenesis would be enhanced in the presence of ample ARA and inhibited when ARA is limiting. [13]

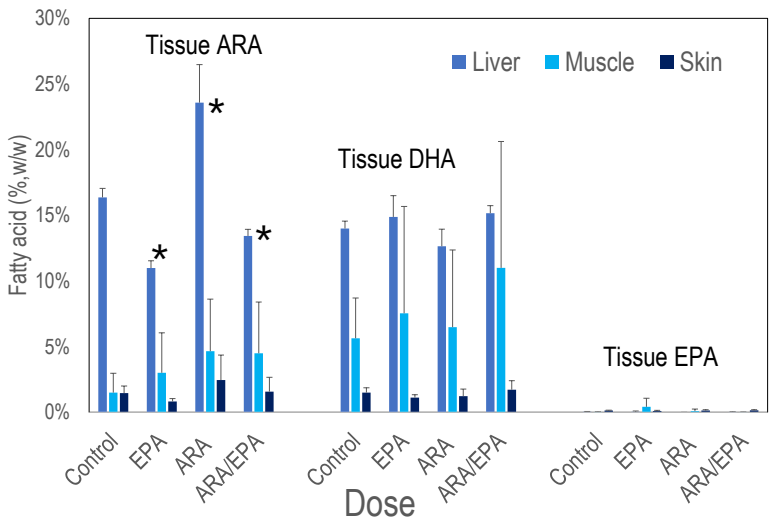


Figure 3. HUFA levels in selected tissue of control and gavaged mice. ARA responds to dosing while neither DHA nor EPA shows any discernable pattern. EPA levels remain below 0.4% even in EPA mice.

EPA/DHA doses higher than 20 g/d have been used on a sustained basis (months to years) in humans for the treatment of traumatic brain injury,[14] 12 g/d to 15 g/d in hypertriglyceridemia [15] or hypertensive patients,[16] and in at least one pregnant woman who gave birth successfully,[17] and all doses were well tolerated. Enhanced bleeding times might be expected based on observations in individuals with high circulating EPA levels, though a recent meta-analysis showed mild effects up to 4 g/d.[18] Our doses of DHA and EPA on a human calorie-equivalent basis are under 15 g/d (7.9 to 14.6 g/d, Table 1). The DHA level that was effective at suppressing all tumorigenesis is 12.2 g/d. This is about 3-fold the normal chronic dose of prescription Lovaza™ used on chronically to treat hypertriglyceridemia.

We observed subjectively in both EPA and DHA groups that the skin/fur was more oily than other groups. Neither EPA nor DHA levels in skin/fur were exceptionally high, which might have been expected from results in guinea pigs that excrete excess ω3 ALA via the skin.[19, 20] D-DHA is a pharmaceutical preparation with D substituted for H in the ten *bis*-allylic positions of DHA[21, 22] which renders it highly resistant to reactive oxygen species (ROS) initiated oxidation *in vivo* [5, 23].

D-DHA is transported identically to the naturally occurring isotopologues of DHA and, thus, at steady state should reach similar concentrations in all body pools. HUFA production is generally suppressed in tumorigenesis by somatic changes as well as other mechanisms,[10] resulting at least in part in the reduction of the formation ROS and consequent downstream cell death. In the present studies, D-DHA was as effective as regular DHA in blocking tumor formation at similar doses. Together, these results suggest that the mechanisms of action of D-DHA and DHA are both ROS-independent. This provocative result requires follow-up.

These results are suggestive of broader efficacy against metastatic proliferation. HUFA-based signaling is ubiquitous throughout mammalian tissue. With ω6 ARA enhancing, and ω3 DPA/DHA/D-DHA blocking, tumorigenesis, the mechanism may be a yin yang where a balance

strongly favoring the ω3s shuts off anomalous proliferative signaling. Since this is not specific to a molecular lesion driving a primary cancer type, it may apply more widely than NB and MYC-driven/implicated cancers.

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