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RETINOIDS IN EXPERIMENTAL NEUROBLASTOMA THERAPY

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To my beloved family

ABSTRACT

Retinoids are analogues of vitamin A, with documented activity against various malignant cell types. Neuroblastoma is a childhood tumour of the sympathetic nervous system that shows a complex clinical and biological heterogeneity, often with poor outcome despite intensive multimodal therapy.

The aim of the thesis was to investigate effects of retinoid treatment *in vitro* on human neuroblastoma cells, and *in vivo* on human neuroblastoma xenografts in nude rats. The ultimate goal was to find a new retinoid treatment for children with neuroblastoma.

Oral treatment with 9-*cis* RA *in vivo* resulted in a significant inhibition of neuroblastoma tumour growth, but with major toxic side effects. Further experiments showed that 9-*cis* RA might not be suitable for clinical use in children with neuroblastoma, because of its short half-life, low bioavailability and toxic profile in rats.

Ro 13-6307 was established to be a morphologically differentiating retinoid, able to reduce proliferation and induce G1 growth arrest in both MYCN amplified and non-amplified neuroblastoma cell lines *in vitro*. Further experiments showed that oral Ro 13-6307 could inhibit neuroblastoma tumour growth *in vivo* with limited toxicity. *In vitro* and *in vivo* results indicated that Ro 13-6307 was at least as effective as the clinically established retinoid 13-*cis* RA. These results demonstrate that Ro 13-6307 is a potential retinoid for clinical oral therapy of children with neuroblastoma.

Despite promising results demonstrating that fenretinide induces apoptosis in neuroblastoma cells *in vitro*, no significant reduction in neuroblastoma tumour growth was observed after oral treatment with fenretinide *in vivo*. Five different doses were evaluated, but no significant inhibiting effect on tumour growth or morphological changes were found in treated compared to untreated tumours. Other alternatives for fenretinide administration should be investigated in future experimental and clinical studies.

Proton magnetic resonance spectroscopy was found to be a suitable method for detecting metabolic alterations in neuroblastoma cells *in vitro* undergoing fenretinide-induced apoptosis. It was possible to monitor the kinetics in the treatment response and to distinguish between fenretinide-sensitive and -resistant cells. These findings suggest that proton magnetic resonance spectroscopy is a potential clinical non-invasive tool to monitor early tumour response to retinoid treatments.

In conclusion, retinoids were shown to inhibit growth of human neuroblastoma cells *in vitro* and *in vivo*, however the effect depends on the retinoid in use. Dosing, scheduling, and toxicity are important factors determining the therapeutic efficacy of retinoids *in vivo*. Ro 13-6307 may be a retinoid for future clinical therapy of children with neuroblastoma.

Key words: Vitamin A, retinoids, neuroblastoma, 9-*cis* RA, Ro 13-6307, differentiation, apoptosis, growth inhibition, 13-*cis* RA, fenretinide, proton magnetic resonance spectroscopy, pharmacokinetic.

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ABBREVIATIONS

^1H MRS	Proton magnetic resonance spectroscopy
AP	Alkaline phosphatase
ATRA	All- <i>trans</i> retinoic acid
AUC	Area under the time-concentration curve
BDNF	Brain-derived neurotrophic factor
Cl	Clearance
C_{max}	Concentration maximum
CRABP	Cellular retinoic acid-binding protein
CRBP	Cellular retinol-binding protein
FACS	Fluorescence activated cell sorting
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
INSS	International neuroblastoma staging system
LOH	Loss of heterozygosity
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
MTD	Maximum tolerated dose
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum bromide
PCD	Programmed cell death
RA	Retinoic acid
RARE	Retinoic acid response elements
RARs	Retinoic acid receptors α , β , γ
RBP	Retinol-binding protein
ROS	Reactive oxygen species
RXR _s	Retinoic X receptors α , β , γ
$T_{1/2}$	Elimination half-life
tCho	Total choline
TUNEL	Terminal deoxy-nucleotidyl transferase nick end labelling

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. Ponthan F, Borgström P, Hassan M, Wassberg E, Redfern CPF, Kogner P. The vitamin A analogues: 13-*cis* retinoic acid, 9-*cis* retinoic acid and Ro 13-6307 inhibit neuroblastoma tumour growth *in vivo*. *Medical and Pediatric Oncology* 36:127-131, 2001.
- II. Ponthan F, Kogner P, Bjellerup P, Klevenvall L, Hassan M. Bioavailability and dose-dependent anti-tumour effects of 9-*cis* retinoic acid on human neuroblastoma xenografts in rat. *British Journal of Cancer* 85:2004-2009, 2001.
- III. Ponthan F, Johnsen JI, Klevenvall L, Castro J, Kogner P. The synthetic retinoid Ro 13-6307 induces neuroblastoma differentiation *in vitro* and inhibits neuroblastoma tumour growth *in vivo*. *International Journal of Cancer*, in press, 104: 2003. Published online 15th of January 2003.
- IV. Ponthan F, Lindskog M, Karnehed N, Castro J, Kogner P. Evaluation of anti-tumour effects of oral fenretinide (4-HPR) in rats with human neuroblastoma xenografts. *Oncology Reports*, in press, 2003.
- V. Lindskog M, Ponthan F, Jarvet J, Karnehed N, Gräslund A, Kogner P. Biochemical responses to fenretinide treatment of neuroblastoma cells as monitored with proton magnetic resonance spectroscopy (¹H-MRS). Manuscript.

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INTRODUCTION

GENERAL INTRODUCTION

Neuroblastoma is a childhood tumour of the sympathetic nervous system with an extraordinary clinical and biological heterogeneity. The most favourable subset of this embryonic tumour may differentiate or regress due to apoptosis after no or minimal therapy. However, the majority of neuroblastomas are aggressive metastatic tumours with poor clinical outcome despite intensive multimodal therapy. The observation that neuroblastomas may occasionally spontaneously regress by differentiation and/or apoptosis to a benign tumour phenotype has generated considerable interest in agents able to modify these important biological processes.

Vitamin A and its analogues (retinoids) are involved in cell proliferation, differentiation and apoptosis of normal tissue during the embryonic development. Furthermore, retinoids can modulate these cellular processes in a variety of cancer cell types *in vitro*, including neuroblastoma.

In this thesis I have investigated treatment effects and toxicity profiles of several retinoids on neuroblastoma growth *in vitro* and *in vivo*. The intention was to find new treatment strategies for children with neuroblastoma who currently have low probability to be cured with the present treatment combination of cytotoxic drugs, irradiation and surgery.

CHILDHOOD CANCER IN GENERAL

Cancer in childhood is rare, but it is the leading cause of death from disease in children. The aetiology of childhood cancer is in many respects unknown and it differs from cancer in adults. In many neoplasms of adulthood, a multistep process has been shown to take place during tumour development and progression (reviewed in Hanahan and Weinberg 2000). In paediatric tumours, only few or even single initiation events, such as specific translocations and subsequent oncogene activation, or deletions leading to loss of tumour suppressor function, are responsible for tumour development. There are approximately 300 cases per year in Sweden, which means that one out of 450 children are diagnosed with a malignant disease before the age of fifteen. Among childhood cancer, leukaemia is the most common, comprising about 30% of the total number closely followed by brain tumours that accounts for almost 28% (Gustafsson et al, 2000), (Figure 1).

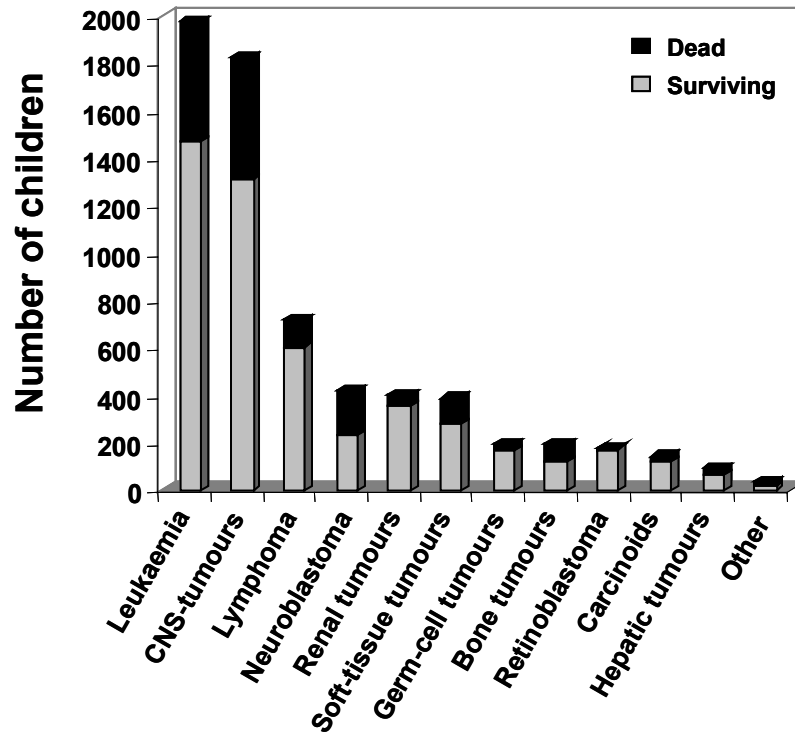


Figure 1. *Distribution of childhood cancer in the Nordic countries, 1985-1994.* 6559 children below the age of fifteen were followed for a maximum of 12.5 years. The upper part of the bar represents the number of deaths (n=1660) in each diagnostic group.

There has been a remarkable improvement in the over-all survival of childhood cancer during recent years, and today three out of four children are cured. However, there are malignancies such as neuroblastoma, medulloblastoma, osteosarcoma and Ewing's sarcoma where the probability of cure is below 60% (Gustafsson et al, 2000). Although, most children with cancer are cured, some of them suffer from harmful irreversible side effects caused by the current treatment that is a combination of chemotherapy, irradiation and surgery. Increased knowledge about cytotoxic drugs and their mechanism of action, the decreased use of irradiation and mutilating surgery minimise these late adverse effects. However, the importance of finding new therapies with less harmful side effects remains.

NEUROBLASTOMA

Neuroblastoma is the most common extracranial solid tumour in childhood cancer (Brodeur et al, 2000). It accounts for 6% of all childhood malignancies before the age of fifteen, which means approximately 15-20 cases of neuroblastoma per year in Sweden (Gustafsson et al, 2000). The disease is the most common cancer in infancy, with a peak of incidence during the first year of life where 50% of the neuroblastomas are diagnosed. The neuroblastic tumours, including neuroblastoma, ganglioneuroblastoma and ganglioneuroma, are derived from primordial neural crest cells, destined for the adrenal medulla and the sympathetic nervous system. The primary tumours of neuroblastoma most often arise in the adrenal medulla, or paravertebrally in the sympathetic trunk (Figure 2).

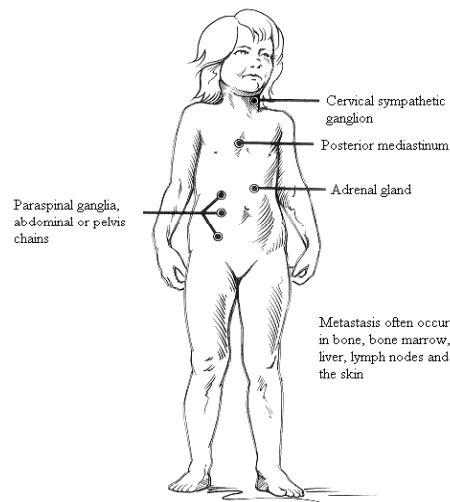


Figure 2. Common locations of neuroblastoma. Primary neuroblastoma tumours arise along the sympathetic nervous chain and in the adrenal glands.

At time of diagnosis neuroblastoma is staged according to the international neuroblastoma staging system (INSS), (Brodeur et al, 1993), staging 1-4 (Table 1). Neuroblastoma shows a wide diversity of clinical behaviour and biological heterogeneity. Some tumours spontaneously regress (Everson 1964) or differentiate to a benign ganglioneuroma (7% of all neuroblastomas), whereas others may be cured with chemotherapy. However, the majority of neuroblastomas (50%) are aggressive metastatic tumours with poor clinical outcome despite

intensive multimodal therapy involving chemotherapy, irradiation, surgery, and high-dose chemotherapy followed by stem cell rescue. The most important and well-established prognostic variables of neuroblastoma are clinical stage (Table 1), age at diagnosis and histopathology (Brodeur et al, 1997a; Shimada et al, 1999). The prognosis is better for children under the age of 1, and for those with low stage disease.

Table 1. International Neuroblastoma Staging System (INSS).

Stage 1	Localised tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopy (nodes attach to and removed with the primary tumour may be positive).
Stage 2A	Localised tumour with incomplete gross excision, representative ipsilateral lymph nodes negative for tumour microscopy.
Stage 2B	Localised tumour with or without complete gross excision; with ipsilateral nonadherent lymph nodes positive for tumour. Enlarged contralateral lymph nodes must be negative microscopically.
Stage 3	Unresectable unilateral tumour infiltrating across the midline ¹ , with or without regional lymph node involvement; or, localised unilateral tumour with contralateral regional lymph node involvement; or, midline tumour bilateral extension by infiltration (unresectable) or by lymph node involvement.
Stage 4	Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver and/or other organs (except as defined for stage 4S).
Stage 4S	Localised primary tumour (stage 1, 2A or 2B) with dissemination limited to skin, liver and/or bone marrow ² (limited to infants below the age of 1).

¹The midline is defined as the vertebral column.

²Marrow involvement in stage 4S should be minimal, i.e. below 10% of nucleated cells. More extensive marrow involvement would be considered to be stage 4. Multifocal primary tumours are followed by a subscript M (e.g., 3_M).
Data according to Brodeur et al, 1993

The biological hallmark of neuroblastoma is the complexity of the genetic alterations, and the use of them as biological markers independently of clinical features (reviewed in Bown 2001). So far, no single genetic change common to all neuroblastomas has been identified. However, there are several types of specific chromosomal abnormalities frequently associated with neuroblastoma such as, deletion of genetic material at chromosome arms 1p, 11q, and 14q, gain of genetic

material at 17q and MYCN amplification. Furthermore, it has been shown that ploidy is a strong prognostic marker (Look et al, 1984). Triploid (3n) tumours are associated with low stage disease in younger children with favourable outcome, whereas diploid (2n) and tetraploid (4n) tumours are associated with unfavourable prognostic markers such as MYCN amplification and 1p deletion, and poor outcome (Hayashi et al, 1989; Kaneko et al, 1987; Look et al, 1991).

The importance of the short arm of chromosome 1 (1p) in neuroblastoma genetics was identified in 1977 (Brodeur et al, 1977). Since then, other cytogenetic studies have confirmed the high frequency of deletions and other rearrangements of 1p (Gilbert et al, 1982). It was subsequently established that loss of 1p in neuroblastoma tumours correlated with unresectable and metastatic disease, whereas localised and clinically favourable tumours had an intact chromosome 1 (Caron et al, 1996; Franke et al, 1986). Furthermore, it was shown that chromosome 1p deletion was associated with MYCN amplification, where 62% of neuroblastoma tumours with loss of heterozygosity (LOH) were MYCN amplified compared to 3% of tumours with intact chromosome 1p (Fong et al, 1989).

The occurrence of chromosome 17 abnormalities in neuroblastoma was first described in 1984 (Gilbert et al, 1984). Later it was shown, that in most neuroblastoma cell lines and primary tumours with 1p deletions, the lost 1p material was replaced by a part of the long arm of chromosome 17 (17q) (Caron et al, 1994; Savelyeva et al, 1994; Van Roy et al, 1994). Gain of chromosome 17q has been associated with unfavourable prognosis in neuroblastoma (Abel et al, 1999; Bown et al, 1999; Caron 1995; Lastowska et al, 1997). However, it is unclear whether gain of 17q has a prognostic role of its own or just in combination with loss of 1p.

There is evidence that tyrosine kinase receptor genes (trk) play an important role in the biology and clinical behaviour of neuroblastomas (Brodeur et al, 1997b). Several independent studies suggest that high expression of trkA is an indicator of favourable outcome (Borrello et al, 1993; Kogner et al, 1993; Nakagawara et al, 1993; Suzuki et al, 1993), inversely associated with MYCN amplification (Nakagawara et al, 1992), and when co-expressed with the low affinity neurotrophin receptor p75 (Kogner et al, 1993) or in the absence of MYCN amplification (Nakagawara et al, 1993), defining a subset of neuroblastomas with excellent outcome regardless of age or stage. TrkB is preferentially expressed in tumours with MYCN amplification (Nakagawara et al, 1994) and may be co-expressed with the ligand BDNF (brain-derived neurotrophic factor). TrkC is expressed primarily in lower stage tumours with favourable outcome (Ryden et al, 1996; Yamashiro et al, 1996), similar to trkA (Svensson et al, 1997).

MYCN amplification is the most important biological marker in clinical use of neuroblastoma today. Currently, MYCN is the only tumour genetic feature used as a basis for treatment stratifications in neuroblastoma clinical trials (Matthay et al, 1998; Schmidt et al, 2000). Amplification of MYCN is well known to correlate with advanced stage and poor outcome (Brodeur et al, 1984; Brodeur et al, 1986; Nakagawara et al, 1987a), and the association appears to be independent of clinical stage (Nakagawara et al, 1987b; Seeger et al, 1985) and age (Rubie et al, 1997). However, the exact molecular mechanism of how MYCN amplification and other chromosomal abbreviations contribute to the aggressive behaviour of neuroblastoma tumours remains unclear (reviewed in Westermann and Schwab 2002).

RETINOIDS

Retinoids are naturally occurring and synthetic analogues of vitamin A (retinol) (Table 2). They are required for many fundamental life processes, including pattern formation during embryogenesis, vision, reproduction, haematopoiesis, bone development, cellular differentiation, proliferation and apoptosis (DeLuca and Zile 1975; Summerbell and Maden 1990). Furthermore, retinoids are involved in many important processes in the developing and the mature nervous system (Hunter et al, 1991; Sucof and Evans 1995; Webster et al, 1986). Animals are not capable of *de novo* synthesis of retinoids, thus the primary dietary sources of retinol are carotenoids from vegetables and retinyl esters from animal tissues, which each is then converted in intestinal cells to retinol (Blomhoff et al, 1990). Retinol is transported in the blood bound to a carrier protein, called the retinol-binding protein (RBP) (Kanai et al, 1968). The intracellular carriers for retinol and retinoic acid (RA) are believed to be two classes of intracellular retinoid-binding proteins, the cellular retinol-binding protein (CRBP I and II), (Ong 1984) and cellular retinoic acid-binding proteins (CRABP I and II), (Bailey and Siu 1988; Bailey and Siu 1990).

Because of the involvement of vitamin A and its analogues in many important biological processes, retinoids have been suggested to play a potential role in treatment and prevention of malignancies. Therefore, during the last decades, retinoids have been used in numerous oncological studies with impressive results achieved in particular in patients with acute promyelocytic leukaemia (Huang et al, 1988).

Table 2. Retinoid structure, binding and activation.

Retinoid		Binding			Activation			
		RAR	RXR		RAR		RXR	
		IC ₅₀ ¹ (nM)	IC ₅₀ (nM)	%	EC ₅₀ ² (nM)	% max	EC ₅₀ (nM)	% max
ATRA 	α	7	10 ⁴	40	6.7	100	26	98
	β	5			2.8	100	1500	160
	γ	6	10 ⁴	30	3.5	100	4000	140
9-cis RA 	α	13	70		13	100	5	100
	β	17			14	98	20	100
	γ	15	95		18	98	720	100
Ro 136307 	α	7.4	10 ⁴	48	0.7	107	160	100
	β	5.7			0.7	94	300	75
	γ	7.4	10 ⁴	49	2.3	72	10 ⁴	0
13-cis RA³ 	α							
	β							
	γ							
Fenretinide 	α							
	β							
	γ							

¹ IC₅₀ = The retinoid concentration at which binding of labelled retinoic acid is inhibited by 50%.

² EC₅₀ = The retinoid concentration required to obtain 50% of the maximum response (at 1000 nM).

³ 13-*cis* RA needs to be isomerised to ATRA for biological activity (Repa et al, 1993). Data according to Apfel et al, 1992 and Apfel et al, 1995 and unpublished data kindly provided by Dr. Michael Klaus at Hoffmann-La Roche, (Basel, Switzerland).

RETINOID RECEPTORS AND SIGNALLING PATHWAYS

Retinoids exert most of their effects by binding to specific receptors and modulating gene expression (Figure 3). The retinoid receptors are members of the nuclear steroid/thyroid hormone superfamily of receptors (Evans 1988; Mangelsdorf et al, 1995), with which they share common structural and functional properties.

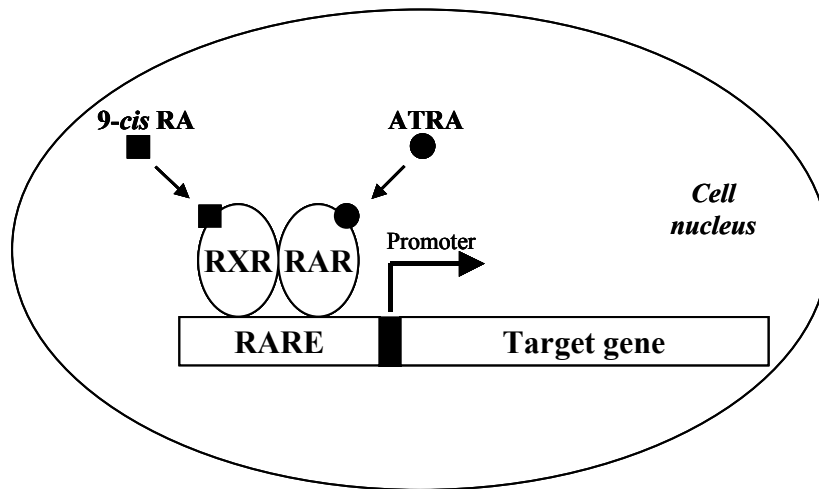


Figure 3. Model of the mechanism of action for retinoids binding to receptors and interacting with DNA. Retinoids bind to the RARs (ATRA) or RXRs (9-cis RA) that usually function as heterodimers, causing conformational changes that promote DNA binding. The RAR/RXR dimers regulate transcription of target genes by binding specific sequences in the gene promoter that are known as retinoic acid response elements (RARE).

The diversity of retinoid-induced signalling pathways is mediated by at least six retinoid receptors that fall into two subfamilies: retinoic acid receptors (RARs) α , β , γ (Brand et al, 1988; Krust et al, 1989; Petkovich et al, 1987), and retinoic X receptors (RXRs) α , β , γ (Leid et al, 1992; Mangelsdorf et al, 1990; Mangelsdorf et al, 1992; Yu et al, 1991). Each of the subtypes (α , β , γ) is encoded by a different gene, and multiple isoforms have been described for both RARs and RXRs, generated by different promoter usage and alternative splicing (Chambon 1996; Leroy et al, 1991; Liu and Linney 1993; Nagata et al, 1994; Nagpal et al, 1992; Zelent et al, 1991). These isoforms exhibit tissue-specific and developmental stage-specific expression, implying that they each have distinctive functional roles. In common with other nuclear receptors, retinoid receptors are characterised by three major domains based on conserved sequence and function. The N-terminal domain is the most variable region and contains amino acid residues, which appear important for transcriptional activation. The DNA-binding domain is the most conserved region and contains two zinc fingers (Freedman 1992) that are involved in recognition of specific DNA sequences (retinoic acid response elements, RARE) and in activation of target genes. The C-terminal ligand-binding domain is involved in ligand binding, dimerisation and transactivation (Chambon 1996;

Evans 1988; Mangelsdorf et al, 1995). Nuclear receptors generally function as dimers. RARs form heterodimers exclusively with RXRs, while RXRs can form heterodimers with RARs and several other nuclear receptors including thyroid hormone receptors and the vitamin D receptor (Chambon 1996; Mangelsdorf 1994). In addition, RXRs can bind RARE as homodimers with high affinity (Ng et al, 1995). The number of possible combinations of dimeric complexes that may be formed adds to the diversity of the retinoid action. Furthermore, the specific retinoid action is determined by the type of nuclear receptor present, its mRNA isoforms, and by the protein-protein interactions with other transcription factors.

APOPTOSIS AND RETINOIDS

Apoptosis represents a meticulously controlled, highly efficient and sophisticated system for removing aberrant cells from the surrounding environment. Apoptosis is essential for maintaining the homeostasis, and deviations from the physiological levels of cell death can have devastating consequences resulting in either proliferative or degenerative disorders (Thompson 1995). Apoptosis is distinguished from necrosis by certain morphological criteria (Kerr 1971) that are characterised by condensation of the nuclear chromatin, nucleosomal fragmentation of DNA, and formation of apoptotic bodies, which eventually are eliminated by phagocytic macrophages (Kerr et al, 1972; Wyllie et al, 1980). Caspases (cysteine aspartate-specific proteases) are proteases important for apoptosis (Nunez et al, 1998), which exist in cells as inactive pro-enzymes. Upon activation, caspases are able to cleave various cellular substrates leading to the biochemical and morphological changes that are characteristic of apoptosis (Kothakota et al, 1997). There are two principal pathways of apoptosis signalling transduction (Figure 4). The death-receptor mediated pathway is activated by receptor-ligand binding, resulting in activation of caspases-8 and subsequent activation of the caspase cascade leading to apoptosis. In the receptor-independent pathway (mitochondrial pathway), intracellular biochemical events affect the mitochondria, resulting in cytochrome *c* release, and the formation of the apoptosome. This results in activation of caspase-9 and subsequent activation of the caspase cascade leading to apoptosis.

In recent years, attention has been devoted to the ability of retinoids to induce apoptosis (Kucharova and Farkas 2002; Nagy et al, 1998). The link between retinoids and cell death was originally an outgrowth of studies on the cellular basis of retinoid teratogenesis (Sulik and Dehart 1988). Since then, retinoids have been shown to induce apoptosis in many tumour-derived cell systems (Delia et al, 1993;

Kalemkerian et al, 1995; Krupitza et al, 1995; Martin et al, 1990; Melino et al, 1994; Oridate et al, 1995). The apoptotic activity is primarily associated with “atypical retinoids” such as fenretinide (Ulukaya and Wood 1999), but high-doses of natural retinoids may also induce apoptosis. Studies on fenretinide-induced apoptosis indicate that this retinoid can function in both retinoic acid receptor-dependent and -independent pathways (i.e. atypical retinoid) (Delia et al, 1997; Fanjul et al, 1996).

There are other suggested pathways of retinoid-induced apoptosis (Altucci and Gronemeyer 2001; Kucharova and Farkas 2002; Wu et al, 2001), however, the exact mechanism of action is poorly understood.

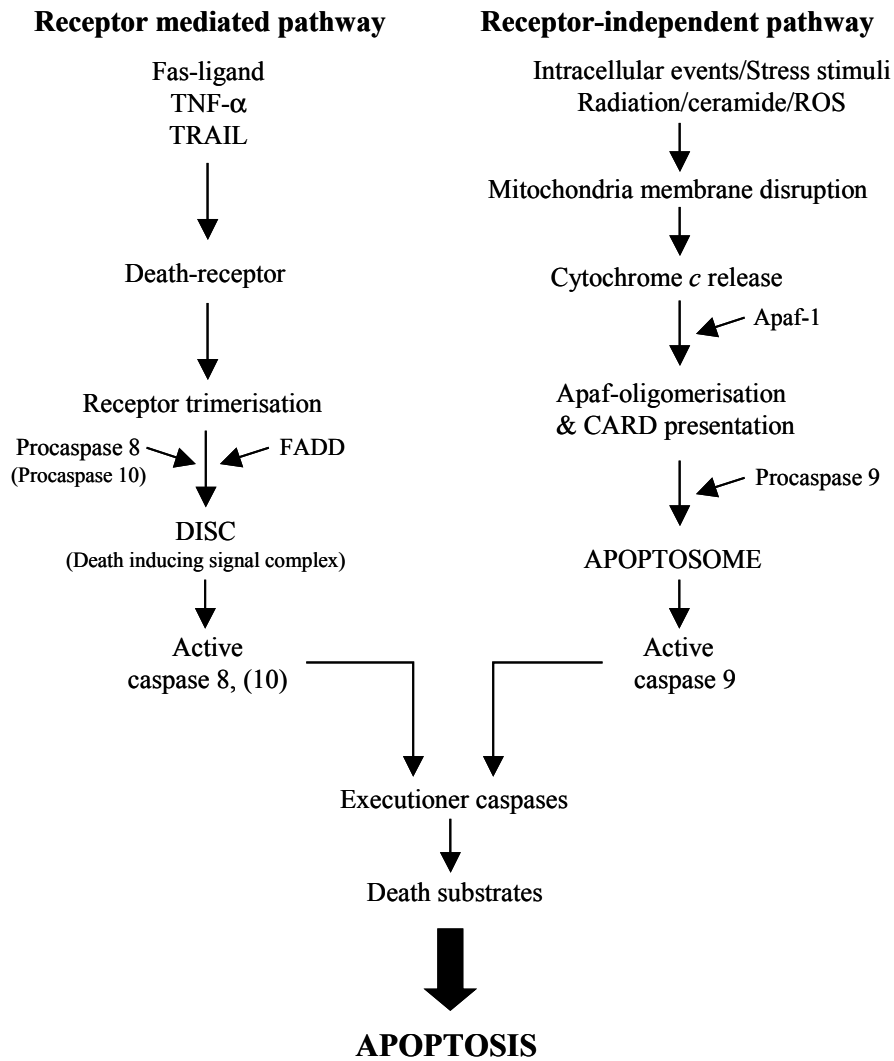


Figure 4. Schematic diagram of the major apoptotic pathways. In the receptor-mediated pathway (left), death-ligand binds to its death-receptor, resulting in trimerisation, DISC formation and activation of caspase 8. In the receptor-independent pathway (right), intracellular biochemical events and stress disrupt the mitochondrial membrane, trigger cytochrome *c* release, initiate apoptosome formation, which leads to activation of caspase 9. The initiator caspases (8, 9, 10) then activates the executioner caspases that cleave the death substrates, which eventually results in apoptosis.

RETINOIDS AND NEUROBLASTOMA DIFFERENTIATION

Non-proliferating and differentiated cells can arise from various leukaemias carcinomas, melanomas and neuroblastomas *in vitro* and *in vivo* either spontaneously or after induction by various agents (Reiss et al, 1986). Retinoids have been recognised to have major effects on cellular differentiation of many cell types, including promyelocytic leukaemia (Breitman et al, 1981) and neuroblastoma (Reynolds et al, 1991; Sidell 1982; Thiele et al, 1985). The differentiation is characterised by alterations in morphology, biochemical activity and gene expression. In neuroblastoma cells treated with RA, these changes are similar to those of maturing neural crest cells (Tsokos et al, 1987), including inhibited growth, extensive neurite outgrowth, synapse formation with distant cells (Sidell et al, 1983), induction of several neurone specific proteins (Hill and Robertson 1997; Melino et al, 1991; Reynolds and Maples 1985) and genes associated with cell cycle, cell shape and neurotransmission (Matsuo and Thiele 1998; Truckenmiller et al, 2001). Furthermore, an alternative pathway to neuronal differentiation, a “flat epithelium-like or substrate-adherent” phenotype has been consistently described in neuroblastoma cells treated with RA (Sidell et al, 1986; Wainwright et al, 2001). Recently, this phenotype has been correlated with retinoid-induced cell senescence (Roninson and Dokmanovic 2003; Wainwright et al, 2001).

RETINOIDS IN NEUROBLASTOMA

Neuroblastoma has the highest rate of spontaneous regression of any human tumour (Everson 1964). These clinical observations have stimulated numerous of studies on neuroblastoma differentiation and apoptosis, especially with agents like retinoids (Reynolds 2000; Reynolds and Lemons 2001) that have been shown to modify these biological processes during the normal development (Summerbell and Maden 1990).

All-trans retinoic acid

All-trans retinoic acid (ATRA) is a naturally occurring retinoid that binds and transactivates the RAR receptors with high affinity (Allenby et al, 1993; Apfel et al, 1995), (Table 2). *In vitro*, ATRA has been shown to be one of the most potent inducers of differentiation in human neuroblastoma cell lines (Haussler et al, 1983; Pahlman et al, 1984; Sidell 1982; Sidell et al, 1983). Furthermore, treatment of both MYCN gene-amplified and non-amplified cell lines led to a marked decrease in MYCN RNA expression and arrested cell proliferation (Reynolds et al, 1991; Thiele et al, 1985). However, there are observations indicating that neuroblastoma

cells treated with ATRA may become more resistant to chemotherapeutic drugs (Lasorella et al, 1995) and irradiation (Ronca et al, 1999).

In nude mice treated with ATRA, the tumour formation was reduced when the retinoid was given five days before tumour cell injection and continued for fourteen days thereafter (Abemayor et al, 1990). Furthermore, treatment with ATRA for 32 days inhibited progressive tumour growth of already established tumours (Abemayor 1992). ATRA has been included in phase I studies in children, where the maximum tolerated dose (MTD) was 60 mg/m² with a continuous schedule (Smith et al, 1992), and 90 mg/m² with an intermittent schedule (Adamson et al, 1997). Furthermore, pharmacokinetic studies demonstrated a relatively short half-life (45 min) and a marked decrease in drug levels after several days of therapy (Smith et al, 1992). In addition, only one child out of eight with refractory neuroblastoma had an objective response of 6 months duration in the trial using the intermittent schedule (Adamson et al, 1997). In a phase II trial, 13 patients with metastatic neuroblastoma were treated with an intermittent schedule of ATRA (30 mg/m²/12hours) for 7 days followed by 7 days rest, but all patients still had progressive disease after 2 months of therapy (Michon et al, 1996).

13-*cis* retinoic acid

13-*cis* retinoic acid (13-*cis* RA) is a synthetic retinoid and the -*cis* isomer of ATRA (Table 2). 13-*cis* RA does not bind directly to RARs, but its isomerisation to ATRA leads to activation of the retinoid receptors (Klug et al, 1989; Repa et al, 1993; Veal et al, 2002). The *in vivo* pharmacokinetic properties of 13-*cis* RA are different from ATRA, with a longer half-life (5 h as apposed to 45 min for ATRA), a higher achievable peak level (7.4 µM, as compared to 1 µM for ATRA), and a more stable steady state level of 13-*cis* RA (Khan et al, 1996; Villablanca et al, 1995) in contrast with the decrease in drug levels after several days of therapy with ATRA (Smith et al, 1992). 13-*cis* RA has been reported to induce morphological differentiation, growth arrest and down regulation of MYCN gene expression of neuroblastoma cells *in vitro*, at least at the same magnitude as ATRA or even superior to ATRA in some neuroblastoma cell lines (Reynolds et al, 1994).

A clinical effect of oral 13-*cis* RA has been reported in certain patients with advanced and/or relapsed neuroblastoma tumours (Kogner et al, 1994; Reynolds et al, 1991), although continuous treatment with 13-*cis* RA (100 mg/m², MTD) against progressive neuroblastoma in a phase II trial showed no significant therapeutic effect (Finklestein et al, 1992). In a recent randomised trial, children

with high-risk neuroblastoma showed a significantly improved event-free survival when treated with an intermittent schedule of high-dose 13-*cis* RA for two weeks every fourth week during 6 months (160 mg/m², MTD), after high-dose therapy and autologous bone marrow transplantation (Matthay et al, 1999). However, in another recent clinical study, no survival advantage was demonstrated for a similar group of patients in clinical remission, receiving continuous 13-*cis* RA at low doses (0.75 mg/kg/day) for up to 4 years (Kohler et al, 2000). These clinical trials indicate that dosing, scheduling, timing and tumour load at start of treatment are important factors in determining the therapeutic efficacy of 13-*cis* RA (Matthay and Reynolds 2000).

Because of the significantly improved event free survival with high-dose pulses of 13-*cis* RA (Matthay et al, 1999), this intermittent regimen is presently included in treatment protocols targeting minimal residual disease (MRD). However, patients treated with 13-*cis* RA may suffer from toxic side effects such as, dry skin, dry mucous membrane, conjunctivitis, hypercalcaemia, and in rare cases even neurological syndromes (Kohler et al, 2000; Olson 1983; Villablanca et al, 1995). Moreover, neuroblastoma tumours from most patients manifest innate or acquired RA resistance (Reynolds et al, 2000; Reynolds and Lemons 2001), and new retinoids are searched for to overcome this resistance.

9-*cis* retinoic acid

9-*cis* retinoic acid (9-*cis* RA) is a naturally occurring retinoid that binds and transactivates both the RAR and the RXR receptors with high affinity (Allenby et al, 1993; Heyman et al, 1992), (Table 2). Furthermore, 9-*cis* RA can induce homodimer formation of the retinoid X receptor (Zhang et al, 1992). *In vitro*, 9-*cis* RA has been shown to induce differentiation, growth arrest and down regulation of MYCN gene expression in neuroblastoma cell lines during continuous treatment (Han et al, 1995; Lovat et al, 1994). In addition, neuroblastoma cells short-term treated with 9-*cis* RA for five days followed by subsequent washout was found to undergo apoptosis (Lovat et al, 1997a). Furthermore, the morphological differentiation and the antiproliferative effect induced by 9-*cis* RA were more pronounced than in neuroblastoma cells treated with ATRA (Han et al, 1995; Lovat et al, 1997b). These different treatment effects caused by the two retinoids might be due to the differences in retinoid receptor binding.

Ro 13-6307

Ro 13-6307 is a synthetic retinoid developed by Hoffmann-La Roche (Basel, Switzerland) that binds to RARs with similar affinity as ATRA but activates the RAR receptors at lower concentrations (Apfel et al, 1992), (Table 2). In addition,

Ro 13-6307 is able to transactivate RXR α and RXR β (Michael Klaus, personal communication). Ro 13-6307 has been shown to induce differentiation in haematopoietic cell lines (Peck and Bollag 1991). However, to our knowledge, this retinoid has not been used in any therapeutic studies on neuroblastoma *in vitro* or *in vivo*, except for the studies included in this thesis.

Fenretinide

Fenretinide (N-(4-hydroxyphenyl)retinamide) has been identified as a synthetic retinoid able to induce apoptosis in neuroblastoma cell lines *in vitro* (Di Vinci et al, 1994; Mariotti et al, 1994), (Table 2). Recent studies have demonstrated that fenretinide does not induce differentiation of neuroblastoma cells *in vitro*, but is more effective in inducing apoptosis and necrosis than ATRA, 13-*cis* RA or 9-*cis* RA (Lovat et al, 2000b; Maurer et al, 1999). Furthermore, fenretinide has been shown to be highly active against neuroblastoma cell lines resistant to ATRA and 13-*cis* RA (Reynolds et al, 2000). The mechanisms involved in fenretinide-induced cell death of neuroblastoma cells are complex and probably involving several overlapping pathways (Wu et al, 2001). Fenretinide-induced apoptosis has been suggested to be caspase-dependent, involving cytochrome *c* release mediated by specific retinoic acid receptors (Lovat et al, 2000a; Lovat et al, 2002). However, oxidative stress via the induction of reactive oxygen species (ROS) and the stress-induced transcription factor GADD153, has been shown to mediate key events involved in fenretinide-induced apoptosis of neuroblastoma cells (Lovat et al, 2000b; Lovat et al, 2002; Maurer et al, 1999). Recent studies also suggest that a p53-independent pathway of fenretinide-induced cytotoxicity may operate through increased intracellular levels of the lipid secondary-messenger ceramide (Maurer et al, 1999; Maurer et al, 2000).

Fenretinide has been studied in two phase I trials, where children with neuroblastoma received oral fenretinide in daily doses ranging from 350-3300 and 100-4000 mg/m², respectively (Villablanca et al, 2002, Garaventa et al, 2002). The maximum tolerated dose was indicated to be 2475 mg/m² (Villablanca et al, 2002). Both of these phase I trials indicated a plateau effect in terms of absorption and/or plasma concentrations at an oral dose level of 2300-2475 mg/m².

ANIMAL MODELS

The use of animals in scientific studies is constantly debated. A great number of investigations can be performed using cell lines. However, some experiments have to be performed in a complete biological system, which cannot be mimicked in cell culture systems. Using animal models can provide these biological systems. In

addition, animal models offer opportunities to evaluate treatment effects, and most importantly discover toxic side effects of new drugs before they are used therapeutically in humans. An ethics committee for animal research must approve all animal experiments, in order to minimise unnecessary and painful experiments on animals.

In this thesis we used a human neuroblastoma xenograft model in athymic nude rats (Nilsson et al, 1993) to evaluate effects of retinoid treatment. The tumours we investigated were established as subcutaneously localised tumours from a human neuroblastoma cell line (SH-SY5Y) originally derived from a primary tumour in a patient with neuroblastoma (Biedler et al, 1973). The xenograft model we used, offered possibilities to establish subcutaneous tumours from other neuroblastoma cell lines with other genetic alteration such as MYCN amplification, but with variable reproducibility. However, the SH-SY5Y tumour model was stable and reproducible. Hence, this cell line was used to evaluate the treatment efficacy both *in vivo* and *in vitro*.

It is possible to establish metastatic animal models of human neuroblastoma by intravenous (Bogenmann 1996), intra aortal (Engler et al, 2001), or intra cardiac injections (Martinez et al, 1996) of human neuroblastoma tumour cells in nude mice or rats. Recently, five metastatic orthotopic (adrenal) human xenograft models of neuroblastoma were characterised, where human neuroblastoma cell lines were adrenally injected to form spontaneous metastases (Khanna et al, 2002). However, it is difficult to establish a reproducible metastatic pattern with this approach, and the metastases do not always occur in the same organs as in children with disseminated neuroblastoma. Furthermore, there are difficulties of evaluating treatment response during therapy with non-invasive tools.

An advantage with a subcutaneous xenograft tumour model is that subcutaneous tumours are easy to evaluate for treatment response during the experiments without using any invasive methods. Furthermore, it offers possibilities to easily inject therapeutic drugs directly into the tumours and to take tumour biopsies during treatment. However, a disadvantage with such a model is that the tumours are not located where the neuroblastoma tumours usually are located in humans.

There are murine animal models of neuroblastoma. A syngeneic metastatic mouse model was established by intravenous injection of GD2-positive hybrid mouse neuroblastoma cells (Lode et al, 1997). By intramediastinal injection of the same cell line (NXS2) a syngeneic non-metastatic mouse model for thoracic neuroblastoma was obtained (Corrias et al, 2002). Another metastatic model was established by creating transgenic mice that over expressed MYCN in neuroectodermal cells, which led to the development of neuroblastoma (Weiss et

al, 1997). Murine models offer opportunities to evaluate immunotherapeutic approaches of neuroblastoma therapy, because of the use of animals with intact immune system (Lode et al, 1998). However, the targeted neuroblastoma tumours in these models are not of human origin (mouse neuroblastoma in mice).

AIMS OF THE STUDY

The aim of the present study was to investigate retinoids as new treatment strategies for children with neuroblastoma, who currently have low probability to be cured with the present treatment including a combination of cytotoxic drugs, irradiation and surgery.

To accomplish this, the following specific aims were elaborated:

- Investigate treatment effects of retinoids, such as differentiation and/or apoptosis, in human neuroblastoma cells *in vitro*.
- Evaluate tumour-inhibiting effects of retinoid treatment *in vivo*, on human neuroblastoma tumours established as xenografts in nude rats.
- Elucidate toxic side effects and their correlated to retinoid treatment.

MATERIALS AND METHODS

“Though this be madness, yet there’s method in it”

Shakespeare, Hamlet

ETHICS

All animal studies performed were approved by the regional ethics committee for animal research in accordance with the Animal Protection Law (SFS1988:534), the Animal Protection Regulation (SFS 1988:539) and the Regulation for the Swedish National Board for Laboratory Animals (SFS1988:541).

CELL LINES

Two different neuroblastoma cell lines were used for the studies presented in this thesis. The adrenergic MYCN non-amplified neuroblastoma cell line SH-SY5Y (Biedler et al, 1973) was cultured in Eagle’s minimum essential medium (Gibco, Paisley, Scotland), and the MYCN amplified neuroblastoma cell line SK-N-BE(2) (Biedler and Spengler 1976) was cultured in RPMI-1640 (Gibco). Cells were grown at 37°C in a humidified atmosphere of 5% CO² in air. All medium used was supplemented with fetal bovine serum (10%), L-glutamine (2 mM), penicillin G (100 IU/ml) and streptomycin (100 µg/ml) (Gibco). SH-SY5Y cells resistant to 3 µM of fenretinide SH-SY5Y-R (kindly provided by Dr. Penny Lovat), (Lovat et al, 2000a), was cultured under the same conditions, with the addition of 3 µM fenretinide to the Eagle’s minimum essential medium every other week during culturing. All cells used were free from mycoplasma as detected by DNA staining.

HUMAN NEUROBLASTOMA XENOGRAFT MODEL

Animals

Athymic nude rats (Rowett rnu/rnu or HsdHan: RNU-rnu, Harlan Netherlands) at the age of 5-10 weeks with an approximate weight of 150-275 g were used for xenografting. No animals had to be excluded from any of the studies performed with the exclusion criteria applied, namely development of an open wound over the tumour.

Xenografting

Establishment of neuroblastoma xenografts was performed as previously described (Nilsson et al, 1993). A suspension of SH-SY5Y cells, 100x10⁶ cells/ml (viable cells), was prepared, and animals were anaesthetized. Twenty million cells suspended in 0.2 ml medium, were subcutaneously injected in each hind leg using

a 23-gauge cannula. The procedure was carefully performed, not to pierce the muscle fascia, and not to lose cells by leakage from the injection site.

Quantification of tumour growth

When tumour take was evident on palpation and/or visible, the tumour length (along the tumour long axis) and width (perpendicular to the long axis) were measured with a calliper every second day. Tumour volume was calculated by length x width² x 0.44 (Wassberg et al, 1997). The true tumour weight was recorded at autopsy. Tumour volume index was calculated using the measured volume divided with the volume measured at tumour take at start of treatment.

DETERMINATION OF 9-*CIS* RA IN PLASMA BY HPLC

Sampling and extraction procedure

Male Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden) with the average weight of 250 g were given a single intravenous or oral dose of 9-*cis* RA 30-45 mg/kg. Blood was collected by cardiac exsanguination in tubes containing sodium heparin (100 IU/ml, 0.05 ml/ml of whole blood, Karolinska Pharmacy, Sweden) and two animals were sacrificed at each time point. Samples were collected from untreated animals and from post injection of 9-*cis* RA (15 min to 8 hours). The blood was immediately centrifuged and plasma was stored at -70°C until analysis, which was performed within 3 weeks from sampling.

In a 10 ml glass tube, 25 µl of the internal standard was added. After addition of 0.5 ml plasma and 0.1 ml phosphate buffer, pH 7, (0.025 M KH₂PO₄ and 0.04 M Na₂HPO₄*2H₂O) the compounds were extracted for 5 min with 3 ml of a diethyl ether-ethyl acetate (50/50, v/v) mixture by vortex mixing. After centrifugation at 2000g for 10 min at 4°C, the organic phase was evaporated to dryness. The residue was dissolved in 90 µl methanol and transferred to an injection vial with cap, for HPLC analysis.

Chromatographic conditions

HPLC analysis was performed using a LKB 2150 pump equipped with an auto sampler (Perkin-Elmer ISS-100) and a variable-wavelength UV detector Spectromonitor (LDC/Milton Roy). The analytical column, a Prodigy ODS (3) silica column (150 x 4,6 mm) with 3 µm particles (Phenomenex, California, USA) was fitted with a guard column (Nova-Pak C18, Waters, USA). Data were acquired and analysed using System Gold (Beckman Instruments Inc). An isocratic gradient was prepared with the final composition of 52.87% methanol, 28.47% acetonitrile, 16.66% water, 1.66% tetrahydrofuran and 0.34% acetic acid.

The mobile phase was degassed by ultrasonic treatment before HPLC analysis. The flow rate was 1 ml/min and the UV detection was carried out at 350 nm. A 65 µl aliquot of each sample was auto-injected and data were collected during 30 min. The total time between injections was 32 min.

Pharmacokinetic analysis

Concentration-time data for 9-*cis* RA and its metabolite (4-oxo-9-*cis* RA) were adjusted to a one-compartment open model using Gauss-Newton (Levenberg-Hartly) criteria. Parameters such as the distribution volume of the central compartment, the elimination rate constant, the plasma maximum concentration and the microconstants were estimated. Whereas, the clearance (Cl) and the distribution volume at steady state were calculated from the primary parameters. The area under the plasma concentration versus time curve (AUC) was calculated from the model-derived parameters and the elimination half-life was calculated from the slope of the phase of elimination. The pharmacokinetic modelling was performed using WinNonlin version 3.0 (Pharsight, Mountain View, CA, USA).

CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

Cell cycle analysis was performed using single parameter DNA flow cytometry (Castro et al, 1993). For cell suspensions, cells were fixed in 1 ml 4% phosphate buffered formaldehyde (Karolinska Pharmacy, Stockholm, Sweden) at room temperature over night, pelleted and suspended in 1 ml 95% ethanol. For paraffin embedded tumour tissue, sections of 90 µm thickness were cut and deparaffinized (Heiden et al, 2000). Prior to FACS analysis, cells and tissue were rehydrated in distilled water for 1 hour, treated with subtilisin Carlsberg solution (0.1% protease XXIV (Sigma), 0.1 M Tris-HCl pH 7.5, 70 mM NaCl) and stained with DAPI-sulforhodamine solution (8 µM DAPI, 50 µM sulforhodamine 101, 0.1 M Tris-HCl pH 7.5, 70 mM NaCl). Samples were analysed using a PAS II flow cytometer (Partec, Münster, Germany) equipped with a 100 W mercury arc lamp HBO 100. DAPI fluorescence was measured above 435 nm. The multicycle program for cell cycle analysis (Phoenix Flow Systems, San Diego, CA, USA) was used for histogram analysis. At least 10⁴ nuclei of each sample were analysed.

TERMINAL DEOXY-NUCLEOTIDYL TRANSFERASE (TUNEL) REACTIVITY ASSAY

The terminal deoxy-nucleotidyl transferase (TUNEL) reactivity assay (ApopTag, S7100, Intergen, NY) was employed for quantification of apoptosis (Gavrieli et al, 1992), by calculating the number of TUNEL-positive cells with condensed and/or

fragmented nuclei per high-power field (40x magnification). At least 10 fields were scored per section.

ACQUISITION OF PROTON MRS SPECTRA

Prior to spectroscopic analysis, cells were suspended in PBS with 10% D₂O added for field lock. A Varian Inova 600 MHz spectrometer was used to acquire one-dimensional ¹H MRS spectra. Each spectrum was recorded on a cell suspension containing approximately 50 x 10⁶ cells using a 40 μL ¹H MRS tube. The tube was spun in a magic angle nanoprobe enabling spinning of the sample at approximately 1.7 kHz. The residual H₂O signal at ~ 4.75 ppm was suppressed by 1.5 s low-power presaturation. The acquisition parameters included: 90° pulse, 256 transients, acquisition time 1 s, spectral width of 8 KHz, recycle delay of 1.5 s, acquisition time of 1 s, line broadening 1 Hz. All experiments were performed at 25°C and total time to collect each spectrum was 10 minutes. The Fourier-transformed spectra were manually phase-corrected (zero and first order) and a spline baseline correction was applied. Chemical shifts were expressed in ppm using the tCho peak at 3.22 ppm and the characteristic lactate doublet at 1.31 ppm as chemical shift references. Signals were quantified by integrating the area under each of the resonances using the vnmrys software. In order to obtain a relative level of an individual metabolite, the peak area integral was corrected for the number of cells in the sample and expressed in units per 10⁶ cells. The peaks were assigned as described by Willker et al, 1996.

FLUORESCENCE STAINING AND MORPHOLOGICAL CHARACTERISATION

For morphological characterization, one million cells were fixed in 1 ml of 4% phosphate buffered formaldehyde (Karolinska Pharmacy, Stockholm, Sweden) at room temperature over night, and mixed with a staining solution containing acridine orange (25 mg/ml) and Hoechst 33324 (25 mg/ml) (Sigma, St. Louis, MO, USA). Analysis was performed at 500 magnifications in a fluorescence microscope (Leitz, Germany) equipped with different filter blocks. The classification of cells included apoptotic, normal/viable cells, and cells without chromatin content. Cells were considered viable when nuclei were morphologically normal with homogenously distributed blue-stained chromatin (Hoechst) together with homogenous green staining (acridine orange). The highly condensed, peripherally clumped or fragmented chromatin in early apoptotic cells (Kerr et al, 1972) was hyperintensely blue-stained with Hoechst, while acridine

orange demonstrated condensed or fragmented nuclei. Cells with a weak but homogenous green stain in combination with a lack of chromatin staining were considered to represent late apoptotic or necrotic cells. At least 200 cells were scored for each sample analysed.

CYTOTOXICITY ASSAY (MTT)

A tetrazolium salt-based colorimetric assay (Mosmann 1983) was used for quantitative measurement of cell death (apoptosis/necrosis) and inhibited proliferation induced by retinoids and other cytotoxic agents.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum bromide) is cleaved by mitochondrial dehydrogenases in living cells and is converted to formazan. After incubation with retinoids in a humidified atmosphere of 5% CO² in air at 37°C for a certain time, MTT (5 mg/ml) was added and cells were further incubated for 3 hours in dark before formazan-crystals were dissolved by adding 150 µl isopropanol with HCl (3.3 ml 37% HCl per litre isopropanol). The absorbance was measured spectrophotometrically using an ELISA plate reader at 595 nm.

WESTERN BLOTTING

The western blot technique was used to determine different protein levels induced or reduced in neuroblastoma cells after retinoid treatment. Proteins were extracted and protein mixtures were electrophoretically separated in SDS-polyacrylamide gels and transferred to nylon membranes. Membranes were blocked with 5% skimmed dry milk to reduce non-specific binding. Primary antibodies were added to the membrane to identify the protein of interest. The secondary horseradish peroxidase (HRP)-conjugated antibodies were added and the protein of interest was detected using enhanced chemiluminescence.

STATISTICAL ANALYSIS

Statistical analysis was performed using Mann-Whitney U test for two independent samples (paper **I-IV**), Kruskal-Wallis test with multiple comparisons for more than two groups (paper **I-II**), and Student's two-tailed, unpaired *t*-test (paper **V**). Values of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

STUDY I

13-*cis* retinoic acid, 9-*cis* retinoic acid and Ro 13-6307 inhibit neuroblastoma tumour growth *in vivo*.

The aim of this study was to evaluate the neuroblastoma tumour growth inhibiting effects and the toxic side effects of three different retinoids *in vivo*. Nude rats with established human neuroblastoma xenograft tumours were treated orally with 13-*cis* RA, 9-*cis* RA or Ro 13-6307 following a continuous treatment scheduling for 10-12 days or a short-term treatment scheduling, where the animals received retinoids for five days followed by five days washout.

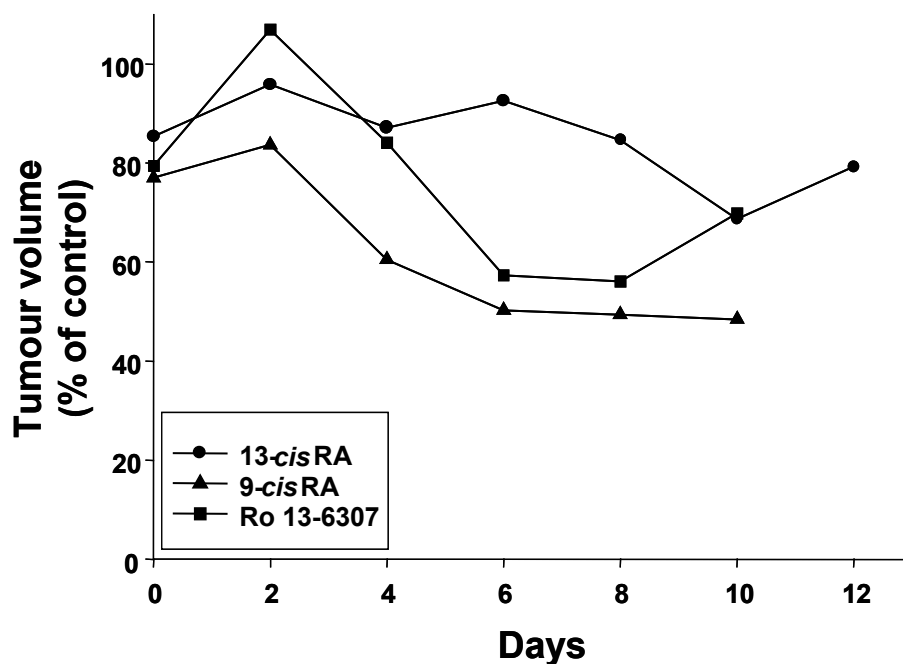


Figure 5. Neuroblastoma SH-SY5Y xenograft tumour volume.

Mean volumes for tumours 0-12 days from start of treatment relative to mean volumes of control tumours (at 100%) are shown for three different treatment groups. Tumours from rats treated with 13-*cis* RA 4 mg/day (●, mean volume±SD day 0: 0.63±0.41 ml, day 10: 4.57 ±0.84 ml, $p<0.025$), 9-*cis* RA 5 mg/day (▲, mean volume day 0: 0.57±0.19 ml, day 10: 3.22±0.48 ml, $p<0.001$), and Ro 13-6307 0.3 mg/day (■, mean volume day 0: 0.59 ±0.05 ml, day 8: 3.00 ±1.48 ml, $p<0.005$).

Tumours from rats continuously treated with 4 mg of 13-*cis* RA for 12 days showed a significant reduction in tumour volume at day 10 ($p < 0.025$) (Figure 5) and significantly lower tumour weights at sacrifice ($p < 0.05$) compared to tumours from untreated rats. Furthermore, rats treated with 13-*cis* RA did not gain weight as much as control animals, but showed no other signs of toxicity (Figure 6). Continuous and short-term treatment with Ro 13-6307 (0.3 mg/day) or 9-*cis* RA (5 mg/day) resulted in reduced tumour volumes at day 8 ($p < 0.005$ and $p < 0.001$, respectively) (Figure 5) and significantly smaller tumours in terms of weight at sacrifice ($p < 0.01$ and $p < 0.005$, respectively) compared to untreated controls. There was no statistically significant difference in tumour-inhibiting effect between the two different treatment schedules, regardless of retinoid used. However, rats treated with Ro 13-6307 or 9-*cis* RA suffered from major toxic side effects with severe weight loss especially for in rats treated continuously (Figure 6).

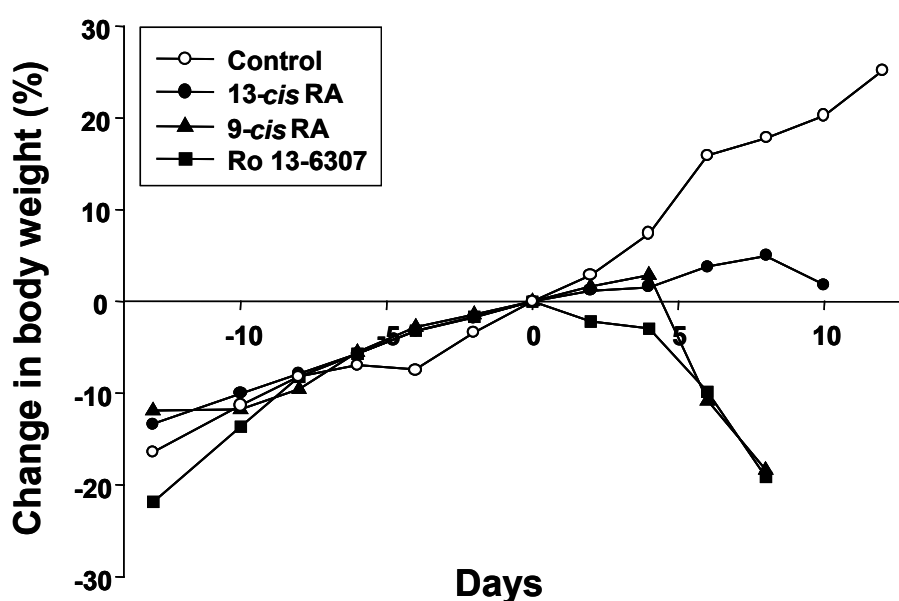


Figure 6. Relative changes in body weight during continuous treatment with 13-*cis* RA (●), 9-*cis* RA (▲), Ro 13-6307 (■) or the control treatment peanut oil (○). All rats regardless of treatment gained less weight than control rats during treatment.

In this study we demonstrated that 13-*cis* RA, Ro 13-6307 and 9-*cis* RA at current doses, were able to inhibit neuroblastoma growth *in vivo* when used as oral

treatment of nude rats with neuroblastoma xenograft tumours. Surprisingly, we found no differences in treatment effect between continuous and short-term treatment as would have been expected considering the *in vitro* results previously reported (Lovat et al, 1997a). However, treatment with both Ro 13-6307 and 9-*cis* RA resulted in major, non-acceptable toxic side effects. Therefore, we concluded that further *in vivo* studies are warranted, including pharmacokinetics and pharmacodynamics, before starting clinical trials in children with neuroblastoma.

STUDY II

Bioavailability and dose-dependent anti-tumour effects of 9-*cis* retinoic acid on human neuroblastoma xenografts in rat.

This study was designed to investigate the bioavailability of 9-*cis* RA in rat, and to further evaluate the anti-neuroblastoma growth effects of 9-*cis* RA *in vivo* observed in paper I.

A pharmacokinetic study was performed where Sprague-Dawley rats received a single intravenous or oral dose of 9-*cis* RA 30-45 mg/kg. Blood was collected at appropriate time points. The concentration of 9-*cis* RA in plasma was measured using an adapted HPLC method for retinoid analysis. The bioavailability of 9-*cis* RA after oral administration was found to be 11% and the half-life ($t_{1/2}$) was approximately 35 min (in the range of 26-43 min) (Table 3).

Table 3. Pharmacokinetic parameters of 9-*cis* retinoic acid in rat.

	<u>Oral administration^a</u>		<u>Intravenous administration^a</u>	
	9- <i>cis</i> RA	4-oxo-9- <i>cis</i> RA	9- <i>cis</i> RA	4-oxo-9- <i>cis</i> RA
AUC ^b (ng/ml x h)	8548	3563	80091	6295
T _{1/2} ^c (h)	0.71	2.1	0.44	2.15
Cmax ^e (ng/ml)			0.11	1.43
Cl ^d (ml/h)	2996	441	120476	738
Tmax ^f (h)	1.05	4.58		3.13

^a 9mg 9-*cis* RA given p.o. or i.v. according to materials and methods.

^b AUC = Area under the time-concentration curve. ^c T_{1/2} = Elimination half-life.

^d Cl = Clearance. ^e Cmax = Maximum concentration. ^f Tmax = Time of Cmax

Nude rats with neuroblastoma xenograft tumours were treated orally with 9-*cis* RA, using two different dose schedules, in order to find a non-toxic but still tumour-effective treatment regimen. Tumours from rats treated with 2 mg of 9-*cis* RA once daily for 12 days, showed no reduction in tumour growth compared to corresponding controls (Figure 7). In contrast, tumours from rats treated with 2.5 mg of 9-*cis* RA twice daily for 10 days, had smaller volumes at day 10 ($p < 0.05$) compared to corresponding controls (Figure 7). However, these tumours were not significantly smaller in terms of tumour weight at sacrifice compared to the same corresponding control tumours. Regardless of treatment schedule rats shown a normal growth curve before and during the experiment, and no other signs of toxicity was observed.

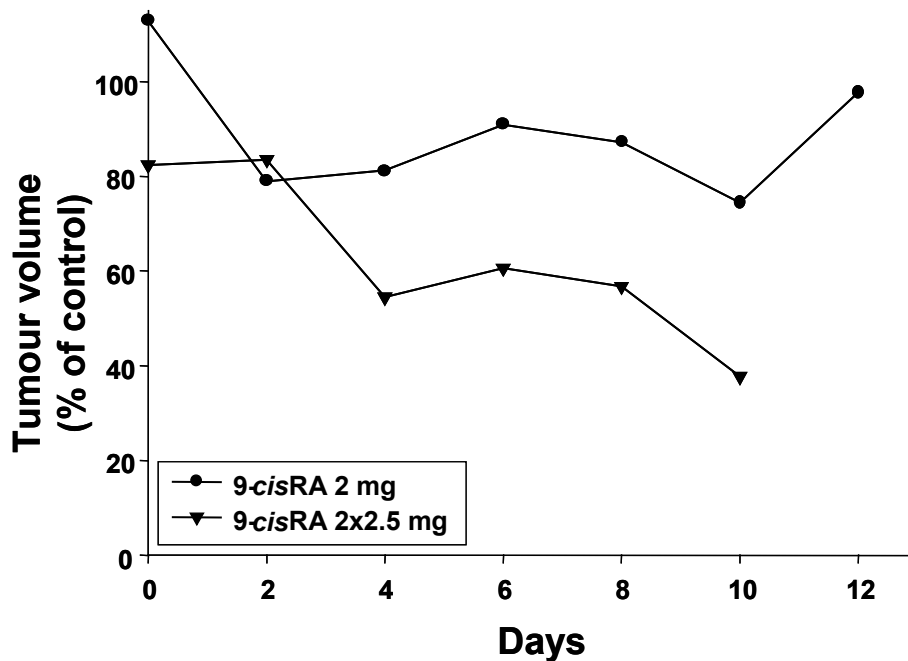


Figure 7. Neuroblastoma SH-SY5Y xenograft tumour volume.

Mean volumes for tumours 0-12 days from start of treatment relative to mean volumes of control tumours (at 100%) are shown for two different treatment groups. Tumours from rats treated with 9-*cis* RA 2 mg/day (●, mean volume±SD day 0: 0.44±0.12 ml, day 12: 4.28 ±2.44 ml) and 9-*cis* RA 2x2.5 mg/day (▼, mean volume day 0: 0.32±0.02 ml, day 10: 2.22±1.41 ml, $p < 0.05$, one-sided probability).

In conclusion, the pharmacokinetic analysis showed a low bioavailability of 9-*cis* RA (11%), probably due to the low absorption in the gastric tract. Furthermore, the half-life of 9-*cis* RA was 35 min, comparable with the half-life of ATRA in rats (Shelley et al, 1982) and in humans (Smith et al, 1992).

In paper I, we concluded that oral treatment with 5 mg of 9-*cis* RA daily resulted in a significant reduction in neuroblastoma tumour growth but with major toxic side effects. In this study, the non-toxic schedules of 2 mg of 9-*cis* RA daily, or 2.5 mg of 9-*cis* RA twice daily, were not effective against neuroblastoma tumour growth *in vivo*. Combining these results we conclude, that 9-*cis* RA inhibits neuroblastoma tumour growth *in vivo* in a dose dependent manner. Furthermore, it seems likely that the peak concentration (C_{max}) rather than the AUC is important for the growth inhibiting effect of 9-*cis* RA. However, the toxicity profile, the short half-life and the low bioavailability of 9-*cis* RA *in vivo*, limit the potential use of this retinoid for clinical oral therapy of neuroblastoma in children.

STUDY III

The synthetic retinoid Ro 13-6307 induces neuroblastoma differentiation *in vitro* and inhibits neuroblastoma tumour growth *in vivo*.

The present study aimed to further evaluate the effect of the synthetic retinoid Ro 13-6307 found in paper I, on the inhibition of neuroblastoma growth *in vivo*. In addition, we investigated the effects of this retinoid on neuroblastoma growth *in vitro* using SK-N-BE(2) and SH-SY5Y cells. Both the *in vitro* and the *in vivo* experiments with Ro 13-6307 were performed and compared with data obtained simultaneously using 13-*cis* RA.

SH-SY5Y and SK-N-BE(2) neuroblastoma cells continuously treated with Ro 13-6307 showed inhibited growth, induced morphological differentiation (Figure 8) and an accumulation of cells in G1-phase of the cell cycle. SH-SY5Y and SK-N-BE(2) cells short-term treated (treatment with retinoids for five days and without retinoids for the following nine days) with Ro 13-6307 showed similar results. Ro 13-6307 was at least as effective as 13-*cis* RA, and had more pronounced effect on the aggressive MYCN amplified cell line SK-N-BE(2), regarding induction of differentiation and G1 arrest.

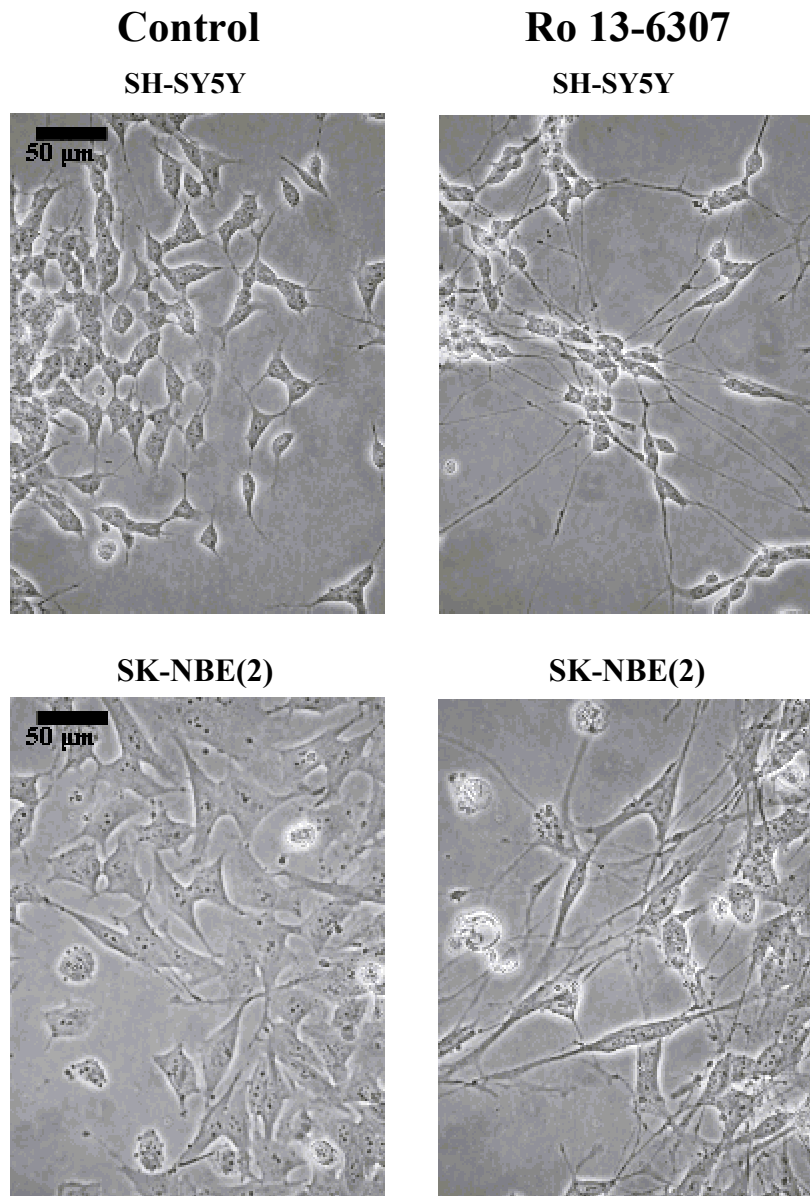


Figure 8. Morphology of *SH-SY5Y* and *SK-N-BE(2)* neuroblastoma cells treated with $1\mu\text{M}$ of Ro 136307 versus control.

To investigate whether Ro 13-6307 could enhance treatment effects achieved with 13-*cis* RA, neuroblastoma cells were sequentially treated with 13-*cis* RA for 5 days followed by 5 days of Ro 13-6307 and 4 days washout, or treated with a combination of both retinoids. No additive treatment effect was found in SH-SY5Y cells regarding growth inhibition and number of cells arrested in G1, using the combined treatment or the sequential treatment with 13-*cis* RA and Ro 13-6307 compared to treatment with the retinoids alone. In contrast, the growth inhibition of SK-N-BE(2) cells, was more effective using the combination of 13-*cis* RA and Ro 13-6307. Furthermore, in the sequentially treated SK-N-BE(2) cells the number of cells arrested in G1 increased when Ro 13-6307 was added. The morphological differentiation of both cell lines was more pronounced in cells continuously treated with both retinoids compared to continuous treatment with either Ro 13-6307 or 13-*cis* RA alone. No apoptosis was detected in either of the cell lines regardless of treatment scheduling.

Nude rats with neuroblastoma xenograft tumours were treated orally once a day, with two different doses of Ro 13-6307, in order to find a non-toxic but tumour-effective treatment. Tumours from rats treated with 0.12 mg of Ro 13-6307, were significantly smaller in terms of tumour volume (Figure 9), (day 4, $p < 0.001$, day 12, $p < 0.025$) and tumour weight ($p < 0.05$) compared to corresponding controls. Tumours from rats treated with 4 mg of 13-*cis* RA showed a significant reduction in tumour volume at day 8 ($p < 0.025$) and day 10 ($p < 0.05$), but not at day 12 compared to corresponding control tumours (Figure 9). Furthermore, there was no significant reduction in tumour weight after 12 days of therapy with 13-*cis* RA compared to corresponding controls. On the other hand, at day 12, tumours from rats treated with 0.12 mg of Ro 13-6307 were smaller than tumour from rats treated with 4 mg of 13-*cis* RA, both in terms of tumour volume and tumour weight ($p < 0.05$, one-sided probability).

Tumours from rats treated with 0.08 mg of Ro 13-6307 for 10 days, showed no reduction in tumour growth compared to corresponding controls (Figure 9). All rats treated with Ro 13-6307 or 13-*cis* RA, gained less weight than control rats during treatment, but showed no other signs of toxicity.

In summary, we demonstrated that Ro 13-6307 is able to induce morphological differentiation, G1 arrest, and inhibited cell growth in both MYCN non-amplified SH-SY5Y cells, and in MYCN amplified SK-N-BE(2) cells *in vitro*.

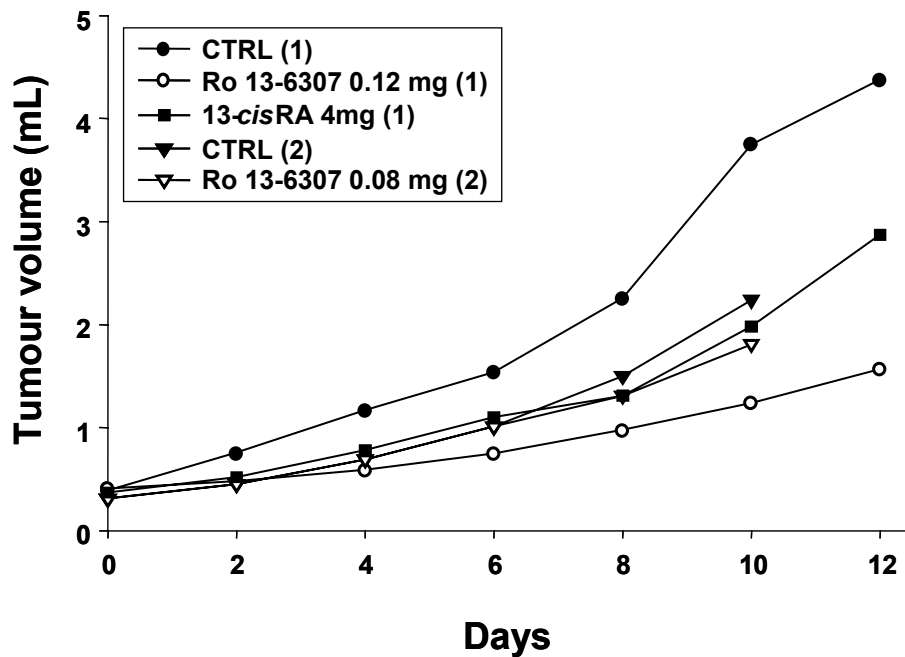


Figure 9. Neuroblastoma SH-SY5Y xenograft tumour volumes in nude rats. Mean volumes for tumours 0-12 days from start of treatment are shown for five different treatment groups. Tumours from rats treated with Ro 13-6307 0.12 mg/day (○, mean volume±SD day 0: 0.41±0.12 ml, day 12: 1.57 ±0.54 ml, p<0.025), 13-cis RA 4 mg/day (■, mean volume day 0: 0.37 ±0.05 ml, day 12: 2.87 ±1.35 ml) and corresponding controls (●, mean volume day 0: 0.39±0.07 ml, day 12: 4.38 ±2.87 ml). Tumours from rats treated with Ro 13-6307 0.08 mg/day (▽, mean volume day 0: 0.31±0.01 ml, day 10: 1.81 ±0.29 ml) and corresponding controls (▼, mean volume day 0: 0.31±0.01 ml, day 10: 2.24±0.84 ml).

Furthermore, 0.12 mg of Ro 13-6307 daily was shown to be an effective anti-neuroblastoma treatment, with limited toxic side effects. In addition, Ro 13-6307 was shown to be more effective in inducing tumour growth inhibition *in vitro* and *in vivo*, than the already clinically established retinoid 13-cis RA. Therefore, we conclude that Ro 13-6307 is a candidate retinoid for neuroblastoma therapy in children. This therapy could either be administrated as an additional retinoid subsequently after treatment with 13-cis RA, in combination with 13-cis RA or as a single retinoid depending on toxicity and pharmacokinetic data in humans.

STUDY IV

Evaluation of anti-tumour effects of oral fenretinide (4-HPR) in rats with human neuroblastoma xenografts.

Recent reports have demonstrated that fenretinide is able to induce growth arrest and a mixed apoptosis/necrosis in neuroblastoma cells *in vitro* (Di Vinci et al, 1994; Lovat et al, 2000a; Mariotti et al, 1994; Maurer et al, 1999).

The aim of this study was to evaluate the ability of fenretinide to inhibit neuroblastoma tumour growth *in vivo*. In three different experiments, nude rats with human neuroblastoma xenograft tumours were continuously treated orally, once daily for 10 days with five different doses of fenretinide ranging from 2.5-75 mg/rat (10-300 mg/kg).

Neuroblastoma xenografts from rats treated with 2.5-75 mg of fenretinide daily, showed no statistically significant reduction in tumour volume compared to corresponding control tumours at day 10. In terms of tumour weight, a significant difference was found in the first experiment comparing tumours from rats treated with 10 mg of fenretinide with corresponding control tumours. However, in the following two experiments with higher doses of fenretinide, no significant difference in tumour weight was detected (Figure 10).

Because of a possible decrease of tumour viability in treated tumours, tumour weights were adjusted for the viable tumour fraction. However, no statistically significant difference could be observed in tumours from treated and untreated animals. Other possible intra tumoural differences such as tumour morphology, cell cycle distribution and number of apoptotic cells were analysed, but no significant differences could be observed in treated compared to untreated tumours. All animals irrespectively of treatment dose showed a normal weight gain before and during the experiments.

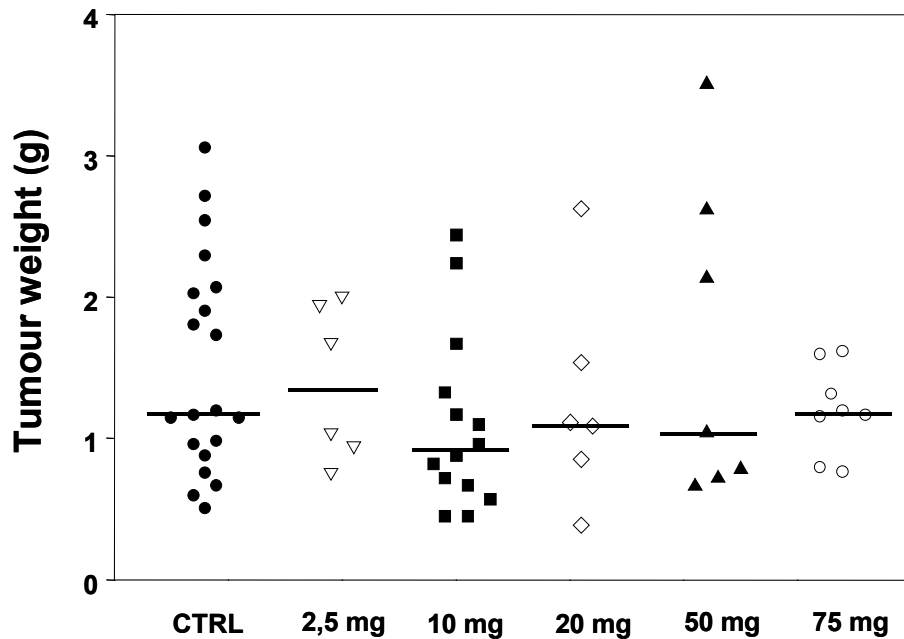


Figure 10. *Neuroblastoma SH-SY5Y xenograft tumour weights.* Tumour weights at sacrifice are shown for six different treatment groups. Tumours from rats treated with fenretinide 2.5 mg/day (∇), 10 mg/day (\blacksquare), 20 mg/day (\diamond), 50 mg/day (\blacktriangle), 75 mg/day (\circ) and controls (\bullet). Horizontal bars represent the median in each treatment group.

We conclude that, despite the promising *in vitro* results demonstrating that fenretinide induces apoptosis in neuroblastoma cells, this study showed no significant reduction in neuroblastoma tumour growth *in vivo* after oral treatment with fenretinide. Despite the five different doses of fenretinide ranging from 10-300 mg/kg used, which are comparable with doses used in clinical phase I trials, still no significant anti-neuroblastoma tumour effect could be detected. Furthermore, we could not detect any intra tumoural treatment effects in this study. Therefore, we suggest that other modalities of drug administration should be used in future experimental and clinical studies with fenretinide.

STUDY V

Biochemical responses to fenretinide treatment of neuroblastoma cells as monitored with proton magnetic resonance spectroscopy (^1H -MRS).

Magnetic resonance imaging (MRI) is an increasingly used method for diagnosis staging and evaluation of treatment response in childhood tumours, including neuroblastoma (Hiorns and Owens 2001; Siegel et al, 2002). MRI can be complemented by the use of proton magnetic resonance spectroscopy (^1H MRS), which allows non-invasive sampling of detailed biochemical information on a large number of intracellular compounds including lactate, phospholipids and amino acids from tissues otherwise difficult, or impossible to access (Negendank 1992). Using ^1H MRS, individual molecules may be identified based on their inherent individual frequency fingerprints (chemical shift from water). A molecular configuration that enables a high level of proton mobility is a prerequisite for detection with ^1H MRS.

The aim of this study was to investigate the ability of detecting biochemical alterations in intact neuroblastoma cells *in vitro* during fenretinide treatment, using ^1H MRS. Furthermore, we evaluated the use of ^1H MRS technique for non-invasive monitoring of the kinetics in the treatment response.

In order to monitor responses specific to fenretinide treatment, we used a neuroblastoma cell line (SH-SY5Y) sensitive to fenretinide, and a subclone of the same cell line with acquired resistance to fenretinide (SH-SY5Y-R, (Lovat et al, 2000a). A typical ^1H MRS spectrum of untreated SH-SY5Y neuroblastoma cell is shown in Figure 11.

We observed significant increases in mobile lipid/choline ratios, as early as six hours post treatment preceded the onset of cell death, which reached its maximum at 48 hours. This increase in lipid/choline ratios was specific for fenretinide-sensitive cells (Figure 12A). Contrary, SH-SY5Y-R cells displayed an early significant drop in the same metabolite ratios (Figure 12B). At 48 hours a 60-70% cell death was detected in sensitive cells concomitant with a significant relative decrease for the majority of ^1H MRS observable metabolites (Table 4). Specifically, a significant drop in lactate, choline compounds and macromolecules/mobile lipids was observed. The relative levels of these three metabolites were not significantly altered in the SH-SY5Y-R subclone.

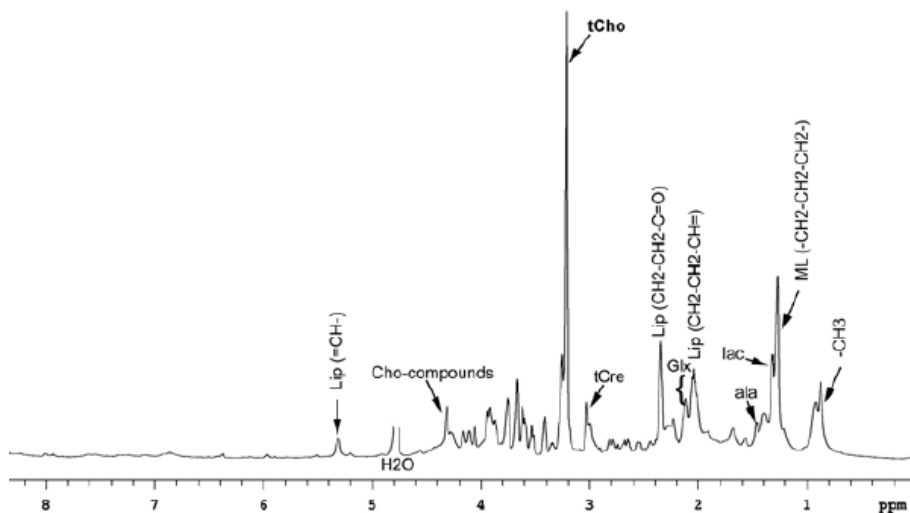


Figure 11. ^1H MRS spectrum of untreated SH-SY5Y human neuroblastoma cells. The prominent tCho peak (3.22 ppm) together with the characteristic lactate doublet (1.33 ppm) was used as chemical shift references. TCho = total choline, tCre = total creatine, Lip = lipids, Glx = glutamine/glutamate, ala = alanine, lac = lactate, ML = methylene (-CH₂-) proton in mobile lipids, -CH₃ = methyl protons in mobile lipids and macromolecules.

Table 4. ^1H MRS observable metabolites in human neuroblastoma cells.

<u>Chemical shift^a</u> <u>(ppm)</u>	<u>Corresponding metabolite(s)^d</u>	<u>SH-SY5Y^b</u>	<u>SH-SY5Y-R^c</u>
3,22	tCho (PCho + Cho + GPC)	-48% (p<0.01)	NS
1,33	lac	-63% (p<0.001)	NS
0,9	lip (-CH ₃); macro molecules	-60 (p<0.001)	NS

^aThe chemical shifts are assigned in accordance with Willker et al.

^bSH-SY5Y cells treated for 48h with 3 μM fenretinide. Signal intensities compared to untreated SH-SY5Y cells.

^cSH-SY5Y-R cells treated for 48h with 3 μM fenretinide. Signal intensities compared to untreated SH-SY5Y-R cells.

^dAbbreviations: Cho=choline, Lac=lactate, Lip=lipids.

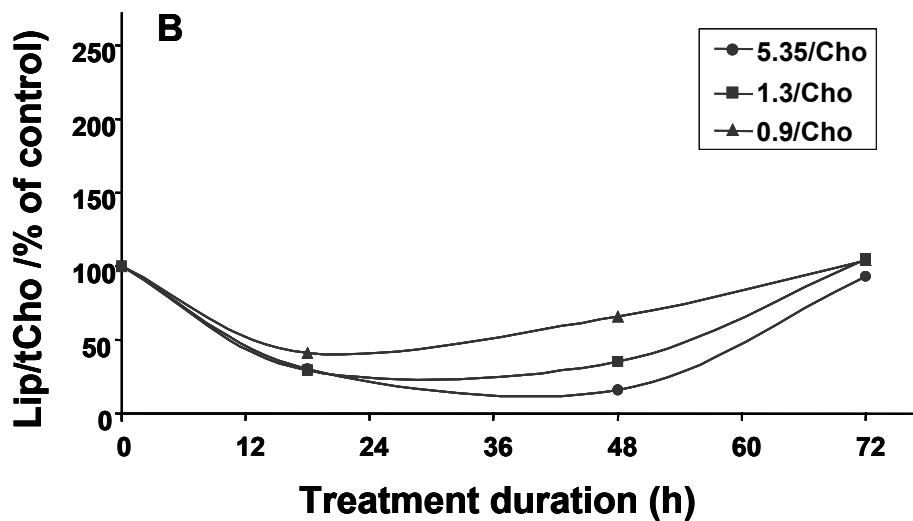
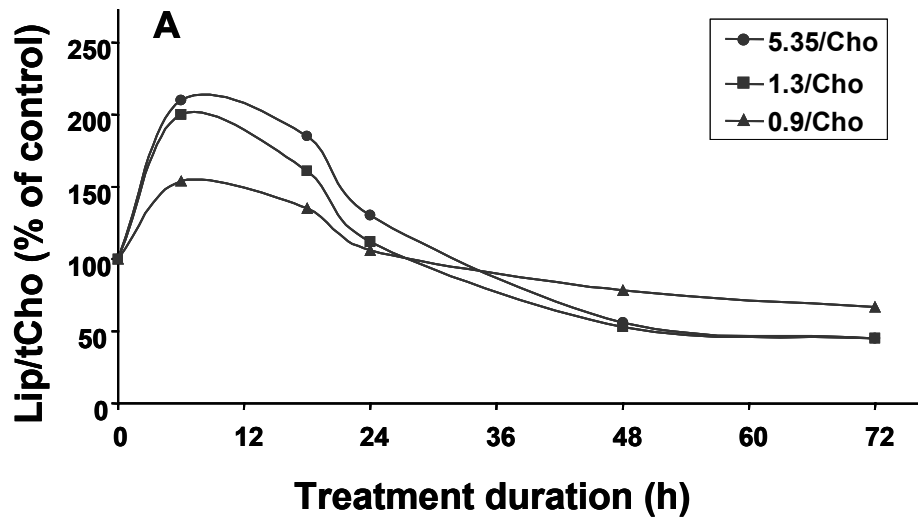


Figure 12A, B. Lipid/choline (tCho) ratio dynamics during fenretinide treatment of SH-SY5Y and SH-SY5Y-R neuroblastoma cells monitored by ^1H MRS. Lipid peaks at 1.3 ppm (■), at 0.9 ppm (▲), and at 5.35 ppm (●).

A. A transient increase in several lipid resonances (normalized to tCho content) is observed at early time points followed by a steep decrease after 24 hours in fenretinide-sensitive SH-SY5Y cells.

B. In contrast, marked decreases in lipid/tCho ratios in fenretinide-resistant SH-SY5Y-R cells are observed early after fenretinide exposure but recover to base line values at 72 hours.

In conclusion, we demonstrated the feasibility of using proton magnetic resonance spectroscopy for detecting metabolic alterations in intact neuroblastoma cells *in vitro* during fenretinide treatment. In addition, we showed that early metabolic changes, preceding morphological cell death, take place in sensitive neuroblastoma cells but not in cells with an acquired resistance to fenretinide. Furthermore, these initial events are followed by a significant drop in relative levels of several key metabolites paralleling morphological apoptosis and necrosis in responsive cells. Our findings suggest that ^1H MRS could be useful as a clinical non-invasive tool to monitor tumour response to fenretinide treatment.

GENERAL DISCUSSION

Neuroblastoma is a childhood tumour of the sympathetic nervous system with a complex clinical and biological heterogeneity, where the probability of survival varies from 30-90%. In the present thesis four different retinoids and their treatment effects on human neuroblastoma cells *in vitro* and *in vivo* were investigated. The aim was to find a new retinoid treatment, preferably for those children who currently have low chance to be cured.

The animal experiments included in this thesis, were performed using a human neuroblastoma xenograft model previously utilised to evaluate treatment effects, including 13-*cis* retinoic acid and other therapeutic drugs (Borgstrom et al, 1999; Jorgensen et al, 2000; Kogner et al, 1997; Wassberg et al, 1997). A subcutaneous xenograft model was chosen to investigate neuroblastoma tumours of human origin, and to be able to evaluate treatment effects during the experiments without using any invasive methods. However, the majority of neuroblastomas in children are aggressive metastatic tumours with poor clinical outcome. Therefore, non-invasive tools to monitor treatment effects in metastatic tumours are needed to be able to use experimental animal models closer to the clinical situation in children.

Proton magnetic resonance spectroscopy (^1H MRS) allows non-invasive sampling of biochemical information on a large number of intracellular compounds (Negendank 1992). Evidence of the ability to detect apoptosis in tumours *in vivo*, employing ^1H MRS, is increasingly emerging (Hakumaki et al, 1999). In addition, it was recently shown that the mobile lipid-choline-ratio is an accurate *in vivo* surrogate marker of the viable tumour fraction (inversely correlated) in neuroblastoma xenografts (Lindskog et al, 2003). In paper V we showed that ^1H MRS could be used as a method for detecting metabolic alterations in intact neuroblastoma cells *in vitro*, treated with fenretinide. Furthermore, it was possible to monitor the kinetics in the treatment response and to distinguish between cells sensitive to fenretinide treatment and those with acquired fenretinide-resistance. Thus, ideally, proton resonance spectroscopy could be used as a non-invasive tool providing biological surrogate markers that may distinguish between proliferating, differentiated, viable and apoptotic/necrotic tumour tissue, and offer opportunities to monitor treatment effects and tailor treatment for individual patients.

Recently, a method for quantitative measurement of tyrosine hydroxylase mRNA for detection of neuroblastoma cells in blood and bone marrow from patients with neuroblastoma was developed (Trager et al, 2003). This method may be used as an alternative non-invasive tool to monitor treatment effects during

therapy and for early and sensitive detection of relapsing disease during follow-up. In animals, this method (Trager et al, 2003) allowed the distinction between rats receiving tumour cells i.v. and s.c. respectively, with the development of wide spread metastatic disease or not.

In this thesis, tumour volume (ml) during the experiment, and tumour weight (g) at the end of the experiment were used to evaluate treatment effects of retinoids. Furthermore, the tumours were examined morphologically to estimate whether the tumour tissue was viable or necrotic. Each *in vivo* experiment needed an individual control group because of possible alterations in tumour growth between different experiments, probably caused by environmental alterations concerning cell culturing and animal breeding. Unfortunately, this made it difficult to compare actual tumour weights and tumour volumes between one experiment and the other. Since retinoids for clinical use are administered orally in oil-based forms, all retinoid treatments were orally administered and dissolved in peanut oil. Furthermore, oral therapy is preferred because it facilitates outpatient treatment.

The results obtained from papers **I** and **II** indicated that 9-*cis* RA could not be considered as the optimal candidate retinoid for oral treatment of neuroblastoma, since no tumour growth inhibiting effect with acceptable toxicity was detected. It is possible that 9-*cis* RA would be effective using other modes of administration. However, the short half-life of 9-*cis* RA and the indication that the toxicity and the tumour growth inhibiting effect mainly depend on the peak concentration (C_{max}), minimise this possibility.

In paper **IV** we evaluated five different doses of fenretinide for oral treatment in xenografted rats, however, none of them showed any tumour growth inhibiting effect. Furthermore, we were not able to detect any intra tumoural changes in the treated tumours compared to untreated tumours. However, in contrast to treatment with 9-*cis* RA, fenretinide did not induce any toxic side effects. In addition, fenretinide has been shown to function by both retinoic acid receptor-dependent and -independent mechanisms (Lovat et al, 2000a; Lovat et al, 2002; Maurer et al, 1999; Sun et al, 1999) and to be more potent in inducing apoptosis in neuroblastoma cells *in vitro* (Lovat et al, 2000b; Maurer et al, 1999), compared to 9-*cis* RA and other naturally occurring retinoids. These features may indicate that fenretinide still is a promising retinoid for neuroblastoma therapy but other modifications of drug administration should be investigated. Interestingly, there are ongoing studies indicating that intravenously administered liposome-entrapped GD2-targeted fenretinide has anti-neuroblastoma tumour-inhibiting effects *in vivo* in mice (L. Raffaghello, personal communication). Furthermore,

fenretinide intraperitoneally administrated together with safingol was shown to prolong the survival of mice with neuroblastoma xenografts (Maurer et al, 2002).

It should be emphasized that our *in vivo* experiments, target established neuroblastoma tumours (start volume, 0.3 ml, approximately corresponding to 16 days after tumour cell inoculation), in contrast to the usual way of using retinoids for cancer prevention (reviewed in Lippman and Lotan 2000; Torrisi and Decensi 2000) and treatment of minimal residual disease (MRD) (Matthay et al, 1999). There are animal studies reporting anti-tumour growth activities of both 9-*cis* RA (Christov et al, 2002; Shalinsky et al, 1995) and fenretinide, (Pienta et al, 1993; Pollard and Luckert 1991) but in these studies, the oral drug treatment started the same day or shortly after tumour cell inoculation. The reason why retinoids preferably are included in neuroblastoma studies targeting MRD is most certainly explained by the indication that cells differentiated by retinoid treatment, may become more resistant to cytotoxic drugs (Lasorella et al, 1995) or irradiation (Ronca et al, 1999). However, as studies of synthetic retinoids with other effects than induction of differentiation are emerging (Lovat et al, 2000b; Maurer et al, 1999; Meister et al, 1998; Ponzoni et al, 1995), these assumptions may have to be reconsidered. This is further supported by the findings that some of these retinoids seem to have a synergistic effects together with cytotoxic drugs (Lovat et al, 2000b).

13-*cis* RA is used in the treatment of childhood neuroblastoma, targeting minimal residual disease (Matthay et al, 1999). Results from paper **I** and **III** showed that the synthetic retinoid Ro 13-6307 was able to inhibit human neuroblastoma tumour growth in nude rats at in least the same magnitude and with similar toxicity as 13-*cis* RA. This is most likely due to that 13-*cis* RA and Ro 13-6307 have similar mechanisms of action. Both retinoids induce differentiation, and they bind to the same retinoid receptors (RARs, 13-*cis* RA via isomerisation to ATRA) with similar affinity, although Ro 13-6307 activates the receptors at lower concentrations. Even if a combined phase I/phase II trial in children with neuroblastoma would find that Ro 13-6307 has anti-tumour effects with limited toxicity, it is doubtful whether Ro 13-6307 would be established as a retinoid for clinical use. A phase III trial comparing the two retinoids should consider that Ro 13-6307 could either be administrated as an additional retinoid subsequently after treatment with 13-*cis* RA, in combination with 13-*cis* RA or as a single retinoid instead of 13-*cis* RA. Such a trial would require a large group of patients and will therefore have to be coordinated all over Europe.

In the majority of *in vitro* and *in vivo* experiments in this thesis the SH-SY5Y cell line was used. It would be of interest to perform similar experiments using

other neuroblastoma cell lines with different biological features, in particular with MYCN amplification. Although, all cell lines established *in vitro* are derived from unfavourable high-risk neuroblastoma tumours, there are significant differences, not in ultimate outcome but in clinical behaviour (time to relapse and/or death) between MYCN amplified and non-amplified neuroblastoma tumours in the high-risk subset (Lastowska et al, 2001). Moreover, the use of a metastatic orthotopic xenograft model instead of a subcutaneous xenograft model in the *in vivo* experiments with retinoids, might perhaps better mimic the clinical situation in children with neuroblastoma. Subsequently, clinical investigations in children with neuroblastoma must be performed, to elucidate treatment effects and toxicity of retinoids.

Retinoids exert most of their effects by binding to the RAR and RXR receptors. The number of receptors, distinct receptor functions, tissue-expression patterns (in normal and tumour tissue), ligand specificities, functional redundancy and regulation of multiple pathways make retinoid-signalling highly complex. RARs form heterodimers with RXRs and mediate classic retinoid activity, while RXRs are more promiscuous and heterodimerise with several other members of the steroid receptor superfamily. There are different retinoid receptor expression patterns in clinical neuroblastoma tumours (Cheung et al, 1998; Farid et al, 2000). A way of developing a targeted retinoid therapy for neuroblastoma could be to analyse tumour tissue for expression of RARs and RXRs (Rana et al, 2002) in order to design a specific retinoid treatment for the receptor expression pattern of the individual tumour. In addition, there is increased interest in developing synthetic retinoid ligands with greater selectivity than that of the natural retinoids. These receptor-selective synthetic retinoids increase the possibility of finding a specific retinoid for treatment of individual patients.

In summary, although neuroblastoma is a rare disease many children have poor outcome despite intensive multimodal therapy. This increases the necessity of finding new better treatment strategies.

CONCLUSIONS

- 9-*cis* RA reduced neuroblastoma growth *in vivo* in a dose-dependent manner. The peak concentration of 9-*cis* RA rather than the AUC is believed to be important for the tumour growth inhibiting effect.
- 9-*cis* RA had a short half-life and a low bioavailability *in vivo* after oral administration. The anti-tumour growth effective dose scheduling of 9-*cis* RA *in vivo* induced major toxic side effects. These features limit the potential use of this retinoid for clinical therapy of children with neuroblastoma.
- The synthetic retinoid Ro 13-6307 inhibited proliferation, induced morphological differentiation and induced G1 growth arrest in both MYCN amplified and non-amplified neuroblastoma cells *in vitro*.
- Oral treatment with 0.12 mg of Ro 13-6307 daily was shown to be an anti-neuroblastoma tumour growth effective therapy *in vivo*, with limited toxic side effects.
- Ro 13-6307 was shown to be at least as effective in inducing tumour growth inhibition both *in vitro* and *in vivo*, as 13-*cis* RA.
- Ro 13-6307 is a candidate retinoid for neuroblastoma therapy in children.
- Oral treatment with fenretinide induced no therapeutically significant anti-neuroblastoma tumour growth effects *in vivo*. No intra tumoural treatment effects of fenretinide could be detected.
- Other forms than oral administration of fenretinide dissolved in oil should be used in future experimental and clinical studies.
- Proton magnetic resonance spectroscopy was shown to be a suitable method for detecting metabolic alterations in intact neuroblastoma cells *in vitro* during fenretinide treatment.
- Proton magnetic resonance spectroscopy may be a useful non-invasive clinical tool to monitor early tumour response to fenretinide treatment.

SAMMANFATTNING PÅ SVENSKA

A-VITAMIN SOM BEHANDLING AV NEUROBLASTOM

Neuroblastom är en tumör i det självstyrande sympatiska nervsystemet vilken främst drabbar små barn. Neuroblastomtumörer uppkommer i binjurarna eller i nervvävnad i den sympatiska gränssträngen bredvid ryggraden. Tumören visar stor variation beträffande klinisk bild och biologiskt beteende. Den kan ibland uppvisa spontan differentiering (mognad) eller regress tack vare apoptos (programmerad celldöd). Neuroblastom är i annat fall den mest svårbotade barntumören och uppvisar dålig prognos trots intensiv behandling med cytostatika, kirurgi och strålning. Upptäckten att vissa neuroblastom kan differentiera eller gå i apoptos har stimulerat forskningsintresset kring ämnen som kan reglera dessa viktiga biologiska processer.

A-vitamin och dess varianter, retinoider, är ämnen som är viktiga för bildandet av många olika vävnader under fosterutvecklingen. Dessutom har studier på celler (*in vitro*) visat att behandling med retinoic acid (RA) kan vara verksamt mot flera typer av cancerceller genom att inducera differentiering och/eller apoptos.

Syftet med min avhandling var att studera retinoid-inducerade effekter i humana neuroblastomceller *in vitro*, samt att utvärdera behandlingseffekter av retinoider *in vivo*. Dessa studier *in vivo* utfördes genom att råttor med nedsatt immunförsvar, s.k. nakna råttor, utvecklade neuroblastom efter injektion av humana neuroblastomceller. Dessa tumörer behandlades med olika retinoider. Det övergripande målet var att hitta en ny alternativ retinoidbehandling för barn med svårbotade neuroblastom.

Oral behandling med 9-*cis* RA *in vivo*, hämmade tillväxten av neuroblastom, men hade allvarliga toxiska biverkningar hos råttorna bland annat i form av kraftig viktnedgång. Efter ytterligare experiment drogs slutsatsen att 9-*cis* RA är olämplig som oral behandling av barn med neuroblastom på grund av dess korta halveringstid, låga biotillgänglighet och toxiska egenskaper vid försök med råttor.

Behandling med den syntetiska retinoiden Ro 13-6307 *in vitro* hämmade tillväxten och inducerade differentiering samt G1-stopp i cellcykeln för neuroblastomceller både med och utan amplifiering av onkgenen MYCN. Oral behandling med Ro 13-6307 *in vivo* visade att denna retinoid kunde minska tumörväxten i råttor utan svåra toxiska biverkningar. Ro 13-6307 visade sig även vara minst lika effektiv som den redan etablerade retinoiden 13-*cis* RA både vid behandling *in vivo* som *in vitro*. Dessa resultat visar att Ro 13-6307 är en retinoid

som möjligen skulle kunna användas för oral behandling av barn med neuroblastom.

Trots att den syntetiska retinoiden fenretinid kan inducera apoptos i neuroblastomceller *in vitro*, gav oral behandling med fenretinid i fem olika doser ingen minskad tumörväxt *in vivo*. Dessutom kunde inga morfologiska förändringar upptäckas i de behandlade tumörerna jämfört med obehandlade tumörer. Fenretinids förmåga att minska tumörväxt *in vivo* bör därför ytterligare utredas genom nya att beredningsformer och andra administrationssätt prövas.

Proton-NMR-spektroskopi visade sig vara en passande metod för att påvisa biokemiska förändringar i neuroblastomceller behandlade med fenretinid *in vitro*. Det biokemiska mönstret skilde sig mellan neuroblastomceller känsliga för eller resistenta mot fenretinidbehandling. Det var möjligt att tidigt se biokemiska förändringar och följa kinetiken hur tumörcellerna svarade på fenretinidbehandlingen. Dessa resultat indikerar att proton-NMR-spektroskopi skulle kunna användas för att tidigt upptäcka och följa fenretinid-inducerade behandlingseffekter i neuroblastomtumörer *in vivo*, utan att man behöver göra några ingrepp på barnet.

Sammanfattningsvis visar studierna, som ingår i min avhandling, att retinoider kan hämma tillväxten av neuroblastomceller både *in vitro* och *in vivo*, men att den erhållna effekten beror på vilken retinoid som används. Tumörkänslighet, dosering och toxiska biverkningar är viktiga faktorer som avgör om retinoider är användbara mot neuroblastom *in vivo*. Ro 13-6307 kan vara en retinoid för neuroblastombehandling i framtiden.

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