Therapeutic Strategies Targeting Hepatocellular Carcinoma

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PREFACE

The project was done in the laboratories of the Cancer Drug Research Laboratory at McGill University located at the McGill University Health Center Campus (MUHC). The laboratory is currently located at the McGill University Health Center Campus Research Institute (MUHC-RI). At the beginning of the project, the laboratory had been located at the old campus of the Royal Victoria Hospital. The research project has been supported by the Liver Disease Research Center at King Saud University, Riyadh, Saudi Arabia. The project was directly and continuously supervised by Dr. Bertrand Jean-Claude. The generation of idea and planning of experiments was done by Bilal Marwa. The conduction of experiments was done by Dr. Bilal Marwa with initial supervision and training by lab members of the Cancer Drug Research Laboratory. The synthesis and chemical analysis of the compound AB-02 was done by Masters Student Alaa Baryyan. The synthesis and chemical analysis of the compound AL-13-51 was performed by Dr. Anne-Laurre Larroque. The thesis was written by Dr. Bilal Marwa and was revised by Dr. Bertrand Jean-Claude. The French abstract was translated by Dr. Benoit Thibault from the English abstract written by Dr. Bilal Marwa.

ABSTRACT

Hepatocellular carcinoma (HCC), the most common type of liver cancer, contributes to a significant portion of cancer-related mortality worldwide. Over the past few decades, the incidence of HCC and its disease-specific mortality have been increasing. Molecularly targeted therapy (MTT) constitutes a relatively new treatment modality that has been shown to improve the rates of survival in different kinds of cancers including HCC. In advanced cases of HCC, sorafenib is the only systemic agent associated with survival benefit. Sorafenib is a kinase inhibitor of several receptor kinases including RAF serine/threonine kinase and VEGFR tyrosine kinase amongst others.

The dismal outcome of advanced HCC despite the use of sorafenib warrants the development of other agents that either augment the action of sorafenib or have a more potent effect. The primary objective of our project is to introduce potential therapeutic strategies involving molecularly targeted agents that would be effective in inhibiting the growth of HCC cells. The identification of such strategies could serve as a rationale for the design of novel molecules or the design of promising clinical trials.

In our experiments, we have tried combinations that include inhibition of pathways that are involved in hepatocarcinogenesis, including simultaneous inhibition of different pathways which have known interactions. We used the growth inhibition assay sulforhodamine B (SRB) assay to determine the growth inhibitory potency of different agents, including novel agents developed in our laboratory. We compared their potency alone and in combinations, both in equimolar and equi-effective combination ratios. We also used Western blot to identify the activity of signaling pathways in HCC cells and changes occurring in response to treatment with different agents and combinations.

Our results show that the addition of the MAPK/ERK Kinase (MEK) inhibitor selumetinib to sorafenib is associated with a synergistic effect on inhibiting the proliferation of HCC cell lines. We also found that the inhibition of the HGF receptor MET using crizotinib was effective and synergistic with sorafenib on cell lines that express the MET receptor. Components of both the MAPK pathway and the HGF/MET pathway are involved in resistance to sorafenib, and thus their inhibition along with using sorafenib could lead to potentiation of its action. In our experiments, the triple combination that includes sorafenib, selumetinib, and crizotinib led to effective synergy in all cell lines.

We conclude that the combination of sorafenib with agents that inhibit one or more of its resistance pathways could be an effective strategy for the treatment of hepatocellular carcinoma. Further studies are needed to prove the effects of combinations of kinase inhibitors in vivo, particularly those including the standard of care agent sorafenib with either selumetinib, crizotinib, or both. A single molecule (combi-molecule) that inhibits both MET and MEK can be an effective potentiating agent to the action of sorafenib on hepatocellular carcinoma.

RESUME

Le carcinome hépatocellulaire (CHC), cancer du foie le plus courant, contribue significativement à la mortalité causeé par le cancer dans le monde. Depuis les dernières décennies, l'incidence du CHC et sa mortalité associée ont augmenté.

Les thérapies moléculaires ciblées (TMC) constituent une modalité de traitement relativement nouvelle qui a été montrée comme étant capable d'améliorer les taux de survie de différents cancers tels que le CHC. Dans les cas avancés de CHC, le sorafenib est le seul agent systémique associé à un bénéfice sur la survie. Le sorafenib est un inhibiteur de kinase ciblant, entre autres, la sérine/thréonine kinase RAF et le récepteur à activité tyrosine kinase VEGFR.

Le sombre pronostic des CHC aux stades avancés, malgré l'utilisation du sorafenib, met en évidence la nécessité de développer d'autres agents capables soit d'améliorer l'action du sorafenib, soit d'induire un effet thérapeutique supérieur à ce traitement. L'objectif principal de notre projet est d'introduire de nouvelles stratégies thérapeutiques potentielles impliquant d'autres thérapies ciblées capables d'inhiber de manière efficace la croissance des cellules de CHC. L'identification de telles stratégies pourrait servir de rationnel à la mise au point de nouvelles molécules ou d'essais thérapeutiques prometteurs.

Dans nos expériences, nous avons testé des combinaisons comprenant l'inhibition de voies de signalisation impliquées dans la carcinogenèse hépatique, l'inhibition simultanée de voies présentant des interactions connues. Nous avons utilisé un test d'inhibition de croissance à la sulforhodamine B (SRB) afin de déterminer le potentiel inhibiteur de différents agents, tels que les nouvelles molécules développées dans notre laboratoire. Nous avons comparé la capacité d'inhibition de croissance de ces agents seuls et en combinaison, selon des ratios de combinaison équi-molaires et équi-effectifs. Nous avons réalisé des Western Blot afin d'identifier l'activité de

voies de signalisation dans les cellules de CHC et leurs modulations en réponse au traitement avec les différents agents et les combinaisons.

Nous résultats ont montré que l'addition du selumetinib, inhibiteur de la kinase MAPK/ERK (MEK), au sorafenib est associée à un effet synergique sur l'inhibition de la prolifération des lignées cellulaires de CHC. Nous avons également démontré que l'inhibition du récepteur au HGF/MET à l'aide de crizotinib, était efficace et synergique avec le sorafenib sur les lignées cellulaires exprimant le récepteur MET. Les composants de la voie MAPK et de la voie HGF/MET sont impliqués dans la résistance au sorafenib, et leur inhibition en association avec le sorafenib pourrait donc permettre la potentialisation de son action. Dans nos expériences, la triple combinaison incluant sorafenib, selumetinib et crizotinib a présenté un effet synergique dans toutes les lignées cellulaires.

Nous concluons que la combinaison du sorafenib avec des agents inhibant une ou plusieurs de ses voies de résistance pourrait être une stratégie efficace pour le traitement du CHC. Des études approfondies sont nécessaires pour prouver les effets de thérapies d'inhibition combinées de kinases in vivo, en particulier combinant le sorafenib, molécule de référence dans ce cancer, et le selumetinib, le crizotinib, ou ces deux molécules. Une molécule unique (combi-molecule) inhibant à la fois MET et MEK pourrait être un possible agent potentialisateur de l'action du sorafenib sur le CHC.

LIST OF ABBREVIATIONS

AASLD	American Association for the Study of Liver Diseases
AAT	Alpha-1 Anti-Trypsin
AFB	Alfa-Feto Protein
AJCC	American Joint Committee on Cancer
AKT	Alpha serine/threonine-protein kinase
ALBI	Albumin-Bilirubin Score system
ALT	alanine transferase
AML	Acute Myeloid Leukemia
APC	Adenomatous Polyposis Coli gene
AXIN1	Axis inhibition protein 1
BAD	Bcl-2-associated death promoter
BCLC	Barcelona Clinic Liver Cancer Group
BSA	Bovine Serum Albumin
CDK	Cyclin-Dependent Kinase
CHC	Carcinome hépatocellulaire
CI_{50}	Combination Index at IC_{50}
COG	Children's Oncology Group
CT	Computed Tomography
CTNNB1	Gene encoding for β-Catenin
DFG	Asparagine-Phenylalanine-Glycine Motif
5dFU	5-Deoxy-Flouro-Uridine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
4E-BP1	Gene encoding for eIF4E
EGF	Epidermal Growth Factor
EGFR	Epithelial Growth Factor Receptor
eIF4E	Eukaryotic translation initiation factor 4E
EMT	Epithelial to mesenchymal transition
ERK	Extracellular Signal-Regulated Kinase
FAK	Focal Adhesion Kinase
FOXO3	Forkhead box O3 Protein
5-FU	5-Flouro-Uracil
Gab2	GRB2-associated-binding protein 2
GF	Growth Factor
GTPase	Guanosine triphosphatase
HBV	Hepatitis B virus
HBx	Hepatitis B x gene
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C virus
HGF	Hepatocyte Growth Factor (also called scatter factor)
HHC	Hematochromatosis
IC_{50}	Inhibitory concentration leading to 50% of growth

iSH2	Inter-Src homology 2 domain
JAK	Janus Kinase
JNK	c-Jun N-terminal kinases
MAPK	Mitogen Activated Protein Kinase
MDR1	Multiple Drug Resistance Gene 1
MET	A trancellular kinase receptor of the HGF ligand
miRNA	Micro-ribonucleic acid
MRI	Magnetic Resonance Imaging
mTOR	Mechanistic target of Rapamycin
MTT	Molecularly Targeted Therapy
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NSCLC	Non-Small Cell Lung Carcinoma
PBS	phosphate buffered saline
PDK1	Phosphoinositide-dependent kinase-1
PDK2	Phosphoinositide-dependent kinase-2
PI3K	Phosphoinositide 3-kinase or Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
RAF	An intracellular kinase (Origin: Rapidly Accelerated Fibrosarcoma)
RFA	Radiofrequency ablation
RNA	Ribonucleic acid
RTK	Receptor Tyrosine Kinase
SBDD	Structure based drug design
SBRT	Stereotactic body radiotherapy
SFRPs	Secreted frizzled-related proteins
SOCS1	Silencer of cytokine signaling 1
SOX1	Sex determining region Y-box 1
SRB	Sulforhodamine b
STAT	Signal Transducer and activator of transcription
TACE	Transarterial chemoembolization
TMC	Thérapies moléculaires ciblées
TP53	Tumour Protein p53
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
Wnt	A group of signaling proteins (Origin: Wingless-related integration site)

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Chapter 1 Introduction

1 Preface

Advances in medicine and health care over the past few decades have led to decreased global human morbidity and mortality due to disease ultimately leading to improvement in the lifespan and quality of life of humans. While the average life expectancy of a human being was around 30 years of age throughout history until the nineteenth century, the current life expectancy worldwide is around 71.[1,2] This effect is attributed mainly to health care advancements that led to decreased human mortality through rapid identification and management of diseases as well as multiple lines of prevention.

The improvement in prevention, identification and therapy of disease has been largely made possible through the generation and communication of meaningful information. The effective process of information transfer and exchange of ideas has led to great contributions in technology tools, building on compiled knowledge and using existing pieces of information to design new experiments and produce increasingly meaningful pieces of information, collectively referred to as data. Those factors all, with the inspiring dedication of people at various points of time and place, contributed to improvements of patient care.

Cancer is a major contributor of mortality and morbidity at the current time. Of the 7.4 billion people currently living on the face of Earth, about 1.6 million people are diagnosed with cancer,[3] and the world loses 8.4 million people to cancer each year. Traditionally, cancer has been thought of as a terminal disease leading to inevitably rapid death. Attempts to cure cancer surgically are centuries old, with evidence from ancient civilizations showing references to surgical removal of tumors.[4] By the beginning of the twentieth century, scientists had begun to assess the use of radiation for cancer therapy. By the middle of the century, Sidney Farber

introduced the idea of giving systemic agents, chemotherapy, to inhibit the proliferation of rapidly dividing cells.[5]

The mortality due to cancer, however, was still quite high until around 1980s and the 1990s when better cancer diagnosis and better treatment regimens were introduced.[6,7] The new century brought about a new paradigm of cancer treatment. Novel agents were discovered and developed, which target specific characteristics of cancer cells leading to specific lethal and inhibitory effects that spare normal tissues. Those novel agents are approved in various types of cancers, of which liver cancer is one of the main diseases that rely largely on a novel molecularly targeted therapy: sorafenib.

Our project studies a specific type of cancer that occurs in the liver, hepatocellular carcinoma. The upcoming parts of this introduction will include an in-depth review of the current knowledge about hepatocellular carcinoma, its epidemiological and clinical features, pathogenesis, standard of care management, and a discussion of advances directed at limiting its impact with particular emphasis about history of liver cancer research and drug discovery and development.

2 Hepatocellular Carcinoma

2.1 Epidemiology and Etiology

Liver Cancer is one of the most lethal types of cancer globally, particularly in adult men. It is the second most common cause of cancer-related mortality in men worldwide. [8,9] Hepatocellular carcinoma (HCC) is the most common type of liver cancer, contributing to 75 % of cases. It affects men three times more than women, and is more common in older individuals, although the rates in younger people are on the rise. Every year, HCC is responsible for the death of between 250,000 to 1,000,000 people worldwide, which is strikingly close to the rate of incidence of the disease, highlighting its aggressive nature.[10]

Most cases of HCC (up to 80%) occur in the background of liver cirrhosis. The most common risk factor for the development of HCC is infection with hepatitis B virus (HBV), with or without the development of cirrhosis[8] The other main risk factors for HCC are chronic hepatitis C virus (HCV) infection, alcoholic hepatitis and non-alcoholic fatty liver disease (NAFLD). Less common risk factors of HCC include conditions that lead to cirrhosis such as hemochromatosis (HHC), Alpha1 Antitrypsin (AAT) deficiency, autoimmune hepatitis, porphyria and Wilson disease. Cirrhosis of any type carries an increased risk of HCC.[8] Exposure to the environmental toxin aflatoxin is another strong risk factor related to HCC and thought to contribute independently to its pathogenesis. The toxin contaminates several food products such as corn, soybean and peanuts.[11]

HCC is most commonly found in developing countries, particularly due to the high prevalence of HBV in those areas. However, there is an increase in the incidence of HCC in developed countries as well as an increase in the rate of HCC-related mortality.[12,13] The rise of HCC rates in developing countries can be attributed to the increase of infection rates of Hepatitis C and increasing rates of alcoholic hepatitis and obesity-related non-alcoholic fatty liver disease (NAFLD). In fact, the latter three conditions cause the majority of cases of HCC in developing countries. HCV-related HCC is the cause of cancer-related death that is increasing at the fastest rate. [16]

Active steps that can be implemented to limit the increasing rates of mortality due to HCC are continuously considered by public health sectors as well as clinical and basic biomedical professionals. These steps include: (1) measures to prevent the development of HCC by controlling risk factors or decreasing the risk of HCC development in individuals at risk, (2) surveillance for early detection of HCC at a curable stage, and (3) improving the treatment given to patients

diagnosed with HCC either by (a) better stratification of patients for better selection of treatment modalities to be offered to patients to increase cure rates or extend survival, or by (b) introduction of novel therapeutic strategies into the treatment of HCC. The latter is the category to which we wish to contribute.

2.2 Clinical Features, Diagnosis, and Management of HCC

Typical HCC cases either show up in follow-up clinic appointments in asymptomatic patients with cirrhosis who come in with an abnormal test in the form of imaging or bloodwork, or in emergency departments with rapidly developing ascites, nausea and vomiting, abdominal pain or upper gastrointestinal bleeding.[18] Diagnosis of HCC is usually done on the basis of radiological images in the form of computed topography (CT) images or magnetic resonance imaging (MRI) scans.[19,20] Once the diagnosis is made, a patient would be classified based on one of the HCC classification systems that would give information on the prognosis and would dictate the next step in the management of the patient.[21]

The management of HCC depends both on characteristics of the tumor and the extent of liver disease. Several algorithms and criteria were developed to guide the decisions taken based on the tumor stage and prognosis. One of the most common algorithms is the Barcelona Clinic Liver Cancer (BCLC) staging system.[20] Other classification systems are the albumin-bilirubin (ALBI) score system,[23] and the TNM (tumor, node and metastasis) system developed by the American Joint Committee on Cancer (AJCC).[24]

Early tumors with smaller sizes with adequate liver reserve can be surgically resected, with a good curable potential.[20] Patients with relatively less available liver reserve due to more extensive liver disease or larger tumors may be potential candidates for orthotopic liver transplant.[25] Patients who do not meet the criteria for liver transplant may have locally delivered

therapeutic modalities with radiographic guidance. Those include ablative therapy – including radiofrequency ablation (RFA), percutaneous acetic acid ablation, cryoablation – and transarterial chemoembolization (TACE) or radioembolization. Patients with locally advanced or metastatic HCC are not amenable for any of the local modalities of treatment and the only approved therapeutic modalities would be sorafenib, as described below. Some patients with both extensive liver disease and a locally advanced tumor may not be amenable to any specific antineoplastic therapeutic modality and would receive palliative therapy.[26]

Radiation therapy has also been attempted in patients with HCC since the tumor is radiosensitive. However, it is not routinely used since the liver is an extremely radiosensitive organ, and radiation may cause life-threatening toxicity to the liver. One of the arising modalities is the use of stereotactic body radiotherapy (SBRT), which minimizes exposure of normal liver tissue to radiation.

Advanced HCC that extends beyond what is amenable to local therapy has been challenging for scientists and treating physicians. The median survival for those cases remains less than one year despite the use of numerous agents in clinical trials. The treatment of HCC is complicated by the fact that it is a relatively chemoresistant tumour.[27] This may be due to the frequent activation of mechanism of drug that lead to drug elimination. Furthermore, the treatment of HCC is complicated by its association with advanced liver disease in a majority of cases, which limits the use of toxic chemotherapeutic agents. Lastly, the heterogeneity of the biology of HCC cases results in a variety of responses to chemotherapeutic agents, which leads to difficulty in having a significant rate of effective response in each of the conducted trials.[28]

Currently, the only systemic agent that has successfully increased the median survival compared to placebo is the multi-kinase inhibitor, sorafenib. Sorafenib is the only systemic agent

associated with survival benefit compared with placebo. Sorafenib is a kinase inhibitor of several receptor kinases include RAF serine/threonine kinase and VEGFR tyrosine kinase amongst others. Details about the properties of sorafenib as well as history of discovery and development of sorafenib will be discussed thoroughly in the following section. Until the approval of sorafenib in 2007, there had been no systemic agent that is known to improve survival in patients with inoperable hepatocellular carcinoma.

3 Molecular Pathways and Pathogenesis of HCC

The process of hepatocarcinogenesis can be learned by understanding the consecutive steps that lead eventually to the development of HCC. We will review the mechanism of initiation of HCC from its risk factor conditions and subsequently discuss the different pathways that are aberrant in each of the different stages during the development of the disease.

3.1 Hallmarks of Hepatocarcinogenesis

Similar to the process of carcinogenesis in other types of cancer, hepatocarcinogenesis is preceded by an event that leads to genomic instability or disruption of the regulation of gene expression. The resulting flexibility in the genome and gene expression profiles of cells would lead to the promotion of properties that give cells a proliferative and growth advantage. This process results in clonal evolution of highly proliferative cells that increasingly acquire more hallmarks of cancers. Genome instability is thought to be one of the most crucial hallmarks of cancer that mediate and promote the other hallmarks.[29,30]

The initiating event can achieve this modifiability either through a defect in chromosomal stability, the mechanisms of DNA repair, or an epigenetic modification either through methylation of promotors of tumor suppressor genes or regulation of other genes through chromatin modulation or upregulation of miRNAs. These aberrations are either caused by a stressor to cells that adds

pressure to adapt in order to get protected from its damaging effects, or a pathogen that directly disrupts genome stability and/or deregulates gene expression. It is thought that multiple stressors act synergistically as initiating events to lead to genomic and transcription vulnerability. Subsequent colonies contain aberrations that lead to inhibition of tumour suppressor genes and up-regulation of genes that facilitate growth, progression and the spread of their malignant progenies.

3.2 Cirrhosis and HCC

The fact that 80% of HCC cases develop in the background of chronic liver disease points towards a strong relationship between the two conditions. It is thought that the chronic inflammation which accompanies chronic liver disease plays a role as a link towards hepatocarcinogenesis.[31] Hepatocyte injury leads to necrosis and is subsequently followed by an inflammatory response mediated by key players of the innate immunity. The cellular and tissue environment of liver disease is abundant in inflammatory cells and their secreted cytokines. Many of the cytokines that are abundant in the inflammatory stroma are pro-proliferative, and cause new liver cells to regenerate. The cycle of hepatocyte damage and repair continues and the background inflammation becomes recurrent and causes the formation of micro and macro-nodules. The increased inflammation and subsequent scarring is a feature of cirrhosis. At those setting, more hepatocytes are regenerating, causing gene expression differences that lead to selection for cells with a growth preference. At this stage, epigenetic variations dominate more than genomic alterations, and the resulting gene expression changes are thought to cause a large part of the initiation process of hepatocarcinogenesis.[32,33] Subsequent colonies contain numerous genomic and epigenetic aberrations in various pathways, some of which are characterized with more aggressive disease.

3.3 Genomic Profiles and Gene Expression Profiles in Hepatocellular Carcinoma

To elaborate on the process of hepatocarcinogenesis, several studies have compared the genomic and gene expression profiles of tumour samples compared to normal background liver tissue from the same patient. The genes identified include pathways involved with genomic stability, regulation of transcription, regulation of metabolism, cellular proliferation, apoptosis and survival, cell migration, T-cell regulation and others A discussion of each of the most frequently aberrant pathways in HCC will be presented in the next section. In one of the gene expression profile analyses, it was found that there are differences in the profiles of different cancers based on their etiologic factors. In particular, components of pathways related to immune evasion and cytoskeleton are more frequently deregulated in HCV-related HCC, while in HBV-related HCC aberrations were more common in cell matrix interactions.

3.4 Cellular Pathways Involved in Hepatocarcinogenesis

3.4.1 *Wnt/ßCatenin Pathway*

The Wnt/ß-catenin pathway contains the most commonly mutated components found in the genomes of HCC patients. It is one of the survival pathways that is activated in several types of cancers. It is activated as the Wnt ligand binds to the transmembrane receptor frizzled. Subsequently, the breakdown of ß-catenin by APC and axis is inhibited, thus allowing ß-catenin to translocate into the nucleus and cause upregulation of several genes involved in cancer progression.[41] A common genetic aberration in HCC is an activating mutation in CTNNB1 or inactivating mutations in axis or APC.[42] Another common mutation in HCC is a mutation in Axis Inhibiting Protein (AXIN1). Epigenetic alteration of negative regulators of CTNNB1 are reported, such as SFRPs (Secreted Frizzled-Related Protein) and SOX1 (SRY-Box1). Though the role of the

Wnt/ß-catenin pathway is huge in HCC, pharmacologic inhibitors or modulators of the pathway have not been successfully developed to become available for HCC patients.

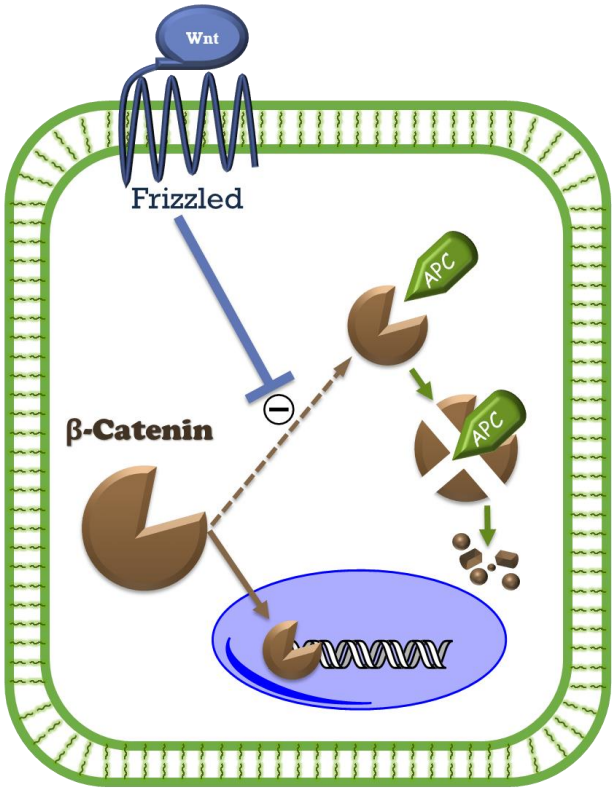


Figure 1 Wnt/ß-catenin pathway

3.4.2 ***P53 and cell cycle pathway***

Another common mutated gene in patients with HCC is the TP53 gene, which encodes the *P53* protein. TP53 is the most commonly mutated gene in human cancer, and its role in protecting the integrity of DNA and preventing neoplasia has been extensively studied.[43] The carcinogenic mechanisms of aflatoxib-B1 (AFB) as well as hepatitis B and C have been linked to alterations in p53 at the genetic, protein or functional level.[31,44] In particular, HCC due to AFB has a high frequency of TP53 mutations at Ser-249. The mechanism of development of TP53 249Ser mutation has been suggested by several studies and include direct mutagenicity of AFB and the production

of mutagenic oxidative species. A common observation is that the frequency of TP53 249Ser is increased in areas where there is both increased exposure to AFB and endemic infection with HBV.[44] The two factors are thought to synergistically affect TP53. HBx gene from HBV transcribes into a protein that affects the function of p53 and increases instability of the genome. The role of free radicals as an endogenous mutagen that causes alteration in TP53 is established and is found in HCC related to chronic inflammation as well as other chronic liver conditions.

The function of p53 protein, which is sometimes referred to as "the guardian of the genome", has been studied extensively since its discovery in 1979.[45] The p53 protein detects DNA damage and other cellular conditions associated with excessive cellular proliferation signals including hypoxia, shortened telomeres and cellular stress. Once the inciting injury is detected, p53 induces an arrest in the cell cycle at G1 by increasing the expression of p21, an inhibitor of cyclin dependent kinase (CDK). p53 also induces DNA repair pathways such as GADD45, and if DNA repair was successful, p53 levels decrease and the cell is allowed to progress through the cell cycle.[43] Otherwise, p53 persistence will lead to the activation of senescence or pro-apoptotic pathway such as BAX, which will prevent the cell with unfixed genome to survive, thus limiting the spread of damaged DNA.

3.4.3 ***HGF/MET***

One of the main pathways involved in hepatocarcinogesis consists of the Hepatocyte growth factor (HGF, also called scatter factor) and its receptor MET.[46] The HGF/MET pathway plays an important role in the regulation of cellular proliferation, survival, invasion, and motility as well as mediating epithelial to mesenchymal transformation (EMT), and thus is responsible for several of the characteristics of invasive cancers.[47] Upon binding to its ligand, MET receptor undergoes autophosphogrylation at its Tyrosine sites Y1349 and Y1356.[48] Subsequently, a group

of docking proteins (including Gab2 and SOS) are recruited and phosphorylated at the receptor site where they in turn allow other proteins to bind and modulate various downstream pathways involved in different functions: FAK and other focal adhesion proteins like PYK2 and paxillin which function to increase cell motility, the proliferative intracellular pathways MAPK and JNK, the JAK/STAT pathway, and the survival pathway PI3K/AKT amongst other pathways. The collective function of downstream effects modulated by HGF/MET are thought to contribute to the ability of cells to metastasize: increased ability to mobilize efficiently using its cytoskeleton, invade the surrounding tissues and increased ability to dislocate and scatter.[47] Aberrant MET signaling and the overexpression of MET are characteristic of more aggressive cancer cells.

Around 40-70% of cases of HCC have MET overexpression, most commonly in the form of increased transcription of the *MET* gene.[46] In addition, several mutations affecting the MET gene are reported in HCC with varying frequencies. Detection of MET overexpression in HCC tissue biopsies can be used as a marker for the tumor aggressiveness. Testing can be done using immunohistochemistry staining using specific antibodies.

Crizotinib

As discussed in section 4.5.3 above, the HGF/MET pathway is one of the important pathways in carcinogenesis particularly in HCC. The 3 MET inhibitors that have been assessed for use in HCC are cabozantinib, tivantenib and foretinib. One of the MET inhibitors that are currently available clinically is crizotinib. It is of interest to our laboratory since our laboratory has studied its pathway interactions[93] and there were attempts to modify it chemically to improve its pharmacology.

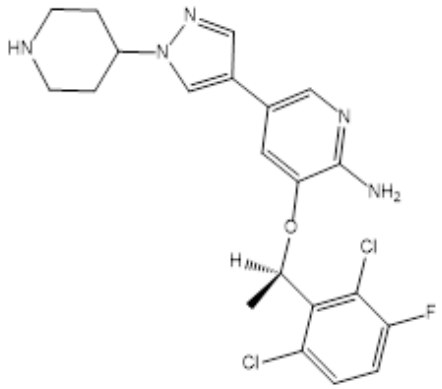


Figure 2 Crizotinib

Crizotinib is a tyrosine kinase inhibitor of MET and ALK that is currently clinically used in the treatment of ALK-rearranged Non-small Cell Lung Cancer (NSCLC). Cirzotinib was developed as a MET inhibitor but its use was redirected towards its action against ALK because of the oncogenic dependency of NSCLC tumours harbouring ALK-rearrangments and the substantial cytoreductive effect produced by inhibiting the ALK pathway.

Pfizer initiated a project to discover a potent inhibitor of MET using structure based drug design (SBDD). The choice of MET as a target was because of the importance of downstream effects mediated by HGF/MET in producing the invasive properties of cancer, and because of the frequent finding of aberrant or deregulated MET in many types of cancer.[94] As described by Cui *et al*.[95], the process of optimization of a MET receptor inhibitor started with modulation of the indoline group of molecules derived from the wide-spectrum kinase inhibitor, sunitinib. The process resulted in the production of a series of lead molecules containing 3-substituted indolin-2-one group, which were optimized to have maximal MET inhibitory activity. The subsequent lead molecule, named PHA-665752, was used to build a cocrystal including the inhibitor with the receptor MET using crystallography. The model gave information about parts of the lead inhibitor

that were inefficiently scaffolded and led to the development of a group of 5-Aryl-3-benzyloxy-2-aminopyridine molecules. Eventually the series were optimized to produce the current structure of crizotinib.[95]

Crizotinib, initially called PF-2341066 was developed by Pfizer and was found to selectively inhibit MET. It was shown to inhibit the phosphorylation of MET and the malignant properties induced by MET including proliferation, migration, and invasion[96]. The effects of crizotinib were further investigated[97] and included inhibiting proliferation, induction of apoptosis, and inhibition of downstream targets of MEK including STAT3, ERK and AKT.

During the evaluation of crizotinib as a novel drug, it was found to have activity against ALK. Thus, it was evaluated for inhibition of tumours that exhibit ALK receptor derangements including anaplastic large cell lymphomas, NSCLC and neuroblastoma[96,98].

Crizotinib has never been evaluated for use against hepatocellular carcinoma *in vivo*. There have been reports about hepatotoxicity in patients taking crizotinib. Grade 3-4 elevations in the liver enzyme alanine transferase (ALT) occurred in the first 2 months of treatment in about 15% of patients in 2 Phase 3 clinical trials[99,100]. Case reports of fulminant acute hepatic failure that lead to death in both cases were also reported.[101] Hepatotoxicity could be the reason crizotinib was not tested in HCC.

3.4.4 ***EGFR***

The epidermal growth factor (EGF) and its receptor (EGFR), in addition to related families of proteins, comprise one of the most commonly studied signaling pathways in cancer cells that is commonly disrupted in various types of cancers.[49,50] The EGFR is part of the ErbB family of receptors, which also includes the human epidermal receptor 2 (HER2) receptor subtype. The

presence of abnormalities related to EGFR is characteristic of a wide variety of subtypes of cancer, and specific mechanisms of disruption can serve as biomarkers to predict response to treatment and thus help decide on the choice of therapeutic regimen to be used. Examples include the use of the mutations L858R, L861Q, G719X and deletions in exon 18 of the EGFR gene as markers for response of non-small cell lung cancer (NSCLC) patients to treatment with EGFR inhibitors like erlotinib and gefitinib.[51,52]

The majority of cases of HCC have positive staining for EGFR using IHC, and about half of the cases have increased *EGFR* gene copy number.[53] In particular, most cases of fibrolamellar HCC have increased expression of EGFR as shown by IHC staining for the receptor. It is unclear whether the status of EGFR and its gene have any correlation with disease aggressiveness and prognosis. Some reports have detected some missense point mutations in the *EGFR* gene and deletion patterns that were recognized in other forms of cancer (particularly NSCLC) are found in HCC.[54]

Preclinical studies evaluating the effects of using EGFR inhibitors on hepatocellular carcinoma showed that they have an antiproliferative activity as well as an effect on inducing cell cycle arrest and apoptosis on cell lines.[55] In vivo, treating mice with gefitinib decreased the rates of development of HCC, suggesting the possibility of using the inhibitor as a form of prophylactic treatment in patients at risk of developing the disease.[56] Nevertheless, a phase 2 trial found that the use of gefitinib was ineffective in HCC. One possible cause of the lack of response of HCC cells to gefitinib was suggested by Giannelli *et al.*[57], who suspected a role played by one of the components of the basement membrane, Laminin-5 (Ln-5), that promotes several aggressive characters of cancer cells. The study showed that Ln-5 restored activation of p-ERK 1/2 and p-AKT following inhibition by gefitinib. Another EGFR inhibitor, erlotinib, was shown to have a

modest benefit in controlling HCC in a phase 2 trial[58], but the phase 3 trial SEARCH which evaluated its use as an adjuvant to sorafenib showed no survival benefit compared to sorafenib plus placebo.[59]

3.4.5 ***MAPK***

The intracellular mitogen activated protein kinase (MAPK) pathway is one of the key intracellular pathways that promote cellular proliferation.[60] Its components serve as second messengers transporting signals from activated membrane receptors to various proteins that lead to regulation of nuclear transcriptions. Several different MAPK pathways have been identified that follow a similar linear pattern, of which the one that is commonly described is the Ras-Raf-MEK-ERK Pathway. Ras is a GTPase that detects signals from G-coupled membrane receptors, and activates Raf. Subsequently, RAF activation leads to phosphorylation activation of MEK, which in turn activates ERK. Activated (phosphorylated) ERK then translocates into the nucleus and activates two of the transcription factors, c-Jun and c-Fos, which act to increase the expression of several genes that are implicated in cellular proliferation (Figure 2).[61] Similar other MAPK pathways use a similar linear scheme where a GTPase protein activates a MAPK kinase kinase kinase (MAPK3K), which in turn activates a MAPK kinase kinase (MAPK2K). The latter ultimately activates MAPK protein that directly phosphorylate proteins involved with cellular processes.

The involvement of the MAPK pathway in carcinogenesis is clear in various types of cancer, particularly HCC, where it contributes to different processes in hepatocarcinogenesis and to resistance to treatment.[62] Although in HCC the presence of specific mutations in RAS or RAF are not common,[42] the pathway is activated in more than half of the cases, and various aberrations in other related proteins that lead to MAPK activation are reported commonly in HCC. The

inhibition of the distal components of the MAPK pathway led to a strong inhibitory effect on liver cancer cell lines.[63] The current standard of care agent in the treatment of HCC, sorafenib, is an inhibitor of RAF, and will be discussed in detail in section 4 below. The MEK inhibitor selumetinib is also discussed below in further details in section 5.2.

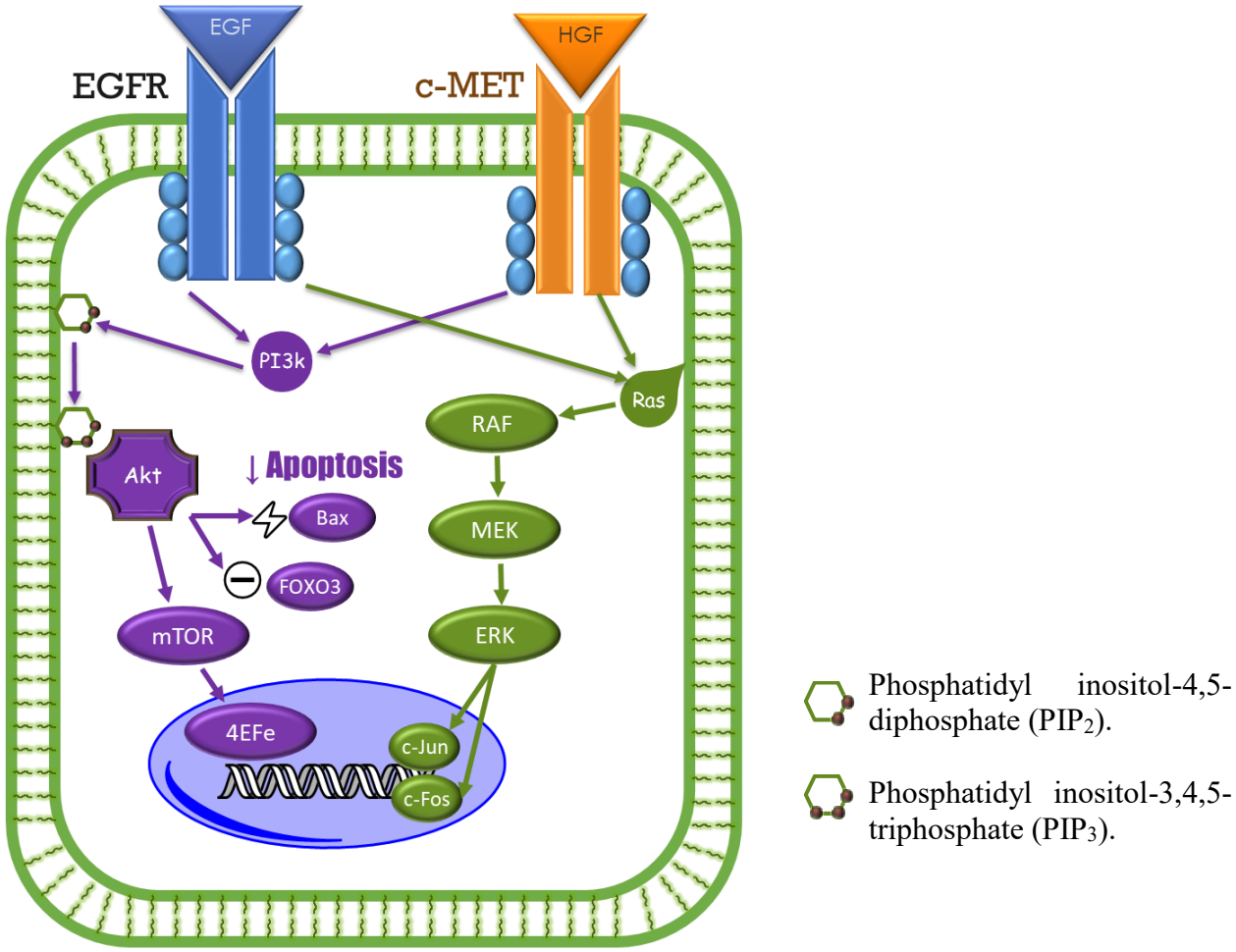


Figure 3 Interactions between EGFR, MET and intracellular pathways

Selumetinib

Selumetinib has been initially discovered by Array biopharma but was later licensed to AstraZeneca for further development.[102] It showed promising pre-clinical activity with IC_{50} for MEK inhibition of 10-14 nmol/L. It showed growth inhibitory activity against human cancer cell lines and xenografts of human tumors in mice including a wide-spectrum of cancer subtypes. It

was tested on hepatocellular carcinoma[63] and was found to inhibit growth on primary liver HCC cells. In addition, selumetinib induced apoptosis and resulted in tumor shrinkage of human HCC-mice xenografts. Phase 1 studies were done to evaluate the safety of selumetinib and to determine the MTD.[103] Interestingly, selumetinib caused complete remission in one of the patients with metastatic melanoma as found by one of the phase I study.[104] Several phase II trials evaluated selumetinib with promising data in metastatic papillary thyroid cancer[105] and advanced melanoma harboring BRAF V600E mutation. It showed an improvement in survival of patients with uveal melanoma.[106] Selumetinib was tested in metastatic biliary cancers with an objective response of tumor 1shrinkage in 3 of the 28 patients and stable disease in 17.[107] Response was seen only in patients with immunohistochemistry showing phosphorylated ERK. Selumetinib was also evaluated in patients with HCC as a monotherapy or in combination with sorafenib.[108] Alone, selumetinib was not shown to have much effect on tumor size by imaging or prolongation of time to progression.[109] A phase Ib trial evaluating selumetinib with sorafenib showed some encouraging anti-tumor activity with a median overall survival of 14.4 months.[108]

3.4.6 ***PI3K/Akt and mTOR***

The phosphoinositide 3-kinase (PI3K)/Akt pathway is the other important intracellular pathway that plays a role in cellular proliferation and survival.[64] It has been found to be frequently altered in a wide variety of cancers, and has been mentioned frequently as a mediator of the carcinogenic potential of several effector proteins or a cause of resistance to therapeutic modalities.

PI3K has three main classes, but the most commonly described is class I, and more specifically the subclass IA.[65] Class IA PI3K is composed of a p110 catalytic subunit and a p85 regulatory subunit. The p85 subunit inhibits the activity of p110 at basal state by binding to it

through inter-src homology 2 (iSH2) domain. Upon activation of a receptor (an RTK or a cytokine receptor), PI3K is recruited to the membrane and is activated (the phosphorylated tyrosine residues on the receptor bind to the SH2 domain on PI3K, recruiting it and releasing the inhibition on the p110 subunit.) Activated PI3K acts on one of the components of the phospholipid cell membrane called phosphatidyl inositol-4,5-diphosphate (PIP_2). PI3K adds a phosphate group to the 3'-carbon of PIP_2, to make phosphatidyl inositol-3,4,5-triphosphate (PIP_3). In turn, the PIP_3 recruits Akt near the cell membrane, and recruits another protein PDK1 that activates it. In order for Akt to be active, it needs to be phosphorylated by PDK1 (that is also recruited to the cell membrane) and PDK2 (that is present in the cytoplasm). Akt is then fully active and can be released into the cytosol to act on its downstream targets.

Akt has three main downstream effects: (a) it activates the mammalian target of rapamycin (mTOR), (b) activates the pro-apoptotic protein, Bcl-2-associated death promoter (BAD), and (c) inhibits the anti-apoptotic transcription factor FOXO3a. As a result, the activation of Akt leads to inhibition of apoptosis and thus it is considered a survival pathway. The other downstream effect of Akt activation is the activation of mTOR, which regulates various cellular functions including cellular proliferation, metabolism, autophagy and cytoskeletal organization. One of the main downstream targets of mTOR is the eukaryotic translation initiation factors (eIF4E). mTOR releases the inhibition of eIF4E by phosphorylating eIF4E-binding protein 1 (4E-BP1). Phosphorylated 4E-BP1 cannot bind to eIF4E, allowing it to proceed to the nucleus and promote genes that play a role in cell survival, cell cycle progression and metastasis.[66] This pathway is particularly important in the process of carcinogenesis (Figure 3)

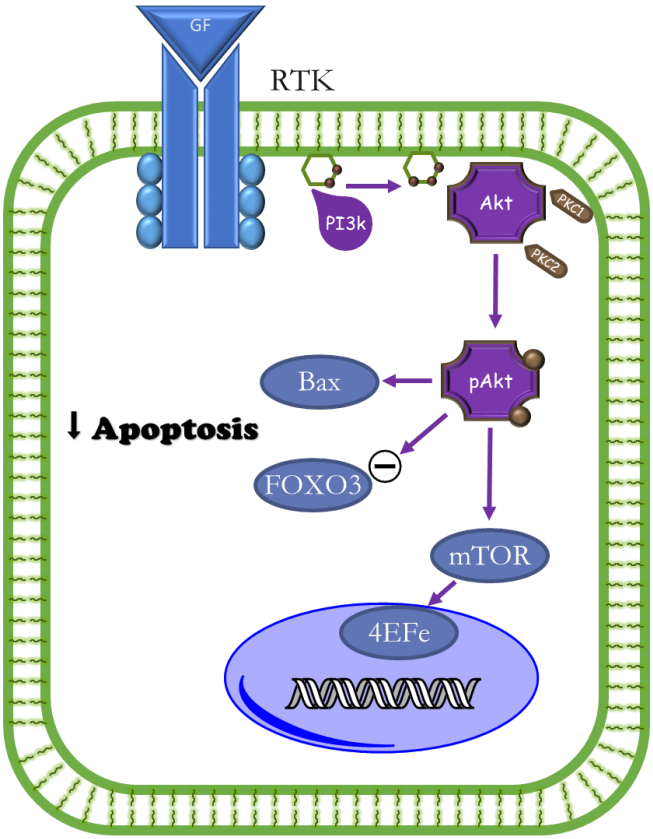


Figure 4 Mechanism of action of the PI3K pathway

The inhibition of mTOR by small molecule inhibitors has been of particular interest in the treatment of cancer, as its inhibitors temsirolimus and everolimus are approved for the use in specific conditions in cancer treatment. Everolimus was shown to have an antitumour effects in patients with HCC as shown by disease control rates of 63% and 44% in a phase 1 and phase 1/2 trial, respectively.[67,68] The EVOLVE-1 is a phase 3 trial that was conducted to assess the use of everolimus in patients who failed sorafenib, but did not show an increased overall survival rate.[69]

3.4.7 *JAK/STAT*

The Janus kinase (JAK)/ Signal transducer and activator of transcription (STAT) is one of the intracellular pathways that enhances proliferation and mediates cellular effects in response to cytokines secreted from the cell's microenvironment. It is a commonly deregulated pathway in

several types of cancers particularly in relation to inflammation surrounding tumor cells.[70,71] Mutations in JAK are rare in solid cancers, but have been reported frequently in myeloproliferative disorders, where one of the JAK inhibitors have shown a highly potent effect and is approved as a standard of care.[72,73]

Under basal condition, each JAK is associated with a subunit of a cytokine receptor, and is in an inactive form. When cytokine receptors are activated by their respective ligands, they undergo dimerization where two subunits of the receptor come together, while simultaneously bringing each of the intracellular JAKs to each other. Interactions of both JAK proteins results in their phosphorylation (by cross-phosphorylation or auto-phosphorylation). The phosphorylated sites then form a docking site for the attachment of the SH2-domain of downstream effectors, including STAT proteins. Once recruited near the cell membrane, STAT is phosphorylated by JAK, and subsequently activated STAT travels into the nucleus to increase the transcription of target genes.[74] In addition to STAT, JAK can lead to recruitment and subsequent activation of Src, PI3K, and Raf, explaining the interactions with the different intracellular pathways.[75]

The activation of JAK/STAT is thought to be an important component of hepatocarcinogenesis relevant to the inflammation-derived cellular proliferation.[31] Direct JAK mutations have been reported in cases of HCC, but it is an uncommon occurrence. One of the common mechanism of deregulation of the JAK/STAT pathway occurs through a protein called Silencer of cytokine signaling 1 (SOCS).[76] The *SOCS1* gene is a frequently found to be epigenetically silenced in HCC through methylation of its promoter.[77] SOCS1 contains an SH-2 domain which binds to JAK in competition with typical downstream targets of JAK, inhibiting their phosphorylation.

4 Sorafenib

Because of its importance and relevance to the topic of HCC, sorafenib is discussed below with in-depth details. The story of sorafenib discovery is an example showing how rational drug design can lead to a clinically useful agent.

4.1 The Discovery of Sorafenib

The initial project that led to the discovery of sorafenib started by Bayer and Onyx Pharmaceuticals in 1994 to identify inhibitors of Raf-1 [78]. Their choice of Raf-1 as a target was due to several reasons based on previously revealed evidence. Disruption of signaling through Raf kinase had already been found frequently in a wide variety of cancers [79]. In addition, a study by Kasid *et al*.[80] had shown that interfering with Raf-1 gene using antisense oligonucleotides would be effective in inhibiting the growth of different human tumor xenografts in athymic mice. Bayer and Onyx validated the importance of Raf-1 as a target by using MEK constructs to disrupt signaling from Raf to ERK on xenografts from several types of human cancers harbouring *K-ras*. They have found that mice expressing the construct survived twice longer than controls lacking it[81].

Bayer and Onyx performed high-throughput screening of molecules that inhibit Raf-1, and that yielded around 200,000 molecules. Lead molecules were subsequently chemically modified to obtain the most optimum inhibitory molecule[78,82]. Lead molecules contained urea, which is a functional group composed of two amine groups connected by a carboxyl group. Initial molecules included sulfur containing aromatic urea structures (3-thienyl urea), but were found to have half-maximal inhibitory concentration (IC_{50}) values of more than 1 μM. Another group of molecules containing bis-aryl urea groups (urea groups connecting two aromatic rings) were shown to have better IC_{50} values. Those were optimized to achieve better binding, and this led to the development of sorafenib (BAY 43-9006), which has an IC_{50} for inhibition of Raf-1 of 6 nM (Figure 4).

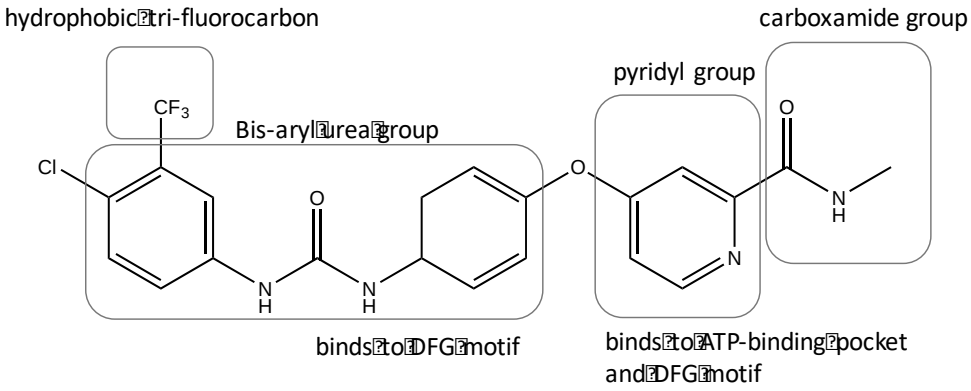


Figure 5 Structure of sorafenib

4.2 The Structure of Sorafenib

The sorafenib molecule is one of the bis-aryl urea molecules that inhibits Raf-1 kinase through a type 2 binding mode where it binds to both the ATP binding pocket and the allosteric asparagine-Phenylalanine-Glycine (DFG) loop that is exposed when the kinase is in an inactive conformation.[83] Thus, it recognizes the inactive form of the kinase,[84] when the DFG loop is outside the pocket (DFG-out conformation). This was shown in X-ray crystallographic studies of sorafenib interactions with Raf-1 as well as B-Raf wild type and V600E mutated B-Raf.[85] Sorafenib is localized in the hinge region between the two lobes of Raf-1, binding to ATP adenine pocket through the distal pyridyl ring where it interacts with amino acid residues Trp530 and Phe583. The DFG motif interacts with sorafenib at 2 of its sites, the distal pyridyl ring and the central phynyl ring. At the other end of the molecule there is a lipophilic ring formed by the triflouromethyl group that interacts with a hydrophobic pocket formed when the kinase is in the inactive form[85] (Figure 4 and 5).

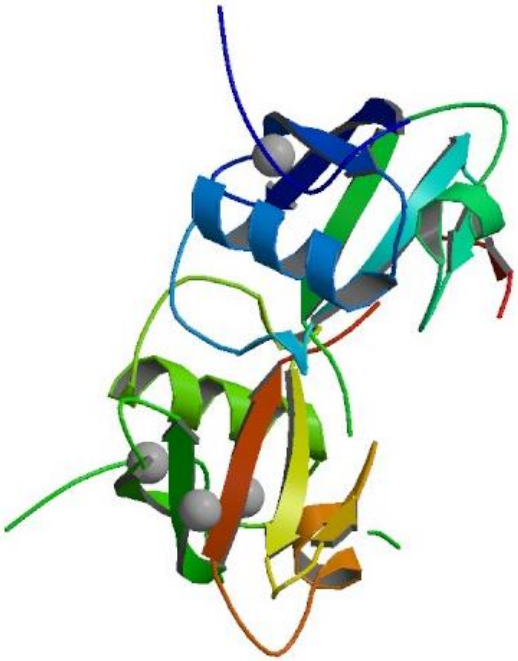


Figure 6 Structure of Raf (Source: protein database)

4.3 Pharmacodynamics of Sorafenib

Biochemical and pharmacodynamics studies investigating the mechanism action of sorafenib revealed more effects than those mediated by Raf-1 inhibition[86]. In vitro biochemical assays showed the inhibitory action of sorafenib on a wide variety of kinases. In addition to inhibition of the serine/threonine isoforms of Raf, including Raf-1 and both wild-type and V600E mutated B-Raf, it also inhibits several receptor tyrosine kinases, including VEGFR, PDGFR, c-kit, Flt-1 and RET[78,87] (figure 6). The Ras/Raf/MAPK pathway was inhibited by sorafenib in several cell lines of different types of cancer expressing the mutant KRAS gene, V600E BRAF and wild-type BRAF, including colon, pancreas and breast tumour cell lines, as well as hepatocellular carcinoma cell lines[88]. Dose-dependent induction of apoptosis was also seen in response to sorafenib in those cell lines. Furthermore, Sorafenib caused growth inhibition of human tumour xenografts in athymic

mice. The effects of sorafenib on angiogenesis have been shown by reduction in microvessel area and density in xenograft models.[87]

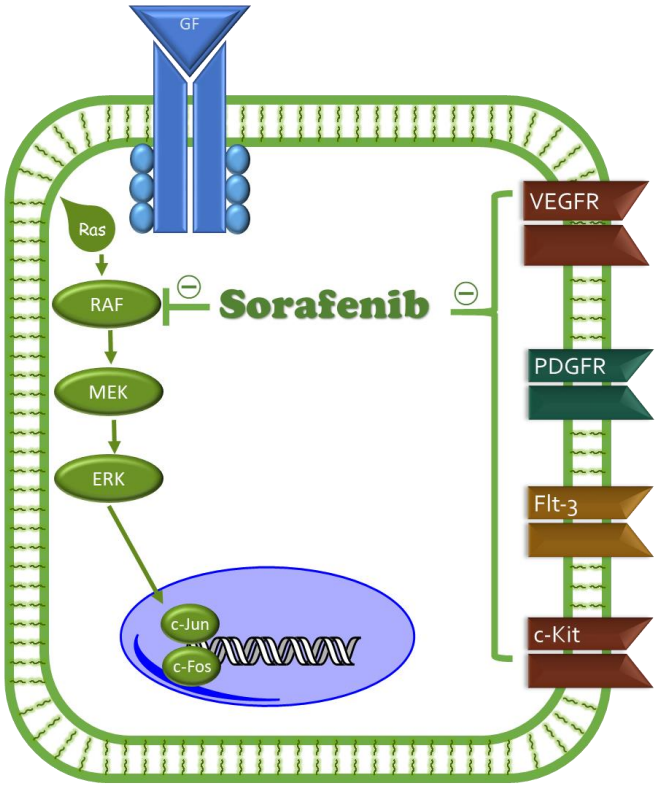


Figure 7 Action of Sorafenib

4.4 Clinical Development of Sorafenib

4.4.1 *Phase I Clinical Trials*

Following preclinical studies, including safety studies in dogs, the investigation of sorafenib moved into phase 1 clinical trials in July 2000[78]. Initially, four trials were conducted that used different schedules of sorafenib in a variety of different types of cancers[89]. The starting dose was 50 mg orally daily, and the optimum regimen was found to be 400 mg bid, where sorafenib was well tolerated. Dose-limiting toxicities included grade 3 diarrhea and fatigue at 800 mg bid, and grade 3 skin toxicity at 600 mg bid. There were no reported cases of severe hematological,

cardiovascular, hepatic or renal toxicities. However, hypertension was observed in 5-11% of cases across the four phase 1 trials.[89]

More clinical trials were done to assess the use of sorafenib in combination, and showed that sorafenib can be well-tolerated in combination with other systemic anticancer agents without a significant increase in toxicity. Agents that were tested in combination with sorafenib include oxaliplatin, 5-flourouracil and leuocovorin, paclitaxel/carboplatin, gemcitabine, doxorubicin, taxotere, CPT-11, dacarbazine, gefitinib and interferon[78].

The children's oncology group (COG) conducted a phase I study to evaluate the safety, pharmacokinetics and pharmacodynamics of sorafenib in children with different solid tumours and leukemias[90]. They concluded that sorafenib can be tolerated in children at a dose of 200 mg/m2/dose bid in solid tumours and 150 mg/m2/dose bid in leukemias. Two children with AML harbouring FLT3 Internal tandem duplication had a remarkable decrease in the number of blast cells in bone marrow to less than 5%. Preliminary results from the study also showed promising activity of sorafenib against pediatric solid tumours as 14 patients sustained stable disease for 4 or more cycles[90].

4.4.2 ***Phase 2 and 3 Clinical Trials***

A phase II clinical trial was done on 137 patients with inoperable hepatocellular carcinoma where they were given sorafenib 400 mg twice daily[91]. Results showed that 46 of patients (33%) had stable disease for 16 weeks or more, while partial response was achieved by 3 patients and 8 patients had minimal response. The next step was to move to phase III clinical trials to evaluate sorafenib as a first-line agent in the management of unresectable hepatocellular carcinoma. The SHARP (Sorafenib HCC Assessment Randomized Protocol) study[92] was a phase III clinical trial where 602 patients with treatment-naïve, unresectable HCC were enrolled and randomized into

either sorafenib 400 mg bid or placebo. Results showed a significant increase in overall survival rates in the sorafenib arm compared to the placebo arm (10.7 months versus 7.9 months, $P<0.001$).

The use of sorafenib in hepatocellular carcinoma was approved by the FDA in November 2007. An evaluation of the cost-effectiveness of the use of sorafenib for hepatocellular carcinoma in Canada using a Markov model showed that its use is more cost-effective than best supportive care for advanced hepatocellular carcinoma[14].

5 Models in HCC Research

Modeling hepatocellular carcinoma in the laboratory has been an ongoing challenge Many of the cell lines used for HCC-related experiments are infected with hepatitis B or hepatitis C virus, and thus need special protective equipment to be handled. Of the most common virus-free cell lines, HepG2 is a paediatric liver tumour, and SK-HEP-1 as well as Huh7 are hemochromatosis-related HCC cell lines. The 3 cell lines have major differences and have been characterized and described in the literature.

HepG2 is a widely used well-established liver cancer cell line that is derived from a 15 year-old Caucasian young man from Argentina. The origin of the cell line is not clear as whether it represents the more common hepatocellular carcinoma or the childhood tumour hepatoblastoma[117]. HepG2 has been characterized by several studies. Its gene expression profile has been studied[118] and showed dysregulation of a variety of genes and network analysis of up-regulated genes showed dysregulation of genes assigned related to “cell cycle, cell-to-cell signaling and interaction, cellular assembly and organization” as well as genes related to “DNA Replication, Recombination, and Repair”. Disruption of STAT signaling was also seen amongst networks of down-regulated genes. HepG2 has wild type p53, but mutant Rb and mutant N-ras,

which points towards the importance of the MAPK pathway in HepG2. Also, HepG2 has been found to have constitutive activation of MET.[119]

Besides being studied as an HCC model, HepG2 has been widely used in the process of drug development for *in vitro* characterization of drug metabolism due to the expression of a wide variety of drug metabolizing enzymes[120].

Another commonly used cell line is SK-HEP-1, which was developed in the Memorial Sloan-Kettering Cancer Center as a model for the study of hepatocellular carcinoma. It was derived from a 52-year-old man with hematochromatosis related hepatocellular carcinoma. As described above, HHC is a disease that causing chronic liver fibrosis as a consequence of disposition of iron in liver cells. It is an important cause of non-viral HCC. The fact that it is not virally contagious makes it an attractive choice as a model for HCC.

The cellular origin of SK-HEP-1 has been a subject of debate. The cell line was derived from an ascetic sample and thus it is not definitely originating from the liver. The nature of SK-HEP-1 growth looks like the growth of a mesenchymal cell. The hypotheses are that either cells from SK-HEP-1 originated from tumour stroma or from cells that have underwent the process of EMT.

The Huh7 is another non-virus-related HCC cell line that has also been increasingly interesting to liver cancer scientists. It was first obtained from a 57-year-old Japanese male with hepatocellular carcinoma. Gene expression profile sequencing showed mutation of the HFE gene consistent with hematochromatosis, despite the lack of data about the iron status in the patient.[121] One of the unique characteristics of Huh7 is that it is self-sufficient in terms of growth factor secretions, mostly related to its carcinogenic function of secreting mitogens. Due to its good

replicative potential, Huh7 has been used for transfection with hepatitis C virus to study its virology as well as virus-related HCC.

6 Combi-molecules

The concept of dual targeting using rationally designed combi-molecules that are composed of the active groups of two or more "parent" drugs that are combined chemically. Different types of co-targeting have been described. Our group has described thoroughly different examples of combi-molecules that are used in oncology by targeting multiple carcinogenic pathways.[122] Two types of combi-molecules have been described which are classified on the basis of the presence or absence of effect before and after the hydrolysis of the molecule.[123,124] Type I combi-molecules are inactive before hydrolysis into both its constituents and thus will need to be broken down near their targets before they can produce their respective actions. Type II Combi-molecules exhibit actions represented by its constituents without hydrolysis into its two moieties. They typically stay as stable molecules for their action. More recently, our group have identified a new type of combi-molecules, type III, where the action is intact before and after hydrolysis.[93] The intact combi-molecule acts on both targets without hydrolysis, but is still able to be hydrolyzed into its 2 bioactive moieties. Although the first two types have more frequently been described in the context of an alkylating agent and a tyrosine kinase inhibitor, the first type III molecules described included only kinase inhibitor moieties.

7 Rationale and Approach

Our project aims to study therapeutic strategies against hepatocellular carcinoma. The focus of the investigations that we conducted were to determine signaling pathways to be targeted for effective drug combination against HCC. We aimed to find an effective combination of treatments in order to inspire the synthesis of new combi-molecules. Discovering a promising

combination may also point towards a clinical trial that can be designed based on pre-clinical data showing the efficacy of an investigated combination.

The objectives of our study were to:

1. screen for strategies that will lead to effective inhibition of the growth of HCC cell lines by using combination treatment or through the introduction of available clinical drugs or potential novel molecules.
2. characterize the effects of novel treatment strategies on HCC cell.

Chapter 2 Combination Therapy Targeting Multiple Sorafenib Resistance Pathways is Potentially an Effective Therapeutic Strategy Against Hepatocellular Carcinoma

1 Introduction

The discovery and development of effective agents that halt the growth of hepatocellular carcinoma (HCC) and thus reduce its increasingly high rates of mortality is an unmet need. Despite the efforts that were put into the field of HCC drug discovery and development, the outcomes have been frustratingly scarce. The hopes are that subsequent clinical trials are designed more rationally on the basis of rich and solid preclinical data. For this reason, there have been investments of efforts and resources into the production of preclinical experiments.

Currently, the only systemic agent that is approved for treatment of advanced-stage HCC is the multi-kinase inhibitor and anti-angiogenic agent, sorafenib. One of the main targets of sorafenib is Raf-1 which is a main component of the MAPK pathway. The interactions of the MAPK pathway with other intracellular pathways are complex but are also strong and clearly evident. Thus, it is encouraging to identify specifically the kinds of intracellular pathway interactions that are present in HCC cells, and the effects of inhibition of their components on cellular proliferation.

Our study was done to identify the intracellular interactions and study what the effects of adding kinase inhibitors are to the activity of those pathways in the hope of designing a molecule that has a strong inhibitory activity on multiple pathways that ultimately lead to enhanced inhibition of HCC proliferation. We took a step further and attempted testing two agents designed in our laboratory that involve simultaneous inhibition of various pathways. Both agents are combi-molecules that are designed to inhibit two different kinase proteins simultaneously.

2 Methods and Materials

The experiments were done on three established liver cancer cell lines that have been used and well-characterized by other investigators as described in the literature: HepG2, Huh7 and SK-HEP-1 cell lines. The main differences between the three cell lines are mentioned in table 2.1 below. The cell lines HepG2 and SK-HEP-1 were received generously from Dr. Peter Metrakos's lab as frozen vials in DMEM and 5% DMSO. The cell line Huh7 was generously obtained from Dr. Jean-Jacques Lebrun's laboratory in DMEM-grown petri dishes.

Table 1 Liver cancer cell lines.

HepG2	**SK-HEP-1**	**Huh7**
15-year-old boy Liver Cancer	Liver cancer cell line from a 52-year-old man with HCC	Hemochromatosis (HHC)-related HCC
Hepatocellular origin, but controversial disease: well differentiated HCC vs. hepatoblastoma	Derived from ascites samples suspected endothelial origin	Hepatocellular origin
Characteristic growth in clusters, resembling the tissue structure of hepatocytes forming bile canaliculi	mesenchymal morphology and behavior	Epithelial, hepatocyte-like morphology and behavior
Doubling time: approx. 48 hours	Doubling time: 30 hours	Doubling time: 30 hours

All cell lines were grown in DMEM media that is supplied with fetal bovine serum (FBS), HEPES buffer and enriched with glutamine. Three types of antimicrobial agents were used in the media: gentamicin, liposomal amphotericin B and ciprofloxacin. Cells were kept in a sterile environment at 37°C with 5% Carbon dioxide (CO_2).

To determine the potency of agents to inhibit the growth of HCC cell lines, the sulforhodamine B (SRB) assay was used. Cells were plated on 96-well plates and were left to attach for one to two days. The cells were then treated with serial concentrations of the drug being tested. They were left in the incubator at 37 °C for 5 days, after which 50% trichloroacetic acid

(TCA) was added and left to fix the cells for 2 hours. The cells were washed with running water and were left to dry overnight. SRB dye was subsequently added and left to stain the cells for a few hours. The plates were washed again with acetic acid and left to dry overnight. On the last day, 10 microM tris base is added and the plates were read using plate reader ELx808 at 492 nm. The software used for analysis was GraphPad Prism 6.0, where a curve-of-best-fit was constructed and used to calculate the concentration that produces 50% of inhibitory effect (IC_{50}).

Treatments with single agents as well as with selected combinations of multiple agents were assessed. Combinations in an equimolar ratio were performed first, and the combination potency was compared to the efficacy of each drug alone. Equi-effective combinations were subsequently analyzed to confirm the synergy where present.

For analysis of synergy of two agents in combination, we used the method described in Suman Rao's thesis.[125] We initially determine the fold-difference (κ) between the potency of both agents as reflected by the dose of drug causing 50% of cellular growth inhibition (IC_{50}). If $\kappa > 6$, indicating unbalanced combination, the synergy of the agents in combination wouldn't reliably be assessed by equimolar combinations as the potency of the equimolar combination would represent the potency of the more potent agent alone. In those cases, the synergy would be evaluated using equi-effective combinations and the Chou-Talalay method would be used, as described below.

Chou-Talalay method[126] was used to calculate the combination index (CI_{50}) in order to assess for the presence of synergy, addition or antagonism in the combination. The ratio of concentrations used in each treatment is based on the potencies of the components of the treatment. The formula $\gamma 1/\gamma 2$ is used. Different concentrations of drugs were used while maintaining the ratio.

CI_{50} was calculated using the formula $CI_{50} = \gamma 1c/\gamma 1 + \gamma 2c/\gamma 2$. In order to determine the potency at different concentrations, isobolograms were constructed using CI10 to CI90 that were

calculated in the similar manner to the one described above to measure CI_{50}, with corresponding IC10 to IC90s respectively. For values of combination indices (CI_{50} mainly but also CI10 to CI90), values below 1 indicated synergy, value of 1 indicated additive effect and a value above 1 indicated antagonism.

For assessment of membrane and intracellular receptor activities and pathways, we used the Western blot technique of radio-immunoblotting of proteins. The techniques used were derived from literature and experience from our lab. Cells were plated in 6-well plates and left to attach for 1 day. They were treated with respective treatment that was left on the cells for 2-hours and 24-hours. At the end-point of treatment, cells were washed twice with PBS and were stored at -20 C until harvest. To harvest the cells, a RIPA (radio-immunoprecipitation assay) buffer was used including a protease inhibitor called PMSF. After adding 50 microL of RIPA buffer with PMSF to each well, cells scraped out mechanically. The well contents were collected and eventually centrifuged. The supernatant was separated and stored at 20 C until the time of protein quantification.

Next, the protein content of cells was quantified using the Bradford Assay. A known amount of protein in the form of bovine serum albumin (BSA) was added in different concentrations to a fixed amount of a dye solution based on Commasie brilliant blue G-250 dye (Bio-Rad® Protein Assay Reagent). After assembling 96-well plates with different known concentrations of BSA as well as the different samples to be tested, a fixed amount of dye is added to all wells and the calorimetric absorbance is tested using plate reader ELx808 at 600 nm. All samples were subsequently diluted to have equal concentrations of protein. Laemelli buffer solution containing SDS-PAGE, 2-mercaptoethanol, bromphenol blue, and glycerol was added to make each of the final sample solutions.

Next, we used pre-casted gels for protein electrophoresis. A 10-well pre-casted gel was used with a graded mesh (BioRad® Gel). Samples were loaded at respective wells and placed in an electrophoresis generator with voltages of 80-150V for about 1-2 hours while submerged with a Running buffer. The resulting gel would be expected to have proteins migrated down the gel according to their molecular weight and would have a protein ladder. Next, proteins on the gel would be transferred to a bovine serum albumin membrane activated with methanol. The transfer takes place in a transfer buffer that includes tris base, glycine and methanol. An electrophoresis generator drives the transfer from the gel to the membrane using 100V for 1 and a half hour.

The membranes are then placed in TBS solution containing 20 mM Tris-HCl, 137 mM NaCl, 0.1 with a detergent containing polysorbate 20 (Tween 20 – Arcos organics®). They are saturated with milk for at least 45 minutes before the primary antibody is left to incubate overnight at 4 C. Next, the primary antibody is washed away and a secondary

antibody is placed for 1.5 hour in room temperature. A detection solution (ECL) is used to detect secondary antibodies. Detection is used using radiographic films. To strip membranes and prepare them for the next antibody testing, a Restore Stripping buffer (Thermo Scientific, Rockford, IL, United States) is used for 30 minutes.

3 Results

3.1 Single drug treatments

Single drug treatments were performed initially to determine their potency on the liver cancer cell lines. Results are shown in table 3.1. Drug screening included 10 small molecule kinase inhibitors and 3 cytotoxic agents. Amongst kinase inhibitors, we have seen particularly high potency of sorafenib on HepG2, selumetinib on HepG2 and crizotinib on HepG2 and SK-HEP-1. We also found that sunitinib was effective in both HepG2 and SK-HEP-1. Amongst cytotoxic agent,

we found that 5-FU had a moderate potency on both cell lines but it's derivative, 5-dFU was potent on SK-HEP-1 but not HepG2. doxorubicin was very potent at nanomolar concentrations on both HepG2 and SK-HEP-1. Sorafenib, Crizotinib and selumetinib had modest potency on Huh-7.

Table 2 Potency measures (IC_{50} values) for different compounds on liver cancer cell lines.

	Target	**HepG2 IC_{50}(μM)**	**Sk-Hep-1 IC_{50}(μM)**	**Huh7 IC_{50}(μM)**
Sorafenib	Raf-1, …	0.841 +/- 0.228	3.154 +/- 0.578	2.451 +/- 0.189
Gefitinib	EGFR	11.349 +/- 1.187	5.520 +/- 0.735	8.340 +/- 0.889
RB10	EGFR	20.74 +/- 3.99	29.17 +/- 4.94	
Crizotinib	MET	0.984 +/- 0.184	0.703 +/- 0.169	2.110 +/- 0.134
Dasatinib	Src	8.726 +/- 2.407	2.204 +/- 0.620	
Selumetinib	MEK	0.0248 +/- 0.017	1.264 +/- 0.426	2.298 +/- 0.494
Ruxolitinib	JAK	10.09 +/- 1.650	19.07 +/- 3.19	
Sunitinib	VEGFR, …	0.824 +/- 0.050	0.731 +/- 0.083	
Imatinib	c-kit, …	6.612 +/- 2.426	11.863 +/- 0.792	
Erlotinib	EGFR	2.65 +/- 1.45	13.075 +/- 1.591	
5-FU	DNA synthesis	2.750 +/- 0.685	5.054 +/- 0.781	
5-dFU	DNA synthesis	28.82 +/- 1.40	0.654 +/- 0.158	
Doxorubicin	DNA intercalation	0.00640 +/- 0.008	0.00556 +/- 0.000371	

3.2 Crizotinib and its combinations on liver cancer cells

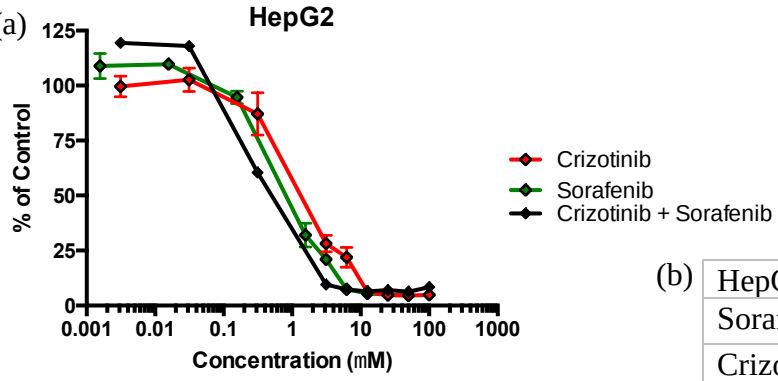
The METinhibitor crizotinib was found to have a potent effect on the inhibition of the growth of liver cancer cell lines HepG2, SK-HEP-1, and Huh7 with IC_{50} values of 0.98 μM, 0.70 μM, and 2.11 μM, respectively. The inhibition should effective interactions as shown below.

3.2.1 *Combination of Sorafenib and Crizotinib on liver cancer cells*

Both sorafenib and crizotinib were potent on the cell line HepG2 with similar IC_{50} values ($\lambda_{Sorafenib}$ = 0.84 μM $\lambda_{crizotinib}$= 0.98 μM, κ=1.17) and had similar IC_{50} values on Huh7 ($\lambda_{Sorafenib}$ = 2.45 μM $\lambda_{crizotinib}$= 2.32 μM, , κ=1.15). But on SK-HEP-1, crizotinib was more potent ($\lambda_{Sorafenib}$ = 3.15 μM $\lambda_{crizotinib}$= 0.70 μM, , κ=4.49). On HepG2 and Huh7 where the fold-difference is small, there is improvement of potency, indicating that the equimolar combination is effective (HepG2:

$\lambda_{Sorafenib+rizotinib}$ = 0.30 μM, ε = 0.76; Huh7 $\lambda_{Sorafenib+rizotinib}$ = 1.02 μM, ε =2.02). However, the combination was not more potent than crizotinib alone on SK-HEP-1 ($\lambda_{Sorafenib+rizotinib}$ = 1.75 μM, ε = 13.7).

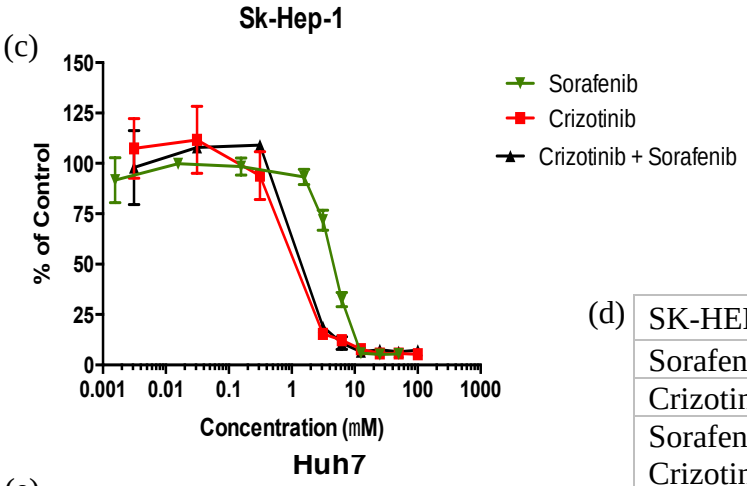
(a)



(b)

HepG2 equimolar combination			
Sorafenib	0.84 μM	Ratio (κ)	1.17
Crizotinib	0.98 μM		
Sorafenib + Crizotinib	0.30 μM	Potency Index (ϵ)	0.76

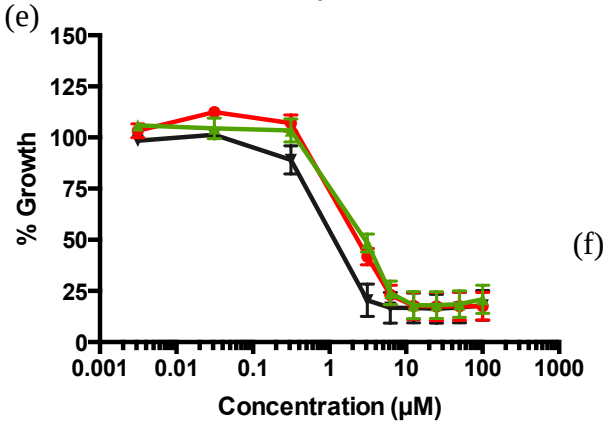
(c)



(d)

SK-HEP-1 equimolar combination			
Sorafenib	3.15 μM	Ratio (κ)	4.49
Crizotinib	0.70 μM		
Sorafenib + Crizotinib	1.75 μM	Potency Index (ϵ)	13.7

(e)



(f)

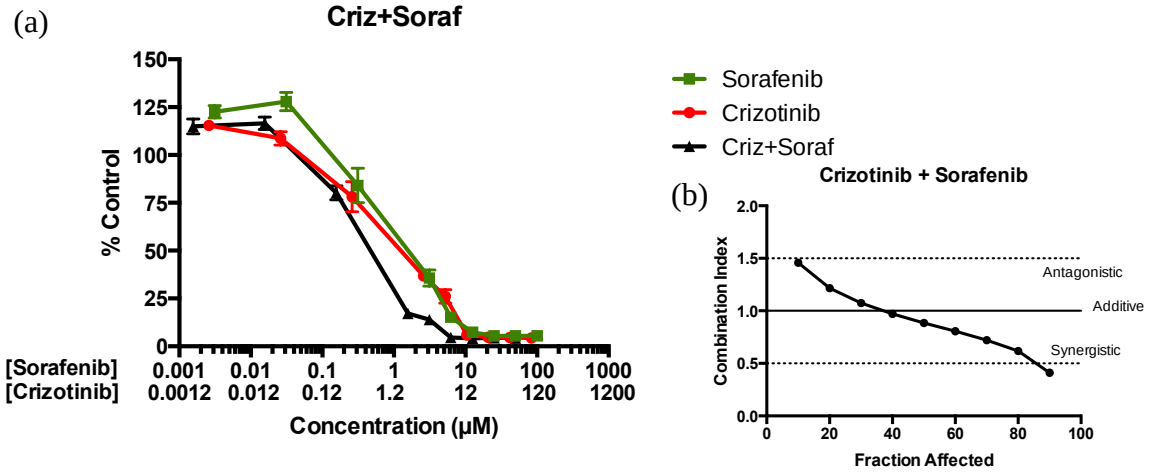
Huh7 equimolar combination			
Sorafenib	2.45 μM	Ratio (κ)	1.15
Crizotinib	2.32 μM		
Sorafenib + Crizotinib	1.02 μM	Potency Index (ϵ)	2.02

Figure 8 (a)-(f): Equimolar combination of sorafenib and crizotinib on the liver cancer cell lines.

Equi-effective combinations of crizotinib and sorafenib were attempted on all 3 cell lines to confirm the effectiveness of the combination and assess the synergy between the agents. The ratio used on HepG2 was 1.2, the ratio on SK-HEP-1 was 4.6, and the ratio was 1:1 on Huh7.

On HepG2, the IC_{50} of sorafenib in combination ($c\lambda_{Sorafenib}$) was 0.29 µM and that of crizotinib ($c\lambda_{crizotinib}$) was 0.35 µM. Results showed mild synergy between crizotinib and sorafenib on HepG2 only with a CI_{50} of 0.88. Isobologram graph showed that the combination of crizotinib and sorafenib was synergistic at higher concentrations and additive (or even mildly antagonistic) at lower concentrations.

(a)



(b)

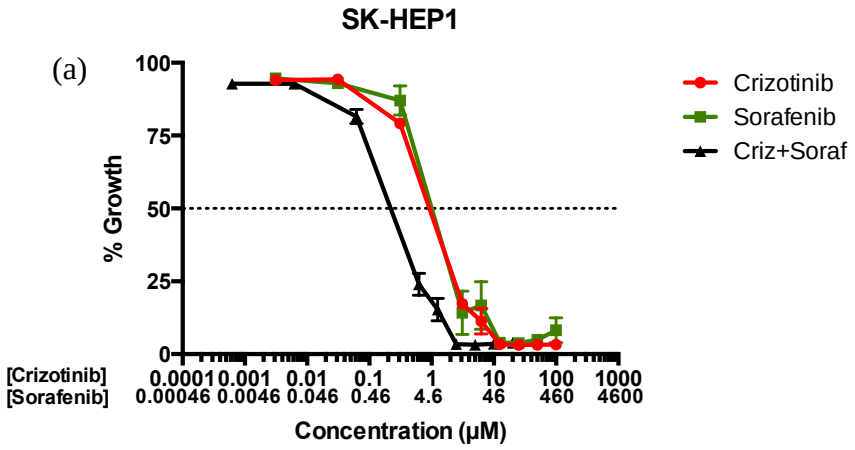
(c)

	Crizotinib	Sorafenib
Alone	0.98 µM	0.84 µM
In Combination	0.35 µM	0.29 µM
CI_{50}	0.88	

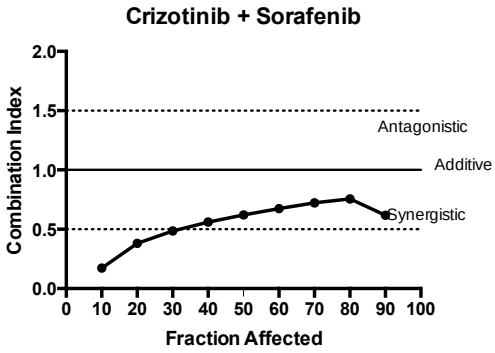
Figure 9 (a)-(c) Equi-effective combination of sorafenib and crizotinib on HepG2

SK-HEP-1, the IC_{50} of sorafenib in combination ($c\lambda_{Sorafenib}$) was 0.94 µM and that of crizotinib ($c\lambda_{crizotinib}$) was 0.13 µM. Results showed good synergy between the two agents on SK-HEP-1 with a CI_{50} of 0.34. On isobologram, synergy was seen on all concentration ranges.

(a)



(b)



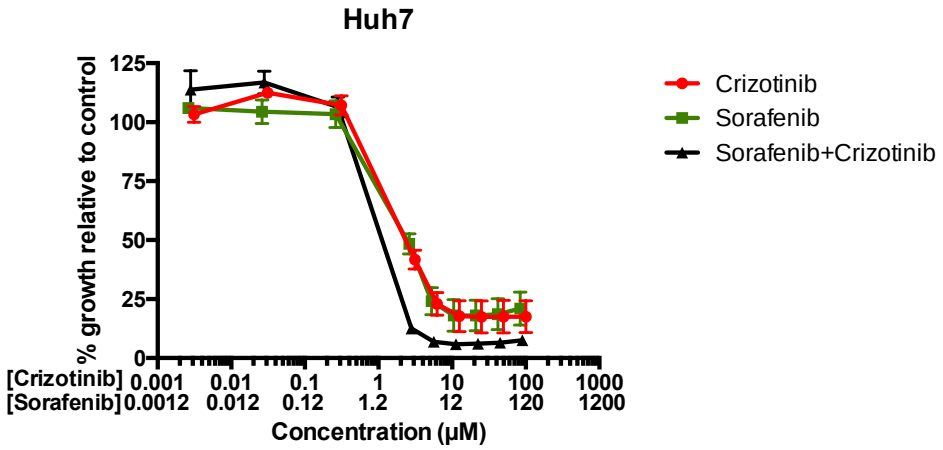
(c)

Equi-effective combination on SK-HEP-1		
	Crizotinib	Sorafenib
Alone	0.70 μM	3.15 μM
In Combination	0.13 μM	0.94 μM
CI_{50}	0.34	

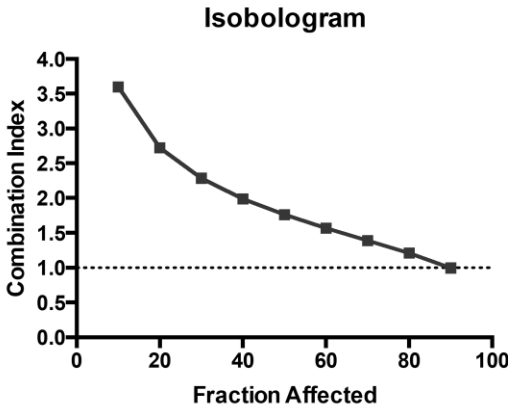
Figure 10 (a)-(c) Equi-effective combination of sorafenib and crizotinib on SK-HEP-1

On Huh7, the IC_{50} of sorafenib in combination ($c\lambda_{Sorafenib}$) was 1.89 μM and that of crizotinib ($c\lambda_{crizotinib}$) was 1.54 μM, both of which show more potency compared to each agent alone. However, the combination index CI_{50} was 1.45 and on isobologram, the interaction was mostly antagonistic but becomes additive at higher concentration.

(a)



(b)



(c)

Equi-effective combination on Huh7		
	Crizotinib	Sorafenib
Alone	2.11 μM	2.45 μM
In Combination	1.54 μM	1.89 μM
CI_{50}	1.45	

Figure 11 (a)-(c) Equi-effective combination of sorafenib and crizotinib on Huh7

3.2.1.1. Western Blotting

Western blot done on SK-HEP-1 following stimulation with EGF for 24 hours was done. Treatment with crizotinib, sorafenib and the combination was given. The doses used were each agent's respective IC_{50} value. With any of the treatments used, pEGFR, seems to be only modestly inhibited at 2 hours. However, in 24 hours, pEGFR seems to have been re-activated in cells treated with sorafenib. This re-activation seems to be inhibited by the addition of crizotinib. The re-activation effect of pEGFR is not shown on cells treated with crizotinib in 24 hours. The MAPK pathway, demonstrated by pERK, seems to have been inhibited by sorafenib more than crizotinib. Maximum inhibition occurs in 24 hours by both agents. The pI3k pathway activity was shown as

activity of pAKT. Crizotinib seems to have maximum inhibitory effect on pAKT, and seems to improve sorafenib's inhibition of pAKT at 2 hours and 24 hours. However, crizotinib seems to inhibit pAKT alone to a higher degree than crizotinib and sorafenib.

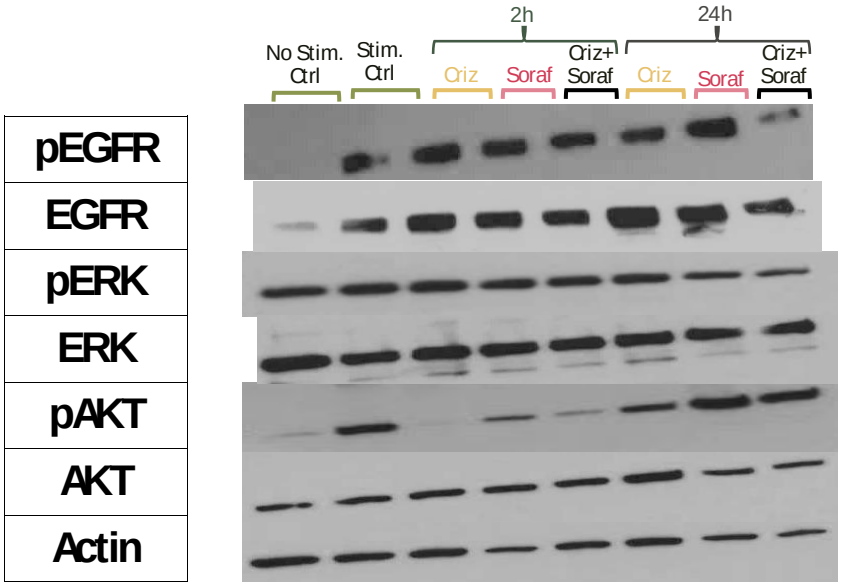


Figure 12 Western Blot on SK-HEP-1 including treatment with crizotinib, sorafenib, or both

Treatment with crizotinib, sorafenib and the combination was done on HepG2 cells. The doses used were the IC_{50} values. pEGFR, seems to be inhibited more by sorafenib and more by the combination at 2 hours and 24 hours. In 24 hours, pEGFR seems to have been re-activated in cells, but the re-activation is blocked by the combination. Total EGFR is reduced with treatment with crizotinib and sorafenib, in 2 hours. The MAPK pathway, demonstrated by pERK, seems to have been inhibited by sorafenib more than crizotinib, but with added inhibition when the combination was used. Maximum inhibition occurs in 24 hours by both agents. Total ERK was decreased by the combination of crizotinib and sorafenib in 2 hours. The pI3k pathway activity was shown as activity of pAKT. Crizotinib seems to have maximum inhibitory effect on pAKT on 2 hours, and seems to improve sorafenib's inhibition of pAKT at 2 hours. However, the inhibition of pAKT was not seen in 24 hours. Unfortunately, no data exists for the effect of treatment on total Akt.

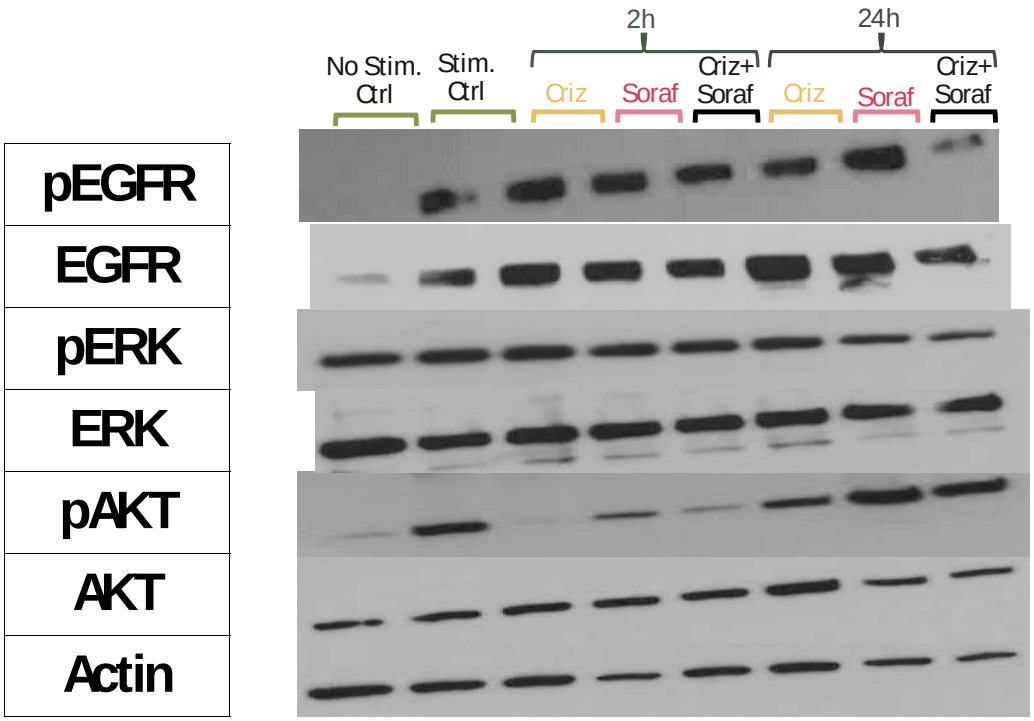
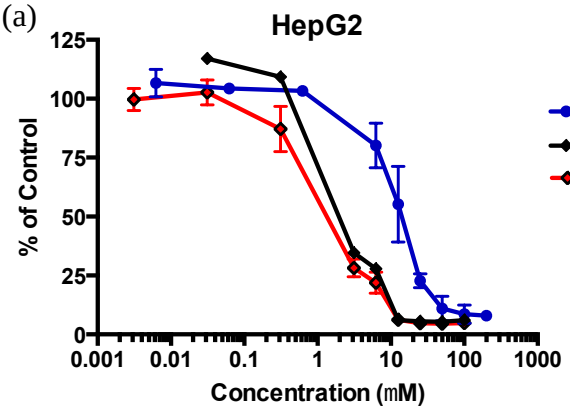


Figure 13 Western Blot on HepG2 including treatment with crizotinib, sorafenib, or both

3.2.2 ***Simultaneous inhibition of MET and EGFR***

Crizotinib was more potent on all cell lines than gefitinib. HepG2 ($\lambda_{crizotinib}$= 0.98 µM, $\lambda_{gefitinb}$ = 11.34 µM, κ=11.54), SK-HEP-1 ($\lambda_{crizotinib}$= 0.70 µM, $\lambda_{gefitinb}$= 5.52 µM, κ=7.89), and Huh7 ($\lambda_{crizotinib}$= 2.11 µM, $\lambda_{gefitinb}$ = 8.34 µM, κ=3.95). The fold-difference between the potency of crizotinib and gefitinib (κ) was more than 6 on HepG2 and SK-HEP-1. Thus, as expected, the IC_{50} of the combination was resembling the IC_{50} of crizotinib alone on HepG2 and SK-HEP-1 as follows: HepG2: $\lambda_{crizotinib+gefitinib}$ = 0.91 µM, ε =11.60; SK-HEP-1: $\lambda_{crizotinib+gefitinib}$ = 0.81 µM, ε = 10.17. However, the fold-difference was less than 6 on Huh7 and the combination was more potent than either drug alone. (Huh7 $\lambda_{crizotinib+gefitinib}$ = 0.56 µM, ε = 1.41).

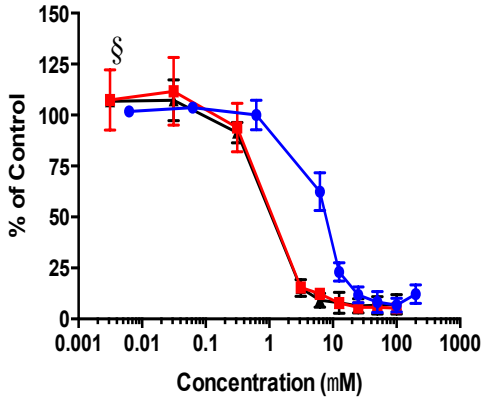
(a)



(b)

HepG2 equimolar combination			
Crizotinib	0.98 µM	Ratio (κ)	11.5
Gefitinib	11.35 µM		
Crizotinib + Gefitinib	0.91 µM	Potency Index (ϵ)	11.6

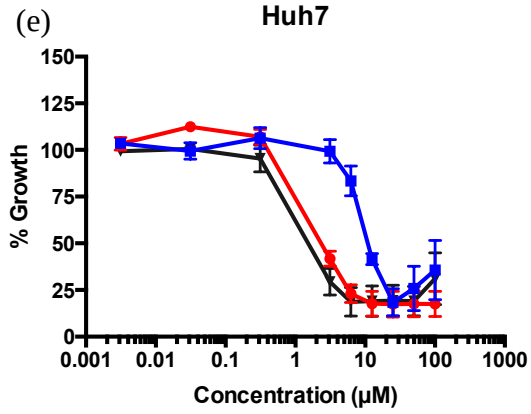
(c)



(d)

SK-HEP-1 equimolar combination			
Crizotinib	0.70 µM	Ratio (κ)	7.89
Gefitinib	5.52 µM		
Gefitinib + Crizotinib	0.81 µM	Potency Index (ϵ)	10.17

(e)



(f)

Huh7 equimolar combination			
Crizotinib	2.11 µM	Ratio (κ)	4.0
Gefitinib	8.34 µM		
Gefitinib + Crizotinib	0.56 µM	Potency Index (ϵ)	1.41

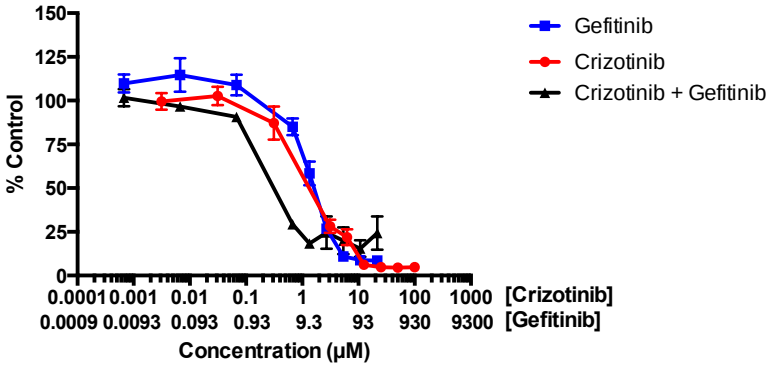
Figure 14 Equimolar combination of sorafenib and crizotinib on the liver cancer cell lines.

Equi-effective combinations of crizotinib and gefitinib were also tested HepG2 and SK-HEP-1 cell lines. On HepG2, the ratio used between gefitinib and crizotinib was 9.3. The IC_{50} of crizotinib in combination ($c\lambda_{crizotinib}$) was 0.40 µM and that of gefitinib ($c\lambda_{gefitinib}$) was 4.37 µM. The resulting combination index (CI_{50}) was 0.89. Isobologram graph showed that the combination of crizotinib and gefitinib was synergistic at most concentrations except lower concentrations where it is additive.

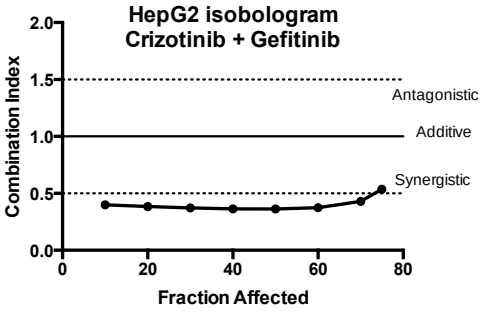
On SK-HEP-1, the IC_{50} of crizotinib in combination ($c\lambda_{crizotinib}$) was 0.22 µM and that of gefitinib ($c\lambda_{gefitinib}$) was 1.44 µM. The resulting combination index (CI_{50}) was 0.44. Isobologram graph showed that the combination of crizotinib and gefitinib was synergistic at all concentrations.

Thus, equi-effective Combination of Crizotinib with Gefitinb is synergistic, pointing towards EGFR and MET pathways cross-talk.

(a)



(b)

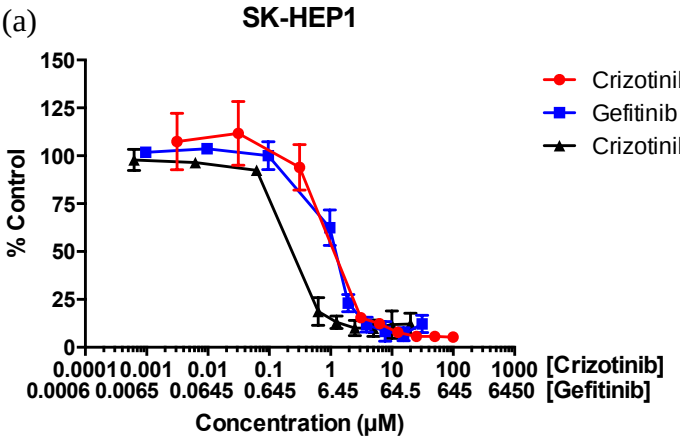


(c)

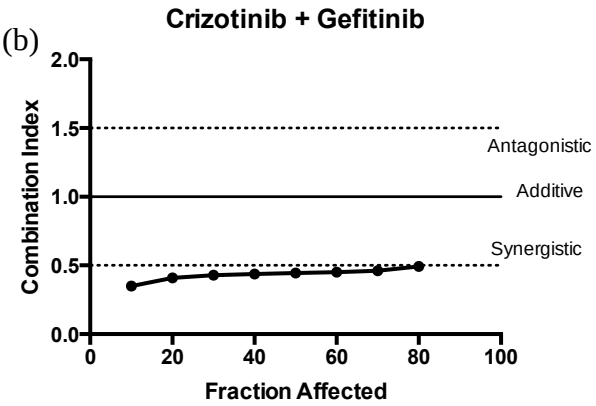
Equi-effective combination on HepG2		
	Crizotinib	Gefitnib
Alone	0.98 µM	11.35 µM
In Combination	0.21 µM	1.95 µM
CI_{50}	0.316	

Figure 15 (a)-(c) Equi-effective combination of gefitinib and crizotinib on the HepG2

(a)



(b)



(c)

Equi-effective combination on SK-HEP-1		
	Crizotinib	Gefitinib
Alone	0.70 μM	5.52 μM
In Combination	0.22 μM	1.44 μM
CI_{50}	0.47	

Figure 16 (a)-(c) Equi-effective combination of gefitinib and crizotinib on Sk-Hep1

3.3 Activity of the MEK inhibitor Selumetinib against HCC

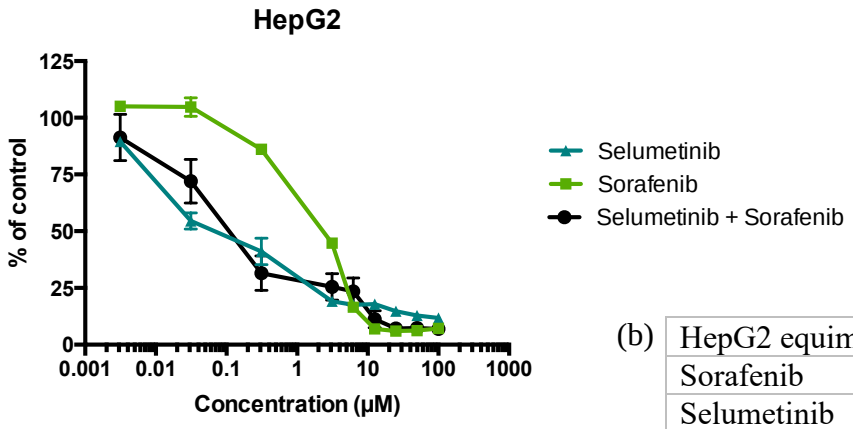
Selumetinib is a MEK inhibitor that was used in our experiments alone and in several sets of combination. Selumetinib was potent on our cell lines particularly HepG2, as shown in table 1 above. Below, we investigate the combination of selumetinib with other kinase inhibitors.

3.3.1 *Selumetinib with sorafenib*

Selumetinib was much more potent than sorafenib on HepG2 ($\lambda_{sorafenib}$= 0.84 μM, $\lambda_{selumetinib}$= 0.025 μM, κ=33.6), but the fold-difference was less than 6 on SK-HEP-1 ($\lambda_{sorafenib}$= 3.15 μM, $\lambda_{selumetinib}$= 1.26 μM, κ=2.5), and on Huh-7 ($\lambda_{sorafenib}$= 2.45 μM, $\lambda_{selumetinib}$= 2.30 μM, κ=1.09).

Equimolar combinations were done to assess effectiveness on all 3 cell lines. On HepG2, the potency of the combination ($\lambda_{sorafenib+selumetinib}$) was 0.046 μM which represents the potency of selumetinib alone. On SK-HEP-1, $\lambda_{sorafenib+selumetinib}$ was 1.07 μM, with $\varepsilon = 2.96$, indicating a mild increase in potency compared to the potency of each drug alone. A more profound effectiveness of the combination was shown with Huh7, where $\lambda_{sorafenib+selumetinib}$ was 0.48 μM and $\varepsilon = 0.44$, indicating increased potency with the combination compared to either drug alone.

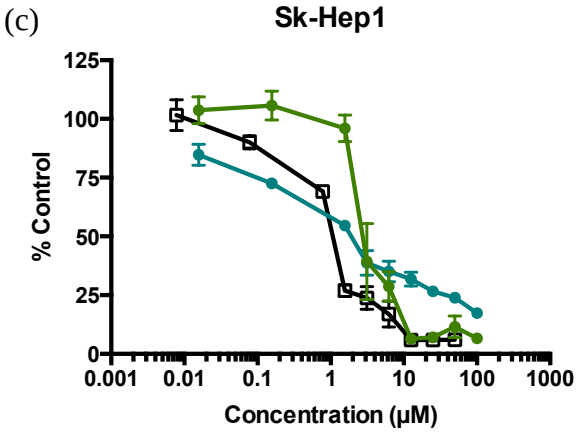
(a)



(b)

HepG2 equimolar combination			
Sorafenib	0.84 μM	Ratio (κ)	33.6
Selumetinib	0.025 μM		
Sorafenib + Selumetinib	0.046 μM	Potency Index (ϵ)	64.9

(c)

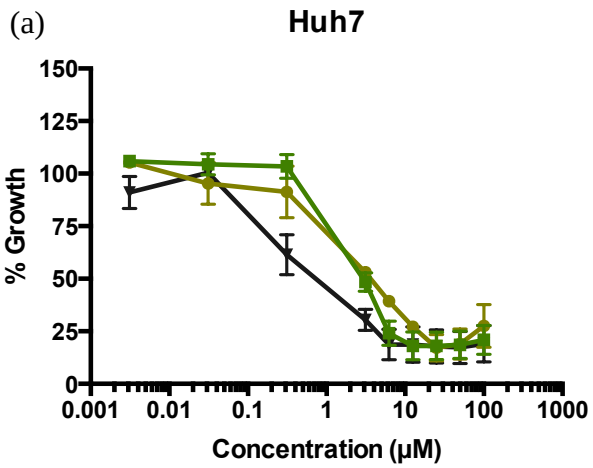


(d)

SK-HEP-1 equimolar combination			
Sorafenib	3.15 μM	Ratio (κ)	2.5
Selumetinib	1.26 μM		
Sorafenib + Selumetinib	1.07 μM	Potency Index (ϵ)	2.96

Figure 17 (a)-(d) Equimolar combination of selumetinib and sorafenib on liver cancer cell lines

(a)



(b)

Huh7 equimolar combination			
Sorafenib	2.45 μM	Ratio (κ)	1.1
Selumetinib	2.3 μM		
Sorafenib + Selumetinib	0.48 μM	Potency Index (ϵ)	0.44

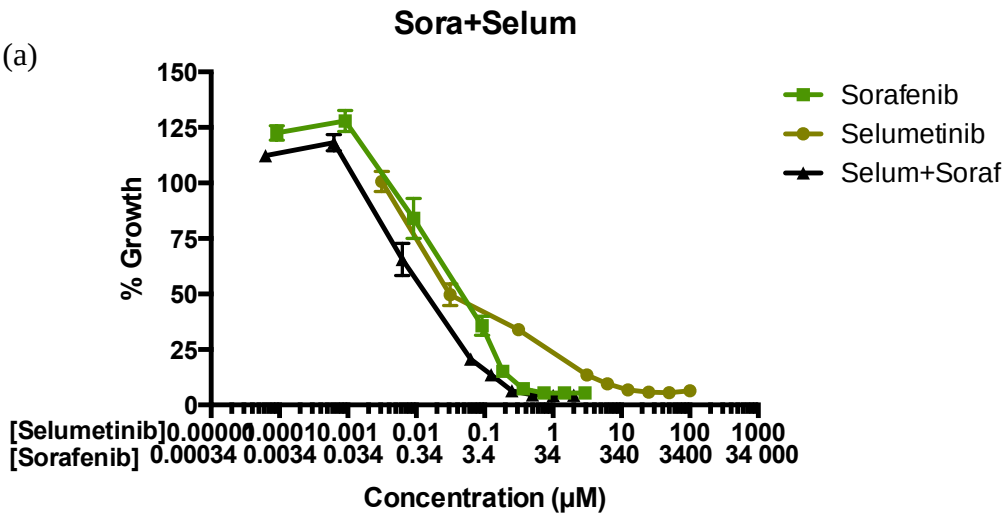
Figure 18 (a)-(b) Equimolar combination of selumetinib and sorafenib on liver cancer cell lines

Equi-effective combinations were done to assess the synergy between sorafenib and selumetinib on the 3 cell lines. On HepG2, the IC_{50} of sorafenib in combination ($c\lambda_{sorafenib}$) was 0.32 μM and that of selumetinib ($c\lambda_{selumetinib}$) was 0.008 μM. The resulting combination index (CI_{50}) was 0.65, indicating synergy. Isobologram graph showed that the combination of sorafenib and selumetinib was synergistic at all concentrations.

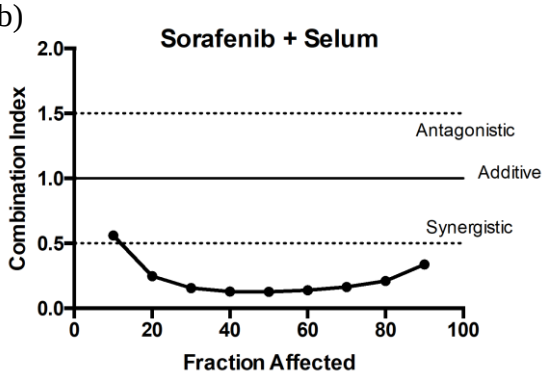
On SK-HEP-1, the IC_{50} of sorafenib in combination ($c\lambda_{sorafenib}$) was 0.77 μM, showing a great increase in potency compared to sorafenib alone. The IC_{50} of selumetinib ($c\lambda_{selumetinib}$) was 0.31 μM. The resulting combination index (CI_{50}) was 0.28, indicating synergy. Isobologram graph showed that the combination of sorafenib and selumetinib was synergistic at almost all concentrations.

On Huh7, the IC_{50} of sorafenib in combination ($c\lambda_{sorafenib}$) was 0.38 μM, showing a great increase in potency compared to sorafenib alone. The IC_{50} of selumetinib ($c\lambda_{selumetinib}$) was 0.34 μM. The resulting combination index (CI_{50}) was 0.30, indicating synergy. Isobologram graph showed that the combination of sorafenib and selumetinib was synergistic at almost all concentrations.

(a)



(b)

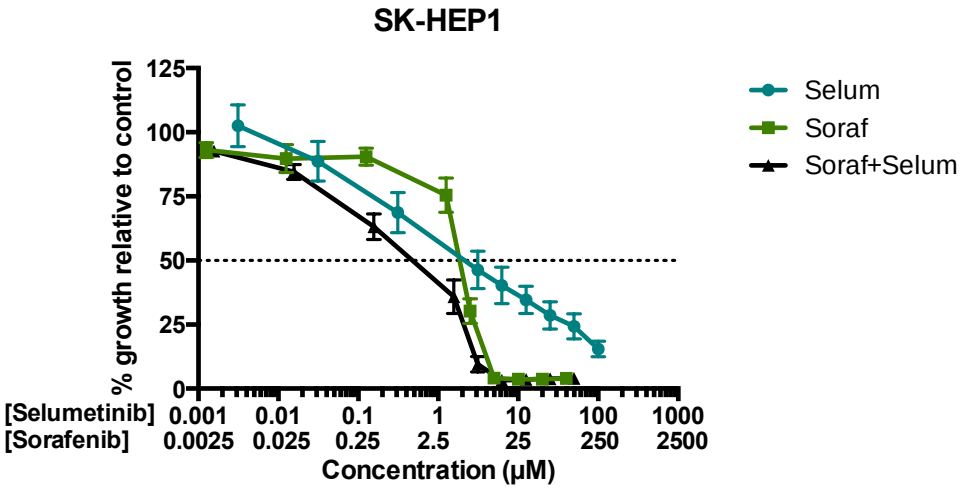


(c)

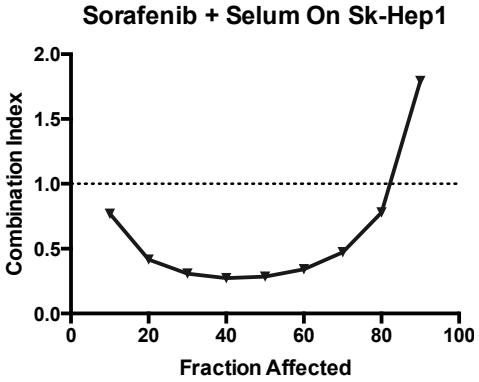
Equi-effective combination on HepG2		
	Selumetinib	Sorafenib
Alone	0.0248 μM	0.841 μM
In Combination	0.00804 μM	0.322 μM
CI_{50}	0.653	

Figure 19 (a)-(c) Equi-effective combination of selumetinib and sorafenib on HepG2

(a)



(b)

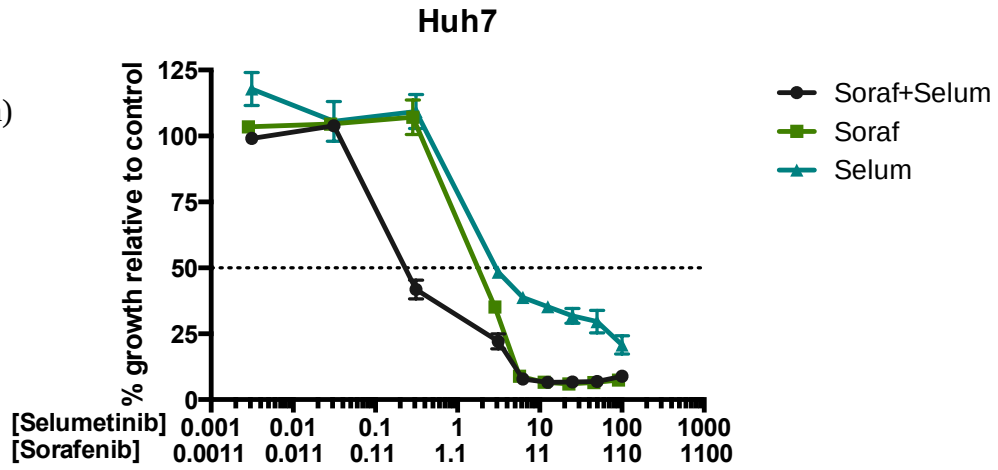


(c)

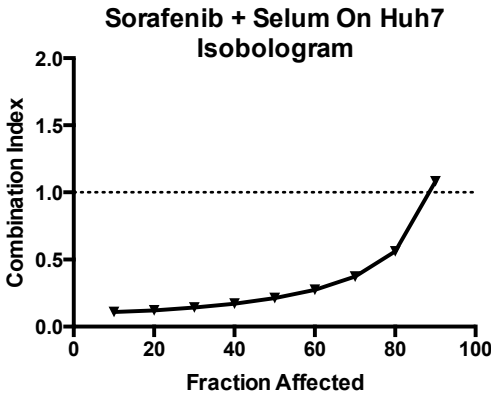
Equi-effective combination on SK-HEP-1		
	Selumetinib	Sorafenib
Alone	1.26 μM	3.15 μM
In Combination	0.31 μM	0.77 μM
CI_{50}	0.28	

Figure 20 (a)-(c) Equi-effective combination of selumetinib and sorafenib on SK-HEP-1

(a)



(b)



(c)

Equi-effective combination on Huh7		
	Selumetinib	Sorafenib
Alone	0.0248 μM	0.841 μM
In Combination	0.00804 μM	0.322 μM
CI_{50}	0.653	

Figure 21 (a)-(c) Equi-effective combination of selumetinib and sorafenib on Huh7

3.3.2 ***Selumetinib with crizotinib***

On HepG2, the IC_{50} of selumetinib ($\lambda_{selumetinib}$= 0.025 μM) is much higher than that of crizotinib ($\lambda_{crizotinib}$ = 0.98 μM), with a ratio (κ) of 39.6 As expected, the potency of the combination ($\lambda_{selumetinib+crizotinib}$) was 1.75, which was weaker than the potency of either drug alone.

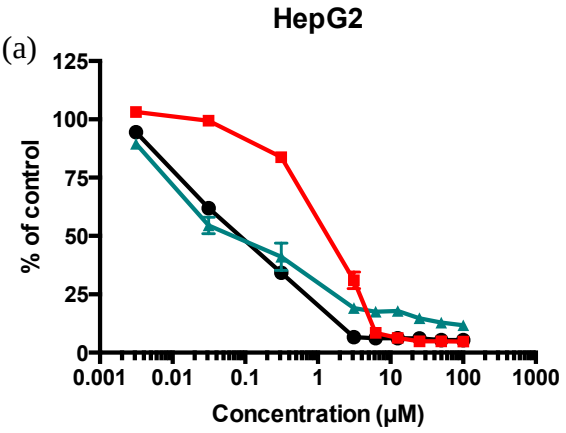
However, On SK-HEP-1, the difference between the potency of both agents is small, ($\lambda_{crizotinib}$= 0.70 μM, $\lambda_{selumetinib}$= 1.26 μM, κ=1.8). The potency of the combination ($\lambda_{crizotinib+selumetinib}$) was 0.81 μM, with a potency index ε = 3.23, indicating a possible effective combination, though the potency is not lower than the potency of crizotinib alone.

On Huh7, the potencies of crizotinib and selumetinib were very similar ($\lambda_{crizotinib}$= 2.11 μM, $\lambda_{selumetinib}$= 2.30 μM, κ=1.1). Equimolar combination was effective with combination IC_{50} ($\lambda_{crizotinib+selumetinib}$) of 0.90 μM, which is more potent than either drug alone. The potency index ε was 0.96, further indicating that the synergy is effective.

Equi-effective combinations were done to assess the synergy between crizotinib and selumetinib on the 3 cell lines. On HepG2, the IC_{50} of crizotinib in combination ($c\lambda_{crizotinib}$) was 0.74 μM and that of selumetinib in the same combination ($c\lambda_{selumetinib}$) was 0.018 μM, both showing slightly more potency than the agent alone. The resulting combination index (CI_{50}) was 1.50, indicating an additive or slightly antagonistic interaction. Isobologram graph showed that the combination of crizotinib and selumetinib was antagonistic at lower concentrations and synergistic at higher concentrations.

On SK-HEP-1, the IC_{50} of crizotinib in combination ($c\lambda_{crizotinib}$) was 0.28 μM and that of selumetinib in the same combination ($c\lambda_{selumetinib}$) was 0.54 μM, both showing more potency than either drug alone. The resulting combination index (CI_{50}) was 0.38 indicating a synergistic effect. Isobologram graph showed that the combination of crizotinib and selumetinib was mostly synergistic except at lower concentrations.

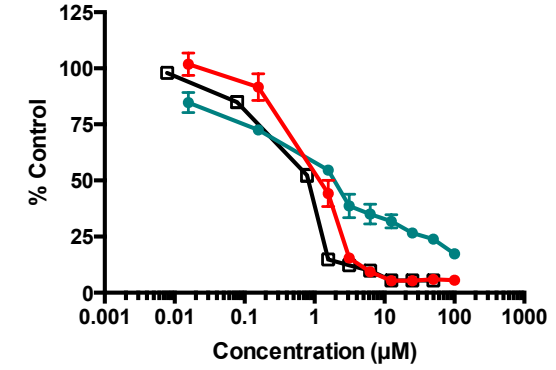
(a)



(b)

HepG2 equimolar combination			
Crizotinib	0.98 μM	Ratio (κ)	39.6
Selumetinib	0.025 μM		
Crizotinib + Selumetinib	1.75 μM	Potency Index (ϵ)	2877

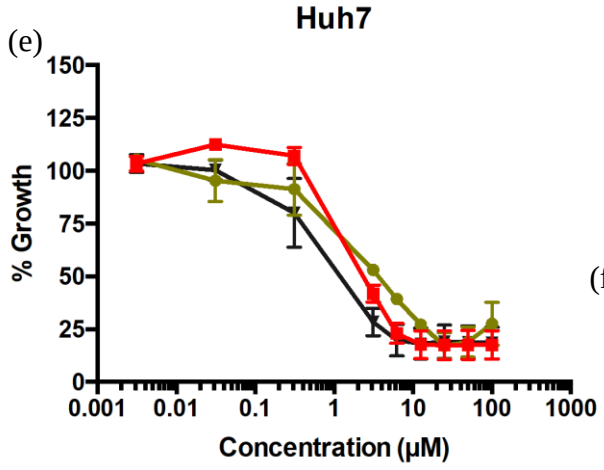
(c)



(d)

SK-HEP-1 equimolar combination			
Crizotinib	0.70 μM	Ratio (κ)	1.8
Selumetinib	1.26 μM		
Crizotinib + Selumetinib	0.81 μM	Potency Index (ϵ)	3.23

(e)



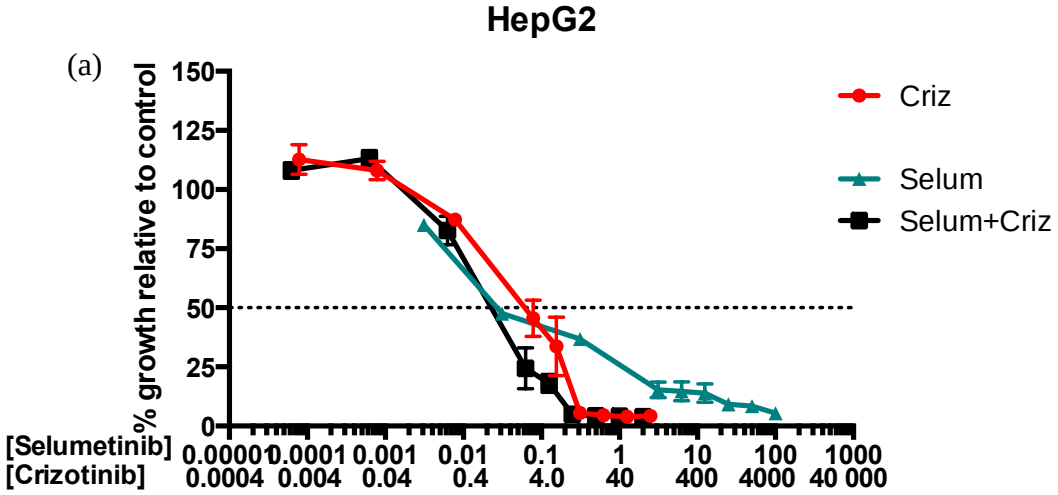
(f)

Huh7 equimolar combination			
Crizotinib	2.11 μM	Ratio (κ)	1.1
Selumetinib	2.30 μM		
Crizotinib + Selumetinib	0.90 μM	Potency Index (ϵ)	0.96

Figure 22 (a)-(f) Equimolar combination of selumetinib and crizotinib on liver cancer cell line

On Huh7, the IC_{50} of crizotinib in combination ($c\lambda_{crizotinib}$) was 1.54 μM and that of selumetinib ($c\lambda_{selumetinib}$) was 1.88 μM. The resulting combination index (CI_{50}) was 1.47, indicating additive or antagonistic interaction. Isobologram graph showed that the combination of crizotinib and selumetinib was antagonistic at lower concentrations and additive or synergistic at higher concentrations.

(a)



(b)

Equi-effective combination on HepG2		
	Crizotinib	Selumetinib
Alone	0.98 μM	0.025 μM
In Combination	0.32 μM	0.008 μM
CI_{50}	0.651	

(c)

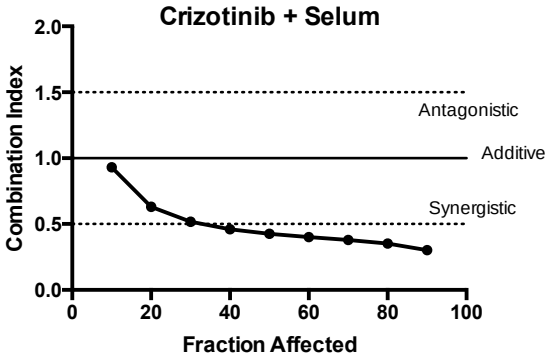
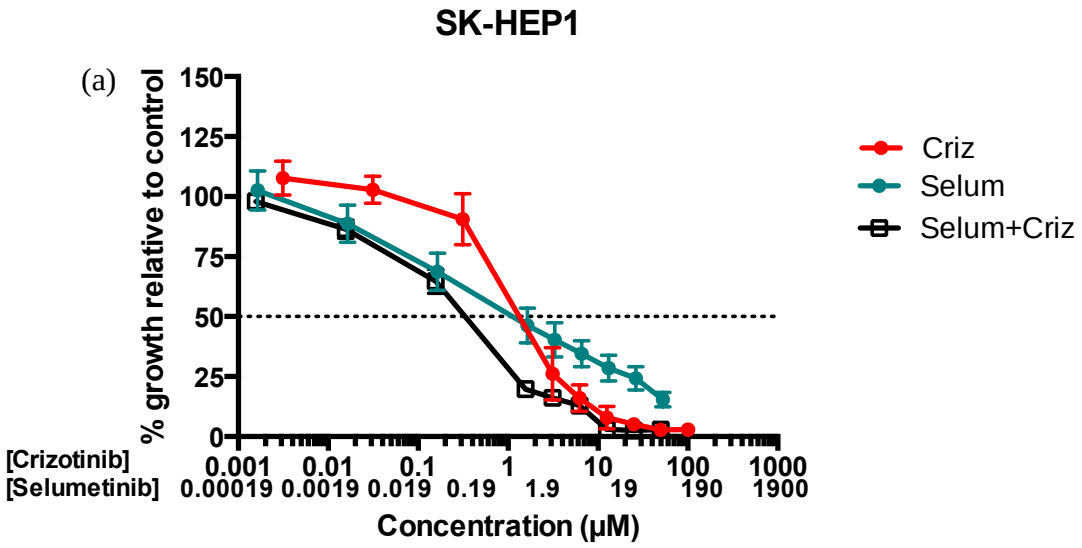


Figure 23 (a)-(c) Equi-effective combination of selumetinib and crizotinib on HepG2

(a)



(b)

Equi-effective combination on SK-HEP-1		
	Crizotinib	Selumetinib
Alone	0.70 μM	1.26 μM
In Combination	0.28 μM	0.54 μM
CI_{50}	0.38	

(c)

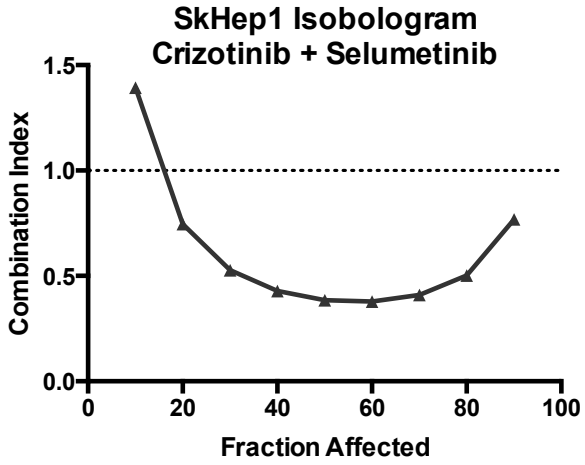
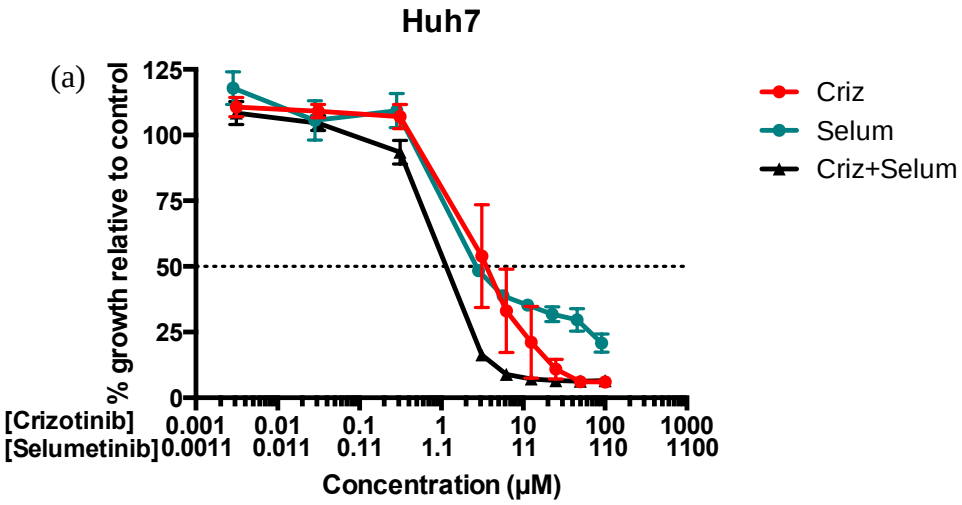


Figure 24 (a)-(c) Equi-effective combination of selumetinib and crizotinib on SK-HEP-1

(a)



(b)

Equi-effective combination on Huh7		
	Crizotinib	Selumetinib
Alone	2.11 μM	2.30 μM
In Combination	1.54 μM	1.88 μM
CI_{50}	1.47	

(c)

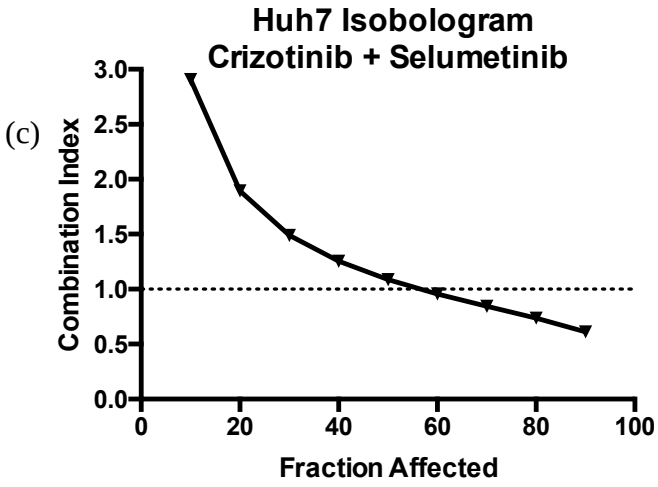


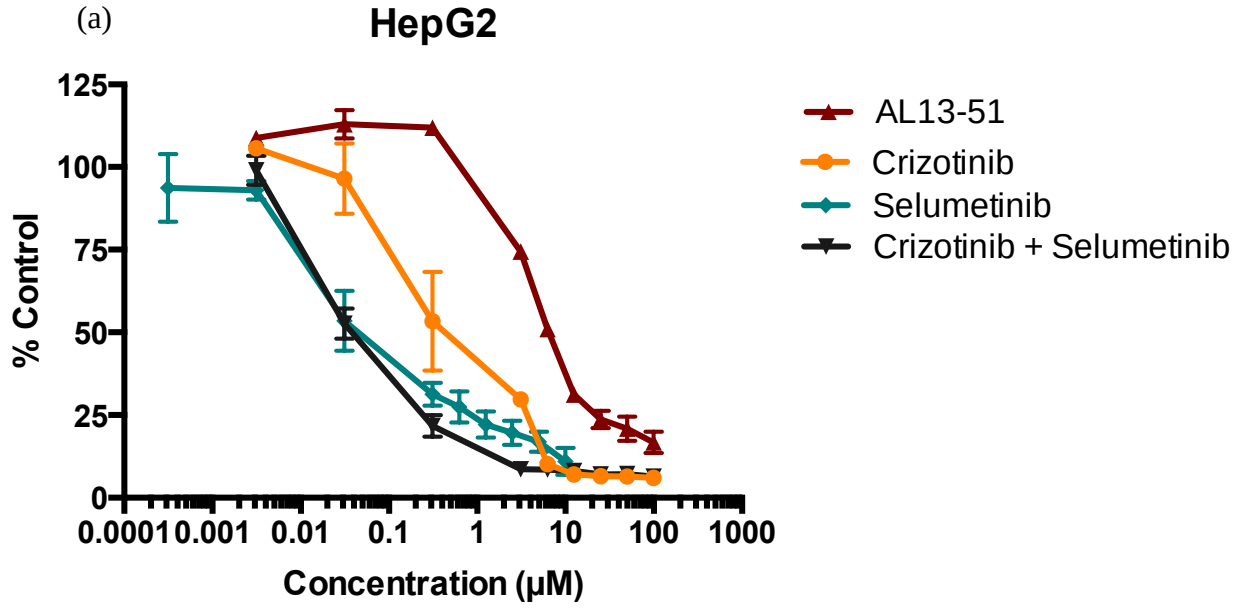
Figure 25 (a)-(c) Equi-effective combination of selumetinib and crizotinib on Huh7

3.3.2.1. The combi-molecule AL-13-51

The combi-molecule AL-13-51 was synthesized by our laboratory by Anne-Lorre Larroque. It is composed of residues of crizotinib and selumetinib.

The molecule was tested on both HepG2, SK-HEP-1 and Huh7 cell lines and its potency was compared to either molecule, crizotinib or selumetinib, alone, as well as the equimolar combination of the two. Either agent alone and the combination were all more potent than the novel combi-molecule. The combi-molecule showed minimal if any growth inhibitory effect on Huh7

(a)

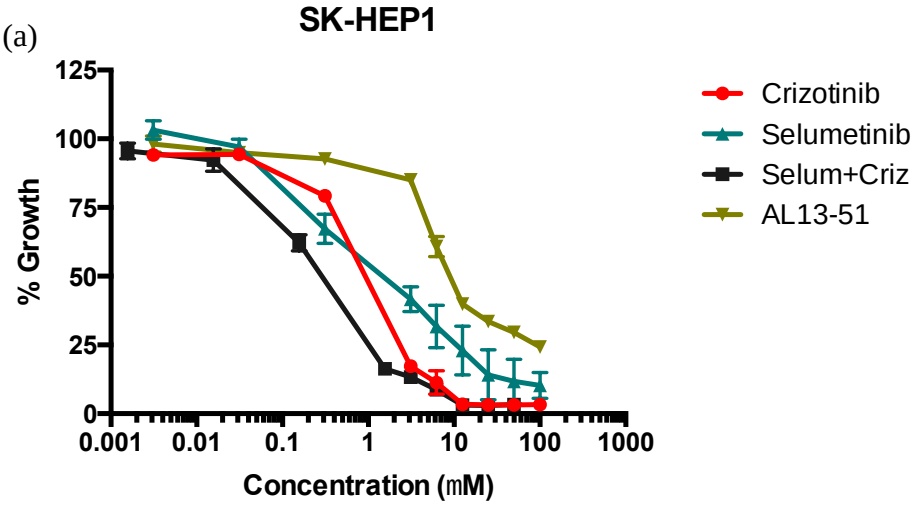


(b)

HepG2 equimolar combination			
Crizotinib	0.98 µM	Selumetinib	0.025 µM
Crizotinib + Selumetinib	1.75 µM	AL 13-51	4.16 µM

Figure 26 (a) -(b) The new molecule AL13-51 on HepG2

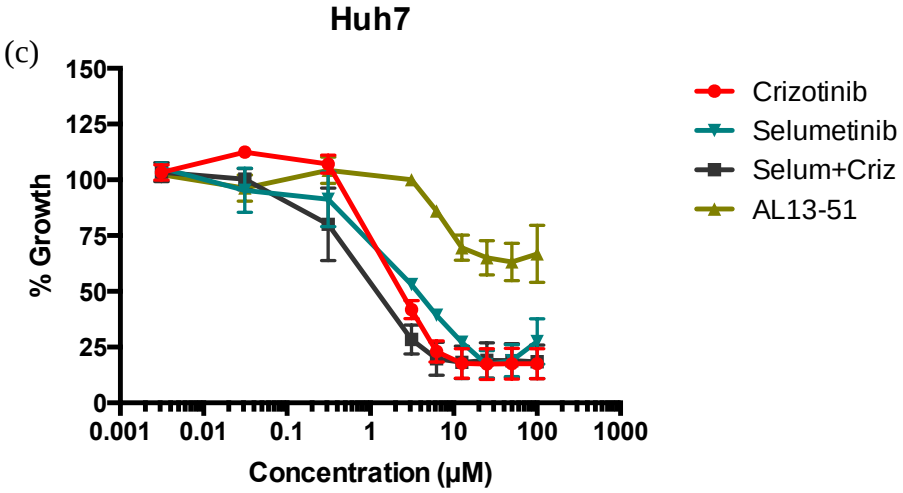
(a)



(b)

SK-HEP-1 equimolar combination			
Crizotinib	0.70 µM	Selumetinib	1.26 µM
Crizotinib + Selumetinib	0.81 µM	AL 13-51	6.31 µM

(c)



(d)

Huh7 equimolar combination			
Crizotinib	2.11 µM	Selumetinib	2.30 µM
Crizotinib + Selumetinib	0.90 µM	AL 13-51	(too high)

Figure 27 (a) - (f) The new molecule AL13-51 on Sk-Hep1 and Huh-7

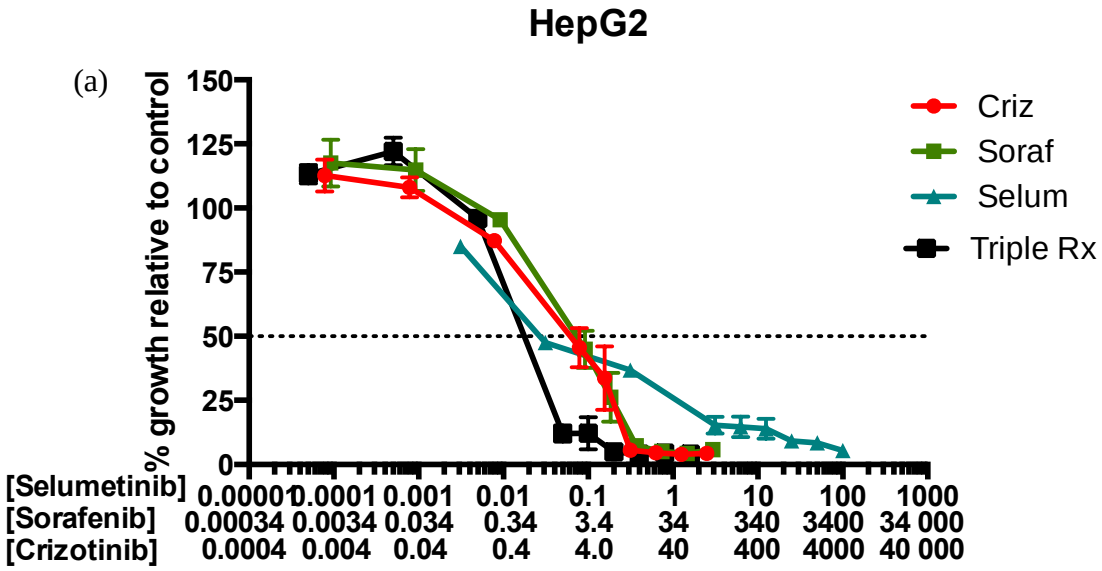
3.3.3 ***Triple Combinations***

To assess the effectiveness of the triple combination of sorafenib, crizotinib and selumetinib, an equi-effective combination treatment was used on the liver cancer cell lines HepG2, SK-HEP-1 and Huh7.

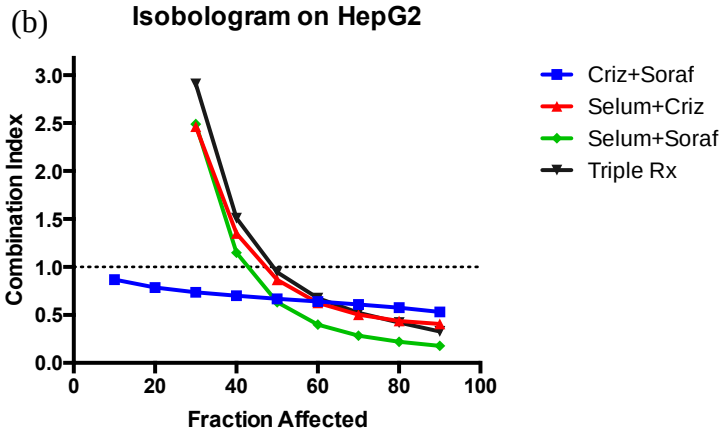
On HepG2, the respective IC_{50} values of sorafenib, selumetinib and crizotinib were as follows: ($\lambda_{sorafenib}$ = μM 0.841 μM, $\lambda_{selumetinib}$= 0.025 μM, $\lambda_{cirzotinib}$ = 0.98 μM). Combination was done with concentrations relative to selumetinib as follows: sorafenib:selumetinib:crizotinib = 1 : 34 : 40. The IC_{50} of each drug in the triple combination was as follows: ($c\lambda_{sorafenib}$ = μM 0.730 μM, $c\lambda_{selumetinib}$= 0.021 μM, $c\lambda_{cirzotinib}$ = 0.859 μM), showing a mild increase in potency of each drug alone. However, the combination index (CI_{50}) was 2.6, indicating that the combination is antagonistic. Isobologram shows that the combination is mostly antagonistic but is synergistic at high concentrations.

On SK-HEP-1, the respective IC_{50} values of sorafenib, selumetinib and crizotinib were as follows: ($\lambda_{sorafenib}$ = 3.154 μM, $\lambda_{selumetinib}$= 1.264 μM, $\lambda_{crizotinib}$ = 0.703 μM). Combination was done with concentrations relative to selumetinib as follows: sorafenib:selumetinib:crizotinib = 1 : 1.9 : 4.6. The IC_{50} of each drug in the triple combination was as follows: ($c\lambda_{sorafenib}$ = 0.832 μM, $c\lambda_{selumetinib}$= 0.334 μM, $c\lambda_{crizotinib}$ = 0.181 μM), showing a mild increase in potency of each drug alone. However, the combination index (CI_{50}) was 2.6, indicating that the combination is antagonistic. Isobologram shows that the combination is mostly antagonistic but is synergistic at high concentrations.

(a)



(b)

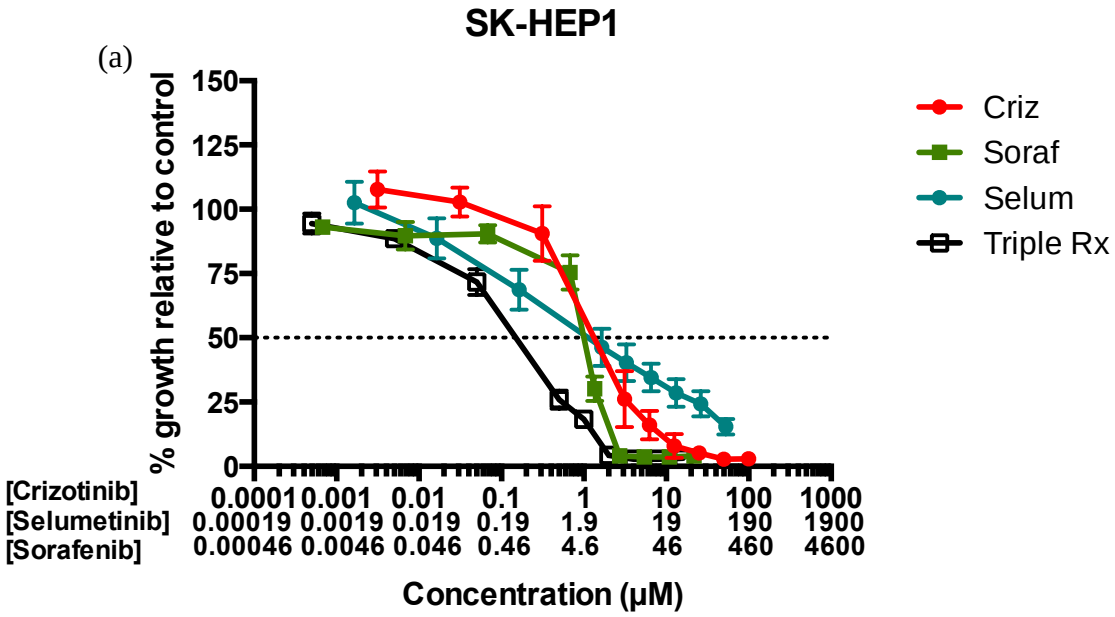


(c)

Equi-effective Triple Combination on HepG2			
	Selumetinib	Sorafenib	Crizotinib
Single	0.025	0.841	0.984
In comb.	0.021	0.730	0.859
CI_{50}	2.608		

Figure 28 (a)-(c) Equi-effective combination of selumetinib, sorafenib and crizotinib on HepG2

(a)



(b)

Equi-effective Triple Combination on SK-HEP-1			
	Criz	Selum	Soraf
Single	0.703	1.264	3.154
Combined	0.181	0.344	0.832
CI_{50}	0.793		

(c)

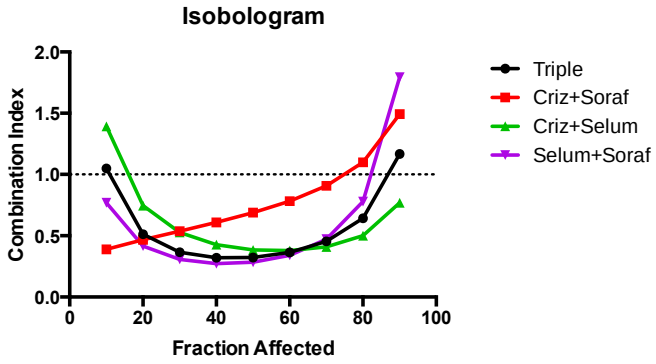
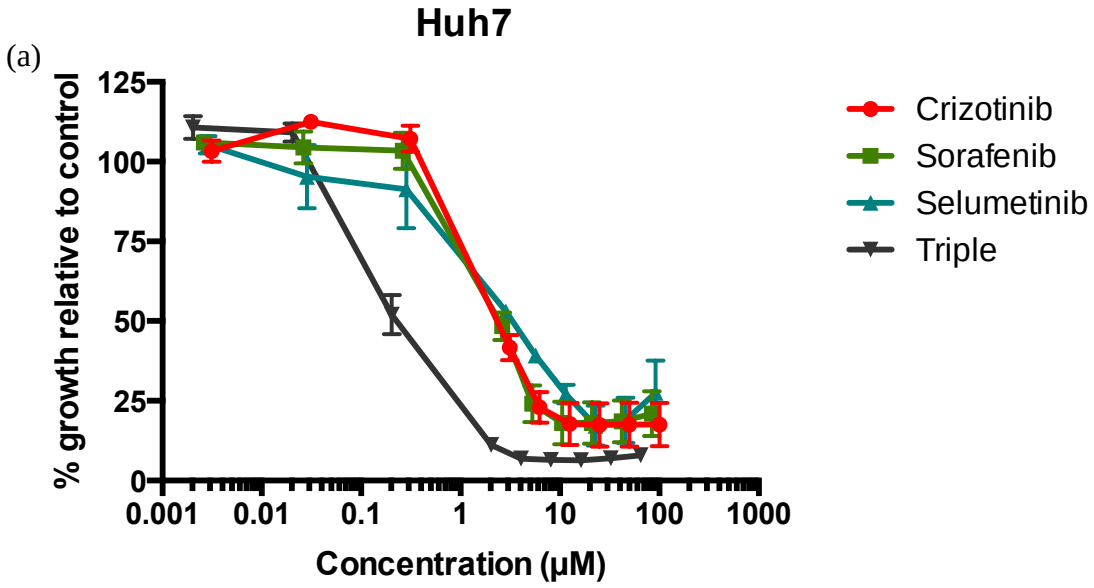


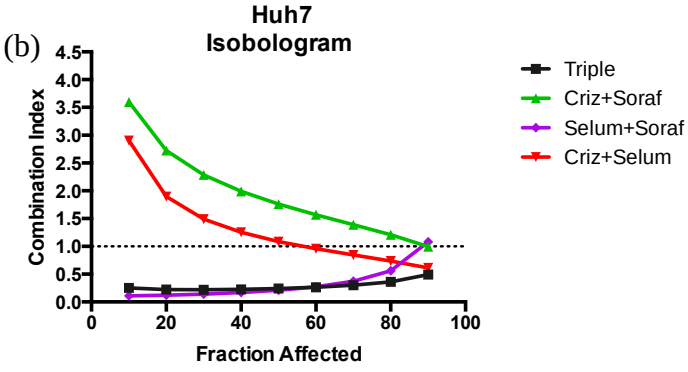
Figure 29 (a)-(c) Equi-effective combination of selumetinib, sorafenib and crizotinib on SK-HEP-1

On Huh7, the respective IC_{50} values of sorafenib, selumetinib and crizotinib were as follows: ($\lambda_{sorafenib}$ = µM 2.54 µM, $\lambda_{selumetinib}$= 2.30 µM, $\lambda_{cirzotinib}$ = 2.11 µM). Combination was done with concentrations relative to selumetinib as follows: sorafenib: selumetinib : crizotinib = 1 : 1 : 1. The IC_{50} of each drug in the triple combination was as follows: ($c\lambda_{sorafenib}$ = 0.314 µM µM, $c\lambda_{selumetinib}$= 0.283 µM, $c\lambda_{cirzotinib}$ = 0.257 µM), showing a large increase in potency compared to each drug alone. The combination index (CI_{50}) was 0.37, indicating that the combination is synergistic. Isobologram shows that the combination is synergistic at all concentrations ranges.

(a)



(b)



(c)

Equi-effective Triple Combination on Huh7			
	Criz	Selum	Soraf
Single	2.11	2.298	2.541
Combined	0.257	0.283	0.314
CI_{50}	0.368		

Figure 30 (a)-(c) Equi-effective combination of selumetinib, sorafenib and crizotinib on Huh

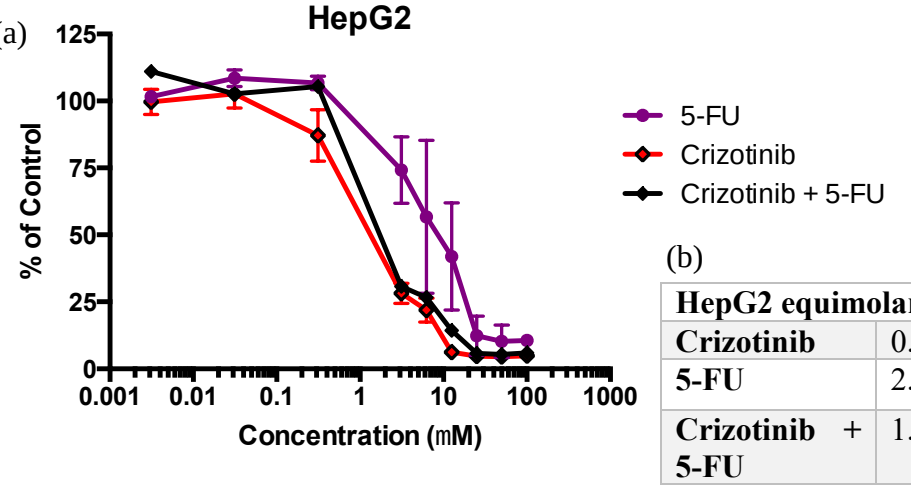
3.4 Combinations of kinase inhibitors with 5-FU

Because of the favorable structure of 5-FU for chemical modification and integration into novel combi-molecules, and because of the interactions that are present between the DNA synthesis pathway and kinase-mediated signaling pathways, we have tried combining 5-FU with the kinase inhibitors sorafenib, crizotinib and gefitinib. We assessed the synergistic potential of the two-drug treatments on inhibiting the growth of HepG2 and SK-HEP-1 cell lines.

3.4.1 *Combination of 5-FU and crizotinib*

In both HepG2 and SK-HEP-1, the potency of crizotinib was higher than 5-FU; HepG2 ($\lambda_{crizotinib}$= 0.98 μM, $\lambda_{5\text{-}FU}$ = 2.75 μM, κ=2.79) and SK-HEP-1 ($\lambda_{crizotinib}$= 0.70 μM, $\lambda_{5\text{-}FU}$ = 5.05 μM, κ=2.48), but the fold difference is less than 6. Equimolar combination potency measure (IC_{50} values, $\lambda_{Crizotinib+5\text{-}FU}$) are closer to the potency of crizotinib alone (SK-HEP-1: $\lambda_{Crizotinib+5\text{-}FU}$ = 1.13 aμM, ε = 4.57; HepG2 $\lambda_{Crizotinib+5\text{-}FU}$ = 1.75 μM, ε =6.78). The potency index ε is high in both cell lines, and on growth inhibition chart below, the combination graph represents that of crizotinib alone.

(a)

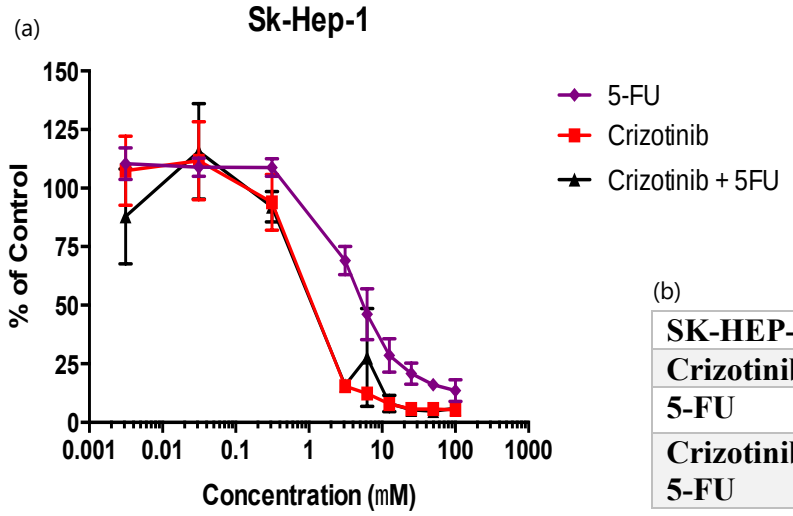


(b)

HepG2 equimolar combination			
Crizotinib	0.98 μM	Ratio (κ)	2.78
5-FU	2.75 μM		
Crizotinib + 5-FU	1.75 μM	Potency Index (ϵ)	6.78

Figure 31 (a)-(b) Equimolar combination of crizotinib and 5-FU on HepG2

(a)



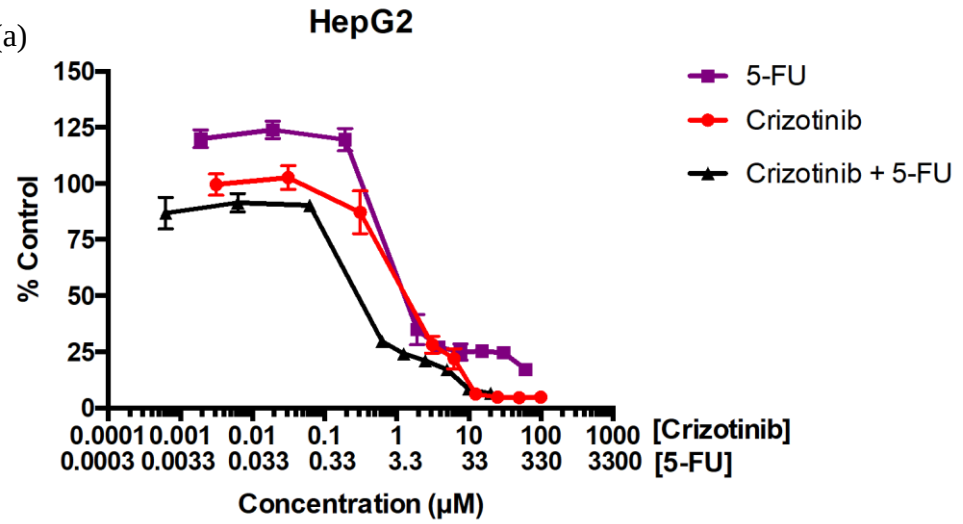
(b)

SK-HEP-1 equimolar combination			
Crizotinib	0.70 µM	Ratio (κ)	2.48
5-FU	5.05 µM		
Crizotinib + 5-FU	1.13 µM	Potency Index (ϵ)	4.57

Figure 32 (a)-(b) Equimolar combination of crizotinib and 5-FU on SK-HEP-1

Thus, we proceeded with equi-effective combination experiments which showed synergy between 5-FU and crizotinib on HepG2 with a CI_{50} of 0.69 and on SK-HEP-1 with a CI_{50} of 0.522. Isobologram showed that the combination of 5-FU and crizotinib was synergistic at lower concentrations and antagonistic at all concentrations except at lower concentrations on SK-HEP-1 where there is possible additive effect.

(a)



(b)

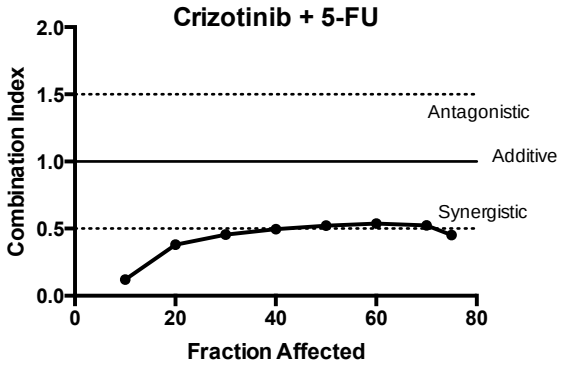
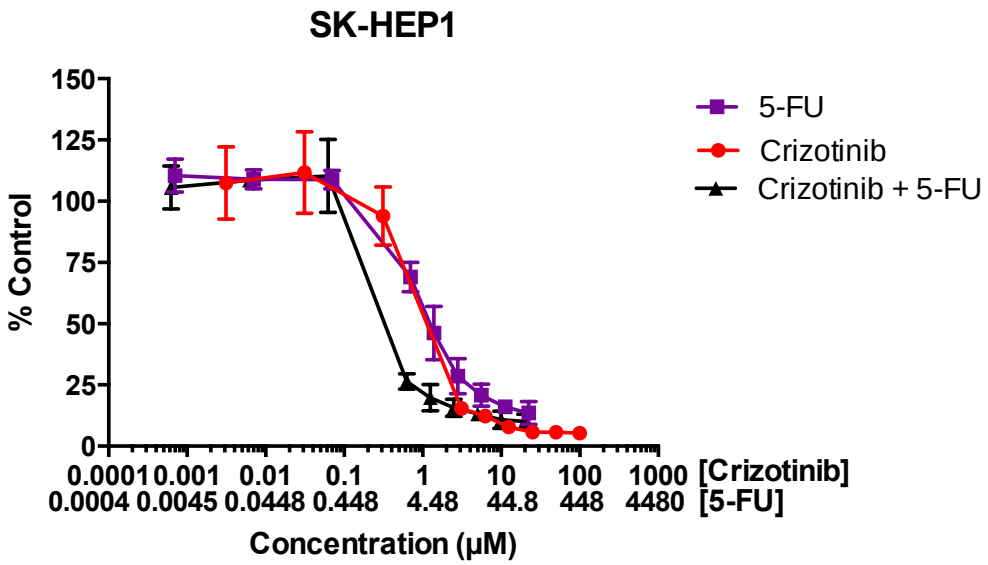
Equi-effective combination on HepG2		
	Crizotinib	5-FU
Alone	0.98 μM	2.75 μM
In Combination	0.341 μM	1.116 μM
CI_{50}	0.691	
Isobologram		

Figure 33 (a)-(b) Equi-effective combination of crizotinib and 5-FU on HepG2

(a)



(b)

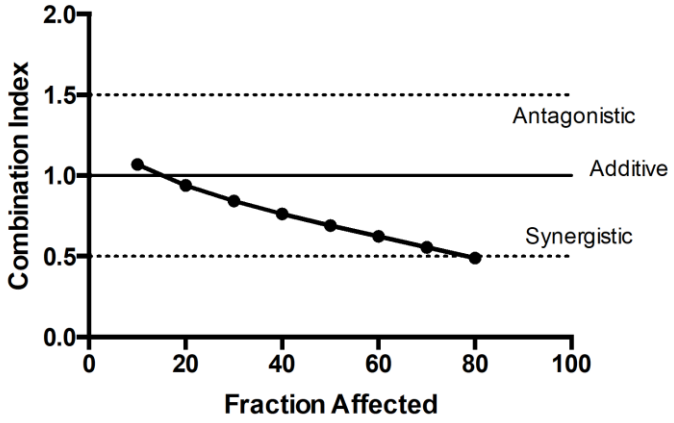
Equi-effective combination on SK-HEP-1		
	Crizotinib	5-FU
Alone	0.70 µM	5.05 µM
In Combination	0.325 µM	1.455 µM
CI_{50}	0.522	
Isobologram		

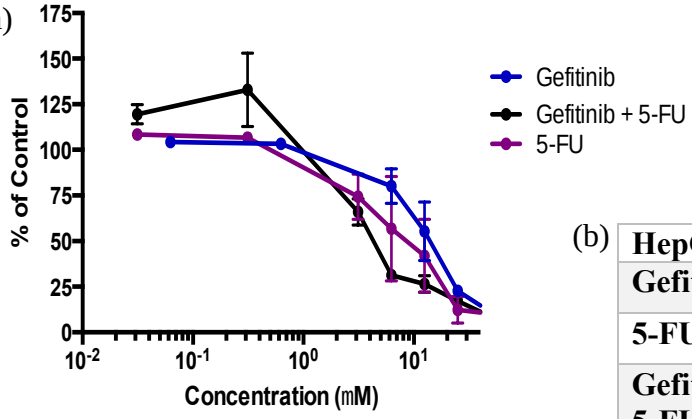
Figure 34 (a)-(b) Equi-effective combination of crizotinib and 5-FU on HepG2 and SK-HEP-1

3.4.2 ***Combination of 5-FU with gefitinib***

The interaction between 5-FU and the EGFR pathway has been well-identified (see section 6.3.1 above). On both HepG2 and SK-HEP-1 cell lines, 5-FU is more potent than gefitinib. HepG2 ($\lambda_{gefitinib}$= 11.35 µM, $\lambda_{5\text{-}FU}$ = 2.75 µM, κ=4.12) and SK-HEP-1 ($\lambda_{gefitinib}$= 5.52 µM, $\lambda_{5\text{-}FU}$ = 5.05 µM,

κ=1.09). The equimolar combination experiments done with 5-FU and gefitinib showed a minimal increase in potency of 5-FU compared to 5-FU alone SK-HEP-1: $\lambda_{gefitinib+5\text{-}FU}$ = 2.90 μM, ε = 2.52; HepG2 $\lambda_{gefitinib+5\text{-}FU}$ = 3.46 μM, ε =6.44

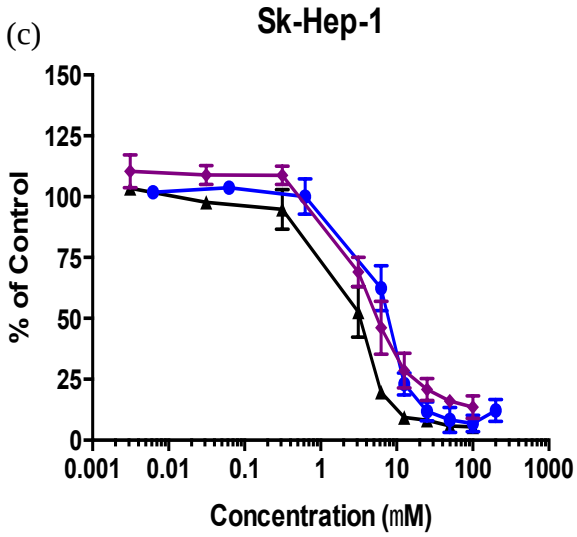
(a)



(b)

HepG2 equimolar combination			
Gefitinib	11.35 μM	Ratio (κ)	4.12
5-FU	2.75 μM		
Gefitnib + 5-FU	3.45 μM	Potency Index (ϵ)	6.44

(c)

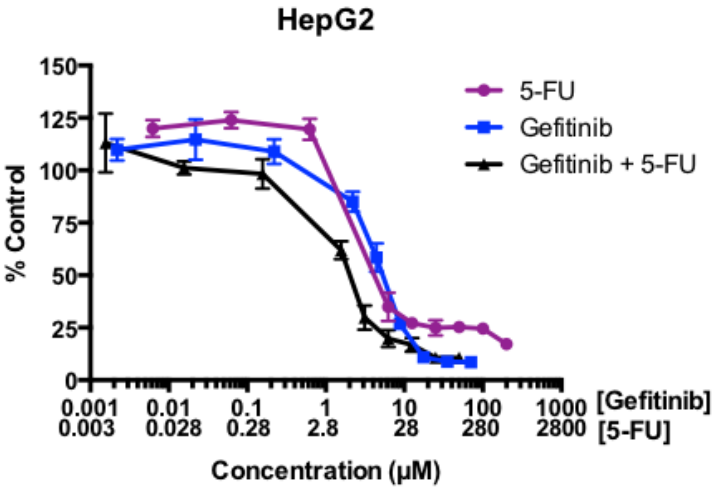


(d)

SK-HEP-1 equimolar combination			
Gefitinib	5.52 μM	Ratio (κ)	1.09
5-FU	5.05 μM		
Gefitinib + 5-FU	2.90 μM	Potency Index (ϵ)	2.52

Figure 35 (a)-(d) Equimolar combination of gefitinib and 5-FU on HepG2 and SK-HEP-1

The equi-effective combination between 5-FU and gefitinib showed no synergy but an additive interaction of 5-FU and gefitinib on both cell lines as shown below.



HepG2	5-FU	Gef
Alone	2.711	11.87
Comb.	1.632	4.651
CI_{50}	0.994	

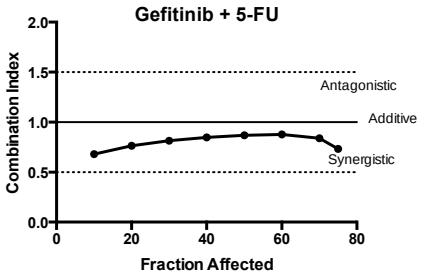
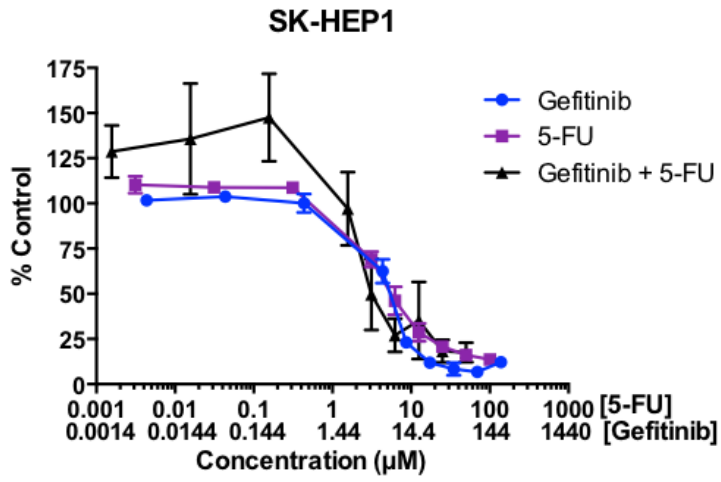


Figure 36 Equi-effective combination of gefitinib and 5-FU on HepG2



SkHep1	5-FU	Gef
Alone	3.889	6.93
Comb.	1.442	2.861
CI_{50}	1.235	

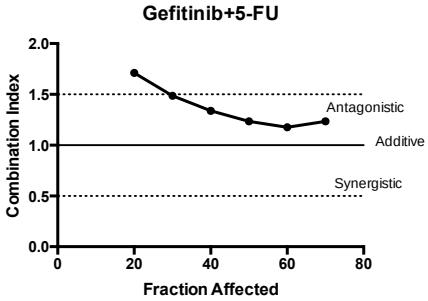
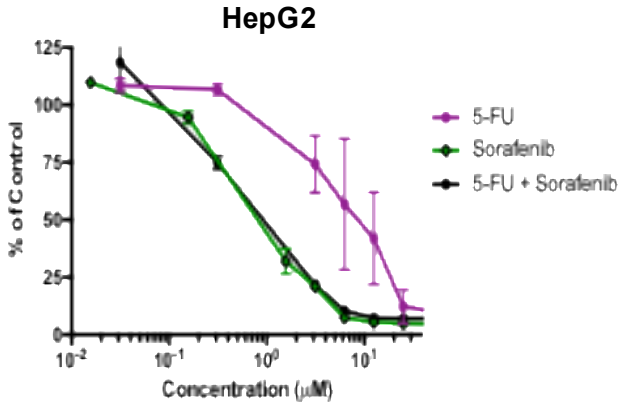


Figure 37 Equi-effective combination of gefitinib and 5-FU on SK-HEP-1

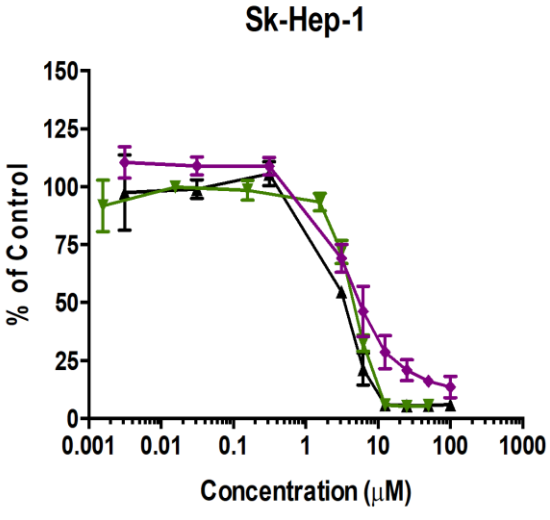
3.4.3 *Combination of 5-FU with sorafenib*

Sorafenib was more potent than 5-FU on both HepG2 (λ_s= 0.84 µM, λ_f = 2.75 µM, κ=3.27) and SK-HEP-1 (λ_s= 3.15 µM, λ_f = 5.05 µM, κ=2.45) cell lines but the fold difference (κ) was less than 6 in both cell lines. Equimolar combinations were done and showed increased potency of both cell lines (SK-HEP-1: $\lambda_{Sorafenib+5\text{-}FU}$ = 3.224 µM, ε = 4.07; HepG2 $\lambda_{Sorafenib+5\text{-}FU}$ = 0.48 µM, ε =2.46). The difference is shown. Despite κ>6 and ε>5, the activity of the combination on HepG2 is closer to the potency of sorafenib suggesting that the effect could be mostly due to sorafenib.



HepG2 equimolar combination			
Sorafenib	0.84 µM	Ratio (κ)	3.27
5-FU	2.75 µM		
Sorafenib + 5-FU	0.84 µM	Potency Index (ϵ)	2.46

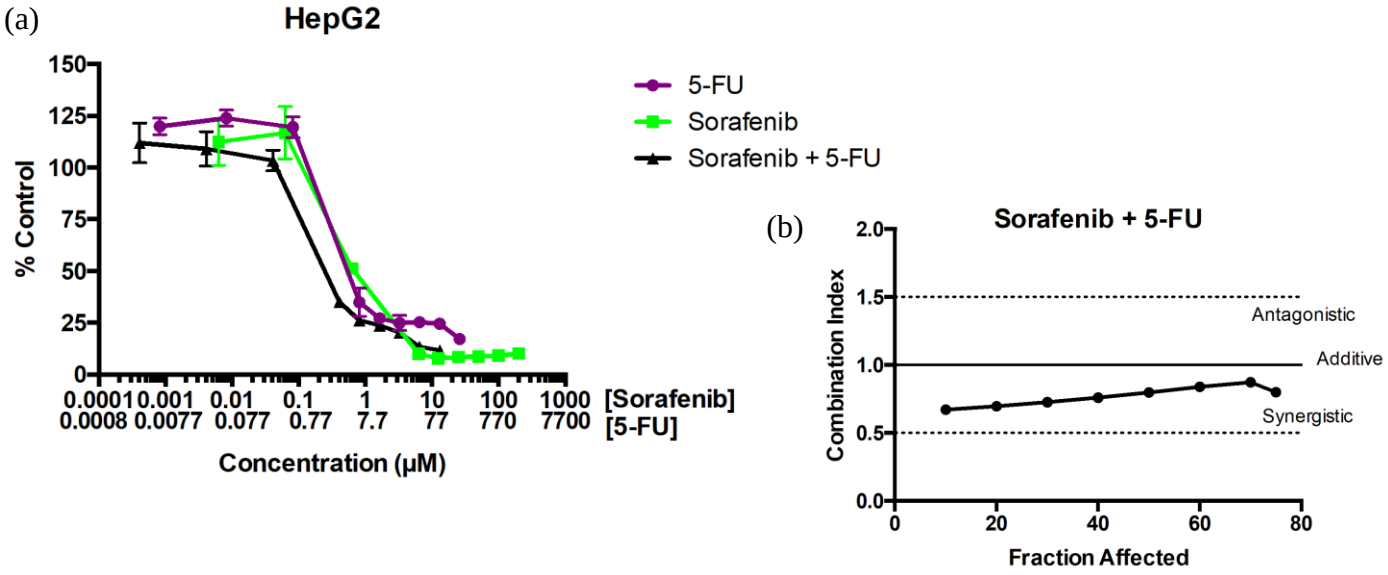
Figure 38 Equimolar combination of sorafenib and 5-FU on HepG2



SK-HEP-1 equimolar combination			
Sorafenib	3.15 µM	Ratio (κ)	2.45
5-FU	5.05 µM		
Sorafenib + 5-FU	3.22 µM	Potency Index (ϵ)	4.07

Figure 39 Equimolar combination of sorafenib and 5-FU on Sk-Hep1

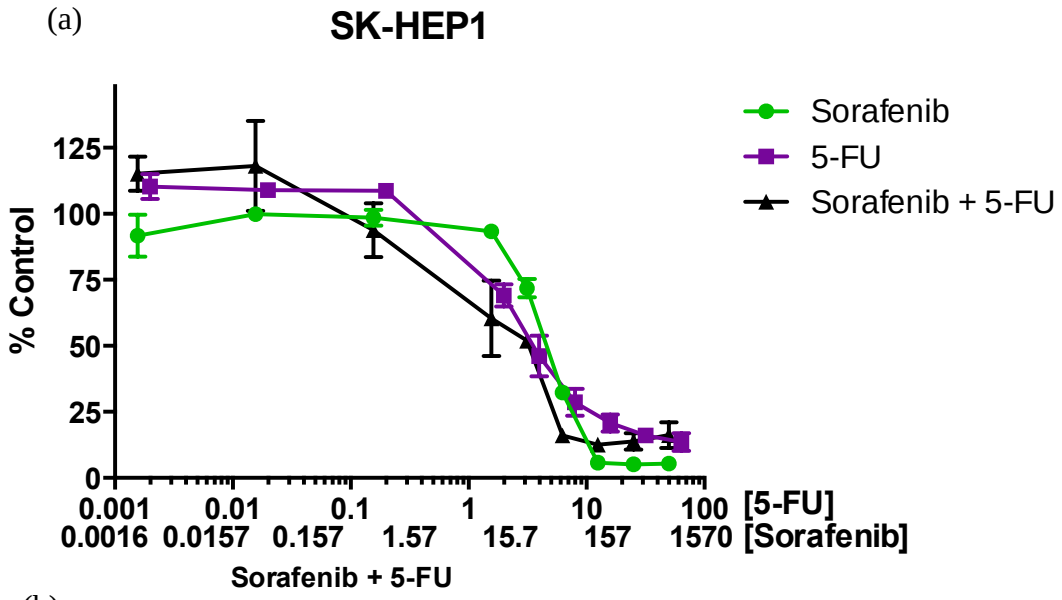
We proceeded with equi-effective combination experiments with ratios of 7.7 and 1.57 on HepG2 and SK-HEP-1, respectively. On HepG2, the IC_{50} of sorafenib in combination ($c\lambda_{Sorafenib}$) was 0.18 µM and that of 5-FU ($c\lambda_{5\text{-}FU}$) was 2.71 µM. Results showed mild synergy between 5-FU and sorafenib on HepG2 only with a CI_{50} of 0.76. CI_{50} on SK-HEP-1 of the combination was 1.068, indicating likely additive effect on combination. However, the isobologram showed that the combination of 5-FU and sorafenib was synergistic at lower concentrations and antagonistic at higher concentrations.



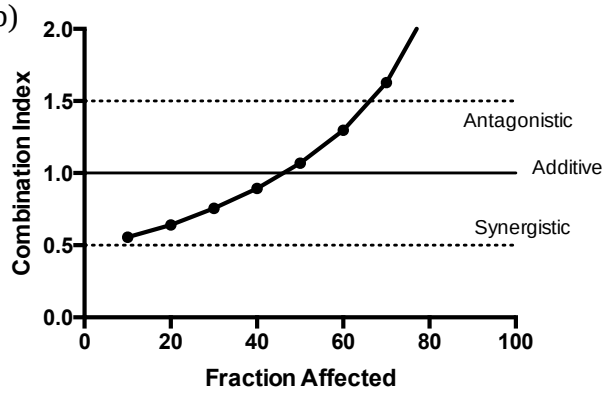
Equi-effective combination on HepG2		
	Sorafenib	5-FU
Alone	0.84 µM	2.75 µM
In Combination	0.18 µM	2.71 µM
CI_{50}	0.76	

Figure 40 (a)-(c) Equi-effective combination of sorafenib and 5-FU on HepG2

(a)



(b)



(c)

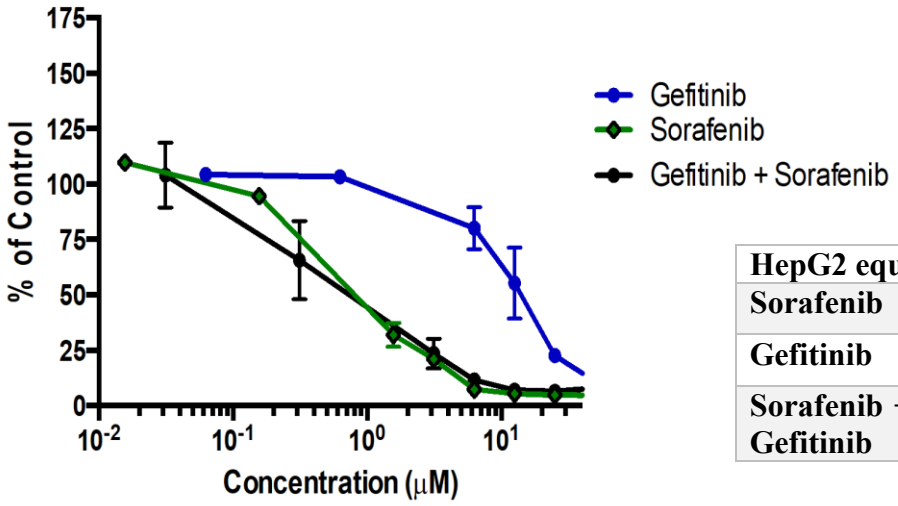
Equi-effective combination on SK-HEP-1		
	Sorafenib	**5-FU**
Alone	3.15 μM	5.05 μM
In Combination	1.39 μM	2.18 μM
CI_{50}	1.07	

Figure 41 (a)-(c) Equi-effective combination of sorafenib and 5-FU on SK-HEP-1

3.5 Combinations of sorafenib with inhibitors of EGFR

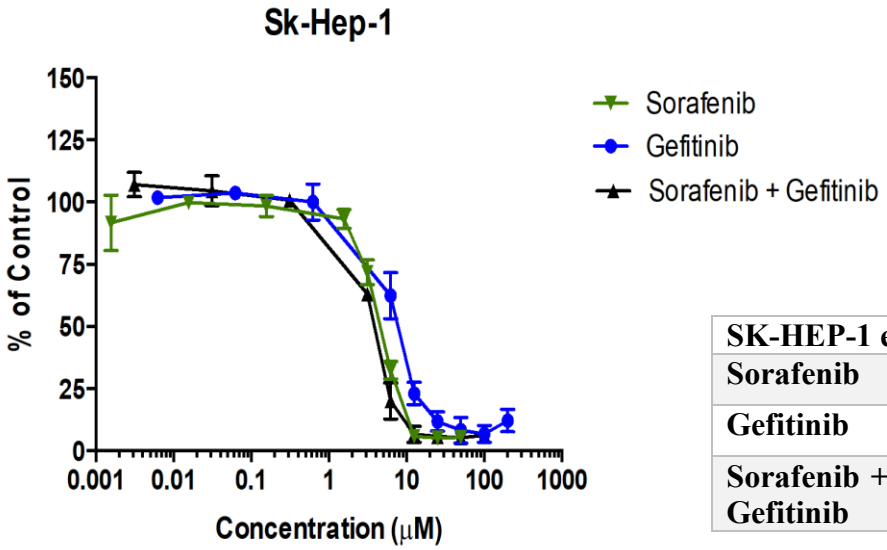
3.5.1 *Combination of Sorafenib and Gefitinib on liver cancer cells*

Sorafenib is more potent than gefitinib on all 3 liver cancer cells. HepG2 ($\lambda_{sorafenib}$= 0.84 μM, $\lambda_{gefitinib}$= 11.35 μM, κ=13.50); SK-HEP-1 ($\lambda_{sorafenib}$= 3.15 μM, $\lambda_{gefitinib}$= 5.52 μM, κ=1.75); Huh7 ($\lambda_{sorafenib}$= 2.32 μM, $\lambda_{gefitinib}$= 8.31 μM, κ=3.59). The value of κ was high in HepG2 but was below 6 on SK-HEP-1 and Huh7. Equimolar combination treatment done on all cell lines showed no improvement in potency in either cell lines with combination IC_{50} ($\lambda_{sorafenib+gefitinib}$) values closer to the IC_{50} of sorafenib ($\lambda_{sorafenib}$). HepG2 $\lambda_{sorafenib+gefitinib}$ = 0.88 μM, ε =15.1; SK-HEP-1: $\lambda_{sorafenib+gefitinib}$ = 3.50 μM, ε = 3.05; Huh7: $\lambda_{sorafenib+gefitinib}$ = 3.157 μM, ε = 6.25. Although on SK-HEP-1, epsilon is below 5, the combination IC_{50} is not lower than IC_{50} of sorafenib on the same cell line, indicating no increased potency in response to the combination.



HepG2 equimolar combination			
Sorafenib	0.84 μM	Ratio (κ)	13.5
Gefitinib	11.35 μM		
Sorafenib + Gefitinib	0.88 μM	Potency Index (ϵ)	15.1

Figure 42 Equimolar combination of sorafenib and Gefitinib on HepG

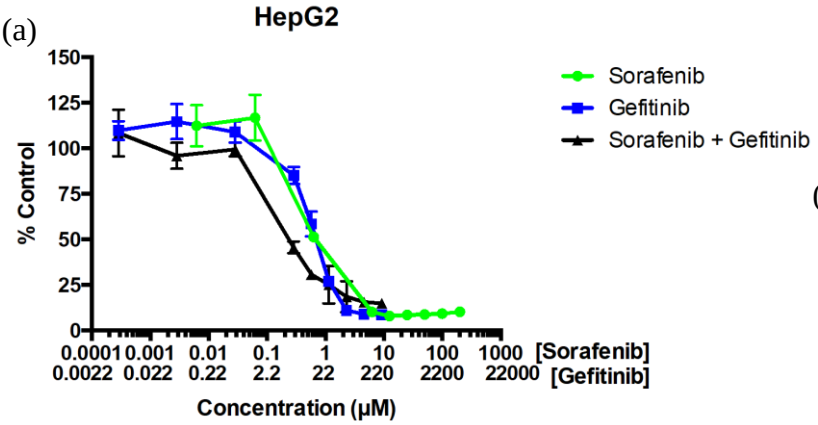


SK-HEP-1 equimolar combination			
Sorafenib	3.15 μM	Ratio (κ)	1.75
Gefitinib	5.52 μM		
Sorafenib + Gefitinib	3.50 μM	Potency Index (ϵ)	3.05

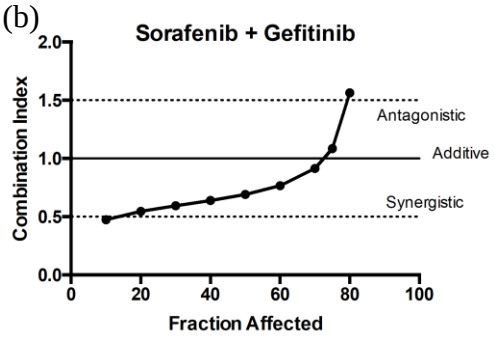
Figure 43 Equimolar combination of sorafenib and Gefitinib on SK-HEP-1

Equi-effective combination of sorafenib and gefitinib was done on HepG2 and SK-HEP-1. The ratios used were 22 and 1 on HepG2 and SK-HEP-1. On HepG2, the potency of sorafenib in combination ($c\lambda_{sorafenib}$) was 0.188 μM, which indicates more potency than sorafenib alone. The potency of gefitinib in combination ($c\lambda_{gefitinib}$) was 4.09 μM, which also indicates more potency than gefitinib alone. The CI_{50}, calculated as ($c\lambda_{sorafenib}/\lambda_{sorafenib} + c\lambda_{gefitinib}/\lambda_{gefitinib}$) was 0.610, indicating a synergistic interaction. Isobologram charting showed that the combination was synergistic amongst most concentration ranges except at higher concentrations of both drugs. On SK-HEP-1, $c\lambda_{sorafenib}$ was 2.70 μM (slightly more potent than sorafenib alone) and $c\lambda_{gefitinib}$ was also 2.70 μM, since the ratio used was 1:1. Gefitinib was more potent in combination than alone. However, CI_{50} on SK-HEP-1 was 1.105, indicating an additive interaction. Isobologram charting showed that the interaction was additive across all concentration ranges.

(a)



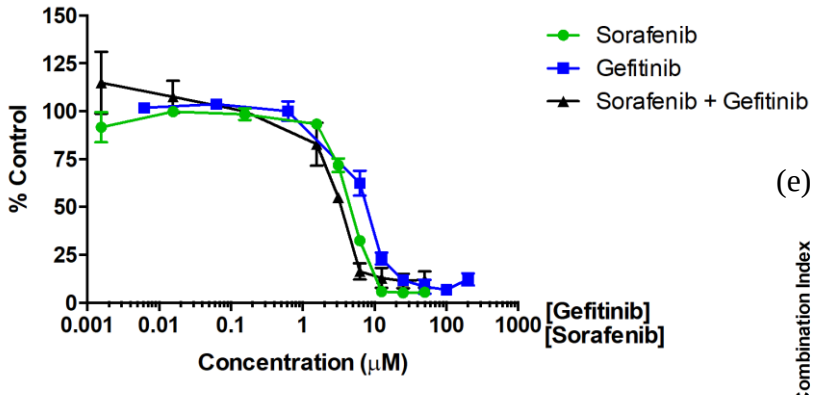
(b)



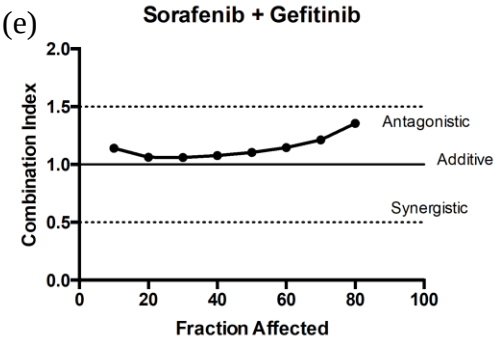
(c)

Equi-effective combination on HepG2		
	Sorafenib	Gefitinib
Alone	0.84 μM	11.35 μM
In Combination	0.188 μM	4.09 μM
CI_{50}	0.61	

(d)



(e)



(f)

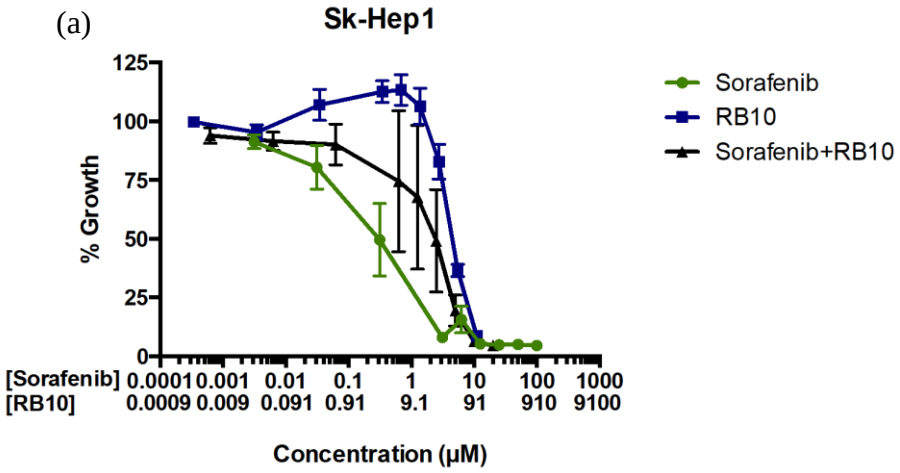
Equi-effective combination on SK-HEP-1		
	Sorafenib	Gefitinib
Alone	3.15 μM	5.52 μM
In Combination	2.70 μM	2.70 μM
CI_{50}	1.105	

Figure 44 (a)-(f) Equi-effective combination of sorafenib and Gefitinib on HepG2 and SK-HEP-1

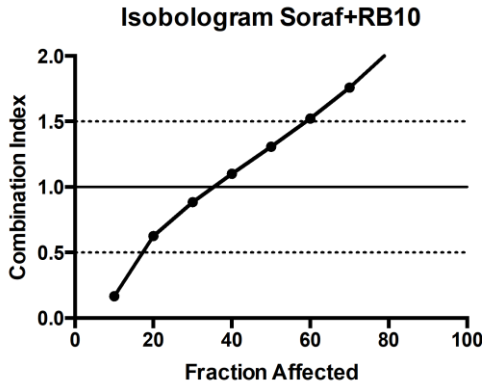
3.5.2 *Combination of Sorafenib and RB10 on liver cancer cells*

RB10 was designed and synthesized in our laboratory. Steps of production of RB10 were done as published earlier. RB10 has potent inhibitory effect of EGFR tyrosine kinase. However, It had a weak effect on HepG2 and SK-HEP-1 cell lines with IC_{50} values of 20.74 µM and 29.17 µM, respectively. Due to the large fold-difference between sorafenib and RB10, it was not surprising for the interaction to represent sorafenib alone. We assessed the interaction using equi-effective combination that showed CI_{50} of 1.61 on SK-HEP-1, which most likely indicates antagonistic interaction. Isobologram charting showed variable combination index values but mostly antagonistic except at lower concentrations.

(a)



(b)



(c)

Equi-effective combination on SK-HEP-1		
	Sorafenib	RB10
Alone	3.15 µM	29.17 µM
In Combination	2.56 µM	15.35 µM
CI_{50}	1.61	

Figure 45 (a)-(c) Equi-effective combination of sorafenib and RB10 on SK-HEP-1

3.5.3 *The combi-molecule AB2*

Based on the strong effects of RB10 on inhibition of EGFR, the combi-molecule AB2 was designed with both sorafenib and RB10 residues. The experiment was conducted on both HepG2 and SK-HEP-1, and compared to RB10, sorafenib and the equimolar combination. However, the molecule was not effective on either cell line as shown below. A negligible amount of inhibition of growth was achieved with maximum dose of AB2 of 100 μM.

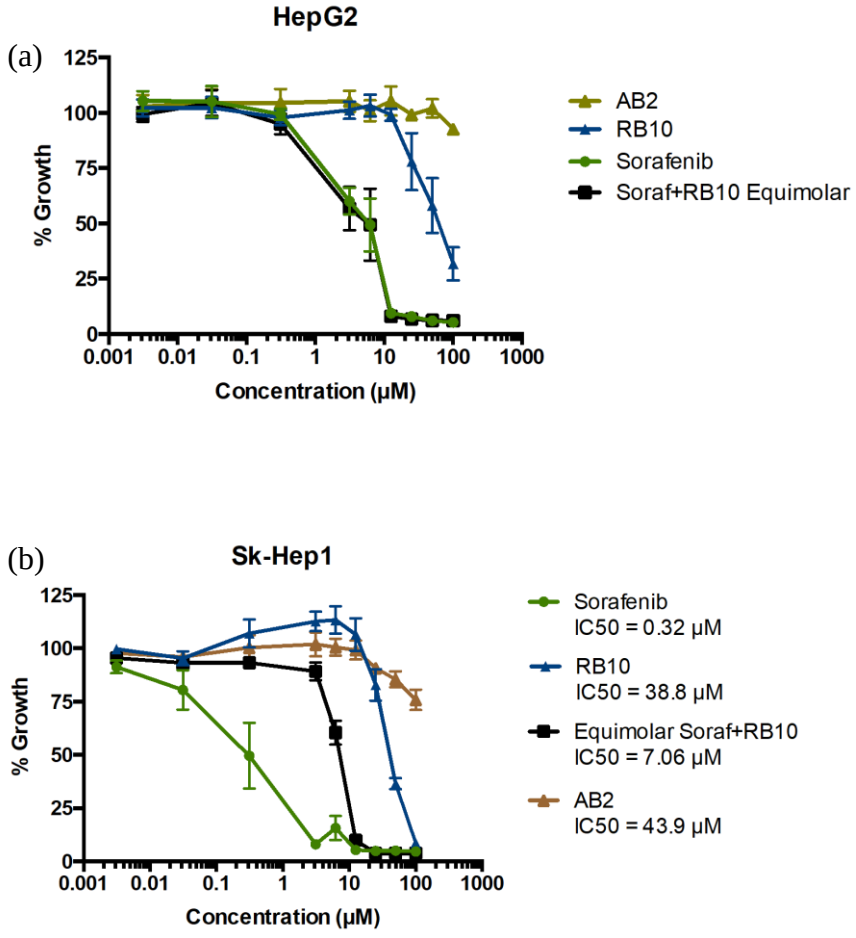
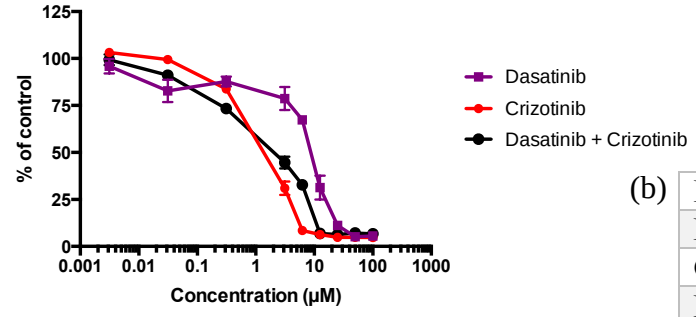


Figure 46 (a)-(b) The combi-molecule AB2 on HepG2 and Sk-Hep1 compared to sorafenib alone, RB10 alone, and their equimolar combination

3.6.1 *Src inhibition in HCC*

The Src inhibitor, dasatinib, was used to determine its potency against the cell lines HepG2 and SK-HEP-1 both alone and in combination. Dasatinib was more effective on SK-HEP-1 ($\lambda_{dasatinib}$ = 2.20 μM) than on HepG2 ($\lambda_{dasatinib}$ = 8.72 μM). The weaker potency of dasatinib on HepG2 and the large difference between its potency and the potency of crizotinib, sorafenib and selumetinib leads to ineffectiveness of equimolar combinations where they would represent the potency of the more powerful agent alone. However, the potencies of dasatinib and gefitinib on HepG2 ($\lambda_{dasatinib}$ = 8.72 μM, $\lambda_{gefitinib}$ = 11.4 μM) are close together, with a ratio κ of 1.3. The combination IC_{50} ($\lambda_{dasatinib+gefitinib}$) was 9.79 μM, and the potency index ϵ was 2.6, indicating a possible effective combination

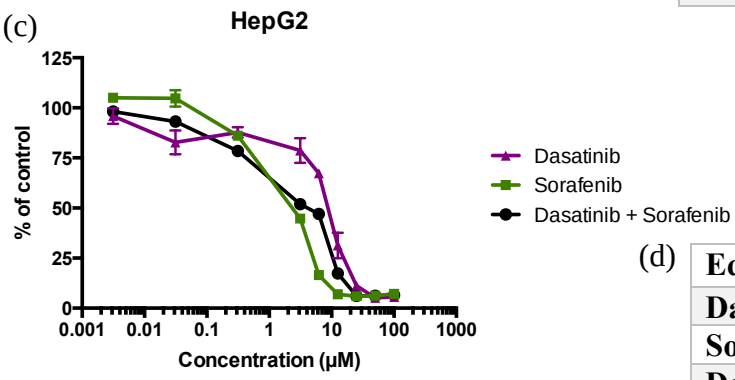
(a)



(b)

Equimolar combination on HepG2			
Dasatinib	8.72 μM	Ratio (κ)	8.9
Crizotinib	0.98 μM		
Dasatinib + Crizotinib	2.04 μM	Potency Index (ϵ)	20.4

(c)

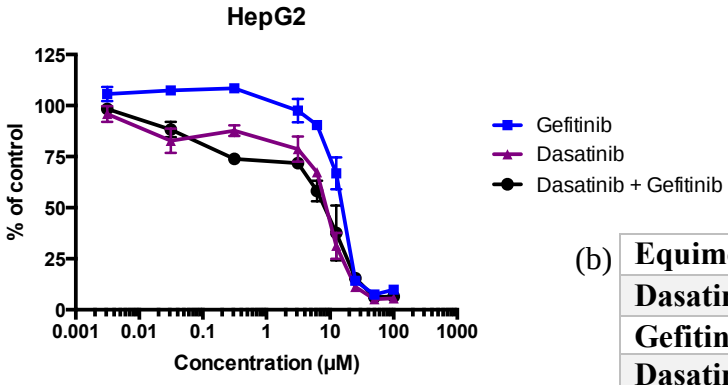


(d)

Equimolar combination on HepG2			
Dasatinib	8.72 μM	Ratio (κ)	10.4
Sorafenib	0.84 μM		
Dasatinib + Sorafenib	4.30 μM	Potency Index (ϵ)	58.2

Figure 47 (a)-(d) Equimolar combinations of dasatinib with crizotinib, sorafenib, or gefitinib on HepG2

(a)



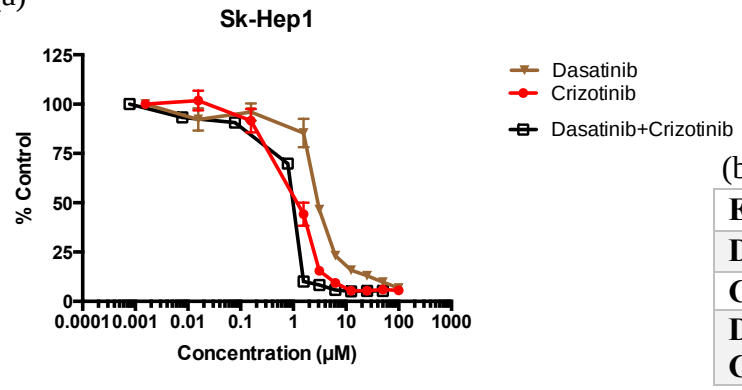
(b)

Equimolar combination on HepG2			
Dasatinib	8.72 μM	Ratio (κ)	1.3
Gefitinib	11.3 μM		
Dasatinib + Gefitinib	9.79 μM	Potency Index (ϵ)	2.58

Figure 48 (a)-(b) Equimolar combinations of dasatinib with crizotinib, sorafenib, or gefitinib on HepG2

On SK-HEP-1, equimolar combination of dasatinib with sorafenib, crizotinib, selumetinib and gefitinib was performed and is shown below. The IC_{50} values of Crizotinib and dasatinib on SK-HEP-1 were as follows: ($\lambda_{dasatinib}$ = 2.20 μM, $\lambda_{crizotinib}$ = 0.70 μM, κ = 3.1), and the IC_{50} of the combination ($\lambda_{dasatinib+crizotinib}$) was 0.94 μM with a potency index (ϵ) of 5.5, indicating an ineffective combination. The IC_{50} values of Sorafenib and dasatinib on SK-HEP-1 were as follows: ($\lambda_{dasatinib}$ = 2.20 μM, $\lambda_{sorafenib}$ = 3.15 μM, κ = 1.4), and the IC_{50} of the combination ($\lambda_{dasatinib+sorafenib}$) was 1.72 μM with a potency index (ϵ) of 1.9, indicating a possible effective combination. The IC_{50} values of Gefitinib and dasatinib on SK-HEP-1 were as follows: ($\lambda_{dasatinib}$ = 2.20 μM, $\lambda_{gefitinib}$ = 5.52 μM, κ = 2.5), and the IC_{50} of the combination ($\lambda_{dasatinib+gefitinib}$) was 1.49 μM with a potency index (ϵ) of 2.4, indicating a possible effective combination. Finally, the IC_{50} values of Selumetinib and dasatinib had IC_{50} values on SK-HEP-1 were as follows: ($\lambda_{dasatinib}$ = 2.20 μM, $\lambda_{selumetinib}$ = 2.29 μM, κ = 1.0), and the IC_{50} of the combination ($\lambda_{dasatinib+sorafenib}$) was 0.94 μM with a potency index (ϵ) of 0.87, indicating a highly possible effective combination.

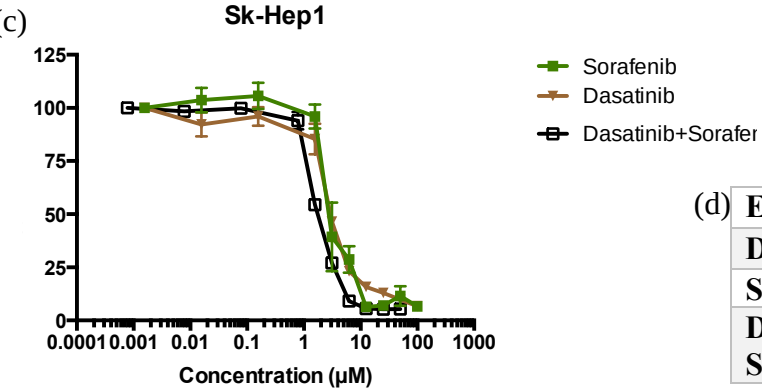
(a)



(b)

Equimolar combination on SK-HEP-1			
Dasatinib	2.20 μM	Ratio (κ)	3.1
Crizotinib	0.70 μM		
Dasatinib + Crizotinib	0.94 μM	Potency Index (ϵ)	5.53

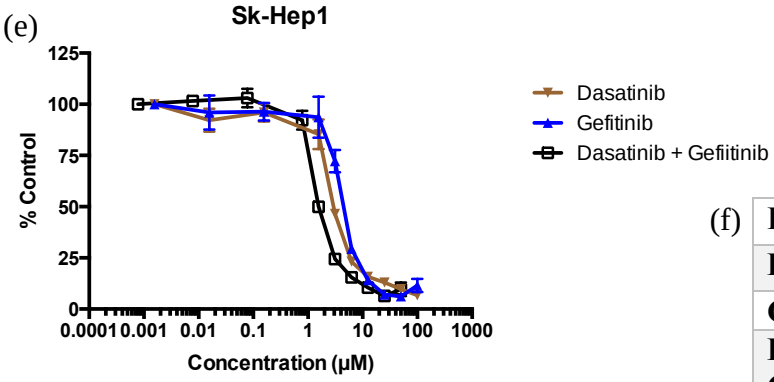
(c)



(d)

Equimolar combination on SK-HEP-1			
Dasatinib	2.20 μM	Ratio (κ)	1.4
Sorafenib	3.15 μM		
Dasatinib + Sorafenib	1.72 μM	Potency Index (ϵ)	1.9

(e)



(f)

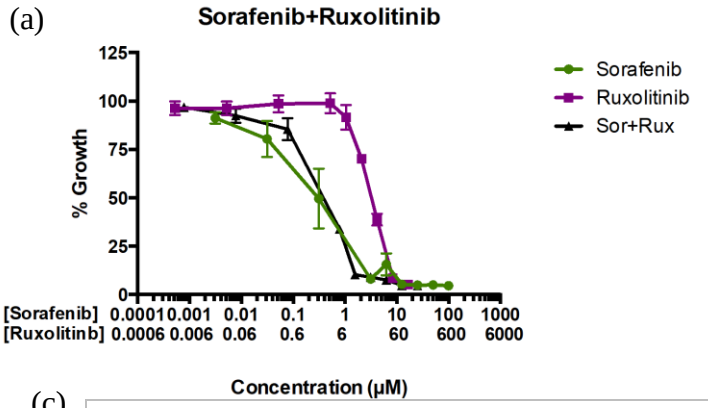
Equimolar combination on SK-HEP-1			
Dasatinib	2.20 μM	Ratio (κ)	2.5
Gefitinib	5.51 μM		
Dasatinib + Gefitinib	1.49 μM	Potency Index (ϵ)	2.37

Figure 49 (a)-(f) Equimolar combinations of dasatinib with crizotinib, sorafenib and gefitinib on SK-HEP-1

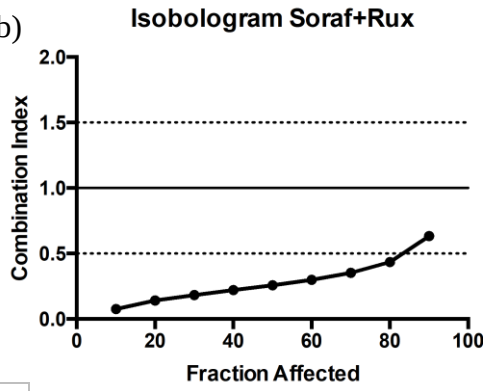
3.6.2 *JAK inhibition in HCC*

The JAK inhibitor ruxolitinib was used in the liver cancer cell lines HepG2 and SK-HEP-1, alone and with sorafenib or the EGFR inhibitor RB10. Ruxolitinib had a weak to moderate potency on both cell lines, with IC_{50}s as follows: HepG2 $\lambda_{ruxolitinib}$: 10.1 μM, and SK-HEP-1 $\lambda_{ruxolitinib}$: 19.1 μM. The combination of sorafenib and sorafenib was tested on SK-HEP-1. Sorafenib was more potent than ruxolitinib on SK-HEP-1($\lambda_{sorafenib}$= 3.15 μM, $\lambda_{ruxolitinib}$ = 19.1 μM, κ=6.0), Equimolar combinations showed a mild increase in the potency of combination ($\lambda_{Sorafenib+ruxolitinib}$ = 2.59 μM, ε = 5.79). However, the value is close enough to the potency of sorafenib and with the borderline high κ and ϵ values, the effect could be mostly due to sorafenib. Thus, we proceeded with equi-effective combination experiments with a ratio of 6. The IC_{50} of sorafenib in combination ($c\lambda_{Sorafenib}$) was 0.43 μM and that of ruxolitinib ($c\lambda_{ruxolitinib}$) was 2.60 μM. Results showed significant synergy between ruxolitinib and sorafenib on SK-HEP-1 only with a CI_{50} of 0.274. Isobologram graphing showed synergy across all concentrations.

(a)



(b)



(c)

Equi-effective combination on SK-HEP-1		
	Sorafenib	Ruxolitinib
IC_{50} Alone	3.154 μM	19.07 μM
IC_{50} in combination	0.434 μM	2.60 μM
CI_{50}	0.274	

Figure 50 Equi-effective combination of Sorafenib and Ruxolitinib on SK-HEP-1

4 Discussion

Although combination therapy has been used often in chemotherapeutic regimens, combinations of small molecule kinase inhibitors have not become very common yet in the clinical practice. Nevertheless, experience in the case of dual targeting of components of the BRAF pathway (using RAF and MEK inhibitors) have been proven successful.[127,128] Also, pre-clinical and early clinical data show that it may be a promising approach in cases where targets are identified and rationally chosen.[129,130] Many cancer treatment protocols use the combination of chemotherapeutic and molecularly targeted therapy. However, in the field of liver cancer, several studies (as shown in the introduction section) have failed to show evidence of increased benefit with the use of multiple systemic agents.[22]

Our study investigated the use of several agents and several combinations against liver cancer. We have found that different agents act on liver cancer cell lines differently, which can be explained by the different genetic make-up of the cell lines. There is a huge interest amongst cancer scientists and oncologists in the field of personalized medicine which carries great potentials for the next generation of cancer treatment. Characterizing different molecular therapeutic strategies would help optimize regimens prescribed to each patient based on the make-up of their tumours.

We found that the interaction between sorafenib and crizotinib was somehow effective, but not consistently. The interaction can be explained by inhibiting the reactivation of MET that can occur as a result of sorafenib. Through inhibition of MET, the use of crizotinib can also lead to inhibition of both the PI3K/AKT and the MAPK pathway. While AKT activation is a resistance pathway of sorafenib, its inhibition by crizotinib can also explain the synergistic interaction. There were reports of interactions between sorafenib and the MET inhibitor tivantinib that showed an additive relationship.

Western blot data from our experiment showed a possible synergistic inhibitor action on the phosphorylation of EGFR with treatment with crizotinib and sorafenib particularly after 24 hours. The effect can be explained as a reversal of the reactivation of EGFR following 24 hours of sorafenib treatment. The increase in thickness of pEGFR band following sorafenib treatment is not present when crizotinib is in the combination.

The interaction between EGFR and MET was found to be effective in previous reports.[119] The results were not surprising in our experiments where we saw a synergistic interaction between gefitinib and crizotinib. The combination has been investigated and was shown to reverse gefitinib resistance in NSCLC cells,[131] but has not been attempted in a clinical trial.

We investigated the use of EGFR inhibition by gefitinib combined with sorafenib, but the results were not promising. EGFR depends greatly on MAPK and PI3K pathway to transmit its signals into the nucleus and promote proliferation. With inhibition of the MAPK pathway with sorafenib, the inhibition of EGFR may not be very useful as one of its main downstream pathways is already inhibited. Sorafenib and erlotinib have been investigated in HCC during the phase III SEARCH trial[59] but did not show improvement in survival compared to sorafenib alone.

Another potent interaction that we found is the interaction of sorafenib with selumetinib. The combination was consistently synergistic in all 3 cell lines tested. Both agents inhibit targets on the MAPK pathway ant the resulting combined inhibition of the pathway could be the reason for the strength of the combination. The combination of sorafenib and MEK inhibitors was found to be effective in pre-clinical studies[132] and was shown to be promising in a phase Ib trial in HCC.[108] The interest in strong inhibition of the MAPK pathway is because it is downstream of many upstream pathways involved in carcinogenesis. The caveat thought about strong inhibition of one pathway is that it may promote the development of other resistance pathways as a result

which would include other intracellular kinase pathways that are independent of the MAPK pathway.

Selumetinib has also shown some promise when combined with crizotinib on SK-HEP-1 and HepG2, but inconsistently synergistic on Huh7. The equimolar combination of crizotinib and sorafenib though was potent. MET acts through both intracellular pathways MAPK and PI3K/AKT. It potently promotes cellular survival and inhibits apoptosis through the PI3K/AKT. Thus, crizotinib inhibits both pathways. With the combination, Selumetinib provides additional inhibition of MAPK pathway including activity that is mediated by other upstream pathways other than MET. Crizotinib inhibits MET with its downstream pathways, including PI3K/AKT which is not inhibited by selumetinib.

The promising results of the crizotinib-selumetinib combination pointed towards synthesizing a combi-molecule composed of crizotinib and selumetinib residues in our laboratory. Our group has experience synthesizing similar types of molecules and testing it in vivo and in vitro. The new molecule, AL 13-51, showed activity against HCC cell lines but when compared to either selumetinib or crizotinib alone or their equimolar combination, it did not show increased potency. Optimization of the molecule would require characterization of activity of the molecule against HGF and MEK. Also, a useful strategy could be to chemically model the molecule *in silico* to help suggest ideas of design change to optimize the activity of the molecule.

Seeing the potential of the sorafenib-crizotinib combination in inhibiting sorafenib resistance pathways, the sorafenib-selumetinib pathway in inhibiting the MAPK pathway potently, and the crizotinib-selumetinib pathway in inhibiting both MAPK and downstream effects of MET, we introduce the idea of a triple combination including sorafenib, crizotinib, and selumetinib. The triple combination was tested in all 3 cell lines and showed promise in 2 of the 3 cell lines with a

very strong synergy. Further steps are needed to characterize the molecular responses and changes in signaling pathways in response to triple therapy.

The use of pyrimidine analogues with kinase inhibitors has been investigated in the literature[133]. Our laboratory has studied the interactions between pyrimidine analogues and the EGFR pathway, and several combi-molecules have been designed that include pyrimidine analogues and the EGFR pathway.[134] 5-Flourouracil (5-FU) is one of the clinically available pyrimidine inhibitors that are currently used in many cancers including pancreatic and colon cancer and has been used for HCC.[111] In our study, we found that the combination of 5-FU with crizotinib was synergistic with both cell lines tested, pointing towards an interaction of the survival pathway mediated through MET with the DNA synthesis pathway. When combined with 5-FU, the EGFR inhibitor gefitinib was at least additive (or slightly synergistic) on HepG2, but this effect was not seen on SK-HEP-1. We also found an additive or a mildly synergistic effect between sorafenib and 5-FU. Trials done with sorafenib and 5-FU on HCC didn't show improvement in survival.[114]

Our experiment also tested synergistic interactions involving the Src inhibitor dasatinib. The intracellular kinase Src interacts with several pathways involved in proliferation including EGFR, the MAPK pathway and the PI3K/AKT pathway. As expected, dasatinib showed an interaction with gefitinib leading to improved potency, which was an interesting interaction that led to the development of a combi-molecule in our laboratory.[93] The interaction between dasatinib and sorafenib or dasatinib and crizotinib did not show potency but may need to be evaluated further.

The interactions between sorafenib and the JAK-STAT pathway were reported in the literature. Sorafenib leads to inactivation of STAT3 through activation of its silencer, SHP-2.[76,135] STAT3 also mediates resistance to several kinase inhibitors, including sorafenib. Thus, we studied the effects of using sorafenib in addition to the JAK inhibitor ruxolitinib. We did find strong

synergy when tested on SK-HEP-1. Further investigations of the interactions between the JAK-STAT and MAPK inhibition would be warranted particularly to investigate the changes in signaling pathway in response to the combination.

The main limitation of our study is that it is based on in vitro experiments on liver cancer cell lines, which do not resemble all the biological properties of the tumour, like tumour initiation, microenvironment and angiogenesis. In vitro experiments, however, are useful for screening of several compounds, and for mechanistic studies at particular time points of investigations.

Our study has investigated a number of combinations to study the interactions between different kinase inhibitors on HCC cell lines. We suggest the eventual introduction of combination therapy to the treatment regimen of HCC with preceding studies characterizing the numerous different combination options that can be introduced in order to conclude the ideal combination in each case. Ideally, different combinations would be ideal for different patients depending on the characteristics of their tumours as well as their health and tolerance status in general.

Chapter 3 Conclusion and Contribution to Knowledge

Patients with hepatocellular carcinoma (HCC) are mostly ones who have faced a long and difficult way through their underlying disease of hepatitis and liver disease, with consequent morbidity and illness due to insufficient liver function. That, combined with the dismal prognosis of the disease, is understandably a painful and hopeless tragedy. Being able to provide them with hope that a developing therapy could save them from imminent mortal sickness would be the biggest gift that can be provided. Through history, similar people suffering from a variety of diseases were provided that gift of the opportunity of treatment that would relieve their suffering and provide them with hope.

Our work that is documented in this thesis added pieces of knowledge that we hope would help developing a successful therapeutic strategy to patients with advanced HCC. We built our hypothesis on existing pieces of knowledge that HCC is responsive to the kinase inhibitor sorafenib. We also built it on previous experience with combination treatment where simultaneous blockade of pathways with mutual cross-talk leads to a synergistic effect. We were guided by the recommendations of the American Association for the Study of Liver Diseases and Journal of the National Cancer Institute (AASLD-JNCI) to try agents in combination with sorafenib in the first-line setting.[136]

Part of our contribution to knowledge in the field of HCC treatment is that combining kinase inhibitors with sorafenib could be an effective strategy, including inhibitors of MET, MEK, and JAK. This idea can be used as a basis for the design of trials using already existing inhibitors of MET, MEK, and JAK kinases combined with sorafenib. Several agents have been developed against MET and are in different phases of clinical trials in a variety of neoplasms. In HCC, two MET inhibitors that are good potentials to be used with sorafenib include cabozantinib[138] and tivantinib.[139]

Our experiments also contributed to more knowledge about the kinase inhibitor Crizotinib, which is already approved in the clinic is for non-small cell lung cancer (NSCLC) with ALK-rearrangements due to its activity against ALK.[100] Crizotinib has not been tried in HCC despite its action on MET inhibition. Our results show that crizotinib has an effect on the inhibition of HCC cell lines that is comparable to the effect of sorafenib. Novel combi-molecule that include MET inhibitory action may be developed and later tested against HCC, particularly in cases where MET is overexpressed.

Another strategy that is suggested by our is the combination of sorafenib with another kinase inhibitor along the MAPK pathway. Tandem inhibition of the MAPK pathway has been shown effective in melanoma, where the combination of BRAF inhibitor with a MEK inhibitor produces more favourable results than BRAF inhibition alone.[128] Tandem inhibition of the MAPK pathway in HCC can be achieved by a MEK inhibitor, such as selumetinib, to sorafenib. Although a clinical trial has shown that selumetinib has no objective effect on untreated advanced HCC,[109] it has not been assessed in combination with sorafenib. In our study, we saw that the combination of sorafenib with selumetinib was promising in the 3 cell lines tested. A combi-molecule that inhibits RAF and MEK may be effective against HCC.

Finally, a strong strategy we suggest would be the triple combination of Raf-MEK-MET inhibition. It is a novel combination that provides multiplied inhibition of the MAPK pathway, which is important for tumour growth as well as inhibition of other intracellular pathways activated by MET. By having a wide spectrum of activity amongst pathways, the combination blocks the potential development of resistance pathways and thus may lead to higher rates of success.

In conclusion, in our study we screened for effective therapeutic strategies against hepatocellular carcinoma and identified a few possible strategies that may be developed further

into more pragmatic ideas for treatment of HCC. Those strategies need more mechanistic investigations to reveal the cells' behaviour in response to treatment. More importantly, need to be verified in vivo and accordingly, it would suggest a potential therapeutic strategy that can be tested in clinical trials. Within the vast space of what can be investigated in the field of HCC treatment, we hope that the information we presented can be used to inspire more discovery and eventual development of a practical therapeutic approach that would benefit sick patients around the world.

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