Modulation of fenretinide induced cell death in neuroblastoma
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Summary and future perspectives
Chapter 7
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Neuroblastoma is the most common solid extracranial tumor in children. The prognosis for children suffering from neuroblastoma is highly dependent on their age at the time of diagnosis and the stage of the disease. Current therapy is based on treatment with $^{131}$I-labeled Meta-iodobenzylguanidine ($^{131}$I-MIBG), chemotherapeutic drugs, surgery, and autologous stem cell transplantation (ASCT). The introduction of immunotherapy with the tumor specific disialoganglioside (GD2)-antibody improved the event free survival of high risk patients who were in complete remission after induction phase and ASCT with 20% (at 2 years).1-3 Despite such aggressive therapeutic strategies the prognosis for patients suffering from high risk neuroblastoma is still poor.4,5 Therefore, new and effective therapeutic strategies are needed. In this thesis, we investigated the possible role for the synthetic retinoid fenretinide in the treatment of neuroblastoma. In this chapter, the results of our research are summarized, followed by final conclusions and suggestions for further research and clinical practice. For the specific interpretations, limitations and conclusions of particular investigations, reference is made to the corresponding chapters.

Summary

Cytotoxic effects

Chapter 2 describes the various effects of fenretinide on a panel of neuroblastoma cell lines, comprising MYCN single copy and MYCN-amplified cell lines. Loss of cell viability and induction of apoptosis was observed in all cell lines with no apparent cell cycle arrest. The effect of fenretinide was also tested on spheroids, which is a tumor model of the avascular state of metastases. Both the observed cytotoxic effects of fenretinide on avascular spheroids and the anti-angiogenesis effect of fenretinide6,7 suggest that fenretinide might be an effective chemotherapeutic drug for the treatment of micrometastases. The effective concentrations of fenretinide inducing cytotoxicity and apoptosis were comparable with achievable plasma concentrations in vivo.8,9 Reactive oxygen species (ROS) production was observed already after 1 hour of fenretinide incubation. The anti-oxidant Trolox was able to prevent the accumulation of ROS and the loss of viability due to fenretinide in five out of six cell lines. Therefore, our results suggest that the production of ROS is one of the main apoptosis inducing mechanisms in neuroblastoma cell lines treated with fenretinide. The major cellular source of ROS is the mitochondrial respiratory chain.10 Since the generation of ROS by fenretinide proved to be very fast it is conceivable that fenretinide interferes directly with the mitochondrial respiratory chain.11 In line with this hypothesis is the fact that a profound decrease in the
mitochondrial membrane potential ($\Delta \psi_m$) was observed after incubation with fenretinide. This decrease in the $\Delta \psi_m$ might lead to cytochrome c release, which in turn will activate caspase 9 to initiate the downstream processes of apoptosis.

Chapter 3 describes an in-depth analysis of the role of mitochondria in fenretinide-induced ROS formation and the effects of fenretinide on the mitochondrial respiratory chain. In this chapter we demonstrated that fenretinide-induced ROS is of mitochondrial origin. With a specific mitochondrial ROS probe we detected ROS of mitochondrial origin. Subsequently, we inhibited this production with a specific mitochondrial antioxidant. Furthermore, the fact that Rho zero osteosarcoma cells (cells without a functional mitochondrial respiratory chain) were unable to produce as much ROS as their control cells following fenretinide incubation indicates that for ROS production to occur, a functional mitochondrial respiratory chain is required. We investigated the effect of fenretinide on the mitochondrial respiratory chain. Our results demonstrated a major role for complex II in the generation of ROS, because ROS production was reduced by the complex II inhibitors Carboxin and thenoyltrifluoroacetone (TTFA). An analysis of the electron flux through the mitochondrial respiratory chain in situ was performed. This in situ analysis showed a strong concentration-dependent decrease in ATP synthesis in mitochondria selectively respiring on either the complex I substrate malate (plus glutamate) or the complex II substrate succinate (plus rotenone). Parallel measurement of either the complex I-mediated production of aspartate (from malate) or the complex II-mediated production of malate (from succinate) showed a decrease in synthesis of both products. Combining fenretinide with the uncoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) demonstrated that the inhibiting effect did not take place at complex V, as the inhibition in aspartate and malate synthesis by fenretinide was not alleviated by CCCP. These data suggest that fenretinide inhibits the mitochondrial respiratory chain between Co-enzyme Q and complex IV (cytochrome c oxidase) (Fig. 1).

Depolarization of the mitochondrial membrane potential was not attenuated by antioxidants, nor was the inhibition of fenretinide on the mitochondrial respiratory chain scavenged by Trolox. This indicated that in neuroblastoma cells the inhibition of the mitochondrial respiratory chain and the mitochondrial depolarization do not lead to ROS production, suggesting these two mechanisms of fenretinide might act independently. At low concentrations, the cytotoxicity of fenretinide was exerted through the generation of mitochondrial ROS at complex II, whereas high concentrations of fenretinide inhibited the mitochondrial respiratory chain. In addition, this inhibition of the mitochondrial respiratory chain coincided with the disappearance of the mitochondrial membrane potential.
Oxidation of the specific mitochondrial phospholipid cardiolipin (CL) by fenretinide has been suggested, resulting in inhibition of complex IV of the mitochondrial respiratory chain, and possibly triggering cytochrome c release. In line with the knowledge that cardiolipin peroxidation can take place as a result of mitochondrial induced ROS-production and that the subsequent release of cytochrome c would initiate the apoptosis cascade, we analyzed cardiolipin peroxidation in neuroblastoma, as described in chapter 4.

After incubating neuroblastoma cells with fenretinide and H₂O₂ we did not identify any oxidized CL. As enrichment of the cardiolipin with poly unsaturated fatty acid acyl chains might lead to increased peroxidation, we successfully enriched our cells with the poly unsaturated fatty acid acyl chain, linoleic acid (18:2). However, this enrichment did not lead to CL-oxidation in neuroblastoma cells either. In contrast to what others reported we concluded that in our neuroblastoma cells and in our leukemia cell line no oxidation of cardiolipin was apparent. Surprisingly, monolysocardiolipin (MLCL) was identified in the CL spectra after incubation with fenretinide. The increase of MLCL might reflect a disturbed mitochondrial homeostasis. Additionally, the decrease in CL content may be mediated in part by degradation to MLCL. This might be of importance, because MLCL migrates towards the outer membrane, likely via contact sites where it interacts with pro-apoptotic peptides such as Bax and tBid.
Modulation of the ROS-inducing effect

As described above, fenretinide-induced apoptosis is the result of ROS induction. Therefore, the cellular redox state, reflecting the intracellular antioxidant defense, might be a key-factor in protecting the cells from ROS-induced cytotoxicity and surviving the drug insult. Manipulation of this protection mechanism could be a way to enhance the cytotoxicity induced by fenretinide. In chapter 5, we investigated the induction of ROS by fenretinide, while limiting the capacity to scavenge this ROS by reducing the levels of glutathione (GSH), an important cellular antioxidant, with buthionine sulfoximine (BSO). This resulted in an increased cytotoxic effect of the fenretinide-BSO-combination in ROS-producing cell lines. A moderate upregulation of catalase was observed after incubation with the fenretinide-BSO-combination. Nevertheless, this upregulation was not sufficient to protect neuroblastoma cells against the cytotoxic effects of the combination of fenretinide and BSO. Other antioxidant enzyme activities (superoxide dismutase (SOD) and glutathione peroxidase (GPx)) were not affected by fenretinide-BSO-treatment. The beneficial effect of the fenretinide-BSO-combination was confirmed in spheroids by profound growth retardation, as a result of decreased proliferation and increased apoptosis. Overall, we concluded that the combination of fenretinide and BSO has a beneficial effect on cytotoxicity in neuroblastoma monolayers as well as spheroids of neuroblastoma cells with ROS producing properties. Since both fenretinide and BSO have been tested in clinical trials and no dose limiting toxicity has been observed,8, 25 the fenretinide-BSO-combination could be a potential combination therapy for neuroblastoma. In contrast to our results, recently published data suggests that in leukemia the antitumor effects induced by fenretinide are independent of glutathione modulation.26

Chapter 6 describes the investigation of the effects of the combination of fenretinide and Hsp90 inhibitor 17-allylaminogeldanamycin (17AAG) on cell lines and spheroids. Hsp90 is a molecular chaperone protein that is induced in response to cellular stress and stabilizes client proteins involved in proliferation and anti-apoptotic signaling. It is, for instance, involved in AKT activation after oxidative stress induction. All cell lines of our panel were sensitive to the cytotoxic effects of both fenretinide and 17AAG. The IC50 for 17AAG is lower than the achievable clinical plasma levels, measured in pediatric patients with various tumors, including neuroblastoma.27-30 The combination of both drugs appeared to be synergistic with respect to cytotoxicity in two out of four cell lines. In spheroids, profound growth retardation was observed in the presence of either 17AAG or fenretinide and increased growth retardation was observed when treated with the fenretinide-17AAG-combination. Hsp70 expression was strongly increased in a concentration dependent manner after treatment with 17AAG. The expression of Hsp70
was of particular interest since this molecular chaperone has a well-documented anti-apoptotic function and its increase may reduce 17AAG induced cytotoxicity. These increased levels of Hsp70 indicated induction of the cellular stress response following Hsp90 inhibition.

**General discussion and future perspectives**

As described in this thesis, fenretinide has a strong potency to induce apoptosis in neuroblastoma and an important mechanism underlying apoptosis induction by fenretinide is the production of mitochondrial ROS. By combining fenretinide with other potential drugs this ROS-inducing effect can be modulated in order to increase the cytotoxicity of fenretinide in neuroblastoma.

**Further elucidation of the apoptosis inducing mechanism**

There is now an increased awareness that tumor-initiating cells (TICs) are present in multiple malignancies, including neuroblastoma, which are responsible for sustained tumor growth, progression, relapse and metastases. An interesting feature of these TICs is that they seem to mirror more closely the phenotype and genotype of primary tumors compared to cultured cell lines. One of the reasons for the lack of efficacious treatments might be the inability of existing drugs to target these TICs. Because of the latter fact, it is important to know if fenretinide is active against those cells. Therefore, it might be interesting to investigate the effects of fenretinide in tumor initiating cells (TICs). It has been stated that cells might be resistant to fenretinide under hypoxic conditions and that this resistance might be the result of autophagy, which is induced in an attempt to survive the drug insult of fenretinide. There is ambivalence in the literature concerning the role of autophagy in cancer. Autophagy has been shown to play an important role in tumor suppression via the tumor suppression gene Beclin 1. On the other hand, autophagy can induce cell survival in response to metabolic stress. Pharmacological inhibition of autophagy was shown to enhance the cytotoxicity of chemotherapeutic agents and to promote tumor regression. It has been stated that drug interactions might differ in spheroids compared to monolayers, because of hypoxic conditions. It would be interesting to investigate whether fenretinide induces autophagy in our spheroid model and whether the combination of fenretinide with an autophagy inhibitor might be beneficial, in order to increase the cytotoxic effect of fenretinide.

**Enhancing the cytotoxic effect**

A potent mechanism of the apoptosis induction is the production of ROS and the
inhibition of the mitochondrial respiratory chain. Therefore, it would be interesting to investigate combinations of fenretinide with drugs that are involved in either of those mechanisms. Previously, it has been shown that MIBG induces inhibition of the mitochondrial respiratory chain at complex I. Furthermore, its efficacy might be enhanced by induction of oxidative stress, because it might lead to increased uptake of MIBG. In order to trigger this latter effect, it might be interesting to investigate MIBG in combination with fenretinide.

Investigating the combination of fenretinide with n3-polyunsaturated fatty acid (n3-PUFA) might be interesting as enrichment with n3-PUFAs causes replacement of the anti-oxidant arachidonic acid, which makes the cell more vulnerable for oxidative stress and apoptosis. This vulnerability is also caused by n3-PUFA’s downregulation of cyclooxygenase-2 (COX-2), which is often overexpressed in tumors and related to apoptosis resistance of cancer cells. Furthermore, long chain n3-PUFAs with 5 or 6 double bonds are easily oxidized, and n3-PUFA-derived peroxidation products are considered crucial for the cytotoxicity of these fatty acids for cancer cells and their ability to inhibit cancer cell growth. Moreover, their peroxidation may sensitize cells to oxidative stress. As fenretinide is known to induce ROS, it might increase the oxidation of n3-PUFA and thus its sensitizing effect. Both PUFAs and fenretinide have been shown to be active in vincristine resistant cell lines. In that respect it might also be interesting to investigate a combination of the two drugs.

Although results from xenograft models are not necessarily predictive of clinical efficacy, such models do overcome a number of limitations of the in vitro models used in our studies, including, to a varying extent, the impact of drug distribution and drug clearance. As there are a number of potential pitfalls when extrapolating in vitro data to the clinic, the data we presented on two ROS modulating combinations should be investigated in a xenograft model.

To determine its future role in treatment protocols, the combination of fenretinide with established chemotherapeutics should be investigated to identify favorable combinations. Some of those are already tested in neuroblastoma, amongst others cisplatinum, etoposide, carboplatin and vincristin. Fenretinide interacts synergistically with cisplatinum, etoposide and carboplatin. Nevertheless, vincristine and cisplatinum tested with fenretinide in a xenograft model did not confirm this synergistic effect. The question to address is whether combinational therapy of fenretinide with other standard chemotherapeutics will be potent in neuroblastoma, i.e. melphalan, doxorubicin, and the alkylators ifosfamide and dacarbazine. Recent evidence has highlighted the antagonistic effects of 13-cis-retinoic acid (RA) on the cytotoxic potential of chemotherapeutic agents including etoposide, topotecan, and doxorubicin in a panel of neuroblastoma cell lines. Thus, it
is important to investigate whether fenretinide could replace 13-cis-RA in order to overcome the antagonistic effect of 13-cis-RA with some standard chemotherapeutics. It has been suggested that fenretinide incorporated in anti-GD2 targeted immunoliposomes increases cytotoxicity in neuroblastoma cells and xenografts. This combination might be promising for high risk neuroblastoma patients.

**Implications for clinical use**

This study strongly suggests a role for fenretinide in the treatment of neuroblastoma. Fenretinide is a safe drug that only seems to induce reversible nightblindness; no other dose-limiting toxicity has been observed. The inhibitory concentrations in our study are comparable with achieved plasma levels in phase I trials. Because fenretinide is already being tested in phase III trials in different tumors, clinical use in neuroblastoma might be near. Investigations should be performed on how and when to incorporate fenretinide in the current treatment protocols. Data from a phase II trial suggested that fenretinide is active against neuroblastoma. The capsule formula used in this trial, however, is suboptimal due to poor bioavailability, which has prompted development of new formulas.

Regarding cell death, the results of fenretinide in neuroblastoma cells are positive. Because the fact that fenretinide has some sensitizing properties with other chemotherapeutics, there might be a place for fenretinide in the induction phase of the treatment protocols to enhance the effect of the standard chemotherapeutics. Since fenretinide is a mild drug, with no dose-limiting cytotoxicity, it might also be an excellent drug for prolonged treatment and thus might be useful in the maintenance phase. It is widely accepted that solid tumors require neo-vascularization for growth beyond a very small size (2–3 mm³) and for metastatic spread. Regarding the effectiveness of fenretinide in spheroids and its anti-angiogenesis effects, therapy with fenretinide could be especially effective against minimal residual disease (MRD).

As mentioned in the introduction, one of the developments in the treatment of neuroblastoma over the past 10 years has been the addition of 13-cis-RA for the treatment of high-risk disease. However, approximately 50% of the patients either do not respond or relapse after 13-cis-RA therapy. Neuroblastoma cells shown to be resistant to 13-cis-RA are sensitive to fenretinide. For this reason fenretinide has been incorporated in a phase II trial for treatment of MRD.

Pre-treatment of neuroblastoma cell lines with retinoic acid results in a significant decrease in the apoptotic response to fenretinide. This might be mediated by up-regulation of Bcl-2 and the inhibition of pro-apoptotic fenretinide signaling pathways by retinoic acid. The interaction between retinoic acid and fenretinide could have important
implications for the scheduling of fenretinide in therapeutic protocols for neuroblastoma. Additionally, cytotoxicity of fenretinide has been observed in cell lines of relapsing neuroblastoma after 13-cis-RA therapy. Fenretinide remained cytotoxic in cell lines that were resistant to all-trans-retinoic acid (ATRA), 13-cis-RA, and etoposide. This suggests that fenretinide might be active and thus useful in high risk neuroblastoma patients that are resistant to these therapies. Although the effects of fenretinide are promising in vitro, an important challenge to its successful transformation from bench to bedside is posed by its bioavailability.

**Implications for further research:**
The cytotoxic effects of fenretinide should be investigated in TICs, because those cells closely retained characteristics of its original tumors.
It must be established whether fenretinide induces autophagy in order to determine the benefits of combining fenretinide with an inhibitor of autophagy.
The combination of fenretinide and MIBG should be examined as a likely means to increase apoptosis by a strong induction of ROS, reported for both drugs.
The combination of fenretinide with n3-polyunsaturated fatty acid should be examined in vincristine resistant neuroblastoma cell lines.
Xenograft studies should be performed with the BSO-fenretinide and the 17AAG-fenretinide combination.
Further investigation of fenretinide with standard chemotherapeutics should be performed in order to establish a possible role for fenretinide in induction phase of current treatment protocols.

**Implications for clinical practice**
In short, fenretinide might be a promising drug for the treatment of high risk neuroblastoma: During the induction phase, in order to sensitize commonly used chemotherapeutics. During the maintenance phase, to prevent relapse, especially in tumors resistant to retinoic acid.
Fenretinide’s mild systemic toxicity suggests a promising role during maintenance therapy. First, however, the problems regarding bioavailability should be solved.
References


Chapter 7


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Summary and future perspectives


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Chapter 7


