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**EFFECTS OF MEVALONATE PATHWAY INHIBITION
ON HIGH-RISK NEUROBLASTOMA CANCER STEM CELLS**

A Dissertation in
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by

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ABSTRACT

Neuroblastoma is a pediatric solid tumor of the sympathetic nervous system. High-risk patients have a five-year survival rate of about 50%. These high-risk tumors are characterized by treatment resistance and recurrence, due to a population of cancer stem cells (CSCs) that retain characteristics of stem cells present during sympathoadrenal development. Consequently, a single CSC can reform heterogeneous tumors through the process of self-renewal and differentiation into bulk tumor cells.

The mevalonate pathway is upregulated in high-risk neuroblastoma and produces isoprenoid intermediates required for post-translational modifications of small GTPases. These modifications ensure proper protein localization and these proteins are regulators of signaling pathways associated with CSC function and survival. Utilizing three high-risk neuroblastoma cell lines (SK-N-BE(2)-C, SH-SY5Y, and SK-N-AS), we observed a time and dose-dependent decrease in cell viability in response to statin treatment and variable responses to downstream mevalonate pathway inhibitors. Interestingly, fluvastatin treatment induced cellular morphology changes consistent with differentiation. This prompted the development of the spheroid model for high-risk neuroblastoma cell lines.

High-risk neuroblastoma spheroids demonstrated multiple CSC characteristics including plasticity, self-renewal, CSC-associated gene and protein expression, drug resistance, and invasion. In all three cell lines we observed dose-dependent reductions in spheroid formation, self-renewal, and invasion with fluvastatin or zoledronate treatment. We did not observe reductions of spheroid cell viability, or apoptosis. Supplementation with isoprenoids revealed that in the SH-SY5Y and SK-N-AS spheroids these effects occur primarily through a geranylgeranylation-dependent mechanism. By contrast, supplementation of the SK-N-BE(2)-C spheroids revealed that the other branches of the pathway were critical for this cell line.

The investigation on GTPases affected in the SH-SY5Y and SK-N-AS spheroids showed alterations in RhoA, RhoB, total Rap1a/Rap1b, and CDC proteins. In contrast to previous publications, we did not find that the hedgehog pathway is constitutive in the high-risk neuroblastoma spheroid model. Identification of signaling pathways associated with these effects is a key future direction.

These results support the potential development of mevalonate pathway inhibitors for treatment of high-risk neuroblastoma. Additionally, the spheroid model developed can be utilized for studying other pediatric and adult CSC populations.

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List of Abbreviations

3D	three dimensional
AD	Alzheimer's Disease
ADRN	noradrenergic/adrenergic
AKT	protein kinase B
ALK	anaplastic leukemia kinase
AML	acute myeloid leukemia
ANOVA	analysis of variance
ASCT	autologous stem cell transplant
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCL2	B-cell leukemia/lymphoma 2
BNC1	basonuclin 1
BTRC	beta-transducin repeat containing E3 ubiquitin protein ligase
CAR	chimeric antigen receptor
CD	cluster of differentiation
CD114	cluster of differentiation 114
CD133	cluster of differentiation 133, pentatransmembrane glycoprotein
prominin-1	
CD24	cluster of differentiation 24
CD34	cluster of differentiation 34
CDC42	cell division control protein 42 homolog
cDNA	copy DNA

CML	chronic myeloid leukemia
CNS	central nervous system
CO ₂	carbon dioxide
COG	Children's Oncology Group
CREBBP	cAMP response element-binding protein binding protein
CSCs	cancer stem cells
CSNK1	casein kinase 1
DFMO	difluoromethylornithine
DGBP	digeranylbisphosphonate
DHH	Desert Hedgehog
DISP	dispatched gene
DMAPP	dimethylallyl pyrophosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid
EFS	event free survival
EGF	epidermal growth factor
ELDA	extreme limiting dilution analysis
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum

FBXW11	F-box and WD repeat domain containing 11
FDA	Food and Drug Administration
FDFT1	farnesyl-diphosphate farnesyltransferase 1
FGF	fibroblast growth factor
FOH	farnesol
FOXA2	Forkhead box A2
FPP	farnesyl pyrophosphate
FPTase	farnesyl protein transferase
FTI	farnesyltransferase inhibitor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS1	Growth arrest specific 1
GD2	ganglioside G2
GD3	ganglioside G3
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GGOH	geranylgeraniol
GGPP	geranylgeranyl pyrophosphate
GGPTase	geranylgeranyl protein transferase
GGTI	geranylgeranyltransferase inhibitor
GLI	glioma-associated oncogene
GPP	geranyl pyrophosphate
GSK3	glycogen synthase kinase 3
GTPase	guanosine triphosphate
HHIP	Hedgehog interacting protein

HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HMGCS1	3-hydroxy-3-methylglutaryl-coenzyme A synthase 1
I-MIBG	iodine meta-iodobenzylguanidine
I-type	intermediate cells
IC ₅₀	inhibits 50%
ICMT	isoprenylcysteine carboxylmethyltransferase
IDRFs	image defined risk factors
IFT	intraflagellar transport protein
IHH	Indian Hedgehog
IL-2	interleukin-2
INPC	International Neuroblastoma Pathology Classification
INRGSS	International Neuroblastoma Risk Group Staging System
INSS	International Neuroblastoma Staging System
IPP	isopentenyl pyrophosphate
kDa	kilodalton
LDL	low-density lipoprotein
LPPS	lipoprotein-poor fetal calf serum
LRP2	low-density lipoprotein receptor-related protein 2
MAPK	mitogen-activated protein kinase
MEM	minimum essential media
MES	mesenchymal
MES buffer	2-(N-morpholino)ethanesulfonic acid
MIBG	metaiodobenzylguanidine

MKI	mitotic karyorrhexis index
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MSK	mitogen and stress-activated protein kinase
MTSS1	metastasis suppressor 1
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
Mut	mutation
N-type	neuroblast-type cells
NEAA	non-essential amino acids
NeuN	neuronal nuclei
NOR	noradrenergic/adrenergic
ORR	objective response rate
PBS	phosphate buffered saline
PHOX2B	Paired-like homeobox 2b protein
PR	partial response
PRKA	Protein kinase A
PRKX	protein kinase x-linked
PTCH1	Patched 1
qRT-PCR	quantitative reverse transcription – polymerase chain reaction
RA	retinoic acid
Rac	ras-related C3 botulinum toxin substrate protein
RAP	reversible adaptive plasticity
Rap1	Ras related protein 1
Rap1GAP	Rap1 GTPase activating protein

RAS	rat sarcoma protein
RCE1	RAS-converting CAAX endopeptidase 1
RET	Rearranged during transfection protein
Rho	RAS homolog
RIPA	radio-immunoprecipitation assay
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S-type	substrate adherent cells
SCAs	segmental chromosome alterations
SCF	stem cell factor
SCID	severe combined immune-deficient
SEM	standard error of the mean
SHH	Sonic Hedgehog
SMO	Smoothened
Sox2	sex determining region Y-box 2
SQLE	squalene epoxidase
STIL	SCL/TAL interrupting locus
STK36	serine threonine kinase 36
SUFU	suppressor of fused
TICs	tumor initiating cells
tRNA	transfer RNA
wt	wildtype
ZIC	Zic family zinc finger

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Chapter 1

Neuroblastoma

Introduction

Neuroblastoma is a pediatric solid tumor of the sympathetic nervous system. It is the most common cancer in infants (younger than one year old) in the United States with most children diagnosed between the ages of one and two years old and about 90% of cases diagnosed prior to age five.^{1,2} It is the most common extracranial solid tumor in children in the United States with about 700 to 800 new cases each year and accounts for approximately 8% of pediatric cancers globally and in the US.^{2,3} Neuroblastoma patients are stratified by risk groups and while low and intermediate risk-group patients have survival rates above 90% the five-year survival rate for high-risk neuroblastoma remains around 50%.³

Pathogenesis and Genetic Factors

Neuroblastoma is an embryonic neuroendocrine cancer meaning it arises from the stem cells of the sympathetic nervous system, specifically the sympathoadrenal cells of the neural crest.⁴⁻⁶ During normal fetal development the neural crest cells, a type of mesenchymal stem cell, differentiate to form the sympathetic chain, the suprarenal sympathetic ganglion, and the neuroendocrine cells of the adrenal gland.^{5,6} When development goes wrong, due to a combination of genetic and epigenetic factors, these neural crest cells can undergo malignant transformation, leading to development of neuroblastoma tumors.⁶⁻¹⁰ These tumors are characterized by heterogenous populations of mesenchymal (stem) and adrenergic (differentiated)

cell states, that can interconvert, leading to highly plastic tumors.⁶⁻¹⁰ The role of these cancer stem cells (CSCs) will be discussed in more detail in Chapter 2.

Most of the genetic aberrations that contribute to formation of neuroblastoma are sporadic with only about 2-3% of cases being heritable.⁶ The most common familial mutations are activation of anaplastic leukemia kinase (*ALK*) gene or inactivation of the gene for paired-like homeobox 2b protein (*PHOX2B*).^{11,12} Interestingly, sporadic neuroblastoma tumors have low rates of recurrent somatic mutations and only a few known oncogenic drivers.^{13,14} Chromosomal aberrations are significantly more frequent in sporadic neuroblastoma (about 90% of tumors) and the kind of aberration is associated with disease prognosis.¹⁴ Whole chromosome gains frequently result in hyperdiploidy, defined as a deoxyribonucleic acid (DNA) index greater than one, and are associated with more favorable outcomes.¹⁵ Segmental chromosome alterations (SCAs), which are gains and/or losses of chromosome segments, are associated with poor prognosis.^{6,16-18} The most frequent genetic aberration in neuroblastoma is the gain of chromosome 17q and loss of chromosome arms 1p and/or 11q are also recurrent.^{6,14} The most common somatic mutations are *MYCN* amplification (20%), *myc* overexpression (10% high-risk), and activation of *ALK* (6-12%) with mutations also seen sporadically in telomere maintenance genes, activation of the rat sarcoma protein (RAS) – mitogen-activated protein kinase (MAPK) pathway, and alterations in ras-related C3 botulinum toxin substrate protein (Rac)/ RAS homolog (Rho) pathway regulators.^{6,13,19-24} The epigenetic landscape of neuroblastoma is characterized by alterations in DNA methylation, disruption in histone modifications and chromatin composition, and expression of non-coding ribonucleic acids (RNAs).²⁵

Staging and Risk Stratification

At the time of diagnosis patients are stratified into risk groups based on a combination of tumor staging and prognostic factors according to the Children's Oncology Group (COG) criteria.³ The COG criteria consider tumor stage, age at diagnosis, and biological features including *MYCN* amplification, histology, hyperdiploidy, and SCAs, to place children into one of three risk groups: low, intermediate, and high risk.³

There are two staging systems for neuroblastoma: the International Neuroblastoma Staging System (INSS) and the International Neuroblastoma Risk Group Staging System (INRGSS). The INSS was established in 1993 and staged tumors based on potential for surgical resection, location of tumor relative to the midline structures, and lymph node involvement.²⁶ The INRGSS was established in 2004 and uses the absence (L1) or presence (L2) of image-defined risk factors (IDRFs) as predictors for primary tumor resectability.²⁷ The INRGSS designates tumors with distant metastasis as M unless the metastatic spread follows a specific pattern seen in infants less than 18 months of age with spread limited to the bone marrow (<10% involvement), liver and skin in which case the designation is MS.²⁷ Approximately 50% of patients present with metastasis at the time of diagnosis.^{3,28} The most common metastatic sites are the bone marrow, bone and lymph nodes.²⁸

As discussed above the presence of unfavorable biological features including *MYCN* amplification, and SCAs are correlated with increased clinical risk, whereas the presence of hyperdiploidy is associated with lower clinical risk.^{6,13}

The histology of neuroblastoma tumors is classified based on the International Neuroblastoma Pathology Classification (INPC) that considers age of the patients as well as histological features to classify tumors as favorable or unfavorable.²⁹⁻³¹ The histological features considered are the mitosis karyorrhexis index (MKI), grade of differentiation, and the presence of

Schwannian stromal cells. MKI is a measure of the cells undergoing mitosis (proliferation) and karyorrhexis, which is the fragmentation of condensed chromatin, and a low MKI ($<100/5000$ cells) is more favorable than a high MKI ($>200/5000$ cells). Tumors are graded as undifferentiated, poorly differentiated, or differentiated based on the appearance of the tumor cells.³² Tumors that are undifferentiated are characterized by the small round blue cell appearance of mesenchymal stem cells while tumors that are differentiated are characterized by mature ganglion or adrenergic cell appearance.³² Histologically neuroblast tumors are categorized as one of four tumor types: neuroblastoma (Schwannian stroma-poor), ganglioneuroblastoma intermixed (Schwannian stroma-rich), ganglioneuroblastoma nodular (mix of Schwannian stroma-rich/dominant and stroma-poor), and ganglioneuroma (Schwannian stroma-dominant).²⁹⁻³¹

Data from COG analysis indicates that at the time of diagnosis approximately 45% of patients have high-risk disease, 25% have intermediate-risk disease, and 30% have low-risk disease.³ At the time of diagnosis, the majority (74%) of high-risk patients are above the age of 18 months with metastatic disease.³ COG reported that in this high-risk patient population no current biomarkers are prognostic, and the event-free survival and overall survival remain low at $46.1\% \pm 1.6\%$ and $59.4\% \pm 1.5\%$ respectively.³

Treatment

Treatment for neuroblastoma uses a risk-adapted first-line therapy approach, meaning treatment is dependent on the patient's risk status.⁶ This approach has led to a de-escalation of therapy in patients with low- and intermediate-risk disease with continued increased overall survival rates for all risk groups.^{3,6} Throughout treatment the International Neuroblastoma Response Criteria is used to assess overall response as either complete response, partial response, minor response, stable disease or progressive disease.²⁸

Treatment for low-risk patients is minimal as many of these patients present with spontaneous tumor regression.^{33–37} These patients are monitored for symptom development and changes in the tumor presentation.^{33–37} Observation without surgical intervention is sufficient for infants less than 12 months old with adrenal tumors.³⁵ Children above the age of 12 months old with L1 tumors without *MYCN* amplification often undergo surgical resection followed by observation regardless of whether resection was complete or partial.^{33–37} Patients with L2 tumors have a full work-up for accurate risk assessment. There are ongoing clinical trials by COG (NCT02176967) to determine if patients with L2 tumors who are younger than 18 months old, with favorable tumor characteristics and no clinical symptoms can be safely observed without other intervention. Asymptomatic patients with MS disease and favorable tumor characteristics can also be observed.³³ Chemotherapy use is limited to patients with symptomatic disease, or unresectable progressive disease following surgery.^{33,37}

Treatment of intermediate-risk patients uses a response-based approach with a combination of chemotherapy and surgical tumor resection, and as needed radiotherapy.³⁷ Patients are treated with complete surgical resection and between two and eight cycles of neoadjuvant chemotherapy.^{37,38} Patients with more favorable tumor characteristics receive either two or four cycles while patients with unfavorable tumor characteristics receive eight cycles.^{38,39} Chemotherapy drugs used include carboplatin, cyclophosphamide, doxorubicin and etoposide.³⁷ There are ongoing trials to further determine which subsets of intermediate-risk patients can be treated with minimal chemotherapy in conjunction with surgical resection.^{37,39,40} Radiotherapy is used for patients with progressive disease during treatment with chemotherapy and/or patients with unresectable progressive disease following chemotherapy.³⁷

Treatment of high-risk neuroblastoma uses a multimodal approach of induction chemotherapy with tumor resection, followed by consolidation high-dose chemotherapy with autologous stem cell transplant, radiotherapy, and subsequent maintenance therapy of

differentiation therapy and immunotherapy.^{6,37} Induction chemotherapy regimens vary on the chemotherapy drugs used but have a common goal of reducing metastatic disease burden and improving primary tumor resection safety. Induction chemotherapy drugs include cyclophosphamide, topotecan, cisplatin, etoposide, vincristine, doxorubicin, carboplatin, and thiotepe.^{6,41,42} Additionally, ALK inhibitors may be incorporated into the regimen for patients with ALK alterations enrolled in a COG clinical trial.⁴³ Objective response rates (ORRs), defined by at least a partial response (PR), for induction chemotherapy are between 71-85%.^{44,45}

Standard-of-care for surgical tumor resection involves maximal safe resection rather than complete surgical excision due to the frequent involvement of vital structures such as airways and major blood vessels.^{6,37} Consolidative radiotherapy is also delivered preoperative to the primary tumor bed.⁴⁶ The COG regimen also includes radiotherapy to metastatic lesions following induction chemotherapy.^{6,47}

Following induction chemotherapy and surgical resection consolidation with tandem cycles of myeloablative chemotherapy and an autologous stem cell transplant (ASCT) occurs.^{6,37} A COG clinical trial reported improved 3 year-event-free survival (EFS) rates in patients receiving two cycles of myeloablative chemotherapy followed by ASCT (3-yr EFS 61.6%) rather than one cycle (3-yr EFS 48.4%).⁴⁸ This therapy is associated with high rates of long-term side effects and acute organ toxicities.⁶

Maintenance therapy consists of isotretinoin (13-*cis*-retinoic acid) and anti-GD2 antibodies (dinutuximab).^{6,37} Isotretinoin induces differentiation in neuroblastoma cells however despite appearing to increase EFS and OS the effects were not statistically significant in a COG clinical trial.^{49,50} The use of dinutuximab, which targets a cancer specific glycolipid, in conjunction with isotretinoin improves EFS rates (66%) compared to isotretinoin alone (46%) in initial COG clinical trials.⁵¹ Long-term follow-up showed increased 5-year OS with combination therapy (73.2%) compared to isotretinoin alone (56.6%).⁵²

In December 2023, the FDA approved eflornithine (difluoromethylornithine (DFMO)), an ornithine decarboxylase inhibitor, for continuation therapy of high-risk neuroblastoma.³⁷ Ornithine decarboxylase is critical for polyamine biosynthesis and a metabolic dependency in *MYCN* driven neuroblastoma cells.^{53,54} Post immunotherapy patients in remission who received DFMO had increased 4-year EFS rates (84%) compared to matched patients who received traditional maintenance therapy (73%).^{54,55}

Despite these intensive multimodal therapies relapse and/or refractory high-risk disease is common. Approximately 10-15% of patients have progressive disease during induction therapy and an additional 40% relapse after initial response.^{49,56} Salvage chemotherapy regimens report ORR of only 15-20% and long-term cure is unlikely.^{57,58} Patients with relapsed and/or recurrent high-risk neuroblastoma are commonly enrolled in clinical trials. A 2018 analysis of patients enrolled in clinical trials found that the median time to first relapse was 18.7 months, and from the time of early-phase trial enrollment the median time to disease progression was only 58 days.⁵⁹ Out of the 383 patients in this analysis only 33 patients survived long-term.⁵⁹

Considering the substantial relapse and/or refractory disease risk there are a significant number of novel therapies in clinical trials and at various stages of research. These include use of anti-GD2 (ganglioside G2) antibodies for recurrent/relapsed disease and/or during induction chemotherapy regimens.⁶⁰⁻⁶³ As well as the development of chimeric antigen receptor (CAR)-expressing T cells against GD2 and other extracellular neuroblastoma markers.⁶⁴ An anti-GD2/ganglioside G3 (GD3) vaccine has also been developed.⁶⁵ The use of ¹³¹I-metaiodobenzylguanidine (MIBG) therapy to deliver targeted radiation to neuroblastoma cells during induction chemotherapy is being studied in phase III clinical trials in high-risk neuroblastoma.⁶⁶ MIBG is taken up by neuroendocrine cells of the body, including neuroblastoma cells, and acts as a delivery system for radioactive iodine. Targeted therapies remain limited due to the low rate of somatic mutations in high-risk neuroblastoma but are being developed. New

generation ALK inhibitors are in early stages of clinical trials.^{43,67,68} Targeting MYCN and myc, which can also be overexpressed, requires indirect disruption of the signaling pathways through a protein AURORA-A.^{69,70} Inhibitors of AURORA-A are also in early phases of clinical trials.^{71,72}

For surviving neuroblastoma patients, long-term toxicities are seen across risk-groups.^{73–}
⁷⁶ 30% of patients experience neurological deficits, 19% experience musculoskeletal abnormalities, and 16% experience learning and psychological difficulties.^{73–75} High-frequency hearing loss occurs in 10% of low-risk patients, 44% of intermediate-risk patients, and 52% of high-risk patients.^{74,75} Due to the intensive therapy high-risk patients also experience short stature (50%), dental abnormalities (29%), endocrine disorders (28%), hypothyroidism (20%), cardiac insufficiency (16%), hypogonadism (14%), and secondary malignancy (10%).^{73–76}

Conclusion

There is a clear need for the development of novel therapies for the treatment of high-risk neuroblastoma. Due to the age of the patients involved it is critically important to consider quality of life and long-term effects of potential treatment. In Chapter 2 we will discuss the neuroblastoma CSCs. The mevalonate pathway as a potential therapeutic target in high-risk neuroblastoma will be discussed in Chapter 3. The observed effects of mevalonate pathway inhibition in high-risk neuroblastoma and the development of a spheroid model for the study of the CSC population are the focus of Chapter 4. Chapter 5 covers the use of this spheroid model for the investigation of mevalonate pathway inhibitors in the context of neuroblastoma CSCs. Chapter 6 covers preliminary mechanism of action of mevalonate pathway inhibition in neuroblastoma CSCs and Chapter 7 and 8 discuss future directions and conclusions.

Chapter 2

Cancer Stem Cells and the Stemness of Neuroblastoma

Introduction

Neuroblastoma is an embryonal tumor arising from the pluripotent stem cells of the neural crest that fail to undergo normal sympathoadrenal development. The persistence of a CSC population in high-risk neuroblastoma tumors is reflected by the histological and pathological features previously discussed. CSCs are associated with poor patient prognoses, increased tumor aggressiveness, metastatic behavior, treatment resistance and relapse.^{9,77–83} Understanding the nature of these CSCs and their influence on tumor biology and treatment response is critical for development of high-risk neuroblastoma treatment options.

The Cancer Stem Cell Model

The CSC model suggests there is a population of undifferentiated cells within a malignant tumor that can differentiate into the bulk heterogeneous tumor cell population, can maintain their stem cell population through self-renewal, and contribute to treatment resistance and metastasis (Figure 1).^{6,79,80,84–86} CSCs are also frequently called tumor initiating cells (TICs). Early evidence of CSCs was observed and documented initially in hematological malignancies.⁸⁴ Since then, CSCs have been identified in a range of solid tumors including breast cancer, glioblastoma, and pediatric cancers including neuroblastoma, osteosarcoma, and medulloblastoma.^{9,79,80,84–87}

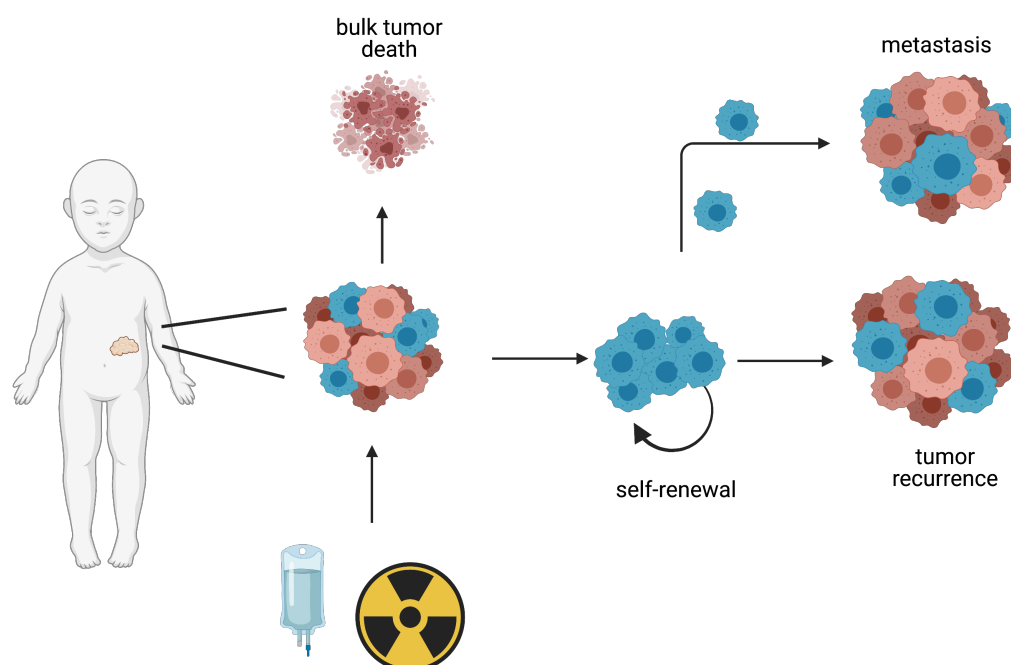


Figure 1: Neuroblastoma cancer stem cells.

This diagram is representative of the CSC model for neuroblastoma tumors. The neuroblastoma tumor is a heterogenous tumor with bulk tumor cells depicted in various shades of salmon/red and CSCs depicted as blue cells. With treatment, depicted as the intravenous fluids bag and radiation symbol, the bulk tumor cells die. CSCs are more treatment resistant and can persist and undergo processes like self-renewal, tumor regeneration, and metastasize. Created with BioRender.com.

Neuroblastoma Cancer Stem Cells

One of the earliest documented proposals for a neuroblastoma CSC population was by J. Robert Cassady in 1984. He hypothesized that there were two clinical patient groups; one with few or no tumor stem cells and a second with many tumor stem cells.⁸⁸ Tumors in the first clinical group were most often found in younger patients and while the tumor cells were malignant, they were not stem cells and could progressively mature or disappear.⁸⁸ This contrasted with tumors in the second clinical group which were most often found in older patients and contained high numbers of these tumor stem cells.⁸⁸ At the time this hypothesis could not be tested but is now supported by modern day evidence.

Histological and Clinical Support for Cancer Stem Cells

The stem-like characteristics of neuroblastoma tumors and the potential origin from undifferentiated neuronal pathways have long been recognized and support the existence of a CSC population.

The stem-like characteristics of neuroblastoma were documented well prior to the proposal of a population of CSCs with James Homer Wright coining the term neuroblastoma in 1910 based on observations that tumor cells from patients mimicked those of undifferentiated nerve cells, neuroblasts.⁸⁹ Subsequently, in the late 1960s and 1970s pathologists recognized the prognostic implications of the degree of differentiation of neuroblastoma tumors and the correlation of presence of maturation with patient survival.^{90,91} By the 1990s the histological characteristics of neuroblastoma tumors were widely accepted as prognostic and the INPC, discussed in Chapter 1, was adapted in 1999 for risk stratification.^{30,32} Prognostically favorable histological features suggest a loss of stem-like characteristics within the tumor as cells appear

differentiated with increased amounts of Schwannian stroma. This contrasts with high-risk neuroblastoma tumors that maintain stem-like characteristics such as high MKI, lack Schwannian stroma and are composed of undifferentiated cells. Furthermore, treatment resistance and frequent relapse observed in high-risk neuroblastoma patients is indicative of a CSC population that can reestablish and maintain bulk tumor mass despite highly intensive treatment regimens.⁸³

Early Neuroblastoma Characterization

Characterization of neuroblastoma tumor cells and *in vitro* cell lines further supports the existence of CSCs. In the 1980s/1990s there were significant research efforts to characterize the heterogeneous cell populations in neuroblastoma tumors, which led to the identification of three cell types based on their morphological appearance and behavior in traditional adherent cell culture.^{90,92} The first two cell types identified were “N” and “S.” N-type cells or neuroblasts were small, rounded cells with neurite outgrowths that grew as loosely adherent cells in culture with properties of noradrenergic neuron signaling.^{90,92} S-type or substrate adherent cells were more epithelial or fibroblastic in cell culture and lacked neuronal features.^{90,92} A third cell type, I-type, or intermediate cells were later discovered and were noted as morphologically and biochemically between N- and S-type cells.⁹⁰ Many parental cell lines were found to contain mixed populations of N- and S-type cells, with some parental lines such as SK-N-BE(2) containing all three cell types.^{90,93,94} The SK-N-SH parental cell line was studied based on the apparent N/S cell type interconversion observed as it was postulated that this transdifferentiation of cell type was indicative of the changes observed clinically during spontaneous regression.⁹² Results published in 1987 on this topic identified cell surface antigens unique to the N- and S-type cells and that the transdifferentiation observed is a controlled alternative differentiation indicative of the stem-like nature of the neuroblastoma cells rather than an uncoordinated random process.⁹³

Further characterization of the I-type cells found that this population of cells are capable of self-renewal as well as multi-lineage differentiation, likely representing a population of malignant CSCs.^{90,95} I-type cells are positive for c-kit and cluster of differentiation (CD) 133, a surface antigen also found on hematopoietic cancer stem cells.⁸⁷ Additionally, I-type cells were the most tumorigenic in a nude mouse xenograft model, followed by N-type cells and lastly S-type cells.⁸⁷ The continued characterization of N- and S-type cells found that while these cell types were also multipotent, they were limited to specific lineages of neural crest development. N-type cells were capable of neuronal like differentiation and S-type cells were capable of glial or Schwannian cell like differentiation.^{90,95}

By the early 2000s the presence of cancer stem cells within neuroblastoma tumors was a significant area of research. I-type cells could be detected in 15 immunostained tumor samples and the frequency of I-type cells correlated with recurrence.⁸⁷ Additionally, a “side population,” a term at the time for a subset of stem cells, were detected in the majority of samples of neuroblastoma cells isolated from patients with relapsed disease.⁹⁶ These stems cells were noted to have *ex vivo* proliferation, asymmetrical division, and expression of drug exporter proteins and c-kit.⁹⁶

Hsu et al. (2013) proposed CD114 as a marker of early (pre-migratory) neural crest cells to identify neuroblastoma CSC populations.⁹⁷ A population of CD114+ cells were detected in all neuroblastoma cell lines tested, including S or N type lines, and these CD114+ cells could rapidly differentiate and produce heterogenous populations with predominantly CD114- cells with a small fraction of CD114+ cells *in vitro* and *in vivo*.⁹⁷ They found that CD114+ cells were enriched in patient populations who had received multiple rounds of chemotherapy or who had relapsed and the CD114+ cells gene expression was consistent with early neural crest derivation.⁹⁷ Interestingly, these CD114+ cells did not express CD133, indicating that the stem-like subpopulations identified in various studies are also heterogenous.⁹⁷

Modern Day Characterization

The use of N-, S- and I- type cell characterization was a historically useful descriptor of cell lines morphological phenotype and some fundamental biological processes of the cell lines. It was however limited as the model originated in extensively cultured cell lines and has since been updated based on molecular characterization.⁸² In 2017, two research groups published findings establishing two distinct cell types within patient samples and neuroblastoma cell lines: adrenergic/noradrenergic and mesenchymal.^{81,82} Noradrenergic/adrenergic (NOR or ADRN) neuroblastoma cells are CD133 negative, express genes involved in adrenergic (neuronal) differentiation, and make up the bulk tumor volume.^{81,82} Mesenchymal (MES) neuroblastoma cells are CD133 positive, express genes associated with mesenchymal stem cells, and are a smaller subset of tumor cells.^{81,82} These cell types display plasticity and the ability to form heterogeneous tumors.^{81,82,98} Interestingly, ADRN/NOR cells were more tumorigenic in mouse models, with both cell types producing heterogeneous tumors, however MES cells were more chemo resistant *in vitro* and were enriched in post-chemotherapy and relapsed patient biopsies.⁸² Ongoing research is being done to understand these cellular identities, much of it with the goal to identify biomarkers and drug targets for subsets of high-risk patients and detect the presence of aggressive subpopulations of tumor cells.

Neuroblastoma Cell of Origin

Neuroblastoma tumors originate during sympathoadrenal development when precursor cells undergo malignant transformation and fail to differentiate, therefore it follows that a population of cells with the tumor maintain a highly stem-like phenotype. The specific precursor cell(s) that give rise to neuroblastoma tumors has not yet been identified.

Jansky et al. (2021) compared the transcriptomes of various cell types throughout adrenal development with those of neuroblastoma tumor cells.⁹ Neuroblastoma tumor cells have high similarity to cells of the developing fetal adrenal gland.⁹ Low and intermediate risk tumors have a higher abundance of late (mature) neuroblast-like tumor cells whereas high-risk tumors have a higher abundance of cycling (developing) neuroblast-like tumor cells.⁹ This suggests that low-risk tumors may arise later in the fetal development pathway. It was also noted that *MYCN* amplified and high-risk tumors had increased reservoirs of additional malignant multipotent cells of the adrenal pathway compared to low-risk tumors.⁹ These similarities were also observed in high-risk neuroblastoma cell lines with substantial populations of Schwannian cell precursor-like cells and early progenitor-like cells in the mesenchymal SK-N-AS cell line.⁹ These findings strongly support the presence of CSCs in neuroblastoma tumors and cell lines.

Cancer Stem Cells and Spheroid Culture

Alternative cell culture techniques that enable the study of the CSC population are an emerging area of active research. Investigation on the potential role for CSCs in relapsed disease found that neuroblastoma cells isolated from tumors and/or bone marrow aspirates, a common metastatic site, could be cultured as spheres in neural crest stem cell conditions.⁸⁰ While low and high-risk patient samples both formed primary spheres, tumor spheres from high-risk patients could be maintained with passaging while the low-risk tumors formed adherent culture when passaged. The bone marrow aspirates of two high-risk patients in remission took the most time (8 weeks vs median 2 weeks) but did form tumor spheres. High-risk tumor spheres, including those from patients in remission, formed primary tumors and metastatic disease when injected into the adrenal fat pad of immunocompromised mice; as few as 10 high-risk unsorted tumor sphere cells were required.⁸⁰ All the tumor spheres formed expressed the neural crest progenitor markers

fibronectin and nestin. A subpopulation of tumor sphere cells expressing CD24 and CD34 were present in high-risk tumor spheres and cell selection for these markers enriched the tumor formation in immunocompromised mice.⁸⁰ It was subsequently shown that neuroblastoma cell lines express neural stem cell markers in various abundances and are also capable of tumor sphere formation in neural crest stem cell media.⁷⁹ The use of spheroid culture alters the protein expression of high-risk neuroblastoma cell lines compared to adherent cell culture, in a manner that is more consistent with the three dimensional (3D) tumor microenvironment.⁷⁷ Increased glycolytic proteins, which are consistent with the Warburg effect, increased heat shock proteins, which are associated with inhibition of apoptosis, angiogenesis, and chemoresistance, and alterations in cytoskeletal proteins were observed.⁷⁷

The ability for neuroblastoma cells to be cultured in both adherent cell culture (anchorage-dependent) and non-adherent (anchorage-independent) sphere cultures is due to reversible adaptive plasticity (RAP).⁹⁹ RAP describes the mechanism by which neuroblastoma cells, *in vitro* and *in vivo*, maintain the ability to convert between phenotypes in response to changes in the environment such as drug treatment, or hypoxia.⁹⁹ It was demonstrated that in response to treatment mouse tumors temporarily transition phenotypes towards that seen in anchorage independent cell culture, and that when treatment was withdrawn the tumor reestablished a mixed heterogenic phenotype.⁹⁹ Mahller et al. argues that plasticity also contributes to the lack of a consistent antigen marker for neuroblastoma CSCs and that functional assays, such as sphere-formation, are critical.⁷⁹

The use of spheroid cultures to identify potential therapeutics for treatment of high-risk neuroblastoma by targeting the CSCs is a relatively new area of pharmacology. Liu et al. (2016) characterized sphere-forming cells isolated from primary TH-MYCN (*MYCN* amplified) mouse neuroblastoma tumors.⁷⁸ These sphere-forming cells were capable of self-renewal *in vitro* and *in vivo* with generation of heterogeneous tumors like those of the parental tumors. Gene expression

analysis found that sphere-forming cells were less differentiated than the bulk parental primary tumor cells and were enriched for two biosynthesis pathways: serine-glycine synthesis pathway and cholesterol biosynthesis pathway.⁷⁸ When either pathway was inhibited sphere-forming cell growth, and tumor formation were inhibited. Further investigation in human neuroblastoma cell lines found that these metabolic pathways are required for growth of high-risk cell lines, regardless of *MYCN* status. Investigation on the clinical relevance of these findings found that metabolic pathway genes were expressed at higher levels in high-risk neuroblastoma tumors than low-risk tumors and that higher expression was associated with decreased event-free survival.⁷⁸ This study highlights the utility of sphere-forming assays in identification of high-risk neuroblastoma therapeutic targets. The cholesterol biosynthesis pathway, also known as the mevalonate pathway, will be discussed further in Chapter 3.

Conclusion

Neuroblastoma tumors have long been recognized for their stem-like properties and the potential for a CSC population to be driving the treatment resistance and relapse in high-risk patients. The high degree of cellular heterogeneity between patients and within individual patient tumors is a lasting hallmark of the embryonic origin. This combined with the observed plasticity of neuroblastoma cells has complicated the identification of CSCs while simultaneously supporting their existence. Spheroid culture models provide a means to analyze the functional effects of therapeutics on the CSC population and stem-like characteristics of neuroblastoma tumor cells in a more biologically relevant system than adherent cell culture. The development of a spheroid culture system to analyze the effects of mevalonate pathway inhibition on neuroblastoma CSCs will be discussed further in Chapter 4.

Chapter 3

The Mevalonate Pathway

Introduction

The mevalonate pathway, also known as the cholesterol biosynthesis pathway or the isoprenoid pathway, has been implicated in the growth and survival of high-risk neuroblastoma cells.^{100–105} Inhibition of this pathway induces differentiation and alters stem-like features of neuroblastoma cells in traditional adherent cell culture through reductions in protein geranylgeranylation.^{102,103} The use of spheroid cultures enables us to study the effects of inhibition on the neuroblastoma CSC population specifically and is the focus of this dissertation.⁷⁷

Altered Metabolism – A Hallmark of Cancer

One of the hallmarks of cancer is a reprogramming of cellular metabolism in tumor cells. One effect, known as the Warburg effect, describes the shift towards aerobic glycolysis that occurs and is enriched for in hypoxic conditions in the tumor microenvironment.^{106–108} This shift is less energy efficient for the production of adenosine triphosphate (ATP) than the mitochondrial oxidative phosphorylation but enables the shunting of glycolytic intermediates to other metabolic pathways.¹⁰⁶ These pathways can then use the intermediates for production of other macromolecules such as proteins and amino acids.¹⁰⁹

Additionally, it has been shown that high-risk neuroblastoma tumors have an upregulation of multiple mevalonate pathway genes identified as metabolic oncogenes.^{110,111} As

discussed in Chapter 2, the Han-Fei Ding lab found that the genes 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (*HMGCS1*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*), and squalene epoxidase (*SQLE*) are upregulated in high-risk neuroblastoma compared to low-risk neuroblastoma and that higher expression was associated with advanced stage and lower event-free survival.^{78,112} Additionally, this lab found that expression of farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*) the gene that encodes the enzyme of the first committed step of the cholesterol branch is associated with high-risk neuroblastoma and worse patient prognosis.¹¹² *HMGCR*, and *SQLE*, catalyze the rate-limiting step of the mevalonate pathway and the rate-limiting step of the cholesterol branch respectively.¹¹³ Both genes have been identified as metabolic oncogenes in solid tumors. Induced expression of *HMGCR* induces transformation in mouse embryonic fibroblast cells and increases growth in both transformed and nontransformed cells *in vitro* soft agar cultures and *in vivo* xenograft mouse models.¹¹⁰ In breast cancer *SQLE* overexpression is associated with poor prognosis and pharmacological inhibition can decrease breast cancer cell viability.¹¹¹ A recent study has also found that serum total cholesterol levels in neuroblastoma patients is a risk factor for recurrence and death among children.¹¹⁴

The Mevalonate Pathway

The mevalonate pathway (Figure 2) produces isoprenoids that serve as precursor molecules for macromolecules such as cholesterol, ubiquinone, and dolichol, as well as post-translational modifications for proteins in the RAS superfamily of GTPases.¹¹³ The first step of the pathway synthesizes 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) from three acetyl-CoA molecules via the HMG-CoA synthase enzyme.^{113,115} HMG-CoA is then reduced by *HMGCR* in the rate limiting step of the pathway to form mevalonate.^{113,115} Mevalonate is then converted into

isopentenyl pyrophosphate (IPP), a five-carbon chain compound. IPP can be isomerized to dimethylallyl pyrophosphate (DMAPP), which is used in production of transfer RNA (tRNA), or further condensed to form geranyl pyrophosphate (GPP), a ten-carbon chain compound.^{116,117} The addition of another IPP molecule to GPP produces the isoprenoid farnesyl pyrophosphate (FPP), a fifteen-carbon compound.¹¹⁸ GPP and FPP are both synthesized by the enzyme FPP synthase.¹¹⁸ At this point the pathway branches. FPP can be used for the synthesis of other molecules including dolichol, utilized for production of squalene which drives sterol synthesis, used for further isoprenoid synthesis, or used for the prenylation of proteins.^{119,120} The addition of another IPP to FPP by the enzyme geranylgeranyl pyrophosphate synthase generates the twenty-carbon chain isoprenoid geranylgeranyl pyrophosphate (GGPP), used for the prenylation of proteins.¹¹⁹ Prenylation of proteins is carried out by the protein prenyltransferases, farnesyl protein transferase (FPTase) and geranylgeranyl protein transferase (GGPTase) I and II for FPP and GGPP respectively.^{121–125}

The mevalonate pathway can be targeted therapeutically at multiple steps (Figure 2). Statins inhibit HMGCR through binding of the enzyme active site which sterically preventing HMG-CoA binding.^{126,127} This class of compounds was originally isolated from fungal metabolites and lovastatin was approved for the treatment of hypercholesterolemia in 1987.^{128,129} The structure of statins can be broken into three parts: an HMG-CoA analogue, a hydrophobic ring structure, and side groups.¹³⁰ The side groups influence the solubility of these compounds and statins can be categorized as either lipophilic or hydrophilic based on this.¹³⁰ Lipophilic statins, including lovastatin and fluvastatin, can easily enter cells and are the focus of this dissertation.¹³⁰ Important to note that lovastatin and simvastatin have intact lactone rings that are enzymatically hydrolyzed *in vivo* to their active form and require activation for *in vitro* use. Treatment with statins initiates a feedback loop which leads to increased production of HMGCR, however this does not appear to diminish potential therapeutic use in cancer cells.^{128,131} Statins

have also been found to synergize with other chemotherapy compounds for the treatment of cancer.^{132–134}

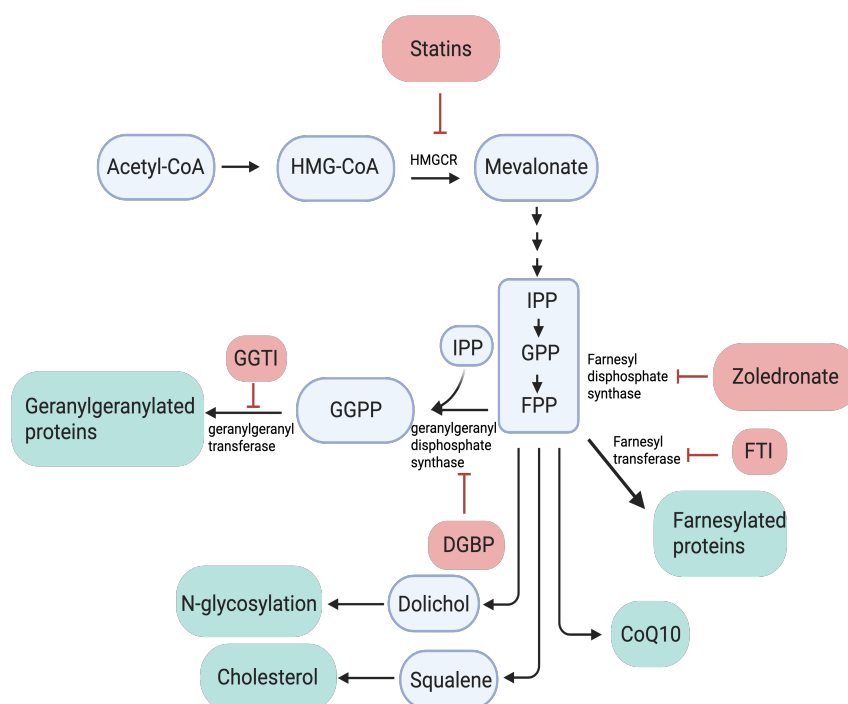


Figure 2: The mevalonate pathway.

The mevalonate pathway, alternatively called the cholesterol biosynthesis pathway or the isoprenoid biosynthesis pathway. This pathway produces lipid molecules of increasing carbon chain lengths known as isoprenoids (IPP: isopentenyl pyrophosphate 5-carbons, GPP: geranyl pyrophosphate 10-carbons, FPP: farnesyl pyrophosphate 15-carbons, and GGPP: geranylgeranyl pyrophosphate 20-carbons). FPP and GGPP are utilized for protein post-translational modification. FPP can be further processed for production of larger biolipids including CoQ10, cholesterol, and dolichol. This pathway can be targeted at multiple steps. Statins inhibit HMG-CoA reductase (HMGCR), while the bisphosphonates zoledronate and digeranylbisphosphonate (DGBP) ‘inhibit *synthase* enzymes. The transfer of isoprenoids onto proteins can also be blocked with transferase inhibitors (FTI: farnesyl transferase inhibitor and GGTI: geranylgeranyl transferase inhibitor). Created with BioRender.com.

Bisphosphonates, including zoledronate (zoledronic acid) and digeranylbisphosphonate (DGBP), inhibit isoprenoid production downstream of mevalonate production.^{135–137}

Bisphosphonates are a class of compounds that mimic pyrophosphoric acid, that have a high affinity for the bone matrix.¹³⁸ Research has shown these compounds can block osteoclast activity, which are the cells that break down bone tissue, during cancer metastasis.^{135,136,138}

Additionally, these compounds are cytotoxic to cancer cells themselves through inhibition of the mevalonate pathway.^{135,136,138} Zoledronate, a nitrogen-containing bisphosphonate, inhibits FPP synthase and was approved for the treatment of bone metastasis in breast and prostate cancer patients in 2001.¹³⁵ DGBP is an inhibitor of geranylgeranyl diphosphate synthase, that blocks production of GGPP without effecting FPP production.^{139,140}

Protein prenylation is critical for the proper function of signaling proteins including the small GTPases of the Ras and Rho families and can be therapeutically targeted with transferase inhibitors as well as upstream inhibitors including statins and bisphosphonates.^{121,141,142} The addition of the lipid portion of FPP or GGPP onto the C-terminal of these GTPases enables proper trafficking to cellular membranes.¹²¹ The C-terminal of these proteins contains a conserved CAAX domain, of which the AAX amino acid group determines whether FPP or GGPP will be utilized.¹⁴³ Over 750 human proteins contain this CAAX domain, however only a small percentage have been found prenylated *in vivo*.¹⁴⁴ Following protein synthesis, the protein prenyltransferases add the lipid onto the cysteine residue of the CAAX domain in the cytosol.¹⁴⁵ Further processing of the CAAX domain by proteolysis and methylation by RAS-converting CAAX endopeptidase 1 (RCE1) and isoprenylcysteine carboxymethyltransferase (ICMT), two endoplasmic reticulum (ER) membrane enzymes, occurs and proteins can be properly trafficked within the cell.^{143,145} Treatment with lovastatin can alter the expression of small GTPases with

increased total protein levels reported for the GTPases: Ras, Ras-related protein 1 (Rap1), RhoA, and RhoB.¹⁴⁶

The ability to target the mevalonate pathway at multiple steps enables thorough mechanistic studies on what branch of the pathway is critical for the function and survival of CSCs. Additionally, downstream products of the pathway can be supplemented for mechanistic assessment. Human cells can convert the alcohol forms of FPP and GGPP, farnesol (FOH) and geranylgeraniol (GGOH), into their respective isoprenoids for use in protein prenylation.¹⁴⁷ The enzyme that catalyzes this has not yet been identified.

The Mevalonate Pathway and Neuroblastoma

Statins

The role of the mevalonate pathway in neuroblastoma cells and potential inhibition as a therapeutic target has been studied for decades both in the context of cancer research as well as in the fields of neurodevelopment and neurodegenerative diseases due to neuroblastoma cell lines' utility as a model system.

Initial research in the early 1980s on neuroblastoma and the mevalonate pathway utilized the murine neuroblastoma cell line Neuro2A.^{148,149} When these cells were treated with activated lovastatin, they appeared to undergo morphological differentiation with extension of neurite outgrowths and reduced DNA synthesis.^{148,149} Investigation on the role of sterol content in these cells suggested that the primary effects of mevalonate pathway inhibition was due to an unidentified product of the mevalonate pathway, not cholesterol.¹⁴⁸ Interestingly, these papers were published only months prior to the identification of prenylated proteins as a product of the mevalonate pathway.^{150–153}

Further research in mouse models of neuroblastoma on the mechanism of induced differentiation found that the morphological changes of neurite-like outgrowths appeared quickly, within four hours of treatment with statins, whereas reductions in cell proliferation and cell cycle arrest were not observed prior to 24 hours of treatment.¹⁵³ Additionally, adding back low-density lipoprotein (LDL) cholesterol, dolichol, ubiquinone, and/or isopentenyl adenine could not rescue cells from these effects of lovastatin treatment.¹⁵³ Only the addition of mevalonate blocked differentiation induced by lovastatin.¹⁵³

In the 1990s this work expanded in human neuroblastoma cell lines. In 1996 researchers noted that a panel of human neuroblastoma cell lines had elevated HMGCR messenger RNA (mRNA) levels compared to non-neuronal tumor derived cell lines and that human neuroblastoma cell lines displayed variable sensitivity to treatment with activated lovastatin that was not correlated to *MYCN* amplification status.¹²⁷ Sensitive cell lines displayed morphological changes consistent with those observed in murine lines including initial cell flattening and neurite formation followed by cell rounding, detachment and cytotoxicity.¹²⁷ Interestingly the most sensitive cell lines did not show increased HMGCR mRNA levels following lovastatin treatment, indicating that the common feedback loop may not be functional.¹²⁷ Alternatively, nonresponsive and intermediately sensitive cells lines did show increased HMGCR mRNA levels post treatment, indicating a functional feedback system.¹²⁷ This research also noted that when human neuroblastoma cell lines were treated with retinoic acid (RA) HMGCR mRNA levels were also reduced, strengthening support for the use of mevalonate pathway inhibition as a therapeutic option in neuroblastoma.¹²⁷

These findings were consistently repeated in the early/mid 2000s with activated lovastatin treatment in additional human neuroblastoma cell lines, as well as additional cancer types including other embryonal pediatric cancers such as medulloblastoma, rhabdomyosarcoma, and hepatoblastoma.^{100,101,104,105} Treatment of these pediatric tumor types with lovastatin induces

variable responses with some cell lines displaying high sensitivity and others nonresponsive.¹⁰⁰ Neuroblastoma cell lines that are responsive display time and dose-dependent effects and have been found to undergo apoptosis following 48 or 72-hour treatment with lovastatin as well as decreased mitochondria activity and reduced colony formation, all of which can be rescued with the addition of mevalonate.^{100,104,105,154} Researchers also found that combination treatment of lovastatin or simvastatin with another differentiating agent such as cyclic-AMP or dimethyl sulfoxide (DMSO) enhanced the induced effects.^{154,155}

Throughout the past decade research on the potential use of statins as treatment for specifically high-risk neuroblastoma has continued. As previously discussed, the Han-Fei Ding lab identified the mevalonate pathway as critical for the growth and survival of high-risk neuroblastoma cell lines regardless of *MYCN* amplification status.⁷⁸ Specifically, they used a panel of five high-risk neuroblastoma cell lines (CHLA-90, IMR32, SHEP1, SK-N-AS, and SK-N-BE(2)) treated with simvastatin for up to six days and observed a dose- and time- dependent decrease in cell number.⁷⁸ Consistent with prior results with lovastatin treatment the cell lines showed variable sensitivity to simvastatin.⁷⁸ In 2017 Alizadeh et al. characterized the response of multiple solid tumor cell lines to simvastatin treatment including the high-risk neuroblastoma cell line SH-SY5Y.¹⁰² These researchers noted a dose- and time-dependent treatment response with induction of cell death through the intrinsic apoptosis pathway consistent with previous findings.¹⁰² Importantly, they expanded on previous addback experiments in the context of cancer research via supplementation with mevalonate, cholesterol, FPP or GGPP. Consistent with previous findings supplementation with cholesterol did not rescue neuroblastoma cells treated with simvastatin but supplementation with mevalonate did confirming the pathway inhibition.¹⁰² The addition of FPP or GGPP also rescued SH-SY5Y cells treated with simvastatin indicating the loss of prenylation as the key mechanism of action in these cells.¹⁰²

Further investigation on the mechanism of action of statin treatment in high-risk neuroblastoma cell lines by Stokes et al. (2020) found that mesenchymal cell lines, specifically SK-N-AS and NLF, were highly sensitive to mevalonate pathway inhibition by lipophilic statins (lovastatin, simvastatin and fluvastatin).¹⁰³ The hydrophilic statins tested (pravastatin and rosuvastatin) had little effect and *MYCN* amplification status did not affect statin sensitivity.¹⁰³ Consistent with previous findings FPP or GGPP could rescue mesenchymal cell line viability however only treatment with a geranylgeranyltransferase inhibitor (GGTI-298) recapitulated the reduced cell viability observed with statin treatment, whereas a farnesyltransferase inhibitor (FTI-277) had minimal effect.¹⁰³ Additionally, western blot analysis for RAS prenylation in the NLF cell line demonstrated a band shift with lovastatin treatment consistent with the loss of prenylation.¹⁰³ Together these results demonstrated that high-risk mesenchymal neuroblastoma cell lines were sensitive to statin treatment due to the resulting loss of protein prenylation.

Additionally, Kuzyk et al. found that simvastatin treatment of SK-N-AS cells induces alterations in cellular metabolism prior to induction of apoptosis, which occurs around 48 hours post-treatment.¹⁵⁶ These alterations included impaired mitochondrial function during acute treatment that by 24-hours was recovered, and a significant impairment in glycolysis with 24-hour treatment.¹⁵⁶ Based on these findings Anderson et al. tested the potential benefit of combination treatment of SK-N-AS cells with simvastatin and two chemotherapies doxorubicin and cisplatin.¹⁵⁷ Pre-treatment of these cells with simvastatin increased the cytotoxic effects of doxorubicin while co-treatment of these two compounds did not.¹⁵⁷ Conversely pre-treatment of these cells with simvastatin did not increase the cytotoxic effects of cisplatin while co-treatment of simvastatin and cisplatin resulted in an increased cytotoxic effect.¹⁵⁷

The Han-Fei Ding lab has continued to investigate the potential utility of statin treatment for high-risk neuroblastoma, with a focus on *MYCN* amplified neuroblastoma. They found that the mevalonate pathway is critical for tumor growth in the commonly used TH-MYCN mouse

model of neuroblastoma.¹¹² Additionally, they treated multiple neuroblastoma cell lines including *MYCN* amplified (BE(2)-C, IMR5, SMS-KCNR) and non *MYCN* amplified cell lines (SHEP1 and SK-N-AS) with simvastatin and pitavastatin for four days and consistent with previous findings noted a dose-dependent variable sensitivity to statin treatment in these high-risk neuroblastoma cell lines.¹¹² They also treated with prenylation transferase inhibitors FTI-277 and GGTI-298 and noted that treatment with FTI-277 did not have a significant effect but GGTI-298 inhibited cell survival and proliferation.¹¹² In the context of *MYCN* amplified high-risk neuroblastoma they have also found that knockdown of the cholesterol branch of the mevalonate pathway reduces tumor growth in mouse models, treatment with simvastatin downregulates mitotic genes, and that statin sensitivity is regulated by the FOXM1 transcription factor.¹¹² Interestingly, they also noted that caffeine treatment synergizes with statin treatment by reducing the feedback response in *MYCN* amplified and non-amplified cell lines.¹¹²

A recent publication by Correia et al. found that treatment of the high-risk neuroblastoma cell line SH-SY5Y with atorvastatin under both normal cell culture conditions and chemically induced hypoxia varied indicating an important role of the tumor microenvironment to statin response.¹⁵⁸ Taken together these results indicate that there is significant evidence of lovastatin cytotoxicity and induced altered differentiation of high-risk neuroblastoma cell lines due to the loss of protein prenylation, specifically geranylgeranylation, in traditional cell culture and neuroblastoma mouse models. These findings support the hypothesis that statin treatment could affect the CSC population in high-risk neuroblastoma, however this has not been previously investigated and is the focus of this dissertation.

In the context of neurodegenerative diseases researchers found that the closed-ring form of mevastatin could reduce differentiated mouse neuroblastoma cell viability through inhibition of proteasome activity, which was reversible with the addition of mevalonate.¹⁵⁹ This effect was not observed with the open-ring structure statin, pravastatin.¹⁵⁹ Additionally, in an Alzheimer's

disease (AD) model activated simvastatin and lovastatin treatment of mouse neuroblastoma cells altered the GTPase mobility on western blot consistent with the loss of prenylation and disrupted GTPase membrane localization.¹⁶⁰ In a different AD model using mouse neuroblastoma cells atorvastatin induced a time and dose-dependent effect on neurite outgrowths and the expression of a glycoprotein relevant to AD that could be rescued with FOH addbacks.¹⁶¹ Alternatively in a mevalonate kinase-deficiency model using human SH-SY5Y neuroblastoma cells the addition of GGOH rescued lovastatin induced apoptosis.¹⁶² Additionally, statins have been found to exhibit neuroprotective effects on undifferentiated human neuroblastoma cells in models of neuroinflammation due to varying induced microglia immune responses.¹⁶³ Taken together these results highlight the need to study neuroblastoma CSCs in a relevant model system as statin treatment can induce variable responses based on cell culture system.

Bisphosphonates

The potential use of zoledronate for the treatment of high-risk neuroblastoma was investigated based on the cytotoxic effects reported in other tumor cell lines and the potential efficacy against osteoclast activity during bone metastasis. Dickson et al. reported that human neuroblastoma cell lines (NB-1691, CHLA-255, and SK-N-AS) were sensitive to zoledronate treatment in a time- and dose-dependent manner, consistent with the findings associated with statin treatment.¹⁶⁴ The cell lines displayed varying sensitivity to zoledronate treatment with induction of cell-cycle arrest and apoptosis observed within 24 hours and increased reductions in cell counts at 48 and 72 hours.¹⁶⁴ Unlike statin treatment, morphological differentiation with neurite outgrowth occurred only with prolonged treatment rather than acute treatment.¹⁶⁴ The addition of FPP to CHLA-255 cells treated with zoledronate could rescue the cells from induced apoptosis. Zoledronate treatment reduced the number of osteoclasts formed when neuroblastoma

cells were co-cultured with human bone marrow stromal cells. Additionally, this research showed that in a mouse model of bone metastasis zoledronate treatment reduced the formation of osteolytic lesions and tumor growth. These findings were consistent with those seen by Peng et al., who also noted a reduction in the formation of osteolytic lesions and tumor growth with zoledronate treatment of a bone invasion neuroblastoma tumor mouse model.¹⁶⁵ Within this tumor model they further identified inhibition of proliferation and increased apoptosis within tumor cells within the bone of zoledronate treated mice, however minimal effects were observed on tumor cells outside of the bone matrix.¹⁶⁵ In the context of preestablished bone lesions treatment with only zoledronate did not have therapeutic benefits however combination treatment of zoledronate with cyclophosphamide and topotecan improved mouse survival.¹⁶⁵ Additionally, they also found that zoledronate treatment inhibited CHLA-255 cell proliferation and induced apoptosis, which was increased with combination treatment with cyclophosphamide.¹⁶⁵ Zoledronate treatment of these neuroblastoma cells also led to a decrease in membrane-associated Ras and decreased signaling of the downstream protein kinase B/extracellular signal-regulated kinase 1/2 (Akt/ERK1/2) pathway.¹⁶⁵

Zoledronate treatment of neuroblastoma cells has also been investigated in the context of enhanced immune cell recognition. $\gamma\delta$ T-cells target cancer cells with increased IPP levels, which zoledronate treatment can induce due to blocking the production of FPP.¹⁶⁶ When neuroblastoma cell lines were pretreated with zoledronate researchers noted an increased $\gamma\delta$ T cell induced lysis.¹⁶⁷ In 2012, Nishio et al. investigated this in the context of neuroblastoma cancer stem cells using a spheroid model.¹⁶⁸ They found that both adherent neuroblastoma cell lines and sphere cultures pretreated with zoledronate had increased susceptibility to $\gamma\delta$ T cell lysis, which could be blocked with statin pretreatment indicating that this effect is due to zoledronate activity on mevalonate pathway enzymes.¹⁶⁸ Additionally, *in vivo* $\gamma\delta$ T cells could inhibit tumor growth of

neuroblastoma sphere forming cells pretreated with zoledronate.¹⁶⁸ These data further supports the potential use of zoledronate for the treatment of high-risk neuroblastoma and highlights the importance of further work featured in this dissertation on the effects of zoledronate treatment in neuroblastoma cancer stem cells.

Clinical Trials

Multiple statins are approved by the Federal Drug Administration (FDA) for use in children for the treatment of familial hypercholesterolemia including fluvastatin, simvastatin, and lovastatin at doses of 20-80 mg/day for fluvastatin and 10-40 mg/day for simvastatin and lovastatin.¹⁶⁹ These compounds are typically well tolerated with minimal concerns for long-term side effects despite long-term daily use by patients.¹⁶⁹ A phase 1 clinical trial in 2022 looked at the potential safety of high-dose simvastatin for the treatment of pediatric relapsed and/or refractory solid and central nervous system (CNS) tumors reported a maximum tolerated dose of 100 mg/m²/dose with a peak plasma concentration of 62 ng/ml $\pm$ 1.5.¹⁷⁰ Patients in this trial received simvastatin with combination chemotherapy cyclophosphamide and topotecan.¹⁷⁰ There have been no additional clinical trials on the use of statins for the treatment of high-risk neuroblastoma.

Zoledronate is not FDA approved for use in children for any indication, however it has been approved for use in adults and there have been multiple prior clinical trials and ongoing trials for the potential use in high-risk pediatric solid tumors. The first reported phase 1 clinical trial results in 2011 found that combination treatment of recurrent/refractory neuroblastoma with zoledronate and cyclophosphamide was well tolerated with maximum tolerated dose of zoledronate to be 4 mg/m².¹⁷¹ A phase 1 clinical trial for refractory neuroblastoma with combination treatment of zoledronate and subcutaneous Interleukin-2 (IL-2) found that while

well tolerated by patients the treatment led to an increased level of circulating immunosuppressive CD4+ T cells, that may prevent beneficial effects of the combination treatment.¹⁷² A preliminary trial of four patients in Korea reported that high-dose ¹³¹I-MIBG followed by $\gamma\delta$ T-cell-depleted haplo-HCT and post-transplant immunotherapy with zoledronate and IL-2 was well tolerated.¹⁷³ There are two ongoing clinical trials in the US for treatment of relapse/refractory neuroblastoma related to the potential use of zoledronate. One (NCT05400603) aims to investigate the use of allogeneic expanded $\gamma\delta$ T cells in combination with dinutuximab, temozolomide, irinotecan and zoledronate. The other (NCT02508038) investigates the safety of transplantation with a haploidentical donor peripheral blood stem cell graft depleted of TCR $\alpha\beta$ + cells and CD19+ cells in conjunction with post-transplant zoledronate treatment. Additionally, physicians have used zoledronate off-label for the treatment of recurrent/refractory neuroblastoma patients and found it to be very well tolerated.¹⁷⁴

Conclusion and Hypothesis

Substantial previous literature to suggests that mevalonate pathway inhibition may be a therapeutic target in high-risk neuroblastoma.^{100–102,104,105} This pathway is upregulated in patient tumors and high-risk neuroblastoma cells lines.^{78,112} Inhibition of this pathway appears to induce effects on differentiation status and cell viability through alterations in protein geranylgeranylation.^{102,103,127} These findings however, are slightly sporadic in what cell lines and pathway inhibitors are utilized, which complicates further preclinical and clinical research. The work in this dissertation prioritized clinical relevance of chosen cell lines and inhibitors used to address this issue.

We hypothesized that treatment of high-risk neuroblastoma cell lines with mevalonate pathway inhibitors, representative of patients with poor clinical outcomes, would induce

differentiation and cell death through loss of protein prenylation. In Chapter 4, we characterized the effect of mevalonate pathway inhibition using a profile of statins and downstream pathway inhibitors in a panel of neuroblastoma cell lines.

Chapter 4

Mevalonate Pathway Inhibition Targets High-Risk Neuroblastoma Cell Lines

Abstract

As discussed in previous chapters there is substantial evidence that treatment of high-risk neuroblastoma with mevalonate pathway inhibitors may be a viable therapeutic option. Based on this we hypothesized that treatment of high-risk neuroblastoma cell lines with mevalonate pathway inhibitors would induce differentiation and cell death through loss of protein prenylation. We intended to use a clinically relevant panel of cell lines and pathway inhibitors for this study to address the gaps in previous research. This work in a traditional adherent cell culture model led us to the development of a spheroid model to enrich for the neuroblastoma CSC population. This spheroid model was utilized to investigate the impact of mevalonate pathway inhibition on the CSC population of high-risk neuroblastoma cell lines. The use of spheroid models to study the CSC population is an emerging field in pediatric oncology and at the time of project inception there were limited publications on its utility for high-risk neuroblastoma.

Introduction

Neuroblastoma is the most common infantile cancer and at the time of diagnosis approximately 45% of patients have high-risk disease.^{1-3,28} For patients with low and intermediate risk disease treatment advances have successfully raised the overall survival rate to above 90%.³ Despite these advances and the utilization of intensive treatment regimens high-risk neuroblastoma has overall survival rates between 50-60%.³ The majority of high-risk patients, at

the time of diagnosis, are above the age of 18 months and already present with metastatic disease, likely due to the presence of a population of aggressive CSCs.^{3,87,90,95}

An important consideration for the preclinical research of potential therapeutics of high-risk neuroblastoma is the clinical relevance of cell culture models. According to the COG there is a lack of clinical prognostic biomarkers to distinguish subpopulations of high-risk patients.³ As such, selection of relevant cell lines and model system for *in vitro* studies on potential therapeutics is more difficult. Russo et al. performed kinase expression profiling of high-risk neuroblastoma patient samples and cell lines.¹⁷⁵ This study defined a subset of high-risk neuroblastoma tumors termed “ultra-high-risk,” indicating that death from disease occurred within 18 months of diagnosis.¹⁷⁵ They identified a unique set of 27 differentially expressed kinases termed “Kinase-27” that were associated with this prognosis. Analysis of the kinase profiles of commonly used neuroblastoma cell lines identified SK-N-AS, SH-SY5Y, and SK-N-BE(2)-C as reliable *in vitro* models of ultra-high-risk neuroblastoma.

As previously mentioned, a challenge for the development of novel therapeutics for treatment of high-risk neuroblastoma is the identification of viable therapeutic targets. As discussed in Chapter 1, neuroblastoma lacks significant gene mutation burden, which is often the focus of targeted therapy for aggressive cancers.¹³ Alterations in cellular metabolism is an emerging hallmark of cancer, with the potential for targeted therapy.¹⁰⁷ In neuroblastoma specifically, the genes associated with the mevalonate pathway are upregulated in high-risk neuroblastoma patient tumors and inhibition of the pathway can alter cell viability *in vitro* as discussed in Chapter 3.^{78,112} There is substantial prior literature on the utility of mevalonate pathway inhibitors against adherent neuroblastoma cell lines, however much of it is disjointed in inhibitor and cell lines chosen, limiting our understanding of potential clinical utility.

Our lab first sought to determine the potential induction of differentiation and cell death in adherent high-risk neuroblastoma cell lines by mevalonate pathway inhibitors in a systemic

fashion. We utilized three high-risk neuroblastoma cell lines that are representative of patients with the worst prognosis and to profile the response of these cell lines to multiple statins and mevalonate pathway inhibitors.

For most of this work we focused on non-*MYCN* amplified cell lines SH-SY5Y and SK-N-AS, but also utilized SK-N-BE(2)-C cells, which have *MYCN* amplification, at times for comparison. The SH-SY5Y and SK-N-AS cell lines were chosen based on their representation of ultra-high-risk disease and their distinct mutation and genotype profiles (Table 1) that are clinically relevant.^{78,102,103,175} Additionally, SH-SY5Y is one of the most utilized cell lines in both neuroblastoma and neurodegenerative research. SH-SY5Y cells were derived from the bone marrow of a Stage 4, four-year-old female patient and are N-type, noradrenergic cells with an activating *ALK* mutation, and gain of chromosome 17q.^{82,176} SK-N-AS cells were derived from the bone marrow of a Stage 4, eight-year-old female patient and are S-type, mesenchymal cells with wildtype *ALK*, loss of chromosome 1p and gain of chromosome 17q.^{82,176} SK-N-BE(2)-C cells were originally derived from the bone marrow of a Stage 4 twenty-five-month-old patient and are I-type, noradrenergic cells with wildtype *ALK*, loss of chromosome 1p, and loss of one copy of chromosome 17 resulting in loss of function of *p53*.^{82,112,176}

Cell Line	N, S, or I type	MESN or NOR	<i>ALK</i>	Chromosome 1p	Chromosome 17	<i>MYCN</i> status
SH-SY5Y	N	NOR	Mut	Normal	Gain	wt
SK-N-AS	S	MESN	wt	Loss	Gain	wt
SK-N-BE(2)-C	I	NOR	wt	Loss	Single copy loss, p53 inactive	amplified

Table 1. High-risk neuroblastoma cell line characteristics.

SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C are established high-risk neuroblastoma cell lines. Their cell type designations (N, S or I type and mesenchymal or noradrenergic) are indicated as well as the status of *ALK* and common chromosomal abnormalities. N: neuronal/neuroblastic. S: non-neuronal/substrate adherent. I: Intermediate. MESN: mesenchymal. NOR: noradrenergic. Mut: mutation, wt: wildtype. Modified from Green, M.V., Reed, J.L., Zielinski, C.D., and Neighbors J.D. Inhibition of the Mevalonate Pathway Targets Neuroblastoma Stem Cells. *Pharmacology*. 2025.¹⁷⁷

Methods

Cell lines and reagents

SK-N-BE(2)-C, SH-SY5Y and SK-N-AS cells were obtained from ATCC and all experiments were performed within 15 passages of receipt. For adherent cultures SH-SY5Y and SK-N-BE(2)-C cells were maintained in a 1:1 mixture of Minimum Essential Media (MEM) (Gibco) and F-12 medium (Gibco) with 10% fetal bovine serum (FBS) added (Hyclone, from ThermoFisher Scientific). SK-N-AS cells were maintained in Dulbecco's Modified Eagle

Medium (DMEM) with 10% FBS and 0.1 mM Non-Essential Amino Acids (NEAA) (Gibco). All cells were maintained at 37°C and 5% carbon dioxide (CO₂.)

Fluvastatin was purchased from Selleck Chemicals, reconstituted in DMSO to 100 mM and stored at -20°C before use. This stock was then diluted to 10 mM immediately before dilution to final concentration in complete cell culture media, as above. Zoledronic acid was obtained from Selleck Chemicals and reconstituted in 0.5 M sodium hydroxide to 10mM and stored at -20°C before use. It was also diluted to final concentration in complete cell culture media immediately prior to use. FOH and GGOH were obtained from ThermoFisher Scientific and used at 30 µM and 20 µM respectively. Lovastatin and Simvastatin were obtained from Abcam and Selleck Chemicals and activated prior to use as described.¹⁷⁸ DGBP was obtained from Terpenoid Therapeutics Inc. and reconstituted to 10 mM before final dilution in complete media for use. GGTI-286 was obtained from Calbiochem and reconstituted to 10 mM before final dilution in complete media for use.

Metabolic Activity Assays

3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assays were carried out as previously described for adherent SK-N-AS and SK-N-BE(2)-C cell lines.¹⁷⁹ SK-N-AS cells were plated at 10,000 cells/well and SK-N-BE(2)-C cells were plated at 5,000 cells/well (24 hour) and 2,500 cells/well (48 hour) and allowed to attach overnight before treating. After 20 and 44 hours of treatment 10 µL of 5 mg/mL MTT reagent (in deionized water) was added to each well. At 24 and 48 hours of treatment stop solution (10% 1 N HCL, 10% Triton X-100, 80% 2-propanol) was added and plates were incubated overnight. Absorbance was read at 570 and 690 nm. For the adherent SH-SY5Y and all spheroid cultures they were carried out as described but with the following changes. Adherent SH-SY5Y cells were plated at 20,000 cells/well and

spheroid cells were plated at 50,000 cells/well in 50 μ L of complete spheroid media and then treated with 50 μ L of 2x treatment media as noted before incubation. At 44 hours of treatment, 10 μ L of 5 mg/mL MTT reagent (in deionized water) was added to each well. At 48 hours of treatment 50 μ L of DMSO was added to each well and plates read at 570 nm on SpectraMax i3x plate reader (Molecular Devices).

Crystal Violet Assays

Adherent SH-SY5Y cells were seeded at 40,000 cells/well, adherent SK-N-AS cells were seeded at 10,000 cells/well and SK-N-BE(2)-C cells were seeded at 5,000 cells/well (24 hours) and 2,500 cells/ well (48 hours) in complete media and incubated overnight. The following day complete medium was removed, and cells were treated with the indicated compounds freshly diluted in complete medium and incubated for indicated time points (24 or 48 hours). At the end time point plates were spun down and fixed with 100% methanol for 10 minutes at -20°C and washed with deionized water. 50 μ L of 0.5% crystal violet solution was added to each well and incubated for 20 minutes at room temperature followed by sufficient washes with deionized water to remove excess crystal violet solution. Plates were then let dry at room temperature and absorbance detected at 590 nm on SpectraMax i3x plate reader (Molecular Devices).

Sphere Model Development Assays

Spheroids were generated in three test media. The first being the complete medium described above for adherent cultures for the cell lines. The second test medium being composed of DMEM/Ham's F12 (3:1), anti-anti (1:100; Gibco), human fibroblast growth factor (FGF) basic (Invitrogen) and epidermal growth factor (EGF) (Gibco) both at 20 ng/mL, heparin (5 μ g/mL;

Sigma), and 1x B-27 (Gibco).⁷⁷ The third test medium being a standard neurosphere media composed of DMEM/Ham's F12 (1:1), 20 ng/mL EGF, 5 µg/mL insulin (Sigma Aldrich), 10 ng/mL FGF, 0.4% bovine serum albumin (Research Products International), and 1x B-27. Media were prepared fresh for each experiment and 1x B-27 was added immediately before use. All spheroid cultures were maintained at 37°C and 5% CO₂.

Initiation of sphere cultures was done by harvesting of adherent cells with 0.25% trypsin followed by trypsin neutralization with complete medium and cell counting. Cells were resuspended at a density of one million cells/mL in spheroid medium. For the initial SK-N-BE(2)-C trial these cells were then plated in an ultra-low attachment 96-well plate with PBS in all outer wells to prevent evaporation. Cells were plated at 1000 cells/well, 300 cells/well and 10 cells/well with 6 wells for each condition for all three trial medias. This plate was incubated at 37°C and 5% CO₂ for up to two weeks. For the spheroid trial with all three cell lines cells were harvested and resuspended as above. Cells were then plated at 1000 cells/well, 500 cells/well, 100 cells/well, 50 cells/well, 20 cells/well, 10 cells/well and 5 cells/well in the second trial sphere media for a total of 15 wells/condition. These plates were incubated for two weeks at 37°C and 5% CO₂.

Statistical Analysis

All data shown are representative of at least 2 independent experiments. Data points shown are presented as mean ± SEM unless otherwise indicated. Statistical analysis of MTT and crystal violet assays were performed with GraphPad Prism 10. Data was analyzed with two-way ANOVA followed by Tukey's post hoc for multiple comparisons. A p-value < 0.05 was considered significant. P-values were calculated for *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Statistical significance is noted in figure legends.

Results

Mevalonate pathway inhibition alters cell viability of adherent high-risk neuroblastoma cell lines

To investigate cytotoxicity of mevalonate pathway inhibition in these high-risk neuroblastoma cell lines we performed a series of metabolic activity and crystal violet assays. To begin adherent cells were treated with a range of doses of either lovastatin, simvastatin or fluvastatin for time periods of 24 or 48 hours. Lovastatin and simvastatin were activated into their active form prior to use in cell assays.^{130,178} Consistent with previous literature there was minimal effect on metabolic activity of SH-SY5Y and SK-N-AS cell lines treated with statins for 24 hours (Figure 3 A-B). With 48-hour statin treatment there was a dose-dependent reduction in the metabolic activity in both cell lines that was consistent between the three statins (Figure 3 D-E). In the SH-SY5Y cell line the reduction was significant for all three statins and the concentration that inhibits 50% (IC₅₀) of the metabolic activity with 48-hour treatment is 4.0 μ M for lovastatin, 7.4 μ M for simvastatin, and 1.9 μ M for fluvastatin. The SK-N-AS cell line is less sensitive to 48-hour statin treatment than the SH-SY5Y cell line. 48-hour treatment of SK-N-AS cells with lovastatin or simvastatin did not reduce metabolic activity below 50% and treatment with fluvastatin only did significantly with doses above 10 μ M. SK-N-BE(2)-C cells displayed some sensitivity to 24-hour simvastatin and fluvastatin treatment with higher doses (30 – 100 μ M) but were not sensitive to 24-hour lovastatin treatment (Figure 3 C). 48-hour treatment of SK-N-BE(2)-C cells with any of the three statins appeared to reduce metabolic activity but this was not statistically significant (Figure 3 F). These results were consistent with those from crystal violet staining for 24- and 48-hour statin treatment (Figure 4 A-D) of SK-N-AS and SK-N-BE(2)-C cells.

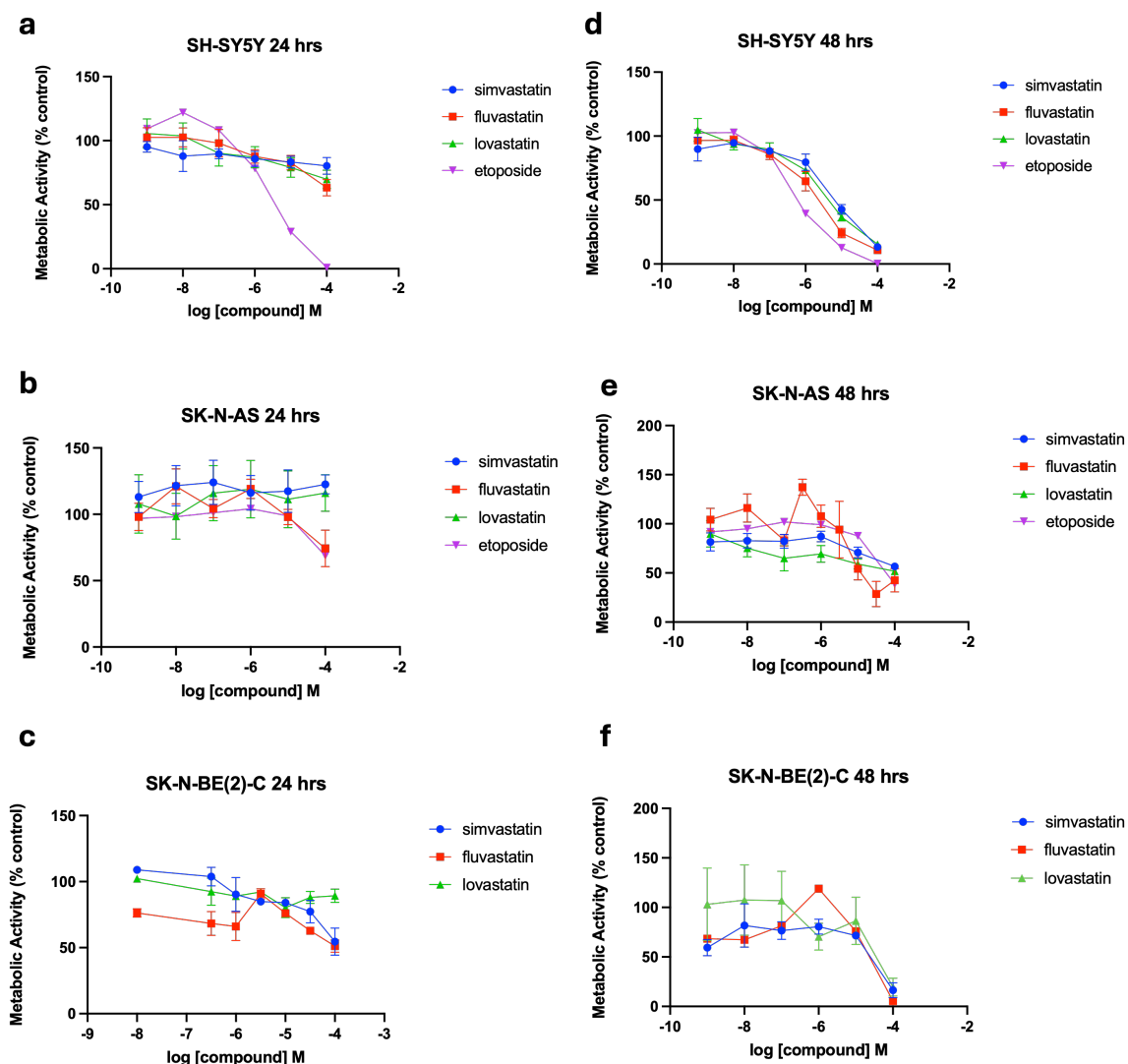


Figure 3: Statin treatment reduces the metabolic activity of adherent high-risk neuroblastoma cell lines.

Statin response was determined by 24- and 48-hour MTT assay in adherent cultures of SH-SY5Y (A, D), SK-N-AS (B, E), and SK-N-BE(2)-C (C, F) cell lines. Data shown as mean $\pm$ SEM, n = minimum 3 independent experiments for SH-SY5Y and SK-N-AS cell lines. SK-N-BE(2) cell line n = 2 independent experiments. Statistical analysis (not shown on graphs) was done with two-way ANOVA and Tukey's post hoc for multiple comparisons.

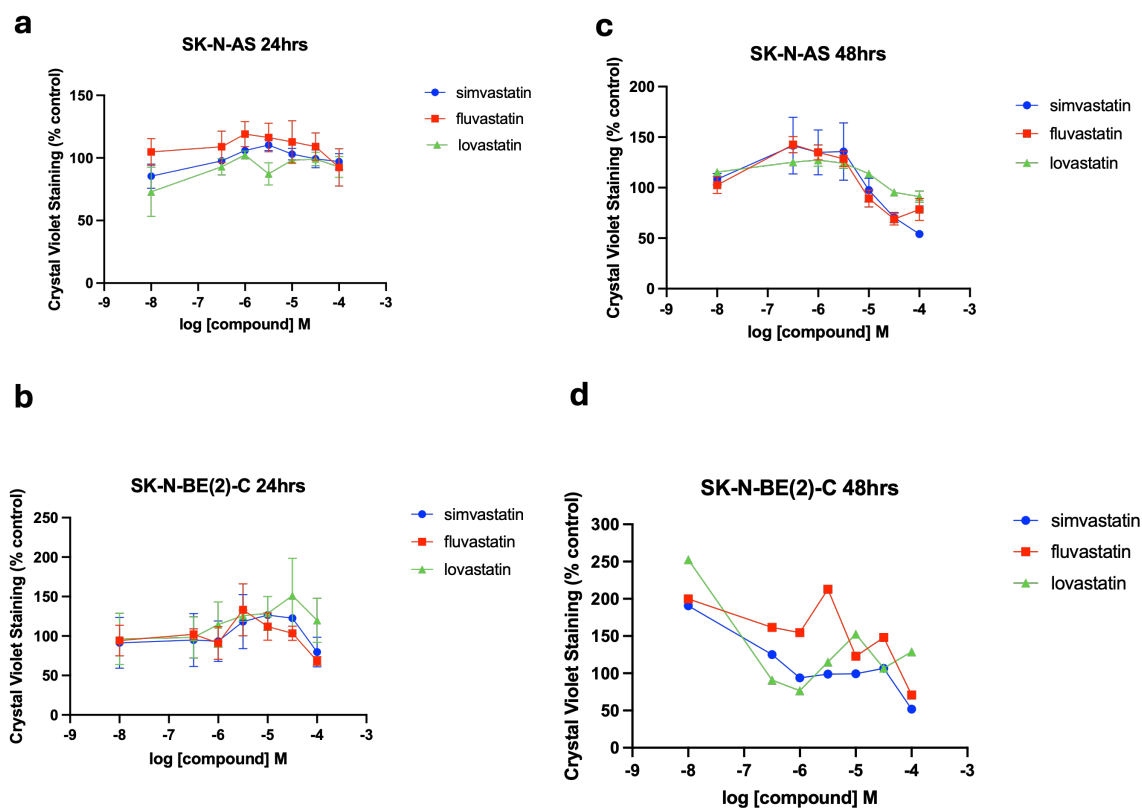


Figure 4: Statin treatment reduces cell viability of adherent high-risk neuroblastoma cell lines.

Cell response was determined by crystal violet staining of adherent cells after 24- and 48-hour statin treatment in SK-N-AS (A,C) and SK-N-BE(2)-C (B,D) cell lines. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments for SK-N-AS cells. SK-N-BE(2) cell line $n = 2$ independent experiments for 24 hours and $n = 1$ for 48-hour experiment. Statistical analysis (not shown on graphs) was done for SK-N-AS cell line with two-way ANOVA and Tukey's post hoc for multiple comparisons.

Based on the prior literature neuroblastoma cells are likely sensitive to mevalonate pathway inhibition due to loss of protein geranylgeranylation because of blocked isoprenoid synthesis.^{102,103,156} To investigate this in these high-risk neuroblastoma cell lines we treated adherent cultures with downstream inhibitors of the mevalonate pathway. Treatment of SH-SY5Y cells with bisphosphonates, DGBP and zoledronate, or a transferase inhibitor GGTI-286 did not significantly alter metabolic activity with 24-hour treatment (Figure 5 A). Similarly, 48-hour treatment with DGBP and GGTI-286 did not significantly affect the metabolic activity of the adherent SH-SY5Y cells (Figure 5 B). There was a dose-dependent reduction in metabolic activity of SH-SY5Y cells with 48-hour treatment of zoledronate, similar to the reduction observed with fluvastatin treatment (Figure 5 B). Treatment of SK-N-AS cells with bisphosphonates, DGBP or zoledronate did not significantly alter metabolic activity at 24 or 48 hours (Figure 5 C-D). There was a significant dose-dependent reduction in SK-N-AS metabolic activity with treatment with GGTI-286 at both 24-and 48-hour timepoints (Figure 5 C-D). Interestingly, crystal violet staining of both SH-SY5Y and SK-N-AS cells treated with GGTI-286 showed a significant dose-dependent reduction in staining at both 24 and 48 hours (Figure 6 A-D). Treatment with bisphosphonates did not significantly reduce crystal violet staining in either SH-SY5Y or SK-N-AS cells, consistent with the observations of metabolic activity (Figure 6 A-D).

As noted above, the SK-N-AS cell line represents the mesenchymal subtype of high-risk neuroblastoma is dependent on protein geranylgeranylation.^{102,103} To further investigate this, we performed addback experiments with supplemental FOH or GGOH used to rescue protein prenylation in the presence of fluvastatin treatment. It is important to note that cells can utilize FOH and GGOH for protein prenylation but cannot integrate FOH into further steps of the mevalonate pathway as this would require the availability of additional IPP molecules that statin treatment blocks formation of. Additionally, we also supplemented cells with mevalonate to

confirm on-target pathway effects of fluvastatin. Fluvastatin was chosen as the statin to move forward with investigating as all three cell lines displayed sensitivity with 48-hour treatment, eliminates the need for activation prior to use, and of the three has a lower potential side effect profile as it does not cross the blood brain barrier. In SK-N-AS cells addition of mevalonate or GGOH significantly reduced 48-hour fluvastatin-induced reductions in metabolic activity but FOH did not (Figure 7).

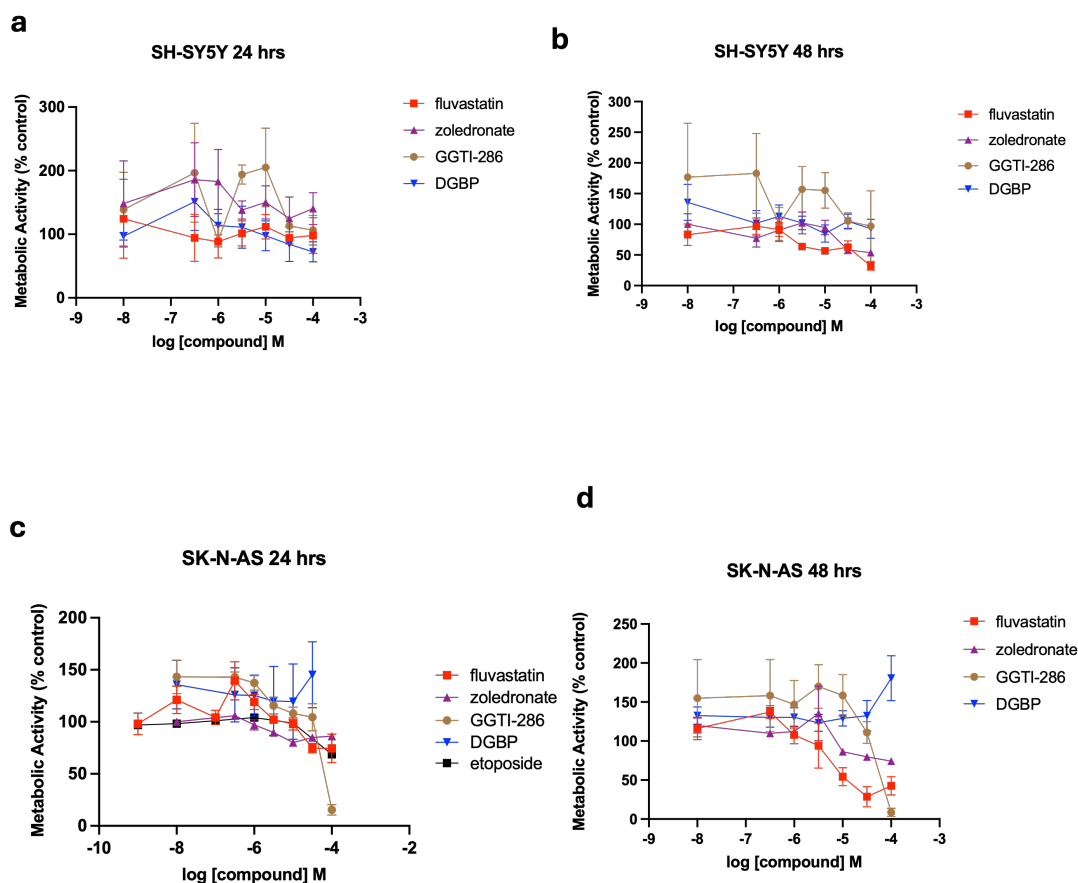


Figure 5: Mevalonate pathway inhibition impacts the metabolic activity of adherent high-risk neuroblastoma cell lines.

Cell response to indicated compounds was determined by 24- and 48-hour MTT assay in adherent cultures of SH-SY5Y (A-B), SK-N-AS (C-D) cell lines. Data shown as mean $\pm$ SEM, n = minimum 3 independent experiments. Statistical analysis (not shown on graphs) was done with two-way ANOVA and Tukey's post hoc for multiple comparisons.

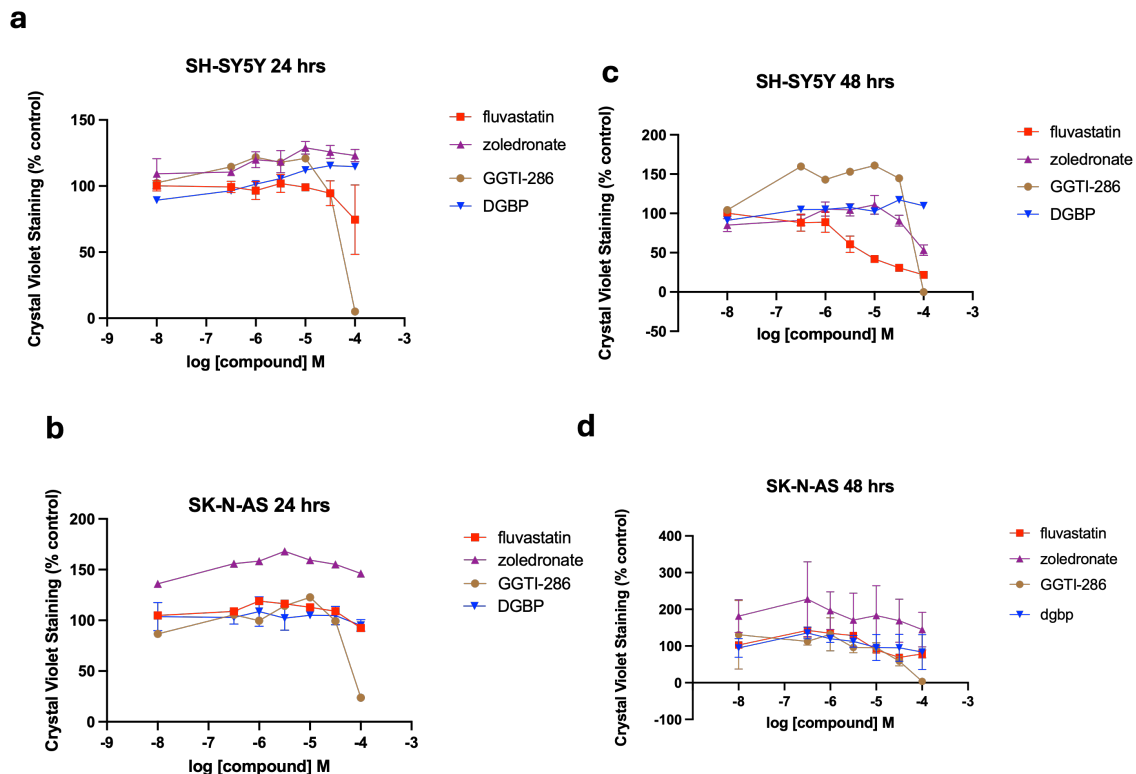


Figure 6: Geranylgeranyl transferase inhibition reduces cell viability of adherent high-risk neuroblastoma cell lines.

Cell response to indicated compounds was determined by crystal violet staining of adherent cells after 24- and 48-hour treatment in SH-SY5Y (A-B), and SK-N-AS (C-D) cell lines. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments. Statistical analysis (not shown on graphs) was done with two-way ANOVA and Tukey's post hoc for multiple comparisons.

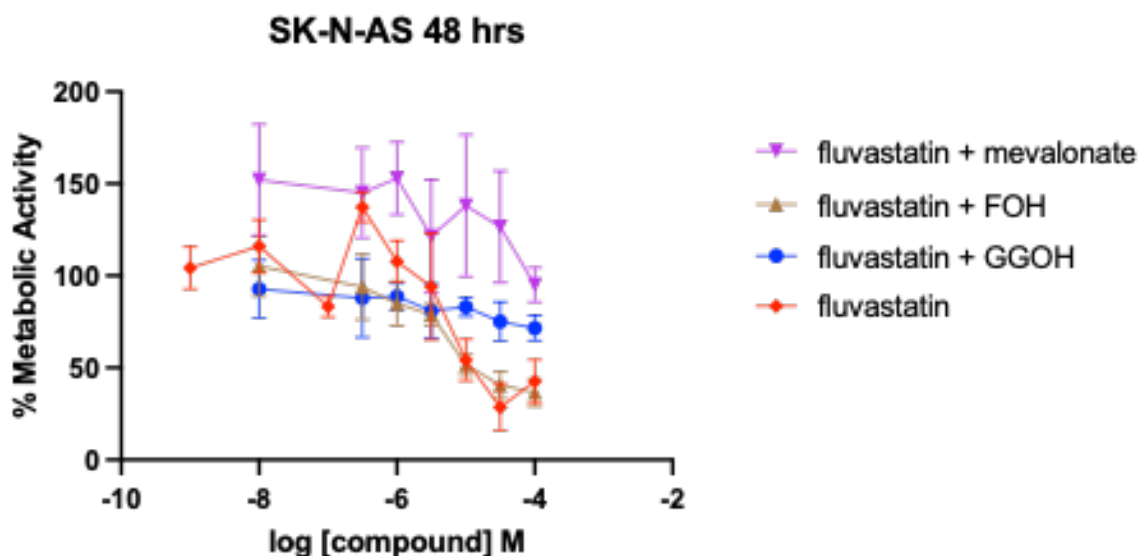


Figure 7: Fluvastatin reduces metabolic activity of adherent high-risk neuroblastoma cell lines through reductions in the geranylgeranyl branch of the mevalonate pathway.

Cell response to treatment with fluvastatin in combination with indicated compound. Response was determined by 48-hour MTT assay in traditional adherent culture. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments. Statistical analysis (not shown on graphs) was done with two-way ANOVA and Tukey's post hoc for multiple comparisons.

Mevalonate pathway inhibition induces differentiation phenotype in high-risk neuroblastoma

Previous studies on the inhibition of the mevalonate pathway in neuroblastoma cell lines with statins described phenotypic changes to adherent cells consistent with differentiation.^{127,153}

These phenotypic changes precede loss of cell viability and included cell body rounding and neurite extension.^{104,127,153,154} To investigate if we saw similar phenotypic changes in our high-risk neuroblastoma cell lines, SH-SY5Y and SK-N-AS, we imaged cells after 24-hour fluvastatin treatment of low confluency plates. In both cell lines we observed a dose-dependent effect on cell body rounding and neurite extension, with increasing fluvastatin doses increasing the observed

effects (Figure 8). In the SH-SY5Y cell line this effect is also observed with 48-hour fluvastatin treatment. Interestingly, in SK-N-AS cells the effect is lost with 48-hour treatment. This is consistent with the previous cell viability findings suggesting that the SH-SY5Y cell line is more susceptible to inhibition of the mevalonate pathway with statin treatment when cultured in traditional adherent models.

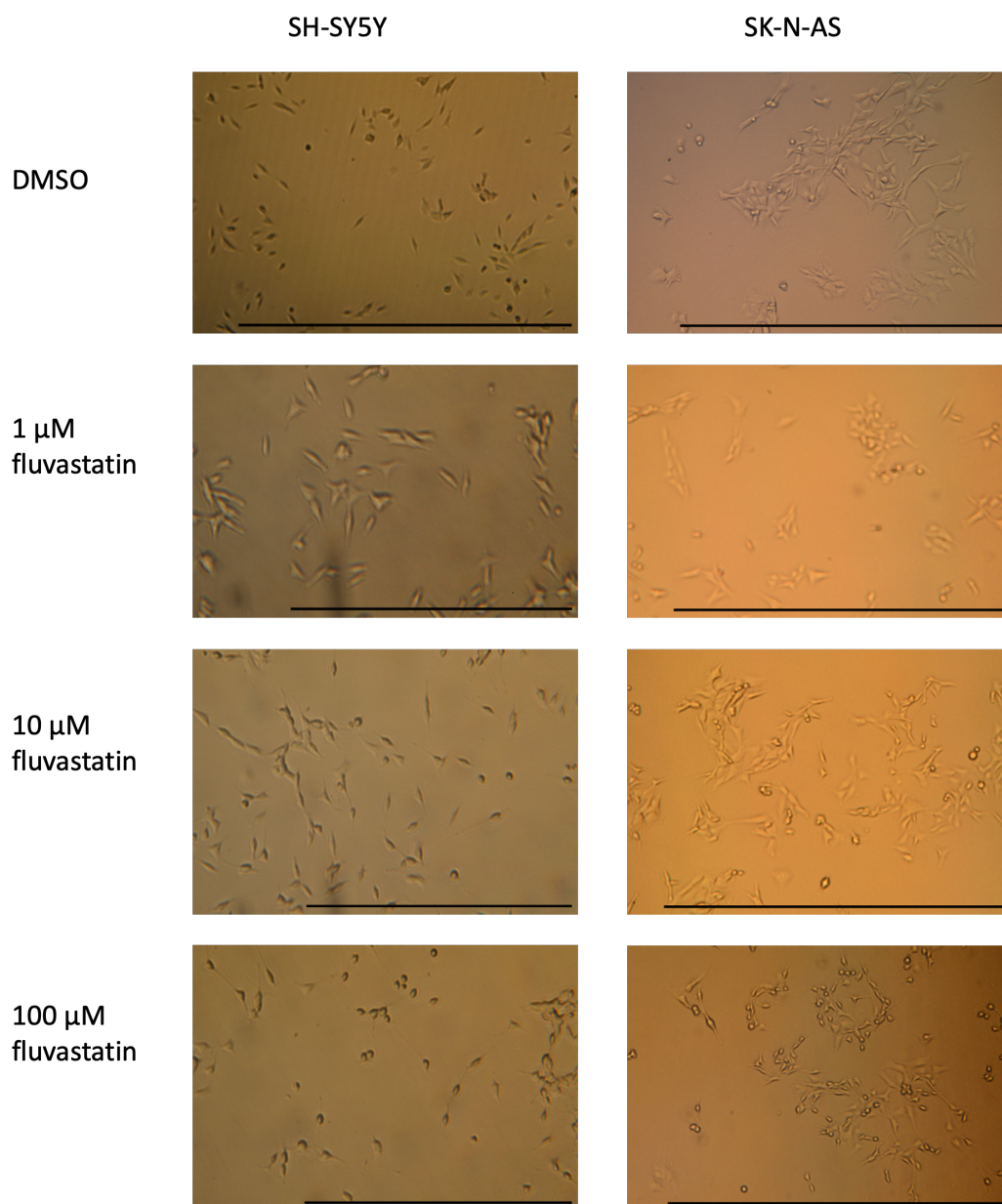


Figure 8: Induction of a differentiation phenotype by mevalonate pathway inhibition in adherent high-risk neuroblastoma cell lines.

Morphological changes in response to 24-hour treatment with fluvastatin at the indicated concentrations and time were recorded in SH-SY5Y and SK-N-AS adherent cell lines. These images were not quantified. Scale bars 1 mm.

High-Risk Neuroblastoma Spheroid Model Development

As discussed in Chapter 2, CSCs are likely to contribute to the aggressiveness of high-risk neuroblastoma and can be found in patient samples as well as high-risk cell lines.^{79,80} Based on our findings that inhibition of the mevalonate pathway has observable effects on the phenotype of high-risk neuroblastoma cell lines in traditional adherent culture we aimed to develop a spheroid model for the use in pharmacological assays. Neuroblastoma cells demonstrate reversible adaptive plasticity (RAP), which should enable successful culture in both adherent and spheroid settings, however at the time of this project there were few publications on the use of spheroid culture with our chosen cell lines.⁹⁹

We began trial spheroid culture with the SK-N-BE(2)-C cell line. In standard cell culture conditions with complete growth media these cells grow as a mixture of adherent and suspension cells indicative of active RAP. These cells were plated in an ultra-low attachment 96-well plate with three test media at three cell densities (1000 cells/well, 300 cells/well, and 10 cells/well) to observe sphere growth. Loose aggregates with cell-cell definition were not considered spheres and spheres were defined as well rounded cellular aggregates with little cell-cell definition and a smooth surface. The first test media used was the standard complete growth medium for this cell line, which resulted in aggregate formation without sphere formation over the course of two weeks. The second test medium used was a formulation by Kumar et al. and did result in sphere formation at all tested cell densities with decreasing sphere counts at the lowest density as expected.⁷⁷ The third test medium used was a neurosphere formulation previously used in our laboratory for the growth of glioblastoma spheres.¹⁸⁰ This medium also resulted in SK-N-BE(2)-C sphere formation but to a lesser extent than the Kumar et al. medium at the 300 cells/well and 10 cells/well densities. Based on these observations we moved forward with the second medium formulation with our additional cell lines.

To investigate the growth of SK-N-BE(2)-C, SH-SY5Y, and SK-N-AS cells in the spheroid medium chosen, we plated cells in ultra-low attachment 96-well plates at decreasing cell per well densities and observed sphere formation over the course of two weeks. We noted continue growth in the plates of all three cells lines on Day 7 without discoloration in the media and lack of indicators of cell death such as debris in the media. We counted the number of wells with sphere formation on Day 9 and Day 14 (Table 2). In both the SK-N-BE(2)-C and SH-SY5Y cell lines we noted significant sphere formation on Day 9 without evidence of media discoloration or cell debris at all cell densities tested. In both cell lines all the wells with the highest initial plating densities of 1000 cells/well and 500 cells/well had multiple spheres that formed by Day 9. Additionally, we observed a decrease in sphere size as the initial plating cell density decreased. By Day 14 both cell lines had discoloration of their media and presence of cell debris in higher density wells, potentially indicating that some spheres have begun to break apart. In the SK-N-AS cell line spheres were not present on Day 9 however aggregates were observed and by Day 14 there was significant sphere formation at the higher cell densities. The lower cell densities tested did not produce spheres consistently in this cell line. Additionally, we were able to passage spheres and force self-renewal from single cell-populations of all three high-risk neuroblastoma cell lines, hallmarks of CSC populations. Based on these findings we moved forward with the application of pharmaceutical based assays using this novel spheroid model.

Wells with sphere formation							
Day 9	1000 cells/well	500 cells/well	100 cells/well	50 cells/well	20 cells/well	10 cells/well	5 cells/well
SH-SY5Y	15	15	15	15	15	12	3
SK-N-AS	Aggregates 15	Aggregates 15	Small aggregates in 15	Small aggregates in 15	Small aggregates in 8	Small aggregates in 4	0
SK-N-BE(2)-C	15	15	15	15	15	15	5
Day 14							
SH-SY5Y	15	15	15	15	15	12	8
SK-N-AS	15	15	8	7	1	0	2
SK-N-BE(2)-C	15	15	15	15	15	15	6

Table 2: Spheroid growth trial.

Results of the spheroid growth trial of untreated SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C cells cultured in ultra-low attachment 96-well plates for up to 14 days with the number of positive wells counted on Day 9 and Day 14. 15 wells were plated for each cell density tested in all cell lines. At the time of plating wells were checked to confirm cells deposited evenly at low cell/well densities. Aggregate formation on Day 9 was noted for the SK-N-AS cell line as this cell line did not have sphere formation at this time point. Spheres were defined as rounded aggregates of cells with a smooth surface and poor cell-to-cell definition. If aggregates appeared loose or with cell-to-cell clear definition they were noted as aggregates.

Mevalonate pathway inhibition effects on high-risk neuroblastoma sphere cell viability

Prior literature on CSCs indicates that this population of cells is more resistant to treatment with cytotoxic small molecules than other cancer cells therefore we investigated the sensitivity of our high-risk neuroblastoma sphere forming cells to mevalonate pathway inhibitors. We did not observe a significant reduction in the metabolic activity of SH-SY5Y spheroid cells with fluvastatin or zoledronate treatment at either 48- or 72-hours, with a slight reduction observed with 96-hour fluvastatin treatment (Figure 9 A-C). We also did not observe a significant

reduction in the metabolic activity of SK-N-AS spheroid cells with 48-, 72-, or 96-hour treatment with fluvastatin or zoledronate (Figure 9 D-F). These results suggest that the sphere cultures of these cell lines are more resistant to treatment with mevalonate pathway inhibitors than their adherent counterparts.

The 48-hour spheroid results presented here were originally published in Green, M.V., Reed, J.L., Zielinski, C.D., and Neighbors J.D. Inhibition of the Mevalonate Pathway Targets Neuroblastoma Stem Cells. *Pharmacology*. 2025.¹⁷⁷

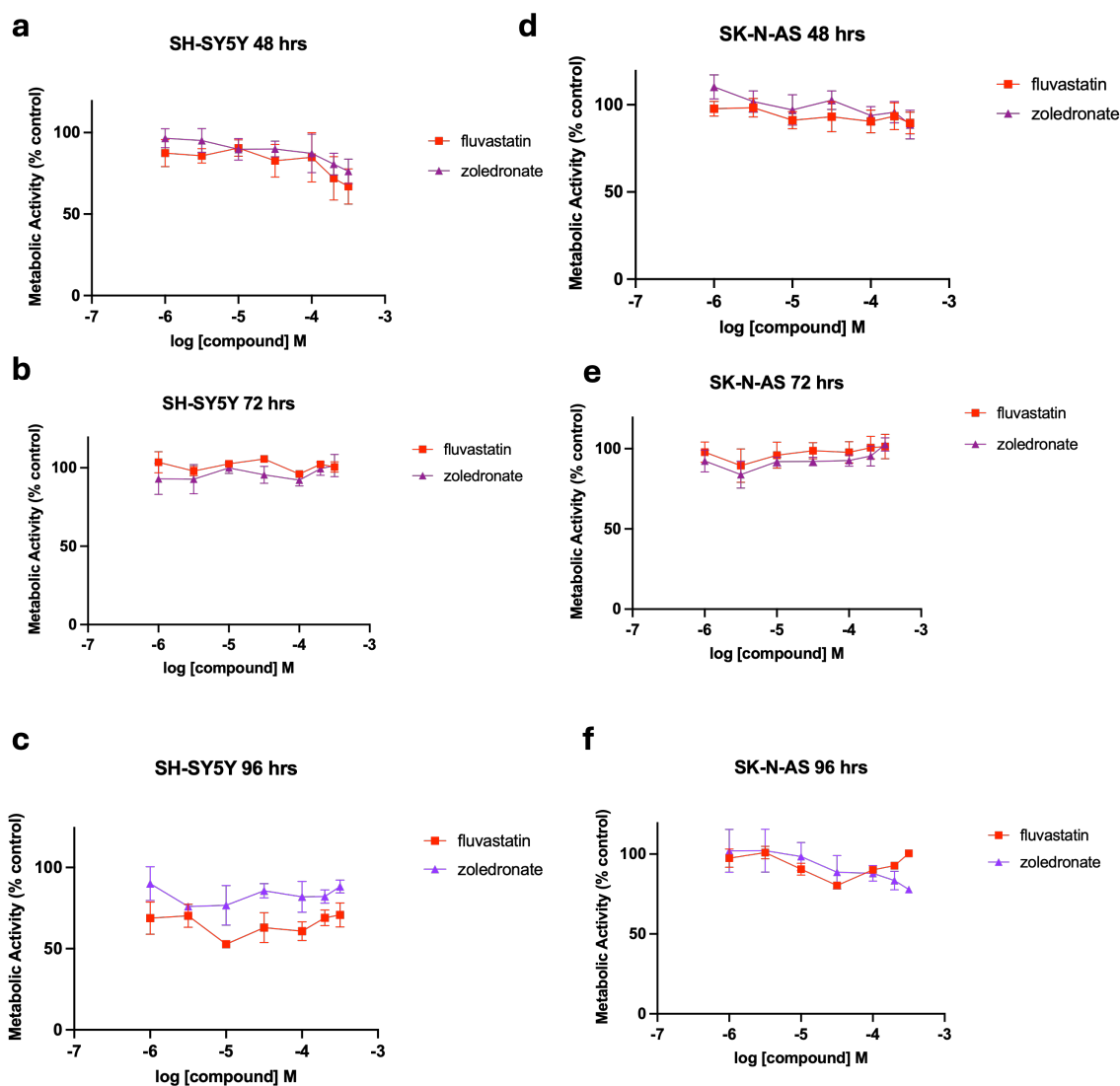


Figure 9: Mevalonate pathway inhibition does not reduce the metabolic activity of high-risk neuroblastoma spheroids.

Compound response was determined by 48-, 72- and 96-hour MTT assay with spheroid cultures of SH-SY5Y (A-C) and SK-N-AS (D-F) cell lines. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments. Statistical analysis (not shown on graphs) was done with two-way ANOVA and Tukey's post hoc for multiple comparisons.

Discussion and Conclusion

Treatment of high-risk neuroblastoma remains an ongoing clinical struggle despite the advancements in recent decades that have improved prognoses for patients in the low and intermediate risk classifications.³ This is thought to be due to the presence of CSCs within the tumor that are resistant to current treatment options and drive recurrence and metastasis.^{9,77–83,98} Research on this topic has been limited by the available model systems and a lack of unique therapeutic targets within neuroblastoma cancer stem cells. Our work aimed to bridge these gaps.

The mevalonate pathway has shown potential as a therapeutic target for aggressive cancers, including high-risk neuroblastoma in which it appears to alter differentiation status prior to induction of cytotoxicity.^{78,100,102–105,112,154,155} Additionally, the inhibitors of this pathway are known to be safe in pediatric populations. Because we investigated the use of mevalonate pathway inhibitors in carefully selected high-risk neuroblastoma cell lines.

For the majority of our work, we selected the SK-N-AS and SH-SY5Y high-risk neuroblastoma cells with the SK-N-BE(2)-C cell line utilized at indicated times for comparison and specific growth benefits. The SK-N-AS and SH-SY5Y cell line are representative of the approximately 60% of high-risk neuroblastoma patients lacking *MYCN* amplification but with genetic profiles consist with those of highest risk.¹⁷⁶ The SK-N-BE(2)-C cell line has *MYCN* amplification as well as a mixed growth (adherent and suspension) phenotype in traditional culture.¹⁷⁶ For development of novel model systems this cell line proliferates quickly and is highly responsive to manipulation of growth conditions allowing for quicker optimization and assay development.

As much of the prior work on mevalonate pathway inhibition was sporadic on inhibitor selection we profiled three common statins in our selected cell lines: simvastatin, fluvastatin, and lovastatin. Treatment outcomes were mostly consistent between the tested statins in the three cell

lines tested. Fluvastatin was therefore chosen for further study based on a few advantages. Firstly, it does not require activation prior to use in cell-based assays, eliminating a source of potential human error during drug handling.¹³⁰ Additionally, due to the molecular structure of fluvastatin it cannot cross the blood-brain barrier reducing the potential off-target effects to a pediatric patient's developing CNS.¹³⁰

Consistent with prior literature we did observe a time and dose-dependent decrease in cell viability with statin treatment in our chosen cell lines when cell viability was analyzed with MTT and crystal violet assays. MTT assays are readouts of the metabolic activity of a cell population and crystal violet assays stain adherent live cells. Changes in either are correlated with cell viability but can occur independent of one another. 24-hour treatment had minimal effect on cell viability of the high-risk neuroblastoma cell lines, but effects were observed with 48-hour treatment. The SH-SY5Y cell line was more sensitive to statin treatment with IC₅₀ values in the low micromolar concentration range while the SK-N-AS cell line only saw approximately 50% reductions with even the highest doses tested. These findings are consistent with prior literature that found neuroblastoma cell lines had variable sensitivity to mevalonate pathway inhibition with lovastatin.^{100,101,127,154}

Interestingly, in the high-risk neuroblastoma cell lines we observed a variable response in cell viability to treatment with downstream mevalonate pathway inhibition. When we investigated the metabolic activity and crystal violet staining of SH-SY5Y and SK-N-AS cells in response to 24-hour treatment with bisphosphonates we did not observe reductions in cell viability, however the SH-SY5Y cell line becomes sensitive with increased time, as effects on metabolic activity were observed with 48-hour zoledronate treatment. Cell viability appeared to increase with some bisphosphonate treatments (Figures 5 and 6) and we hypothesize this may be caused by imbalances in the prenylated proteins that regulate cell growth. Prenylated proteins regulate both oncogenic (increase growth) and tumor suppressor (restrict growth) signaling pathways in cancer

cells. If there is a smaller population of proteins regulating tumor suppressor signaling pathways than the population regulating oncogenic signaling, then treatment with prenylation inhibitors may result in the elimination of functional proteins associated with the tumor suppressor pathways while some functional proteins remain that drive oncogenic signaling. As the dose is increased this remaining population is eventually eliminated and the oncogenic signaling is disrupted, resulting in eventual loss of cell viability.

Treatment with GGTI-286 resulted in decreased crystal violet staining at both 24 and 48-hour treatment timepoints in both SH-SY5Y and SK-N-AS cell lines but only affected the metabolic activity of the SK-N-AS cell lines. This suggests that the response triggered in the SH-SY5Y cell line by GGTI-286 may impact adhesion to the well surface through a cell line specific mechanism. Our observations in the SK-N-AS cell line are consistent with those reported for mesenchymal neuroblastoma cells. These findings overall indicate that the cellular response to mevalonate pathway inhibition in high-risk neuroblastoma is as variable and dynamic as the cell lines themselves.

During our profiling of the cell viability response to mevalonate pathway inhibition with statins we noted unique phenotypic changes in the SH-SY5Y and SK-N-AS cell lines consistent with those correlated with differentiation status of neuroblastoma cells.¹⁵⁴ As previously discussed, the CSCs in neuroblastoma tumors, which are a population of undifferentiated cells, are thought to drive the aggressiveness of this cancer. As such differentiation is the current target of maintenance therapy for high-risk patients following stem cell transplants. Current treatment regimens use isotretinoin, which induces differentiation through HMGCR, and a targeted immunotherapy, with the goal of delaying recurrence with an overall survival rate of 73%.^{49–52} A novel differentiating agent DFMO was recently approved for continuation therapy for *MYCN* amplified high-risk patients.^{37,53–55} Our findings led us to investigate the utilization of mevalonate pathway inhibition in non-*MYCN* amplified high-risk neuroblastoma CSC populations.

To investigate the effect of mevalonate pathway inhibition on the CSC population in high-risk neuroblastoma cell lines we developed a spheroid-based culture method that we could adapt for our pharmacological assays. We had previous experience with glioblastoma sphere models for this purpose and used those protocols as a starting point. At the time of project inception there were a handful of papers on this topic with the most recent from the Hong Fei Ding lab with successful culture of mouse derived *MYCN* amplified sphere cultures.⁷⁸ Our first spheroid growth attempt utilized the SK-N-BE(2)-C cell line and showed rapid sphere formation in ultra-low attachment plates in a media formulation by Kumar et al..⁷⁷ When SH-SY5Y and SK-N-AS cells were subsequently plated in this media, they too formed true spheres within 14 days. Interestingly, some of the aggregates that formed during the growth assays would continue to sphere development while others would break apart into cell debris in all three cell lines. Of the three cell lines the SK-N-BE(2)-C spheres grew the quickest and largest, followed by the SH-SY5Y spheres, with SK-N-AS spheres being the slowest to form. Cultures of all three cell lines' spheres could be "passaged" and continue to grow for multiple generations in new plates/medium. Replating of sphere cells, as whole spheres or dissociated into single cell suspensions, in traditional adherent dishes or flasks, returned the cells to their typical adherent growth status. This shows that all three high-risk neuroblastoma cells lines demonstrate RAP and maintain the ability to alter growth in response to their environment.

This spheroid model system has limitations. The spheroid model is initiated from traditional cell culture, which overtime have genetic differences from newly patient derived samples. It also is limited to one parental cell type during formation, which contrasts with the microenvironment of patient tumors that feature numerous tumor cell types, structural cells, immune cells, blood vessels and more.

The first pharmacological assay we attempted using this spheroid model were metabolic activity assays. We were unsure if the MTT solution would be processed by the cells of the

sphere as we did not know how metabolically active these cells are but we did observe the traditional color change indicative of metabolic activity in the dissociated spheroid cells. The assay operates using the same protocol as our traditional suspension cell lines but in an ultra-low attachment plate and with the same day DMSO solubilization as overnight incubation with STOP solution caused the entire well to turn purple and cloudy which give unusable readings.

Interestingly, we found that the spheroid cultures of SH-SY5Y and SK-N-AS cells were more resistant to fluvastatin treatment than the adherent cultures and remained unaffected by zoledronate treatment at all time points tested. This is consistent with expectations for spheroid culture models to enrich for the cancer stem cell population of cell lines which are more chemo resistant than the bulk tumor cells. This reduction in sensitivity was not due to difficulty of the compounds accessing cells within the interior of spheroids as spheroids were dissociated prior to plating. Importantly, for us this validated the utility of this model in a traditional pharmacological assay.

Chapter 5

Inhibition of the mevalonate pathway targets neuroblastoma stem cells

Preface

This chapter is modified from the author accepted manuscript of the following publication:

Green, M.V., Reed, J.L., Zielinski, C.D., and Neighbors J.D. Inhibition of the Mevalonate Pathway Targets Neuroblastoma Stem Cells. *Pharmacology*. 2025.

Michelle V. Green conceptualized this project, planned and carried out the experiments and statistical analysis, and wrote the manuscript. J.L.R. contributed to experimental design and spheroid experiments with M.V.G. and C.D.Z. contributed to spheroid experiments. J.D.N. contributed to experimental design and edited the manuscript.

Abstract

Introduction:

High-risk neuroblastoma is a difficult to treat cancer with high rates of recurrence and treatment resistance, likely driven by a population of cancer stem cells. Multiple previous studies have suggested that the use of mevalonate pathway inhibitors, such as statins and bisphosphonates, may have anticancer activity against high-risk tumors.

Methods:

We aimed to observe the use of mevalonate pathway inhibitors against the cancer stem cell population of high-risk neuroblastoma cell lines SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C using spheroid-based assays.

Results:

We observed significant reductions in spheroid formation capability, stem cell frequency, and stemness properties of these neuroblastoma cancer stem cells with fluvastatin and zoledronate treatment.

Conclusion:

Treatment with mevalonate pathway inhibitors leads to reduced neuroblastoma cancer stem cell function through reductions in protein geranylgeranylation.

Introduction

Neuroblastoma is the most common malignancy in infants and the most common extracranial solid tumor in children in the United States, accounting for approximately 8% of pediatric neoplasms globally.^{1,2} At the time of diagnosis patients are stratified into low, intermediate, and high-risk groups based on multiple factors such as age, tumor stage, *MYCN* amplification, and chromosome abnormalities.^{181,182} Most patients who are over the age of one at the time of diagnosis, of whom about 70% have metastatic spread, are classified as high-risk.¹⁸³ Medical advancements have improved outcomes for low and intermediate risk patients however, despite intensive therapy regimens high-risk patients have a high chance of relapse and a five-year survival rate of only around 40-50%.^{1,183,184}

The mevalonate pathway (also called the cholesterol synthesis pathway or isoprenoid pathway) plays a key role in cancer signaling, and several cancers are known to co-opt this metabolic pathway. Significantly higher expression of mevalonate pathway genes is found in patient samples of high-risk neuroblastoma compared to non-high risk patient samples and has been correlated with decreased event-free survival.⁷⁸ The key intermediates in the mevalonate

pathway (Figure 10 A), including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are both precursors to larger compounds such as steroids and dolichols and important for proper function of key signaling proteins, including small GTPases of the Ras and Rho families.^{141,142} These proteins are dependent on post-translational modification with FPP and GGPP respectively, for proper membrane localization and signaling.¹²¹ Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR), which catalyzes the first step in the mevalonate pathway.¹²⁶ Bisphosphonates, including zoledronate (zoledronic acid), inhibit later steps in the pathway. Both classes of compounds lead to decreased intracellular production of the isoprenoids FPP and GGPP, which can result in protein mis-localization and disruption of cellular signaling cascades.

There is significant evidence that inhibitors of this pathway such as statins and bisphosphonates may have antineoplastic effects on high-risk neuroblastoma.^{100,101,127,154} Neuroblastoma cell line sensitivity to statins, specifically lovastatin, was initially reported in the 1980s, and interestingly, researchers noted differentiation-like phenotypic changes upon treatment.^{148,149,153} These changes included neurite-like outgrowths that mimicked the differentiation induced by retinoic acid (RA) treatment, which is an established therapy used in neuroblastoma and that the lovastatin and RA-induced changes were both mediated by HMGCR.¹²⁷ There was a range of sensitivity within neuroblastoma cell lines and this sensitivity was linked to the ability of cell lines to upregulate HMGCR when treated, as well as the RA responsiveness.^{101,127}

The use of zoledronate has been shown to have potential for treatment of neuroblastoma bone metastasis through induction of immune regulation and angiogenesis, however some *in vivo* animal models suggest the drug may be absorbed too quickly.^{164,165,171,185} A phase 1 study in neuroblastoma patients found that zoledronate was well tolerated in conjunction with cyclophosphamide treatment and there are ongoing trials in additional high-risk pediatric tumor

types on the utility of combination therapy.¹⁷¹ Zoledronate treatment may also sensitize cancer cells to immune responses, specifically the V γ 9V δ 2 T cells, which can inhibit *in vitro* cell growth in adherent and sphere culture, and *in vivo* mouse models.^{168,172,173,185,186} There are ongoing clinical efforts to use zoledronate in conjunction with immunotherapy to sensitize the tumor-initiating cells (TICs) to a population of T cells in the patient.^{168,172,173}

Targeting TICs, also known as cancer stem cells (CSCs), may be a viable option for the treatment of high-risk neuroblastoma. These CSCs are thought to contribute to treatment resistance, metastasis, and relapse of many aggressive cancers such as breast cancer, leukemia, pediatric brain cancer, and neuroblastoma.^{79,80,84–86} Neuroblastoma tumors are comprised of cells along a spectrum of differentiated adrenergic like cells and undifferentiated mesenchymal-like cells, with high-risk and relapsed tumors comprised of more of the undifferentiated population.⁶ Neuroblastoma CSCs are a population of self-renewing, highly proliferative, undifferentiated cells and the presence of these CSCs in patients is correlated with worse prognoses.^{79,80} This population of cells likely contributes to the intratumor heterogeneity and plastic nature of high-risk neuroblastoma tumors.⁸⁰ Spheroid cultures of neuroblastoma patient derived TICs and cell-lines have been shown to enrich for the CSCs, but these models have not yet been widely utilized for investigating the role of the mevalonate pathway in human cancer cell lines.^{77,102}

The mevalonate pathway has been implicated in the growth of mouse model of *MYCN* amplified neuroblastoma spheroids, which show an upregulation of the pathway in the sphere-forming cells.⁷⁸ The sensitivity of neuroblastoma cell lines in adherent cultures to mevalonate pathway inhibition appears to be independent of *MYCN* amplification status and is driven by *HMGCR* expression.^{78,103} Investigation into the mechanism of action of statins in standard undifferentiated neuroblastoma cell lines has found that lovastatin induces apoptosis through inhibition of the mevalonate pathway that results in loss of protein prenylation and reductions in membrane localization of Ras and Rho family proteins.^{100,160} This mechanism of action was

observed in the high-risk SH-SY5Y cell line, as well as in breast, lung, and brain cancer cell lines, and confirmed to occur across the mesenchymal subtype of neuroblastoma cell lines, including in SK-N-AS cells.^{102,103}

In this work we aimed to investigate the use of mevalonate pathway inhibitors, fluvastatin and zoledronate, against multiple high-risk neuroblastoma cell lines in a spheroid model (shown in Table 1). The majority of this work used the SH-SY5Y and SK-N-AS cell lines as these cell lines are not *MYCN* amplified, which is lacking in most high-risk patients. The *MYCN* amplified cell line, SK-N-BE(2)-C, was used to repeat critical experiments in this additional high-risk subtype. All of these cell lines have genetic profiles like those of patients that die within 18 months of diagnoses.^{175,187} The spheroid model enriches for the CSC population and provides a means to assess the impact of mevalonate pathway inhibition in this critical subset of cells.

Methods

Experimental Model Details

Cell lines and reagents

SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C cells were obtained from ATCC, and all experiments were performed within 15 passages of receipt. For monolayer cultures, SH-SY5Y and SK-N-BE(2)-C cells were maintained in a 1:1 mixture of Minimum Essential Media (MEM) (Gibco) and F-12 medium (Gibco) with 10% fetal bovine serum (FBS) added (Hyclone, from ThermoFisher Scientific). SK-N-AS cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 0.1 mM Non-Essential Amino Acids (NEAA) (Gibco). All cells were maintained at 37°C and 5% CO₂.

Spheroids were generated and maintained in spheroid medium composed of DMEM/Ham's F12 (3:1), anti-anti (1:100; Gibco), human FGF basic (Invitrogen) and EGF (Gibco) both at 20 ng/mL, heparin (5 µg/mL; Sigma), and 1x B-27 (Gibco).⁷⁷ Media was mixed fresh for each experiment and 1x B-27 was added to spheroid media immediately before use. For spheroid cultures with GDNF (Sigma) was added at 10 ng/mL to spheroid media immediately before use. All spheroid cultures were incubated at 37°C and 5% CO₂.

Fluvastatin was obtained from Selleck Chemicals and reconstituted in DMSO to 100mM and stored at -20°C before use. This stock was then diluted to 10mM immediately before dilution to final concentration in complete cell culture media, as above. Zoledronic acid was obtained from Selleck Chemicals and reconstituted in 0.5 M sodium hydroxide to 10mM and stored at -20°C before use. It was also diluted to final concentration in complete cell culture media

immediately prior to use. FOH and GGOH were obtained from ThermoFisher Scientific and used at 30 μ M and 20 μ M respectively.

Method Details

Spheroid forming assays

Cells were plated at 200 cells/ well in given concentrations of compounds in the spheroid media in low adhesion 96-well plates (Corning Costar) and incubated. Plates were incubated for 11 days before quantification for SH-SY5Y cells, 14 days before quantification for SK-N-AS cells and 10 days before quantification of SK-N-BE(2)-C cells. At these time points there was maximum spheroid formation without significant cell death. PBS was used in all outside wells to reduce evaporation. For counting, spheroids were defined as rounded aggregates of cells with a smooth surface and poor cell-to-cell definition. Loose aggregates of well-defined cells were not included in spheroid counts. This was based on methods previously described.¹⁸⁸

For spheroid forming assays results are presented as box plots, with whiskers representing the minimum and maximum values and the box representing quartiles. Spheroid counts were carried out with a minimum of 60 wells counted per condition per independent experiment and the experiment was repeated a minimum of 3 times. Spheroid count data was analyzed with two-way ANOVA followed by Dunnett's post hoc test for multiple comparisons for Figure 1 and with a two-way ANOVA followed by Tukey's post hoc test for multiple comparisons for Figures 3 and 8. Dunnett's post hoc was originally used for comparison of treatment to control but further multiple comparison analysis required the inclusion of Tukey's post hoc.

Extreme limiting dilution assays

SH-SY5Y cells and SK-N-AS cells were plated at 1000, 200, 100, 20, 10, 5, and 1 cell(s)/well in 200 μ l spheroid media in low adhesion 96 well plates (Corning Costar) with indicated concentration of compounds. Plates were incubated for 11 or 14 days as described above before quantification. PBS was used in all outside wells to reduce evaporation. For counting, spheroids were defined as above. For these assays, 240 replicate wells were counted for cells plated at 1000, 200, and 100 cells/well, 480 replicate wells were counted for cells plated at 20, 10, and 5 cells/well and a minimum of 70 replicate wells were counted that were verified to contain a single cell at plating. Analysis was done using the online extreme limiting dilution analysis (ELDA) webtool that uses maximum likelihood estimations in generalized linear models to determine the spheroid forming cell frequency followed by chi-square tests for significance.¹⁸⁹ This method was adapted from ELDA analysis in other cancers.^{189,190} ELDA results are presented as 95% confidence intervals.

Spheroid invasion assays

SH-SY5Y cells, SK-N-AS cells, and SK-N-BE(2)-C cells were plated in 200 μ l spheroid media in low adhesion round bottom 96 well plates (Corning Costar) and grown for 3 to 5 days. Spheroids were transferred to new 96-well plates containing GeltrexTM basement membrane matrix with indicated compounds and spheroid transfer was confirmed under microscope. Spheroids were incubated for 14 days and imaged on Day 0, 7, and 14 with an ECHO Revolve Microscope. SK-N-BE(2)-C spheroids were also imaged in Day 3. This method was modified from previously described protocols.¹⁹¹ The total spheroid area was quantified using the Measurement feature in Adobe Photoshop with a scale bar of 890 μ m from the ECHO Revolve

Microscope.¹⁹² Data analyzed with two-way ANOVA with Tukey's post-hoc for multiple comparisons.

Cell viability assays

MTT assays were carried out as previously described,¹⁷⁹ with the following exception. Spheroid cells were plated at 50,000 cells/well in 50 μ L of complete spheroid media and then treated with 50 μ L of 2x treatment media as noted before incubation. At 44 hours of treatment, 10 μ L of 5 mg/mL MTT reagent (in water) was added to each well. At 48 hours of treatment 50 μ L of DMSO was added to each well and plates read at 570 nm on SpectraMax i3x plate reader (Molecular Devices).

Spheroid self-renewal assays

For self-renewal assays cells were cultured as described above for spheroid formation assays and grown for 5 days. On Day 5 spheroids were harvested and washed 3x with 1x DPBS (Gibco) before subject to enzymatic digestion with 0.25% Trypsin-EDTA (Gibco) for 2 minutes with vigorous pipet mixing. Trypsin was neutralized with complete adherent cell culture media followed by centrifugation to remove the trypsin media solution. The pellet was then resuspended, cells were counted and resuspended to a final concentration of 10 cells/mL. 100ul of the single-cell suspension was plated into 96-well ultra-low-attachment round bottom plates (Corning Costar) and single-cell wells were confirmed to achieve a minimum of 120 wells per replicate with a minimum of 3 biological replicates performed. Spheroids were grown for 14 days. Fresh spheroid media (with or without treatment) was added on Day 7. For counting, spheroids were defined as above, and 120 wells counted per condition each replicate with 3

replicates for SK-N-AS spheroids and 4 replicates for SH-SY5Y spheroids. For SK-N-BE(2)-C 3 ultra-low attachment round bottom plates were plated per condition tested with a minimum of 45 wells per replicate and a total of 4 replicates performed. Data analyzed via two-way ANOVA with Sidak's post-hoc for multiple comparisons.

Quantitative real-time PCR

Spheroids were grown as described above with the indicated concentrations of fluvastatin and zoledronate for 72 hours. RNA was harvested on ice using RNeasy Mini Kit (Qiagen). Synthesis of cDNA from total RNA was performed following manufacturer's instruction using superscript III reverse transcriptase (Invitrogen).

Quantitative RT-PCR was performed to confirm expression of stemness related genes using Taqman gene expression assays. Briefly cDNA samples were combined with Taqman Gene Expression Master Mix (Applied Biosystems) and Taqman primers and plated in hard-shell PCR plates 96-well thin wall (BioRad). The thermal protocols were provided by Applied Biosystems and are available upon request. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method, whereas the mean fold change = $2^{-(\text{average } \Delta \Delta C_t)}$ was assessed using the mean difference in the ΔC_t between the gene and the internal control *GAPDH*. Genes assayed include: *nestin*, *Sox2*, *RBFOX3*, and *GFAP* (Table 3). *GAPDH* had a standard deviation of 1.91 and 2.74 for SH-SY5Y and SK-N-AS respectively.

Statistical significance was assessed for all genes assayed using ΔC_t values via two-way ANOVA with Bonferroni's correction with GraphPad Prism 10.

Gene Symbol	Gene Name	Gene Aliases	Assay ID	ThermoFisher Scientific Catalogue #
<i>GAPDH</i>	glyceraldehyde-3-phosphate dehydrogenase	G3PD, GAPD, HEL-S-162cP	Hs02786624_g1	4331182
<i>NES</i>	nestin	Nbla00170	Hs04187831_g1	4331182
<i>SOX2</i>	SRY-box 2	ANOP3, MCOPS3	Hs01053049_s1	4331182
<i>RBFOX3</i>	RNA binding protein, fox-1 homolog 3	FOX-3, Fox3, HRNBP3, NEUN	Hs01370653_m1	4331182
<i>GFAP</i>	glial fibrillary acidic protein	ALXDRD	Hs00909233_m1	4331182

Table 3: *Taqman*[®] gene expression assays for qRT-PCR.

PCR primers used.

Immunoblotting

Spheroids were grown as described above and maintained in 6-well ultra-low attachment plates (Corning Costar). Prior to treatment spheroids were harvested and replated at a 1:3 density in a new 6-well plate with the indicated concentrations of fluvastatin and zoledronate for up to 5 days. Spheroids were harvested in RIPA buffer with protease inhibitors and protein quantification was performed by Pierce BCA assay (ThermoFisher). Equal amount of protein (5 – 10 µg) were loaded on precast 4-12% bis/tris gels (Invitrogen) and run at 175 V for 60-90 minutes in MOPS or MES buffer (ThermoFisher). MOPS buffer was used for proteins larger than 50 kilodalton (kDa) and MES buffer was used for those smaller than 50 kDa. Transfer was carried out on ice at 25 V for 90 minutes onto a polyvinylidene fluoride membrane. Total protein stain was done with No-Stain™ Protein Labeling Reagent (Invitrogen), visualized on a Bio-Rad Chemidoc, and quantified for total protein normalization in Image Lab (Bio-Rad). Membranes were then blocked with 1x ScanLater buffer (Molecular Devices), probed with primary antibodies overnight (1:500 dilution), rinsed with ScanLater wash buffer and then probed with ScanLater Eu-conjugated secondary antibodies (Molecular Devices). Dry membranes were imaged with a SpectraMax i3x with a Western blot cartridge (Molecular Devices). Membranes were stripped with Restore™ Plus Western Blot Stripping Buffer for 10 minutes and reimaged on the SpectraMax i3x to ensure removal of the first antibody used. These membranes were then blocked, reprobed for the indicated antibodies overnight, washed, incubated with secondary antibody and imaged on the SpectraMax i3x. All full-size western blot images are available upon request. Quantification was done on ImageJ with statistical analysis (two-way ANOVA with Sidak's post-hoc for multiple comparisons) performed with GraphPad Prism 10.

Primary antibodies included: apoptosis cocktail (ab136812), cell cycle cocktail (ab139417), RhoB (CST 63876S), CDC42 (ab187643), Rap1a/Rap1b (CST 4938S), RhoA (CST 2117S), Ras (CST 3339S), Rac1/Rac2/Rac3 (ab282581), nestin (CST 73349S), sox2 (CST 3579S), E-cadherin (CST 14472), pan-cadherin (CST 4068S), and vimentin (CST 5741S).

Quantification and Statistical Analysis

All data shown are representatives of 2-5 independent experiments. Data points shown are presented as mean $\pm$ SEM unless otherwise indicated. Statistical analysis and figure generation was performed with GraphPad Prism 10 unless otherwise indicated. Adobe Photoshop was used to quantify the total spheroid area and generate some of the panels of Figure 6. A p-value < 0.05 was considered significant, and all p values were corrected for multiple comparisons with appropriate post-hoc tests as noted. P-values were calculated for * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Statistical details for each experiment can be found in the method details section as well as the figure legends.

Results

Mevalonate pathway inhibition alters spheroid formation of high-risk neuroblastoma

To investigate the role of the mevalonate pathway (Figure 10 A) in high-risk neuroblastoma cancer stem cells we performed a spheroid formation assay in SH-SY5Y, SK-N-AS and SK-N-BE(2)-C cell lines. The ability for cells to survive in this serum-free suspension culture is related to the plasticity of these undifferentiated stem-like cell populations.^{80,99} A range of concentrations of fluvastatin and zoledronate were used. In both non-*MYCN* amplified cell lines a dose-dependent effect was observed (Figure 10 B-C). After 11 days, the SH-SY5Y cell line spheroid forming capability was reduced by 1 μ M fluvastatin and 10 μ M zoledronate treatment and nearly eliminated by 10 μ M fluvastatin and 30 μ M zoledronate treatment (Figure 10 B). The range how many spheroids formed in each well was altered with 3 μ M zoledronate treatment in the SH-SY5Y cell line as well but the effect on median number of spheroids per well was minimal.

After 14 days, the SK-N-AS cell line spheroid forming capability was significantly reduced by 1 μ M fluvastatin and 3 μ M zoledronate treatment and further decreased with increased doses of 10 μ M fluvastatin and 10 μ M and 30 μ M zoledronate treatment (Figure 10 C). The SK-N-AS cell line took longer to form true spheroids in the culture system and the spheroids appeared smaller than those of the SH-SY5Y cell line as well.

After 10 days, the *MYCN*-amplified SK-N-BE(2)-C cell line spheroid forming capability was significantly reduced by treatment with fluvastatin and zoledronate in a dose-dependent manner (Figure 10 D). Treatment with 1 μ M fluvastatin and 3 μ M zoledronate did result in a significant reduction in the number of spheres formed per well and treatment with increased doses furthered this observed effect (Figure 10 D). The SK-N-BE(2)-C cell line grew spheres in a

similar timeline to the SH-SY5Y cell line however did not produce as many spheroids per well. The SK-N-BE(2)-C spheroids appeared larger than those observed for the other cells lines, however this was not quantified in this assay.

To determine if the reduction in spheroid formation was related to reductions in the frequency of spheroid-forming cells in the population, an extreme limiting dilution analysis (ELDA) was performed in SH-SY5Y and SK-N-AS spheroids. For this assay SH-SY5Y and SK-N-AS cells were plated at decreasing cell densities down to a single cell per well and the quantity of spheroid positive wells were counted after 11 or 14 days, respectively. This was then used to calculate the frequency of the spheroid forming cells in the SH-SY5Y and SK-N-AS cell populations. Over the course of 11 days there was a significant dose-dependent reduction in spheroid forming cell frequency with 1 μ M and 10 μ M fluvastatin in the SH-SY5Y cells (Figure 10 E). There was significant reduction in spheroid forming cell frequency with 30 μ M zoledronate in the SH-SY5Y as well, however 3 μ M zoledronate did not have an effect. Over the course of 14 days there was a significant dose-dependent reduction in spheroid forming cell frequency with 1 μ M and 10 μ M fluvastatin as well as 3 μ M, 10 μ M and 30 μ M zoledronate in the SK-N-AS cell line (Figure 10 F).

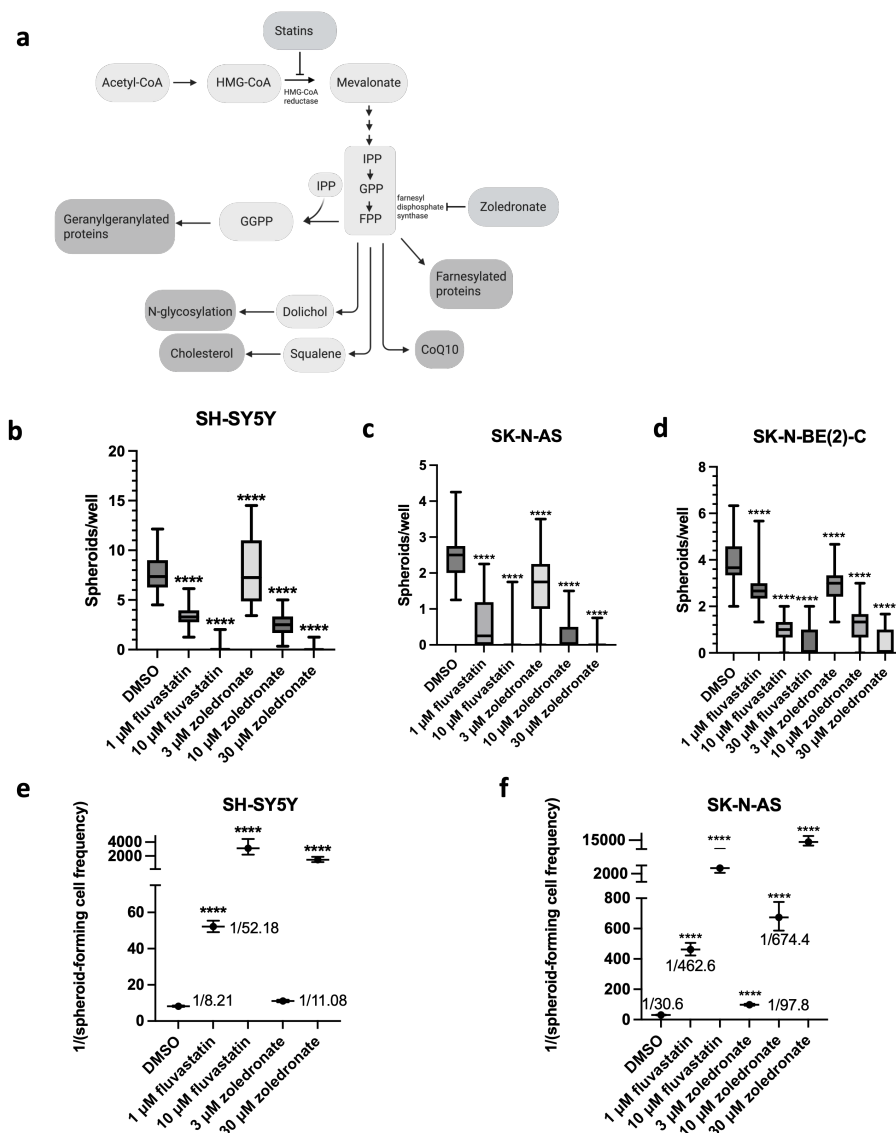


Figure 10: Mevalonate pathway inhibition alters neuroblastoma spheroid formation.

(A) The mevalonate pathway. IPP: isopentenyl pyrophosphate. GPP: geranyl pyrophosphate. FPP: farnesyl pyrophosphate. GGPP: geranylgeranyl pyrophosphate. Created in BioRender. Green, M. (2023) BioRender.com/y01q648. (B-D) Spheroid formation assays of SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C in the presence and absence of indicated concentration of fluvastatin and zoledronate. Spheroid counts were carried out with a minimum of 60 technical replicate wells

counted per condition for each of 3 independent experiments. Data analyzed via two-way ANOVA with Dunnett's post-hoc, α 0.05. Data shown as box and whisker plots with the whiskers representing minimum and maximum values and the box representing quartiles. (E-F) Frequency of spheroid-forming cells determined by extreme limiting dilution assays of SH-SY5Y and SK-N-AS cell lines. Upper and low bars represent the 95% CI. Analysis was done with the ELDA webtool. **** $p < 0.0001$

Altered spheroid formation may be due to loss of protein prenylation

There is substantial evidence in the literature that inhibition of the mevalonate pathway disrupts cellular function through the loss of protein prenylation including farnesylation and geranylgeranylation.^{100,154} Specifically, in neuroblastoma it is thought that loss of geranylgeranylation results in the reported effects in adherent cell culture.^{100,101,154} Therefore, we supplemented SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C spheroids with 20 μ M geranylgeraniol (GGOH) and 30 μ M farnesol (FOH) to observe if this could rescue fluvastatin and zoledronate induced reductions in spheroid forming capabilities. GGOH and FOH are the alcohol forms of the isoprenoids GGPP and FPP, respectively. The use of GGOH or FOH rather than GGPP or FPP for supplementation utilizes a yet unidentified kinase system that phosphorylates the prenyl groups and introduces the substrates downstream of the enzymes inhibited by fluvastatin or zoledronate.¹⁴⁷

Over the course of 11 days, we observed that neither GGOH nor FOH supplementation fully rescued the reduction in spheroid forming capabilities induced by 10 μ M and 1 μ M fluvastatin treatment in SH-SY5Y cells (Figure 11 A). Similarly, over the course of 11 days we observed that supplementation with either GGOH or FOH could not rescue spheroid forming capabilities of SH-SY5Y cells when treated with 30 μ M zoledronate (Figure 11 B). The reduction in spheroid forming capabilities of SH-SY5Y cells induced by 10 μ M zoledronate was partially

rescued by supplementation with GGOH but not FOH and supplementation with GGOH and FOH increased spheroid forming capabilities of this cell line in the presence of 3 μ M zoledronate (Figure 11 B).

Over the course of 14 days, we observed that supplementation with GGOH could rescue the reduction in spheroid forming capabilities of the SK-N-AS cell line in the presence of 10 and 1 μ M fluvastatin (Figure 11 C). Interestingly, supplementation with FOH further decreased the spheroid forming capabilities in SK-N-AS cells treated with fluvastatin. Supplementation with GGOH also partially rescued the spheroid forming capabilities of SK-N-AS cells in the presence of 10 μ M zoledronate and fully restored it with 3 μ M zoledronate treatment (Figure 11 D).

Over the course of 10 days, we observed that neither supplementation with GGOH nor FOH could fully rescue the reduction in spheroid formation capabilities induced by fluvastatin or zoledronate treatment in SK-N-BE(2)-C cells (Figure 11 E-F). The reduction in spheroid formation induced by fluvastatin at all tested doses and 3 μ M and 10 μ M zoledronate was partially rescued with supplementation of GGOH. The supplementation with FOH further decreased the reduction in spheroid formation induced by treatment with all doses of both fluvastatin and zoledronate tested (Figure 11 E-F).

To observe if there was similar restoration of spheroid forming cell frequency, an ELDA with 20 μ M GGOH and 30 μ M FOH was performed in both SH-SY5Y and SK-N-AS cell lines. We found that supplementation with GGOH was able to partially rescue spheroid-forming cell frequency in the presence of low-dose mevalonate pathway inhibitors in both SH-SY5Y and SK-N-AS cells (Figure 11 G-H). Additionally, when SH-SY5Y spheroids were treated with an alternative bisphosphonate, digeranylbisphosphonate (DGBP) at 30 μ M, we saw a significant reduction in spheroid forming cell frequency (Figure 11 G). This compound, DGBP, is a direct inhibitor of geranylgeranyl diphosphate synthase and thus directly lowers GGPP levels without

effecting the levels of FPP.^{139,140} We also saw a significant reduction in the spheroid forming cell frequency of SH-SY5Y cells when treated with a geranylgeranyltransferase inhibitors GGTI-286 also at 30 μ M (Figure 11 G). These results suggest that the reduction in protein geranylgeranylation is driving the effects on neuroblastoma cancer stem cells.

(A-F) Spheroid formation assays of SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C in the presence and absence of indicated concentration of fluvastatin and zoledronate with addition of farnesol (FOH)

and geranylgeraniol (GGOH) as indicated. Spheroid counts were carried out in triplicate with a minimum of 60 replicate wells counted per condition. Data analyzed via two-way ANOVA with Dunnett's post-hoc, α 0.05. Data shown as box and whisker plots with the whiskers representing minimum and maximum values and the box representing quartiles. **(G-H)** Extreme limiting dilution assays of SH-SY5Y and SK-N-AS to determine the frequency of spheroid-forming cells in the presence of indicated compounds. Upper and low bars represent the 95% CI. Analysis was done with the ELDA webtool. ** $p \leq 0.01$ **** $p \leq 0.0001$

To investigate if the mevalonate pathway inhibitors were cytotoxic to the neuroblastoma spheroids, we performed metabolic activity assays and probed for apoptosis and cell cycle protein markers in the SH-SY5Y and SK-N-AS spheroid. There was no significant reduction in the metabolic activity of the spheroids in either cell line in the MTT assay (Figure 12 A-B). For protein analysis the lowest doses 1 μ M fluvastatin and 3 μ M zoledronate were used to treat second generation SH-SY5Y and SK-N-AS spheroids over the course of 5-days. The higher doses of fluvastatin and zoledronate eliminated the ability of spheroids to be cultured in high enough quantities to successfully harvest protein for analysis. There was no significant induction of cell cycle arrest or induction of apoptosis in either SH-SY5Y or SK-N-AS spheroids with either fluvastatin or zoledronate treatment (Figure 12 C-G). There was a significant reduction in total procaspase 3 protein levels in SH-SY5Y spheroids however there was no evidence of cleaved caspase 3 or cleaved PARP. These findings indicate that the mevalonate pathway inhibitors are impacting the functionality of the neuroblastoma cancer stem cells rather than significantly impacting their cell viability.

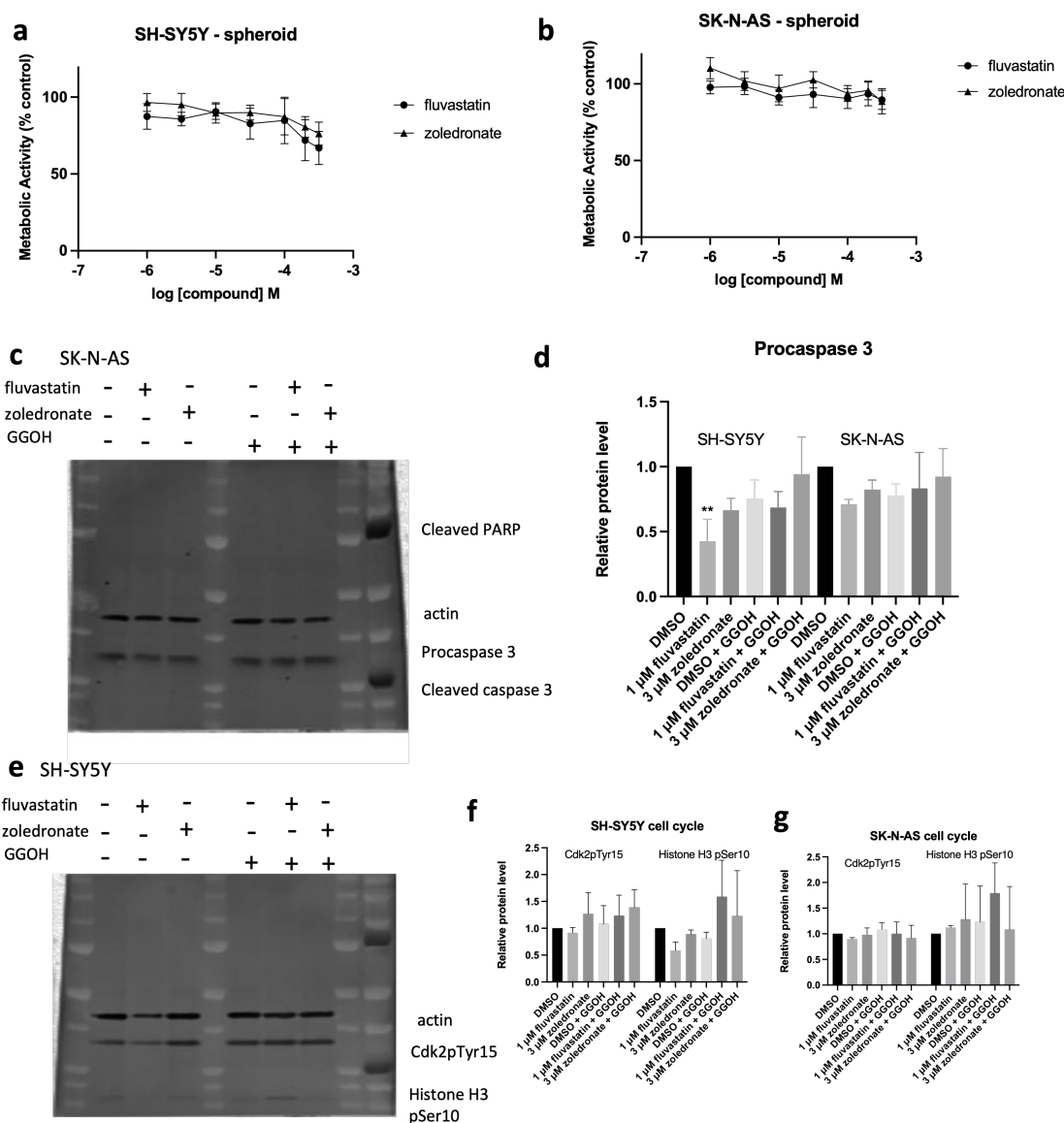


Figure 12: Mevalonate pathway inhibition does not alter neuroblastoma spheroid cell viability.

(A-B) Compound response was determined by 48-hour MTT assay in adherent and spheroid cultures of SH-SY5Y and SK-N-AS cell lines. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments. (C-G) Western blot analysis of induction of apoptosis (C-D) and cell cycle (E-G) arrest in neuroblastoma spheroids treated with indicated concentrations of fluvastatin and zoledronate in the presence and absence of GGOH addback. Representative blot images shown. Graphs representative of 3 independent experiments presented as mean $\pm$ SEM. Data analyzed by two-way ANOVA with Sidak's post-hoc for multiple comparisons.

To determine which of the small GTPases may be involved in these observed effects we treated SH-SY5Y and SK-N-AS second-generation spheroids with 1 μ M fluvastatin and 3 μ M zoledronate over the course of 5-days with or without supplementation of GGOH. We observed that treatment of SH-SY5Y and SK-N-AS spheroids with fluvastatin resulted in a decrease in the protein level of RhoB, which was rescued with GGOH supplementation (Figure 13 A-B). In both SH-SY5Y and SK-N-AS spheroids there was a slight reduction in the RhoB protein level following zoledronate treatment, however it was not statistically significant. In the SH-SY5Y spheroids, we observed that fluvastatin treatment resulted in a decrease in the protein level of RhoA, CDC42, a Rho family GTPase, and a decrease in the total Rap1a/Rap1b protein level, which were partially rescued with the addition of GGOH (Figure 13 A). In the SK-N-AS spheroids we observed that fluvastatin treatment resulted in a decrease in the protein level of total Rap1a/Rap1b, which was rescued with the addition of GGOH (Figure 13 B). We did not observe changes in CDC42 protein levels in SK-N-AS spheroids (Figure 13 B) and there was a slight decrease in RhoA levels with fluvastatin treatment in SK-N-AS spheroids that was not significant. Zoledronate treatment did not alter protein levels of RhoA, CDC42, or total Rap1a/Rap1b in either SH-SY5Y or SK-N-AS spheroids. Rap1 is the only member of the Ras family of GTPases that is geranylgeranylated rather than farnesylation and has two isoforms Rap1a/Rap1b both of which are implicated in cancer signaling. Rap1 demonstrates shifts on western blotting dependent on prenylation status.¹⁹³ We observed that the ratio of active (prenylated) Rap1 to total Rap1 may have visually decreased in response to fluvastatin and zoledronate treatment in SH-SY5Y spheroids and increased with GGOH supplementation however this was not significant (Figure 13 A). We did not observe a change in this ratio in SK-N-AS spheroids with either treatment. Fluvastatin treatment of SH-SY5Y and SK-N-AS spheroids did appear to slightly reduce the amount of Rac protein however this was not

significant (Figure 13 A-B). Zoledronate treatment did not significantly alter Rac protein levels in SH-SY5Y or SK-N-AS spheroids. Total Ras protein levels were not significantly altered in SK-N-AS spheroids (Figure 13 B) with either fluvastatin or zoledronate treatment. Total Ras protein levels in SH-SY5Y spheroids seemed to decrease with fluvastatin treatment however this was not significant and zoledronate treatment increased total Ras protein levels in a manner that was not rescued with GGOH supplementation (Figure 13 A). The use of only lower treatment doses for efficient protein harvest may limit the effects observed on protein levels and work is ongoing to further improve the spheroid harvest with higher doses and to investigate the signaling pathways modulated by these small GTPases.

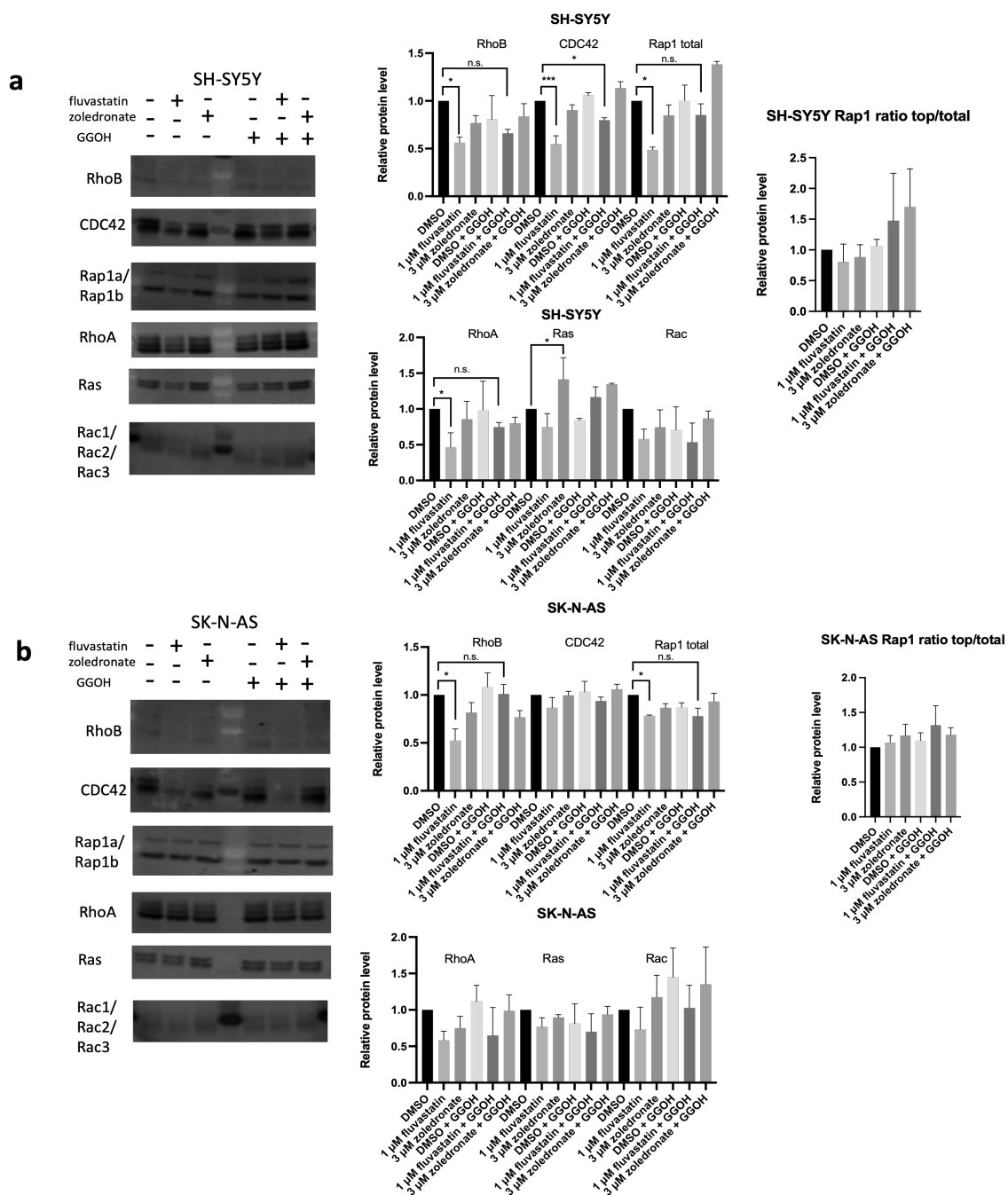


Figure 13: Mevalonate pathway inhibition effects on a subset of small GTPases.

(A-B) Western blot analysis of indicated small GTPases in SH-SY5Y and SK-N-AS spheroids treated with the indicated concentrations of fluvastatin and zoledronate in the presence and absence

of GGOH for 5 days. Representative blot images shown. Graphs representative of 3 independent experiments presented as mean $\pm$ SEM. Data analyzed by two-way ANOVA with Sidak's post-hoc for multiple comparisons.

Mevalonate pathway inhibitors alter stemness of neuroblastoma spheroids

Current treatment of high-risk neuroblastoma includes the use of retinoic acid (RA) to induce differentiation, in a HMGCR-dependent mechanism, however RA-resistance and relapse remain major concerns.¹²⁷ Therefore, we next investigated if the observed effects on neuroblastoma spheroids were associated with alterations in the differentiation/stemness of the CSCs. To do this we looked at 2 common stemness markers, nestin and Sox2 (sex determining region Y-box 2), and two differentiation associated markers neuronal nuclei protein (NeuN) (neuronal differentiation) and glial fibrillary acidic protein (GFAP) (glial differentiation factor) in neuroblastoma spheroids.^{79,154,194} Using qRT-PCR we did not detect NeuN or GFAP mRNA in spheroids from either cell line under any treatment condition, indicating that the mevalonate pathway inhibition was not inducing neuronal or glial differentiation. Treatment with either fluvastatin or zoledronate did not significantly alter the mRNA or protein expression of the stemness markers nestin or Sox2 (Figure 14 A-E). This indicates that the reductions in spheroid functionality occur prior to significant induction of differentiation of neuroblastoma cancer stem cells.

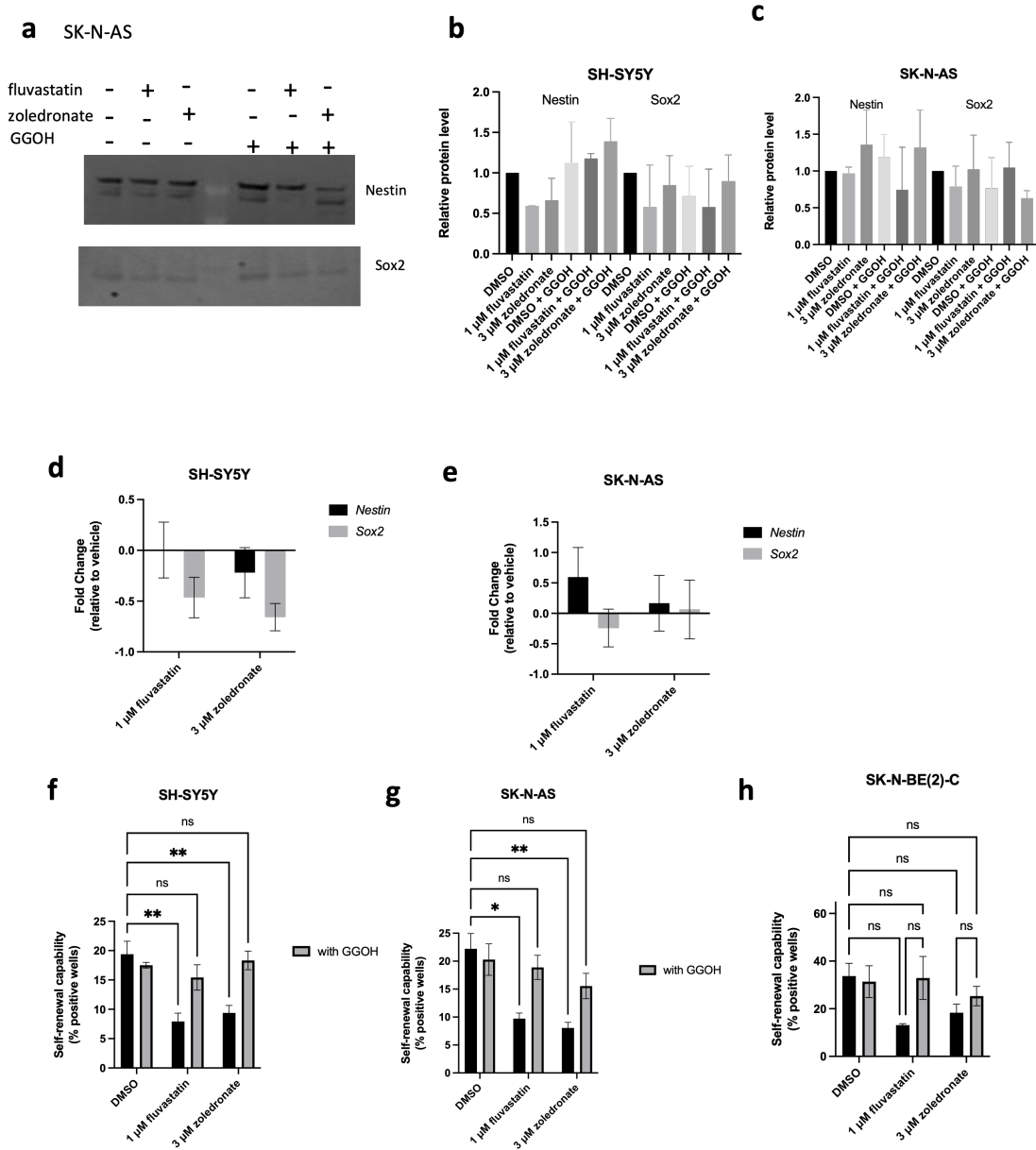


Figure 14: Mevalonate pathway inhibition impact on stemness characteristics of neuroblastoma spheroids.

(A-C) Western blot analysis of neuroblastoma differentiation associated proteins nestin and sox2. SH-SY5Y and SK-N-AS spheroids were treated with the indicated concentrations of fluvastatin, zoledronate and GGOH and harvested after 5 days. Representative blot images shown. Graphs representative of 3 independent experiments. Data analyzed by two-way ANOVA with Sidak's post-hoc for multiple comparisons. (D-E) qRT-PCR analysis of neuroblastoma differentiation

associated genes. Graphs represent fold change with vehicle treatment = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method. **(F-H)** Self-renewal analysis of SH-SY5Y, SK-N-AS and SK-N-BE(2)-C spheroids. Graphs representative of 3 independent experiments with a minimum of 120 wells counted per condition each replicate for SH-SY5Y and SK-N-AS spheroids. Graphs representative of 3 independent experiments with a minimum of 45 wells counted per condition each replicate for SK-N-BE(2)-C spheroids. Graphs presented as mean $\pm$ SEM. Data analyzed via two-way ANOVA with Sidak's post hoc. * $p \leq 0.05$ ** $p \leq 0.01$

An additional marker for stemness is the ability of spheroid cells to self-renew or regrow their population from a single-cell suspension.⁸⁰ For this assay we grew primary spheroid cultures and then disassociated them into single-cell suspension in the presence or absence of fluvastatin or zoledronate treatment. The spheroid cultures were allowed to grow, and positive wells were counted after 14 days. For both non-MYCN amplified cell lines treatment with fluvastatin and zoledronate reduced the self-renewal capability and this was rescued with GGOH supplementation (Figure 14 F-G). In the SK-N-BE(2)-C cells a similar trend was observed with fluvastatin and zoledronate treatment but this was not significant (Figure 14 H). These results demonstrate that mevalonate pathway inhibition can block the functionality of individual spheroid cells to both form primary spheroids as well as maintain the spheroid population in both SH-SY5Y and SK-N-AS cell lines.

Neuroblastoma CSCs also contribute to the metastatic potential of high-risk tumors therefore we then investigated the effect that treatment with mevalonate pathway inhibitors has on the invasive properties of neuroblastoma spheroids.¹⁹¹ To do this, primary spheroids were grown and then seeded in a basement membrane matrix, which mimics the native 3D environment of a tumor, with or without mevalonate pathway inhibitors and incubated for two weeks. There was a significant decrease in the invasion of SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C (Figures 15-17) spheroids with fluvastatin treatment at all doses tested. The fluvastatin

treatment eliminated the presence of the peripheral invading cells surrounding the central sphere core and resulted in a significant decrease in the total spheroid area in all three cell lines compared to the control spheroids on Day 7 and Day 14 (Figures 15-17). There was a significant dose-dependent decrease in the invasion of the SK-N-AS spheres with zoledronate treatment observed on both Day 7 and Day 14 (Figure 16). The reduction in the outer peripheral invading cells was present with high-dose treatment but could not be observed with the lower doses, however the total spheroid area was still significantly reduced (Figure 16). There was also a dose-dependent reduction in the invasion of SH-SY5Y cells with zoledronate treatment however the lower-doses tested were less effective in this cell line with the lowest dose not significantly reducing the presence of the outer peripheral invading cells or total spheroid area over the course of 14 days (Figure 15). There was also a dose-dependent reduction in the invasion of SK-N-BE(2)-C cells with zoledronate treatment (Figure 17). The lowest zoledronate dose tested did not reduce the total spheroid area or the presence of the invading cells surrounding the central sphere cores however the higher doses did significantly reduce both over the course of 14 days (Figure 17). Additionally, we imaged SK-N-BE(2)-C spheroids on an additional day, day 3, and noted that initial growth was not impacted by either treatment with fluvastatin or zoledronate but the dose-dependent impacts were present over the course of 7 and 14 days (Figure 17).

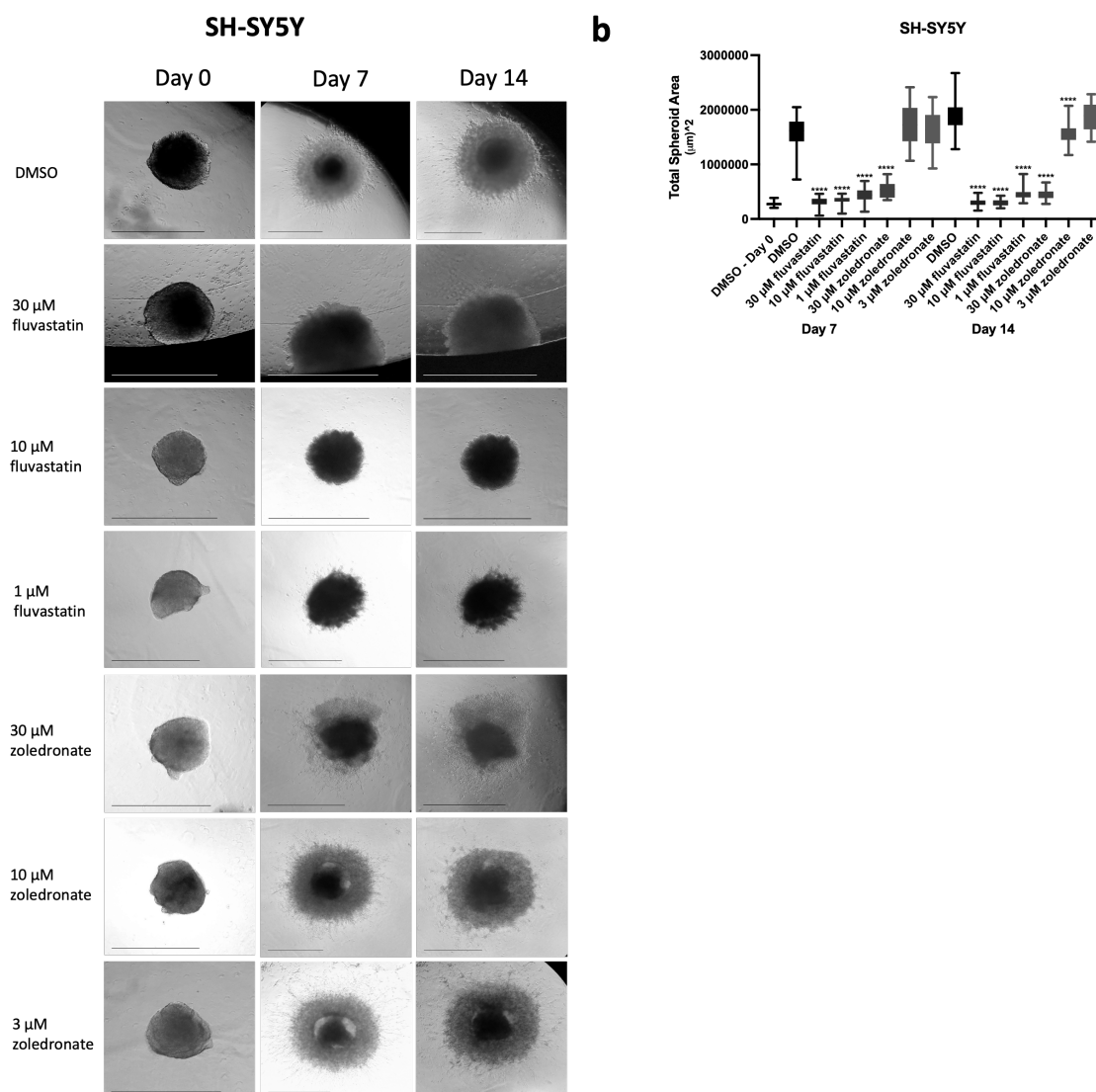


Figure 15: Mevalonate pathway inhibition impact on spheroid invasion SH-SY5Y.

(A) Representative images of SH-SY5Y spheroids on day 0, 7 and 14 with indicated treatments in an ECM invasion assay. Scale bars 890 μm . **(B)** Quantification of the total spheroid area of SH-SY5Y spheroids on day 0, 7 and 14 with the indicated treatments. Graphs representative of 3 independent experiments with a minimum of 24 wells counted per condition each replicate. Data analyzed via two-way ANOVA with Tukey's post hoc. ** $p \leq 0.01$ *** $p \leq 0.001$ **** $p \leq 0.0001$. Data shown as box and whisker plots with the whiskers representing minimum and maximum values and the box representing quartiles.

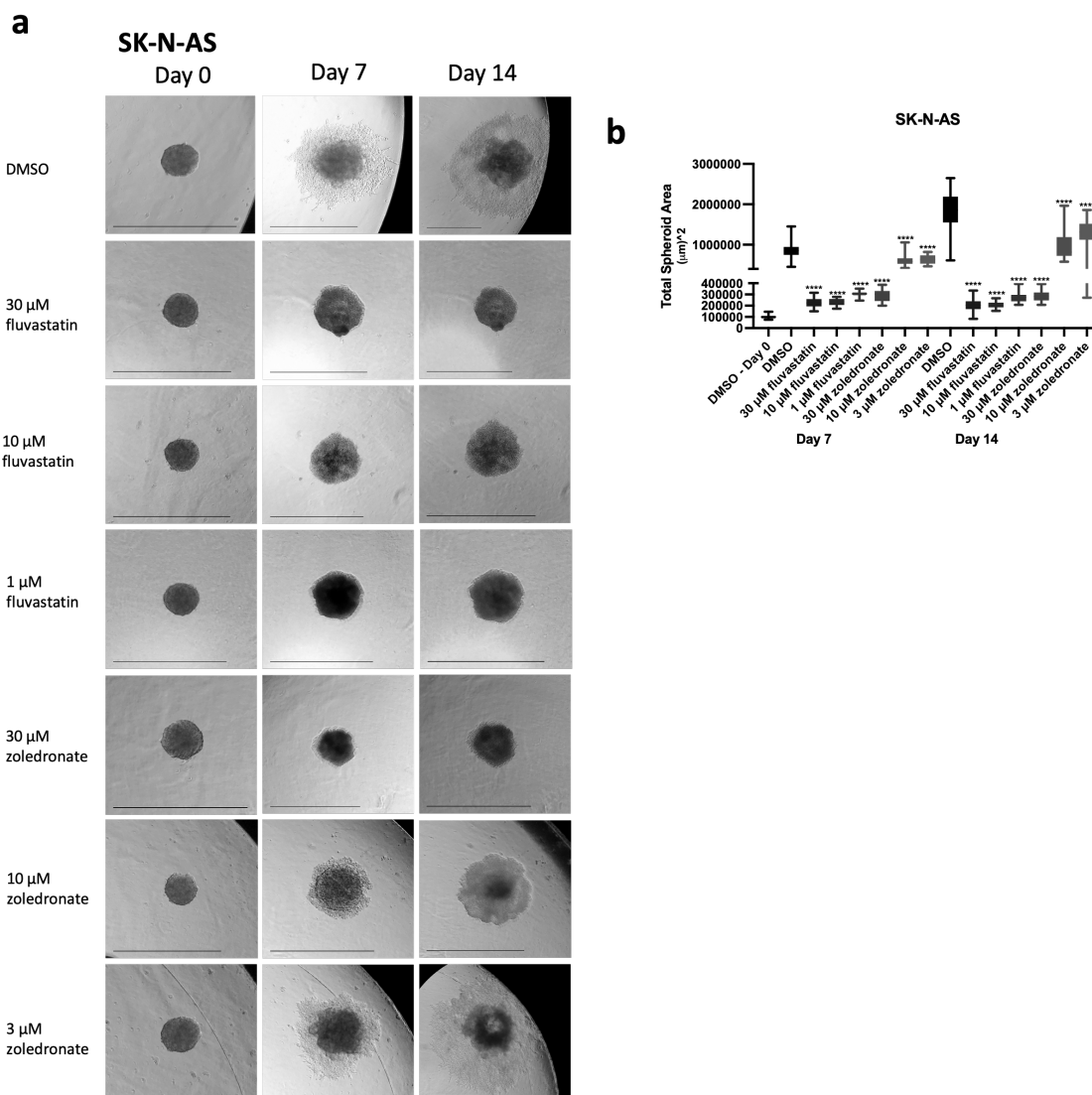


Figure 16: Mevalonate pathway inhibition impact on spheroid invasion SK-N-AS.

(A) Representative images of SK-N-AS spheroids on day 0, 7 and 14 with indicated treatments in an ECM invasion assay. Scale bars 890 μm . **(B)** Quantification of the total spheroid area of SK-N-AS spheroids on day 0, 7 and 14 with the indicated treatments. Graphs representative of 3 independent experiments with a minimum of 24 wells counted per condition each replicate. Data analyzed via two-way ANOVA with Tukey's post hoc. ** $p \leq 0.01$ *** $p \leq 0.001$ **** $p \leq 0.0001$. Data shown as box and whisker plots with the whiskers representing minimum and maximum values and the box representing quartiles.

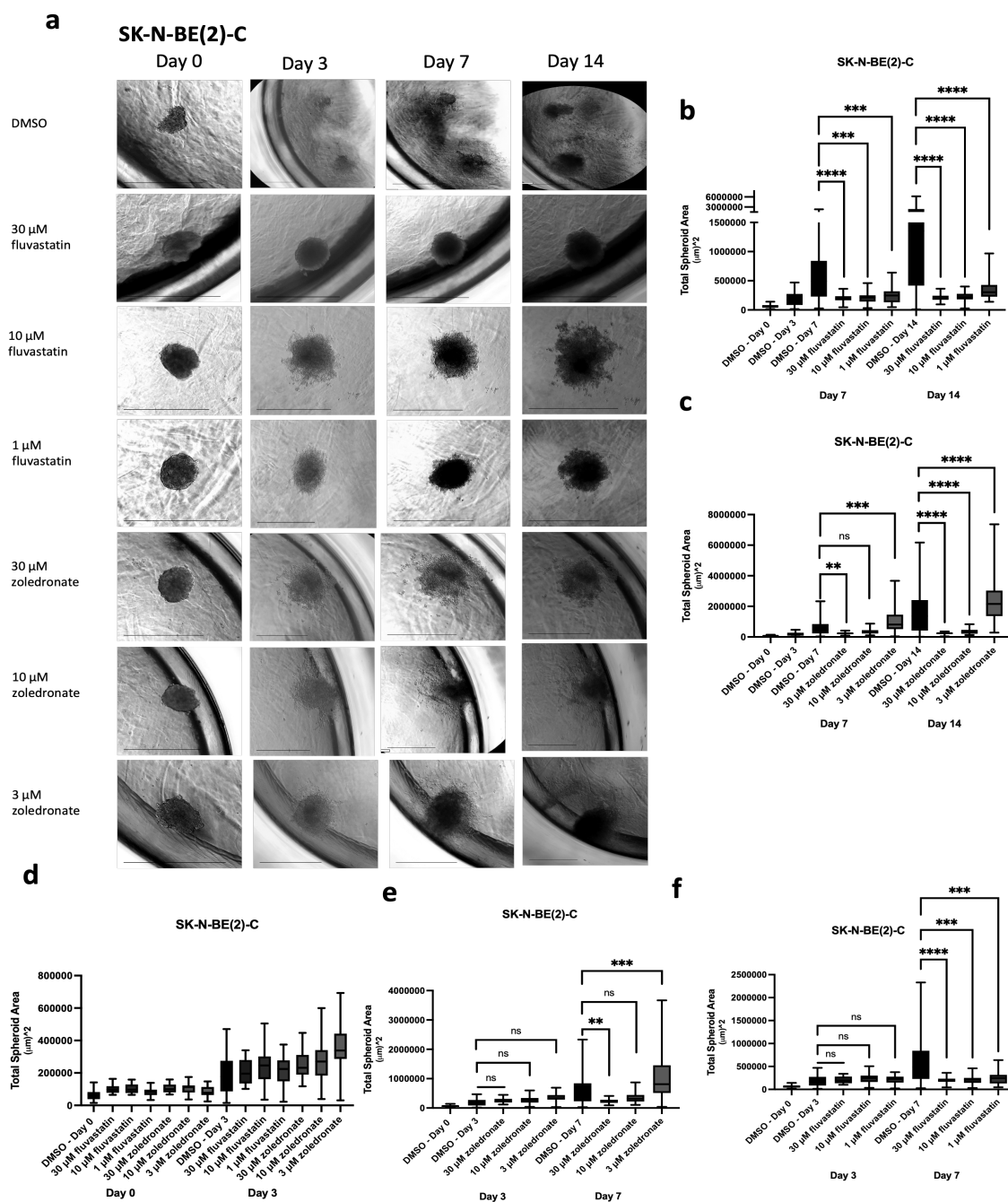


Figure 17: Mevalonate pathway inhibition impact on spheroid invasion SK-N-BE(2)-C.

(A) Representative images of SK-N-BE(2)-C spheroids on day 0, 3, 7 and 14 with indicated treatments in an ECM invasion assay. Scale bars 890 μ m. (B-F) Quantification of the total spheroid

area of SK-N-BE(2)-C spheroids on day 0, 3, 7 and 14 with the indicated treatments. Graphs representative of 3 independent experiments with a minimum of 24 wells counted per condition each replicate. Data analyzed via two-way ANOVA with Tukey's post hoc. ** $p \leq 0.01$ *** $p \leq 0.001$ **** $p \leq 0.0001$. Data shown as box and whisker plots with the whiskers representing minimum and maximum values and the box representing quartiles.

To further investigate the potential mechanism of action resulting in decreased spheroid invasion we looked at the protein levels of vimentin and pan-cadherin. Vimentin is a marker of epithelial-mesenchymal transition (EMT), involved in metastasis and involved in the spheroid forming capabilities of breast cancer spheroids.¹⁹⁵ Cadherins are adhesion proteins, of which E-cadherin (epithelial) and N-cadherin (neural) are the most well studied within cancer as a switch from E-cadherin to N-cadherin is associated with EMT.¹⁹⁶ We did not detect E-cadherin in either SH-SY5Y or SK-N-AS spheroids (Figure 18) and did not observe significant alterations in total vimentin or pan-cadherin levels in either SH-SY5Y or SK-N-AS spheroids treated with low dose fluvastatin or zoledronate treatment (Figure 19 A-B).

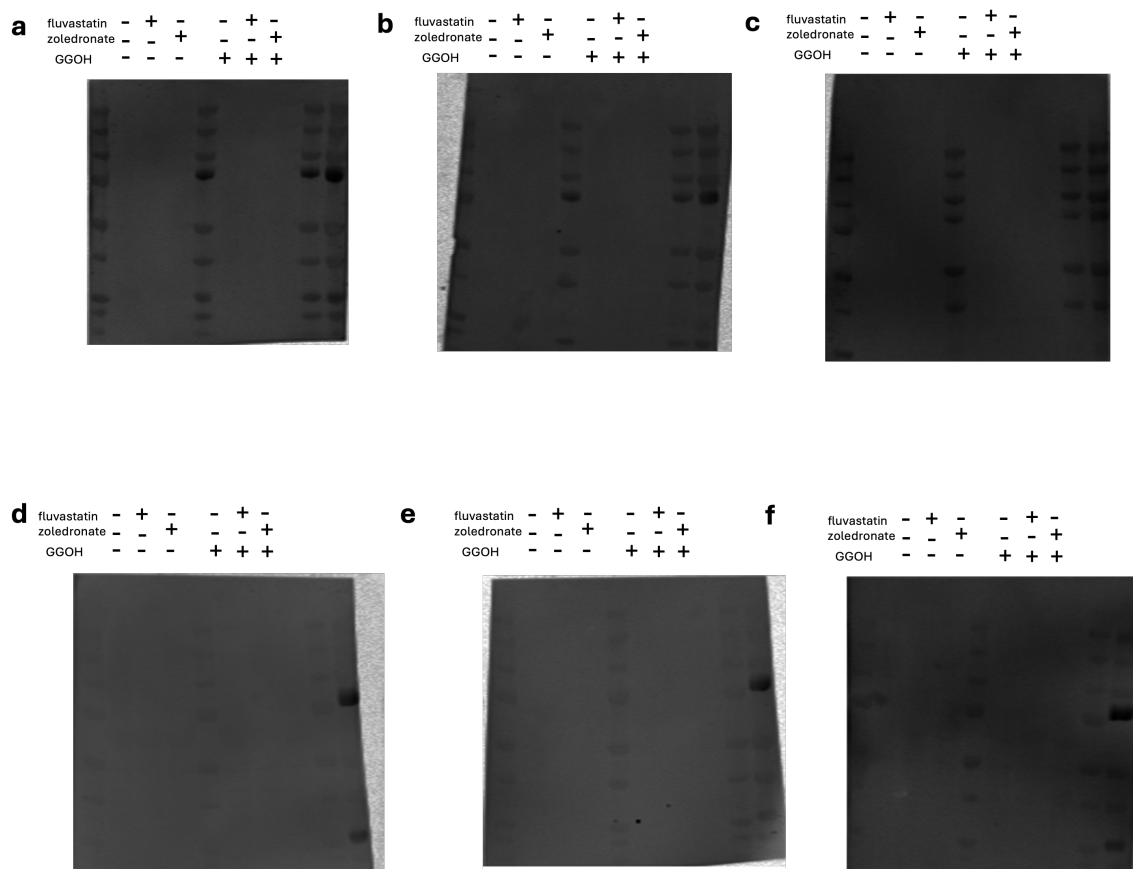


Figure 18: E-cadherin was not detected in high-risk neuroblastoma spheroids.

(A-C) E-cadherin SH-SY5Y full blot images. **(D-F)** E-cadherin SK-N-AS full blot images.

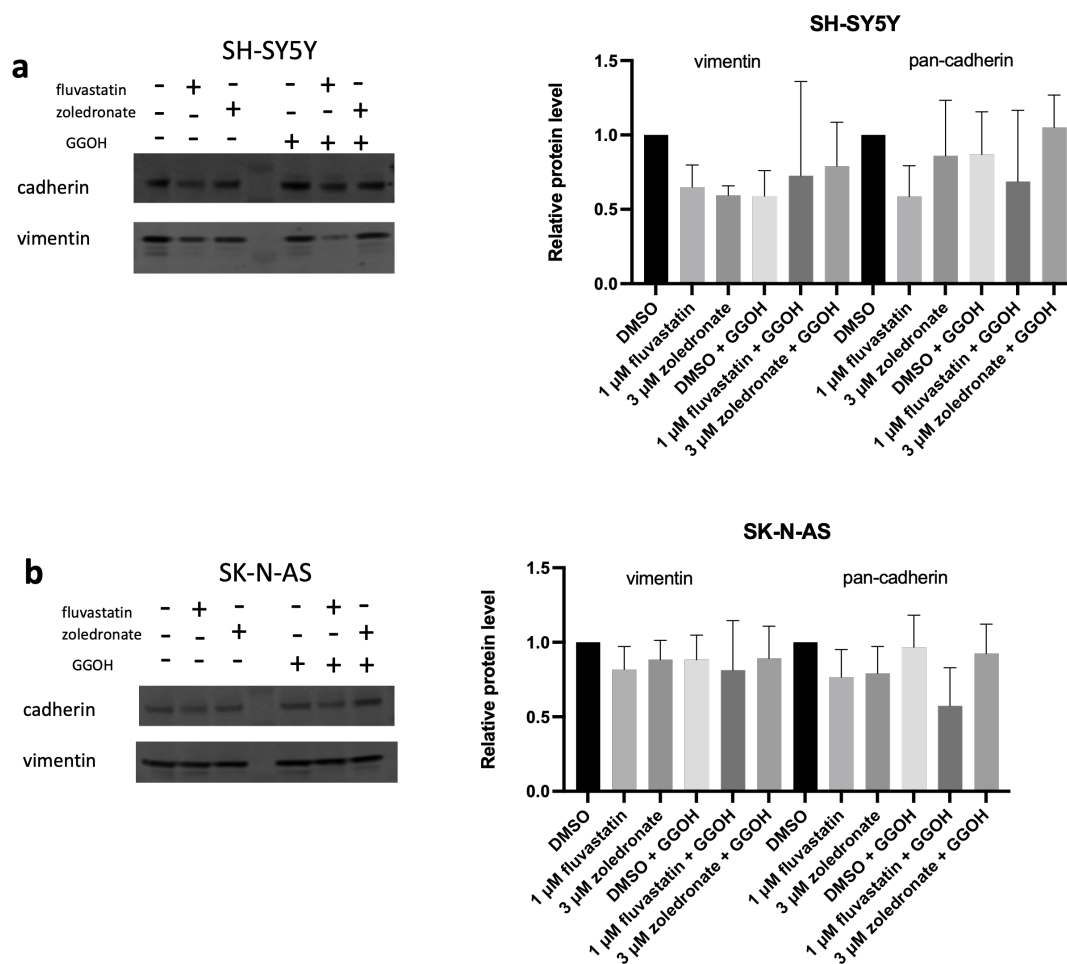


Figure 19: Low-dose mevalonate pathway inhibition does not affect EMT-associated proteins.

(A-B) Western blot analysis of EMT-associated proteins: vimentin and pan-cadherin. SH-SY5Y and SK-N-AS spheroids were treated with the indicated concentrations of fluvastatin, zoledronate and GGOH and harvested after 5 days. Representative blot images shown. Graphs representative of 3 independent experiments and data shown as mean $\pm$ SEM. Data analyzed by two-way ANOVA with Sidak's post-hoc for multiple comparisons.

Mevalonate pathway inhibitors alter spheroid formation in the presence of oncogenic factors

Finally, we were interested in if the effects of mevalonate pathway inhibitors on neuroblastoma cancer stem cells persist with alterations in signaling within the spheroid environment. Extracellular signaling molecules such as glial cell-line derived neurotrophic factor (GDNF) have been implicated in neuroblastoma cell proliferation, oncogenic signaling including non-canonical hedgehog pathway signaling, and can contribute to the maintenance of stemness.^{197–199} Interestingly, alterations in mevalonate pathway signaling can influence canonical hedgehog pathway signaling therefore we tested whether the addition of GDNF alters the observed effects in our spheroid model.²⁰⁰ SH-SY5Y and SK-N-AS spheroids were grown as above with the addition of 10 ng/mL GDNF to the culture media. The presence of GDNF did not prevent the reduction in maximum spheroid formation induced by treatment with fluvastatin in either cell line (Figure 20 A and D). The presence of GDNF however did prevent the reduction in maximum spheroid formation induced by 3 μ M zoledronate in SH-SY5Y spheroids (Figure 20 B). Conversely the presence of GDNF further reduced the maximum spheroid formation of SK-N-AS cells when treated with zoledronate (Figure 20 E). Additionally, the presence of GDNF did not alter the reduction in self-renewals induced by treatment with fluvastatin or zoledronate treatment in either cell line (Figure 20 C and F). These results demonstrate that the effects of the mevalonate pathway inhibition on neuroblastoma spheroids persist in the presence of this external oncogenic signaling.

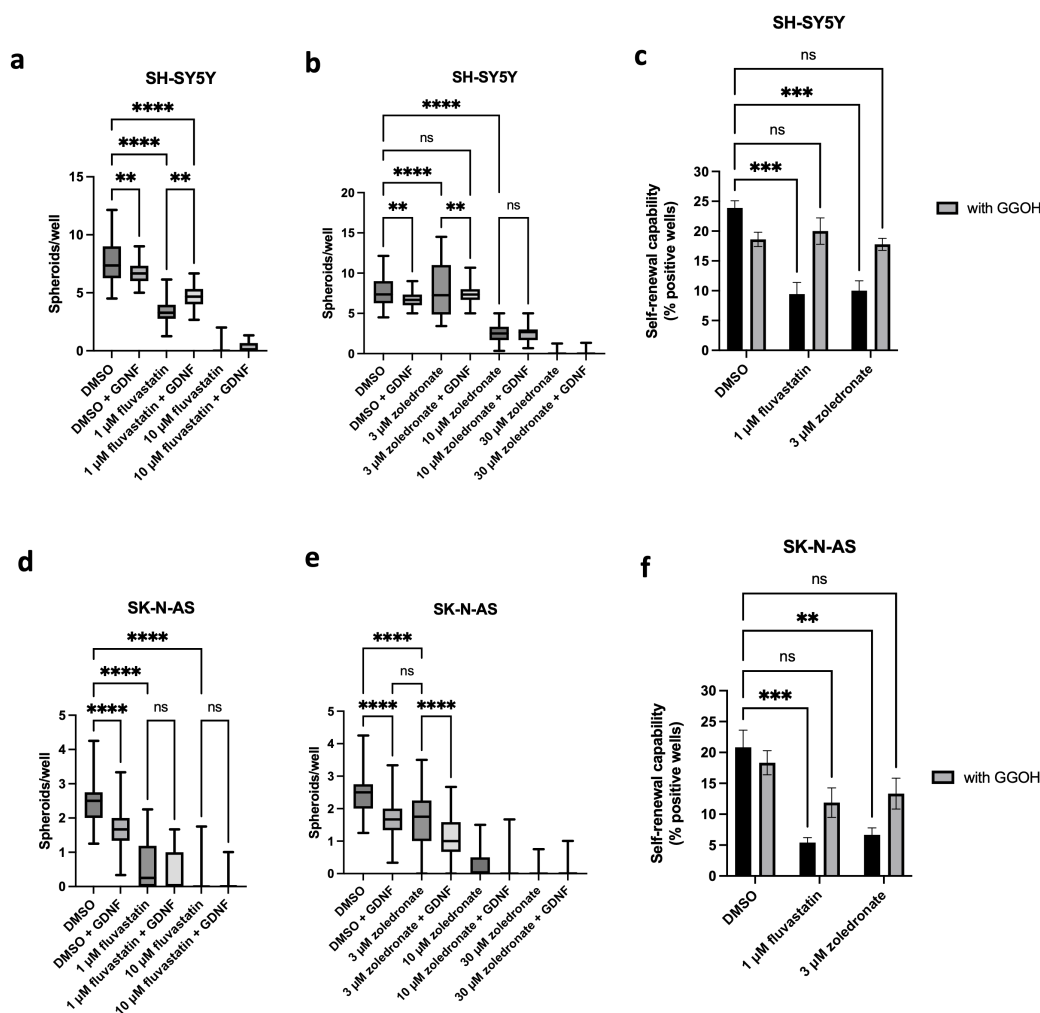


Figure 20: Mevalonate pathway inhibition alters spheroid formation in the presence of the oncogenic ligand GDNF.

(A-D) Spheroid formation assays in SH-SY5Y and SK-N-AS in the presence and absence of indicated compounds with the addition of GDNF to the spheroid growth media. Spheroid counts were carried out in triplicate with a minimum of 60 replicate wells counted per condition. Data analyzed via two-way ANOVA with Dunnett's post-hoc, α 0.05. Data shown as box and whisker plots with the whiskers representing minimum and maximum values and the box representing quartiles. **(E-F)** Self renewal capability assays in SH-SY5Y and SK-N-AS spheroids with the addition of GDNF to the spheroid growth media during primary and secondary spheroid growth. Graphs representative of 3 independent experiments with a minimum of 120 wells counted per condition each replicate and presented as mean $\pm$ SEM. Data analyzed via two-way ANOVA with Sidak's post hoc. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Discussion and Conclusion

In the present study, we analyzed the effect of mevalonate pathway inhibition on neuroblastoma cancer stem cells using a spheroid culture model of two high-risk neuroblastoma cell lines SH-SY5Y and SK-N-AS (Figure 21). Critical experiments were also replicated in the *MYCN* amplified SK-N-BE(2)-C cell line. These cell lines collectively represent a diverse genetic background of high-risk neuroblastoma tumor patients (Table 1). For this study we chose to focus on two compounds, fluvastatin and zoledronate, that act at different steps within the mevalonate pathway (Figure 10 A). Fluvastatin inhibits HMGCR and due to its chemical structure is unable to pass through the blood brain barrier.^{127,201} This is preferred for the potential use in treatment of neuroblastoma since most tumors exist outside of the central nervous system (CNS) and it minimizes impact of the compound on the developing CNS. Similarly, zoledronate, belongs to the class of compounds known as nitrogen bisphosphonates that inhibit the enzyme FPP synthase and are highly absorbed by the bone, which is a common site of neuroblastoma metastasis.¹⁶⁵ Fluvastatin and zoledronate are both FDA approved for their use in hypercholesteremia and hypercalcemia of malignancy treatment respectively.^{202,203}

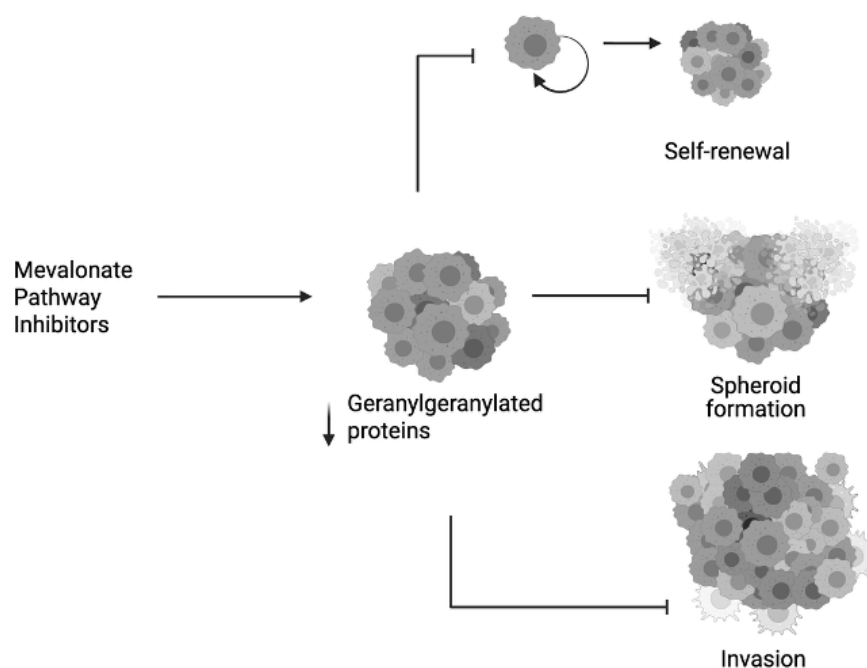


Figure 21: Inhibition of the mevalonate pathway targets neuroblastoma stem cells through reductions in protein geranylgeranylation.

Mevalonate pathway inhibition decreases protein geranylgeranylation in neuroblastoma stem cells and decreases their spheroid formation, invasion, and self-renewal capabilities. Created in BioRender. Green, M. (2025) <https://BioRender.com/m03f164>

Cancer stem cells, also known as tumor initiating cells, have been isolated from neuroblastoma patient tumors and are thought to contribute to the tumor heterogeneity, drug resistance, and aggressiveness.^{78,80,194} The use of a spheroid culture model was chosen as it enriches for this cancer stem cell population of high-risk neuroblastoma and better captures the 3D nature of neuroblastoma tumors than traditional adherent *in vitro* cell culture.^{79,80,204} We did observe differences between the cell lines used in the spheroid model system. The SH-SY5Y and

SK-N-BE(2)-C spheroids were quicker to form and typically had higher numbers of spheroids per well or growth condition than the SK-N-AS spheroids grown in identical conditions. As shown in Table 1, both SH-SY5Y and SK-N-AS cell lines lack *MYCN* amplification but are known to be sensitive to mevalonate pathway inhibition due to their reliance on small GTPases in signaling cascades.^{78,102,103} The SK-N-BE(2)-C cell line has *MYCN* amplification and has also been shown to be sensitive to mevalonate pathway inhibition.¹¹² The SH-SY5Y cell line is noradrenergic and neuronal (N) type, while the SK-N-AS is mesenchymal and non-neuronal (S) type, and the SK-N-BE(2)-C cell line is noradrenergic and an intermediate (I) type.^{82,176,205} Additional differences between the cell lines include loss of chromosome 1p in SK-N-AS and SK-N-BE(2)-C lines and mutated ALK in SH-SY5Y.^{176,205} Both SK-N-AS and SH-SY5Y have gain of chromosome 17q while the SK-N-BE(2)-C cell line has a single copy lost with inactive *p53*.¹⁷⁶ These differences may contribute to the observations in spheroid growth. Further studies on the genetic changes induced with spheroid culture would be warranted. The induction of spheroid formation and continued growth in that culture model demonstrates the plasticity of these high-risk cell lines, a stemness characteristic.⁹⁹

In this work, we observed that treatment with either fluvastatin or zoledronate reduced the spheroid formation capability of SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C cells and that this occurred at concentrations below those needed to achieve cytotoxicity, induce cell cycle arrest, or induce apoptosis. Importantly, we noted that this reduction could be rescued with the addition of GGOH, which rescues protein geranylgeranylation lost by treatment with both compounds in both non-*MYCN* amplified cell lines. In the *MYCN* amplified SK-N-BE(2)-C cell line the addition of GGOH could partially rescue the spheroid formation reduction induced by fluvastatin and zoledronate treatment. We noted a drastic decrease in the self-renewal capability of spheres with low-dose fluvastatin and zoledronate treatment in both non-*MYCN* amplified lines as well and this was once again rescued with the addition of GGOH. The importance of geranylgeranylation

in this effect was further confirmed by specific inhibition of GGPP synthase by DGBP, and inhibition of geranylgeranylation by transferase inhibitor GGTI-286 which both decreased spheroid forming frequency in SH-SY5Y cells. In the SK-N-BE(2)-C spheres there was a significant decrease in the self-renewal capability as well and similarly to the spheroid formation assays the supplementation with GGOH was insufficient to fully rescue this effect. These findings are consistent with previous reports that *MYCN* amplified cell lines also rely on the cholesterol branch of the mevalonate pathway. The additional finding in the SK-N-BE(2)-C cell line that supplementation with FOH in the presence of fluvastatin or zoledronate further reduced spheroid forming capability should also be highlighted.

Two proteins, farnesyl protein transferase (FPTase) and geranylgeranyl protein transferase type I (GGPTase), are responsible for the post-translational modifications of small GTPases with FPP and GGPP respectively.¹²¹ These proteins transfer the lipid portion of FPP and GGPP respectively to the C-terminal of the Rho family protein. The C-terminal of the target proteins contains a conserved CAAX amino acid structure that is recognized by the transferases. The cysteine (C) is the ultimate sight of modification being alkylated on the sulfur atom. The AAX amino acid group designates whether FPP or GGPP will be used for the alkylation and is ultimately cleaved off and the carboxy terminal of the cysteine is methylated to afford the ester.¹⁴³ It is estimated that there are over 756 potential members of the human proteome that carry this signature CAAX motif, however, only a small percentage of them have been demonstrated to be prenylated *in vivo*.¹⁴⁴ Prior literature on high-risk neuroblastoma adherent cell lines indicated that mevalonate pathway inhibitors act through a geranylgeranylation dependent mechanism.^{78,100,102–104,154} Our results are consistent with these findings in the non-*MYCN* amplified cell lines SH-SY5Y and SK-N-AS. In the SK-N-BE(2)-C cell line the geranylgeranyl dependent mechanism likely contributes to the observed effects however the potential for the involvement of the cholesterol branch of the pathway warrants further investigation. Additionally, we hypothesize

this cell line may undergo alternative farnesylation when treated with mevalonate pathway inhibitors with FOH supplementation leading to increased cell death.

Additionally, we saw a dose-dependent decrease in the invasiveness of SH-SY5Y, SK-N-AS, and SK-N-BE(2)-C spheroids with fluvastatin treatment. Zoledronate treatment decreased the invasion of SK-N-AS spheroids in a dose-dependent manner and high-dose treatment decreased the invasion of SH-SY5Y and SK-N-BE(2)-C spheroids. The observation in SK-N-BE(2)-C spheroid invasion that initial growth over the course of 3 days was not impacted by treatment but that continued growth over the course of 7 and 14 days was dose-dependently impacted is also very interesting. This suggests that treatment with mevalonate pathway inhibitors have a prolonged effect on neuroblastoma CSCs. Taken together our findings suggest that the CSC population of high-risk neuroblastoma is targetable with mevalonate pathway inhibitors. The loss of spheroid formation, reduced spheroid-forming cell frequency, reduced self-renewal and impaired invasiveness are all markers of impaired CSC function. Treatment with fluvastatin and zoledronate thus appear to reduce the functionality of high-risk neuroblastoma CSCs through a primarily geranylgeranylation dependent mechanism.

Investigations into the mechanism of action of statins have utilized both standard undifferentiated neuroblastoma cell lines and purposefully differentiated cells due to the potential neuroprotective effects in neurodegenerative disease. In differentiated neuroblastoma cell lines, the closed-ring form of lovastatin can act through inhibition of the proteasome to reduce cell viability.¹⁵⁹ Statins also have neuroprotective effects that vary by statin due to differences in the induced microglia immune responses.¹⁶³ As previously discussed, the mechanism of action of statins in undifferentiated cell lines, including SH-SY5Y and SK-N-AS, is through the inhibition of protein prenylation and subsequent loss of function of Ras and Rho family proteins.^{102,103} As mentioned, there is also evidence that the cholesterol branch of the mevalonate pathway is required for high-risk neuroblastoma cell growth specifically *MYCN* amplified cell lines such as

SK-N-BE(2)-C.¹¹² Interestingly, the spheroid culture model used does not have cholesterol lipids in the media formulation. Further studies to tease out the mechanism of action of mevalonate pathway inhibition within in the cancer stem cell population using specific inhibitors of the prenyl transferases and the cholesterol branch are being planned.

Investigation on the specific GTPases effected in the neuroblastoma CSCs was impacted by limitations in protein harvest from spheroid cultures. We did observe potential changes in the levels of CDC42, RhoA, RhoB, and Rap1 GTPases, which varied by cell line. Additionally, given the differences observed in effected GTPases between the two cell lines future experiments to elucidate the specific GTPases responsible using genetic manipulations are warranted as well as development of localization assays within the spheroid model.

In addition to the need for further mechanistic studies, we recognize the limitations of our spheroid model. This model does not enable evaluation of the immune response or the tumor microenvironment as the spheroids are formed from a single adherent cell line. We are interested in the expansion of this model through co-culture systems and incorporation in *in vivo* mouse models in the future. As previously discussed, we also ran into difficulties with protein and RNA harvest with higher doses of the inhibitors and are working on how to improve these methods for future studies. This study also did not directly compare our spheroid findings to complementary adherent assays; however, our results are consistent with prior reported findings in adherent culture of both cell lines used. Additionally, we are interested in expanding this work with primary cultures from patient samples to investigate the applicability of these findings on patient cancer stem cells.

Additionally, we investigated if the results observed were maintained in the presence of an oncogenic signaling factor GDNF. GDNF signals through binding with the receptor tyrosine kinase RET that can induce signaling through multiple oncogenic pathways including MAPK, PI3K/AKT, and non-canonical hedgehog signaling.^{197,206} SH-SY5Y and SK-N-AS spheroids

cultured with the addition of GDNF remained sensitive to the observed effects of fluvastatin and zoledronate treatment on the spheroid formation and self-renewal capability. This suggests that the suspected loss of protein geranylgeranylation induced by these compounds is sufficient to block oncogenic signaling within the tumor microenvironment. It also demonstrates the utility of the spheroid model for addition of growth factors unique to various tumors within the context of pharmacological studies.

Importantly our results demonstrate the utility for cancer spheroid methods to be used to observe phenotypic effects on cancer stem cell populations. At doses below those reported to substantially induce antiproliferative effects in traditional adherent lines we observed reductions in spheroid formation, self-renewal, and invasiveness. These findings suggest that impacting the cancer stem cell populations through mevalonate pathway inhibition could be a viable therapeutic target in high-risk neuroblastoma.

Chapter 6

Investigation into signaling pathways impacted by mevalonate pathway inhibition in high-risk neuroblastoma spheroids

Abstract

High-risk neuroblastoma spheroid cultures are susceptible to inhibition of the mevalonate pathway likely due to loss of critical protein geranylgeranylation.¹⁷⁷ These observed changes in sphere cell function occur at doses of fluvastatin and zoledronate below those which can induce cytotoxicity and that may be achievable in a clinical setting.¹⁷⁷ Therefore, we aimed to identify the mechanism behind this loss of function by investigating the potential signaling pathways disrupted in neuroblastoma spheroids when the mevalonate pathway is inhibited.

Introduction

Neuroblastoma tumors likely originate from neural crest cells during early embryonic development therefore many of the signaling pathways associated with normal early human development are also associated with this cancer.⁶ During normal development stem cell characteristics are lost as neural crest cells migrate and differentiate down their standard lineages.^{6,9} In mature tissues and organs, a small population of stem cells, with regulatory pathways intact remains. During neuroblastoma tumor development neural crest cells fail to properly differentiate, and CSC populations are maintained.^{6,9,79} This gives rise to highly plastic and aggressive tumors with persistence of developmental related signaling pathways.

As previously discussed in Chapter 1, patients with low-risk and some intermediate-risk tumors can be monitored or conservatively treated due to spontaneous tumor differentiation and/or regression that occurs in these populations.^{6,33–37} If high-risk tumors can be therapeutically induced towards these lower-risk states this could improve patient outcomes and response rates to available therapies. Current maintenance therapy with isotretinoin and eflornithine attempts to do just this by induction of differentiation and disruptions in cellular metabolism respectively.^{6,37,49,50} Our findings in Chapters 4 and 5, suggest that fluvastatin and/or zoledronate could be similarly used to target the neuroblastoma cancer stem cell population.¹⁷⁷ We observed reductions in multiple cancer stem cell functions that we suspect are due to disruptions in one or more differentiation associated signaling pathways.

We have shown that treatment with mevalonate pathway inhibitors on high-risk neuroblastoma spheroids can reduce the functionality of cancer stem cells through the loss of protein prenylation at doses potentially achievable in clinic. As discussed previously prenylation is a post-translational modification critical for the function of small GTPases as effectors in cellular signaling pathways.^{121,141,142} Farnesylation and geranylgeranylation of GTPases, based on their C-terminus sequence, enable proper intracellular trafficking of these proteins to the cell membrane.¹²¹ Once at the membrane these proteins interact with receptor proteins and downstream signaling proteins as part of intracellular signaling cascades.

We found that in both the mesenchymal SK-N-AS cell line and the adrenergic SH-SY5Y cell line this reduced functionality appears to predominantly be caused by loss of geranylgeranylation specifically. Supplementation with GGOH, which can be utilized for protein prenylation, rescued the functionality of spheroid cells in formation assays and self-renewal assays. Additionally, protein analysis identified changes in GTPases that are commonly geranylgeranylated in these spheroid cells with fluvastatin and zoledronate treatment. In both SK-N-AS and SH-SY5Y spheroids fluvastatin treatment led to a significant decrease in total levels of

RhoB and Rap1a/Rap1b, with rescue with GGOH supplementation and a similar trend observed with zoledronate treatment. In the SH-SY5Y spheroids treatment with fluvastatin also significantly decreased the total protein levels of RhoA and CDC42, with a similar trend observed in SK-N-AS spheroids for RhoA that was not significant. Zoledronate treatment did not alter RhoA and CDC42 levels in either cell line. Additionally, treatment with fluvastatin led to slight reductions in Rac and Ras protein levels in SH-SY5Y spheroids and a slight reduction in Rac protein level in SK-N-AS spheroids. Zoledronate treatment did not alter Rac protein levels in either cell line but did increase total Ras protein levels in SH-SY5Y spheroids only.

Developmental signaling pathways implicated in high-risk neuroblastoma include the Wnt, hedgehog, and RAS/MAPK signaling pathways.^{23,207,208} Inhibition of the mevalonate pathway may cause changes in these pathways both directly due to loss of protein post-translational modifications and indirectly through changes in signaling protein binding partners and protein complexes.

One of the pathways associated with sympathoadrenal development that has been implicated in high-risk neuroblastoma is the hedgehog signaling pathway.²⁰⁷ Canonically, when the ligand Sonic Hedgehog (SHH) binds to the receptor Patched 1 (PTCH1) this inactivates the receptor's inhibitory function on the transmembrane protein Smoothened (SMO) which triggers an intracellular protein signaling cascade resulting in the activation of the GLI1 transcription factor.²⁰⁹ This pathway has been found to be active in neuroblastoma tumors and neuroblastoma adherent cell lines.^{197,209–213} Inhibition of the canonical signaling pathway has been shown to reduce high-risk neuroblastoma cell lines cell viability and proliferation, induce cell cycle arrest and apoptosis, and reduce the colony formation in soft agar assays.^{209,211–213} Interestingly, canonical activation of the pathway has been observed in neuroblastoma cell lines in response to hypoxia.²¹⁰ Adherent neuroblastoma cell lines have also been found to express SHH with endogenous activation of the pathway.²¹³ Additionally, the small GTPases RhoA, Rac1 and Rap1

have been associated with this signaling pathway.^{214,215} In neuroblastoma cell lines non-canonical hedgehog signaling has also been observed through GDNF activation of the receptor rearranged during transfection (RET), which leads to activation of AKT, inhibition of GSK3 β and activation of GLI1.¹⁹⁷ In human brain samples RET binds Rap1 GTPase activating protein (Rap1GAP) and in a rat adrenal tumor line, PC12, was found to regulate neurite outgrowth.²¹⁶ Treatment of adherent high-risk neuroblastoma cells with hedgehog pathway inhibitors reduced the population of cells expressing CD133 and CD15 stem cell markers with a reduction in neurosphere formation.²¹⁷

Based on these findings and observations we hypothesized that mevalonate pathway inhibition induced effects on high-risk neuroblastoma spheroids were associated with changes in the hedgehog signaling pathway.

Methods

Cell lines and reagents

SH-SY5Y and SK-N-AS cells were obtained from ATCC and all experiments were performed within 15 passages of receipt. Spheroids were generated and maintained in spheroid media composed of DMEM/Ham's F12 (3:1), anti-anti (1:100, Gibco), human FGF basic (Invitrogen) and EGF (Gibco) both at 20 ng/mL, heparin (Sigma) 5 µg/mL, and 1x B-27 (Gibco).⁷⁷ Media was mixed fresh for each experiment and 1x B-27 was added immediately before use. All spheroid cultures were incubated at 37°C and 5% CO₂.

Fluvastatin was obtained from Selleck Chemicals and reconstitute in DMSO to 100mM and stored at -20°C before use. This stock was then diluted to 10mM immediately before dilution to final concentration in spheroid media. Zoledronic acid was obtained from Selleck Chemicals and reconstituted in 0.5 M sodium hydroxide to 10mM and stored at -20°C before use. It was also diluted to final concentration in spheroid media. GGOH was obtained from ThermoFisher and used at 20 µM.

Quantitative real-time PCR

Spheroids were grown as described above with the indicated concentrations of fluvastatin and zoledronate for 72 hours. RNA was harvested on ice using RNeasy Mini Kit (Qiagen). Synthesis of cDNA from total RNA was performed following manufacturer's instruction using superscript III reverse transcriptase (Invitrogen).

Quantitative RT-PCR was performed to investigate the expression of hedgehog pathway related genes using the Taqman Array, Human Hedgehog Fast 96-well (Applied Biosystems)

system. The functionality of the array was confirmed with positive control medulloblastoma cells that express this pathway (unpublished data).

For the hedgehog pathway analysis briefly the cDNA samples were combined with Taqman Gene Expression Master Mix (Applied Biosystems) and added to the appropriate wells of the plate. The thermal protocols were provided by Applied Biosystems. The thermal protocols were provided by Applied Biosystems. Relative gene expression was calculated using the $2^{-\Delta Ct}$ method, whereas the mean fold change = $2^{-(\text{average } \Delta\Delta Ct)}$ was assessed using the mean difference in the ΔCt between the gene and the internal control GAPDH. Genes assayed include: *E2F1*, *STIL*, *SMO*, *GLI1*, *SUFU*, *CSNK1G2*, *PRKACB*, *SHH*, *PTCH1*, *BTRC*, *PTCH2*, *ZIC3*, *LRP2*, *IFT88*, *MTSS1*, *IFT52*, *RAB23*, *CSNK1G1*, *BNC1*, *CREBBP*, *FOXA2*, *GLI2*, *CSNK1E*, *GAS1*, *MYF5*, *GSK3 β* , *CSNK1G3*, *DHH*, *HHIP*, *CSNK1D*, *STK36*, *DISP2*, *DISP1*, *PRKACA*, *ZIC2*, *ZIC1*, *FBXW11*, *BCL2*, *GLI3*, *CSNK1A1*, *PRKX*, *PRKY*, *PRKACG*, and *IHH*. For the hedgehog pathway analysis *GAPDH* had a standard deviation of 0.43 and 2.00 for SH-SY5Y and SK-N-AS respectively. Statistical significance was assessed for all genes assayed using ΔCt values via two-way ANOVA with Bonferroni's correction with GraphPad Prism 10.

Immunoblotting

Spheroids were grown as described above and maintained in 6-well ultra-low attachment plates (Corning Costar). Prior to treatment spheroids were harvested and replated at a 1:3 density in a new 6-well plate with the indicated concentrations of fluvastatin and zoledronate for up to 5 days. Spheroids were harvested in RIPA buffer with protease inhibitors and protein quantification was performed by Pierce BCA assay (Thermo Fisher). Equal amount of protein (5 – 10 µg) were loaded on precast 4-12% bis/tris gels (Invitrogen) and run at 175 V for 60-90 minutes in MOPS or MES buffer (Thermo Fisher). MOPS buffer was used for proteins larger than 50 kilodalton (kDa) and MES buffer was used for those smaller than 50 kDa. Transfer was carried out on ice at 25 V for 90 minutes onto a polyvinylidene fluoride membrane. Total protein stain was done with No-Stain™ Protein Labeling Reagent (Invitrogen), visualized on a Bio-Rad Chemidoc, and quantified for total protein normalization in Image Lab (Bio-Rad). Membranes were then blocked with 1x ScanLater buffer (Molecular Devices), probed with primary antibodies overnight (1:500 dilution), rinsed with ScanLater wash buffer and then probed with ScanLater Eu-conjugated secondary antibodies (Molecular Devices). Dry membranes were imaged with a SpectraMax i3x with a Western blot cartridge (Molecular Devices). Membranes were stripped with Restore™ Plus Western Blot Stripping Buffer for 10 minutes and reimaged on the SpectraMax i3x to ensure removal of the first antibody used. These membranes were then blocked, reprobed for the indicated antibodies overnight, washed, incubated with secondary antibody and imaged on the SpectraMax i3x. All full-size western blot images are available upon request. Quantification was done on ImageJ with statistical analysis (two-way ANOVA with Sidak's post-hoc for multiple comparisons) performed with GraphPad Prism 10.

Primary antibodies included: MSK1, pMSK1, pGSK3β, GSK3β, pAKT.

Results

Mevalonate pathway inhibition does not significantly alter hedgehog signaling in high-risk neuroblastoma spheres

The hedgehog pathway, through both canonical and non-canonical signaling, has been shown to be active in high-risk neuroblastoma cell lines.^{197,209,212} Additionally, this pathway has been associated with the small GTPases that we have found to be modulated in high-risk neuroblastoma spheroids when treated with mevalonate pathway inhibitors.^{177,214,215} Therefore, we investigated if the observed effects in high-risk neuroblastoma spheroids were associated with alterations in hedgehog pathway signaling. To do this we looked at 44 hedgehog and developmental associated genes using qRT-PCR. Treatment with fluvastatin or zoledronate, with or without GGOH supplementation did not significantly alter mRNA levels of any assayed genes (Figures 22-33). The expression of assayed genes has not been previously characterized in high-risk neuroblastoma spheroids.

In SH-SY5Y and SK-N-AS spheroids we did not detect *SHH* but did detect the two genes Desert Hedgehog (*DHH*) and Indian Hedgehog (*IHH*), that encode alternative ligands for canonical pathway signaling. In SK-N-AS spheroids expression of *IHH* was less consistent with about 50% of the samples processed having nondetectable levels. In both SH-SY5Y and SK-N-AS spheroids we detected expression of *SMO* and in SH-SY5Y spheroids we detected gene expression of both isoforms of the receptor PTCH (*PTCH1* and *PTCH2*). In SK-N-AS spheroids only *PTCH1* was expressed. Looking at the pathway transcription factors, in both SH-SY5Y and SK-N-AS spheroids we detected expression of *GLI1*. *GLI2* was detected in all SH-SY5Y spheroid samples but only detected in about 50% of SK-N-AS samples. Conversely *GLI3* was detected in SK-N-AS spheroid samples but not detected in SH-SY5Y spheroid samples. Gene

expression of the developmental transcription factor *E2F1* was detected in both SH-SY5Y and SK-N-AS spheroids but *ZIC 1*, *ZIC 2*, and *ZIC3* were not expressed in either spheroid line.

Gene expression of intracellular hedgehog pathway signaling proteins SCL/TAL₁ interrupting locus (*STIL*), suppressor of fused (*SUFU*), glycogen synthase kinase 3 beta (*GSK3β*), Hedgehog interacting protein (*HHIP*) and the GTPase *Rab23* was detected in both SH-SY5Y and SK-N-AS spheroids. In SH-SY5Y and SK-N-AS spheroids the hedgehog pathway target gene B-cell leukemia/lymphoma 2 (*BCL2*) was detected but the genes basoonuclin 1 (*BNC1*) and Forkhead Box A2 (*FOXA2*) were not expressed. Both SH-SY5Y and SK-N-AS spheroids expressed the genes *DISP1/2*, which encode the cellular membrane proteins responsible for hedgehog ligand secretion. The co-receptor gene *LRP2* was detected in SH-SY5Y spheroids but not SK-N-AS spheroids. Both spheroids expressed the co-transcription factor genes for *MTSS1*, *CREBBP*, and *GAS1*, which modulate transcription with GLI.

RNA expression of beta-transducin repeat containing E3 ubiquitin protein ligase (*BTRC*) and F-box and WD repeat domain containing 11 (*FBXW11*), which are proteins involved in degradation of hedgehog related proteins, was detected in SH-SY5Y and SK-N-AS spheroids. In a similar vein the regulators Intraflagellar Transport Protein 88 (*IFT88*) and Intraflagellar Transport Protein 52 (*IFT52*) were also detected in both SH-SY5Y and SK-N-AS spheroids.

The protein kinases serine threonine kinase 36 (*STK36*) and protein kinase x-linked (*PRKX*) genes were detected in both SH-SY5Y and SK-N-AS spheroids. The gene expression of three subunits of Protein Kinase A (PRKA) (*PRKACB*, *PRKACA*, and *PRKACG*) were also detected in both SH-SY5Y and SK-N-AS spheroids. Additionally, six members of the Casein Kinase 1 family (*CSNK1G2*, *CSNK1G1*, *CSNK1E*, *CSNK1G3*, *CSNK1D*, and *CSNK1A1*) were detected in both high-risk neuroblastoma spheroid models.

Therefore, while we did not observe impacts to hedgehog associated genes with mevalonate pathway inhibition of high-risk neuroblastoma spheroids, we did characterize the

expression of the hedgehog signaling pathway related genes in this high-risk neuroblastoma spheroid model.

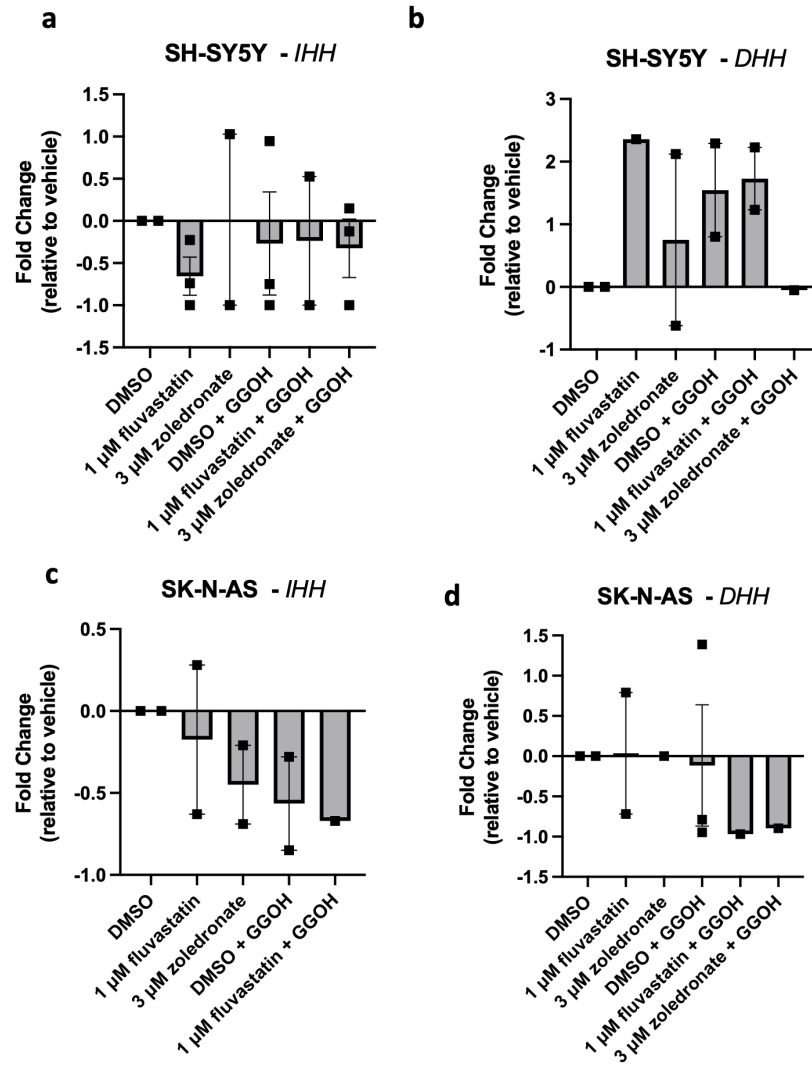


Figure 22: Mevalonate pathway inhibition effects on hedgehog pathway ligands.

(A-D) qRT-PCR analysis of hedgehog pathway ligands. SH-SY5Y and SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

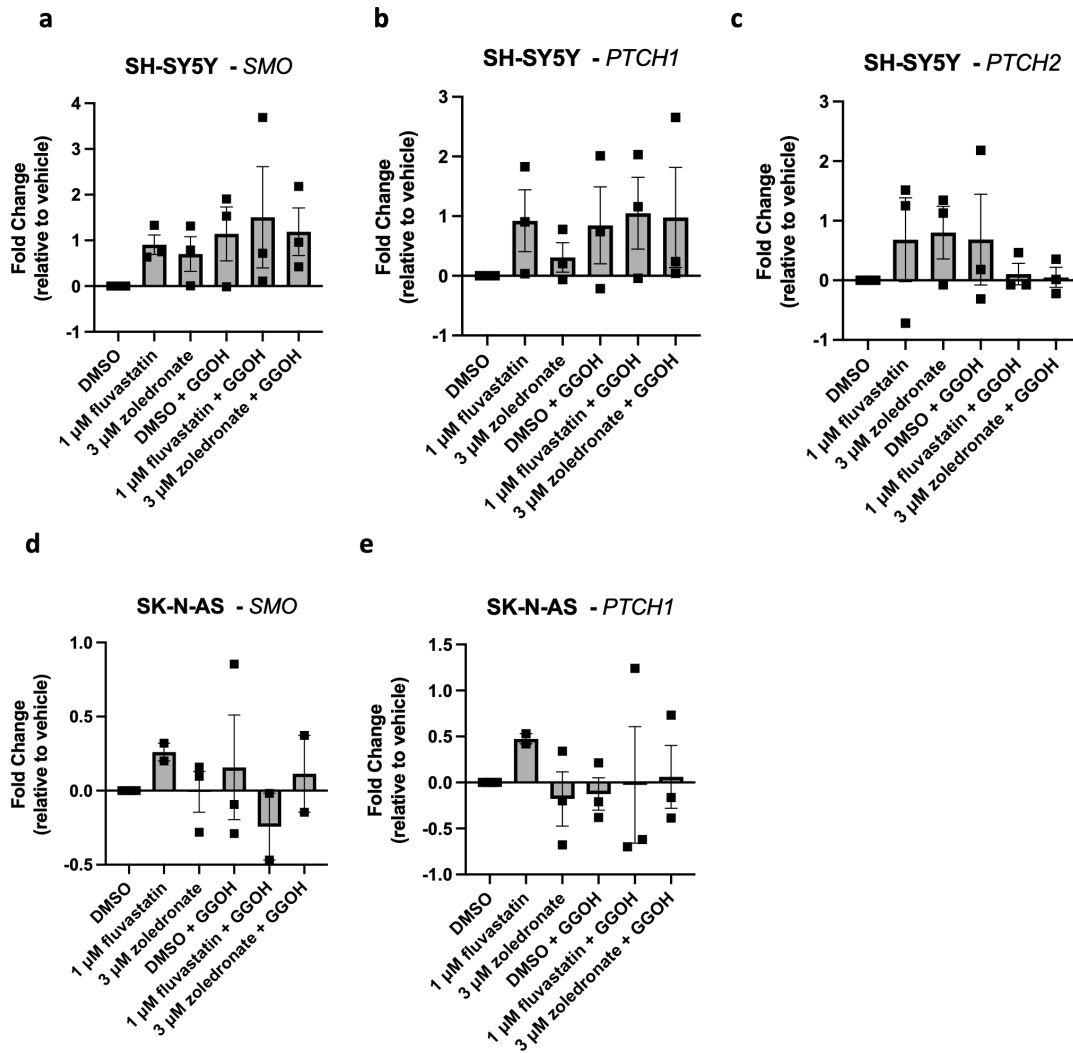


Figure 23: Mevalonate pathway inhibition effects on hedgehog pathway cell membrane proteins.

(A-E) qRT-PCR analysis of hedgehog pathway proteins localized to the cell membrane. SH-SY5Y and SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

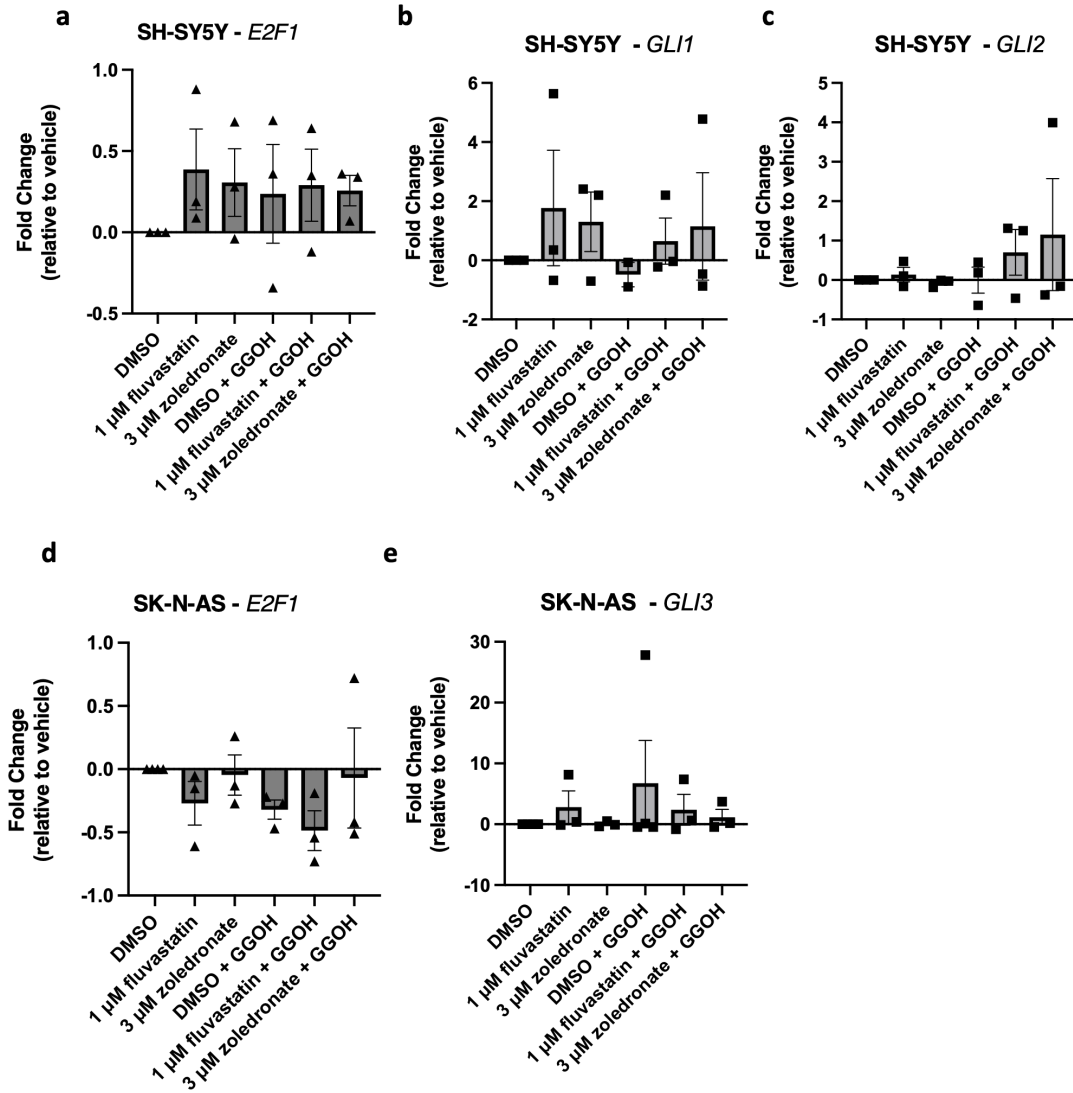


Figure 24: Mevalonate pathway inhibition effects on hedgehog pathway and developmental related transcription factors.

(A-D) qRT-PCR analysis of hedgehog pathway and developmental related transcription factors. SH-SY5Y and SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

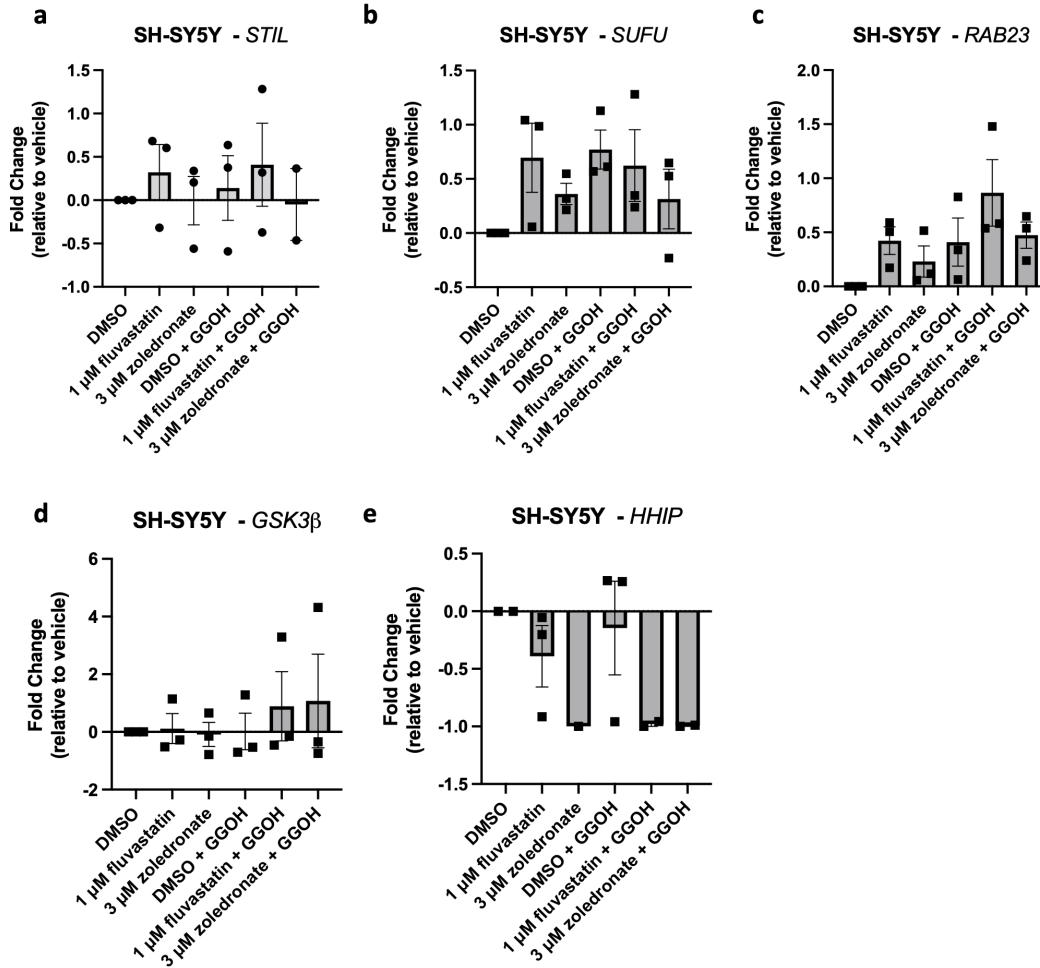


Figure 25: Mevalonate pathway inhibition effects on hedgehog pathway related intracellular signaling proteins – SH-SY5Y.

(A-D) qRT-PCR analysis of hedgehog pathway related intracellular signaling proteins. SH-SY5Y spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

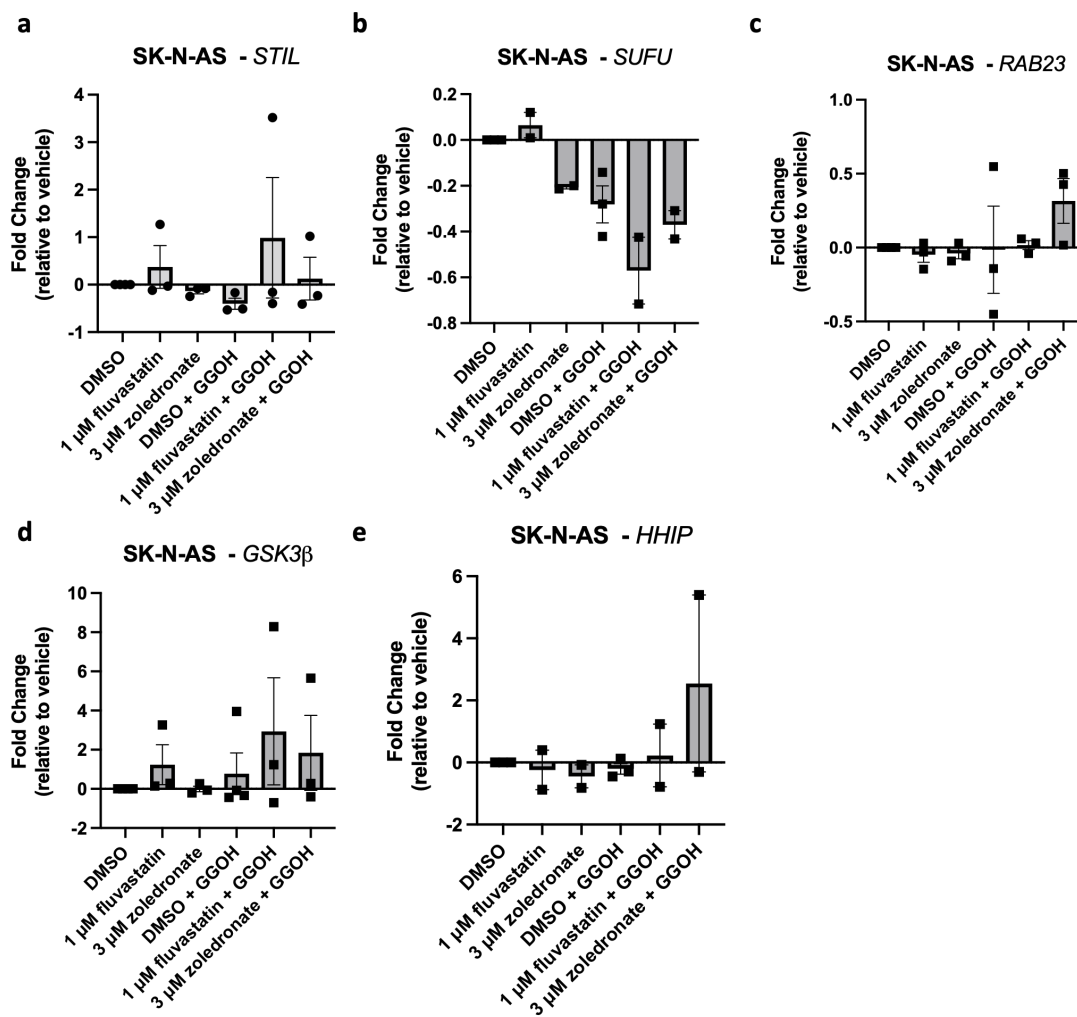


Figure 26: Mevalonate pathway inhibition effects on hedgehog pathway related intracellular signaling proteins – SK-N-AS.

(A-E) qRT-PCR analysis of hedgehog pathway related intracellular signaling proteins. SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

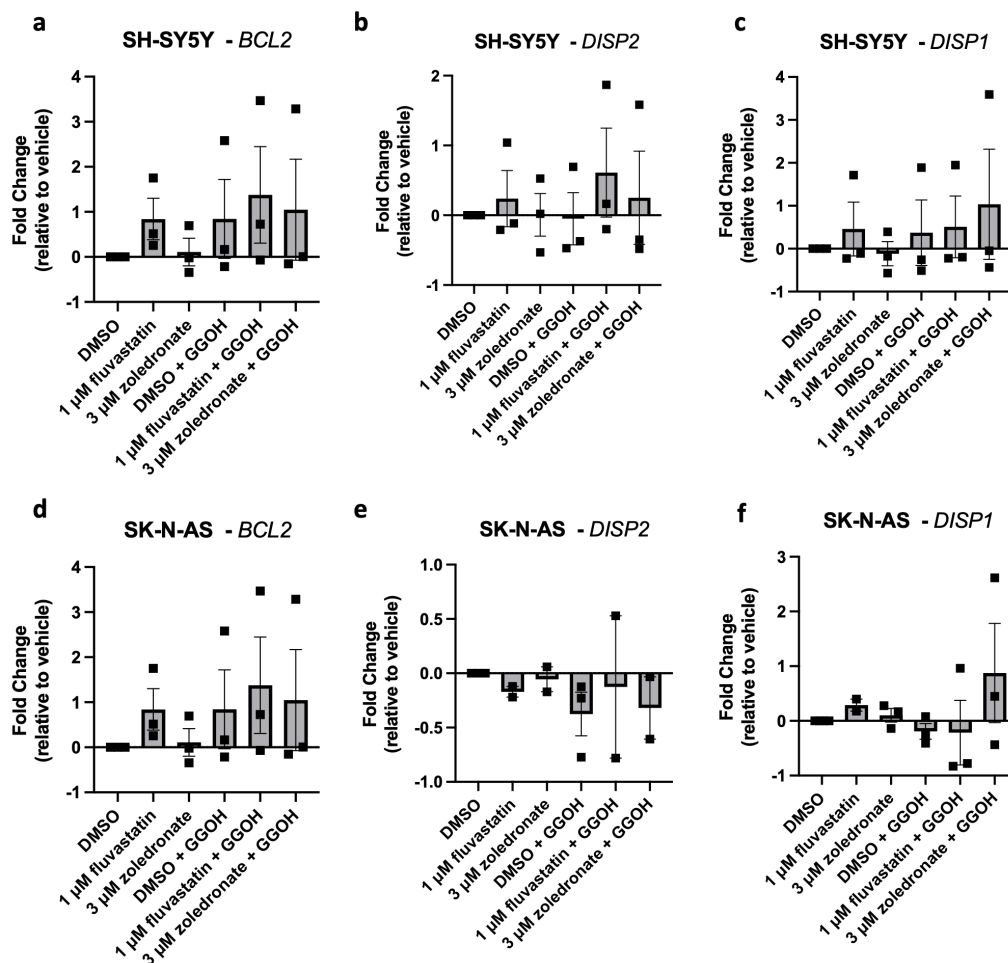


Figure 27: Mevalonate pathway inhibition effects on hedgehog pathway target genes and secretion proteins.

(A-E) qRT-PCR analysis of hedgehog pathway target genes and secretion proteins. SH-SY5Y and SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

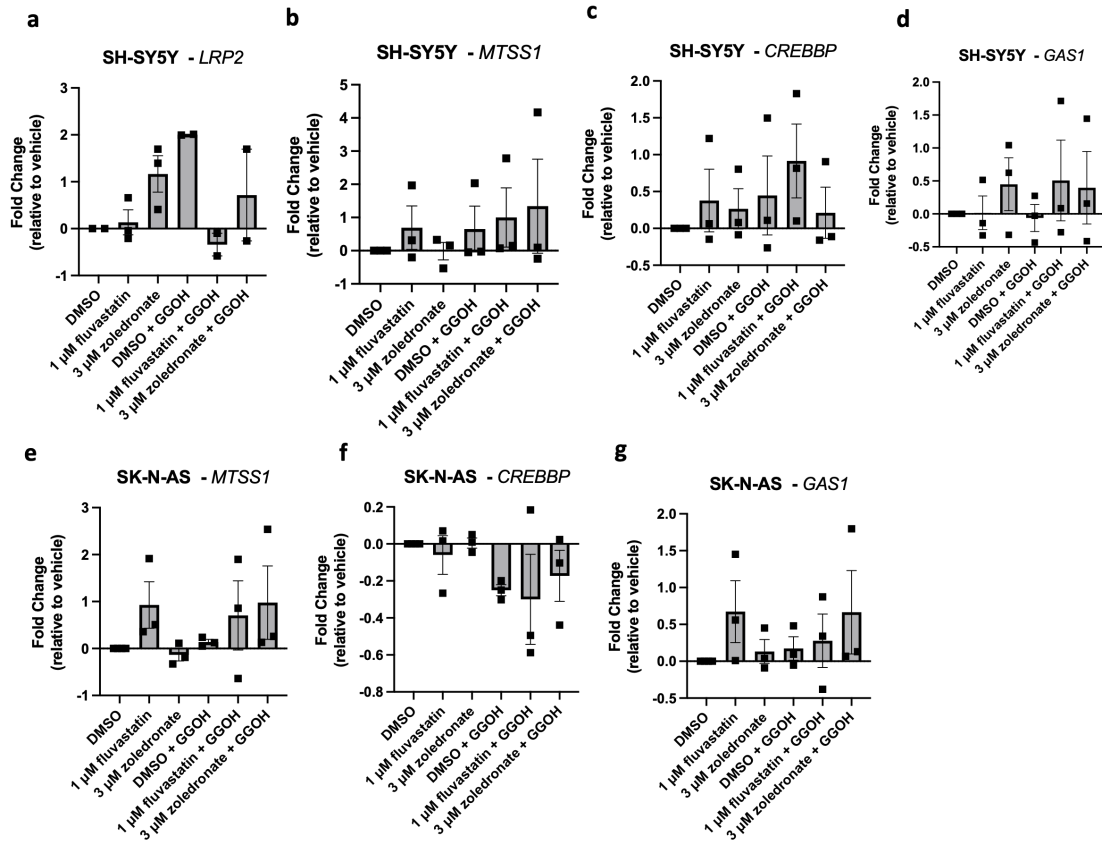


Figure 28: Mevalonate pathway inhibition effects on hedgehog pathway co-receptor and co-transcription factors.

(A-E) qRT-PCR analysis of hedgehog pathway proteins that can interact and function with pathway receptors and transcription factors. SH-SY5Y and SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

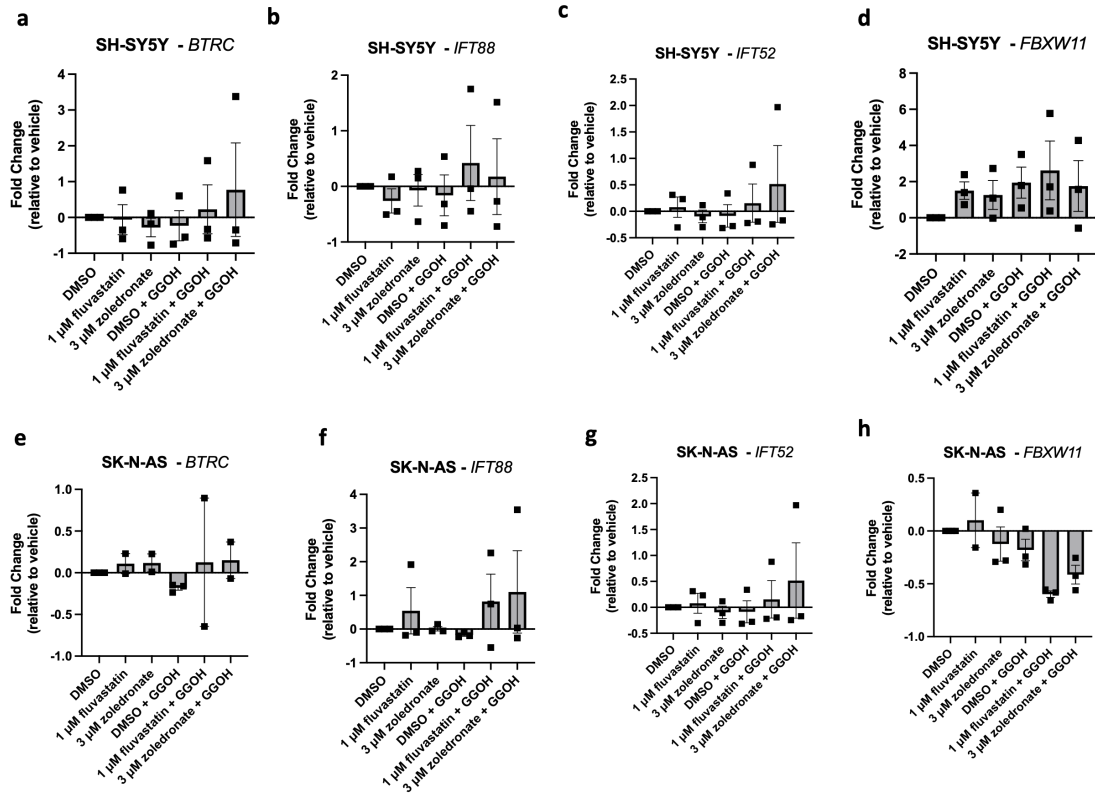


Figure 29: Mevalonate pathway inhibition effects on hedgehog pathway regulatory proteins.

(A-E) qRT-PCR analysis of hedgehog pathway regulatory proteins. SH-SY5Y and SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

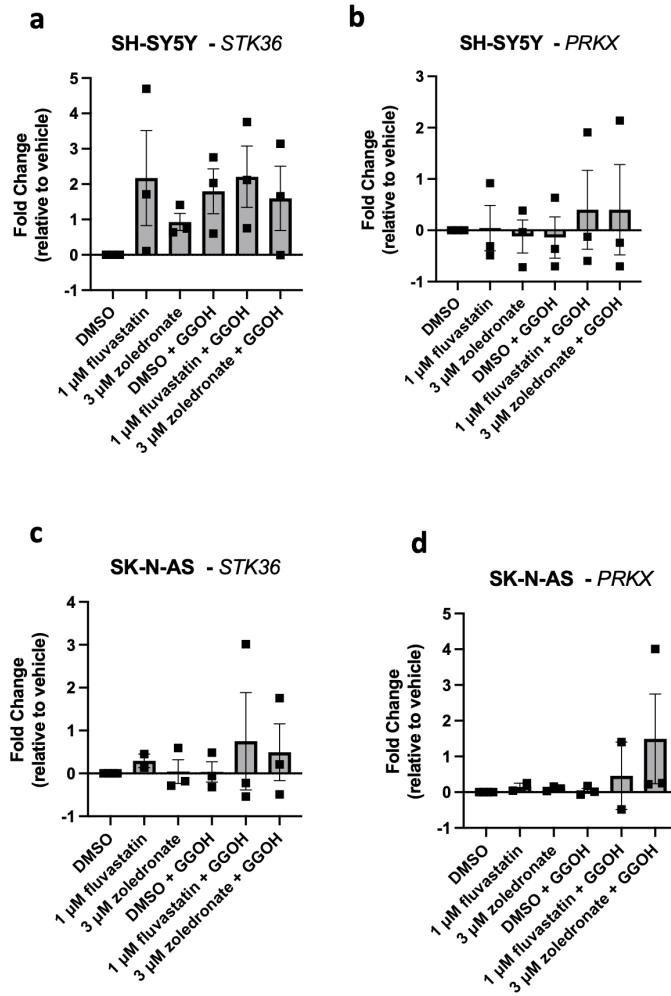


Figure 30: Mevalonate pathway inhibition effects on protein kinases.

(A-E) qRT-PCR analysis of hedgehog pathway protein kinases. SH-SY5Y and SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

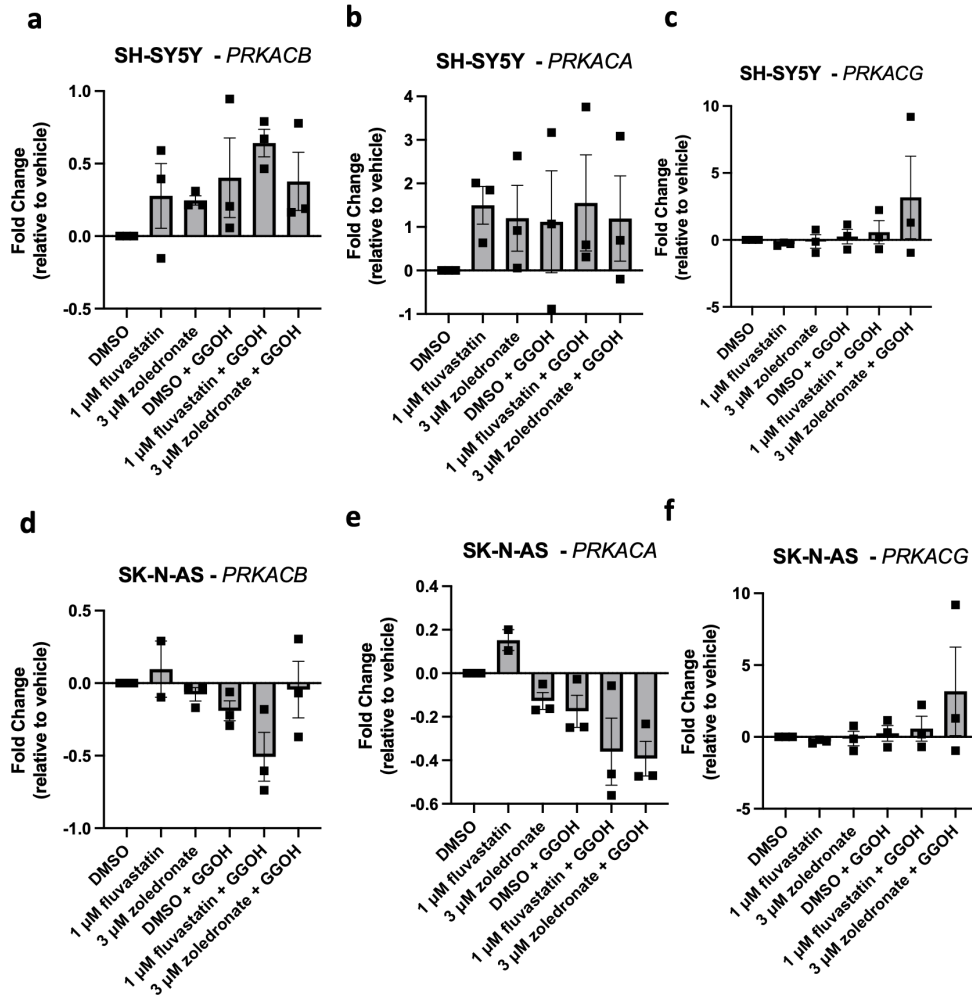


Figure 31: Mevalonate pathway inhibition effects on Protein Kinase A subunits.

(A-E) qRT-PCR analysis of hedgehog pathway protein kinase A. SH-SY5Y and SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

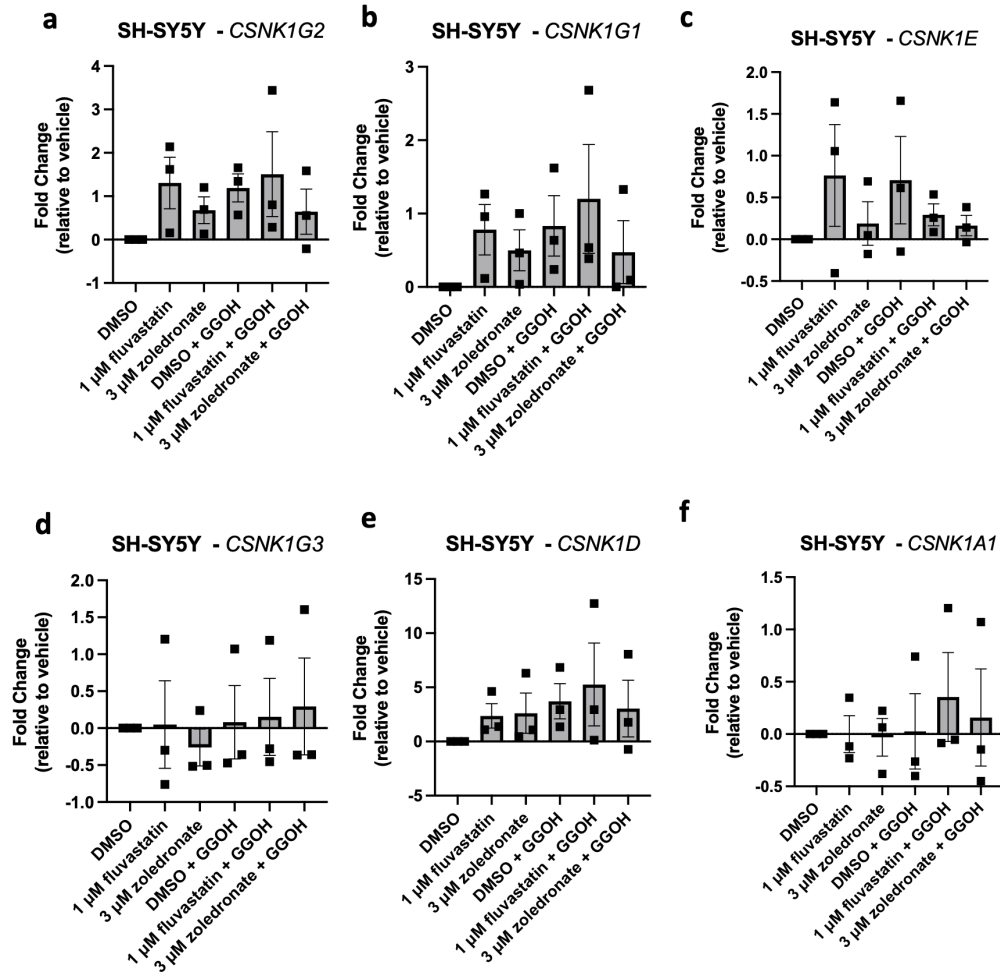


Figure 32: Mevalonate pathway inhibition effects on CSNK1 – SH-SY5Y.

(A-E) qRT-PCR analysis of hedgehog pathway associated kinase family CSNK1. SH-SY5Y spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

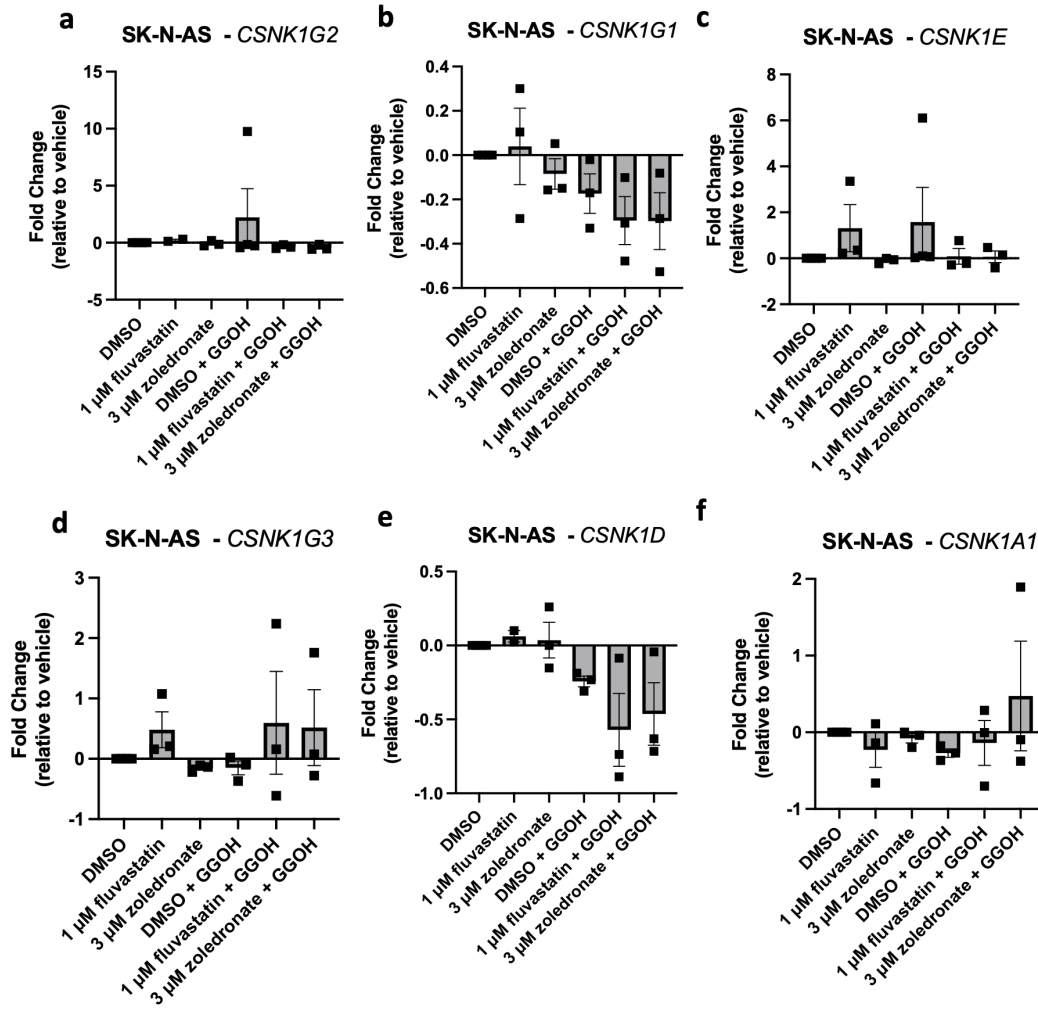


Figure 33: Mevalonate pathway inhibition effects on CSNK1 – SK-N-AS.

(A-E) qRT-PCR analysis of hedgehog pathway associated kinase family CSNK1. SK-N-AS spheroids were treated with indicated concentrations of fluvastatin, zoledronate, and GGOH and RNA was harvested after 72 hours. Graphs represent fold change with vehicle = 0 following two-way ANOVA with Tukey's correction. GAPDH was used as an internal control gene. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method.

Mevalonate pathway inhibition does not significantly alter protein levels of tested intracellular signaling molecules

As previously discussed, there are multiple signaling pathways associated with sympathoadrenal development that could be associated with the observed effects of mevalonate pathway inhibition in high-risk neuroblastoma spheroids.^{6,23,208} For preliminary investigation into this we profiled a few common proteins that are downstream/intracellular signaling components of developmental and neuroblastoma related pathways.

The intracellular signaling molecule GSK3 β is one such protein. As previously mentioned, it can signal through non-canonical hedgehog pathway signaling and is a component of the WNT signaling pathway.^{197,218} With fluvastatin and zoledronate treatment we did not observe any significant impact on RNA expression in either SH-SY5Y or SK-N-AS spheroids however the activity of GSK3 β is also regulated at the protein level, through phosphorylation. Phosphorylation at serine 9 inactivates the protein. We performed western blots to investigate the potential impact of mevalonate pathway inhibition on the total and phosphorylated levels of GSK3 β in high-risk neuroblastoma spheroids. For SH-SY5Y and SK-N-AS spheroids we consistently observed two bands on western blots for GSK3 β and phos- GSK3 β (Figure 34). We did not observe any significant change in the ratio of phosphorylated to total GSK3 β , or the total of either form of GSK3 β individually, with treatment with either fluvastatin or zoledronate treatment in either SH-SY5Y or SK-N-AS spheroids. We also investigated AKT, as it is an upstream kinase of GSK3 β and did not observe any significant effect in total AKT levels in either SH-SY5Y or SK-N-AS spheroids (Figure 35).¹⁹⁷ We were unable to successfully probe for phosphorylated AKT or Mitogen- and Stress-activated protein kinase (MSK) in the high-risk neuroblastoma spheroids.

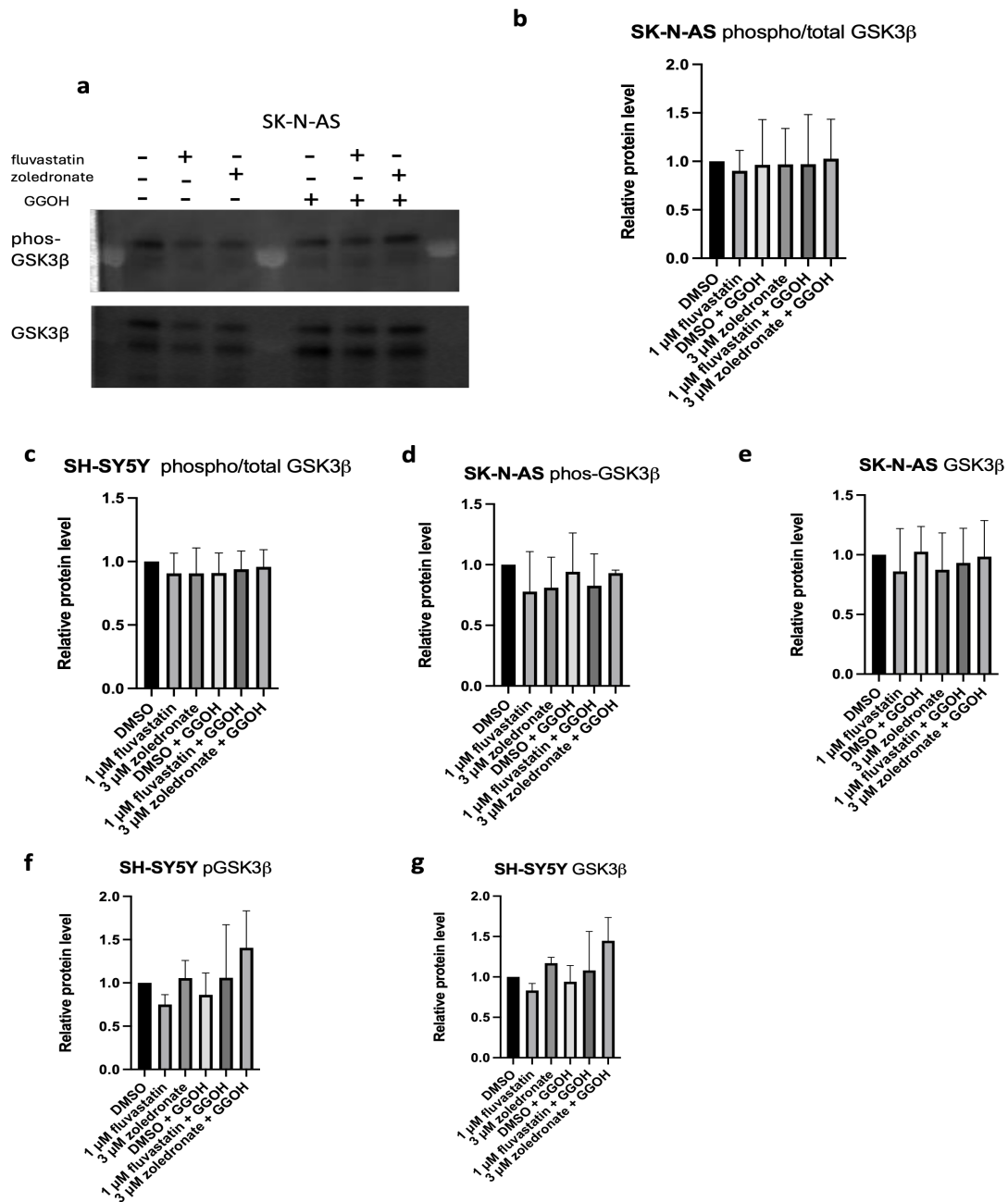


Figure 34: Mevalonate pathway inhibition effects on GSK3β in high-risk neuroblastoma spheroids.

(A-G) Western blot analysis of GSK3β protein levels in SH-SY5Y and SK-N-AS spheroids treatment with mevalonate pathway inhibitors. Spheroids were treated with the indicated concentrations of fluvastatin, zoledronate, and GGOH and incubated for up to 5 days.

Representative blot images shown. Graphs representative of 3 independent experiments presented as mean $\pm$ SD. Data analyzed by two-way ANOVA with Sidak's post-hoc for multiple comparisons.

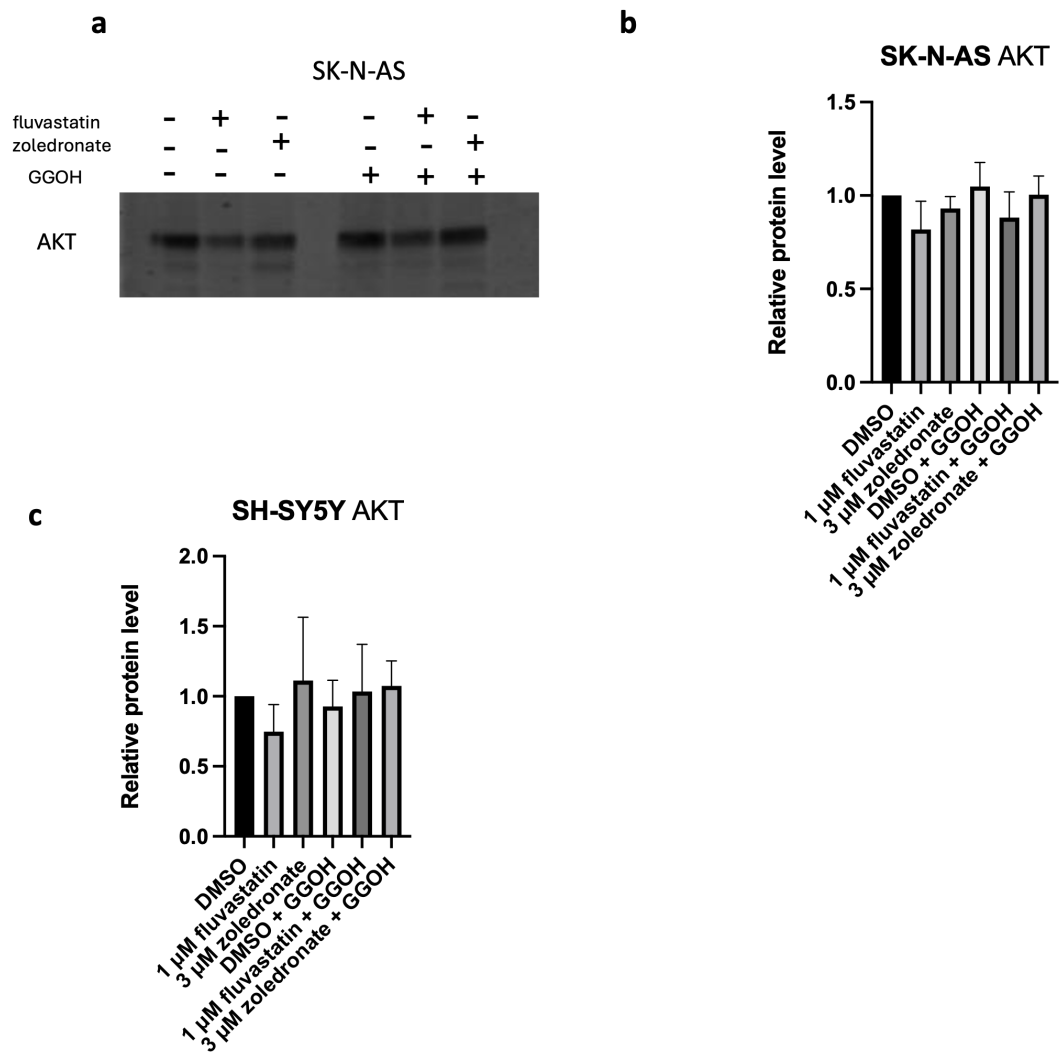


Figure 35: Mevalonate pathway inhibition effects on AKT in high-risk neuroblastoma spheroids.

(A-G) Western blot analysis of AKT protein levels in SH-SY5Y and SK-N-AS spheroids treatment with mevalonate pathway inhibitors. Spheroids were treated with the indicated concentrations of fluvastatin, zoledronate, and GGOH and incubated for up to 5 days. Representative blot images shown. Graphs representative of 3 independent experiments presented as mean $\pm$ SD. Data analyzed by two-way ANOVA with Sidak's post-hoc for multiple comparisons.

Discussion and Conclusion

Neuroblastoma cancer stem cells are hypothesized to originate from the developing sympathoadrenal neural crest cells during embryonic development and therefore likely retain aberrant signaling of developmental associated signaling pathways.⁶ This population of tumor cells is associated with the highly aggressive nature of high-risk neuroblastoma as CSCs contribute to drug resistance, metastasis, and tumor recurrence.^{78–80} We have developed a high-risk neuroblastoma model for the preclinical study of this CSC population that can be utilized for pharmacological analysis of drug candidates.

In Chapters 4 and 5 we showed that treatment of high-risk neuroblastoma spheroids with mevalonate pathway inhibitors induces a functional effect on the CSC population.¹⁷⁷ Investigation into the mechanism by which mevalonate pathway inhibitors have this functional effect found that multiple GTPases were potentially involved, suggesting the alteration of one or more signaling pathways. We hypothesized the hedgehog signaling pathway may be associated with the observed alterations, due to its implication in adherent neuroblastoma cell lines and association with multiple affected GTPases, that were altered with treatment of high-risk neuroblastoma spheroids with mevalonate pathway inhibitors.^{197,213–217}

The role of this pathway in neuroblastoma spheroids had not yet been investigated. In this present study we did not identify significant changes in hedgehog pathway-associated gene expression. We also noted important differences in the expression of hedgehog-associated gene expression in our spheroid model compared to prior literature on adherent cell lines. In adherent model systems high-risk neuroblastoma has been found to express the SHH ligand providing endogenous activation of the pathway.^{210,211,213–217} We did not detect gene expression of *SHH* in either SH-SY5Y or SK-N-AS spheroids, expression of the other ligands *DHH* and *IHH* was limited, and target genes were not activated. This indicates that in our spheroid model of high-risk

neuroblastoma the hedgehog pathway was not active. This pathway may require exogenous addition of ligands such as GDNF for activation, which is a condition we have observed mevalonate pathway inhibitors to target.

We also did not observe mevalonate pathway inhibition induced changes in GSK3 β , AKT or MSK1 in either SH-SY5Y or SK-N-AS spheroids. This analysis was likely hindered by the ability to harvest sufficient protein from the spheroids when treated for longer times or with increased doses. Continued research utilizing flow cytometry for protein analysis of neuroblastoma spheroids would be very interesting.

In this present study, we attempted to analyze the effect of mevalonate pathway inhibition of high-risk neuroblastoma on developmental signaling pathways. We were unsuccessful in identifying what signaling pathway(s) is responsible for the observed effects in Chapter 4 and 5. This is likely in part due to the mevalonate pathway inhibitors impacting multiple signaling pathways in a cell due to their broad effects on protein prenylation and due to the large number of developmental pathways and signaling pathways implicated in cancer. RNA sequencing to capture the full scope of what occurs in the high-risk neuroblastoma spheroids with mevalonate pathway treatment would be valuable.

CSCs pose a tricky therapeutic target specifically those of embryonal malignancies like neuroblastoma. These CSCs lack substantial mutational burden and the signaling pathways utilized by the cancer are likely needed for childhood development. Identification of compounds such as fluvastatin, that are able to exert functional effects on the CSC below the level to induce cell death are likely key to addressing this therapeutic need.

Chapter 7

Future Directions

Introduction

The studies presented in this dissertation provide insights into the functional effects of mevalonate pathway inhibition on high-risk neuroblastoma CSCs in a novel high-risk neuroblastoma spheroid model system. This opens the door for more questions on the utilization of mevalonate pathway inhibition in neuroblastoma, the utilization of the spheroid model for additional cancer types, and what the mechanism underlying the observed effects are. In this chapter we will discuss these novel research directions.

Combination Therapy: Mevalonate Pathway Inhibitors and Chemotherapy

Mevalonate pathway inhibition of high-risk neuroblastoma SH-SY5Y and SK-N-AS spheroids did not reduce spheroid cell viability, induce cell cycle arrest, or induce apoptosis.¹⁷⁷ The results however do indicate that mevalonate pathway inhibition has a functional effect on neuroblastoma spheroids as seen through reduction in spheroid formation, reduction in self-renewal, and inhibition of spheroid invasion. Investigation into the question of if mevalonate pathway inhibition with fluvastatin and zoledronate can sensitize neuroblastoma spheroids to chemotherapy treatment would be interesting. This is a research topic of significant clinical importance as novel therapeutics for pediatric cancer are most commonly used in combination with existing therapies rather than standalone therapy.

We did do some preliminary experiments on this topic. SK-N-AS and SH-SY5Y spheroids were grown as discussed in prior chapters and MTT assays for cell viability were done with combinations of fluvastatin and zoledronate and cisplatin and cyclophosphamide. When SK-N-AS spheroids were treated for 48 hours with cisplatin and 10 μ M fluvastatin or zoledronate there appeared to be a reduction in metabolic activity compared to cisplatin treatment alone (Figure 36). The impact of combination treatment with fluvastatin and zoledronate with cisplatin and cyclophosphamide in SK-N-AS spheroids requires further testing. We did not optimize dose or treatment length for this preliminary data. This experiment was done once in SH-SY5Y spheroids, therefore results are extremely limited for potential interpretation. Additional cell viability assays and expansion to other chemotherapies and radiation would be beneficial as preclinical work.

It would also be interesting to study the combination of mevalonate pathway inhibitors *in vivo* with chemotherapy but also immunotherapy against tumors formed from neuroblastoma spheroids. Zoledronate is currently in clinical trials for use in patients, but we are lacking a clear guidance for what subset of high-risk patients it would be beneficial for and what treatment it is optimal to be used in combination with. Utilizing the spheroid model in conjunction with combination therapy could provide very valuable insight into clinical utility of these compounds.

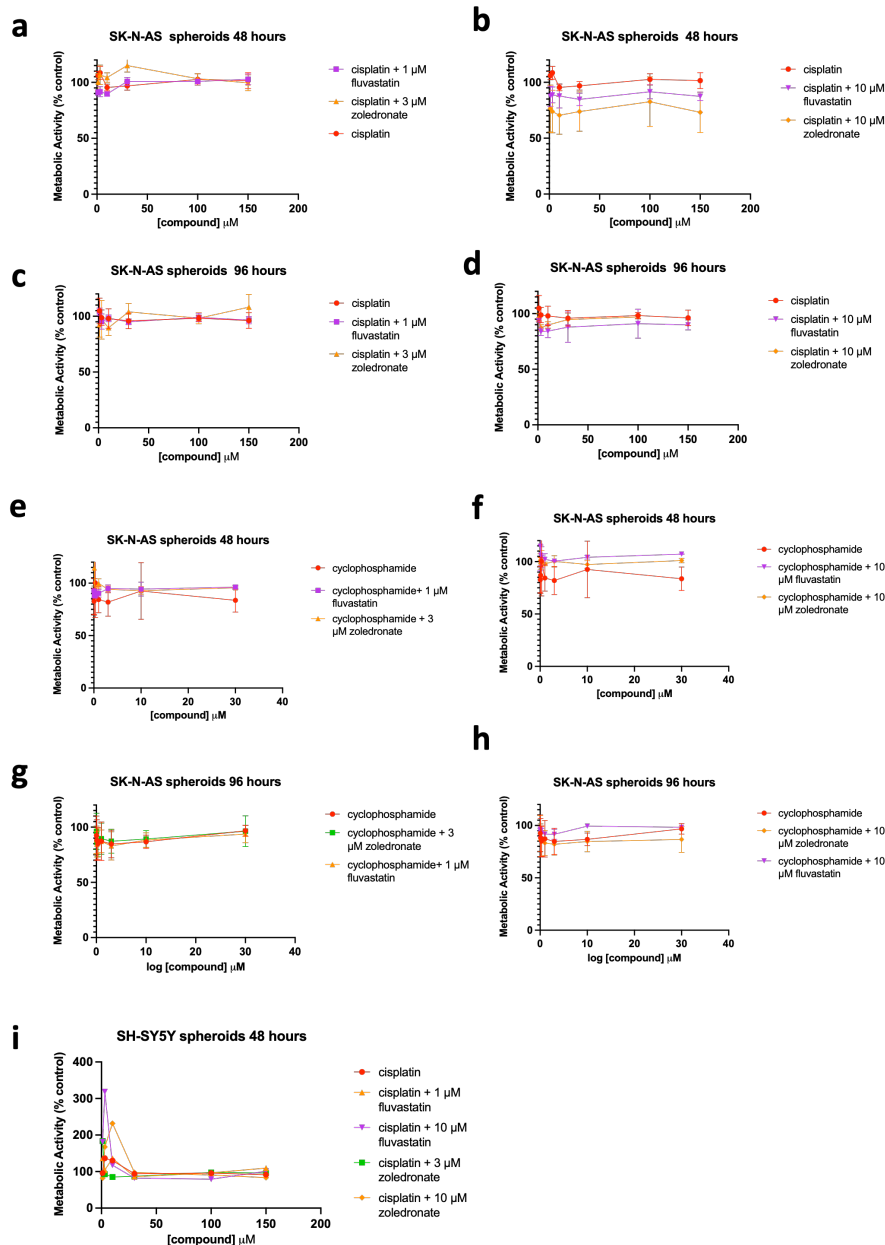


Figure 36: Combination treatment of high-risk neuroblastoma spheroids with mevalonate pathway inhibitors and chemotherapy.

(A-I) Compound response was determined by 48 hour and 96 hour MTT assays in spheroid cultures of SK-N-AS and SH-SY5Y cell lines. Data shown as $\pm$ SEM, $n = 3$ independent experiments for SK-N-AS spheroids. Data for SH-SY5Y is representative of one replicate.

Neuroblastoma Spheroid Pathway Analysis

As discussed in Chapter 6 we could not identify the signaling pathway or pathways that are associated with the observed effects of mevalonate pathway inhibition in our high-risk neuroblastoma spheroids. Our findings indicate that the hedgehog pathway is inactive in high-risk neuroblastoma spheroids, since spheroid cells did not endogenously express SHH. We also observed that mevalonate pathway inhibition of high-risk neuroblastoma spheroids had effects when GDNF was added to the spheroid model.¹⁷⁷ Further analysis of whether GDNF stimulates non-canonical hedgehog signaling in high-risk neuroblastoma spheroids is warranted.¹⁹⁷

This analysis would also benefit from the development of a flow-cytometry based protein assay for better detection of low-level protein expression.

Further pathway analysis would benefit from RNA sequencing to get a better overall understanding of the signaling pathways active in neuroblastoma spheroids. Many of the pathways that have been implicated in development and adherent neuroblastoma cell culture are likely relevant to the spheroid model, however as we observed with the hedgehog pathway there are likely important differences we are unaware of at this time. Sequencing would not only assist in understanding the mechanism by which mevalonate pathway inhibitors have the observed effects but also for identifying additional signaling pathways as potential therapeutic targets in CSCs.

Sequencing could be used to inform further experiments to tease out the differences in cell line response to mevalonate pathway inhibition. As we observed, the cells lines and spheroids displayed variable sensitivity to downstream inhibitors and variable responses to GGOH and FOH supplementation. Identification of differences in expression of mevalonate pathway related genes would help understand these variable responses. Further use of targeted inhibitors, such as

GGTIs and FTIs, and genetic manipulations would also help to tease out the response of high-risk neuroblastoma spheroids to mevalonate pathway inhibition.

Confocal Microscopy Of Spheroids

The current invasion assay for spheroids described in Chapter 5 involves the manual imaging and analysis of hundreds of images by hand and is limited to only two-dimensional images of the spheroids. Additionally, the ability to monitor the growth characteristics of spheroids and counting of spheroids for spheroid formation analysis and ELDA would benefit from improved imaging. Investment in the imaging systems for confocal microscopy of 96-well plates would be beneficial to these experiments through 3D automated imaging and enable the broader application of this protocol in other cancer models in our laboratory.

Embryonal Malignancies And Spheroids

The spheroid model system developed and described in this work has utility for the study of other malignancies. Our lab has previously used spheroids for glioblastoma work and the protocols developed were built from those. The utility for embryonal malignancies, like neuroblastoma, and other pediatric cancers such as medulloblastoma especially should be highlighted and considered. The CSC population within neuroblastoma and other embryonal cancers is often highly dynamic in response to the environment the cell lines are cultured in and the best model system would likely be one that closely mimics the tumor environment.⁷⁹

We have done some preliminary work with the utilization of spheroid models for culturing other pediatric cancers. Specifically, we cultured the medulloblastoma DAOY cells in spheroid medium, using the same formulation as described here for neuroblastoma. These cells

readily formed spheroids and we observed a dose dependent decrease in maximum spheroid formation with simvastatin and lovastatin treatment (Figure 37). We also observed a decrease in the self-renewal capability of these spheroids with simvastatin or lovastatin treatment (Figure 37). The protocol used for this cell line was identical to that for neuroblastoma spheroids.

Further work in this CSC model with additional cancer types, specifically pediatric and embryonal cancer, can be done to analyze the pharmaceutical effects of the various compounds.

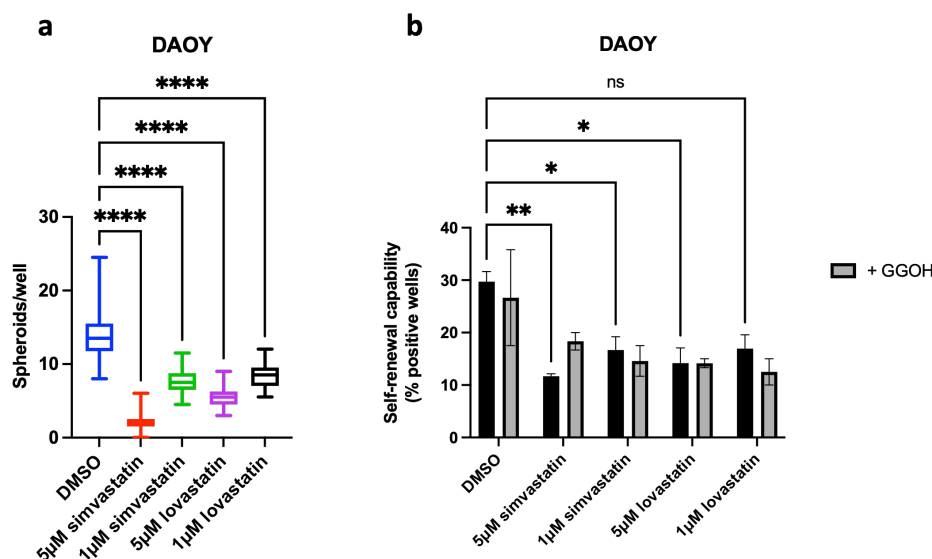


Figure 37: Mevalonate pathway inhibition of medulloblastoma spheroids.

DAOY cells were grown as described for neuroblastoma spheroids. (A) Spheroid formation assay of DAOY in the presence of indicated concentrations of simvastatin and lovastatin. Spheroids were plated at 200 cells/well and spheroid counts were carried out in duplicate with 60 wells counted per condition. Data analyzed via one-way ANOVA with Dunnett's post-hoc α 0.05. Data shown as box and whisker plots with the whiskers representing minimum and maximum values and the box representing quartiles. (B) Self-renewal assay of DAOY spheroids. Graph representative of two independent experiments with 120 wells counted per condition each replicate. Graphs presented as mean $\pm$ SEM. Data analyzed via two-way ANOVA with Sidak's post hoc. * $p \leq 0.05$ ** $p \leq 0.01$

Conclusions

The work presented in this dissertation can be applied in many ways to research moving forward. The preclinical development of mevalonate pathway inhibitors for use in combination with chemotherapy and/or radiation could be very informative to the ongoing clinical trials in this field. As well as the signaling pathway analysis for elucidating the characteristics of the neuroblastoma spheroids and the mechanism by which mevalonate pathway inhibitors block their CSC functions. The immediate application of the spheroid model to projects in our laboratory is perhaps the most obvious next step forward. We are actively developing this model for other ongoing projects in the lab.

Chapter 8

Conclusions

Introduction

This body of work has contributed to our understanding of mevalonate pathway inhibition functional effects on the neuroblastoma CSC population through the development and utilization of a novel spheroid model. There is a stark contrast between neuroblastoma patients: low-risk patients often do not require medical intervention as their tumor(s) can spontaneously differentiate while high-risk patients who receive every treatment available and still have a five-year survival rate of about 50%.^{3,6} There is a critical need to identify therapeutics that can target the population of CSC that drive the aggressiveness of these tumors. By developing more of an understanding of how mevalonate pathway inhibitors affect this population investigators can determine the potential utility of statins, zoledronate, and other inhibitors, as chemotherapeutics in neuroblastoma patients. This chapter summarizes our results on the effects of mevalonate pathway inhibition of neuroblastoma CSCs and provide context on the findings.

Mevalonate Pathway Inhibition Targets Neuroblastoma Cancer Stem Cells

This work began with the hypothesis that treatment of high-risk neuroblastoma cell lines with mevalonate inhibitors, representative of patients with poor clinical outcomes, would induce differentiation and cell death through loss of protein prenylation. There was substantial prior literature on this topic however it was a bit sporadic with cell line(s) and inhibitor(s) chosen

therefore we aimed to profile multiple statins and downstream inhibitors in our chosen cell lines.^{78,102,103,112,127,154,156}

For this work we utilized three high-risk neuroblastoma cancer cell lines, SH-SY5Y, SK-N-AS and SK-N-BE(2)-C, that are representative of patients with the very poor prognoses and death within 18 months of diagnosis.¹⁷⁵

In adherent cell culture these cell lines are sensitive to mevalonate pathway inhibition with three statins tested (lovastatin, simvastatin and fluvastatin) with SH-SY5Y being the most sensitive and SK-N-AS cells being least sensitive, which is consistent with previous literature.^{100-102,104,127,154} We chose fluvastatin to use moving forward as the potential side-effect profile is improved as it cannot cross the BBB into the developing CNS. Prior literature also found that neuroblastoma cells were sensitive to inhibition of the mevalonate pathway due to loss of protein geranylgeranylation.^{102,103} When adherent cells were treated with bisphosphonates, DGBP or zoledronate, we did not see significant reductions in cell viability, but we did see a reduction in cell viability when treated with GGTI-286. Perhaps the most intriguing observation was the differentiation phenotype that fluvastatin treatment induced in SH-SY5Y and SK-N-AS cells, suggesting that targeting this pathway could influence the CSC population.

As discussed in Chapter 4 we developed a spheroid model of these high-risk neuroblastoma cell lines to use for further pharmacological analysis of mevalonate pathway inhibitors. When these methods were being developed, we used existing glioblastoma spheroid protocols and adjusted growth conditions and assay design as needed.¹⁸⁰ Spheroid models are more common now, but we hope the insights discussed in Chapter 4 may be helpful for future research on novel cancer types.

In Chapter 5 we utilized this high-risk neuroblastoma spheroid model to investigate the impact of mevalonate pathway inhibition on the neuroblastoma CSC population.¹⁷⁷ We found that mevalonate pathway inhibition with fluvastatin or zoledronate of SH-SY5Y, SK-N-AS and SK-

N-BE(2)-C spheroids reduced spheroid formation in a dose-dependent manner that was likely due to altered protein prenylation. The variations observed between cell lines are likely influenced by what branch of the mevalonate pathway these cell lines are dependent upon. SK-N-AS spheroid formation was rescued with GGOH supplementation, consistent with prior literature on the mesenchymal subtype.¹⁰³ SH-SY5Y spheroid formation was partially rescued with GGOH supplementation with some increased spheroid formation observed with FOH. SK-N-BE(2)-C spheroid formation reduction was rescued with supplementation of GGOH but supplementation with FOH further decreased spheroid formation.¹⁷⁷

Importantly, we observed that mevalonate pathway inhibition with low-dose fluvastatin (1 μ M) or low-dose zoledronate (3 μ M) led to a reduction in the self-renewal capability of all three high-risk neuroblastoma spheroids.¹⁷⁷ Low-dose fluvastatin treatment also led to a significant reduction in the invasion of all three high-risk neuroblastoma spheroids. Low-dose zoledronate was sufficient to reduce SK-N-AS spheroid invasion but 10 μ M zoledronate was required to observe this effect in SH-SY5Y and SK-N-BE(2)-C cells. These effects were observed without evidence of cell cycle arrest or apoptosis indicating that the mevalonate pathway inhibitors exert functional effects on this cell population.

Investigation on the specific GTPases that are impacted in high-risk SH-SY5Y and SK-N-AS spheroids found that there are changes in total levels of RhoB, Rap1a/Ralp1b, RhoA, CDC42 and Rac that vary between the cell lines.¹⁷⁷ One of the developmental pathways, the hedgehog signaling pathway, was screened for gene expression changes following mevalonate pathway inhibition with fluvastatin and zoledronate in SH-SY5Y and SK-N-AS spheroids. We did not identify any significant changes in the gene expression of any hedgehog associated genes. We also noted differences in the signaling pathway activation in our spheroid model and previous literature reports. Our spheroid model did not substantially express the pathway ligands, and we did not detect target gene expression, both of which have been identified in adherent cell lines

and hypoxic conditions. This suggests that other signaling pathways associated with high-risk neuroblastoma cell lines likely are altered in this spheroid model. Further research on this is critical for understanding what signaling pathways are potential therapeutic targets for high-risk neuroblastoma CSCs and for investigation of the mechanism of mevalonate pathway inhibition.

Conclusion: Providing Context

High-risk neuroblastoma is an aggressive form of pediatric cancer with five-year survival rates of about 50%.³ This is despite intensive treatment approaches with multiple rounds of high-dose chemotherapy with autologous stem cell transplant, radiation, differentiation therapy and immunotherapy.³⁷ A population of cells within the tumor known as CSCs are thought to be responsible for this aggressiveness, as discussed in Chapter 2.^{79,80} Potential treatment options that can target this population of cells are desperately needed. The work presented here investigates the potential utility of mevalonate pathway inhibitors against this population through use of a spheroid model. The cell lines chosen for this work took clinical relevancy into account as they represent patients with extremely poor prognoses and the inhibitors chosen took side-effect profiles and relevancy to clinical use into account. Fluvastatin is FDA approved for use in pediatric cancers and showed consistent impacts to high-risk neuroblastoma spheroid function at a dose likely achievable in patients. Zoledronate is FDA approved and in current clinical trials for the treatment of neuroblastoma. Understanding the variable responses to these compounds observed in cell lines could be informative for what patient populations may benefit from the addition of statin treatment to current therapies. More work is needed to understand the effects of mevalonate pathway inhibitors on high-risk neuroblastoma spheroids however this work is a positive step in the fight against pediatric cancer.

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- Weissenrieder JS, Weissenkampen JD, Reed JL, **Green MV**, Zheng C, Neighbors JD, Liu DJ, Hohl RJ. RNAseq reveals extensive metabolic disruptions in the sensitive SF-295 cell line treated with schweinfurthins. *Sci Rep*. 2022 Jan 10;12(1):359. doi: 10.1038/s41598-021-04117-7. PMID: 35013404; PMCID: PMC8748991.
- Weissenrieder JS, Reed JL, **Green MV**, Moldovan GL, Koubek EJ, Neighbors JD, Hohl RJ. The Dopamine D2 Receptor Contributes to the Spheroid Formation Behavior of U87 Glioblastoma Cells. *Pharmacology*. 2020;105(1-2):19-27. doi: 10.1159/000502562. Epub 2019 Oct 23. PMID: 31645049; PMCID: PMC10777736.
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