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Inhibition of Cancer Stem Cells by Glycosaminoglycan Mimetics

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Inhibition of Cancer Stem Cells by Glycosaminoglycan Mimetics

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University

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LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
ALDH	Aldehyde dehydrogenase
APC	Adenomatous polyposis coli
ATCC	American Type Cancer Culture
BRAF	Rapidly Accelerated Fibrosarcoma-B
CAF	Cancer activated/associated fibroblast
CCL	Chemokine ligand
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CIMP	CpG island methylator phenotype
CIN	Chromosome instability
CMS	Consensus molecular subtype
CRC	Colorectal cancer
CSC	Cancer stem-like cell
DAPI	Diamidino phenylindole
DCTD	Division of Cancer Treatment and Diagnosis
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminitraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FAP	Familial adenomatous polyposis

FBS	Fetal bovine serum
FDA	Food and Drug Administration
FIT	Fecal immunochemical test
FOBT	Fecal occult blood test
FU	Fluorouracil
GAG	Glycosaminoglycan
GDP	Guanosine diphosphate
GI	Gastrointestinal
GTP	Guanosine triphosphate
HDR	High dose rate
HINT	Hydropathic interaction
HNPCC	Hereditary non-polyposis colorectal cancer
HS06	Heparan sulfate hexasaccharide
HSPG	Heparan sulfate proteoglycan
HTS	High throughput screening
IBD	Inflammatory bowel disease
IGF	Insulin-like growth factor
IRI	Irinotecan
KRAS	Kirsten rat sarcoma virus protein
LGR5	Leucine rich repeat containing G-protein coupled receptor 5
LS	Lynch syndrome
mAb	Monoclonal antibody
MMR	Mismatch repair
MSI	Microsatellite instability
MSI-L	Microsatellite instability low
MSI-H	Microsatellite instability high
MSS	Microsatellite stability
MTT	Dimethylthiazol diphenyltetrazolium
MYC	**My**elo**c**ytomatosis
NCI	National Cancer Institute

NET	Neuroendocrine tumor
NHB	Non-Hispanic black
NHW	Non-Hispanic white
NIH	National Institute of Health
NSGM	Non-saccharide glycosaminoglycan mimetic
NYR	Non yet recruiting
OCT4	Octamer-binding transcription factor 4
OX	oxaliplatin
PBS	Phosphate buffered saline
PDB	Protein database
PDX	Patient-derived xenograft
PI3K	Phosphoinositide 3-kinase
PJS	Peutz Jegher Syndrome
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
SCM	Stem cell media
SCNA	Somatic copy number alteration
SFF	Spheroid formation frequency
SMAD	Mothers against decapentaplegic homolog
TGF	Transforming growth factor
TNM	Tumor, node, metastasis
UC	Ulcerative colitis
UICC	Union for International Cancer Control
VA	Veterans Affairs
VEGF	Vascular endothelial growth factor
WNT	**W**ingless-related I**nt**egration Site

ABSTRACT

INHIBITION OF CANCER STEM CELLS BY GLYCOSAMINOGLYCAN MIMETICS

By Connor O'Hara

A thesis submitted in fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2019

Director: Dr. Umesh R. Desai

Alfred and Frances Burger Professor of Medicinal Chemistry

Inhibition of Cancer Stem Cells by Glycosaminoglycan Mimetics

In the United States cancer is the second leading cause of death, with colorectal cancer (CRC) being the third deadliest cancer and expected to cause over 51,000 fatalities in 2019 alone.[1] The current standard of care for CRC depends largely on the staging, location, and presence of metastasis.[2] As the tumor grows and invades nearby lymph tissue and blood vessels, CRC has the opportunity to invade not only nearby tissue but also metastasize into the liver and lung (most commonly).[3] The 5-year survival rate for metastasized CRC is <15%, and standard of care chemotherapy regimens utilizing combination treatments only marginally improve survival.[3-5] Additionally, patients who have gone into remission from late-stage CRC have a high risk of recurrence despite advances in treatment.[6-7]

The Cancer Stem-like Cell (CSC) paradigm has grown over the last 20 years to become a unifying hypothesis to support the growth and relapse of tumors previously regressed from chemotherapy (Figure 1).[8] The paradigm emphasizes the heterogeneity of a tumor and its microenvironment, proposing that a small subset of cells in the tumor are the source of tumorigenesis with features akin to normal stem cells.[9] The CSCs normally in a quiescent state survive this chemotherapy and "seed" tumor redevelopment.[10] First observed in acute myeloid lymphoma models, CSCs have since been identified in various other cancers (to include CRC) by their cell surface antigens and unique properties characterizing them from normal cancer cells.[11-12] These include tumor initiation, limitless self-renewal capacity to generate clonal daughter cells, as well as phenotypically diverse, mature, and highly differentiated progeny.[13-14]

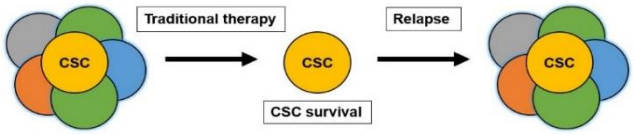


Figure A. Cancer stem cell paradigm of relapse.

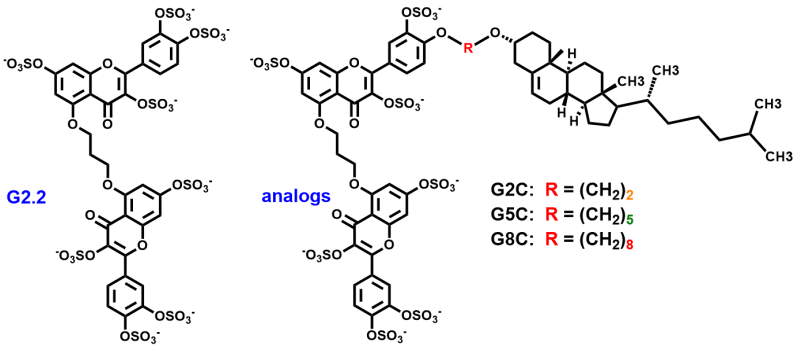


Figure B. Structure of G2.2 and lipid modified analogs.

Previously our lab has identified a novel molecule called G2.2 (Figure 2) from a unique library of sulfated compounds showing selective and potent inhibition of colorectal CSCs in-vitro.[15] G2.2 is a mimetic of glycosaminoglycans (GAGs) and belongs to a class of molecules called non-saccharide GAG mimetics (NSGMs). Using a novel dual-screening platform, comparisons were made on the potency of G2.2 in bulk monolayer cells, primary 3D tumor spheroids of the same cell line, and subsequent generations of tumor spheroids. This work has shown in-vitro the fold-enhancement of CSCs when culturing as 3D tumor spheroids. Spheroid culture serves as a more accurate model for the physiological conditions of a tumor, as well as the functional importance of upregulating CSCs. Evaluation of G2.2 and other NSGMs was performed in only a few cell lines, developing a need to better understand the ability of G2.2 to inhibit spheroids from a more diverse panel of cancer cells to better understand G2.2's mechanism.

The last few decades have seen the advancement in fundamental biological and biochemical knowledge of tumor cell biology and genetics.[16] CRC, in particular, has served as a useful preclinical model in recapitulating patient tumor heterogeneity in-vitro.[17] Recent work has characterized the molecular phenotypes of CRC cell lines in a multi-omics analysis, stratifying them into 4 clinically robust and relevant consensus molecular subtypes (CMS).[18-19] Our work was directed to screen a panel of cells from each of the molecular subtypes and characterize the action of G2.2 and 2nd generation lipid-modified analogs, synthesized to improve the pharmacokinetic

properties of the parent compound. Four NSGMs, namely G2.2, G2C, G5C, and G8C (Figure 2) were studied for their ability to inhibit the growth of primary spheroids across a phenotypically diverse panel.

Primary spheroid inhibition assays were performed comparing the potency of new NSGMs to G2.2. Fifteen cell lines were evaluated in a panel of colorectal adenocarcinoma cell lines with several cell lines representing each CMS. Primary spheroid inhibition assays revealed 3 distinct response with regard to G2.2's ability to inhibit spheroid growth. Cells from CMS 3 and 4, which display poor clinical prognosis, metabolic dysregulation, and enhanced activation of CSC pathways, showed the most sensitivity to G2.2 (mean IC_{50} = 89 ± 55 μM). Mesenchymal CMS 4 cell lines were over 3-fold more sensitive to treatment with G2.2 when compared to CMS 1 cell lines. Resistant cell lines were composed entirely of CMS 1 and 2 (mean IC_{50} = 267 ± 105 μM). In contrast, all lipid-modified analogs showed greater potency than the parent NSGM in almost every CRC cell line. Of the three analogs, G8C showed the greatest potency with a mean IC_{50} of less than 15 μM. Of the CRC spheroids studied, HT-29 (CMS 3) was most sensitive to G8C (IC_{50} = 0.7 ± 0.2 μM).

Table A. Spheroid inhibition potency of G2.2 and analogs.

Compound	HT-29 IC_{50} (μM)	Panel Average IC_{50} (μM)
G2.2	28 ± 1	185 ± 55
G2C	5 ± 2	16 ± 15
G5C	8 ± 2	63 ± 19
G8C	0.7 ± 0.2	6 ± 3

To evaluate the selectivity of NSGMs for CSC spheroid inhibition, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) cytotoxicity assays were performed on monolayer cell culture, and

the fold-selectivity of NSGM for spheroids was analyzed. Data shows that NSGMs preferentially target CSC-rich spheroids compared with monolayer cellular growth, with G2.2 having over 7-fold selectivity for spheroid conditions. This fold selectivity was enhanced in CMS 3 and 4, supporting the idea that G2.2 targets a mesenchymal and stem-like phenotype. To further validate this selectivity, limiting dilution assays were performed across the panel to determine the tumor-initiating capacity of each cell line. Cell lines which showed a sensitive response to G2.2 were over 2-fold more likely to develop into spheroids, validating the previous hypothesis. Further characterization was performed analyzing the changes G2.2 induced on CSC markers, as well as the basal expression of a unique pair of cancer cells. Western blots showed a reduction in self-renewal marker across all CMS after treatment with G2.2, and that cell lines sensitive to G2.2-treatment overexpress mesenchymal and stem-like markers. G2.2-resistant cell lines show an epithelial phenotype, lacking this expression.

The positive results observed in these studies enhance the understanding of G2.2 and analogs, and further evaluation with additional cell lines of various tissues would improve the knowledge thus far gained. However, all experiments described take valuable time to perform and analyze. Thus, there became a need to develop a high-throughput screening (HTS) platform for our assays that standardized analysis and enhanced productivity. Initial development of the method for this assay are underway, and recent evidence from these evaluations of breast cancer spheroids suggests that G2.2 and analogs may be tissue-specific compounds for the treatment of cancer. Future work entails refining the application of this method for evaluation of the NCI-60 (National Cancer Institute) tumor cell panel.

Overall, these results make several suggestions concerning the NSGMs evaluated against the panel. First, G2.2 selectively targets CSCs with limited toxicity to monolayer cells of the same cell line. Further, G2.2 has the greatest potency with CMS 3 and 4, whose mesenchymal phenotypes are associated with poor clinical prognosis and enrichment of CSCs. Supporting evidence include that sensitive cell lines are highly tumorigenic and show enhanced expression of mesenchymal/CSC markers compared to resistant cell lines. Lipid-modification of G2.2 enhances in-vitro potency against spheroid growth, with nM potency reached in the most sensitive cell lines. Evidence in the development of a HTS platform also suggests these NSGMs show tissue specificity to cancers of the intestine. Further work characterizing the mechanism of NSGMs in a broader multi-tissue panel will enhance our understanding of the compounds as a potential therapy to dramatically improve patient survival through specific targeting of tumorigenesis.

1. CANCER AND CANCER STEM CELLS

a. Clinical Impact of Colorectal Cancer

Cancer continues to be a leading cause of death both in the United States as well as in the world, with approximately 606,880 predicted deaths in the United States alone during 2019.[20] Of these deaths, there are several sites in the human body where cancer is more likely to occur than others. These include lung, colorectal, breast and prostate – the four most common types of cancers.[1, 20-21] Recent years have seen dramatic reductions in cancers as a whole, with the rates of lung cancers steadily declining thanks to reduction in smoking in the United States. There have additionally been improved rates in screening and early detection that have improved outcomes for many diagnosed with cancer.[1] Still, with an aging population of baby-boomers and increasingly poor lifestyle choices among younger generations the expectation for many cancers is to increase.

Colorectal cancer (CRC) is currently a 2nd most commonly diagnosed cancer, as well as a 2nd leading cause of cancer deaths with 101,420 deaths predicted for 2019 in the United States.[1] Like most other forms of cancer, it is generally diagnosed in patients over 50 years of age. For the population older than 50, the mortality trends for CRC have decreased thanks to increased screening efforts and changes in risk factors (obesity, high consumption of processed meats, heavy alcohol consumption, smoking).[22] For the younger generations the rates of diagnosis and mortality are increasing however, as these aforementioned screening efforts are frequently

neglected until symptoms appear later in life. At this point in development, cancer is much more likely to be diagnosed at a later stage with the potential to have migrated to other locations in the body. The 5-year survival rate for CRC drops dramatically once the tumor metastasizes, and is much more difficult to treat.[23] As a result, it is important that the public take the necessary efforts to reduce their exposure to common risk factors and take advantage of early screening and detection methods that might be able to catch colorectal neoplasia before it further develops into a malignancy. To better understand how CRC develops and is eventually diagnosed, the following discussion will present the epidemiology and pathology of the disease.

i. Colorectal Cancer Epidemiology and Pathology

1. Epidemiology of Colorectal Cancer

Colorectal cancer (CRC) will cause approximately 51,020 deaths in the United States for both males and females, as predicted by compiled and fitted data from the National Center for Health Statistics (See Figure 1).[20] Although the likelihood of developing CRC is similar in both sexes, men have a higher chance of development of the malignancy. CRC is the 2nd leading cause of death from cancer in both sexes combined, behind only lung cancer.[1, 21] The trend in development of CRC varies depending on the age of the patient, predisposed health risks, lifestyle, and socioeconomic status. The birth-to-death chance of CRC in men and women is approximately 1 in 24, while in populations over 60 years old the chances of development continue to increase several fold each decade.[24] It is recommended by the NIH that people over the age of 55 continue to be screened as regularly as afforded to detect and treat CRC as early as

possible. This effort significantly raises the chances for survival, and early screening and detection efforts continue to become more affordable and convenient.

Mortality trends for CRC have decreased in a tremendous fashion today compared with several decades ago, largely due to efforts to decrease risk factors, more effective treatment options for patients, and the use of preventative screening measures. Most of these changes are seen in the population aged 55 and older, who are at the greatest risk of development. There is a more alarming trend seen in the population younger than 55 years old, where CRC has increased by 1% per year each year.[4, 23] Although CRC is a heterogeneous disease, there are several risk-factors in addition to age that can attribute to an increased chance of development.

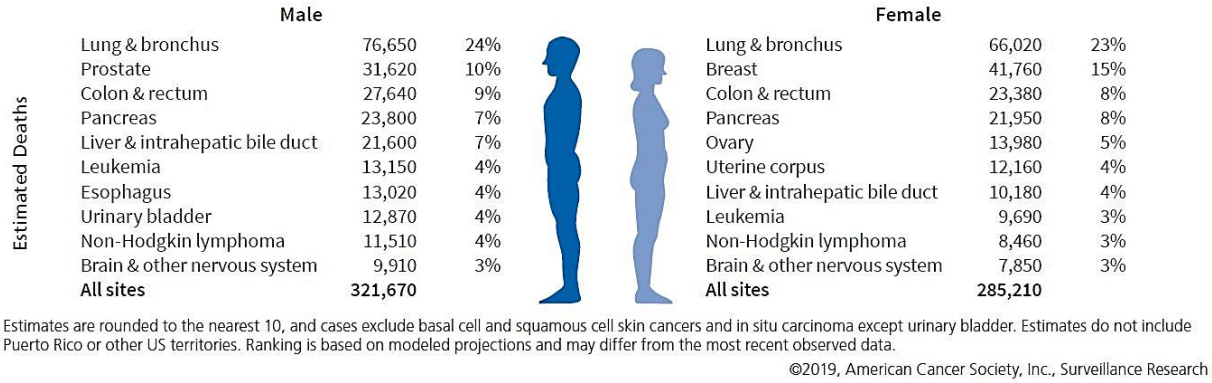


Figure 1. Estimated incidence of mortality by cancer type, American Cancer Society Surveillance Research 2019.

Many of these risk factors can be changed early enough in life to reduce incidence rates before old age. As indications of CRC are usually not seen until after age 50, it is important to make efforts to tilt the scale in your favor prior to indications. Behavioral risk factors like

obesity, diet, alcohol consumption, and amount of regular exercise have been shown to contribute to CRC incidence.[23, 25-28] With diet, consumption of red meats and processed meats in excess of 100 g per day have been established with approximately a 20% elevated risk of CRC development. Lack of physical exercise and obesity have shown to be greater contributing risk factors, with consumption of greater than 3 standard drinks per day to be the most significant of the behavioral factors.[29-31] It is important not only for CRC but other cancers and disease states that these factors be limited and steps taken to lower your risks.

More significant than behavioral risk factors are those which are hereditary as well as other medical factors that put someone in an elevated chance of developing CRC. There are two well-known hereditary risk factors in addition to a family history of CRC that have been shown to enhance the risk: familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) also known as Lynch Syndrome.[22-23, 32] People with these conditions not only have a greater chance of development over the course of their life, but tend to develop CRC at a much younger age than the normal patient. HNPCC develops from an inherited set of DNA mismatch repair genes, and FAP from mutation in the adenomatous polyposis coli gene (APC).[33-34] Respectively, the age of onset for each is near 45 years old compared to teenage years/young adult life.

In addition to mutations in genes that cause a direct risk for CRC, there are other health conditions that elevate the risk of development. Diabetic patients with Type 2 diabetes are at around a 30% elevated risk, which in addition to the prevalence of obesity in Type 2 diabetics contributes to malignant colorectal neoplasia.[35-37] Those with either form of Inflammatory

Bowel Disease (IBD), such as Ulcerative Colitis (UC) or Chrohn's Disease are at approximately double the risk of developing CRC compared to the average person. Any person with one of these pre-existing medical conditions should consult with their doctor about screening for CRC at an earlier age.

There have been several studies performed by government agencies that indicate socioeconomic and ethnic implications in CRC development.[1, 38-39] According to the American Cancer Society, non-hispanic blacks (NHB) are about 20% more likely to develop CRC and nearly 40% higher to die as a result of CRC compared to non-hispanic whites (NHW).[20-21, 38-39] There are a variety of reasons that are believe to attribute to this. Data from the United States Census Bureau on Income and Poverty in the United States from 2002 – 2017 show that the percentage of NHB earning $15,000.00 or less is about double that of NHW, as well as the percentage of NHB living in poverty during 2016 – 2017 being double that of NHW. Access to insurance and reasonable healthcare is an important step in being eligible to afford screening and preventative measures for CRC, and without those initiatives efforts to combat CRC statistics regress several decades.

Due to the generally slow growth of benign colorectal polyps to develop into a larger malignant polyps, there is ample opportunity for those with any of the risk factors for CRC to take action and seek care. There are both visual examinations as well as stool tests that can indicate to healthcare providers whether you are positive or at increased risk of developing CRC.[40-42] Many of these tests, such as fecal immunochemical tests (FIT) or fecal occult blood tests (FOBT) can be performed in the comfort of your home and are covered by Medicare. Other

more invasive visual examinations, which can be performed in a physician's office, are also covered by most insurances and are more accurate in determining malignant polyps. Following the recommended guidelines for age when screening is appropriate can reach a tumor before it has the opportunity to grow and invade other tissues, prolonging both quality and length of life for patients. Findings from the New England Journal of Medicine suggest that removing adenomatous polyps by colonoscopic methods prematurely can reduce mortality from CRC by about 53%.[43] With more extensive and sophisticated detection methods available at affordable prices, there is no reason why further steps cannot be taken to increase the percentage of individuals screened and continue to lower the incidence rates of CRC.

2. Pathology of Colorectal Cancer

MOLECULAR PATHOLOGY

Due to the increasingly affordable and convenient methods of DNA sequencing and analysis, the last several decades have expanded to healthcare providers the opportunity to characterize CRC not only in the traditional surgical and histopathological manners, but also with mutational status and genomic instability.[44-46] There are several genes that are linked to the development and progression of CRC, divided into oncogenes, proto-oncogenes, or tumor suppressor genes.[47-49] Any form of genetic mutation or epigenetic mutation to these regulators will produce either gain of function or loss of function changes that have a physiological impact on the progression of this heterogeneous disease. Although there are a number of complex changes that occur at the molecular and genetic level to induce tumorigenesis of CRC, this

section will focus in broad terms on the well-known genetic mutations that are related to CRC development and how they induce the disease.[50-51] A more detailed examination of all these changes and more will be covered in Section 1.d. (Consensus Molecular Subtypes).

CRC is a gastrointestinal cancer with a varied and complex pathology dependent on a composition of factors that ultimately lead to tumorigenic development. Although there are many potential drivers of this cancer, several hypothesis driven mutations have been well studied in CRC development and found in a large percentage of patients with CRC.[52] All of these gene mutations are somatic, inherited through passing of familial genes. This however does not categorize these sporadic CRCs as hereditary forms of CRC (LS aka HNPCC, FAP, PJS, etc.).[40, 49] The genetic mutations of CRC can be divided into separate pathways that group their mechanism, such as those of genomic instability, inactivation of tumor suppressor genes, activation of oncogenic pathways, and growth factor pathways.

Examples of somatic mutations that effect genetic stability are those mutations that repress the actions of normal DNA mismatch repair (MMR) machinery.[53-54] Normal cell function has a variety of proteins that assist when DNA base pairs are either damaged or not appropriately matched to their corresponding nucleotide base, as well as insertions and deletions. The process is highly conserved in eukaryotes, with dysregulation of these repair genes occurring in approximately 15% of patients with non-familiar CRC and over 50% of patients with hereditary LS (HNPCC).[55] The genes responsible for MMR detected in the majority of these genomic instable mutations include MLH1, MSH2, and MUTYH, and are associated with an accelerated pace of development in malignant CRC along the adenoma-carcinoma sequence.[56-57]

DNA MMR mutations are commonly associated with hypermethylation of promoter sequences on DNA that silence the transcription of those MMR machinery epigenetically. The saw-tooth like serrated sessile polyps previously discussed generally have silence repair mechanisms that lead to fold-more mutations which heighten malignancy.[56, 58] Using immunohistochemistry staining researchers can identify loss of any number of these proteins. By location, the prevalence of MMR mutations are commonly found in those malignant polyps in the proximal colon, and have a greater tendency to be found in an older female patient.[59-61]

Adenocarcinomas are the most prevalent form of CRC, forming from mutations in the adenomatous tissues that act as intestinal glandular cells. The genetic mutation that is most frequently seen in adenomatous CRC is that of tumor suppressor adenomatous polyposis coli (APC).[51-52, 62] This gene is responsible for regulation of cell growth, and dysregulation allows for rapid and unregulated growth of these glandular cells in the large intestine to form adenomas. APC is part of the β-catenin degradation complex, whose function is to control levels of the Wnt-signaling protein β-catenin.[63-64] When this APC gene is mutated and the APC protein is not transcribed, β-catenin is not repressed and there is a constitutive overexpression of the Wnt pathway that initiates nuclear transcription of cellular activation and induces this malignant growth profile.[65-67] However, generally the mutation of APC is only part of the requirement for tumorigenesis and another "insult" is required for full development. This is why patients with FAP that have a significant predisposition to this mutation should be prepared for surgical removal of both adenomatous polyps and sometimes resections of the entire large intestine.[68-69]

TP53, tumor protein 53, is a gene that transcribes a 53 kDa protein p53 that acts as a tumor suppressor.[50, 70] P53 is responsible for the regulation of the cell cycle arrest and consequently cellular growth, and when mutations to TP53 occur cells can rapidly divide. This is usually the second and subsequent factor in mutation of APC that leads to the development of CRC, and the two are largely seen together in the course of molecular pathology for this disease.[32, 34, 49]

A third step in early development in the adenoma-carcinoma sequence is the mutation and inactivation of the transforming growth factor β pathway (TGF-β).[45, 71] Approximately 30% of somatic mutations in CRC have inactivation of TGFBR2, a serine/threonine protein kinase, caused by frameshift mutations induced by MMR deficiency.[57, 72] PTEN is an additional tumor suppressor protein that can act as a third step in development, and acts to repress the PI3K family of tumor oncogenes that contribute to CRC development.[69, 73] Other oncogenes, like BRAF and KRAS induce pro-cancer signaling cascades at constitutive levels in CRC, and many patients also show expression of them as putative biomarkers for prognosis and treatment approach.[74] There is significant cross-talk and cross-signaling the further in development a colorectal malignancy proceeds, often complicating the understanding of tumor initiation. However, the more genetic markers available the more knowledge is gained by physicians in determining what therapeutic approaches will work and be best tolerated by the patient to repress their cancer. The observations made in the 21st century by researchers and physicians regarding the observable phenotypic differences in colon cancer established a precedent, by which further validation of the subtypes of the disease could establish more selective and sensitive treatments targeting specific factors in tumor initiation and progression. The following section will elaborate on the evidence

from pathological data gathered to refine the heterogeneous CRC and stratify it into molecular subtypes.

b. Consensus Molecular Subtypes

Since the late 2000's there has been increasing focus on the advancement of early diagnostic and prognostic markers for CRC. Analysis in the clinic have attempted to progress passed the traditional TNM staging methods and histopathological imaging to better understand how the molecular markers of CRC pathology can assist with more accurate prognosis and response to treatment among patients with various types of CRC. From those years until now, there have been considerable refinement in basic tumor biology and pharmacology for CRC.[47, 71, 75] Despite advances in target-based therapies, there is still somewhat limited information to clinically validate molecular markers for treatment selection.[76-77] The use of consensus molecular subtypes (CMS) increasingly is used to refine the understanding of how a patient will respond to therapy, and steps that can be taken to potentially increase disease-free survival.[18, 45] Over the years, the pieces of this puzzle have continued to come together to create a more firm grasp on the overall picture of CRC tumorigenesis and development for the benefit of the patient. It is hopeful that these markers, from morphology to genetic and epigenetic factors to protein expression, can aid in the development of more personalized treatments that improve the standards of care for CRC.

i. Molecular Phenotypes of CRC

Early evaluations of the genetic characteristics of CRC showed strong correlation with the disease and specific mutations in either tumor suppressor proteins or oncogenes that enhance tumorigenesis. Mutations were commonly seen in tumor suppressor adenomatous polyposis coli (APC), where adenomatous polyps develop from. This protein is observed in over 90% of CRC patients, and plays an active role in regulation of cell cycle.[45, 50-51] When somatic mutations occur in APC, an additional protein β-catenin (key downstream signal in the Wnt-signaling pathway) is not degraded. β-catenin forms a complex with Wnt protein and leads to transcription of growth factors and proteins responsible for anchorage-dependent cell adhesion, among other anabolic biological functions.[63, 78-80] Mutated APC is not able to quench cellular growth and results in the uncontrolled proliferation of the glandular cells that give rise to adenomas in the intestine. Although this mutation served as a consistent marker in most cases of CRC, the clinical significance in terms of treatment response was lacking during initial research.

An additional protein mutation common in ~ 30% of patients is that of oncogene KRAS.[59, 81-82] KRAS acts as a signaling switch in cell growth and signaling, and when mutations occur on chromosome 12 where it is located, generally there is a loss of negative regulation leading to proliferative growth and cancer development. This is viewed as a secondary mutation following APC/Wnt signaling that proceeds in the adenoma-carcinoma sequence to the development of more intermediate adenomas. Mutations of KRAS reduce the ability of GTPase proteins to inactivate hydrolysis of GTP to GDP, further compromising signaling pathways important in regulation of cellular growth.[83-84] KRAS status has entered the clinic recently in staging protocol for physicians as a predictive biomarker for CRC and a deciding factor for

treatment.[2] Patients with mutated KRAS shows a remarkable lack of response compared to wild-type (wt) KRAS to both small molecule and monoclonal antibody anti-epidermal growth factor receptor (anti-EGFR) therapy.[74, 85-86] About 99% of patients with this mutation do not response well or at all to this treatment, supported by multiple clinical trial evaluations in the late 2000's. This marker has served conclusively to direct physician's choice of treatment in the clinic and continues to be validated.

Other mutations see commonly in CRC include those of tumor suppressor gene TP53, which encodes for protein p53 that is a regulator of DNA damage and repair in addition to acting as a checkpoint inhibitor to limit proliferative growth. SMAD4, an essential transcription factor and regulator in the TGF-β signaling pathway, is often mutated in CRC resulting in loss of regulation in epithelial cell growth and increased risk of tumor formation.[87-88] On the contrary, mutations in proto-oncogenes like those of signal transduction regulator protein BRAF, will also induce a molecular predisposition for malignant polyp formation. In CRC, the V600E missense mutation of BRAF is shown to enhance the tumorigenic signaling of various oncogenic growth factors and increase the physical size of adenomas.[33, 50, 59] These among others are the variety of somatic mutations that are observed in sporadic CRC, contributing to the initiation and development of the heterogeneous disease state

Fingerprinting of the genes of tumors from CRC have created another class of genetic alterations responsible for some of the observed somatic mutations previously discussed.[58] One of the most studied genotypes of CRC is that of the deficient mismatch repair (MMR) machinery found in approximately 15% of all patients.[58, 89] This subgroup of patients has mutations or

epigenetic silencing of the proteins normally responsible for the correction of frameshift mutations in the genome. MMR proteins, such as MLH1 and MSH2, counter errors in DNA replication in a sequential process prior cell division. There is a strong relationship between CpG island methylator phenotype (CIMP) and deficient MMR, where enhanced methylation of promoter sequences for these repair proteins cause a silencing event that decreases the transcription of the repair machinery.[50, 90-91] When they are not transcribed, they cannot respond to mutations and are among the mutator phenotype that is responsible for the permanent and tumorigenic alterations of tumor suppressor genes and upregulation of oncogenes.

The molecular classification of tumors can be segmented on the status of changes to the short and repetitive sequences of nucleic bases called microsatellites. Tumors can be defined as microsatellite instable (MSI) if they show instability in 40% or more of microsatellite marker regions, and microsatellite stable (MSS) if none of the markers show instability.[90, 92] Further, MSI tumor profiles can either be MSI-hi or MSI-lo, characterizing the frequency of instability in the regions. Deficiencies in MMR machinery can lead to the development of MSI CRC. It has been shown in Stage 2 patients with CRC that MSI is a marker indicating poor response to flouropyrimidine drug therapy. Patients that show MSI have a better response to surgery alone, and tend to also have a more positive prognosis. In contrast, patients with MSS show a poor prognosis comparatively but have tumors that do respond to flouropyrimidine therapy.[57, 93-94]

The other 85% of patients with sporadic CRC who lack the MSI that results from deficient MMR have a different form of genetic instability known as chromosomal instability (CIN).[17, 71, 83] This is featured by the amplification or translocation and thereof loss of the entire

function of the chromosome. Individuals with CIN also tend to have MSS, and generally have a poor prognosis. The wide variety of alterations at the level of genomic stability that then affect the appropriate transcription of genes that regulate cellular growth not only have improved the understanding of how malignant polyps form in CRC, but also improve the classification of this complex disease to redefine how physicians can improve the prognosis for patients with more advanced and aggressive stages. The following section will discuss the aggregation of data from all of these classifications over several years has led to the stratification of CRC into categorical subtypes.

ii. Stratification of CRC based on Multi-omics Analysis

In the last two decades large amount of data have been gathered from clinical research efforts to group CRC according to mutational status, genomic instabilities, morphology, prognosis, and response to treatment. The response to this work has been meta-scale multi-omics analysis of CRC, trying to develop trends and correlations that would improve the clinical response to this malignancy that for many years had been thought of as homogenous. Originally, CRC was divided into groups based on genomic stability, including MSI (MSI-H/MSI-L), MSS, CIN, CIMP. It was discovered that tumors with tumors with deficient MMR were MSI-H, poorly differentiated, found in the proximal side of the colon, and were clinically distinct from those having MSS. The identification of mutations that correlated with these genomic events further elucidated the pathology of CRC.

The addition of gene expression based profiling in conjunction with genomic instability produced incomplete subtypes that attempted to characterize CRC. Recently, there has been

great efforts to define these molecular subtypes, and understand the associations of each with patient survival. Although there are several stratifications that are proposed to stratify the disease, there is a general consensus that each characterizes CRC into robust and clinically relevant molecular subtypes. Many of these studies were performed in-vitro using patient derived colorectal cancer cell lines, which have been shown previously to recapitulate the main subtypes of CRC at the genomic levels in the clinic. A consortium of scientists published recently the interconnectivity between the subtype classification systems of independent research bodies, concluding on expression of 4 consensus molecular subtypes of CRC. Further studies showed the multi-level data integration from those findings through DNA, RNA, and protein profiling to enhance the use of CRC as a supported preclinical model system for drug discovery efforts in cancer. Shown below in Figure 2 are the findings from these studies.[18, 45]

CMS1 MSI immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Figure 2. The representation of CRC based on 4 consensus molecular subtypes characterizing various biological differences. These phenotypic variances include microsatellite instability (MSI), Cytosine-p-Guanosine island methylator phenotype (CIMP), somatic copy number alterations (SCNA), mutations in BRAF, KRAS, and TGF-β intracellular signaling proteins, as well as mutations to the genes for Wnt and Myc signaling pathways.

The molecular subtypes defined were stratified into the following: CMS 1 with high levels of MSI and immune activation, CMS 2 showing traditional adenoma-carcinoma signaling pathway elevation, CMS 3 with high metabolic dysregulation and KRAS mutational status, and CMS 4 mesenchymal growth profile and worse overall survival. Cell lines can be profiled during screening to better explain any pharmacological response to treatments, and potentially improve treatment decisions in therapy. From the data published in 2015 in *Nature*, researchers in Norway were able to do just that using the consensus molecular subtype system.[18-19]

Using integrated data analysis 34 colorectal cancer cell lines derived from patients were profiled with targeted deep sequencing of the kinome and other relevant oncogenes, DNA copy number profiles, principle component analysis of gene microarrays and reverse phase protein microarrays. Findings from this work, shown in Figure 3, identified potential target genes for each genomic instability profile and attempted to pinpoint factors important for the distinction of each molecular subtype.[19]

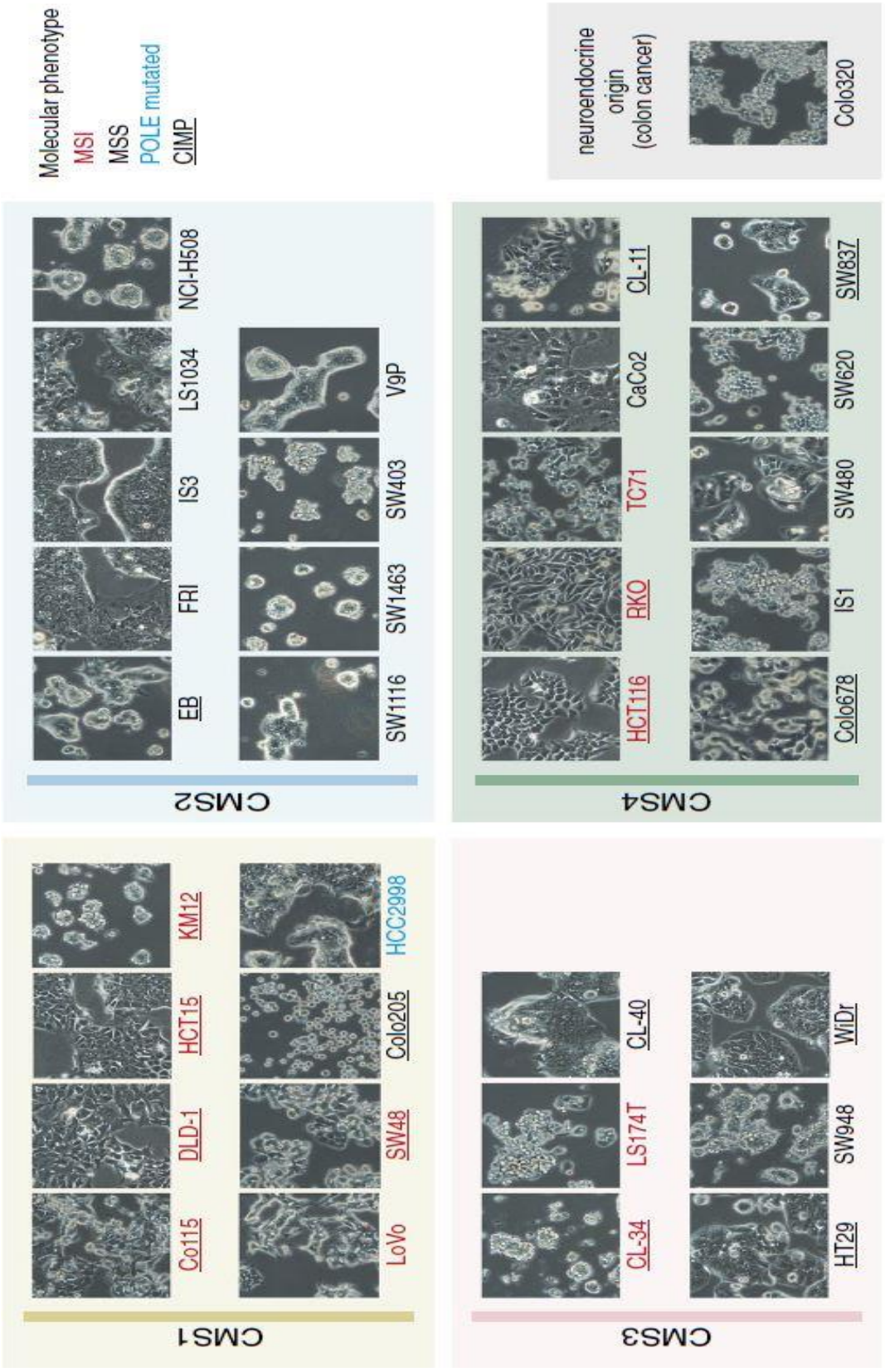


Figure 3. The stratification of 34 patient derived colorectal cancer cell lines by their subtype consensus molecular subtype.

This work continues to be useful in the preclinical and clinical settings, where researchers are able to identify promising agents for anti-cancer therapy and better characterize their mechanism of action and therapeutic potential.

c. Anti-cancer Therapies

Over the last few decades, we have seen vast improvements in the methods used by physicians to treat CRC. Not only have the detection and imaging improved the staging and characterization of tumor to guide therapy, but the tools and training for colorectal surgeons has also improved. Therapies to target CRC have shifted from cytotoxic drugs to more direct and targeted therapies that dramatically improve the life expectancy of a patient as well as their quality of life.[5, 75, 95] As we continue to develop improved therapy that focuses on specific disease initiating agents, the tumor microenvironment, and the body's own immune system to aid in both protection and defense, the classical approaches to treatment of cancer remain foundational to treatment. As we develop a better understanding of drug mechanisms and tumor cell biology, our knowledge about cancer development and progression will expand the modalities of treatment and further improve treatment of CRC. The following discussion will focus on the main modes of treatment in CRC, and briefly discuss how they are used and when.

Common Approaches to Treat CRC

i. Modalities

The development of CRC is a progressive process, where a benign adenomatous polyp will grow over the course of a decade or more to become a large and malignant colorectal tumor.[40, 96] Depending on the initial staging of a tumorous polyp, the medical team attending will need to discuss the best plan and approach for treatment. This will not only depend on the stage of the tumor (TNM or UICC), but also the histology and morphology, the exact location where initial tumor resides, and the molecular profile of pathology in relation to certain genetic mutations and chromosomal stability. Generally speaking, there exists three different modalities in treating CRC, including surgery, chemotherapy, and radiation therapy, and hormone therapy. Each one has a specific and intended use, either to treat tumors at the primary site or abroad, or to simply provide palliative care to improve the lifespan of the patient.

Surgery is routine and typically performed for all early stage CRC (Stages 1-3), and also in later stage CRC where previous therapy has reduced the size of intestinal and nodal tumors.[43, 97-99] The success of surgery in treatment largely depends on the size of the tumors, and the step in development it exists when caught. Tumors restricted to the primary site or locally generally have a higher rate of success, while those that are in advanced stage CRC have significant rates of relapse and recurrence with aggressive qualities after surgery.[100] Patients with hereditary conditions like Familial Adenomatous Polyposis (FAP), who have many polyps, may require resection of the entire colon and colostomy depending on the status and location of the tumor (Figure 4).[69]

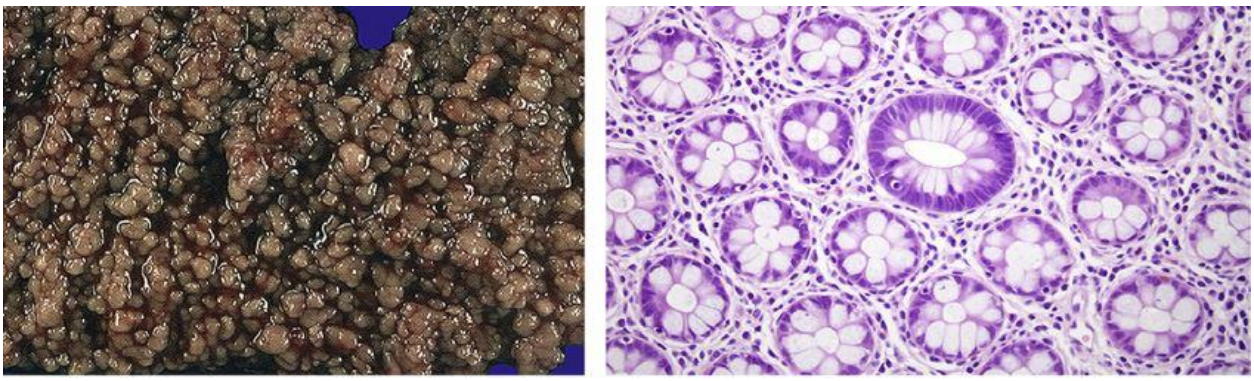


Figure 4. Familial adenomatous polyposis macro-(left) and microscopic sectioning (right), highlighting the large quantity of colorectal polyps associated with the disease. Patients with FAP often required entire resection of the colon to prevent malignant formations.

Polypectomy is the surgical removal of one or more polyps, and should be performed on any polyp that warrants.[43, 101-102] This often requires thorough review by both a pathologist and colorectal surgeon to develop a fitting approach and plan for the operation. Using the wiring of the colonoscope which an electric current can pass through, the surgeon will lasso the polyp and excise it from the intestinal wall. There are specific criteria for which this removal is performed, determining the distance of surrounding tissue that should also be removed to prevent relapse. The procedure is generally easier to perform in the rectum, sigmoidal and distal portion of the colon as it is more easily accessible through the anus. Proximal colon polyps are more difficult to view in both colonoscopy and laparoscopy, and as a result surgery in this region is often less successful in removing all of the cancerous tissue. Risk factors for surgical resection including involvement of the submucosa and morphology of the polyp (pedunculated or sessile) can be used as predictive measures for adverse outcome following surgery.[6, 30, 103]

A colectomy is a more advanced procedure that requires resection of the entire large intestine. This may be due to the extent to which polyps occupy the intestinal wall, or the aggressive qualities of several polyps over a broad course of tissue in the colon.[104-105] This operation is usually performed for patients with hereditary CRC that has a predisposition to develop into an aggressive and metastatic cancer.[40, 69, 96] Often a colostomy or other form of stoma is utilized to create an alternative exit for waste product from the bowels. This like any surgical procedure has the possibility for adverse events outside the relapse of tumor to occur. This can include bacterial infections, poor physical recovery, prolapse of other internal organs, development of a hernia, and others.[105] Regardless, early stage CRC combined with enhanced imaging, staging and surgical procedures have shown to dramatically reduce the mortality of the disease and improve the disease-free recovery of patients. Surgical methods for CRC will remain a golden standard of therapy, with improvements in methods and enhancement of techniques by more experienced colorectal surgeons improving both quality of life and life-expectancy.

In addition to surgery, radiation therapy is also a common and traditional form of therapy for many cancers.[106-108] Although it is rarely used in the treatment of colon cancer, it is used with some frequency in the treatment of rectal cancer as an adjuvant therapy along with chemotherapy or as a neoadjuvant agent to assist in the reduction of tumors.[3, 109-110] In some cases, external beam radiation therapy is used to treat advanced metastatic colon cancer where surgery is not feasible, and is given as a neoadjuvant therapy to increase the therapeutic potential of chemotherapy agents. This tactic is used to achieve optimal symptom control in patients with dire symptoms, with a variety of local ablation and embolization options for the radiotherapist to

employ (precision stereotactic body radiation, HDR-brachytherapy, selective internal radiation, etc.). Patients with liver metastasis may benefit from an improvement in tumors size when radiotherapy is used adjuvant to chemotherapy.[111] The results of many studies evaluating radiation therapy are examined with great scrutiny across the field, and many feel that the negative side effects of this form of therapy outweigh the benefits. The effects of radiation over time build up in a patient to give GI problems, fatigue, and inflammation that may all be chronic or even further develop other forms of cancer over time.

Chemotherapy is a more common and pronounced modality for treatment of colorectal cancer, and is used in both colon and rectal cancer at most stages. Generally not used but for in regional and distant CRC, it is sometimes used in early stage CRC that shows aggressive qualities with invasive potential and poor prognostic features.[5, 112-113] Neo-operative chemotherapy is used in an attempt to reduce the size of polyps prior to surgery, with the hopes of improving the success of disease-free surgical procedure. Early chemotherapeutic agents used in clinic were cytotoxic agents that interfered with cellular replication and DNA synthesis. These agents were termed “kinetic poisons”, due to the tactic of targeting the proliferative cellular growth seen commonly in cancer.[112-113] As a result, other fast growing cells in the body, such as those in the GI system, blood, and skin, are also targeting and killed. This leads to a variety of side effects that are manifested as the cancer is being treated.

Regardless of the negative side effects of cytotoxic chemotherapies, they continue to be used today in the clinic due their effectiveness at reducing tumor volume and improving the metastatic profile of patients with CRC. Additionally, cytotoxic therapies are not the only

chemotherapies for use in patients. Chemotherapy can further be divided into targeted-therapies, which are directed at specific proteins and growth factors that are found to be involved in the mechanism of disease for CRC and cancer in general (EGFR, VEGF).[5, 12, 75, 86] These targeted therapies can further be divided into their patient response based on the molecular phenotype specific to the patient, with different mutational status directing the efficacy of a particular therapy in the patient (Figure 5).[114] Because of their specificity, they tend to have fewer and less severe side effects compared with cytotoxic drugs. These targeted agents are both small molecules and biologics (recombinant proteins and monoclonal antibodies), some of them being top grossing FDA-approved agents for oncology. Commonly, chemotherapeutic regimens are established with multi-modal therapy that do not simply use one anti-cancer drug but are rather comprised of multiple drugs. Several studies over the course of decades have shown that drugs in combination show better patient response when compared to monotherapy, and this has become a standard of care for CRC patients.[81, 115-117] In the next section we will focus on chemotherapeutic regimens for the treatment of CRC and the process of selecting therapies for patients.

ii. **Common Chemotherapy Drugs/Regimens Used in the Clinic**

The use of chemotherapeutic regimens in CRC is generally used only for late stage cancer that shows an aggressive, invasive, or metastatic nature. For patients that have regional lymph node involvement or distant organs being impacted by the malignancy, or even in patients that have localized neoplasia that show of potential for poor recovery with surgery alone, there are a variety of chemotherapeutic agents that are routinely used by a colorectal oncologist. The discovery and development of new therapeutic markers as well as the stratification of CRC into

clinically relevant molecular subtypes offers a refined perspective when determining what treatment will and will not work for a given patient.

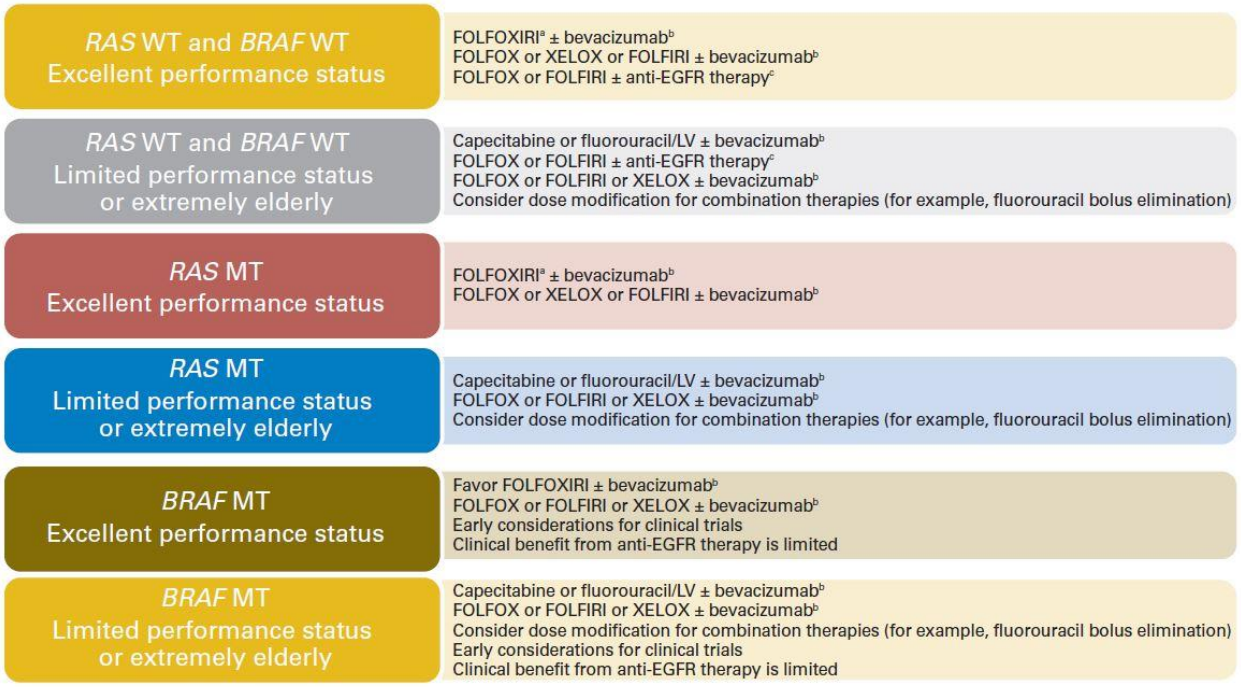


Figure 5. Stratification of chemotherapy regimens based on molecular phenotypes.

Traditionally and continually, cytotoxic chemotherapy continues to be a mainstay in the therapeutic regimen. Pyrimidine analogs such as 5-fluorouracil (5-FU) and platinum derivatives such as oxaliplatin (OX) are some of the most prescribed compounds for the treatment of CRC despite the toxic side effects of the molecules (Figure 6).[100, 113, 118] Mechanistically, 5-FU blocks the action of an enzyme responsible for synthesizing thymidine, causing the cell unable to replicate the DNA it needs to divide and undergo a thymine-less death via apoptosis. OX is also an agent that prevents cellular division via interruption of DNA replication, acting as an alkylating agent that cross links to DNA strands and induces apoptosis.[119-120] The two agents are

typically concomitant in the clinical setting acting as a combination therapy as 5-FUOX. This is one of the most commonly employed adjuvant chemotherapies, and has shown improved toxicity to cancer cells when given with folate analog leucovorin (stabilizes the enzyme that synthesizes thymidine, Figure 6). The combination of the 3 agents together is referred to as FOLFOX, and shows improved progression-free survival in patients.[118, 121]

An additional small molecule used frequently in CRC is the compound irinotecan (Figure 6), a topoisomerase 1 inhibitor that inhibits DNA replication and cell division. Irinotecan can also be given as a combination therapy with other agents such as in FOLFIRI (5-FU, leucovorin, and irinotecan) or as FOLFOX (5-FU, leucovorin, and oxaliplatin).[81, 122] Capecitabine, a prodrug of FU, is rapidly metabolized in the body to act as its active pyrimidine analog and used for CRC as well as other forms of cancer. In addition to compounds working as doublets to reduce tumor development, triplets can be used for sometimes improved performance as in FOLFOXIRI (FU, leucovorin, oxaliplatin, and irinotecan).[112] Depending on the patient response, one of these strategies may be more effective than another. Despite the positive attributes these chemotherapies have on cancer cell death, their nature as kinetic poisons have their fair share of negative side effects and symptoms.

Targeting the fastest growing cells in the body, all of the above mentioned compounds also destroy normal cells in the GI tract as well as epithelial cells on skin and in the scalp. This leaves patients feeling nauseous frequently on the medicine and causing significant hair loss, a defeating and humbling consequence of therapy. Patients will have difficulty in healing cuts, experience loss of appetite, and frequently feel fatigued. As a result of the negative side effects

of these more traditional chemotherapies, there have been extensive efforts in the 21st century attempting to shift focus in the clinic to targeted therapy that direct their action on specific factors influencing tumorigenesis.

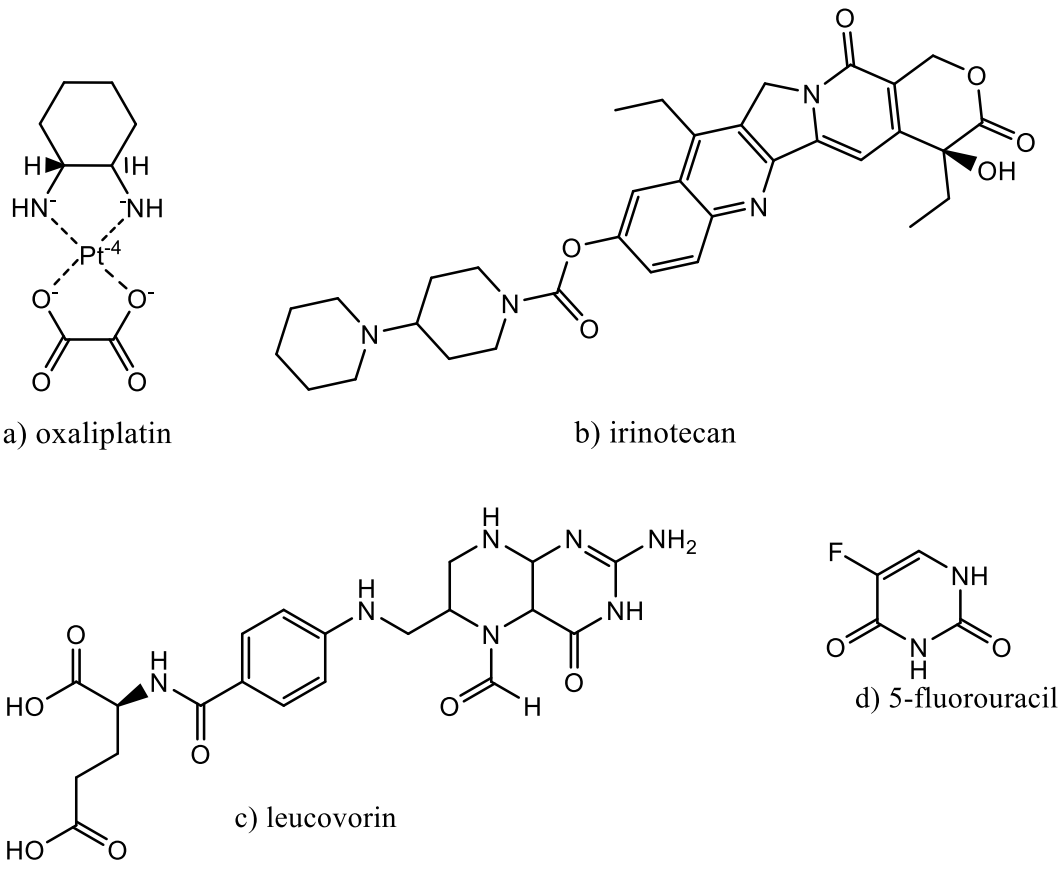


Figure 6. Structures of some common chemotherapeutic agents used in the treatment of colorectal cancer, often in multi-drug combination therapy regimens.

Most of these targeted therapies are biologics, such as monoclonal antibodies (mAb) or recombinant proteins. They can be divided into their mechanism of action and their targets, for CRC so far being either vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR).[16, 86] Bevacizumab is a first in line mAb targeting a particular isoform VEGF-

A, and currently grosses more than any other CRC therapy as Genentech's *Avastin* with nearly 7 billion USD annually.[121, 123] VEGF is an important growth factor for cancer cells in regard to both angiogenesis and metastasis. By inhibiting this growth factor, bevacizumab cuts off the supply of both blood and nutrients toward the tumor as well as inhibits formation of new vasculature. Cetuximab and panitumumab are both EGFR targeting mAbs, and are effective at targeting the PI3K and Ras-mutated pathways that are upregulated in CRC.[84, 113, 124] Recombinant protein aflibercept also targets VEGF, however has effects on both isoforms A and B as well as other growth factors.

The biological therapies used in the clinic for CRC provide additional improvements in therapy that prolong the life of patients, in some instances by doubling that survival time. They are often used as either first in line or secondary treatments alongside the previously mentioned chemotherapies. Cetuximab has been shown to improve progression free survival alongside FOLFIRI in patients with KRAS wt, as well as bevacizumab showing improvements in overall survival and progression free survival alongside FOLFOX or FOLFIRI in patients with KRAS mutant or proximal KRAS wt tumors.[74, 125] The molecular characterization of CRC aided early on in the evaluation of anti-EGFR inhibitors like cetuximab not being effective in mutant KRAS, who instead are treated with anti-VEGF compounds.

Despite the enhanced survival of patients undergoing treatment with targeted therapies, the most promising responses are shown when used together with traditional chemotherapy. This still gives rise to the negative side effects that degrade the patient experience, and the profound costs of combination/targeted treatments leaves an overwhelming financial burden and

stress to both the patient and their family. Additionally, target-directed therapy in CRC has shown only to improve the overall and progression free survival by maximally double the time compared to control.[95, 114] This may be only a period of 2 years with advanced or metastatic disease, and is only delaying the inevitable. It is important to consider that the tumor has a complex microenvironment composed of many different forms of cells, some of which will be able to rejuvenate the state of a tumor after some time even with treatment from these agents. The following section will take a look at the controversial yet supported theory of stem-like cancer cells and their effects on tumorigenesis.

d. Cancer Stem Cell Paradigm

The research community has taken steps in recent years to focus on refining the basic principles of tumor cell biology to gain a more refined understanding of initiation, development, and progression. In this work, there has been major breakthroughs in defining the molecular pathology of tumorigenesis and the complexity of the malignant disease state. Perhaps the most fundamental was the shift in perspective of the microenvironment of the tumor from a simple stochastic model of a relatively homogenous set of cells that undergo various sequential mutations to produce a tumor, to the more hierarchical model that explains origin in a heterogeneic tumor microenvironment with tumor initiating cells.[9, 126] The latter, although still largely misunderstood, has shown promise in the last decade with the identification of such tumor initiating cells in a variety of cancer tissues.[10, 95] The next few sections will attempt to explain the notion of cancer stem cells in the tumor microenvironment, their purpose, and the role they may play in the clinic to improve upon efforts of disease-free survival as we progress to develop personalized therapy for patients.

i. The Tumor Microenvironment

Early "reductionist" views of tumor biology laid aim on the concept that tumor cells were deranged cells arising from normally functioning cells that had mutational drivers leading them to a state of uncontrolled growth and proliferation. They were evasive and resistant to therapy, extremely motile and had upregulation in various signaling pathways as a result of their mutations.[126] Although these concepts at large still hold true today, there is a much more complex system of cells that are functioning in fluid states of functionality in a tumor than were expected even 15 years ago. Traditional views of the cell were that of relative homogenous mutant cell populations that could be targeted by addressing the specific mutation initiating their existence.[127] Over the last few decades our views have shifted to a heterotypic biological basis with a collaboration of various tumor initiating cells, primary cancer cells, poly-clonal daughter cells, and more functioning with plasticity in a microheterogeneic tumor microenvironment (Figure 7).[9] This tumor environment various from patient to patient, tissue to tissue, and even tumor to tumor intra-patient.

There are varying degrees of opinion on the roles of different micro-regions and their function in relation to tumorigenesis. Many of the emerging concepts of both cellular and extracellular factors are still not well understood, and the relationships between them requires an enhanced understanding of this systemic and communal effort. For example, the role of the microbiota in tumor progression and development for gastrointestinal cancers like CRC is still unknown, although there is significant evidence in support of regulation in these diseases.[128-129] What is known, however, is that a great deal of plasticity and adaptation takes place with various sensory mechanisms that respond to direct environmental factors.

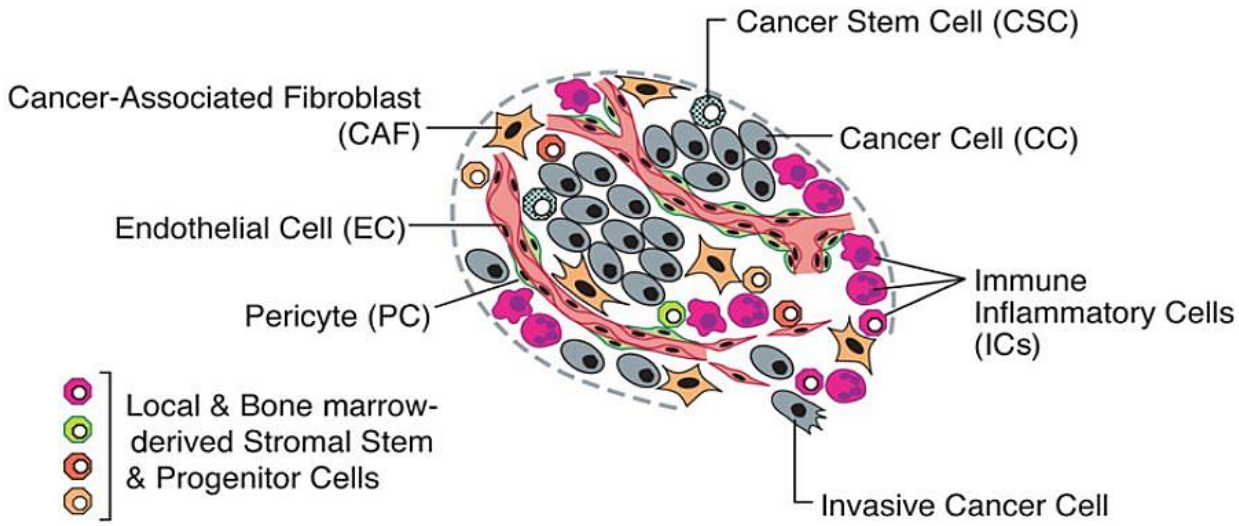


Figure 7. Model of the heterogeneic tumor cell model with various interactions.

There are a variety of paracrine and endocrine signaling events that occur in the tumor environment that are mediated by seemingly non-participating cellular members in the tumor and surrounding tissue. Cancer-activated fibroblasts (CAFs) assist to remodel the extracellular matrix, which house infinite combinations of glycoproteins and glycolipids responsible for angiogenic signaling and pro-tumor inflammatory response to stimulate cancer cell proliferation.[130] Epithelial cells may be induced to undergo transition into mesenchymal cells from the tumor stroma to advance and metastasize into nearby tissue, invading cell walls and entering the lymphatic system and blood vessels.[131] Exosomes secreted from the tumor rich of tumorigenic proteins and signaling molecules influence nearby tissue to enhance tumorigenesis.[132] Various cytokine receptors such as CXCR4 expressed in elevated levels by CAFs enhance the epithelial-mesenchymal transition and promote metastasis.[133]

There are subsets of cells in the tumor environment that possess tumor initiating capabilities and are able to seed the growth of a tumor even after chemotherapy. The epithelial cells of a tumor function to aid in the development of new vasculature and enhance tumor angiogenesis. Various tumor inflammatory cells, which one may think should aid in the destruction of cancerous cells, aid the growth and proliferation of the tumor by releasing proangiogenic growth factors such as epidermal growth factor (EGF) and VEGF proteins.[114, 134] Given all of the functionally and structurally different cellular and non-cellular factors working in a communal effort to support the sustainment of a tumor, there are both many challenges and opportunities for researchers to evaluate which switches are key to regulation. Of them, the tumor initiating cells known as cancer stem cells pose as a promising candidate for therapeutic targeting, and have shed new light on the understanding of tumor progression in recent years.

ii. Evidence of Cancer Stem-like Cancer Cells

The idea of stem-like cancer cells (CSCs) originates much earlier than is commonly presented in literature of the topic in the 21st century. Early work by Hamburger and Salmon attempt to develop a protocol to enrich human tumor stem cells in a suspended matrix, where they note that the only real evidence of efficacy in anti-cancer therapy is whether compounds can effectively kill a target population of cells with colony forming capacity.[135-136] The idea that there was a subpopulation of tumor forming cells was not a new concept even 50 years ago, however it was the limitations of sorting, separating and identifying such populations that limited the understanding of these early developmental cancer cells. There were also differences in opinion as to whether these colony forming tumor cells arose from normal stem cells that underwent mutation to dysregulate the conventional development and become a cancer stem cell

with limited differentiated capacity, or whether many different cell types including epithelial cells underwent mutation and were transformed into a tumor initiating capacity.[11]

In the late 90's this conversation had new light shed on it with work performed to better understand the tumorigenesis of leukemia cells. Dick and Bonnet were able to separate various colonies of acute myeloid leukemia cells based on different cell-surface antigens and seed them into severe immune deficient mice.[11] What was discovered was that only a specific group of primitive cells, those high in CD34/CD38 antigens, were able to transform into leukemic tumors in vivo. Not only this, but that those very primitive cells shared many of the phenotypic characteristics of normal stem cells as well as those of stem cells in patient tumor populations. These cells showed great potential for self-renewal, and were markedly different from the non-tumorigenic qualities of the majority of other cancer cells with various antigenic phenotypes. This work prompted a seemingly practical method for isolation of "tumor initiating cells" in other forms of cancer to include solid tumors.[11]

Over the next decade, many different attempts were made to characterize and expand upon the work done in leukemia models, using the principles of normal stem cell biology to better understand the tumorigenic qualities of tumor initiating cells.[137-138] Epithelial breast cancer cells rich in CD44 and low in CD24 (cluster of differentiation proteins) showed the impressive ability to seed new tumors in-vivo using murine models in seeding densities as low as 200 cells.[139] Glioblastomas were shown to carry reduced cell populations elevated in antigen CD133 that not only initiated tumor proliferation but also produced daughter cells that were both neuronal and glial, indicating that these tumor initiating cells not only could selectively develop

a tumor in-vivo but produce differentiated progeny that lacked this ability.[140] Work performed in the intestinal epithelial cells located in the intestinal crypts showed an enhance motility and proliferative capability that was lost as the cells migrated from the crypt to the intestinal lumen.[141] This promoted the notion that the intestinal crypts provided a unique stem cell niche environment where tumor initiating cells such as colon cancer stem cells could be generated.

The model proposed for these CSCs is shown below in Figure 8. The main characteristics of tumor initiating cells is that of limitless self-renewal capacity, where a CSC is able to renew to other initiating tumor cells that share the same potency and are able to continue regeneration of the tumor.[137-138] This was observed in many of the above models in that xenografts harvested from the mice showed a population of cells with the same antigenic markers and profile of the tumor initiating cells seeded originally.[11] The second principle of these CSCs is that they confer the ability to differentiate into phenotypically different progeny with a limited potency. That the daughter cells of this population will go on to differentiate and specialize within the tumor cell population to enhance the development and sustaining properties of the malignancy.[137-138, 142] Additionally, it was proposed that tumor initiating CSCs would preferentially survive treatment by traditional chemotherapy and remain behind to reconstitute a tumor that otherwise would have seemed to regress with treatment.

Today the CSC paradigm is more generally accepted in the scientific community. Efforts have been underway since the early 2000's to identify signaling pathways that are unique to CSCs and can be used in therapeutic targeting of cancer to enhance the efficacy of traditional therapy.[143-144] The unique challenges with CSCs is, as observed in the previous text, the wide

variety of cell surface antigens used to distinguish tissue-specific colony forming cancer cells. This makes it difficult to categorize CSCs through use of common or universal markers. Additionally, basic understanding about the stem cell biology of tumor initiating CSCs is still not well understood. Despite this, continued advances are being made in the field to better isolate and characterize these cells from a tumor cell population for use in the clinic, where the benefits of a therapy targeting CSCs are apparent.

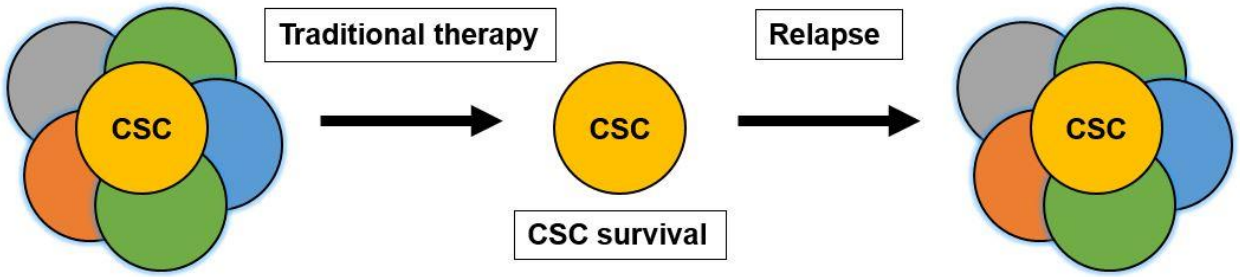


Figure 8. Basic proposed model of the CSC paradigm, in which a chemo-resistant subpopulation regenerates tumor growth following treatment.

iii. Clinical Implications of CSCs

The evolution of cancer therapy from traditional chemotherapies to target-based therapy has enhanced the overall survival and disease-free survival for many patients with a variety of cancers. These cancers span multiple tissues and numerous sites and in patients with a diverse staging.[145] All of these factors play a role in the efficacy of treatment and the expected outcomes and prognosis for a patient. Despite the advances in treatment, a large percentage of those diagnosed with cancer will relapse and die as a result of the disease. In CRC, that number is estimated to be near 20% for those after a curative surgical resection.[146] In more dire blood

cancers like lymphoma, patients with peripheral T-cell lymphoma may expect up to recurrence rates of up to 75%.[147] These unfortunate circumstances often cloud the many successes that have been made in treating cancers, however it also provides us an opportunity to reflect on why current treatments only marginally improve patient outcomes and what other paradigms are validated to focus efforts.

Many of the biologic compounds previously mentioned in the chemotherapeutic regimens used in clinic do in fact prolong survival in patients. However this may only be several months of time, where the disease still ultimately proves fatal. Immunotherapy and target-based biologic therapy are often used together with traditional chemotherapy to improve the patient response.[112, 148] These traditional therapies, such as those impairing the DNA replication of cancers cells and inducing apoptosis, are kinetic poisons that have serious clinical side effects. Because they target the rapidly dividing cells, they are also largely only targeting the cells that are mature and differentiated. These cells compose the bulk of a tumor, however are not responsible for initiating tumorigenesis. The result of this therapy is, after some time, the recurrence and relapse of the original tumor in some cases with a more aggressive nature that reconstitutes the disease in full.

The CSC paradigm or theory of tumor initiating cells proposes a unifying hypothesis to explain this tumor relapse, and expanded efforts in the last decade have attempted to define the mechanisms and signaling pathways that can serve as therapeutic targets implicating CSC development. Unfortunately, there is a complex network of intra- and intercellular signaling pathways and cross-talk between implicated factors that complicate mechanistic understandings

of CRCs.[149-150] The opportunity still awaits those who can uncover the secrets of these mechanisms the chance to reduce cancer mortality to a minimum if not completely. Either as part of an adjuvant therapy regimen or prior to traditional therapy, CSC-targeting compounds can destroy cells responsible for the self-renewal of tumor thereby depleting the survival of cancer after other treatments. This opportunity, although challenging, provides ample opportunity for researchers to improve patient therapy and reduce a global health burden.

e. Targeting Cancer Stem Cells

Efforts to understand how measures can be taken to target CSC populations in a tumor environment were initially elusive, as there was a lacking in the understanding of basic tumor cell biology and the involvement of stem cell biology to this system. There was inconclusive evidence as to how these subpopulations of cells could be isolated from the tumor for studying their characteristics, and a diverse range of cell surface markers complicate research attempting to identify tumor-specific populations. However, efforts to characterize the molecular subtypes of cancers improved the implication of CSCs in those pathways and provided key insight as to the involvement of epithelial mesenchymal transition, genomic instability, metabolic changes and signaling pathways that regulate CSC development and tumorigenesis. In the following sections, we will discuss the putative markers that have been accepted to indicate tumor initiating cells, the signaling pathways CSCs use to seed tumor development, and therapies in preclinical and clinical development attempting to specifically target this subtype of tumor cells in the hopes of improved therapy.

i. Putative Markers of CSCs

Characterization of CSCs was first initiated with the early studies on acute myeloid leukemia that first separated tumor initiating cells from other non-initiating cells in the blood via cell surface antigens.[11] Since then, many other cancerous tissues have separated and sorted cells with various cell surface antigenic markers that initiate tumor development in their own tissue. These initial studies found that only leukemia cells that were $CD34^{+}/CD38^{-}$ would initiate the tumor in-vivo.[11] CD34, a cell surface phosphoglycoprotein, indeed has been found on hematopoietic and early development epithelial stem like cells and has been shown to be required for activation of tumorigenesis.[151] Many of the other putative markers of a mesenchymal and stem-like tumor initiating cell are also cluster of differentiation proteins (CD, Table 1).[152] These glycans found as transmembrane and cell surface antigens play a large role in the glycocalyx for intracellular signaling and recognition, and many have been implicated in the development of tumors and CSCs.

Table 1. Putative CSC markers identified by cancer tissue, many of which are cluster of differentiation proteins (CD).

Tumor Type	Putative CSC Markers
Colon	CD133, CD44, CD166, EpCAM, CD24, CXCR4, CEA, LGR5, ALDH-1
Head and Neck	CD44, ALDH, YAP1, BMI-1
Leukemia	CD34, CD123, $CD38^{-}$, $CD90^{-}$
Breast	ESA, CD44, CD24, ALDH-1
Liver	CD133, CD49, CD90
Brain	CD133, BCRP1, A2B5, SSEA1
Lung	CD133, ABCG2
Prostate	CD44, CD133
Pancreatic	CD133, CD44, EpCAM, CD24, ABCG2

One of the most well cited markers for CSCs is the transmembrane glycoprotein CD133, which has been identified across many different tissues for its expression in tumor initiating cells.[153] Although the exact role of CD133 remains unclear, it has been of relative debate due to its findings expressed in normal stem cells and during pro-angiogenic cellular responses.[154] Still, for several tissue types it is regarded as a useful marker for isolation of these CSCs, to include colorectal cancers and other GI cancers. Colon cancer stem cells have quite a variety of markers characterizing them, as it is one of the most well studied in-vitro cancers for its use in preclinical model systems.[155] The remainder of this discussion will focus on those markers with highest degree of affiliation with stem-like properties of CRC tumor initiating cells.

Pluripotent transcriptional factors like OCT4, SOX2, and NANOG have been shown to indicate stem-like phenotype in a range of cancer tissues to include CRC.[95, 156] OCT4 is involved in early embryonic development and tumorigenesis, with elevated expression in the epithelial mesenchymal transition of colonic crypt, and has been found in the colonic epithelial cells of CRC tumor samples at enhanced levels.[157] In colorectal adenocarcinomic polyps, levels of SOX2 were shown to maintain a state of pluripotent undifferentiation in the crypt and be essential for CRC development from adenomas.[158] Transcriptional factor NANOG, which is regulated by levels of OCT4/SOX2, influences the pluripotent state of epithelial cells of CRC and is shown to correlate with a poor prognosis in the clinic.[155]

At the intestinal colonic crypt where the stem cell niche is found along with the previously mentioned markers, aldehyde dehydrogenase (ALDH) is also shown consistently in many of the crypt bases.[159] As the CRC is either treated with chemotherapy or progresses

through the adenoma-carcinoma sequence there has been shown an overexpression of ALDH-1 cells that are believed to be tumor initiating.[160] Cells with intense expression of ALDH-1 show enhanced capacity for self-renewal, resistance to treatment, and a poor clinical prognosis. In addition to the upregulation of ALDH-1 cells following treatment, several other markers such as CD166, CD44, and G-protein coupled receptor LGR5 are also elevated. LGR5 is unique among them, that it is not a cell surface glycoprotein, and that it is only expressed in the colonic base crypt cells.[161-162] Found in and around Paneth cells, which are key players in mucosal defense of the intestines, LGR5 cells have been implicated in stem cell maintenance and secretion of growth factors such as EGF and TGF-β. LGR5 knockdown cells have shown to cause tumor regression, with the opposite effects upon reinstatement of $LGR5^+$ cells.[162]

Other markers, such as transcriptional factor of proto-oncogenes c-Myc, are upregulated in the Wnt signaling pathway that is shown to be essential for the traditional adenoma-carcinoma sequence of cancer development.[163] c-Myc is involved in stem cell renewal and differentiation of normal stem cells, and may very well play the same role in tumor initiating CSCs. CD44, also elevated in Wnt signaling, is yet another cell surface glycoprotein overexpressed in the development of carcinomic polyps and functions under normal biological conditions as a migration and pluripotency. CD44 positive cells can replace the necessity of CD133 in CRC and are some of the more aggressive cells in tumorigenesis.[14, 160, 164] All of these markers serve to indicate cells requiring a lower seeding density to initiate in-vivo tumor development, many of which correspond with a particular signaling pathway that is enhanced or key to the development of cancer and specifically CRC. We will now examine those pathways that are alleged to signal tumorigenicity in CRC.

ii. Signaling and pathways for CSC Development and Regulation

The mechanisms for cell signaling in the development and regulation of cancer can be quite similar regardless of the tissue involved. As such, there are a great deal of pathways that have been exposed as significant to this end, largely due to the genomic profiling and overexpression of oncogenes that are found throughout many cancers. The following pathways have been implicated in the development of CRC with an emphasis on the role in regulation of CSCs and their maintenance.

One of the main drivers of intestinal cancer is the mutation of tumor suppressor APC previously discussed in the molecular phenotype of CRC. This mutation leads to unrepressed signaling of the Wnt protein that leads to unmediated cellular proliferation (Figure 9).[137] However, there is striking evidence to suggest that Wnt-signaling is involved in the tumor initiating capacity of CSCs in the colon.[51, 66] Since Wnt-signals are important for normal self-renewal and proliferation of normal pluripotent stem cells, and it is overexpressed in early-phase CRC development, it is reasonable to think it is a key pathway. Furthermore, cells lacking the intestinal specific LGR5 CSC marker have shown a repressed Wnt-signaling. Additionally, there is significant cross-talk between Wnt protein and downstream effectors and the TGF-β signaling pathway, another marker in early development of CRC and the adenoma-carcinoma sequence.

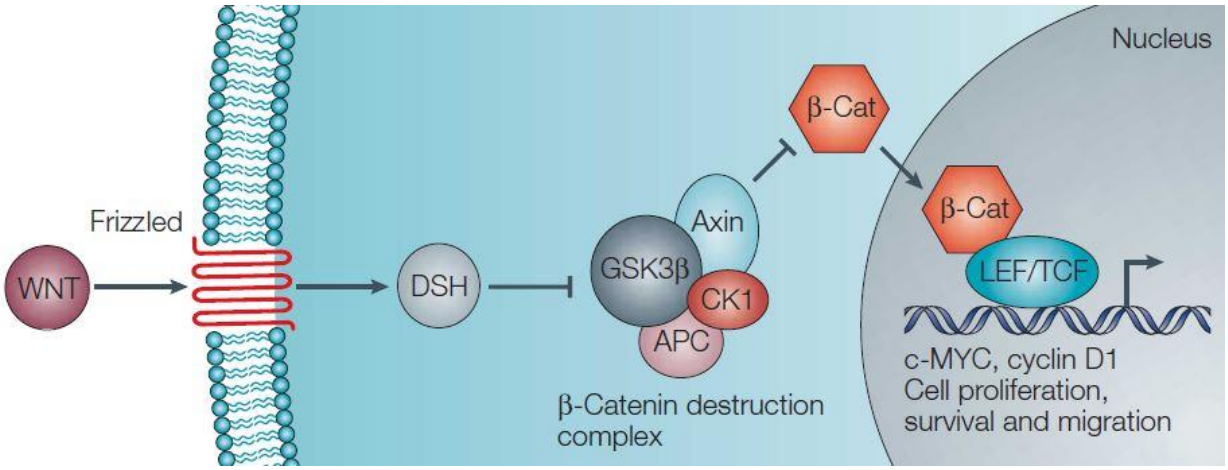


Figure 9. Wnt-signaling pathway in CRC development and CSC self-renewal regulation.

Additional pathways such as the NOTCH signaling pathway have shown to be important for many aspects of cancer development relative to CSCs and tumorigenesis. This pathway is relatively complex and has multiple known physiological functions, and has enhanced cross-talk with Wnt and TGF-β signaling pathways. Due to the wide extent of cellular communication that NOTCH is implicated in, many processes from regulation of CSCs to antineoplastic and immunological regulation is implicated.[143] The Hedgehog pathway has also shown that ligands secreted in its signaling are important for CSC regulation and embryonic development, and that agents targeting this pathway reduce the expression of CSC markers in prostate cancers in clinic.[144, 164-165] The difficulty in targeting these specific pathways remains the mechanism in doing so and the systemic tumor response to their inhibition. A more complete understanding of their biology is necessary to improve selective and sensitive CSC targeting.

Since proteins like c-Myc and other self-renewal regulators such as BMI-1 are shown to be enhanced in the expression of tumor initiating CRC cells, it is also plausible to evaluate markers of the epithelial-mesenchymal transition (EMT) that arise during cancer cell metastasis

and the CSC-niche of the tumor microenvironment. The overexpression of TGF-β and EGF signaling pathways rich in CSCs has shown to also cause dissociation of the cell-cell adhesion complexes important in epithelial cells leading to motility and invasion from the intestinal crypts where CSCs are found. Cells that are mesenchymal in phenotype and un-differentiated overexpress these EMT markers, like the embryonic cytoskeleton protein vimentin and the N-cadherin found in aggressive and stem-like glioblastomas.

An altered metabolic state is common among cancers, and CRC shows CSCs important for tumorigenesis that depend on the mutational status of KRAS protein to engage in tumor initiation and regulation of their own metabolism.[8, 13, 155] The metabolic reprogramming of the stem-cell niche mediated through KRAS and EMT state are important contributors to the metastatic state of tumors and the progression of tumors. There is major difficulty in isolating whole pathways that are unique to CSCs as compared to normal stem cells or early progenitor cancer cells.[166] Many of the pathways described are involved in complex cross-talk and regulation, where the shutdown of one growth factor leads to the upregulation and continued maintenance of pathways by another. In the last two decades there have been enhanced efforts to identify CSC-specific pathways that can be targeted to improve therapy selection, many of which are hindered by a lack of basic understanding. In the next section we will discuss these efforts in the pharmaceutical and research communities, and attempt to highlight both the successes and failures and what is still to be learned.

iii. Agents Under Development to Target CSCs

In the last 5 years there have been a variety of agents enter preclinical and clinical trials for the targeting of CSCs in a tumor population (Table 2). Many of these trials are still ongoing, and several of them were withdrawn due to unforeseen failures in specificity and proof of concept. Several of them experienced drawbacks in the toxicity of normal stem cell systems and healthy nearby cells. Others yet were not able to address the improvisation of the plasticity that is native to CSCs. These tumor initiating cells are able to adapt and generate mechanisms to combat the loss of one specific signaling pathway, with significant and complex cross-talk among key players. Additionally, the quiescent and dormant state that many stem-like tumor initiating cells undergo in response to therapy makes them a difficult target for therapeutic compounds. There is a demand for new insights and understanding of the tumor stem cell biology and mechanisms for survival that need to be addressed before any successful targeting of CSCs can be ultimately successful.

Much of the work performed in industry has been through companies like AbbVie and Boston Biomedical.[167-168] The former, although having major setbacks in their program clinically, have made major financial investments on several smaller companies aimed at identifying CSC specific therapies.[169] The latter, also with a recent halt in clinical progression of their Phase 3 molecule Napabucasin (Figure 10) targeting pancreatic stem cell pathways, still has several other alternative clinical trials underway with CSC agents. The same molecule,

Napabucasin, remains in Phase 3 evaluation for combination therapy targeting CSC pathway in metastatic CRC (NCT02753127).[170] Additionally, Boston Biomedical has several phase 1-2 studies underway and active with compound Amcasertib (Figure 11), an agent purported to target CSC kinase factors in the NANOG pathway of CSC regulation (NCT02232633).

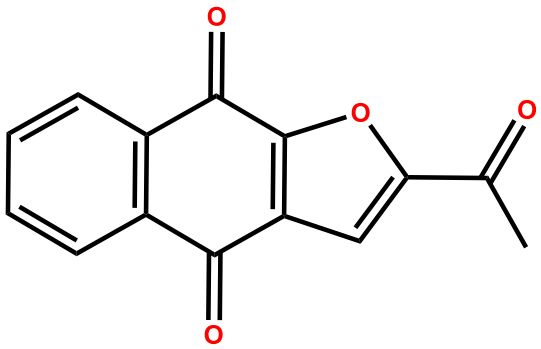


Figure 10. Structure of clinical compound Napabucasin (2-acetylbenzo[f][1]benzofuran-4,9-dione), currently under clinical trials for the treatment of metastatic CRC sponsored by Boston Biomedical. Napabucasin, also known as BBI-608, has secondary outcome measurements aimed at evaluating CSCs.

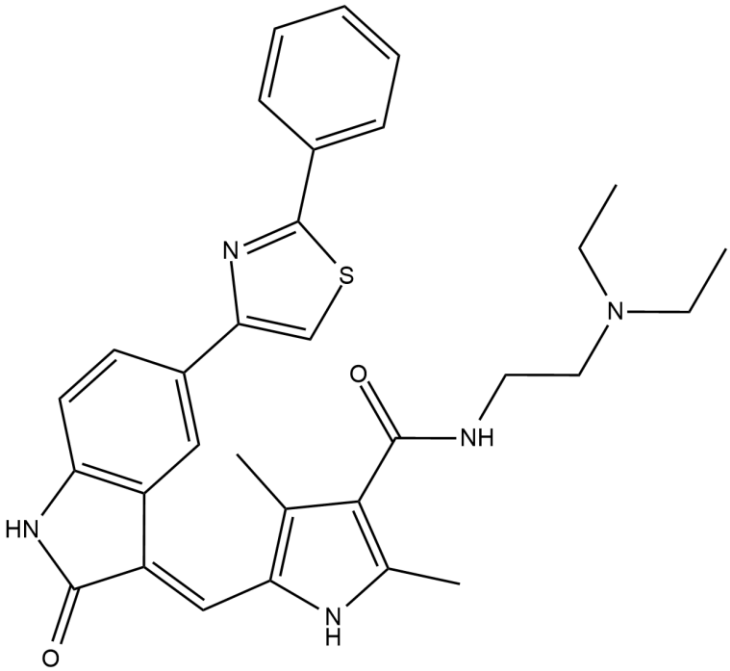


Figure 11. Structure of CSC pathway targeting molecule Amcasertib under development by Boston Biomedical. Amcasertib is alleged to alter the NANOG pathway of CSC self renewal and pluripotency, leading to suppression of various kinase signaling cascades. It is being evaluated in patients with hepatobiliary cancer who have exhausted all other means of therapy.

Table 2. List of a Portion of Clinical Trials Evaluating anti-CSC Therapy

Trial Number	Phase	Status	Condition
NCT02753127	3	Active	Metastatic CRC
NCT01781455	1	Active	Advanced Solid Tumors
NCT02483247	1b/2	Active	Advanced Solid Tumors
NCT02279719	1b/2	Active	Advanced hepatocellular carcinoma
NCT02432326	1b	Active	Advanced Solid Tumors
NCT02467361	1b/2	Active	Advanced Solid Tumors
NCT02024607	1b/2	Active	Advanced GI Cancer
NCT02432326	1b	Active	Advanced Solid Tumors
NCT01553851	2	Completed	Oral Squamos Cell Cancer
NCT01190345	2	Completed	Breast Cancer
NCT01579812	2	Completed	Advanced Ovarian/Fallopian Cancer
NCT02370238	2	Active	Metastatic Triple Neg. Breast Cancer
NCT01088815	2	Completed	Metastaic Pancreatic Adenocarcinoma
NCT03949283	3	NYR	Recurrent Platinum Resistance Ovarian Cancer
NCT03548571	3	Recruiting	Glioblastoma

In addition to the clinical trials mentioned above, there have been other clinical trials attempting to target and regulate CSCs in patients with various cancers elsewhere. There has been evidence to support the idea that anti-Diabetic medications like metformin and the TZD class of compounds regulate CSCs by inducing differentiation and selective apoptosis, respectively.[171-172] Although the effects of these compounds as anti-cancer agents is promising, it is unclear the mechanism or function they have in CSCs. Another agent, Reparixin, went through a window of opportunity trial to investigating the CSC inhibition in breast cancer, although a limited group of participants led to early termination (NCT01861054). The proposed anti-CSC mechanism of this agent was the activation prevention of CXCR1/2, which has been implicated in CSCs previously.[173]

In the realm of academia there have also been extensive efforts over the last couple decades to identify both characterization and pathway targeting of CSCs, with many agents showing promising data. Toden and researchers have shown using in-vitro spheroid inhibition assays as well as PDX models in-vivo that oligomeric proanthocyanidins (Figure 12) inhibit the tumor forming capacity of CRC cells and reduce the expression of putative CSC markers in different CRC cell lines.[174] It is believed that these molecules are targeting the NOTCH and Hippo pathways, however the dose dependent cytotoxicity in normal cancer cell culture suggest these agents may not be as selective to CSCs as desired.

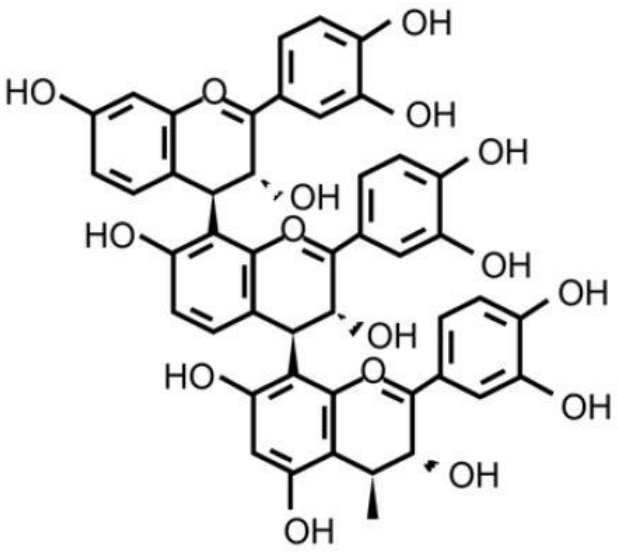


Figure 12. Structure of template oligomeric proanthocyanidins (OPCs) that have been shown to reduce tumorigenic properties of CRC xenografts and alter the CSC pathway in CRC cells. OPCs represent a naturally occurring molecules found in a variety of fruits and vegetables with anti-cancer properties, and the oligomeric form allows for improved solubility and bioavailability compared to the polymers found in nature.

These are just some examples of the various academic and pharmaceutical efforts to pursue therapy targeting CSC pathways in the clinic to improve patient survival.[144, 175-176] Although setbacks include the financial resources necessary to carry compounds through late phase testing, a lack of biological context to the mechanisms and inner workings of CSC pathways in tumors, and lack of specific and sensitive markers for tumor initiating cells in a

cancer cell population, there are endless opportunities ahead to uncover potential pathways and targets. The notion that a tumor can be depleted through the targeting of this colony forming subpopulation will hopefully be better understood and addressed in the coming years.

f. Preclinical CSC Models

CSCs have been shown to represent a smaller population of cells within a tumor environment, varying from tissue to tissue. In some cases, this population can be as low as 0.1-1% of the over cell population.[12, 152, 164] In order to evaluate the performance of CSC targeting agents, there must be a mechanism to enrich the population of CSCs for in-vitro as well as in-vivo experiments. There are several methods used for each stage in evaluation, and specific criteria employed in each assay to achieve the criteria for as authentic a selectivity assay as possible. Using the previously established models to enrich for CSCs based on putative markers, as well as physical platforms and chemically induced platforms researchers can evaluate the efficacy of potential therapies in targeting and eliminating tumor initiating CSCs. The following sections will describe the most noted methods and how they are used in preclinical models.

i. 3-D In-vitro Culturing Platforms

The use of 3-D cell culturing platforms for anti-cancer research has been a widely used strategy and still novel technique since its introduction in the 1980s and earlier as a means to recapitulate the tumor microenvironment.[135, 177] In-vivo tumors, as they grow, develop a highly complex and multifaceted display of cells that contribute to growth, progression, survival and

response to therapy. These 3-D models were originally intended to serve as a structural and physiological mimic of the in-vivo tumor, a closer connection to the conditions observed in the human body from plastic plates.[127] The idea that 3-D models allowed for the development of microregions of a cell culture that exhibited gradients of critical metabolites meant that the effects of chemotherapeutics could be more accurately studied for their effect on real tumors. It was observed over the years that the structural mimicry of 3-D models also displayed functional mimicry as the development of tumor microenvironment expanded and multi-cellular tumor model became validated.

Flash forward 20 years to the late 2000s when the CSC paradigm showed growing popularity and the 3-D cancer culture platform resurged as a practical and novel model. Studied reported that culturing cells under anchorage independent conditions in-vitro allowed the colon cancer models, termed colonospheres, to grow in a floating sphere that overexpressed putative tumor initiation pathways and many stem cell markers.[10, 178] In fact, this same work showed an 80-fold increase in CD44 positive population of cells when grown as colonospheres as opposed to traditional culture.[80] Additionally, cells that were previously treated with chemotherapeutic agents seemed to develop into colonospheres without the need for large seeding density, suggesting that enrichment may also be achieved by dosing culture with chemotherapy.

A variety of platforms developed to both culture cells in a 3-D model that would enhance the physiological relevance of in-vitro work as well as induce the overexpression of CSCs. Each of the various models where consistent with the growth of round, free floating cultures that enhanced the expression of CSC markers and thus tumor initiating cells. The multicellular

spheroid model plated cells in non-adherent plates followed by various aggregation techniques that, after suspension on agarose-based media, would develop without adhering into tumor spheroids.[179] The hanging drop method became particularly convenient and popular, with a small drop of cells being aliquoted onto plastic and then inverted to allow for suspended growth into spheroidal conditions. This method, unlike others, generally used fetal bovine serum as a supplement to the media.

Other methods cultured cells via a platform that contained various thickness of fibrin media.[180] This mechanical method grew cells in a fibrin gel and that allowed for suspended spheroidal growth, and showed that low density of these cells were enriched in tumor initiating cells far more capable of developing tumors in-vivo. Advances on this scaffold allowed for more refined fibrous platforms that enhanced the EMT pathway, resistance to chemotherapeutic agents, and in a means that would be simple and scalable.[181] The stiffness of these mechanical matrices were implicated in the ability of tumor initiating CSCs to develop, with expression of tumor initiating stemness markers being regulated by the polyacrylamide media resistance.[182]

Still different platforms also showed evidence of the enrichment of CSCs that would improve tumor development in-vivo without the need for a mechanical media but rather a chemically inducing media. The use of serum free media supplanted with a variety of growth factors identified in the CSC niche would allow cells to grow on non-adherent plates as 3-D tumor spheroids that enhanced tumorigenesis. Studies showed that putative CSC markers like ALDH-1 and CD44 were upregulated when cells were cultured under these conditions, and that when injected into immunodeficient mice tumors formed more efficiently than cells cultured

under conventional means at lower densities.[183] 3-D tumor spheroids can also be harvested from tissue of in-vivo tumor development and exhibit remarkable in-vitro tumor growth that improves the validation of preclinical models in a cost effective and convenient platform. Additionally, in research focusing on tumors of the intestinal tract can derive 3-D forming, CSC rich colonies from the intestinal crypts where cellular organoids can be harvested.[184-185] These organoids feature the complexity and heterogeneic nature of in-vivo tumors and are reproducible in the in-vitro use of evaluating therapies that may inhibit tumor initiation.

Regardless of the platform used to develop a 3-D cancer culture model, the use and versatility of these models have been both validated and accepted as a more refined and accurate preclinical model to evaluate anti-cancer therapy. Tumor spheroids recapitulate the complexity of the tumor microenvironment by the establishment of a physiologically relevant structure with microregions of nutrient gradients. Additionally, spheroidal models enhance both the overall population of CSCs and tumor initiating cells via putative stemness markers and improve upon the success of in-vivo tumor development and tumorigenesis in immunocompromised animal models. These efforts continue to be used to evaluate not only improved anti-cancer therapy selection for clinical study, but also for selective targeting of a CSC rich population of cells.

ii. Enhancing the Population of CSCs for Evaluation

Previously described were methods used to develop 3-D culture methods that enhance the preclinical validation of anti-cancer therapies through both structural and functional mimicry of in-vivo tumors. This platform is also commonly used to overexpress CSCs, since many research efforts to develop suspended spheroids also notice elevated levels of CSC markers and stemness

pathways. There are additional means of enhancing the population of CSCs and tumor initiating cells that have yet to be described in detail, and serve as alternative approaches in developing selective CSC agents. The use of in-vivo xenograft models, the chemical enrichment of CSC pathways, as well as low dose chemotherapy have both been shown to elevate colony forming tumorigenic cells in overall cell population, and will be here be described in more detail.

The transplantation of cells grown in culture to an immunocompromised mouse has long been a model to evaluate the potential of stem-like tumor initiating cells to develop as well as a model to evaluate therapy that specifically target CSCs.[11] These xenograft implants have been used extensively to study potential CSC markers and evaluate pathways by which CSC regulation and signaling occur. Interestingly, when any cell is cultured in the patient-derived xenograft model (PDX) there is also a large scale enhancement (initially) of stem like cells with strong tumor initiating capacity.[186-187] The population of these cells show intense staining of CSC markers compared even to those cultured under spheroidal growth in-vitro. This allows for more efficient production of CSCs to later be evaluated from harvesting PDX tumors for novel therapies. The nutrient rich environment of the in-vivo model allows for the unique signals and growth factors in the stem cell niche to be utilized without research bias or presumption. For this reason, many have found PDX models to be the ideal and most validated model for evaluating anti-CSC agents.

In addition to PDX models, there is a large basis of work to support the enrichment of CSCs by stimulating the pathways reported to be involved in tumorigenesis and tumor initiating cell signaling. Addition of insulin-like growth factor 1 (IGF-1) was shown to enhance the

population of CSCs in-vitro via a Wnt/β-catenin mediated mechanism, and that downregulated IGF-1R suppressed not only the Wnt-signaling but reduced the tumorigenic effects of cancer cells.[188-190] Other findings propose that enhancing the NOTCH signaling pathway via an EGFR dependent activation enhanced the proportion of ALDH positive cells, another putative marker of CSCs.[143-144] These and other pathways, such as Hedgehog and EMT have been targeted to enhance stem like progenitor cells. By stimulating the progression of cells from an epithelial state to a mesenchymal and invasive phenotype, an enhancement of CSC markers were observed.

Another property reported in earlier sections of the CSC phenotype was the resistance to traditional chemotherapy. Normal cellular death and apoptosis processes do not work in CSCs, who lack a rapidly dividing nature. It has been proposed that in fact these cells are in a state of observant quiescence, feeding off of the environmental stimulus to determine whether activation is needed. With this theory, researchers have been able to overexpress the population of CSCs via treatment of both xenografts and cell culture models with low dose chemotherapeutic drugs that would deplete mature and differentiated cells leaving behind only a population of tumor initiating cells.[191] Although this enrichment is effective initially, over some period of time the differentiation process will resume and the CSCs will yet again become only a subpopulation. This can be said for each of the methods discussed, as 3-D tumor spheroids will eventually reach a size large enough that the percentage of CSCs will again be depleted. These are simply the best methods utilized to date for analysis of agents specifically targeting tumor initiating cells, with animal PDX models the most expensive and most fulfilling. In the future there is anticipation that new methods for enrichment of these progenitor cells can be developed for a more comprehensive analysis.

g. Glycans in Cancer

Glycans and glycosaminoglycans (GAGs) serve a wide variety of important physiological functions, many of which are essential for normal development.[192] When these processes, such as cell signaling or enzymatic function, are interrupted the development of disease states are inevitable. GAGs have been implicated in the development of many cancers, with increasing evidence to elucidate their roles in the development and progression. Specific GAG degrading enzymes, such as Heparanase, serves an enzymatic role under normal function and is shown to play a very specific role in pro-angiogenic vascularization and metastases. Other glycosylation events are much more broad, and a complete understanding this regulation is lacking. Briefly we will discuss the role of GAGs in cancer as well as their involvement in tumorigenic pathways for CSCs.

i. Role of Glycans in Cancer Signaling and Tumorigenesis

Glycans play a large role in the key pathological steps of the progression, development, survival and advancement in cancers. Structurally, they can form from many different macromolecular arrangements that add a great deal of complexity to understanding their exact mechanisms. Glycosylation and establish glycosidic linkages to fats as in glycolipids, that help to both stabilize the phospholipid bilayer of the exterior cell wall as well as to serve in extracellular signaling events in the glycocalyx that surrounds the outside of the membrane in a dwarfing shield.[193] Alternatively, glycosidic linkage can connect glycans and proteins to form proteoglycans, which have strong evidence or dysregulation in cancer signaling. Such evidence is supported by tumor specific antibodies that target antigenic epitopes on the surface of tumor

cells. Addition of glycans to a protein or lipid increase the molecular complexity of the macromolecule, giving cells and tissue a specific phenotype and functional diversity.

There are several key changes in glycans that are observed frequently in cancers. One of which is the "sialylation", or the addition of a sialyic acid monomers to the end of a polypeptide chain. Dysregulated glycosylation in cancer typically involves an increase in the levels of sialyl Lews x antigens, whose altered products play a role in cellular recognition and cell signaling.[194-195] Other glycans, like chondroitin sulfate and heparin sulfate, assist to regulate the binding of cancer cells to the external tumor microenvironment. Enzyme heparanase is responsible for cleaving heparan sulfate proteoglycans (HSPG) in the extracellular matrix (ECM), and has a fundamental role in both angiogenesis and metastases.[196] In cancer, it has been shown that heparanase cleavage of HSPG in the ECM releases a variety of pro-angiogenic cytokines and matrix metalloproteinases that allow for cleavage of the cell-cell adhesion complex and the eventually motility and migration of cancer cells from the primary tumor site.[134, 197]

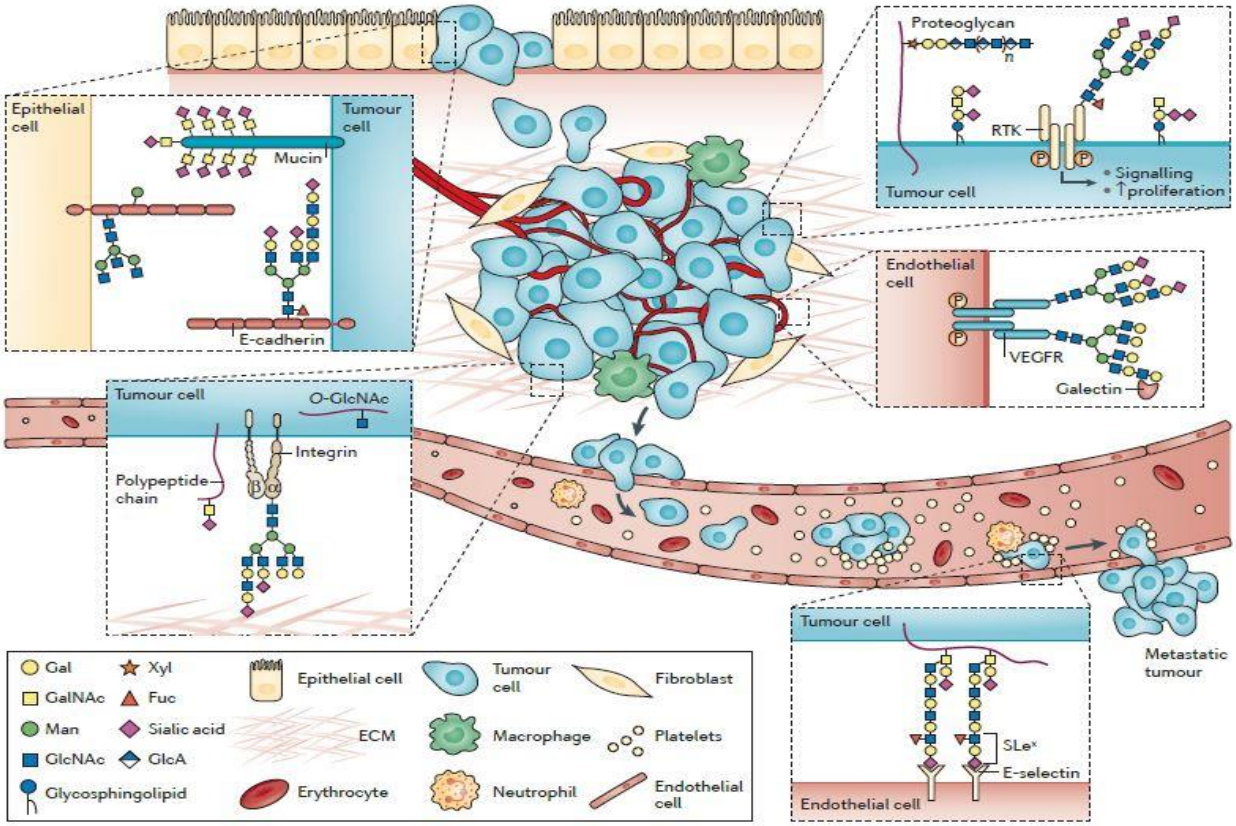


Figure 13. Various roles of glycans in the development and progression of cancer.[196]

Figure 13 above illustrates a diagram that highlights many of the various roles that glycans play in the development and progression of cancer.[198] From the aberrant signaling that occurs from oncogenic mutations that enhancing the proliferative cell signaling and tumor growth, to the interaction of polypeptide GAGs with the fibronectin and stromal layer of the tumor microenvironment, glycans are consistently elevated and found in a distinctive pattern in cancerous tumors compared with normal tissue.[199] The intestinal crypts of CRC are found to be rich with an enhanced expression of sialylation, and the metabolic shift of cancer cells for glycolysis relies on glycan metabolites as nutritional sensors for maintenance. The precise functions of many of the GAGs involved in tumorigenesis are still being uncovered and

evaluated, however it is apparent from the work already performed that changes in glycosylation have an effect on virtually every major step in the development of cancer. Determining how to regulate these processes would be key to developing additional therapeutic pathways for anti-cancer agents.

ii. Significance of Glycans to CSCs

In addition to the progression and regulation of normal cancerous cells, glycans and GAGs have been associated long term with the development and tumorigenesis resulting from CSCs and other tumor initiating progenitor cells.[193] Previously mentioned was the fact that the colonic crypt and basement membrane was home to stem-like precursor cells and CSCs that displayed elevated levels of sialylation that was not emphasized in normal colonic tissue samples.[141, 194] To supplement this glycan marker of stemness, research has shown that oncogenic signaling pathways are elevated during the aberrant morphological changes in epithelial cells. These cells have a synthetic pathway marked by N-glycan glucosyltransferases, and have been linked to tumorigenesis via cell surface proteoglycans in several tissues and models.[194, 200]

Glycans are implicated in various roles in many diseases to include cancer. There are mechanisms previously identified that enable researchers to attempt to characterize and evaluate the glycomics of tumors and potentially target them. The identification of tumor initiating stem cells were first shown in leukemia models by filtering cells based on those cell surface transmembrane glycoproteins as antigenic epitopes.[11] This lead to the identification of many other putative markers of CSCs that also are cell surface glycans.[160, 193, 201] Based on the

evidence of the intricate involvement of glycans in the initiation, development, signaling, progression, invasion, and survival of both normal malignant cancer tissue and those or progenitors and CSCs, it is reasonable to propose that with novel and specific compounds a new anti-cancer and anti-CSC therapy could be derived from the targeting of implicated glycans.

2. Glycosaminoglycan (GAG) Mimetics

a. GAG Mimetics

Glycosaminoglycans (GAGs) are a complex and diverse class of glycans that are responsible for innumerable physiological function and biological processes.[202-203] These linear polysaccharides are composed of repeating disaccharide units of either N-acetylglucosamine/N-acetylgalactosamine and a form of uronic acid such as glucuronic acid or iduronic acid.[204] They are the most abundant form of polysaccharide in the human body, and have functions ranging from the defense and structure of the extracellular matrix to the lubrication of joints for movement. Due to the wide variety of roles they play in the human body, it is reasonable that in recent decades there is such a keen interest in understanding the glycomics of human life and the value that GAGs may play in both therapy and as a means of supporting novel therapy. There are two GAGs in particular that have been at the center of the development of this mimicry, namely heparan sulfate and the classic anticoagulant heparin. The former making a large portion of the extracellular matrix and surrounding glycocalyx, with important roles outside of those mentioned in pro-angiogenic factors in cancer.[192] The latter, an essential medication enhancing a

variety of blood-related disorders. We will briefly discuss the attempts to mimic both the structure and function of these compounds for therapeutic improvement.

As previously demonstrated, heparan sulfate plays an intrical part of the ability in tumor cells to support neovasculature growth and advance beyond the primary site to invade nearby and distant tissues.[197] Figure 14 provides the structure of heparan sulfate in both major and minor disaccharide forms.[204] Despite the well documented role in tumorigenesis, heparan sulfate also plays a major role in other disease states and mimetics of it are under development for potential therapy. Many different viruses, to include dengue virus (serious tropical illness), show some degree of affinity for heparan sulfate for viral attachment and entry mechanisms. Because of this, researchers are working to design sulfated polysaccharides and small molecule mimics of heparan sulfate to ameliorate the spread of dengue virus and improve treatment options.[205] Additional studies by other researchers with the same molecule used for dengue show that this sulfated oligosaccharide mimetic of heparan sulfate inhibits cellular infection rates of the herpes simplex virus with only a tetrasaccharide portion of the mimetic necessary for anti-viral effects.[206] This work supports the idea that biological response is very sensitive to specific sequences of GAGs and that with the identification of those sequences potent and effective therapies can be produced.

In addition to the roles heparan sulfate may play in regard to anti-viral mechanisms, there are also roles in various for the GAG with intracellular signaling, wound healing, and other structural repair that have therapeutic applications. GAG like heparan sulfate have many different protein binding partners upon which they exhibit physiological activity. One research

team showed that a specifically modified sulfation pattern would produce a heparan sulfate polysaccharide mimetic that prevented inflammatory related chemokine signaling events by binding to CCL5 with comparable binding to heparan sulfate.[207] This work supports the use of heparan sulfate mimetics for various roles as anti-inflammatory compounds for clinical consideration.

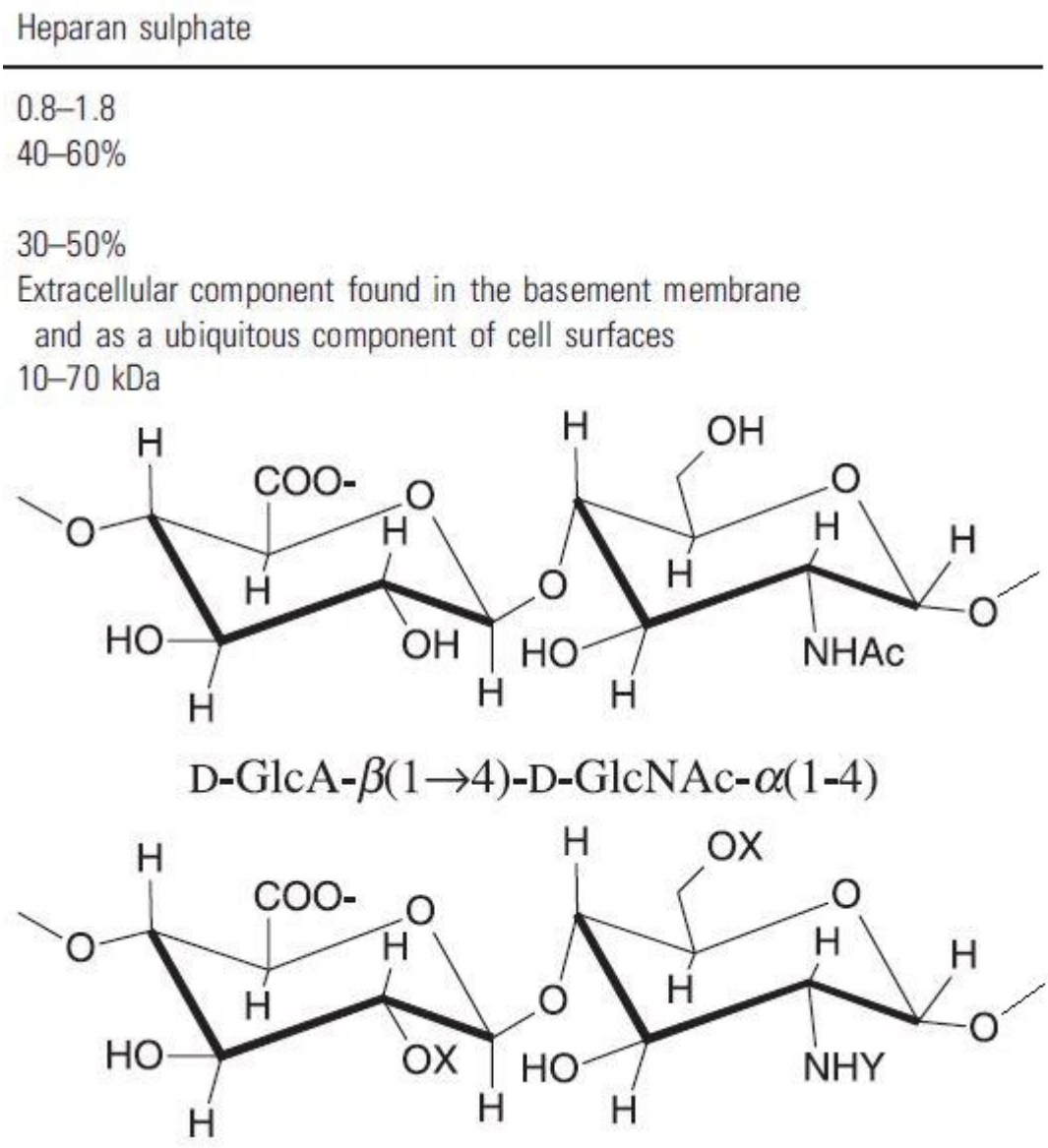


Figure 14. Structure and characteristics of heparan sulfate glycosaminoglycan.

The endothelial vasculature and fatty acid regulation play key roles in various metabolic disorders like diabetes and obesity as well as cardiovascular disease.[208] GAGs found in the blood system play a large role in this signaling and regulation, attempting to maintain a homeostasis. Researchers have shown that small molecule heparan sulfate mimetics are able to induce protective effects against the oxidative stress caused by lipid-induced endothelial dysfunction.[209] They attributed the biological function to the key sulfate pattern also displayed by GAGs allowing for key ionic interactions with Akt/NOS pathway oxidases that restore endothelium vasodilation and attenuate radical oxygen species from oxidase activity. The evidence in this research proposes that readily synthesized and sulfated small molecule heparan sulfate mimetics can restore the homeostasis of endothelial dysfunction and serve as potential therapies for metabolic disease that plagues western culture.

Heparan sulfate GAGs also play a fundamental role in the structure of various tissues and organ systems, and extensive research efforts have looked at the development of heparan sulfate mimetics to serve as therapeutics. It has been shown that heparan sulfate polysaccharide mimetics with various monomeric substitutions (largely sulfate groups) stimulated the growth of musculature precursors and enhanced the rate of myogenesis in wound repair.[210] Other researchers attempted to develop a therapy to impact the reduced rate of wound healing in diabetics with the treatment of diabetic mice with a heparan sulfate mimetic reduced the rates of ulceration, enhanced ulcer healing, and enhance the breaking strength of fibers at the wound.[211] Uniquely, researchers in France displayed the heparan sulfate mimicry of a synthetic polysaccharide in the restoration of extracellular matrix in gingival tissue of the mouth to combat the bacterial degradation of periodontitis.[212]

Each of these examples explores the use of heparan sulfate GAG mimetics for the therapeutic treatment of viral, bacterial, and metabolic disorders that GAGs play a role in regulation. Although they all vary in structure, with some being full polysaccharides and others non-saccharide small molecules, they each gave strong evidence of function to reverse the consequences of the disorder via a similar means. What was key to the physiological function of every compound discussed was the key position and pattern of sulfate groups that directed sensitive and specific ionic interactions with binding partners and host tissue.

One of the most well-known GAGs that has served as an integral part of the clinical medicine cabinet for the past century is the anti-coagulant heparin. It has remained on the World Health Organization's List of Essential Medicines due to its impact in the clinic to relieve deep vein thrombosis and severe clotting events in the vasculature.[204] Heparin binds to antithrombin and activates it to then have a dramatic and enhanced ability to inhibit factor Xa, or thrombin in the coagulation cascade.[213] One of the most fascinating features of heparin, is that there is only a pentasaccharide sequence that is responsible for all of the enzymatic activity (Figure 15). Of this pentasaccharide, there are only a few key sulfate groups that dictate this activity, with a central 3-O sulfate moiety primarily responsible for function of heparin.

The pentasaccharide sequence shown above only accounts for approximately 30% of the total polymer of heparin, and has caused some serious clinical incidents in regard to purity.[214] However, its function as an essential anti-coagulant in clinic maintains its value. The notion that only a pentasaccharide is responsible for the biological activity, and then in that specific sulfate groups dictated this action, specificity, and selectivity, promoted the investigation by the

research community for the identification of heparin mimetics that could achieve these effects in a more "drug-like" manner without the undesirable and unpredictable side effects of natural GAG therapy. This began with the cleavage of the polymer into small fragments hoping to isolate smaller low molecular weight heparins, like Enoxaparin.[215-216] Other heparin analogs and mimetics have since been made and are clinically available to treat clotting, however many still experience undesirable side effects that limit their use in the clinic.[217-218]

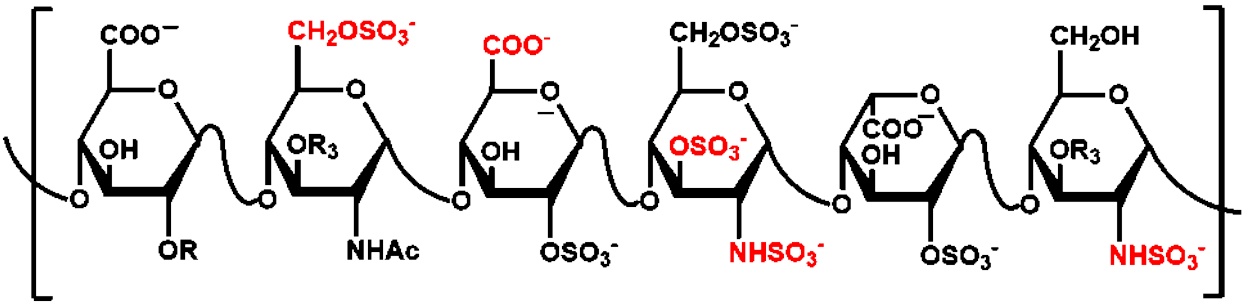


Figure 15. Structure of a heparin oligomer containing the essential pentasaccharide sequence required for anti-coagulant activity shown with key functional groups highlighted in red.

Many new heparin mimetics for anticoagulant activity have focused on the idea of allosteric inhibition of an additional factor in the anticoagulant cascade, factor XIa. Researchers identified an oligomer that bound to the heparin binding site of XIa with nM potency and approximately 200-fold selectivity for this coagulation factor.[219] This work showcased the potential for heparin mimetics as new therapeutics to target a safer and effective coagulation target for clinical use. The role of heparin also goes beyond the realm of anti-coagulants, with other heparin mimetics being developed to target other heparin binding proteins. One research effort shows that heparin oligosaccharides mimic the binding of heparin to FGF, and could potentially be used as an anti-inflammatory or agent of tissue repair.[220]

Although only heparan sulfate and heparin mimetics were discussed here, there are many other functionally important GAGs that mediate biological activity for a variety of disease states. In the following sections, we will discuss the impact of GAG mimetics in the role in regulating cancer. The two classes of mimetics will be broken up into the saccharide mimetics and non-saccharide small molecule mimetics, both of which in studies from our lab have shown valuable anti-cancer and anti-CSC effects. The principles of their application in cancer follows what was previously known and addressed in Chapter 1 on the roles of glycans in cancer, compounded with the models of GAG mimetic specificity and sensitivity to treating disease based on key positions of sulfation. The following content serves as the underlying basis and motivation for the work performed in this thesis.

b. Saccharide-based GAG Mimetics for Cancer Treatment

As was discussed in Chapter 1 from an assortment of reviews on the involvement of GAGs and other glycans in cancer, there are a wide variety of specific interactions that these polymers play in development and progression of a tumor. If the interactions could be modified by a mimetic of the same kind of molecule, a new treatment paradigm could be opened for anti-cancer compounds. With a specific saccharide length and key positions of sulfation, screening of heparan sulfate oligomers may prove to find such activity. Due to the advances in the CSC paradigm and the unresolved cancer relapse and progression from traditional chemotherapy, it was proposed that oligomer mimetics of natural GAG CSC-regulating activity could be

identified in a small library of oligomers derived from heparan sulfate, chondroitin sulfate, and dermatan sulfate.[221]

Screening was performed on a colorectal adenocarcinoma cell line that previously was shown to display high CSC expression when cultured under spheroid conditions.[222] The results of this study (Figure 16) indicated that, like the anticoagulant activity of heparin, there was a specific length of GAG that was responsible for the anti-cancer and anti-CSC effects.[221] The hexasaccharide, referred to as HS06, showed the greatest inhibition of primary spheroids compared to oligomers of lesser and greater length (ranging from a disaccharide to 38 units).

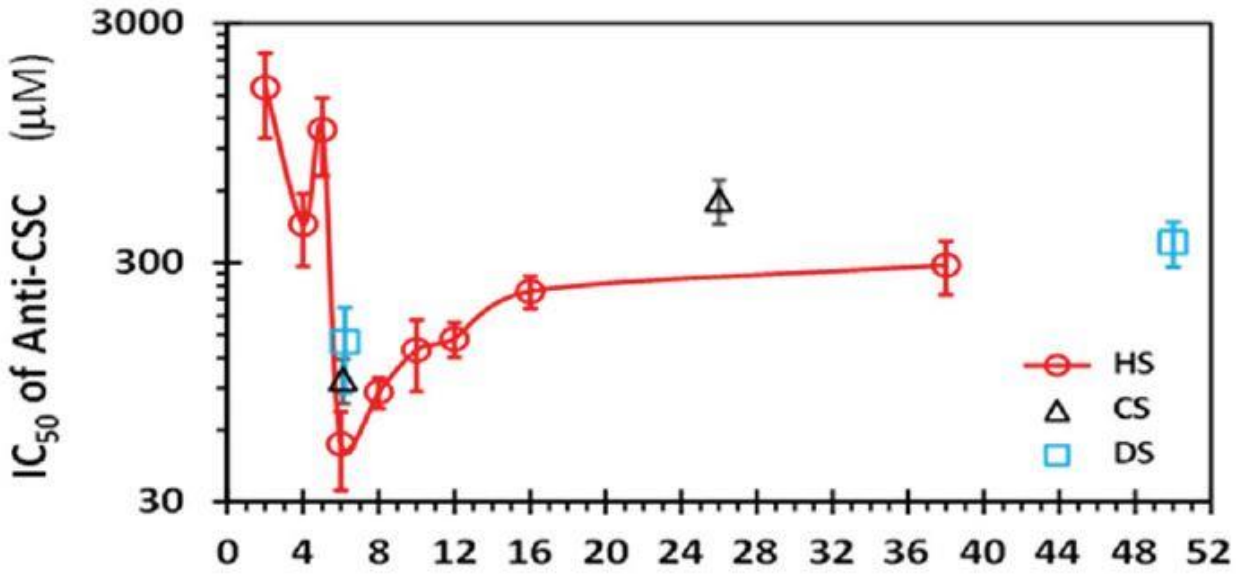


Figure 16. Plot showing the inhibition potency of oligomers of varying length and composition to CSC spheroid growth in HT-29 cells.

Further evidence from this study showed that HS06 inhibited the self-renewal capabilities of CSCs and reduced the expression of putative colon CSC markers such as LGR5, c-MYC, and

CD133 (Figure 17). Comparatively, HS06 did not show inhibition of cancer cells grown under normal cell culture, suggesting that the compound exhibited selectivity for CSCs compared to more differentiated cells that would compose the bulk of the tumor.[221] These promising results highlighted that there is in fact a unique length dependency of heparan sulfate oligomer mimetics to specific anti-CSC activity. The difficulty in utilizing saccharide-based mimetics of GAGs is still the lack of purity and difficulty in synthesis due to the microheterogeneity of potential structures even in a hexasaccharide sequence. Efforts need to be made to develop small-molecule GAG mimetics that mirror the biological functions of GAGs and oligomers like HS06 with greater synthetic ease and reproducibility. Compounds that can achieve this have a greater role in the clinic and drug-like therapeutics with pharmacological control. This led research efforts to design synthetic small-molecule GAG mimetics for anti-cancer evaluation.

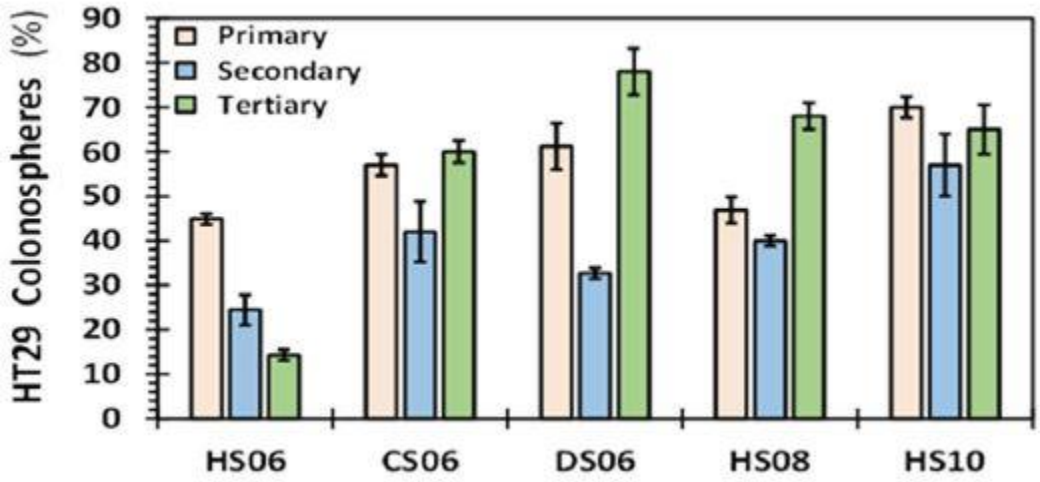


Figure 17. Inhibition of subsequent generation of primary CRC CSC-spheroids by various oligomer mimetics of heparan sulfate.

c. Non-saccharide Glycosaminoglycan Mimetics (NSGMs) for Cancer Treatment

There is compounding evidence on the role of polyphenolic molecules such as flavonoids in their role as anti-cancer molecules.[223-225] Generally found in a wide varieties of plants and fruits, they are considered a natural product to protect against cancer development and progression. Quercetin is one of the largest studied flavonoids for this principle, along with the natural phenol curcumin.[224, 226] Acting as metabolites in diet, the addition of sulfate groups makes them polar and soluble, easier to be excreted. The sulfation of these polyphenolic natural products has been studied at large for contributions to anti-cancer therapy.[225, 227] Interestingly, they are similar in structure to the small oligomer mimetics of GAGs. It was proposed that these compounds could then serve as templates to which specific sulfation patterns could be produced to mitigate anti-cancer and specifically anti-CSC activity that was observed in compounds like HS06.

The development of a library of sulfated NSGMs with various aromatic and polyphenol structure that could be commercially purchased encouraged the possibility that one out of many scaffolds could induce anti-CSC effects as was seen in HS06. With varying degrees of sulfate groups and at varying positions, this library of 53 compounds that belonged to 12 different scaffolds was evaluated for its ability to inhibit CSCs of CRC (Figure 18).[15] A novel dual screening approach (Figure 19) was used to demonstrate selectivity of compounds for CSC-rich spheroids as opposed to normal cultured CRC cells, as well as to show a consistent inhibition of development in subsequent spheroid cultures without further treatment. Of the 53 compounds, one in particular demonstrated the most potent and selective inhibition of CSCs, called G2.2

(Figure 20).[15] G2.2 is a dimeric quercetin scaffold with 8 strategically placed sulfate groups mediating ionic and polar interactions.

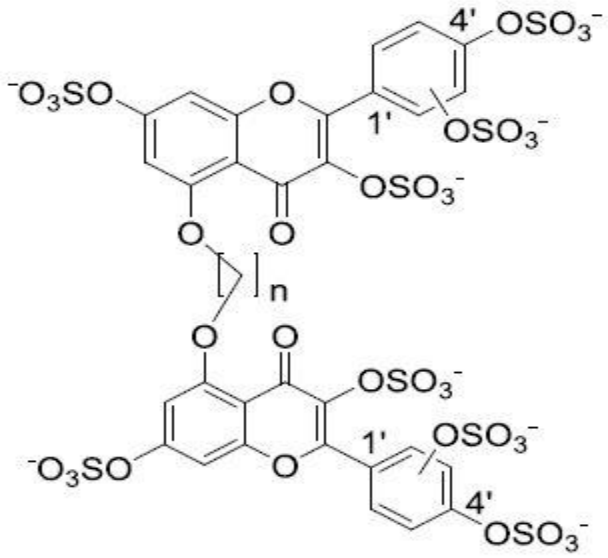


Figure 18. Scaffold 2 of NSGM structures defined below:

G2.1: n = 2; 3'- OSO_3^-
G2.2: n = 3; 3'- OSO_3^-
G2.3: n = 2; 2'- OSO_3^-
G2.4: n = 4; 3'- OSO_3^-
G2.5: n = 5; 3'- OSO_3^-
G2.6: n = *trans*-2-butene; 3'- OSO_3^-

These are only representations from 1 of the 12 scaffolds.

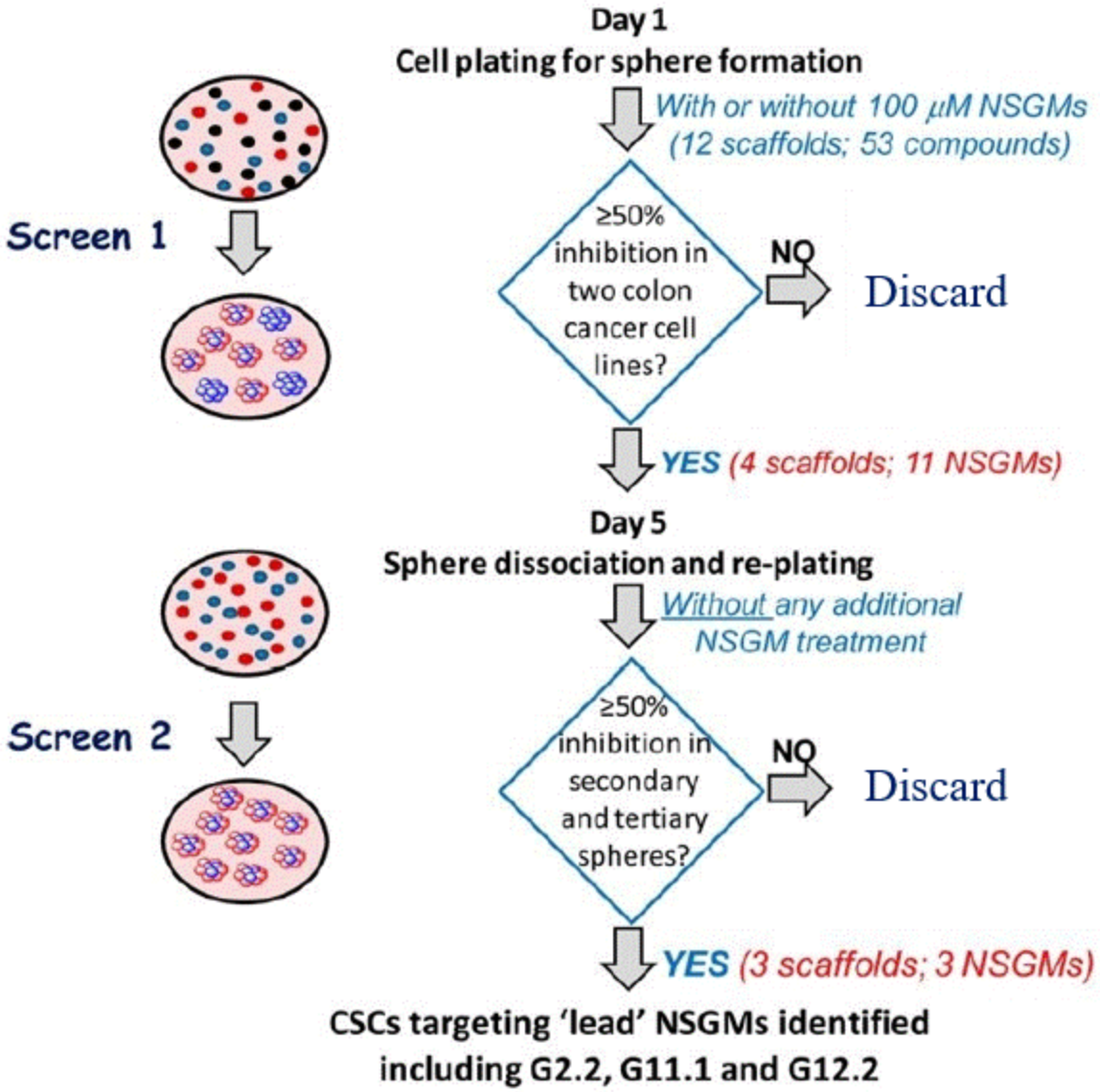


Figure 19. Novel dual screening approach used to identify agents potent in primary spheroid inhibition as well as limiting self-renewal properties of CSCs in subsequent secondary spheroid inhibition. Compounds able to inhibit 50% or more of primary spheroids proceeded to Screen 2, where primary spheroids were dissociated and seeded again as secondary spheroids without additional treatment. Those compounds able to continue inhibition of 50% or more of secondary spheroids were successful in the dual screening approach.

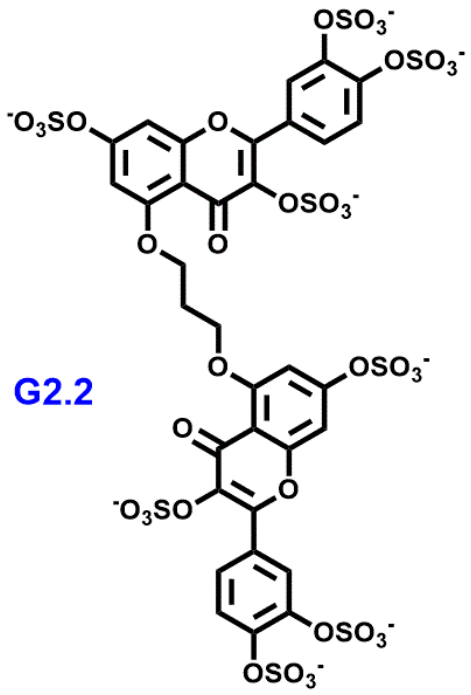


Figure 20. Structure of non-saccharide glycosaminoglycan mimetic (NSGM) G2.2. G2.2 is a dimer of a highly sulfated quercetin scaffold, with the number and position of sulfate groups being key to its anti-CSC actions. In dual screening evaluation of G2.2, it outperformed other NSGMs in both primary spheroid inhibition and secondary spheroid inhibition assays.

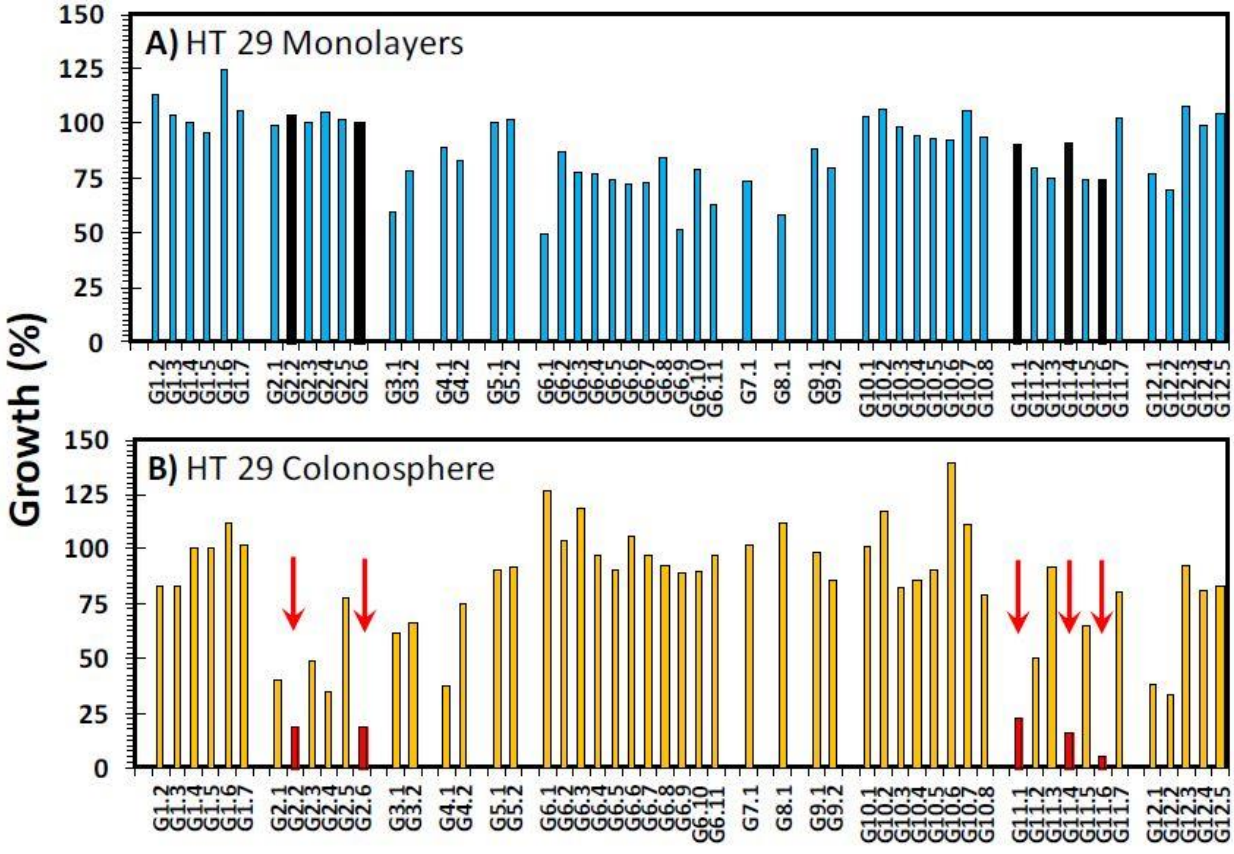


Figure 21. Differential targeting of monolayer and spheroidal culture by NSGMs.

To further support the selective inhibition of CSC-rich spheroids, G2.2 was evaluated for the effect it had on the expression of putative CSC and stemness markers for CRC as well as cell-induced apoptosis. G2.2 showed a near 40% reduction in the expression of many putative CSC and stemness markers for CRC (CD44, CD133, CXCR4, EpCAM, LGR5) across the board compared to other inactive NSGMs. G2.2 was identified from this study as the most potent and selective NSGM, and was used as a template for understanding interactions of GAG mimetics on anti-cancer and anti-CSC activity.[15] Further studies with this same molecule were performed to identify a mechanism of action and evaluate the NSGM in-vivo. These studies revealed that G2.2 selectively inhibited CRC xenografts rich induced by CSCs with dose dependency and dramatically reduced the tumor size in-vivo over a 3 week period (Figure 22).[228] Furthermore, G2.2 showed robust inhibition of CSC markers and self-renewal factors in treated xenografts.

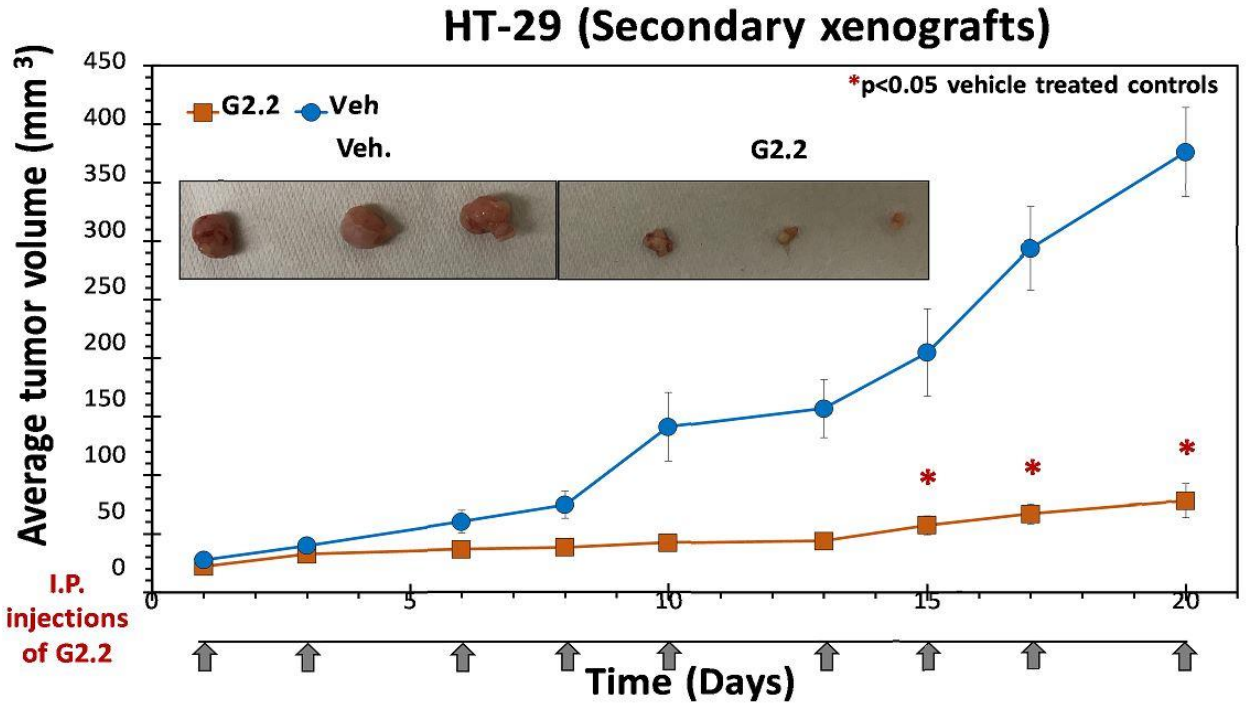


Figure 22. G2.2 inhibits growth of HT-29 xenografts in murine model ~ 5-fold compared to vehicle over 3 weeks.

This work, collectively suggests several important concepts applicable to the work in this thesis. The first, that tumor initiating cells such as CSCs are a recurrent problem in standard treatments that only effect the bulk of a tumor and leave CSCs behind to relapse. Next, CSCs can be enhanced in-vitro using 3-D spheroid models that allow researchers to characterize anti-CSC actions of novel compounds. Additionally, both cancer and CSC function and activity is regulated to some degree by glycans and GAGs. GAG mimetics have shown to alter cancer activity in the research community, and are useful as potential therapeutics for cancer. All of the above statements have largescale support in the literature. Through studies with heparan sulfate mimetic HS06, there has been evidence that the oligomer functions to regulate CSCs in CRC in-vitro. This is postulated to be a result of its unique hexasaccharide sequence with key sulfation to direct specific anti-cancer effects. Further, a NSGM G2.2, mimics the effects observed in HS06 to be a potent and selective inhibitor of CSCs in CRC. G2.2 has been shown in other work to be structurally very similar to HS06, and the anti-CSC effects have been further validated in PDX models.[229]

These findings support the idea that a sulfated small molecule GAG mimetic can act both in-vitro and in-vivo to inhibit the survival of CSCs and potentially serve as anti-cancer therapy to reduce the proliferation of a tumor in clinic. The remaining work will discuss the methods, experimental results, analysis and conclusions observed in defense of this thesis.

3. G2.2 Selectively Targets CSCs

a. Rationale

i. Background of CRC Cell Lines Used in Panel

The purpose of the work performed in this thesis was to evaluate the potency of leading NSGM compound G2.2 across a panel of CRC cell lines that represented various molecular subtypes and molecular phenotypes. G2.2 had previously been studied in a handful of cell lines, and by expanding the variety of cells screened we might be able to better understand both the mechanism by which G2.2 acts as well as to characterize a preference of G2.2 for a particular subset of CRC. Screening in a larger panel would also further validate any findings of CSC-targeting actions of G2.2. The panel chosen (Table 3) consisted of patient-derived continuous tumor cells of both CRC and 1 GI cell line of the neuro-endocrine origin (NET), with the large majority being colorectal adenocarcinoma cells.

All of the cell lines studied were found to be tumorigenic in nude mice, usually forming tumors within a 3 week period. There is a pair of cells studied with particularly interesting qualities and relationship. Cell line SW480 was established from a primary adenocarcinoma of the colon in a 50 year old Caucasian male with expression of EGF, TGF-β, carcinogenic embryonic antigen (CEA), as well as oncogene mutations in p53 and Myc. Cell line SW620 was established from the same patient 1 year later derived from lymph node metastasis. This cell line expressed only small quantities of CEA and consisted of small spherical cells lacking microvilli.

This would be a unique pair to evaluate activity of NSGMs on since the molecular phenotype would be dramatically altered in the metastatic tumor, yet with genetic identify remaining constant.

Cell Lines	CMS	Phenotype	TP53	KRAS	BRAF	PIK3CA	PTEN	MSI	CIMP
COLO 205	1	MSI Immune	p.Y107fs; p.Y103fs	wt	p.V600E	wt	wt	MSS	+
KM-12	1	MSI Immune	p.P72fs; p.H179R	wt	p.P403fs	wt	p.G129X; p.K267fs	MSI	+
LOVO	1	MSI Immune	wt	p.G13D; p.V14A	wt	wt	wt	MSI	-
HCT15	1	MSI Immune	p.S241F	p.G13D	wt	p.E545K; p.D549N	wt	MSI	+
LS1034	2	Canonical	p.G245S	p.A146T	wt	wt	wt	MSS	-
NCI-H508	2	Canonical	p.R273H	wt	p.G596R	p.E545K	wt	MSS	-
SW1116	2	Canonical	p.A159D	p.G12A	wt	wt	wt	MSS	-
HT-29	3	Metabolic	p.R273H	wt	p.V600E; p.T119Sc	wt	wt	MSS	+
WiDr	3	Metabolic	p.R273H	wt	p.V600E; p.T119Sc	wt	wt	MSS	+
LS174T	3	Metabolic	wt	p.G12D	p.D211Gc	p.H1047R	wt	MSI	-
HCT116	4	Mesenchymal	wt	p.G13D	wt	p.H1047R	wt	MSI	+
SW620	4	Mesenchymal	p.R273H; p.P309S	p.G12V	wt	wt	wt	MSS	-
SW480	4	Mesenchymal	p.R273H; p.P309S	p.G12V	wt	wt	wt	MSS	-
RKO	4	Mesenchymal	wt	wt	p.V600E	p.H1047R	wt	MSI	+
CNDT2.5	NET	NA	NA	NA	NA	NA	NA	NA	NA

Table 3. Characteristics of cell panel used for screening; Representation of the cell line, consensus molecular subtype (CMS), mutational status of common CRC genes, and the microsatellite and chromosomal stability status. Cell line CNDT2.5 is a NET that was not characterized in the referenced study but used in this evaluation.

ii. Hypothesis of G2.2 Screening

The hypothesis for the screening of G2.2 against a panel of cancer cells was that G2.2 would continue to be a potent and selective inhibitor of CSC-rich spheroids across the panel. G2.2 would display a selectivity in the potent inhibition of 3D-spheroids, lacking such potency in the inhibition of monolayer culture of highly differentiated 2D traditional cell culture. These 3D spheroids, with the rich expression of tumor initiating CSCs, would emphasize the selective

targeting of G2.2 for CSCs. This hypothesis was extrapolated from and based on the previous work performed in the lab.

b. Materials and Methods

Cell Culture.

All patient derived cancer cells were obtained from ATCC with cell line authentication performed by either supplier or performed in house. The cells used in the study were maintained in 10 cm tissue culture treated plates (USA Scientific) as monolayer culture in the following media per supplier instructions: Dulbecco's Modified Eagle Medium – Nutrient Mixture F-12 (DMEM:F-12; cell lines HT-29, HCT116, WiDr, KM12,), Eagle's Minimum Essential Medium (EMEM; cell lines LS174T, RKO), RPMI-1640 Medium (cell lines LS1034, NCI-H508, Colo-205, HCT15), Kaighn's Modification of Ham's F-12 Medium (F-12K; cell line LoVo), and Leibovitz's L-15 Medium (L-15; cell lines SW620, SW480, SW1116). Complete growth medium was prepared for all with addition of 10% fetal bovine serum (FBS; Gibco) and 1% streptomycin/penicillin (AA; Gibco). The cells were passaged using trypsin containing ethylenediaminetraacetic acid (EDTA, Gibco) before they reached 70% confluency.

Cell Proliferation Assay (Selectivity).

Cell proliferation was evaluated by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT cell proliferation assay. For cell lines evaluated, approximately $2.5x10^3$ cells/100 μL/well were plated in 96-well tissue culture treated plates. After overnight incubation at 37° C. with 5% CO_2 vehicle (control) or NSGM was added at the desired concentration and the cells

were further incubated for 60-72 h. At the end of the incubation, 30 µL of 5 mg/mL MTT solution (Sigma) made in phosphate buffered saline (PBS; Gibco) was added to each well and incubated for a minimum of 2-3 hr. until crystal formation was observed. Following this, cell culture media and MTT solution were discarded from each well. Next, 100 µL of dimethyl sulfoxide (DMSO, Sigma) was added drop wise to each well and the mixture was gently aspirated to ensure crystals completely dissolved to give homogenous solution. Finally, the plate was placed on the spectrophotometer and absorbance values were read at 590 nm. Growth inhibition was calculated as percent of control.

Primary (1°) Spheroid Inhibition Assay.

For primary spheroid formation, cells (no more than 6 passages) were plated in non-treated, low adhesion, 96-well plates (USA Scientific) at a cell density ranging from 100 cells/100 µL/well – 300 cells/100 µL/well depending on optimum density for cell growth. Cells were plated in stem cell media (SCM) that consisted of DMEM:F12:AA (Gibco), supplemented with 1xB27 (Gibco; 20 ng/mL epidermal growth factor (EGF; Sigma), 10 ng/mL fibroblast growth factor (FGF; Sigma)). After a brief period of incubation, vehicle (control) or NSGM at the desired concentration were aliquoted to each well (conditions plated in technical triplicate for each sample). On day 3-8 (spheroid formation for control wells varied for each cell line and were monitored), the numbers of spheroids ranging from 50-150 µm in diameter were counted using a phase contrast microscope and percent inhibition was calculated compared to control.

Limiting Dilution Assay.

The limiting dilution assay was performed with the intent to evaluate the spheroid formation frequency (propensity) under spheroid culture conditions used in primary spheroid growth. Cell lines were plated in low-adhesion 6-well non-treated plates (USA Scientific) using previously defined stem cell media conditions at approximately 1000 cells/well. After a period of 3-5 days (depending on rate of growth for primary spheroids) the cells were harvested from the plate. Cells were washed in phosphate buffered saline (PBS; Gibco) and mechanically dissociated with vigorous pipetting. Once fully dissociated into single cells, cells were counted manually using Trypan Blue stain (Fisher Scientific, 0.4%) and hemocytometer to determine density. Cells were then seeded into 96-well non-treated plates (USA Scientific) at varying cell densities (1 cell/well to 128 cells/well) under triplicate conditions. After 3 days, the formation of secondary spheroids was evaluated with a simple yes/no indication at that density. Analysis of the tumor initiating frequency in-vitro was calculated using the Extreme Limiting Dilution Analysis software (http://bioinf.wehi.edu.au/software/elda/). Cell lines that are able to form spheroids at lower seeding densities are said to have greater spheroid formation frequency and tumor initiating capacity.

Western Blotting Analysis.

Western blot analysis was performed according to the standard protocol described in the literature. Briefly, cells were plated in serum-free SCM in a low adhesion 6-well plate to obtain spheroids. Mature spheroids were treated on day 4 after plating, with vehicle or NSGMs for indicated time and cells were solubilized in lysis buffer (20 mM Na3PO4, 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 2.5 mM Na3VO4) containing protease (Roche) as well as phosphatase

inhibitor cocktails (Sigma). Following centrifugation at 10,000 g for 20 min, the supernatant was used for Western blot analysis. In all analyses, protein concentration was determined by the Bio-Rad Protein Assay kit (Bio-Rad). Approximately 25 μg of protein was separated by polyacrylamide gel electrophoresis and was transferred to PVDF membrane (Bio Rad). Blocking was done with 5% bovine serum albumin (Sigma) for 1 h followed by overnight incubation with primary antibody (dilution 1:1000): anti-CD44 (Cell Signaling), anti-LGR5 (Origene), anti-CD133 ((Miltenyi Biotec), anti-BMI-1(Millipore), anti-c-MYC (Millipore), anti-Vimentin (Cell Signaling), anti-N-Cadherin (Cell Signaling), anti-E-Cadherin (Cell signaling), and anti-β-Catenin (Cell Signaling). This was followed by incubation with appropriate secondary antibody and protein bands were visualized using the enhanced chemiluminescence detection system and imaged with LAS-3000 Imaging System (FUJIFILM). Densitometry was determined by ImageJ analyzer software and results were calculated as relative intensity compared to control. All experiments were performed at least in duplicate conditions, triplicate where applicable.

NSGM Synthesis

NSGM G2.2 was synthesized as previously described and supplied for this study as needed.[15, 228]

c. Experiment Results

i. Primary Spheroid Inhibition in CRC Panel

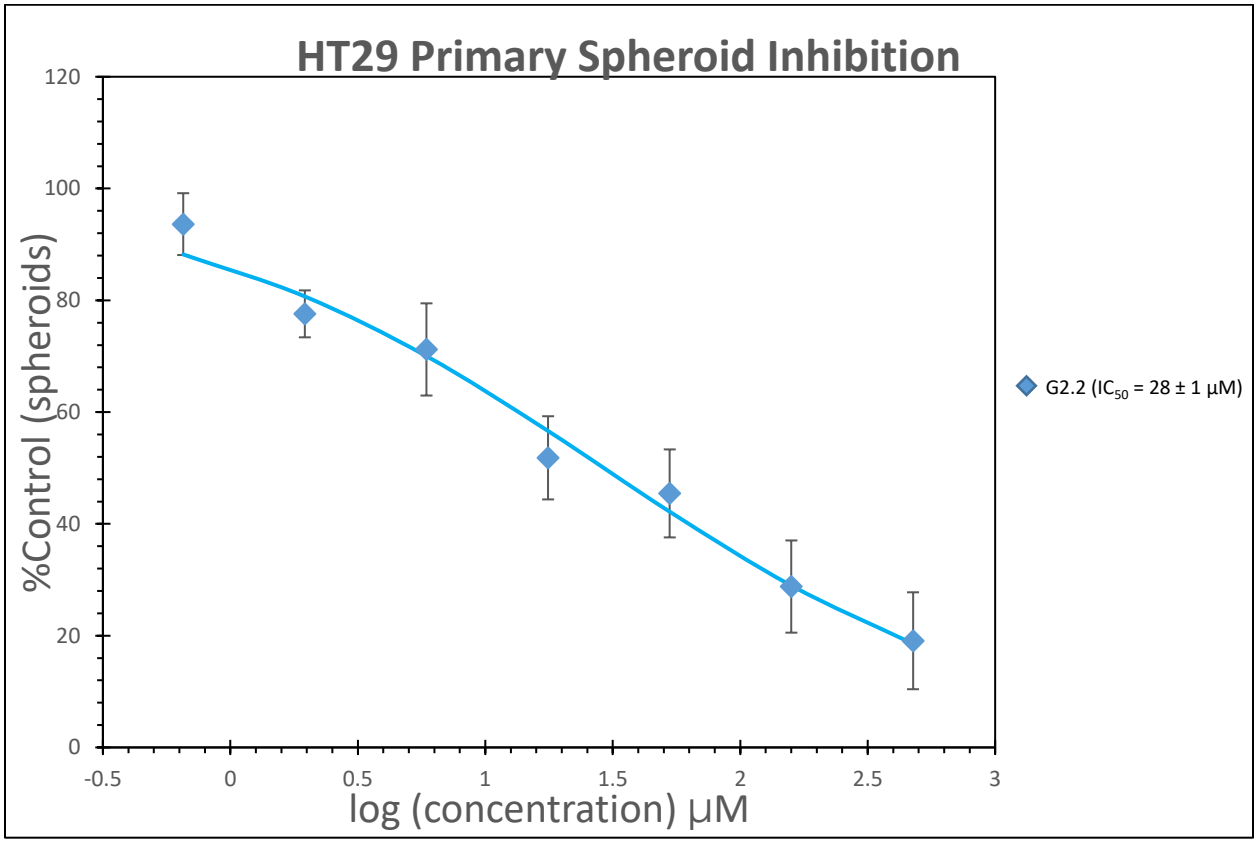


Figure 23. Primary spheroid inhibition curve of NSGM G2.2 on HT-29 (CMS 3) CRC cells.

The first cell line to be screened was in HT-29 cells. This was previously evaluated and proposed to be a good indication of assay competency. From these results (performed in triplicate, 1 biological replicate), screening proceeded with the remaining 14 cell lines. The next few pages will show figures from cell lines representing different CMS, followed by a summarization graph of all cell lines screened under spheroid conditions. Data tables will follow

including standard error. Graphs were made in Excel based off of data calculated from Prism (GraphPad).

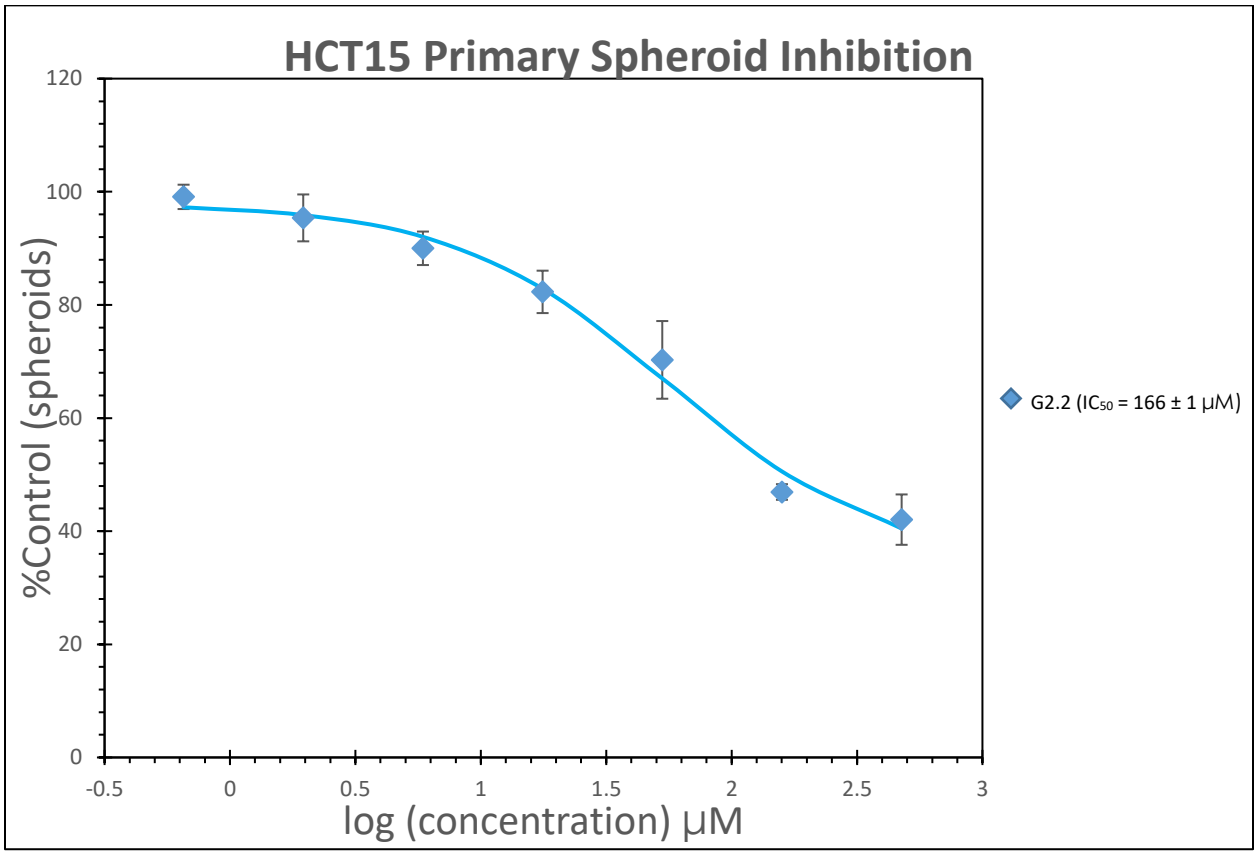


Figure 24. Primary spheroid inhibition curve of NSGM G2.2 on HCT15 (CMS 1) CRC cells.

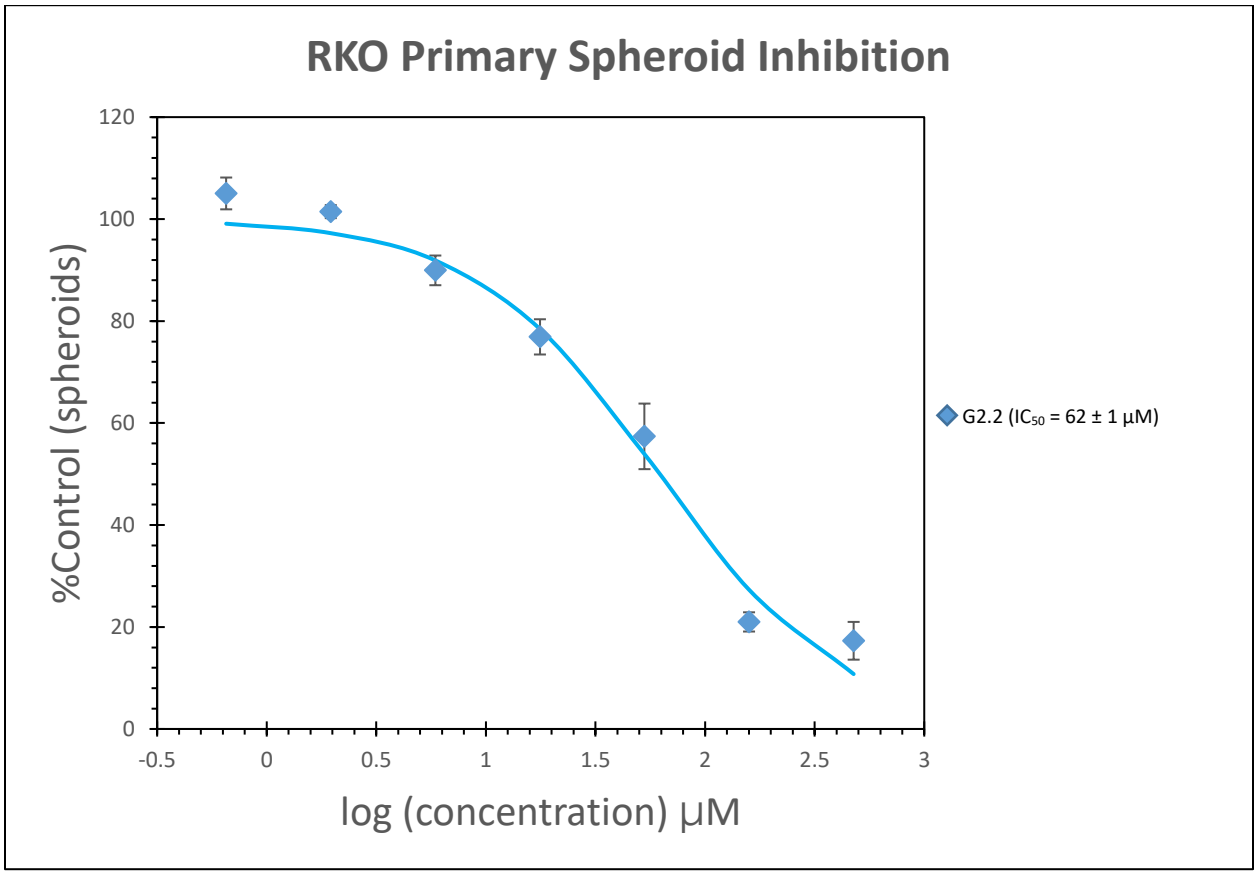


Figure 25. Primary spheroid inhibition curve of NSGM G2.2 on RKO (CMS 4) CRC cells.

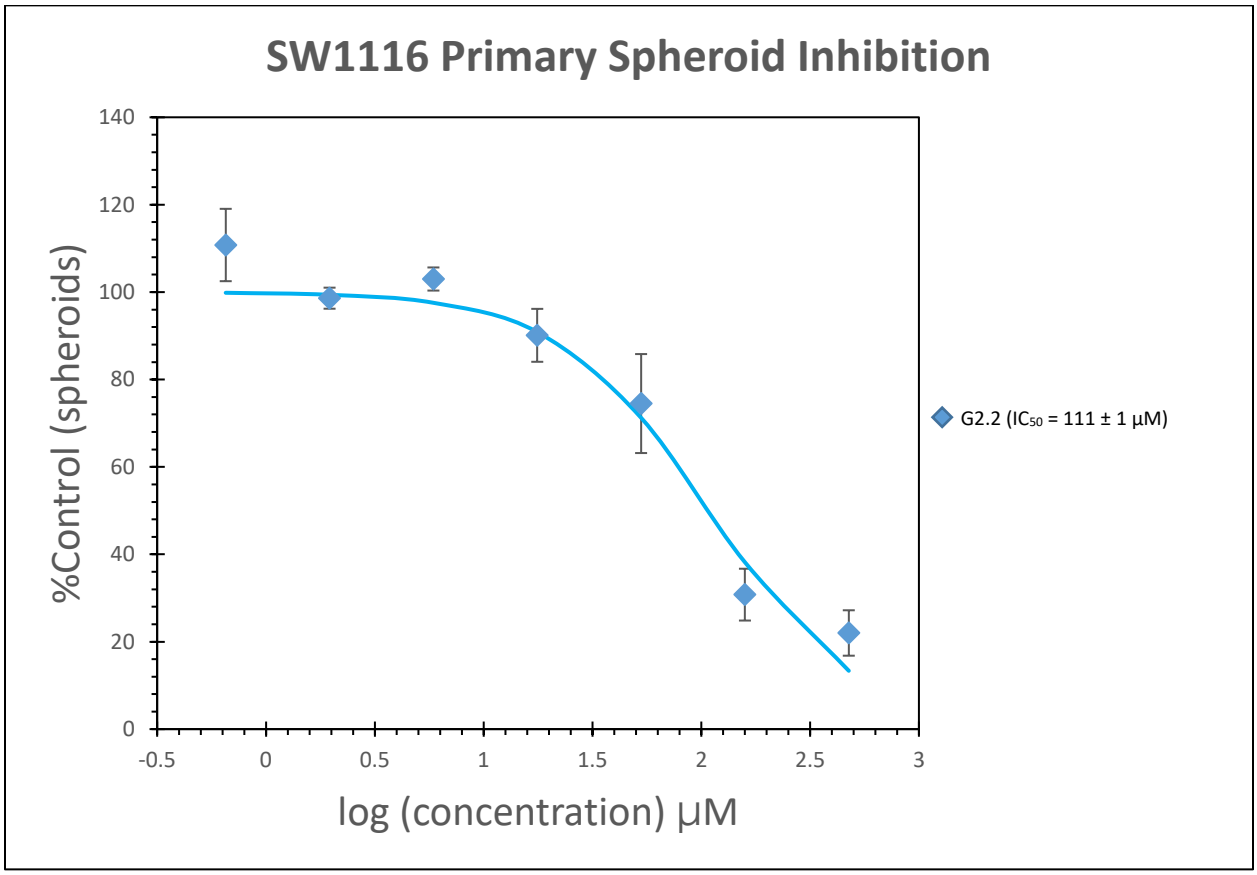


Figure 26. Primary spheroid inhibition curve of NSGM G2.2 on SW1116 (CMS 2) CRC cells.

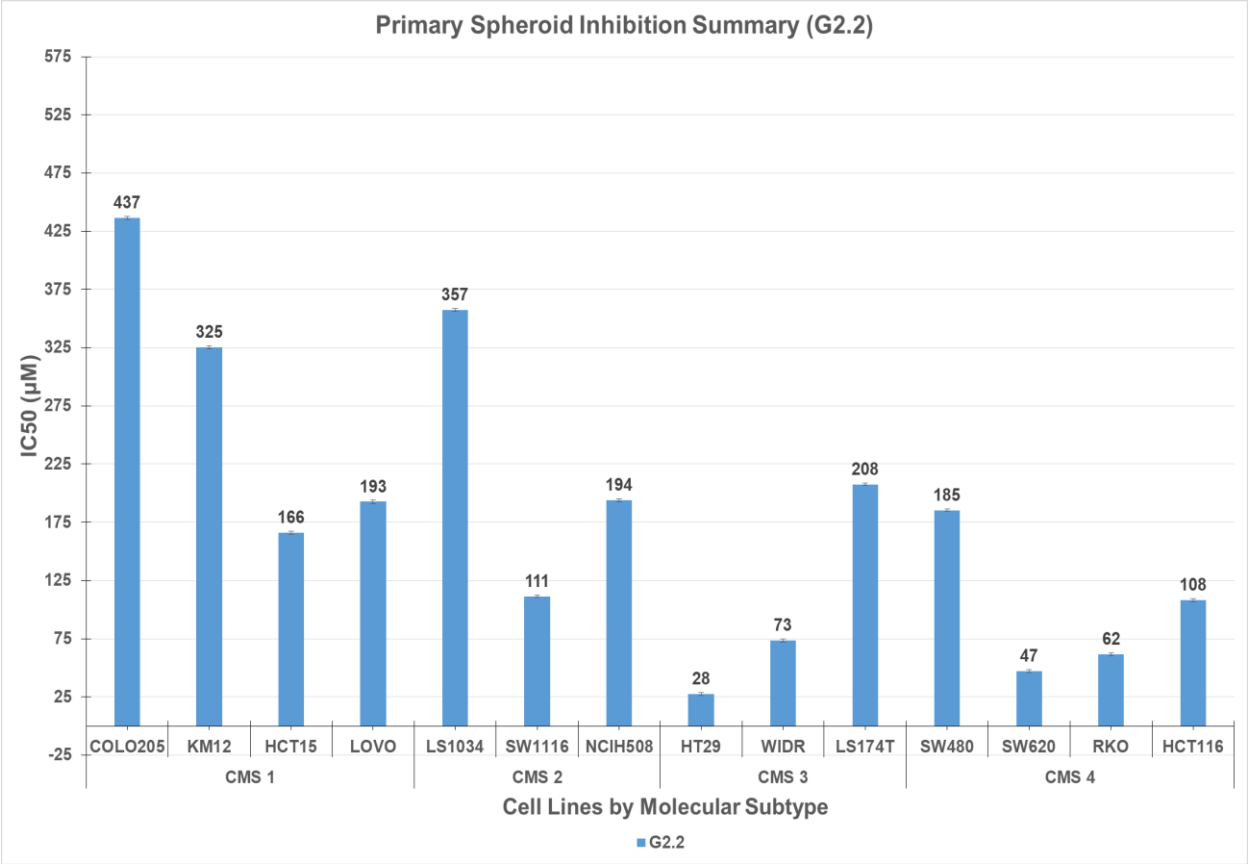


Figure 27. Summary of primary spheroid inhibition potency values by cell line for NSGM G2.2.

Table 4. Table of primary spheroid inhibition potency values (µM) determined from standard sigmoidal 3-pt. curve in GraphPad Prism with standard deviations (± 1 SE)

Primary Spheroid Inhibition Values (µM)								
	CMS 1				CMS 2			
	COLO205	KM12	HCT15	LOVO	LS1034	SW1116	NCIH508	
G2.2	437 ± 1	325 ± 1	166 ± 1	193 ± 1	357 ± 1	111 ± 1	194 ± 1	
	CMS 3			CMS 4				NET
	HT29	WIDR	LS174T	SW480	SW620	RKO	HCT116	CNDT2.5
G2.2	28 ± 1	73 ± 1	208 ± 1	185 ± 1	47 ± 1	62 ± 1	108 ± 1	553 ± 1

ii. Spheroid Selectivity (2-D Cytotoxicity)

To evaluate the selectivity of G2.2 toward CSC-rich 3-D spheroid culture conditions, NSGM was evaluated at the same concentrations as in spheroid inhibition assays on traditional 2-D cell culture growth of cell lines selected to represent each CMS. The data below show comparatively the primary spheroid inhibition potency against the concentration of NSGM inhibiting 50% growth of monolayer culture.

Table 5. Table of primary spheroid inhibition potency values compared to the 50% toxicity value from MTT assay data for NSGM G2.2

	Primary Spheroid IC50 Values (µM)			
	KM12	**LS1034**	**HT29**	**SW620**
G2.2	325.4	357.3	27.56	46.96
	Monolayer Culture 50% Toxicity (µM)			
	KM12	**LS1034**	**HT29**	**SW620**
G2.2	125	>476	>476	>476

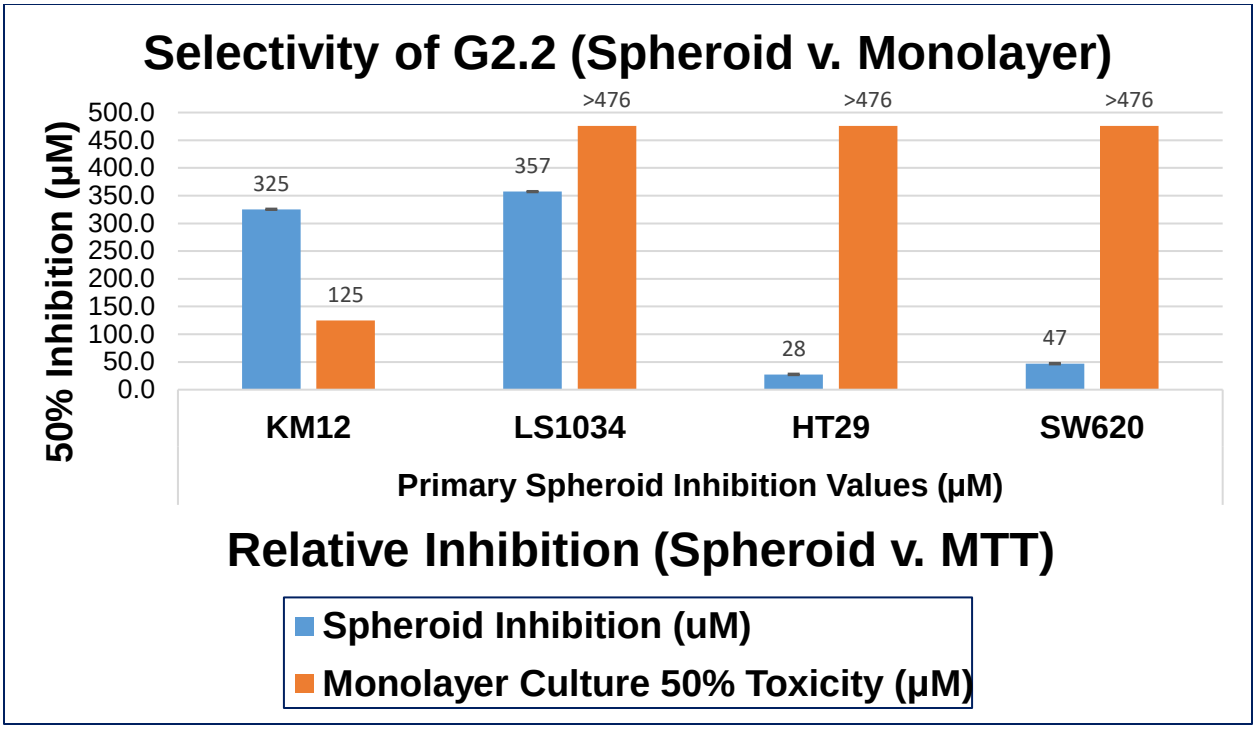


Figure 28. Bar graph comparing the potency of NSGM G2.2 to inhibit primary spheroids (blue) vs. the concentration required for 50% toxicity (red) in monolayer culture of respective cell line. Note that ~ 476 µM was the highest concentration evaluated and therefore the toxicity of G2.2 in monolayer cells LS1034, HT-29, and SW620 could be higher than shown.

iii. Limiting Dilution Assay Results (Spheroid Formation Frequency)

The limiting dilution assay is performed to evaluate the tumor initiating properties of various cell lines by assessment of spheroid growth. Cells are harvested from primary spheroid conditions and seeded as secondary spheroids (self-renewal) at varying cell densities. After a pre-determined growth period, wells are evaluated for the presence of spheroids. Those cell lines that are able to generate spheroids at a lower seeding density have a greater spheroid formation frequency and enhanced tumorigenic properties in-vitro. The following table is a summary of

the results obtained by Dr. Chetna Sharon, who directed, executed, and analyzed the experiment with the assistance of Connor O'Hara.

Table 6. Table of results from limiting dilution assay as well as mean potency of G2.2 by cell line.

Cell Line	CMS	Fraction	%	G2.2 IC50
COLO-205	1	1/4.1	24.3	436.5
KM-12	1	1/5.7	17.5	325.4
LOVO	1	1/16.7	6	192.7
HCT15	1	1/2.5	40.8	165.9
SW1116	2	1/25.1	4	111.4
LS1034	2	16-Jan	6.4	357.3
NCI-H508	2	1/1.8	54.6	194.1
HT-29	3	1/2.8	35	27.56
WiDr	3	1/2.8	35.2	73.38
LS174T	3	1/1.9	52	207.6
HCT116	4	1/3.1	32	108.1
SW620	4	1/2.5	40	46.96
SW480	4	6-Jan	16.5	185.4
RKO	4	1/3.2	31.4	61.54
CNDT2.5	NET	1/12.5	8	552.7

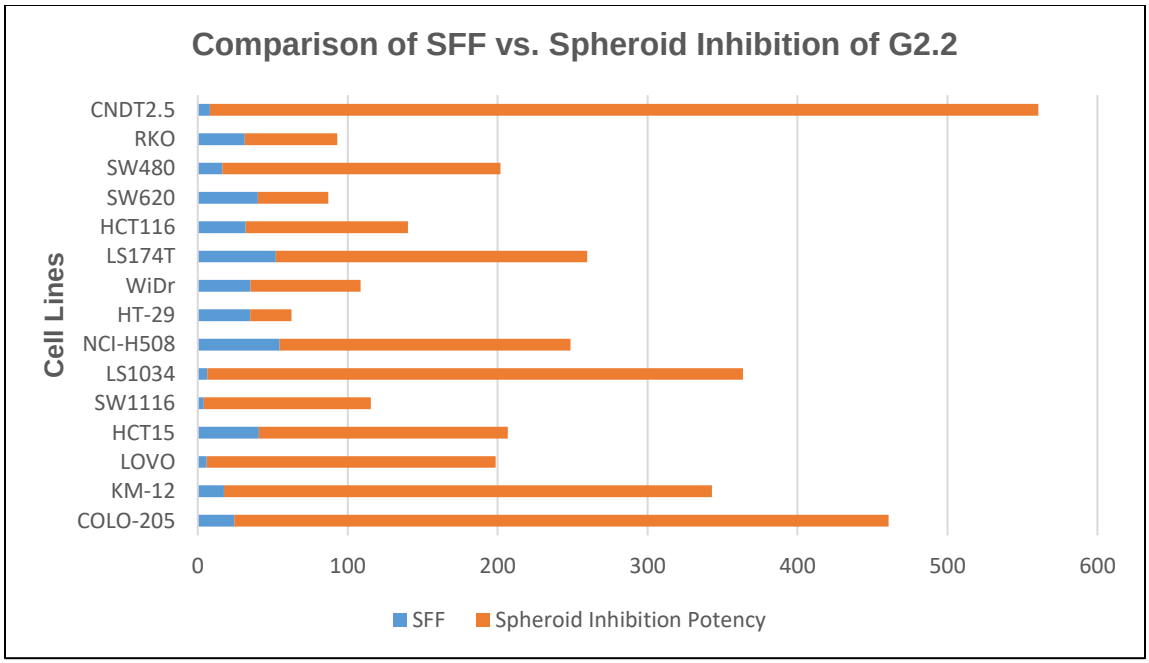


Figure 29. Graph of SFF values (blue) compared to 50% spheroid inhibition values (orange) to help visualize the comparison between potency of G2.2 and tumorigenicity of cell lines.

iv. Mechanistic Studies

1. Characterization of CSC/EMT Markers of Cells

After observing the effects of G2.2 treatment across the panel under spheroid conditions and select cell lines under monolayer conditions, we hoped to better understand the mechanism by which G2.2 was working and the phenotype of the cells G2.2 was acting on. The following figures represent the protein expression of CSC markers after spheroid treatment of G2.2 vs. control, and the basal protein expression of EMT markers on cells under monolayer culture to better characterize the actions of G2.2.

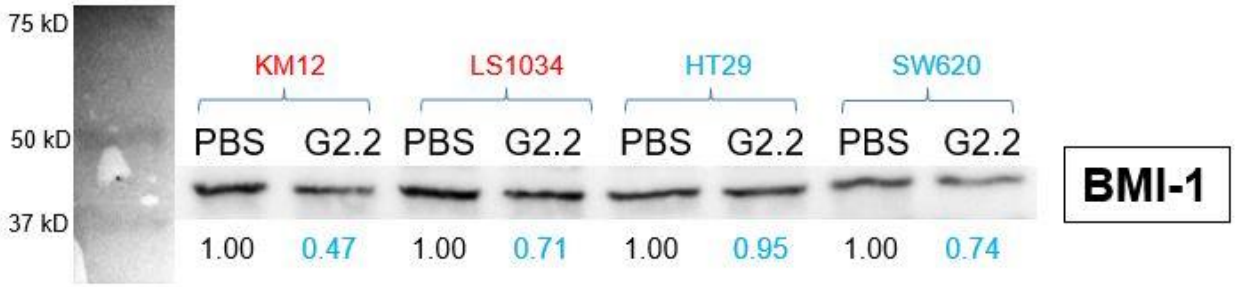


Figure 30. Western blot of cell lines from each CMS (1-4) showing reduction in expression of CSC self-renewal marker BMI-1 after treatment of G2.2 (100 μM) compared to vehicle (PBS). All samples were cultured in 10-cm non-treated plates, and protein expression adjusted based on the relative density of housekeeping protein GAPDH.

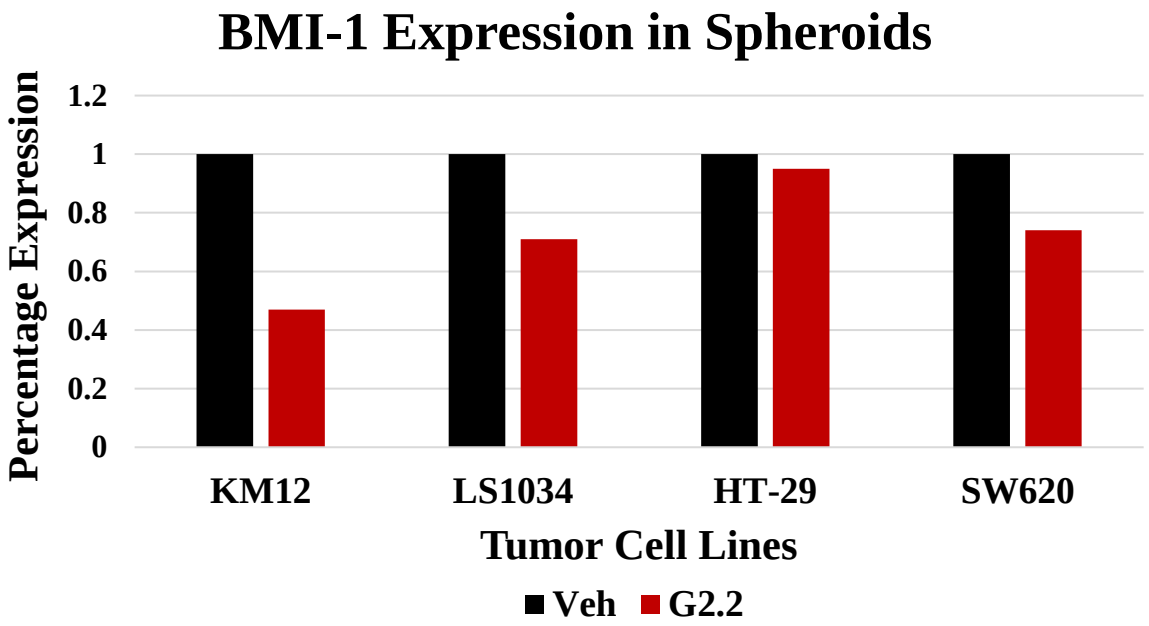


Figure 31. Bar graph of western blot of cell lines from each CMS (1-4) showing reduction in expression of CSC self-renewal marker BMI-1 after treatment of G2.2 (100 μM) compared to vehicle (PBS). All samples were cultured in 10-cm non-treated plates, and protein expression adjusted based on the relative density of housekeeping protein GAPDH. This data represents one membrane of protein expression in single replicate from samples run on 1 polyacrylamide gel.

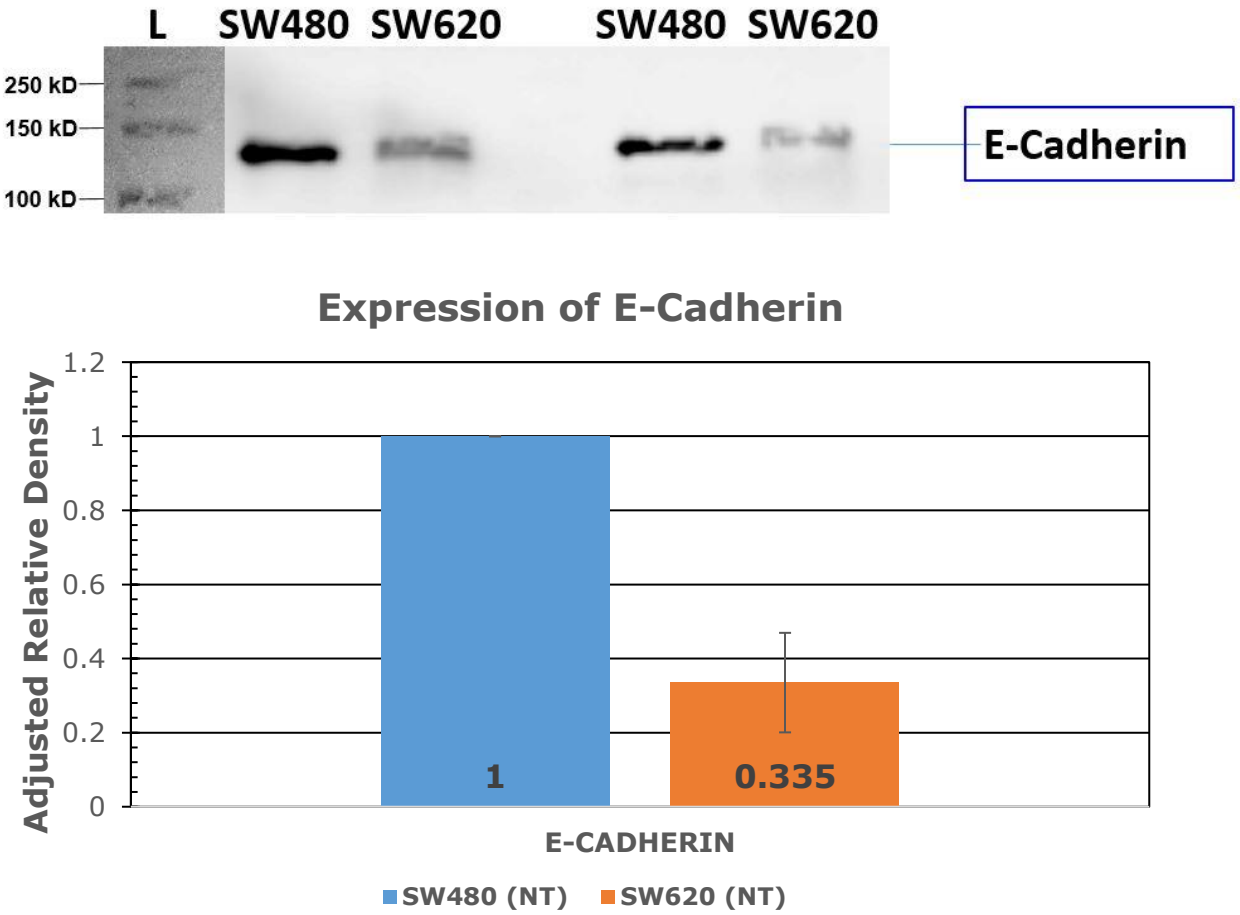


Figure 32. Western blot and bar graph of non-treated samples of SW480/SW620 CRC cells showing their basal expression of E-Cadherin, a marker of epithelial cell-cell adhesion. SW480 cells are derived from the primary site of a patient with CRC, whereas SW620 are derived from that same patient 1 year later at a lymph node metastasis. Sample densities were adjusted based on the loading control Cyclophilin B.

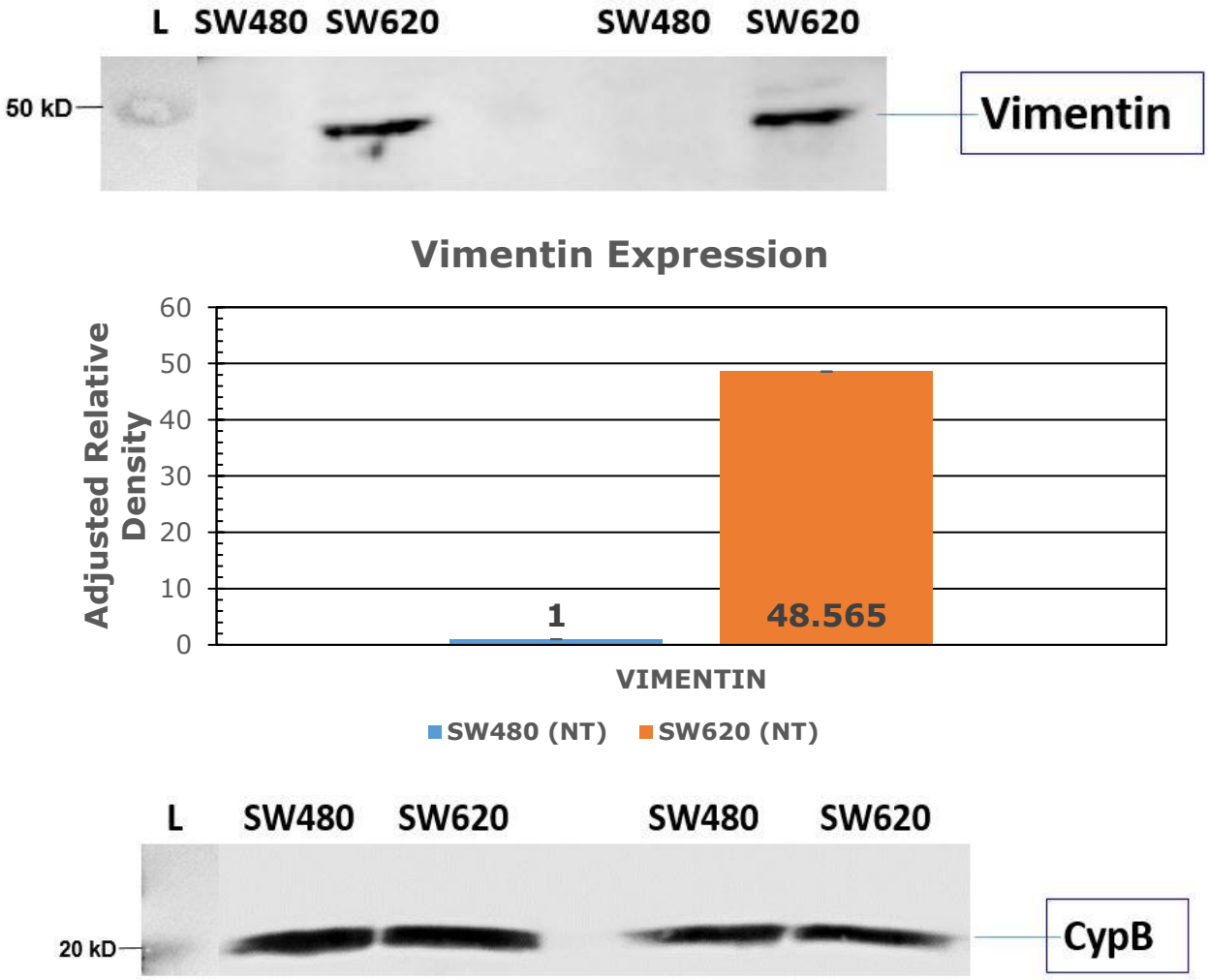


Figure 33. Western blot and bar graph of non-treated samples of SW480/SW620 CRC cells showing their basal expression of Vimentin, a mesenchymal and developmental filament protiein. SW480 cells are derived from the primary site of a patient with CRC, whereas SW620 are derived from that same patient 1 year later at a lymph node metastasis. Sample densities were adjusted based on the loading control Cyclophilin B (also shown). Proteins E-Cadherin and Vimentin were evaluated on different portions of membrane from the same polyacrylamide gel.

Figure 34. Western blot of non-treated samples of SW480/SW620 CRC cells showing their basal expression of N-Cadherin, a marker of mesenchymal development. No densitometry could be accurately performed on these samples, however it is clear to see the marked intensity of N-Cadherin in SW620 samples compared to non-existent expression in SW480.

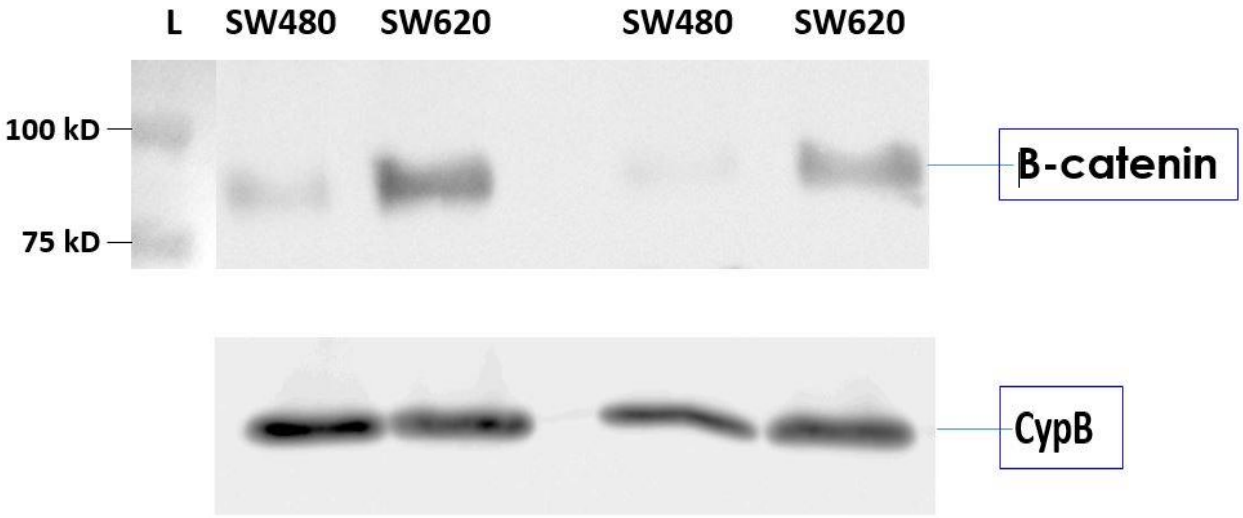


Figure 35. Western blot of non-treated samples of SW480/SW620 CRC cells showing their basal expression of β-Catenin, a downstream effector in Wnt signaling pathway implicated in early embryonic development and tumorigenesis. Although no densitometry was taken for these samples, there is marked expression intensity of the protein in SW620 samples with minimal expression in SW480 sample, indicating the mesenchymal nature of SW620 cells. Expression of housekeeping Cyclophilin B indicates appropriate loading of each sample.

d. Analysis and Discussion

i. Primary Spheroid Inhibition in a Panel of CRC Cells

Primary spheroid inhibition assays of CSC-rich 3-D tumor spheroids revealed a response profile that correlated with molecular phenotype of different cell lines, suggesting a selective targeting mechanism. Across the panel of cells screened, G2.2 displayed a mean IC_{50} = 203 μM (SE = ± 44 μM, SD = ± 152 μM) and a median IC_{50} = 184 μM (SEM = ± 55 μM). In the most sensitive cell line, HT-29 (CMS 3), G2.2 had a potency of ~ 28 μM (SD = ± 1 μM). In the least sensitive cell line that belonged to a molecular subtype identified, Colo-205 (CMS 1), G2.2 held a potency of ~ 434 μM (SD = ± 1 μM).

These results suggest that the effects of G2.2 can be divided into 3 unique responses (Figure 32): a sensitive response (4 cell lines), a moderately resistant response (7 cell lines), and a resistant response (4 cell lines; $x < 100$ μM $< y < 300$ μM $< z$). Cell lines from the sensitive response group were composed of all CMS 3 (HT-29, WiDr) and CMS 4 (RKO, SW620) cell lines. Cell lines in the resistant group were composed of all CMS 1 (KM-12, Colo-205) and CMS 2 (LS1034) cell lines, as well as the NET GI tumor CNDT2.5. Cells in the sensitive group held a mean IC_{50} = 52 μM (SD = ± 20 μM), while cells in the resistant group held a mean IC_{50} = 418 μM (SD = ± 101 μM).

The results observed in the primary spheroid inhibition assays show that G2.2 preferentially targets cells belonging to CMS 3/4, which are observed to have enrichment of CSC phenotype and signaling pathways as well as poor clinical prognosis. G2.2 was over 7-fold more potent in

cell lines that represented this phenotype, in comparison to the canonical and more immunogenic cells of the other molecular subtypes.

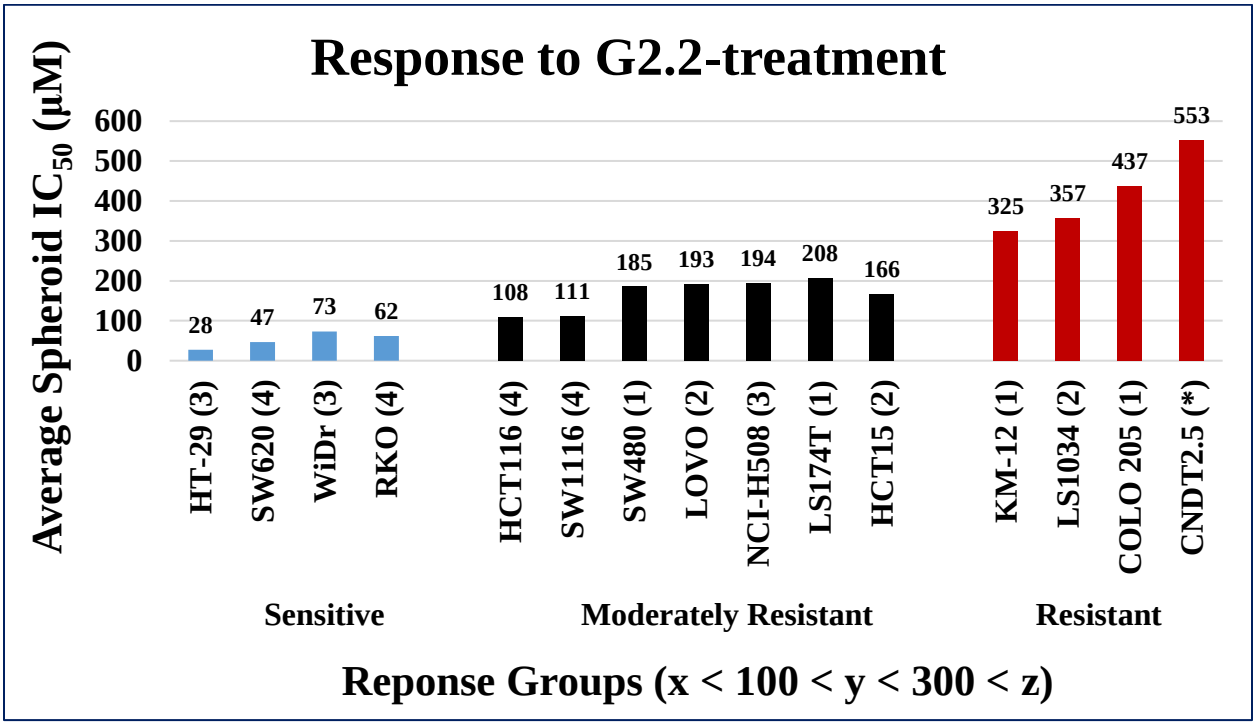


Figure 36. Graphical representation of the 3 unique responses of cells in the spheroid panel to treatment with G2.2. Cells were divided based on their sensitivity to the NSGM; the cells that were resistant to G2.2 were composed of CMS 1/2, while cells sensitive to G2.2 were composed of CMS 3/4 phenotype.

ii. Spheroid Selectivity (2-D Cytotoxicity)

In selectivity assays measuring toxicity of compounds in representative monolayer cancer cell cultures, limited toxicity was observed compared to the IC_{50} values in corresponding spheroid cells. The results of the monolayer cytotoxicity assays revealed that in 75% of the cell lines

screened, there was a preferential targeting of cells under the CSC-rich spheroidal conditions. The toxicity of G2.2 on monolayer complete growth media culture appeared to depend upon the molecular phenotype and CMS of the cell line evaluated. Cells from CMS 3 and 4, which have CSC-characteristics and poor clinical prognosis compared to CMS 1 and 2, were over 10-fold more sensitive to G2.2 when cultured under spheroidal conditions compared to the highly differentiated monolayer conditions. This indicates that there is some specific CSC-dependent mechanism that G2.2 is acting, or that cells from CMS 3/4 have some particular phenotypic indicator that is involved in the mechanism of action G2.2 has as an anti-cancer and anti-CSC compound.

iii. Limiting Dilution Assay Results (Spheroid Formation Frequency)

Results from limiting dilution assays indicate that, on a broad scale, cell lines sensitive to G2.2 treatment are up to 2-fold more likely to develop into tumorigenic spheroids than cells belonging to a resistant response group. When cell lines are organized based on their response to SFF, 80% of those cells are sensitive to G2.2 and make up CMS 3/4 (Figure 36). In comparing the average SFF to the average spheroid potency between the 3 response profiles, the G2.2 sensitive cells have over a 2-fold greater tumor initiating potential or SFF (35.4 ± 3.5). Interestingly, cells belonging to the moderately resistant profile ($300 > IC_{50} > 100$ μM) are composed over every CMS and had a much wider range of SFF values from the limiting dilution assay (Figure 34). Some of the cells, like NCI-H508, HCT-15, and LS174T are able to develop rapidly into spheroids and generate beautiful spheres in-vitro. With the exception of LS174T (CMS 3), those cells all belong to CMS 1/2, which do not have the tumorigenic CSC phenotype observed in the

other subtypes. More characterization of the differences observed within the moderately resistant class of cells would be key to understanding the subtle differences that direct these properties and the response to G2.2.

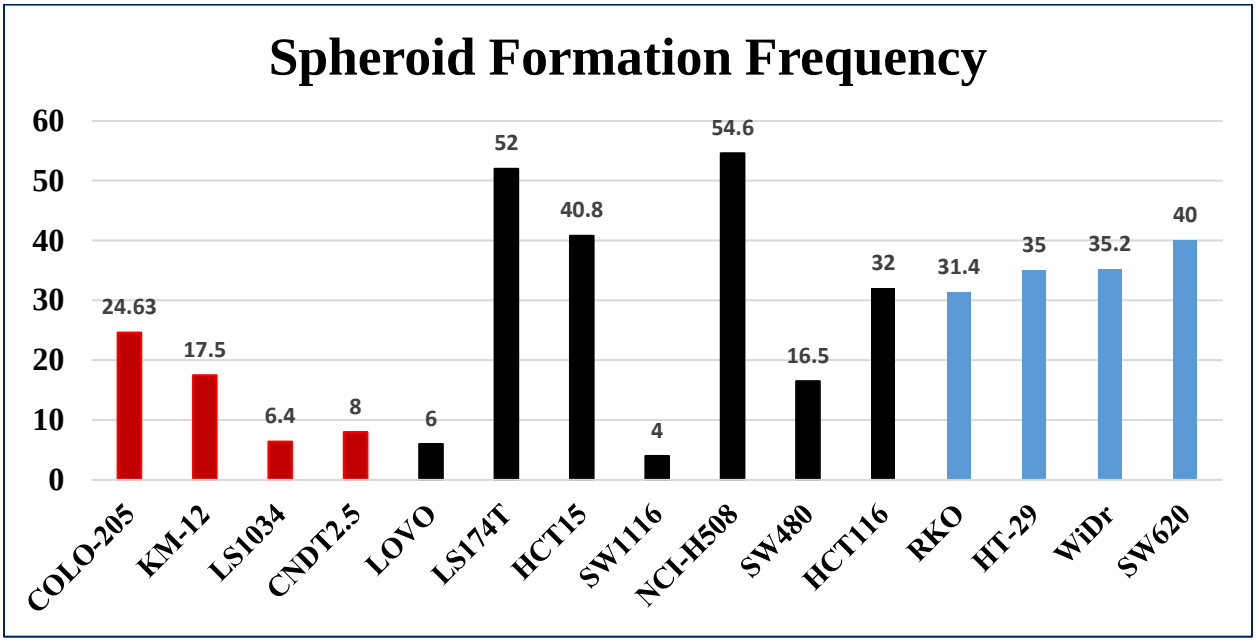


Figure 37. Graphical representation of the spheroid formation frequency determined by the limiting dilution assay. Overall, the cells that showed a sensitive response to G2.2 treatment (blue) were able to readily initiate tumor spheroid development, compared to those cells of the resistant response to G2.2 (red) which had relatively poor tumor initiating capacity.

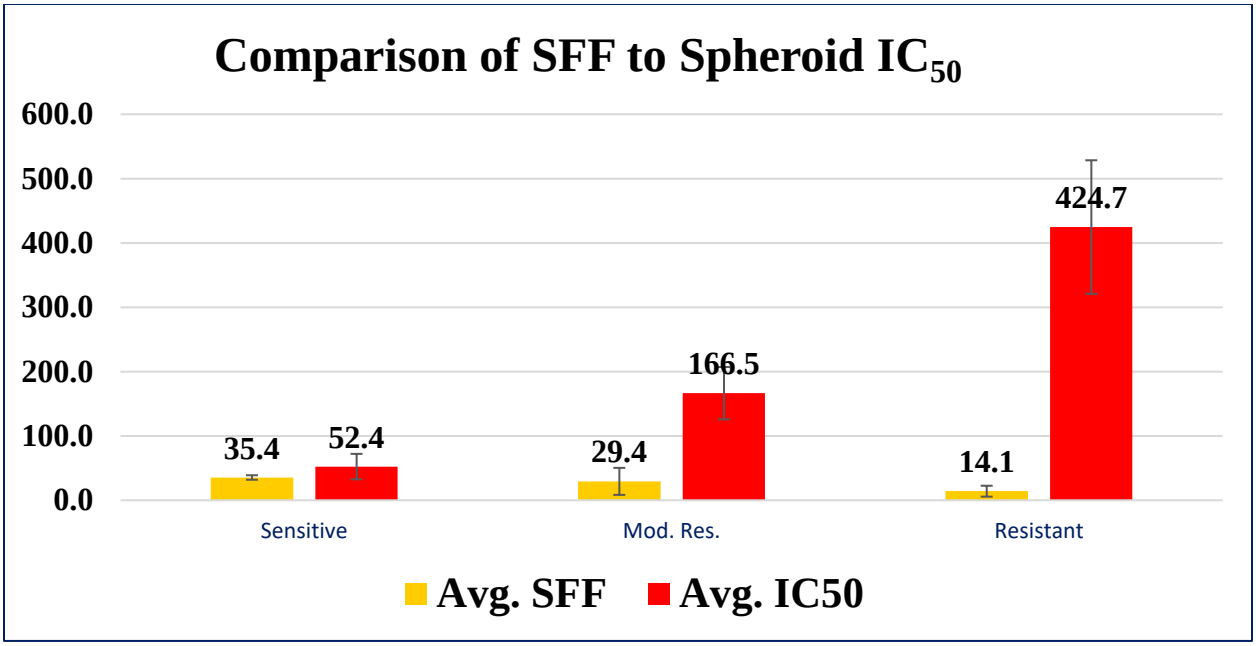


Figure 38. Graphical representation comparing the mean SFF (yellow) of each G2.2 response group to mean potency (red) of each group. STD Error = ± 1 SE. Sensitive response to G2.2 (< 100 μM), moderately resistant response to G2.2 (> 100 μM < 300 μM), and resistant response to G2.2 (> 300 μM) were clear across the panel to distinguish the response of each cell to G2.2 treatment.

iv. Mechanistic Studies

1. Characterization of CSC/EMT Markers of Cells

The western blotting data for expression of various markers of CSC phenotype and EMT profile revealed some very interesting observations. The self-renewal marker BMI-1 was observed to be under-expressed in all cell line samples treated with 100 μM G2.2, with line SW620 downregulated ~ 26% compared to cell treated with vehicle control (PBS). It is possible that the response would be even more pronounced if a more appropriate v/v of drug was aliquoted to the

10-cm plate that spheroids were grown and treated. During primary spheroid inhibition assays a 5 μL aliquot is added to 100 μL of SCM, resulting in the drug being 4.76% v/v in solution. In the treatment of spheroids, G2.2 is aliquoted to 10 mL of SCM. Even if mathematically the treatment of 5 μL a highly concentrated G2.2 reaches the desired concentration for treating the spheroids, the v/v% will be minimal in solution and the distribution of that drug may not be as efficient as required to observe significant changes to large scale protein expression. For this reason, in the future, aliquot of drug for treatment should be no less than 5% v/v of the total solution. Regardless, it was observed that treating each of the representative cell lines (CMS 1-4) with G2.2 decreased the expression of self-renewal marker BMI-1 that has been cited to contribute to the tumorigenic state of cancer and the CSC phenotype.

In the non-treated samples examining the basal protein expression in SW480/SW620 cells, promising results were observed. SW480/SW620 represent a unique patient-derived cell pair that allow very clear and validated phenotypic observations that, in this work, support the overall hypothesis that G2.2 targets CSCs and tumor initiating phenotypes. SW620 cells are derived from the metastatic lymph node site from the same patient that SW480 primary site tumor was harvested 1 year earlier. SW620 cells show ~ 3-fold reduced expression of epithelial cell-cell adhesion marker E-Cadherin while also showing over 48-fold enhanced expression of mesenchymal marker Vimentin. Additionally, SW620 showed intense expression of mesenchymal N-Cadherin as well as tumorigenic marker β-Catenin. G2.2 is ~ 4-fold more potent in primary spheroid inhibition assays in cell line SW620 as compared to primary tumor site SW480 cells. Overall, the protein expression observed from western blotting reinforces the

idea that G2.2 is a CSC-targeting agent with a preference in-vitro for cells that share a mesenchymal and stem-like phenotype.

e. Conclusions

The studies carried out evaluating the effects of NSGM G2.2 on CSC spheroids of CRC enhanced our understanding how G2.2 works. G2.2 remained a selective compound, specifically targeting 3D spheroids rich in CSCs with fold decreased potency in monolayer cell culture lacking CSC enrichment in respective cell lines. G2.2 was identified not to be potent in every cell line evaluated, but rather in a group of cells belonging to a molecular phenotype that is rich in CSC-characteristics and signaling as well as the tumorigenic outcomes that give poor clinical prognosis. Further, in this phenotype G2.2 is also incredibly selective at inhibiting the growth of 3D CSC-rich spheroids compared to differentiated monolayer cells. Those same cells also have fold-enhancement in their ability to generate spheroids, with high tumor initiating capacity. Western blots revealed that cells sensitive to G2.2 treatment are enriched in mesenchymal markers and stem-like profile. Treating spheroids with G2.2 reduced to various degrees the expression of self-renewal markers that are key in CSC regulation, as well as markers of mesenchymal stem-like growth and signaling. G2.2 has shown to be both a potent and selective inhibitor of CSCs, and further studies will be carried out attempting to better characterize these observations.

4. LIPID-MODIFIED ANALOGS SHOW IMPROVED POTENCY

a. Rationale

i. Synthesis of Lipid Modified Analogs

Previous studies in the lab revealed that G2.2 was a potent scaffold for which structural modification could be made to improve upon anti-cancer properties. It was discovered in PK work that G2.2 did not have the bioavailability that would be desired in an orally acting cancer therapy. As a result, efforts to synthesize analogs of G2.2 that would improve systemic bioavailability and enhance the "drug-like" PK as well as to enhance the potency of the NSGM were underway. Chemists in the lab synthesized analogs with various linkers connecting the dimeric G2.2 scaffold, as well as lipophilic moieties extending off of G2.2 to achieve these goals. With the hypothesis that G2.2 was likely targeting a transmembrane growth factor, a lipophilic addition to G2.2 would perhaps enhance the binding event of G2.2 to a protein within the phospholipid bilayer of the membrane. The resulting compounds, G2C, G5C, and G8C, have the addition of a cholesterol moiety replacing a terminal sulfate group on one of the monomers of G2.2 with alkyl linker of various length between the G2.2 dimer and the cholesterol. The structures of these analogs are shown in Figure 39 below.

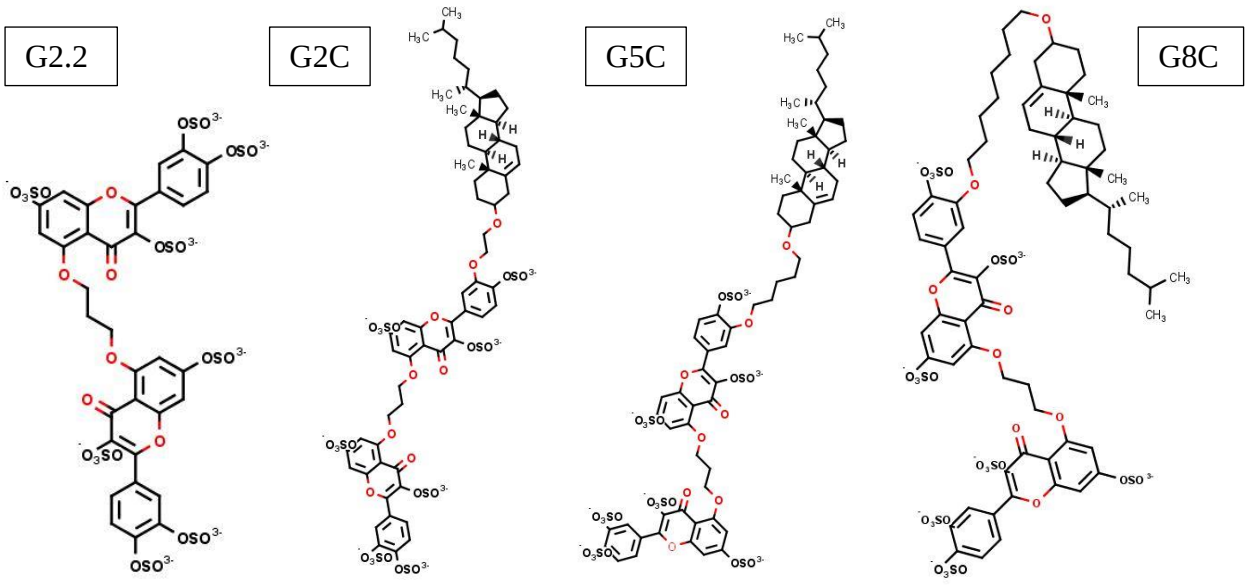


Figure 39. Structures of G2.2 and lipid modified analogs. Synthesized in the Desai Lab by Morla, S. and Afosah, D. K.

i. Hypothesis

The hypothesis for this study was that through addition of the lipophilic moiety on to the scaffold of G2.2, we would observe an improved performance in-vitro in targeting CSCs. It was proposed that there may also be some degree of chain length dependency with optimal potency, and that a trend would be observed in evaluating the length of the linker between G2.2 and cholesterol and the anti-CSC properties of the NSGM.

b. Materials and Methods

Cell Culture.

All patient derived cancer cells were obtained from ATCC with cell line authentication performed by either supplier or performed in house. The cells used in the study were maintained in 10 cm tissue culture treated plates (USA Scientific) as monolayer culture in the following media per supplier instructions: Dulbecco's Modified Eagle Medium – Nutrient Mixture F-12 (DMEM:F-12; cell lines HT-29, HCT116, WiDr, KM12,), Eagle's Minimum Essential Medium (EMEM; cell lines LS174T, RKO), RPMI-1640 Medium (cell lines LS1034, NCI-H508, Colo-205, HCT15), Kaighn's Modification of Ham's F-12 Medium (F-12K; cell line LoVo), and Leibovitz's L-15 Medium (L-15; cell lines SW620, SW480, SW1116). Complete growth medium was prepared for all with addition of 10% fetal bovine serum (FBS; Gibco) and 1% streptomycin/penicillin (AA; Gibco). The cells were passaged using trypsin containing ethylenediaminetraacetic acid (EDTA, Gibco) before they reached 70% confluency.

Cell Proliferation Assay (Selectivity).

Cell proliferation was evaluated by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT cell proliferation assay. For cell lines evaluated, approximately $2.5x10^3$ cells/100 µL/well were plated in 96-well tissue culture treated plates. After overnight incubation at 37° C. with 5% CO_2 vehicle (control) or NSGM was added at the desired concentration and the cells were further incubated for 60-72 h. At the end of the incubation, 30 µL of 5 mg/mL MTT solution (Sigma) made in phosphate buffered saline (PBS; Gibco) was added to each well and incubated for a minimum of 2-3 hr. until crystal formation was observed. Following this, cell

culture media and MTT solution were discarded from each well. Next, 100 µL of dimethyl sulfoxide (DMSO, Sigma) was added drop wise to each well and the mixture was gently aspirated to ensure crystals completely dissolved to give homogenous solution. Finally, the plate was placed on the spectrophotometer and absorbance values were read at 590 nm. Growth inhibition was calculated as percent of control.

Primary (1°) Spheroid Inhibition Assay.

For primary spheroid formation, cells (no more than 6 passages) were plated in non-treated, low adhesion, 96-well plates (USA Scientific) at a cell density ranging from 100 cells/100 µL/well – 300 cells/100 µL/well depending on optimum density for cell growth. Cells were plated in stem cell media (SCM) that consisted of DMEM:F12:AA (Gibco), supplemented with 1xB27 (Gibco; 20 ng/mL epidermal growth factor (EGF; Sigma), 10 ng/mL fibroblast growth factor (FGF; Sigma)). After a brief period of incubation, vehicle (control) or NSGM at the desired concentration were aliquoted to each well (conditions plated in technical triplicate for each sample). On day 3-8 (spheroid formation for control wells varied for each cell line and were monitored), the numbers of spheroids ranging from 50-150 µm in diameter were counted using a phase contrast microscope and percent inhibition was calculated compared to control.

NSGM Synthesis

NSGM analogs of G2.2 were synthesized as previously described and supplied for this study as needed.

c. Experiment Results

i. Primary Spheroid Inhibition in a Panel of Colorectal Cancer Cells

Screening of the lipid-modified analogs proceeded in the same manner as for G2.2. Cells were seeded as primary spheroids and the potency of each NSGM was determined on its ability to inhibit the formation of spheroids (50 – 150 μm) was compared against vehicle (PBS). The following will show data from select cell lines followed by a summary table and figure overviewing the responses of the lipid modified analogs against positive control G2.2. It should be noted that, due to the hypothesis of improved performance, lipid modified analogs were evaluated at lower potencies than G2.2 (476 μM – 30 nM). It should also be noted that G2.2 was evaluated on the same plate as the analogs in triplicate, and that all spheroid inhibition experiments were performed on the same biological replicate (G2.2 and analogs).

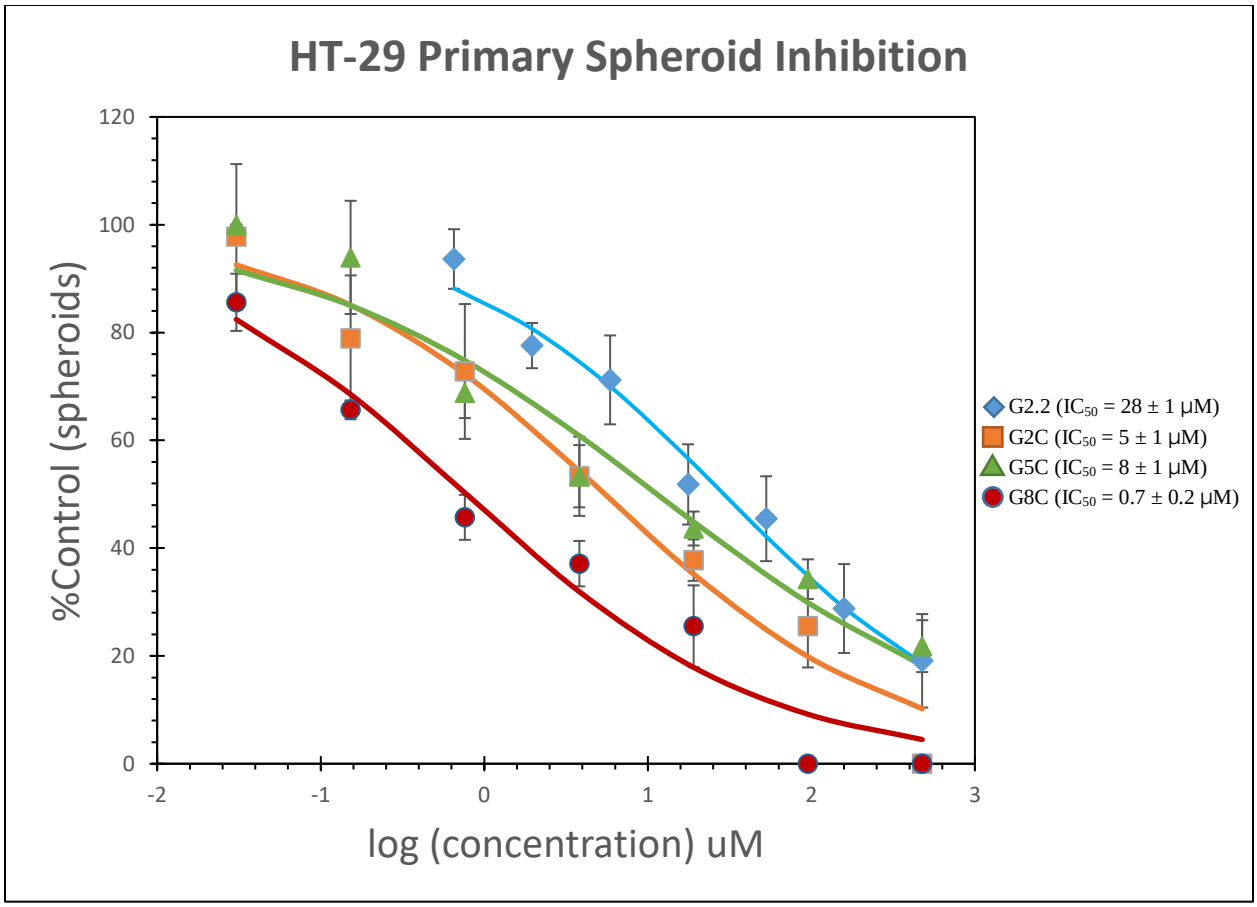


Figure 40. Primary spheroid inhibition curve of HT-29 (CMS 3) cells treated by G2.2 (PC) and lipid modified analogs (SD = ± 1 SE)

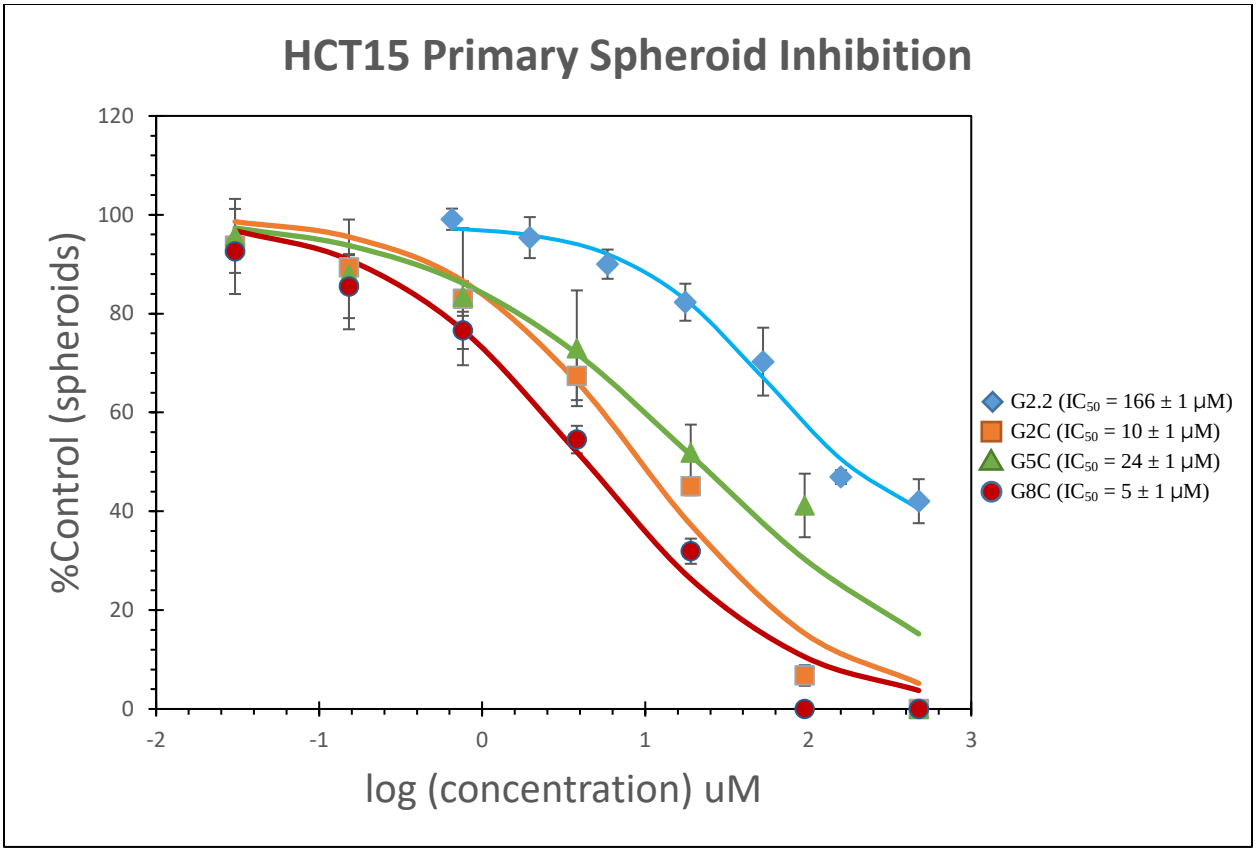


Figure 41. Primary spheroid inhibition curve of HCT15 (CMS 1) cells treated by G2.2 (PC) and lipid modified analogs (SD = ± 1 SE)

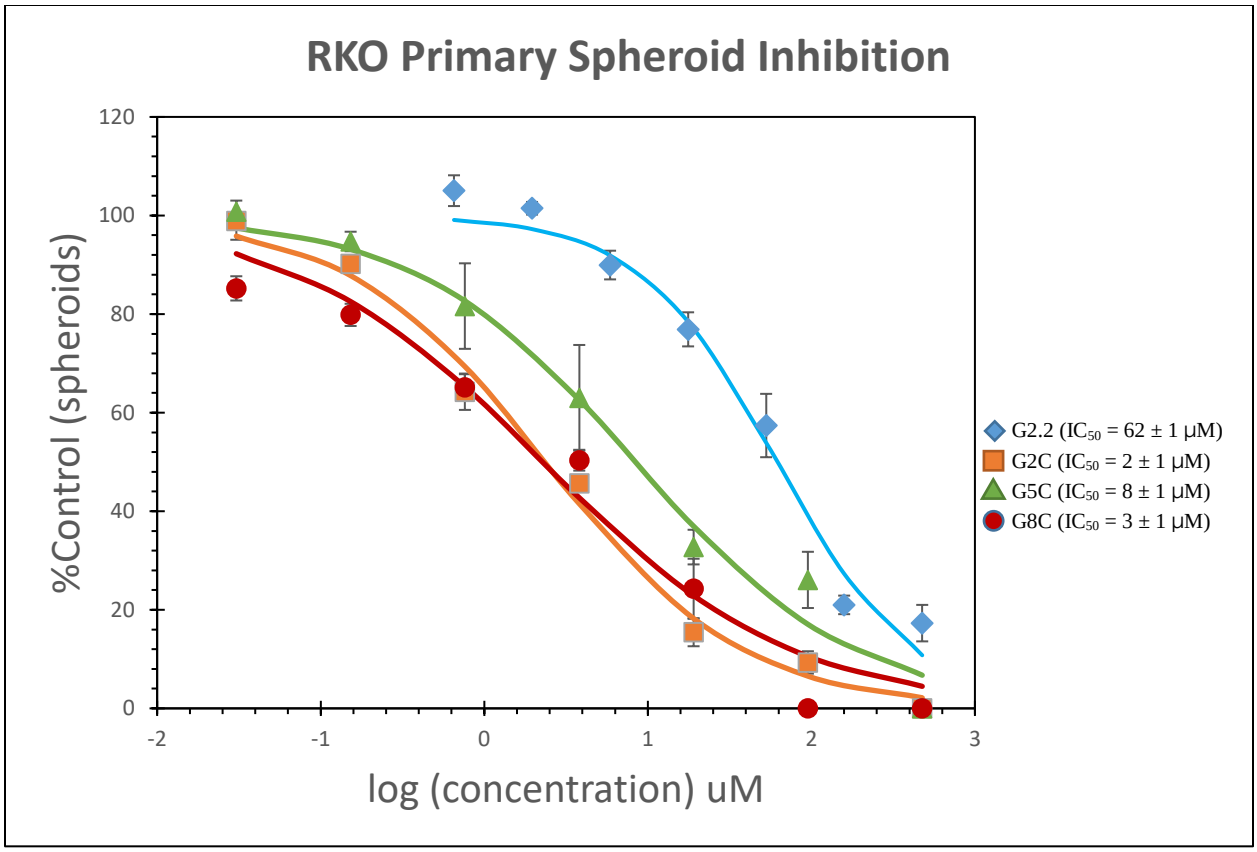


Figure 42. Primary spheroid inhibition curve of RKO (CMS 4) cells treated by G2.2 (PC) and lipid modified analogs (SD = ± 1 SE)

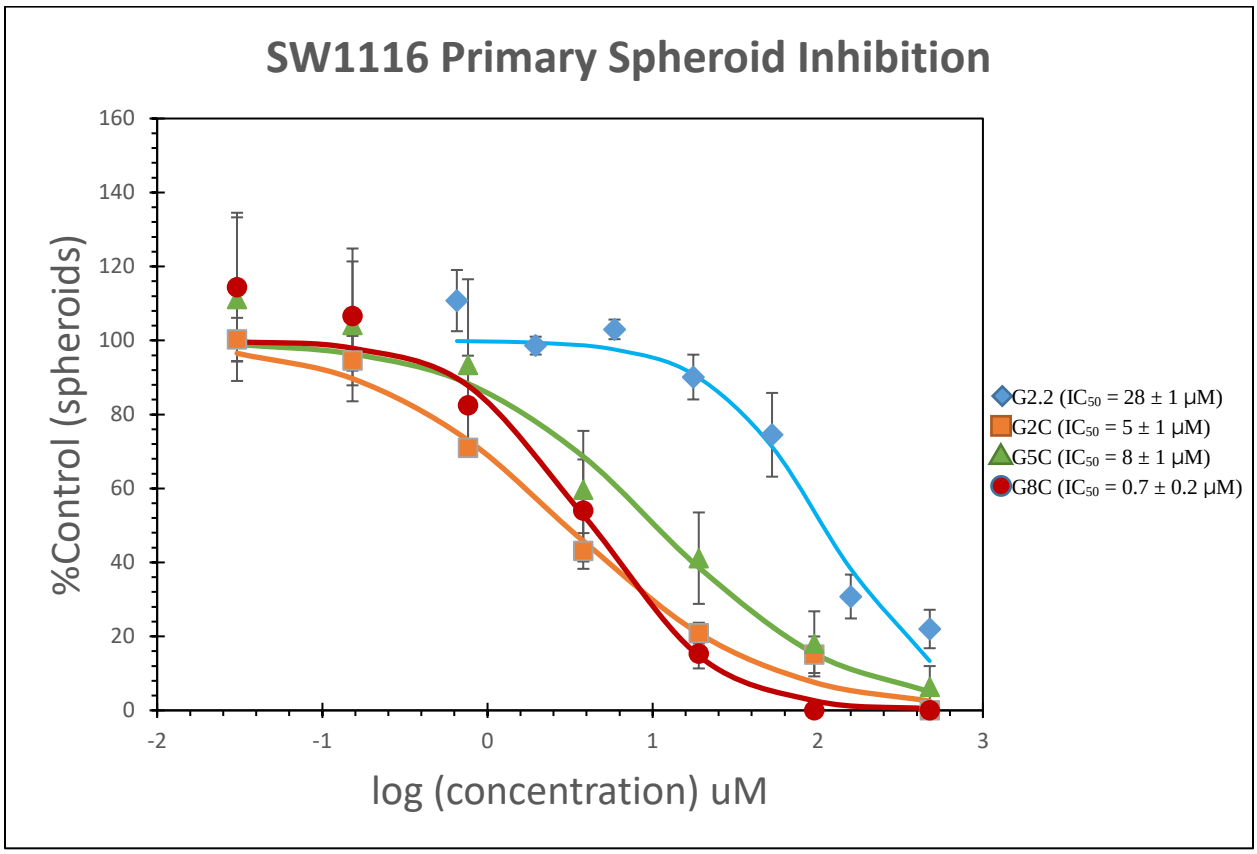


Figure 43. Primary spheroid inhibition curve of SW1116 (CMS 2) cells treated by G2.2 (PC) and lipid modified analogs (SD = ± 1 SE)

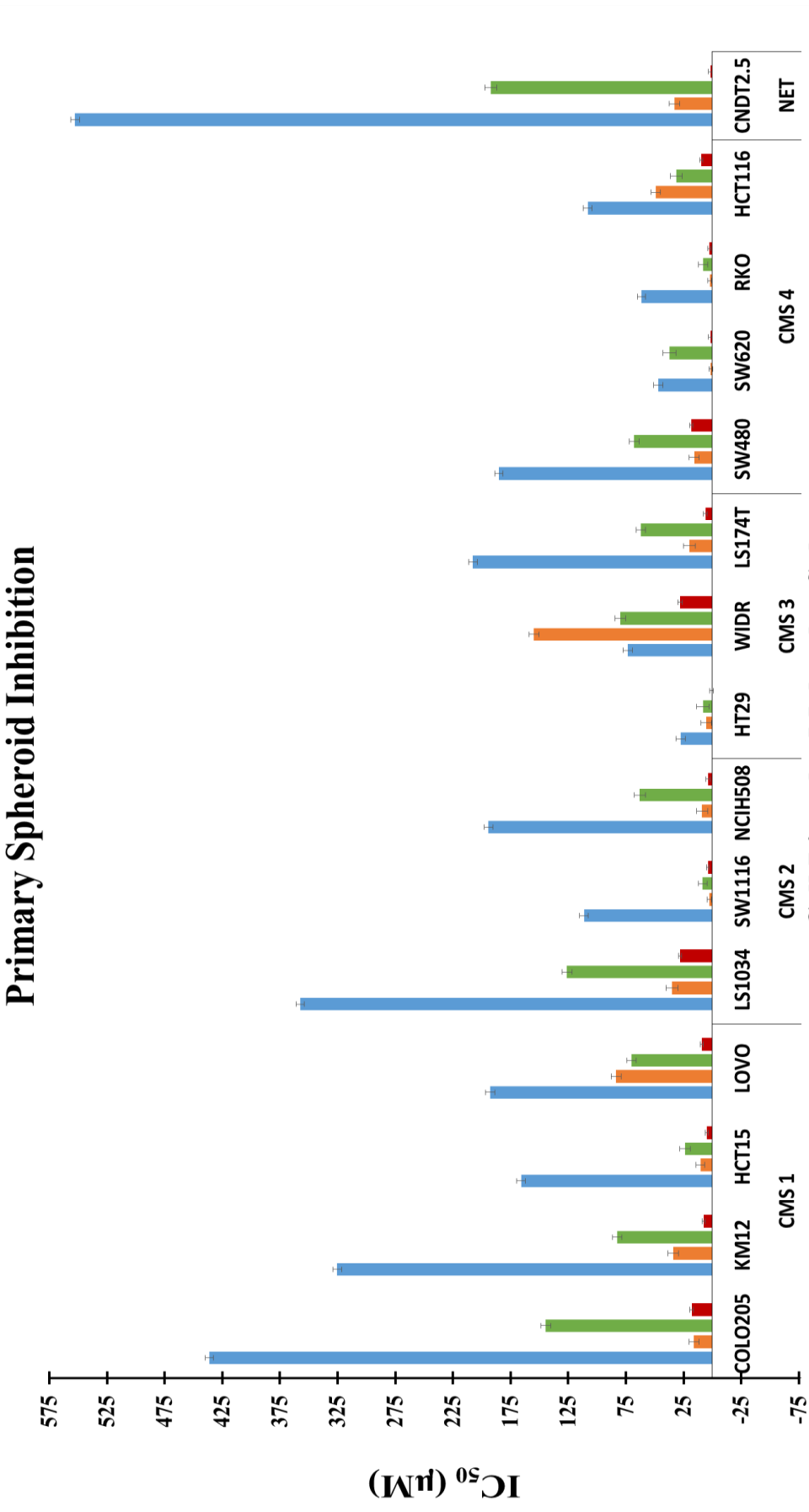


Figure 44. Summer of primary spheroid inhibition curves evaluating G2.2 (PC) against lipid modified analogs (SD = ± 1 SE). Cells are arranged by their CMS.

Primary Spheroid Inhibition Values								
	CMS 1				CMS 2			
	COLO205	KM12	HCT15	LOVO	LS1034	SW1116	NCIH508	
G2.2	437 ± 1	325 ± 1	166 ± 1	193 ± 1	357 ± 1	111 ± 1	194 ± 1	
G2C	16 ± 1	34 ± 1	10 ± 1	84 ± 1	35 ± 2	3 ± 1	9 ± 2	
G5C	145 ± 1	83 ± 1	24 ± 2	70 ± 1	126 ± 1	8 ± 1	63 ± 2	
G8C	18 ± 1	7 ± 2	5 ± 1	9 ± 1	28 ± 1	4 ± 1	4 ± 2	
	CMS 3			CMS 4				NET
	HT29	WIDR	LS174T	SW480	SW620	RKO	HCT116	CNDT2.5
G2.2	28 ± 1	73 ± 1	208 ± 1	185 ± 1	47 ± 1	62 ± 1	108 ± 2	553 ± 1
G2C	5 ± 2	155 ± 2	20 ± 2	16 ± 1	1 ± 0.2	2 ± 1	49 ± 1	33 ± 2
G5C	8 ± 2	80 ± 1	62 ± 1	68 ± 1	37 ± 2	8 ± 1	31 ± 2	192 ± 2
G8C	0.7 ± 0.2	28 ± 2	6 ± 1	18 ± 1	2 ± 1	3 ± 1	10 ± 1	2 ± 1

Table 7. Primary spheroid inhibition potency table (μM) of G2.2 and lipid analogs.

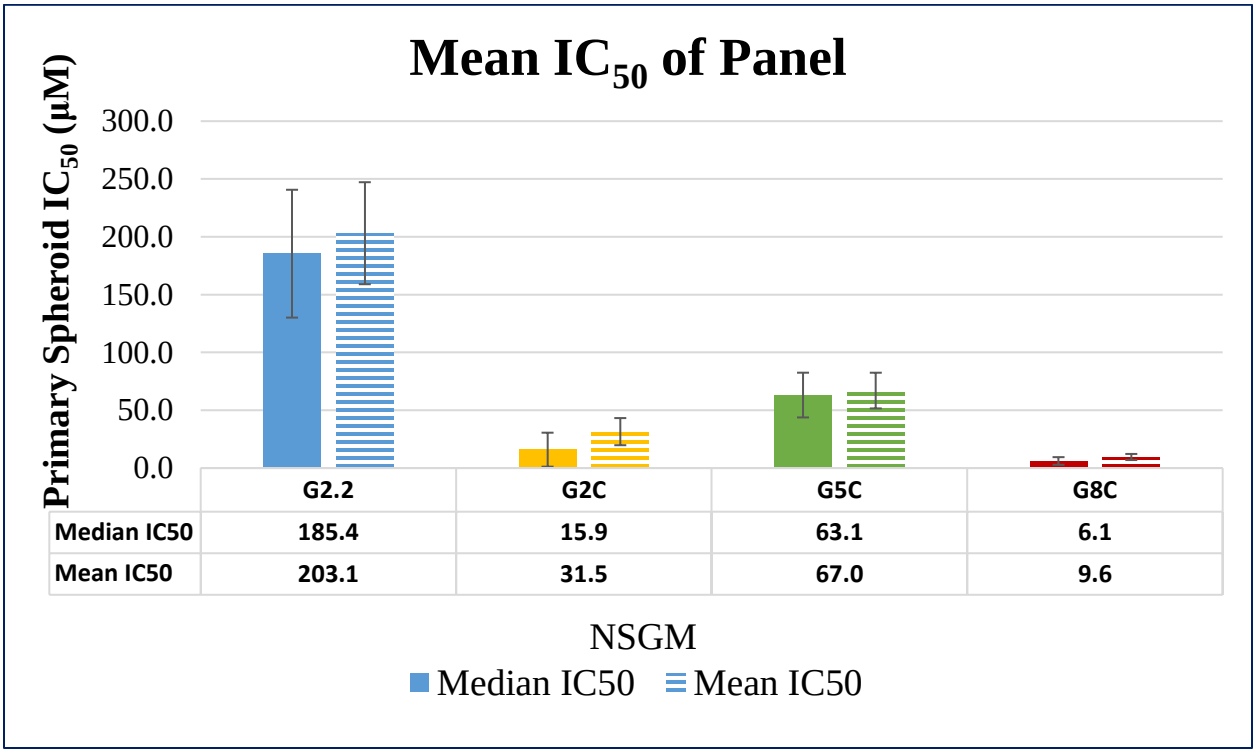


Figure 45. Mean/Median spheroid inhibition potency table (μM) of G2.2 and lipid analogs.

ii. 2-D Growth Inhibition (MTT cytotoxicity)

To evaluate the selectivity of G2.2 toward CSC-rich 3-D spheroid culture conditions, NSGM was evaluated at the same concentrations as in spheroid inhibition assays on traditional 2-D cell culture growth of cell lines selected to represent each CMS. The data below show comparatively the primary spheroid inhibition potency against the concentration of NSGM inhibiting 50% growth of monolayer culture.

	Monolayer Culture 50% Toxicity (µM)					Primary Spheroid IC50 Values (µM)			
	KM12	LS1034	HT29	SW620		KM12	LS1034	HT29	SW620
G2.2	125	476	476	476	G2.2	325.4	357.3	27.56	46.96
G2C	20	100	70	80	G2C	33.97	35.04	5.46	1.36
G5C	225	150	476	476	G5C	82.56	126.2	8.22	37.23
G8C	40	100	320	200	G8C	7.46	27.79	0.73	1.81

Table 8. Comparison of spheroid inhibition potencies (right) with monolayer cytotoxicity values (left). The cytotoxicity values are defined as the concentration required to inhibit 50% culture.

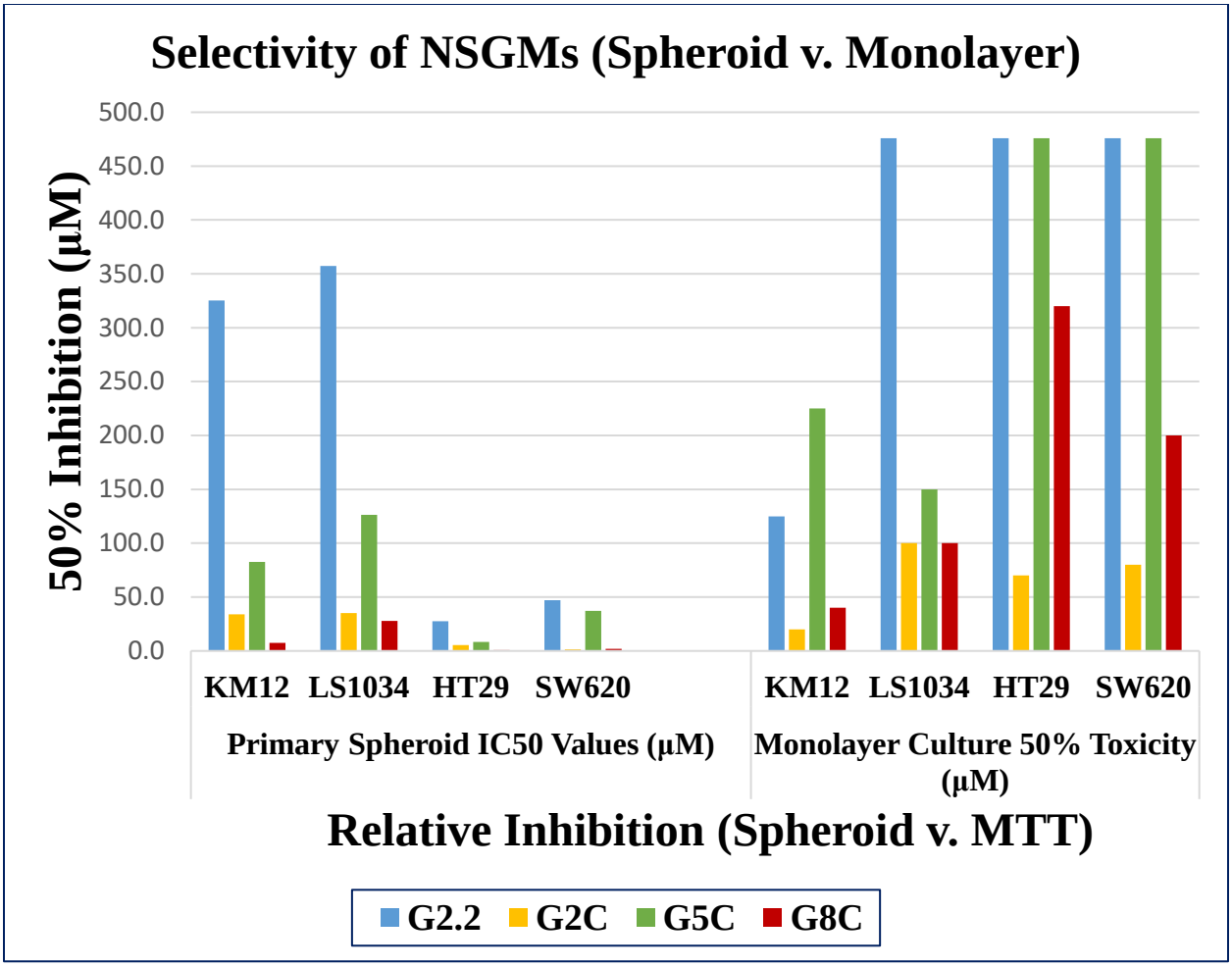


Figure 46. Graphical representation of the average spheroid inhibition potencies and monolayer cytotoxicity values.

d. Analysis and Discussion

i. Primary spheroid inhibition in a panel of colorectal cancer cells

The results of the primary spheroid inhibition assay revealed that, with the exception of cell line WiDr, the lipid modified analogs showed greater potency in every other cell line evaluated. G8C was the most potent of the analogs, with a median IC_{50} = 6 ± 3 μM (mean IC_{50} = 9 ± 2 μM). G2C was close behind, with a median IC_{50} = 15 ± 14 μM (mean IC_{50} = 31 ± 11 μM). The wide variability in inhibition potency with G2C is observed with cell line SW620 (CMS 4) having near 1 μM inhibition potency while cell line WiDr (CMS 3) having ~ 150 μM inhibition potency. G5C was easily the least potent analog of G2.2 with a median IC_{50} = 63 ± 19 μM (mean IC_{50} = 67 ± 15 μM). For lipid modified analogs, there was no clear trend as to the potency values. No particular molecular subtype fared better or worse, with a more sporadic resistance observed that seemed to be cell line-specific rather than by CMS (as seen in G2.2). However, there was over a 30-fold improved potency in cell line HT-29, the most sensitive to G2.2 treatment. On average, lipid modified analogs were anywhere from 3- to 30-fold more potent than G2.2 across the panel, which supports the hypothesis that the structural modification would enhance potency. More research into the mechanistic changes of proteins deemed targets of G2.2 would need to be performed to understand if these analogs were in fact operating via a completely different mechanism of action not only from G2.2, but from each other.

ii. Selectivity assays (MTT cytotoxicity)

Cytotoxicity evaluation using MTT assay revealed some interesting information about the selectivity of lipid modified analogs compared to G2.2. G2C was the most toxic against differentiated monolayer cell culture, with a 50% toxicity observed on average at ~ 70 μM. G2C was also ~ 3-fold more toxic to monolayer cell culture than G8C, the next most toxic analog. G8C had an average observed 50% toxicity near 170 μM, however was far less toxic to monolayer cells from the molecular subtypes with CSC-phenotype. In fact, in the most sensitive cell line to G8C treatment (HT-29) G8C was ~ 400-fold more selective to the CSC-rich spheroid culture. This helps support the idea that lipid modification of G2.2 still preserves selectivity for spheroidal growth and CSCs. G5C was the only lipid modified analog in the panel to exceed the maximum concentration (476 μM) within any significant toxicity observed in a cell line (which occurred in HT-29 and SW620 cells). In fact, both cell lines where this event occurred were those of CSC phenotype (CMS 3/4). Across the entire panel, G5C was observed to induce 50% toxicity at over 300 μM in monolayer conditions. This suggests that, although not as potent as the other analogs, G5C also is a selective CSC-targeting NSGM. Further evaluation of each of these analogs across the entire panel would enhance our understanding of the sensitivity of these agents under traditional monolayer culture.

e. Conclusions

After reviewing the evidence presented in this work to evaluate lipid modification to G2.2, it is apparent that this action has improved the overall potency of the compound several fold. Although G2.2 remains less toxic across the panel to the differentiated monolayer cells, there

was fold-selectivity with those cells belonging to stem-like molecular phenotypes in every NSGM evaluated. Do to the varying potency with no specific trends, it is reasonable to think that the lipid modified analogs have the capacity to act in a different mechanism than G2.2. As the work to identify a specific mechanism of G2.2 is still pending, this would need to be further studied from the results of G2.2. Further work to understand how a range of structural modifications impact G2.2, with more traditional structure-based drug design methods would enlighten our work and refine the essential structural motif other than sulfate group location that is required to improve upon G2.2.

5. DEVELOPMENT OF HTS PROTOCOL

a. Rationale

The development of a HTS protocol for the work presented in Chapters 3 and 4 arose after the findings form these experiments. As of now, 15 cell lines have been screened with these agents, all from CRC. There have been important observations as to the varying responses of G2.2 and analogs to specific molecular phenotypes, and promising results have been shown. However, as the number of cell lines evaluated increases the more that can be learned from these studies. Additionally, the effects of G2.2 and analogs has not yet been observed in cancers of different tissues (exception of pancreatic). It would be important to understand whether G2.2 and analogs were selective to CRC or whether they would exhibit potency in another cancerous tissue. So far, the correlation between spheroid inhibition potency and an additional factor (e.g. mutation status, SFF, genetic stability, etc.) has only produced weak relationships. It is hopeful that, as the

number of cell lines screened increases, so too will the potential correlations leading to a more fruitful understanding of the observations seen in previous studies. Despite the promising results seen so far, the process of performing primary spheroid assays takes a significant amount of time. From the time to culture the cells to confluency, to the growth of control spheroids, to the manual data collection and observation, a great deal of effort is involved. Therefore it has become necessary for the development of a HTS protocol to enhance the productiveness of this research and to produce reproducible data that can be evaluated via an autonomous process. The NCI-60 cell panel (Figure 42), which has been used in cancer research in-vitro for extensive periods of time, was acquired to represent 9 different cancer tissue types with 60 total cell lines for evaluation.

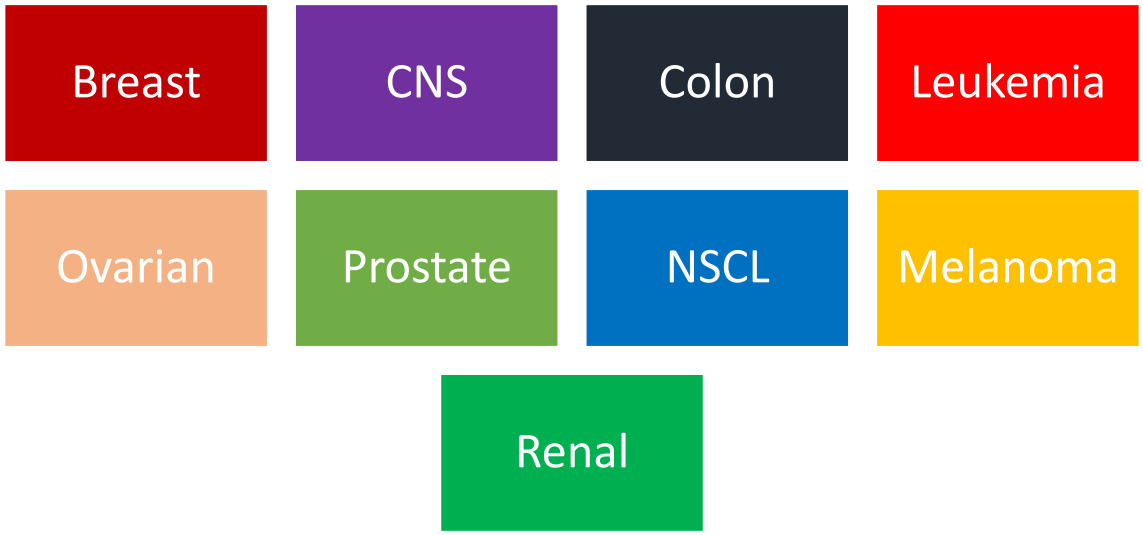


Figure 47. 9 different tissue types composing 60 patient derived cancer cells in the NCI-60 Human Tumor Cell Lines panel.

b. Materials and Methods

Cell Culture.

All patient derived cancer cells were obtained from NCI DCTD Tumor Repository with cell line authentication performed by either supplier or performed in house. The cells used in the study were maintained in 10 cm tissue culture treated plates (USA Scientific) as monolayer culture in the following media per supplier instructions: Dulbecco's Modified Eagle Medium – Nutrient Mixture F-12 (DMEM:F-12; cell lines HT-29, HCT116, WiDr, KM12,), Eagle's Minimum Essential Medium (EMEM; cell lines LS174T, RKO), RPMI-1640 Medium (cell lines LS1034, NCI-H508, Colo-205, HCT15), Kaighn's Modification of Ham's F-12 Medium (F-12K; cell line LoVo), and Leibovitz's L-15 Medium (L-15; cell lines SW620, SW480, SW1116). Complete growth medium was prepared for all with addition of 10% fetal bovine serum (FBS; Gibco) and 1% streptomycin/penicillin (AA; Gibco). The cells were passaged using trypsin containing ethylenediaminetraacetic acid (EDTA, Gibco) before they reached 70% confluency.

Primary (1°) Spheroid Inhibition Assay.

For primary spheroid formation, cells (no more than 6 passages) were plated in non-treated, low adhesion, 96-well plates (USA Scientific) at a cell density ranging from 100 cells/100 μL/well – 300 cells/100 μL/well depending on optimum density for cell growth. Cells were plated in stem cell media (SCM) that consisted of DMEM:F12:AA (Gibco), supplemented with 1xB27 (Gibco; 20 ng/mL epidermal growth factor (EGF; Sigma), 10 ng/mL fibroblast growth factor (FGF; Sigma)). After a brief period of incubation, vehicle (control) or NSGM at the desired concentration were aliquoted to each well (conditions plated in technical triplicate for each sample). On day 3-8 (spheroid formation for control wells varied for each cell line and were

monitored), the numbers of spheroids ranging from 50-150 μm in diameter were counted using a phase contrast microscope and percent inhibition was calculated compared to control.

HTS Assay

For the high throughput screening assay, spheroids are treated as previously described in the primary spheroid inhibition assay. After control well spheroids have growth into an appropriate size (50 – 150 μm), plates are sealed with parafilm and transported to the facility with a Cytation 5 (Cytek) multi-mode plate reader and imager. NucBlue fluorescent dye (Thermo Fisher) is mixed 1:1 with the SCM already in the spheroid plate. Spheroids are then incubated at 37° Celsius (5% CO_2) for a period of 45 mins. – 1.5 hours. After incubation, plates are removed and placed inside the Cytation plate reader with the lid removed. Flourescent imaging is performed at a wavelength of 460 nm (DAPI) to record the emission of blue light from the spheroids. Post-image processing include deconvolution and image stitching, and the output of the image and cytometry is exported as an excel file. Filters used include a plug that refines the window of counting to only include the inside of the well, as well as size exclusion criteria that only counts spheroids between 50 – 150 μm.

c. Experiment

Initial experiment began with the observation of spheroid inhibition of G2.2 and lipid modified analogs against MCF-7 breast cancer cell line. This cell line had previously been used for in-vitro evaluations on other projects, and was readily available for use in this study. Additionally, it had previously been observed that MCF-7 cells grew into easily distinguishable and aesthetic

spheroids. The following figures show the preliminary data observed from this plate of MCF-7 spheroids, with treatment of NSGM performed in triplicate conditions.

i. Primary Spheroid Inhibition Evaluations

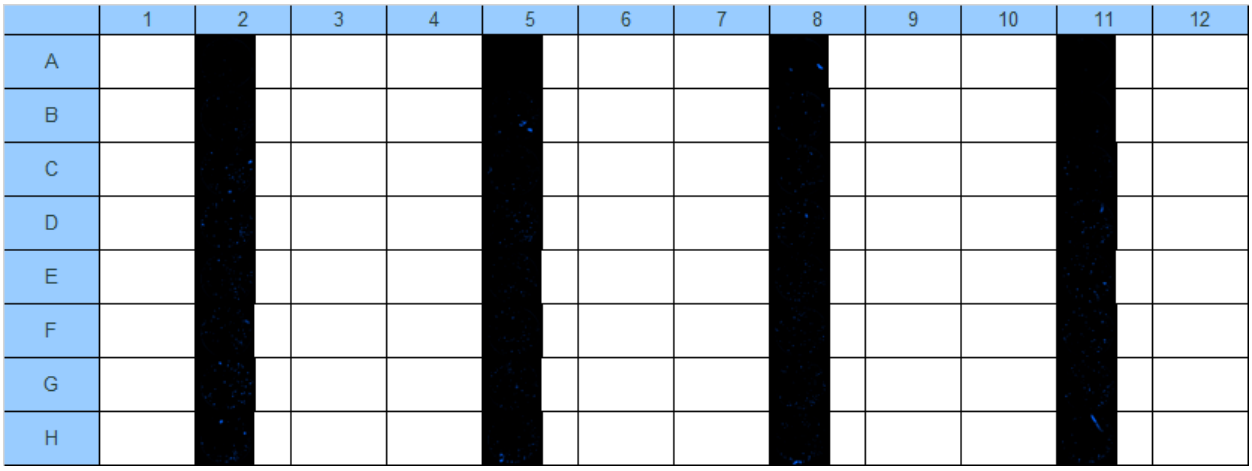


Figure 48. Exported analysis of images from the whole plate scanning of Cytation 5 multi-mode plate reader on the primary spheroid inhibition of NSGMs on MCF-7 breast cancer cell line.

	1	2	3	4	5	6	7	8	9	10	11	12
A	?????	10	?????	?????	0	?????	?????	2	?????	?????	0	?????
B	?????	24	?????	?????	20	?????	?????	18	?????	?????	3	?????
C	?????	57	?????	?????	22	?????	?????	30	?????	?????	17	?????
D	?????	70	?????	?????	70	?????	?????	40	?????	?????	50	?????
E	?????	56	?????	?????	41	?????	?????	44	?????	?????	57	?????
F	?????	57	?????	?????	44	?????	?????	51	?????	?????	49	?????
G	?????	99	?????	?????	49	?????	?????	60	?????	?????	59	?????
H	?????	41	?????	?????	58	?????	?????	46	?????	?????	41	?????

Figure 49. Exported analysis of cytometry from the whole plate scanning of Cytation 5 multi-mode plate reader on the primary spheroid inhibition of NSGMs on MCF-7 breast cancer cell line.

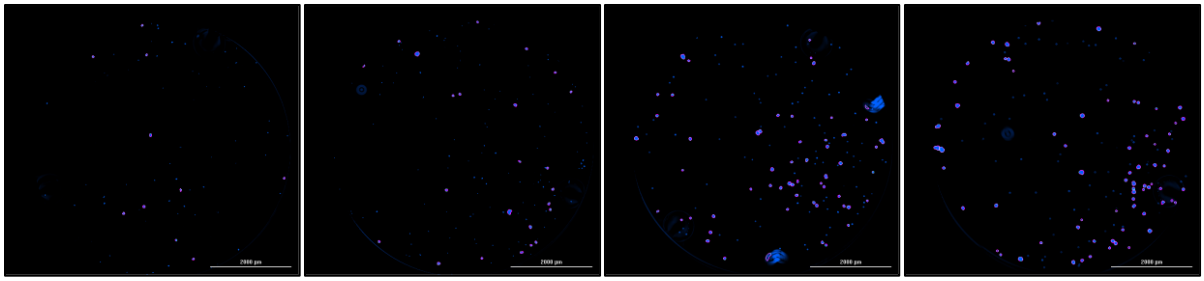


Figure 50. Exported images from the whole plate scanning of Cytation 5 multi-mode plate reader on the primary spheroid inhibition of NSGMs on MCF-7 breast cancer cell line. From left to right: Well A2 (316 µM G2.2), Well B2 (100 µM G2.2), Well C2 (31 µM G2.2), Well D2 (10 µM G2.2).

ii. Imaging Parameter Development

Initial imagine parameters used phase contrast and brightfield imaging, sometimes in combination. Additionally, it was attempted that Z-stacking could be performed across the whole plate to enhance the detail on the spheroids for easy observance. There was no plug used initially, and cytometry would be performed across the entire image. Additionally, it was not until recent that the use of NucBlue stain was administered to plates prior to imaging. Below are various figures representing the different parameters used in protocol development.

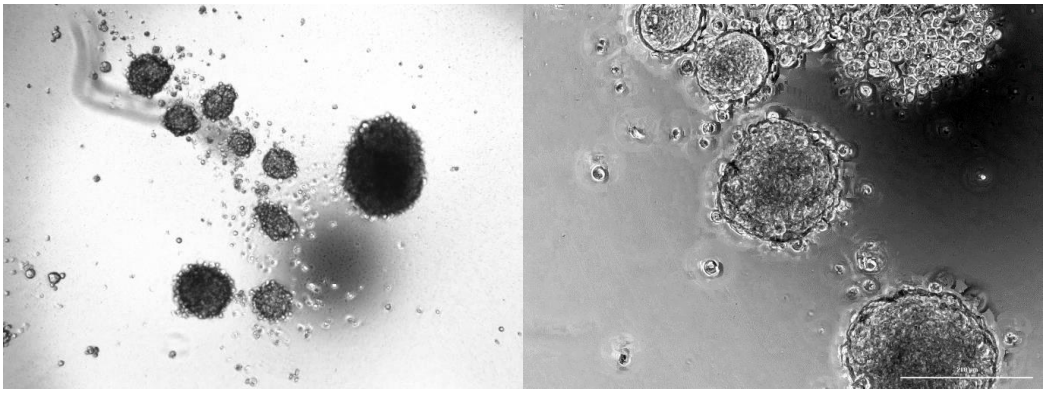
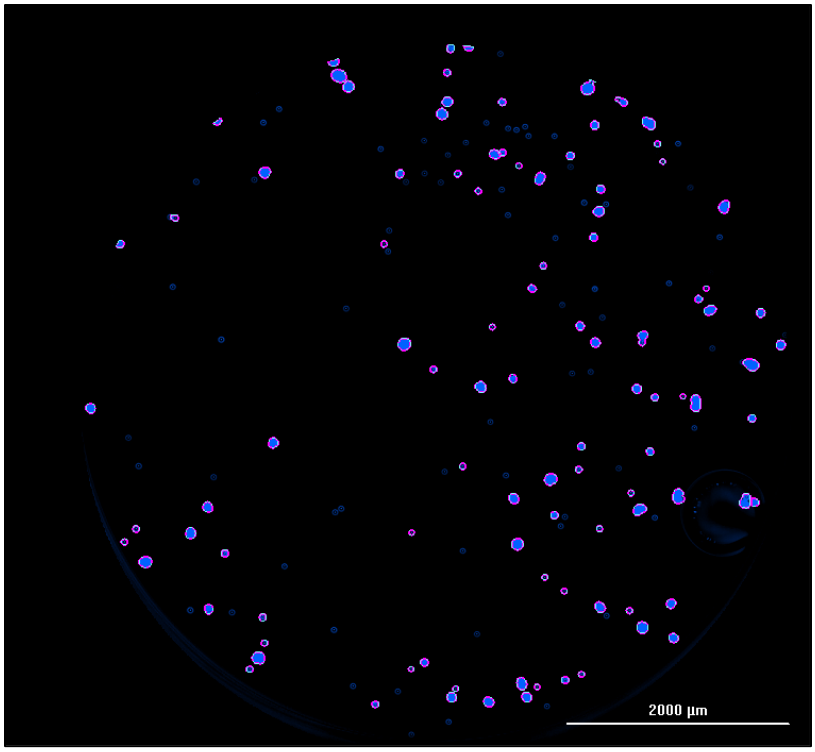


Figure 51. Brightfield images of non-treated HT-29 spheroids with Z-stacking at 10X.



Name	Value
Detection Threshold	5069
Cell Count	99
Object Size	81.9
Object Mean[Deconvolved [Stitched[Tsf[MCF 7 NucBlue:DAPI 377,447]]]]	19126
Cell Count[spheroids 50-75 um]	49
Cell Count[spheroids 100-150 um]	23
<Execution time>	219

Figure 52. Fluorescent image of MCF-7 spheroids treated with 316 nM G2.2, and the summary table exported from Cytation 5 with mean cytometry data.

d. Discussion

After spending considerable time with the plate reader, it has become apparent that this will be a useful tool in establishing a HTS protocol for automating the platform of our primary spheroid analysis and data validation. In the early stages of development, a full understanding of the operations and fine tuning of images were not known, yet since then a more thorough understanding of the basic functions and most useful image processing have become known. Several experimental protocols have been established and saved to progress forward with in this development. The plug used for cytometry has relatively good accuracy in limiting only the intra-well images for counting, and the resolution of the images are good. It is easy to filter for

specific size spheroids, and the raw metrics provided by the software are incredibly useful for further understanding the physical characteristics of the spheroids evaluated. Additionally, this method can be followed by anyone with little experience, and provides for validated detection and counting.

Some limitations of the assay at this point in development include the non-specificity of the NucBlue stain used in imaging. This stain is able to penetrate the 3D spheroid and emits blue fluorescence at DAPI excitation emission (~ 400 nm) when the dye binds to DNA from a living cell. However, it will stain any living cell and not only spheroids. The size exclusion filter allows for a work-around with this limitation, however it does not prevent the inaccurate counting of a single cell that may have swollen with media to be 50 μm in size. Additionally, the cytometry feature will often count clouds of blue stain that reflect from the bubbles in the well. This can lead to erroneous cytometry. Furthermore, the detail on the spheroids with fluorescence is not as clear to indicate 3D spheroid as in brightfield or phase contrast. Yet the latter two options, with the meniscus effect, produce incomplete images of the wells that are not able to be counted properly.

i. What has been accomplished?

At this point in development, there have been some promising data and achievements. First, the basic understanding of the plate reader and imager have been completed, allowing for more advanced applications of the wide-array of functions this instrument has. Secondly, we have been able to identify and count spheroids from a primary spheroid inhibition assay and produce

quality images and metrics for further evaluation. The addition of specific and sensitive fluorescent staining has enhanced the optics of the protocol as well. Further, in initial studies with MCF-7 breast cancer tissue, it appears that the NSGMs are not nearly as potent inhibitors as they were in CRC spheroids. This may indicate a degree of tissue selectivity that would need to be further evaluated.

ii. Where do we proceed?

Further studies attempting to refine the HTS protocol for primary spheroid inhibition analysis will attempt to enhance the spheroid-sensitivity of both optical parameters as well as stains used for imaging. It would be important to cross-validate the number of spheroids counted via the automated platform with what can be done manually, and any improvement to the detail of spheroids in post-analysis images would be ideal for user reviewing. There are about 52 cell lines that have not yet been screened, and it is hopeful that spheroid parameters can be developed to analyze the potency of G2.2 and other analogs via this HTS protocol. This will enhance our understanding of the activity of our agents and produce a greater set of data to make correlations with.

e. Conclusions

Overall, there is a vital need for the development of a HTS protocol to improve the output and productiveness of the work that lies ahead. The results from these initial experiments show great promise in utilizing this instrument, and in the future a great deal of work will be spent trying to refine and shape the parameters to achieve a convenient and reproducible system of analysis.

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