LIPID MEDIATORS IN THE PREVENTION AND THERAPY OF NEUROBLASTOMA

Helena Gleissman

Stockholm 2009
All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by E-Print, Stockholm.
Front cover by Dr Karl Harrison, 3dChem.

© Helena Gleissman, 2009
Till Mamma

“If we knew what it was we were doing, it would not be called research, would it?”

-Albert Einstein
ABSTRACT

Neuroblastoma is a cancer of the nervous system that mainly affects infants and young children. It is the most common solid extracranial tumor of childhood, and accounts for almost 10% of all childhood cancer deaths. Despite intense multimodal treatment consisting of surgery, chemotherapy, radiotherapy, and stem cell rescue, survival is only 50% in the high-risk group, and the overall survival is around 70%. We therefore need to improve existing treatment protocols and search for new medications.

Inflammation drives cancer growth and targeted therapy that dampens inflammatory responses is anti-proliferative. We have previously shown that the inducible COX-2 enzyme that converts the omega-6 fatty acid arachidonic acid (AA) to various inflammatory prostaglandins is upregulated in neuroblastoma tissue. We have also shown that celecoxib, a selective inhibitor of COX-2, may inhibit neuroblastoma growth.

This thesis shows that celecoxib both prevents tumor formation and reduces tumor growth in a neuroblastoma xenograft rat model. In addition, celecoxib enhances the effect of the chemotherapeutic drugs irinotecan and doxorubicin. By immunohistochemistry, we detected reduced proliferation and inhibited angiogenesis in tumors from animals treated with celecoxib, either by gavage or by an enriched diet.

Omega-3 fatty acids oppose the effects of omega-6 fatty acids such as AA and have been implicated in cancer treatment and prevention. Omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are precursors of anti-inflammatory compounds. From EPA, a different series of prostaglandins are produced, and from DHA, resolvins and protectins are produced, which are potent pro-resolving lipid mediators essential for the clearance of inflammatory cells and mediators at an injured site.

In this thesis I show that DHA is toxic to neuroblastoma cells both in vivo and in vitro. In vivo, DHA is able to delay time to tumor development and reduce tumor growth in neuroblastoma xenograft models. In vitro, DHA acts by inducing mitochondrial-dependent apoptosis. We also show that neuroblastoma cells convert DHA to hydroperoxy and hydroxy fatty acids through both enzymatic and non-enzymatic mechanisms. However, DHA is not converted to resolvins or protectins in neuroblastoma cells. DHA also potentiates the effect of other cytotoxic drugs such as chemotherapeutics, arsenic trioxide, and non-steroidal anti-inflammatory drugs (NSAIDs).

In summary, this thesis shows that inhibiting the omega-6 fatty acid pathway and enhancing the omega-3 fatty acid pathway are both possible new strategies for neuroblastoma treatment, and suggests these compounds to be tested as novel therapy for children with neuroblastoma in clinical trials.
LIST OF PUBLICATIONS


ADDITIONAL PAPERS


*These authors contributed equally
4.2.1 Reduction of tumor burden alone and in combination with cytostatic drugs.............................................27
4.2.2 Celecoxib as both prevention and treatment.........................28
4.2.3 Celecoxib inhibits proliferation and suppresses angiogenesis .................................................................................30
4.3 DHA in vitro (Paper I and III).................................................................31
4.3.1 Cytotoxicity alone and together with cytostatic drugs, NSAIDs, and arsenic trioxide...........................................31
4.3.2 Elucidation of metabolic pathways...........................................34
4.4 DHA in vivo (Paper IV).................................................................36
4.4.1 Neuroblastoma xenografts...........................................................36
4.4.2 Transgenic mice carrying the MYCN oncogene.....................38
5 Summary and conclusions.............................................................................39
6 Future perspectives.................................................................................40
7 Sammanfattning på svenska........................................................................42
8 Acknowledgements......................................................................................43
9 References......................................................................................................49
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-H(p)DHA</td>
<td>17-hydro(per)oxy-docosahexaenoic acid</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>As₂O₃</td>
<td>Arsenic trioxide</td>
</tr>
<tr>
<td>BSO</td>
<td>L-Buthionine-sulfoximine</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DPA</td>
<td>Docosapentaenoic acid</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxyicosatetraenoic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Endothelial growth factor receptor</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas liquid chromatography</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H(p)ETE</td>
<td>Hydro(per)oxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>ICAM/VCAM</td>
<td>Intracellular/vascular cell adhesion molecule</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LNA</td>
<td>Linolenic acid</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipoxigenase</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>mPGEs-1</td>
<td>Microsomal PGE synthase 1</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MYCN</td>
<td>V-myc myelocytomatosis viral related oncogene, neuroblastoma derived</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NDGA</td>
<td>Nordihydroguaiaretic acid</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroid anti-inflammatory drug</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>PD1</td>
<td>Protectin D1</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PLA</td>
<td>Phospholipase A</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisomal proliferator activation receptor</td>
</tr>
<tr>
<td>PPTP</td>
<td>Pediatric Preclinical Testing Program</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Radical oxygen species</td>
</tr>
<tr>
<td>RvD1</td>
<td>Resolvin D1</td>
</tr>
<tr>
<td>TgMyc</td>
<td>Transgenic mouse carrying the MYCN oncogene</td>
</tr>
<tr>
<td>TVI</td>
<td>Tumor volume index</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxanes</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

This thesis focuses on neuroblastoma, a malignant tumor of early childhood, and on more effective strategies for treatment. The conventional therapy of today has a 70% success rate at best. The novel treatment modalities that have been explored in this thesis concern fatty acids and their metabolism. Fatty acids, once thought of as solely an energy source in our bodies, have proven to be highly active molecules. They can act as ligands in signal transduction, as transcription factors that regulate protein synthesis, and as membrane components that regulate the fluidity, permeability, and dynamics of cell membranes.

In addition, fatty acids are precursors to a wide range of different lipid mediators that regulate inflammatory responses and metabolic pathways. Most fatty acids can be synthesized in the body, but not all. The essential precursors of all omega-3 fatty acids, linolenic acid (LNA), and of all omega-6 fatty acids, linoleic acid (LA), must be obtained from the diet. Thus dietary habits, and especially intake of fat, do more to the body than just influence weight and waist circumference. Dietary habits affect the system as a whole, down to gene-level, and both the amount of fat and the kind of fat we eat can have profound and significant effects on health. Daily fat intake can actually be a matter of life and death.

The overall impact of proper daily fat intake for neuroblastoma patients remains to be investigated, but the data presented in this thesis shows that it can be of significance. Inhibiting the omega-6 while enhancing the omega-3 metabolic pathways reduces neuroblastoma growth in vitro and in vivo, as shown by in vitro studies on human neuroblastoma cells as well as by animal studies. In addition, this thesis proposes possible mechanisms behind the observed effects, and presents the way that lipid mediators and enzyme inhibitors of the metabolic pathways of interest can augment the effect of cytostatic and cytotoxic drugs.

1.1 NEUROBLASTOMA

Neuroblastoma is a cancer of early childhood. It predominately affects infants below one year of age. The median age at diagnosis is 18 months, 75% are diagnosed before four years of age, and less than 10% are diagnosed during school age. Older children and adolescents have been diagnosed with neuroblastoma, but this is very rare (Gustafsson, et al 2007). The tumor arises from precursor cells of the sympathetic nervous system in the neural crest, which is a transient embryonal structure that arises from the ectoderm during closure of the neural tube. The neural crest consists of a population of multipotent progenitor cells that will differentiate into peripheral sensory neurons, cells in the enteric nervous system, Schwann cells, pigment cells, and parts of the craniofacial skeleton. Tumors are typically localized in sympatho-adrenal tissue, i.e. along the spinal cord in the sympathetic ganglia, or in the adrenal medulla (Fig. 1). Prognostic factors for neuroblastoma include age at diagnosis, stage, and genetic aberrations such as MYCN amplification, 1p and 11q deletion, and 17q gain (Brodeur 2003, Johnsen, et al 2009, Maris, et al 2007). Patients are allocated into low, intermediate, and high risk
groups according to the International Neuroblastoma Risk Group (INRG) classification system (Table 1) (Cohn, et al 2009), and the survival in the high risk group in Sweden is 54.5% (Träger, et al 2009). The choice of treatment for patients with neuroblastoma differs widely depending on what risk group the individual patient belongs to. High risk neuroblastomas require aggressive and multi-modal treatment consisting of induction chemotherapy, surgery, myoablative chemotherapy, stem cell rescue, and irradiation as well as novel therapies such as retinoids and immune therapy. The chemotherapeutic drugs of choice are vincristin, cisplatin, etoposide, and doxorubicin. Despite this harsh therapy, many patients are non-responsive and cannot be cured. Less intensive therapy is required in the lower risk groups, and in the special case of stage 4S, no treatment is needed since these tumors often regress spontaneously. The overall survival of neuroblastoma patients is about 70%; thus 30% of the children do not survive, making neuroblastoma the most deadly solid extracranial tumor of childhood. Therefore, the importance of finding novel treatment strategies is evident (Johnsen, et al 2009).

Figure 1. Neuroblastoma primary tumors derived from the neural crest arise in the sympathetic nervous system including the adrenal medulla, sympathetic ganglia and paraganglia. Neuroblastomas mainly metastasize to lymph nodes, bone and bone marrow, and in infants there is often also spread to liver and subcutaneous tissue.

Table 1. The INRG staging system for neuroblastoma.

<table>
<thead>
<tr>
<th>INRG Stage</th>
<th>Age (months)</th>
<th>Histologic Category</th>
<th>Grade of Tumor Differentiation</th>
<th>MYCN</th>
<th>11q Aberration</th>
<th>Ploidy</th>
<th>Pretreatment Risk Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1/L2</td>
<td></td>
<td>GN maturing; GNB intermixed</td>
<td>NA</td>
<td>NA</td>
<td>Salmonoid</td>
<td>K</td>
<td>Very low</td>
</tr>
<tr>
<td>L1</td>
<td></td>
<td>Any, except GN maturing or GNB intermixed</td>
<td>NA</td>
<td>NA</td>
<td>Salmonoid</td>
<td>K</td>
<td>Very low</td>
</tr>
<tr>
<td>L2</td>
<td>&lt; 18</td>
<td>Any, except GN maturing or GNB intermixed</td>
<td>NA</td>
<td>No</td>
<td>No</td>
<td>E</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>≥ 18</td>
<td>GNB nodular; neuroblastoma</td>
<td>NA</td>
<td>Yes</td>
<td>Yes</td>
<td>H</td>
<td>Intermediate</td>
</tr>
<tr>
<td>M</td>
<td>&lt; 18</td>
<td>NA</td>
<td>Hyperdiploid</td>
<td>F</td>
<td>Low</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt; 12</td>
<td>NA</td>
<td>Diploid</td>
<td>I</td>
<td>Intermediate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 to &lt; 18</td>
<td>NA</td>
<td>Diploid</td>
<td>J</td>
<td>Intermediate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt; 18</td>
<td>Amp</td>
<td>Diploid</td>
<td>O</td>
<td>High</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 18</td>
<td>Amp</td>
<td>Diploid</td>
<td>P</td>
<td>High</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>&lt; 18</td>
<td>NA</td>
<td>Yes</td>
<td>Q</td>
<td>High</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amp</td>
<td>Yes</td>
<td>R</td>
<td>High</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


1.2 EICOSANOIDS AND DOCOSANOIDS

“Eicosa” and “docosa” are Greek words meaning twenty and twenty-two, respectively. Eicosanoids and docosanoids are small and short-lived hormone-like molecules formed from fatty acids that regulate numerous processes in the body. They consist of 20 or 22 carbon backbones with various side chains and differently positioned double bonds. The main groups of eicosanoids are prostaglandins (PGs), tromboxanes (TXs), and lipoxins, and the main groups of docosanoids are resolvins and protectins.

1.2.1 Precursors and production

The precursors of all eicosanoids and docosanoids are these polyunsaturated fatty acids (PUFAs): Arachidonic acid (20:4, n-6, AA), Eicosapentaenoic acid (20:5, n-3, EPA), and Docosahexaenoic acid (22:6, n-3, DHA). These fatty acids are in turn the result of desaturation of LA and LNA by Δ5- and Δ6-desaturases that remove two hydrogen atoms, and of elongation by elongases that add two carbon atoms (Fig. 2). The omega-6 and the omega-3 fatty acids compete for the same desaturases and elongases, but the omega-3 family members are the preferred
substrates (Biondo, et al 2008). However, since the conversion from LA and LNA is low, PUFAs are best obtained from the diet (Burdge and Calder 2005). Once consumed, they are incorporated into phospholipids of cell membranes and the distribution is tissue-dependent. For example, the brain, retina, and testes are especially enriched in DHA, while most other tissues have a surplus of AA (Piomelli, et al 2007). When needed, these fatty acids are released from the cell membrane by the enzyme phospholipase A2 (PLA2). The PLA2-activity is tightly regulated by Ca²⁺ and phosphorylation (Kudo and Murakami 2002), and is increased in response to factors such as inflammatory stimuli (Yedgar, et al 2006). The free fatty acids are then available for conversion to a panel of different lipid mediators by the enzymes cyclooxygenase (COX), lipoxygenase (LOX), and cytochrom P450 monooxygenase.

**Figure 2.** Elongation and desaturation of omega–6 and omega–3 PUFAs.
1.2.1.1 Enzymatic activity

The enzymatic conversion of fatty acids mediated by COX actually consists of two sequential activities that are interdependent but located on opposite sides of the protein: cyclooxygenase and peroxidase. The cyclooxygenase active site abstracts a hydrogen molecule from arachidonate, and then two molecules of oxygen are stereospecifically inserted, forming an endoperoxide/peroxyl radical that is ultimately reduced to the hydroperoxide PGG2. The PGG2 is then reduced by the heme peroxidase forming PGH2, which is released and serves as a precursor for the synthesis of downstream eicosanoids, including prostacyclin (PGI2) and TXs. The LOX reaction is initiated by activation of the resting enzyme (by a small amount of hydroperoxide) by oxidation of the ferrous heme to the active ferric state, which may catalyze hydrogen abstraction from arachidonate. Cytochrome P450 is an enzyme that catalyzes a monooxygenase reaction that transfers one oxygen molecule via a direct hydroxylation mechanism. Cytochrome P450 can act on PUFAs via several different mechanisms of oxygenation, including bisallylic hydroxylation, epoxidation, and hydroxylation of the ω-side chain (Fig. 3) (O'Donnell, et al 2009).

Figure 3. The three families of eicosanoid-generating enzymes. Lipoxygenases (LOX) generate hydro(per)oxyeicosatetraenoic acids (HpETEs, HETEs), hydro(per)oxyoctadecadienoic acids (HpODEs, HODEs, from linoleate), as well as leukotrienes (LTs), lipoxins, and hepoxilins. Cylooxygenases (COX) generate prostaglandin H2 (PGH2), the precursor for all prostaglandins (PGs) and thromboxane (TX). Cytochrome P450 isoforms generate epoxy fatty acids (EETs) and 20-HETE and metabolize PGH2 to form prostacyclin (PGI2) and TX. O'Donnell et al, 2009. Reprinted, with permission, from Lipid Signaling Protocols, DOI: 10.1007/978-1-60327-115-8_1. Springer.
1.2.1.2 Arachidonic acid and its downstream metabolites

AA is converted by COX to PGs of the 2-series and to TXs (collectively termed prostanoids), which all contain a five-membered cyclopentane ring. It can also be converted by LOX to leukotrienes (LTs) of the 4-series, lipoxins, hydroperoxy eicosatetraenoic acids (HpETEs), hydroxyl eicosatetraenoic acids (HETEs), and hepoxilins. These compounds differ structurally and are generally straight chain oxidized lipids. Furthermore, AA is converted by cytochrome 450 or cytochrome 450-induced radical oxygen species (ROS) to epoxygenase products (EETs), AA ω/ω-1 hydroxylase products (HETEs), LOX-like products (HETEs), and primary free radical oxidation products (HpETEs) (Fig. 3) (Biondo, et al 2008, Ellis, et al 2007, Gonzalez-Periz and Claria 2007, O'Donnell, et al 2009).

1.2.1.3 Docosahexaenoic acid, eicosapentaenoic acid, and their downstream metabolites

EPA is converted by COX to PGs of the 3-series, and by LOX to LTs of the 5-series, and to lipoxins. LOX can also convert both EPA and DHA to resolvins of the E- and D-series, respectively. Furthermore, LOX can convert DHA to protectins (more detailed discussion below).

1.2.2 Eicosanoid signaling and biological effects

Eicosanoids signal predominantly via activation of 7-transmembrane domain G protein-coupled receptors. Receptors for eicosanoids include EP (PGE2), IP (prostacyclin), TP (TXs), CysLT1/T2 (LTs), and ALX (lipoxins). Some eicosanoids can mediate signaling by nuclear receptors or transcription factors; for example, the peroxisomal proliferator activation receptor family (PPARs) is activated or inhibited by HETEs, either directly through ligand binding or by activation of MAP kinase-dependent PPAR phosphorylation (Yu, et al 1995). HETEs from 12- or 15-LOX can stimulate numerous signaling pathways in several cell types, including mesangial, smooth muscle, endothelial, and adrenal glomerulosa cells. Pro-inflammatory pathways that are activated by 12(S)-HETE include MAP kinases, Ras translocation, CREB, and protein kinase C. Finally, EETs and 20-HETE can modulate the activity of potassium channels in the vascular smooth muscle, leading to the regulation of vessel tone (O'Donnell, et al 2009).

Generally, eicosanoids that are formed from the omega-6 fatty acid AA are pro-inflammatory, while eicosanoids and docosanoids formed from the omega-3 fatty acids DHA and EPA are anti-inflammatory. The reality is not as simple as stated here, however; the anti-inflammatory lipoxins that are formed from AA are a good example of an exception to this rule. The role of eicosanoids as inducers and regulators of inflammatory response has been the most studied (Khanapure, et al 2007). Recently, the docosanoids have been studied more extensively, and it has become evident that resolution of inflammation, a process previously thought to occur passively, is governed by the production of docosanoids - that is, the resolvins (Farooqui, et al 2007).
Another important effect of the eicosanoids, especially the PGs, is protection of the gastrointestinal mucosa by stimulatory action on gastric mucus and bicarbonate secretions (Brzozowski, et al 2005). This is the mechanism behind the gastrointestinal adverse side effects such as gastric ulcers seen in patients on long-term aspirin treatment (aspirin is a COX-inhibitor, as discussed later.) PGs also exert smooth muscle cell contraction in the placenta during labor in childbirth and are used therapeutically to induce labor.

1.2.3 Eicosanoids and docosanoids in cancer

Many studies have shown that certain eicosanoids can accelerate cancer growth, and that enzymes responsible for conversion of fatty acids to eicosanoids are upregulated in malignant tissue compared to normal (Krishnamoorthy and Honn 2008). This is especially true for AA and the COX enzyme, which are both found in abundance in most cancer tissues examined (Koki and Masferrer 2002). In addition, receptors for PGE\textsubscript{2} and LTB\textsubscript{4} are often upregulated (Hull, et al 2004, Massoumi and Sjolander 2007). However, not only is AA content higher in cancer tissue (Dong, et al 2006), but the DHA and EPA content is also usually lower (Kokoglu, et al 1998, Martin, et al 1996, Reynolds, et al 2001). Therefore, the accelerated cancer growth could be an effect of both increased omega-6 content and decreased omega-3 content. This ratio of omega-6 to omega-3 is often discussed and believed to be more important than the absolute levels of a particular fatty acid. In western society, this ratio is about 10-20:1, while in other cultures, and also historically, this ratio has been 1-2:1 (Simopoulos 2006). It has been postulated that this might partly explain why the incidence of the endemic diseases of our time, such as coronary heart disease, inflammatory diseases, metabolic diseases, and cancers, are increasing in the western world.

Since these fatty acids are essential, and the only factor with substantial impact on this ratio is which foods are eaten, changed dietary habits must be responsible for this alteration in the omega-6/omega-3 balance. In the Japanese population, whose traditional diet includes much fish, the incidence of certain cancers such as breast cancer has increased along with a more “westernized” lifestyle and food consumption (Marugame, et al 2006). Since this observation was made, several studies have pointed out that omega-3 fatty acid consumption is associated with decreased cancer risk of the breast, prostate, and colon (Courtney, et al 2007, Fradet, et al 2009b, Smith-Warner, et al 2006, Thiebaut, et al 2009). Whether this effect is mediated by the actual DHA or by the DHA-derived docosanoids has not yet been clarified.

1.3 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS)

Non-steroidal anti-inflammatory drugs, NSAIDs, comprise a chemically diverse group of compounds, including salicylates, propionic acids, pyrazoles, acetic acids, oxicams, fenamates, and naphthyl-alkanones (Reuben 2007). Since the isolation of salicylic acid from willow bark in 1829, NSAIDs have become an important part of
the pharmaceutical treatment of pain and inflammation. The term "non-steroidal"
is used to distinguish these drugs from steroids, which have a similar eicosanoid-depressing anti-inflammatory action. All NSAIDs reduce the heat, redness, swelling, and pain of inflammation by inhibiting both isoforms of the COX enzyme, thereby blocking the production of PGs, the major agents of inflammation. As the use of NSAIDs increased, adverse side effects, mainly gastrointestinal problems, became evident (Harris and Breyer 2006, Ong, et al 2007). These effects occur partly because of decreased levels of protective PGs in the gut mucosa. These protective PGs stimulate gastric mucus and bicarbonate secretions, increase the gastric microcirculation, and enhance formation of mucosal sulfhydryl compounds (Brzozowski, et al 2005).

The premise that COX-1 performs cellular "housekeeping" functions for normal physiological activity and is the predominant isoform expressed in platelets and the GI tract, whereas COX-2 acts at inflammatory sites, led to the development of COX-2 selective inhibitors, the coxibs. However, some adverse effects from the presumed COX-1 inhibition remained with the coxibs, and in addition, cardiovascular events were observed which led to the withdrawal of rofecoxib (Vioxx) from the market in 2004. However, celecoxib is still on the market as Celebra or Celebrex (Half and Arber 2009).

1.3.1 Celecoxib – the facts

Celecoxib was the first COX-2 specific inhibitor (coxib) approved by the United States Food and Drug Administration (FDA) in December 1998 (Reuben 2007). Celecoxib is a sulfonamide, extensively distributed into tissues and metabolized by the cytochrome P450 2C9 isoenzyme with a half-life of 11 hours (Pitt, et al 2002). Celecoxib is nine times more efficient in inhibiting COX-2 than COX-1 (compared to 80 times more for Rofecoxib) (Ong, et al 2007). One of the keys to developing COX-2 selective drugs is the larger active site of COX-2, which makes it possible to engineer molecules too large to fit into the COX-1 active site but still able to fit the COX-2. The larger active site of COX-2 is partly due to a polar hydrophilic side pocket that forms because of substitution of Ile523, His513, and Ile434 in COX-1 by Val523, Arg513, and Val434 in COX-2.

The bulky sulfonamide group in celecoxib prevents the molecule from entering the COX-1 channel. Within the hydrophilic side pocket of COX-2, the oxygen of the sulfonamide group interacts with Hist90, Arg513, and Gln192 and forms hydrogen bonds (Michaux and Charlier 2004). The mechanisms triggering cardiovascular toxicity of COX-2 inhibitors are not entirely understood. The prevailing hypothesis involves a disruption in the balance of prostacyclin and TXA2. TXA2 causes platelet aggregation, vasoconstriction, and smooth muscle proliferation, while prostacyclin is a vasodilator that inhibits platelet aggregation and vascular proliferation. Platelets, which express only COX-1, are the primary producers of TXA2. Prostacyclin is manufactured primarily in endothelial cells in response to COX-2. Non-aspirin, non-selective NSAIDs reversibly inhibit COX-1 and COX-2, thereby maintaining relative homeostasis of prostacyclin and TXA2. Selective COX-2 inhibitors, however, predominantly inhibit platelet prostacyclin production,
shifting the balance in favor of TXs and therefore thrombosis (Ardoin and Sundy 2006).

1.3.2 Areas of application

Celecoxib is licensed for use in osteoarthritis, rheumatoid arthritis, acute pain, painful menstruation and menstrual symptoms, and to reduce the number of colon and rectal polyps in patients with familial adenomatous polyposis. (As reported in FASS 2008 and by FDA at www.fda.gov).

1.3.3 Celecoxib in cancer

COX-2 is upregulated in a variety of pre-malignant lesions such as oral leucoplakia, actinic keratosis, prostatic intraepithelias neoplasia, and carcinoma in situ of the bladder and breast. It is also upregulated in several malignant tissues and invasive tumors. Usually the level of expression correlates to invasiveness and metastatic spread (Koki and Masferrer 2002). This has led to studies hypothesizing that COX-2 and the increased levels of eicosanoids that are produced enhance tumor growth, and that inhibition of this pathway could be a treatment strategy in malignancy. This hypothesis proved correct, and now literally thousands of experimental and clinical studies have suggested a role for COX-2 in neoplasia, including hyperproliferation, transformation, tumor growth, invasion, and metastasis, and show that blocking COX-2 can prevent or delay these events (Koki and Masferrer 2002). Indeed, coxibs have been shown to inhibit tumor growth through many mechanisms that will be discussed below, and they can also potentiate the activity of cytotoxic drugs, hormones, and radiotherapy (Gasparini, et al 2003).

A prospective randomized trial in patients with familial adenomatous polyposis showed that celecoxib suppresses formation of polyps and causes existing polyps to regress (Steinbach, et al 2000). As a result of this study, celecoxib obtained approval from the US Food and Drug Administration as preventive therapy in these patients. Clinical testing of celecoxib in other cancers is ongoing (as reported by NIH at www.clinicaltrials.gov).

The mechanisms proposed for the celecoxib-induced tumor growth inhibitory effects are many. First, celecoxib is antiangiogenic by induction of proangiogenic factors such as vascular endothelial growth factor (VEGF), inducible nitric oxide synthase, interleukins 6 and 8, and TIE2 (angiopoietins 1-2 receptor) (Dannenberg, et al 2001, Gately and Li 2004, Masferrer, et al 2000). Second, celecoxib induces apoptosis by increasing concentrations of unesterified AA. This fatty acid modulates mitochondrial permeability and release of cytochrome c (Chan 2002, Johnsen, et al 2004). Unesterified AA also causes cell cycle arrest by decreasing expression of cyclins A and B1 and through expression of the cell cycle inhibitory proteins p21WAF1 and p27KIP1 (Grosch, et al 2001). Third, celecoxib inhibits invasiveness, metastatic spread, and adhesion by reducing expression of glycosyltransferases, type I sialyl Lewis antigens, MMP1-2, and CD44 (Kakiuchi, et
al 2002, Tsujii, et al 1997). Last, celecoxib lowers the estrogen levels through reduced PGE₂-induced aromatase transcription (Brueggemeier, et al 1999). Hence, there are both COX-dependent and -independent mechanisms behind celecoxib-induced tumoricidal effects.

1.4 OMEGA-3 FATTY ACIDS

The one essential omega-3 fatty acid is LNA, from which both EPA and DHA are formed. EPA and DHA contain different numbers of double bonds, but the first double bond from the methyl end of the carbon chain is always situated between carbons three and four. LNA can primarily be found in leafy green vegetables, walnuts, and canola oil. By the enzymes Δ-6-desaturase, Δ-5-desaturase and elongase, LNA is converted to EPA and DHA. However, this conversion only takes place to a limited extent, and occurs more in women than in men (Burdge and Calder 2005). The primary source of EPA and DHA for humans is fatty fish such as salmon, herring, and mackerel. These fish are rich in omega-3 fatty acids because they consume photosynthetic and heterotrophic microalgae of the genus Schizochytrium that produce EPA and DHA, which become increasingly concentrated in organisms as they move up the food chain (Hammond, et al 2001).

1.4.1 Docosahexaenoic acid – the facts

DHA (\textit{all-cis-docosa}-4,7,10,13,16,19-hexaenoic acid) has a chain length of 22 carbons that contains six double bonds, which makes it the longest chain and most unsaturated fatty acid commonly found in biological systems (Fig. 4). In the human body, it is either aquired from the diet or it is derived from EPA via docosapentaenoic acid (DPA) as an intermediate. This has been thought to occur through an elongation step followed by the action of Δ-4-desaturase. It is now considered more likely that DHA is biosynthesized via a 24-carbon chain intermediate, followed by β-oxidation. Thus, EPA is twice elongated yielding 24:5 n-3, then desaturated to 24:6 n-3, then shortened to DHA. This pathway is known as Sprecher’s shunt (Voss, et al 1991). In humans, DHA is especially enriched in neural tissue. It comprises 40% of the PUFAs in the brain, 60% of the PUFA in the retina, and 50% of the weight of the neurons’ plasma membrane (Singh 2005). It is esterified into phospholipids embedded in cell membranes, especially of phosphatidylethanolamine (PE) and phosphatidylserine (PS), preferably in sn-2 position (Piomelli, et al 2007).

In membranes containing DHA, the packing is distorted by steric restrictions associated with the presence of multiple rigid double bonds; that is, the bent shape of this fatty acid prevents a perfect fit in the membrane. This is thought to significantly alter many basic membrane properties including acyl chain order and fluidity, phase behavior, elastic compressibility, permeability, fusion, flip-flop, and protein activity (Stillwell and Wassall 2003).
Once taken up by cells or released from the cell membrane, DHA acts as a ligand to certain nuclear receptors, such as the PPAR-γ (Gani and Sylte 2008, Zapata-Gonzalez, et al 2008) and the RXR receptor (de Urquiza, et al 2000, Lengqvist, et al 2004). In conjunction with these receptors that act as transcription factors, DHA helps regulate various biological functions ranging from lipid metabolism and homeostasis to cell differentiation and cell death (Berquin, et al 2008, Sun, et al 2008, Zand, et al 2007). Many of the receptor-mediated effects of DHA are still unexplored.

In addition, DHA has been shown to influence signal transduction of a variety of pathways. For example, DHA activates the Jak/Stat pathway; downregulates protein kinase C, Ras, ERK and NF-κB; sustains phosphorylation of EGFR; and influences the Bcl-2 family of proteins regulating cell growth (Berquin, et al 2008). Furthermore, DHA can modulate the translation machinery by reducing intracellular Ca²⁺ stores (Jude, et al 2006).

Figure 4. all-cis-docosa-4,7,10,13,16,19-hexaenoic acid, DHA, 22:6 n-3. 
Provided by Dr Karl Harrison, 3dChem.

1.4.2 Areas of application

In 1970 the pioneers of omega-3 fatty acid research, Dr. Dyerberg and Dr. Bang from Denmark, visited Greenland on an expedition to understand how the Inuits could eat a high-fat diet and still have one of the lowest death rates from cardiovascular disease in the world (Dyerberg and Bang 1979). Their discovery that the Inuits had favorable blood lipids resulted in a publication in *Lancet* in 1971 (Bang, et al 1971). Not until some years later had Dr. Dyerberg and Dr. Bang analyzed all blood samples on an old gas chromatogram and found two fatty acids, DHA and EPA. This was the birth of omega-3 fatty acid research. Since then, mainly through dietary studies, DHA has been associated in beneficial ways with an enormous range of human afflictions including cancer, heart disease, rheumatoid arthritis, asthma, lupus, alcoholism, visual acuity, kidney disease, respiratory disease, peroxisomal disorders (Zellweger’s Syndrome), dermatitis, psoriasis, cystic fibrosis, ...
schizophrenia, depression, neurologic and brain development, malaria, multiple sclerosis, and even migraine headaches. In fact, it is difficult to find any human disorder where omega-3 fatty acids have not been tested (Riediger, et al 2009, Stillwell and Wassall 2003).

The common denominator that might explain the beneficial effects of omega-3s and DHA in particular in this great variety of diseases and symptoms is its anti-inflammatory properties. Until recently, it was unknown how DHA exerted these anti-inflammatory effects. One postulated reason is that DHA replaces AA in cellular membranes, and hence less AA is available for conversion by COX and LOX to pro-inflammatory eicosanoids. Furthermore, DHA competes with AA for binding sites on the COX enzyme, and is actually the preferred substrate (Tapiero, et al 2002). These indirect mechanisms for inhibiting inflammatory responses seem reasonable and have proven to be correct, but a huge step was taken towards understanding DHA's beneficial effects when resolvins and protectins were identified in 2002 (Serhan, et al 2002).

Resolvins of the D-series are produced from DHA by the enzymes 5-LOX and 15-LOX, or by a COX-enzyme that has been acetylated by aspirin (Serhan, et al 2004). Protectins are also formed via LOX-mediated pathways (Serhan 2007) (Fig. 5). These lipid mediators, and also resolvins of the E-series (EPA-derived), powerfully clear inflammation by clearing neutrophils and macrophages from inflammatory sites. Actually, they are essential for resolution of an inflammatory response, a process that was formerly believed to occur passively (Serhan, et al 2008). These newly discovered substances are currently in clinical testing (as reported by Resolvyx at www.resolvyx.com). However, many of the mechanisms behind the positive effects observed by DHA and EPA are still elusive.

1.4.3 DHA in cancer
1.4.3.1 Prevention studies

**Figure 5.** The DHA metabolome.

Reprinted, with permission, from the Annual Review of Immunology, Volume 25 © 2007 by Annual Reviews.

A
A
R
R

A
A
Reprinted, with permission, from the Annual Review of Immunology, Volume 25 © 2007 by Annual Reviews.
Mechanisms underlying the tumor preventive characteristics of DHA are not clearly understood. Some of the first steps required for tumor establishment are adhesion and angiogenesis. DHA has been shown to inhibit adhesion (Victory, et al 2007), probably by down-regulating Rho GTPase, inhibiting cytoskeleton reorganization (Yi, et al 2007), and reducing ICAM-1 and VCAM-1 protein expression (Goua, et al 2008). DHA also decreases TNF{\textalpha}-induced monocyte rolling, adhesion, and transmigration (Schaefer, et al 2008). These effects might also be applicable to tumor cells in the process of adhering to tissue sites. DHA has also been shown to reduce angiogenesis (Rose and Connolly 1999), probably by decreasing VEGF levels (Victory, et al 2007).

An observational study that supports this theory of cancer prevention by omega-3 fatty acids was carried out among the native Inuit population of Alaska (Lanier, et al 2003). In this study, childhood cancer incidence was analyzed from 1969 to 1996, and apart from the increased number of hepatocellular carcinomas in this population due to Hepatitis B infection (a phenomenon that disappeared after initiation of a vaccination program), the rate of childhood cancer was significantly lower compared to a North American population. Specifically, the incidence of neuroblastoma was reduced tenfold (0.8/million vs 8/million). The Inuit population of Alaska pursue a lifestyle where fish and seal meat are the main commodities, and their DHA levels are several-fold higher than in Caucasians (Dewailly, et al 2001, Lucas, et al 2004), where the omega-3/omega-6 ratio has dropped dramatically over the past decades (Simopoulos 2002). Other clinical studies on adult cancer have shown that omega-3 is strongly associated with a decreased risk of aggressive prostate cancer (Fradet, et al 2009) and renal carcinoma (Wolk, et al 2006).

1.4.3.2 Therapeutic studies

In another study, DHA was given as single therapy to nude mice xenografted with BxPC-3 pancreatic cancer cells. DHA was also combined with curcumin, a dual COX-2 and 5-LOX inhibitor. Tumor growth was inhibited by DHA as single therapy, and the inhibitory effect increased with the combination of DHA and curcumin. To my knowledge, this is the only study that has been published investigating the effects of DHA in established xenografts (Swamy, et al 2008).

The suggested mechanisms behind the therapeutic effects of DHA in cancer are suppression of biosynthesis of proinflammatory molecules due to replacement of AA in cell membranes, influence on transcription factor-activated gene expression via receptor-ligand binding, influence on signal transduction via protein interaction, mechanisms that alter hormone-stimulated growth via lipid homeostasis, and mechanisms that involve the production of free radicals and ROS due to lipid peroxidation (Berquin, et al 2008, Larsson, et al 2004, Pauwels and Kairemo 2008). In addition, DHA might provide a protective function in cells with a high concentration of DHA embedded in their membranes, whereas cells with a low concentration of DHA are susceptible to DHA-mediated cytotoxicity (Siddiqui, et al 2008).

One therapeutic study in breast cancer patients where DHA was combined with the chemotherapeutic drugs epirubicine, cyclophosphamide, and 5-fluourouracil emphasizes that an inter-individual uptake and incorporation of DHA alters the
treatment response. Patients were supplemented with DHA daily during the chemotherapy cycles and could then be divided into high and low incorporating groups based on the DHA levels in plasma and red blood cells. The high incorporating group was characterized by longer overall survival and delayed time to tumor progression compared to the low incorporating group (Bougnoux, et al 2006). There are currently several ongoing clinical trials where DHA or omega-3s are being tested for cancer prevention, support, or therapy (Berquin, et al 2008).

DHA as a treatment strategy is often combined with chemotherapeutic drugs since DHA most likely enhances cytotoxic effects of these drugs (Biondo, et al 2008) (Paper I).

1.5 CAN CELECOXIB AND DHA BE GIVEN TO CHILDREN?

A pharmacokinetic study on celecoxib in children shows that the levels used in vitro are achievable in plasma, and that a dose of 250 mg/m² is tolerated well (Stempak, et al 2002). The information on pharmacokinetics of DHA in children is limited. However, DHA supplementation to children has been done in different studies with doses up to several grams per day, resulting in increased plasma levels and without adverse side effects (Germano, et al 2007, Koletzko, et al 2009, Voigt, et al 2001). Both celecoxib and DHA supplementation effectively alters plasma lipid composition in children, an important prerequisite for any attempt to validate these experimental findings in children with neuroblastoma.
2 AIMS

PUFAs and their downstream products can influence cancer growth. Neuroblastoma cells are deficient in DHA while eicosanoid-producing enzymes are upregulated. Thus, the purpose of this project was to investigate the effect of modulating the omega-6 pathway by blocking the COX-2 enzyme, and modulating the omega-3 pathway by adding exogenous DHA to neuroblastoma cells.

The specific aims of the project were to:

✓ Investigate the effect of celecoxib, a COX-2 specific inhibitor, on neuroblastoma development in vivo, as an adjuvant to current treatment protocols.

✓ Investigate whether DHA could inhibit neuroblastoma growth in vitro, and study the mechanisms behind the DHA-induced cell death.

✓ Investigate whether DHA could enhance the cytotoxicity of other cytostatic and cytotoxic drugs, such as chemotherapeutic agents, COX-inhibitors, and arsenic trioxide (As2O3).

✓ Investigate the fate of DHA when added to neuroblastoma cells to elucidate which compound in the metabolic pathway exerts the cytotoxic effects.

✓ Investigate whether DHA could prevent tumor development and inhibit tumor growth in vivo.

The studies were performed on neuroblastoma cell lines, in neuroblastoma xenografts, and in transgenic mice spontaneously developing neuroblastoma.
3 METHODS

The methods used in this thesis are here described briefly. For more information, please see the individual papers.

3.1 IN VITRO

3.1.1 Human tumor cell lines

The tumor cell lines used were established from high-risk patients. For a detailed description of the characteristics of the cell lines, see table 2.

The following panel of cell lines was used: SK-N-BE(2), SH-SY5Y, SK-N-AS, SK-N-DZ, IMR-32, and SK-N-SH. For control purposes the MRC-5 lung fibroblast cell line was used. Cells were grown in RPMI 1640 [SK-N-BE(2)], or in Eagle’s MEM (all others) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 IU/mL penicillin G, and 100 µmol/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Cells were split twice weekly, and harvested in log-phase for experimental use.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MYCN amp</th>
<th>MDR phen</th>
<th>p53 mut</th>
<th>1p del</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-BE(2)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>No</td>
<td>No/Low</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>SK-N-DZ</td>
<td>Yes</td>
<td>Moderate</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>IMR-32</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Amp: amplification; MDR: multidrug resistance; phen: phenotype; mut: mutation; del: deletion.

3.1.2 Cell viability assays

3.1.2.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay

Cells were seeded in 96-well plates and allowed to attach overnight. The following day, the medium was changed to a fresh medium containing selected compounds and drug combinations as indicated for each experiment. Plates were then incubated for 24-96 hours. At final time points, the medium was replaced by serum-free medium containing 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT). Cells were incubated for three hours, and acidified isopropanol was then added to dissolve crystals. After the cells were shaken overnight
at 4°C, absorbance was measured using a microplate reader at 570 nm. The survival ratios between the treated cells compared to the controls were calculated.

3.1.2.2 Clonogenic assay

Cells were seeded in petri dishes and allowed to attach for at least six hours before start of treatment. The medium was changed to a fresh medium containing the compound or drug of interest and incubated at 37°C. Treatment was discontinued after four to seven days by adding a fresh drug-free medium to the dishes, which were then allowed to incubate for another 7-14 days with one additional medium change at day 14. At the end of the experiment, plates were rinsed with phosphate buffered saline (PBS) and fixed in formaldehyde, and colonies were stained with Giemsa. Colonies containing at least 50 cells were counted manually under a light microscope. For each treatment, the surviving fraction was calculated as the ratio of the mean plating efficiency (PE) of treated cells over the PE of untreated control cells.

3.1.2.3 Trypan blue exclusion

To monitor long-term cell growth, cells were seeded in 175 cm² or 80 cm² flasks and treated with indicated drugs for up to 12 days. The medium was replaced with a fresh medium and drug as often as needed for the drug of use. At final time points, cells were harvested and pooled with detached cells recovered from the medium. Cell number and viability were examined by trypan blue dye exclusion, using light microscopy.

3.1.2.4 Fluorometric microculture cytotoxicity assay (FMCA)

384-well microtiter plates were prepared with drug solutions in duplicate at ten times the desired final drug concentration. The plates were stored at −70°C until use and protected from light during all experimental steps. Cell suspensions were seeded into the drug-prepared microtiter plates at a cell density of 5,000 cells per well and incubated for 72 hours at 37°C. After incubation, the fluorometric microculture cytotoxicity assay was done using the automated Optimized Robot for Chemical Analysis (Orca, Beckman Coulter, Fullerton, CA), programmed through the software SAMI (Beckman Coulter). The plates were washed; fluorescein diacetate (Sigma-Aldrich) was added; and after 50 min of incubation, fluorescence generated was measured at 485/520 nm in a fluorometer in Fluoroscan II (Labsystems Oy, Vantaa, Finland). The fluorescence is proportional to the number of intact cells in the well. A successful assay required a ratio of >5 between the signal in the control wells and the blank wells and a coefficient of variation of <30% in the control wells.

3.1.3 Fluorescence-activated cell sorting analysis

3.1.3.1 Measurement of the mitochondrial membrane potential

Cells were seeded in six-well plates and treated with selected compound for 24-72 hours. The medium was then changed to a medium containing
tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, OR) and cells were incubated for ten minutes. Cells were then rinsed with PBS, harvested, and analyzed directly for mitochondrial transmembrane potential using TMRE. Analysis was done on the FL2 channel on a FACSCalibur flow cytometer, using Cell Quest Pro software (Becton Dickenson, CA).

### 3.1.3.2 Measurement of cell cycle distribution by propidium iodide staining

Cells were seeded in six-well plates and treated with compound as indicated. Cell cycle distribution was evaluated by propidium iodide-staining of cells recovered from the wells by trypsinization and pooled with nonadherent cells recovered from the culture medium. Fluorescence resulting from excitation at 488 nm with a 15-mW argon laser was monitored at 585 ± 21 nm using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

### 3.1.3.3 Measurement of cell cycle distribution by DAPI staining

After treatment in six-well plates, the cells were harvested and washed in PBS, then fixed in 4% phosphate-buffered formaldehyde (Karolinska Pharmacy) at room temperature overnight, pelleted, and suspended in 95% ethanol. Prior to FACS analysis, cells were rehydrated in distilled water for 1 hr, treated with subtilisin Carlsberg solution (Sigma-Aldrich), and stained with DAPI-sulforhodamine solution. Samples were analyzed using a PAS II flow cytometer (Partec, Münster, Germany). DAPI fluorescence was measured above 435 nm. The multicycle program for cell cycle analysis (Phoenix Flow Systems, San Diego, CA) was used for histogram analysis.

### 3.1.3.4 Measurement of apoptosis by annexin V staining

Cells were grown in six-well plates and treated with selected drug for 24-72 hours. Cells were harvested, washed in PBS, and stained with annexin V according to the manufacturer’s protocol (Annexin V Apoptosis detection kit, Sigma-Aldrich, Solna, Sweden). FACS analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) in the F1 channel with CellQuest software (Phoenix Flow Systems, CA).

### 3.1.4 Fluorometric measurement of radical oxygen species

Intracellular reactive oxygen species (ROS) were detected using an oxidative-sensitive fluorescent probe, DCFH-DA (Molecular Probes). Cells were seeded into 96-well plates and incubated for 6-24 hours with indicated compound. After incubation, medium was replaced by PBS containing 10 μM DCFH-DA and cells were incubated at 37°C for 60 min. Intracellular ROS generation was measured using a microplate fluorescence reader at 485 nm excitation and 530 nm emission wavelength.
3.1.5 Immunoblotting

Treated cells were harvested and pelleted and proteins were extracted in RIPA buffer (Cell Signaling, Beverly, MA) containing a protease inhibitor (Roche Diagnostics, Stockholm, Sweden). Protein concentrations were measured using Bradford reagent (BioRad, Sundbyberg, Sweden). Equal quantities (50-150 µg) were separated by SDS-PAGE, transferred to nylon membranes (Millipore Inc, Solna, Sweden), and probed with a primary antibody diluted in 1% BSA or 5% dry milk in TBS-T. The secondary antibody (conjugated with horseradish peroxidase, Cell Signaling) was diluted in 5% dry milk in TBS-T, and Pierce Super Signal (Pierce, Rockford, IL) was used for chemiluminescent detection.

3.1.6 Immunohistochemistry

Proliferation was evaluated in 5-µm deparaffinized tumor sections by staining for Ki-67. The specific Ki-67 antibody was incubated overnight at 4°C, before a SuperPicture polymer detection kit with appropriate secondary antibodies was used together with a diaminobenzidine tetrahydrochloride substrate chromogen system to visualize immunopositivity. Proliferation was assessed by counting the number of Ki-67 positively stained nuclei and the total number of cells in four representative regions in three tumors at x400 magnification. The results are expressed as the proportion of positively stained cells.

Angiogenesis was evaluated in 5-µm deparaffinized tumor sections by staining for biotinylated Bandeirea simplicifolia-1 (BS-1) lectin to highlight endothelial cells. BS-1 was incubated overnight at 4°C. Detection was performed with ABComplex conjugated to horseradish peroxidase. Sections were developed using diaminobenzidine tetrahydrochloride, counterstained with Harris' hematoxylin, and mounted with Kaiser's glycerol gelatin. Cell pellets of bovine endothelial cells were used as a positive control and omission of BS-1 as a negative control. Three tumor slides per treatment group and four fields per slide were quantified for vessel density at x200 magnification. The results are expressed as average number of microvessels per field.

3.1.7 Lipid extraction

3.1.7.1 Extraction of plasma samples for detection of celecoxib

Samples were prepared by adding 25 µL of 0.1 M phosphate buffer solution (pH 2.4) and 50 µL of internal standard (valdecoxib, 4 µmol/L) to 50 µL of plasma in a 4-mL polypropylene tube (Sarstedt, Darmstadt, Germany). To the same tube, 1 mL of n-hexane/diethyl ether (1:1, v/v) was added as the extractant. The tubes were capped, mixed by vortexing for 1 min, and centrifuged at 3,000 x g for 150 s. The supernatant was transferred to a second set of clean tubes and evaporated to dryness under a stream of nitrogen at 40°C. The residue was then reconstituted in 100-µL mobile phase.
3.1.7.2 Extraction of lipids from cell cultures

Cells were harvested, washed in PBS, and counted using trypan blue exclusion. Ten to twenty million cells were suspended in 1 mL PBS for short incubations (30 min), or clear RPMI medium for longer incubations (4 hours). Cells were incubated with designated compounds at 37°C shielded from light. Reaction was stopped with two volumes of ice-cold methanol and samples were stored at -80°C for at least two hours to allow protein precipitation. Samples were then centrifuged at 2000 rpm for 10 min to remove any precipitation, after which internal standards 5(S)-HETE-d₈ (2 ng) or PGE₂-d₄ (2 ng) were added (Cayman Chemical, Ann Arbor, MI, USA). Samples were concentrated (Savant SpeedVac AES1010, Ramsey, MN, USA) and 4 mL of water and 200 µL methanol were added to the concentrate. The resulting clear supernatants were acidified with 2 M hydrochloric acid to pH 3.5 and immediately applied to C18 SPE cartridges that had been preconditioned with 20 mL methanol followed by 20 mL water (Waters Corp, Milford, MA, USA). The cartridges were washed with 4 mL water and 10 mL hexane in succession. Finally, the hydroxy fatty acids were eluted with 8 mL methyl formate. The extraction procedure was performed using a vacuum manifold (Waters Corp, Milford, MA, USA). The vacuum was adjusted so that individual drops could be seen from each cartridge. The organic solvent was evaporated under a fine stream of nitrogen, and the residue was dissolved in 100 mL methanol, flushed with nitrogen, and stored at -80°C awaiting LC-MS/MS analysis.

3.1.7.3 Extraction of lipids from blood plasma and tissues

To analyze lipids in plasma and tissues, we used a direct transesterification method described previously (Lepage and Roy 1986). Briefly, blood plasma (100 µL) or homogenized tumor tissue (50-100 mg) was added to 2 mL of a methanol/toluene solution (4:1) containing an internal standard (pentacosanoic acid methyl ester, 25 µg/mL) and the anti-oxidant BHT (10 µg/mL) in a glass tube. 200 µL acetyl chloride was then slowly added during magnetic stirring. Before the lid was closed tightly, nitrogen gas was blown into the tube for 30 seconds to remove remaining oxygen. The tubes were then incubated at 100°C for 60 minutes. After the tubes had cooled, 5 ml 6% K₂CO₃ was added slowly, followed by 1 mL heptane. The solution was mixed thoroughly and centrifuged 10 min at 3000 rpm. The methanol/toluene phase was then collected and analyzed for DHA content by GLC and GC-MS.

3.1.8 Liquid chromatography - tandem mass spectrometry (LC-MS/MS)

3.1.8.1 Analysis of celecoxib in plasma

The chromatographic system consisted of a Waters Acquity UPLC system with an autosampler and a binary solvent delivery system (Waters, Milford, MA). Liquid chromatography was done on a 50 x 2.1-mm Waters Acquity BEH C18 1.7-mm column. Mass spectrometry was performed using a Micromass Quattro Premier (Waters, Manchester, United Kingdom) tandem mass spectrometer. The system was controlled by MassLynx version 4.0. The isocratic mobile phase consisted of
80% methanol in 2 mM aqueous ammonium acetate with a flow rate of 0.2 mL/min. Ionization was achieved using electrospray in both positive and negative ionization modes. The injection volume was 2 µL and the injection interval was 1.2 min. LC-MS/MS was done with an autosampler temperature of 8°C, a desolvation gas temperature of 280°C, a source temperature of 120°C, a desolvation gas flow of 800 L/h, a cone gas flow of 40 L/h, a collision gas pressure of 3 x 10^-3 mbar (argon), and ion energies of 0.9 V for both quadrupoles. Quantitative analysis was done in the multiple reaction monitoring (MRM) mode with the following transitions: m/z 380→316, 380→296, and 382→362 for celecoxib, and m/z 313→210 and 313→118 for valdecoxib. The dwell time was set to 80 ms for each transition.

### 3.1.8.2 Analysis of DHA-derived lipid mediators in cell suspension

LC-MS/MS analysis was performed with an Agilent 1100 series HPLC paired with an ABI Sciex Instruments 3200 Qtrap linear ion trap quadrupole mass spectrometer equipped with an Agilent Eclipse Plus C18 column (4.6mm x 50mm x 1.8µm). Instrument control and data acquisition were performed using Analyst™ 1.4.2 software. The mobile phase consisted of methanol/water/acetic acid (60/40/0.01;v/v/v) and was ramped to 80/20/0.01 (v/v/v) after 5 minutes, 95/5/0.01 (v/v/v) after 8 minutes, and 100/0/0.01 after 14 minutes to wash the column. Ion pairs from previously reported MRM methods were used for profiling and quantitation of lipid mediators and internal standards. The criteria of matching retention time and at least six diagnostic ions to synthetic standards were used for positive identification of compounds of interest. Quantitation was performed using standard calibration curves for each compound and recovery was calculated using deuterated internal standards (PGE2-d4 or 5(S)-HETE-d8.)

### 3.1.9 Gas liquid chromatography – mass spectrometry (GLC-MS)

Identification of methyl-esterified fatty acids in plasma samples and tissue extracts from animals treated with DHA was performed by GC-MS using a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph fitted with a capillary column of 5% phenylmethylsiloxane (length, 12 m; film thickness, 0.33 µm). Helium at a flow rate of 30 cm/s was used as the carrier gas. The oven temperature was raised from 120°C to 300°C at a rate of 10°C/min. A Hewlett-Packard model 5890 gas chromatograph equipped with a flame ionization detector and a capillary column of Equity-1701 (Supelco, Bellefonte; length, 30 m; film thickness, 0.25 µm) was used for quantitative determination of DHA. Helium at a flow rate of 25 cm/s was used as the carrier gas. The oven temperature was raised from 210°C to 280°C at a rate of 4°C/min. Peaks corresponding to the methyl esters of DHA and pentacosanoic acid (internal standard) were integrated with respect to peak height using a Hewlett-Packard model 3396 integrator. Standard curves obtained by injection of known mixtures of DHA and pentacosanoic acid methyl esters gave an average response factor of 0.97 from DHA relative to methyl ester pentacosanoic acid.
3.1.10 High-performance liquid chromatography (HPLC)

Incubates of 100 µM DHA with cells suspended in 10 mL of medium were extracted with diethyl ether and the product was methyl-esterified by treatment with diazomethane. Analysis by normal-phase HPLC using a column of Nucleosil 50-5 (250x4 mm, eluted with 2-propanol-hexane (0.6:99.4, v/v) at a flow rate of 2 mL/min) revealed a peak of material absorbing at 234 nm (effluent volume, 15 mL). This material was collected and further analyzed by chiral-phase HPLC using a Chiralcel OB-H column (250x4.6 mm, eluted with 2-propanol-hexane (3:97, v/v) at a flow rate of 0.5 mL/min. As references, the methyl esters of 17(S) and 17(R)-hydroxydocosa-4Z,7Z,10Z,13Z,15E,19Z-hexaenoic acids were used. The elution volumes were 10.3 mL (17(S) enantiomer) and 11.5 mL (17(R) enantiomer).

3.2 IN VIVO

Two animal models were used: one xenograft model in athymic rats, and one transgenic mouse model that carries the human MYCN oncogene (TgMyc).

3.2.1 Xenograft rat model

Male athymic rats HsdHan™:RNU- Foxn1nu (Harlan, Horst, the Netherlands), four to six weeks old, were used for all in vivo xenograft experiments. Rats were housed and maintained in laminar flow cabinets under specific pathogen-free conditions and given sterile water and food ad libitum. SH-SY5Y and [SK-N-BE(2)] xenografts were established by injection of 20x10⁶ human neuroblastoma cells subcutaneously in both flanks under general anesthesia (Isoflurane 2-4%). Treatment was started either before tumor cell injection or when tumors had established and grown to a volume of 0.2-0.3 mL. Tumor growth was measured with digital calipers and the volume was calculated by the formula (width²*length)*0.44. The animals were followed for 12 days following tumor take, or in the case of no tumor development up to 60 days. At sacrifice the rats were euthanized in carbon dioxide and cardiac blood was drawn, heparinized, and spun at 3000 rpm, and the plasma and blood cell component were stored at -80°C. The tumor was removed, weighted, divided, and fixed in 4% paraformaldehyde and snap frozen in liquid nitrogen before storage at 4°C or -80°C, respectively.

3.2.2 Transgenic mouse model

Transgenic TgMyc mice were obtained from the Mouse Model of Human Cancer Consortium (MMHCC) repository. This model is based on targeted expression of the MYCN oncogene under control of the mouse tyrosine hydroxylase promoter in the neural crest during development (Weiss, et al 1997). These mice predominantly develop abdominal, but also rarely thoracic tumors, at a median of 50 days of age. The tumors develop in sympato-adrenal tissue including paraspinal ganglia (Hansford, et al 2004), and grow rapidly and aggressively.
Prior to arrival, the strain was backcrossed 16 generations to the 129x1/SvJ strain. Animals were housed in macroclone cages with access to water and standard rodent pellet (R36, Lantmännen, Skebo, Sweden) ad libitum. Breeders (7-8 weeks) were put on a DHA-enriched diet (1% DHASCO in R36, 1g/kg/day, Lantmännen), one week prior to breeding. Breeders and pups were continuously fed the 1% DHASCO diet throughout their lifespan, while controls received a standard diet.

All animals (controls and treated) underwent abdominal palpations three times weekly to assess tumor development. Based on previous experience, animals were followed for tumor development until a minimum age of 25 weeks.

Upon tumor development, the animals were monitored closely and euthanized upon signs of discomfort, tumor-related problems, or heavy tumor burden. At sacrifice, the animals were weighed and euthanized in carbon dioxide. Cardiac blood was drawn, heparinized, and spun at 3000 rpm, and plasma and blood cell components were stored at -80°C until analysis. The tumor was removed, weighed, divided, and fixed in 4% paraformaldehyde and snap frozen in liquid nitrogen before storage at 4°C or -80°C, respectively. The data collected for each animal was date of birth, time to tumor take, date of termination, tumor weight and volume, and tumor burden (tumor weight as percentage of body weight).

3.3 STATISTICAL ANALYSIS

3.3.1 Treatment effects of single drugs in vitro

The two-tailed nonparametric Mann-Whitney U-test was used to test statistical significance of the data obtained from independent treatment groups. A p value <0.05 was considered to be significant.

3.3.2 Calculation of synergistic effects of drug combinations in vitro

Data was analyzed using the median-effect method of Chou and Talalay (Chou and Talalay 1984) using the software CalcuSyn (version 2, Biosoft). Each dose-response curve (individual agents as well as combinations) was fitted to a linear model using the median effect equation, allowing calculation of a median effect value $D$ (corresponding to IC$_{50}$) and slope. Degree of fit was assessed using the linear correlation coefficient and $r > 0.85$ was set as the criterion for a successful analysis.

The extent of interaction between the drugs was expressed using the combination index (CI) for mutually exclusive drugs: $CI = (d_1 / D_1) + (d_2 / D_2)$, where $D_1$ and $D_2$ represent the concentration of drugs 1 and 2 alone required to produce a certain effect, and $d_1$ and $d_2$ are the concentration of drugs 1 and 2 in combination required to produce the same effect. A CI close to 1 indicates an additive effect; CI significantly lower than 1 was defined as synergy; and CI significantly higher than 1 as antagonism. Mutual exclusivity was assumed. One-sample t tests were used to determine if the mean CIs differed from 1.
3.3.3 Calculation of synergistic effects of drug combinations on tumor growth in vivo

One-way and two-way ANOVAs were used to analyze tumor volumes at day 12. Data was log transformed and normality was checked by transforming all groups to a mean of 0 and testing the combined data using the Kolmogorov-Smirnov (Lilliefors) test. Dunnett's test was used to compare celecoxib delivered by oral gavage with each dietary concentration. Kaplan-Meier analyses and log-rank tests were used to investigate the effect of celecoxib on tumor development. All statistical tests were two-sided and carried out using SPSS version 11 or Systat version 10 (SPSS, Inc., Chicago, IL).

3.3.4 Effects of single drugs on tumor growth in vivo

Time to tumor take was measured and tumor-free survival probability was calculated according to Kaplan-Meijer and compared using log rank tests. Treatment effect was evaluated by comparing tumor weights and volumes using 2-sided Student's \( t \) tests for independent samples, and with a revised form of the Pediatric Preclinical Testing Program (PPTP) (Houghton, et al 2007). For each tumor and day, the tumor volume index (TVI) was calculated (volume/initial volume). The TVI at the last day of the treatment was then translated into an overall treatment response (OTR): partial response (PR), stable disease (SD), or progressive disease (PD). The tumor growth delay (TGD) was calculated by dividing the individual time to an event (days until the TVI ≥ 4) with the median time to events in the control group. The TGD was then further incorporated into the overall treatment effect (OTE) and resulted in a final score for each individual animal. The final median score for each treatment group was then translated back into an OTE for each treatment group (Table 3).

<table>
<thead>
<tr>
<th>TVI</th>
<th>OTR</th>
<th>TGD</th>
<th>OTE</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤4</td>
<td>PR</td>
<td>≤ 1.5</td>
<td>PR1</td>
<td>0</td>
</tr>
<tr>
<td>&gt;1.5</td>
<td>PR</td>
<td>&gt; 1.5</td>
<td>PR2</td>
<td>1</td>
</tr>
<tr>
<td>&gt;4≤6</td>
<td>SD</td>
<td>≤ 1.5</td>
<td>SD1</td>
<td>2</td>
</tr>
<tr>
<td>&gt;1.5</td>
<td>SD</td>
<td>&gt; 1.5</td>
<td>SD2</td>
<td>3</td>
</tr>
<tr>
<td>&gt;6≤20</td>
<td>PD</td>
<td>≤ 1.5</td>
<td>PD1</td>
<td>4</td>
</tr>
<tr>
<td>&gt;1.5</td>
<td>PD</td>
<td>&gt; 1.5</td>
<td>PD2</td>
<td>5</td>
</tr>
</tbody>
</table>

TVI: Tumor volume index; OTR: Overall treatment response; TGD: Tumour growth delay; OTE: Overall treatment effect; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Table 3. Scoring and staging of neuroblastoma xenografts based on tumor volume index and time to event, according to a revised form of the PPTP.
4 RESULTS AND DISCUSSION

4.1 CELECOXIB IN VITRO (PAPER II)

4.1.1 Cytotoxicity of celecoxib alone and in combination with cytostatic drugs

Previous work in our group has shown that celecoxib is toxic to neuroblastoma cells in vitro by mechanisms including induction of the intrinsic apoptotic pathway (Johnsen, et al 2004). In this study we used two cell lines, SH-SY5Y and SK-N-BE(2), to study potential synergistic or additive effects of celecoxib in combination with the chemotherapeutic drugs doxorubicin, etoposide, irinotecan, cisplatin, and vincristine (Fig. 6). Both cell lines were incubated with increasing concentrations of celecoxib and a chemotherapeutic drug, and survival was measured by the FMCA assay. Celecoxib induced a synergistic or additive cytotoxic effect in SH-SY5Y and SK-N-BE(2) neuroblastoma cells when used in combination with doxorubicin, etoposide, irinotecan, or vincristine. In contrast, celecoxib and cisplatin had antagonistic effects in both cell lines. Staining cells with propidium iodide and analysis of cell cycle distribution by flow cytometry revealed that the combination of celecoxib and chemotherapeutic drugs indeed induced apoptosis to a higher extent than each drug alone.

Figure 6. Chemical structures of the chemotherapeutic drugs used in combination with celecoxib.
Provided by Dr Malin Wickström, Uppsala University.
Others have shown that celecoxib in combination with chemotherapeutic drugs work in therapeutic synergy in vitro and in vivo, such as in the cases of taxanes (Olsen 2005), paclitaxel and carboplatin (Sandler and Dubinett 2004), and etoposide and FEC (5-fluorouracil, epirubicin, cyclophosphamide) (Arun and Goss 2004). This may occur because chemotherapeutic drugs can promote transcription of the COX-2 gene and stabilize the COX-2 messenger RNA transcript. If a tumor does not have high COX-2 to start with, treatment will thus induce it. This leads to increased production of PGs, which have been implicated in tumorigenesis. Blocking COX-2 at the same time as chemotherapy is administered may therefore improve clinical efficacy of these drugs (Olsen 2005). In hormone-dependent cancers such as breast cancer, celecoxib can act in synergy with exemestane, an aromatase inhibitor, and thereby prevent tumor growth (Chow, et al 2005).

The antagonistic effect of celecoxib on cisplatin has been reported previously in human ovarian cancer cell lines (Bijman, et al 2008). Cisplatin is a platinum-based chemotherapeutic drug used to treat various types of cancers. Platinum complexes are formed in cells, which bind and cause cross-linking of DNA, ultimately triggering apoptosis. Addition of celecoxib to cisplatin treatment has shown less formation of platinum-DNA adducts, indicating that celecoxib somehow prevents the cross-linking process. However, published data on the interaction between COX-2 inhibitors and platinum compounds is not consistent. Some studies show synergistic effects of cisplatin and celecoxib (Kilic, et al 2008), while others report a null result (Gonzalez-Cortijo, et al 2008). Different cell lines, culture conditions, durations, and doses of treatment can be factors responsible for the observed variation.

### 4.2 CELECOXIB IN VIVO (PAPER II)

#### 4.2.1 Reduction of tumor burden alone and in combination with cytostatic drugs

Nude rats with established neuroblastoma xenografts were treated with celecoxib alone (10 mg orally daily by gavage), or in combination with doxorubicin (1 mg/kg i.p. every third day), irinotecan (2 or 0.75 mg/kg i.p. every second day), or etoposide (10 mg/kg i.p. for 5 days). Tumor growth was monitored for 12 days. Celecoxib alone induced a 43% decrease in tumor growth. Irinotecan as a single agent showed an 86% decrease, and the drugs combined reduced tumor growth by 93%. This was the most pronounced growth inhibitory effect observed. Treatment with doxorubicin alone induced a 42% reduction in tumor growth, and doxorubicin in combination with celecoxib reduced tumor growth by 65% compared to controls. Celecoxib did not enhance the tumor-inhibiting effect of etoposide at the evaluated dose level. As discussed above, these results are in agreement with previous work on combinational effects of celecoxib and chemotherapeutic drugs in vitro.

Irinotecan is a semisynthetic analog of camptothecin and is cleaved by cellular carboxylesterases in vivo, thereby producing the active compound 7-ethyl-10-hydroxycamptothecin (SN-38). Irinotecan/SN-38 interacts with cellular
topoisomerase I (Topo-1) and single-stranded DNA breaks, forming reversible Topo-1/SN-38/DNA cleavable complexes. Collision of these complexes with the advancing replication forks produces irreversible double-stranded DNA breaks and cell death (Hsiang and Liu 1988). Although irinotecan is a powerful and widely used drug, tumor cells can develop irinotecan resistance (Xu and Villalona-Calero 2002) by activating the transcription factor NF-κB (nuclear factor κB) (Cusack, et al 2000). COX-inhibitors have been shown to inhibit NF-κB activation by specific binding and inactivation of the protein responsible for nuclear translocation of NF-κB, explaining why irinotecan and celecoxib might work in synergy (Wang, et al 1999). In addition, celecoxib has been shown to reduce gastrointestinal side effects of irinotecan in pre-clinical testing (Javle, et al 2007).

Doxorubicin is an anthracyclin that intercalates DNA and inhibits macromolecular biosynthesis. Doxorubicin has also been reported to interact directly with the cell membrane and to inhibit the enzyme helicase, which unwinds DNA for replication. One of the primary targets for doxorubicin is thought to be the enzyme topoisomerase II, where inhibition of DNA re-ligation results in the induction of protein-associated strand breaks in DNA (Fornari, et al 1994). Celecoxib is thought to enhance the efficacy of doxorubicin by inactivating NF-κB (van Wijngaarden, et al 2007) and inhibiting glycoprotein P, the product of the multiple drug resistance (MDR) gene (Awara, et al 2004). Enhanced effects of doxorubicin by celecoxib have been shown for many cancers (Hashitani, et al 2003).

Some studies, however, have detected no synergistic or additive effect of celecoxib on doxorubicin and irinotecan (El-Rayes, et al 2008, Xu, et al 2009), nor even upregulation of multidrug-resistant proteins upon celecoxib treatment (Gradilone, et al 2008). This implies that different cancer forms may respond differently to celecoxib, and that some combinations should even be avoided.

Furthermore, coxibs have been shown to also act in synergy with irradiation (Park, et al 2008). The mechanisms underlying the radiation-enhancing effects of COX-2 inhibitors include an accumulation of cells in the G2/M phases of the cell cycle that are considered to be sensitive to irradiation, a reduction of PG-induced immunosuppressive activity caused by antitumor immunologic responses capable of potentiating tumor responses to radiation, and direct effects on tumor neovascularization (Milas, et al 1999). This interesting way in which celecoxib could be clinically relevant in neuroblastoma has not yet been tested in our lab.

### 4.2.2 Celecoxib as both prevention and treatment

For the purpose of prevention, rats were randomly assigned to receive 250, 500, 1500 or 2500 ppm celecoxib in the diet starting two to seven days before tumor cell injection. The animals continued to receive the celecoxib-enriched diet until sacrifice 12 days after tumor development, or until 40 days after tumor cell injection in case of no tumor development. The median time to tumor take, defined as the number of days for a tumor in an animal to reach a volume of 0.3 mL, was delayed in all treatment groups compared with tumors in the control group and was statistically significant for rats treated with 500 and 2500 ppm of celecoxib (log-rank
tests, p=0.03 and p<0.001, respectively). There was a significant dose-dependent delay in tumor development in rats treated with up to 2500 ppm of celecoxib (p<0.001). Fifty percent of rats receiving the highest dose of celecoxib, 2500 ppm in their diet, had still not developed tumors at the end of the experiment, 60 days after tumor cell injection, although they had been without treatment since day 40 after tumor cell injection.

For therapeutic purposes, nude rats with established neuroblastoma tumors were randomly assigned to receive 250, 500, 1500 or 2500 ppm celecoxib in the diet, or 10 mg/kg/day by gavage for 12 days. At sacrifice, mean tumor volumes were reduced by 30% to 65% compared with controls. Tumors in rats receiving celecoxib through oral gavage grew significantly slower compared with tumors in control animals (p=0.025). There was no significant difference in tumor growth inhibition when celecoxib was given as a daily bolus dose compared with celecoxib provided in the diet at any concentration (p>0.25). From these results we cannot conclude that a high peak plasma concentration per se is important for the observed antitumor effects in vivo. Clearly, continuously providing celecoxib in the diet, resulting in a steady-state plasma concentration, was also an effective treatment regimen. By supplementing the diet with 250 to 2500 ppm of celecoxib, we obtained plasma concentrations up to 6.373 nM, which is within the same range as in children receiving 250 mg/m² celecoxib orally twice daily (Stempak, et al 2002). All oral celecoxib treatments were nontoxic and induced tumor growth inhibition at plasma levels that could be obtained in these children and at a lower concentration than is needed to suppress neuroblastoma cell growth in vitro (Johnsen, et al 2004, Johnsen, et al 2005).

Hence, celecoxib is a potent drug that alone can both prevent tumor formation and inhibit growth of already established tumors. The mechanism behind the tumoricidal effects is, again, inhibition of NF-κB (van Wijngaarden, et al 2007) and Glycoprotein P (Kang, et al 2005). Furthermore, celecoxib inhibits PGE₂, which otherwise leads to promotion of angiogenesis [increased VEGF, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) production], an anti-apoptotic mechanism (via increased Bcl-2 and AKT activity), stimulation of tumor metastasis by increasing matrix metalloproteinases, and decreased immune surveillance, or decreased cytokine production and NK activity (Chu, et al 2004).

Large clinical prevention studies have been carried out, especially in patients with a high risk of colon cancer. In these studies, celecoxib reduces the risk of colon cancer depending on the dose, but some studies show a slight increase in adverse side effects, including renal and hypertensive events, gastrointestinal ulceration, and hemorrhage events (Bertagnolli, et al 2009). These side effects, however, seem to occur only in patients who are already at high risk for such cardiovascular events (Chan 2003, Solomon, et al 2008). Therefore, celecoxib can probably be administered safely to a majority of the population, and especially to young children, who seldom suffer from cardiovascular problems. However, celecoxib might not be recommended for a subset of patients.
4.2.3 Celecoxib inhibits proliferation and suppresses angiogenesis

As early as the 1980s, research established that PGs possess angiogenic properties (Form and Auerbach 1983). The ability to induce angiogenesis is essential to enable most solid tumors to grow beyond 2–3 mm in diameter. Tumor cells ensure their own growth by secreting vascular growth factors that stimulate angiogenesis, such as VEGF, bFGF, and PDGF. Among the growth factors identified so far, VEGF seems to be the most prominent and the most important factor for tumor angiogenesis (Marme 1996).

To elucidate the action of celecoxib and because inhibition of angiogenesis can contribute to a reduced cell proliferation, tumors were stained with the proliferation marker Ki-67 and the endothelial cell marker BS-1 and analyzed by two-way ANOVA with respect to drug and celecoxib effects. Treatment with celecoxib resulted in a significant decrease in cell proliferation (percentage of Ki-67–positive cells; p<0.001). There was a significant effect of drug treatment (p=0.001), but only doxorubicin gave a significant reduction in proliferation compared with control (P<0.001). The interaction term was significant (p=0.04), indicating a difference in effect of celecoxib between drug treatments: celecoxib had a significant effect in reducing proliferation of tumors in control or doxorubicin-treated animals (p<0.006), but not in irinotecan-treated animals (p=0.65).

There was also a significant effect of celecoxib in reducing microvessel density (p=0.031; treatment, celecoxib interaction nonsignificant, p>0.2). There was a 55% decrease in microvessel density in tumors from animals receiving celecoxib compared with tumors from animals receiving no treatment (p=0.01). For the other drug combinations, the decrease in microvessel density was not significant compared with celecoxib as a single-drug treatment with the present sample size. There was no significant difference in microvessel density between tumors from rats receiving celecoxib by gastric feeding and those from animals receiving celecoxib in the diet (p>0.16).

Stimulation of angiogenesis is considered one of the most important mechanisms by which COX-2 and PGs support tumor growth (Leahy, et al 2000, Liao, et al 2007). COX-2 regulates PG production and the angiogenic factors VEGF and FGF. The expression of VEGF has been reported to be weak in tumor cells lacking COX-2 (Williams, et al 2000). In addition, COX-2-generated PGs can enhance bFGF-induced angiogenesis through induction of VEGF (Majima, et al 2000), or PGs can stimulate IL-6 production, which in turn stimulates VEGF production (Rak, et al 1996). Thus, the antiangiogenic effect of celecoxib can be explained by its COX-2 dependent mechanisms. Furthermore, inhibition of COX-2 by both nonselective and selective NSAIDs decreases migration of vascular endothelial cells as well as endothelial tube formation (Masferrer, et al 2000).

These results together with our findings further confirm that COX-2 plays a crucial role in tumor-associated angiogenesis and suggest that celecoxib may have potential for use as an antiangiogenic agent in neuroblastoma treatment.
4.3 DHA IN VITRO (PAPER I AND III)

4.3.1 Cytotoxicity alone and together with cytostatic drugs, NSAIDs, and arsenic trioxide

4.3.1.1 DHA causes cell cycle arrest and affects the mitochondrial membrane potential

A panel of neuroblastoma cell lines was treated with DHA at different concentrations and durations. All treatment effects were compared to cells treated with the monounsaturated fatty acid oleic acid (OA) and to untreated controls. A significant dose-dependent decrease in neuroblastoma cell survival was observed in all of the five neuroblastoma cell lines tested. In contrast to DHA, OA had limited effect on neuroblastoma cell survival. This suggests that the highly unsaturated structure of DHA is important for its tumoricidal effects. To investigate the mechanisms behind the observed cytotoxicity, we performed cell cycle distribution analysis by FACS using DAPI staining and observed a sub-G1 peak in the DHA-treated cells, as an indicator of cell death, and an increase in G2, which indicates cell cycle arrest. We also investigated the mitochondrial membrane potential by TMRE-staining in cells treated with DHA, since mitochondrial membrane leakage is one of the first events in cell death (Halestrap 2009). DHA induced depolarization of the mitochondrial transmembrane potential in all neuroblastoma cell lines investigated. Cyclosporin A (CsA), an inhibitor of the mitochondrial membrane transition pore (Leytin, et al 2009), prevented mitochondrial depolarization and significantly attenuated the cytotoxicity induced by DHA. DHA also reduced the clonogenic potential of neuroblastoma cells.

Numerous studies show that DHA induces dose-dependent apoptosis of cancer cells. Serini et al. (Serini, et al 2009) have reviewed many suggestions of mechanisms that seek to explain this phenomenon, including both the intrinsic and the extrinsic pathway (Arita, et al 2001, Narayanan, et al 2001). DHA modifies the expression of Bcl-2 family proteins by increasing the levels of the pro-apoptotic proteins Bak and Bcl-xS and reducing those of the anti-apoptotic proteins Bcl-2 and Bcl-xL (Calviello, et al 2005, Danbara, et al 2004, Yamagami, et al 2009). In agreement with our results, others have also shown that DHA induces cytochrome c release from mitochondria and mitochondrial membrane depolarization (Arita, et al 2001). We believe that the apoptosis seen in our cell lines upon DHA treatment is a result of an apoptosis-signaling cascade that occurs through the intrinsic pathway.

4.3.1.2 DHA increases levels of oxidative stress

The initial event in the oxidative metabolization of all PUFAs is hydrogen abstraction. This occurs at an increased rate when the internal redox balance of a cell is seriously disturbed and the production of initiating species, such as ROS, cannot be sufficiently suppressed, a common scenario in tumor cells (Pelicano, et al 2004). The most common ROS are hydroxyl radicals, superoxide radicals, anions, alkoxy radicals, peroxyl radicals, singlet oxygen, ozone, and hydrogen peroxide (Jahn, et al 2008). Intracellular accumulating ROS leads to disruption of the
mitochondrial membrane potential, to release of cytochrome c with consecutive activation of the caspase cascade, and, ultimately, to programmed cell death through apoptosis (Chen, et al 1996). The glutathione (GSH) system (GSSG/2GSH) is considered to play a central role in maintaining cellular redox balance (Schafer and Buettner 2001) by scavenging radicals formed by oxidation.

Since DHA is a highly unsaturated PUFA, it is susceptible to peroxidation. We investigated whether DHA treatment affected the amount of ROS detected in neuroblastoma cells. OA was again used as a control. We used a fluorometric method to measure ROS, and could see a transient induction of ROS in neuroblastoma cells treated with DHA. We then treated neuroblastoma cells with DHA in the presence or absence of the GSH-depleting agent BSO (Anderson, et al 1999), and saw that BSO significantly enhanced the cytotoxic efficacy of DHA in all neuroblastoma cell lines tested. Strengthening this theory, N-acetylcystein (NAC), a precursor of GSH, and α-tocopherol, a radical scavenger, protected neuroblastoma cells from DHA-induced cytotoxicity. As mentioned above, OA did not exert the same cytotoxic effects on neuroblastoma cells as DHA, which further strengthens the theory that the unsaturated structure of DHA, which makes it more prone to peroxidation, is partly responsible for its cytotoxicity.

4.3.1.3 DHA enhances the effect of cytotoxic compounds

The effects of combined treatment of DHA with cytotoxic drugs (doxorubicin, irinotecan, and cisplatin) on neuroblastoma cell survival were evaluated in chemosensitive cells (SH-SY5Y) and in cells with a MDR phenotype [SK-N-BE(2)] (Keshelava, et al 2001). Previous data suggests that MDR cancer cells may be more sensitive to PUFA than their non-MDR counterparts (Weber, et al 1994). These drugs were chosen because they are commonly included in treatment protocols for children with neuroblastoma (doxorubicin, cisplatin) or have shown promising activity in both preclinical neuroblastoma studies and clinical trials in patients with refractory tumors (irinotecan) (Furman, et al 1999). DHA significantly enhanced the cytotoxicity of all three drugs in both cell lines at all time points.

The mechanism whereby DHA enhances the effects of chemotherapeutic drugs is not clear, but many speculations have been reviewed by Biondo et al. (Biondo, et al 2008). These include:

- Effects on membrane-associated signal transduction, such as decreased Ras-, PI3K/AKT-, Her-2/neu- signaling, and changes in lipid raft composition
- Lipid peroxidation by drug potentiation, which stimulates formation of oxygen free radicals
- Inhibition of chemotherapy-induced NF-κB
- Enhanced drug uptake by altering membrane properties and decreasing production of MDR proteins
• Effects on mediators of apoptosis, such as increased expression of the antiapoptotic protein Bcl-2

Intensive chemotherapy, one of the most important treatment options in neuroblastoma patients, is associated with adverse side effects that in addition to bone marrow depression include ototoxicity and nephrotoxicity (cisplatin), cardiotoxicity (anthracyclines), and severe intestinal toxicity (irinotecan) (Hecht 1998, Kremer, et al 2002). Experimental data indicates that omega-3 PUFAs may exert a protective effect on the gastrointestinal mucosa (Cha, et al 2005) which could be relevant if combined with, for example, irinotecan. In rats, DHA has been shown to partly protect normal bone marrow from the adverse effects of chemotherapy (Atkinson, et al 1997). On the basis of our present in vitro findings and the reported in vivo toxicity studies, DHA might improve the therapeutic utility of several cytotoxic agents commonly used in the treatment of childhood neuroblastoma.

We also evaluated DHA in combination with diclofenac and celecoxib, and in terms of cytotoxicity, these compounds worked in synergy in neuroblastoma cells. This effect may occur because cells supplemented with DHA incorporate the DHA into cell membranes at the expense of AA (Pauwels and Kairemo 2008). Hence, the long-term result is that less AA is available for COX conversion to the eicosanoids that have shown to increase proliferation of cancer cells (Krishnamoorthy and Honn 2008). Combining this phenomenon with direct COX-inhibition may add to COX-inhibition-induced cytotoxicity, as also discussed below).

Arsenic trioxide (As$_2$O$_3$) has shown promising effects in experimental models of drug resistant neuroblastoma (Karlsson, et al 2004, Ora, et al 2000, Woo, et al 2006), and is being evaluated in clinical trials in children (G.C.F. Chan, personal communication). Since DHA is prone to peroxidation and As$_2$O$_3$ has been shown to induce an increased production of ROS, we investigated whether DHA could affect the sensitivity of neuroblastoma cells at a clinically relevant dose of As$_2$O$_3$. A low concentration of DHA potently enhanced the cytotoxicity of As$_2$O$_3$ in all neuroblastoma cell lines tested. This sensitization was prevented by the addition of vitamin E. These findings agree with those of Sturlan et al., who demonstrated that DHA could circumvent acquired resistance to As$_2$O$_3$ in hematological cell lines by mechanisms involving ROS (Sturlan, et al 2003).

In response to oxidative stress induced by ROS-inducing agents, PUFAs undergo free-radical chain reaction breakdown. This causes the formation of toxic lipid peroxidation products which, in turn, synergize with the ROS induced by the oxidative agent to induce tumor cell apoptosis (Germain, et al 1998).
4.3.2 Elucidation of metabolic pathways

4.3.2.1 Neuroblastoma cells metabolize DHA to hydro(per)oxy fatty acids via both enzymatic and non-enzymatic mechanisms

While much research has focused on the metabolism of the omega-6 fatty acid AA to different eicosanoids such as PGs and TXs, the metabolism of omega-3 fatty acids, EPA and DHA, has been somewhat forgotten. Only recently were the bioactive products of DHA, namely resolvins of the D-series and the protectins, identified (Serhan, et al 2002). These novel lipid mediators are essential for the resolution of inflammation, a process previously thought to take place passively. DHA is converted through several steps to resolvins and protectins via hydroperoxy and hydroxy fatty acid intermediates through LOX or acetylated COX enzymes. Formation of the intermediates can also occur via autoxidation.

Since no research has been done regarding these compounds in cancer cells, we started by determining the metabolism of DHA in neuroblastoma cells using two human neuroblastoma cell lines, SK-N-BE(2) and SH-SY5Y. Lipids in supernatants from cells incubated with DHA, in the absence or presence of the pan-LOX inhibitor NDGA, were extracted and then analyzed by LC-MS/MS. Both neuroblastoma cell lines synthesized the intermediates 17-hydroxy-(H)DHA, 14-HDHA, 7-HDHA and 4-HDHA, but no resolvins were produced, and very low levels of protectins were detected. Previous studies in our lab have shown that neuroblastoma cells express 5-LOX (Sveinbjornsson, et al 2008). We also performed immunoblotting and detected presence of 15-LOX. Therefore, NB cells contain both enzymes necessary for converting DHA to resolvins and protectins, but this conversion does not take place. In the presence of NDGA, an 18% and 12% decrease occurred in levels of 17-HDHA and 14-HDHA, respectively. Steric analysis revealed that biologically derived 17-HDHA separated into two peaks due to the 17(S) enantiomer (64%) and the 17(R) enantiomer (36%), further suggesting that hydroxy fatty acids at least partly are produced by 15-LOX mediated enzymatic conversion. Enzymatic conversion yields a compound in (S) configuration only, while other pathways such as autoxidation yields a racemic mixture.

4.3.2.2 DHA-derived hydroperoxy fatty acids induce apoptosis in neuroblastoma cells

To analyze the effect of DHA and its metabolites on neuroblastoma cell survival, SH-SY5Y and SK-N-BE(2) cells were incubated with DHA, 17-hydroperoxy(Hp)-DHA, or 17-HDHA, and cell survival was measured using the MTT assay. All compounds induced cytotoxicity according to time and dose. 17-HpDHA had significant cytotoxic potency with an IC50 of 3-6 µM at 72 hours, compared to 12-15 µM for DHA. 17-HDHA was less toxic, with an IC50 of 25 µM after 72 hours incubation. Thus, some of the DHA-mediated cytotoxic effects observed in neuroblastoma cells occur because of the conversion of DHA to the more cytotoxic metabolite 17-HpDHA. Reduction of this compound to 17-HDHA reduces this effect.

Immunoblotting to detect PARP-cleavage and FACS analysis using Annexin V staining showed that DHA and 17-HpDHA, but not 17-DHA, induce apoptosis in
neuroblastoma cells. Resolvin D1 (RvD1) and protectin D1 (PD1) did not affect
neuroblastoma cell survival. Addition of the anti-oxidant α-tocopherol (0.0-5.0 nM)
to the growth medium in combination with 17-HpDHA protected cells from the
17-HpDHA-induced cytotoxicity according to dose, which further supports the
hypothesis that lipid peroxidation is involved in DHA-mediated growth inhibition
and cell death described by us and others (Girotti 1998) (Paper I).

Hence, DHA probably has the capacity to induce cytotoxicity in neuroblastoma
cells by the intracellular formation of hydroperoxy fatty acids and by competing
with AA for incorporation into the membrane phospholipids. DHA could also
induce cytotoxicity by binding to catalytic sites of elongases, desaturases, and COX-
2 (Larsson, et al 2004), as described above and as hypothesized by Siddiqui et al.
(Siddiqui, et al 2008) in a review on DHA’s many oxidation products. To my
knowledge, only one study has investigated the action of DHA hydroperoxides in
neuroblastoma cells, but this study examined only exogenously supplied and a non-
sterospecific DHA hydroperoxy fatty acid (Liu, et al 2008). This DHA-derived
hydroperoxide induced apoptosis in neuroblastoma cells through several apoptotic
hallmarks including nuclei condensation, DNA fragmentation, poly (ADP-ribose)
polymerase cleavage, and increased activity of caspase-3. In addition, release of
cytochrome c, increased Bcl-2 expression, and attenuation of mitochondrial
membrane potential was observed. This data agrees with ours and indicates that
DHA hydroperoxides may induce apoptosis in human neuroblastoma cells, which
may be mediated by the mitochondrial (intrinsic) pathway.

4.3.2.3 DHA decreases the level of PGE2-secretion and augments the cytotoxic
effects of celecoxib

As previously mentioned, DHA is incorporated into cell membranes at the expense
of AA, which leads to less formation of AA-derived PGE2. To investigate if this
holds true for neuroblastoma cells, SK-N-BE(2) cells were incubated with DHA,
17-HDHA, RvD1, or PD1, and the amount of PGE2 secretion was analyzed by LS-
MS/MS. DHA reduced the PGE2-levels by >50%, but 17-HDHA, RvD1, and
PD1 did not affect PGE2 levels. The reduction of PGE2 is thus mediated by DHA-
incorporation and not its downstream metabolites. Several studies have shown that
supplementation of omega-3 PUFA to cells, animals, and humans reduces the AA-
derived eicosanoids, and our results agree with these studies (LeBlanc, et al 2008,

We have previously shown that COX-2 and microsomal PGE synthase 1 (mPGES-
1), the enzymes responsible for converting AA to PGE2, are highly expressed in
neural tumors and that inhibition of these enzymes has profound effects on the
survival of these tumors (Baryawno, et al 2008, Johnsen, et al 2004), (Paper II). We
therefore investigated the effect of combining DHA with the COX-2 specific
inhibitor celecoxib in neuroblastoma cells and show that these compounds induced
synergistic cytotoxicity. Other studies on the combination of celecoxib and DHA
have shown similar additive or synergistic effects in various human cancer cell lines.
Mechanisms seem to be both COX-2-dependent, such as blockage of COX-2 and
inhibition of AA metabolism (Swamy, et al 2004), and COX-2 independent, such as
induction of heat shock proteins (Narayanan, et al 2006) and modulation of NF-kB
4.4 DHA IN VIVO (PAPER IV)

4.4.1 Neuroblastoma xenografts

In two sets of experiments, DHA was given to athymic rats carrying neuroblastoma xenografts. DHA was given orally by gavage in the form of DHASCO oil containing 40% DHA. DHA was also incorporated into the diet at 5% and 16%. The DHA-dose given orally by gavage was 0.5 and 1.0g/kg/day. The enriched diets gave a DHA-intake of about 3 and 9 g/kg/day, respectively. DHA incorporated into the diet was given both as prevention before tumor cell injection and as treatment at tumor take. DHA by gavage was given as treatment only.

A pharmacokinetic study was performed to establish the distribution and elimination rates of DHA given as one oral dose (1.0 g/kg) to non-tumor-bearing rats. The half-life of DHA was found to be six hours with a maximum concentration of 274 µg/mL at one hour.

4.4.1.1 Reduction of tumor burden, gavage vs diet

To evaluate the potential of DHA in the treatment of neuroblastoma, rats with established SK-N-BE(2) neuroblastoma xenografts were given a daily dose of DHA by gavage for 12 days starting at tumor take (0.3 mL). Tumor growth was monitored every other day and evaluated using a modified version of the PTPP evaluation system. Median tumor volume index at the end of the experiment (day 12) was 3.72 for animals receiving a high dose of DHA, 5.47 for animals receiving a low dose of DHA, and 9.48 in control animals. This corresponded to partial response grade 1 (PR1), stable disease grade 1 (SD1) and progressive disease grade 2 (PD2) for the different groups, respectively. The median DHA levels in plasma were increased from 88.7 µg/mL in the control group to 136.0 and 122.4 µg/mL in the low and high dose DHA groups, respectively (p<0.03 for each group compared to control). The median DHA levels in the tumor tissue increased even more drastically from 0.246 µg/mg in the control group to 0.443 and 0.668 µg/mg in the low and high dose DHA groups, respectively (p<0.04 for each group compared to control). The majority of the tumors in the treatment groups responded to therapy, while two tumors in each group did not. In the tumors that did not respond to treatment, the DHA levels were 0.22 and 0.36 µg/mg (low dose) and 0.58 and 0.41 µg/mg (high dose), all values being lower than the median for each group.

One study in breast cancer patients further emphasizes this inter-individual uptake, incorporation, and response. Patients were supplemented with DHA daily during chemotherapy and could then be divided into high and low incorporating groups based on the DHA levels in plasma and red blood cells. The high incorporating group was characterized by longer overall survival and delayed time to tumor progression compared to the low incorporating group (Bougnoux, et al. 2006).

In animals receiving a DHA-enriched diet, no significant differences in tumor weight, tumor volume, or stage of disease were observed at the end of the experiment in any of the treatment groups compared to the control group, as evaluated by the modified PPTP evaluation system. The median level of DHA in plasma of control rats was 94.1 µg/mL, and DHA supplementation increased this
level to 269.6 µg/mL (pre-treatment with 16 % DHA diet), 237.5 µg/mL (5% DHA diet), or 298.9 µg/mL (16% DHA diet), p<0.002 for each group compared to the control group.

Hence, the levels of DHA in plasma were similar in both experiments, but tumor reduction was only seen in animals treated with DHA by gavage. Tumor reduction did not occur in animals on a DHA-enriched diet, where DHA is accumulated more slowly and over time. This indicates that repeated peaks in the plasma concentration may be necessary to mediate the tumoricidal effects. Intracellular DHA is converted to 17-hydroperoxy-DHA enzymatically or by autoxidation, and this lipid mediator can induce apoptosis of neuroblastoma cells (Paper III). This effect, however, is dose-dependent, and anti-oxidants such as α-tocopherol can protect cells from hydroperoxy fatty acid-induced cell death. Tumor cells often have dysfunctional redox systems and can be vulnerable to oxidative stress (Pelicano, et al 2004). Therefore, a bolus gavage of DHA most likely generates an unmanageable surplus of toxic lipid mediators, whereas small amounts over time, as provided by the DHA-enriched diet, can be scavenged. The observed variation in DHA uptake correlates well with other studies showing differences in DHA incorporation due to dissimilar rates of metabolism, enzymatic activity, background diet, age, and gender (Arterburn, et al 2006, Childs, et al 2008, Rusca, et al 2009, Bougnoux, et al 2006).

4.4.1.2 Prevention and treatment

To investigate the potential of DHA in tumor prevention, athymic rats were fed a diet containing 16% DHASCO starting seven days prior to injection of SH-SY5Y neuroblastoma cells. Control animals received a standard diet. Time to tumor take (0.3 mL) was monitored in both groups after tumor cell injection. In the DHA-supplemented group the mean time to tumor take was 25 days, compared to 18 days in the control group (p=0.002). One rat receiving a DHA-enriched diet did not develop tumors.

At tumor take, the control animals were further randomized into three treatment groups: untreated controls, 5% DHASCO-enriched diet, or 16% DHASCO-enriched diet. The pre-treated animals continued to receive the 16% DHASCO-enriched diet. Tumor growth was monitored continuously and the tumor weight was measured at sacrifice, 12 days after tumor take. As stated above, no significant differences in tumor weight, tumor volume, or stage of disease were observed at end of this experiment in any of the treatment groups, compared to the control group.

The observation that a DHA-enriched diet is potent enough to delay or prevent tumor establishment, but not to reduce growth of already established tumors, has also been reported in a model of mammary carcinogenesis (Noguchi, et al 1997).

Mechanisms underlying the tumor preventive characteristics of DHA are not clearly understood. Some of the first steps required for tumor establishment are adhesion and angiogenesis. DHA has been shown to inhibit adhesion (Victory, et al 2007), perhaps by down-regulating Rho GTPase, inhibiting cytoskeleton reorganization (Yi, et al 2007), and reducing ICAM-1 and VCAM-1 protein
expression (Goua, et al 2008). DHA also decreased TNF-alpha-induced monocyte rolling, adhesion, and transmigration (Schaefer, et al 2008). These effects might also be applicable to tumor cells that are in the process of adhering to tissue sites. DHA has also been shown to reduce angiogenesis (Rose and Connolly 1999), probably by decreasing VEGF levels (Victory, et al 2007). These data suggest that DHA treatment may be effective in delaying or preventing neuroblastoma formation, and may be clinically useful for maintenance therapy in minimal residual disease.

4.4.2 Transgenic mice carrying the MYCN oncogene

In humans, amplification of the MYCN oncogene in neuroblastoma predicts poor prognosis and resistance to therapy (Henry, et al 2005). We therefore evaluated the effects of DHA supplementation using TgMyc mice that spontaneously develop aggressively neuroblastoma-like tumors originating from the neural crest (Weiss, et al 1997). A total of 74 animals (18 litters) carrying the MYCN transgene were given a DHA-enriched diet (1% DHASCO) throughout their entire life, starting even in utero as mating pairs were put on the diet prior to mating. These DHA-fed animals were compared to a control group consisting of 121 animals (40 litters) given a standard diet. Out of these animals, 55 animals (74%) in the DHA-group and 83 animals (69%) in the control group developed tumors. The median time to tumor take was 56 days in the DHA-group and 50 days in the control group. These differences were not significant. The plasma and tumor DHA-levels were significantly elevated in animals given the DHA-enriched diet. Animals on the standard diet had a median plasma DHA level of 78.0 µg/mL compared to treated animals with 450.0 µg/mL, p<0.001. Median levels of DHA in tumor tissue increased from 0.297 µg/mg in controls to 1.218 µg/mg in treated animals, p<0.001. Despite this efficient uptake and incorporation, DHA did not appear to influence the growth of these aggressive MYCN-driven neuroblastomas. No differences between the groups in body weight, tumor weight, tumor weight as percent of body weight, or time to sacrifice from tumor take were observed.

A secondary, but noteworthy, finding was that the breeders on the DHA diet gave birth to more pups per litter, on average 6.6 pups compared to 4.8 pups per litter in the control group (p=0.0004). This may be of importance in other fields of research. There is no consensus in the literature regarding the effect of DHA or omega-3 supplementation on fertility. Some studies show that DHA-enrichment decreases rates of successful implantation of the fertilized egg in the endometrium (Wakefield, et al 2008). On the other hand some studies show that spermatozoa from asthenozoospermic, oligozoospermic, and oligoasthenozoospermic men had lower levels of DHA than those from normozoospermic men (Aksoy, et al 2006, Tavilani, et al 2006). Our result supports the hypothesis that DHA enhances fertility, but we have not pursued this issue further.
5 SUMMARY AND CONCLUSIONS

In this thesis, novel agents involved in eicosanoid and docosanoid synthesis have been tested in vitro and in vivo for neuroblastoma prevention and therapy. The COX-2 inhibitor celecoxib alters eicosanoid production by blocking conversion of AA to prostaglandins and thromboxanes. The omega-3 fatty acid DHA alters presence of eicosanoids by replacing AA in cell membranes, and is the precursor of docosanoids. These compounds shift the balance of lipid mediators, and thereby affect cell proliferation and growth, as indicated below:

In vitro:

✓ Celecoxib acts in synergy with chemotherapeutic drugs in terms of neuroblastoma cytotoxicity.

✓ DHA acts in synergy with chemotherapeutic drugs, NSAIDs and arsenic trioxide in terms of neuroblastoma cytotoxicity.

✓ Neuroblastoma cells express 15-LOX.

✓ DHA is converted to 17-HpHDA and 17-HDHA by neuroblastoma cells.

✓ Neuroblastoma cells do not produce resolvins or protectins from DHA.

In vivo:

✓ Celecoxib delays tumor development and potentiates the cytotoxic effects of irinotecan and doxorubicin in human neuroblastoma xenografts.

✓ Celecoxib reduces proliferation and angiogenesis of neuroblastoma tumors.

✓ DHA delays tumor development and reduces tumor growth in human neuroblastoma xenografts.
The data presented in this thesis, together with previous studies from other research groups, show that both celecoxib and DHA have preventive and therapeutic effects on neuroblastoma. They can also be safely administered to children in doses required to obtain therapeutic effects. Studies on celecoxib are already ongoing in several cancer trials in adults, and the use of DHA in cancer trials is emerging.

Since the pharmacokinetic profiles of these drugs have been established in vivo and to some extent in patients, I believe that enough is known to initiate phase I/II studies on children with neuroblastoma or other cancers. Due to the small number of patients, a multi-center study would be necessary to draw conclusions of statistical significance.

Both celecoxib and DHA have been shown to enhance the cytotoxicity of certain chemotherapeutic drugs. Therefore, the addition of DHA or celecoxib to conventional chemotherapy during either induction or maintenance therapy could prove useful.

The easiest way of administrating DHA is probably as oil capsules for older children, or by spoon or mixed with food for younger children. In children with cancer, the DHA could be given along with parenteral nutrition. Alternatively, physicians could adjust the lipids in the current formulas of parenteral nutrition to contain more omega-3 and less omega-6. Since DHA showed high potential in delaying and even preventing tumor formation in our and other studies, the most probable area of application would be during maintenance chemotherapy after surgery, rather than for therapeutic purposes during induction therapy. Because DHA does not seem to be harmful or to promote tumor growth, trials investigating this effect would be valuable and not associated with high risks.

Celecoxib seems to be very potent even as a single drug in cancer therapy, and is probably a candidate in both therapeutic and preventive settings. Since COX-2 is overexpressed in almost all neuroblastoma tumors investigated, celecoxib is a candidate drug for all neuroblastoma patients requiring treatment. If celecoxib augments the cytotoxicity of chemotherapeutics, it could also reduce chemotherapy-induced side effects by decreasing the dose needed.

These drugs could also be used in the future for primary prevention. Since long-term use of celecoxib has been associated with various side effects, however, it may not be the ideal drug for prevention. DHA, on the other hand, is found in the diet and has not been shown to be harmful. The study of Alaskan Inuits mentioned earlier implies a correlation between fatty fish intake and lower incidence of neuroblastoma. Whether this is a causal relationship remains to be studied, and a study could be done by, for example, supplementing pregnant and lactating women with DHA and performing a long-term follow-up of their children. This is, of course, a large-scale study that requires extensive resources. Even if this intervention does not change the neuroblastoma and other cancer incidences of...
Swedish or European children, the evidence of the beneficial effects of omega-3 intake are convincing enough for it to be ethically approved, and other parameters of interest, such as neurodevelopmental markers and incidence of asthma and allergy, could be measured concomitantly.

I hope that the work in this thesis can continue to later involve clinical trials, and that the future holds better prognoses for children with neuroblastoma and other forms of cancer, perhaps due to some of the pathways and mechanisms presented here.
Neuroblastom är en tumör i det perifera nervsystemet, utanför hjärnan och ryggmärgen, som främst drabbar spädbarn och barn i de yngre åldrarna. Förutom hjärttumörer är detta den vanligaste solida tumören hos barn, 6% av all barncancer, och den orsakar drygt 9% av alla barncancerdödsfall. Trots intensiv terapi i form av kirurgi, strålning, cytotstatika och stamcellsskörd är överlevnaden otillräcklig i högriskgruppen, ca 50%, medan den totala överlevnaden hos alla barn med neuroblastom är ca 70%. Det finns därför ett stort behov av att förbättra nuvarande behandlingsprotokoll och hitta nya mediciner.

Inflammation kan öka tillväxt av cancer och riktad terapi som dämpar olika inflammatoriska komponenter verkar tillväxthämmande. Vi har tidigare visat att enzymet COX-2, som omvandlar arakidonsyra (AA) till inflammatoriska prostaglandiner, är uppreglerat och aktivt i neuroblastomtumörer. Vi har vidare visat att selektiva inhibitorer av COX-2, såsom celecoxib, hämmar neuroblastomtillväxt in vitro.

Denna avhandling visar att celecoxib både hindrar tumöruppkomst och minskar tumörtillväxt i en rättmodell av neuroblastom. Dessutom förstärker celecoxib effekten av cytotstatika, t.ex. irinotecan och doxorubicin. Analyser av tumörsnitt visade både minskad celldelning och minskad nybildning av blodkärl hos djur som behandlats med celecoxib via sondmatning eller en berikad diet.

Fleromättade omega-3 fettsyror har föreslagits som användbara i cancerbehandling och prevention. Omega-3 fettsyror som docosahexaensyra (DHA) och eicosapentaensyra (EPA) har motsatt effekt till omega-6 fettsyror som t.ex. AA, genom att verka anti-inflammatoriskt. Från EPA bildas en annan sorts prostaglandiner, och från DHA bildas resolviner och protektiner, alla med anti-inflammatoriska egenskaper.

I detta arbete visar vi DHA kan orsaka programmerad celldöd hos neuroblastomceller in vitro. Vi visar också att neuroblastomceller omvandlar DHA till hydroperoxy- och hydroxyfettsyror via både enzymatiska och icke-enzymatiska vägar. DHA omvandlas däremot inte till resolviner eller protektiner i neuroblastomceller. DHA förstärker även effekten av cytotstatika, arseniktrioxid och andra anti-inflammatoriska läkemedel, t.ex. just celecoxib. I samma djurmodell som ovan kan DHA försena tumörutveckling och minska tumörtillväxt.

Sammanfattningsvis visar vi med studierna i denna avhandling att blockering av omega-6 fettsyror, eller behandling med omega-3 fettsyror, båda kan vara möjliga nya strategier för neuroblastomterapi, och föreslår därför att celecoxib och DHA testas i kliniska studier för barn med neuroblastom.
8 ACKNOWLEDGEMENTS

Under mina fem år på barncancerforskningsenheten har jag omgetts av många personer som i stort och smått på olika sätt bidragit till att göra min doktorandtid till en otroligt stimulerande, lärorik, utvecklande, och inte minst rolig och härlig fas i mitt liv. Jag vill tacka er var och en för att jag fått förmånen att arbeta tillsammans med er och lära känna er. Jag är tacksam för er hjälp, inspiration och vänskap.

Först och främst, mina handledare och nuvarande samt före detta gruppmedlemmar:


Magnus Lindskog, min bihandledare och kära vän. Det var med dig det började, hela projektet. Tack för stöd och uppmuntran från dag ett när jag var grön på lab och ny i gruppen. Vårt arbete har i allra högsta grad varit ”fun-saturated”(!) och jag uppskattar verkligen våra diskussioner om life and science. Låt dem fortsätta!

Lotta Elfman, vår eminenta BMA och min skrivbordsgranne. Din hjälpsamhet, din vilja att vara involverad och din inställning att ”ingensting är omöjligt” har gjort arbetet på labbet genomförbart och dessutom roligt. Tack för härliga skratt, trevliga samtal, gott fikabröd och gulliga födelsedags-sms. Du tänker på alla. Vad vore vi utan dig?


**Ebba Palmberg**, min doktorandkollega och även blivande läkarkollega (du ligger ju lite före, men ändå…). Du är en entusiast som bidrar med många nya planer och idéer i vår grupp! Det är kul att få vidga vyerna. Vi får se till att i framtiden diskutera strategier för det här med att hinna med både läkeri och forskeri!

**Catarina Träger**, min doktorandkollega och skrivrumskompis. Dessutom är du redan erfaren barnonkolog, och jag tycker att ditt bidrag till gruppen är jurteviktigt just därför! Kul att ha fått en massan klinisk input av dig. Och också värdefullt med våra relationsdiskussioner på rummet sena kvällar när vi inte orkat jobba mer…


**Frida Ponthan**, före detta gruppmedlem, nu utvandrad forskare i Newcastle. Du var mitt ankare i labbet från första början. Tack för hjälpen med ALLT. Det var du som lärde mig att celler, magnetomrö rarare, blottingapparater och datorer precis som människor behöver ”kärlek och tålamod”. Sen fixar sig allt. Man kan inte bli upplärd av nån bättre. Tack och lycka till över there!

Jag vill vidare tacka mina medarbetare på barrncancerforskningsenheten:


Staffan Eksborg, vår stygge apotekare med koll på farmakokinetiken. Snacka om någon som ställer upp ögonblickligen oavsett. Så fort jag behövt hjälp har du kommit rusandes upp på BCFE med redan färdiggrande figurer i högsta hugg, i TIFF-format såklart. Tack så mycket för din input vid djurförsöken!


Stort tack till hela gänget på BCFE, som alla bidrar till gemenskapen med både intellektuella och vanvettiga diskussioner: Martina, Annika, Julio, AnnaCarin, Carina, Åsa, Malin, Elisabet, Christer, Göran, Mats, Stefan H och Stefan S. TACK!

Mina kollegor och samarbetspartners på KI och i Uppsala:


Anna och Lena-Maria, gruppens adoptivdoktorander. Det har varit så kul att ha er som extra gruppmedlemmar! Tack för roliga events tillsammans, för härligt resesällskap, för långa samtal och ventilering vad gäller labbande, att vara doktorand och försöka hinna med allt annat i livet på samma gång (som ni båda verkar klara galant!). Låt samkvämen fortsätta!

Dali, Cissi och Xin på nollan. You always greet me with happy smiles, and you are always eager to help out in times of crisis! Thanks for great assistance in the lab and nice conversations over lunch and cups of tea.

Malin och Dieter borta i Uppsala. Tack för härliga konferensresor och andra vetenskapliga events tillsammans i neuroblastomvärlden. Malin, tack för din hjälp med FMCA i Uppsala, härliga snack under somrarna i djurhuset, och för figurerna till denna bok. Ni har båda varit ovärdeliga för mig under disputationsprocessen också (mer än ni vet om). Vilka praktextemplar att ha som förebilder!

My collaborators at Harvard:

Charles N Serhan, professor at Brigham and Women’s Hospital. Thanks so much for letting me visit the lab and try my ideas out. All of them were probably not sane, but it was all worth it, since my visit to you taught me loads about science and research with an international perspective. Thanks for taking the time to help me with small and big things, and for your hospitality to invite me to your home.

All co-workers in the Serhan lab: Rong, Padmini, Kim, Lisa, Antonio, Sriram, Kie, Lucy, Matt, Tim, Gaby, Stacey, Gina, Sungwhan, and Mary. Thanks for greeting me with enthusiasm, introducing me to the lab work patiently, taking care of me socially, and just being great friends. I miss you all and hope to see you again soon!

My editor in Boston:

Sylvia Pearce, the woman who speaks a little of all languages! Thanks for tremendous work with this thesis. Thanks for sitting up days and nights to finish it on time, at the same time as being patient with me and my questions and comments. I am so lucky to have met you. I found a great editor and a marvellous friend, all in one person!

Mina allra bästaste vänner:


Min makalösa familj:


Alexander, min minsta lillebror, som förvisso är störst till växten. De minsta skola bliva de största, sägs det! Din inställning att allt ordnar sig kan ibland vara frustrerande, men ack


Lena, Björn, Henrik, Åsa, Peter, Nanna, Olle och Ruben, min underbara extrafamilj som tar hand om mig som en prinsessa. Tack för ett välkommande hem, er omtanke och er uppmuntran med detta arbete. Tack för roliga kvällar med tabuspel och bastubad som välbehövliga pauser i skrivandet. Tack till Nanna och Olle för alla fina teckningar och vitsippor. De gjorde susen när jag skulle jobba hårt hela dagen! Jag uppskattar er så världigt mycket, och ser fram emot härliga sommarveckor i Hagen!


“Never let a problem to be solved, get in the way of a person to be loved.”

-Thomas S Monson
9 REFERENCES


“I’m glad I did it, partly because it was worth it, but mostly because I shall never have to do it again.”

-Mark Twain