Modulation of fenretinide induced cell death in neuroblastoma

Cuperus, Roos

Citation for published version (APA):
Cuperus, R. (2014). Modulation of fenretinide induced cell death in neuroblastoma
General introduction
General Introduction

Neuroblastoma is a childhood tumor that, despite intensive therapy, has a poor prognosis especially in high risk patients. Therefore, new therapeutic options are needed to improve the outcome of these patients. The research described in this thesis attempts to unravel the apoptosis-inducing mechanism of fenretinide in neuroblastoma and, in order to increase its anti-tumor effects, we investigated possibilities for combination therapy. This introduction will describe various clinical aspects associated with neuroblastoma, the role of oxidative stress in cancer in general and subsequently an overview of the effects of fenretinide on neuroblastoma. Finally, the background of combination therapy in general and its specific application to neuroblastoma with fenretinide will be discussed.

Neuroblastoma

Origin and clinical presentation

Neuroblastoma is a tumor originating in the neural crest during fetal development. Failure of affected neural crest cells to differentiate is the first step towards malignant transformation. As the neural crest normally develops into the adrenal gland and the sympathetic nervous system, the tumor can manifest itself throughout the paravertebral sympathetic ganglia and the adrenal gland. The adrenal gland is the most common primary site, followed by abdominal, thoracic, cervical, and pelvic sympathetic ganglia. In the family of neuroblastic tumors, neuroblastoma is the most aggressive tumor followed by ganglioneuroblastoma and ganglioneuroma.\textsuperscript{1,2} Clinical presentation is highly variable and depends on the site of the primary tumor, the presence of metastatic disease or paraneoplastic symptoms.\textsuperscript{3} Neuroblastoma can metastasize to lymph nodes, bone marrow, bone, liver, and skin. Bone metastasis tends to appear in the orbit. Therefore, periorbital ecchymoses (raccoon eyes) are a classical sign of disseminated neuroblastoma.\textsuperscript{4}

Epidemiology

Neuroblastoma is the third most common pediatric tumor, accounting for approximately 8% of childhood malignancies. The incidence of neuroblastoma per year is 10.5 per million in children under the age of 15.\textsuperscript{5,7} Despite the low incidence, neuroblastoma is responsible for approximately 9% of cancer deaths in children.\textsuperscript{8} The majority of patients with neuroblastoma are diagnosed at the age of 0 to 4 years, with a median age of 17 months.\textsuperscript{9} Forty percent of the patients are under 1 year of age at time of diagnosis, and less than 5% are over the age of 10 years. In the Netherlands 25 new cases are diagnosed every year.
Chapter 1

**Diagnosis, pathology, staging and risk stratification**

At diagnosis, the presence of elevated concentration of catecholamine-derivates in urine is investigated as well as the presence of tumor cells in the bone marrow. Neuroblastoma cells are detected using immunocytology with anti-GD2, as GD2 (disialoganglioside) is present on the surface of neuroblastoma cells. Imaging at diagnosis is performed through scanning with \(^{123}\)I-labeled Meta-iodobenzylguanidine (MIBG) to evaluate disseminated disease. Finally, the characteristic histopathological features and genetic makeup of neuroblastoma are assessed. According to the histological classification, neuroblastoma is the most aggressive type of the neuroblastic tumor family. Genetic aberrations are discussed below in part 1.4. Prognosis and treatment are highly dependent on staging and risk classification.

The International Neuroblastoma Staging System (INSS) was revised in 1993. As the INSS is a post-surgical staging system, the International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pretreatment risk classification. A new staging system (INRGSS) based on clinical criteria and tumor imaging was developed in 2009. In the INRGSS, the extent of disease is determined by the absence or presence of image-defined risk factors (IDRFs) and/or metastatic tumor cells at the time of diagnosis, before any treatment (Fig. 1).

Risk classification is subsequently based on a combination of clinical and biological variables. At present, risk classification of neuroblastoma is not uniform between different groups. However, all risk classification systems use age, stage and MYCN copy number as risk factors. Histopathological grading is also used; all European groups include neuroblastoma and ganglioneuroblastoma in the treatment protocols for malignant neuroblastoma. Ganglioneuroma are classified as a benign disease and are not included in treatment protocols.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment</td>
</tr>
<tr>
<td>L2</td>
<td>Locoregional tumor with presence of one or more image-defined risk factors</td>
</tr>
<tr>
<td>M</td>
<td>Distant metastatic disease (except stage MS)</td>
</tr>
<tr>
<td>MS</td>
<td>Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow</td>
</tr>
</tbody>
</table>

*Figure 1.* INRG Staging System. Figure adapted from Monclair et al JCO 2009.
The INRG has proposed a risk classification into four categories, in line with the INRGSS (see Fig. 1); very low risk, low risk, intermediate risk, and high risk, based on INRG tumor stage, age, histology, differentiation, \textit{MYCN} amplification, chromosome 11q status, and DNA ploidy (Fig. 2).\textsuperscript{15, 16} This classification is a proposal and thus, has not yet been incorporated in all protocols.

<table>
<thead>
<tr>
<th>INRG</th>
<th>Age (months)</th>
<th>Histologic Category</th>
<th>Grade of Tumor Differentiation</th>
<th>\textit{MYCN} Aberration</th>
<th>Ploidy</th>
<th>Pretreatment Risk Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1/L2</td>
<td>Any, except GN maturing, or GNB intermixed</td>
<td>NA</td>
<td>Amp</td>
<td>A Very low</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>Any, except GN maturing, or GNB intermixed</td>
<td>NA</td>
<td>Amp</td>
<td>B Very low</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>&lt;18</td>
<td>Any, except GN maturing, or GNB intermixed</td>
<td>NA</td>
<td>Amp</td>
<td>C Very low</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;18</td>
<td>GNB nodular; neuroblastoma</td>
<td>NA</td>
<td>Amp</td>
<td>D Low</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Differentiating</td>
<td>No</td>
<td>Amp</td>
<td>G Intermediate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poorly differentiated or undifferentiated</td>
<td>No</td>
<td>Amp</td>
<td>H Intermediate</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>&lt;18</td>
<td>NA</td>
<td>Hyperdiploid</td>
<td>F Low</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;12</td>
<td>NA</td>
<td>Diploid</td>
<td>I Intermediate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 to &lt;18</td>
<td>NA</td>
<td>Diploid</td>
<td>J Intermediate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;18</td>
<td>Amp</td>
<td>O High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;18</td>
<td>Amp</td>
<td>P High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>&lt;18</td>
<td>NA</td>
<td>No</td>
<td>C Very low</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>Amp</td>
<td>Q High</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textbf{Figure 2.} Classification of neuroblastoma tumors according to the INRG classification system. Pretreatment risk group H has two entries. Diploid (DNA index 1.0); hyperdiploid (DNA index > 1.0, including near-triploid and near-tetraploid tumors); very low risk (5-year EFS 85%); low risk (5-year EFS 75% to 85%); intermediate risk (5-year EFS 50% to 75%); high risk (5-year EFS 50%). GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified; L1, localized tumor confined to one body compartment and with absence of image-defined risk factors (IDRFs); L2, locoregional tumor with presence of one or more IDRFs; M, distant metastatic disease (except stage MS); MS, metastatic disease confined to skin, liver and/or bone marrow in children 18 months of age (for staging details see text and Monclair et al\textsuperscript{14}); EFS, event free survival. Figure adapted from Cohn et al.\textsuperscript{13}
Chapter 1

The risk stratification used by the Dutch Childhood Oncology Group (DCOG) Neuroblastoma Disease Committee is identical to that of the German Pediatric Oncology and Hematology group (GPOH) (Fig. 3).

**Genetics**

Neuroblastoma is a very heterogeneous disease and genetic aberrations and mutations have been shown to predict clinical behavior and outcome.\(^1\)\(^6\), \(^1\)\(^7\) Some of these aberrations have been designated to be prognostic tumor markers: *MYCN* amplification, DNA-ploidy, chromosome 1p loss of heterozygosity, chromosome 11q loss, and chromosome 17q gain.\(^1\)\(^6\), \(^1\)\(^8\)-\(^2\)\(^3\) Chromothripsis, massive genomic rearrangements, have been associated with unfavorable prognosis.\(^2\)\(^4\) In addition, structural aberrations in neuritogenesis genes have been found in high-stage tumors.\(^2\)\(^4\)

Amplification of the oncogene *MYCN* is one of the most important genetic abnormalities and associated with advanced disease and bad outcome.\(^2\)\(^5\), \(^2\)\(^6\) Because of its prognostic impact, *MYCN* amplification is used in most risk stratification protocols (Fig. 2 & 3). *MYCN* amplification is found in 30% to 40% of stage 3 and 4 neuroblastoma and in only 5% of localized or stage 4s neuroblastoma.\(^2\)\(^7\), \(^2\)\(^8\) In neuroblastoma, hyperdiploidy of tumor cells is associated with a better response to chemotherapy than diploidy.\(^2\)\(^9\) Loss of tumor suppressor regions has been reported in neuroblastoma with the most frequently affected regions located on the chromosome arm 1p and 11q; both are associated with bad outcome. In almost all tumors with *MYCN* amplification, chromosome 1p is lost, but loss of 1p also occurs in *MYCN* single-copy cases. Loss of chromosome 11q is inversely correlated with *MYCN* amplification.\(^3\)\(^0\)-\(^3\)\(^2\) 17q arm is gained in almost all high grade neuroblastoma.\(^3\)\(^3\)
Mutations of anaplastic lymphoma kinase (ALK) gene were found in 6-10% of neuroblastoma and are thought to have an activating function, and are associated with an unfavorable prognosis. ALK mutations account for most cases of hereditary neuroblastoma, although they are also observed in sporadic cases. Germline mutations, such as the PHOX2B oncogene mutation, are associated with cases in which neuroblastoma appears in combination with other congenital diseases such as M Hirschsprung or central hypoventilation syndrome.

Prognosis and treatment

Prognosis strongly depends on the risk stratification; the 5 years event-free survival rate is >85%, 75-85%, 50-75%, and 65% in the very low, low, intermediate, and high risk group, respectively. Spontaneous remission is possible in stage 4s (see Fig. 3). Despite improvements in the identification of the clinical, biological, and genetic parameters that are associated with high-risk disease at diagnosis, there have only been modest advancements in therapeutic efficacy for high risk patients the last decades.

In general, treatment protocols are divided into three different phases, namely; induction of remission, consolidation of remission, and a maintenance phase to eradicate minimal residual disease (MRD). The DCOG-treatment regimen is based on the GPOH-treatment strategy. The schedule for high risk patients is shown in Fig. 4. The medium risk protocol is the same as the high risk protocol without upfront MIBG therapy and without high-dose chemotherapy with stem cell transplantation. In low risk patients, a wait-and-see strategy is followed. However, when necessary (e.g. tumor progression, organ dysfunction) mild chemotherapy is applied.

As neuroblastoma arises from the sympathetic nervous system, the majority of neuroblastoma display the norepinephrine transporter. Meta-Iodobenzylguanidine (MIBG) is a neurotransmitter analogue that can be radioactively labeled with $^{131}$I for treatment.

**Figure 4.** Overview of neuroblastoma high risk treatment protocol S = surgery, N5 (cisplatin, etoposide, vindesine), N6 (vincristine, dacarbazine, ifosfamide, doxorubicin) = chemotherapy cycles, MIBG = $^{131}$I-MIBG treatment, MEGA +ASCT = myeloablative high-dose chemotherapy (melphalan, etoposide, carboplatin) with autologous stem cell transplantation, 13-cis-RA = 13-cis-retinoic acid, 6 cycles, followed by 3 months break and 3 more cycles.
or with $^{123}$I for diagnostics. The radiopharmacon shows uptake in 90% of neuroblastoma. The Dutch Childhood Oncology Group included two radioactive labeled $^{131}$I- MIBG treatments upfront in the induction treatment protocol for high risk patients. In different studies high-dose chemotherapy showed to be beneficial compared to maintenance or induction chemotherapy in high risk neuroblastoma. This is now part of the standard treatment protocol for high risk patients. The response to induction therapy is correlated with the longterm outcome.

Treatment during the maintenance phase consists in the application of the differentiating agent 13-cis-retinoic acid (13-cis-RA) with the intent of eradicating minimal residual disease (MRD). In the 1980s, research suggested that the tumor specific GD2 might be a target for therapy with GD2-antibody in neuroblastoma. Recently, the Children’s Oncology Group (COG) published their positive results on the combination of 13-cis-RA and immunotherapy with ch14.18, a monoclonal antibody against the tumor specific GD2. In patients who were in complete remission after induction phase and autologous stem cell transplantation (ASCT) this immunotherapy improved the event-free and overall survival with 43% (measured 2 years after treatment). This therapy is only performed in the USA; patients from the Netherlands are referred for this treatment.

Over half of the high risk patients thought to be in complete remission eventually relapse. Until now, cure is extremely rare for relapsed neuroblastoma. Patients who appear to be cured during initial therapy can subsequently develop long-term sequelae related to treatment; including hearing loss, cardiac dysfunction, infertility, and second malignancies. At present, different new therapeutical strategies are being tested, amongst others a group of small molecules, which are medicines targeting tumor specific genetic aberrations. There is a tendency to focus on more personalized approaches to treatment.

**Oxidative stress**

**Oxidative stress in general**

Oxidative stress is defined as the result of an imbalance between the production rate of reactive oxygen species (ROS) and the antioxidant defense capacity. Oxidative stress can result from either diminished levels of antioxidants, or increased production of ROS. The consequences of oxidative stress can include adaptation by up-regulating the defense system, cell injury, or cell death. ROS might react with biological molecules and thereby leave its “fingerprint”. The three main classes of macromolecules which have been identified representing this fingerprint are lipids, DNA, and proteins.
ROS are generated in all cells by mitochondrial respiration and redox enzymes. Oxygen reduction by the mitochondrial respiratory chain, as well as metal-ion-catalyzed reactions, generates a wide diversity of highly reactive metabolites of oxygen and nitrogen. These products mainly include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO·), which can be formed either from O$_2^-$ and H$_2$O$_2$ (Haber–Weiss reaction) or from a metal ion (Fe$^{2+}$, Fe$^{3+}$) and H$_2$O$_2$ (Fenton reaction). Oxidative stress in general is associated with aging and pathologic conditions such as cancer and neurodegenerative disease.

### Oxidative stress in cancer

Oxidative stress has been shown to play a major role in the genesis, progression, and malignancy of a number of cancers. Current evidence supports the hypothesis that cancer cells are characterized by enhanced ROS generation. Although the exact mechanism of induced ROS is not clear, several lines of evidence suggest that this ROS production is induced after the expression of genes associated with tumor transformation, such as Ras, Bcr-Abl, and c-Myc; resulting in a state of perpetually elevated stress. Additionally, deregulation of antioxidant enzymes was observed (reviewed in). The increase of ROS may promote cell proliferation and survival. It has been established that increased ROS may lead to the activation of the phosphoinositide 3-kinase (PI3K)/AKT survival pathway, which reflects a cellular response to the ROS-induced stress in the attempt to survive the drug insult. However, when the increase of ROS reaches a certain level (the threshold), it may overwhelm the cellular antioxidant capacity and trigger the cell-death process (Fig 5). Therefore, cells with higher basal ROS generation seem to be more dependent on the antioxidant system and more vulnerable to further oxidative stress. A further increase of oxidative stress by using exogenous ROS-generating agents or drugs
that disable the endogenous antioxidant system may preferentially increase ROS above the threshold, leading to cell death. In contrast, normal cells may be better able to tolerate such exogenous oxidative stress because of their low basal ROS outputs and normal metabolic regulations.\textsuperscript{67, 69} Because cancer cells exhibit increased ROS compared with normal cells, this redox difference provides a biochemical basis for the development of new therapeutics with high selectivity.\textsuperscript{67}

**Fenretinide in cancer**

Fenretinide is frequently mentioned for its detrimental effect on tumors. It is this aspect of the drug that will be discussed in this chapter. Other important features, such as its anti-inflammatory effects\textsuperscript{70, 71} and its propensity to prevent both obesity and insulin resistance\textsuperscript{72} will not be discussed here.

**Retinoids**

It has been established that retinol mainly controls epithelial differentiation. Experiments performed in the 1970s on vitamin A deficient patients with keratinized epithelia demonstrated that retinol administration leads to skin normalization and cell differentiation. Since most cancers are epithelia-derived carcinomas, vitamin A was taken into consideration as a potential anti-tumor agent.\textsuperscript{73} Retinoids include active metabolites of vitamin A (retinol) as well as synthetic derivates and are essential regulators of cell growth, differentiation, and cell death. Retinoids restore regulation of differentiation and growth in some premalignant and malignant cells \textit{in vitro} and \textit{in vivo}. This observation has led to their development as chemopreventive drugs.\textsuperscript{74-77} The anti-tumor effects of retinoids were first described in the 1970s in a bladder cancer rodent model.\textsuperscript{78} The retinoids \textit{all-trans}-retinoic acid (ATRA) and \textit{13-cis}-RA have been shown to induce differentiation and growth inhibition in neuroblastoma cells through its interaction with retinoid receptors.\textsuperscript{47, 79} Dose-limiting toxicity of \textit{13-cis}-RA has been observed and includes hypercalcemia and combinations of skin, gastrointestinal, and hematopoietic toxicities. Prevalence of advanced bone age in a cohort of neuroblastoma patients who received \textit{13-cis}-RA was also described.\textsuperscript{79, 81} Treatment with \textit{13-cis}-RA has been shown to increase the 3 year event-free survival in children with advanced-stage neuroblastoma.\textsuperscript{45} Therefore, treatment with \textit{13-cis}-RA is now standard therapy in treatment of medium and high risk neuroblastoma, as described above. It should be noted that retinoic acid abolishes the cytotoxic effects of some chemotherapeutics.\textsuperscript{82}
**Fenretinide**

Fenretinide (N-(4-hydroxyphenyl) retinamide) (4-HPR) is a synthetic retinoic acid derivative which, unlike 13-cis-RA, induces apoptosis rather than differentiation in a variety of tumor cell models and xenografts. Clinically, fenretinide is well tolerated and it is currently used in clinical trials for neuroblastoma and other tumors. A significant factor in its development for use in neuroblastoma therapy is that fenretinide is synergistic regarding cytotoxicity in combination with some chemotherapeutic drugs.

**Apoptosis induction**

The exact mechanism underlying the apoptosis-inducing properties of fenretinide is not yet fully understood. There is evidence that fenretinide induces apoptosis through both retinoic acid receptor (RAR)-dependent and reactive oxygen species (ROS)-dependent pathways. These will be discussed in turn.

**Retinoic acid receptors**

Fenretinide is a selective activator of retinoic acid receptors, in particular RAR-β and RAR-γ. RAR-β stimulates the induction of cell-cycle inhibitors and thus proliferation inhibition. The RAR activation by fenretinide is 10 times less than that by retinoic acid. The RAR-mediated pathway, however, might be important in fenretinide-induced apoptosis in neuroblastoma. Nevertheless, other studies in many cell systems, including neuroblastoma, indicate that apoptosis in response to fenretinide seems to be RAR-independent.

**Reactive oxygen species**

The induction of ROS by fenretinide might be the critical factor underlying the ability of fenretinide to induce apoptosis. The effector mechanisms and sources of fenretinide-induced ROS are not yet fully elucidated. There is evidence that the endoplasmic reticulum and/or the mitochondria might play a role in this ROS induction.

It has been suggested that oxidative stress induced by fenretinide in neuroblastoma is mediated by increased 12-lipoxygenase (12-LOX) activity. Downstream of 12-LOX the induction of growth-and-DNA-damage is a consequence of ROS. The subsequent induction of the pro-apoptotic protein Bak accompanies mitochondrial cytochrome c release and caspase-3-dependent apoptosis. Induction of the lipid second messenger ceramide has been reported to be the result of fenretinide-induced ROS production (Fig. 6). It has been suggested that up-regulation of the ceramide-ganglioside signaling pathway might lead to induction of endoplasmic reticulum (ER)-stress. The increase of ceramide...
Chapter 1

The production of ROS leads to mitochondrial cytochrome c release followed by caspase activation and apoptosis induction. There is also evidence that ROS leads to ceramide increase followed by apoptosis induction.

Figure 6. Proposal of the mechanism of action of fenretinide induced apoptosis in neuroblastoma cells. The production of ROS leads to mitochondrial cytochrome c release followed by caspase activation and apoptosis induction. There is also evidence that ROS leads to ceramide increase followed by apoptosis induction.

after fenretinide treatment in neuroblastoma might promote cell death by a combination of apoptosis and necrosis through p53-independent pathways. The pro-death c-Jun N terminal kinase (JNK) is also activated by sphingosine-1-phosphate activation of extracellular signal regulated kinases (ERK1/2) as a result of ceramide increase. Thus, this activation of JNK has been implicated in fenretinide-induced apoptosis.

It has been described, in ovarian cancer cells, that fenretinide enhances AF1q expression levels. This upregulation is an important event in the fenretinide signalling cascade that leads to apoptosis through ROS generation, ER-stress response, and JNK activation.

Other evidence suggests that in leukemia cells not ceramide but dihydroceramide is induced after fenretinide treatment, the role of dihydroceramide has not yet been elucidated.

Some members of the Bcl2 family (BBC3 also known as PUMA) are key regulators of apoptotic pathways, they are influenced by ER stress and conversely can regulate both ER stress-induced apoptosis and mitochondrial mediated apoptosis (for the latter see below). In neuroblastoma, an early induction of BBC3 has been observed in fenretinide treated cells. Furthermore, fenretinide activated the NFkB pathway, this pathway is also implicated in ER-stress-induced apoptosis.

The major cellular sources of ROS are the mitochondrial respiratory chain, ROS-generating enzymes such as xanthine oxidase and NADPH oxidase, and the phospholipase A2-activated arachidonic acid metabolism. The inability of inhibitors of xanthine oxidase, NADPH oxidase, and phospholipase A2 to suppress fenretinide-induced ROS generation excludes these ROS-producing systems from being major sources of fenretinide-induced ROS in cervical cancer cells. As oxidative stress-inducing agents have
been shown to increase the permeability of the inner mitochondrial membrane and antioxidants effectively prevent mitochondrial membrane permeabilization, it is to be expected that there is a relation between ROS production, mitochondrial membrane permeabilization, and apoptosis.\textsuperscript{119-122} This permeabilization of the inner mitochondrial membrane is considered to be one of the mechanisms by which proapoptotic proteins are released from mitochondria, including cytochrome $c$ and apoptosis-inducing factor (AIF).\textsuperscript{123} Cytochrome $c$ has been demonstrated to be involved in the activation of a caspase cascade (Fig. 6), whereas AIF has been shown to directly trigger apoptosis.\textsuperscript{118} Increase in the mitochondrial membrane permeability, release of cytochrome $c$, and induction of the intrinsic apoptosis pathway have been established as key events in fenretinide-induced cell death in some cells.\textsuperscript{124-126} The initial evidence for involvement of caspases in fenretinide-induced apoptosis includes the activation of the effector caspase-3, which results in cleavage of poly (ADP-ribose) polymerase (PARP), caspase-8 and caspase-9.\textsuperscript{118, 127, 128}

**Other anti-tumor effects of fenretinide**

Apoptosis induction is not the only mechanism through which tumor growth in cancer is inhibited. Here some other fenretinide-induced processes resulting in inhibition of tumor growth will be discussed briefly.

**Inhibition of angiogenesis**

Angiogenesis, the neoformation of vessels, is an important phenomenon facilitating primary tumor growth and metastatic dissemination to distant sites. Several factors contribute to the enhanced capacity to form new vessels, leading to extensive tumor cell survival and tumor expansion.\textsuperscript{129} Anti-angiogenic strategies mainly aim to inhibit vessel construction in its early stages and/or to normalize newly formed vessels.\textsuperscript{130} This activity is associated with mechanisms directly targeting endothelial cell functions or interfering with the production, release and activation of specific growth factors and cytokines regulating the angiogenic process, thus creating local conditions in the micro-environment unfavorable to new vessel formation.\textsuperscript{73} Experimental evidence indicates that fenretinide might also be active because of an anti-angiogenic effect.\textsuperscript{73, 131} Inhibition of vascular endothelial growth factor (VEGF) and fibroblast growth factor was observed in neuroblastoma treated with fenretinide, resulting in anti-angiogenic effects.\textsuperscript{131, 132}
Chapter 1

Inhibition of tumor invasion

The ability of cancer cells to metastasize to distant organs and to establish new sites of tumor formation by locally altering the tissue microenvironment poses a great obstacle to the treatment of malignant cancer. The metastatic process crucially requires cell motility into and out of blood or lymphatic vessels and invasion of basement membranes.\(^7\) There is evidence that tumor invasion is inhibited by fenretinide in different tumors.\(^13\) It has been reported that inhibitory effects of fenretinide on the formation of macro- and micro-metastasis in an \emph{in vivo} metastatic model of neuroblastoma was the result of fenretinide incorporation in GD2-targeted immunoliposomes.\(^13\)

Clinical trials in neuroblastoma

Several clinical trials have been performed to evaluate the activity and efficacy of fenretinide on different malignancies, both in prevention and therapeutic settings. A list of ongoing trials can be found on www.clinicaltrials.gov. In phase I trials in neuroblastoma the major clinical dose-limiting side effect of fenretinide is reversible night blindness (nyctalopia) resulting from a decrease in circulating retinol levels and its specific transport protein (retinol-binding protein (RBP4)).\(^91\) Drug plasma levels of fenretinide are comparable with apoptosis inducing concentrations in cell lines. Prolonged disease stabilization and regression have been reported.\(^88,\) \(^13\) In a phase II trial, data suggested that fenretinide is active against neuroblastoma. The capsule formula used in this trial, however, is suboptimal due to poor bioavailability. Moreover, due to the large amount of capsules the treatment was challenging to administer to children.\(^13\)

Bioavailability and plasma levels

Thus, it is necessary to improve bioavailability to establish the role of fenretinide therapy in high risk neuroblastoma. As fenretinide is a hydrophobic compound, it is generally recommended to administer the drug with high fat meals to increase its bioavailability.\(^13\) Fenretinide cannot easily cross the intestinal membrane and, therefore, has a limited oral bioavailability.\(^13,\) \(^14\) \(^11\) This phenomenon has hampered its clinical assessment. Pharmacokinetics of fenretinide confirmed that steady-state drug concentrations are achievable in the range associated with \emph{in vitro} activity.\(^13\)

Several formulas of fenretinide are being tested in different tumors \emph{in vitro} and \emph{in vivo}. In general, all those formulas are based on the idea that, because of its hydrophobic character, fenretinide should be packaged in a liposomal structure in order to optimize pharmacokinetics and feasibility of administration.\(^14\) For example, fenretinide packaged
in LYM-X-SORB (LXS), a lipid matrix technology powder, is tolerated without dose-limiting toxicity while higher plasma levels are achieved (New Approaches in Neuroblastoma Therapy-trial (NANT)). An intravenous fenretinide emulsion formulation is also being tested in ongoing adult cancer trials and a pediatric neuroblastoma (NANT) trial. Results from these studies will help to determine whether higher fenretinide plasma levels are tolerable and anti-tumor activity is thereby improved.

Another strategy is to combine fenretinide with a compound that enhances its plasma levels, as is performed with a combination of ketoconazole and fenretinide in mice. It has been suggested that inhibition of the hepatic cytochrome p450 enzyme CYP by ketoconazole results in increased systemic fenretinide levels. At normal clinical doses the combination with fenretinide may increase systemic exposure to fenretinide in humans. Therefore, treatment with ketoconazole is being combined with fenretinide/LXS oral powder in an ongoing phase I trial.

**Combination therapy**

**In general**

Because combining chemotherapeutic agents is an established way to improve drug-efficacy, combinations are now the standard treatment of metastatic cancer, including neuroblastoma. The triad “synergism – additivity – antagonism” is used to describe the effects of combination treatment compared to that observed for the monodrugs. Although a number of definitions for these terms are used, the most cited definition and the most used method to evaluate synergism is that of Chou and Talalay. The favorable outcomes of synergism are 1) increasing the efficacy of the therapeutic effect, 2) decreasing the dosage but increasing or maintaining the same efficacy to avoid toxicity, 3) minimizing or slowing down the development of drug resistance, and 4) providing selective synergism against target (or efficacy synergism) versus host (or toxicity antagonism). Additivity and antagonism are generally less prominent in the literature. Additivity refers to outcomes where the combined effect is the sum of both effects taken separately; antagonism means that the combined effect is less than the sum of each (and possibly less than either separately).

**Fenretinide in combination with other drugs in neuroblastoma**

Numerous studies are testing fenretinide in combination with other anti-tumor compounds in order to synergistically increase its cytotoxic activity. Here, a distinction can be made between studies testing experimental drugs and studies testing drugs that are currently part of regular treatment protocols. According to the latter, synergism has been
observed when fenretinide is combined with cisplatin, etoposide, or carboplatin. Though the exact mechanism is unclear, the generation of free radicals by fenretinide might be the key property causing the synergistic response.\textsuperscript{160} Fenretinide and vincristine and cisplatin have been tested in a xenograft neuroblastoma model. The combination of fenretinide with those chemotherapeutics does not support earlier data suggesting favorable effects of these combinations.\textsuperscript{161, 162} The combination of fenretinide and cisplatin has also been tested in ovariun cells, which resulted in synergistic growth inhibition.\textsuperscript{163} Subsequently, in a phase I trial performed on adults with different tumors, the combination of fenretinide with cisplatin and paclitaxel has been tested. The study demonstrated that it is safe and feasible to combine fenretinide with those chemotherapeutics.

**Modulation of ROS induction**

This thesis further investigates whether the effects of fenretinide can be increased by modulating its ROS-inducing capacities. Two existing strategies show promise, inhibition of glutathione (GSH) and Hsp90 respectively. They will be discussed briefly in turn.

Cellular antioxidants protect cells from ROS-induced damage. GSH is an important cellular reducing agent.\textsuperscript{164} Buthionine Sulfoximine (BSO) is a strong inhibitor of \( \gamma \)-glutamylcysteine synthetase (\( \gamma \)-GCS), which is the rate-limiting enzyme in the production of GSH, both \textit{in vivo} and \textit{in vitro}.\textsuperscript{165, 166} In some studies the cytotoxic effects of BSO have been attenuated by antioxidants.\textsuperscript{166} In a phase I trial with BSO, performed on patients with various tumors, only minimal and no dose-limiting toxicity was observed. A significant decrease of GSH, measured in peripheral mononuclear cells, was achieved in patients receiving BSO.\textsuperscript{165}

Another site for modulation of the ROS inducing effect is heat shock protein 90 (Hsp90), a chaperone protein involved in maintaining the conformation, stability, activity, and cellular localization of several key oncogenic client proteins. Many of these client proteins are regulatory proteins or perform key functions in proliferation or modulation of apoptosis.\textsuperscript{167, 168} Hsp90 activates the associated client protein to either bind ligand or to be phosphorylated during signal transduction, as has been described for AKT.\textsuperscript{169} Inhibition of Hsp90, therefore, should abrogate the AKT survival pathway and sensitize cancer cells to certain anticancer agents that may otherwise activate the AKT protective mechanism.\textsuperscript{170, 171} Several recent studies suggest that human cancer cells are very sensitive to inhibition of Hsp90, because Hsp90 is overexpressed in various tumor cells, including neuroblastoma.\textsuperscript{172} 17-allylamino-geldanamycin (17AAG) exerts its antitumor effect by binding the N-terminal ATPase domain of Hsp90 to inhibit its chaperone function.\textsuperscript{170} Preclinical tests have shown that 17AAG is non-toxic to normal cells, even though it is highly active against tumor cells.\textsuperscript{171, 173} Cytostasis, cell-cycle arrest,
and apoptosis as a result of inhibition of RAS-RAF-pathway and PI3K/AKT-pathway have been observed following 17AAG incubation in colon carcinoma and leukemia.\textsuperscript{174, 175} Furthermore, 17AAG shows a potent synergistic toxicity when combined with other chemotherapeutics.\textsuperscript{175-183} This highlights the potential use of 17AAG as single agent and as a sensitizer.

**Aim and scope of this thesis**

Despite intensive therapy, the prognosis for high risk neuroblastoma is still poor. New therapeutic options are needed to improve the outcome of high risk patients. Fenretinide is a promising new therapy currently tested in different tumors, including neuroblastoma. The aim of this thesis is to unravel the apoptosis-inducing mechanism of fenretinide in neuroblastoma and to identify targets for combination therapy.

In chapter 2 we describe the cytotoxic effects, growth inhibition, and apoptosis of fenretinide on a panel of neuroblastoma cells, monolayers, and spheroids. Additionally we describe our findings regarding ROS generation and mitochondrial membrane depolarization. Chapter 3 develops an in depth analysis of the mechanism of action of fenretinide, illustrated by the inhibition of the mitochondrial respiratory chain at the level of complex II by fenretinide. In chapter 4 we report the effects of fenretinide on the unique mitochondrial phospholipid cardiolipin. In chapter 5 we investigate the effect of the combination of fenretinide and BSO on GSH levels, antioxidant enzyme levels, ROS production, and viability in neuroblastoma in order to increase the efficacy of fenretinide regarding ROS-production. In chapter 6 we present our findings on the combination of fenretinide with Hsp90 inhibitor 17-AAG. Chapter 7 provides a short summary and discusses the implications of the results for future clinical application and research.
Chapter 1

References


General introduction


70 H. Yu, M. Valerio, and J. Bielawski, Fenretinide inhibited de novo ceramide synthesis and proinflammatory cytokines induced by Aggregatibacter actinomycetemcomitans. J. Lipid Res. 54 (2013) 189-201.


Chapter 1


107 A.L. Anding, J.S. Chapman, D.W. Barnett, R.W. Curley, Jr., and M. Clagett-Dame. The unhydrolyzable fenretinide analogue 4-hydroxybenzylretinone induces the pro-apoptotic genes GADD153 (CHOP) and Bcl-2-binding component 3 (PUMA) and apoptosis that is caspase-dependent and independent of the retinoic acid receptor. Cancer Res. 67 (2007) 6270-6277.


Chapter 1


125 N. Hail, Jr. and R. Lotan, Mitochondrial respiration is uniquely associated with the prooxidant and apoptotic effects of N-(4-hydroxyphenyl)retinamide. J. Biol. Chem. 276 (2001) 45614-45621.


