Panchoo, Marlyn., Role of Scavenger Receptor Class B Type 1 in High-Risk Neuroblastoma. Doctor of Philosophy (Biochemistry and Cancer Biology), November 2017, 89 pages, 4 tables, 23 figures.

Neuroblastoma (NB), the most common extra-cranial childhood cancer in the United States is responsible for 15% of all pediatric cancer deaths. Due to advancements in treatment approaches, survival in low- and intermediate-risk NB patients now exceeds 90%. However, patient outcome for high-risk NB still remains poor with an overall survival of less than 50%. Nearly all high-risk NB patients present with metastatic disease at diagnosis and are unresponsive to intense chemotherapy, radiotherapy or aggressive surgery. Recently, the high-density lipoprotein (HDL) receptor, scavenger receptor class B type 1 (SR-B1), has emerged as an important indicator of cancer progression and patient outcome. Moreover, cancerous cells exhibit a higher expression of SR-B1 than normal non-malignant cells. SR-B1 is mainly responsible for the selective uptake of cholesteryl ester (CE) from HDL but also mediates reverse cholesterol transport.

In this study, the expression of SR-B1 was identified on high-risk NB cells. Blocking of SR-B1 diminished cell proliferation, migration and invasion and induced apoptosis. Additionally, inhibition of SR-B1 reduced CE content in high-risk NB cells. Finally, high expression of SR-B1 in NB biopsy samples correlated with poor patient outcome. Taken together, this study identified SR-B1 expression as a potential regulator of high-risk NB progression linked to changes in cellular cholesterol metabolism. These findings also identify SR-B1 as a potential target for treatment of high-risk NB.

ROLE OF SCAVENGER RECEPTOR CLASS B TYPE 1 IN HIGH-RISK NEUROBLASTOMA

DISSERTATION

Presented to the Graduate Council of the

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in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Marlyn Panchoo, B.S., M.S.

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Dedicated to Amelia…

In memory of my grandmother, Cecilia Panchoo

You're always in our hearts.

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"*To God be the Glory, Great things He has done*".

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LIST OF ABBREVIATIONS

ACAT	Acyl-coenzyme A: Cholesterol Acyl Transferase
ALK	Anaplastic Lymphoma Receptor Tyrosine Kinase
apoA-I	Apolipoprotein A-I
apoB	Apolipoprotein B
ATCC	American Type Culture Collection
ATRX	A-thalassaemia/mental Retardation Syndrome X-linked
BCA	Bicinchoninic Acid
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CCK-8	Cell Counting Kit-8
ccRCC	Clear Cell Renal Cell Carcinoma
CE	Cholesteryl Ester
CHO	Chinese Hamster Ovary
CLA	CD36-and-LIMPII analogous-1
CML	Chronic Myelogenous Leukemia
COPD	Chronic Obstructive Pulmonary Disease
CT	Computerized Tomography
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GD2	Ganglioside 2

GEO	Gene Expression Omnibus
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
H&E	Hematoxylin and Eosin
HDL	High-Density Lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HRNB	High-Risk Neuroblastoma
HVA	Homovanillic Acid
IDRFs	Image-defined Risk Factors
IL-2	Interleukin-2
IMDM	Iscove's Modified Dulbecco's Medium
INRG	International Neuroblastoma Risk Group
INRGSS	International Neuroblastoma Risk Group Staging System
INSS	International Neuroblastoma Staging System
ITS	Insulin-Transferrin-Selenium
IV	Intavenous
kDa	Kilodalton
KOH	Potassium Hydroxide
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
MAPK	Mitogen-Activated Protein Kinases
MIBG	Meta-iodobenzylguanidine
MKI	Mitosis-Karyorrhexis Index
MRI	Magnetic Resonance Imaging
mTOR	Mechanistic Target of Rapamycin
MYCN	Neuroblastoma-derived v-*myc* avian myelocytomatosis viral related oncogene

NADH	Nicotinamide Adenine Dinucleotide
NB	Neuroblastoma
NGF	Nerve Growth Factor
NPC	Nasopharyngeal Carcinoma
NSE	Neuron-Specific Enolase
NT	Neuroblastic Tumors
NT3	Neurotrophin-3
PBS	Phosphate-Buffered Saline
PDZK1	PSD95/Drosphilia dises–large/tight junction protein ZO1 containing 1
PHOX2B	Paired-like Homeobox 2b
PI	Propidium Iodide
PI3K	Phosphatidylinositol-3-Kinase
PSA	Prostate Specific Antigen
RER	Rough Endoplasmic Reticulum
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SR-B1	Scavenger Receptor Class B Type 1
VLDL	Very Low-Density Lipoprotein
VMA	Vanillylmandelic Acid
XRT	External-beam Radiotherapy

CHAPTER 1

INTRODUCTION

1.1. Neuroblastoma

1.1.1. History and Epidemiology

In 1864, Dr. Rudolf Virchow was the first to identify a type of neuroblastic carcinoma [1]. Though he described the lesion as a "glioma", his findings led to studies by physicians who went on to further identify the pathology and neural cell origin of NB [1-3]. In 1910, Dr. James H. Wright published an article describing a then unrecognized tumor consisting of undifferentiated nerve cells which he named neuroblastoma (NB) [3]. Since then understanding of the development of NB has dramatically increased leading to advancement in therapeutics and patient survival [4-11].

In North America, NB accounts for 7% of all cancers diagnosed in children between the ages of 0 to 14 years and is responsible for 15% of all childhood cancer deaths [12]. Approximately 700 cases of NB are diagnosed each year, with 1 case per every 7000 live births [7,13,14]. The average age at diagnosis is less than 12 months old and 90% of cases are diagnosed by the age of 5 years [14,15]. NB in patients over 30 years is rare with nearly 0.2 cases per every million people per year [16]. About 50% of NB tumors arise in the adrenal gland but NB also occurs at sites such as the neck, chest, abdomen, and spinal column [13]. Although studies have examined whether there are environmental factors that may be related to whites

having a significantly higher incidence of NB as compared to children of other races, or boys having a slightly higher incidence than girls, no compelling risk factor has been discovered [17].

1.1.2. Development and Diagnosis

NB develops from neural crest cells and can be found anywhere along the sympathetic nervous system [12]. During embryogenesis normal neural crest cells migrate close to the neural tube and develop into a wide range of cell types such as peripheral neurons, Schwann cells, glia and cells of the adrenal medulla (Figure 1.1) [18,19]. It is widely accepted that NB arises when neural crest cells remain undifferentiated at sites along the sympathoadrenal chain [19]. NB cells often take on an appearance like Schwann cells (S-type cells) or immature neurons of the sympathetic nervous system (N-type cells) [12].

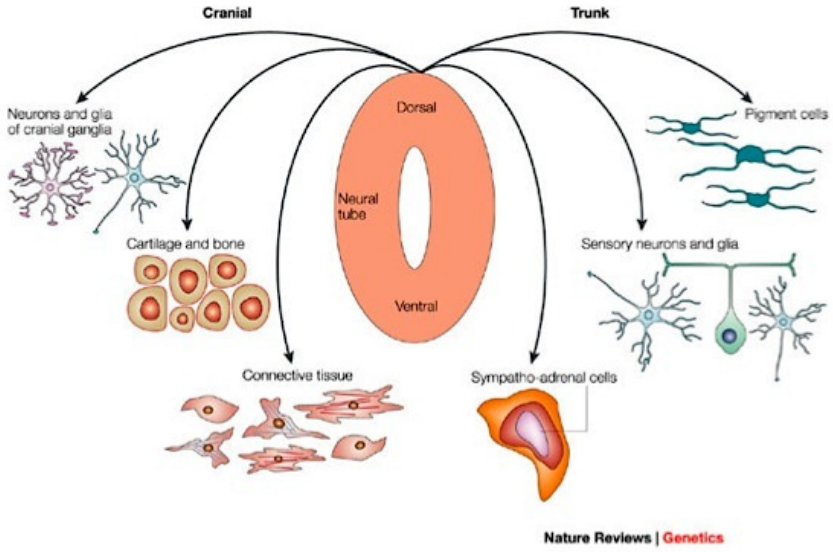


Figure 1.1. Neural Crest Cell Derivatives: During embryogenesis pluripotent normal neural crest cells migrate from the dorsal to ventral aspect of the neural tube and differentiate into various cell types in response to local signals. Neural crest cells in the trunk develop into cell types such as melanocytes and sensory neurons while neural crest cells in the cranial region form connective tissue, cartilage and bone. Adapted from Knecht, *Nature Reviews Genetics* (2002) [20]

NB is a complex heterogeneous disease whose clinical presentation varies widely depending on the location, metabolic by-products and metastasis of the primary tumor. Patients with localized tumors are often asymptomatic and discovery of tumors are usually incidentally [6]. Patients with disseminated disease however, present with symptoms such as bone pain and pancytopenia [21,22]. The most common presentation of NB is an abdominal mass. Other signs and symptoms of NB includes paralysis (caused by the tumor compressing the spinal cord), fever, hypertension, anemia, subcutaneous nodules (caused by skin metastases) and the presence of miosis, ptosis, and anhidrosis (Horner syndrome) [23].

The internationally recommended criteria for NB diagnosis is based on either: 1) light microscopy (with or without immunohistology or electron microscopy) of tumor tissue indicating unequivocal presence of pathology, along with increased urine or serum catecholamine metabolites; or 2) trephine biopsy or bone marrow aspirate showing presence of unequivocal tumor cells such as syncytia or clumps of imunocytologically positive cells and increased urine or serum catecholamine metabolites [24].

Diagnostic evaluation of NB is via biopsy, tumor imaging and assessment of urine and serum catecholamines and metabolites [4,24]. A biopsy of the tumor and bone marrow, in the case of metastatic disease, is needed to identify factors such as *MYCN* oncogene status, DNA ploidy and 1p chromosomal deletion [24]. In addition, tumor samples are needed to assess tumor histopathology and subsequent risk and treatment stratification [23,24]. Computerized tomography (CT) scans and magnetic resonance imaging (MRI) are used to visually localize tumors and identify paraspinal tumors via MRI. Meta-iodobenzylguanidine (MIBG) scintigraphy utilizes radioisotopes of iodine (I-131 or I-123) to locate tumor, metastases and residual tumor

tissue after surgical resection [6,25]. MIBG localizes to adrenergic tissues [26]. Elevation in urine, and in special cases, serum catecholamine metabolites indicate the presence of NB disease. A study in Japan showed that elevation of the urinary catecholamine metabolites, vanillylmandelic acid (VMA) and homovanillic acid (HVA), 2.5 standard deviations above the mean per milligram of excreted creatinine is appropriate for diagnosing NB [27].

Furthermore, the involvement of pathologists who are knowledgeable about pediatric cancers is required for NB diagnosis. The typical hematoxylin and eosin (H&E) staining with light microscopy, by itself, is insufficient to diagnose NB [23]. The small round blue cells observed from H&E staining are morphologically similar to Ewing sarcoma, rhabdomyosarcoma and non-Hodgkin's lymphoma [23,24,28]. Thus the use of immunologic reagents such as neuron-specific enolase (NSE) and ganglioside 2 (GD2) antibodies along with cytogenetic analysis of the tumor is recommended for correct diagnosis [24,29,30].

1.1.3. Classification and Staging

The Shimada system of histopathological classification of neuroblastic tumors (NTs) was originally developed in 1984 [31]. Dr. Hiroyuki Shimada proposed the classification of NTs into categories based on stromal development and content, age at diagnosis, degree of differentiation, and the mitosis –karyorrhexis index (MKI) [31]. The International Neuroblastoma Pathology Classification was established in 1999 to regulate the terminology and criteria used for prognostic assessment of the morphological features of NTs [32]. The assignment of favorable or unfavorable prognosis is determined based on the aforementioned histological features and the patients' age (Table 1.1.) [31-34].

Table 1.1. Prognostic Evaluation of Neuroblastic tumors based on the International Neuroblastoma Pathology Classification: Patients with neuroblastic tumors are classified based on the morphological features of the tumor, level of tumor differentiation and patient's age.

International Neuroblastoma Pathology classification		**Original Shimada classification**	**Prognostic group**
Neuroblastoma	(Schwannian stroma-poor)		
Favorable		Favorable	Favorable
<1.5 years	Poorly differentiated or differentiating & low or intermediate MKI tumor		
1.5–5 years	Differentiating & low MKI tumor		
Unfavorable		Unfavorable	Unfavorable
<1.5 years	a) Undifferentiated tumor [a]		
	b) High MKI tumor		
1.5–5 years	a) Undifferentiated or poorly differentiated tumor		
	b) Intermediate or high MKI tumor		
≥5 years	All tumors		
Ganglioneuroblastoma, intermixed	(Schwannian stroma-rich)	Stroma-rich Intermixed	Favorable[b]
Ganglioneuroma	(Schwannian stroma-dominant)		
Maturing		Well differentiated (favorable)	Favorable[b]
Mature		Ganglioneuroma	
Ganglioneuroblastoma, nodular	(composite Schwannian stroma-rich/stroma-dominate and stroma-poor)	Stroma-rich nodular (unfavorable)	Unfavorable[b]

MKI: mitosis karyorrhaxis index
Adapted from Shimada, Cancer (1999)[32], and P. D. Q. Pediatric Treatment Editorial Board (2017)[4]
[a]*Rare subtype, especially diagnosed in this group.*
[b]*These prognostic groups are not related to the age of the patient.*

Another clinical variable used to predict patient outcome is stage of disease. The International Neuroblastoma Staging System (INSS) was established in 1988 to develop a worldwide system for determining stage of NB tumors [35,36]. Tumors are classified into five stages, stage 1, stage 2A, stage 2B, stage 3, stage 4 and stage 4S, based on post clinical evaluation and ease of surgical resection [24,35]. Stage 1 tumors are localized, completely resectable and show no lymph node involvement. Stage 2A and 2B are also localized tumors but with incomplete excision and, in the case of stage 2B lymph node involvement. Stage 3 tumors are unresectable having spread across the midline of the body and may or may not involve lymph nodes. Stage 4 tumors have metastasized to organs and distant lymph nodes. The final stage, stage 4S, are localized tumors as characterized in stage 1, 2A or 2B with metastasis to the skin, liver and/or bone marrow only and are present in infants less than 1 year old and almost always spontaneously regress [24,36,37]. Identifying the correct stage is essential as NB stage determines the treatment protocol administered. The complexity and diversity of NB and its implications for treatment have initiated substantial research into the biological and genetic factors that drives this disease.

1.1.4. Molecular Biology

NB cells are characterized by a variety of somatic genetic mutations, which have been extensively studied [38-41]. Most NBs are sporadic and occur in children who have no family history of the disease. However, less than 2% of NBs are inherited from parents. Familial NB occurs when mutations in the signaling pathway that regulate the sympathoadrenal lineage are passed from parent to child [19,42]. The paired-like homeobox 2b (PHOX2B), which regulates neuronal differentiation and cell cycle exit, was the first mutation identified in familial NB. The

anaplastic lymphoma receptor tyrosine kinase (*ALK*) gene is another common lesion present in familial NB. *ALK* gene amplification and somatic *ALK*- activating mutations are common in sporadic NB along with α-thalassaemia/mental retardation syndrome X-linked *(ATRX*) mutations [19].

A major genetic alteration in NB is *MYCN* amplification. It occurs in 20% of NB tumors and is an indicator of poor prognosis [19]. *MYCN* is a member of the *MYC* proto-oncogene family, and was first identified as amplified DNA with homology to viral *myc* in NB cell lines [40,43]. *MYCN* is a transcription factor that regulates the growth, differentiation, and survival of the cells in the nervous system and is also present in the neural crest [19,40]. Deletion of the short arm of chromosome 1(1p) is the most notable loss of heterozygosity (LOH) observed in NB. 1p deletion is present in about 30% of NB cases and correlates to both progressive disease and *MYCN* amplification however, whether deletion of 1p can independently be an indicator of prognosis remains a debated issue [44]. Other genetic alterations in NB includes deletion of 11q, gain of copies of 17q and DNA ploidy usually observed in low risk tumor [44]. Moreover, the neurotrophic receptors and their ligands: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) regulate neural cell growth, survival and differentiation. Tyrosine kinases of the NGF family *TRK-A*, *TRK-B* and *TRK-C* have been implicated in NB prognosis. In the presence of NGF, Trk-A expressing neuroblasts differentiate into mature neural cells [45]. As a result, a high level of Trk-A is considered a prognostic marker of favorable outcome in NB [24,38,46]. Conversely, expression of Trk-B and BDNF are associated with *MYCN* amplification and high-risk NB tumors. These factors together establish an autocrine survival loop that enhances proliferation, chemoresistance and metastasis [45,46].

1.1.5. Risk Stratification and Treatment

Assessment of risk in NB requires an examination of histopathological and biological features and INSS stage. The International Neuroblastoma Risk Group (INRG) task force was established in 2004 [47] to develop a common approach for pretreatment NB risk assessment. Unlike INSS, International Neuroblastoma Risk Group Staging System (INRGSS) staging is based on "image-defined risk factors" (IDRFs) such as encasement of the brachial plexus and infiltration of adjacent organs or structures (Table 1.2) [48]. INRGSS is divided into four stages: L1- localized tumor without any IDRFs, L2- localized tumor with one or more IDRFs, M- metastatic tumor and MS- metastatic tumor in children less than 18 months old, with metastases restricted to the skin, liver and/or bone [49].

The stratification of NB into low-, intermediate- and high-risk groups uses prognostic factors such as stage, age, *MYCN* amplification status, tumor differentiation, histologic category, and chromosomal and DNA alterations (Figure 1.2) [50]. Low-risk patients have about a 95% chance of survival. Low-risk tumors are either stage 1, 2, or 4S with non-amplified *MYCN* and are completely resectable. Intermediate-risk patients may have stage 2, 3 or 4 tumor with no *MYCN* amplification [50]. High-risk patients have a chance of survival of less than 50% with stage 2, 3 or 4 tumors and amplified *MYCN* [19,50,51]. The prognosis is poor for children diagnosed after age 1 as they present with wide-spread metastasis at the time of diagnosis [38].

Table 1.2. Image-Defined Risk Factors (IDRFs) in Neuroblastic Tumors

Ipsilateral tumor extension within two body compartments Neck-chest, chest–abdomen, abdomen-pelvis.
Neck Tumor encasing carotid and/ or internal jugular vein Tumor extending to base of skull Tumor compressing trachea
Cervico-thoracic junction Tumor encasing brachial plexus roots Tumor encasing subclavian vessels and/or vertebral and/or carotid artery
Thorax Tumor encasing the aorta and/or major branches Tumor compressing the trachea and/or principal bronchi Lower mediastinal tumor, infiltrating costo-vertebral junction between T9 and T12
Thoraco-abdominal Tumor encasing the aorta and/or vena cava
Abdomen/pelvis Tumor infiltrating the of porta hepatis and/or hepatoduodenal ligament Tumor crossing the sciatic notch Tumor invading renal pedicle
Intraspinal tumor extension provided that. More than one third of the spinal canal is invaded and/or the perimedullary leptomeningeal space is obliterated, or spinal cord signal is abnormal
Infiltration of adjacent organs/structures Pericardium, diaphragm, kidney, liver, duodeno-pancreatic block, mesentery.
Encasement of major vessels by tumor Vertebral artery, internal jugular vein, subclavian vessels, carotid artery, aorta, vena cava, major thoracic vessels, branches of the superior mesenteric artery at its root and the coeliac axis, iliac vessels

Adapted from Monclair, J. Clin. Oncol. (2009)[48]

Risk	*MYCN* amplification	Stage*	Age at diagnosis	Overall survival (%)	Current treatment approach
Low risk	No	4S	<12 months	>91 ± 2‡	Supportive care
	No	Locoregional	≤21 years	>95†	Surgery ± chemotherapy§
Intermediate risk	No	4	<18 months	89 ± 2‡	Surgery and moderate intensity chemotherapy
High risk	Yes	Locoregional	≤21 years	53 ± 4‡	Dose-intensive chemotherapy, surgical resection of residual primary tumour, radiation to primary and resistant metastatic sites, myeloablative therapy with autologous stem cell rescue, anti-GD2 immunotherapy and 13-*cis*-retinoic acid
	Yes	4	<18 months	29 ± 4‡	
	Yes or no	4	≥18 months and ≤21 years	31 ± 1‡	
	No	4	≥12 years	<10‖	

**Stage 4 represents metastatic disease and stage 4S represents special pattern in infants with resectable primary tumor (See text).* † *Survival at 5 years was based on large international retrospective analysis before the era of immunotherapy.* § *Although most locoregional NBs are treated with surgery alone for low-risk disease, a small subset requires chemotherapy because of unfavorable biology.*

Figure 1.2. Neuroblastoma Patient Survival based on Risk Groups: Stage, age at diagnosis and *MYCN* amplification status influences NB patient survival. Patients with low-risk disease have no amplification of *MYCN*, are diagnosed within their first year of life and have an overall survival of about 91%. However, patients with high-risk disease have less than 50% chance of survival, have amplified *MYCN* and are older than 1 year old when diagnosed. Treatment of high-risk disease includes intensive radiotherapy, chemotherapy and surgery. Adapted from Cheung, *Nature Reviews Cancer* (2013) [19]

Whether the tumor is low-, intermediate- or high-risk determines NB treatment approach. Treatment of low-risk patients involves surgical resection or observation. The overall 5-year survival for low-risk patients is about 95% [52]. Chemotherapy with or without surgical resection, observation and radiotherapy in difficult cases is the standard treatment for intermediate-risk patients [4]. Treatment of high-risk NB is aggressive and includes surgery and 5-6 cycles of induction chemotherapy, myeloablative therapy and stem cell transplantation, and post-consolidation immunotherapy and radiotherapy to treat residual disease (Figure 1.3) [51,53].

Despite these aggressive treatments the survival of high-risk NB patients remains below 50% [23,51]. Hence, the importance of understanding the molecular factors that drive this debilitating disease.

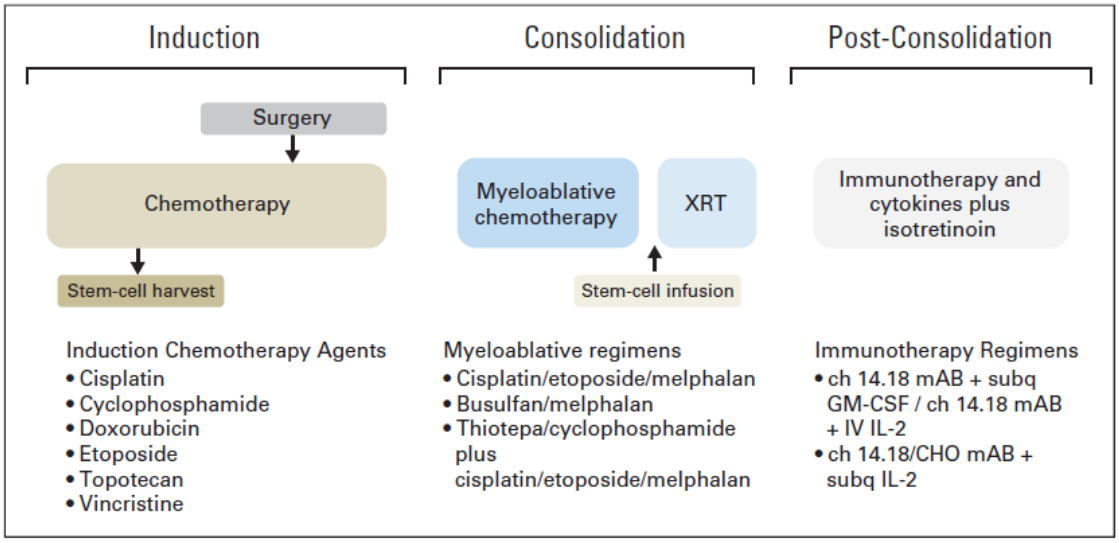


Figure 1.3. Current High-Risk Neuroblastoma Standard-of-Care Treatment Approach: Treatment of high-risk NB disease is divided into three phases of therapy: Induction, Consolidation and Post-Consolidation. The induction phase includes high doses of chemotherapeutic agents as well as surgery. During the consolidation phase high-dose chemotherapy along with stem-cell transplantation and external-beam radiotherapy [XRT]) is administered. The post-consolidation phase focuses on treatment of residual disease as half of all patients relapse after clinical remission. The post-consolidation regimen involves anti-ganglioside 2 (GD2) immunotherapy, cis-retinoic acid and cytokines. ch, chimeric; CHO, Chinese hamster ovary; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-2, interleukin-2; IV, intravenous; mAB, monoclonal antibody; Adapted from Pinto, *J. Clin. Oncol.* (2015) [51]

1.2. Scavenger Receptor Class B Type I

1.2.1. Structure and Function

The scavenger receptor class B type 1 (SR-B1) is a 509-amino acid, ~ 82kDa plasma membrane glycoprotein protein that contains a large 5-6 cysteine residues extracellular domain, two transmembrane domains and N- and C-terminal cytoplasmic domains (Figure 2.1) [54,55]. Multiple sites of N-linked glycosylation accounts for the difference in amino acid predicted mass (~57kDa) and observed mass of ~82kDa from immunochemical analysis of SR-B1 [56]. The four terminal residues (EAKL) on the C-terminal cytoplasmic domains associate with the PDZK1 adaptor protein that is essential for SR-B1 stability and signaling [57-59]. SR-B1 is the murine high-density lipoprotein receptor [56] to the human homolog CD36-and-LIMPII analogous-1 (CLA-1) [54].

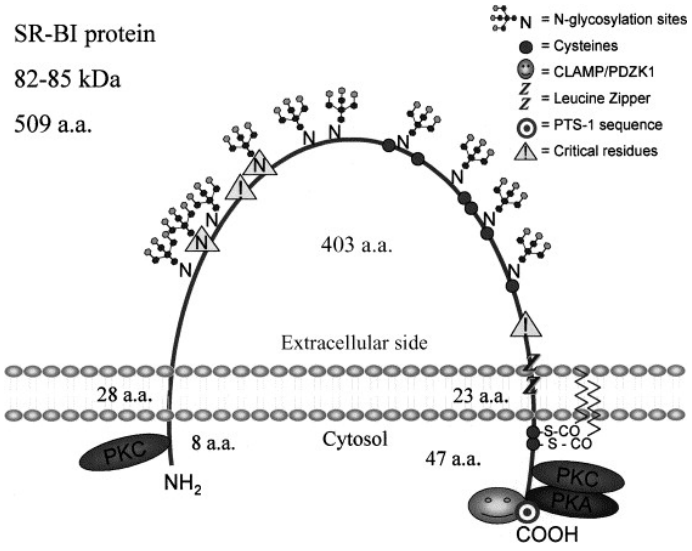


Figure 1.4. SR-B1 Protein Structure: Schematic shows N- glycosylated large extracellular domain, two transmembrane domains and N- and C-terminal cytoplasmic domains. Mutations at sites indicated by triangles leads to loss of selective lipid uptake or loss of N-glycosylation and development in the Golgi apparatus. Adapted from Rhainds, *Int J Biochem Cell Biol.* (2004) [60]

SR-B1 is recognized as the high-density lipoprotein (HDL) receptor as it facilitates the uptake of cholesteryl ester (CE) from HDL [54,56,60,61]. However, SR-B1 also has the ability to bind to a variety of other ligands such as low-density lipoprotein (LDL), acetylated LDL, oxidized LDL and very low-density lipoprotein (VLDL) [54]. The receptor-mediated uptake of CE from HDL drives reverse cholesterol transport (RCT) where excess cholesterol from peripheral tissues are returned to the liver, and is either used to synthesize steroid hormones or is excreted via the bile duct [62]. This removal of excess cholesterol is an important protective mechanism against the development of atherosclerosis [63-65].

Moreover, SR-B1 influences the cholesterol content of the plasma membrane and regulates the bidirectional flow of lipids between the cell and HDL [66,67]. Knockout of SR-B1 in female mice resulted in abnormal HDLs, dysfunctional oocyte and infertility [68] , while treatment of dendritic cells with anti-SR-B1 antibodies inhibited the uptake of the hepatitis C virus [69]. SR-B1 also induced phagocytosis of apoptotic cells via a signaling pathway involving mitogen-activated protein kinases (MAPK) [70]. Additionally, SR-B1 is found to be concentrated in cholesterol-rich micro-domains of the plasma membrane called caveolae [54,59] and SR-B1 expression is prominent in steroidogenic tissues as well as tissues with important roles in cholesterol metabolism such as adrenal, ovary, testes and liver tissues [56,60,71]. SR-B1 is also implicated in the pathogenesis chronic obstructive pulmonary disease (COPD) [72] and binding of HDL to SR-B1 triggers several signaling (Akt, MAPK) pathways through recruitment of the non- receptor tyrosine kinase src [58,73,74].

1.2.2. Mechanism of Action

Unlike LDL cholesterol transport, that results in endocytosis of LDL and the LDL receptor (LDLR) [54], the selective uptake of cholesteryl ester (CE) from HDL occurs in a two-step process: 1) binding of the CE- rich HDL to SR-B1 and 2) transfer of the lipid into the plasma membrane without the internalization of the HDL molecule (Figure 1.5.) [54,55]. Binding occurs through direct protein-protein interaction between the amphipathic helix of HDL apolipoprotein A-I (apoA-I) and the extracellular domain of SR-BI [75,76]. The CE then passively diffuses from HDL into the plasma membrane down its concentration gradient [75]. The binding of HDL to SR-B1 provides the best alignment to ensure efficient transfer of lipid, since the conformation of the wild-type apoA-I regulates the binding to SR-B1 receptor but does not alter the effectiveness of the lipid transfer [77]. The efflux of free cholesterol to HDL is influenced both by the binding of HDL to SR-B1 and the capability of the receptor to disturb the arrangement of the free cholesterol molecules in the cell membrane [77].

1.2.3. SR-B1 and Cancer

As the key receptor responsible for the selective uptake of CE from HDL, the investigation of the role of SR-B1 in cancer development has received increasing attention [78,79]. Generally, high expression of SR-B1 is present on tissues such as the liver, which regulates cholesterol levels in the body and steroidogenic tissues such as the gonads, and adrenal glands, which require cholesterol as a precursor for steroid hormone synthesis [55]. However, cancer cells exhibit high proliferative rates and thus require an ample supply for cholesterol for membrane biosynthesis [80]. One of the ways cells obtain cholesterol is via SR-B1 and HDL [81]. Thus, an abundant expression of SR-B1 has been observed on breast, lung, ovarian and

prostate cancer tissues [71,82-86]. Moreover, nasopharyngeal carcinoma (NPC), melanoma and clear cell renal cell carcinoma (ccRCC) also exhibited enhanced SR-B1 expression [85,87,88].

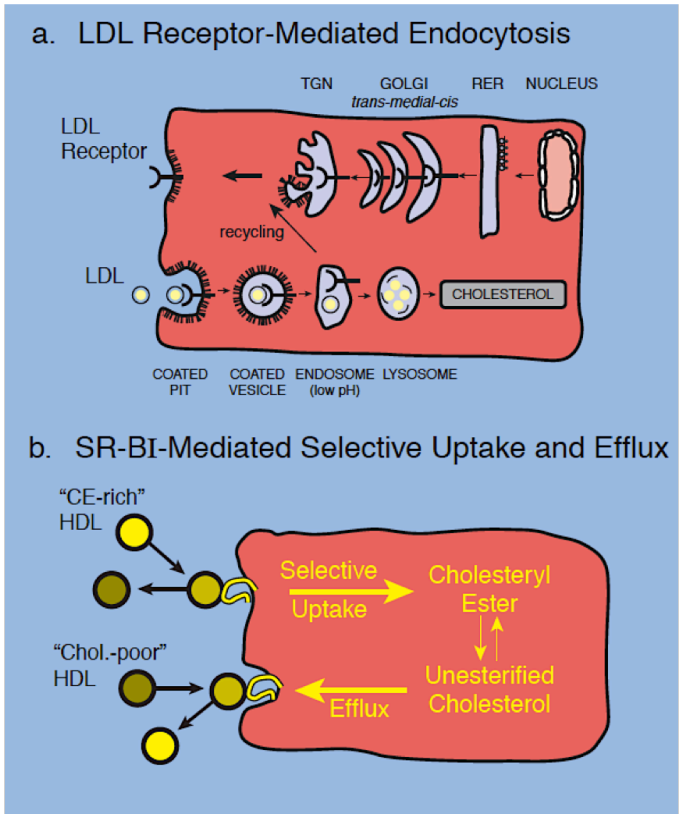


Figure 1.5. Receptor-mediated Lipoprotein Cholesterol Transfer: A) Low-density lipoprotein (LDL) receptors synthesized in the rough endoplasmic reticulum (RER) are transported via the Golgi apparatus to coated pits on the cell surface. Apolipoprotein B (apoB) on LDL binds to the LDL receptor. The LDL-receptor complex is endocytosed into vesicles, which is then converted to low pH endosomes and induces LDL receptor dissociation and recycling back to the cell surface. Hydrolytic enzymes degrade LDL releasing its cholesterol to the cell. **B)** Apolipoprotein A-I (apoA-I) on high-density lipoprotein (HDL) binds to SR-B1 with high affinity which mediates the selective uptake of its cholesteryl ester (CE). **Lysosomal degradation of the lipoprotein molecule is not involved in selective uptake; rather, the lipid-depleted ("Chol.-poor") HDL particle is liberated from the cells.** Chol. - Cholesterol. Adapted from Krieger, *Annu Rev Biochem.* (1999)[54]

In breast cancer, SR-B1 was linked to increased intra-tumor CE accumulation and breast cancer aggressiveness [89]. Additionally, high SR-B1 expression correlated with reduced progression-free survival in ccRCC and can potentially serve as a diagnostic and independent prognostic marker in ccRCC [85]. SR-B1 was identified as a potential biomarker of NPC with 75% of patient biopsies and all NPC cell lines tested overexpressing SR-B1 [88]. Furthermore, SR-B1 has been shown to be associated with prostate cancer progression and creation of a castration-resistant phenotype [83]. In a breast cancer study, knockdown of SR-B1 reduced cell proliferation and migration and diminished HDL-promoted activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway reducing tumor growth [82]. Danilo et al. also showed that SR-B1 inhibition with a pharmacological agent lowered cell signaling and proliferation in breast cancer [82]. Similarly, Cao et al. showed that a mutant HDL receptor inhibited breast cancer cell proliferation [90]. Finally, SR-B1 knockdown reduced secretion of the prostate specific antigen PSA [84]. Taken together these studies confirm SR-B1's role in cancer progression.

1.3. Cholesterol

1.3.1. Cholesterol Metabolism

Cholesterol is an extremely important biological molecule [81]. It is the major component of the cell membrane and is the precursor for the synthesis of essential vitamins, steroids and bile acids. Cells fulfill their need for cholesterol by either synthesizing cholesterol or taking up cholesterol from lipoproteins. Lipoproteins, as the name suggests, are lipid and cholesterol carrier molecules. Low-density lipoproteins (LDL) and high-density lipoproteins (HDL) are the major transporters of lipids to the cell [91]. A critical balance in cholesterol level is important,

as too high a level of cholesterol is toxic to cells. When the level of cholesterol is elevated, the rate-limiting enzyme, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, is down-regulated/degraded reducing cholesterol synthesis, exogenous lipid uptake is halted, and extra cholesterol is converted to cholesteryl esters (CE) by the enzyme acyl-coenzyme A: cholesterol acyl transferase (ACAT) [92,93]. The resulting cholesteryl esters are stored in cytoplasmic lipid droplets. High levels of CEs are present in steroidogenic tissues, such as the adrenal gland and the gonads, which require a reservoir of cholesterol as a precursor for the synthesis of steroid hormones [94].

1.3.2. Cholesterol and Cancer

Lipid metabolism plays a significant role in cancer biology as cancer cells alter their metabolism to support their growth rate [95-99]. Lipids such as cholesterol and phospholipids are essential components of the cell membrane and high rates of proliferation exhibited by tumor cells demands a substantial quantity of lipids to ensure an available pool of cholesterol for membrane biosynthesis [96,98,100]. Recently, dysregulated lipid metabolism has emerged as a common feature of many human cancers [82,89,101-103].

There is increasing evidence that supports HDL as a source of cholesterol during tumor development [78,82]. Epidemiologic studies identify low levels of HDL and HDL-cholesterol in cancer patients and suggest HDL levels as markers of cancer risk and development [95,104,105]. A frequent observation in cancer patients are altered cholesterol levels both in the plasma and tumor tissues [106-108]. Analysis of lung cancer tissue revealed a 2-fold and 3.5-fold increase in total cholesterol and cholesteryl ester content, respectively, when compared to normal lung tissue. Also, lung cancer patients' serum HDL levels were strikingly reduced [106]. A review of

serum lipid and lipoprotein levels in a group of pediatric cancers revealed that HDL- cholesterol levels increased during clinical remission after therapy suggesting that HDL levels are potentially linked to tumor growth [109]. Associations between serum lipid and lipoproteins and cancers such as breast [108], prostate [103], and gynecologic cancers [104] have been identified and the altered HDL levels were independent of cardiovascular and inflammatory disease [96].

Furthermore, activation of the cholesterol synthesis pathway due mainly to increased expression of sterol regulatory element binding factors was observed in human high-risk NB via transcriptional profiling. Reduction of cell survival after treatment with statins suggests that this metabolic program is essential for the growth and tumorigenicity of high-risk NB cells [110]. The genes involved in cholesterol synthesis pathways were also activated in high-risk NB [110]. Hence it's plausible to suppose that there may be a link between regulation of tumor cell growth and cholesterol metabolism [111,112].

1.3.3. Cholesterol Metabolism and SR-B1

SR-B1 plays an important role in the regulation of the bidirectional flow of cholesterol between the cell and HDL and thus has an impact on cholesterol metabolism. For example SR-B1 overexpression increased intratumor cholesterol accumulation [89] and HDL- CE delivered to the cell via SR-B1 was efficiently and rapidly hydrolyzed in comparison to uptake via CD36 scavenger receptor [113]. Additionally, in African green monkey kidney COS-7 cells, transfection with SR-B1 increased esterification of free cholesterol from HDL suggesting that the observed increase in cholesterol esterification observed was due to an increased pool of free cholesterol and that SR-B1 did not specifically promote cholesterol esterification [114]. The expression of SR-B1 in this model leads to reallocation of cholesterol to parts of the cell

membrane, which then promotes the flux of free cholesterol between the cells and lipoproteins [114].

1.3.4. Acyl-coenzyme A: Cholesterol Acyltransferase

Cholesterol acquired from both de novo synthesis as well as exogenous uptake from lipoproteins move within the cells to the ER where it is converted to cholesteryl esters (CE) by the enzyme acyl-coenzyme A: cholesterol acyltransferase (ACAT) [115,116]. ACAT exists as two isoenzymes in mammals, ACAT1 and ACAT2 each encoded by a different gene [116]. These endoplasmic reticulum (ER) membrane bound enzymes convert excess cholesterol to CE utilizing long chain fatty acyl-CoA [115]. ACAT1 is more prevalent and is found in many different tissues and cell types while ACAT2 is less common and is found in the small intestine and liver [117,118].

The ACAT enzymes produce CE, which are stored in lipid droplets in the cytoplasm. Though the level of CE is low in many cell types, steroidogenic tissues such as the adrenal gland and ovary have higher levels of CE as repository of cholesterol for steroid hormone production [93]. CE is also present in the plasma, in hepatic very low-density lipoprotein (VLDL) and chylomicrons of the intestine. In atherosclerosis, accumulation of CE produces foamy macrophage cells that narrow arteries signaling the initial stages of this disease [119]. Given its role in atherosclerosis and other diseases, ACAT was identified as a drug target.

Most ACAT inhibitors are analogs of the long chain fatty acyl CoA and their hydrophobicity allows them to enter the lipid bilayer and get in contact with residues that are critical for ACAT enzymatic activity [93]. Avasimibe (CI-1011) is an orally administered selective ACAT inhibitor developed by Pfizer for prospective treatment of atherosclerosis and

other diseases [120]. In *in vitro* studies avasimibe showed a good safety profile and reduced cellular CE content without increasing intracellular free cholesterol [121]. However, avasimibe Phase III clinical trials were ended for unspecified reasons.

As mentioned above, ACAT inhibitors were identified as potentially useful in treating atherosclerosis and Alzheimer's disease [122,123]. In atherosclerosis, avasimibe was able to reduce the CE content of foam cells and cause plaque regression in animal models [93]. However, in human clinical trials, avasimibe failed to reduce the progression of atherosclerosis and CE rich macrophage foam cells [123] . Moreover, in Alzheimer's disease ACAT inhibition was shown to shrink extracellular amyloid –β (A-β) protein aggregate plaques, though the exact mechanism is still unclear [93,122].

In cancer, high expression of ACAT was observed which paved the way for the potential use of ACAT inhibitors as treatment for cancer [124]. Inhibition of cholesterol esterification with avasimibe significantly restricted the proliferation of chronic myelogenous leukemia (CML) cells. Additionally, combination of avasimibe and the tyrosine kinase inhibitor, imatimib resulted in significant synergistic inhibition of CML cell proliferation [101]. In pancreatic cancer, high levels of ACAT1 correlated with poor survival and pharmacological inhibition or knockdown of ACAT reduced cell growth and metastasis in an orthotropic mouse model [125]. Similarly, avasimibe reduced growth in breast and prostate cancer and glioblastoma cells [103,124,126,127].

1.4. Significance and Hypothesis

1.4.1. Significance

SR-B1 regulates bidirectional movement of cholesterol between the cell and HDL and has been linked to progression of many cancers. Since many cancer cells alter their cholesterol metabolism to ensure an ample supply of lipids for membrane biogenesis and cholesterol synthesis pathway genes are activated in high-risk NB, elucidation of the role of SR-B1 in NB cholesterol metabolism will enhance scientific knowledge and impact therapeutic approaches.

The regulation of the size of the cholesterol pool is essential, as too much cholesterol is toxic to the cells. As a result, the ACAT enzyme converts excess cholesterol into cholesteryl ester (CE) that is stored in cytoplasmic lipid droplets. In some cancers, ACAT expression is increased and the use of ACAT inhibitors suppressed tumor cell proliferation. Analysis of ACAT activity and ACAT inhibition in high-risk NB will impact the potential use of ACAT inhibitors as treatment for high-risk NB. Furthermore identification of SR-B1 as a potential prognostic indicator of NB patient survival may significantly improve risk stratification and therapeutic approaches.

The enigmatic nature of NB continues to be a challenge for clinicians and researchers. The clinical behavior of NB ranges from spontaneous regression to relentless progression even with intensive multimodal therapy. Over the decades, understanding of the biology of NB through identification of molecular and genetic factors that drive NB has resulted in more effective therapies and improved patients outcomes. However, survival has remained poor for high-risk NB patients. Consequently, there is a critical need for the discovery of other factors that promote NB development, to enhance the patients' quality of life and length of survival. Here the HDL receptor, SR-B1, is identified in NB as a pivotal regulator of tumor aggressiveness.

This knowledge potentially implicates HDL as a source of cholesterol in NB progression and will aid in the discovery of novel targets and therapeutic approaches for high-risk NB treatment.

1.4.2. Hypothesis

SR-B1 is overexpressed in high-risk NB and drives its progression.

The specific aims to test this hypothesis are:

Specific Aim 1

To investigate the association of SR-B1 protein expression with NB aggressiveness

Here the expression of SR-B1 was identified in NB cells. Additionally, anti-SR-B1 antibody was used to inhibit SR-B1 and changes in cell proliferation, migration and invasion were investigated. Inhibition of SR-B1 reduced cell survival, migration and invasion.

Specific Aim 2

To determine the association between SR-B1 expression and cholesterol metabolism in NB

The effect of SR-B1 expression and inhibition on cholesterol metabolism was studied. Furthermore, ACAT activity and the effect of delipidated serum on SR-B1 expression, cell survival and cholesterol metabolism were determined. Moreover, HDL addition rescued cell survival.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Culture

SH-SY-5Y and normal human astrocytes cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA). SMS-KCN, SMS-KCNR and SK-N-BE (2) cell lines were obtained from the Children's Oncology Group (Lubbock, TX). The normal human astrocytes cell line was maintained in DMEM medium containing 10% FBS and100 U/mL Penicillin-Streptomycin. NB cells were cultured in RPMI 1640 medium supplemented with 2 mM L-Glutamine, 10% fetal bovine serum (FBS) and 100 U/mL Penicillin-Streptomycin at 37 °C in 5% CO_2. Confluent flasks were subcultured twice weekly and seeded at a ratio of 1:5.

2.2. Materials

Western blot antibodies for SR-B1 and beta actin were purchased from Abcam (Cambridge, MA). Anti-rabbit secondary antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit IgG control antibody was purchased from Vector Laboratories, Inc. (Burlingame, CA). The inhibitory anti-SR-B1 antibody was custom produced by Rockland Immunochemicals Inc. (Limerick, PA) using the following peptide sequence CRPGNSGPKGQKGEKGSGN, residues 325-342 from the extracellular domain of the SR-B1 receptor [128]. This antibody was sterilized with 0.45µM Polyvinylidene Fluoride (PVDF) filter and protein concentration was determined by the bicinchoninic acid (BCA assay) [129]. Anti-

SR-B1 antibody stock concentration is 3.56 mg/mL. The anti-SR-B1 antibody acts as an inhibitor by obstructing the interaction between high density lipoprotein (HDL) and the SR-B1 receptor [130].

2.3. Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer. Next, the protein concentration of the supernatants were determined using the BCA assay [129] and 20μg protein from each sample was resolved by electrophoresis in SDS acrylamide gel. After electrophoresis, the gels were transferred to a nitrocellulose membrane using the iBlot™ Gel Transfer Device (Thermo Fisher Scientific; Waltham, MA). The membrane was then probed with respective primary antibodies at 1:1000 dilution and conjugated secondary antibodies using the iBind Western System (Thermo Fisher Scientific; Waltham, MA). After this, the immunocomplexes were detected using SuperSignal™ West Femto Maximum Sensitivity chemiluminescent substrate (Thermo Fisher Scientific; Waltham, MA). To quantitate protein bands ImageJ software was used [131]. Results were reported as ratio of SR-B1 to actin.

2.4. Cell Proliferation Assay

Cells were plated at a density of 5 x 10^4 cells /mL in RPMI 1640 medium supplemented with 2 mM L-Glutamine, 10% FBS and 100 U/mL Penicillin-Streptomycin (complete media) in 96 well plates. At each time point, 10μL of a water-soluble tetrazolium salt solution from the Cell Counting Kit -8 (CCK-8) (Dojindo Molecular Technologies, Inc.; Rockville, MD) was added to respective wells and incubated for four hours at 37 °C in 5% CO_2. In live cells, NADH reduced this solution and produced an orange-color formazan dye. The amount of the formazan

dye formed is directly proportional to the number of live cells. Absorbance was then measured at 450nm using a microplate spectrophotometer (PowerWave; Biotek Instruments Inc.).

2.5. Migration and Invasion Assay

In 60mm dishes, 5 x10^5 cells were plated and allowed to attach overnight. The next day fresh media containing various concentrations of inhibitory anti-SR-B1 antibody in complete media were added to cells and incubated for 72 hours. After treatment, cells were serum starved overnight in IMDM media. The following day cells were harvested, counted and 5 x10^4 cells were added to the upper chamber of a 24-well 8µm non-coated (migration) or BioCoat Matrigel (invasion) transwell chamber (Corning Inc., Tewksbury, MA) in IMDM + 1% insulin-transferrin- selenium (ITS). The bottom wells contained IMDM + 10% FBS + 1% ITS as chemoattractant. Cells were incubated overnight at 37^{o}C in 5% CO_2. Then cotton swabs were used to remove the non-migrating or non-invading cells from the inside of the chambers. The membranes were fixed with 70% ethanol and then stained with 0.2 % crystal violet. Light microscopy was used to enumerate the number of stained cells in five random fields of each insert at 10X magnification. Migration and invasion percentage was calculated using the formulas below:

1) $$\left(\frac{\text{Average number of cells in each field per insert}}{\text{Area of microscope viewing field}}\right) \times \text{Entire area of the Transwell insert} = \textit{Total number of cells per insert}$$

2) $$\left(\frac{\text{Total number of cells per insert}}{\text{Number of cells seeded}}\right) \times 100 = \underline{\textbf{Percent Migration or Invasion}}$$

2.6. Apoptosis Assay

Cells were plated at 0.1 x 10^6 cells/mL in 100mm dishes and allowed to attach overnight. Then various concentrations of inhibitory anti-SR-B1 antibody in complete media were added to cells and incubated for 72 hours. After treatment, media containing potential apoptotic cells were collected along with trypsinized cells. Harvested cells were counted and washed twice in 1X PBS. 1 x 10^6 cells were resuspended in an Annexin V-FITC and propidium iodide solution and incubated in the dark at room temperature for 15 minutes [132]. Stained cells were immediately analyzed using Cellometer K2 (Nexcelom Bioscience, Lawrence MA) a fluorescence detection and image-based cell counting device [132]. Scatterplot of the results were generated using the FCS Express 6 (De Novo Software) (Glendale, CA). For each treatment group percent apoptotic cells are presented ± standard deviation (SD).

2.7. Nile Red Assay

2 x10^5 NB cells were cultured in 35 mm glass-bottomed dishes and then fixed with 4% paraformaldehyde (PFA). Cells were washed in PBS and then stained with 300 nM Nile red (9-diethylamino-5H-benzo [α] phenoxazine-5-one). Nile red is a fluorescent dye that differentially stains polar lipids (phospholipids) and neutral lipids (cholesterol esters (CEs) and triglycerides). Polar lipids display a red emission while neutral lipids display yellow. The red emission was observed at 540nm excitation while the yellow emission was observed at 460 nm excitation. Images were captured at 40X magnification. Corrected total cell fluorescence was calculated using ImageJ.

2.8. Cholesterol Esterification

A complex of ^{14}C-oleate and bovine serum albumin (BSA) was prepared by mixing 0.7mg of potassium hydroxide (KOH) with 100 µL of 1.85 MBq of ^{14}C-oleic acid and the ethanol evaporated. A solution of 0.75 mL PBS (without Ca^{2+} and Mg^{+}) and 2.12 mg fatty acid-free BSA was added and mixed by vortexing. 2µCi/mL of this solution was added to cells and incubated for 4 hours. After incubation cells were trypsinized and collected. Lipids were extracted with cold acetone and neutral lipids separated by thin layer chromatography. Incorporation of ^{14}C-oleate into cholesteryl esters was measured via a beta counter [133]. Protein was determined using the BCA assay.

2.9. Cholesterol Assay

Hexane: isopropanol (v/v 3:2) solution was added to 1 x 10^6 cells. The samples were sonicated, then centrifuged and protein content of the pellet was determined via BCA assay. The cholesterol extracted was dried under nitrogen and redissolved in a small volume of isopropanol. The Cholesterol E and Free Cholesterol E from Wako Chemicals USA, Inc. (Richmond, VA, USA) was used to determined cholesterol content. 2µL of sample was placed into wells of 96-welled plate and 200µL of the respective buffers were added to the wells. The plate was incubated at 37^{o}C for 5 minutes. Absorbance was then measured at 595nm using a microplate spectrophotometer (PowerWave; Biotek Instruments Inc.).

2.10. Patient Survival Probability

The Tumor Neuroblastoma-Kocak-649- custom-ag44kcwolf data set [37] (GEO accession number GSE45547) was used to create overall and event-free Kaplan-Meier survival

curves on the R2: Genomics Analysis and Visualization Platform (Amsterdam, the Netherlands) [129]. The data set consisted of gene expression profiles for 649 NB patient tumor samples. The cut-off modus "scan" in R2 was used to identify the threshold that best separated low and high SR-B1 gene expression. In the order of expression, the Kaplan scanner used every increasing expression value as a cutoff to generate two groups and tested the p-value in a log-rank test, identifying the most significant expression cutoff for analysis of survival. Survival data was not available for 173 of the samples and were omitted from analysis.

2.11. Statistical Analyses

All statistical tests were performed using GraphPad Prism7 and data is expressed as mean ± standard deviation (SD) for experiments in duplicates. Significance of treatments was determined using Student's t-test or one-way Anova, post hoc test (Turkey). $p < 0.05$ was considered statistical significant.

CHAPTER 3

RESULTS

3.1. Association of SR-B1 protein expression with NB aggressiveness

High expression of the scavenger receptor class B type 1 (SR-B1) has been observed in cancers of the breast, prostate, ovary and skin [71,82,83,86,87]. In the above-mentioned cancers, the high expression of SR-B1 is often correlated with cell aggressiveness, poor patient outcome and cellular cholesterol regulation [82,83,87,89]. In this section, we determined the association of SR-B1expression with cell growth rate and migration and the effect of SR-B1 inhibition on cell survival and apoptosis in neuroblastoma (NB).

3.1.1 SR-B1 is expressed on NB cells

The protein expressions in a panel of NB cell lines were examined using Western blot analysis. Table 3.1 shows the characteristics of the patients and tissue that gave rise to the NB cell lines. SR-B1 was expressed at different levels in NB cell lines tested but was barely expressed in normal human astrocytes (Figure 3.1). Three cell lines with varying levels of SR-B1 expression (Figure 3.1) were selected. Specifically SMS-KCN and SMS-KCNR which were obtained from an 11-month old boy before and after chemotherapy [134] and SH-SY-5Y a thrice subclone of SK-N-SH obtained from a 2 year old girl post therapy [135].

Table 3.1. Characteristics of Neuroblastoma Cell Lines

Cell Lines	SMS-KCN	SMS- KCNR	SH-SY-5Y	SK-N-BE (2)	BE(2)C
Gender	Male	Male	Female	Male	Male
Stage	4	4		4	4
Age at diagnosis (months)	11	11	48	24	24
Phase of Therapy	Diagnosis	Progressive Disease	Progressive Disease	Progressive Disease	Progressive Disease
MYCN Status	Amplified	Amplified	Non- Amplified	Amplified	Amplified
Primary Tumor Site	Adrenal gland	Adrenal gland	Upper Chest	Brain	Brain
Source of Culture	Primary tumor	Bone Marrow	Bone Marrow	Bone Marrow	Bone Marrow
Karyotype	del(1)(p34), t(17;20)(q21or 22;q13)	del(1)(p34), t(17;20)(q21or 22;q13), t(6;?)(q21or22 ;?)	47,XX,der(1)(1pter→;1q25::1q25→1q11::1q44→1q25::1q25→1qter),+7,der(9)(9pter→9q34::7q22→7qter),der(22)(22pter→22q13::17q21→17qter)	t(1;2)(p22;p21),t(3;17)(p21;q21), Monosomy (17,18), variable rearrangements involving (4,6,9,10,11,and/or 20)	
Doubling Time (Hours)	109	72	27	30	18
References	[134,136]	[134,137]	[135,138,139]	[28,137,140,141]	[142-144]

Diagnosis: cells lines derived from samples of patients before treatment
Progressive Disease: cell lines derived from patients who relapsed after chemotherapy

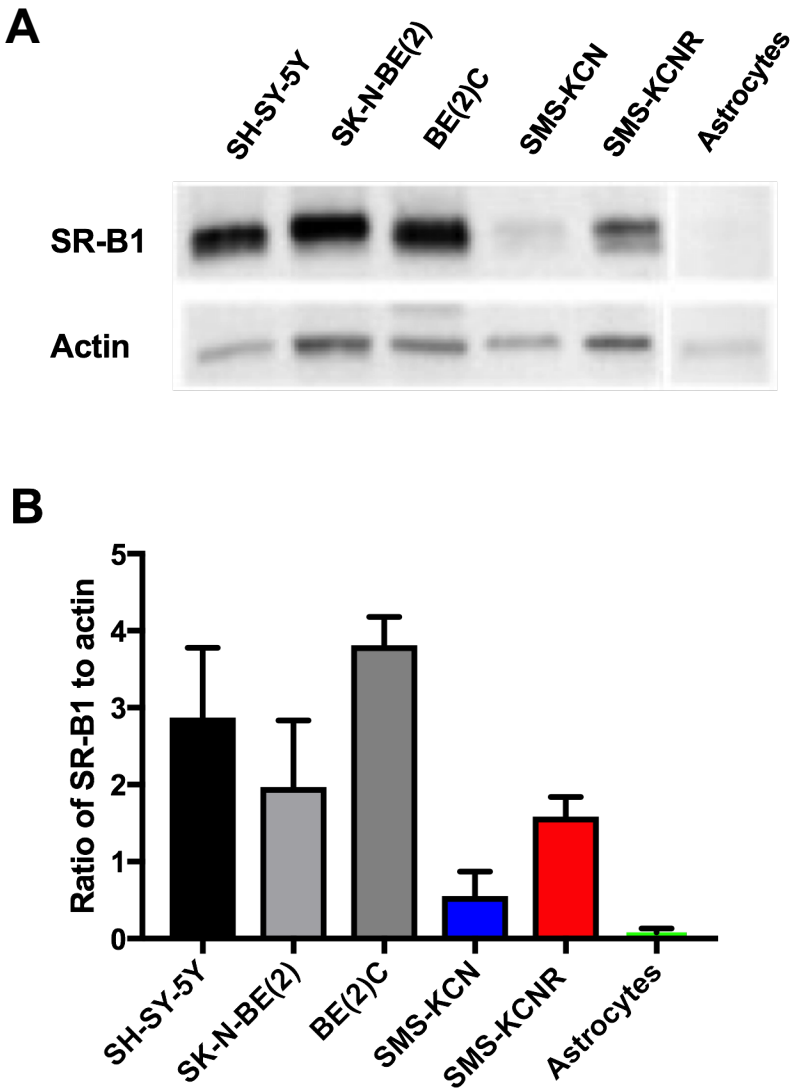


Figure 3.1. SR-B1 expression in NB cells and normal human astrocytes: A) Representative Western blot image. B) Quantitation of SR-B1 expression. SR-B1 was expressed at varying levels in NB cells. SMS-KCN was designated low SR-B1 expresser, SMS-KCNR -moderate SR-B1 expresser and SH-SY-5Y high SR-B1 expresser. Astrocytes were used as a representative normal non-malignant cell and its SR-B1 expression was negligible. Results shown are representative of three independent experiments.

3.1.2. Association of SR-B1 expression with cell proliferation and migration

SR-B1's ability to regulate cell cholesterol has been suggested as the link between SR-B1, HDL and cell proliferation [66,112]. Comparison of the three cell lines showed no significant difference in proliferation during the first 24 hours. However, after 24 hours SH-SY-5Y cells were growing at a faster rate than both SMS-KCN, SMS-KCNR. SH-SY-5Y had the highest level of SR-B1 expression and showed a more elevated growth curve than both SMS-KCN and SMS-KCNR (Figure 3.2). A search of the literature showed doubling times of 109, 72 and 27 hours for SMS-KCN, SMS-KCN, SH-SY-5Y, respectively [136,137,139,144] which were comparable to our observed growth rate for these cells. This finding suggested that there is likely an association between SR-B1 expression and cell growth rate.

More than half of high-risk NB patients present with extensive metastases at the time of diagnosis [38,145]. Given that migration is one of the initial stages of metastases, the migration of NB cells with different levels of SR-B1 protein expression was examined. The high SR-B1 expresser (SH-SY-5Y) showed markedly higher migration rates than the lower SR-B1expressing cell lines suggesting an association between SR-B1 expression and migration (Figure 3.3)

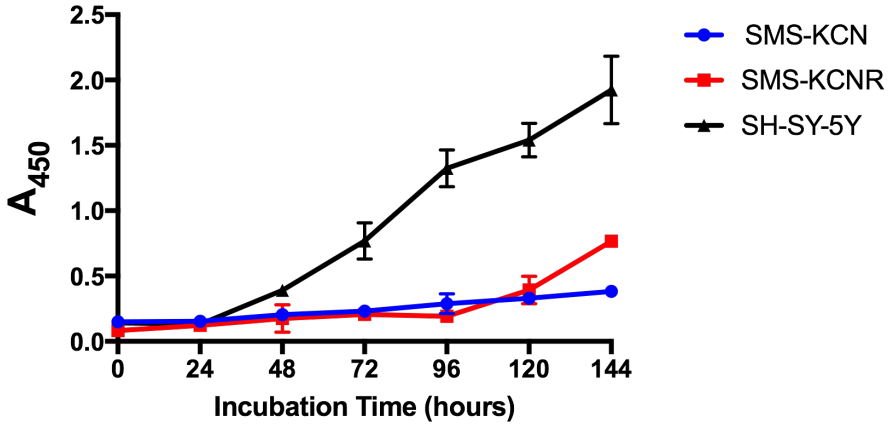


Figure 3.2. Cell proliferation curve NB cells: Cells were seeded at a density of 0.5 x 10^5 cells/mL. At indicated times cells were incubated with Cell- Counting Kit-8 (CCK-8) solution for 4 hours and absorbance of the orange formazan produced was measured at 450 nm. Linear regression analysis of the curve was performed and the slopes of the proliferation curves were compared ±SE. SMS-KCN- slope of 0.0017±0.0001; SMS-KCNR- slope of 0.009 ±0.001; SH-SY-5Y- slope of 0.014 ±0.001; High SR-B1 expressing cell line (SH-SY-5Y) showed higher proliferation than other cell lines. The proliferation rate of these cell lines was comparable to doubling times reported in Table 3.1. Results shown are representative of two independent experiments done in quadruplets.

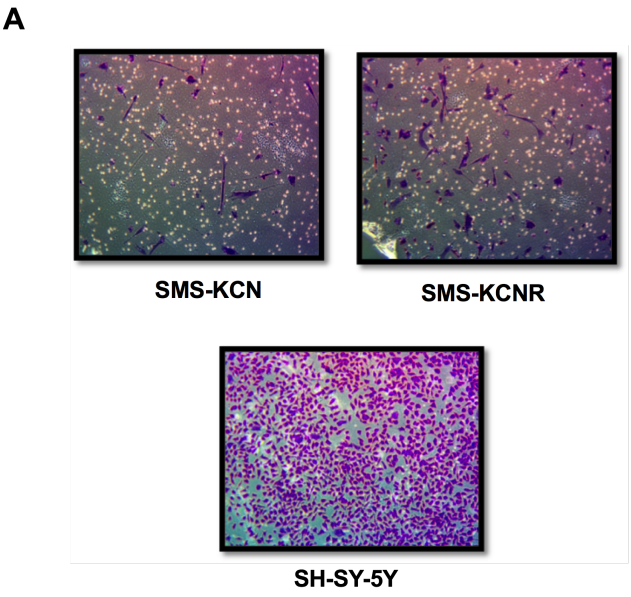
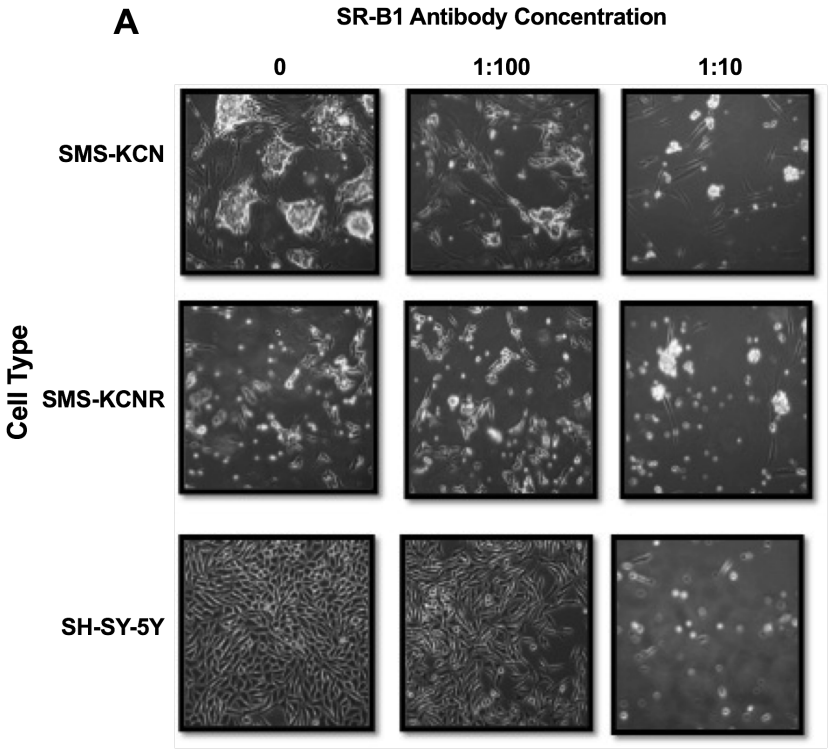
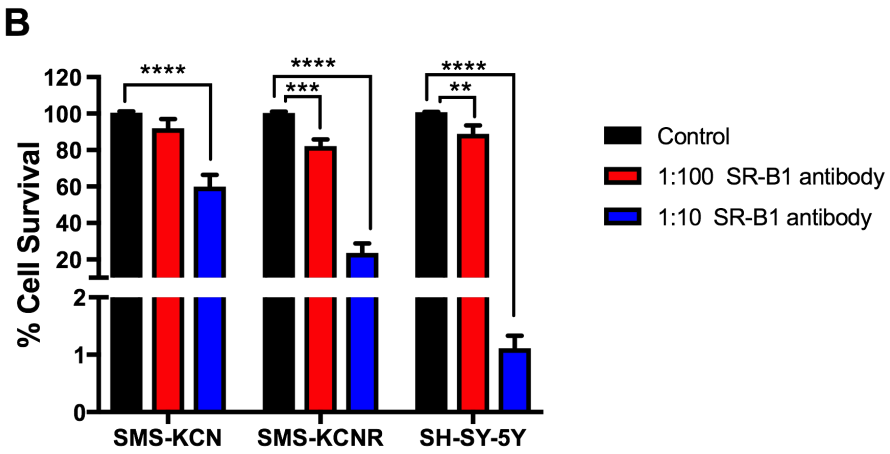


Figure 3.3. Migration of NB cells: Cells were seeded at 1 x 10^5 cells/mL and grown in RPMI containing 10% FBS for 72 hours. Next, cells were serum starved for 24 hours then 50,000 cells were added to the upper well of the migration chamber. Cells were allowed to migrate overnight. The next day the cells were stained with crystal violet and counted. A) Representative images of migrated cells (purple spots) at 10X magnification. B) Comparison of cell migration: The high SR-B1 expressing cell line (SH-SY-5Y) showed significantly greater migration than the low (SMS-KCN) and moderate (SMS-KCNR) SR-B1 expressing cell lines. Similarly migration of SMS-KCNR was greater than SMS-KCN; n=3, ***p=0.0001

3.1.3. Effect SR-B1 inhibition on cell survival, migration and invasion

To demonstrate a direct relationship between SR-B1 expression and cell aggressiveness, the effect of SR-B1 inhibition on cell survival, migration and invasion was investigated. Thus far, this study has shown that high SR-B1 expression is correlated with enhanced cell proliferation and migration. Anti-SR-B1 antibody raised against the extracellular portion of the SR-B1 receptor was used to inhibit SR-B1. The anti-SR-B1 antibody works as an inhibitor by blocking the interaction between high-density lipoprotein (HDL) and SR-B1 [56,130]. The protein concentration of the stock anti-SR-B1 antibody was 3.56 mg/mL and dilutions of 1:100 and 1:10 of antibody were used in experiments. As shown in figure 3.4, inhibition of SR-B1 reduced cell survival in the three cell lines. The morphology of the cells changed from elongated into a circular shape after SR-B1 inhibition (Figure 3.4A). The 1:10 SR-B1 antibody concentration showed the most significant reduction in survival in SH-SY-5Y cells. SMS-KCNR showed ~ 30% survival with 1:10 SR-B1 antibody concentration while SMS-KCN showed 60% cell survival under the same conditions (Figure 3.4B). To exclude the toxicity of the antibody solution as a possible cause for reduced survival and further observations, the cells were incubated as described above in IgG control antibody at the same protein concentration of the 1:10 SR-B1 antibody (0.356 mg/mL). Figure 3C shows that the IgG control antibody did not reduce the survival of the NB cells proving that the antibody solution was not toxic to the cells. Likewise, inhibition of SR-B1 also significantly reduced cell migration and invasion, which was more pronounced in the 1:10 SR-B1 antibody treatment (Figure 3.5). Taken together this suggests that SR-B1 is a regulator of cell survival, migration and invasion.


A
SR-B1 Antibody Concentration
0
1:100
1:10
Cell Type
SMS-KCN
SMS-KCNR
SH-SY-5Y


B
% Cell Survival
120
100
80
60
40
20
2
1
0
****
***
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SMS-KCN
SMS-KCNR
SH-SY-5Y
Control
1:100 SR-B1 antibody
1:10 SR-B1 antibody

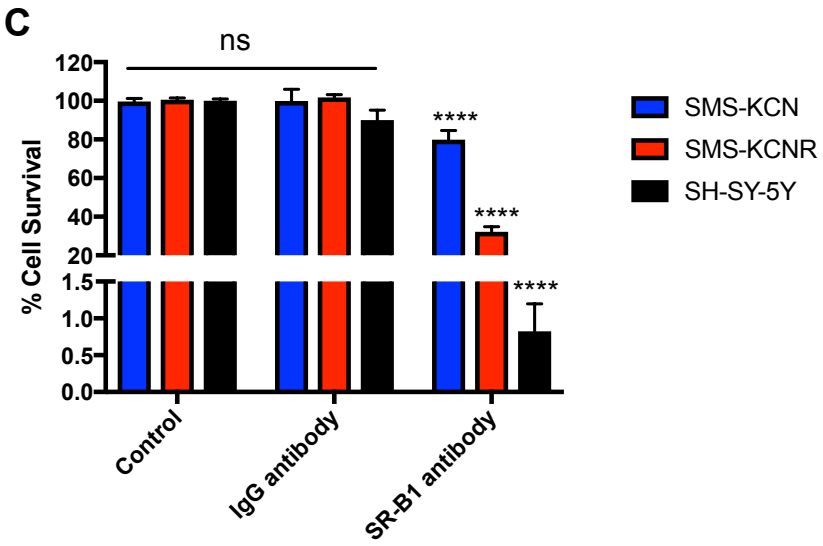
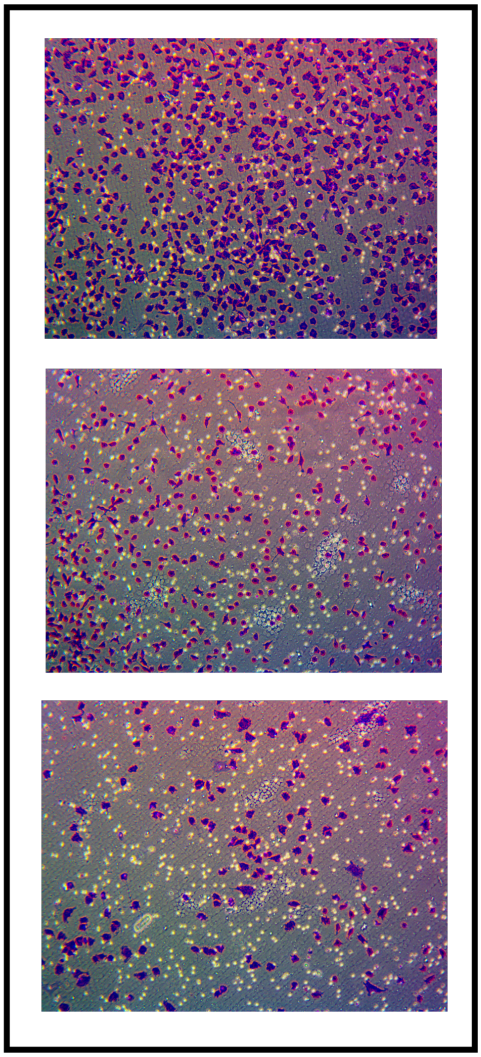


Figure 3.4. SR-B1 reduces NB cell survival: Cells were incubated in medium containing 10% FBS and indicated treatments for 72 hours. At the end of treatment, cells were incubated with Cell- Counting Kit-8 (CCK-8) solution for 4 hours and absorbance of the orange formazan product was measured at 450 nm. A) Representative light microscopy images of cells after treatment, 10X magnification. B) Cell survival bar graph: Significant reduction in cell survival of all cell lines tested after SR-B1 inhibition. The high SR-B1 expressing cell line was most responsive with only ~1% survival at 1:10 anti-SR-B1 antibody treatment. C) Cell survival bar graphs: IgG antibody protein concentration was matched to the 1:10 anti-SR-B1 antibody dilution (0.356 mg/mL). No significant differences in IgG antibody and control were identified showing that the antibody solution was not toxic. Control indicates RPMI media supplemented with 10% FBS; n=4, *p= 0.03, **p= 0.002****p<0.0001, ns -non-significant.

A



Control

1:100 SR-B1 Antibody

1:10 SR-B1 Antibody

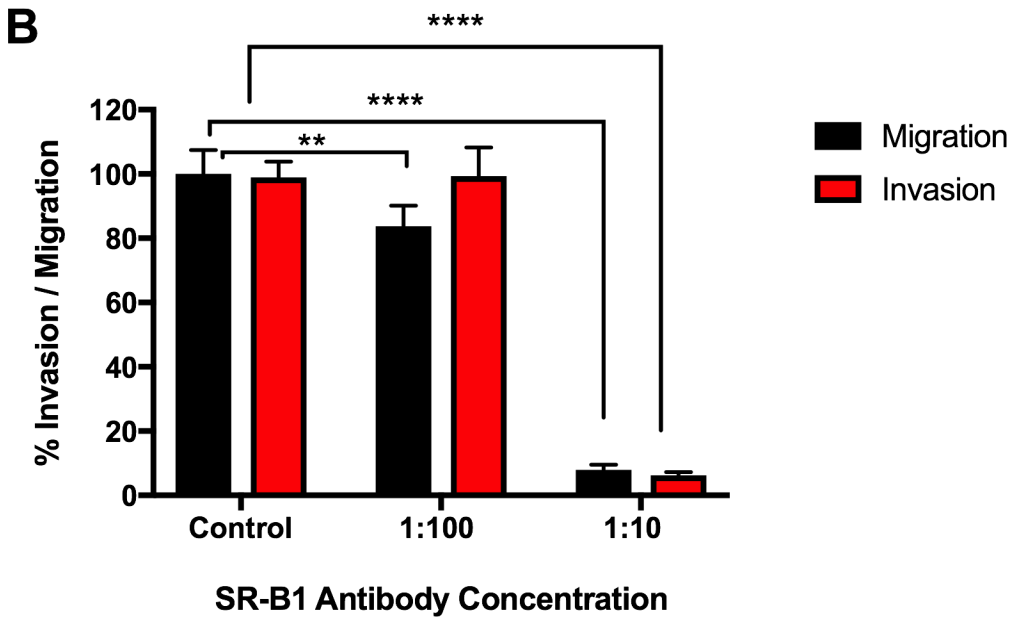
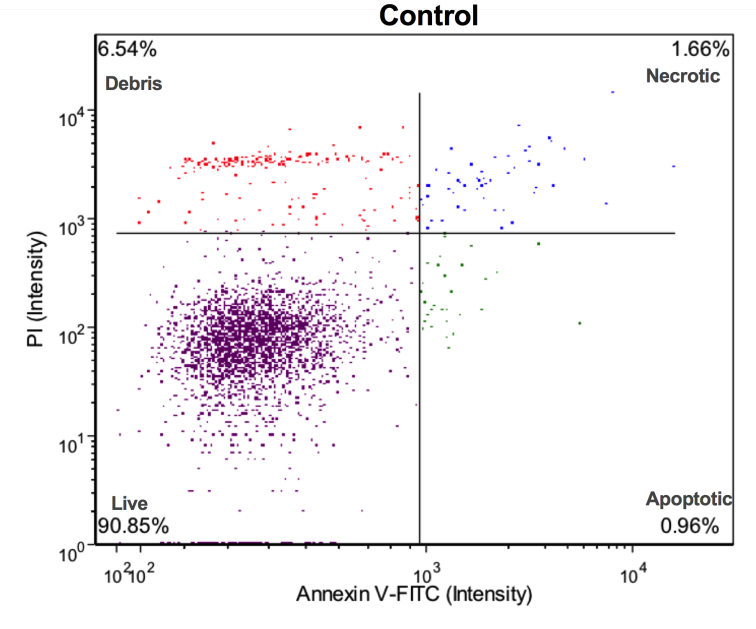


Figure 3.5. SR-B1 inhibition reduces cell migration and invasion: SH-SY-5Y cells were seeded at 1 x 10^5 cells/mL and grown in RPMI containing 10% FBS in the presence or absence of various concentrations of the anti-SR-B1 antibody for 72 hours. Next, cells were serum starved for 24 hours then 50,000 cells were added to the upper well of the migration or invasion chamber. Cells were allowed to migrate overnight. The cells were stained with crystal violet and counted. A) Representative images of migrated cells (purple spots) at 10X magnification. B) Quantitation of migrated and invaded SH-SY-5Y cells treated as indicated. SH-SY-5Y cell line was selected based on its high expression of SR-B1 and its elevated response to SR-B1 inhibition. Control indicates RPMI media supplemented with 10% FBS; n=3, **p=0.002, ****p<0.0001

3.1.4. SR-B1 inhibition induces apoptosis in NB cells

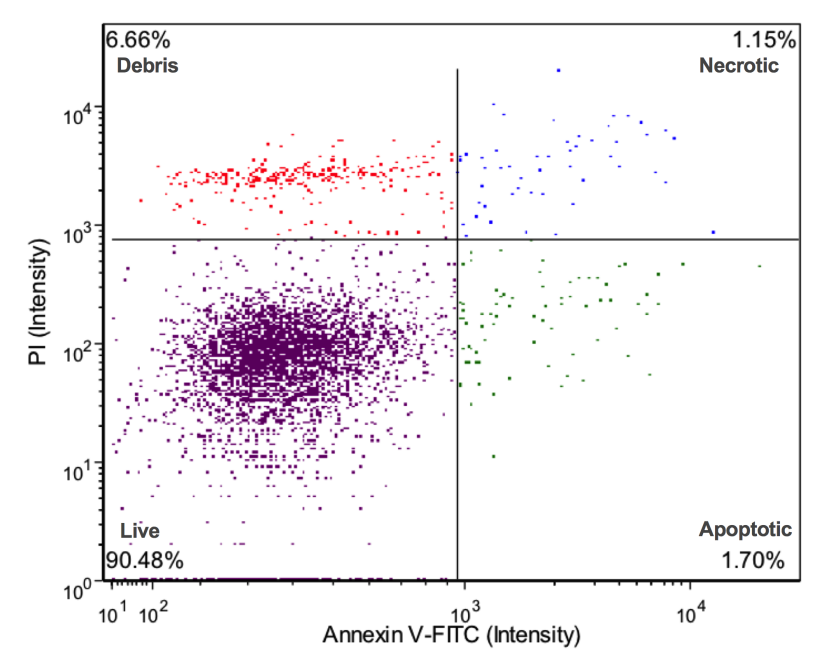
One of the many ways tumor cells facilitate their continued development is through evasion of programmed cell death, apoptosis [80]. Here, the effect of SR-B1 inhibition on apoptosis in NB cells was investigated. Given the robust response of the SH-SY-5Y to 1:10 SR-B1 treatment, this high SR-B1 expressing cell line was used for subsequent experiments. Cells were incubated with the different treatments for 72 hours. Then the cells were harvested and stained using Annexin V –FITC and propidium iodide (PI). Annexin binds to phosphatidylserine (PS), which translocates from the internal to the external leaflet of the plasma membrane during early apoptosis. PI is a membrane exclusion dye that only binds to the DNA of cells with compromised membranes. Figure 3.6 A-C presents scatterplots of the results. The bottom left quadrant contains live cells with intact membranes; the bottom right contains apoptotic cells with partially weaken cell membranes and is stained mostly by Annexin V –FITC; the upper right quadrant contains necrotic cells with extremely weaken membranes which are stained by both Annexin V –FITC and PI; the upper left quadrant of the scatterplot represents debris, which are cells whose membrane are almost completely destroyed and thus is stained by PI only. The stained cells were analyzed using image cytometry [132]. The results indicate a significant increase in the percent of apoptotic cells after treatment with 1:10 SR-B1 antibody (Figure 3.6). This further confirms the hypothesis that the SR-B1 receptor is critical to NB cells.

A



B

1:100 SR-B1 antibody



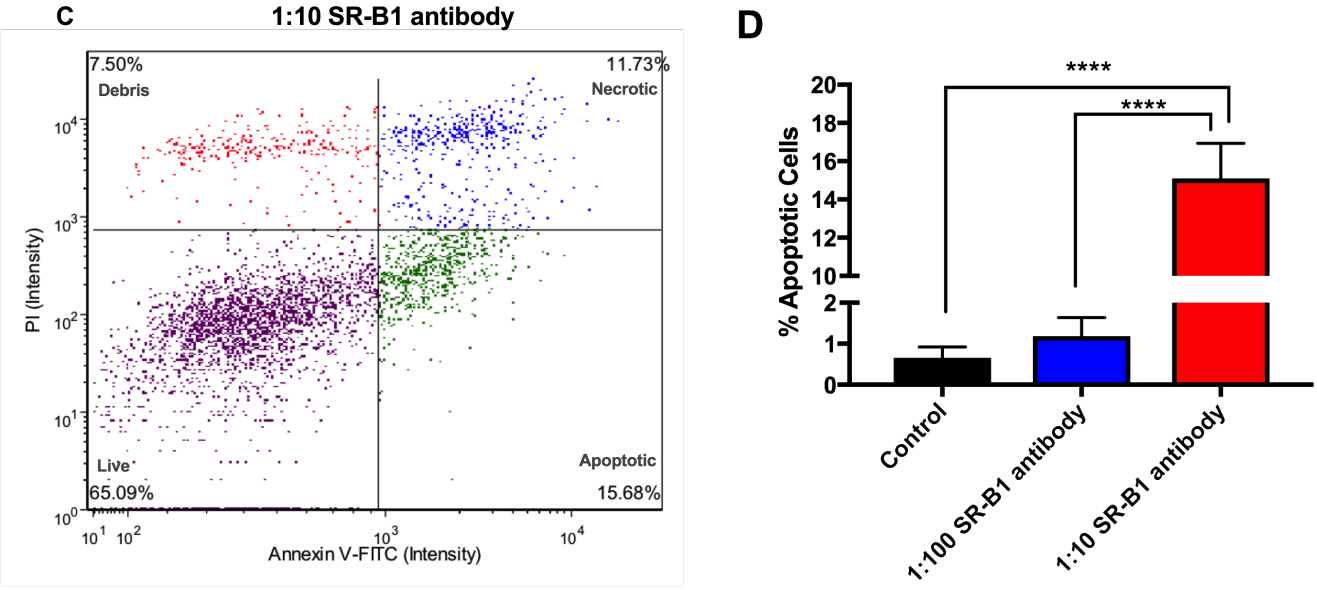


Figure 3.6. SR-B1 inhibition induces apoptosis: Cells were plated at 0.1 x 10^6 cells/mL in 100mm dishes and allowed to attach overnight. Then various concentrations of inhibitory anti-SR-B1 antibody in complete media were added to cells and incubated for 72 hours. After treatment with 1 x 10^6 cells were harvested then resuspended in Annexin V-FITC and propidium iodide solution and incubated for 15 minutes at room temperature in the dark. Stained cells were immediately analyzed using Cellometer K2, a fluorescence detection and image-based cell counting device. Scatterplot of the results were generated using the FCS Express 6 (De Novo Software). A), B) and C) Representative scatterplots of Annexin –FITC and PI stained SH-SY-5Ycells after SR-B1 antibody treatment; D) Quantitation of apoptotic cells represented as a bar graph; Control indicates RPMI supplemented with 10% FBS. Results shown are representative of two independent experiments done in triplicate; ****$p<0.0001$

3.2. SR-B1 regulates cholesterol metabolism in NB

Neoplastic transformations are often accompanied by altered lipid metabolism and changes in plasma lipoproteins concentration [96,146]. The high-density lipoprotein (HDL) receptor, scavenger receptor class B type 1 (SR-B1) is implicated in the observed lipid reprogramming in cancerous tissues [78]. By regulating the selective uptake of cholesteryl ester (CE), SR-B1 is able to control cell cholesterol content [82]. In the previous section, high expression of SR-B1 was identified on high-risk neuroblastoma (NB) cells. Given the link between SR-B1, cholesterol and cancer, the influence of SR-B1 on cholesterol metabolism in NB was studied further.

3.2.1. Intracellular lipid droplets are present in NB cells

The accumulation of cytoplasmic lipid droplets occurs in many diseases including cancer [146]. To identify the presence of cytoplasmic lipid droplets in high-risk NB cells, the selective fluorescent stain Nile red was used [147]. The Nile red dye differentially stains neutral lipids such as cholesteryl esters and triglycerides and polar lipids such as phospholipids. Neutral lipids appear yellow while polar lipids appear red. As shown in figure 3.7, intracellular lipid droplets are present in high-risk NB cells.

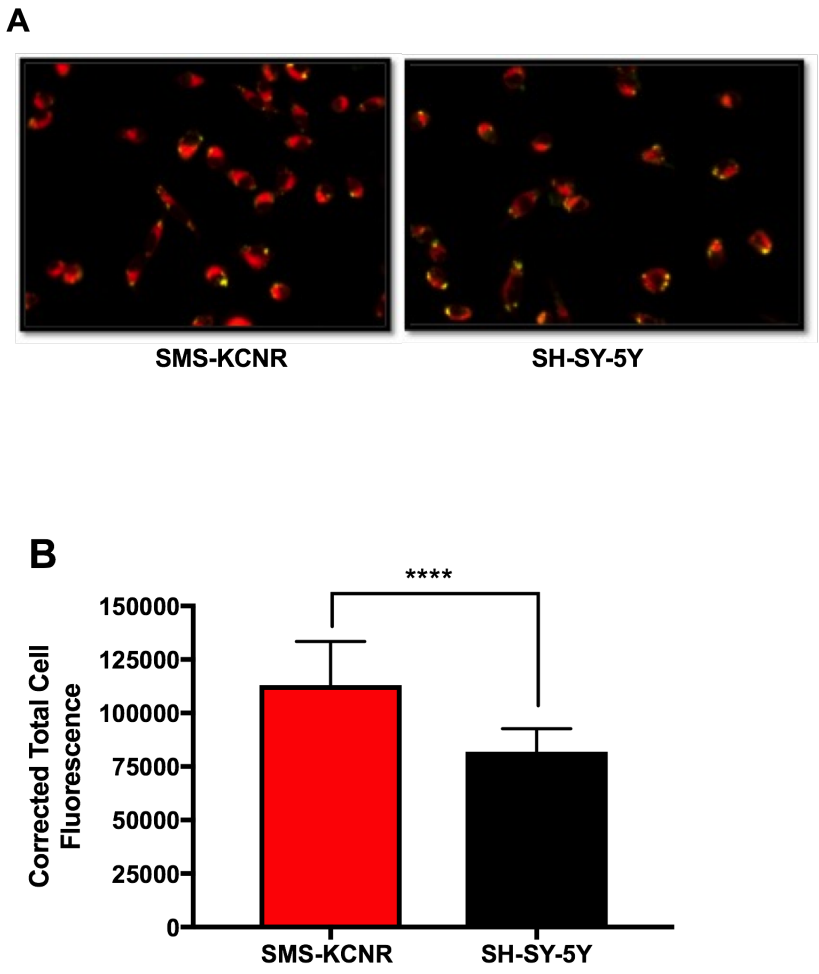


Figure 3.7. Intracellular lipid accumulation in NB: Cells were incubated overnight in complete media then stained with Nile red. Nile red is a fluorescent dye that differentially stains polar lipids (phospholipids) and neutral lipids (cholesterol esters and triglycerides). Polar lipids appear red while neutral lipids appear yellow. Images were captured at 40X magnification. Corrected total cell fluorescence was calculated using ImageJ. A) Representative Nile red images; B) Quantitation of images in A); n=20, ****$p<0.0001$

3.2.2. High levels of cholesterol esterification in high SR-B1 expressing cells

After confirming the accumulation of cholesteryl esters (CE), the next step was to figure out the factors responsible for this observation. The enzyme acyl-coenzyme A: cholesterol acyltransferase (ACAT) converts excess cholesterol to CE which is stored in lipid droplets [93]. The activity of the enzyme responsible for cholesterol esterification (ACAT) was determined in the NB cells. The levels of ACAT activity in high-risk NB cells were measured using ^{14}C oleate. The high SR-B1 expressing cell line, SH-SY-5Y showed significantly more that cholesterol esterification that the cells with lower SR-B1 expression (Figure 3.8). SR-B1 expression is potentially positively associated with ACAT activity.

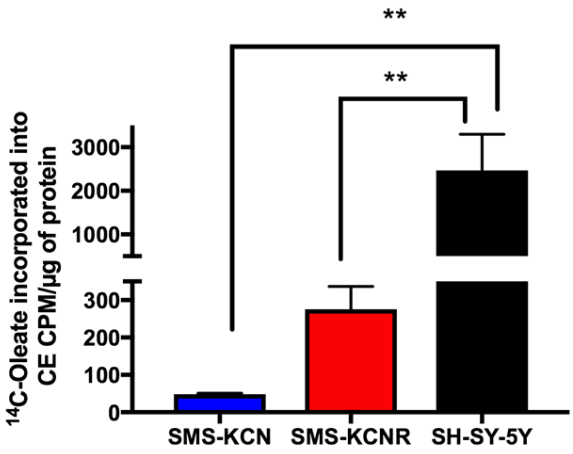


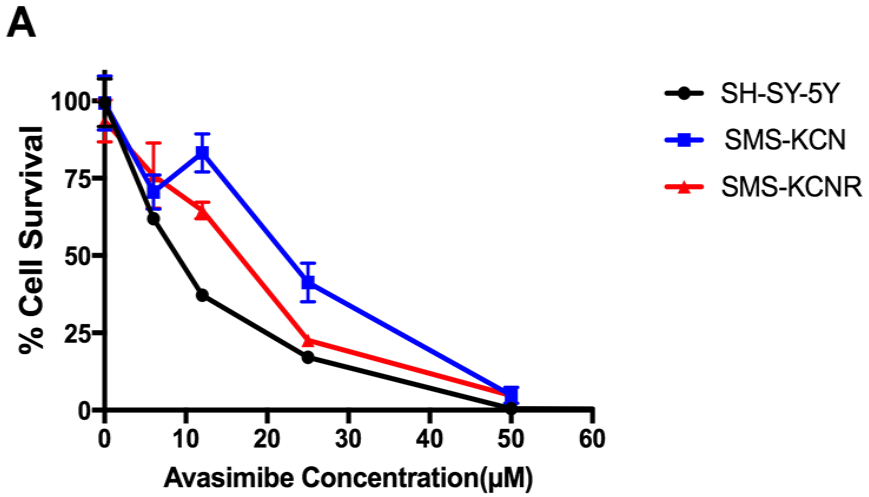
Figure 3.8. ACAT activity in NB cells: Cells were incubated in complete media containing 2µCi/mL of ^{14}C oleate for 4 hours. Lipids were extracted with cold acetone and neutral lipids separated by thin layer chromatography. Incorporation of ^{14}C-oleate into cholesterol esters per milligram of protein was measured using a beta counter and BCA assay. High SR-B1 expressing cell line showed higher ACAT activity than the low and moderate SR-B1 expressing cells; n=2, ***p $\leq$0.003

3.2.3. Inhibition of ACAT decreases cell survival and induces apoptosis NB

In some cancers, treatment with ACAT inhibitors reduced cell growth [101]. Here the effect of the ACAT inhibitor, avasimibe –a long chain fatty acyl CoA analog, was examined. Avasimibe had the greatest effect on SH-SY-5Y cells (high-SR-B1 expresser) with an observed half maximal inhibitory concentration (IC_{50}) of 9.6μM (Figure 3.9). Avasimibe IC_{50} of SMS-KCN and SMS-KCNR was 24.4μM and 17.4μM, respectively. Since the concentration of avasimibe needed to kill half of the low (SMS-KCN) and moderate (SMS-KCNR) SR-B1 expressing cells was extremely high, the SH-SY-5Y cells were used for subsequent experiments. Treatment of SH-SH-5Y cells with 8.5μM of avasimibe increased the concentration of apoptotic cells three- fold (Figure 3.10).

3.2.4. SR-B1 inhibition produces a greater reduction in cell survival than ACAT inhibition

The observation that inhibition of SR-B1 or ACAT reduced cell survival prompted the analysis of the effect of both SR-B1 and ACAT inhibition on cell survival. The results showed that inhibition of both ACAT and SR-B1 did not produce an additive killing effect. However as shown in figure 3.11, the inhibition of SR-B1 alone decreased cell survival to a greater degree than ACAT inhibition only. The cells dependence on an exogenous source of cholesterol for survival potentially exceeds its reliance on cholesterol esterification. This finding further confirmed the importance of SR-B1 in NB.



Cell Line	IC_{50} of Avasimibe (μM)
SMS-KCN	24.4 ± 1.6
SMS-KCNR	17.43 ± 3.5
SH-SY-5Y	9.6 ± 0.6

Figure 3.9. ACAT inhibition reduces cell survival: Cells were incubated in medium containing 10% FBS and treated with various concentrations of the ACAT inhibitor, avasimibe for 72 hours. At the end of treatment, cells were incubated with Cell- Counting Kit-8 (CCK-8) solution for 4 hours and absorbance of the orange formazan produced was measured at 450 nm. A) Cell survival plots; B) Table showing calculated IC_{50} of avasimibe for cell lines: IC_{50} was calculated using non-linear regression analysis-log (inhibitor) vs. response equation in GraphPad Prism 7; n=5

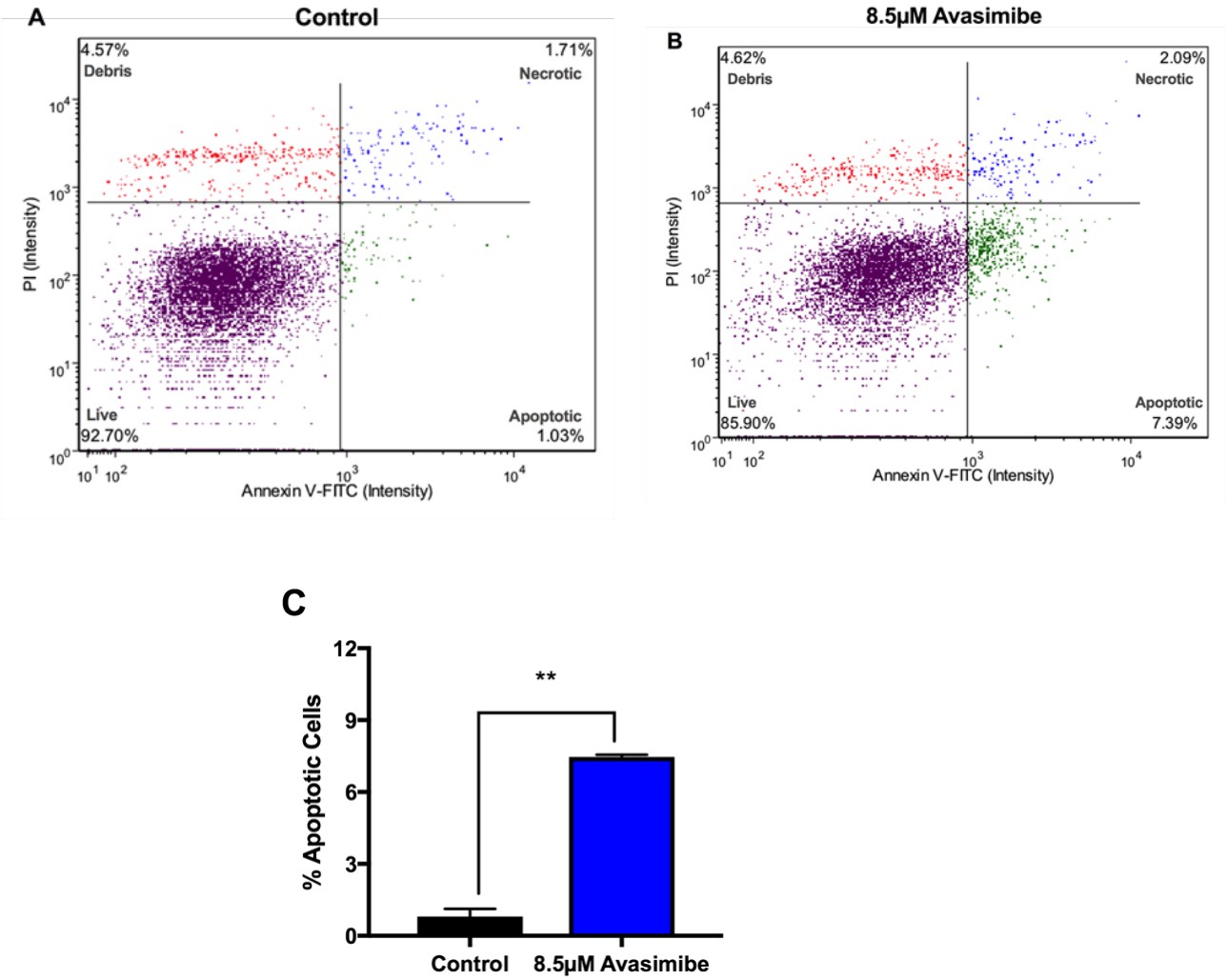


Figure 3.10. ACAT inhibition induces apoptosis: SH-SY-5Y cells were plated at 0.1 x 10^6 cells/mL and allowed to attach overnight. Then incubated in complete media containing 8.5µM avasimibe for 72 hours. After treatment, 1 x 10^6 cells were harvested then resuspended in an Annexin V-FITC and propidium iodide solution and incubated at room temperature for 15 minutes. Stained cells were immediately analyzed using fluorescence detection and an image-based cell counting device. Scatterplots of the results were generated using the FCS Express 6 (De Novo Software). A) Control plots - RPMI supplemented with 10% FBS. B) 8.5µM avasimibe treated plot of Annexin –FITC and PI stained SH-SY-5Ycells after SR-B1 antibody treatment; C) Quantitation of apoptotic cells represented as a bar graph. The percent of apoptotic cells is significantly higher in avasimibe treated cells versus control; n=3, ** p=0.001

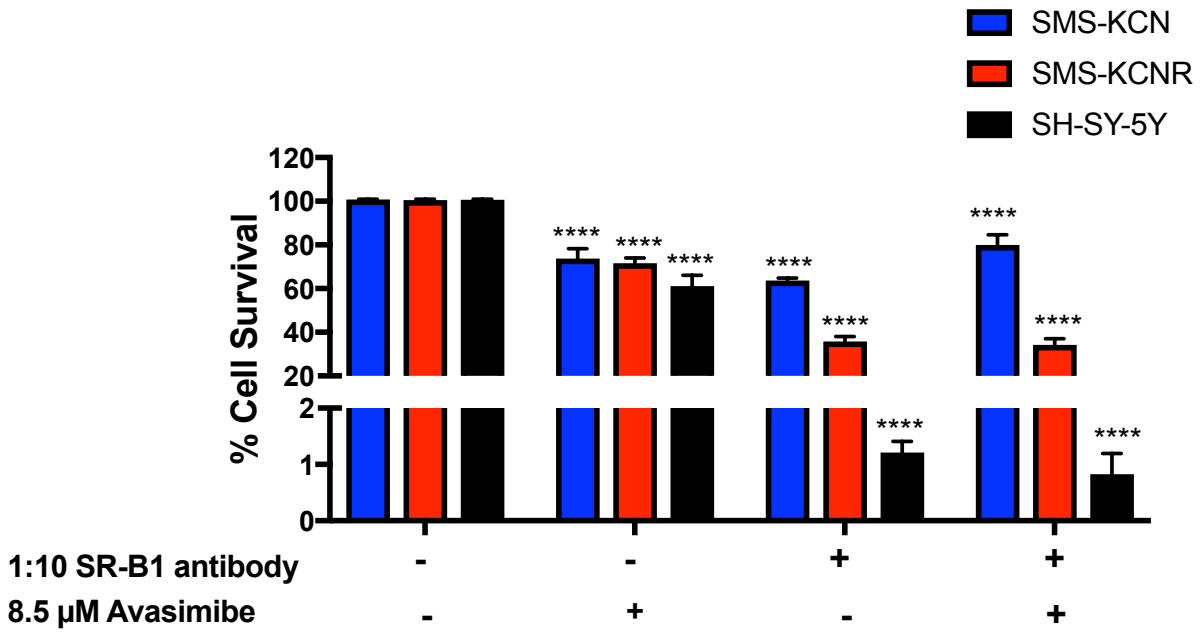


Figure 3.11. Effect of SR-B1 and ACAT inhibition on cell survival: Cells were incubated in medium containing 10% FBS and indicated treatments for 72 hours. At the end of treatment, cells were incubated with CCK-8 solution for 4 hours and absorbance of the orange formazan produced was measured at 450 nm. SR-B1 inhibition results in a greater decrease in cell survival than ACAT inhibition; n=4, ****p<0.0001

3.2.5. SR-B1 inhibition decreases cellular cholesterol content

Several reports showed that inhibition or knockdown of SR-B1 reduced cell cholesterol content [82,84]. Here anti-SR-B1 antibody was use to block SR-B1 activity for 72 hours then cholesterol content of the cells were examine. As shown in figure 3.12, there was a significant reduction in cellular cholesterol fractions between the control and SR-B1 treated cells. This

suggests that the anti-SR-B1 antibody blocks the majority of the high affinity interactions between HDL and SR-B1 on the SH-SY-5Y cells which, inhibited cellular uptake of HDL-cholesterol thus reducing cellular cholesterol content.

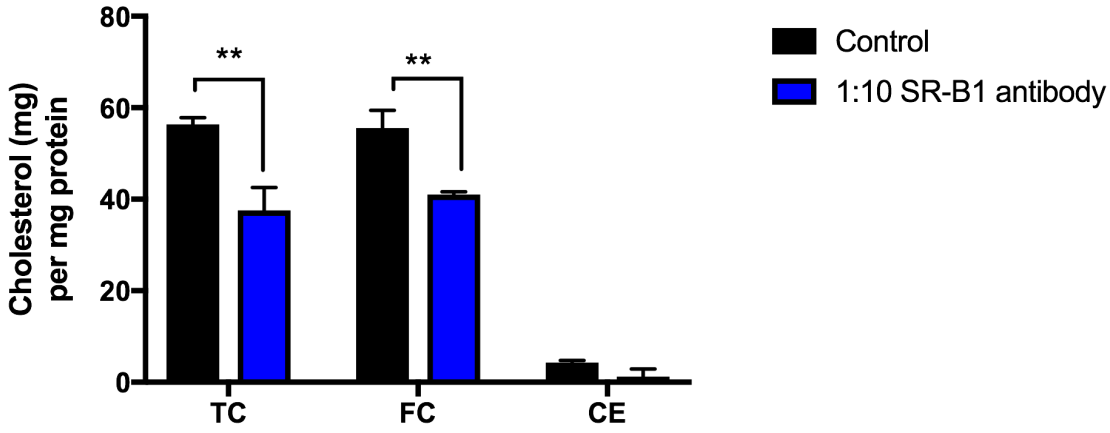


Figure 3.12. SR-B1 inhibition reduces cellular cholesterol content: Cells were treated as indicated for 72 hours. Then cholesterol was extracted from 1 x10^6 SH-SY-5Y cells with hexane: isopropanol (v/v 3:2) solution and total and free cholesterol measured using Wako cholesterol assays kit. Protein content was determined with BCA. The cholesterol content in the cell after SR-B1 inhibition was significantly reduced when compared to untreated control cells. Control indicates RPMI media supplemented with 10% FBS; n=3, **p<0.007
TC- total cholesterol, FC-free cholesterol, CE – cholesteryl ester

3.2.6. Effect of delipidated serum on NB cells

A report by Uda et al. found that deprivation of CE from exogenous sources in CEM-CCRF lymphoblastic cell line decreased cell survival and that adding LDL or HDL rescued cell proliferation [148]. Given that NB cells express relatively high levels of SR-B1 and that SR-B1 facilitates uptake of HDL-cholesterol, we determined the effect of lipid depletion on cell survival, migration, invasion and cholesterol content. Figure 3.13 shows that culturing of cells in media containing 10% delipidated serum (DFBS) decreased cell survival by ~50%. Similarly a reduction in cell migration was also observed when the culture medium was depleted of lipids (Figure 3.13B).

When cancer cells are stressed, in this case by growth in lipid free serum, alterations in cell physiology occur [80]. As shown in figure 3.14, cholesterol content of the SH-SY-5Y cells was significantly reduced when cultured in DFBS. In figure 3.15, Western blot analysis shows increased SR-B1 expression in SH-SY-5Y cells (high-SR-B1 expresser) after 72 hours incubation in DFBS. This suggests that these cells may have increased their SR-B1 expression in an attempt to capture HDL-cholesterol [149]. Conversely no change in SR-B1 expression was observed in the low and moderate SR-B1 expressing cell lines possibly indicating that these cell lines may utilize mechanisms other than SR-B1 to obtain cholesterol.

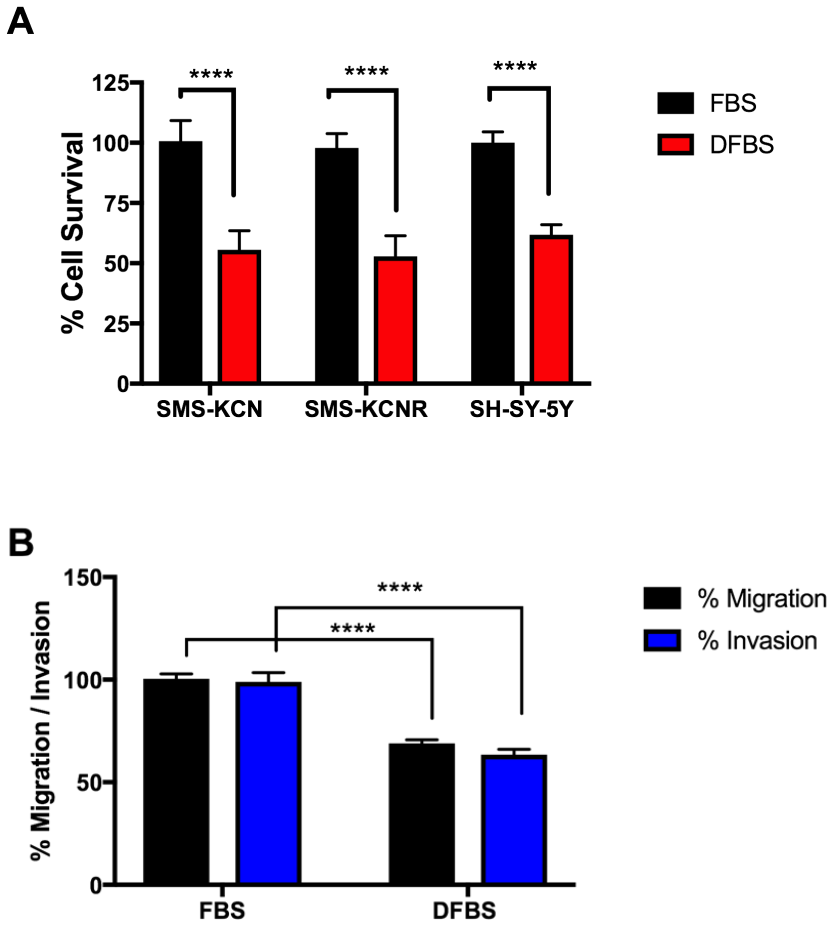


Figure 3.13. Effect of lipid depleted serum on NB cells: A) Reduced cell survival of cells in DFBS: Cells were incubated in FBS or DFBS for 72 hours. Then cell survival was determined using the cell counting kit-8 (CCK-8); n=10 ****p<0.0001 B) Reduced cell migration of SH-SY-5Y cells in DFBS: Cells were incubated in FBS or DFBS for 72 hours then serum starved for 24 hours. The next day 50,000 cells were added to the upper well of the migration or invasion chamber. Subsequently, cells were allowed to migrate overnight. Next, the number of migrated or invaded cells were determined after staining the membrane with crystal violet; n=5 *p=0.03; FBS-Fetal Bovine Serum; DFBS- Delipidated Fetal Bovine Serum.

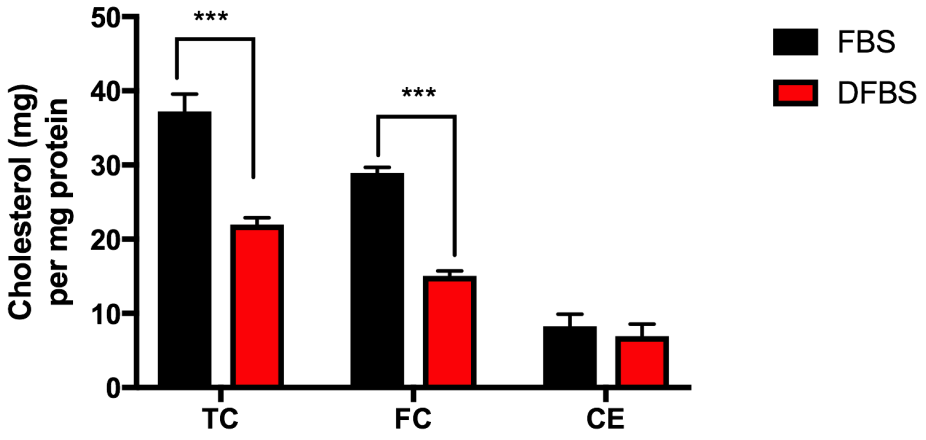


Figure 3.14. Delipidated serum reduces cellular cholesterol content: Reduced cholesterol content in SH-SY-5Y cells: Cells were incubated in DFBS for 72 hours. Then cholesterol was extracted from 1 x10^6 SH-SY-5Y cells with hexane: isopropanol (v/v 3:2) solution. Total and free cholesterol was measured using Wako cholesterol assay kits. Cholesteryl ester content was calculated by subtracting free cholesterol content from total cholesterol. Protein content was determined from the cell pellet via BCA; n=3 ***p=0.0001 FBS-Fetal Bovine Serum; DFBS-Delipidated Fetal Bovine Serum; TC- total cholesterol; FC-free cholesterol; CE – cholesteryl ester

3.2.7. Effect of lipoprotein addition on cell survival

In the previous section, it was shown that excess CE accumulated in SH-SY-5Y cells. Cellular CE originates from either esterification of free cholesterol to CE by the ACAT enzyme, or via import from exogenous lipoproteins [81]. Here, the ACAT inhibitor avasimibe and DFBS were used to study the effect of HDL addition on cell survival. Interestingly, addition of HDL did not significantly improve SMS-KCNR (moderate SR-B1 expresser) cell survival (figure 3.16A). However, as shown in figure 3.17B, addition of HDL increased SH-SY-5Y (high SR-B1

expresser) cell survival in either the presence or absence of ACAT inhibition. This suggests a possible link between SR-B1 expression and the dependence of cells on an extracellular source of cholesterol. The SH-SY-5Y cells expressed higher levels of SR-B1 than SMS-KCNR, which may account for the reduced response to HDL addition in the lower SR-B1 expressing cell line. Higher SR-B1 expression may indicate a greater need for extracellular cholesterol and thus may be a potential target for therapy.

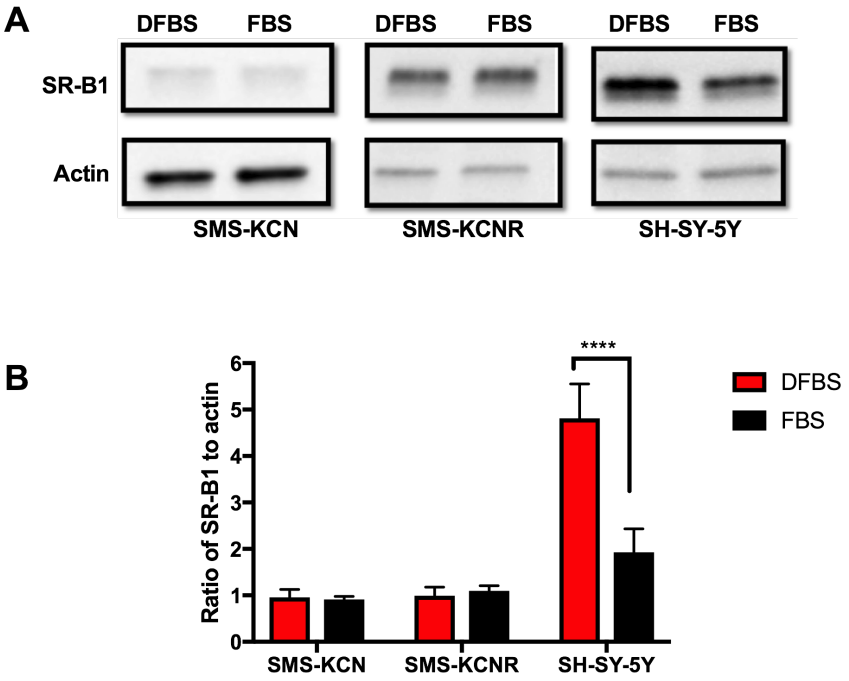


Figure 3.15. Effect of lipid depleted serum on SR-B1 expression: A) Representative Western blots of SR-B1 expression after 72-hour incubation in FBS or DFBS B) Quantitation of SR-B1 expression. The expression of SR-B1 increased in DFBS treated SH-SY-5Y cells. SR-B1 expression in SMS-KCN and SMS-KCNR was not altered by DFBS treatment; n=3, ****$p<0.0001$, FBS-Fetal Bovine Serum, DFBS- Delipidated Fetal Bovine Serum.

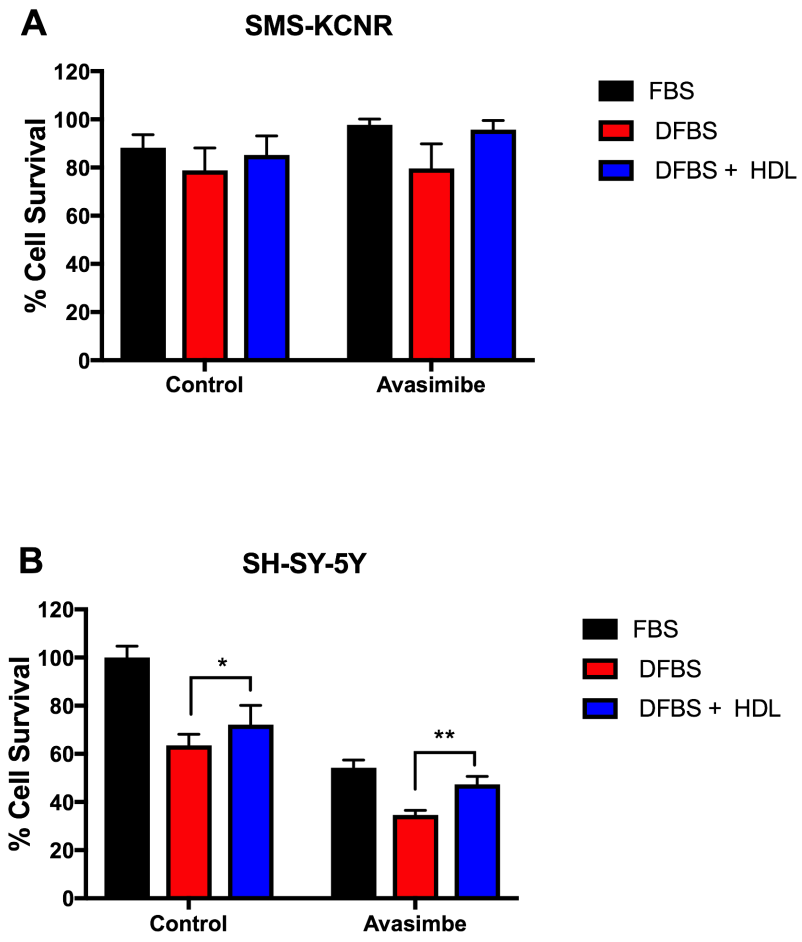


Figure 3.16. Effect of HDL addition on cell survival: Cells were grown in FBS, DFBS or DFBS + HDL for 72 hours then cell survival was measured using the cell counting kit-8 (CCK-8) A) SMS-KCNR and B) SH-SY-5Y cell survival in the presence and absence of ACAT inhibition and HDL. The addition of HDL rescued the survival of SH-SY-5Y cells (high SR-B1 expresser) but in SMS-KCNR cells (moderate SR-B1 expresser) addition of HDL had no significant effect on cell survival. This suggests that the high SR-B1 expressing cell line is more dependent on HDL-cholesterol while the moderate SR-B1 cell line may be dependent on other sources of cholesterol such as LDL; n=3 *p=0.045, **p=0.003; FBS-Fetal Bovine Serum; DFBS- Delipidated Fetal Bovine Serum; HDL- High Density Lipoprotein: LDL- Low Density Lipoprotein

High SR-B1 expression correlates with poor NB patient outcome

In the preceding sections, SR-B1 has been implicated in cell proliferation, migration, invasion, apoptosis and cholesterol metabolism in NB cells. In this section the association between SR-B1 and patient prognosis in NB tissue biopsies was examined. Studies in breast and prostate cancer revealed a higher level of SR-B1 in cancerous tissues as compared to non-malignant tissue and this correlated with tumor aggressiveness and poor prognosis in patients [83,84,86,89].

To determine whether the expression of SR-B1 correlates with event-free and overall survival in patients with NB, we utilized existing RNA microarray data on the R2: Genomics Analysis and Visualization Platform to generate survival curves (Figure 3.17). Table 3.2 shows the characteristics of the patients in the Tumor Neuroblastoma-Kocak-649- custom-ag44kcwolf data set [37]. The International Neuroblastoma Staging System (INSS) classifies tumors into four stages based on clinical evaluation and ease of surgical resection of the tumor [24]. Stage 4 tumors have metastasized to the liver, bone, distant lymph nodes and other organs and are designated as high-risk [19]. Most of the tumors in this data set were stage 4 and were located in the adrenal gland. Analysis of patient survival data by SR-B1 gene expression showed that high SR-B1 expression in 35% of the NB patients and correlated with a significant decrease in event-free and overall patient survival [37,129]. The definition of high and low expression was computer-generated based on the threshold that provided the best distinction between the two populations of SR-B1 gene expression. Moreover, analysis of SR-B1 expression and survival of high-risk patients in this data set also showed a correlation between high SR-B1 expression and poor outcome (Figure 3.18). High-risk patients with low SR-B1 expression had an overall survival probability that was twice higher than that of high-risk patients with high SR-B1

expression. Thus, it is plausible that SR-B1 may be a prognostic indicator of poor outcome in NB and a potential target for therapeutics.

Table 3.2. Patient clinicopathological characteristics*

Variable	N=649 (%)
Age	
Less than 18 months	414 (64)
More than 18 months	235 (36)
Gender	
Female	268 (41)
Male	344 (53)
Unknown	37 (6)
International Neuroblastoma Staging System (INSS) [24]	
Stage 1	153 (24)
Stage 2	113 (17)
Stage 3	91 (14)
Stage 4	214 (33)
Stage 4S	78 (12)
Primary Tumor Localization	
Abdomen	135 (21)
Neck/Chest	69 (11)
Adrenal Glands	276 (43)
Unknown	169 (26)

**Adapted from Kocak et al., Cell Death Dis, 2013[37]*

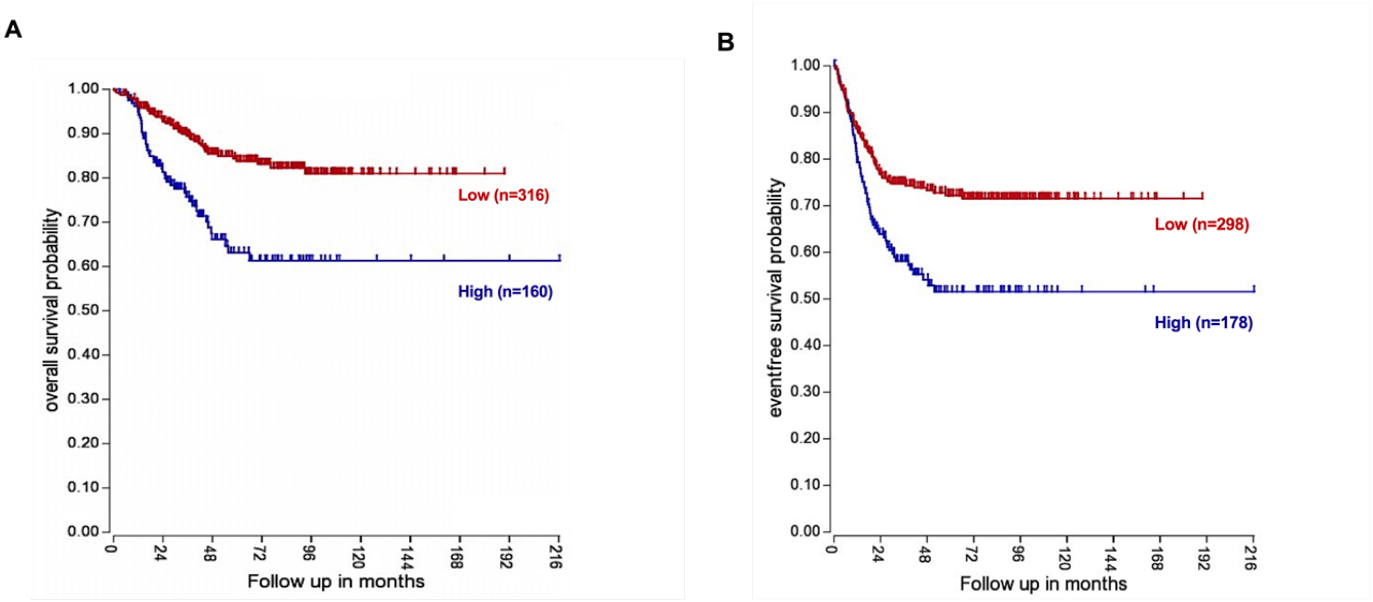


Figure 3.17. High SR-B1 expression is correlated with reduced survival: A) Overall survival Kaplan-Meier curve; chi=20.40, degrees of freedom=1, $p=6.3 \times 10^{-6}$; B) Event-free survival Kaplan-Meier curve. chi=14.72, degrees of freedom=1, $p=1.2 \times 10^{-4}$.Curves were generated with R2: Genomics Analysis and Visualization Platform using Tumor Neuroblastoma-Kocak-649-custom-ag44kcwolf data set. Overall survival describes the length of time the patient survived after diagnosis. Event-free survival describes the length of time the patient remains free of cancer-related adverse events after the end of primary treatment; n=476

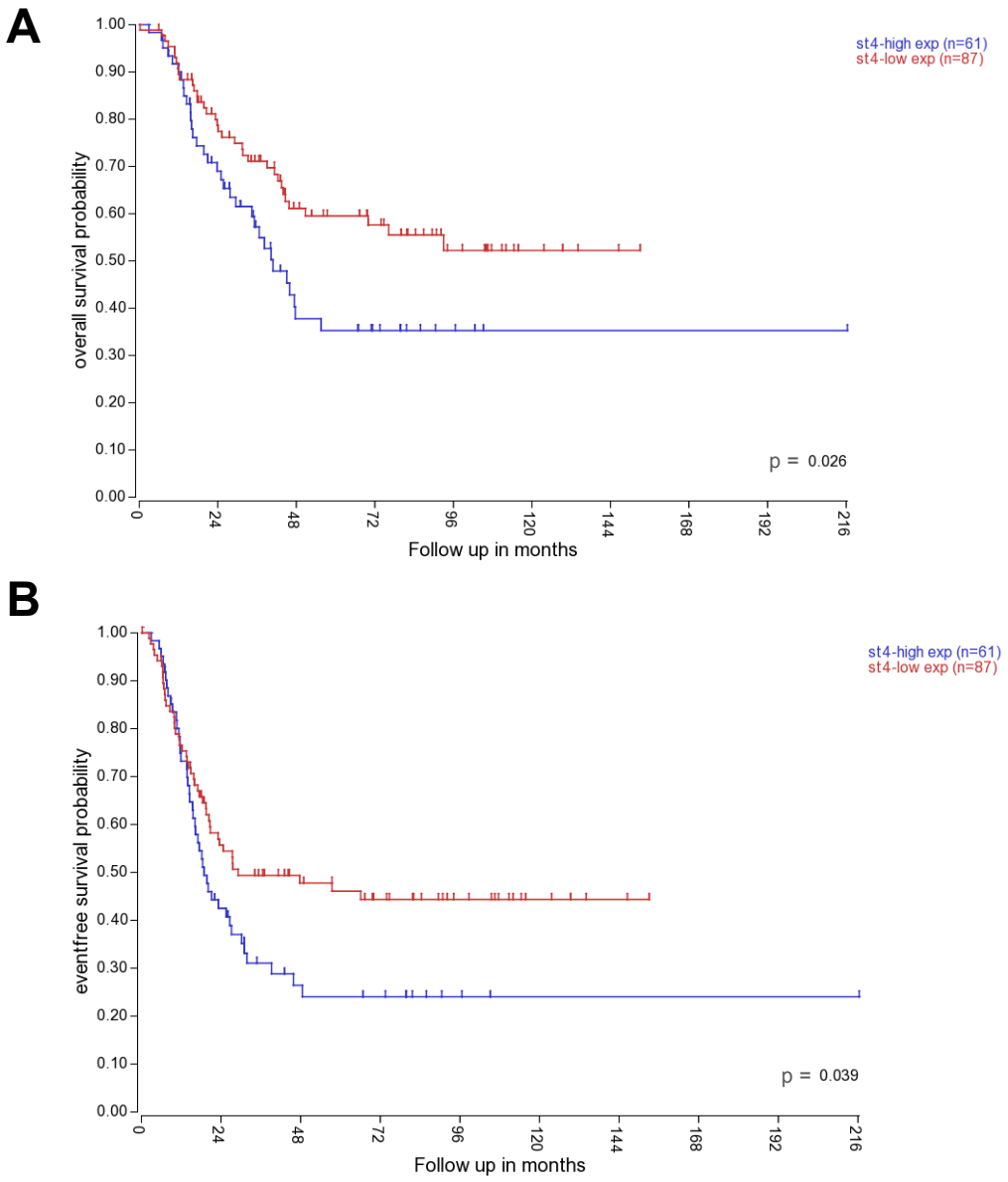


Figure 3.18. High SR-B1 expression is correlated with reduced survival in high-risk (stage 4) NB: A) Overall survival Kaplan-Meier curve; chi=4.98 df=1 p=0.026, B) Event-free survival Kaplan-Meier curve; chi=4.24, df=1, p=0.039. Curves were generated with R2: Genomics Analysis and Visualization Platform using Tumor Neuroblastoma-Kocak-649- custom-ag44kcwolf data set; n=148

CHAPTER 4

DISCUSSION AND CONCLUSION

The aim of this study was to identify the role of scavenger receptor class B type 1 (SR-B1) in neuroblastoma (NB). The findings of this study show that inhibiting SR-B1 reduces cell proliferation, migration, invasion and cholesterol content. Moreover, culturing of cells in lipid free serum resulted in decreased cell survival, which was reversed by the addition of high-density lipoprotein (HDL). Finally, this study showed a correlation between high expression of SR-B1 and poor patient outcome. These findings suggest a link between SR-B1 and NB progression and indicate that SR-B1 may serve as a potential therapeutic target.

Association of SR-B1 expression with NB cell aggressiveness

SR-B1 is a membrane protein that binds HDL facilitating the transport of cellular cholesterol, a critical component for tumor cell proliferation and metastasis [66,79,102,146]. Furthermore, SR-B1 is overexpressed in a variety of cancers [71,82,83,85-87]. In our study, SR-B1 was expressed to varying degrees in all NB cell lines tested, while it was lower in normal human astrocytes. The results are broadly consistent with observations of SR-B1 expression in other malignancies. In nasopharyngeal carcinoma cell lines high expression of SR-B1 was observed [88]. Shahzad et al. confirmed these findings and showed higher expression of SR-B1 in different cancerous tissues but not in normal tissues tested except in the liver [71].

The link between SR-B1 and cell proliferation and aggressiveness, observed in other cancers, prompted an investigation to determine whether SR-B1 plays similar roles in NB. Firstly, three NB cells were selected based impart on their level of SR-B1 expression. SMS-KCN and SMS-KCNR cell lines were obtained from an 11-month old male at diagnosis and post chemotherapy, respectively and showed differential expression of SR-B1. SH-SY-5Y, a thrice sub-line of SK-N-SH obtained from a 2-year old girl post therapy, showed the highest level of SR-B1 expression [135]. The proliferation and migration of SMS-KCN, SMS-KCNR and SH-SY-5Y was also compared. SH-SY-5Y hereafter referred to as the high SR-B1 expresser, had the steepest growth curve and greatest cell migration percentage. While the moderate SR-B1 expresser (SMS-KCNR) and lower SR-B1 expresser (SMS-KCN) showed growth and migration patterns that corresponded with their SR-B1 expression.

In the present study, a role for SR-B1 in the proliferation, migration and invasion of NB was established. Consistent with results obtained in other cancers, inhibition of SR-B1 reduced cell survival, migration and invasion. In a breast cancer study, knockdown or pharmacologic inhibition of SR-B1 also inhibited cell proliferation, migration and invasion [82]. Similarly in prostate cancer, knockdown of SR-B1 reduced cell viability [84]. In our current study, anti-SR-B1 antibody against the extracellular domain of the SR-B1 receptor was utilized as the inhibitor, to limit or eliminate SR-B1 function. Earlier studies have successfully used anti-SR-B1 antibody to inhibit SR-B1 function [56,130,150,151]. Using DiI HDL, Temel et al. showed that SR-B1 antibody inhibited the uptake of DiI HDL in a dose-dependent manner [130]. SR-B1 antibody treatment blocks the high–affinity interactions between HDL and SR-B1 disrupting its function [130,150]. Furthermore, this study showed that inhibition of SR-B1 induced apoptosis in NB

cells. Taken together, these results describe for the first time, specific roles for SR-B1 in NB cell survival, migration and invasion and induction of apoptosis.

SR-B1 regulates cholesterol metabolism in NB

SR-B1 and HDL are well-known for their roles in the development of atherosclerosis and the process of reverse cholesterol transport where HDL carries excess cholesterol from peripheral cells and transports them to the liver for recycling or excretion [62]. In addition, SR-B1 and HDL function in many other cellular processes such as initiation of cell signaling [73], regulation of cholesterol membrane content [54] and phagocytosis of apoptotic cells [70]. Although high expression of SR-B1 has been observed in numerous malignancies [83,86-88] and correlated with regulation of cholesterol metabolism in breast cancer [82,89], the effect of SR-B1 on cholesterol metabolism in NB is yet to be elucidated. Liu et al. revealed the metabolic reprogramming of the serine and cholesterol pathways in NB and suggested metabolic gene expression may be a prognostic indicator for NB [110]. However, neither SR-B1 expression nor its role in NB cholesterol metabolism was investigated sufficiently.

Altered cholesterol metabolism via intracellular accumulation of lipids was observed for the first time in NB cells. This study also identified ACAT activity in NB cells and showed that inhibition of that enzyme with avasimibe reduces cell survival and induces apoptosis. These results are broadly consistent with results obtained in glioblastoma cell lines where avasimibe treatment reduced cell survival and induced cell cycle arrest and apoptosis [126]. We demonstrated that inhibition of both ACAT and SR-B1 did not produce an additive effect; rather the results indicate that SR-B1 may have a greater effect on cell survival than avasimibe.

A study by Uda et al. showed that CE deprivation reduced cell proliferation and suggested that leukemia cells required a lipoprotein source of CE for survival [148]. A limitation

of this study was that the role of the expression of lipoprotein receptors such as SR-B1 was not examined [148]. To bridge this gap, we used NB cell lines with varying SR-B1 expression to determine the effect of lipid deprivation on cell survival, migration, invasion and cholesterol content. As expected, culturing of the cells in delipidated FBS (DFBS) reduced cell survival, migration and invasion as well as diminished cell cholesterol content. Remarkably, lipid deprivation increased expression of SR-B1 in the high SR-B1 expresser cell line, while SR-B1 expression remained unchanged in the low and moderate SR-B1 expressing cell lines. These findings indicate that lipid deprivation may be associated with upregulation of SR-B1 in an attempt to obtain exogenous HDL-cholesterol. Furthermore, this is the first study to show that addition of HDL rescues NB cell survival. It should be noted that HDL addition had no effect on cell survival in the low and moderate SR-B1 expressing cell lines. This data further suggests that a high expression of SR-B1 indicates an increased dependence on an exogenous source of cholesterol, most likely originating from HDL. Our study also found that inhibition of SR-B1 reduced cholesterol content in the NB cells. This observation is new in the NB field and concurs with studies in breast cancer that showed knockdown of SR-B1 reduced cellular cholesterol content [82].

Association between SR-B1 expression and patient outcome

Studies in a number of cancers revealed an association between tumor SR-B1 expression and poor patient prognosis [84-87]. In the present study, SR-B1 gene expression in NB patient biopsies was analyzed using Tumor Neuroblastoma-Kocak-649- custom-ag44kcwolf data and the R2: Genomics Analysis and Visualization Platform [37,129]. High SR-B1 expression was observed in 35% of samples and correlated with reduced patient overall and event-free survival.

Further analysis of this dataset using survival data of the high-risk patients also showed an association between high SR-B1 expression and poor overall and event free survival. These results indicate for the first time the potential prognostic value of SR-B1 in NB.

Neuroblastoma (NB) is often described as an enigmatic disease due to the common variability in patient outcomes. Some NB tumors mature into benign ganglioneuroma while others may spontaneously regress [38]. However, patients older than 12 months have an overall poor prognosis as they frequently present with extensive metastatic disease at diagnosis [19]. The identification of genetic features of NB such as *MYCN* amplification, 1p chromosome deletion or DNA ploidy have greatly impacted NB classification and treatment approaches. Over the years other features such as neurtrophin receptors expression, p53 status, and Akt2 have all provided additional insight into the mechanisms that drive NB [152]. Here SR-B1 is presented as another factor that is associated with NB progression and clinical outcome.

Our findings are anticipated to contribute to the development of SR-B1 as a therapeutic target for NB. The high expression of SR-B1 by NB cells along with its dependence on HDL for cholesterol offers an avenue for novel and potentially improved therapy. In one study, reconstituted high density lipoprotein (rHDL) containing the drug fenretinide was more effective at reducing the viability of NB cells and showed a marked protective effect on non-malignant retinal cells [153]. The targeted delivery of the drug via the SR-B1 receptor diminished off-target toxic effects [153]. Furthermore, a HDL mimetic peptide nanoparticle was shown to inhibit growth of high SR-B1 expressing nasopharyngeal carcinoma [88].

Future studies include knockdown of SR-B1 in an orthotopic NB mouse model to examine the effect of SR-B1 on tumor growth. Additionally, the use of statins to inhibit de novo synthesis of cholesterol may provide insight into the effect of cholesterol synthesis inhibition on

SR-B1 function. Future larger studies with neuroblastoma tissue examining SR-B1 expression as well as analysis of the serum lipoprotein in NB patients to determine if the high SR-B1 expression observed is associated with reduce levels of plasma HDL is recommended.

Conclusions

In summary, the results suggest that SR-B1 expression is associated with NB cell proliferation and migration. Additionally, inhibition of SR-B1 suppressed cell proliferation, migration and invasion, and enhanced apoptosis in NB. SR-B1 potentially regulates cellular cholesterol content and is up regulated in some NB cells during periods of CE deprivation. Taken together, these findings identify SR-B1 as an important regulator of NB progression and a potential therapeutic target for treatment of neuroblastoma.

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