Evaluation of Galectin-1 as a Target for Therapy in Neuroblastoma

A dissertation
Submitted in partial fulfilment of the requirements for the award of
Doctor rer medic
in
Medical Sciences
in the Faculty of Medicine at the University of Duisburg-Essen

Submitted by
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2011
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Tag der mündlichen Prüfung: 10. Mai 2011
FOREWORD

Galectin-1 is a protein involved in different facets of tumour progression. This thesis analyses the impact of galectin-1 as a target for therapy of neuroblastoma, the most common extracranial solid cancer in childhood. It also focuses on the use of novel therapies and animal models in preclinical investigations of galectin-1 downregulation that could possibly contribute to improve the current poor survival of patients with the most aggressive relapsing form of the disease.

Essen, January 2011

*Let me tell you the secret that has led me to my goal.*
*My strength lies solely in my tenacity*

*Louis Pasteur*
# TABLE OF CONTENTS

1. **INTRODUCTION**...........................................................................................................7
   1.1. Neuroblastoma........................................................................................................7
      1.1.1. Biology of Neuroblastoma...............................................................................7
      1.1.2. Clinical Presentation, Diagnosis and Staging.................................................8
   1.2. Targeted Treatment of Neuroblastoma: An Overview..........................................12
      1.2.1. Histone Deactylase Inhibitors.......................................................................14
         1.2.1.1. Suberoylanilide hydroxamic acid.........................................................15
         1.2.1.2. Valproic Acid.......................................................................................15
      1.2.2. Temozolomide...............................................................................................15
      1.2.3. Farnesylthiosalicylic Acid............................................................................16
   1.3. Mouse Models of Neuroblastoma...........................................................................16
      1.3.1. Xenograft Models.........................................................................................17
      1.3.2. Orthotopic Metastatic Models.......................................................................17
      1.3.3. Transgenic Models.......................................................................................18
   1.4. Galectin-1............................................................................................................19
   1.5. Objectives.............................................................................................................23

2. **MATERIALS AND METHODS**..................................................................................24
   2.1. Materials...............................................................................................................24
      2.1.1. Chemicals and Consumables.......................................................................24
      2.1.2. Buffers and Reagents...............................................................................26
      2.1.3. List of Primers and Oligonucleotide Sequences........................................27
      2.1.4. Enzymes..................................................................................................28
      2.1.5. Commercial Kits......................................................................................28
      2.1.6. Antibodies.................................................................................................29
      2.1.7. Cell Culture Materials..............................................................................29
         2.1.7.1 Medium for Bacteria Cell Culture.........................................................29
         2.1.7.2 Bacteria Strain and their Characteristics............................................30
         2.1.7.3 Neuroblastoma Cell Lines and their Origins........................................30
      2.1.8. Equipment..................................................................................................30
      2.1.9. Surgical Equipment...................................................................................31
Introduction

2.2. Methods.............................................................................................................32
  2.2.1 Cell Cultures................................................................................................32
  2.2.2 Drug Formulation and Administration....................................................32
  2.2.3 Proliferation/cytotoxicity Assay.................................................................32
    2.2.3.1 MTT Proliferation Assay.................................................................33
    2.2.3.2 xCELLigence Cellular Analysis.........................................................33
  2.2.4 shRNA Regulated Galectin-1 Expression..................................................33
  2.2.5 siRNA Transfection.................................................................................34
  2.2.6 Reversed Transcriptase PCR Analysis......................................................34
  2.2.7 Co-Immunoprecipitation.........................................................................35
  2.2.8 Western Analysis......................................................................................35
  2.2.9 Cell Cycle Analysis by Flow Cytometry...................................................36

2.3. Animal Experiments.....................................................................................36
  2.3.1 Ethics and Animal Care............................................................................36
  2.3.2 Microsurgical Procedure.........................................................................36
  2.3.3 Mouse Grafting and Treatment Schedules..............................................37
  2.3.4 Histology..................................................................................................37
  2.3.5 Immunohistochemistry...........................................................................37
  2.3.6 Statistical Analysis..................................................................................38

3. RESULTS............................................................................................................39
  3.1. Transient siRNA/shRNA Directed Galectin-1 Downregulation.....................39
  3.2. Histone Deacetylase Inhibitors Inhibit Proliferation of
       Neuroblastoma Cells in vitro........................................................................42
  3.3. Effects of Farnesylthiosalicylic Acid and Temozolomide on
       Neuroblastoma Cell Growth in vitro...........................................................45
  3.4. Effects of Combining Temozolomide to Farnesylthiosalicylic
       Acid in Neuroblastoma Cells in vitro..........................................................46
  3.5. Galectin-1 Co-localises with HRas in Neuroblastoma Cells.........................52
  3.6. Galectin-1 Silencing in Neuroblastoma does not apparently
       Potentiate the Anti-proliferative Activity of HDAC Inhibitors,
       FTS or TMZ in vitro....................................................................................53
  3.7. The Anti-proliferative effect of Galectin-1 Downregulation is not
       Accompanied by Changes in Cell Cycle Distribution...............................63
Introduction

3.8. Steps Towards an Orthotopic Neuroblastoma Tumour Model…………65
3.9. FTS does not Show Antitumour Activity as a Single Agent but
Chemosensitises Experimental Neuroblastoma to the
Effects of TMZ……………………………………………………………….67
3.10. TMZ or Galectin-1 Downregulation Increase the Survival of
Neuroblastoma Tumour-bearing Mice but do not act
synergistically……………………………………………………………….69
3.11. The Tumour Growth Inhibitory Effect of TMZ and Galectin-1
Silencing is not Primarily Caused by Reduced Cell
Proliferation…………………………………………………………………71

4. DISCUSSION……………………………………………………………………….73
4.1. In Vitro Studies………………………………………………………………74
4.2. HDAC Inhibitors and Galectin-1 Silencing………………………………. 75
4.3. TMZ, FTS And Galectin-1 Silencing in vitro and in vivo………………... 76

5. CONCLUSION………………………………………………………………………80

6. REFERENCES………………………………………………………………………81

7. APPENDIX………………………………………………………………………98

8. ACKNOWLEDGMENTS…………………………………………………………100

9. CURRICULUM VITAE…………………………………………………………….102
1. INTRODUCTION

1.1. NEUROBLASTOMA

Neuroblastoma, a neoplasm of the sympathetic nervous system, is the second most common extracranial malignant tumour of childhood and the most common solid tumour of infancy (Park et al., 2008). It was first described by the German physician Rudolf Virchow in 1864 and later named by James Homer Wright in 1910. Ever since, neuroblastoma has continued to bewitch paediatric oncologists due to its enigmatic clinical behaviour. On the lower end of the scale, the tumour will undergo spontaneous regression or differentiation to a more benign tumour with very minimal or no therapy whereas on the extreme end, the tumours progress or relapse despite aggressive multimodality therapy.

The incidence rate of neuroblastoma is 10.5 cases per million children who are less than 15 years old (Park et al., 2008). It accounts for about 8 - 10% of all childhood cancers and for 15 % of childhood cancer mortality and is reported to be the second most common cause of death in children only preceded by accidents (Maris, 2007; Castel et al., 2007). The incidence of Neuroblastoma is similar across the world with boys showing a slightly higher frequency than girls. Only speculations have been put forward for this discrepancy.

1.1.1. Biology of Neuroblastoma

Neuroblastoma arises from neural crest cells which is a pluripotent highly migratory cell population that arises at the junction of the neural tube and the dorsal ectoderm. The neural crest cells differentiate into a variety of cell types derivatives which include the peripheral nervous system, chromaffin (endocrine) cells of the adrenal medulla and paraganglia, pericytes, pigment cells and the facial skeleton (Park et al., 2008; Mora & Gerald, 2004). Neuroblastoma can arise anywhere along the sympathetic nervous system but 65 % of primary tumours arise in the abdomen with the medulla of the adrenal gland being predominant (Park et al., 2008).

The aetiology of neuroblastoma is still mainly unknown, but environmental factors seem unlikely (Brodeur, 2003). Familial forms of NB are rare, accounting for about 10 % of all cases. Recently, the anaplastic lymphoma kinase (ALK) was identified
as a predisposing factor for familiar neuroblastoma (Mosse et al., 2008) and has also shown to be mutated in sporadic neuroblastomas (Caren et al., 2008). Activating germline and somatic mutation in ALK was found as well as amplification and in-gene rearrangements, all which correlated to more advanced disease (Caren et al., 2008; Mosse et al., 2008).

NB can also occur in patients affected with other neural crest disorders or malignancies, such as the heritable disease neurofibromatosis (von Recklinghausen disease), where neurofibromatous tumours develop, and Hirschsprung disease, with loss of ganglia in the colon, are associated with neuroblastoma (Brodeur, 2003, Park et al., 2008). In addition, a germline mutation and constitutional mutations in the \textit{PHOX2B} gene has been shown to correlate with hereditary or multifocal NB (Bourdeaut et al., 2005; Van Limpt et al., 2003). This gene is also the major disease gene for congenital central hypoventilation disorder (Ondine’s Curse, a lack of autonomic respiratory control) which shows a hereditary co-occurrence with both neuroblastoma and Hirschsprung disease (Bourdeaut et al., 2005). Interestingly, there seems to be an under representation of neuroblastoma among patient with Downs Syndrome (Brodeur, 2003).

1.1.2 Clinical Presentation, Diagnosis and Staging

The clinical presentation of neuroblastoma is as heterogeneous as the tumour and depends upon the site of tumour origin, metastatic spread and the presence of paraneoplastic syndromes (Maris et al., 2007). The spectrum of pediatric neuroectodermal tumours ranges from undifferentiated, truly malignant neuroblastomas, via ganglioneuroblastomas to well-differentiated, mostly benign ganglioneuromas (van Noesel & Versteeg, 2003). The current diagnosis for neuroblastoma is defined by histological presence of tumour cells in biopsy of bone marrow aspirate, assessment of catecholamines or catecholamine metabolites in urine or serum, and possible metastatic spread and tumour size is monitored by computer tomography, magnetic resonance imaging and/or scintigraphy (Maris et al., 2007; Park et al., 2008).

At the time of diagnosis neuroblastoma is staged according to the international neuroblastoma staging system (INSS) (Brodeur et al., 2004), staging 1-4 (Table 1). The most prognostic features in neuroblastoma are age, stage and tumour
biology (Brodeur et al., 1997; Schimada et al., 1999). Children aged < 18 months and are at a lower stage have a more favourable outlook than older infants and those at a higher stage with the exception of stage 4S (S = special).

The INSS is a surgically based staging system, and as such patients with locoregional disease can vary significantly based on degree of surgical resection which is dependent on the skill of the surgeon that decides how extensive the excision can be thereby increasing the risk for staging patients more towards high-risk treatment, hence the risk of over treating patients is also increased. In addition, patients with localised disease who will not undergo surgery cannot be properly staged. Given these limitations, the International Neuroblastoma Risk Group (INRG) addressed this problem in 2009 with a new staging system which is based on Image Defined Risk Factors (IDRF) that strictly evaluates the resectability. In this system the patients are divided into three stages, localised L1 or L2 depending on if the tumours have IDRF or not, metastatic stage M and the special metastatic stage MS (Monclair et al., 2009). The INRG stage is then further weighed into the INRG classification system together with other prognostic factors to achieve a pre-treatment risk stratification system (Cohn et al., 2009). In the MS stage, that was previously called 4S, infant patients present with small localised tumours and metastases to the liver, skin and/or bone marrow. This is the group with in which there is spontaneous tumour regression.

Five to ten percent of NBs that are detected regress without treatment, which is the highest frequency observed in any tumour type. Clinically, regression of NB can be manifested as a complete disappearance of the tumour or differentiation into a benign ganglioneuroma. Relapse may develop following spontaneous regression, but is considered a rare event (Haas et al., 1988).

A consistent pattern of biological factors that describes these patients has not been established, although low MYCN levels and high TrkA expression are considered favourable. The three current hypotheses on the mechanism of regression are that it is due to an immunological attack on the tumour, spontaneous differentiation/maturation, or that there is a delay in the developmental time-switch for apoptosis (Papac, 1996; Hellstrom & Hellstrom, 1971; Cole, 1981; Pritchard & Hickman, 1994). This intriguing clinical behaviour of
Introduction

NB has stimulated research on differentiation- and apoptosis-inducing agents for the treatment of the disease.

Table 1: International Neuroblastoma Staging System (INSS) [Brodeur et al, 1993]

<table>
<thead>
<tr>
<th>Stage 1</th>
<th>Localised tumour with complete gross excision with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically (nodes attached to and removed with the primary tumour may be positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 2A</td>
<td>Localised tumour with incomplete gross resection; representative ipsilateral nonadherent lymph nodes negative for tumour microscopically</td>
</tr>
<tr>
<td>Stage 2B</td>
<td>Localised tumour with or without complete gross excision with ipsilateral nonadherent lymph nodes positive for tumour; enlarged contralateral lymph nodes must be negative microscopically</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Unresectable unilateral tumour infiltrating across the midline$^a$, with or without regional lymph node involvement, localised unilateral tumour with contralateral regional lymph node involvement, or midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, or other organs (except as defined for stage 4S)</td>
</tr>
<tr>
<td>4S</td>
<td>Localised primary tumour (as defined for stage 1, 2A, or 2B) with dissemination limited to skin, liver, or bone marrow$^b$ (limited to infants &lt; 1 year of age)</td>
</tr>
</tbody>
</table>

$^a$ The midline is defined as the vertebral column. Tumours originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column.

$^b$ Marrow involvement in stage 4S should be minimal (i.e. < 10% of total nucleated cells identified as malignant on bone marrow biopsy or marrow aspirate). More extensive marrow involvement should be considered to be stage 4. The metaiodobenzylguandine scan (if performed should be negative in the marrow).
The biological hallmark of neuroblastoma is the complexity of the genetic alterations. These can be used as biological markers independently of clinical features (reviewed in Bown 2001). There has been no single genetic change common to all neuroblastomas identified till date. 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 17q 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 discussed that co-occurrence with loss of 1p might account for this association and that 17q gain is not an independent prognostic marker.

Amplification of the proto-oncogene MYCN is the most prototypic genetic aberration in neuroblastomas and is found in 20–25% of all neuroblastomas and
Introduction

usually results in 5-400 gene copies per cell corresponding with high levels of protein expression (Seeger et al., 1988; Brodeur & Fong, 1989). MYCN was identified as a gene homologous to c-MYC and overrepresented in neuroblastomas (Schwab et al., 1983).

MYCN amplification is associated with advanced disease as well as with an unfavorable prognosis in infants with a lower stage of disease (Brodeur et al., 1984; Seeger et al., 1985) and further correlates with resistance to certain treatment modalities (Livingstone & Mairs, 1997) and the association appears to be independent of clinical stage (Nakagawara et al., 1987; Seeger et al., 1985) and age (Rubie et al., 1997). Therefore, MYCN status is usually assessed in patients due to its strong prognostic value and is used worldwide for treatment stratification (Berthold et al., 1997).

Neurotrophin receptors represent another important factor responsible for regulating the malignant transformation of NB cells. Neurotrophin signalling plays a central role in normal neuronal development and is mediated by the Trk family of tyrosine kinases (Barbacid, 1995). Expression patterns of the neurotrophin receptors (TrkA, TrkB and TrkC) correlate with the biological and clinical features of NB and the degree of neuroblast differentiation (Brodeur et al., 1997). TrkA is the cognate receptor for the nerve growth factor (NGF), and developing neurons tend to differentiate in response to the ligand or die by apoptosis when NGF is withdrawn (characteristics of a dependence receptor). Consequently, high TrkA expression is correlated with a favourable prognosis of NB and is also inversely correlated to MYCN-amplification, and virtually absent in high-risk patients (Kogner et al, 1993; Nakagawara et al., 1993; Brodeur et al., 2009). By contrast, full length TrkB, the receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4, is highly expressed in MYCN-amplified advanced-stage aggressive tumours and is thought to mediate autocrine survival in tumours which simultaneously express BDNF (Nakagawara et al., 1994).

1.2. Targeted Treatment of Neuroblastoma: An Overview

In recent years, treatment protocols for neuroblastoma have been directed based on ‘risk-group’ criteria, which take into consideration various biological predictors of tumour behaviour (Table 2). In treating the low-risk patients, surgery is used as
the primary approach in order to minimize the risk of chemotherapy-related morbidity (Kushner et al., 1996). The survival rate for these patients is 95% (Alvarado et al., 2000; Perez et al., 2000). Stage 4S patients constitute a special group of patients who often undergo spontaneous regression in the majority of cases and also have a low risk of progression to advanced-stage disease, thus these patients are observed rather than operated (Nickerson et al., 2000). Intermediate-risk patients usually undergo surgery followed by moderate-dose multimodal chemotherapy. It has been shown that children lacking MYCN amplification had a 93% 3-year event-free survival (EFS) rate while children with MYCN amplification only had a 10% 3-year EFS rate, again pointing at the clinical significance of this marker (Schmidt et al., 2000). The majority of NB patients belong to the high-risk-group. Treatment for high-risk patients can be divided into four phases: 1) induction therapy (chemotherapy); 2) Local control maintenance using a combination of aggressive surgical resection and administration of radiotherapy 3) consolidation therapy (myeloablative chemotherapy and stem cell rescue (e.g. autologous bone marrow transplantation (ABMT)) and; 4) minimal residual disease therapy (e.g. administration of retinoids or targeted molecules directed against GD2 on NB cells (see below)). Up to date, the standard treatment for children with high-risk NB is intensive myeloablative therapy supported by autologous hematopoietic stem-cell transplantation and the administration of retinoids (this follow-up therapy might be long and might include immune therapy). The overall survival has improved for high-risk NB patients over the last decades, but cure rates remain low (10-20% long-term survival) despite intensive multimodal therapy (Brodeur & Maris, 2002). Given that conventional chemotherapeutics are nearing their maximum potential with regards to efficacy and patient tolerance, a myriad of novel therapeutic approaches have been investigated over the past several years. The identification of new active agents or drug combinations is essential to improving the prognosis for these patients.
Table 2: Neuroblastoma risk groups based on clinical and biological features (Brodeur, 2003)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Low</th>
<th>Intermediate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>All</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2A,B</td>
<td>&lt;1 year, or 1–21 years and MYCN non-Amp, or 1–21 years and MYCN Amp, FH</td>
<td>None</td>
<td>1-21 years with MYCN Amp with UH</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>&lt;1 year and MYCN non-Amp, or 1–21 years and MYCN non-Amp with FH</td>
<td>0–21 years and MYCN Amp, or 1–21 years and MYCN non-Amp with FH</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>&lt;1 year and MYCN non-Amp</td>
<td>&lt;1 year and MYCN Amp, or 1–21 years</td>
</tr>
<tr>
<td>4S</td>
<td>MYCN non-Amp; FH; hyperploid</td>
<td>MYCN non-Amp; UH; diploid</td>
<td>MYCN Amp</td>
</tr>
</tbody>
</table>

Amp, amplified; FH, favorable histology; UH, unfavorable histology

1.2.1. Histone Deacetylase Inhibitors

Modulation of gene expression through targeting epigenetic events with DNA methyltransferase inhibitors and histone deacetylase (HDAC) inhibitors is one of the emerging strategies in cancer therapy and has demonstrated significant clinical activity in hematological malignancies. HDACIs have been shown to exert potent anticancer activities inducing differentiation, cell cycle arrest, apoptosis, increase immunogenicity and inhibition of angiogenesis of cultured cancer cells. Notably, a high efficacy of these drugs has been selectively revealed in malignant cells rather than in normal cells (Emmanuelle et al., 2008). Histone deacetylase agents have also shown preclinical activity against neuroblastoma (Deubzer et al., 2007; Furchert et al., 2007). A handful of HDACi are now being tested in phase I/II clinical trials both as monotherapy and in combination with other chemotherapeutic regiments and radiotherapy.
Introduction

1.2.1.1. Suberoylanilide hydroxamic acid (SAHA)

SAHA (vorinostat, Zolinza®) has already been approved as a second line treatment for patients with refractory, persistent or relapsed cutaneous T Cell Lymphoma. Clinical investigations have provided evidence that SAHA exhibits a high therapeutic potential for different forms of tumours at doses that are well tolerated by patients (Kelly et al., 2005). In addition, SAHA seems to exert antitumour effects in a synergistic manner with various compounds such as anticancer drugs (Ocker et al., 2005; Dey, 2006).

1.2.1.2. Valproic acid (VPA)

Valproic acid is now an established drug for the treatment of epileptic seizures (absence, tonic-clonic [grand mal]), and complex partial seizures) and mania in bipolar disorder (Bowden and Singh, 2005). VPA has shown potent antitumour effects in a variety of in vitro and in vivo systems, by modulating multiple pathways including cell cycle arrest, apoptosis, angiogenesis, metastasis, differentiation and senescence. These effects seem to be cell type specific which may depend also on the level of differentiation and the underlying genetic alterations. VPA has demonstrated strong anti cancer activity in NB studies (Cinatl et al., 1996; Michealis et al., 2004; Blaheta et al., 2007).

1.2.2. Temozolomide (TMZ)

TMZ is a second generation methylating imidazotetrazine compound (TZC) which undergoes chemical degradation at physiological pH to the cytotoxic metabolite 3-methyl- (triazen-1-yl)imidazole-4-carboxamide (MTIC) (Tsang et al, 1991). TMZ was approved by the Food and Drug Administration (FDA) in the US, as well as in Europe, for the treatment of anaplastic astrocytoma and other high grade gliomas in an adjuvant setting. Several studies with TMZ have been recently completed in paediatric malignancies and other tumour entities (De Sol et al., 2006). Temozolomide is considered to exert its toxic effects primarily by generating O6-methylguanine in DNA (Tisdale et al., 1987; Wedge et al., 1996). Quite recently, a phase II clinical trial of Irinotecan and Temozolomide in children with relapsed or
refractory NB showed the clinical benefit in these patients. Several studies in other cancers have revealed that TMZ is a pro-autophagic and anti-angiogenic drug.

1.2.3. Farnesylthiosalicylic Acid (FTS)

FTS, Salirasib (S-trans, trans-farnesylthiosalicylic acid), is a Ras farnesylcysteine mimetic that has been shown to selectively disrupt the association of active Ras proteins with the plasma membrane, impairing Ras membrane anchorage and function. FTS was shown to efficiently diminish Ras signaling (by disrupting galectin-1-HRAS association) and attenuation of cell growth in vitro and in vivo in various human malignancies including pancreatic, lung and other cancers (Rotblat et al., 2008). Phase I clinical studies have been carried out on solid tumours and phase II trials including a Phase I/II study dosing salirasib + gemcitabine for the treatment of newly diagnosed pancreatic cancer patients at Memorial Sloan-Kettering as well as a Phase II trial treating both treatment naïve and previously treated lung cancer patients with salirasib mono-therapy are currently be carried out (Tsimberidou et al., 2009). Evidence of the antitumour activity of FTS in NB demonstrates that it causes NB growth arrest by disrupting the cooperation between Ras and MYCN (Yaari et al., 2005).

1.3. MOUSE MODELS OF NEUROBLASTOMA

The limited number of patients, which are currently often enrolled simultaneously in both national and international studies for the treatment of paediatric malignancies, poses a challenge as how to use these as wisely as possible (Ablett et al., 2004). In addition, as the cure rates in childhood cancer increase the number of available patients shrinks, and the eligible patients are often heavily pretreated so that the majority of them are not responding to chemotherapy (Balis et al., 2009). Modelling human cancer in animals is a field under constant development, and as much as these models can aid us both in unravelling the pathogenesis of the disease and aid in selection of therapies, there are drawbacks and considerations to be taken into consideration. These models may not be perfect in every aspect mimicking the patient child, but it is the best replacement available.
1.3.1. Xenograft Models

In this model system, any type of tumour tissue (primary or cell lines) of any origin is implanted, usually s.c., in immunodeficient mice or rats, and after latency period growing tumours are established and the animals can be assigned to treatment schedules (Morton et al., 2007). The cells can be chosen to mimic a specific feature of the disease or a subgroup of the patients. For instance, Keratoepithelin was overexpressed in NB cell lines and implanted in nude mice to monitor tumour progression (Becker et al., 2006). The s.c. neuroblastoma xenografts have been the base for a wealth of publications and testing of conventional and novel therapies, and it is beyond the scope of this text to make a synoptic review of this subject.

1.3.2. Orthotopic and Metastatic Models

Orthotopic growth, meaning in its “right place”, has been described for several cancer types, and can provide additional valuable data in parallel or in addition to s.c. xenografts. Metastatic models have their further use in the fact that they represent the principal clinical challenge, i.e. eradicating disseminated disease. For neuroblastoma, the orthotopic sites used are in and around the adrenal gland, whilst metastatic models are reported with by i.v. injection (peripheral or into the aorta). In addition, orthotopic models can also metastasize (something s.c. xenografts very rarely do) especially in an NK-cell deficient model. The orthotopic neuroblastoma model was first described in 1994/95 (Flickinger et al., 1994; Judware et al., 1995). In 2000 it was further developed by GFP-expressing cells (implanted near the adrenal gland), enabling the metastases to be detected by RT-PCR and the visualisation and measuring of the tumour by magnetic resonance imaging (MRI) (Moats et al., 2000). In 2001, a metastatic neuroblastoma model was described, where cells injected i.v. resulted in the development of both a primary adrenal mass and distant metastases to bone and liver (Engler et al., 2001). In an attempt to address the differences between heterotopic s.c. tumours and orthotopic tumours, a study in 2002 found that the orthotopic tumours showed more relevant biology (Khanna et al., 2002). In 2005,
orthotopic growth of neuroblastoma cells in the adrenal gland pointed out the importance of a correct microenvironment, as these tumours reproduced the abnormal vascularisation and metastatic growth of patients (Joseph et al., 2005). The orthotopic and metastatic model have the same practical requirements as the heterotopic model as they also use immunodeficient mice, except in syngeneic models. The use of non-invasive techniques to follow and measure tumour growth and disease dissemination is a requirement.

1.3.3. Transgenic Models

The earliest report of a transgenic model of neuroblastoma was in the early 90s where in an effort to study a polyoma virus antigen under the thymidine kinase promotor, these mice developed neuroblastoma-like tumours and preneoplastic lesions in the adrenal medulla (Aguzzi et al., 1990). This discovery was followed by others making transgenic mice with different constructs and seemingly by chance ending up with neuroblastoma-like tumours (Iwamoto et al., 1993; Pecori Giraldi et al., 1994; Servenius et al., 1994; Skalnik et al., 1991). However, Weiss and colleagues have genetically engineered a transgenic mouse that overexpresses the MYCN proto-oncogene under the control of the tyrosine kinase promoter, and these animals have a high prevalence of neuroblastoma (Weiss et al., 1997). The model showed that MYCN by itself could drive the tumourigenesis of neuroblastoma and that the gene dosage of MYCN was a direct contributor, as homozygous mice showed increased incidence and shorter latency of tumour formation (Weiss et al., 1997). Further, the model was shown to be metastatic and that the tumours displayed syntenic gains and losses of genetic material that can be observed in patients (Weiss et al., 1997). The transgenic mice were then shown to further have amplified the transgene in the tumours and, interestingly, no correlation between MYCN expression and transgene dosage or tumour latency was observed (Norris et al., 2000). It has also been shown that whilst normal litter mates develop hyperplasia in the ganglia that spontaneously regress this was delayed and incomplete in the transgenic mice, and the conclusion was put forward that the improper expression of MYCN drives tumourigenesis by disturbing
the normal process when these cells should be eradicated (Hansford et al., 2004). The histology of tumours from these animals (Moore et al., 2008) and characteristics of cell lines established from the tumours (Cheng et al., 2007) showed high similarity to human neuroblastoma.

Several studies have further confirmed that genomic events in these mice are syntenic to those observed in patients (Hackett et al., 2003; Lastowska et al., 2004; Weiss et al., 2000). There has been a number of treatment studies performed on these animals, including MYCN antisense oligonucleotide (Burkhart et al., 2003), anti-angiogenic TNP-470 (Chesler et al., 2007), methionine aminopeptidase inhibitor (Morowitz et al., 2005), retinoids (Liu et al., 2005), phosphatidylinositol 3 kinase (PI3K) inhibitor (Chesler et al., 2006), the histone deacetylase inhibitor Trichostatin A (Kuljaca et al., 2007; Liu et al., 2007), and cyclophosphamide (Chesler et al., 2008). Several genetic targets have been shown to be deregulated in the model, including the multidrug resistance-associated protein (MRP) where treatment sensitivity can be restored by the novel MRP1 inhibitor Reversan (Burkhart et al., 2009). The ornithine decarboxylase 1 (ODC1) can be targeted using the suicide inhibitor DFMO (Hogarty et al., 2008; Rounbehler et al., 2009). Haploinsufficiency of the putative tumour suppressor Clustrin (Chayka et al., 2009) and transcription factor and potential oncogene HMGA1 (high mobility group A1 protein) (Giannini et al., 2005) has also been reported. Most recently, it has also been presented that expression of PHOX2B plays an important role in the pathogenesis of neuroblastoma in these mice (Alam et al., 2009).

1.4. GALECTIN-1

Galectins are a phylogenetically conserved family of lectins defined in 1994 as a shared consensus of amino-acid sequences of about 130 amino acids and the carbohydrate recognition domain (CRD) responsible for β-galactoside binding (Barondes et al., 1994).

Galectin-1 (gal-1), the first protein to be discovered in the family is a 14 kDa lectin expressed ubiquitously in mammalian organism and has been reported to be expressed in many tumour types including astrocytoma, melanoma and prostate, thyroid, colon, bladder and ovary carcinomas (reviewed by Danguy et al., 2002).
Interestingly, in most cases such expression correlates with the aggressiveness of these tumours and the acquisition of metastatic phenotype. Many studies have demonstrated the involvement of gal-1 in various aspects of tumourigenesis and it has been described as a promising cancer target (see figure 1, reviewed in Liu & Rabinovich, 2005; Rabinovich, 2005; Salatino et al., 2008).

Galectin-1 function has been found to be intricately linked to essential biological processes in tumour cells. It can act as an extracellular matrix protein to modulate cell adhesion (Hughes, 2001) as well as intracellularly by interaction with H-ras causing Ras membrane anchorage and cell transformation (Paz et al., 2001). Galectin-1 directs the transport of farnesylated H-ras to the Golgi apparatus in various systems (Belanis et al., 2008). Inhibition of H-ras/Galectin interaction by small molecule inhibitors, such as farnesylthiosalicylate, (Paz et al., 2001) has proved anticancer activity in various model systems and is now proceeding to clinical development. Additionally, galectin-1 knockdown has been shown to synergistically potentiate the actions of Temozolomide in a mouse metastatic melanoma model thus emphasising the role of galectin-1 in sensitizing tumour cells to chemotherapy (Mathieu et al., 2007). Galectin-1 also regulates complex signalling pathways involved in tumour–host interaction (Juszczynski et al., 2007; Rodig et al., 2008) and angiogenesis (Thijssen et al., 2006). By forcing activated T cells to undergo apoptosis (He & Baum, 2004), Gal-1 expression has been hypothesized to contribute to the tumour-immune escape. Inhibition of Gal-1 function resulted in T-cell mediated tumour rejection (Rubinstein et al., 2004). Gal-1 has been shown to contribute to tumour angiogenesis in a mouse melanoma model (Le Mercier et al., 2008a), and selective peptide inhibitors to Gal-1 have been developed that show profound antiangiogenic activity in vivo (Thijssen et al., 2007).
Fig 1: Contribution of galectin-1 to tumour progression. Galectin-1 interacts with oncogenic HRAS and contributes to membrane anchorage of HRAS and tumour transformation. In addition, this protein modulates cell growth, cell adhesion and cell migration, thereby affecting the process of tumour metastasis. Furthermore, recent evidence indicates that tumour cells secrete substantial levels of galectin-1 to evade T-cell-mediated responses. (from Rabinovich, 2005).
In gliomas, galectin-1 expression is elevated in patients with poor outcome, and moreover, galectin-1 expression was enhanced at the invasive front and stimulated glioma cell migration (Strik et al., 2007). In colon cancer, galectin-1 expression was also positively correlated with disease progression. Galectin-1 is regulating inflammatory responses as well as the induction of T-cell apoptosis by interaction with CD3/CD7 (Toscano et al., 2007) and modulation of cell adhesion, at least in part, by the interaction with the beta-1 unit of integrin (reviewed by Liu and Rabinovich, 2005).

Galectin-1 also emerged as an interesting target from both transcriptome and proteome studies of NB. Galectin-1 mRNA is up-regulated in patients with aggressive, relapsing NB (Schramm et al., 2005) and the protein was up-regulated in an in vitro model of aggressive, TrkB-expressing NB (Sitek et al., 2005). Previous work has identified gal-1 as important for migration and invasion of NB cells. In this study, the effects of blocking galectin-1 function on cell motility have been shown. Interfering with gal-1 functions reduced invasiveness and migration of human NB cells in vitro and these aggressive features were tightly linked to the expression and activation of the neurotrophin receptor, TrkB that is associated with poor prognosis in NB (Cimmino et al., 2009). Given the contribution of galectin-1 to tumour growth and metastasis, it is predicted that inhibitors of galectin-1 will find their way into cancer clinical trials, leading to delays in tumour progression and improvements in overall survival.
1.5. OBJECTIVES

The aim of the present work is to evaluate galectin-1 as a drug target for the treatment of neuroblastoma. Previous work has identified gal-1 as important for migration and invasion of NB cells. Invasion and metastasis are among the most important problems to be addressed in NB therapy. While treatment of the primary tumour is feasible, dissemination of the disease is often fatal. However, the precise \textit{in vivo} functions of galectin-1 are currently unclear.

The specific goals of this work are:

- Analyze the consequences of galectin-1 inhibition in NB experimental tumours
- Study the effects of promising novel NB therapies after galectin-1 knockdown i.e. HDAC inhibitors, temozolomide and farnesylthiosalicylic acid.
- Establish an orthotopic metastatic model of neuroblastoma
2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals and Consumables

<table>
<thead>
<tr>
<th>Chemical/Consumable</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 bp DNA Marker</td>
<td>Invitrogen, Karlsruhe, Germany</td>
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<tr>
<td>2-Mecaptoethanol</td>
<td>Sigma, Diesenhofen, Germany</td>
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<tr>
<td>Acetic acid</td>
<td>Sigma, Diesenhofen, Germany</td>
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<tr>
<td>Acetone</td>
<td>Sigma, Diesenhofen, Germany</td>
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<tr>
<td>Blasticidin</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Bromphenol blue</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>DMSO</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Dry milk powder</td>
<td>Carl Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Ethanol</td>
<td>J.T. Baker, Deventer, Netherlands</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>Serva Electrophoresis GmbH, Heidelberg, Germany</td>
</tr>
<tr>
<td>Farnesylthiosalicylic Acid</td>
<td>Cayman Chemicals, Ann Arbor, USA</td>
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<tr>
<td>Glucose</td>
<td>Carl Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Sigma, Diesenhofen, Germany</td>
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<tr>
<td>Glycine</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
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<td>Isopropanol</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>Kanamycin Sulfate</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Whatman paper</td>
<td>Biorad, München, Germany</td>
</tr>
<tr>
<td>Methanol</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>Novex® sharp Pre-stained Protein Marker</td>
<td>Invitrogen, Karlsruhe, Germany</td>
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<td>NP-40</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>NUPAGE® 4-12% Bis-Tris Gels</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Sigma, Steinheim, Germany</td>
</tr>
<tr>
<td>PBS</td>
<td>Gibco, Eggenstein, Germany</td>
</tr>
<tr>
<td>Protein A/G PLUS-Agarose</td>
<td>Santa Cruz Biotechnology, Inc., Heidelberg, Germany</td>
</tr>
<tr>
<td>Material</td>
<td>Supplier</td>
</tr>
<tr>
<td>------------------------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>Proteinase Inhibitor</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>siPORT™ NeoFX™ Transfection Agent</td>
<td>Applied Biosystems/Ambion, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>Carl Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Sodium Dodecylsulphate (SDS)</td>
<td>GERBU Biotechnik GmbH, Gaiberg, Germany</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Fluka Chemie GmbH, Buchs, Germany</td>
</tr>
<tr>
<td>Suberoylanilide Hydroamic Acid (SAHA)</td>
<td>Cayman Chemicals, Ann Arbor, USA</td>
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<tr>
<td>Sucrose</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>Tocris Bioscience, Ellisville, USA</td>
</tr>
<tr>
<td>Tris Base</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>Tris Hydrochloride</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
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<td>Triton X-100</td>
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<tr>
<td>Trypton/Peptone</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>Tween 20 Polysorbate</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>Valproic Acid</td>
<td>Sigma, Diesenhofen, Germany</td>
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<tr>
<td>Yeast extract</td>
<td>Roth, Karlsruhe, Germany</td>
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<tr>
<td>Bradford Reagent</td>
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</tr>
<tr>
<td>Tryptone</td>
<td>Carl Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>PeqGOLD Universal Agarose</td>
<td>PeqLab; Erlangen, Germany</td>
</tr>
<tr>
<td>Agar</td>
<td>Carl Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>ISOTON® II Diluent</td>
<td>Beckman Coulter GmbH, Krefeld, Germany</td>
</tr>
<tr>
<td>Xylol</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>Amersham Hybond™ ECL™</td>
<td>Amersham Biosciences Europe GmbH, Freiburg, Germany</td>
</tr>
<tr>
<td>Fast Green FCF</td>
<td>Sigma, Diesenhofen, Germany</td>
</tr>
<tr>
<td>MOPS</td>
<td>Carl Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>EDTA</td>
<td>Feinbiochemica GmbH &amp;Co, Heidelberg, Germany</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
</tbody>
</table>
# Materials and methods

<table>
<thead>
<tr>
<th>Material/Reagent</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD Matrigel™ Basement Membrane Matrix. Cat No. 356231</td>
<td>BD Biosciences, Bedford, MA, USA</td>
</tr>
<tr>
<td>DAKO Pen</td>
<td>DAKO Denmark A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>Isofluran</td>
<td>Delta Select GmbH, Dreieich, Germany</td>
</tr>
<tr>
<td>Bepanthen</td>
<td>Bayer Vital GmbH, Leverkusen, Germany</td>
</tr>
<tr>
<td>Betaisodona</td>
<td>Mundipharma GmbH, Limburg, Germany</td>
</tr>
<tr>
<td>0.9 % Saline</td>
<td>Fresenius Kabi, Bad Homburg, Germany</td>
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<tr>
<td>Xylazin 2%</td>
<td>Ceva Tiergesundheit GmbH, Düsseldorf, Germany</td>
</tr>
<tr>
<td>Ketamin 10%</td>
<td>Ceva Tiergesundheit GmbH, Düsseldorf, Germany</td>
</tr>
<tr>
<td>RIMADYL®</td>
<td>Pfizer GmbH, Karlsruhe, Germany</td>
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## 2.1.2. Buffers and reagents

<table>
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<tr>
<th>Buffer/Reagent</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Fast Green Staining Solution</td>
<td>0.5% Fast Green FCF, 20% Methanol, 10% Glacial acetic acid</td>
</tr>
<tr>
<td>PBST</td>
<td>0.02% Tween 20 in PBS</td>
</tr>
<tr>
<td>BLOTTO</td>
<td>5% Fat dry milk powder in PBST buffer</td>
</tr>
<tr>
<td>TAE buffer (50 x)</td>
<td>242 g Tris pH 8.5, 57.1 mL Glacial acetic acid, 100 mL 0.5 M EDTA in 1 liter H₂O</td>
</tr>
<tr>
<td>4x NUPAGE LDS Loading buffer</td>
<td>424 mM TRIS-HCl, 564 mM TRIS Base, 8% LDS, 2.04 mM EDTA, 40% Glycerin, 0.88 mM Coomassie, 700 mM Phenol red</td>
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<tr>
<td>Ripa Buffer</td>
<td>50 mM Tris-HCl pH 7.4; 150 mM NaCl,</td>
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### Materials and methods

<table>
<thead>
<tr>
<th>Lysis Buffer</th>
<th>Buffer components</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% NP-40; 1% Tween 20; 0,5% Doc (Doox)</td>
<td>50 mM Tris-HCl pH 8,0; 150 mM NaCl, 1% NP-40; 5mM EDTA</td>
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<tr>
<td>NP-40 Lysis Buffer</td>
<td>50 mM MOPS, 50 mM TRIS Base, 0,1% SDS, 1 mM EDTA</td>
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<tr>
<td>20x MOPS-SDS Running Buffer</td>
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#### 2.1.3. List of primers and oligonucleotide sequences

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<th>Primer Name</th>
<th>Primer Sequence</th>
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<tr>
<td>H1 Forward Sequencing Primer</td>
<td>5'-TGTTCTGGGAAATCACCATA-3'</td>
</tr>
<tr>
<td>M13 Reverse Primer</td>
<td>5'-CAGGAAACAGCTATGAC -3'</td>
</tr>
<tr>
<td>Galectin-1 shRNA sense</td>
<td>5'-CAC CGC GCC AGC AAC CTG AAT CTC AAC GAA TTG AGA TTC AGG TTG CTG GCG-3'</td>
</tr>
<tr>
<td>Galectin-1 shRNA reverse</td>
<td>5'-AAA ACG CCA GCA ACC TGA ATC TCA ATT CGT TGA GAT TCA GGT TGC TGG CGC-3'</td>
</tr>
<tr>
<td>Human Galectin-1 Forward Primer</td>
<td>5'-CTCTCGGGTGAGTCTTCTG-3'</td>
</tr>
<tr>
<td>Human Galectin-1 Reverse Primer</td>
<td>5'-ACGAAGCTCTTACGCAGGG-3'</td>
</tr>
<tr>
<td>Mouse Galectin-1 Forward Primer</td>
<td>5'-GTGTGCATCACCCTTCCAGG-3'</td>
</tr>
<tr>
<td>Mouse Galectin-1 Reverse Primer</td>
<td>5'-TGCCCTTATTGGGGCTACAGG-3'</td>
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<tr>
<td>Human GAPDH Forward Primer</td>
<td>5'-CACCCATGGCAATCCCATGGCA-3'</td>
</tr>
<tr>
<td>Human GAPDH Reversed Primer</td>
<td>5'-TCTAGACGGCGATCTCCAGG-3'</td>
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<tr>
<td>Mouse GAPDH Forward Primer</td>
<td>5'-GTGAAGGTCGGTTGAACG-3'</td>
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<tr>
<td>Mouse GAPDH Reversed Primer</td>
<td>5'-GGTGAAGACACCATGACTC-3'</td>
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### 2.1.4. Enzymes

<table>
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<tbody>
<tr>
<td>GoTaq® DNA Polymerase</td>
<td>Promega GmbH, Mannheim, Germany</td>
</tr>
<tr>
<td><em>Taq</em>-DNA Polymerase</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>SuperScript® II Reverse Transcriptase</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>RNaseOUT™ Recombinant Ribonuclease Inhibitor</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Ribonuclease H</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Random Primers</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>My-Budget dNTP-mix</td>
<td>Bio-Budget Technologies GmbH, Krefeld, Germany</td>
</tr>
</tbody>
</table>

### 2.1.5. Commercial kits

<table>
<thead>
<tr>
<th>Kits</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>RNeasy Micro Kit</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>QIAGEN Plasmid Mini Kit</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>QIAfilter Plasmid Midi Kit</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>peqGOLD Plasmid Miniprep Kit</td>
<td>PEQLAB, Erlangen, Germany</td>
</tr>
<tr>
<td>VECTASTAIN ABC Kit (Standard)</td>
<td>Vector Laboratories, Inc, Burlingame, CA, USA</td>
</tr>
<tr>
<td>ImmPACT™ DAB</td>
<td>Vector Laboratories, Inc, Burlingame, CA, USA</td>
</tr>
<tr>
<td>Amersham ECL Plus Western Blotting Detection Reagents</td>
<td>Amersham Biosciences Europe GmbH, Freiburg, Germany</td>
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### 2.1.6. Antibodies

<table>
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<th>Antibody Description</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>Goat Anti-human Galectin-1</td>
<td>1:200 in PBST</td>
<td>R&amp;D Systems, Wiesbaden-Nordenstadt, Germany</td>
</tr>
<tr>
<td>Rabbit Monoclonal Antibody against HRAS (Clone Y132)</td>
<td>1:250 in PBST</td>
<td>Origene, Rockville, MD, USA</td>
</tr>
<tr>
<td>Rabbit polyclonal Ki-67 Antibody</td>
<td>1:250 in goat serum</td>
<td>Abbiotec, San Diego, CA, USA</td>
</tr>
<tr>
<td>Rabbit polyclonal CD31 (N-term) Antibody</td>
<td>1:300 in goat serum</td>
<td>Abbiotec, San Diego, CA, USA</td>
</tr>
<tr>
<td>Mouse Monoclonal GAPDH Antibody, clone 6C5</td>
<td>1:2000 in PBST</td>
<td>Millipore GmbH, Schwalbach/Ts., Germany</td>
</tr>
<tr>
<td>Rabbit anti-goat IgG-HRP: sc-2922</td>
<td>1:10000 in PBST</td>
<td>Santa Cruz Biotechnology Inc, Heidelberg, Germany</td>
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<tr>
<td>Polyclonal Goat anti-mouse-HRP. P0447</td>
<td>1:5000 in PBST</td>
<td>DAKO GmbH, Hamburg, Germany</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG-HRP sc-2004</td>
<td>1:1000 in PBST</td>
<td>Santa Cruz Biotechnology Inc, Heidelberg, Germany</td>
</tr>
<tr>
<td>Polyclonal Goat Anti-Rabbit Immunoglobulins/Biotinylated Code No. E 0432</td>
<td>1: 1000 in goat serum</td>
<td>DAKO GmbH, Hamburg, Germany</td>
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### 2.1.7. Cell culture Materials

<table>
<thead>
<tr>
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<th>Source</th>
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<tr>
<td>DMEM</td>
<td>Gibco, Eggenstein, Germany</td>
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<tr>
<td>RPMI Medium</td>
<td>Gibco, Eggenstein, Germany</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS) Gold</td>
<td>PAA Laboratories GmbH, Cölbe, Germany</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Gibco, Eggenstein, Germany</td>
</tr>
<tr>
<td>Opti®-MEM</td>
<td>Gibco, Eggenstein, Germany</td>
</tr>
<tr>
<td>Trypsine/EDTA</td>
<td>Gibco, Eggenstein, Germany</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>Gibco, Eggenstein, Germany</td>
</tr>
<tr>
<td>Zeocin™</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
</tbody>
</table>
Materials and methods

2.1.7.1. Medium for Bacteria Cell culture

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOC</td>
<td>2% Tryptone, 0.5% Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose</td>
</tr>
<tr>
<td>LB medium</td>
<td>Per litre: 10 g tryptone, 5 g yeast extract, 5 g NaCl</td>
</tr>
<tr>
<td></td>
<td>After autoclave sterilization, Ampicillin (kanamycin 50 µg/mL) was added to 100 µg/mL</td>
</tr>
</tbody>
</table>

2.1.7.2. Bacteria strains and their characteristics

<table>
<thead>
<tr>
<th>Bacteria Strain</th>
<th>Genotype of Bacteria Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echerichia coli DH5αF’</td>
<td>DeoR, endA1, gyrA96, hsdR17(rk–mk+), recA1, relA1, supE44, thi-1, (lacZYA-argFV169), δ80lacZδM15</td>
</tr>
<tr>
<td>Top 10 Escherichia coli</td>
<td>F’mcrA(mrr-hsdRMS-mcrBC); δ80lacZδM15; δlacX74; recA1: deoR; araD139; Δ(ara-leu)7697; galU; galK; rpsL; (StrR); endA1; nupG</td>
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2.1.7.3. Neuroblastoma cell lines and their origins

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<tr>
<th>Cell Line</th>
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<tr>
<td>Kelly</td>
<td>DSMZ GmbH, Braunschweig, Germany</td>
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<tr>
<td>SK-N-BE(2)</td>
<td>DSMZ GmbH, Braunschweig, Germany</td>
</tr>
<tr>
<td>SY5Y_TetR</td>
<td>DSMZ GmbH, Braunschweig, Germany</td>
</tr>
<tr>
<td>NXS2</td>
<td>DSMZ GmbH, Braunschweig, Germany</td>
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2.1.8. Equipment

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<td>ELISA Reader KC Junior</td>
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### Materials and methods

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#### 2.1.9. Surgical Equipment

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<td>HAMILTON microlitre syringe (1701RN) and Needles</td>
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<td>Johnson &amp; Johnson, Neuss, Germany</td>
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<td>Shaving Machine</td>
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<td>Disposable Scalpel (15)</td>
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2.2. Methods

2.2.1. Cell Culture

NB Cell lines were cultured at 37°C, 5% CO₂. Kelly and SK-N-BE(2) were maintained in RPMI 1640 medium with 10% FCS and Penicillin/Streptomycin (Pen/Strep). NXS2 was cultured in DMEM medium with 10% FCS and Pen/Strep. Stable SH-SY5Y cell lines which express the TetR gene (SH-SY5Y_TetR) from the pcDNA™6/TR were maintained in RPMI 1640 medium (10% FCS and Pen/Strep) and 7.5 µg/mL Blasticidin.

2.2.2. Drug Formulations and Administration

TMZ was dissolved in DMSO as a stock solution of 100 mM. The stock solution was diluted with PBS to a final concentration of 5 mg/kg prior to use in vivo. SAHA was dissolved in DMSO to a stock concentration of 2 mM. FTS stock solution of 100 mM in chloroform was prepared aliquoted and stored at -80°C. Prior to use in vitro, the chloroform was evaporated, DMSO (40 µL) is added, the tube is vortexed and RPMI 1640/10% FCS (360 µL) is added. The final DMSO concentration was adjusted to 0.1% in all experiments. For in vivo experiments, the chloroform is evaporated from the FTS stock solution (84 µL aliquot), 8 µL ethanol is added. 7 µL of 1M NaOH is then added followed by addition of 1.48 mL PBS. This gives a final concentration of 2 mg FTS/mL.

2.2.3. Proliferation/Cytotoxicity Assay

Cell proliferation and cytotoxicity after drug treatment and/or siRNA transfection was determined using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) which determines the mitochondrial activity in living cells and also by the xCELLigence system (Roche).
Materials and methods

2.2.3.1. MTT Proliferation Assay

5 x 10^3 cells (SK-N-BE(2) and NXS2) or 1.5 x 10^4 Kelly cells were plated in 96 multiwell plates. Cells were treated/transfected as described at the indicated times and the MTT was performed 72 hours later. The cells were incubated with 1.25 mg/mL MTT for 4 hours at 37°C and then the formazan salts formed was lysed with MTT lysis reagent (10% SDS, 5% glacial acetic acid in DMSO) for one hour. Results were quantified by measuring the absorbance at 570 nm. The concentration of drug required for 50% reduction of cell proliferation (IC\textsubscript{50}) was extrapolated from dose-response curves. Each value represents six replicates, and each experiment was repeated at least three times.

2.2.3.2. xCELLigence Cellular Analysis

The xCELLigence system monitors in real time the electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture plates (termed “E-Plates”) as a quantitative measure of the biological status of cells. This is recorded as a dimensionless parameter termed Cell Index (CI) which is a measure of the cell number, viability, and morphology. The CI of a given well will be zero if there are no cells present and this will increase as more cells attach to the bottom of the plates (electrodes). Any change in the cell morphology, adhesion or viability degree will change the CI. The major advantage of this system over the MTT is that it provides the dynamic monitoring of changes in cell status as opposed to a given end point.

5 x 10^3 SK-N-BE(2) cells were transfected in the 96-well E-plates. After 24 hours the medium was replaced with medium containing the indicated drugs and the cell status was monitored for another 72 hours. The experiment was performed in triplicate for each value and repeated at least twice.

2.2.4. shRNA Regulated Galectin-1 Expression

Plasmids expressing shRNA targetinggalectin-1 was constructed using the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit according to the manufacturer’s manual. In summary, oligonucleotides encoding the sense and antisense strands
of shRNA against galectin-1 (see section 2.1.2) were annealed and cloned into the pENTR™/H1/TO vector to transform TOP10 E. coli. Ten entry colonies were cultured in LB medium containing 50 µg/mL kanamycin. The plasmids DNA were isolated using the peqGOLD Plasmid Miniprep Kit (PeQLab) following the manufacturer’s protocol. The constructs were then sequenced to confirm the presence and correct orientation of double stranded oligonucleotide insert. Glycerol stocks of positives clones were stored at -80°C for further use.

In order to generate stable cell lines that constitutively express the plasmid construct, SH-SY5Y_TetR cells were transfected with 4 µg plasmid DNA using the microporator according to the manufacturer’s protocol. A plasmid expressing GFP was used to control transfection efficiency. The cells were maintained in 7.5 µg/mL Blasticidn and 50 µg/mL Zeocin to maintain selection pressure.

2.2.5. siRNA Transfection

Cell lines were transiently transfected with siRNAs directed against Gal-1 using siPORT™ NeoFX™ Transfection Agent according to instructions provided by the manufacturer (Ambion, Darmstadt, Germany). An alexaFlour-labeled scrambled siRNA (Qiagen) was used to control for transfection efficiency and as a control for Gal-1 mediated effects. Untreated cells and cells transfected with siPORT NeoFX buffer only were included as negative controls.

5 x 10^3 SK-N-BE(2) and NXS2 cells per well or 1.5 x 10^5 cells per well were transfected in 96-well and six-well plates respectively. The final siRNA concentration in all experiments was 30 nM. The galectin-1 expression level was evaluated after five days.

Cells (1.5 x 10^6 cells/75 cm flask) for mouse tumour grafting were transfected on day one. The cells were trypsinised and re-transfected 72 hours (day 4) after the first transfection. The knockdown efficiency was monitored by western analysis after another 72 hours (day 8), which is the time point of mice tumour grafting.

2.2.6. Reversed Transcriptase PCR Analysis

Total RNA from NB cell lines transfected with siRNA and/or treated with drugs was prepared using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) and
subsequently transcribed into cDNA using SuperScript® II Reverse Transcriptase and RNaseOut (Invitrogen). Relative quantification of Gal-1 mRNA expression was achieved both using Taqman Assay Hs00355202_m1 (Applied Biosystems, Foster City, CA, USA) and the standard curve based method. For the semi quantitative RT-PCR analysis, the cDNA was amplified and subjected to electrophoretic separation on a 2% agarose gel containing 0.5 mg/mL ethidium bromide. The PCR products were detected using UV transillumination, photographed and the ratios of galectin-1 to GAPDH were densitometrically determined using the BioDocAnalyze live software (Biorad). All assays were carried out in triplicate. Galectin-1 mRNA expression was normalized to the averaged GAPDH expression in each sample. Data are presented as mean values ± SD.

2.2.7. Co-Immuno precipitation

Cells were washed with cold PBS and lysed using the NP-40 lysis buffer supplemented with Protease inhibitor (Roche). Approximately 3 mg whole cell lysate (evaluated by Bradford assay) were adjusted to 500 µL and precleared with protein A/G PLUS-Agarose before being immunoprecipitated overnight with 2,5 µg of antibody and 50 µL protein A/G PLUS-Agarose. The precipitates were washed with lysis buffer, eluted with SDS sample buffer and then subjected to SDS-PAGE and western immunoblotting as described below.

2.2.8. Western Analysis

Cell lines were plated or transfected as described above (1.5 x 10^5 cells/well) in six welled plates one day before treatment with indicated drugs. After 72 hours of treatment with drugs at indicated concentrations or with control vehicle, the cells were washed in cold PBS and lysed using the Ripa lysis buffer containing protease inhibitor followed by high speed centrifugation. Cell lysates (10 – 20 µg) were separated on NuPAGE BisTRIS Gel (4-12% Polyacrylamide-Gradient) and blotted onto nitrocellulose membranes. Membranes were blocked for one hour in BLOTTO (5% skimmed milk in PBS supplemented with 0.02% Tween-20). Membranes were incubated overnight with primary antibody at 4°C. After washing three times with PBST (PBS with 0.02% Tween-20), the blots were incubated for
one hour with horseradish peroxidase-conjugated secondary antibody at room temperature. Detection of the bands was done with enhanced chemiluminescence ECL plus kit (Amersham).

2.2.9. **Cell Cycle Analysis**

Parallel experiments were performed as described for western analysis for the assessment of cell cycle distribution by fluorescence-activated cell sorting (FACS) analysis at 24, 48 and 72 hours after treatment with drugs. Cells were harvested, centrifuged, washed with cold PBS and then fixed in ice-cold 70% ethanol at 4°C overnight. After centrifugation at 1200 rpm for 5 minutes at 4°C, cells were re-suspended in PBS supplemented with 0.2-0.5 mg/mL RNase and then incubated for one hour at 37°C. The cells were stained with 10 µg/mL Propidium Iodide and the cell cycle phase distributions were analysed with a FACS flow cytometer (Bechman-Coulter Epics XL).

2.3. **Animal Experiments**

2.3.1. **Ethics and Animal Care**

All the in vivo experiments described in this study were performed with locally bred A/J and nu/nu mice on the basis of authorisation number 9.93.2.10.34.07.313 of the Animal Ethics Committee. The mice were used according to institutional guidelines when they were 7-10 weeks old.

2.3.2. **Microsurgical Procedures**

A/J mice were anaesthetised using 0.1 mL/10g of the anaesthesia formulation (5 mL 0.9% NaCl, 1 mL 10% Ketamine, 0.25 mL Xylazine) intraperitoneally (ip). Fur on the left side of the body between the fore and the hind legs was shaved and prepped with betaisadone. Bepanthen was applied to the eyes to maintain eye moisture and protection against irritants. An incision was made over the left retroperitoneal space to expose the adrenal gland and a total of 6 x 10⁵ NXS2 cells in 10 µL PBS was injected into the left adrenal gland with a disposable needle (Hamilton) on a 10µL Hamilton syringe. The incision was sutured with a 5.0 Vicryl® (Johnson and Johnson). Each animal was subcutaneously administered 0.02-0.04
mL of Temgesic. The mice were kept warm during anaesthesia, surgery and recovery on a thermal plate at 37°C and if necessary, anaesthesia was maintained using intranasal administration of Isoflurane. During the first week after surgery or as long as was dimmed necessary, the animals were supplied with 4 mg/kg Rimadyl® diluted in 0.9% NaCl to alleviate the pains. The health of the mice was closely monitored every day for the first two weeks and three times a week thereafter until mice were sacrificed.

2.3.3. Mouse Grafting and Treatment Schedules

A/J mice were shaved and the skin decontaminated with betaisadone at the site of injection and 2 x10^5 NXS2 cells in 200 µL Matrigel were injected s.c. into the right flank of each recipient mouse. In other experiments, galectin-1 siRNA transfected cells [2 x 10^5 Kelly or 1.0 x10^7 SK-N-BE(2)] in 200 µL matrigel were injected s.c. into the right flank of nude mice. The mice were randomly assigned into one of the experimental groups with six mice in each group. The indicated treatment schedules were initiated one after mouse tumour grafting. The experimental protocols are detailed in the legends of the results section. The tumours were measured everyday for the synografts and three times a week for the xenografts using a digital calliper. Tumour volume was calculated by using the formula: V_{tumour} = Length x width x height. Animals were sacrificed when the tumour volume reached or exceeded 1 cm^3.

2.3.4. Histology

Mice were killed by cervical dislocation and the tumours were excised. The lungs, kidney and liver of mice that were orthotopically grafted were also removed for assessment of tumour metastasis. These samples were fixed in 4% paraformaldehyde and then embedded in paraffin wax for conventional hematoxylin-eosin staining or immunohistochemical analysis.

2.3.5. Immunohistochemistry

Four-micrometer sections were deparaffinised and hydrated by xylene. The slides were then incubated for 30 minutes in 3% H_2O_2 in methanol to quench the activity of any endogenous peroxidase. After washing, the slides were blocked in 10%
goat serum in PBS for one hour at RT followed by overnight incubation at 4°C with Ki67 antibody. The slides were then washed thrice with PBS and incubated with secondary antibody for 1 h at RT. Subsequently the sections were rinsed and the antigen-antibody binding was visualised by means of the avidin-biotin complex (ABC) method using DAB (3,3’-diaminobenzidine) as chromogen. Immunoreactions were visualized with the ABC complex diluted 1:50 in phosphate-buffered saline (Vectastain, Vector, Burlingame, CA, USA). The number of positive cells was scored and graphed by averaging three repeated assessments.

2.3.6. Statistical Analysis

Results are expressed as mean values with standard deviations (SD). A two-tailed unpaired t-test was used to analyse the statistical difference between treatment groups in all in vitro assays. Survival analyses were carried out by means of Kaplan–Meier curves and the log-rank test. All statistical tests were carried out using GraphPad Prism™ software (GraphPad™, San Diego, Calif., USA). P < 0.05 was considered as a statistically significant result.
Results

3. RESULTS

3.1. Transient siRNA/shRNA Directed Galectin-1 Downregulation

The necessity of a gene or protein for a particular biological function is often investigated by loss-of-function studies. RNAi has the potential to mediate the long- or short-term silencing of gene expression at the DNA, RNA and/or protein level (Martin & Caplen, 2007). The expression level of galectin-1 was analysed in a panel of NB cell lines (Fig 2). Galectin-1 is abundantly expressed in all cell lines analysed except in Kelly cells which show very minimal amount of galectin-1 protein expression.

Fig 2: Expression of Galectin-1 in Neuroblastoma cell lines: 40 µg of protein extract of each cell was loaded per lane and the blot was probed with galectin-1 antibody. The blot was stripped and re-probed with antibody against GAPDH as loading control.

Galectin-1 shRNA was used in order to stably and specifically knockdown galectin-1 in SH-SY5Y that stably expresses the Tet repressor to enable conditional tetracycline-regulated galectin-1 expression. Transient induction shows ~35% of galectin-1 reduction compared to non-induced transfectants (Fig 3). Generation of stable transfected clones was not possible because the cells ceased growing or died after about six weeks in selection medium. Complete removal of the selection pressure did not change cell growth characteristics.
Results

In other experiments, a specific anti-galectin-1 siRNA maximally reduced the galectin-1 mRNA level in the cell lines, Kelly, SK-N-BE(2) and NXS2 4 days post transfection in comparison to untreated cells (Fig 4). Galectin-1 protein expression was greatly reduced in NXS2 cells while there seem to be virtually no decrease in galectin-1 protein levels in SK-N-B(2) (Fig 5A). However, re-transfection of the cells showed an overwhelming decrease in galectin-1 protein in both SK-N-BE(2) and NXS2 cells 8 days (day of mice tumour grafting) after the first transfection (Fig 5B).

Fig 3: Inducible galectin-1 shRNA mediated transient knockdown of galectin-1. SH-SY5Y-TetR cells were transfected with pENTR™H1/TO entry constructs containing shRNA targeting galectin-1 or with a GFP-expressing plasmid vector and grown in medium containing zeocin and blasticidin for selection of transfectants. Untransfected and cells transfected with transfection reagent alone (blank control) were used as controls. 24 hours after transfection, the medium was replaced with medium containing 1 µg/mL tetracycline. Total RNA was extracted after 72 hours induction with subsequent cDNA synthesis. Galectin-1 mRNA was quantified using real time RT-PCR normalised to GAPDH as house-keeping gene.
Fig 4: Galectin-1 siRNA knockdown of galectin-1 mRNA expression: NB cell lines were transfected with galectin-1 (Gal-1) or scrambled (scr) siRNA in 96 well plates. After 24 hours, the medium was changed and cells were grown for another 72 hours upon which total RNA was extracted. cDNA was synthesized and used for real time RT-PCR analysis of gal-1 mRNA expression normalized to GAPDH.
Results

3.2. HDAC Inhibitors inhibit proliferation of NB cells in vitro

Previous studies have shown that HDAC inhibitors inhibit NB cell proliferation and apoptotic cell death (Deubzer et al., 2007; Michealis et al., 2004). The ability of HDAC inhibitors VPA and SAHA to inhibit NB cell proliferation was assessed using the MTT assay. NB cell lines were treated with varying concentrations of VPA (0, 0.4, 0.8, 1.2, 1.6, 2.0 mM) or SAHA (0.1, 0.25, 0.5, 0.75, 1.0 µM) for 72 hours and dose-response curves were plotted with compound concentration against percentage of viable cells (Fig 6A and B). Compounds showed antiproliferative activity in a dose dependent manner with VPA and SAHA IC\textsubscript{50} values at 1.1, 1.19, 1.2 mM and 0.57, 0.8, 0.85 µM for Kelly, SK-N-BE(2) and NXS2 cells respectively.

Fig 5: Western blot analysis depicting the expression levels of galectin-1 protein after gal-1 siRNA transfection. Cells were transfected with gal-1 or scr siRNA or left untransfected (NT). Protein extracts were collected on day 4 (A) or the cells were trypsinised, re-transfected and grown for another 72 hours (day 8) before protein extraction (B). 10 µg of protein was loaded per lane and GAPDH blots were used to assess equal loading in each experiment.
Fig 6: Effect of HDAC inhibitors on proliferation of NB cell lines. The cells were treated with the indicated doses of VPA (A) and SAHA (B) for 72 hours. Effect on proliferation was assayed by MTT as described in materials and methods. Untreated cells served as controls and were set to 100%.
Fig 7: HDACi upregulate the expression of galectin-1 in NB cells. Cells were treated with 0.1% DMSO or the IC$_{50}$ concentrations of HDACi as indicated or left untreated (NT) for 72 hours. cDNA was synthesised from extracted RNA and galectin-1 mRNA level was measured by semi-quantitative RT-PCR. Data represent mean ± SD of three independent experiments A = NXS2: B = SK-N-BE(2): C = Kelly.
Results

Next, experiments were performed to examine if the treatment of NB cells with HDACi alters the expression of galectin-1. Contrary to our belief that HDACi downregulate galectin-1 expression, the results show that HDACi actually increase the expression of galectin-1 in NB cells (Fig 7). VPA appears to be a more potent regulator of galectin-1 expression than SAHA since it shows a higher up-regulation of galectin-1.

3.3. Effect of FTS and TMZ on Neuroblastoma Growth in vitro

In similar experiments to those previously described above single agent TMZ (0, 40, 80, 120, 160, 200 µM) had weak effect on the proliferation of all NB cell lines tested with a growth inhibition of ~68 - 85% at a concentration of 200 µM (Fig 8). On the other hand Kelly and SK-N-BE(2) were resistant to FTS treatment (0, 20, 40, 60, 80, 100 µM) with an IC50 > 100 µM whereas NXS2 was more sensitive to FTS with an IC50 of 49 µM.
Results

3.4. Effects of Combining TMZ to FTS in NB Cells *in vitro*

The combination of drugs in various human cancer regimens is usually more effective than mono-drug therapy. Also it has been reported that FTS sensitizes human melanoma to the effects of Dacarbazine which is an Imidazotetrazine compound like TMZ *in vivo* (Halaschek-Wiener *et al.*, 2003). The potential synergistic or additive potential of FTS to TMZ was evaluated. To pursue this goal, NB cells were treated with a clinically achievable dose (100 µM) of TMZ (Kanzawa *et al.*, 2004) alone and in combination to a single concentration of FTS. The concentration that approximates to the IC$_{50}$ (50 µM) as determined by MTT was used for NXS2. The concentration of 75 µM was chosen for use in SK-N-BE(2) and Kelly cells because it is the concentration that has been mostly used in most publications (Kloog *et al.*, 2005). Combining FTS to TMZ resulted in a statistically significant additive growth-inhibitory effects in both human cell lines tested when

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![Figure 8: Proliferative response of NB cell lines to single agent TMZ or FTS. Cell lines were seeded in 96 well plates and treated with indicated concentrations of drugs or untreated (control) 24 h later for 72 h. The proliferation was evaluated using the MTT assay.](attachment:image)
compared with control (SK-N-BE(2), 72.18±2.64 % of control, P = 0.0069 and Kelly, 65.1±1.36 % of control, P= 0.0064) but not in NXS2 cells (Fig 9). However only SK-N-BE(2) resulted in a statistically significant reduction in cell survival when compared to single agent FTS with P = 0.0298 but not with TMZ, P= 0.0927. The difference in combination therapy to mono-therapy did not reach statistical significance in both NXS2 and Kelly cells.
Fig 9: Anti-proliferative effect of combination of TMZ and FTS in NB cells. Cell lines were seeded in 96 well plates and treated with FTS alone 50 µM for NXS2 cells or 75 µM for SK-N-BE(2) and Kelly cells, or in combination with 100 µM TMZ or left untreated (NT = control) 24 h later for 72 h. The proliferation was evaluated using the MTT assay. Indicated P values compared with blank control (DM = 0.1 % DMSO). A. NXS2. P = 0.0534, single agent TMZ versus combination group. B, SK-N-BE(2). P = 0.0927, combination group versus TMZ. C. Kelly. P = 0.2877, combination group versus TMZ.
Results

Next the expression of galectin-1 after treatment with TMZ or FTS or drug combination was evaluated in parallel experiments to those performed above to explore the mechanisms of the various drugs cytotoxicity in relation to galectin-1 expression.

Fig 10: Effect of TMZ and FTS on galectin-1 expression in NB cells determined by semi quantitative RT-PCR. A, NXS2 and B, SK-N-BE(2) cells
Results

Furthermore the effect of TMZ and FTS to changes in cell cycle distribution was appraised by flow cytometry. TMZ is known to induce G2/M cell cycle arrest in various brain tumours. As shown in Fig 11 TMZ caused a minimal increase (~10%) in population at the G2/M Phase and a decrease in the population at the G1 in all NB cell lines though this changes was not statistically significant (P > 0.05, TMZ Vs DMSO control). FTS however did not show any changes in cell cycle distribution of cells or apoptosis as depicted in the cell population at the sub G1 Phase in all cell lines. This result is surprising in the case of NXS2 where FTS shows strong growth-inhibitory effects as depicted by proliferation assay. This might suggest that decrease in cell viability by FTS in NXS2 might not be attributed to changes in cell cycle. Combination of TMZ and FTS did not alter these results in all cell lines tested.
Results

Fig 11: Flow cytometric analysis of cell cycle distribution after treatment with TMZ and FTS. Cells were seeded for 24 h in 6 well plates and treated for 72 hours with 100µM TMZ alone, 50 µM FTS alone (A = NXS2) or 75 µM FTS (B = SK-N-BE(2) and C = Kelly), or in combination. Data shown are representative of three independent experiments.
Parallel experiments were also performed for the analysis of galectin-1 expression after treatment with compounds in order to assess cell cycle changes in relation to galectin-1 expression (Fig 12).

![Western Blot](image)

**Fig 12**: Impact of TMZ and FTS treatment to galectin-1 expression in NXS2 and SK-N-BE(2) cells as judged by Western Blot analyses. A= untreated cells, b = 0.1 % DMSO, c and d = 25 and 50 µM or 50 and 75 µM FTS for NXS2 and SK-N-BE(2) respectively, e = 100 µM TMZ, f = combination treatment.

FTS and TMZ cause only slight changes in the expression of galectin-1 protein level in SK-N-BE(2) and NXS2 cells. This indicates that the TMZ-induced higher expression of galectin-1 in NXS2 cells at the mRNA level is not observed at the protein level.

### 3.5. Galectin-1 co-localises with HRAS in Neuroblastoma

Previous reports have demonstrated the association of galectin-1 to HRAS in transformed endothelial cells and the potential of FTS to disrupt this association (Rotblat *et al.*, 2004). To further explore the mechanism of action of FTS in NB cells, the interaction of Galectin-1 and HRAS was examined by co-immunoprecipitation. HRAS was immunoprecipitated (IP) using anti-HRAS antibody and then blotted with anti-galectin-1 antibody. The results show that HRAS co-immunoprecipitates with galectin-1 in both NXS2 and SK-N-BE(2) (Fig 13) suggesting that the effect of FTS in NB cells is not dependent on the galectin-1/HRAS interaction.
Results

Fig 13: Galectin-1 co-immunoprecipitates with HRAS in NB cells. Protein extracts were immunoprecipitated with HRAS monoclonal antibody and the blotted with galectin-1 antibody. Cell lysate from whole protein extracts was used as positive control and the eluant after IP (IPctrl) was used as negative control.

3.6. Galectin-1 silencing in Neuroblastoma does not potentiate the antiproliferative activity of HDAC Inhibitors, FTS or TMZ in vitro

Galectin-1 knockdown has previously been shown to increase the anti-tumour effects of cytotoxic, pro-autophagic and pro-apoptotic drugs both in vitro and in vivo (Le Mercier et al., 2008b). We have also recently demonstrated the effects of galectin-1 on NB cells migration (Cimmino et al., 2009) which in turn has been shown to sensitise cells to cytotoxic drugs, hence the power of galectin-1 silencing to sensitise NB cells to the anti-tumour effects of HDACi, FTS and TMZ was examined in vitro.
Results

Fig 14: Effect of galectin-1 knockdown and treatment with VPA. NB cells were transfected with siRNA against galectin-1 (gal-1 si) or scrambled siRNA (scr si) and/or treated with the IC₅₀ concentration of VPA for 72h. Statistical analysis was performed by t-test of six independent experiments. Untransfected and DMSO vehicle treated cells were included as controls. Anti-gal-1 plus VPA was tested against scr si plus VPA. There is a significant decrease in proliferation using the combinatory treatment when compared to VPA as a single agent only for SK-N-BE(2) cells (P = 0.0029). This is not the case with NXS2 cells, P = 0.1742 and Kelly cells, P = 0.3591).
Results

Galectin-1 knockdown did significantly inhibit cell proliferation in SK-N-BE(2) (P = 0.0294) but not in NXS2 (P = 0.0611) and Kelly (P = 0.1051) cells when compared with scrambled siRNA treated (control) group. Decreasing galectin-1 in SK-N-BE(2) cells resulted in a greater VPA-induced anti-proliferative effects (P= 0.0029) in comparison to cells treated with scrambled siRNA and VPA (Fig 14). However, there were no significant differences in proliferation in NXS2 and Kelly cells between the two groups.
Results

On the other hand galectin-1 silencing did not cause a marked difference in the proliferation of NB cells to the effects of SAHA (Fig 15) in comparison with mock transfected cells treated with SAHA. However, there was a significant sensitivity of galectin-1 knockdown to effects of SAHA in NXS2 cells (P = 0.0012). Furthermore, silencing galectin-1 greatly reduced the percentage of viable cells when treated to TMZ than treatment with TMZ alone in all NB cells tested with NXS2 displaying the most pronounced effect. Also, decreasing endogenous expression of galectin-1 weakly lowered the viability of NB cells when treated with TMZ (NXS2, P = 0.0227; SK-N-BE(2), P = 0.0878) when compared to cells treated with scrambled siRNA plus TMZ.

Fig 15: Proliferative effects of galectin-1 knockdown and treatment with SAHA. Representative data of 6 independent experiments are shown. Gal-1 knockdown + SAHA versus scr si + SAHA (NXS2, P = 0.0660; SK-N-BE(2), P = 0.1903). Gal-1 + SAHA versus single agent SAHA (NXS2, P = 0.0012; SK-N-BE(2), P = 0.2562; Kelly, P = 0.2246).
Results

**NXS2**

![Bar chart showing proliferation results for different treatments.](image1)

- **P = 0.0227**
- **P = 0.0003**

**SK-N-BE(2)**

![Bar chart showing proliferation results for different treatments.](image2)

- **P = 0.0131**
The effects of combining the knockdown of endogenous galectin-1 and FTS was tested in SK-N-BE(2) cells and NXS2 cells. FTS did not significantly reduce cell viability after galectin-1 knockdown in NXS2. Galectin-1 silencing appeared to sensitise SK-N-BE(2) cells to FTS (P = 0.0340) however when compared with scrambled siRNA and FTS treated cells, the effect was modest and not statistically significant (P = 0.0820). Results representative of six independent experiments are shown in Fig 16 below. These results therefore demonstrate that TMZ has very minimal potential in synergy to galectin-1 silencing in NB cells \textit{in vitro}.

Fig 16: Effects of galectin-1 knockdown and treatment with 100 µM TMZ. Galectin-1 downregulation reduces cell proliferation in NB cells. NXS2, P = 0.0227; SK-N-BE(2), P = 0.0878 (TMZ alone Vs si gal-1+TMZ). Comparing siRNA gal-1 plus TMZ knockdown versus TMZ alone; NXS2, P = 0.0003; SK-N-BE(2), 0.0131; Kelly, P = 0.0084.
Fig 17: Effects of galectin-1 knockdown and combination with FTS. The concentration used in NXS2 cells was 50 µM and 75 µM for SK-N-BE(2). FTS does not show a synergistic effect to cell proliferation in NXS2 cells. Anti-gal-1 plus FTS versus scr si plus FTS; NXS2 P = 0.1182; SK-N-BE(2), P = 0.0820. FTS alone compared to FTS plus siRNA gal-1 knockdown; NXS2, P = 0.77364; SK-N-BE(2), P = 0.0340).
Results

Based on the results obtained by MTT assay, the power of downregulating galectin-1 in potentiating the anti-tumour effects of HDACi, TMZ and FTS was also assessed using the XCelligence device (Roche), which has the advantage of real time cell analysis compared to conventional end-point analyses. For this purpose SK-N-BE(2) cells were used in parallel experiments to those described above (Fig 14- 17). The results confirm that knocking down galectin-1 significantly inhibit cell proliferation in SK-N-BE(2) cells as shown in Fig 18 below. This is in contrast to results obtained in melanoma cells where galectin-1 knockdown does not alter cell proliferation in vitro (Mathieu et al., 2007). Again, the results are in agreement that TMZ and FTS at the concentrations used do not inhibit cell growth and also that they do not result in any additive growth-inhibitory effects of galectin-1 silencing (Fig 18 C and D). However XCelligence analysis reveals that there is a strong synergistic effect of galectin-1 downregulation to the cytotoxic effects of SAHA (Fig 18 B) contrary to results obtained by MTT assay. A possible explanation to this might be that SAHA induces a more potent effect on cell attachment/motility than VPA, an aspect which cannot be reflected by MTT assay. Furthermore, galectin-1 silencing does not sensitise SK-N-BE(2) cells to the cytotoxic effects of VPA as depicted previously (Fig 18 A).
Results

B

C

[Graphs showing cell index over time for different treatments: DMSO, scr si, scr si+SAHA, SAHA, gal-1 si, gal-1 si+SAHA, DMSO, scr si, scr si+TMZ, TMZ, gal-1 si, gal-1 si+TMZ.]
Results

Fig 18: Real time measurement of cell proliferation *in vitro* using the XCelligence (Roche) device. The cell index is proportional to the impedance due to cell adhesion and is equivalent to cell viability. Galectin-1 siRNA (blue lines) significantly reduced cell proliferation of SK-N-BE(2) cells. “Controls” used include cells treated with 0.1 % DMSO vehicle control (brick red lines) or cells transfected with scrambled siRNA (scr si) unrelated to any gene (green lines). A, Treatment of cells with gal-1 siRNA and 1.2 mM (IC_{50}) VPA (sky blue lines) did not show any significant reduction in cell viability to cells treated with VPA and scr si whereas B; Significant decrease in cell viability of cells treated with 0.80 µM SAHA (IC_{50}) and gal-1 siRNA (yellow lines) compared to scr si plus SAHA treated cells (yellow lines). C: Treatment of cells with 100 µM TMZ (light red lines) results in no significant decrease in cell proliferation in and also did not result in any significant additive anti-proliferative effect to the gal-1 siRNA (light blue lines) when compared to treatment controls (yellow lines). D; FTS at 75 µM (light red lines) virtually did not alter the cell viability of cells and also did not result in any synergy to the growth-inhibitory effects of gal-1 siRNA (blue and sky blue lines).
3.7. The anti-proliferative activity of galectin-1 downregulation is not accompanied by changes in cell cycle distribution.

The effect of galectin-1 knockdown on changes in cell cycle was examined by flow cytometry. In addition, it was interesting to investigate the effects of galectin-1 knockdown and TMZ treatment since this was chosen to be assessed in vivo. The effect of TMZ on the expression of galectin-1 protein after siRNA treatment was also examined by western blotting (Fig 19). Apparently, TMZ did not change galectin-1 expression after siRNA galectin-1 knockdown. Anti-galectin-1 siRNA did not alter the cell cycle distribution of both NXS2 and SK-N-BE(2) cells (Fig 20) when compared to cells treated with scrambled siRNA. This results show that the anti-proliferative effect of galectin-1 knockdown is not related to any changes in cell cycle. Furthermore, galectin-1 silencing did not cause or enhance the TMZ-induced increase of cells in the G2/M phase in NB cells.

Fig 19: TMZ does not alter galectin-1 expression after knockdown by siRNA in NXS2 and SK-N-BE(2) cells. $1.5 \times 10^5$ NB cells were transfected with anti-galectin-1 sRNA (gal-1 si) or unrelated scrambled siRNA (scr si) in six welled plates. After 24 hours, the cells were treated with 100 µM TMZ or 0.1 % DMSO (DM) vehicle control or left untreated (NT) for 72 h. Protein extracts were then subjected to western analysis. Blots were probed with galectin-1 antibody and later striped and re-probed with anti GAPDH antibody as a loading control.
Fig 20: Galectin-1 knockdown does not change the cell cycle distribution of NB cells. SK-N-BE(2) (A) and NXS2 (B) cells were treated as in Fig 28 and cell cycle analysis were performed by FACS. Error bars are from three independent experiments.
Results

3.8. Steps towards an orthotopic neuroblastoma tumour model

The primary objective was to establish an orthotopic syngeneic NB mouse model presenting disseminated NB in different organs typical of NB metastasis. For this purpose a tumour induced by NXS2 cells in A/J mouse was excised, cut into pieces of approximately 1 mm³ and surgically placed on the adrenal gland of a second A/J mouse (see 2.3.2). After 28 days, the mice were killed and the organs were examined for macroscopic metastases. Hematoxylin-eosin (HE) stained tissue sections from the spleen, kidney and liver were also examined for micrometastases. Although all mice developed tumours at the surgical site, no apparent organ metastases were observed at autopsy (Fig 21). Lack of micrometastases was also confirmed by HE staining (Fig 22).

Fig 21: Macroscopic examination of mouse organs. NXS2 NB cells were injected into the left adrenal gland of AJ mice. Photomicrographs were taken at autopsy of different organs including tumour. A) contra-lateral adrenal gland and kidney. B) Tumour sandwiched between the liver and kidney of injected adrenal gland.
Fig 22: Microscopic examination of mouse organs and tumour. Microscopic examination of HE stained tissue sections reveal no micro metastasis. Tumour sample was also stained for comparison (positive control).
3.9. FTS does not show anti-tumour activity as a single agent but chemosensitises experimental NB to the effects of TMZ

Although NB cells displayed only modest sensitivity to FTS and TMZ \textit{in vitro}, other reports have shown their therapeutic benefit \textit{in vivo} (Le Mercier \textit{et al.}, 2007). Hence, the therapeutic potential of single agent FTS as well as its sensitising effect in combination with TMZ was investigated in a NB syngeneic model. AJ mice (n = 6/group) were injected subcutaneously with NXS2 NB cells and treated with 20 mg/kg FTS (i.p.) alone or 40 mg/Kg TMZ (i.p.) alone every other day, or as combination therapy with single agents administered individually on alternate days (Fig 23). Mice were sacrificed when tumour volume reached 1000 mm$^3$ (primary endpoint) or appeared sickly. FTS did not significantly increase the survival of tumour-bearing mice compared to control group (P = 0.93) whereas TMZ significantly improved the survival of animals when compared to controls (P = 0.03). However the combination of FTS and TMZ neither did not significantly improve the therapeutic benefits of TMZ alone (P = 0.51) nor the survival of mice-bearing tumours when compared with controls (P = 0.05). It should however be emphasised that this experiment was stopped due to treatment related side effects. Hence, the results presented are preliminary, but suggest a “synergistic” effect of FTS and TMZ may exist. At that time, only one out of the six mice of the group receiving the combination treatment but all of the mice in the control and FTS single agent groups had reached the primary endpoint.
Results

Fig 23: Kaplan Meier survival analysis for the evaluation of NB growth inhibition by FTS and TMZ in a NB syngeneic model. AJ mice were injected s.c. on the lower left flank with $5.0 \times 10^6$ NXS2 cells and were treated i.p. with either 20 mg/kg FTS (green) or 40 mg/kg TMZ (red) alone every other day or a combination of both drugs (blue) on alternating days until the experiment was stopped. Control group received PBS/5 % DMSO (black). Treatment was initiated one day after inoculation.
3.10. TMZ or galectin-1 downregulation increase the survival of NB tumour bearing mice but do not act synergistically

Decreasing galectin-1 expression in a mouse melanoma and glioma models have been shown to improve the anti-tumour effects of TMZ (Mathieu et al., 2007; Le Mercier et al., 2008). Thus the potential of galectin-1 to inhibit tumour growth and to sensitise to treatment with TMZ was examined in a NB xenograft model. SK-N-BE(2) cells were transfected with either siRNA directed against galectin-1 or an unrelated scrambled siRNA and grafted s.c. to groups of nu/nu mice (n=6) after eight days. Western analysis confirmed depletion of galectin-1 expression (see Fig 5) compared to untransfected or scrambled siRNA transfected cells. TMZ (40 mg/kg) was then administered i.p. to two groups of mice (one grafted with gal-1 siRNA transfected cells and the other with scrambled siRNA) three times a week for four weeks one day after tumour grafting. Two groups received 5% DMSO in PBS as per TMZ treatment. As seen in Fig 24, decreasing galectin-1 expression alone and TMZ treatment alone significantly increased the survival of tumour-bearing mice when compared to scrambled siRNA plus vehicle control group (P = 0.0069 and P = 0.0037 respectively). However galectin-1 depletion plus TMZ treatment did not result in any additive beneficial effect to the survival of scrambled siRNA plus TMZ treated group (P = 0.0037). These results imply that galectin-1 reduces tumour burden as effective as TMZ but no additive effects to TMZ anti-tumour activity in this NB model.
Results

Fig 24: Effect of decreasing Galectin-1 silencing and response to TMZ in NB xenograft model. 8 – 10 weeks old nu/nu mice were grafted with 1.0 x 10⁷ SK-N-BE(2) cells transfected with either gal-1 siRNA and treated with vehicle control (red line) or TMZ (blue line) or transfected with scrambled siRNA and then treated with vehicle control (black line) or TMZ (green line). TMZ administration began one day post tumour grafting to mice at 40 mg/Kg i.p. three times a week for four weeks. As vehicle control, 5 % DMSO in PBS was used. Mice were sacrificed when tumour volume had reached 1000 mm³. Scr+TMZ Vs gal-1+TMZ groups show no significant difference (log rank test, P = 0.7710).
Results

3.11. The tumour growth inhibitory effect of TMZ and galectin-1 silencing is not primarily caused by reduced cell proliferation

To determine the mechanism that contributes to the tumour growth inhibition following transient galectin-1 knockdown or treatment with TMZ, ki67 staining was performed using fixed tissue sections from SK-N-BE(2) tumours in all treatment groups. As shown in Fig 25 (a) and (b), neither TMZ treatment nor galectin-1 silencing resulted in reduced proliferation when compared to control and scrambled siRNA control groups. Combined TMZ plus galectin-1 knockdown did not result in any change in proliferation compared to scrambled siRNA plus TMZ treated groups.

Fig 25(a): TMZ and Gal-1 silencing anti-tumoural effects are not due to proliferation inhibition in NB mouse xenograft model. Histological sections were obtained from the tumours of the mice in each treatment group. Representative sections from each treatment group are shown. A) scr + control, B) scr + TMZ; C) gal-1 + control; D) gal-1 + TMZ
Fig 25 (b): TMZ and gal-1 silencing does not reduce Ki67 proliferation index in the SK-N-BE(2) NB xenograft model. Histological sections were obtained from the tumours of the mice in each treatment group and Ki67 staining was performed. Average proliferation index of each treatment group was determined by counting positive cells per microscopic field at 100 x magnification. No significant difference in the proliferative index (% of Ki-67 positive cells) was found for the treatment groups (e.g. Scr+ TMZ versus gal-1 + TMZ, P = 0.3812).
Galectin-1 has been shown to be implicated in different aspects of tumour biology, including angiogenesis, invasiveness and tumour immune escape in many tumours (Rabinovich et al., 2005, Camby et al., 2006). We have previously shown that galectin-1 mRNA and protein levels are up-regulated in patients with the aggressive subtype of NB (Schramm et al, 2005, Sitek et al., 2005). Furthermore, galectin-1 blockade in tumour cells has been demonstrated to halt tumour progression (Rubenstein et al., 2007) and also to potentiate the effects of the pro-autophagic drug TMZ (Mathieu et al., 2007). In this study, the effects of galectin-1 loss were elucidated both in vitro and in vivo to evaluate blocking galectin-1 functions as a treatment for aggressive NB. Here we used the well characterised human NB cell lines Kelly (Schwab et al., 1983) and SK-N-BE(2) and also the murine NB cell line NXS2 (Lode et al., 1997) which reflect the more aggressive forms of NB.

Various obstacles exist for the development of therapies for paediatric cancers. The rarity of these diseases is a considerable problem. Neuroblastoma is the most common extracranial paediatric solid tumour, yet only 150 children and adolescents will be diagnosed each year in Germany (Deutsches Kinderkrebsregister, Jahresbericht 2007). This is in sharp contrast to the most common adult solid tumour, lung cancer, with more than 50,000 new cases diagnosed annually (Robert Koch Institut, Verbreitung von Krebserkrankungen in Deutschland 2010). With so few people affected by this disease, there is a reduced market incentive for industry-based drug development. A second challenge, even with drug in hand, is the development of clinical trials adequately powered to address the question of efficacy. Testing combinations of compounds adds yet another layer of complexity. When only a limited number of drugs can be tested, rational selection for testing becomes critical. Thus the need for mouse models in evaluating new combination therapies is an invaluable asset.

Current regimens for high-risk neuroblastoma cure fewer than half of all patients, with many experiencing relapse from minimal residual disease remaining after consolidation with high-dose chemotherapy and autologous hematopoietic stem-
cell transplantation (Matthay et al., 1999). Although some success has been achieved using post-transplantation maintenance therapy with the differentiating agent 13-cis-retinoic acid or with monoclonal antibodies targeting neuroblastoma-specific proteins (Cheung et al., 1998), further advances are needed. Also, it is becoming increasingly evident that conventional chemotherapeutics are approaching or perhaps have already attained their maximum therapeutic potential. Thus, novel therapeutic options are warranted.

In this study, the antitumoural efficacy of TMZ and FTS, both novel therapies, were tested in NB models.

### 4.1. In vitro studies

Three methods were used in order to down-regulate galectin-1 expression i.e. RNAi or chemical inhibition using HDACi. Though siRNA successfully decreased galectin-1 expression in all cell lines tested, we could not develop a stable galectin-1 stably transfected shRNA cell NB cell line since the cells reach senescence after about eight weeks of selection. Both the cells with the plasmid construct and empty vector attained senescence at the same time suggesting that the problem could be the vector hence a different construct using a different vector can be used instead.

Galectin-1 transient silencing induced growth arrest in all NB cells lines indicating that gal-1 plays a regulatory role on cell proliferation in NB. However the mechanisms of this anti-proliferative activity still requires elucidation. FACS analysis of the different cell cycle distribution after galectin-1 depletion did not show any changes to untransfected cells. This is in contrast to what is observed in colorectal cancer where intracellular gal-1 induces cell cycle arrest and apoptosis (Sateli & Rao, 2010). Ironically, reduced expression of galectin-1 expression in both glioma (Le Mercier et al., 2008b) and Melanoma (Mathieu et al., 2007) did not result in any apoptosis or autophagy. These variable results emphasise the delicate role galectin-1 plays in different tumour types and also suggest that the mechanism of galectin-1 induced antitumour effects are tumour or cell type specific.
4.2. HDAC Inhibitors and Galectin-1 Silencing

The pharmacologic inhibition of histone deacetylases (HDAC) is an emerging novel molecular treatment strategy in cancer therapy (reviewed in Yoo & Jones, 2006; Menucci & Pelluci, 2006). HDAC inhibitors have been shown to induce the inhibition of cell growth, migration, apoptosis, and to promote differentiation in several cancer cell lines in vitro, and to exert antitumoural effects in mouse models (Marks et al., 2001; Bolden et al., 2006; Furchert et al., 2007; reviewed Pan et al., 2007). In NB, HDACi have been demonstrated to suppress many tumour-relevant proteins e.g. MYCN, survivin, E2F-1, all of which are found up-regulated in aggressive, high-risk NB (Deubzer et al., 2008). In other studies, inhibitors of HDAC were reported to restore p53 tumour suppressor pathway by increasing p21\textsuperscript{Waf1/Cip1} and activating p53 gene (Condorelli et al., 2008). As a functional consequence, HDACi induce growth inhibition and cell arrest in cultured cells (Toscani et al., 1998; Deubzer et al., 2008). Galectin-1 is negatively regulated by p53 (Puchades et al., 2007; Le Mercier et al., 2008a) and Gal-1 reciprocally has been shown to modify p53 biological functions and increase p21\textsuperscript{Waf1/Cip1} in human glioblastoma. In addition, galectin-1 has been previously been associated with NB cell migration and invasion (Cimmino et al., 2009). We therefore tested the hypothesis that HDACi might also down-regulate galectin-1 expression in NB cells. Functionally, HDACi treatment inhibited cell proliferation, which is an unexpected finding given the previous findings linking galectin-1 expression to a more aggressive NB phenotype. The mechanisms of galectin-1 modulation by HDACi remain unclear. This notwithstanding, this result points to the specific actions of HDACi in transcriptional regulation of gal-1 and indirectly supports the contention that galectin-1 expression is linked to the status of histone acetylation. Moreover, this was the motivation to explore a synergistic effect of down-regulating galectin-1 in combination with HDACi.

It turned out that siRNA-mediated galectin-1 downregulation sensitized only SK-N-BE(2) NB cells to the anti-proliferative activity of SAHA but not to VPA in vitro as monitored by real time viability analyses (XCelligence, Roche). This phenomenon is not observed using the MTT assay, which did not reveal synergy of galectin-1 depletion and HDACi on NB growth inhibition. Whether these results are due to
Discussion

the fact that the MTT assay is an endpoint measurement recording only mitochondrial activity at a given time, whereas the xcelligence “Cell Index” monitors cellular attachment and viability in real-time, remains to be determined. Interestingly, galectin-1 knockdown apparently potentiates the cytotoxic effects of VPA in NXS2 cells but not in Kelly and SK-N-BE(2) cells. This discrepancy might be due to the fact that cell lines are as heterogeneous as the tumours they originate from thus affecting the clinical outcome of different treatment schedules (George et al., 2010). However, these findings however illustrate that a combination of gal-1 blockade and HDACi could be a valuable treatment option in NB and therefore merits further investigation in vivo to fully establish its efficacy.

4.3. TMZ, FTS and Galectin-1 Silencing in vitro and in vivo

Since many of the functions of galectin-1, such as tumour invasiveness or angiogenesis, can be best assessed in a model reflecting the full metastatic capacity, we also aimed to establish an orthotopic model of NB by microsurgery. This NB model should mimic disseminated or relapsing NB and combine the benefits of both orthotopic (intra-adrenal injection of NB cells) and syngeneic (use of murine cell line, NXS2) model potentials to improve the power that illustrates a true clinical situation. Mice developed tumours but the absence of both macro and micro metastasis meant the model was not suitable for its intended use. A plausible explanation of this might be that the amount of cells injected in the adrenal gland was not sufficient to elicit metastasis. Also, there is the possibility that most of the cells injected in the adrenal gland were extruded into the interstitial space due to high pressure from within the adrenal. A good way of improving intra-adrenal intake will be to mix cell suspension with fibrinogen/thrombin for clot formation thus preventing extrusion of cells after injection. The protocol/procedure used in the development of this syngeneic orthotopic model is a promising one since the surgical procedure and tumour development was successful. It should be well noted that this is still a model in building as work is still going on to improve several technical aspects e.g. tagging cells with luciferase and use of MRI to monitor cell growth in life time.
Discussion

TMZ is a pro-autophagic (Kanazawa et al., 2004) and pro-angiogenic (Kurzen et al., 2003; Fischer et al., 2007) drug that has demonstrated anti-tumour activity in a number of tumour types including paediatric tumours. It has also shown anti-tumour activity against preclinical NB mouse models (Middlemas et al., 2000; Houghton et al., 2000; Wagner et al., 2007) and has now been tested in clinical trials for children with high-risk relapsing NB (Wagner et al., 2010; Rubie et al., 2006) hence illustrating its power in NB therapy. Furthermore, stable as well as transient galectin-1 downregulation have been reported to sensitize tumour cells to the effects of TMZ in both melanoma and Glioma models (Mathieu et al., 2007; Le Mercier et al., 2008a; Le Mercier et al., 2008b). With these features in mind, we studied the effect of gal-1 knockdown and treatment with TMZ in NB model systems. It has been previously shown that TMZ increases the expression of gal-1 in gliomas (Mathieu et al., 2007; Le Mercier et al., 2008b). Interestingly, TMZ apparently does not alter the expression of gal-1 in NB cells both at the mRNA and protein levels suggesting that TMZ does modulate gal-1 expression in NB.

Our data show that TMZ (100 µM) does have weak cytotoxic effect against all NB cell lines tested in vitro. This result is consistent with the other reports in melanoma and glioblastoma (GBM). TMZ is known to induce G2/M growth arrest in tumour cells (Kanazawa et al., 2004). In the present study, TMZ induced only a modest shift of the cell cycle to G2/M growth arrest, which was not statistically significant. These results laid the foundation to evaluate the in vivo effects of combining TMZ and galectin-1 downregulation (see below).

FTS is a Ras antagonist that prevents the attachment of Ras to the cell membrane leading to its degradation by disrupting the Hras/gal-1 association (Paz et al., 2001). We therefore assessed the effect of FTS on NB cells in culture. FTS did not inhibit growth of the human NB cells tested but was cytotoxic to the murine NB cell line NXS2. A plausible explanation for this might be that, certain genetic factors inherent in the murine cell line render them susceptible to FTS. This observation in human NB cells is inconsistent with the well established growth inhibitory effect of FTS to other tumour types like glioblastoma (Blum et al., 2006; Amos et al., 2006), Melanoma (Smalley & Eisen, 2002), Prostate cancer (Mcpherson et al, 2003) and Lung cancer (Zundelevich et al., 2007). Taken into consideration the action of FTS on activated Ras and also that these tumours either harbour mutated or activated
Ras, one might be tempted to conclude that FTS is effective only in those tumours in which Ras is essential as a driving. This is further supported by the report that FTS disrupts the interaction between Ras and MYCN leading to reduced expression of both proteins and inhibiting growth of LAN-1 NB cells in vitro. LAN-1 in this study expressed very high amounts of MYCN and active Ras (Yaari et al., 2005). Since the action of FTS also strongly depends on the association of HRas and gal-1, the interaction of gal-1 and HRas was assessed. Our results show that gal-1 interacts with HRas and that FTS treatment of NB cells does not result in any significant expression in gal-1 expression. However a study of the interaction of activated HRas and gal-1 will shed more light as to the actions of FTS in NB. Again FTS alone did not induce any changes in the cell cycle distribution of all cell lines tested indicating that reduced proliferation of NXS2 cells by FTS is not as a result of cell cycle growth arrest.

In addition to the set of mechanisms already identified for FTS antitumour activities, the compound also exerts its effect by impairing angiogenic processes (Blum et al., 2005). Also, FTS sensitized melanoma cells in vivo to the antitumoural effects of Dacarbazine (Halaschek-Wiener et al., 2003). Owing to the fact that TMZ is an imidazotetrazine derivative of Dacarbazine, we hypothesized that FTS will also sensitise NB cells to the antitumour effects of TMZ. This hypothesis was tested in a NB mouse syngeneic model since NXS2 cells had already shown tumour growth inhibitory activity in vitro. Despite the weak cytotoxic effect of TMZ in vitro, it did significantly increase the survival of tumour-bearing mice. When tumours were treated with TMZ and FTS, the combined effect was not different from TMZ monotherapy. Combination therapy did not show any improved effectiveness in localised tumours resulting in no relative improved survival of mice bearing tumours compared to TMZ alone treated group. Only functional assays of NXS2 FTS treated cells can provide hard evidence for this scenario. Bearing in mind the considerable role TrkB plays in NB aggressiveness (Kohl et al., 1984), in activating Ras and our previous finding of gal-1 involvement in TrkB mediated NB aggressiveness, one can suggest that studies of the interplay of these factors might be beneficial in understanding the effects of FTS in NB. Furthermore, we analysed the consequences of galectin-1 knockdown and its power to sensitize NB tumours to the growth inhibitory effect of TMZ in a NB
Discussion

xenograft model, as this has been found to be the most reproducible system in terms of tumour growth and comparability of response parameters. Transient galectin-1 downregulation by siRNA significantly increased the survival of tumour bearing mice as effective as TMZ. However, gal-1 depletion did not improve the survival of tumour bearing mice when treated with TMZ. This result also contrasts what has been described for experimental melanoma and glioblastoma. Additionally, TMZ treatment alone or galectin-1 knockdown was not able to reduce the Ki67 index possibly negating the anti-proliferative role of both gal-1 downregulation and TMZ to be the mechanism of action in tumour growth inhibition in NB. Again, the fact that TMZ increases the expression of gal-1, a phenomenon not observed in the present study, suggest that TMZ might work completely differently in NB.

All in all, the data strongly reinforce the therapeutic potential of galectin-1 loss in NB and also supports the use of TMZ as chemotherapeutic treatment for NB.
5. Conclusion

High-risk NB is a difficult disease to treat and relapsing minimal residual disease is generally resistant to current therapeutic regimens. Overexpression of galectin-1 can be one mechanism by which NB become resistant to chemotherapeutics since its overexpression correlates with aggressiveness of the disease.

In the present study, we first showed that downregulation of galectin-1 resulted in a decreased viability of NB cells in vitro. The potential therapeutic effects of depleting galectin-1 were then demonstrated in an in vivo NB model. Decreasing the expression of galectin-1 in an NB experimental model reduced tumour burden and prolonged the survival of mice-bearing tumours.

Furthermore, the therapeutic potential of both farnesyl-thiosalicylate (FTS) and temozolomide (TMZ) were elucidated. While FTS did not show antitumoral activity except for one NB murine cell line, NXS2, in vitro and in vivo, TMZ was effective in inhibiting growth of NB tumours.

There was no synergistic effect of combining TMZ and FTS or of silencing galectin-1 and treatment with either FTS or TMZ. Further studies on the mechanisms, by which galectin-1 loss causes tumour growth inhibition may result in the design of more effective targeted therapy regimens for NB. For example, gene expression profiling of galectin-1 knockdown in NB cell lines might reveal genes involved in pathways responsible for galectin-1 mediated tumour progression and aggressiveness in NB.

Most importantly, this study has shown that interfering with galectin-1 function impairs tumour growth in all NB models analysed. Since galectin-1 is expressed in all primary NBs and also in gliomas, melanomas and other tumour entities, our conclusion is that galectin-1 is a bona fide cancer target. Galectin-1 downregulation therefore provides an excellent tool for new treatment strategies in children with high-risk NB.
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7. Appendix

List of Tables

Table 1  International Neuroblastoma Staging System ......................................10
Table 2  Neuroblastoma risk groups based on clinical and biological features ..............................................................14

List of Figures

<table>
<thead>
<tr>
<th>Fig 1</th>
<th>Contribution of galectin-1 to tumour progression ........................................21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig 2</td>
<td>Expression of Galectin-1 in Neuroblastoma cell lines ....................................39</td>
</tr>
<tr>
<td>Fig 3</td>
<td>Inducible galectin-1 shRNA mediated transient knockdown of galectin-1 ..................40</td>
</tr>
<tr>
<td>Fig 4</td>
<td>Galectin-1 siRNA knockdown of galectin-1 mRNA expression .................................41</td>
</tr>
<tr>
<td>Fig 5</td>
<td>Western blot analysis depicting the expression levels of galectin-1 protein after gal-1 siRNA transfection ..........................42</td>
</tr>
<tr>
<td>Fig 6</td>
<td>Effect of HDAC inhibitors on proliferation of NB cell lines ............................43</td>
</tr>
<tr>
<td>Fig 7</td>
<td>HDACi upregulate the expression of galectin-1 in NB cells .................................44</td>
</tr>
<tr>
<td>Fig 8</td>
<td>Proliferative response of NB cell lines to single agent TMZ or FTS ........................46</td>
</tr>
<tr>
<td>Fig 9</td>
<td>Anti-proliferative effect of combination of TMZ and FTS in NB cells ....................48</td>
</tr>
<tr>
<td>Fig 10</td>
<td>Effect of TMZ and FTS on galectin-1 expression in NB cells determined by semi quantitative RT-PCR .................................49</td>
</tr>
<tr>
<td>Fig 11</td>
<td>Flow cytometric analysis of cell cycle distribution after treatment with TMZ and FTS ..........................................................51</td>
</tr>
</tbody>
</table>
| Fig 12 | Impact of TMZ and FTS treatment to galectin-1 ..............................................
Appendix

<table>
<thead>
<tr>
<th>Fig 13</th>
<th>Galectin-1 co-immunoprecipitates with HRAS in NB cells</th>
<th>53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig 14</td>
<td>Effects of galectin-1 knockdown and treatment with VPA</td>
<td>54</td>
</tr>
<tr>
<td>Fig 15</td>
<td>Effects of galectin-1 knockdown and treatment with SAHA</td>
<td>56</td>
</tr>
<tr>
<td>Fig 16</td>
<td>Effects of galectin-1 knockdown and treatment with 100 µM TMZ</td>
<td>58</td>
</tr>
<tr>
<td>Fig 17</td>
<td>Effects of galectin-1 knockdown and treatment with FTS</td>
<td>59</td>
</tr>
<tr>
<td>Fig 18</td>
<td>Real time measurement of cell proliferation <em>in vitro</em></td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>using the XCelligence</td>
<td></td>
</tr>
<tr>
<td>Fig 19</td>
<td>TMZ does not alter galectin-1 expression after knockdown by siRNA in NXS2 and SK-N-BE(2) cells</td>
<td>63</td>
</tr>
<tr>
<td>Fig 20</td>
<td>Galectin-1 knockdown does not change the cell cycle distribution of NB cells</td>
<td>64</td>
</tr>
<tr>
<td>Fig 21</td>
<td>Macroscopic examination of mouse organs</td>
<td>65</td>
</tr>
<tr>
<td>Fig 22</td>
<td>Microscopic examination of mouse organs and tumour</td>
<td>66</td>
</tr>
<tr>
<td>Fig 23</td>
<td>Kaplan Meier survival analysis for the evaluation of NB growth inhibition by FTS and TMZ in a NB syngeneic model</td>
<td>68</td>
</tr>
<tr>
<td>Fig 24</td>
<td>Effect of decreasing Galectin-1 silencing and response to TMZ in NB xenograft model</td>
<td>70</td>
</tr>
<tr>
<td>Fig 25(a)</td>
<td>TMZ and Gal-1 silencing anti-tumoural effects are not due to proliferation inhibition in NB mouse xenograft model</td>
<td>71</td>
</tr>
<tr>
<td>Fig 25(b)</td>
<td>TMZ and gal-1 silencing does not reduce Ki67 proliferation index in SK-N-BE(2) NB xenograft model</td>
<td>72</td>
</tr>
</tbody>
</table>
Acknowledgements

I owe my deepest and sincere gratitude to Prof Angelika Eggert for providing me with the extraordinary opportunity to work in her lab and for introducing me to the very intellectually stimulating and fascinating field of cancer biology which has given me a wider perspective on the importance and possibilities of science. I truly appreciate that you never missed the opportunity to remind me that I can accomplish anything that I dream and work to attain. Thanks for the excellent team of people that you always gathered in your lab which provided a nice, friendly and thrilling working environment both in and out of the lab.

I am deeply indebted to my supervisor, Dr Alexander Schramm whose experience, expertise, beneficial insightful suggestions and sensitive reading of the text were crucial to the successful completion of this thesis. Thank you for your guidance and friendship, patience, confidence and optimism throughout our work and most especially for still believing in me during my darkest period of doubt.

Special thanks to Dr Johannes Schulte for the very wonderful introduction to in vivo experimentation. I am much obliging to Dr Kathy Astrahanseff and Dr Harald Stephan for their unflinching encouragement and support in various ways and also for all the enthusiastic discussions on scientific research.

I will forever remain indebted to Manuel Schulze-Dasbeck, Ellen Mahlow, Melanie Bowmann and Sabine Dressmann for the very excellent technical support in a host of techniques.

I also want to express my appreciation to Dr Gero Hilken, Dr Philip Dammann and Christine Krüger for their laudable assistance in all aspects of animal experimentation especially in mouse surgical techniques.

My time at the Uniklinik has been a very rewarding, interesting and discovering journey during which I met a lot of interesting people that have helped me a lot and whose names I would regrettably not be able to mention. Ich danke euch!

Special thanks to both former and present members of the ONCOLAB for their ever friendly and cheerful comradeship and for making my stay here in Germany a pleasant one.
Acknowledgements

Many thanks to the European Union for providing the financial support, without which, this project would not have been realised.

I want to extend special recognition and appreciation to my family and friends for their unending love. Your encouragement and support have served as the basis from which my hope and perpetual optimism for a brighter future originated.

Now to him who is able to do immeasurably more than all we ask or imagine, according to his power that is at work within us.

Ephesians 3:20
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