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The Role of Multi-Drug Resistance Associated Protein 4 and P-glycoprotein in Resistance of Neuroblastoma to Topotecan and Irinotecan

Abstract

High-risk neuroblastoma presents a significant therapeutic challenge because the 5-year survival rate remains less than 30% despite the use of surgery, multi-agent chemotherapy, radiation, and autologous bone marrow transplant. Novel therapeutic modalities are under development. The camptothecin analogs topotecan and irinotecan have been identified as successful cytotoxic agents. For topotecan, pharmacokinetically guided dosing to achieve a systemic exposure associated with preclinical anti-tumor activity in neuroblastoma xenograft models is feasible and has elicited favorable responses in children with high-risk neuroblastoma. However, some children with high-risk disease did not respond to the putatively effective topotecan systemic exposure. These children represent a subset of the disease intrinsically resistant to topotecan. Furthermore, mRNA expression of the adenosine triphosphate (ATP)-binding cassette (ABC) transporters P-glycoprotein (Pgp) and multidrug resistance associated protein 1 (MRP1), which efflux many drugs used in neuroblastoma therapy, has been implicated in poor outcome in neuroblastoma. Therefore, the purpose of our studies was to determine the role of ABC transport protein expression in neuroblastoma resistance to the camptothecin analogs topotecan and irinotecan.

Initially studies focused on determining the expression of ABC transporters for which the camptothecin analogs are substrates in neuroblastoma cell lines. By western blot analysis we demonstrated MRP4 and Pgp expression in neuroblastoma cell lines relatively resistant to topotecan (e.g., NB1691), but not in cell lines sensitive to topotecan (e.g., NB1643). In contrast, MRP1, MRP2, and breast cancer resistance protein (BCRP) expression did not discriminate between sensitive and resistant cell lines. To determine the functional contribution of both MRP4 and Pgp in neuroblastoma, we used RNA interference (RNAi) to silence MRP4 and Pgp expression in NB1691. Long term, stable expression of retroviral vector mediated short hairpin RNA (shRNA) reduced MRP4 and Pgp expression. Isogenic cell lines with reduced expression of MRP4 and Pgp exhibited an increase in sensitivity to both topotecan and SN-38, the active moiety of the prodrug irinotecan. In addition, we overexpressed MRP4 in NB1643, which resulted in increased topotecan resistance.

The NB1691 cell lines with reduced MRP4 expression were subsequently transplanted as xenografts into severe combined immunodeficiency (SCID) mice to determine the effect of MRP4 expression on the *in vivo* response to topotecan. Unexpectedly, MRP4 silencing did not persist *in vivo*, and none of the xenograft models responded to topotecan. However, MRP4 expression was associated with failure to respond to topotecan, supporting the hypothesis that MRP4 mediates resistance to topotecan.

Finally, we determined the ABC transporter expression profile in primary tumor specimens from patients with high-risk neuroblastoma who were treated with pharmacokinetically guided topotecan. Of the 14 specimens studied, MRP4 was expressed in 2 samples, and Pgp was expressed in 4 samples. BCRP was not expressed in any of the neuroblastoma cell lines *in vitro*, but immunohistochemical analysis demonstrated BCRP expression in nine primary neuroblastoma samples. Although we predicted that MRP4 and/or Pgp expression would be associated with failure to respond to topotecan, results of immunohistochemical analysis did not demonstrate such an association.

The results of the *in vitro* studies demonstrate that MRP4 and Pgp confer resistance to topotecan and SN-38. In the xenograft studies, MRP4 expression was associated with failure to respond to topotecan. However, this phenotype was not recapitulated in children treated with topotecan. These results may be confounded by small sample size and timing of sample acquisition. Further investigation of the role of ABC transporters in children with neuroblastoma who receive either topotecan or irinotecan may be warranted. In addition to the camptothecin analogs, patients will receive other drugs effluxed by the ABC

transporters (e.g., doxorubicin, vincristine, etoposide, cyclophosphamide). Therefore, analyzing ABC transporter expression by immunohistochemistry in diagnostic tumor specimens may help to select agents not subject to efflux by ABC transporters expressed in the tumor. However, eliminating drugs effluxed by ABC transporters from the treatment regimen creates a potential gap in therapy and may reduce drug intensity. Therefore, further rational design and development of drugs that evade ABC transporter-mediated efflux, and potentially other resistance mechanisms in neuroblastoma, is also warranted.

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**THE ROLE OF MULTI-DRUG RESISTANCE ASSOCIATED PROTEIN 4 AND
P-GLYCOPROTEIN IN RESISTANCE OF NEUROBLASTOMA TO
TOPOTECAN AND IRINOTECAN**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Patricia Kellie Turner
December 2007

Dedication

I dedicate this work to my parents Patricia and Doug Turner, my sister Kim Turner, and my grandmother Helen Turner. Their constant support, encouragement and unconditional love have always inspired me in all of my endeavors.

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I am indebted to my mother, father, sister, and grandmother for their unconditional love and support. I am eternally grateful to my mentor and role model Dr. Clinton Stewart for his guidance and support. I will always appreciate his patience, the numerous opportunities he has provided, and all of the time he has spent teaching me. Other important teachers in my life whose time and guidance I truly appreciate are the members of my graduate committee: Drs. Mary Danks, Bernd Meibohm, Victor Santana, and John Schuetz. I thank Suzan Hanna, my “Mom” in the lab, for everything that she taught me and for her constant encouragement. I also thank many others who have helped me along the way, including Brad Johnston, Dr. Mark Leggas, Chuck Fraga, Dr. Stacy Throm, Dr. Jiaowang Dong, Dr. Lisa Iacono, Dr. Burgess Freeman, Dr. Bill Zamboni, Dr. Yanli Zhuang, Jun Shen, Geeta Nair, Dr. Feng Bai, Nik Hagedorn, Elaine Hubbard, Dr. Carl Panetta, Dr. Peter Houghton, Chris Morton, Klo Spelshouse, Peter Barta, Seth Dixon, Rebecca Bush, Jessie Morgan, Mark Wilkinson, Dr. Partha Krishnamurthy, Dr. Kelly Mercer, and Dr. Charis Marston.

Abstract

High-risk neuroblastoma presents a significant therapeutic challenge because the 5-year survival rate remains less than 30% despite the use of surgery, multi-agent chemotherapy, radiation, and autologous bone marrow transplant. Novel therapeutic modalities are under development. The camptothecin analogs topotecan and irinotecan have been identified as successful cytotoxic agents. For topotecan, pharmacokinetically guided dosing to achieve a systemic exposure associated with preclinical anti-tumor activity in neuroblastoma xenograft models is feasible and has elicited favorable responses in children with high-risk neuroblastoma. However, some children with high-risk disease did not respond to the putatively effective topotecan systemic exposure. These children represent a subset of the disease intrinsically resistant to topotecan. Furthermore, mRNA expression of the adenosine triphosphate (ATP)-binding cassette (ABC) transporters P-glycoprotein (Pgp) and multidrug resistance associated protein 1 (MRP1), which efflux many drugs used in neuroblastoma therapy, has been implicated in poor outcome in neuroblastoma. Therefore, the purpose of our studies was to determine the role of ABC transport protein expression in neuroblastoma resistance to the camptothecin analogs topotecan and irinotecan.

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The results of the *in vitro* studies demonstrate that MRP4 and Pgp confer resistance to topotecan and SN-38. In the xenograft studies, MRP4 expression was associated with failure to respond to topotecan. However, this phenotype was not recapitulated in children treated with topotecan. These results may be confounded by small sample size and timing of sample acquisition. Further investigation of the role of ABC transporters in children with neuroblastoma who receive either topotecan or irinotecan may be warranted. In addition to the camptothecin analogs, patients will receive other drugs effluxed by the ABC transporters (e.g., doxorubicin, vincristine, etoposide, and cyclophosphamide). Therefore, analyzing ABC transporter expression by immunohistochemistry in diagnostic tumor specimens may help to select agents not subject to efflux by ABC transporters expressed in the tumor. However, eliminating drugs effluxed by ABC transporters from the treatment regimen creates a potential gap in therapy and may reduce drug intensity. Therefore, further rational design and development of drugs that evade ABC transporter-mediated efflux, and potentially other resistance mechanisms in neuroblastoma, is also warranted.

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List of Abbreviations

ABC	ATP-binding cassette
ABCB1	gene member 1 of ABC subfamily B (alias MDR1)
ABCC1-4	gene members 1-4 of ABC subfamily C
ABCG2	gene member 2 of ABC subfamily G (BCRP gene)
ABCP	placenta resistance protein (alias BCRP)
AraC	cytarabine
ATP	adenosine triphosphate
AUC	area under the concentration-time curve
BCRP	breast cancer resistance protein
Bcrp	breast cancer resistance protein, mouse
cAMP	cyclic adenosine monophosphate
CDDP	cisplatin
cGMP	cyclic guanosine monophosphate
CI	continuous infusion
cMOAT	canalicular multispecific organic anion transporter
COG	Children's Oncology Group
CR	complete response
C _t	cycle threshold
CTX	cyclophosphamide
Dauno	daunorubicin
DLT	dose limiting toxicity
DNA	deoxyribonucleic acid

Dox	doxorubicin
dsRNA	double stranded RNA
E ₂ 17βG	estradiol-17-β-D-glucuronide
EST	expressed sequence tag
FACS	fluorescence activated cell sorter
FDA	Food and Drug Administration
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GFP	green fluorescent protein
G-CSF	granulocyte-colony stimulating factor (filgrastim)
i.p.	intraperitoneal
INSS	International Neuroblastoma Staging System
IRES	internal ribosomal entry site
i.v.	intravenous
kDa	kiloDalton
MDR1	gene for multidrug resistance protein 1
Mdr1a/1b	multidrug resistance protein 1a and 1 b, mouse
MEF	murine embryonic fibroblast
6-MP	6-mercaptopurine
mRNA	messenger RNA
Mrp1	multidrug resistance associated protein, mouse
MRP1-4	multidrug resistance associated protein 1-4
Mrp4	multidrug resistance associated protein 4, mouse
MSCV	murine stem cell virus

MTD	maximum tolerated dose
MXR	mitoxantrone resistance protein (alias BCRP)
NB	neuroblastoma
NBD	nucleotide binding domain
OR	objective response
p.o.	<i>per os</i> (oral)
PCR	polymerase chain reaction
PGE ₁₋₂	prostaglandin E 1-2
Pgp	P-glycoprotein
PMEA	9-(2-phosphonylmethoxyethyl) adenine
POG	Pediatric Oncology Group
PR	partial response
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcriptase PCR
SD	stable disease
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA
SN-38	irinotecan metabolite
SNP	single nucleotide polymorphism
6-TG	6-thioguanine
TMD	transmembrane domain

VM-26	teniposide
VP-16	etoposide

Chapter 1. Introduction

Neuroblastoma is the most common extracranial solid tumor of childhood, accounting for 7-10% of all childhood malignancies (1,2). Most patients present with metastatic disease and have a poor prognosis (2,3). Therefore, improving therapy for children with neuroblastoma is a major focus of ongoing preclinical and clinical research. Results of work in one such area, overcoming multi-drug resistance, suggest that it may be one of the many barriers to improving survival in children with this disease. The molecular basis of multi-drug resistance in neuroblastoma is likely to be multifactorial, caused by alterations in DNA repair pathways (4), increased intracellular detoxification/inactivation of drugs (5,6), alterations in cell survival pathways (7,8), decreased binding of drugs to the intended target (9), or increased efflux of chemotherapeutic agents from cells.

Available data suggest that in neuroblastoma cells increased efflux may be a determinant of resistance to chemotherapy. In solid tumor cells, drug efflux can be mediated by members of the ABC transport protein superfamily. P-glycoprotein (Pgp) and multi-drug resistance associated protein 1 (MRP1), both members of the ABC transporter superfamily, are expressed in tumor specimens from children with neuroblastoma (10,11,12). High Pgp and/ or MRP1 expression in neuroblastoma is associated with poor prognosis. Also associated with poor prognosis is MYCN, a transcription factor often amplified in neuroblastoma (13). The observation that MYCN regulates the expression of several ABC transporters suggests two independent

prognostic indicators for poor outcome: MYCN amplification and ABC transporter expression (14).

Several drugs used in neuroblastoma therapy, including doxorubicin, vincristine, and etoposide (15,16), are ABC transporter substrates. The camptothecin analogs topotecan and irinotecan are emerging as useful components of neuroblastoma therapy (15,17) and are also substrates for ABC transporters (18). Therefore, the major focus of this dissertation will be to assess the role of ABC transporter expression in resistance to the cytotoxicity of topotecan and irinotecan *in vitro*, *in vivo*, and in children with neuroblastoma. Expression of ABC transporters for which these camptothecin analogs are substrates will be characterized in neuroblastoma cell lines demonstrating a spectrum of sensitivity to these compounds. Molecular techniques including exogenous overexpression and RNA interference (RNAi) will be used to evaluate the functional contribution of individual transporters to drug resistance *in vitro* by quantitating camptothecin analog sensitivity in isogenic cell lines. In addition, xenografts in mice will be generated using these isogenic cell lines to assess the response to camptothecin analogs. Lastly, primary tumor specimens from patients with high-risk neuroblastoma will be analyzed to identify potential associations between clinical response to topotecan and ABC transporter expression.

1.1 Neuroblastoma

1.1.1 Epidemiology

Neuroblastoma is the most common cancer in children younger than one year (19). Furthermore, neuroblastoma is the most common extracranial solid tumor of

childhood. Neuroblastoma can arise from any tissue of sympathetic origin, but the adrenal gland is the most common primary site. In the United States the annual neuroblastoma incidence among children less than 15 years of age is 9.7 cases per million (20). Neuroblastoma occurs at similar rates in both males and females, but a slightly higher predominance is reported in whites than in blacks (10.2 versus 7.8 cases per million, respectively). Most commonly neuroblastoma occurs in children younger than two years. The median age at diagnosis is 22 months (21). Between 1985 and 1988 the overall 5-year survival for children with neuroblastoma was 58% (1). However, only 34% of children diagnosed with stage 4 neuroblastoma are long-term survivors (22). The biological mechanisms that contribute to the poor survival in children with advanced stage neuroblastoma are a subject of intense research. ABC transporter mediated efflux of chemotherapeutic agents from tumor cells is among the many potential causes of poor survival in children with neuroblastoma. Therefore, the major focus of this dissertation will be on the role of ABC transporters in high-risk neuroblastoma, in particular, the role of these proteins as determinants of sensitivity or resistance to the camptothecin analogs topotecan and irinotecan.

1.1.2 Diagnosis

Patients with neuroblastoma can present with abdominal discomfort, fullness, or pain (23). Hypertension, sweating, flushing, and tachycardia may also be present, depending on tumoral catecholamine secretion. Before 1988 no uniform criteria for neuroblastoma diagnosis, staging, and response evaluation existed, making it difficult to compare clinical studies of neuroblastoma(24). From 1988-1993 the major pediatric oncology groups from the United States, Europe, and Japan proposed consensus criteria

for neuroblastoma diagnosis, staging, and response evaluation (25). Tumor stage and other prognostic indicators (e.g., MYCN status, DNA ploidy, Shimada Histology) are assessed in order to stratify patients into risk groups which determine the course of therapy.

1.1.3 Prognostic indicators and risk adapted therapy

1.1.3.1 Neuroblastoma disease stage as a prognostic indicator

Therapeutic modalities and treatment intensity are determined based upon a child's risk for a poor outcome (i.e., low, intermediate, and high-risk disease). Our study focuses on high-risk neuroblastoma. Factors used to stratify neuroblastoma patients into low, intermediate, or high-risk neuroblastoma (Table 1.1) include disease stage according to the international staging system for neuroblastoma (INSS), a patient's age, *MYCN* gene copy number, Shimada histology, and DNA index (2). The patient's age at diagnosis and INSS stage are the two most important prognostic variables in childhood neuroblastoma. The current standardized INSS defines six possible stages (Table 1.2) of childhood neuroblastoma based upon tumor confinement, resection, metastasis to lymph nodes, bone marrow, liver, or other sites (2). Children with advanced neuroblastoma (i.e., stage 3 or 4) have a poor prognosis.

1.1.3.2 Age at neuroblastoma diagnosis as a prognostic indicator

The correlation between young age at diagnosis (i.e., younger than 12-18 months) and good prognosis is well documented (26). Recent evidence suggests that children younger than 18 months have a more favorable prognosis than older children diagnosed

Table 1.1 Neuroblastoma risk group stratification.

INSS Stage	Age (Years)	MYCN Status	Shimada Histology	DNA Index	Risk Group
1	0-21	Any	Any	Any	Low
2A/2B	<1	Any	Any	Any	Low
	≥1-21	Non-amplified	Any	NA	Low
	≥1-21	Amplified	Favorable	NA	Low
	≥1-21	Amplified	Unfavorable	NA	High
3	<1	Non-amplified	Any	Any	Intermediate
	<1	Amplified	Any	Any	High
	≥1-21	Non-amplified	Favorable	NA	Intermediate
	≥1-21	Non-amplified	Unfavorable	NA	High
	≥1-21	Amplified	Any	NA	High
4	<1	Non-amplified	Any	Any	Intermediate
	<1	Amplified	Any	Any	High
	≥1-21	Any	Any	NA	High
4S	<1	Non-amplified	Favorable	>1	Low
	<1	Non-amplified	Any	=1	Intermediate
	<1	Non-amplified	Unfavorable	Any	Intermediate
	<1	Amplified	Any	Any	High

Source: Castleberry, RP. Neuroblastoma, Eur J Cancer, 33: 1430-1437, 1997.

Table 1.2 International staging system for neuroblastoma.

Stage	Description
Stage 1	Localized tumor confined to area of origin; complete gross resection, $\pm$ microscopic residual disease; ipsilateral and contralateral lymph nodes are tumor negative
Stage 2A	Unilateral with incomplete gross resection; ipsilateral and contralateral lymph nodes tumor negative
Stage 2B	Unilateral with complete or incomplete gross resection; ipsilateral lymph node tumor positive; contralateral lymph node tumor negative
Stage 3	Tumor infiltrates across midline $\pm$ regional lymph node involvement; or unilateral tumor with contralateral node involvement; or midline tumor with bilateral lymph node involvement
Stage 4	Tumor dissemination to distant lymph nodes, bone marrow, liver, or other organ except as in 4S
Stage 4S	Localized primary tumor as for Stage 1 or 2 with dissemination to liver, skin, or bone marrow with $<10\%$ nucleated marrow cells being tumor cells

Source: Castleberry, RP. Neuroblastoma, Eur J Cancer, 33: 1430-1437, 1997.

with neuroblastoma. Specifically, the 5-year event-free survival for 12- to 18-month old children was more than 70-92% compared to 31-38% for older children (27,28,29).

1.1.3.3 *MYCN* amplification as a prognostic indicator

The oncogene *MYCN* located on chromosome 2p23-24 was first identified in neuroblastomas (30), but is also amplified and overexpressed in other tumors including retinoblastoma (31) and small-cell lung cancer (32). *MYCN* amplification is associated with advanced stage neuroblastoma and rapid disease progression. Although the precise molecular reasons for the association between *MYCN* amplification and poor prognosis have not been determined, some of the *MYCN* target genes are involved in cell growth and cell cycle progression (33). Cells overexpressing *MYCN* have shortened cell cycle times (34). *MYCN* overexpression is also associated with increased metastatic potential via reduced attachment to extracellular matrix (35).

Normally *MYCN* is expressed in the developing nervous system. N-Myc, the protein product of the *MYCN* gene, localizes to the nucleus. N-Myc recognizes E-box motifs in the promoter region of target genes and activates transcription (33,36). N-Myc target genes include several ABC transporters which efflux many of the drugs used in neuroblastoma therapy (reviewed in section 1.3).

Norris and colleagues evaluated the relationship between several prognostic indicators in neuroblastoma and outcome, including *MYCN* amplification, age, tumor stage, and *MRP1* expression (10). High *MRP1* mRNA expression was the prognostic indicator most closely associated with outcome. In particular, high *MRP1* levels predicted reduced event-free and overall survival in children with neuroblastoma. After adjusting outcome for *MYCN* amplification in multivariate analysis, *MRP1* expression remained an

independent predictor of survival. Due to the prominence of *MYCN* amplification in neuroblastoma and the molecular relationship between N-Myc and ABC transporter expression, the association between *MYCN* amplification and poor prognosis may be due to reduced intracellular accumulation of drugs.

1.1.3.4 Shimada Histology as a prognostic indicator

The histopathologic features of neuroblastoma can also be used as prognostic indicators. Neuroblastoma histopathology consists of diffuse neuroblastic cell clusters with irregular fibrovascular separations. The Shimada histology classification system was proposed upon retrospective evaluation of untreated primary neuroblastoma specimens from 295 patients for whom at least 2 years of follow-up data were available (37). This system includes evaluation of stromal development and neuroblastic cell maturation/differentiation. Initially specimens are classified as stroma-rich or stroma-poor. Stroma-rich tumors are divided into 3 subgroups: well-differentiated, intermixed, and nodular. Both well-differentiated and intermixed tumors are classified as favorable (37,38,39). Lastly, a nodular appearance confers unfavorable histology. Favorable or unfavorable histology in stroma-poor cases depends on neuroblast differentiation, mitosis-karyorrhexis index (MKI), and age at diagnosis.

1.1.3.5 DNA Index

DNA Index is another feature of neuroblastoma used in risk group stratification to guide therapy for children with neuroblastoma. DNA Index (also called DNA ploidy) is a measure of tumor DNA content, which is measured by FACS analysis of propidium iodide stained nuclei. Aneuploidy is considered a favorable clinical prognostic factor in

neuroblastoma. Patients with diploid or tetraploid tumors have a poor prognosis in comparison to those with triploid tumors (40,41,42,43).

1.1.3.6 Risk group stratification

Heterogeneity is a hallmark of neuroblastoma as demonstrated by variations in age at diagnosis, DNA index, tumor histopathology, MYCN amplification, and other biological features in neuroblastoma. Based on the heterogeneous survival rates for children diagnosed with neuroblastoma, the Pediatric Oncology Group (POG) and Children's Cancer Group designed a risk stratification system to guide risk-related therapy based upon prognosis (2). Stratification is based on five prognostic factors: INSS stage, age at diagnosis, MYCN status, Shimada histology, and DNA index (Table 1.1). This system stratifies children diagnosed with neuroblastoma into three risk groups: low, intermediate, and high-risk disease.

1.1.4 Risk-adapted therapy

1.1.4.1 Low risk patients

Patients with low risk disease have greater than 95% predicted event-free survival (2,44). Low risk neuroblastoma includes all patients with Stage 1 (localized) disease. Localized disease is present in approximately 40% of neuroblastoma cases. In addition, all children with Stage 2 disease are classified as low risk except those older than 1 yr with MYCN amplification and unfavorable histology. In a POG study of 101 children with localized low risk disease treated with surgery as the sole treatment modality, only 9 patients relapsed (44). Therefore, children with low risk localized disease should undergo

surgical resection of the tumor. However, if local recurrence of the tumor is observed, these patients can be treated with further surgery or chemotherapy.

1.1.4.2 Intermediate risk patients

Patients with intermediate risk disease have a 3-year predicted event-free survival greater than 85% (2). Children with intermediate risk disease are stratified according to Table 1.1. Therapy for intermediate risk disease includes surgery and chemotherapy. Despite the favorable outcome for most patients with intermediate risk neuroblastoma, advancements must be made in therapy to reduce acute and long-term toxicities.

1.1.4.3 High-risk patients

The focus of this dissertation will remain on the contribution of ABC transporters to camptothecin analog resistance in high-risk neuroblastoma. Children diagnosed with high-risk neuroblastoma have less than 30% predicted event-free survival. Children with high-risk disease are stratified according to Table 1.1. Currently children diagnosed with high-risk neuroblastoma receive multiple modalities of therapy including cytotoxic chemotherapy, surgery, radiation, and bone marrow transplant.

The current protocol approach to high-risk neuroblastoma therapy includes induction, continuation, and maintenance phases. The goal of induction is to reduce the tumor mass by combining agents such as cisplatin, doxorubicin, etoposide, vincristine, and cyclophosphamide (45). These five drugs are combined to take advantage of different mechanisms of action and non-overlapping toxicities. Moreover, in accordance with the Goldie-Coldman hypothesis, the risk of developing drug resistance is decreased by the use of non-cross-resistant chemotherapeutic agents to achieve rapid cytoreduction (46). In the second phase patients may receive continuation therapy that includes the same

agents used during induction. Alternatively, a myeloablative preparative regimen (e.g., melphalan, etoposide, and carboplatin or melphalan and total body irradiation) followed by autologous stem cell transplantation (47,48) can be used to eradicate residual disease. Due to the high relapse rate in children with high-risk neuroblastoma, the goal of maintenance therapy is to eliminate minimal residual disease. Currently the differentiation inducing agent 13-*cis*-retinoic acid (isotretinoin) is used in maintenance therapy for high-risk neuroblastoma (48).

Due to the extremely poor outcome in children diagnosed with high-risk neuroblastoma, several approaches to improving therapy have been investigated recently or are currently in development. New therapeutic modalities such as ¹³¹I-MIBG (norepinephrine analog that concentrates in sympathetic tissue) or anti-GD2 (antigen highly expressed in neuroectodermal tumors) antibodies are under investigation. For drugs with wide variability in pharmacokinetics such as topotecan, approaches to minimize interindividual variability or to achieve the putatively effective systemic exposure have been evaluated. For example, the pharmacokinetically guided approach to topotecan dosing in children with high-risk neuroblastoma showed an excellent response to single agent topotecan targeted to the putatively effective systemic exposure (49,15). However, chemotherapeutic agents are rarely used as single agents. Due to the favorable outcome in some children with neuroblastoma, pharmacokinetically guided topotecan has also been evaluated in combination with other drugs active in neuroblastoma such as cyclophosphamide.

Despite the extremely favorable response to topotecan in children with high-risk neuroblastoma (60% response rate), the lack of a response in 40% of the children treated

with pharmacokinetically guided topotecan indicates clinical resistance to topotecan in neuroblastoma (15). The mechanisms for this resistance are not completely understood. Identifying the molecular determinants of resistance in children with either intrinsic resistance at therapy initiation or acquired resistance after several cycles of chemotherapy may help to guide selection of the most active treatment regimens for individual patients.

Resistance in neuroblastoma is likely to be a multifactorial phenomenon. For the camptothecin analogs, mutations in drug target (i.e., topoisomerase I) can lead to resistance (50,51). In addition, resistance can be caused by increased intracellular detoxification and alterations in cell death or survival pathways. Finally, ABC transporters that efflux camptothecin analogs can confer resistance.

The studies of ABC transporters in neuroblastoma have generated controversial and conflicting results. High levels of *MRP1* mRNA expression in primary, untreated neuroblastoma are associated with reduced survival and event-free survival in patients with neuroblastoma (10). Moreover, in tumors without *MYCN* amplification, high levels of *MDR1* mRNA expression are associated with poor cumulative survival and event-free survival (11). However, the prognostic significance of P-glycoprotein expression, which is encoded by *MDR1*, in neuroblastoma remains controversial (52). For example, de Cremoux and colleagues have demonstrated that high levels of *MRP1* or *MDR1* did not predict neuroblastoma response in a study of biopsy samples from 29 high-risk patients (53). In contrast Norris and colleagues have demonstrated that *MRP1* is a significant, independent predictor of poor prognosis in a prospective analysis of a large group of children with neuroblastoma (10). Despite the controversial results of studies of the relation between ABC transporter expression in neuroblastoma and clinical outcome, the

drugs that are subject to ABC transporter-mediated resistance are used in the treatment of neuroblastoma. For example, the camptothecin analogs topotecan and irinotecan have anti-tumor activity in children with neuroblastoma, but these drugs are substrates for several of the ABC transporters. Elucidation of the role of ABC transporters in resistance of primary tumors to topotecan and irinotecan may improve our ability to obtain the maximum therapeutic benefit of topotecan and irinotecan in treating neuroblastoma

1.2 Camptothecin analogs are topoisomerase I interactive agents

Camptothecin is a naturally occurring alkaloid isolated from the *Camptotheca acuminata* tree (54). Despite demonstrated anti-tumor activity, the clinical utility of camptothecin sodium is limited due to severe toxicity, including hemorrhagic cystitis characterized by necrotic ulcerations throughout the urinary tract (55,56,57). Other toxicities associated with camptothecin sodium include nausea, vomiting, alopecia, stomatitis, and diarrhea. The dose limiting toxicity (DLT) of camptothecin sodium is myelosuppression, including neutropenia and thrombocytopenia. However, due to the promising anti-tumor properties of camptothecin sodium and the pH dependent toxicity in the urinary bladder, more water-soluble camptothecin analogs topotecan and irinotecan were synthesized. Results of early clinical trials with these compounds demonstrated a more acceptable toxicity profile and retention of the promising anti-tumor activity of the parent compound. Figure 1.1 shows chemical structures of the parent pentacyclic camptothecin as well as topotecan, irinotecan, and SN-38 (the active metabolite of the prodrug irinotecan). The clinical utility of both topotecan and irinotecan has been demonstrated in pre-clinical and clinical studies of several pediatric tumor models, including neuroblastoma.

Figure 1.1 Camptothecin analog chemical structure.

	R1	R2	R3	R4
Camptothecin	H	H	H	H
Topotecan (TPT)	H	OH	$-\text{CH}_2\text{N}(\text{CH}_3)_2$	H
Irinotecan (IRN, CPT-11)	H		H	$-\text{CH}_2\text{CH}_3$
SN-38	H	OH	H	$-\text{CH}_2\text{CH}_3$

Camptothecin analogs undergo reversible pH-dependent conversion between the open-ring carboxylate form and closed-ring lactone form. At physiologic pH (7.4) the carboxylate form of the camptothecin analog predominates; whereas, the lactone form predominates under acidic conditions. The camptothecin analog lactone form is responsible for the pharmacological activity at the site of action by interacting with the target enzyme topoisomerase I.

Topoisomerase I is the molecular target of camptothecin analogs. Topoisomerase I relieves torsional strain in DNA by cleaving of a single DNA strand, permitting the second DNA strand to pass through the break, and then religating the cleaved strand. Camptothecin analogs stabilize these transient DNA-topoisomerase I complexes during DNA synthesis. The replication fork arrests upon collision with the resultant covalent DNA-topoisomerase I complexes, which are thought to generate lethal DNA strand breaks (58).

At physiological pH the α -hydroxylactone E-ring ring system undergoes reversible hydrolysis to form the anionic carboxylic acid moiety (58). *In vitro* studies of camptothecin analogs have demonstrated that the pharmacologically active form is the closed ring lactone form (59). The open E-ring carboxylate conformation has little if any cytotoxic effect (60). Because the lactone form of camptothecin analogs is considered the active conformer, phase I and II trials in children have extensively investigated the pharmacokinetics of topotecan lactone and irinotecan/SN-38 lactone.

1.2.1 Topotecan

Topotecan (9-dimethylaminomethyl-10-hydroxycamptothecin, Hycamtin®) is FDA-approved for treatment of ovarian cancer and small cell lung cancer in adults. Preclinical xenograft studies in mice have demonstrated activity of protracted topotecan administration for treatment of several pediatric solid tumors including neuroblastoma, medulloblastoma, glioblastoma, and rhabdomyosarcoma (61,62). Importantly, a steep topotecan lactone systemic exposure-response relationship has been documented. Zamboni and colleagues investigated the relationship between topotecan systemic exposure and response in a panel of six neuroblastoma xenograft models (61). Mice

bearing subcutaneous neuroblastoma xenografts were treated with a range of topotecan dosages (0.36 to 2 mg/kg) daily for 5 days for 2 weeks [(dx5)2] every 21 days for 3 courses. The investigators determined the topotecan lactone systemic exposure associated with response in a panel of six neuroblastoma xenografts (i.e., NB1382.2, NB1643, NB1691, NB1771, NBEB, and NBSD). Four of the 6 neuroblastoma xenograft models demonstrated a complete response to topotecan dosages associated with a topotecan lactone area under the concentration-time curve (AUC) less than or equal to 88 ng/mL*hr. Both in vivo and in vitro NB1643 was relatively sensitive to topotecan; whereas, NB1691 required a topotecan lactone systemic exposure of 290 ng/mL*hr to achieve a complete response (63). Despite the resistance of NB1691 to topotecan both in vitro and in vivo, the favorable response of four other neuroblastoma xenograft models to topotecan provided a strong basis for further study of topotecan in neuroblastoma clinical trials.

Several topotecan dosage schedules have been evaluated in phase I and II clinical trials in children. A 72 hr continuous topotecan infusion showed minimal anti-tumor activity in neuroblastoma (Table 1.3). A five-day schedule of topotecan was minimally active in neuroblastoma, but interindividual variability in topotecan pharmacokinetics was high (66, 67).

However, topotecan is an S-phase selective agent. Maximizing drug exposure to cells (particularly those with long cell-cycle times and low growth fractions) in S-phase can be achieved using protracted administration schedules such as daily for five days for two to three weeks (68). Studies of topotecan in xenograft models demonstrated the superiority of protracted schedules of administration over high doses administered

Table 1.3 Topotecan clinical trials in children with recurrent or refractory solid tumors including neuroblastoma.

Phase	Dosage	N (n with NB)	MTD (mg/m ² /d)	DLT	Responses in children with NB	Notes	Ref.
I	72 hr CI	27 (9)	1.0 with G- CSF	Myelosuppression	1 CR at 1.3 mg/m ² /day maintained for 8 months; 5 SD		(64)
II	72 hr CI	93 (not reported)	1.3	Myelosuppression	1 CR	Schedule inactive in recurrent/ refractory NB	(65)
I	Dx5 Q 21d 30 min i.v. infusion	40 (9)	1.4 without G-CSF; 2.0 with G- CSF	Neutropenia, thrombocytopenia	3 PR	7-fold range in topotecan lactone systemic clearance	(66)
II	Dx5 Q 21d by 30 min i.v. infusion	20 (13)	1.48	Anemia, neutropenia, thrombocytopenia	2 PR; 4 SD		(67)

Abbreviations: NB- neuroblastoma, MTD- maximum tolerated dose, DLT- dose limiting toxicity, CI- continuous infusion, G-CSF- granulocyte colony stimulating factor, CR- complete response, Dx5 Q 21 d- daily for 5 days every 21 days, PR- partial response, SD- stable disease

Sources: Pratt CB, et al. Phase I study of topotecan for pediatric patients with malignant solid tumors, *J Clin Oncol*, 12: 539-543, 1994.

Blaney SM, et al. Phase II trial of topotecan administered as 72-hour continuous infusion in children with refractory solid tumors: a collaborative Pediatric Branch, National Cancer Institute, and Children's Cancer Group Study, *Clin Cancer Res*, 4: 357-360, 1998.

Tubergen DG, et al. Phase I trial and pharmacokinetic (PK) and pharmacodynamics (PD) study of topotecan using a five-day course in children with refractory solid tumors: A Pediatric Oncology Group Study, *J Ped Hem/Onc*, 18: 352-361, 1996.

Langler A, et al. Topotecan in the treatment of refractory neuroblastoma and other malignant tumors in childhood - a phase-II-study, *Klin Padiatr*, 214: 153-156, 2002.

Intermittently (68). Based on the strength of these preclinical data, Santana and colleagues evaluated the protracted administration with pharmacokinetically guided dosing of topotecan to minimize variability in systemic exposure and achieve the putatively effective topotecan systemic exposure in children.

Santana and colleagues conducted a pilot study of protracted topotecan in children with refractory solid tumors (Table 1.4) (49). Due to the wide interpatient variability in topotecan pharmacokinetics and the documented relationship between topotecan systemic exposure and response, the investigators used a pharmacokinetically guided dosing approach to minimize interpatient variation in topotecan systemic exposure. This approach also removes interindividual variability as a factor contributing to resistance. The single day topotecan lactone systemic exposure evaluated in children was based upon anti-tumor response in xenograft models at topotecan lactone AUC ranging from 88 to 144 ng/mL*hr (68,66,61).

In the pilot Phase I trial the pharmacokinetically guided dosing resulted in achieving the targeted systemic exposure in 78% (46 of 59) of the pharmacokinetic studies (49). The investigators also demonstrated a significant reduction in AUC values outside of the targeted range in comparison to that which would be achieved using a fixed dosing approach based solely on body surface area. Due to the manageable toxicity and favorable responses to pharmacokinetically guided dosing of protracted topotecan, a prospective phase II trial was conducted in previously untreated high-risk neuroblastoma (Table 1.4) (15). The pharmacokinetic targeting success rate was 72%, indicating the superiority of this individualized approach to topotecan dosing over fixed dosing to achieve a desired AUC.

Table 1.4 Clinical trials of topotecan pharmacokinetically guided dosing in children with solid tumors including neuroblastoma.

Phase	Dosage Schedule	N (n with NB)	Population	Single Day Topotecan Lactone Target (ng/mL*hr)	NB Responses	Reference
I	(Dx5)2 Q 24-28 d 30 min i.v. infusion	15 (5)	Recurrent/ refractory solid tumors	Cohort 1: 120-160 Cohort 2: 80-120	Cohort 1: 1 PR Cohort 2: 1 PR	(49)
II	(Dx5)2 Q 28 d 30 min i.v. infusion	30 (30)	Previously untreated high-risk NB	80-120	1 CR 17 PR	(15)

Abbreviations: NB- neuroblastoma, (Dx5)2- daily for 5 days for 2 weeks, Q 24-28 d- every 24 to 28 days, i.v.- intravenous, Q 28 d, every 28 days, PR- partial response, CR- complete response

Sources: Santana VM, et al. A pilot study of protracted topotecan dosing using a pharmacokinetically guided dosing approach in children with solid tumors, Clin Cancer Res, 9: 633-640, 2003.

Santana VM, et al. Improved response in high-risk neuroblastoma with protracted topotecan administration using a pharmacokinetically guided dosing approach, J Clin Oncol, 23: 4039-4047, 2005.

The feasibility of pharmacokinetically guided topotecan dosing determined in the pilot study was confirmed in this phase II study. The 60% response rate (complete and partial responses) was excellent in these previously untreated patients, especially in comparison to studies in relapse patients where the response rate was only 15% (67). The 60% response rate to pharmacokinetically guided topotecan was also more favorable than the 40% response rate observed in a Phase II POG study of topotecan (2 mg/kg administered dx5) in 33 children with previously untreated Stage IV neuroblastoma (3). The higher response rate in the Santana study might be due to several factors including number of days of topotecan, dosage of topotecan, and systemic exposure of topotecan. The patients with refractory tumors on the Langler study (67) might represent a more drug resistant disease subset than the previously untreated population in the Santana study. In the Langler and POG (3) studies, patients received only 5 days of topotecan rather than 10 days over 2 weeks. Moreover, the daily topotecan dosages were lower in the Langler and (median 1.48, range 0.4-1.7 mg/m²) and POG studies (2 mg/m²) than in the Santana study (median 2.7, range 0.95-3.8 mg/m²). However, since topotecan pharmacokinetics were not reported in the Langler or POG studies, direct comparison of topotecan pharmacokinetics cannot be made to those reported by Santana and colleagues. Based on the documented interindividual variability in topotecan pharmacokinetics, substantial variation in topotecan systemic exposure was likely present in the studies using a fixed dosing approach. The approach to topotecan fixed dosing for 5 days every 21 days without dosage adjustment based on a targeted systemic exposure is probably subtherapeutic, especially in comparison to the protracted topotecan schedule targeting a systemic exposure of 80-120 ng/mL*hr. Based on the systemic exposure-response

relationship, interindividual variability in drug exposure likely contributes to some cases of apparent topotecan resistance. However, the pharmacokinetic guided topotecan dosing approach minimizes interindividual variability in topotecan systemic exposure.

Although protracted topotecan with pharmacokinetically guided dosing adjustments to achieve an AUC of 80-120 ng/mL*hr resulted in an excellent response rate in chemotherapy naïve children diagnosed with neuroblastoma, the tumor response rate (60%) was lower than the pharmacokinetic targeting success (72%). The patients who did not respond to pharmacokinetically guided protracted topotecan represent a subset of the disease that is clinically resistant to topotecan.

Resistance to topotecan can be multifactorial. Reduced expression of topoisomerase I, the molecular target of topotecan, can result in topotecan resistance. Topoisomerase I mutations that affect topotecan binding may confer topotecan resistance (50,51). Increased DNA repair via tyrosyl-DNA-phosphodiesterase overexpression can also lead to camptothecin analog resistance(69). Finally, ABC transporter-mediated efflux from the cell may result in topotecan resistance.

1.2.2 Irinotecan

Irinotecan (7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyloxy-camptothecin, Camptosar®), a camptothecin analog, has been evaluated in a variety of pediatric tumors, including neuroblastoma. The prodrug irinotecan has been FDA approved for treatment of metastatic colorectal cancer. Irinotecan is metabolized by carboxylesterases to the active metabolite SN-38. Preclinical and clinical studies have demonstrated that i.v. irinotecan was also active against pediatric tumors including rhabdomyosarcoma,

medulloblastoma, glioblastoma, and neuroblastoma (62,70). Oral irinotecan was active in a panel of six neuroblastoma xenograft models (71).

Vassal and colleagues demonstrated irinotecan activity in a panel of three neuroblastoma xenografts established from patients with poor prognosis (72). IGR-N835 was established from a previously treated patient with *MYCN* amplified stage IV disease lacking *MDR1* expression. IGR-NB3 and IGR-NB8 were derived from two patients with untreated stage III disease, *MYCN* amplification, and *MDR1* overexpression, each of which was refractory to chemotherapy. A complete or partial response to irinotecan (either using a dx5 or q4dx5 administration schedule) was achieved in all 3 xenograft models studied. Although several investigators have demonstrated resistance to irinotecan and SN-38 in Pgp overexpressing cell lines *in vitro* (73,74), the response to irinotecan of xenografts overexpressing Pgp suggests that Pgp did not play a major role in irinotecan resistance in this model. However, to confirm this hypothesis it would be necessary to demonstrate irinotecan efficacy in patients with neuroblastoma that overexpresses Pgp.

In contrast to the dx5 irinotecan dosing approach reported by Vassal et al, Thompson and colleagues demonstrated the anti-tumor activity of protracted (i.e., daily for 5 days for 2 weeks) i.v. irinotecan in a panel of 6 neuroblastoma xenografts (70). The characteristics of each xenograft model are listed in Table 1.5. A single course of irinotecan 10 mg/kg daily for 5 days for 2 weeks induced a complete response in NB1691, NB1643, NBEB, and NB1382.2 tumors. Also, a partial response was achieved in NB1771. However, NBSD, which was derived from a bone marrow aspirate, did not respond significantly to this irinotecan dosage. This might indicate that neuroblastoma bone marrow metastases are more refractory to irinotecan than the primary tumor. The

Table 1.5 Neuroblastoma xenograft features.

Xenograft	Stage	Site	Prior Therapy	MYCN Amplification
NB1691	D	Adrenal	AraC, Dauno, 6-TG, VP-16, 5-azacytidine	Yes
NB1643	D	Retro-peritoneal	None	Yes
NB1382.2	C	Retro-peritoneal	VCR, VP-16, CTX, CDDP, carboplatin	Yes
NB1771	D	Adrenal	None	Yes
NBSD	D	Bone marrow aspirate	CTX, Dox, CDDP, VM-26	Yes
NBEB	D	Adrenal	CTX, Dox, CDDP, VM-26	No

Abbreviations: AraC- cytosine arabinoside, Dauno- daunorubicin, 6-TG- 6-thioguanine, VP-16- etoposide, VCR- vincristine, CTX- cyclophosphamide, CDDP- cisplatin, Dox- doxorubicin, VM-26- teniposide. (Note: stage D is equivalent to current INSS stage 4.)

Source: Thompson J, et al. Efficacy of systemic administration of irinotecan against neuroblastoma xenografts, Clin Cancer Res, 3: 423-431, 1997.

investigators also determined that the irinotecan and SN-38 systemic exposures associated with the minimum effective dose in xenograft models can also be achieved clinically in children with cancer (17). Furthermore, at systemic exposures tolerated in children (75) oral irinotecan elicited anti-tumor responses in the same panel of neuroblastoma xenografts (71).

Several schedules of irinotecan administration have been evaluated in children with solid tumors in phase I trials (Table 1.6). The major toxicities associated with irinotecan were myelosuppression and diarrhea. Favorable responses were observed in several tumor types, for neuroblastoma in particular. Based solely on the phase I clinical data, the optimal irinotecan schedule cannot be determined unequivocally. However, the extensive preclinical studies of Houghton and colleagues have demonstrated the superiority of protracted irinotecan to shorter schedules. Moreover, due to the potential for failure to respond to irinotecan, future clinical trials of irinotecan should include prospective evaluation of factors that might influence anti-tumor response, including expression of ABC transporters in the tumor.

1.3 ABC transporters

The human ABC superfamily of transmembrane proteins contains 49 genes divided into 7 subfamilies (ABCA through ABCG) based upon gene structure, order of domains, and amino acid sequence homology (79). ABC transporters are expressed in both normal and tumor tissues and efflux a variety of endogenous substrates and xenobiotics from cells against a concentration gradient. ABC transporters expressed in normal tissues protect cells from toxic substances. ABC transporters expressed in normal tissues also play a role in the pharmacokinetics (absorption, distribution, metabolism, and

Table 1.6 Irinotecan phase I clinical trials in children with recurrent or refractory solid tumors including neuroblastoma.

Dosage Schedule	N (n with NB)	MTD, DLT	Responses in children with NB	Reference
(Dx5)2 by 1 hr i.v. infusion	23 (5)	20 mg/m ² /day, diarrhea	1 PR	(17)
Dx5 q 21 d by 1 hr i.v. infusion	35 (7)	Heavily pretreated stratum: 39 mg/m ² /day, neutropenia/ thrombocytopenia; Less heavily pretreated stratum: 50 mg/m ² /day, diarrhea	1 PR, 1 SD	(76)
QW for 3W by 1 or 2 hr i.v. infusion	Less heavily pretreated: 48 (5) More heavily pretreated: 33 (11)	600 mg/m ² /dose, delayed diarrhea and cholinergic syndrome for less heavily pretreated; neutropenia for heavily pretreated	1 PR persisted for 5 courses	(77)
Dx3 by 2 hr i.v. infusion q 28d	28 (26)	160-180 mg/m ² /day, myelosuppression (hematopoietic lineages) and diarrhea	4 PR	(78)

Abbreviations: NB- neuroblastoma, MTD- maximum tolerated dose, DLT- dose limiting toxicity, (Dx5)2- daily for 5 days for 2 weeks, Dx5 q 21 d- daily for 5 days every 21 days, QW- every week, 3W- 3 weeks, PR- partial response, SD- stable disease

Sources: Furman WL, et al. Direct translation of a protracted irinotecan schedule from a xenograft model to a phase I trial in children, *J Clin Oncol*, 17: 1815-1824, 1999.

Blaney S, et al. A phase I study of irinotecan in pediatric patients: a pediatric oncology group study, *Clin Cancer Res*, 7: 32-37, 2001.

Vassal G, et al. A phase I study of irinotecan as a 3-week schedule in children with refractory or recurrent solid tumors, *J Clin Oncol*, 21: 3844-3852, 2003.

Mugishima H, et al. Phase I study of irinotecan in pediatric patients with malignant solid tumors, *J Pediatr Hematol Oncol*, 24: 94-100, 2002.

excretion) of drugs that are ABC transporter substrates. Finally, multi-drug resistance via drug efflux is one of the functional consequences of ABC transporter expression in malignant cells.

ATP binding or hydrolysis drives this efflux process. Several mechanisms for extruding substrates from a cell have been proposed, including those of a classical pump, a flippase, a hydrophobic vacuum cleaner, and an ATP switch. According to the classical pump model, ABC transporters remove substrates directly from the cytoplasm to the extracellular space. However, because substrates have been shown to enter the transporter via the membrane and not the cytoplasm, ABC transporters likely function according to an alternate mechanism (80). According to the flippase model, a transporter flips a substrate from the inner membrane leaflet to the outer membrane leaflet from which the substrate could diffuse into the extracellular environment (81). The hydrophobic vacuum cleaner model proposes that the transmembrane domains associate to form a channel in the plasma membrane (82). Upon passive diffusion of a substrate into the lipid bilayer, the transporters detect substrates in the membrane and efflux them from the cell. The ATP switch model is similar to the hydrophobic vacuum cleaner model. Upon substrate binding to a high-affinity site on the transmembrane domain (TMD) of the inner membrane leaflet, two ATP molecules bind the nucleotide binding domain (NBD), inducing a conformational change in the TMD (83). This conformational change exposes the substrate to the extracellular space, and the affinity of the transporter for the substrate is reduced. Hence, the bound drug is released into the extracellular space. Following ATP hydrolysis, inorganic phosphate and ADP are released, and the transporter is restored to its basal conformation.

In addition to similar mechanisms of action, ABC transporters share structural features. Structural domains common to this protein superfamily include a TMD and a highly conserved ATP-binding cassette, which is also called the nucleotide binding domain (NBD). The ATP-binding domains known as Walker A and Walker B motifs in the NBD are separated by 90-120 amino acids (79). A functional transporter is comprised of at least 2 TMDs, each of which contains 6-11 membrane spanning α -helices.

The ABC transporters Pgp, MRP1, MRP2, MRP4, and BCRP may play a role in resistance to topotecan, irinotecan, and SN-38. The role of these transporters in resistance to topotecan, irinotecan, and SN-38 in addition to what is known about their expression in neuroblastoma will be summarized below.

1.3.1 P-glycoprotein

P-glycoprotein (Pgp) was the first member of the ABC transporter superfamily described(84), and it is encoded in humans by the gene *ABCB1*, also known as *MDR1*. The protein product of the *MDR1* gene is a 170 kDa plasma membrane glycoprotein. Pgp has also been shown to be expressed in the membranes of organelles such as the nuclear envelope and Golgi apparatus (85,86). Although the full-length *MDR1* gene was not cloned until 1986 (87), Pgp expression in multi-drug resistant cell lines was first demonstrated in 1976 (84).

Immunohistochemical analysis has demonstrated Pgp expression in various human tissues. Pgp was expressed in the epithelium of the gastrointestinal tract and bronchus, and in vascular endothelium, notably in the blood-brain barrier. Pgp was also expressed in secretory and excretory tissues including bile canaliculi and ductules, renal

tubules, pancreatic ducts, mammary glands, prostate glands, salivary glands, and adrenal glands (88).

1.3.1.1 Pgp substrates

Pgp substrates include a wide variety of structurally diverse, hydrophobic xenobiotics including anthracyclines, anthracenes, vinca alkaloids, epipodophyllotoxins, tubulin polymerizing drugs, HIV-1 protease inhibitors, and fluorophores. Pgp substrates used in neuroblastoma therapy include doxorubicin, vincristine, etoposide, and teniposide (18). In addition, the camptothecin analogs topotecan and irinotecan, which are components of neuroblastoma therapy, have been evaluated as Pgp substrates with conflicting results (73,89,90).

The first studies demonstrating that Pgp confers resistance to topotecan were conducted in drug selected cell lines. Chinese hamster ovary cells selected for colchicine resistance (CH^RC5) that overexpressed Pgp were 15 times more resistant to topotecan than the parental cell line (AuxB1) and 10 times more resistant to SN-38 (89,73). Topotecan and SN-38 accumulation and drug-stabilized topoisomerase I- DNA complexes were reduced in CH^RC5 compared to AuxB1, suggesting reduced intracellular topotecan or SN-38 in CH^RC5 due to Pgp-mediated efflux. However, these were drug selected cell lines, and expression of other transporters that might confer topotecan resistance (e.g., BCRP or MRP1) cannot be ruled out. These studies were performed prior to identification of other drug transporters; hence, the contribution of other ABC transporter families to the results of these studies is unknown.

In contrast to the drug selected cell lines used by Hendricks (89) and Mattern *et al* (73), Jansen and colleagues demonstrated irinotecan and SN-38 resistance in cells

transfected with *MDR1* cDNA (74). A melanoma cell line transfected with *MDR1* was 5 times more resistant than the parental cell line to irinotecan and only 1.4 times as resistant to SN-38. When these same cell lines were implanted as xenografts in mice, virtually no difference was observed in sensitivity to irinotecan, indicating that the *in vitro* and *in vivo* model systems were not equivalent. Specifically, the xenografts had higher topoisomerase I activity than the cell lines *in vitro*, which may account for the lack of resistance observed *in vivo*. Similar to the results of Mattern and colleagues, the *in vitro* level of resistance to topotecan and SN-38 was one to two orders of magnitude lower than for the classical Pgp substrates doxorubicin and vincristine.

Pommier and colleagues used two *in vitro* systems to demonstrate that Pgp confers resistance to topotecan (90). First, KB-V1, a human carcinoma cell line selected for vinblastine resistance, was more resistant to camptothecin, topotecan, and SN-38 than the parental KB-3-1 cell line. In addition to the vinblastine selected cell line, camptothecin analog cytotoxicity was studied in an isogenic system, NIH3T3 and NIH-MDR-G185. The resistant cell line was transfected with a plasmid encoding Pgp and maintained in colchicine. NIH-MDR-G185 showed increased resistance to topotecan, but not camptothecin or SN-38. The investigators did, however, make the important distinction that even though Pgp confers resistance to topotecan, Pgp confers a much higher level of resistance to drugs such as doxorubicin.

1.3.1.2 Pgp expression in neuroblastoma

Multidrug resistance conferred by ABC transporters can be either intrinsic or acquired. Intrinsic resistance of an untreated tumor can be due to expression of ABC transporters. Acquired resistance refers to chemotherapeutic selection of drug resistant

clones (e.g., those expressing ABC transporters). Intrinsic and acquired resistance has been studied in neuroblastoma, and both may have implications for clinical outcomes including response and survival.

Pgp is expressed in many tumor types, especially those that arise from tissues that normally express Pgp. Using three monoclonal antibodies to different Pgp epitopes, Pgp expression has been demonstrated prior to chemotherapy in non-Hodgkin's lymphoma, intestinal leiomyosarcoma, adenocarcinoma of the stomach, colon, kidney, breast, and adrenal cortex (88). In addition, Fojo and colleagues demonstrated that *MDR1* mRNA is expressed in the adrenal gland, adrenal medulla, and in neuroblastoma tumor samples of unspecified stage taken from 3 patients following relapse after treatment with vincristine and doxorubicin (91). Although the investigators did not evaluate the relationship between *MDR1* expression and outcome, several other groups have conducted such analyses in neuroblastoma.

Results of studies evaluating the significance of Pgp mRNA or protein expression in neuroblastoma are inconsistent (92,93,52,10). The inconsistencies may be explained by small study populations, differences in prior therapy, absence of paired samples at diagnosis and relapse, failure to isolate tumor tissue from normal tissue, and the potential lack of correlation between mRNA and protein expression level (94). The most reliable studies using immunohistochemistry to detect protein expression in neuroblastoma biopsies have demonstrated that overall survival is significantly lower in children with tumors that express Pgp at diagnosis (93) and Pgp expression at diagnosis increases the risk of death in neuroblastoma by 60% (52). Therefore Pgp expression in neuroblastoma

is likely an unfavorable feature and may confer poor prognosis in neuroblastoma at least perhaps in an as yet undefined subset of patients (93,52).

1.3.2 MRP1

The human *MRP1* (*ABCC1*) gene contains 31 exons, maps to chromosome 16p13, and encodes a 190 kDa protein. *MRP1* was first identified in a lung cancer cell line selected for doxorubicin resistance without expression of Pgp (95). *MRP1* mRNA expression was ubiquitous in a panel of human tissues (96,97). In addition, western blot and immunohistochemical analysis of normal human tissues demonstrated essentially ubiquitous MRP1 expression (98). MRP1 was expressed in the adrenal gland, lung, heart, skeletal muscle, liver, spleen, kidney, stomach, small and large intestine, prostate gland, testis, ovary, and erythrocytes. However, MRP1 was not observed in endothelium.

1.3.2.1 MRP1 substrates

Some overlap exists between Pgp and MRP1 substrates. As for Pgp, MRP1 transports both endogenous and xenobiotic substrates. MRP1 substrates include glutathione S-conjugates, sulfate conjugates, and glucuronides (Table 1.7) (99). In an isogenic system Chen and colleagues demonstrated an ATP-dependent reduction in intracellular accumulation of both irinotecan and SN-38 in KB-3-1 cells transfected with MRP1 cDNA (100). Furthermore, compared to the parental cell line the MRP1 overexpressing cell line was 7 and 15 times more resistant to irinotecan and SN-38, respectively.

Allen and colleagues knocked out the murine orthologs of human *MRP1* and *MDR1* (*Mrp1*, *Mdr1a*, and *Mdr1b* respectively) in murine embryonic fibroblasts (MEF)

Table 1.7 **Partial list of endogenous and xenobiotic MRP1 substrates.**

Class	Substrate
Drugs	Methotrexate
	Doxorubicin, daunorubicin, epirubicin, idarubicin
	Etoposide
	Vincristine, vinblastine
	Irinotecan, SN-38, topotecan
GSH conjugates	Doxorubicin-SG
	Cyclophosphamide-SG
	Melphalan-SG
	Leukotriene C ₄
	PGA ₂ -SG, PGJ ₂ -SG
Glucuronide conjugates	Etoposide-Gluc
	SN-38-Gluc
	E ₂ 17βG
	Glucuronosylbilirubin
Sulfate conjugates	Estrone-3-sulfate
	Dehydroepiandrosterone sulfate

Source: Deeley RG and Cole SP. Substrate recognition and transport by multidrug resistance protein 1 (ABCC1), FEBS Lett, 580: 1103-1111, 2006.

to examine the contribution of each gene to basal drug resistance to a panel of drugs including topotecan and SN-38 (101). In *Mdr1a/1b* null MEFs, a small (but significant) increase was observed in sensitivity to topotecan and SN-38. However, a much larger increase in topotecan and SN-38 sensitivity was observed in the triple knockout MEFs (*Mrp1* and *Mdr1a/1b* null). Therefore, the camptothecin analogs are better substrates for MRP1 than Pgp.

1.3.2.2 MRP1 expression in neuroblastoma

Because MRP1 transports multiple drugs used in neuroblastoma therapy including the camptothecin analogs, the relationship between MRP1 expression in neuroblastoma and outcome has been examined. Norris and colleagues used RT-PCR to determine the *MRP1* mRNA expression in 60 primary, untreated neuroblastomas (10). Patients who received chemotherapy were treated with regimens including the MRP1 substrates vincristine and doxorubicin. The cumulative survival rate was higher in patients with low *MRP1* expression than in those with high expression (94% vs. 57%, respectively, $P < 0.001$, log-rank test). The 5-year event-free survival (EFS) rate was also higher in patients with low MRP1 expressing tumors than in those with high expression (46% vs. 91%, respectively).

The same group confirmed these findings in a large prospective study of 209 neuroblastoma samples using RT-PCR (12). When all patients were evaluated based upon *MRP1* expression level, 5-year EFS was significantly higher in patients with low *MRP1* expressing tumors in comparison to high *MRP1* expression (76% vs. 40%, $P < 0.001$, log-rank test).

High *MRP1* expression remained a significant predictor of outcome in three subsets of patients. First, in patients with localized disease, those with high *MRP1* had worse EFS than those with low expression (56% vs. 90%, $P=0.001$, log-rank test). Next, children with *MYCN* nonamplified tumors and high *MRP1* had worse EFS than those with low expression (46% vs. 81%, $P<0.001$, log-rank test). Finally, in children with stage 4 disease high *MRP1* was associated with extremely poor EFS compared to those with low expression (14% vs. 44%, $P<0.001$, log-rank test). Therefore, even in patients with favorable prognostic features such as localized disease or nonamplified *MYCN*, *MRP1* expression may be a useful indicator of prognosis that can be used to guide therapy.

MRP1 mRNA expression is a prominent feature in neuroblastoma and may be a strong, independent indicator of poor prognosis. However, due to the essentially ubiquitous MRP1 expression in normal tissue, modulation of tumor-specific MRP1-mediated resistance presents a significant challenge. However, the relationship between MRP1 expression in neuroblastoma and poor prognosis may not be solely attributed to drug efflux. As MRP1 expression is strongly correlated with *MYCN* amplification, the expression of this ABC transporter may be a surrogate marker for another currently unidentified *MYCN* target gene responsible for poor prognosis. Furthermore, these studies have focused on the *MRP1* mRNA expression, and therefore the question remains about the mechanistic implications of the strong correlation between *MYCN* amplification and MRP1 mRNA expression. It has not yet been determined whether MRP1 and *MYCN* have independent roles in neuroblastoma. A mechanistic explanation for the poor prognosis in patients with high *MRP1* expression remains to be elucidated.

1.3.3 *MRP2*

The human *ABCC2* gene contains 32 exons (102) mapping to chromosome 10q24 and encodes a 180 kDa protein with alternative nomenclature that includes cMOAT (for canalicular multispecific organic anion transporter) and MRP2 (multidrug resistance associated protein 2). The MRP2 protein sequence is 48% homologous to MRP1 (103). MRP2 is primarily expressed in the hepatocyte canalicular membrane and mediates the hepatobiliary excretion of organic anions including glutathione and bilirubin glucuronides (104). MRP2 expression has also been demonstrated at the apical membrane of the renal proximal tubules (105).

1.3.3.1 MRP2 substrates

As with Pgp and MRP1, there is considerable overlap in substrate specificity between MRP1 and MRP2. MRP2 substrates include vincristine, vinblastine, methotrexate, cisplatin, doxorubicin, epirubicin, etoposide, cyclophosphamide, irinotecan, and SN-38 (106,107,108). MRP2 both transports and confers resistance to irinotecan and SN-38. First in the HepG2 hepatic cancer cell line expressing MRP2, antisense RNA to *MRP2* reduced MRP2 expression and increased sensitivity to both irinotecan and SN-38 (106). Chu and colleagues used canalicular membrane vesicles isolated from rats to demonstrate the ATP-dependent transport of both the lactone and carboxylate forms of SN-38G and the carboxylate forms of irinotecan and SN-38 (109).

1.3.3.2 MRP2 in neuroblastoma

To date MRP2 protein expression has not been demonstrated in neuroblastoma. Norris and colleagues have demonstrated that *MRP2* mRNA levels in patients with neuroblastoma did not discriminate between treatment success or failure, but analysis of

MRP2 protein expression was not reported (110). However, due to MRP2-mediated transport of drugs used in neuroblastoma therapy including irinotecan/SN-38, vincristine, cisplatin, and doxorubicin, MRP2 protein expression should be further evaluated in neuroblastoma. Although neuroblastoma does not arise from a tissue that normally expresses MRP2, expression of MRP2 in neuroblastoma is possible. Due to the potential for efflux of irinotecan and SN-38 in MRP2-expressing tumor cells, if MRP2 is expressed in neuroblastoma, the relationship between MRP2 expression and response to therapy including MRP2 substrates should be explored.

1.3.4 MRP4

The human *ABCC4* gene maps to chromosome 13q32 (111) and encodes the 170 kDa protein MRP4 (112). The organic anion transporter MRP4 was first identified in 1997 by searching the human expressed sequence tag (EST) database for sequences homologous to the 3'-ends of *MRP1* and *MRP2* (113). Human *MRP4* mRNA expression was demonstrated in lung, kidney, bladder, and gall bladder and in drug resistant cell lines derived from several tumor types including non-small cell lung cancer, small cell lung cancer, lung adenocarcinoma, and promyelocytic leukemia. Tissue distribution studies have shown that MRP4 is also expressed in the adrenal gland, on the apical side of renal proximal tubules and capillaries in the brain, and the basolateral side of the choroid plexus epithelium (114,115,116).

1.3.4.1 MRP4 substrates

MRP4 is an organic anion transporter, and its substrates include both xenobiotics and endogenous substances. MRP4 was identified as a transporter of the nucleoside

analog 9-(2-phosphonylmethoxyethyl) adenine (PMEA) (111). Inverted membrane vesicles were used to identify the endogenous MRP4 substrates cGMP, cAMP, and estradiol 17- β -D-glucuronide (E₂17 β G) (117). Prostaglandin E₁ (PGE₁) and PGE₂ have also been identified as endogenous substrates (118). MRP4 also effluxes 6-mercaptopurine (6-MP) monophosphates, 6-thioguanine (6-TG) monophosphates, and methotrexate (117,119) and confers resistance to cyclophosphamide, but not etoposide, 5-fluorouracil, carboplatin, vincristine, vinblastine, or paclitaxel (120).

Topotecan is an MRP4 substrate (114). In particular, increasing topotecan concentrations reduced ATP-dependent uptake of E₂17 β G into inverted membrane vesicles overexpressing MRP4. Also, Saos2 cells overexpressing MRP4 were 5 times more resistant to topotecan and accumulated less topotecan intracellularly than the parental cell line.

Subsequently Tian and colleagues used stably transfected HepG2 (which endogenously expresses MRP4 (121)) cells overexpressing MRP4 to demonstrate that MRP4 confers resistance to other camptothecin analogs (120). Cells overexpressing MRP4 were 3- to 6-fold resistant to irinotecan lactone. MRP4 conferred 8- to 9-fold resistance to SN-38 lactone and carboxylate. The level of resistance to SN-38 and irinotecan was similar to that observed for the classical MRP4 substrates bis-POM-PMEA and methotrexate. Furthermore, this increase in resistance to both SN-38 and irinotecan was accompanied by a 2- to 4-fold reduction in intracellular accumulation of both irinotecan and SN-38.

Using the same MRP4 overexpressing HepG2 cell line, Tian and colleagues also confirmed that MRP4 transports and confers resistance to topotecan (122). MRP4

overexpression conferred 12-fold resistance to topotecan. The level of topotecan resistance in MRP4 overexpressing cells was similar to that for bis-POM-PMEA. In addition, topotecan intracellular accumulation was reduced in the MRP4 overexpressing cells. Due to the endogenous MRP4 expression in the HepG2 cell line, these data provide information about the contribution of overexpressed MRP4 to camptothecin analog transport and resistance. The data do not give information about the level of resistance conferred by the basal level of MRP4 expression. To determine the contribution of basal MRP4 expression, the gene could be silenced using RNAi. Alternatively, if an inhibitor specific for MRP4 (and not other ABC transporters) existed, it could be used to assess the contribution of MRP4 to camptothecin analog transport and resistance.

1.3.4.2 MRP4 in neuroblastoma

Norris and colleagues reported that *MRP4* mRNA expression was correlated with *MYCN* amplification in neuroblastoma and was an independent predictor of poor outcome (110). Although *MYCN* has not yet been shown to activate MRP4 transcription, the MRP4 promoter contains an E box. Therefore, MRP4 may be a *MYCN* target gene. In addition, MRP4 may confer clinical resistance to drugs used in neuroblastoma therapy, notably the camptothecin analogs. Hence, the contribution of MRP4 expression to camptothecin analog resistance in neuroblastoma will be explored in this project.

1.3.5 *BCRP*

The breast cancer resistance protein (BCRP) is the product of the *ABCG2* gene and maps to chromosome 4q22. BCRP aliases include MXR (for mitoxantrone resistance) and ABCP for (placental ABC transporter). BCRP was identified by Doyle

and colleagues in MCF7AdrVp, a breast cancer cell line selected for doxorubicin resistance that did not express Pgp or MRP1 (123). At the same time Allikmets and colleagues demonstrated BCRP expression in the placenta (124). Shortly thereafter, Maliapaard and colleagues demonstrated BCRP overexpression in an ovarian cancer cell line selected for topotecan resistance (125). BCRP is comprised of 663 amino acids and a single NBD at the amino terminus. BCRP domains are organized NH₂-NBD-TMD-COOH, giving the appearance of a half-transporter in contrast to Pgp and MRP (NH₂-TMD-NBD1-TMD-NBD2-COOH) (123). However, BCRP homodimerization is required for function (126). In addition to expression in placental syncytiotrophoblasts, BCRP is also expressed in the apical membrane of the small intestine epithelium, bile canaliculi, and the apical membrane of endothelial cells in brain vasculature (127).

1.3.5.1 BCRP substrates

BCRP transports a variety of xenobiotics and endogenous substrates (Table 1.8). Specifically, several studies have demonstrated that BCRP has a role in camptothecin analog resistance. The majority of data addressing the substrate specificity of each transporter has been generated using drug selected cell lines. Reduced accumulation and increased resistance to topotecan and SN-38 have been demonstrated in BCRP overexpressing cell lines selected for drug resistance (125,128). Because this selection process can produce numerous genetic and post-translational alterations, the most reliable substrate specificity data is generated in isogenic systems. Houghton and colleagues demonstrated that Saos2 cells transfected with *ABCG2* and overexpressing BCRP are 12-fold and 50-fold more resistant to topotecan and SN-38, respectively, than the vector control cell line (129). All of these studies taken together indicate that BCRP confers

Table 1.8 Partial list of BCRP substrates.

Xenobiotic Substrates	Endogenous Substrates
Mitoxantrone	E ₂ 17βG
Topotecan	Estrone-3-sulfate
Irinotecan, SN-38, SN-38 glucuronide	Dehydroepiandrosterone
Methotrexate, methotrexate polyglutamates	

Source: Litman T, et al. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2), *J Cell Sci*, 113 (Pt 11): 2011-2021, 2000.

resistance to the camptothecin analogs SN-38 and topotecan.

1.3.5.2 BCRP in neuroblastoma

The role of BCRP expression in neuroblastoma has not been studied extensively. In one study of the mRNA expression of ABC transporters in neuroblastoma, BCRP mRNA was detected in neuroblastoma tumor specimens from patients, but the level of mRNA expression was not related to treatment outcome (110). The only other study of BCRP expression in neuroblastoma used immunohistochemistry to demonstrate BCRP protein expression in the xenograft NB1691 (130). Therefore, the extent to which BCRP is expressed in neuroblastoma tumors in children and its role in response to camptothecin analogs is unknown.

In summary, BCRP plays a role in camptothecin analog tumor resistance. Therefore, the contribution of BCRP to camptothecin analog resistance in neuroblastoma will be explored in this project.

1.3.6 *Modifying ABC transporter function*

To determine the role of ABC transporters in drug resistance, the function and/ or expression of the transporter must be modulated. A small molecule chemical modulator can be used to inhibit transport or compete with substrates. Table 1.9 contains a partial list of small molecule chemical modulators of ABC transporters. However, no small molecule chemical inhibitor exists that inhibits a single ABC transporter. These chemical modulators of ABC transporters are therefore more useful in modulating the activity of a variety of transporters rather than in studying the contribution of a single transporter to resistance.

Other useful approaches to investigate the function of specific transporters in neuroblastoma cells or normal tissues include gene knockout, RNA interference (RNAi), and overexpression. Null models in which the gene for a specific ABC transporter has been knocked out can be used both *in vitro* and *in vivo*. For example, differences in drug sensitivity between knockout and wild type MEFs can be determined *in vitro* to determine the contribution of basal ABC transporter expression in normal tissues (101). Knockout mice can be compared to wild-type mice in pharmacokinetic studies. However, this approach is laborious and expensive. Moreover, it does not directly test the function of the human transporter in a human system such as a cell line derived from a human tumor sample. Also, due to the variation in sensitivity of different tumor types to cytotoxic drugs, the contribution of ABC transporters to drug resistance in a particular tumor type should be studied in the tumor or cell line of interest.

Exogenous overexpression of ABC transporters is another useful tool for studying ABC transporter function. This approach has been used consistently to identify

Table 1.9 Chemical modulators of ABC transporters.

Drug	Transporters Inhibited	References
Verapamil	Pgp, MRP1, MRP4	(18,131,132)
Quinidine	Pgp, MRP1, MRP2	(18)
Valspodar	Pgp, MRP1	(18)
MK571	MRP1, MRP2, MRP4, MRP7	(133,134,112,135)
Gefitinib	Pgp, BCRP	(130,136)
GF120918	Pgp, BCRP	(137,138,139,140)
Indomethacin	MRP1, MRP4	(141,118,112)

Sources: Litman T, et al. From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance, Cell and Molecular Life Sciences, 58: 931-959, 2001.

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Table 1.9 (continued)

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Jedlitschky G, et al. The nucleotide transporter MRP4 (ABCC4) is highly expressed in human platelets and present in dense granules, indicating a role in mediator storage, *Blood*, 104: 3603-3610, 2004.

endogenous and xenobiotic substrates and to demonstrate multi-drug resistance. Cell lines overexpressing ABC transporters have the advantage over those with basal expression levels in that differences in substrate accumulation or resistance are more pronounced and easily detected. However, due to protein abundance this approach does not give much information about the role of normal, basal protein expression.

RNA interference (RNAi) can be used to silence the expression of a specific gene. Just as for targeting the disruption of a particular gene locus to knock out a gene, RNAi can be used to study the contribution of basal gene expression to the phenotype of interest.

RNAi was first described as double-stranded RNA (dsRNA) mediated sequence-specific post transcriptional gene silencing in the nematode *Caenorhabditis elegans* (142). The highly conserved RNAi phenomenon of gene silencing has been observed in protozoa, invertebrates, vertebrates, plants, fungi, and algae. RNAi is hypothesized to serve as an antiviral defense mechanism (143,144).

Experimentally, RNAi can be used to reduce or eliminate the expression of a protein by targeting its mRNA for degradation. Dicer, an RNase III-like enzyme cleaves long dsRNA molecules into 21- to 23-nucleotide small interfering RNAs (siRNAs) (145). Subsequently the siRNAs are incorporated into the RNAi-induced silencing complex (RISC). ATP activates the RISC complex to expose the antisense siRNA strand and bind the targeted complementary mRNA (146). Finally, the mRNA is degraded by exonucleases (147,148). Exogenously delivered RNAi used to inhibit ABC transporter function has an advantage over chemical inhibition due to specific targeting of a single gene.

Despite the ability to achieve targeted silencing of a single gene based upon nucleotide sequence complementarity, RNAi can induce nonspecific off-target effects that include the interferon response and concentration-dependent induction of off-target genes(149). The interferon response elicited by introduction of long dsDNA can be circumvented by introduction of siRNAs, which are short dsDNA molecules 21-22 bp in length that specifically target degradation of mammalian genes. Using the minimum effective siRNA concentration eliminates the induction of off-target or “siRNA-response” genes (150).

To achieve gene silencing with RNAi, the construct must be appropriately delivered to the target cell and can be transient or stable. RNAi can be delivered as small interfering RNA (siRNA) or short hairpin RNA (shRNA) oligonucleotides to achieve transient knockdown of a gene. Plasmids or retroviral vectors containing a shRNA cassette under the control of the RNA polymerase promoter (i.e., H1 or U6) can be used to achieve stable expression of RNAi. Brummelkamp and colleagues achieved stable RNAi by including a puromycin resistance cassette in a plasmid containing the H1 promoter and a 19-nt gene specific insert followed by a spacer to form a hairpin and then the reverse complement of the 19-nt sequence (151). Therefore, stable retroviral mediated RNAi will be used to silence ABC transporter expression in neuroblastoma cell lines to determine the contribution of basal expression to *in vitro* and *in vivo* camptothecin analog sensitivity.

1.4 Summary

High-risk neuroblastoma remains a significant clinical challenge despite aggressive and intense therapy. The camptothecin analogs topotecan and irinotecan are

useful components of neuroblastoma therapy. Despite a favorable anti-tumor response rate to pharmacokinetically guided topotecan in children with high-risk neuroblastoma, some patients failed to achieve a clinically meaningful response. Therefore, we postulate that molecular mechanisms of drug resistance were also present in primary neuroblastoma cells. Specifically we propose that Pgp, MRP1, MRP2, MRP4, and BCRP may be expressed in neuroblastoma and may confer resistance to topotecan and irinotecan/ SN-38. The primary hypothesis of this dissertation was that the expression of ABC transporters contributes to clinical resistance of neuroblastoma to the cytotoxicity of topotecan and irinotecan/ SN-38.

1.5 Specific aims

The primary objective of this work was to understand the role of ABC transporters in neuroblastoma resistance to the camptothecin analogs. Potentially relevant ABC transporters include Pgp, MRP1, MRP2, MRP4, and BCRP. To accomplish this objective we will use genetically modified neuroblastoma cell lines, xenograft models, and biopsy specimens from children with high-risk neuroblastoma.

1.5.1 To determine the contribution of ABC transporters to topotecan and irinotecan sensitivity in neuroblastoma cell lines

A panel of neuroblastoma cell lines representing a range of topotecan and irinotecan sensitivities will be evaluated to determine if differential expression of ABC transporters identifies specific transporters that may confer clinical resistance to the camptothecin analogs. RNAi will be used to silence MRP4 and Pgp in a resistant

neuroblastoma cell line. In addition, MRP4 will be overexpressed in a sensitive neuroblastoma cell line.

1.5.2 To determine the role of MRP4 in neuroblastoma xenograft response to topotecan

A murine xenograft model in which MRP4 has been silenced via RNAi will be established. Mice bearing neuroblastoma xenografts with silenced MRP4 will be treated with topotecan to assess the contribution of basal MRP4 expression to topotecan resistance in neuroblastoma *in vivo*.

1.5.3 To determine the role of ABC transporter expression in response to topotecan in children with high-risk neuroblastoma

Immunohistochemistry will be used to characterize the ABC transporter expression in neuroblastoma biopsy specimens from children with high-risk disease. The association between ABC transporter expression and response to pharmacokinetically guided topotecan will be evaluated.

In summary, a subset of children with high-risk neuroblastoma did not respond to camptothecin analog based therapy. ABC transporters (e.g., MRP4 and Pgp) may contribute to this clinical resistance. These *in vitro*, *in vivo*, and clinical studies of neuroblastoma will help to define the role of MRP4 and Pgp in camptothecin analog resistance in neuroblastoma.

Chapter 2. MRP4 and P-glycoprotein confer resistance to topotecan in neuroblastoma

2.1 Introduction

Neuroblastoma is the most common extracranial solid tumor in children. The prognosis for children diagnosed with high-risk neuroblastoma is poor, with 5-year survival less than 30% (152). Topotecan and irinotecan anti-tumor activity in neuroblastoma has been demonstrated in preclinical studies and phase I clinical trials, including studies in children with high-risk disease (64,66,70,71,17,76,153). Furthermore, there is a steep topotecan systemic exposure/ anti-tumor response relationship. However, substantial interindividual variation in topotecan pharmacokinetics exists in children. Topotecan systemic clearance, the determinant of systemic exposure as measured by AUC, varies at least ten-fold in children (49). Therefore, dosing topotecan based solely on body surface area may lead to some anti-tumor responses but also a range of toxic and subtherapeutic exposures.

Minimizing interindividual variability in topotecan systemic exposure with a pharmacokinetically guided dosing approach has been successful in terms of achieving the putatively effective systemic exposure in children with high-risk neuroblastoma (15). In a phase II trial this pharmacokinetically guided dosing approach also resulted in very good anti-tumor activity. Out of 28 children with high-risk neuroblastoma evaluable for a response to pharmacokinetically guided topotecan, 1 complete response and 17 partial responses were observed. However, the ten children with tumors that failed to respond may represent a subset of the disease resistant to topotecan (49,154,155).

Although clinical resistance to topotecan is multi-factorial, ABC transporters have been implicated in multi-drug resistance in neuroblastoma. Pgp and MRP1 have been associated with diminished response of neuroblastoma to chemotherapy (52,156). Many of the drugs used to treat neuroblastoma are MRP1 and/ or Pgp substrates, including doxorubicin, vincristine, and etoposide. However, mechanistic studies validating a link between protein expression of ABC transporters and resistance to chemotherapeutic agents are lacking. Moreover, in neuroblastoma the expression of other members of the ABC transporter superfamily such as MRP2, MRP4, and BCRP has not been well characterized.

We have previously demonstrated that topotecan is an MRP4 substrate (114). Topotecan, irinotecan, and SN-38 are BCRP substrates. Irinotecan and SN-38 are also MRP2 substrates (125,157,158). Several investigators have demonstrated that Pgp confers resistance to topotecan. In this study we used RNAi to reduce MRP4 and Pgp expression in neuroblastoma cell lines to evaluate the effect of ABC transporters on topotecan and irinotecan resistance.

RNAi can be mediated through siRNA or shRNA. Reductions in protein expression mediated by siRNA are transient. In cell culture, this gene silencing persists for 3-5 days (159). Variation in transfection efficiency also limits the application of transient transfection of siRNA. Knock down via transient transfection of siRNA targeting ABC transporters is suitable to test certain phenotypes such as drug accumulation. However, this transient system is not suitable to test longer time-dependent phenotypes such as drug sensitivity using the MTT assay.

In contrast with transient siRNA-mediated downregulation of gene expression, retroviral vector mediated shRNA can persist for long periods of time, due to integration in the host genome. In addition, retroviral vectors can carry selection markers such as puromycin resistance genes or green fluorescent protein (GFP). Continuous selection pressure can be applied by culturing in the presence of puromycin. Alternatively, drug interactions or non-specific effects of selection antibiotics can be avoided using GFP as a selection marker. GFP expressing clones can be sorted by FACS, and GFP expression over time can be monitored via flow cytometry or fluorescence microscopy. But in all cases, reduced protein expression of the shRNA targeted gene must be confirmed.

Thus, the objectives of the present study were to determine whether MRP4 and Pgp confer resistance to topotecan and SN-38 in neuroblastoma. Therefore, we used shRNA to knock down MRP4 and Pgp expression in the cell line NB1691. Furthermore, we overexpressed MRP4 in the cell line NB1643. These in vitro models were used to evaluate intracellular drug accumulation and sensitivity to camptothecin analogs.

2.2 Materials and methods

2.2.1 Chemicals and cell lines

Topotecan, ³H-topotecan, and ¹⁴C-topotecan were generously provided by GlaxoSmithKline (King of Prussia, PA). SN-38, the active component of the pro-drug irinotecan (CPT-11) was provided by Pfizer (New York, NY).

NB1691, NB1643, SKNAS, SKNSH, NBEB (also known as SJNB1), and NBSD (also known as SJNB4) cell lines were kindly provided by Dr. Peter Houghton (St. Jude Children's Research Hospital, Memphis, TN). Saos2 pcDNA and Saos2 MRP4 cell lines

were kindly provided by Dr. John Schuetz (St. Jude Children's Research Hospital). The amphotropic retroviral packaging cell line Phoenix was obtained from ATCC (Manassas, VA). Cells were maintained in RPMI 1640 (Mediatech, Herndon, VA), 10% FBS (HyClone, Logan, UT), and 1% L-glutamine (Mediatech) at 37°C, 5% CO₂.

2.2.2 Transient MDR1 siRNA

Three siRNA constructs targeting MDR1 (Ambion, Austin, TX) were transfected (30 nM) into NB1691 using siPORT Amine (Ambion). The sequence targeting MDR1 exon 4 was selected for further use in stable shRNA studies, and the primers 5'-GATCC-CCGGGAAAAGAAACCAACTGTCTCAAGAGGACAGTTGGTTTCTTTTCCCTTT-TTGAAA-3' and 5'-AGCTTTTCCAAAAAGGGAAAAGAAACCAACTGTCCTCT-TGAGACAGTTGGTTTCTTTTCCCGGG-3' containing *Bgl*III and *Hind*III half-sites were cloned into pSuper.

2.2.3 Stable RNAi by cloning MRP4 or MDR1 shRNA into MSCVGFP

The pSUPER42 (118) vector containing a MRP4 shRNA cassette was generously provided by Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam, Netherlands). The MSCVGFP42 construct was made by cloning a *Sma*I and *Sal*I fragment from pSUPER42 containing the H1 promoter and MRP4 shRNA sequence into *Sal*I and *Hind*III sites of MSCVGFP (160). The MSCV MDR1 shRNA GFP construct was made by cloning a *Sma*I and *Sal*I fragment from pSuperMDR1shRNA into the *Sal*I and *Hind*III sites of MSCVGFP. FuGENE6 (Roche Applied Science) was used to transfect MSCVGFP42, MSCV MDR1 shRNA GFP, or MSCVGFP into Phoenix producer cells. Retrovirus was harvested from Phoenix cells sorted for GFP expression, and NB1691 cells were

transduced with vector alone, the MRP4 knockdown construct MSCVGFP42, or the Pgp knockdown construct MSCV MDR1 shRNA GFP. Clones with stable MRP4 or Pgp knockdown were generated by sorting single NB1691 cells expressing GFP into 96-well plates, expanding, and screening by real-time RT-PCR and Western blot for MRP4 or Pgp expression.

2.2.4 Cloning MRP4 into NB1643

The MRP4 expression vector MSCV MRP4 IRES GFP containing an internal ribosomal entry site permitting co-expression of MRP4 and GFP (kindly provided by Dr. John Schuetz) was transfected into Phoenix cells (161). NB1643 cells were transduced with retrovirus harvested from cells sorted for GFP expression. NB1643 cells overexpressing MRP4 were selected by FACS, expanded, and screened by Western blot for MRP4 expression.

2.2.5 Real-time RT-PCR

RNA was extracted from cells with Tri-Reagent (Molecular Research Center, Cincinnati, OH), and cDNA was synthesized using a mixture of oligo-dT and random hexamer primers with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Real-time PCR analysis was conducted using gene specific primers (Table 2.1) and QuantiTect SYBR Green PCR Kit (Qiagen) on the ABI Prism 7900HT (Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: 15 min at 95°C followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and finally a denaturation stage. The mRNA expression level was determined by the comparative C_t method. Fold-change in mRNA expression level was calculated by evaluating the

Table 2.1 Primer sequences for real-time RT-PCR.

Gene	Primer Sense	Primer Antisense	Reference
MDR1	CTGTATTGTTTGCCACCACGA	AGGGTGTCAAATTTATGAGGCAGT	(162)
MRP4	CAGTACCTCAAAGCTGCAAGTC	CCCAGTATGAAAGCCACCAA	(163)
BCRP	CAGGAGGCCTTGGGATACTT	TGCCACAGCAGTGGAATCT	(164)
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	(163)
β -actin	GAGCACAGAGCCTCGCCTTT	TGACCCATGCCCACCATCAC	

Sources: Wunderlich K, et al. Vasospastic persons exhibit differential expression of ABC-transport proteins, Mol Vis, 9: 756-761, 2003.

Assem M, et al. Interactions between hepatic Mrp4 and Sult2a as revealed by the constitutive androstane receptor and Mrp4 knockout mice, J Biol Chem, 279: 22250-22257, 2004.

Zamber CP, et al. Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine, Pharmacogenetics, 13: 19-28, 2003.

expression $2^{-\Delta\Delta C_t}$ where C_t is the cycle threshold, ΔC_t is the difference in mean cycle threshold between the gene of interest and the normalizing gene (e.g., $C_{t\text{ MRP4}} - C_{t\text{ GAPDH}}$), and $\Delta\Delta C_t$ is the difference between the ΔC_t 's of the reference and test samples (e.g., $\Delta C_{t\text{ NB1691}} - \Delta C_{t\text{ NB1691 4.27}}$).

2.2.6 Western blot

Membrane enriched fractions were isolated from cells by sonicating in homogenizing buffer (100 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, and protease inhibitors, Roche Applied Science, Indianapolis, IN) and centrifugation at 18,000 x g for 15 min. The pellet was resuspended in microsome storage buffer (100 mM KH_2PO_4 , 1 mM EDTA, 20% glycerol, and protease inhibitors), and DNA was sheared by passing through a 27 gauge needle. In some cases, membrane proteins were isolated using the Mem-PER Eukaryotic Membrane Protein Extraction Kit (Pierce, Rockford, IL). Detergents used in the Mem-PER protein isolation were removed using the PAGEprep Advance Clean-Up Kit (Pierce).

Protein was quantitated by Bradford Assay (Bio-Rad, Hercules, CA). Proteins (10 μg per lane) were separated by SDS-PAGE (4-12% Bis-Tris gel, MOPS running buffer, Invitrogen, Carlsbad, CA) and transferred to PVDF membranes. Blots were probed with the polyclonal rabbit-anti-human MRP4, kindly provided by Dr. John Schuetz, or the monoclonal antibodies M₄I-10 (rat anti-human and mouse MRP4, Axxora, San Diego, CA), BXP-21 (mouse anti-human BCRP, Kamiya Biomedical, Seattle, WA), AC-15 (mouse anti-human β -actin, Sigma, St. Louis, MO), and JSB-1 (mouse anti-human Pgp, Signet Labs, Dedham, MA). Proteins were visualized with ECL Plus (Amersham Biosciences, Piscataway, NJ).

Membrane samples that appeared to express MRP4 with a higher molecular weight than the positive control were treated with the enzyme PNGaseF (New England Biolabs, Ipswich, MA) to deglycosylate proteins. For this reaction, 10µg of membrane protein were denatured in the reaction buffer for 10 min at 37°C. Then 500 units PNGaseF was added for a 1 hr incubation at 37°C.

2.2.7 Intracellular topotecan, PMEA, rhodamine 123, and mitoxantrone accumulation

Neuroblastoma cells were plated in 6-well plates (300,000 cells/well) in media without phenol red and allowed to attach overnight. ³H-topotecan or ¹⁴C-topotecan was added (final concentration of 10 µM, ³H-topotecan specific activity 100 dpm/pmol and ¹⁴C-topotecan specific activity 30 dpm/pmol) for 6 hr at 37°C. MRP4 function was assessed by PMEA intracellular accumulation. Bis(POM)³H-PMEA (Moravek Biochemicals, Brea, CA) was added (10 µM) and incubated at 37°C for 20 hr. The media was removed, and cells were washed once in ice-cold PBS. Cells were detached by addition of 0.5 N NaOH and acidified with 1 N HCl. Intracellular ³H-topotecan, ¹⁴C-topotecan, or ³H-PMEA was measured by scintillation counting with normalization to total protein content.

Pgp function was assessed by intracellular rhodamine accumulation in 10⁶ neuroblastoma cells incubated for 30 min with 0.1 mg/mL rhodamine 123 (165). Intracellular fluorescence was determined by FACS. Pgp function in cells expressing GFP, which fluoresces in the same channel as rhodamine 123, was assessed by

intracellular ^3H -mitoxantrone (Moravek Biochemicals) accumulation, as described for radiolabeled topotecan and PMEAs.

2.2.8 MTT assay

Cells were plated in RPMI 1640 without phenol red, 10% FBS, and 1% L-glutamine in 96-well plates (10,000 cells/well) and allowed to attach overnight. Cells were exposed to various concentrations of topotecan (1 nM to 200 μM) or SN-38 (1 nM to 100 μM) for 6 hr, and the media was replaced. Viability was assessed 4 days later by the addition of MTT (Sigma, St. Louis, MO) for 3 hr. Formazan crystals produced only by viable cells were solubilized with 0.04 N HCl in isopropanol. Absorbance was measured at 570 nm with wavelength correction at 650 nm on the μQuant Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT).

2.2.9 Statistical considerations

The drug concentration that inhibited 50% of cell growth (IC_{50}) was calculated according to the Michaelis-Menten equation. Student's t-test with Bonferroni correction was used to determine differences in IC_{50} between control and transduced neuroblastoma cell lines. Dunnett's multiple comparison tests was used to determine differences in intracellular drug accumulation between control and transduced neuroblastoma cell lines. All tests were two-tailed.

2.3 Results

2.3.1 *Functional MRP4 and Pgp expression in neuroblastoma cell lines resistant to camptothecin analogs*

Since expression of ABC transporters confers resistance to topotecan and irinotecan/SN38, we evaluated the expression of Pgp, MRP1, MRP2, MRP4, and BCRP in six neuroblastoma cell lines. We determined that MRP1 was expressed in all neuroblastoma cell lines representing a spectrum of sensitivity to topotecan. In contrast, neither MRP2 nor BCRP was expressed *in vitro* in these six neuroblastoma cell lines. Next, we determined that both MRP4 and Pgp were differentially expressed in the six neuroblastoma cell lines (Figure 2.1). Both MRP4 and Pgp were expressed in NB1691. However, neither MRP4 nor Pgp were expressed in NB1643. SKNAS also expressed MRP4 but not Pgp. SKNSH expressed Pgp but not MRP4.

We also measured intracellular accumulation of the MRP4 substrate PMEA (Figure 2.2A). NB1691 and SKNAS, the two cell lines that expressed MRP4, exhibited the lowest intracellular PMEA accumulation, consistent with the hypothesis that MRP4 was functionally active and effluxing PMEA. In contrast, NB1643 accumulated the highest level of intracellular PMEA.

Likewise, to demonstrate functional expression of Pgp, we measured intracellular accumulation of the Pgp substrate rhodamine 123 (Figure 2.2B). The two Pgp expressing cell lines NB1691 and SKNSH accumulated the least rhodamine intracellularly, suggesting the presence of a functionally active efflux transporter. The highest rhodamine accumulation was observed in NB1643, consistent with the lack of an efflux transporter.

Figure 2.1 MRP4 and Pgp expression in topotecan resistant neuroblastoma cell lines.

The blot was probed with polyclone rabbit-anti-human MRP4 antibody. The negative and positive controls are from Saos2 pcDNA and Saos2 MRP4 cell lysates, respectively. MRP4 is expressed in NB1691 and SKNAS. The lower molecular weight band present in all samples is a non-specific band. Pgp is expressed in NB1691 and SKNSH. NB1643 expresses neither MRP4 nor Pgp.

Figure 2.2 Accumulation of the MRP4 and Pgp substrates PMEA and rhodamine 123 in neuroblastoma cell lines.

PMEA accumulation is the lowest in the MRP4 expressing cell lines in NB1691 and SKNAS. The highest PMEA accumulation was observed in NB1643, which does not express MRP4 (A). Pgp expression in NB1691 and SKNSH leads to reduced intracellular accumulation of rhodamine 123. The highest rhodamine accumulation occurs in NB1643, which lacks Pgp expression (B).

To evaluate whether the expression of ABC transporters was related to topotecan sensitivity in this panel of six neuroblastoma cell lines, we determined the IC₅₀ to a six hr topotecan exposure by MTT assay. The topotecan IC₅₀ was 8 μ M for NB1691 and 0.4 μ M for NB1643 (Figure 2.3A). This 20-fold increased topotecan resistance in NB1691 is accompanied by reduced topotecan intracellular accumulation (Figure 2.3B) in NB1691 compared to NB1643, consistent with drug efflux as a resistance mechanism in NB1691. The next highest topotecan IC₅₀ was observed in SKNSH, which expressed Pgp but not MRP4.

2.3.2 Functional MRP4 knockdown with stably expressed shRNA

Due to MRP4 and Pgp expression in the two neuroblastoma cell lines most resistant to topotecan, and a lack of MRP4 or Pgp expression in the neuroblastoma cell line most sensitive to topotecan, we took two approaches to determine the relative contribution of MRP4 or Pgp to resistance. First, we used RNAi to reduce the expression of either MRP4 or Pgp in NB1691 and determine if there was an increase in sensitivity to the camptothecin analogs. The second approach to determine the contribution of MRP4 to camptothecin analog sensitivity was to overexpress MRP4 in NB1643 and determine if there was an increase in resistance to camptothecin analogs.

Using retroviral mediated expression of MRP4 shRNA, both MRP4 protein and mRNA expression were reduced in NB1691 GFP4.2 clones 7 and 9 compared to both the parental and vector only cell lines (Figure 2.4, Figure 2.5). Functional knockdown of MRP4 was confirmed by increased PMEA accumulation in NB1691 4.2 7 and 4.2 9 (Figure 2.6). Functional knockdown of MRP4 resulted in increased sensitivity to topotecan 4-fold and 2-fold in clones 4.2 7 and 4.2 9, respectively (Figure 2.7).

Figure 2.3 ³H-Topotecan sensitivity and accumulation in neuroblastoma cell lines.

The topotecan IC₅₀ determined by MTT assay after a 6 hr exposure to the drug is 8 μM for NB1691 and 0.4 μM for NB1643 (A). Less ³H-topotecan accumulates intracellularly in the resistant NB1691 compared NB1643 (B).

Figure 2.4 Immunoblot analysis of MRP4 expression in NB1691 cells transduced with MRP4 shRNA.

MRP4 expression was reduced in the NB1691 clones transduced with the retrovirus carrying the MRP4 shRNA and GFP cassettes. β-actin expression is depicted as the loading control.

Figure 2.5 Real-time RT-PCR analysis in NB1691 MRP4 shRNA clones.

MRP4 mRNA expression is reduced in clones 4.2 7 and 4.2 9 compared to the parental cell line and vector control. Values are presented as mean $\pm$ standard deviation.* $P \leq 0.01$ (t-test with Bonferroni correction).

Figure 2.6 PME A intracellular accumulation in NB1691 MRP4 shRNA clones.

Intracellular PME A accumulation was determined after a 20 hr incubation with Bis-POM-PME A. Reduced MRP4 expression in clones 4.2 7 and 4.2 9 results in an increase in PME A intracellular accumulation, indicating functional knockdown of MRP4. Values are presented as mean $\pm$ standard deviation. * $P \leq 0.001$ (Dunnett's test).

Figure 2.7 Topotecan sensitivity in NB1691 MRP4 shRNA clones.

Viability curve derived from MTT assay of NB1691 and clones with reduced MRP4 expression demonstrates increased topotecan sensitivity in clones 4.2 7 and 4.2 9. The 6 hr topotecan IC₅₀ is lower in NB1691 clones with reduced MRP4 expression compared to the parental cell line (inset). Viability is presented as mean $\pm$ standard deviation with the best fit line. IC₅₀ values are presented as mean IC₅₀ estimate (95% confidence interval) (B). * $P \leq 0.01$ (t-test with Bonferroni correction).

Furthermore, knocking down MRP4 expression resulted in 2-fold increased SN-38 sensitivity in clones 4.2 7 and 4.2 9 (Figure 2.8). We also evaluated the sensitivity to the prodrug irinotecan in the clones with reduced MRP4 expression, but there was no difference between the parental control and clones 4.2 7 and 4.2 9 (data not shown).

2.3.3 Functional MRP4 overexpression in NB1643

After demonstrating that topotecan and SN-38 sensitivity increased upon reduction of MRP4 expression, we evaluated the effect of MRP4 overexpression in NB1643. MRP4 protein levels were 1.4- fold higher in NB1643 MRP4 IRES GFP compared to the parental cell line, as determined by densitometry (Figure 2.9). These samples were subjected to enzymatic deglycosylation because we observed slight variation in MRP4 electrophoretic migration between NB1643 and the Saos2 control cells. We reasoned that the observed differences in MRP4 molecular weight among cell lines may be due to differential glycosylation. Protein band intensity increased upon deglycosylation with PNGaseF, indicating that the M₄I-10 antibody may have more specificity for deglycosylated MRP4 than the native glycoprotein.

MRP4 expression in NB1643 resulted in reduced intracellular accumulation of PMEA, indicating the presence of functional MRP4 (Figure 2.10A). PMEA accumulation in NB1643 MSCV MRP4 IRES GFP was 20% lower than in NB1643 ($P \leq 0.01$), proportional to the MRP4 expression level. In the Saos2 MRP4 control cell line, PMEA accumulation was 2-fold lower than the vector control (Figure 2.10B). Functional expression of MRP4 in NB1643 resulted in an increase in camptothecin analog resistance. The 6 hr topotecan IC₅₀ was 7-fold higher in NB1643 MRP4 compared to

Figure 2.8 SN38 sensitivity in NB1691 MRP4 shRNA clones.

Viability curve derived from MTT assay of NB1691 and clones with reduced MRP4 expression demonstrates increased SN-38 sensitivity in clones 4.2 7 and 4.2 9. The 6 hr SN-38 IC_{50} is lower in NB1691 clones with reduced MRP4 expression compared to the parental cell line (inset). Viability is presented as mean $\pm$ standard deviation with the best fit line. IC_{50} values are presented as mean IC_{50} estimate (95% confidence interval). * $P \leq 0.01$ (t-test with Bonferroni correction).

Figure 2.9 Immunoblot analysis of MRP4 expression in NB1643 cells transduced with MSCV MRP4 IRES GFP.

The blot was probed with the monoclonal M₄I-10 antibody. MRP4 is overexpressed in NB1643 cells transduced with MSCV MRP4 IRES GFP. PMEA accumulation is reduced in cells overexpressing MRP4. Sensitivity to topotecan and SN-38 decreases in NB1643 cells overexpressing MRP4.

Figure 2.10 Intracellular PMEA accumulation in NB1643 cells overexpressing MRP4.

MRP4 overexpression in NB1643 results in 20% decrease in intracellular PMEA accumulation, indicating the presence of a functional efflux transporter (A). PMEA accumulation in the control Saos2 cell line was reduced 2-fold in the MRP4 overexpressing cell line Saos2 MRP4 (B). Values are presented as mean $\pm$ standard deviation. * $P \leq 0.005$ (Dunnett's multiple comparison test).

NB1643 (Figure 2.11). The 6 hr SN-38 IC₅₀ was 1.5-fold higher in NB1643 MRP4 compared to NB1643 (Figure 2.12).

2.3.4 Functional Pgp knockdown with stably expressed shRNA

Based upon the preliminary screen of six neuroblastoma cell lines in which Pgp was expressed in the most resistant cell line to topotecan, we also evaluated the effect of reducing Pgp expression in NB1691. The shRNA construct targeting MDR1 mRNA for degradation resulted in a significant decrease in MDR1 expression in clones 1, 2, and 3 (Figure 2.13).

Intracellular mitoxantrone accumulation was used to assess functional Pgp knockdown in the NB1691 MDR1 shRNA clones. Rhodamine 123 could not be used as a probe for Pgp function because rhodamine 123 and GFP fluoresce in the same channel. In the Pgp overexpressing cell line LLCPK1 MDR1, mitoxantrone accumulation was significantly reduced compared to LLCPK1, consistent with mitoxantrone as a Pgp substrate. Mitoxantrone accumulation was significantly higher (2-fold) in NB1691 MDR1 shRNA clones 1, 2, and 3 than in the parental NB1691, consistent with reduced Pgp function (Figure 2.14). Furthermore, topotecan accumulated intracellularly at higher levels in MDR1 shRNA clones 1 and 3 (Figure 2.15A). Further confirming topotecan as a Pgp substrate, topotecan accumulation was reduced in the Pgp overexpressing LLCPK1 MDR1 in comparison to the vector control (Figure 2.15B). Finally, the reduction in Pgp function and increased topotecan accumulation resulted in increased sensitivity to topotecan. The topotecan IC₅₀ was 2- to 5-fold lower than NB1691 in MDR1 shRNA

Figure 2.11 Topotecan sensitivity in NB1643 cells overexpressing MRP4.

Viability curve derived from MTT assay of NB1643, NB1643 IRES, and NB1643 MRP4 demonstrates reduced topotecan sensitivity in the cell line overexpressing MRP4. The 6 hr topotecan IC₅₀ is significantly higher in NB1643 MRP4 compared to the parental cell line (inset). Viability is presented as mean $\pm$ standard deviation with the best fit line. IC₅₀ values are presented as mean IC₅₀ estimate (95% confidence interval). * $P \leq 0.01$ (t-test with Bonferroni correction).

Figure 2.12 SN-38 sensitivity in NB1643 cells overexpressing MRP4.

Viability curve derived from MTT assay of NB1643, NB1643 IRES, and NB1643 MRP4 demonstrates reduced SN-38 sensitivity in the cell line overexpressing MRP4 (mean $\pm$ standard deviation, best fit line). The 6 hr SN-38 IC₅₀ is significantly higher in NB1643 MRP4 compared to the parental cell line. Values are presented as mean IC₅₀ estimate (95% confidence interval) (B). * $P \leq 0.01$ (t-test with Bonferroni correction).

Figure 2.13 Real-time RT-PCR analysis of in NB1691 MDR1 shRNA clones.

MDR1 mRNA expression is reduced in clones 1, 2, and 3 compared to the parental cell and vector control. Values are presented as mean $\pm$ standard deviation.* $P \leq 0.01$ (t-test with Bonferroni correction).

Figure 2.14 Intracellular mitoxantrone accumulation in NB1691 MDR1 shRNA clones.

Reduced Pgp expression in MDR1 shRNA clones 1, 2, and 3 results in an increase in mitoxantrone intracellular accumulation, indicating functional knockdown of Pgp (A). Mitoxantrone accumulation is reduced in the Pgp overexpressing cell line LLCPK1 MDR1 (B). Values are presented as mean $\pm$ standard deviation. * $P \leq 0.001$ (Dunnett's multiple comparison test).

Figure 2.15 Intracellular ^{14}C -topotecan accumulation in NB1691 MDR1 shRNA clones.

Reduced Pgp expression in MDR1 shRNA clones 1 and 3 results in an increase in topotecan intracellular accumulation (A). Topotecan accumulation is significantly reduced in the Pgp overexpressing cell line LLC PK1 MDR1 (B). Values are presented as mean $\pm$ standard deviation. * $P \leq 0.001$ (Dunnett's multiple comparison test).

clones 2 and 3 (Figure 2.16). However, reduced Pgp expression and function did not result in increased sensitivity to SN-38 (Figure 2.17). To determine if this unexpected phenotype was related to an increase in expression of other ABC transporters such as MRP2, we used real-time RT-PCR to evaluate mRNA expression. However, MRP2 was expressed at low levels in all of the cell lines, and no statistically significant difference was noted in expression level among the cell lines (data not shown).

2.4 Discussion

In this study we have shown that ABC transporter expression confers camptothecin analog resistance in neuroblastoma cell lines. Furthermore, reducing ABC transporter expression via shRNA reduces camptothecin analog resistance. The ABC transporters MRP4 and Pgp, which confer resistance to topotecan and SN-38 (89,114,120,122), were expressed and functional in neuroblastoma cell lines relatively resistant to topotecan. In contrast, MRP4 and Pgp were not expressed in NB1643, which is relatively sensitive to topotecan. The differences in sensitivity to topotecan between NB1643 and NB1691 are not likely due to *p53* status because both of these cell lines are wild-type *p53* (166,167,168). These *in vitro* results are consistent with previous *in vivo* xenograft studies in which NB1643 xenografts responded to a topotecan systemic exposure of 52 ng/mL*hr (61). In contrast, a topotecan systemic exposure of 290 ng/mL*hr was required to achieve a complete response in NB1691 xenografts. The results of the current study may explain the mechanisms of camptothecin analog resistance in some neuroblastomas.

Figure 2.16 Topotecan sensitivity in NB1691 MDR1 shRNA clones

Viability curve derived from MTT assay of NB1691 and MDR1 shRNA clones 2 and 3 demonstrates increased topotecan sensitivity in clones 2 and 3. The 6 hr topotecan IC₅₀ is statistically significantly lower in clones 2 and 3 expressing MDR1 shRNA compared to the parental cell line (inset). Values are presented as mean IC₅₀ estimate (95% confidence interval) (B). * $P \leq 0.01$ (t-test with Bonferroni correction).

Figure 2.17 SN-38 sensitivity in NB1691 MDR1 shRNA clones.

Viability curve derived from MTT assay of NB1691 and MDR1 shRNA clones 2 and 3 demonstrates increased SN-38 resistance in clone 3. The 6 hr topotecan IC₅₀ is statistically significantly higher in clone 3 expressing MDR1 shRNA compared to the parental cell line (inset). Values are presented as mean IC₅₀ estimate (95% confidence interval) (B). * $P \leq 0.01$ (t-test with Bonferroni correction).

The initial results of immunoblot analysis of ABC transporter expression in neuroblastoma cell lines representing a spectrum of topotecan sensitivities indicated that MRP4 and Pgp may be partly responsible for resistance to camptothecin analogs in neuroblastoma. Therefore, to determine the individual contribution of MRP4 and Pgp to camptothecin analog resistance, we used RNAi to reduce MRP4 or Pgp expression in NB1691 and evaluate the effect on drug resistance. Knocking down MRP4 expression resulted in reduced MRP4 function, i.e., increased intracellular accumulation of the MRP4 substrate PMEA and topotecan. Reduced MRP4 expression and function also resulted in increased sensitivity to both topotecan and SN-38. Moreover, exogenous MRP4 overexpression conferred topotecan and SN-38 resistance in a neuroblastoma cell line relatively sensitive to topotecan and SN-38.

As observed for MRP4, shRNA to Pgp reduced Pgp expression and function. In NB1691 clones with reduced Pgp expression, intracellular accumulation of mitoxantrone, a probe for Pgp function was increased. Topotecan intracellular accumulation was also increased, resulting in increased sensitivity. However, reduced Pgp functional expression did not result in an increase in SN-38 sensitivity. We reasoned that this may be due to a compensatory increase in expression of an ABC transporter for which SN-38, but not topotecan, is a substrate, such as MRP2. However, MRP2 expression did not differ among the NB1691 MDR1 shRNA clones or controls. Therefore, we postulate that the observed increase in SN-38 resistance in NB1691 MDR1 shRNA clone 3 may be due to increased intracellular inactivation of the drug.

Although neuroblastoma xenograft models and patients exhibit responses to the camptothecin analogs, there is a sensitivity continuum among both patients and

xenografts. For example, NB1691 and NB143 were isolated from patients with stage D neuroblastoma (70). NB1643 was established from a diagnostic tumor sample. In contrast, the patient from whom NB1691 was derived had received prior therapy including cytarabine, daunomycin, 6-thioguanine, etoposide, and 5-azacytidine. This multi-agent chemotherapy may have acted as a selective pressure for a neuroblastoma clonal population that expresses ABC transporters. However, it is also possible that the ABC transporters expressed in NB1691 were present at the onset of disease and before chemotherapeutic selection of resistant clones. Without a paired tumor specimen isolated prior to therapy, it is not possible to determine whether NB1691 represents intrinsic or acquired resistance. Regardless, we have demonstrated that NB1691 is a model of ABC transporter mediated resistance to camptothecin analogs.

The findings that MRP4 and Pgp mediate resistance to the camptothecin analogs in neuroblastoma have several potential applications. A gene therapy approach using RNAi to knock down the expression of ABC transporters in the tumor may increase the sensitivity of neuroblastoma to topotecan or irinotecan. Reducing the expression of ABC transporters at the level of the tumor may also increase the sensitivity to other drugs traditionally used in neuroblastoma, such as doxorubicin, vincristine, etoposide, and cyclophosphamide. In addition to inhibiting ABC transporters by reducing protein expression, drugs designed to inhibit ABC transporters such as valspodar or biricodar may theoretically help to increase tumor levels of cytotoxic drugs. However, MDR modulators have not significantly improved outcome in clinical trials (169,170,171).

In contrast to the MDR modulators biricodar or valspodar, other drugs that were not initially designed as modulators of MDR have been shown to modulate MDR as a

“side effect.” For example, the EGFR tyrosine kinase inhibitor gefitinib also inhibits BCRP and Pgp (130,136). Combining gefitinib (or other similar compounds such as erlotinib) with topotecan or irinotecan can take advantage of the cytostatic effect of gefitinib in addition to its ability to increase tumor levels of cytotoxic compounds. However, gefitinib inhibits ABC transporters expressed in normal tissues (e.g., kidney) important in the pharmacokinetics of topotecan and irinotecan. Therefore, the interaction between camptothecin analogs and EGFR tyrosine kinase inhibitors can be three-fold: increasing camptothecin analog systemic exposure, inhibiting camptothecin analog efflux from malignant cells, and pharmacologic interaction between EGFR inhibition and topoisomerase I interaction.

Finally, the results of the current study may be useful in future trials of individualizing therapy for patients with neuroblastoma. Determining the ABC transporter expression profile on a diagnostic tumor sample can be used to select drugs that are not substrates for the transporters expressed in an individual patient’s tumor. However, the possibility remains that no drugs currently available will be predicted to be effective in some children, especially with high-risk disease. Therefore, further development of drugs that evade the ABC transporters and other resistance mechanisms in children with neuroblastoma is required to improve the survival rate.

Chapter 3. Effect of MRP4 shRNA on NB1691 xenograft response to topotecan

3.1 Introduction

Murine xenograft models are a useful preclinical tool for assessing response of human tumors to chemotherapeutic agents. Anti-tumor response can be measured in xenograft-bearing mice treated with various drugs or drug combinations. Furthermore, various dosages and schedules of drug administration can be compared in murine xenograft models.

A panel of six neuroblastoma xenograft models demonstrated a range of responses to topotecan (155). A complete response to 3 intravenous topotecan courses administered daily for 5 days for two weeks ($[(dx5)2]3$) was achieved in 4 of the 6 xenograft models at a dosage of 0.61 mg/kg. In contrast to the xenografts that responded to topotecan, the relatively resistant neuroblastoma xenograft model NB1691 required a topotecan dosage as high as 2 mg/kg to achieve a complete response. But a partial response to this $[(dx5)2]3$ topotecan schedule was observed in NB1691 at a dosage of 1 mg/kg.

Efflux of topotecan by ABC transport proteins has been implicated in resistance to topotecan. In a screen of ABC transport protein expression of neuroblastoma cell lines cultured *in vitro*, we demonstrated that the relatively resistant NB1691 expressed both MRP4 and Pgp (172), and topotecan is a substrate for both MRP4 and Pgp (114). However, the relatively sensitive NB1643 did not express MRP4 or P-glycoprotein. Therefore, we hypothesized that knocking down MRP4 or P-glycoprotein expression in

NB1691 would increase the sensitivity of NB1691 to camptothecin analogs and yield a response profile similar to NB1643 both *in vitro* and *in vivo*. As described in Chapter 2, the *in vitro* studies of topotecan sensitivity in NB1691 in which shRNA was used to reduce MRP4 expression demonstrated an increase in topotecan sensitivity.

The objective of this study was to evaluate the contribution of MRP4 expression in neuroblastoma to tumor response to topotecan *in vivo*. Therefore, we established the NB1691 MRP4 shRNA clones as xenografts in SCID mice. We measured the xenograft response to topotecan [(dx5)2]3 at dosages previously shown to elicit partial and complete responses in the NB1691 xenograft model.

3.2 Materials and methods

3.2.1 Knockdown via stable shRNA

MRP4 expression in NB1691 was knocked down *in vitro* using shRNA as described in Chapter 2. Single cell clones were selected by FACS, expanded, and characterized by Western blot and *in vitro* cytotoxicity assays. Cells (1×10^8) were harvested and transplanted into the flanks of CB17/Icr female *scid*^{-/-} mice.

3.2.2 Growth inhibition studies

Mice bearing s.c. tumors received topotecan when tumors were ~0.20 to 1 cm in diameter (173). Tumor diameters were measured weekly using Vernier calipers. Assuming tumors to be spherical, tumor volumes were calculated according to the formula $[(\pi/6) \times d^3]$ where d is the mean diameter. Tumor bearing mice were randomized into groups of ten prior to therapy. One group was treated with vehicle control, and a

second group was treated with topotecan. All mice were housed under barrier conditions. All experiments were conducted using protocols and conditions approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee.

3.2.3 *Drugs and formulation*

Topotecan (GlaxoSmithKline, King of Prussia, PA) was formulated in 0.9% saline and administered i.v. (0.05 mL/10g body weight) at a dose of 1 or 2 mg/kg as a short injection (<1 minute) into the lateral tail vein. Mice received topotecan [(dx5)2]3.

3.2.4 *Real-time RT-PCR*

RNA was extracted from xenograft tissue with Tri-Reagent (Molecular Research Center, Cincinnati, OH), and cDNA was synthesized using a mixture of oligo-dT and random hexamer primers with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Real-time PCR analysis was conducted using gene specific primers as described in Chapter 2. Thermal cycling conditions were as follows: 15 min at 95°C followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and finally a denaturation stage. The mRNA expression level was determined by the comparative C_t method. Student's t-test (two-tailed) with Bonferroni correction was used to determine differences in mRNA expression between NB1691 and MRP4 shRNA clones.

3.2.5 *Immunohistochemistry*

Neuroblastoma xenograft tissues were fixed in formalin and embedded in paraffin. Sections (4 µm) were placed on positively charged slides. Slides were heated (60°C) for 30 minutes and cleared of paraffin in CitriSolv (Fisher Scientific, Hampton,

NH) and alcohol gradient solution panel prior to high temperature antigen recovery in citrate buffer pH 6.0. Slides were blocked with 3% hydrogen peroxide in methanol and avidin/ biotin blocking reagent (Vector Labs, Burlingame CA) prior to incubation with the primary antibody. Irrelevant isotype fractions were used as negative controls. Biotinylated secondary antibodies were used (Vector Labs), and the tertiary reagent was streptavidin-horseradish peroxidase (Dako, Carpinteria, CA). Color development was achieved with diaminobenzidine (DAB, Dako), and the slides were counterstained with hematoxylin (Dako).

3.3 Results

As described in Chapter 2, the retroviral-vector mediated MRP4 shRNA reduced MRP4 mRNA and protein expression in NB1691 clones 4.2 7 and 9 *in vitro*. When these clones were transplanted into SCID mice as xenografts, all 4 cell lines (NB1691 parental, GFP1 vector control, and clones 4.2 7 and 9) grew and developed palpable tumors. However, on the second passage *in vivo*, clone 4.2 7 did not develop palpable tumors.

To determine the effect of reducing MRP4 expression in NB1691 on xenograft response to topotecan, mice were treated at a topotecan dosage and schedule that had previously elicited a partial response in NB1691 (1 mg/kg [(dx5)2]3). We hypothesized that reduced MRP4 expression would be associated with a more pronounced antitumor response in comparison to the parental cell line. Unexpectedly, none of the tumors responded to topotecan 1 mg/kg (Figure 3.1). Therefore, we treated the xenografts on the same schedule at a higher topotecan dosage (2 mg/kg). Again, unexpectedly none of the xenografts responded to the higher topotecan dosage (Figure 3.2). NB1691GFP 4.2 7 was

Figure 3.1 NB1691 MRP4 shRNA xenograft response to i.v. topotecan 1 mg/kg [(dx5)2]3.

Tumor volume was measured weekly until tumor volume quadrupled. Mice were treated with vehicle control (A-D) or topotecan daily for 5 days for 2 weeks every 21 days for up to 3 courses (E-H). NB1691 (A, E); NB1691 GFP1 (B, F); NB1691 GFP 4.2 7 (C, G); NB1691 GFP 4.2 9 (D, H). Each curve represents the growth of an individual tumor.

Figure 3.2 NB1691 MRP4 shRNA xenograft response to i.v. topotecan 2 mg/kg [(dx5)2]3.

Tumor volume was measured weekly until tumor volume quadrupled. Mice were treated with vehicle control (A-C) or topotecan daily for 5 days for 2 weeks every 21 days for up to 3 courses (D-F). NB1691 (A, D); NB1691 GFP1 (B, E); NB1691 GFP 4.2 9 (C, F). Each curve represents the growth of an individual tumor.

not included at the 2 mg/kg dosage because the xenografts did not grow after the second passage *in vivo*.

To determine if ineffective MRP4 knockdown was related to failure to respond in the NB1691 xenograft clones, we evaluated the MRP4 mRNA and protein expression in the xenografts. In clone 4.2 7, real-time RT-PCR analysis demonstrated that MRP4 mRNA expression was approximately half the mRNA level in the parental xenograft (Figure 3.3). Whereas, *in vitro*, the MRP4 mRNA expression level in clone 4.2 7 was only 10% of the parental cell line (Figure 2.5). However, in clone 4.2 9 MRP4 mRNA expression was not significantly reduced compared to NB1691 (Figure 3.3), indicating that the MRP4 shRNA did not persist in this clone. Microscopic evaluation of xenograft sections revealed green fluorescence, indicating that the GFP cassette had not been silenced. Immunohistochemical staining of MRP4 in the NB1691 xenografts demonstrated heterogeneous MRP4 expression in all four xenografts (Figure 3.4). Therefore, effective MRP4 knockdown via shRNA did not persist in this *in vivo* xenograft model.

Upregulation of other ABC transporters for which topotecan is a substrate may be responsible for the lack of response in the MRP4 shRNA xenografts. Therefore we also evaluated MDR1 and BCRP mRNA expression. MDR1 mRNA expression was only significantly elevated in the GFP1 vector only xenograft (Figure 3.5). However, the observed level of MDR1 expression in all four xenograft models may be sufficient to confer *in vivo* resistance to topotecan. Real-time RT-PCR analysis demonstrated that BCRP mRNA was expressed at low levels (C_t of approximately 30) in all four xenograft models, and no significant difference was observed in expression level among them (data

Figure 3.3 MRP4 mRNA expression in NB1691 MRP4 shRNA xenografts.

MRP4 mRNA expression is significantly reduced in NB1691 4.2 7 but not 4.2 9. * $P < 0.01$ (t-test with Bonferroni correction).

Figure 3.4 MRP4 is expressed in NB1691 MRP4 shRNA xenografts.

Immunohistochemical analysis of NB1691 MRP4 shRNA xenografts showing heterogeneous membrane staining of MRP4 (brown stain). NB1691 (A); NB1691 GFP1 (B); NB1691 4.2 7 (C); NB1691 4.2 9(D). MRP4 shRNA did not effectively reduce the MRP4 expression in NB1691 xenografts.

Figure 3.5 MDR1 mRNA expression in NB1691 MRP4 shRNA xenografts.

MDR1 mRNA expression is significantly increased in NB1691 GFP1. * $P < 0.01$ (t-test with Bonferroni correction).

not shown). BCRP was not expressed in the four xenograft models as determined by immunohistochemical analysis. Therefore, *in vivo* expression of BCRP does not account for the failure of these xenografts to respond to topotecan.

3.4 Discussion

In order to determine the contribution of MRP4 expression *in vivo* to response to topotecan, we established the NB1691 clones transduced with MRP4 shRNA as xenografts. SCID mice were treated with a protracted topotecan schedule that had previously elicited a partial response in CBA/CaJ mice bearing NB1691 xenografts (61). Unexpectedly, none of the 4 xenograft models responded to 1 or 2 mg/kg topotecan [(dx5)2]3. To determine if MRP4 silencing via shRNA persisted in xenografts, we analyzed MRP4 expression by real-time RT-PCR and immunohistochemistry. MRP4 mRNA expression was higher *in vivo* than *in vitro*. In addition, results of immunohistochemical analysis demonstrated that MRP4 was heterogeneously expressed in all four xenografts. Therefore, effective MRP4 shRNA did not persist *in vivo*. However, expression of MRP4 in these four xenograft models and failure to respond to topotecan are consistent with the hypothesis that MRP4 expression confers resistance to topotecan.

The lack of response to topotecan of the parental NB1691 xenograft may be attributable to variations in tumor growth or response in different strains of mice. The partial response of NB1691 to 1 mg/kg topotecan was observed in CBA/CaJ mice (61). The current study was conducted in SCID mice. Thompson and colleagues demonstrated that NB1691 xenografts in SCID mice were less sensitive to topotecan than in the CBA/CaJ host (174).

Topotecan pharmacokinetics are similar in SCID and CBA/CaJ mice (Dr. Clinton Stewart, unpublished data), so the difference between strains is not attributable to variations in systemic exposure. SCID mice bearing NB1691 xenografts did not respond to oral topotecan 2 mg/kg (Chris Morton, personal communication May 30, 2006). In SCID mice the topotecan systemic exposure after an oral dose of 2 mg/kg is 55 ng/mL*hr (136), approximately equivalent to that achieved by 0.36 mg/kg i.v. topotecan. In CBA/CaJ mice bearing NB1691 xenografts, a partial response was achieved in mice treated with 0.36mg/kg i.v. topotecan, indicating that variations in mouse strain may impact the response of NB1691 xenografts to topotecan.

The NB1691 cell line used to establish xenografts had been maintained in tissue culture. Cell lines maintained in tissue culture are subject to alterations (175) that may influence sensitivity to cytotoxic drugs including topotecan. For example, promoter methylation and silencing of apoptosis-related genes can occur during passage in tissue culture (176). Genetic instability including chromosomal gains, losses, or translocation and gene amplification or deletion can also accumulate during tissue culture passage (176). Enhanced recombinatorial repair of topotecan-induced DNA strand breaks has been implicated in camptothecin analog resistance and may occur during passage over time in culture (177). Inhibition of cell death signaled by topotecan-induced DNA lesions may also result in topotecan resistance. Alternatively, enhanced activity of the DNA repair enzyme MGMT can result in topotecan resistance (178). Any of these alterations may account for the failure to respond to topotecan *in vivo*.

Despite the decreased sensitivity of NB1691 xenografts in SCID mice, we predicted that xenografts derived from the MRP4 shRNA clones would be more sensitive

to topotecan than the parental cell line. However, we did not observe differences *in vivo* response based on MRP4 expression.

Failure of the MRP4 shRNA may be attributed to retroviral vector silencing. Gene expression has been silenced in retroviral vectors containing the MLV LTR. This silencing is mediated by the binding of ELP, an embryonal LTR-binding protein that suppresses transcription of the MLV LTR. However, expression of the MRP4 shRNA cassette in the MSCV retroviral vector that we used was directed by the PCMV LTR in which the ELP binding site has been deleted, decreasing the potential for retroviral vector silencing (160,179). Furthermore, GFP expression in all of the transduced xenografts was visualized with fluorescence microscopy, suggesting that the retroviral vector was not silenced *in vivo*.

The disparity in the effect of MRP4 expression on topotecan sensitivity observed *in vitro* and *in vivo* may also be due in part to differences in the *in vitro* and *in vivo* environments. For example, Mattern *et al* demonstrated that resistance conferred by ABC transporters *in vitro* was not be recapitulated *in vivo* for the camptothecin analogs (73). Pgp expression *in vitro* conferred 15-35 fold resistance to topotecan. In contrast, Pgp expression *in vivo* did not significantly alter topotecan sensitivity.

The hypoxic tumor microenvironment may contribute to the disparity between *in vitro* and *in vivo* sensitivity to topotecan observed in our studies. MRP4 expression may have been induced in the hypoxic tumor microenvironment, overwhelming any RNAi-mediated knockdown in MRP4 expression. Recent *in vitro* studies have demonstrated increased MRP4 mRNA expression in NB1691 after 24 hours of hypoxia (Dr. Stacy Throm, personal communication March 14, 2007). BCRP expression is also induced

under hypoxia via the HIF-1 pathway in placental choriocarcinoma, osteosarcoma, and acute myelogenous leukemia cell lines (180). The hypoxic tumor microenvironment stands in contrast to the oxygen replete *in vitro* environment that permits persistent MRP4 knockdown and differential topotecan sensitivity based on MRP4 expression.

The results of these studies highlight the differences between *in vitro* and *in vivo* environments. While we observed shRNA mediated MRP4 reduction *in vitro* that correlated with increased sensitivity to the camptothecin analogs, the same patterns were not recapitulated in the xenograft models.

Although the goal of these experiments was to determine the effect of MRP4 expression *in vivo* on the response of neuroblastoma to topotecan, the results do not provide an answer to this research question. However, because all of the NB1691 clones studied herein expressed MRP4 and failed to respond to topotecan, the results of these studies are consistent with the hypothesis that MRP4 expression confers resistance to topotecan and may be a factor in failure of some neuroblastomas to respond. Future studies in which MRP4 can be effectively silenced *in vivo* will be required to address this issue definitively.

Chapter 4. ABC transporter expression in neuroblastoma biopsy specimens from children treated with pharmacokinetically guided topotecan

4.1 Introduction

The camptothecin analog topotecan is active against neuroblastoma in preclinical xenograft models. Based on the preclinical activity of topotecan in models of childhood solid tumors, topotecan has been evaluated in phase I and II clinical trials in children. In a phase II trial of topotecan in 30 children with high-risk neuroblastoma, a complete response was observed in 1 child, and partial responses were observed in 17 children for a response rate of 60% (15). Despite this favorable response to topotecan, a higher response rate was predicted based upon the anti-tumor activity of topotecan in neuroblastoma xenograft models. The 40% of children with high-risk neuroblastoma who did not respond to topotecan represent a subset of the population resistant to topotecan.

We have also demonstrated in neuroblastoma cell lines *in vitro* that the ABC transporters MRP4 and Pgp confer topotecan resistance. Thus, the objective of the current study was to evaluate the potential relation between ABC transport protein expression and clinical response to topotecan. Therefore, we evaluated the expression of ABC transporters for which topotecan is a substrate (i.e., MRP4, Pgp, and BCRP) in tumor specimens from children enrolled on the phase II trial of topotecan in high-risk neuroblastoma. We used immunohistochemistry to determine the plasma membrane expression of ABC transport proteins in neuroblastoma samples.

4.2 Materials and methods

4.2.1 Patients

All patients evaluated in this retrospective study were enrolled on a phase II trial of pharmacokinetically guided topotecan at St. Jude Children's Research Hospital (15). Patients enrolled on the phase II trial had previously untreated high-risk neuroblastoma (i.e., INSS stage 3 or 4 disease in children older than 1 year or *MYCN* amplified stage 3 or 4 disease in children younger than 1 year). The St. Jude Institutional Review Board approved this retrospective study.

4.2.2 Tumor specimens

We attempted to evaluate ABC transporter expression in bone marrow aspirates obtained prior to therapy. However, the acid decalcification of these specimens prevented effective antigen retrieval and immunohistochemical detection of the proteins of interest. Therefore, we used formalin-fixed paraffin-embedded tumor specimens obtained during resection of the primary tumor after two cycles of topotecan and standard induction therapy including cyclophosphamide, doxorubicin, etoposide, cisplatin, ifosfamide, and carboplatin.

4.2.3 Definitions of response to pharmacokinetically guided topotecan

Response to pharmacokinetically guided topotecan was evaluated after two cycles and defined by extent of tumor regression. A complete response (CR) was more than 90% regression of the primary tumor and complete resolution of metastatic disease. A partial response (PR) was more than 50% regression of all disease. An objective response

(OR) was 25-50% tumor regression. Stable disease (SD) was less than 25% tumor regression without tumor progression. Patients with a CR or PR were classified as responders, and those with OR or SD were classified as non-responders. Standard radiographic methods (e.g., CT scans, MRI scans) were used to evaluate response.

4.2.4 Immunohistochemistry

Sections (4 μ m) of formalin-fixed paraffin-embedded neuroblastoma were placed on positively charged slides. Slides were heated (60°C) for 30 minutes and cleared of paraffin in CitriSolv (Fisher Scientific, Hampton, NH) and alcohol gradient solution panel prior to high temperature antigen recovery in citrate buffer pH 6.0. Slides were blocked with 3% hydrogen peroxide in methanol and avidin/ biotin blocking reagent (Vector Labs, Burlingame CA) prior to incubation with the primary antibody. MRP4 was probed with the rat anti-human monoclonal antibody M4I-10 (Axxora). BCRP was probed with the rat anti-human and mouse monoclonal antibody BXP-53 (Axxora). Pgp was probed with the mouse anti-human monoclonal antibody JSB-1 (Signet Labs). Irrelevant isotype fractions were used as negative controls. Biotinylated secondary antibodies were used (Vector Labs), and the tertiary reagent was streptavidin-horseradish peroxidase (Dako, Carpinteria, CA). Color development was achieved with DAB (Dako), and the slides were counterstained with hematoxylin (Dako). Human kidney sections were used as positive controls for MRP4, Pgp, and BCRP staining.

Stained slides were visualized under a light microscope at 40x magnification. Only tumors with membrane staining of tumor cells were considered positive for expression of the individual transporters. Staining of endothelial cells within the tumor was not scored as positive.

4.2.5 Statistical considerations

The Fisher exact test was used to determine if the children for whom tumor samples were available were representative of the study population in terms of response to topotecan. The Fisher exact test was also used to determine if ABC transporter expression was a significant indicator of response to topotecan. All tests were two-tailed.

4.3 Results

Neuroblastoma sections were available from 14 out of 28 evaluable patients. The positive and negative controls in human kidney are depicted in Figure 4.1. MRP4, Pgp, and BCRP are expressed in the brush border of the proximal convoluted tubule. No staining was noted in the isotype negative controls.

Out of the 14 available neuroblastoma sections, MRP4 was expressed in 2, Pgp was expressed in 4, and BCRP was expressed in 9 (Table 4.1). Neuroblastomas from four of eight responders expressed at least one of the ABC transporters evaluated. Tumors from four of the six non-responders expressed at least one of the ABC transporters evaluated. The neuroblastoma section from patient nine was representative of all specimens in which MRP4 was not expressed (Figure 4.2A). MRP4 was expressed in the membrane of neuroblastoma cells from patient number seven (Figure 4.2B). Pgp was not expressed in the neuroblastoma specimen from patient 13 (Figure 4.2C). Pgp was localized to the plasma membrane of neuroblastoma cells from patient number ten (Figure 4.2D), but was heterogeneous and localized to only a few regions of the tumor specimen. This pattern of sporadic Pgp staining was representative of all Pgp positive specimens. BCRP was not expressed in the neuroblastoma cells from patient three (Figure 4.2E). The tumor from patient two expressed BCRP (Figure 4.2F).

Figure 4.1 ABC transporter expression in human kidney controls.

Left column: irrelevant IgG negative control. Right column: positive control. Rat IgG (A); M4I-10 (B); Mouse IgG (C); JSB-1 (D); Rat IgG (E); BXP-53 (F). Brown staining in panels B, D, and F indicate localization of MRP4, Pgp, and BCRP to brush border of the proximal tubules. All images are shown at 40x magnification.

Table 4.1 ABC transporter expression in neuroblastoma specimens and response to pharmacokinetically guided topotecan.

Patient Number	Response	MRP4 expression	Pgp expression	BCRP expression
1	PR	-	-	-
2	PR	-	-	+
3	PR	-	-	-
4	PR	-	-	+
5	PR	-	+	+
6	PR	-	+	+
7	PR	+	-	+
8	PR	+	-	-
9	OR	-	-	+
10	OR	-	+	+
11	SD	-	-	-
12	SD	-	-	+
13	SD	-	-	-
14	SD	-	+	+

Abbreviations: MRP4- multi-drug resistance associated protein 4; Pgp- P-glycoprotein; BCRP- breast cancer resistance protein; PR-partial response; OR- objective response; SD- stable disease

Figure 4.2 ABC transporter expression in neuroblastoma tissue obtained during primary tumor resection.

Patient 9: MRP4 negative (A); Patient 7: MRP4 positive (B); Patient 13: Pgp negative (C); Patient 10: Pgp positive (D); Patient 3: BCRP negative (E); Patient 2: BCRP positive (F). All images are shown at 40x magnification.

Despite expression of MRP4, Pgp, or BCRP in neuroblastoma, these ABC transporters were not statistically related to response to pharmacokinetically guided topotecan ($P > 0.05$, Fisher Exact test). There was an essentially even distribution of ABC transporter protein expression among responders and non-responders to topotecan. However, this was a small sample size and may not be sufficiently powered to detect a difference in ABC transporter expression between responders and non-responders.

4.4 Discussion

We used immunohistochemistry to detect the expression of MRP4, Pgp, and BCRP in primary neuroblastoma sections and determined the relation to topotecan response. When compared to methods such as RT-PCR to detect mRNA expression (10,11,12,110) this immunohistochemical approach prevented contamination of ABC transporter expression in normal cells (e.g., vascular endothelium) that may obscure results. This immunohistochemical approach also permitted detection of ABC transporters localized to the plasma membrane of neoplastic cells

Based on the possibility of chemotherapy related induction or selection of ABC transporters, we initially attempted to determine the ABC transporter expression profile in bone marrow aspirates obtained prior to therapy. However, acid decalcification of the bone marrow aspirates prevented antigen retrieval and detection of ABC transporter expression. Due to the availability of formalin-fixed, paraffin-embedded primary tumor tissue we decided to evaluate the ABC transporter expression in these samples. However, these tumors were resected after two topotecan cycles and standard multi-agent induction therapy. In these available samples, expression of MRP4, Pgp, or BCRP was not related to failure to respond to topotecan.

Previous studies have demonstrated that high levels of Pgp and MRP4 mRNA expression are predictive of poor outcome in neuroblastoma. Several factors may explain why a relation between MRP4/Pgp/BCRP expression and the clinical response to pharmacokinetically guided topotecan was not detected in the current study. The ABC transporter expression profile in the primary tumor may have changed as a result of induction therapy. Whereas cyclophosphamide, doxorubicin, and etoposide may be subject to ABC transporter mediated resistance, cisplatin, ifosfamide, and carboplatin may have eliminated the MRP4/Pgp/BCRP expressing clones responsible for failure to respond to topotecan. Although this phenomenon is the rationale for multi-agent chemotherapy, exposure of the neuroblastoma specimens to these drugs may have confounded our results.

Norris and colleagues demonstrated *MRP4* mRNA expression in 100% of 52 neuroblastomas from untreated patients (110). In this population of 52 patients, high MRP4 mRNA expression was a significant indicator of poor survival. The previous studies demonstrating a relation between ABC transporter expression and outcome evaluated mRNA, not protein expression (11,12,10). Although the real-time RT-PCR method permits simple and rapid quantitation of mRNA expression in tumor homogenate, this method does not necessarily isolate tumor tissue from normal tissue such as blood vessels. This is particularly problematic for the ABC transporters, which are expressed in vascular endothelial cells. Furthermore, the tissue must be preserved in order to isolate high quality RNA, and a technique such as laser capture microdissection should be used to isolate neuroblastoma cells from endothelia (181). Immunohistochemistry permits detection of ABC transporter subcellular localization. Cytosolic localization of a

transporter would not likely result in resistance because the transporter is not oriented to efflux substrates from the cell.

Whether response to topotecan predicts long-term outcome such as overall survival or event-free-survival remains to be determined. Evaluating whether the ABC transporter protein expression profile in the primary or metastatic tumor at any point (before, during, or after therapy) predicts outcome may help to improve the response of neuroblastoma to the camptothecin analogs. To accomplish this in a clinical study of children with neuroblastoma, the question about correlation between ABC transporter mRNA and protein expression must first be addressed. ABC transporter expression in the same tumor sample should be evaluated using both real-time RT-PCR and immunohistochemistry. Next, ABC transporter expression should be evaluated before and after therapy to determine the influence of chemotherapy on ABC transporter expression. Finally, a clinical study should be sufficiently powered to determine the relationship between ABC transporter expression and clinical outcome in children with neuroblastoma.

Chapter 5. Summary and future directions

Neuroblastoma is the most common extracranial solid tumor in children. Patients who present with localized disease have excellent predicted survival. However, most children diagnosed with neuroblastoma are at high-risk for a poor outcome because they present with metastatic disease and/ or other unfavorable biological features including *MYCN* amplification. Therapy for children who are diagnosed with high-risk disease can include multi-agent chemotherapy, myeloablation with bone marrow transplant/ stem cell rescue, surgery, and radiation. Despite this intense therapy, five-year survival in children with high-risk neuroblastoma is much less favorable than for those with low- or intermediate-risk disease (30 to 40% compared to 80 to 95%).

One of the approaches recently evaluated for improving the outcome in children with high-risk neuroblastoma is pharmacokinetically guided topotecan dosing (49,15). This approach was based on significant anti-tumor activity of topotecan systemic exposures of 100 ng/mL*hr in neuroblastoma xenograft models. Because topotecan clearance in children varies at least 10-fold and in animal models topotecan anti-tumor activity depends on systemic exposure, topotecan dosage was individualized to achieve a targeted systemic exposure of 80 to 120 ng/mL*hr in children with newly diagnosed high-risk neuroblastoma. Pharmacokinetically guided topotecan dosing was feasible in this population, and the response to this single agent was considered excellent. However, a significant proportion of children (40%) did not respond to topotecan and represent a subset of the disease that is resistant to topotecan.

ABC transporter mediated efflux of topotecan from neoplastic cells has been implicated in topotecan resistance. The ABC transporters for which topotecan is a substrate include Pgp, MRP1, MRP4, and BCRP. ABC transporters also confer resistance to other cytotoxic drugs traditionally used in high-risk neuroblastoma therapy (i.e., doxorubicin, etoposide, and vincristine). Furthermore, MRP1, Pgp, and MRP4 are expressed in neuroblastoma, and high levels of mRNA expression of these transporters is predictive of poor overall survival and event-free survival (10,11,12,110). However, the relation between expression of ABC transport proteins in neuroblastoma cells and anti-tumor response to ABC transporter substrates has not been evaluated.

The major objective of these studies was to evaluate the relation between expression of ABC transport proteins in neuroblastoma and response to the camptothecin analogs topotecan and SN-38. The first specific aim was to determine the contribution of ABC transporters to topotecan and irinotecan/SN-38 sensitivity in neuroblastoma cell lines. After identifying ABC transporters that confer resistance to camptothecin analogs *in vitro*, we evaluated the contribution of ABC transporter expression in neuroblastoma xenografts to topotecan response *in vivo*. Finally, we assessed the relation between expression of ABC transporters in neuroblastoma biopsy specimens and the anti-tumor response to pharmacokinetically guided topotecan.

First, by western blot analysis we screened a panel of neuroblastoma cell lines with a range of sensitivity to topotecan for expression of ABC transporters. NB1691, the most topotecan resistant cell line evaluated, expressed both MRP4 and Pgp. In contrast, NB1643, the most topotecan sensitive cell line evaluated, expressed neither MRP4 nor Pgp. Based upon these results, we determined the effect of reducing MRP4 and Pgp

expression via shRNA in NB1691 on topotecan and SN-38 sensitivity. As expected, topotecan sensitivity increased in NB1691 clones with reduced MRP4 and Pgp protein expression. We also demonstrated that topotecan sensitivity was reduced upon overexpression of MRP4 in NB1643.

To determine if the increased *in vitro* topotecan sensitivity in NB1691 MRP4 shRNA clones translated into increased sensitivity *in vivo*, we established these NB1691 clones as xenografts in SCID mice. Mice bearing neuroblastoma xenografts were treated with topotecan at dosages that had been shown to elicit at least 50% tumor volume regression in NB1691 xenografts (155). Unexpectedly, no tumor regression was observed in any of the xenografts evaluated. Furthermore, no difference was noted in response to topotecan between the parental NB1691 and the MRP4 shRNA clones. Most likely this occurred because MRP4 knock down did not persist *in vivo*.

Because the rationale for this study of ABC transporters in camptothecin analog resistance was based upon the failure to respond to topotecan in 40% of children with high-risk neuroblastoma in a phase II trial, we also retrospectively evaluated the ABC transporter expression profile in neuroblastoma specimens from that phase II trial. Based on the *in vitro* data demonstrating increased sensitivity to topotecan in neuroblastoma clones with reduced MRP4 and Pgp expression, we reasoned that MRP4 and/ or Pgp would be expressed in neuroblastoma tissue from children who did not respond to topotecan. However, results of immunohistochemical detection of ABC transport protein in the membrane of neuroblastoma cells demonstrated that expression of MRP4, Pgp, or BCRP was not related to response to topotecan. Because this analysis could not be conducted on tissue prior to therapy, the results may be confounded by the effects of

multi-agent chemotherapy. Including tumor samples obtained prior to chemotherapy and preserved to ensure optimal antigen recovery in future clinical studies of neuroblastoma may permit a broader assessment of the impact of ABC transporter expression on anti-tumor activity of drugs.

The findings from the *in vitro* and xenograft studies conflict with the results of evaluation of clinical samples from children with neuroblastoma. The *in vitro* studies demonstrated that MRP4 and Pgp confer resistance to topotecan. The xenograft studies pointed towards a potential association between MRP4 expression and failure to respond to topotecan. But the lack of association between ABC transporter expression in the clinical samples and response to topotecan may be confounded by the timing of sample acquisition after two topotecan courses and induction therapy. More than highlighting the limitations of experimental models of neuroblastoma, the implications of our results also highlight the importance of determining ABC transporter expression prior to potential clonal selection by multi-agent chemotherapy in children with neuroblastoma. However, further preclinical xenograft studies and clinical studies will have to be conducted to determine the contribution of ABC transporter protein expression to response to individual drugs and overall outcome in neuroblastoma.

The ABC transporter superfamily is only one class of proteins that influences anti-tumor response to drugs used in neuroblastoma therapy. Novel determinants of response could also be identified using mRNA gene expression arrays or single nucleotide polymorphism (SNP) chip arrays on laser capture microdissected tumor tissue. If tumor samples can be obtained at discrete time points during therapy including at diagnosis, after induction, consolidation, and bone marrow transplant, therapy could

potentially be individualized and optimized based upon changes in gene expression signature.

Further preclinical studies could also be conducted to assess the effect of ABC transporter expression on anti-tumor response in neuroblastoma. For example, a mouse model of neuroblastoma could be developed to address the contribution of ABC transporters to tumor pathology and response to drugs. To accomplish this, the MRP4 deficient mouse (114), for example, could be crossed with the transgenic mouse model overexpressing *MYCN* in neuroectodermal cells(182). Other ABC transporter knock out models including *Bcrp* and *Mdr1a/1b* models could also be crossed with the *MYCN* transgenic mouse. If the progeny of these crosses were viable, neuroblastoma development could be compared to the parental *MYCN* transgenic mouse to assess the role of ABC transporters in tumor formation. If tumors form in the progeny, they could be treated with various drugs including topotecan and irinotecan to determine the effect of ABC transporters on tumor response. But there is a caveat that the absence of an ABC transporter in normal tissues such as the kidneys will alter the pharmacokinetics of the drug. So an appropriate dosage that yields equivalent systemic exposures in the *MYCN* transgenic mouse and the MRP4 knockout/ *MYCN* transgenic progeny must be determined empirically.

As ABC transporter expression is upregulated by hypoxia in osteosarcoma, acute myelogenous leukemia, and choriocarcinoma cell lines(180), the role of hypoxia in resistance to topotecan and irinotecan should be assessed. In future xenograft studies, administration of RNAi *in vivo* (183) may permit more precise control over reduction of ABC transporter expression in the hypoxic tumor microenvironment.

Pharmacokinetically guided topotecan in combination with other agents (e.g., etoposide (184), cyclophosphamide (3), vincristine (185), and gefitinib (130,136)) may be an effective approach to improve long-term survival in high-risk neuroblastoma and other pediatric cancers. In evaluating these combinations, it will be important to use appropriate drug sequences, schedules, dosages, and systemic exposures in order to minimize host toxicity and maximize anti-tumor response. Additional analysis of the role of ABC transport protein expression in clinical response to these combinations may further improve the utility of the camptothecin analogs in neuroblastoma.

In conclusion, our studies show that MRP4 and Pgp confer resistance to topotecan and SN38 in neuroblastoma. We developed an RNAi approach to silencing the expression of these ABC transporters in neuroblastoma to determine the contribution to camptothecin analog resistance. Future studies in children with neuroblastoma will further delineate the clinical relevance of our findings. The application of these data in the care of children with neuroblastoma may ultimately improve clinical outcome.

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Vita

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