

**EFFECTS OF PI3K/AKT/mTOR AND VEGFR PATHWAY INHIBITORS ON
LIVER CANCER STEM CELLS
AND
BIOACTIVITIES OF NOVEL PYRAZOLIC CHALCONE DERIVATIVES ON
LIVER CANCER**

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IN
MOLECULAR BIOLOGY AND GENETICS**

**By
Deniz Cansen Kahraman
December 2017**

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We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

Işık Yuluğ
(Advisor)

Rengül Atalay
(Co-advisor)

Özgür Şahin

Ayşegül Üner

İhsan Gürsel

Ayşe Elif Erson Bensan

Approved for the Graduate School of Engineering and Science:

Ezhan Karaşan
Director of the Graduate School of Engineering and Science

ABSTRACT

EFFECTS OF PI3K/AKT/mTOR AND VEGFR PATHWAY INHIBITORS ON LIVER CANCER STEM CELLS AND BIOACTIVITIES OF NOVEL PYRAZOLIC CHALCONE DERIVATIVES ON LIVER CANCER

Deniz Cansen Kahraman

Ph.D. in Molecular Biology and Genetics

Advisor: Işık Yuluğ

Co-advisor: Rengül Atalay

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Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related mortality, such that it the second most frequent cause of cancer death worldwide. Due to its heterogeneous composition and aggressive behavior, it is resistant to conventional therapies and also Sorafenib and Regorafenib which are FDA-approved multikinase inhibitors targeting pathways involved in angiogenesis and proliferation. The mechanisms behind the acquired resistance to Sorafenib were described as activation of compensatory pathways such as PI3K/Akt/mTOR, JAK-STAT, epithelial to mesenchymal transition (EMT), microenvironment and presence of cancer stem cells. Liver cancer stem cells originate from damaged and transformed hepatic progenitor cells (HPCs) which are found responsible for chemo-resistance, tumor relapse, and metastasis. For this reason, the effects of PI3K/Akt/mTOR inhibitors, Sorafenib and DNA intercalators on the enrichment of LCSCs were investigated. CD133+/EpCAM+ population from HCC cells were analyzed by flow cytometry after treatment with inhibitors, and effective inhibitors against LCSCs were further tested for their potential combinatorial effects together with Sorafenib. It was shown that upon treatment with Sorafenib or DNA intercalators the LCSCs were enriched, whereas Rapamycin (mTOR inhibitor), LY294002 (PI3K inhibitor) were able to inhibit the enrichment of LCSCs and

reduced the CD133+/EpCAM+ population ratio. Combination studies revealed that when cells are treated initially with Rapamycin and then with Sorafenib, both the LCSC ratio and the sphere formation capacity of cells were reduced compared to cells treated with Sorafenib alone. To understand the alterations in gene expression induced by the inhibitors, a large panel of genes involved in regulation of cancer pathways were analyzed using Nanostring nCounter Technology. Systematic pathway analysis using Cytoscape Score Flow algorithm application allowed us to identify differential response genes involved in stemness. It was shown that genes involved in regulation of stem cells (Wnt and Notch pathway) were downregulated upon treatment with Rapamycin and DAPT (Notch pathway inhibitor), yet Sorafenib treatment resulted in differential regulation of these pathways, where JAG1 gene was found to be up-regulated. Interestingly, IL-8 expression was upregulated dramatically upon treatment with Sorafenib, but downregulated upon DAPT or Rapamycin treatment. Inhibition of IL-8 signaling resulted in reduction in both LCSC ratio and sphere formation capacity of HCC cells, which could be indicating the role of IL-8 signaling in the conservation of stemness features of LCSCs. For this reason, blockade of IL-8 signaling was suggested to be a promising therapeutic approach for HCC.

Another topic in this thesis focuses on the potential of VEGFR2 TKIs and quinoids to inhibit both liver cancer cells and liver cancer stem cells. VEGFR TKIs such as Sorafenib, are widely studied for the treatment of many cancers, yet as mentioned above, there are many clinical studies providing the evidence that anti-VEGF or anti-VEGFR therapies lead to stable disease, which is then followed by disease progression in different cancer types. In recent years it has also been shown that antiangiogenic agents are increasing cancer stem cell population via generation of tumor hypoxia. Quinoids, on the other hand, are compounds that are selectively active in hypoxic conditions. Thus, the main aim of this study was to evaluate the bioactivities of compounds from each group on liver cancer cells and also to analyze their effects on the enrichment of LCSCs. Our results have shown that VEGFR2 TKIs were cytotoxic at lower concentrations compared to quinoids. However, it was shown that VEGFR2 TKIs are more likely to enrich LCSC population

whereas some of the quinoids were able to reduce this ratio. With this information, a new concept called “aggressiveness factor”, which defines the potential of a compound to cause more aggressive cancer, was introduced.

In the last part of this thesis, bioactivities of pyrazolic chalcone derivatives on HCC cell lines and their mechanism of action were investigated. Chalcones and pyrazolic structures are well known for their anti-cancer activities. Newly synthesized pyrazolic chalcone derivatives were tested against different cancer cells, and selection based on the IC₅₀ values of compounds was made to analyze their effect on a panel of HCC cells. Results have shown that, compounds **39**, **42**, **49** and **52** were the most effective derivatives which had anti-proliferative activities in less than 5 μ M concentrations. Further investigation of cell cycle progression and cell death mechanisms have revealed that compounds **42** and **52** caused cell cycle arrest at the G2/M phase and induced apoptotic cell death. Also, levels of cell cycle proteins, p21, CDK1, and phospho-CyclinB1 were shown to decrease upon treatment with these compounds.

Keywords: Hepatocellular carcinoma, PI3K/Akt/mTOR, pathway, Sorafenib, liver cancer stem cells, drug resistance, VEGFR-TKI, quinoids, pyrazolic chalcone derivatives, bioactivity, cell cycle, apoptosis

ÖZET

PI3K/AKT/mTOR VE VEGFR SİNYAL YOLAKLARI İNHİBİTÖRLERİN KARACİĞER KANSERİ KÖK HÜCRELERİ ÜZERİNDEKİ ETKİLERİ VE PİRAZOLİK KALKON TÜREVLERİNİN KARACİĞER KANSERİ ÜZERİNDEKİ BİYOAKTİVİTELERİ

Deniz Cansen Kahraman
Moleküler Biyoloji ve Genetik Bölümü, Doktora
Tez Danışmanı: Işık Yuluğ
Tez Eş-danışmanı: Rengül Atalay
Aralık, 2017

Hepatoselüler karsinom (HSK) kansere bağlı ölüm nedenlerinde en ön sıralarda yer almaktadır. Dünyada meydana gelen kanser ölümleri arasında ikinci sırada yer almaktadır. Heterojen yapısı ve agresif davranışı sebebiyle, kemoterapötik ilaçlara ve FDA onaylı, çoğalma ve anjiyojenez yolaklarını hedef alan, multikinaz inhibitörü Sorafenib ve Regorafenib'e karşı dirençlidir. Sorafenib'e karşı gelişen direncin arkasında yer alan mekanizmalar, PI3K/Akt/Mtor, JAK/STAT gibi dengeleyici yolların aktivasyonu, epitelyal mesenkimal geçiş (EMG), mikroçevre ve kanser kök hücrelerinin varlığı olarak açıklanmaktadır. Karaciğer kanser kök hücreleri (KKKH) hasara uğramış ve değişime uğramış hepatik öncü hücrelerden meydana gelmişlerdir ve bu hücreler kemoterapi direnci, tümörün nüksetmesi ve metastazı gibi olaylardan sorumludurlar. Bu sebeple, PI3K/Akt/mTOR yolağı inhibitörleri, Sorafenib ve DNA interkalatörlerinin KKKH üzerindeki etkileri incelenmiştir. Bu inhibitörler ile muamele edilmiş HSK hücre hatlarındaki CD133+/EpCAM+ popülasyonu akış sitometrisi ile analiz edilmiş, KKKH'lere karşı etkili olabilen inhibitörler Sorafenib ile kombinasyon deneylerindeki potansiyelleri belirlenmek üzere test edilmiştir. Sonuçlar göstermiştir ki, Sorafenib veya DNA interkalatörleri ile muamele edilen hücrelerde KKKH'leri zenginleşirken,

Rapamisin (mTOR inhibitörü), LY294002 (PI3K inhibitörü) CD133+/EpCAM+ popülasyonu oranını düşürüp KKKH zenginleşmesine engel olmuştur. Kombinasyon çalışmaları ile hücrelerin öncelikle Rapamisin ile daha sonra ise Sorafenib ile muamele edilmeleri ile , hem KKKH oranının hem de küre oluşturma kapasitelerinin yalnızca Sorafenib ile muamele edilen hücrelere göre azaldığı gösterilmiştir. İnhibitörlerin hücrelerdeki gen ifade seviyelerinde sebep oldukları değişimleri anlamak için, Nanostring nCounter teknolojisinden faydalanarak, kanser yolaklarını regüle eden genlerin analizi yapılmıştır. Cytoscape Score Flow algoritması uygulaması ile gerçekleştirilen sistemik yolak analizi, kök hücreleşme ile ilgili genlerdeki ifade farklılıklarının belirlenmesine yardımcı olmuştur. Rapamisin veya DAPT (Notch yolağı inhibitörü) ile muamele sonrası kök hücrelerin regülasyonunda yer alan genlerin (Wnt ve Notch yolakları) ifadelerinin azaldığı gösterilmiş, fakat Sorafenib ile muamele sonucunda bu yolaklardaki genlerin ifadelerinde farklılıklar (örn. JAG1 geninde artış) gözlemlenmiştir. İlginç bir şekilde, IL-8 ifadesi Sorafenib ile muamele sonucu artış göstermiş, fakat DAPT veya Rapamisin ile muamele sonucu düşüş göstermiştir. IL-8 yolağının inhibe edilmesi, hem KKKH oranında hem de HSK hücrelerinin sfer oluşturma kapasitelerinde azalmaya sebep olmuştur. Bu da IL-8 yolağının KKKH'ların kök hücre özelliklerini koruyucu bir rolü olduğunu işaret ediyor olabilir. Bu sebeple, IL-8 sinyal yolağının bloke edilmesinin HSK terapisi için umut verici bir yaklaşım olduğu belirtilmiştir.

Bu tezde ayrıca, VEGFR2 tirozin kinaz (TK) inhibitörlerinin ve quinoidlerin karaciğer kanseri hücreleri ile beraber KKKH'lerini de inhibe edebilme potansiyelleri ile ilgili çalışmalara odaklanılmıştır. Sorafenib gibi diğer VEGFR TK inhibitörleri çoğu kanser çeşidinin tedavisi için yaygın olarak çalışılmaktadır. Fakat, daha önce de belirtildiği üzere, klinik çalışmalar anti-VEGF veya anti-VEGFR terapilerinin farklı kanser türlerinde hastalığı stabile edip daha sonra ilerlemesine sebep olduğu bulguları içermektedir. Son yıllarda anti-anjiyojenik ajanların tümörde hipoksi oluşturarak kanser kök hücre popülasyonunu arttırdığı gösterilmiştir. Buna karşılık olarak, quinoidlerin hipoksik ortama duyarlı ve seçici aktif bileşiklerdir. Bu sebeple, bu çalışmadaki temel amacımız, bu iki grup bileşiklerin HSK hücreleri üzerindeki biyoaktivitelerini belirlemek

ve aynı zamanda KKKH'lerinin zenginleşmesi üzerindeki etkilerini analiz etmektir. Sonuçlar göstermiştir ki, VEGFR2 TK inhibitörleri quinoidlere göre çok daha düşük konsantrasyonlarda sitotoksik etkilerini göstermektedir. Buna rağmen, VEGFR2 TK inhibitörlerinin KKKH hücrelerini zenginleştirdiği, quinoidlerin bazılarının ise bu oranı azaltabildiği gösterilmiştir. Bu veriler ile, bir bileşiğin daha agresif bir kansere sebep olma potansiyelini tanımlayan ve “agresiflik faktörü” adı verilen yeni bir kavram ortaya konulmuştur.

Tezin son bölümünde ise pirazolik kalkon türevlerinin HSK hücre hatları üzerindeki biyoaktiviteleri ve etki mekanizmaları araştırılmıştır. Kalkonlar ve pirazolik yapılar anti-kanser özellikleri ile bilinen bileşikleridir. Yeni sentezlenmiş 41 adet pirazolik kalkon türevleri ilk önce farklı kanser hücreleri üzerinde test edilmiş, ve aralarından IC₅₀ değerlerine bağlı olarak seçim yapılarak, bileşiklerin etkileri HSK hücre paneli üzerinde test edilmiştir. Sonuçlar, **39**, **42**, **49** ve **52** bileşiklerinin 5 µM dan küçük konsantrasyonlarda anti-proliferatif etkilerini göstermesi ile en etkili türevler olduğunu göstermiştir. Hücre döngüsü ve hücre ölümü mekanizmalarının araştırılması ile **42** ve **52** bileşiklerinin G2/M fazında areste ve apoptotik hücre ölümüne sebep olduğu ortaya çıkmıştır. Buna ek olarak, bileşikler iler muamele sonucunda p21, CDK1 ve fosforile-CyclinB1 gibi hücre döngüsünde yer alan proteinlerin seviyelerinin azaldığı gösterilmiştir.

Anahtar Kelimeler: Hepatoselüler karsinom, PI3K/Akt/mTOR sinyal yolağı, Sorafenib, karaciğer kanseri kök hücresi, ilaç direnci, VEGFR2 TK inhibitörleri, quinoid, pirazolik kalkon türevleri, biyoaktivite, hücre döngüsü, apoptoz



To my precious family...

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Abbreviations

ATP	Adenosine triphosphate
ALDH	Aldehyde dehydrogenase
ATRA	All-trans retinoic acid
ANXA3	Annexin A3
ABC	ATP binding cassette
ABCB5	ATP binding cassette B5
bp	Base pair
BMI1	B-cell-specific Moloney murine leukemia virus integration site 1
BSA	Bovine serum albumin
CSC	Cancer stem cell
CI	Cell index
CXCL1	Chemokine (C-X-C) ligand 1
JNK	c-Jun NH2-terminal Kinase
CDS	Coding sequence
cDNA	Complementary DNA
CTGF	Connective Tissue Growth Factor
CDK	Cyclin-Dependent Kinase
Cq	Quantitative cycle
qRT-PCR	Quantitative Reverse Transcription PCR
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DMSO	Dimethyl Sulphoxide
DTT	Dithiothreitol
ddH ₂ O	Double-distilled water
DOX	Doxorubicin
DMEM	Dulbecco's modified Eagle's medium
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
EMT	Epithelial-mesenchymal transition
EDTA	Ethylenediaminetetraacetic Acid
ECM	Extracellular matrix
FBS	Fetal bovine serum

FGF	Fibroblast growth factor
FLNA	Filamin A
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
GSH	Glutathione
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
g	Gram
HPC	Hepatic progenitor cell
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HNF4 α	Hepatocyte Nuclear Factor 4 alpha
HIF-1 α	Hypoxia-inducible factor 1 alpha
IgG	Immunoglobulin G
IC ₅₀	Inhibitory Concentration 50
ICAM-1	Intercellular Adhesion Molecule 1
IL	Interleukin
IL-6	Interleukin 6
IL-8	Interleukin 8
JAK-STAT	Janus Kinase/Signal Transducer and Activator of Transcription
kDa	Kilo Dalton
KEGG	Kyoto encyclopedia of genes and genomes
LRCC	Label-retaining cancer cells
LCSC	Liver cancer stem cell
LPC	Liver progenitor cell
MACS	Magnetic-activated cells sorting
mTOR	Mammalian target of rapamycin
T _m	Melting temperature
mRNA	Messenger RNA
μ g	Microgram
μ l	Microliter
miRNA	MicroRNA
mg	Milligram
MEM	Minimum essential medium
MAPK	Mitogen-activated protein kinase
MDR1	Multidrug resistance
nm	Nanometer
NEAA	Non-essential amino acid

NF- κ B	Nuclear factor- κ B
Oligo(dt)	Oligodeoxythymidylic Acid
OSM	Oncostatin M
OD	Optical density
OV6	Oval cell marker 6
PTEN	Phosphatase and tensin homolog
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween-20
PI3K	Phosphatidylinositol 3-kinase
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PARP	Poly ADP-ribosyl polymerase
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
PROM1	Prominin 1
ROS	Reactive oxygen species
RT-CES	Real-time cell electronic sensing
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
S6 kinase	S6 kinase
SHP-1	SH2-containing protein tyrosine phosphatase-1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRB	Sulforhodamine B
THY1	Thymus cell antigen 1
TZ	Time zero
TGF- β	Transforming growth factor beta
TCA	Trichloroacetic acid
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween-20
TBE	Tris-borate-EDTA
TAP	Tumor-activated prodrug
TME	Tumor microenvironment
TKI	Tyrosine kinase inhibitor
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A

VEGFR

Vascular endothelial growth factor receptor

4EBP1

4E-Binding Protein 1

5-FU

5-fluorouracil





Chapter 1

Introduction

1.1. Hepatocellular carcinoma

Liver cancer is one of the leading causes of cancer-related deaths worldwide, with 782,000 new cases and 746,000 deaths reported in 2012 [1]. The prognosis for liver cancer is very poor (overall ratio of mortality to the incidence of 0.95) [2]. Liver cancer has several subtypes, but hepatocellular carcinoma (HCC) is the major histological subtype, accounting for 78% of all the liver cancer cases [3]. The etiological factors associated with HCC are chronic infection by Hepatitis B virus (HBV), Hepatitis C virus (HCV), exposure to aflatoxin, excessive alcohol intake, and obesity [4, 5]. HCC development involves several steps, where cirrhosis occurs as a consequence of hepatic

injury, which is followed by continuous cycles of hepatocyte regeneration and cell death. As a result of dysplastic nodule expansion, telomerase reactivation, and genomic instability, cancer occurs.

Depending on the tumor stage, patients with HCC have multiple treatment options such as curative resection, liver transplantation and systemic targeted therapy with Sorafenib (Nexavar, BAY43-9006) or Regorafenib. When transplantation is not an option or the tumor is in advanced stage, the latter is preferred. Sorafenib and Regorafenib is an oral multi-kinase inhibitor approved by FDA which has both anti-proliferative and anti-angiogenic effects on liver cancer cells [6, 7] by inhibiting Raf, VEGFR, and PDGFR kinases (Figure 1.1). Nevertheless, these drugs can prolong patient survival for 2-3 months [8]. Also, the heterogeneous structure of HCC leads to primary or acquired resistance to these therapies. Therefore, discovery of alternative therapies targeting resistance-associated pathways is necessary for effective treatment of HCC.

Multi-kinase inhibition by Sorafenib

Multi-kinase inhibition (X) upstream and downstream in tumor proliferation and angiogenic pathways serves to block tumor growth.

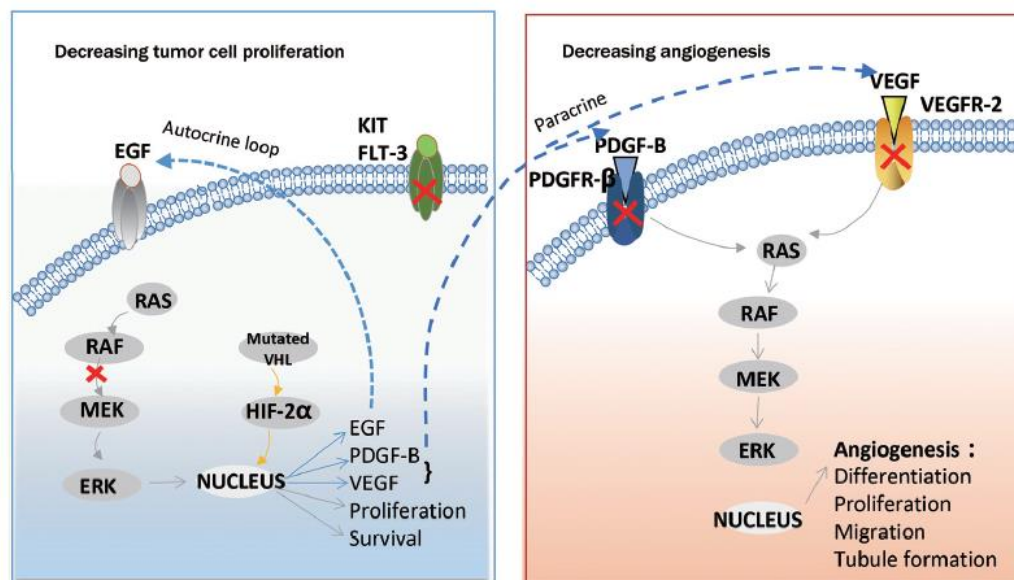


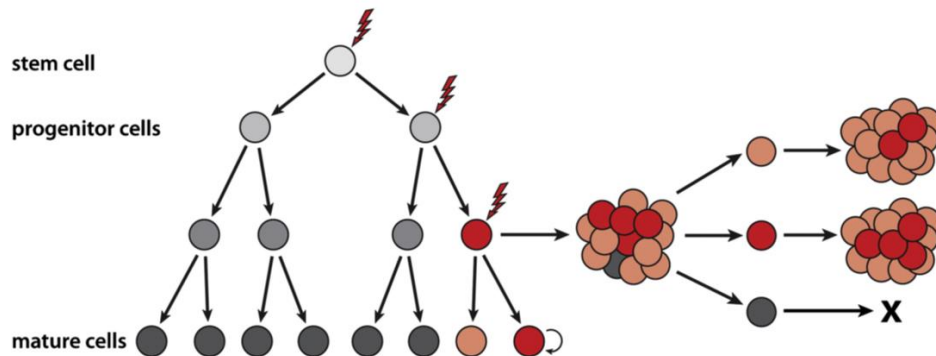
Figure 1.1. Inhibition of multiple targets by Sorafenib. Sorafenib inhibits VEGFR, PDGFR, c-Kit and RET receptor tyrosine kinase signaling and also the downstream

Raf/MEK/ERK pathway to exhibit anti-angiogenic, anti-proliferative effects.
(CR#4239001150971)

1.2. Tumor heterogeneity and Cancer stem cells

Tumor heterogeneity has been defined as the existence of subpopulations of cells within a tumor (intra-tumor) or between tumors of the same subtype (inter-tumor) with different genotypes and phenotypes that acquire distinct biological roles [9, 10]. Traditionally explained, tumor heterogeneity arises from clonal evolution of tumor cells caused by continuous accumulation of genetic/epigenetic changes, or the changes in the tumor microenvironment (clonal evolution model). However, recently another description is made, so-called the “**cancer stem cell hypothesis**”, where tumor heterogeneity is created by a hierarchical organization of tumor cells by stem/progenitor-like cells, known as cancer stem cells (CSCs) [11, 12]. (Figure 1.2) The cancer stem cell model has drawn more attention since resistance to both radiation and chemotherapy, and tumor relapse could be explained reasonably by this theory [13-15]. CSCs are also known as tumor-initiating cells, since they have the potential to self-renew indefinitely, similar to normal stem cells. These cells are highly tumorigenic, metastatic, and resistant to chemotherapy and radiotherapy. Deregulation of the common pathways shared with normal stem cells, such as Wnt, Notch, Hedgehog and advanced DNA repair mechanisms play a vital role in the progression of tumorigenesis. Thus identification of CSC markers and introducing treatment strategies that target CSCs are of vital importance.

A Clonal evolution model



B Cancer stem cell model

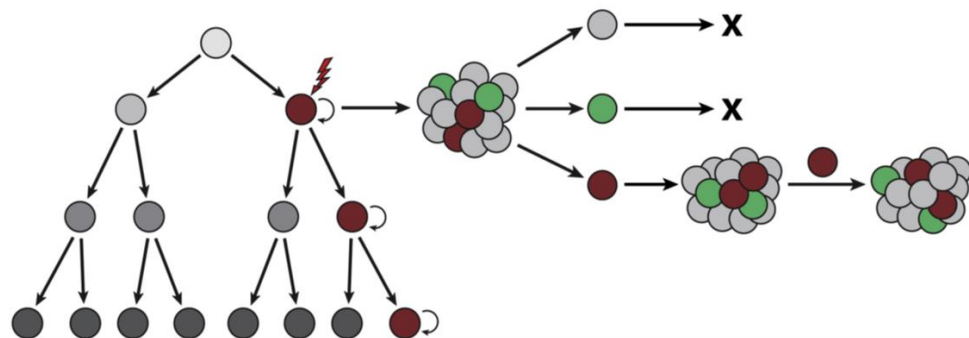


Figure 1.2. Schematic representation of “clonal evolution” and “cancer stem cell” models. A. Clonal evolution model, where mutations cause selective growth advantage (red cell) and tumor cells that arise from this clone have the same tumorigenicity, whereas other cells (grey) are not capable of forming tumors randomly. B. The cancer stem cell model is based on a hierarchical organization of cells. A small sub-population of cells (brown cell) is able to form a new tumor (brown and grey cells) and their differentiation (green cells) leads to the heterogeneity of the tumor.

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1.2.1. Identification of cancer stem cells

There are multiple ways to detect and isolate cancer stem cells from a heterogeneous population of cancer cells. One of them is the expression of specific cell surface markers on CSC population of most of the solid tumors involving breast, lung, colon, prostate, ovary, and liver. These are; CD133 (PROM1), CD44, CD24, EpCAM (epithelial cell adhesion molecule), THY1 and ATP binding cassette B5 (ABCB5) as well as Hoechst 33342 exclusion [16]. For the isolation of CSCs, sorting strategies using fluorescence-

activated cell sorting (FACS) which is capable of sorting via fluorochromes in immunofluorescence staining, and magnetic-activated cell sorting (MACS) which is based on super-para-magnetic and biodegradable microbeads attached to a specific antibody against a CSC marker that allows enrichment of CSCs by running the cells through a column placed in a strong magnetic field, are widely used. Another method is using Aldehyde dehydrogenase (ALDH) which is an intra-cellular marker that is used to isolate CSCs by flow cytometry via the Aldefluor method. Also, Hoechst 33342 dye staining method, which is based on the exclusion of the dye by the ABC transporters of CSCs detected by flow cytometry, is used commonly [17]. Advantages and disadvantages of each method are described in Table 1.1.

Table 1.1. Advantages and disadvantages of common CSC isolation methods.

Isolation	Identification	Advantages	Disadvantages
FACS	CSC specific markers	Multiparameter separation	Low viability of recovered cells-high cost-complexity of equipment-cell suspension solutions only
	ALDH	Stability	specificity
	Hoechst 33342	No need for specific cellular markers	low specificity-lack of purity-do not reflect the entire stem cells-toxicity of dye
MACS		Fast and easy	Monoparameter separation-cell suspension solutions only

1.2.2. Liver cancer stem cells

HCC is a histologically heterogeneous disease and contains a subpopulation of cells that are called liver cancer stem cells (LCSCs). As mentioned before, early in the development of HCC, long term hepatocyte regeneration and inflammation cause changes in the pathophysiology of the liver such as expansion of stem/progenitor cells expand as a result of impaired hepatocyte replication. Also, alteration of the microenvironment leads to the infiltration of lymphocytes which produce and secrete cytokines (Wnt, FGF, PDGF, VEGF, TGF- β) to activate stromal cells to promote the development of CSCs [18]. Nevertheless, there are several possible suggestions about the origin of LCSCs (Figure

1.3) [19, 20]. The first possibility is the liver stem/progenitor cells which have the ability to self-renew and proliferate to form liver progenitor cells (LPCs) [21]. Malignant transformation of these cells is suggested to cause generation of CSCs through long term TGF- β exposure. Yet, transformed adult hepatocytes, which are able to regenerate during liver damage and differentiate into hepatocytes, are also one of the possible origins of LCSCs [22].

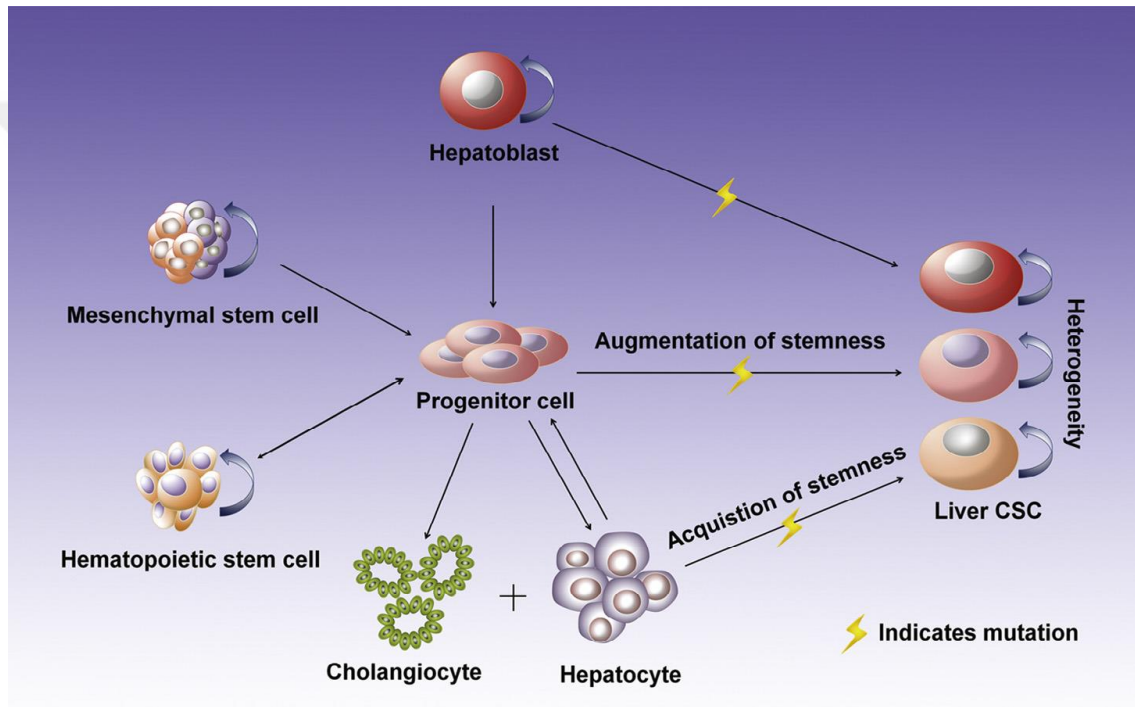


Figure 1.3. Potential origin of LCSCs.
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There are a number of markers to detect LCSCs which are mostly known for their distinct functions and are involved in the maintenance of CSC features (Table 1.2) [18, 23]. These are CD133, EpCAM, CD90, CD44, CD24, CD13 and oval cell marker OV6, as well as Hoechst dye efflux or ALDH activity, that were previously mentioned. Cells that are positive for these markers were shown to have more tumorigenic features than marker-negative cells in immunodeficient mice. For primary HCC, EpCAM, CD90, and CD133 are markers confirmed for the enrichment of CSCs. It has been shown that EpCAM⁺ and CD90⁺ CSCs were different regarding morphology and gene expression. In addition these

two markers were not expressed in primary HCC together, where EpCAM⁺ cells were shown to be associated with high tumorigenic capacity and epithelial stem cell features, whereas CD90⁺ cells had mesenchymal features with high metastatic capacity and activation of c-Kit signaling [24]. EpCAM has been shown to enhance Wnt signaling [25, 26] and CD133 expression maintains stemness through neurotensin/IL-8/CXCL1 signaling [27]. While CD13 takes role in the prevention of cell damage induced by genotoxic reagents [28], CD44 is found to control intracellular level of reduced glutathione (GSH) and regulate ROS defense and tumor growth [29].

Table 1.2. LCSC surface markers and their functions.

Cell surface marker	Function in CSCs	Phenotypes of marker-positive CSCs	Cell lines
CD133	Neurotensin/IL-8/CXCL1 signaling	Tumorigenic, chemoresistant	PLC8024, Huh7, Hep3B, primary HCC
EpCAM	Activation of Wnt signaling	Tumorigenic, invasive, chemoresistant, circulating	Huh1, Huh7, primary HCC
CD44	Regulation of redox status through xCT	Tumorigenic, invasive	PLC, PLC/PRL/5, HLF
CD90	Not reported	Tumorigenic, metastatic, circulating	HepG2, Hep3B, 20, 116, 125, PLC, Huh7, MHCC97L, MHCC97H, primary
CD13	ROS-induced DNA damage reduction	Tumorigenic, cell cycle arrest, chemoresistant	PLC/PRL/5, Huh7, Hep3B
CD24	STAT3-mediated NANOG regulation	Tumorigenic, chemoresistant, metastatic	PLC/PRL/5, HLE, Huh7, primary HCC
DLK1	Not reported	Tumorigenic, chemoresistant	Hep3B, Huh7
OV6	Not reported	Tumorigenic, chemoresistant, invasive, metastatic	Huh7, SMMC7721, primary HCC

Apart from surface markers, the maintenance of CSCs are regulated by different transcription factors, such as Oct4, Sox2, NANOG, c-Myc, Klf4, etc. It has been reported that dysregulation of these factors promotes LCSC propagation and tumorigenesis [30-33].

1.2.2.1. LCSCs for the clinical management of liver cancer

The fact that conventional HCC therapies target differentiated and growing HCC cells, and fails to eliminate CSCs, it has become more important to develop alternative methods to predict patient survival as well as potential therapies that achieve eradication of cancer. At this point, LCSC concept is suggested to contribute and improve HCC treatment greatly.

CSC markers, such as EpCAM has been used for the early diagnosis of lung cancer and to predict patient survival through FDA-approved CellSearch system, which determines the EpCAM+ circulating tumor cells [34]. Similarly, another research has examined CD45-CD90+ cells in blood samples of HCC patients and suggested that the detection of these cells could be a diagnostic method for HCC [35]. Another study has achieved to isolate circulating LCSCs from blood of HCC patients for the diagnosis of HCC. Thus novel diagnostic approaches for HCC might be available which aims to detect circulating hepatoma cells that express LCSC markers.

LCSC existence has been found to correlate with the patient prognosis as well. Research has reported that enhanced cytoplasmic expression of CD133 is a prognostic factor for recurrence of HCC and an indication of poor patient survival especially at the stage III and IVA HCC patients [36]. In addition, high expression of CD90 or OCT4 has been shown to predict a poor prognosis in HCC and that their combination presents higher sensitivity in the prediction of prognosis [37, 38]. CD24, CD44 and ICAM-1 expression have also been reported to correlate with aggressive HCC behavior and poor clinical outcome of patients [39-41]. Apart from the prognostic value of the surface markers, high expression of stemness-associated genes, such as Sox2, Oct4 and NANOG has also been shown to reflect CSC abundance and are suggested to be indicative of poor prognosis in HCC [42, 43]. Altogether, LCSC markers have also been regarded important regarding predicting the prognosis of HCC patients.

Apart from the diagnostic and prognostic potential of LCSCs in HCC, novel therapeutic strategies that aim to eradicate these cells have also been explored by many studies (Figure 1.4) [44, 45]. These strategies include ablation of cell surface markers, blockade of CSC related pathways, and disruption of the microenvironment, self-protection such as anti-immune evasion therapies.

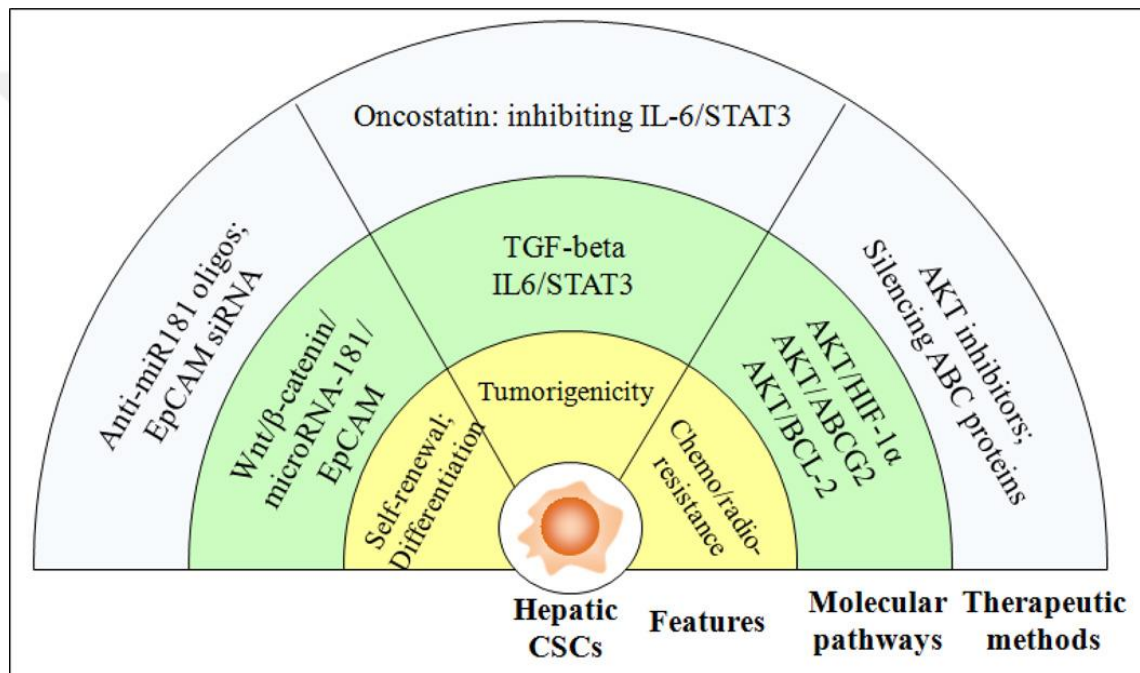


Figure 1.4. Features of LCSCs, deregulated pathways and targeting strategies.
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Studies have revealed that directly targeting the LCSC surface markers such as CD133, CD90, EpCAM, CD44 through the administration of a marker-specific antibody conjugated with a cytotoxic drug or RNAi mediated silencing could potentially inhibit hepatoma cells from propagating and even increased the sensitivity of HCC cells to Sorafenib [46-49].

Another strategy which aims to block the stemness-related pathways, involve several alternatives such as anti-self-renewal, anti-tumor growth, and induction of cell differentiation.

A genome-wide screening study has identified a microRNA (miRNA-181) which directly targets the Wnt/ β -catenin signaling in EpCAM⁺ LCSCs and repress expression of stemness-related genes. Another study has demonstrated that targeting BMI1 with small molecule inhibitors could impair cell viability, reduce migration and sensitized HCC cells to 5-fluorouracil (5-FU) [50, 51]. Apart from impairing self-renewal, many studies are focusing on inhibiting the growth of LCSCs. For instance, targeting PTEN [52], TGF- β family proteins such as IL-6 [53, 54], have been shown to be potential therapeutic strategies for treatment of HCC. In addition, studies inducing differentiation of LCSCs in combination with conventional chemotherapy is suggested to be effective for HCC treatment. For instance, all-trans retinoic acid (ATRA) is found to induce differentiation of EpCAM⁺ LCSCs which was shown by a reduction in the CSC marker expression. In addition, combination of ATRA and cisplatin were shown to have a better therapeutic effect than cisplatin alone [55]. Another study introduced nuclear factor-4 α (HNF4 α), which is a regulator of hepatocyte differentiation and maintenance of hepatic function, to induce differentiation of CD133⁺/OV6 LCSCs [56, 57]. Oncostatin M (OSM), which is a cytokine that activates STAT3 and induces differentiation of hepatoblasts, was shown to differentiate EpCAM⁺ LCSCs. In addition, the combination of OSM and 5-FU was shown to eliminate both LCSCs and non-CSC cells synergistically [58].

Chemoresistance of LCSCs is another issue confronted in clinics which is mainly caused by the quiescent characteristics of LCSCs and their drug efflux ability. Therefore, many studies have identified potential targets to overcome this problem. One of them is the Akt/PKB signaling, which was found to be required for the CD133⁺ LCSCs. Upon inhibition of Akt, the chemoresistance of LCSCs was reported to be eliminated and cells became sensitive to DOX and 5-FU treatment [59]. Lupeol, which is a chemical (triterpene) found in fruits and vegetables, was found to inhibit PTEN/Akt/ABCG2

signaling which leads to suppression of self-renewal mechanisms of CD133+ LCSCs and sensitized them to DOX and Cisplatin treatment [60]. Another study revealed that silencing β -catenin signaling sensitized LCSCs to cisplatin [61].

Targeting the LCSC niche is another strategy which does not involve direct targeting of LCSCs, but effects the stem cell niche factors. For instance, blocking Akt/HIF-1 α /PDGF-BB autocrine signaling was shown to weaken the chemoresistance of LCSCs and liver cancer cells under hypoxic condition [62]. On the other hand, anti-angiogenic therapy including VEGF or VEGFR inhibitors (Vatalanib and Cediranib), and the monoclonal antibody Bevacizumab, and multikinase inhibitors (Sorafenib, Sunitinib, Brivanib, and Linifanib) are found to suppress formation of tumor vasculature and effect self-renewal capacity of CSCs [63, 64]. Yet, their effects on LCSCs remains unclear.

Immunotherapy for CSCs has been an attractive topic for many researchers in recent years. Indeed, there have been studies conducted for glioblastoma [65], ovarian cancer [66], colorectal cancer [67]. A targeted immunotherapy study regarding HCC has revealed that Annexin A3 (ANXA3) which was found to be expressed in LCSCs, once transfected into dendritic cells, caused activation of T cells and killed LCSCs [68, 69]. Another recent study, which involves targeting of CD47 through a specific antibody, revealed that inhibition of CD47 increases phagocytosis of HCC cells by macrophages and suppresses tumor growth in xenograft models of HCC [70]. Therefore, it is suggested that immunotherapy might be an effective option for the eradication of LCSCs.

1.3. Sorafenib resistance

Sorafenib and Regorafenib are the only drugs used to treat advanced HCC which are approved by FDA, and they are effective in prolonging median survival time of HCC patients. Yet, recent studies have revealed that Sorafenib may cause primary resistance or acquired resistance in some patients (Figure 1.5) [71].

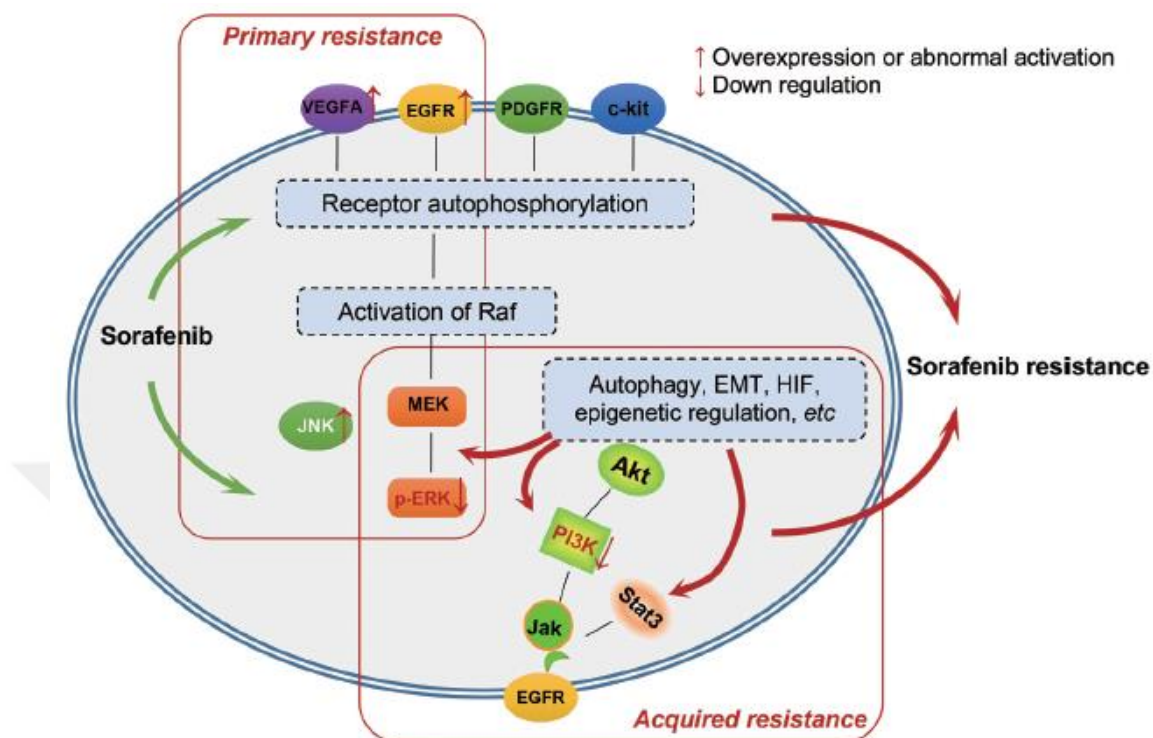


Figure 1.5. Primary and acquired resistance mechanisms to Sorafenib in liver cancer.

(CR#4239001150971)

1.3.1. Primary Sorafenib resistance

Primary resistance to Sorafenib is caused by genetic heterogeneity of HCC where some HCC cells and patients are initially resistant to Sorafenib. Although the exact mechanism that causes primary resistance remains unclear, several explanations may help to understand the mechanism. One of them is the evidence that nearly half of the HCC patients have over-expression or abnormal activation of EGFR or its ligand [72]. Other than EGFR signaling, the Ras/Raf/MEK/ERK pathway might be important for Sorafenib resistance. It is well known that in HCC this pathway is activated and the MAPK levels affect the sensitivity to treatment. It is reported that down-regulation of pERK might be causing Sorafenib resistance [73]. In addition c-Jun N-terminal kinase (JNK), which is also a member of MAPK family, was reported be used as a marker in the prediction of sensitivity Sorafenib treatment [74]. Another candidate biomarker that is suggested by

another study is amplification of VEGFA, which provides a distinct sensitivity to Sorafenib in patients [75].

1.3.2. Acquired Sorafenib resistance

Although Sorafenib is effective in blocking multiple cellular signaling pathways, it has been reported by many studies that it also activates the compensatory pathways and addiction switches at the same time or sequentially in HCC patients. The pathways and mechanisms involved in acquired resistance to Sorafenib are; PI3K/Akt/mTOR and JAK-STAT pathways, epithelial-mesenchymal transition (EMT), hypoxic microenvironment and cancer stem cells. [71, 76]

1.3.2.1. PI3K/Akt/mTOR pathway and Sorafenib resistance

PI3K/Akt and MAPK pathways which are involved in cancer progression and development of HCC are activated or overexpressed in most of the HCC patients. Although Sorafenib targets the MAPK pathway, PI3K/Akt pathway remains unaffected. Thus, PI3K/Akt signaling pathway acts as a compensatory pathway and causes Sorafenib resistance. It has been reported that Sorafenib activates Akt and causes upregulation of its downstream components such as S6K and 4EBP1 in HCC cells. Thus it is suggested that inhibition of Akt may be effective in sensitizing HCC cells to Sorafenib and induce apoptotic cell death [77-79].

1.3.2.2. JAK-STAT pathway and Sorafenib resistance

JAK/STAT pathway is also involved in the regulation of cell proliferation, differentiation, survival, and apoptosis in liver. It has been found that STAT3 is activated in HCC and

that its knockdown resulted in improved therapeutic results on HCC [80]. The activation of SHP-1 by Sorafenib treatment has been reported to inhibit phosphorylation of STAT3 and inhibit JAK/STAT pathway. However in cells that acquire Sorafenib resistance the expression of phosphorylated STAT3 has been shown to increase, which further cause a decrease in the expression of SHP-1 and pSHP-1 levels. Thus it is suggested that abnormal activation of STAT3 might play a role in acquired Sorafenib resistance.[81]

1.3.2.3. Epithelial-mesenchymal transition and Sorafenib resistance

Epithelial-mesenchymal transition (EMT) is the transformation of epithelial cells to a mesenchymal phenotype which is well known as a vital event involved in embryonic development and wound healing. It causes cells to acquire migratory and invasive properties and maintains stemness and causes immunosuppression [82]. It also participates in the progression and invasion of many cancer types including HCC [83, 84]. During EMT, downregulation of E-cadherin and cytokeratins together with the cell-cell adhesion molecules are observed, whereas the transcriptional repressors of E-cadherin (Snail, Twist, and Zeb) are upregulated. These events cause transformation to mesenchymal phenotype, which is reported to be the resistant state of cells to Sorafenib treatment due to upregulation of upstream PI3K/Akt and MAPK pathways. However, there are multiple pathways that regulate, induce and reverse EMT which contribute to the resistance of HCC to Sorafenib (Table 1.3) [85].

Table 1.3. EMT and molecular events involved in the development of Sorafenib resistance in HCC. “-“ stands for negative correlation with Sorafenib efficacy.

Pathways	Molecules	Regulates/induces	Sorafenib efficacy
Wnt	Frizzled, GSK-3 β , β -catenin, TCF	Tumor cell proliferation, metastasis, EMT, alteration of microenvironment	-
TGF β	Smad, TIP30	Cell proliferation, differentiation, apoptosis, EMT, tumor invasion, metastasis	-
RTK-Ras	Raf, MEK, ERK	Tumor microenvironment, tumor development and progression	-
RTK-PI3K	Akt, mTOR	EMT, motility and metastasis	-
Hedgehog	Gli, PTCH1, Snail/Slug	Cancer cell proliferation, metastasis, EMT, stromal fibrosis	-
TNF α	I κ B, NF- κ B	EMT, metastasis, invasion, Twist expression $\uparrow$	-
IL-1 β	IRAK, TRAF, NF- κ B	Tumor cell migration, invasion, angiogenesis, tumor growth, tumor progression, inflammation, EMT	-
IL-6	HSP27, STAT3	Proliferation, EMT, metastasis, inflammation	-
Notch	Snail, Slug, Jagged1	Snail $\uparrow$, Slug, EMT, E-cadherin $\downarrow$, tumor cell migration, invasion, chemoresistance $\uparrow$	-

1.3.2.4. Hypoxic microenvironment and Sorafenib resistance

For the development of tumors, tumor microenvironment (TME) plays a critical role since it is composed of different type of cells and structures including blood vessels, the extracellular matrix (ECM), normal cells (stromal, fibroblasts, immune cells, pericytes, and adipocytes) and signaling molecules [86]. Unfortunately, anti-angiogenic drugs cause contraction of the tumor blood vessels which reduces the blood flow and the oxygen levels. This is called tumor hypoxia, which is closely related with resistance to many anti-cancer drugs including Doxorubicin in HCC [87, 88] since the survival of hypoxic cells

inside solid tumors has increased survival abilities due to their adaptive responses to hypoxia (regulation of genes involved in glucose uptake, metabolism, and proliferation). Sorafenib was found to downregulate the synthesis of hypoxia-inducible factor (HIF)-1 α in HCC cells [89]. However, studies suggested that the adaptive responses may cause expansion of drug-resistant cells and lead to activation of multidrug resistance 1 (MDR1) gene activation, which encodes for P-glycoprotein, further decreasing the concentration of Sorafenib [90]. In addition, while Sorafenib inhibits HIF-1 α synthesis (upstream inducer of VEGF), it causes a hypoxic response switch that relies on the HIF-2 α pathways which activate TGF- α /EGFR pathway and causes resistance of HCC cells [91].

1.3.2.5. Liver cancer stem cells and Sorafenib resistance

As mentioned previously, LCSCs are capable of proliferating, differentiating and are highly resistant to drugs. Acquired resistance to Sorafenib can be explained by the capacity of differentiation of LCSCs into a new tumor by remaining undamaged from Sorafenib treatment and cause cancer recurrence (Figure 1.6) [85]. This was first demonstrated by a study which describes a subset of cancer cells named “label-retaining cancer cells (LRCCs),” which are highly enriched in HCC cells after Sorafenib treatment and displayed more resistance to toxicity of Sorafenib treatment by activating ERK or Akt signaling [92].

Altogether, the mechanisms discussed are essential in overcoming Sorafenib resistance and in designing alternative therapies for HCC patients.

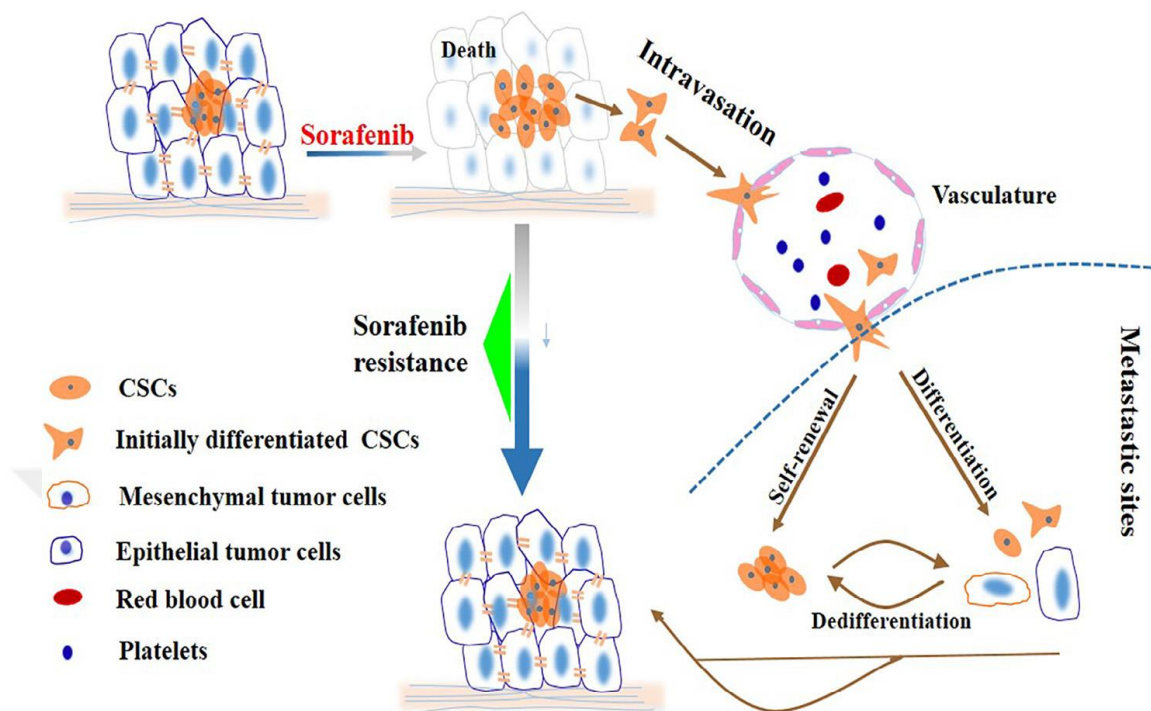


Figure 1.6. Role of liver cancer stem cells in the development of Sorafenib resistance. Upon treatment with Sorafenib, most of the tumor cells are killed, and LCSCs remain. These cells are able to reconstitute the tumor on their own by differentiation leading to tumor relapse and even more malignant tumors. Another mechanism involves the migration LCSCs into blood vessels and metastasis into distal sites to differentiate and form a tumor with acquired resistance to Sorafenib.
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1.4. Quinones and cancer therapy

Quinones are a class of cyclic organic compounds that are derived from aromatic compounds and are known for their anti-tumoral activity [93]. They are used as tumor-activated prodrugs (TAP) to selectively kill tumor cells by getting activated in tumor tissue [94, 95] (Figure 1.7). In fact, mitomycin C is the most popular quinone-based hypoxia-activated prodrug [96]. Since it shows only a modest selectivity to hypoxia, the analog porfiromycin was shown to have higher selectivity. These prodrugs are activated by one-electron reduction and transform into DNA-crosslinking agents that form guanine-guanine crosslinks in the major groove [97]. In fact, a clinical study of porfiromycin in

combination with radiation therapy was reported to be useful in therapy of head-and-neck cancer [98].

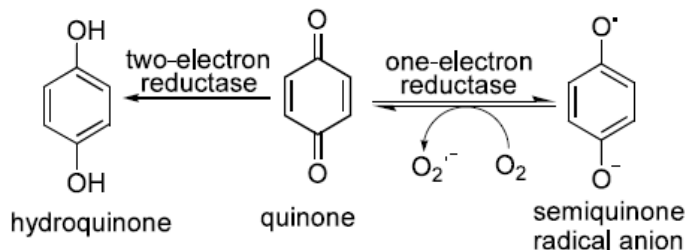


Figure 1.7. Quinone metabolism. Quinones undergo one-electron reduction by cytochrome C P-450 reductase to the semiquinone radical anion which can be oxidized back in normal cells.

In terms of HCC, several studies are emphasizing the potency of quinone derivatives as anti-tumor drugs. For instance, plumbagin, a medicinal plant-derived naphthoquinone, was shown to restrain angiogenesis-mediated tumor growth in HCC through inhibition of PI3K-Akt, VEGF/KDR, and Angiopoietins/Tie2 pathways together with angiogenic factors such as VEGF, bFGFi and CTGF [99]. However, it has been reported that quinones and quinone-like compounds are mainly organotoxic and that minor changes in the side chains of their structure cause variation in the toxicity of the compounds [100, 101]. Therefore, it is critical to model quinoid derivatives that cause minimum possible toxicity and highest inhibitory effect on cancer cells. In addition, the effects of quinones and quinone-containing alkylating agents on cancer stem cells haven't been studied so far.

1.5. Pyrazolic chalcone derivatives as potential anti-cancer drugs

Chalcones are aromatic compounds are highly abundant in many plants such as tea, beer, vegetables and fruits and are reported to be cytotoxic in vitro [102]. They also have anti-inflammatory, analgesic and antipyretic effects and some are shown to have bactericidal,

anti-fungal and insecticidal or antimutagenic activity [103, 104]. Chalcones belong to the flavonoid family which are intermediates in the flavonoid biosynthesis. Their chemical structure which contains a 1, 3-diphenyl, propenone template can be modified, which alters their molecular targets and provides various biological activities. In fact, chalcones having different structural changes have been investigated for their potential in cancer prevention and therapy in many studies (Figure 1.8) [105]. These include chalcones that target the p53 pathway, tubulin polymerization, proteasomal activity, multidrug resistance transporters, or tumor vasculature; modulate cell cycle progression; induce cell death and affect the NF- κ B, androgen, and estrogen receptor pathways.

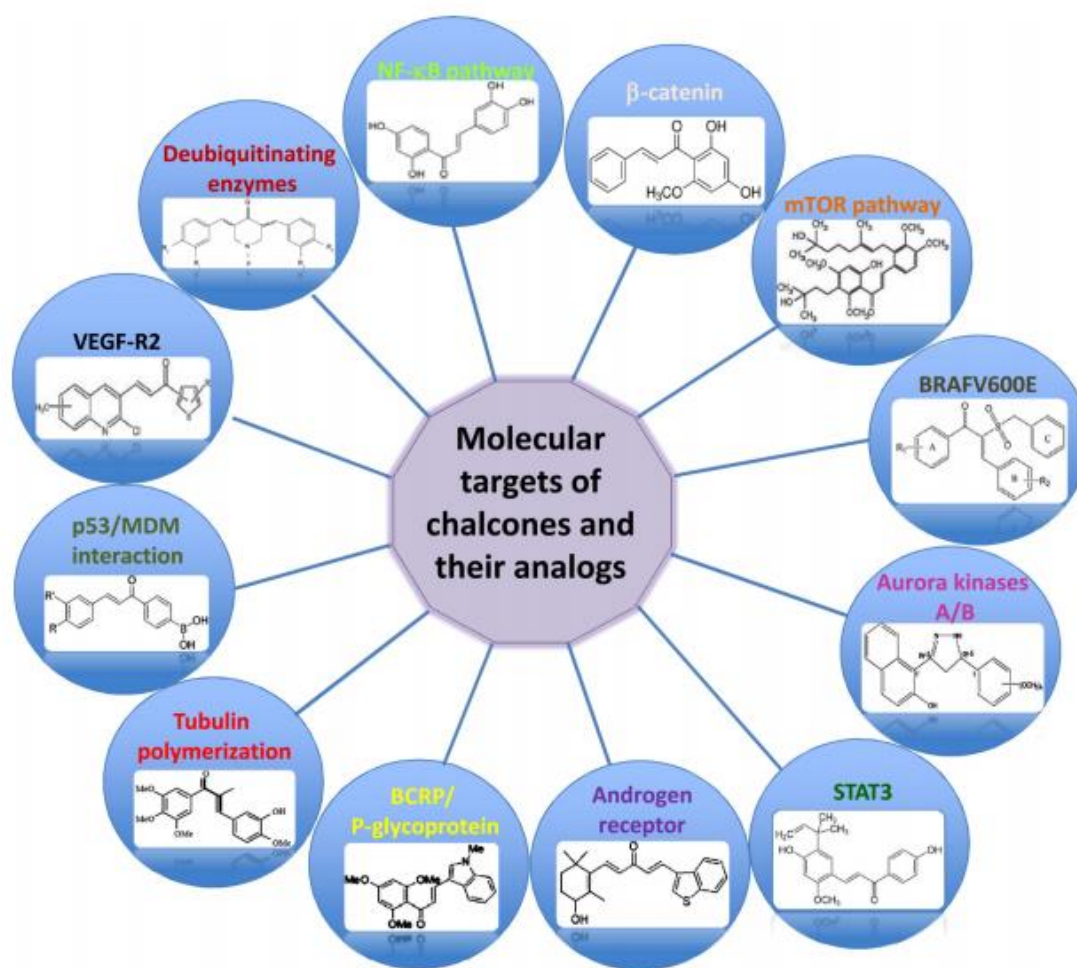


Figure 1.8. Schematic representation of molecular targets of chalcones and their analogs.

(CR#4239010996428)

On the other hand, pyrazolic structures are commonly known for their analgesic, antipyretic and anti-inflammatory actions, they have also been shown to possess a wide range of anti-proliferative, and anti-leukemic activities [106-112]. However, the selectivity of these structures is highly dependent on the type of peripheral substituents of the pyrazole skeleton.

Thus synthesis and determining the antiproliferative effect of novel pyrazolic chalcone derivatives have been under investigation for different cancer types including leukemia, lung, colon and liver cancer. [113, 114]





Chapter 2

Objectives and Rationale

Hepatocellular carcinoma has a very heterogeneous nature, and the onset of cancer progression involves viral infections of the liver by HBV and HCV, exposure to chemicals, alcohol intake and obesity. Upon liver injury, hepatic progenitor cells (HPCs) are damaged and transformed into cells that carry stem cell features, so-called liver cancer stem cells (LCSCs). Known as one of the most important factors that cause chemoresistance, tumor relapse and metastasis, LCSCs have also been reported to be involved in Sorafenib resistance, and they can be identified by their positivity for the cancer stem cell markers (CD133, CD90, EpCAM, CD44, etc.). Therefore, discovery of novel

therapies targeting both tumor cells and liver cancer stem cells is of vital importance. For this purpose, the first and the second part of this thesis comprehensively focuses on the investigation of potential targets and inhibitors that affect the LCSC enrichment and possible combination treatment strategies to overcome Sorafenib resistance (Figure 2.1).

Briefly, in the first part, characterization of cancer stem cells of Huh7 and Hep3B cells were done and a panel of PI3K/Akt/mTOR pathway inhibitors together with Sorafenib and clinically used chemotherapeutics (Doxorubicin and Camptothecin) were tested against HCC cell lines and their CD133+/EpCAM+ (LCSCs) populations, to introduce potential small molecule inhibitors that prevent the enrichment of LCSCs. Additionally, several combinatorial treatment strategies using Sorafenib together with selected inhibitors were performed. A panel of cancer pathways related genes were also analyzed to identify genes that may play a role in the conservation of stem cell features.

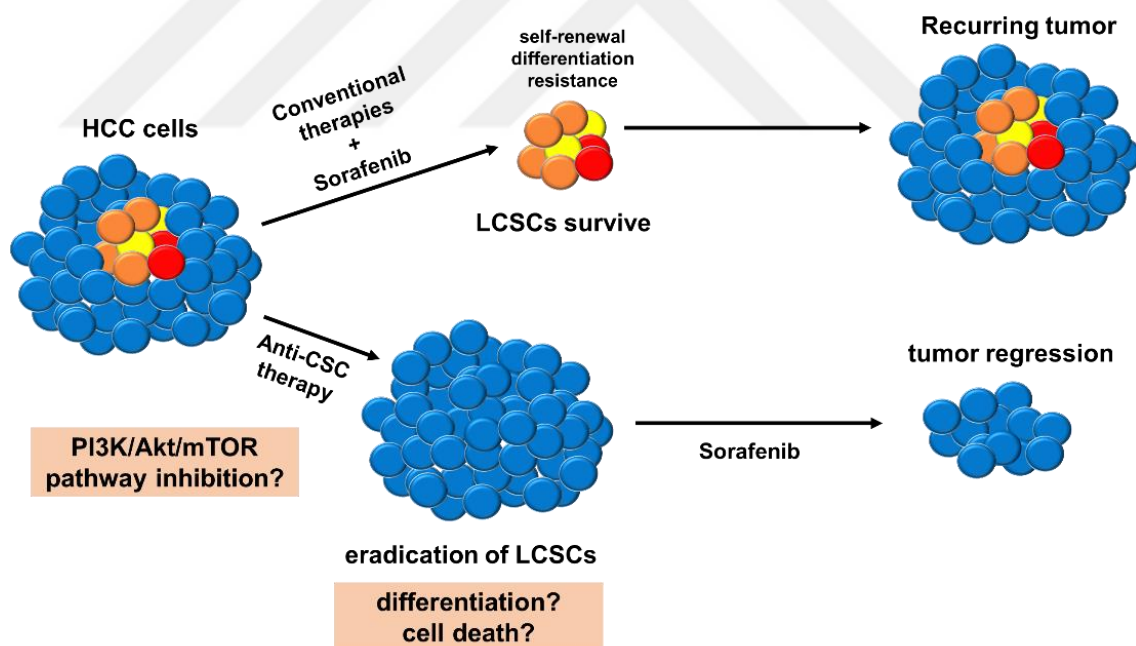


Figure 2.1. Objective of the first part of the study.

The second part of the study focuses on the differential effects of VEGFR2 tyrosine kinase inhibitor derivatives and quinones on liver cancer cells and LCSCs. This part of the study was designed to emphasize the importance of evaluating novel compounds not

only in terms of their cytotoxic potential but also in terms of their ability to eliminate tumor-initiating cells inside the cancer cells. For this reason, compounds that were able to display such effect were accepted to be superior to the compounds that did not fulfill both actions.

The third part of the study focuses on the anti-cancer effects of novel pyrazolic chalcone derivatives on HCC cells and the investigation of underlying molecular mechanisms involved. For this part of the study, initially, all compounds were tested against a panel of cancer cells for their cytotoxic activities. Then, molecules that were potent especially against HCC cells were further tested against a panel of HCC cells. The effects of compounds on cell proliferation, cell cycle and the mechanism of cell death induced were determined. And finally, the affected pathways were analyzed through western blot analysis. Together with all the experimental procedures described, the mechanism by which the pyrazolic chalcone derivatives induce their anti-cancer effect is thereby introduced.



Chapter 3

Materials and Methods

3.1. Materials

3.1.1. Cell culture reagents

All reagents used in cell culture are listed in (Table 3.1) Cell culture petri dishes, plates, cryovials were from Corning Life Sciences Incorporated (USA). Serological pipettes and sealed-cap polycarbonate centrifuge tubes were from Costar Corporation (Cambridge, UK)

Table 3.1. Reagents used in cell culture.

Name	Company	Catalog #	Storage conditions
Dulbecco's modified Eagle's medium (DMEM)	GIBCO, Thermo Fisher Scientific	31885	+4°C
Roswell Park Memorial Institute (RPMI) 1640 medium	GIBCO, Thermo Fisher Scientific	31870	+4°C
DMEM/F12 medium	GIBCO, Thermo Fisher Scientific	21331-02	+4°C
Minimum Essential Medium (MEM)	GIBCO, Thermo Fisher Scientific	10370	+4°C
Penicillin/straptomycin	GIBCO, Thermo Fisher Scientific	15140-122	-20°C
Trypsin-EDTA	GIBCO, Thermo Fisher Scientific	25200	-20°C
Non-essential amino acid (NEAA)	Lonza	BE12-114E	+4°C
Fetal bovine serum (FBS)	GIBCO, Thermo Fisher Scientific	10270	-20°C
L-glutamine	GIBCO, Thermo Fisher Scientific	25030	-20°C
B27 supplement	GIBCO, Thermo Fisher Scientific	10889038	-20°C
Epidermal growth factor (EGF)	Thermo Fisher Scientific	PHG0311	-20°C
Fibroblast growth factor (FGF)	MERCK	F0291	-20°C
Heparin	MERCK	H3149	+4°C
Insulin	MERCK	I-6634	-20°C
Hydrocortisone	MERCK	H0888	-20°C

3.1.2. Kinase inhibitors and DNA intercalators

The kinase inhibitors and DNA intercalators used for the first part of the thesis are listed in Table 3.2. The list involves 10 different inhibitors targeting the PI3K/Akt/mTOR pathway, 1 inhibitor targeting the Notch pathway (DAPT), 1 inhibitor of IL-8 signaling (Reparixin), 2 different DNA intercalators (Doxorubicin and Camptothecin), and Sorafenib, a multikinase inhibitor targeting both VEGFR/PDGFR kinases and RAF/MEK/ERK pathway.

Table 3.2. Inhibitors and DNA intercalators used in the first part of the thesis.

Inhibitor	Target/s	Catalog #
Sorafenib	B-Raf, VEGFR	BAY 54-9085
ZSTK474	PI3K	Selleck chemicals, S1072
PI-103	PI3K, mTOR	Calbiochem, 528100
PI3Ki- α	PI3K- α	Calbiochem, 528116
PI3Ki- β	PI3K- β	Calbiochem, 528113
AKTi-2	Akt2	Calbiochem, 124029
AKTi-1,2	Akt1, Akt2	Calbiochem, 124018
LY294002	PI3K	Calbiochem, 440202
Wortmannin	PI3K	Calbiochem, 681675
Rapamycin	PI3K, mTOR	Calbiochem, 553210
BEZ-235	PI3K, mTOR	Santa Cruz, sc-364429
Camptothecin	DNA intercalator	MERCK, C9911
Doxorubicin	DNA intercalator	SABA, Adrimisin
DAPT	γ -secretase-Notch	Selleck chemicals, S2215
Reparixin	IL-8	Cayman Chemical, CAY17350

3.1.3. Sulforhodamine B (SRB) assay and Real-time cell electronic sensing (RT-CES) reagents

Sulforhodamine B (SRB) sodium salt was from Sigma Aldrich, trichloroacetic acid (TCA) was from MERCK, and Tris was from Amresco. CloneFill Instrument for washing the 96-well plates was from Genetix and the plate reader ELx800 was from Biotek. The Real-time cell analyzer Instrument (single plate) and the E-plates (96-well) were from ACEA Biosciences.

3.1.4. Flow cytometry and antibodies for fluorescence staining

For the flow cytometric analysis BD Accuri™ C6 Cytometer (BD Biosciences) and Novocyte® flow cytometer (ACEA Biosciences) instruments were used. The fluorescence antibodies used are listed in Table 3.3.

Table 3.3. Antibodies for flow cytometry analysis.

Antibody	Cat. no.	Company	Working Dilutions
anti-CD133/1 (AC133)-Biotin	130-090-664	Miltenyi Biotec	1:11 in 5% BSA-1X PBS
anti-CD326 (EpCAM)-FITC	130-080-301	Miltenyi Biotec	1:11 in 5% BSA-1X PBS
anti-CD90-FITC	130-095-403	Miltenyi Biotec	1:11 in 5% BSA-1X PBS
anti-mouse IgG1 isotype control-FITC	130-092-213	Miltenyi Biotec	1:11 in 5% BSA-1X PBS
anti-mouse IgG1 isotype control-Biotin	130-093-018	Miltenyi Biotec	1:11 in 5% BSA-1X PBS
anti-biotin-PE	130-090-756	Miltenyi Biotec	1:11 in 5% BSA-1X PBS
anti-OCT4	2750S	Cell signaling	1:200 in 5% BSA-1X PBS
anti-Nanog	3580S	Cell signaling	1:400 in 5% BSA-1X PBS
anti-mouse-IgG-Alexa 647	ab150083	Abcam	1:200 in 5% BSA-1X PBS

3.1.5. Magnetic cell sorting reagents and materials

All materials used for the magnetic separation of CD133+/EpCAM+ cells are listed in table 3.4.

Table 3.4. Magnetic cell sorting reagents and materials.

Materials and reagents	Cat. no.	Brand
MidiMACS Separator	130-042-302	Miltenyi Biotec
MACS MultiStand	130-042-303	Miltenyi Biotec
LS Separation Columns	130-042-401	Miltenyi Biotec
Anti-biotin Multisort Kit	130-091-256	Miltenyi Biotec
MACS BSA stock solution	130-091-376	Miltenyi Biotec
MACS Separation buffer	130-091-221	Miltenyi Biotec

3.1.6. Nanostring nCounter reagents and instruments

nCounter® Master Kit (NAA-AKIT-012), PanCancer Pathways Panel (XT-CSO-PATH1-12) and the nCounter® FLEX Analysis System (Prep Station and Digital Analyzer) were from nanoString Technologies.

3.1.7. RNA extraction, cDNA synthesis, and polymerase chain reaction (PCR) reagents

RNeasy Mini Kit was from Qiagen (cat.no: 74106). RevertAid First Strand cDNA synthesis kit and PCR reagents; 10X Taq DNA Polymerase Buffer (+ $(\text{NH}_4)_2\text{SO}_4$, - MgCl_2), Taq DNA Polymerase, 2 mM dNTP, 25 mM MgCl_2 , were from Fermentas. DNase I recombinant, RNase-free kit (Roche, cat.no: 04716728001), LightCycler® 480 SYBR Green I Master for quantitative PCR reactions was from Roche Applied Sciences. NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer used for nucleic acid concentration measurements was from Thermo Fisher Scientific. Quantitative PCR was performed using Light Cycler® 96 Real-Time PCR System from Roche Applied Sciences, and for conventional PCR, T100 thermal cycler from Bio-Rad was used.

3.1.8. Oligonucleotides

All primers used in this study were synthesized by Oligomer Biyoteknoloji. The list of primers is given in Table 3.5.

Table 3.5. Primers used in this study.

Genes	Primer Sequences	Amplicon size (bp)
IL-8	F: TCCTGATTTCTGCAGCTCTGT	107
	R: AAATTTGGGGTGGAAAGGTT	
NANOG	F: TACCTCAGCCTCCAGCAGATG	127
	R: TCTTCGGCCAGTTGTTTTCTG	
GAPDH	F: TATGACAACGAATTGGCTAC	115
	R: TCTCTCTTCCTCTTGCTCT	

3.1.9. Agarose gel electrophoresis

Electrophoresis grade agarose was from Sigma Aldrich (A9414). GeneRuler® 50bp DNA ladder was from Fermentas, and EZ-VISION® DNA dye as loading buffer (6x) was from

Amresco. Horizontal electrophoresis equipment and the power supply was from Cleaver Scientific.

3.1.10. Protein gel electrophoresis

Mini-PROTEAN Tetra Cell Systems gel electrophoresis equipment and TGX precast gels, Trans-Blot Turbo Transfer System, 4X Laemmli sample buffer (cat.no: 1610747) were from Bio-Rad. PageRuler Prestained protein ladder (cat. no: 26616) and SuperSignal West Femto Chemiluminescent Substrate (cat. no: PI34095) was from Thermo Fisher Scientific. Phosphatase inhibitor cocktail (PhosStop) and Protease inhibitor cocktail tablet (complete EDTA free) were from Roche Applied Sciences. Bradford reagent was from Sigma Aldrich. Ponceau S was from AppliChem Biochemica. C-Digit® imaging system was from LI-COR Biosciences.

3.1.11. Antibodies for western blot analysis

All primary antibodies used in this study are listed in Table 3.6. Incubation times for each working dilutions indicated are for 2 hours at room temperature.

Table 3.6. Western blot antibody list.

Antibody	Company	Cat. no.	Working dilution
SOX2	Cell signaling	2748S	1:200 in 5%BSA-0.1%TBS-T
NANOG	Cell signaling	3580S	1:200 in 5%BSA-0.1%TBS-T
phospho-Akt (Ser473)	Cell signaling	9271S	1:100 in 5%BSA-0.1%TBS-T
Akt	Cell signaling	9272S	1:300 in 5%BSA-0.1%TBS-T
PARP	Cell signaling	9532S	1:200 in 5%BSA-0.1%TBS-T
Cyclin-B1	BD Biosciences	554177	1:200 in 5%BSA-0.1%TBS-T
phospho-Cyclin-B1 (Ser147)	Bioss Antibodies	bs-3122R	1:200 in 5%BSA-0.1%TBS-T
Cdc2/Cdk1	Calbiochem	PC25	1:200 in 5% BSA 0.1%TBS-T
p21/WAF1/Cip1	Millipore	05-345	1:200 in 5% BSA 0.1%TBS-T
p53	Millipore	05-224	1:200 in 5% BSA 0.1%TBS-T
phospho-p53 (Ser15)	Cell signaling	9286S	1:200 in 5% BSA 0.1%TBS-T
Rb	Santa Cruz	sc-102	1:200 in 5%BSA-0.1%TBS-T
phospho-Rb (Ser807/811)	Cell signaling	9308S	1:200 in 5%BSA-0.1%TBS-T
β -actin	Cell signaling	4967S	1:5000 in 5% milk-0.1% TBS-T

3.1.12. Cell cycle analysis with flow cytometry

MUSE[®] Cell cycle assay kit (cat.no:MCH100106) and MUSE[®] Cell Analyzer was from Millipore.

3.1.13. Immunofluorescence staining reagents

Annexin-V-FLUOS Staining kit (cat. no. 11858777001) was from Roche Applied Sciences, and UltraCruz Mounting Medium For Fluorescence with DAPI (cat. no. SC-24941) was from Santa Cruz. Fluorescence imaging of stained samples was done using Nikon Eclipse 50i microscope.

3.1.14. General reagents

Commonly used chemicals such as ethanol, methanol, and paraformaldehyde used as fixatives were from Sigma Aldrich or Carlo Erba Reagents. Dimethyl sulfoxide (DMSO) (cat.no.A3672) was from AppliChem Biochemica. Tween was from Millipore.

3.2. Solutions and Media

3.2.1. Cell culture solutions

DMEM complete medium:	10% Fetal bovine serum (FBS), 100 units/ml penicillin and streptomycin, 1% non-essential amino acid. Stored at 4°C.
RPMI complete medium:	10% Fetal bovine serum (FBS), 100 units/ml penicillin and streptomycin, 2 mM L-Glutamine. Stored at 4°C.
Sphere formation medium (in DMEM/F12):	1X B27 supplement, 20 ng/ml EGF, 10 ng/ml FGF, 2 µg/ml Heparin, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin. Stored at 4°C.
Freezing medium:	20% FBS, 7% DMSO, 73% complete medium
10X Phosphate buffered saline (PBS):	80 g NaCl, 2 g KCl, 14.4 g Na ₂ HPO ₄ , 2.4 g KH ₂ PO ₄ in 1L ddH ₂ O autoclaved. pH of 10X buffer=6.8. When diluted to 1X working concentration, pH=7.4.

3.2.2. Preparation of inhibitor stocks

All inhibitors (except for Doxorubicin) and compounds used in this study were dissolved in dimethyl sulfoxide (DMSO). Doxorubicin was dissolved in ddH₂O. Stock solutions ranged from 40 mM to 10 mM with respect to their solubility in their solvents and stored at -20°C. Solvents were used as controls for all experiments.

3.2.3. Sulforhodamine B assay solutions

SRB stain solution:	0.04 grams of SRB stain dissolved in 10 ml 1% acetic acid solution.
10% TCA solution:	100% TCA diluted to 10% in cold ddH ₂ O.
10 mM TrisBase:	0.6 grams of Tris dissolved in 500 ml cold ddH ₂ O.

3.2.3. Flow cytometry and immunofluorescence solutions

4% paraformaldehyde:	4 grams of paraformaldehyde dissolved in 100 ml PBS, stirred 4 hours at 58°C, pH= 7.4. Stored at -20°C in dark.
Incubation buffer:	0.5 grams of BSA in 100 ml 1X PBS. Stored at 4°C.
Permeabilization buffer:	ice-cold 90% methanol

3.2.4. Western blotting reagents

NP-40 lysis buffer:	50 mM TrisHCl (pH=8), 150 mM NaCl, 0.1% SDS, 1% NP-40, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail in ddH ₂ O.
10x Tris-buffered saline (TBS):	12.2 grams of Trisma base, 87.8 g NaCl dissolved in 1L of ddH ₂ O. pH=7.6.
1X TBS-Tween (TBS-T)	0.1% Tween-20 in 1X TBS.
Ponceau S:	0.1% (w/v) Ponceau S, 5% (v/v) acetic acid in ddH ₂ O.
Blocking solution:	5% (w/v) non-fat dry milk or bovine serum albumin (BSA) in 0.1% TBS-T

3.3. Methods

3.3.1. Cell culture methods

In this study, a human colon carcinoma cell line (HCT116), a human breast cancer cell line (MCF7), a human breast epithelial cell line (MCF12A), a human fetal lung fibroblast cell line (MRC-5) and 5 different Hepatocellular carcinoma cell lines (Huh7, HepG2, Hep3B, Mahlavu and SNU475) used. HCC cell lines (Huh7, HepG2, and Mahlavu), human breast carcinoma cells (MCF7) and human colon carcinoma cells (HCT116) were grown in complete DMEM, and SNU475 were grown in complete RPMI-1640 media. MCF12A, human breast epithelial cells, were grown in DMEM/F12 media supplemented

with 10% FBS, 1% NEAA, 100 ng/ml EGF, 500 ng/ml hydrocortisone, and 10 µg/ml insulin. MRC-5, human fetal lung fibroblast cells, were maintained in Minimum Essential Medium (MEM) with 10% FBS, 2 mM L-glutamine. All media contained 100 units/ml penicillin and streptomycin and all cells were incubated at 37 °C in a humidified incubator with 5% CO₂.

3.3.1.1. Growth and sub-culturing of cells

All cells were passaged regularly as they reached a confluency of 70-80%. In each passage, the old medium was aspirated, and cells were washed once with PBS. Cells were detached from the surface of the dish by trypsin-EDTA (500 µl for 100 mm dish). As soon as the cells start detaching, they were collected in fresh medium and mixed thoroughly with serological pipettes to make sure the cells were single cell. Cells were then transferred on new culture dishes (1:4-1:10 sub-culturing ratio) with fresh medium and moved back and forth and left to right to make sure the cells were equally distributed. FBS stock solutions were thawed at 4°C and heat-inactivated at 56°C for 30 minutes, then aliquoted into 50 ml falcons to be stored at -20°C. All solutions and media were warmed to 37°C in a water bath before use. In order to seed defined number of cells into cell culture plates/dishes, cells were collected into a falcon tube and mixed well to make sure the cells were homogenously distributed. 10 µl of cells were placed on both sides of a hemocytometer to be counted under the microscope. The number of cells counted in the defined area in both sides were averaged and multiplied by 10⁴ to reach the number of cells in 1 ml of cell suspension. Alternatively, 50 µl of cells were transferred into a 1.5 ml eppendorf tube to be counted by the flow cytometer.

3.3.1.2. Cryopreservation of cells

Cells that reached a confluency of 60-70% and that were exponentially growing, were collected by trypsinization in fresh medium and centrifuged at 15000 rpm for 5 minutes. After the supernatant was discarded, the pellet was resuspended in freezing medium (as previously described) such that 1 ml contained 1×10^6 cells and were transferred into cryovials. Cryovials were put to -20°C for an hour and then kept in -80°C overnight to be placed in liquid nitrogen tanks for long-term storage.

3.3.1.3. Thawing of frozen cells

A frozen cryovial was taken out from the liquid nitrogen tank or -80°C and quickly thawed in a water bath at 37°C . As soon as the cells started to thaw, the suspension was transferred into a 15 ml falcon with 5 ml of fresh culture medium and centrifuged at 15000 rpm for 5 min to remove the DMSO present inside the freezing medium. Once the pellet was resuspended in 10 ml complete medium, cells were mixed well and transferred into culture dishes, distributed evenly and incubated overnight at a 37°C humidified incubator with 5% CO_2 . The next day, cells were checked if they were attached to the surface of the culture dish. To remove the unattached cells from the dish, old medium was aspirated and replaced with fresh complete medium.

3.3.2. Sulforhodamine B (SRB) assay

Cells were seeded into 96-well plates at indicated numbers in Table 3.7 in 150 μl of medium/well.

Table 3.7. Number of cells seeded into 96-well plates for SRB assay.

	Cell line	# of cells/ well (96-well plate)
HCC	Huh7	2000
	HepG2	3000
	Hep3B	3000
	Mahlavu	1000
	SNU475	1000
other	MCF7	2000
	HCT116	3000
	MRC-5	2000
	MCF12A	3000

After 24 hours, the media on top of cells were discarded and replaced with fresh complete media (150µl/well). For the starting concentration desired for each drug, an extra 150 µl of media was added into corresponding wells (total of 300 µl in first wells of the dilution series). Then the desired inhibitor was applied to these wells in triplicates. The amount of inhibitor to be used from the stock solution was calculated using the $M_{stock} * V_{stock} = M_{final} * V_{final}$ formula as follows: 20 mM * 0.6 µl = 40 µM * 300 µl. The volume of starting concentration of the inhibitors was kept constant (0.6 µl) in order to use 1 common DMSO control for 5 different inhibitors. The percentage of DMSO to not exceed 0.2% was critical since higher doses were toxic to cells. 2-fold dilution series were done using a multi-channel pipette adjusted to 150 µl. The dilutions were done such that the highest concentrations were at far ends of the 96-well plate and the lowest concentrations were close to DMSO control wells (Figure 3.1). In cases where the inhibitor was soluble in water, control wells were treated with cell culture grade ddH₂O. Additionally, a time-zero (TZ) plate was prepared at the same day and fixed at the day of treatment in order to detect the initial cell density before drug treatment.

	40µM	20µM	10µM	5µM	2.5µM	DMSO	2.5µM	5µM	10µM	20µM	40µM		
Inhibitor 1	0.6µl					0.6µl	0.6µl					0.6µl	Inhibitor 4
	0.6µl											0.6µl	
	0.6µl											0.6µl	
Inhibitor 2	0.6µl											0.6µl	Inhibitor 5
	0.6µl											0.6µl	
	0.6µl					0.6µl						0.6µl	
Inhibitor 3	0.6µl											0.6µl	Inhibitor 3
	0.6µl											0.6µl	
	0.6µl											0.6µl	

Figure 3.1. Schematic representation of treatment strategy used for SRB assay.

Once the desired incubation time was reached (72h) the media on top of cells were discarded, and cells were washed once with PBS. Then, 50 µl of cold 10% TCA was applied to each well for fixation. After 1 hour at 4°C, TCA was discarded, and wells were washed with ddH₂O at least 4 times, and the plates were air-dried at room temperature. In order to stain the fixed cells with SRB, 50 µl of SRB solution was prepared (as described previously) and applied to each well and incubated for 10 min in dark at room temperature. For the removal of unbound SRB, cells were washed with 1% acetic acid and left for air-drying. Protein-bound SRB was solubilized in 100 µl/well 10 Mm Tris-base and the absorbance of each well was measured at 515 nm using a 96-well plate reader (BioTek). The OD values were used to analyze the effect of each inhibitor on cell proliferation with respect to control group. The formulas used for the calculation of IC₅₀ values for each inhibitor:

$$\% \text{inhibition} = (1 - (\text{AVERAGE}(\text{OD}_{\text{inhibitor}}) / \text{AVERAGE}(\text{OD}_{\text{DMSO}}))) * 100$$

$$y\text{-int} = \text{INDEX}(\text{LINEST}(\text{C\#6:C\#6}, \text{LN}(\text{C\#5: C\#5}), \text{TRUE}, \text{FALSE}), 2)$$

$$\text{slope} = \text{INDEX}(\text{LINEST}(\text{C\#6:C\#6}, \text{LN}(\text{C\#5: C\#5}), \text{TRUE}, \text{TRUE}), 1)$$

$$R^2 = \text{INDEX}(\text{LINEST}(\text{C\#6:C\#6}, \text{LN}(\text{C\#5: C\#5}), \text{TRUE}, \text{TRUE}), 3)$$

$$\text{IC}_{50} = \text{EXP}((50 - y\text{-int}) / \text{slope})$$

(C#: column number)

3.3.3. Real-time cell electronic sensing (RT-CES) system

In order to monitor the cell growth of cells and cytotoxicity of inhibitors RT-CES system was used. Initially, a background reading was done after adding 50 μ l of complete medium to each well of the E-Plate 96. Then cells were seeded into 96-well plates (Huh7: 2000 cell/well, HepG2: 3000 cell/well, Mahlavu and SNU475: 1000 cell/ml) in 100 μ l of medium/well. The proliferation of cells was monitored every 30 minutes for 24 hours. As the cells reach a certain cell index (CI) value (preferentially 0.6-0.8; log phase), the medium was discarded, and 150 μ l of fresh medium was added to each well and cells were treated with indicated concentrations of inhibitors. For the first 24 hours, CI values were recorded every 10 min (short-term drug response) and then every 30 minutes for the remaining 48 hours (long-term drug response). The effect of inhibitors on cell growth was evaluated with respect to DMSO control (CI_{inh}/CI_{DMSO}).

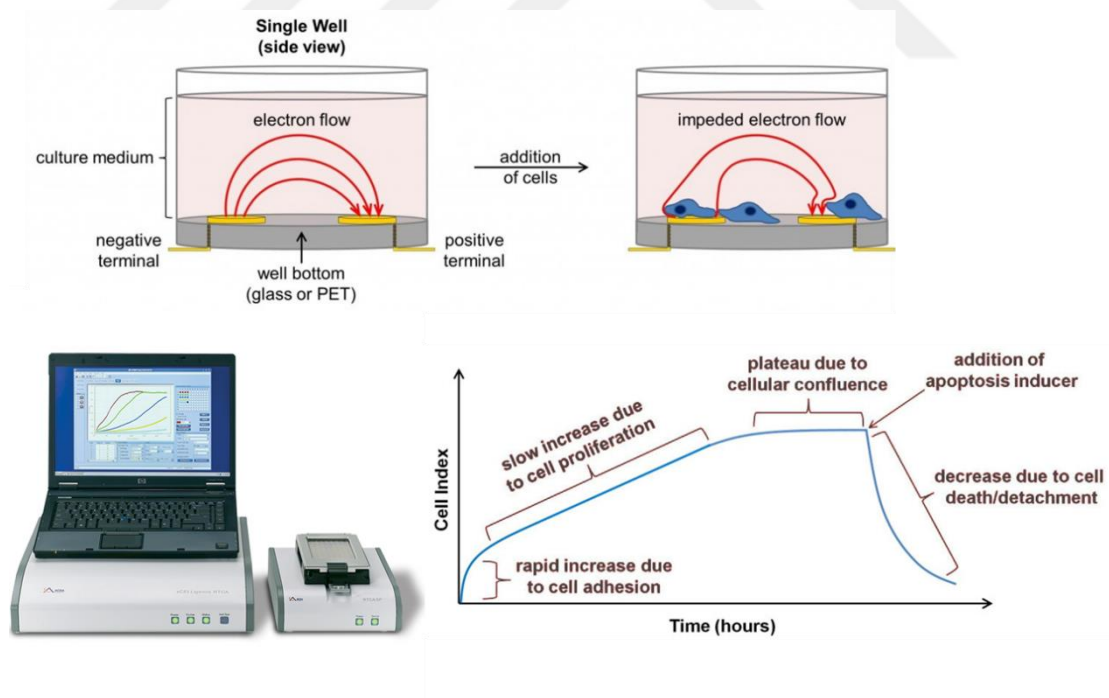


Figure 3.2. Schematic representation of cellular impedance apparatus and the RT-CES system. Cell index values represent the cell growth. The electron flow at the bottom of each well is affected by the number of cells attached. As the number of the cells

covering the electrodes increases, the electrical impedance (Z) increases. This information is converted into CI values by the algorithm of the RT-CES system.

3.3.4. Flow cytometry analysis for detection of liver cancer stem cells

Cells were inoculated into 100 mm culture dishes (Huh7: 200,000, Hep3B: 300,000, Mahlavu: 100,000, and SNU475: 100,000 cells). 24 hours later, old media was discarded, and fresh complete media was added. Cells were treated with the inhibitors (IC₅₀ conc.) for 72 hours. Cells that were detached from the surface of the culture plates were discarded, and remaining cells were collected by trypsinization in a 15 ml falcon tube. Cells were centrifuged at 15000 rpm for 5 minutes, and the supernatant was discarded. Cells were washed once with PBS by centrifugation. The cell pellet was fixed with 4% paraformaldehyde for 20-30 minutes. Then fixed cells were washed once with cold PBS and centrifuged. Anti-CD133/1-Biotin and anti-EpCAM-FITC antibodies (prepared as indicated previously) were added onto Huh7 and Hep3B cells to be incubated for 30 minutes in dark at room temperature. After washing cells with 1X PBS, cells were stained with the secondary antibody, anti-biotin-PE and incubated for another round of 30 minutes in dark at room temperature. Then cells were washed again and resuspended in 100 µl of MACS buffer (incubation buffer) to be analyzed with BD Accuri C6 flow cytometer. For the fluorescence compensation mouse IgG1 isotype control-FITC and mouse IgG1 isotype control-biotin antibodies were used. Mahlavu and SNU475 cells were stained with anti-CD90-FITC antibody only. Since CD133, CD90, and EpCAM are expressed mostly on the membrane of cells, no permeabilization step was necessary. Whereas for the detection of stemness markers, Nanog and OCT4, cells were permeabilized with 90% ice-cold methanol for 10 minutes on ice after the fixation step. Blocking of cells was done using incubation buffer for an hour. Cells were then washed and resuspended in 100 µl of Nanog or OCT4 antibodies for an hour at room temperature. Then cells were washed by centrifugation in 2-3 ml of incubation buffer. Cells were resuspended in secondary antibody, anti-rabbit-IgG-Alexa 647 and incubated for 30 minutes in dark, at room temperature. After a washing cells by centrifugation in 2-3 ml

incubation buffer, cells were resuspended in 200 µl of PBS and analyzed by flow cytometry. As a control for unspecific binding of antibodies, cells stained with only secondary antibody were prepared and compared to that of experimental groups.

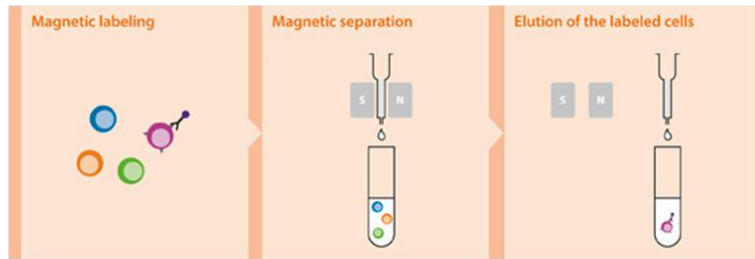
For the analysis of CD133/EpCAM stained cells on the flow cytometry, initially, cells stained for the isotype controls were read to localize the cells negative for both markers and to draw a quadrant on the window (bottom-left), then cells that were stained for one of the markers but not the other (for example: CD133 + isotype control of EpCAM) were read in order to do a fluorescence compensation, which helps eliminate false positive fluorescence signals coming from each fluorochrome. After setting the compensation values only then each sample was analyzed.

3.3.5. Magnetic Cell Separation

For the separation of CD133+/EpCAM+ cells from the heterogeneous population of Huh7 and Hep3B cells, “Multisort Magnetic Separation” technique was used. This method is based on labeling the cells of interest with magnetic microbeads and applying labeled cells to a column placed in a separator which creates the magnetic field. The unlabeled cells pass through while the magnetically labeled cells are retained within the column. The flow-through can be collected as the unlabeled fraction. Once the column is removed from the separator, the magnetically labeled cells can be eluted from the column (Figure 3.3A). In this study, two subsequent positive selection methodology was followed. In this strategy, Multisort Microbeads specific for the first marker allows the first positive selection. Then these cells are prepared for the second positive selection by first releasing the multisort microbeads through enzymatic reaction and then magnetically labeling the cells with microbeads specific for the second marker (Figure 3.3B).

A

Basic principle of magnetic cell separation

**B**

Principle of two subsequent positive selections

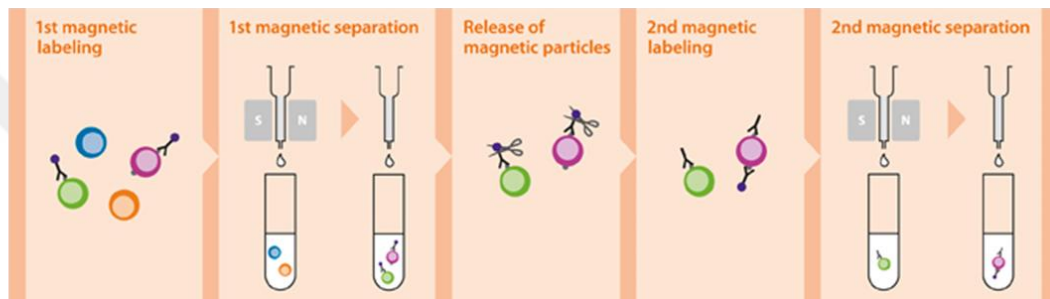


Figure 3.3. Schematic representation of basic principles MACS. A. magnetic cell separation and B. Two subsequent positive selections.

In this study CD133 positive cells were separated initially and then these cells were magnetically labeled with a second microbead that was directed against EpCAM.

Huh7 and Hep3B cells were grown, trypsinized and collected in 50 ml falcons. In order to begin the process with single cell suspension, cells were passed through a cell strainer (BD Biosciences) and then counted as previously described. The number of cells determines the amount of reagents to be used. As recommended by the manufacturer, the amounts mentioned here were for 1×10^7 cells and cells were kept cold throughout the whole process. If the number of cells exceeds 1×10^7 , all reagent volumes and total volumes were scaled up accordingly. Single suspension cells were centrifuged 300xg for 10 minutes, and the supernatant was aspirated. Cell pellets were resuspended in 100 μ l MACS buffer and 10 μ l anti-CD133-biotin antibody to be incubated for 10 minutes in dark at 4-8°C. In order to remove the unbound primary antibody, cells were washed by adding 3-5 ml of buffer and centrifuged at 300xg for 10 minutes. After removal of the

supernatant completely, cell pellets were resuspended in 90 µl of buffer and 10 µl of anti-biotin Multisort Microbeads and incubated for 15 minutes at 4-8°C. In addition, 10 µl of anti-biotin-PE antibody was added to be incubated for an additional 5 minutes in dark at 4-8°C. After a washing step, cells were resuspended in 3 ml of buffer to proceed to magnetic separation. 3ml of buffer was rinsed through the separation column (this step is required to prepare the column). Then cell suspension was applied onto the column. Unlabeled cells were collected in a 15 ml falcon, and the column was washed three times with 3 ml of buffer. The total effluent collected was labeled “CD133 negative cells”. The column was removed from the separator and placed on a separate falcon. 5 ml of buffer was pipetted onto the column, and the magnetically labeled cells were immediately flushed out by firmly applying the plunger. In order to increase purity, cells were passed over a second column. For the removal of Multisort Microbeads, 20 µl of Multisort Release Reagent was added per 1 ml cell suspension and incubated for 10 min at 4-8°C. In order to remove any residual magnetically labeled cells, magnetic separation procedure was repeated. This time, the released fraction was collected and washed. The cells were then resuspended in 60 µl of buffer + 30 µl of Multisort Stop Reagent and 10 µl of anti-EpCAM-FITC antibody (second marker). After 10 minutes of incubation at 4-8°C, cell were washed and resuspended in 90 µl of buffer + 10 µl of anti-FITC microbeads (15 min at 4-8°C). Unbound microbeads were removed by a washing step and cells were resuspended in 3 ml of buffer to proceed to magnetic separation (as described previously). At the end of the second column separation, the flow through contained the CD133+/EpCAM- cells, whereas the cells flushed out from the column contained CD133+/EpCAM+ cells. In order to determine the purity of the separation, a small sample was removed from each fraction and analyzed by flow cytometry.

3.3.6. Sphere formation assay

Sphere formation assay was performed in order to identify stem cells based on their ability to promote in vitro expansion at the single cell level in vitro. In this study, the sphere

formation capacity of HCC cells (Huh7 and Hep3B) treated with different inhibitors/combinations were evaluated. For this purpose, cells were collected and seeded into ultra-low attachment plates (96-well) 400 cells/well in 200 μ l/well of sphere formation medium. In order to help minimize medium evaporation, PBS was added to the first and last columns (column 1 and 12) of the 96-well plate. For each cell line or treatment, cells were seeded into the wells of 2 rows for a total of 20 wells. Then, the upper and lower edges of the 96-well plate were sealed with parafilm to avoid evaporation of medium, and the plate was placed in a 37°C humidified incubator with 5% CO₂. After one-week incubation (without any medium exchange), tumorsphere numbers were counted under a light microscope. The size of spheres was also measured with the help of area measurement tools of the microscope software. Representative images of the spheres were also taken at 10x and 20x magnifications.

3.3.7. RNA extraction and cDNA synthesis

Huh7 cells were seeded onto 100 mm culture dishes (100,000 cell/dish). The next day cells were treated with the indicated inhibitors for 72 hours at IC₅₀ concentrations. For the sequential treatment strategy, cells were treated for an additional 72 hours with the second inhibitor. Cells were trypsinized and collected in 15 ml falcon tubes and centrifuged at 15000 rpm for 5 minutes to remove the medium completely. Supernatant was removed, and cell pellets were washed with 5 ml of PBS and centrifuged again. After removal of the supernatant, pellets were ready for Total RNA extraction with RNeasy Mini Kit. Cell pellets were loosened, and 350 μ l of Buffer RLT was added and pipetted to mix. After making sure that the lysate is homogenized (vortexing for 1 min), 350 μ l of 70% ethanol was added and mixed well by pipetting. Up to 700 μ l of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube. The samples were centrifuged for 15 seconds at 8000 x g, and the flow through was discarded. 700 μ l of Buffer RW1 was added to each spin column and centrifuged for 15 seconds at 8000 x g to wash the spin column membrane. After discarding the flow-through, 500 μ l of Buffer

RPE was added to the spin column and centrifuged for 15 seconds at 8000 x g and the flow through is discarded. The same amount of Buffer RPE was added to spin columns to be centrifuged for 2 minutes at 8000 x g. The spin columns were placed in new 1.5 ml collection tubes, and 30 µl of RNeasy-free water was added directly to the spin column membrane. Centrifugation (1 min at 8000 x g) was done to elute the RNA. RNA samples were then measured for their concentration and A260/A280, A260/A230 ratios were checked for RNA quality and purity using a Nanodrop.

For qRT-PCR analysis, RNA samples were treated with DNase in order to remove genomic DNA. 50 µl of RNA, 10 µl of 10x incubation buffer and 2 µl of Roche DNase were mixed and total volume was completed to 100 µl with ddH₂O. The mixture was incubated in water bath (at 37°C) for an hour. Meanwhile, a small amount of Phase Lock was taken and rubbed to the mouth of an Eppendorf. Then, 100 µl of phenol-chloroform isoamyl-alcohol (P:C:IAA) was added onto the incubated samples and mixed by vortexing to be transferred into tubes with Phase Lock. After 10 minutes of incubation on ice, tubes are centrifuged at 13000 rpm for 20 minutes at 4°C. The aqueous (upper) part of the tube was transferred into a new Eppendorf tube and 10 µl from 3M Sodium acetate following 300 µl absolute ethanol were added to be incubated overnight at -20°C. Then, samples were centrifuged at 13000 rpm for 30 minutes at 4°C. Supernatant was discarded, and the pellet was resuspended in 500 µl ice-cold 70% ethanol and vortexed shortly. After centrifugation at maximum speed for 15 minutes at 4°C, ethanol was removed carefully, and samples are left for air-drying for 30 minutes. Samples are resuspended in 25 µl of RNase free water, and RNA concentration and purity measurements were done.

To confirm that the DNase treatment of RNA samples was successful, a PCR reaction was set up using GAPDH primers. DNase treatment was repeated until genomic DNA contamination was completely removed.

First-strand cDNA synthesis was carried out from 1 µg of total RNA using Oligo dT primers with the RevertAid First Strand cDNA synthesis kit (Fermentas). Initially, in order to synthesize 1 µg of cDNA from each sample, RNA amounts to be used were

calculated based on the concentrations of each RNA sample. Then, 1 μ l of oligo dT was added into the mixture (in a total volume of 12 μ l) and incubated at 65°C for 5 minutes in thermal cycler. The rest of the components (10 mM dNTP, Ribolock, 5x buffer and RevertAid M-MulV RT enzyme) was added into the mix, and incubated at 25°C for 5 minutes and at 42°C for 60 minutes. In order to confirm that the cDNAs were synthesized, conventional PCR was performed using GAPDH primers. PCR conditions were as the following: denaturation (94°C for 2 min), a loop of cycle of 94°C for 30 sec, at T_m: 60°C for 30 sec, 72°C for 30 sec, and final extension at 72°C for 5 minutes.

3.3.8. Expression analysis with Nanostring® nCounter® Technology

The nCounter technology of Nanostring provides a platform that enables quantification of each target of interest based on digital counts and direct molecular barcoding of target genes without any amplification step. This is possible through hybridization of each target with a unique probe pair that consists of a color-coded Reporter probe (which carries the signal on its 5' end) and a Capture probe, which has a biotin moiety on the 3' end. The reporter probe has six positions in which different colors (4 color options) could be used to make unique combinations, which allows a large diversity of probes to be used together in a single well. Thus it is possible to individually resolve and detect and identify target-probe complexes during data collection. After removal of the excess probes by washing steps that involve a two-step magnetic bead-based purification, hybridized probes bind to the nCounter Cartridge to align and immobilize. Data collection is performed using epifluorescence microscopy and CCD capture technology that is built inside the nCounter instrument in order to yield thousands of molecule counts. These images are then processed within the system and presented as values in a format that enables the user to continue with further data analysis. This technology provides many advantages over microarray and high-throughput sequencing since it delivers more sensitive, specific and reproducible results (with lower background noise, a higher dynamic range) that can be easily analyzed by the user. (Figure 3.4)

In this study, gene expression of a panel of “cancer pathways” related genes (“PanCancer pathways” panel) was analyzed. This CodeSet includes 770 genes from 13 cancer-associated canonical pathways including: PI3K, MAPK, STAT, RAS, Cell cycle, DNA damage control, Apoptosis, Hedgehog, Wnt, Transcriptional Regulation, Chromatin Modification, and TGF- β . Total RNA samples were prepared as mentioned previously and hybridization reactions were prepared as follows (Figure 3.5): Aliquots of both Reporter Codeset and Capture ProbeSet reagent were removed from the freezer and thawed at room temperature. A master mix was prepared by adding 70 μ l of hybridization buffer to the tube containing the Reporter CodeSet. The hybridization strip tubes (12) were labeled and cut in half (2 pieces of 6 tubes) and 8 μ l of master mix was and 5 μ l of RNA sample (50 ng) were added to each tube. After mixing the capture ProbeSet by inverting the tube and spinning down the contents, 2 μ l of Capture ProbeSet was added immediately to each tube and capped. After making sure the reagents were mixed (inverting tubes several times), tubes were spun down and immediately placed in the pre-heated 65°C thermal cycler (Bio-Rad). The reactions were incubated for at least 16 hours and placed into the nCounter Prep Station. The next day, nCounter Prep Plates and the cartridges were removed from +4 and -20°C to allow them to equilibrate to room temperature for 10-15 minutes. The Prep plates were centrifuged at 2000 x g for 2 minutes in order to collect all liquids in the bottom of the wells. To initiate a run in the Prep Station, Prep Plates, cartridges, Prep Pack and hybridized samples were placed carefully as recommended by the manufacturer. On the “sample selection” screen, sample positions to be processed were selected. The Prep Station was confirmed to start processing for several hours. When the run was successfully completed, all disposables together with the sample cartridge were removed from the Prep Station. Sample cartridge was sealed with the adhesive film to prevent evaporation and transferred into the Digital Analyzer. The Reporter Library Files (RLF) of the “PanCancer pathways” panel was uploaded to the Analyzer before initiating a run, and the position of the stage where the cartridge was placed was selected on the screen. After completing several hours of running (~4 hours) the data (Reporter Code Count (RCC) file) was transferred to be

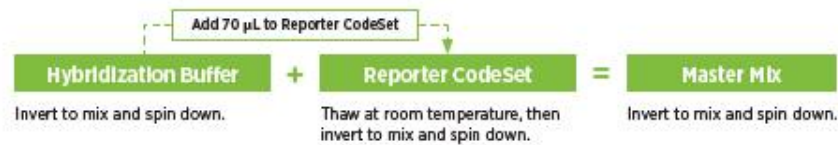
The diagram illustrates a three-step process for DNA microarray analysis using a microfluidic cartridge:

- 1. Solution phase hybridization:** A mixture of **Capture Probe** (blue), **Reporter Probe** (red), and **Target** (blue) DNA sequences forms a **Target-Probe Complex**. This mixture undergoes **solution phase hybridization** within a circular chamber.
- 2. Immobilization and alignment:** The mixture is loaded into an **nCounter Cartridge**. **excess probes removed**, and the **hybridized probes bind to cartridge**. The **complexes are immobilized and aligned on cartridge**, as shown by the DNA strands being fixed in place within the cartridge's wells.
- 3. Data analysis:** The final state shows the DNA complexes aligned on the cartridge. A table summarizes the results:

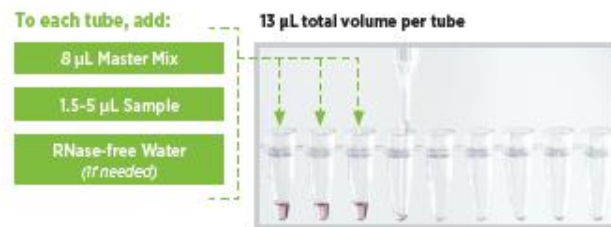
Barcode	Counts	Identity
[Blue, Yellow, Green, Red, Blue, Green]	3	XL5A
[Red, Yellow, Green, Red, Green, Red]	2	FOX5
[Red, Yellow, Green, Red, Green, Red]	1	INSULIN

47

1. Create Master Mix



2. Set up Hybridization Reactions



3. Complete Hybridization Reactions



Figure 3.5. Workflow for nCounter XT Gene Expression Assay.

3.3.9. Cellular pathway analysis using “Pathway Scoring Application”

A Cytoscape pathway analysis application for signal transduction scoring algorithm for cancer pathway analysis, so-called “Pathway Scoring Application”: <http://apps.cytoscape.org/apps/pathwayscoringapplication> [115] was used for modeling the behavior of the treated cells. This application accepts and processes the Log2-transformed normalized gene expression values obtained from the Nanostring nCounter Technology and Advanced Analysis software. Initially, Nanostring nCounter log2 values were transformed into ranked values. The genes were sorted in ascending order, and each log2 value was ranked with reference to all log2 values. Then this information was uploaded to Cytoscape 3.5 software platform where “pathway scoring application” plug-

in was run by integrating “Pathways in cancer (human)” KEGG pathway. The scores assigned to each gene by the algorithm were compared between each experimental group.

3.3.10. Primer design for qRT-PCR

The coding sequences (CDS) of the genes of interest were taken from NCBI (National Center for Biotechnology Information) website: <https://www.ncbi.nlm.nih.gov/gene>. For the designing of the primers, Primer3 Input (version 0.4.0) online web tool: <http://bioinfo.ut.ee/primer3-0.4.0/> (established by Steve Rozen and Helen J. Skaletsky) was used. For each gene, primers were designed such that their length was 18-22 bp, had a GC content of 40-60%, and their melting temperature was around 60°C (with max. 1°C difference between primer pairs). It was also important for the primers to amplify at a region that consists of at least one exon-exon junction, so that if the primer pair amplifies a longer product than expected, this would mean there is a genomic DNA contamination. Then, using the Primer BLAST tool: <https://www.ncbi.nlm.nih.gov/tools/primer-blast/> the designed primer pairs were checked if they can only amplify the desired region but not any other region on another gene. Primers that were used in this study are listed in Table 3.5.

3.3.11. Expression analysis qRT-PCR

Real-time quantitative PCR reactions were performed in triplicates for each gene of interest using 3 µl of 1:20 diluted cDNA template. Master mix for each reaction consisted of 5 µl of SYBR Green, 0.6 from both forward and reverse primers in a total of 10 µl (completed with 0,8 RNase free water). The following program was used: initial denaturation at 95 °C for 10 min, amplification for 45 cycles (95°C 10 sec followed by 60°C 10 sec and 72°C 10 sec), and melting at 95°C 10 sec followed by 65°C for 60 sec and 97°C for 1 sec. In order to calculate primer efficiency a reaction with 1:10, 1:20, 1:40 and 1:80 cDNA dilution series in triplicates for each primer was included. The

standard curve (quantification cycle (Cq) vs. log of starting quantity) was plotted by the Light Cycler[®] 96 Real-Time PCR System, and these values were used to calculate relative expression values. Cq values generated by the system were transformed into relative expression values by using the formula: $E(-\Delta Cq)$. In order to calculate the relative expression between the control and experimental group, the following equation was used: $R = (E_{target})^{\Delta Cq_{target}(control-sample)} / (E_{ref})^{\Delta Cq_{ref}(control-sample)}$.

3.3.12. Agarose gel electrophoresis

2% Agarose was dissolved in TBE buffer in a microwave and poured onto a gel casting apparatus to polymerize. PCR products and the ladder were mixed 1:1 with EZ-VISION[®] DNA dye and loaded onto gels. The gel was run at 120V and bands were visualized under UV trans-illuminator.

3.3.13. Total protein extraction from cancer cells and Bradford assay

Huh7 (200,000 cell), HepG2 (300,000 cell), Mahlavu (100,000 cell) and SNU475 (100,000 cell) were seeded into 100 mm cell culture dishes. The next day, medium was removed and replaced with 6 ml of fresh complete medium with inhibitors or DMSO control in IC₁₀₀ concentrations. After incubating cells for 24 hours, apoptotic cells in the medium was collected into 15 ml tubes, and 5 ml of cold PBS was added on top of cells. Attached cells were collected into the same tubes by scraping the cells from the surface of the dishes. Cells were centrifuged at 1600 rpm for 10 min at +4°C, and the supernatant was discarded. Cells were resuspended in 5 ml ice-cold PBS and centrifuged again. After the supernatant was removed, the cell pellets were lysed using the lysis buffer (described in 3.2.4), $\times 2V_{\text{pellet}}$. By vortexing every 5 min for 30 min on ice. The lysates were centrifuged at 13000 rpm for 20 minutes at +4°C, and the supernatant was collected into separate eppendorf tubes, which contained the proteins. The proteins were stored at -20°C until use. The protein concentrations of the lysates were measured by Bradford assay

following the recommended 96-well protocol. Bovine Serum Albumin (BSA) protein of known concentrations (5, 2.5, 1.25, 0.6, 0.3 mg/ml) and lysis buffer (as blank) was used to draw a standard curve, which is then used to calculate the protein concentrations of each sample with respect to their absorbance values under 595 nm excitation. The samples were diluted 1:3 in a separate tube and 5 µl from each diluted sample and the standards were pipetted into wells of a 96-well plate. Then, on top of all samples, 250 µl of Bradford reagent was added. After 3-5 minutes of incubation on a shaker in dark, OD values were obtained by reading the 96-well plate with the plate reader (BioTek, ELx800). From the standard curve, a linear formula was obtained, which was used to calculate the protein concentrations of each sample.

3.3.14. Western Blot Analysis

For the preparation of the protein samples for SDS-PAGE electrophoresis, a mixture of 25-40 µg of protein + 4x Laemmli buffer + 25 mM DTT in ddH₂O was prepared in a total volume of 12 µl for 15-well gels and 25 µl for 10-well gels. The samples were heated at 95°C for 5-7 minutes for denaturation and incubated on ice until loading into 10% or 12% Tris-Glycine gels in TGX running buffer. Mini-PROTEAN® Tetra Cell Systems and TGX™ precast gels) and transfer system (Trans-Blot® Turbo Transfer System) was used according to the manufacturer's protocol for all the western blotting analysis. The gels were transferred onto PVDF membranes and stained with Ponceau S for several minutes to control the efficiency of transfer. De-staining of Ponceau S was done by washing the membranes with ddH₂O. To proceed with the blocking step, membrane was incubated in blocking solution (5% milk powder in 1x TBS-T (0.1% tween)) on a shaker at 4°C overnight. Then, the membrane was incubated with the primary antibody (1 or 2 hours) at room temperature and washed for 30 min (in 5, 10, 10 and 5 min intervals) with 1x TBS-T(0.1%tween) on a shaker. The secondary antibodies conjugated with horse-radish peroxidase (HRP), were used to incubate membranes for 1 hour at room temperature followed by the same washing procedure. Membranes were then incubated with

SuperSignal West Femto Chemiluminescent substrate for 5 minutes, and proteins were visualized, and band intensities were quantified using C-Digit imaging system (LI-COR).

3.3.15. Immunofluorescence for detection of cell death

HepG2 (90,000 cell/well) and SNU475 (30,000 cell/well) were seeded onto coverslips (12x12mm) placed into 6-well plates. After 24 hours, medium on top of attached cells was aspirated, and fresh 3 ml of complete medium was added. Cells were treated with the compounds using their corresponding IC₅₀ concentrations. After 48 hours of incubation, cells were washed twice with cold PBS and stained with Annexin-V-Fluos labeling solution (100 µl/coverslip) for 15 minutes. After removal of excess dye by washing cells with 1x PBS once, coverslips were mounted onto microscope slides using a single drop of UltraCruz Mounting Medium for Fluorescence with DAPI according to the manufacturer's recommendations. Apoptotic cells were imaged using Nikon Eclipse 50i fluorescence microscope.

3.3.16. Cell cycle analysis with flow cytometry

HepG2 (300,000 cell) and SNU475 (100,000 cell) were seeded into 100 mm culture dishes. After 24 hours, medium on top of cells was aspirated, and 6 ml of fresh medium was added. Treatment of cells was done with the compounds using their corresponding IC₁₀₀ concentrations. After 24h, 48, and 72h incubations, detached (dead) and attached cells were collected in a 15 ml tube via trypsinization. Then, cells were centrifuged at 16000 rpm for 10 minutes at +4°C. Supernatant was discarded, and cells were washed with 5ml of ice-cold PBS and centrifuged at 16000 rpm for 10 minutes. The supernatant was discarded such that 50 µl of PBS remains on top of each pellet. Then, pellets were resuspended in 50 µl of residual PBS by pipetting, and ice-cold 70% ethanol was added onto the suspension dropwise while vortexing. Samples were incubated at -20°C at least for 3 hours for fixation. For staining of the cells with Propidium Iodide solution, cells

were washed with 5 ml of cold PBS twice by centrifugation at 2000 rpm for 5 minutes. After removal of the supernatant, cells were resuspended in 200 μ l of MUSE Cell Cycle reagent and incubated for 30 minutes at room temperature in dark. The cell suspensions are then transferred into 1.5 ml Eppendorf tubes to be analyzed by the MUSE Cell Analyzer. Cell cycle distribution and apoptotic cells were determined and compared among samples by the given percentage values for G0/G1, S, G2/M and Sub-G1 fractions.





Chapter 4

Results

4.1. PART-I: Analysis of PI3K/Akt/mTOR pathway inhibitors on liver cancer stem cells

Initially, magnetically separated HCC cells were analyzed to determine the stem cell characteristics of separated populations. Then, bioactivities of 10 different small molecule kinase inhibitors acting on PI3K/Akt/mTOR pathway against liver cancer stem cells (LCSCs) were elucidated in comparison with Sorafenib, DNA intercalators and a well-known cancer stem cell inhibitor, DAPT. Inhibitors that were effective against LCSCs

were further evaluated for their combinatorial effects together with Sorafenib. Additionally, alterations in expression of cancer pathway related genes were studied to introduce possible molecular mechanisms involved in Sorafenib resistance and potential targets to overcome cancer stem cell enrichment. Additionally, the “pathway scoring algorithm” was also used to determine the enrichment scores of differentially expressed genes. The methodology followed to analyze the differentially expressed genes and to integrate this data into pathway scoring application is summarized in Figure 4.1.

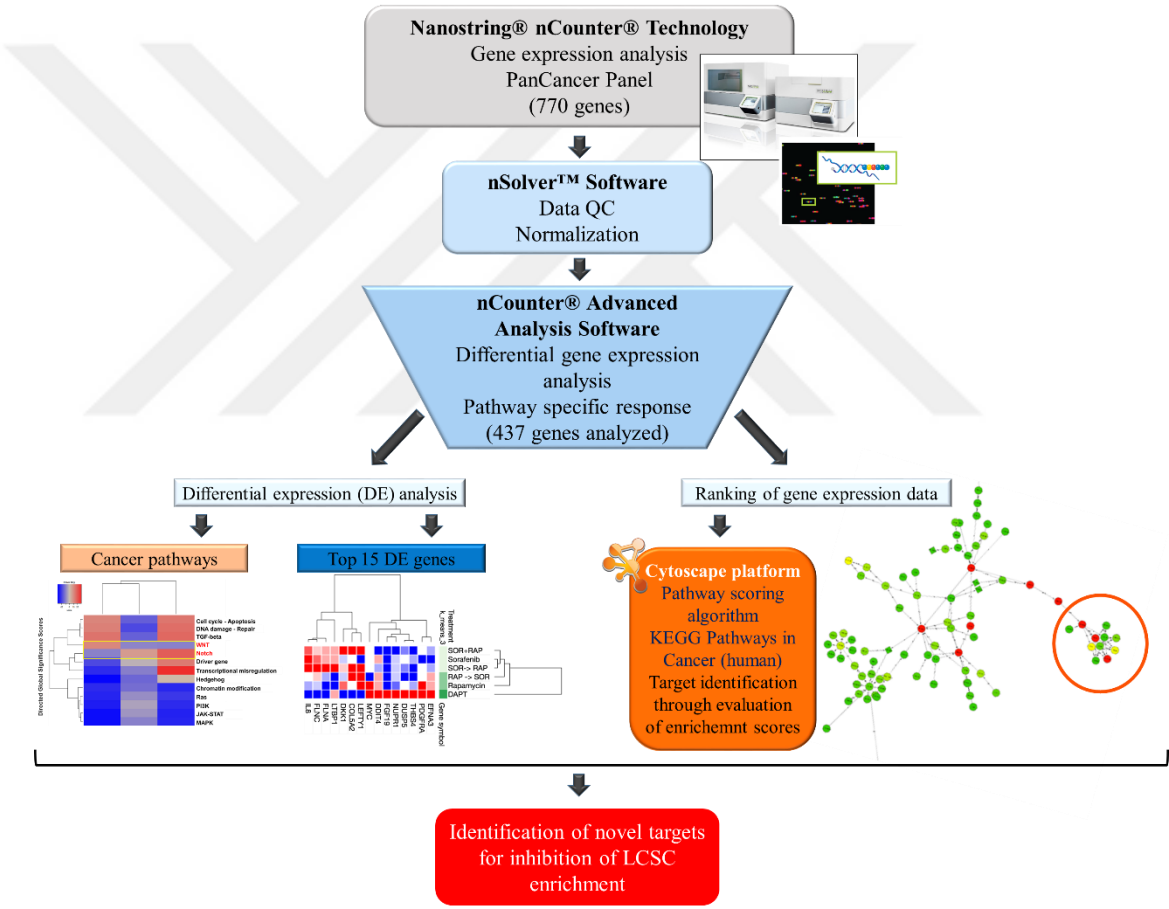


Figure 4.1. Flowchart representing steps followed to analyze the differential expression data and integrate it into the pathway scoring algorithm. (Detailed protocol in methods section).

4.1.1. Expression of LCSC markers in HCC cell lines

Prior to isolation of cancer stem cells from the two epithelial-like (Huh7, Hep3B), and two mesenchymal-like (Mahlavu, SNU475) HCC cells lines, expression of cancer stem cell markers in these cells were identified (Figure 4.2). As mentioned before, CD133 and EpCAM markers were known to be expressed in cells having epithelial origins, whereas CD90 or CD44 were more likely to be expressed in cells with mesenchymal features. Flow cytometry analysis has shown that marker positivity was variable in all cells. 15-30% of Huh7 cells were positive for both CD133 and EpCAM markers, and Hep3B cells that were positive for CD133 (50-70%) were all positive for EpCAM. As expected, these cells were negative for CD90 expression. On the other hand, CD90 positive cells in Mahlavu and SNU475 were only 0.4-0.6 % of the whole population.

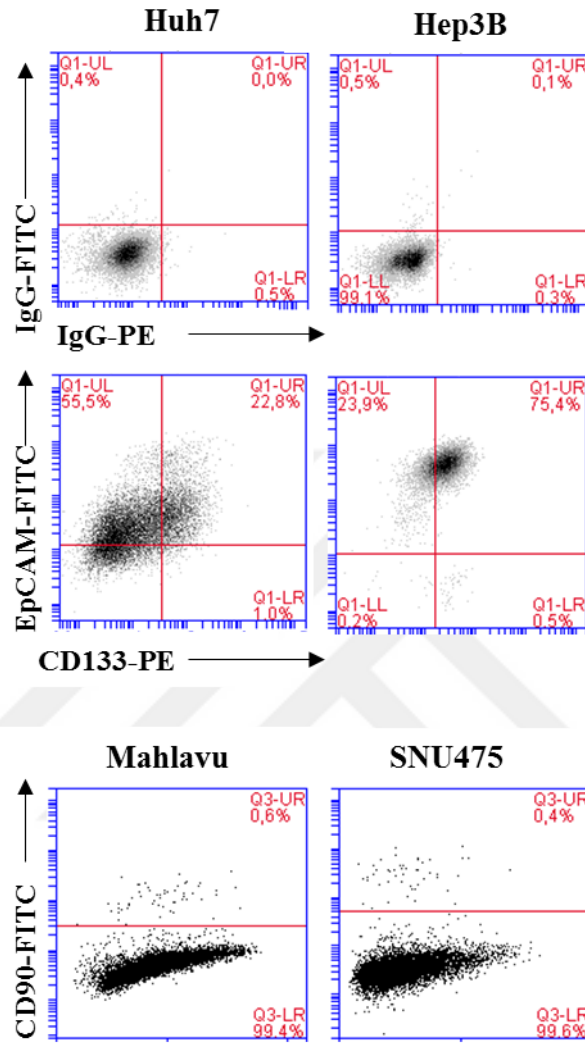


Figure 4.2. Expression of cancer stem cell markers. Representative figures show expression of CD133, EpCAM in epithelial-like (Huh7 and Hep3B) and CD90 expression in mesenchymal-like (Mahlavu and SNU475) HCC cell lines.

4.1.2. Characterization and Isolation of LCSCs in Huh7 and Hep3B cell lines

To characterize the CD133+/EpCAM+ populations isolated from the Huh7 and Hep3B cells, CD133-/EpCAM- and CD133+/EpCAM+ cells were separated using magnetic cell separation method. Flow cytometry analysis before and after separation of CD133+/EpCAM+ cells have shown that the purity of these cells was 85-90% pure

(Figure 4.3). Therefore, the magnetic separation technique was found convenient for the isolation of LCSCs from these cells.

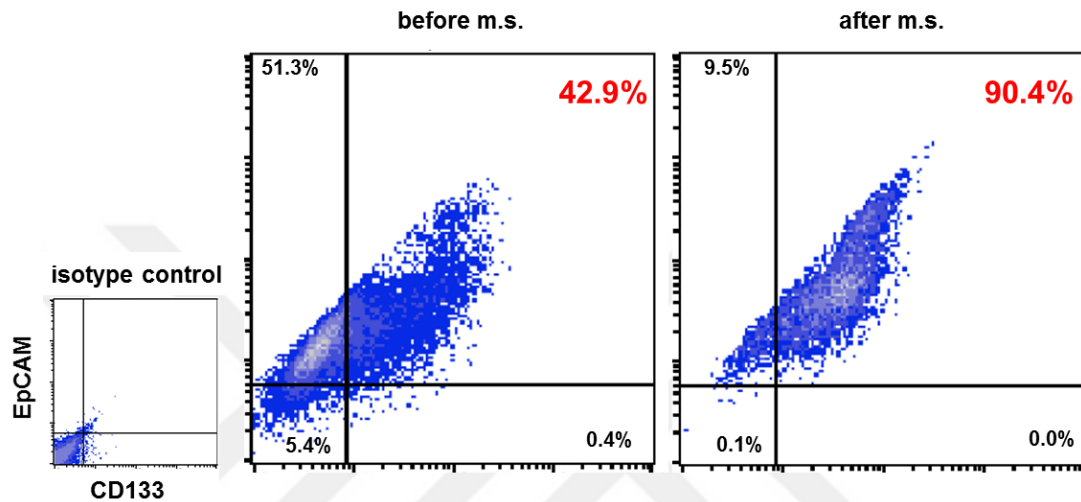
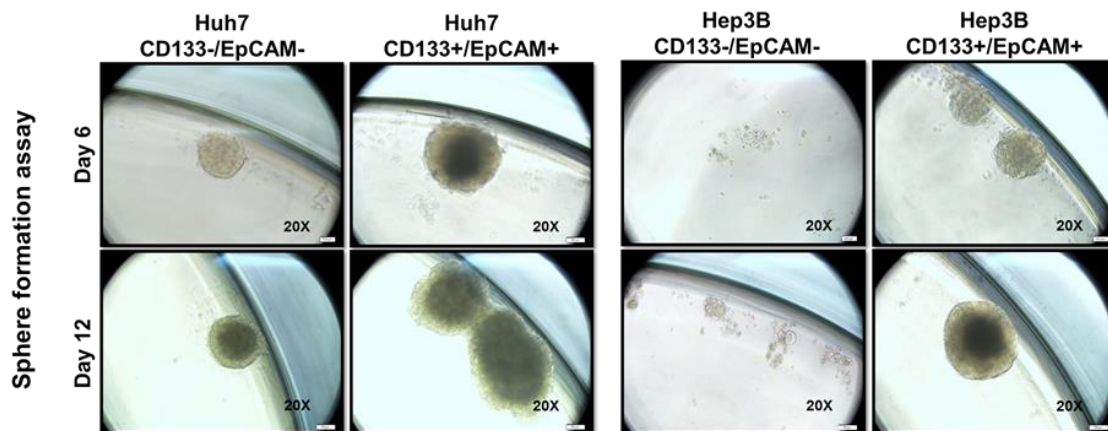


Figure 4.3. The purity of CD133 and EpCAM marker-positive cells before and after magnetic separation shown by flow cytometry. Upper right quadrant indicates the percentage of double positive cells for CD133 and EpCAM.

4.1.2.1. Sphere formation capacity of isolated LCSCs

To determine the stemness features of magnetically separated populations of Huh7 and Hep3B cells (CD133-/EpCAM- and CD133+/EpCAM+), their sphere formation capacities were compared. Results have shown that cells that were positive for both markers were able to form vivid and dense spheres, whereas cells that were negative for both markers were barely able or unable to form spheres (Figure 4.4). In addition, the sphere counts were higher for CD133-/EpCAM- population of Huh7 cells compared to CD133+/EpCAM+ cells.



Average count	Huh7		Hep3B	
	dn	dp	dn	dp
day 6	5±1	12±2	0	4±1
day 12	8±3	18±2	0	7±2

Figure 4.4. Sphere formation capacities of magnetically separated cells. Representative images show spheres formed by CD133-/EpCAM- (dn: double negative) and CD133+/EpCAM+ (dp: double positive) populations of Huh7 and Hep3B cells at days 6 and 12. Average counts of spheres from 12 different wells for two populations at indicated times were given in the table below.

4.1.2.2. Expression of stemness markers in isolated LCSCs

Expressions of well-known stemness factor proteins, Nanog and OCT4 were compared between CD133+/EpCAM+ and CD133-/EpCAM- populations of Huh7 and Hep3B cells to determine if there is a distinct pattern of stemness-related gene expression in separated populations. Flow cytometry results have revealed that both Nanog and OCT4 expressions were higher in CD133+/EpCAM+ population compared to CD133-/EpCAM- cells (Figure 4.5).

Altogether these results have revealed that CD133 and EpCAM markers were suitable for the identification of cells that have cancer stem cell-like features in Huh7 and Hep3B cell lines.

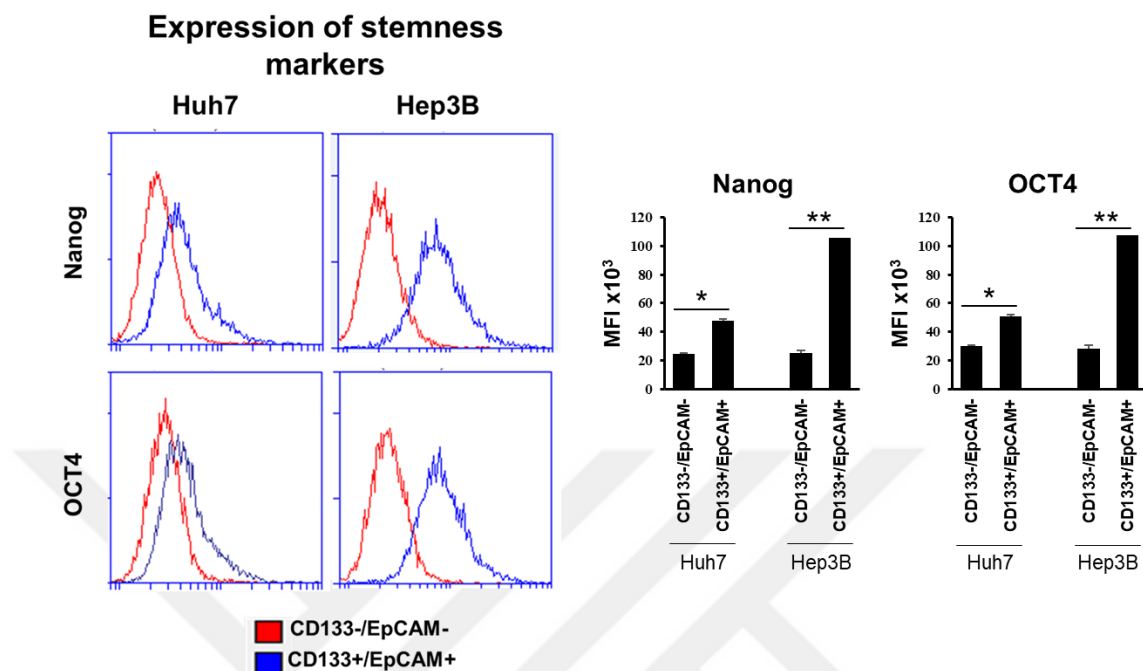


Figure 4.5. Expression of stemness markers in magnetically separated populations. Dot plots showing Nanog and OCT4 expression in (CD133+/EpCAM+ and CD133-/EpCAM-) of Huh7 and Hep3B cells determined by flow cytometry. X-Axis indicates positivity for markers, whereas y-axis indicates cell count. Bar graphs indicate mean fluorescence intensities of Nanog and OCT4 staining in Huh7 and Hep3B cells.

4.1.3. Anti-cancer activities of PI3K/Akt/mTOR pathway inhibitors, Sorafenib and DNA intercalators on HCC cell lines

Bioactivities of 10 different PI3K/Akt pathway inhibitors, along with Sorafenib, DNA intercalators and a well-known cancer stem cell inhibitor, DAPT on HCC cells were determined by NCI-60 SRB assay. The inhibitory concentrations (IC₅₀ values) were evaluated with respect to the DMSO control groups. Results have shown that IC₅₀ values for each inhibitor showed small differences between each cell line tested. Therefore an average IC₅₀ value was assigned to each inhibitor (Table 4.1). These concentrations were used throughout the following experiments explained in this study.

Table 4.1. IC₅₀ values for the inhibitors involved in this study.

Inhibitor	Target/s	*IC₅₀ (μM)
Sorafenib	B-Raf, VEGFR	8.0
ZSTK474	PI3K	7.0
PI-103	PI3K, mTOR	4.0
PI3Ki-α	PI3K-α	0.1
PI3Ki-β	PI3K-β	40.0
AKTi-2	Akt2	20.0
AKTi-1,2	Akt1, Akt2	5.0
LY294002	PI3K	4.0
Wortmannin	PI3K	20.0
Rapamycin	PI3K, mTOR	0.1
BEZ-235	PI3K, mTOR	1.0
**Camptothecin	DNA intercalator	0.1
**Doxorubicin	DNA intercalator	0.1
**DAPT	γ-secretase-Notch	5.0

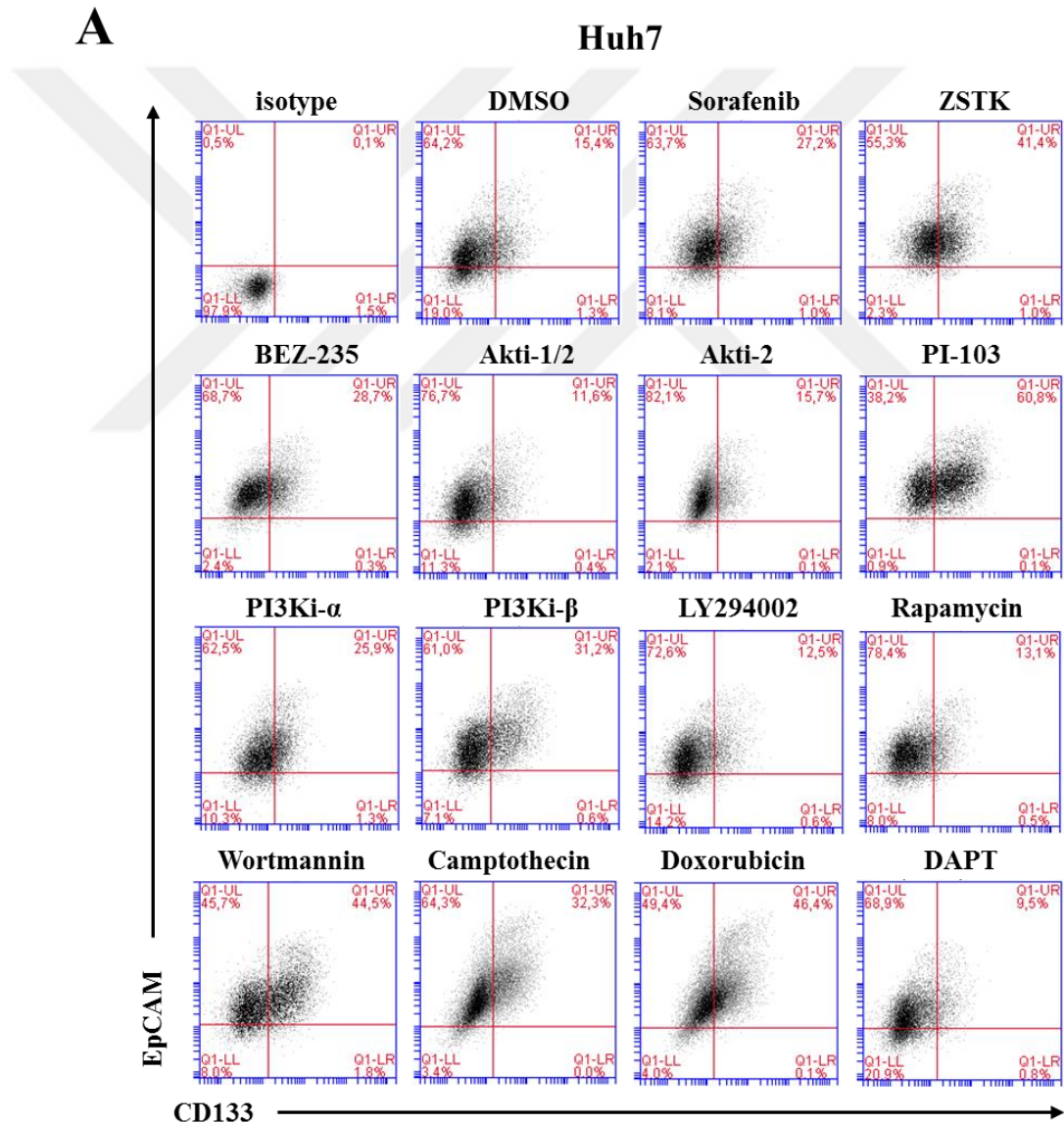
*Average IC₅₀ values of each inhibitor on liver cancer cells. Treatments with all inhibitors were done in triplicates. Average Standard deviations were less than 10% for all values.

** DNA intercalators, Camptothecin and Doxorubicin, and Notch inhibitor DAPT are used as controls in this study.

4.1.4. Differential effects of small molecule inhibitors and DNA intercalators on LCSCs

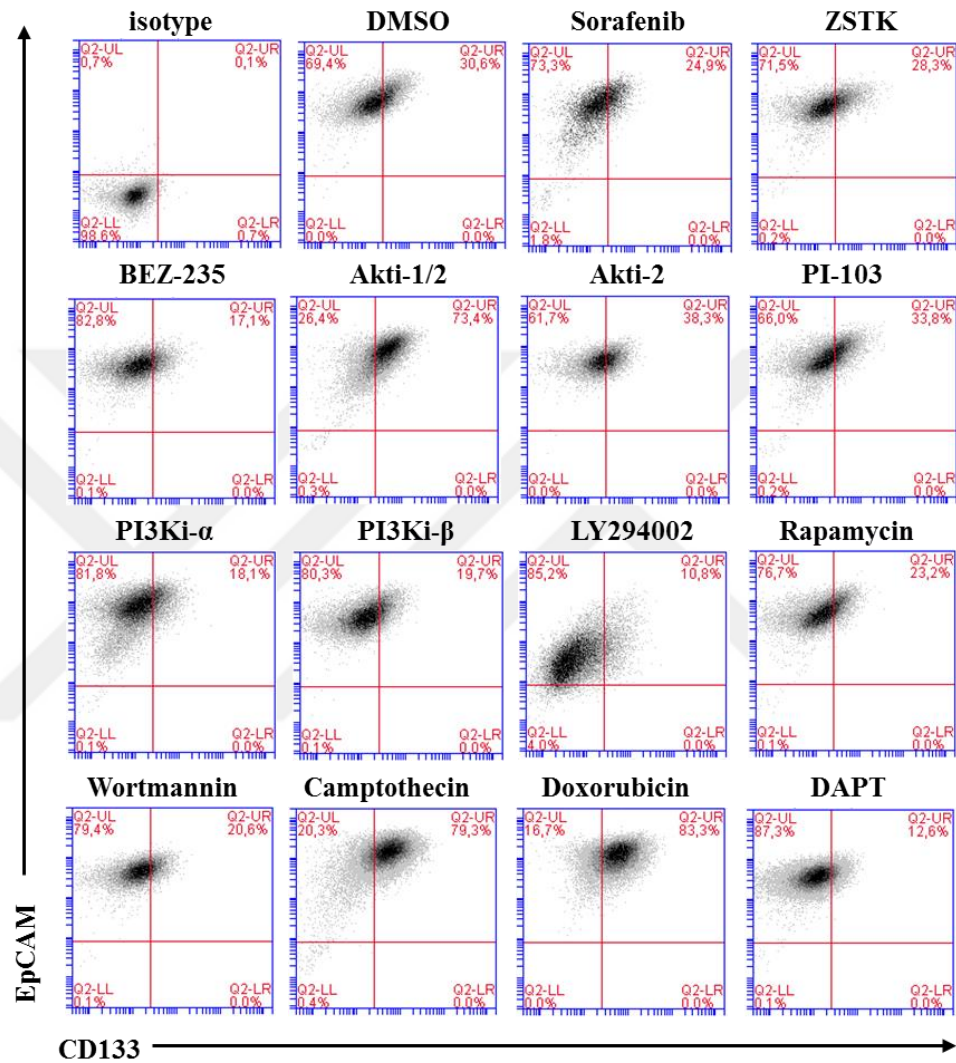
In order to determine the changes in the expression of stem cell markers after treatment with inhibitors listed in Table 1, cells were treated with the corresponding IC₅₀ concentrations for 72 hours. Cells that were able to survive the treatment were collected, stained for LCSC markers to be analyzed by flow cytometry. It was shown that, treatment

of Huh7 and Hep3B cells with Sorafenib or DNA intercalators resulted in enrichment of CD133+/EpCAM+ cells whereas PI3K/Akt pathway inhibitors lead either to the enrichment of this population (ZSTK474, NVP-BEZ235) or did not have any effect on LCSCs (Akti-1,2, Akti-2, PI3Ki- α , PI3Ki- β). Yet, mTOR-inhibitor Rapamycin, PI3K inhibitor LY294002, and Notch pathway inhibitor DAPT were able to reduce the CD133+/EpCAM+ cell population significantly. (Figure 4.6, Figure A1)



B

Hep3B



C

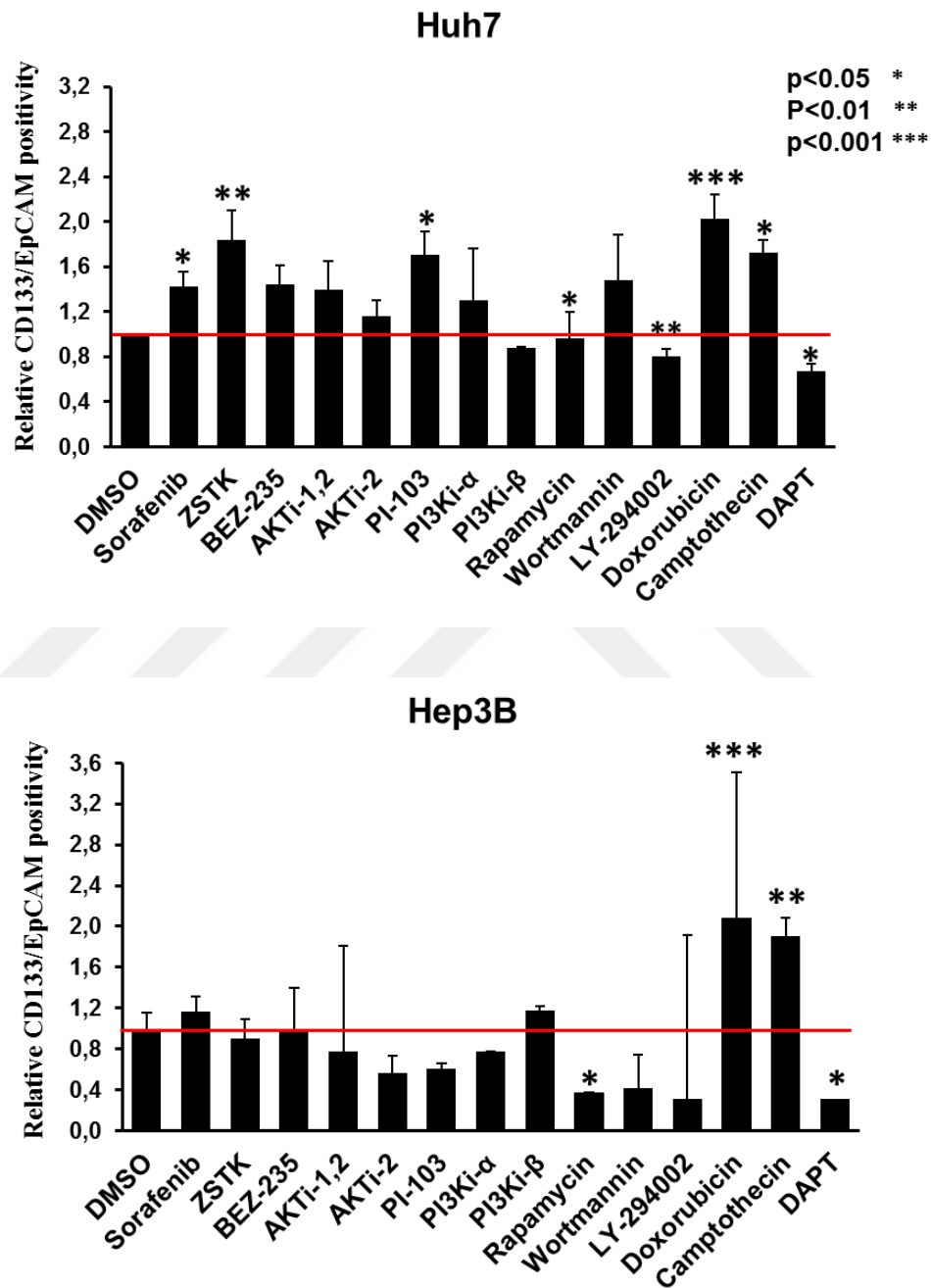
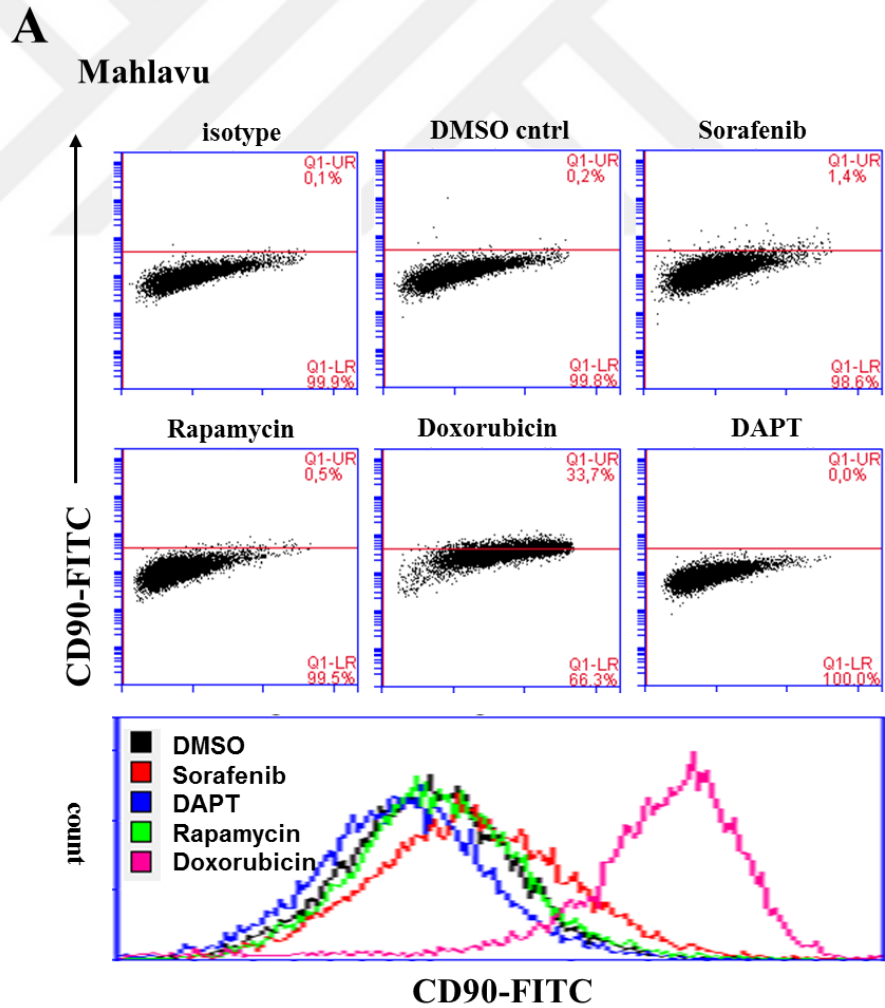


Figure 4.6. Positivity of LCSC markers in Huh7 and Hep3B cells after treatments with inhibitors. Representative images indicating alterations in the positivity of CD133/EpCAM markers in **A.** Huh7 and **B.** Hep3B cells after treatment with IC₅₀ concentrations of inhibitors for 72h. **C.** Bar graphs represent relative CD133/EpCAM positivity with respect to DMSO control group (red line: threshold) for each cell line.

The effects of selected inhibitors, Rapamycin, LY294002, Sorafenib, Doxorubicin, and DAPT were evaluated for mesenchymal-like HCC cells (Mahlavu and SNU475) as well (Figure 4.7, Figure A2). The results have shown that, Sorafenib and Doxorubicin causes a significant increase in CD90 positivity, whereas Rapamycin and DAPT were able to decrease cancer stem cell enrichment.



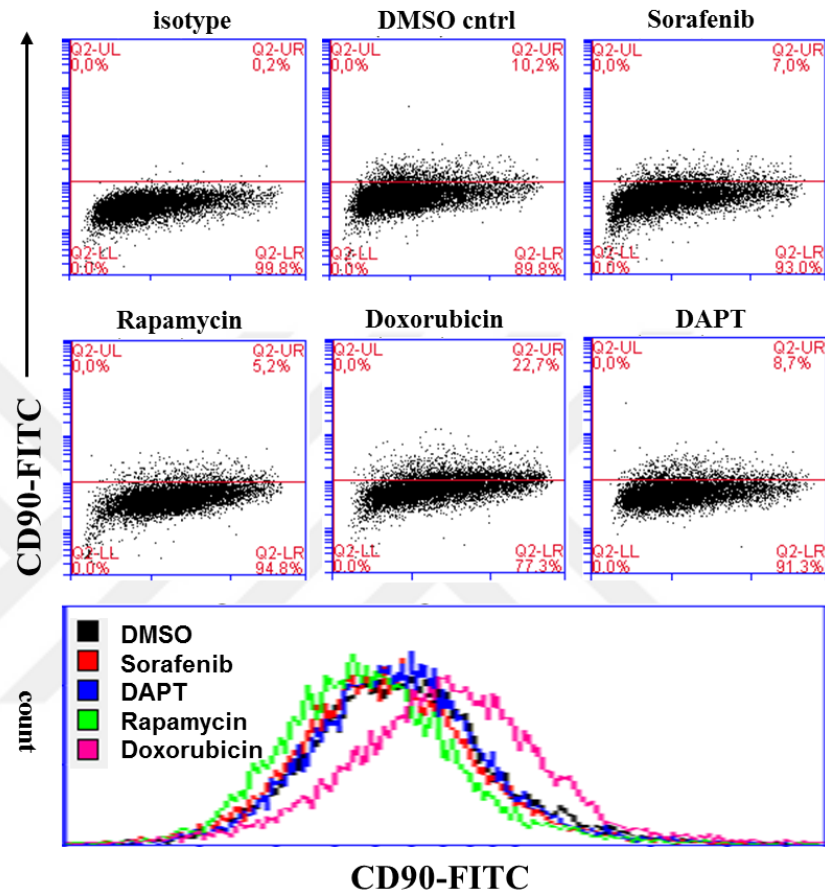
B**SNU475**

Figure 4.7. Positivity of CD90 in Mahlavu and SNU475 cells after treatment with selected inhibitors. Histograms represent relative intensity of CD90 positivity in **A.** Mahlavu and **B.** SNU475 cells; (x-axis) and cell count (y-axis) for each treatment.

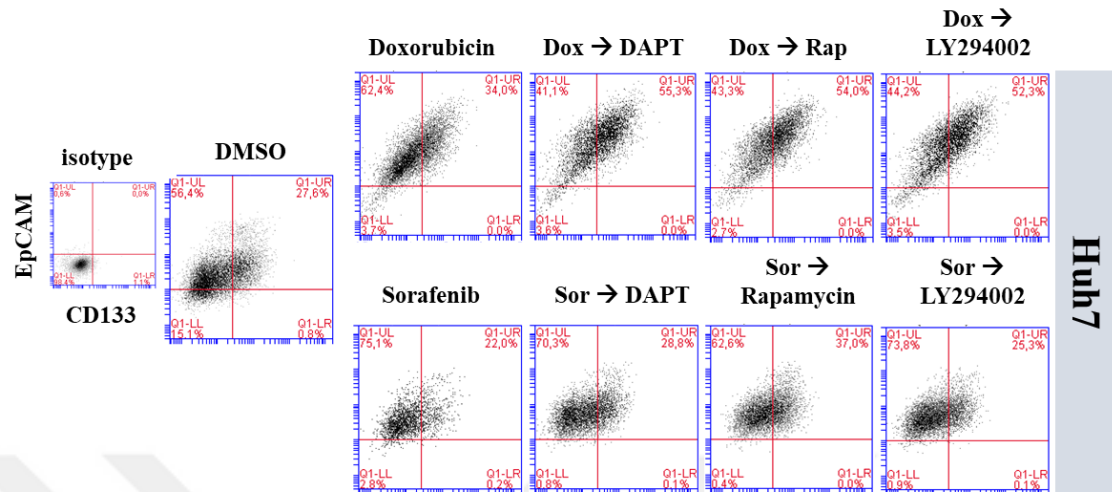
Altogether, it was concluded that Rapamycin was able to prevent enrichment of CSCs both in epithelial and mesenchymal-like HCC cell lines, similar to the effect of DAPT.

4.1.5. Combinatorial treatment of most potent PI3K/Akt signaling pathway inhibitors together with Sorafenib

After evaluating the differential effects of PI3K/Akt/mTOR pathway inhibitors on the enrichment of LCSCs, the potential of combinatorial treatment of HCC cells using selected PI3K/Akt/mTOR pathway inhibitors together with Sorafenib was investigated. For this purpose, two different sequential treatment strategies were followed; a) treatment of cells with Sorafenib or Doxorubicin and then with Rapamycin, LY294002 or DAPT, and b) treatment of cells with Rapamycin or DAPT and then with Sorafenib.

4.1.5.1. Flow cytometry of cells treated following sequential treatment strategies

Results have shown that when cells are treated initially with inhibitors that by themselves cause enrichment of LCSCs, and then with an effective inhibitor (such as Rapamycin, LY294002 or DAPT), the enrichment process of LCSCs were even more enhanced. (Figure 4.8, Figure A3) Therefore, this strategy, named as “sequential treatment A” was tested on Huh7 cells only, since further experiments were not necessary due to the failure of the strategy to inhibit enrichment of LCSCs.

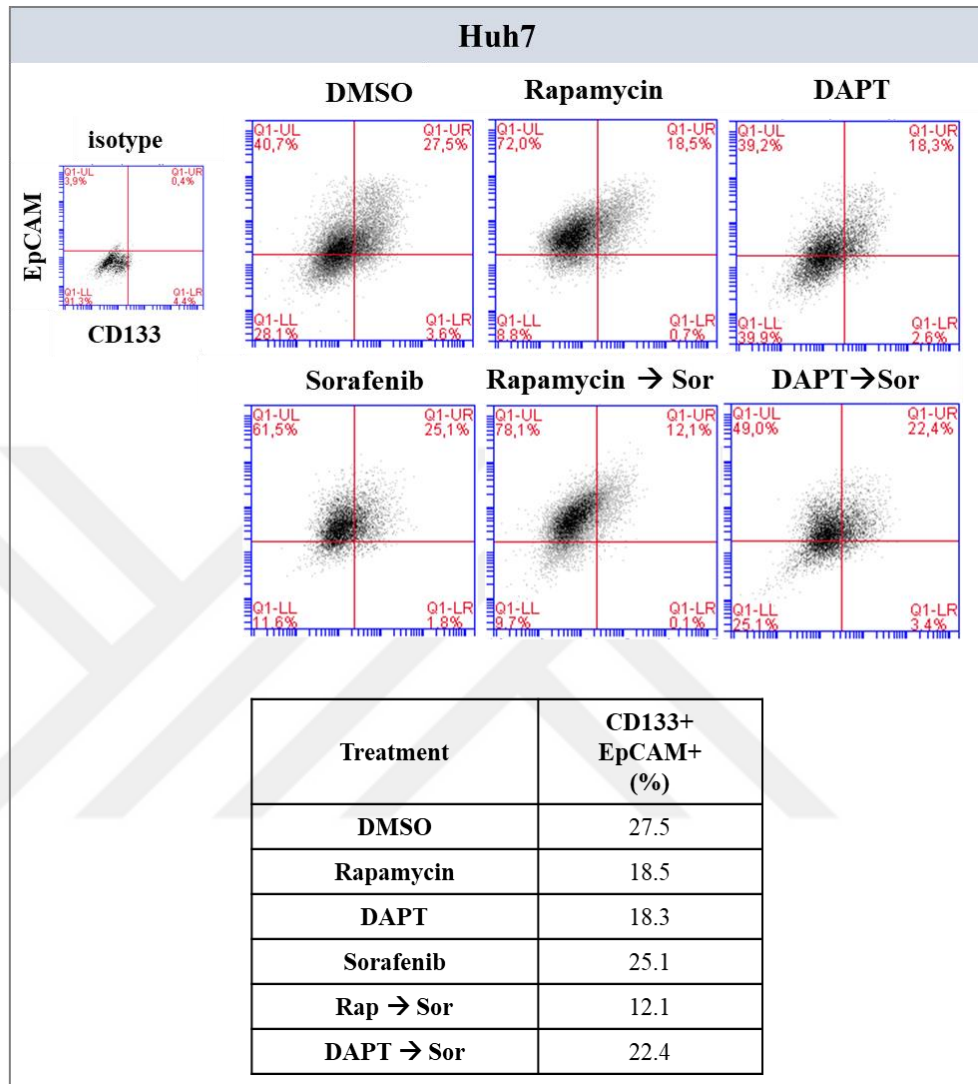


LCSC positivity (%)				
DMSO	DOX	DOX → DAPT	DOX → Rapamycin	DOX → LY294002
27.6	34.0	55.3	55.0	52.3
Sorafenib	Sor → DAPT	Sor → Rapamycin	Sor → LY294002	
22.0	28.8	37.0	25.3	

Figure 4.8. The effect of “sequential treatment strategy A” on LCSCs of Huh7 cells. Representative flow cytometry images and table give percent positivity for CD133 and EpCAM were listed for each treatment. Results of sequentially treated cells were compared to that of 72h of single treatments (Doxorubicin or Sorafenib).

However, when the “sequential treatment strategy B” was applied, it was shown that Rapamycin treatment before Sorafenib decreased number of cells positive for CD133 and EpCAM compared to cells treated with Sorafenib only in both Huh7 and Hep3B cells (Figure 4.9, Figure A4). Thus it was concluded that sequential treatment strategy B was more promising in terms of inhibiting enrichment of LCSCs, which could be addressing an unknown mechanism of Rapamycin on the regulation stemness-related processes.

A



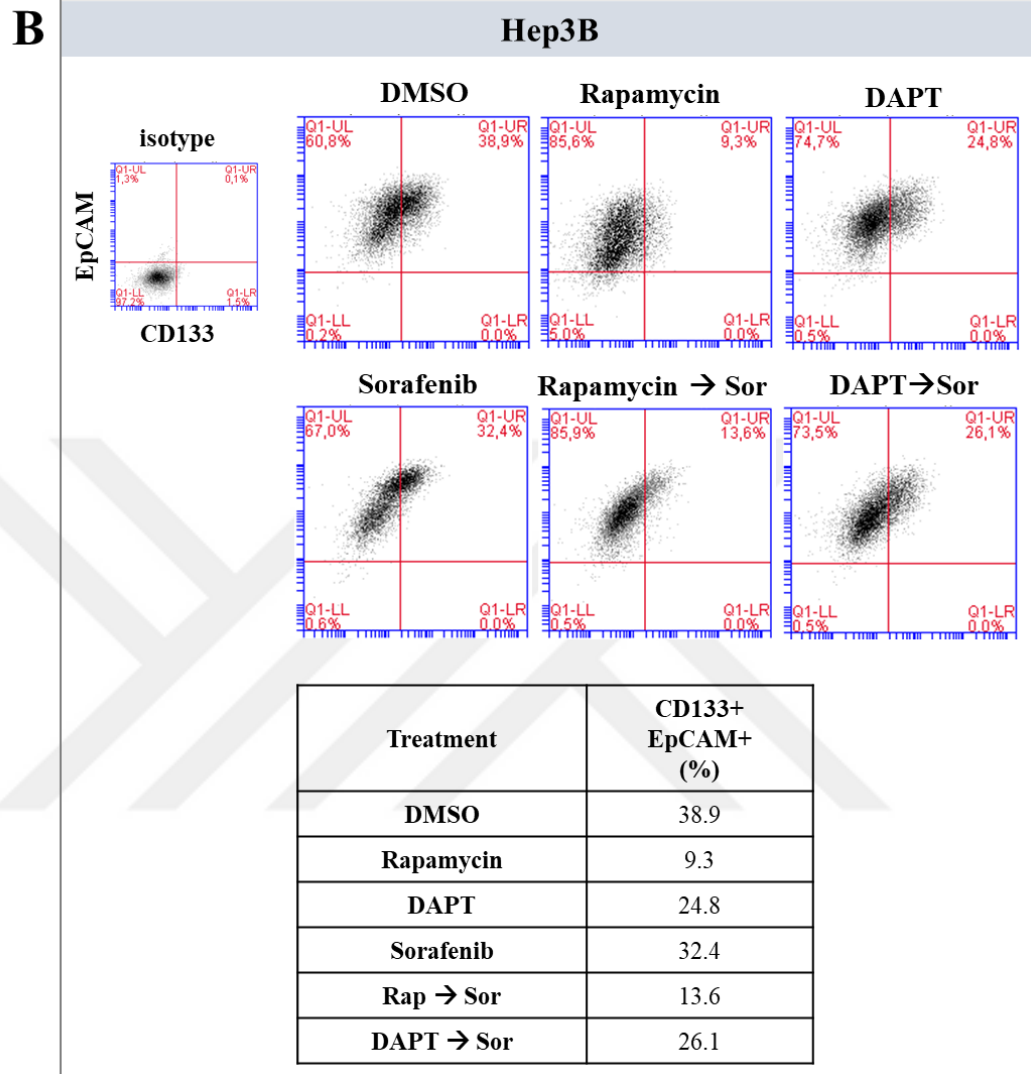


Figure 4.9. Positivity of CD133/EpCAM population of Huh7 and Hep3B cells after “sequential treatment strategy B”. Representative images showing percent positivity of LCSCs in each group of **A.** Huh7 and **B.** Hep3B cells compared with respect to their DMSO controls.

4.1.5.2. Sphere formation analysis of cells treated with Rapamycin, Sorafenib, their combinations and DAPT

To further demonstrate the potential of “sequential treatment strategy B” on regulation of stemness-related events, sphere formation assay was performed (Figure 4.10). The results have shown that, once cells are treated with Rapamycin or DAPT, both the sphere size

and count decreases significantly. Sorafenib treatment alone was shown to increase both the sphere size and the sphere counts. Once cells are treated with Sorafenib prior or at the same time with Rapamycin, the size of the spheres become larger, similar to that of Sorafenib treated cells. On the contrary, when the sequential treatment strategy B is followed, where Rapamycin is given prior to Sorafenib, there is a significant decrease in the size and count of spheres.

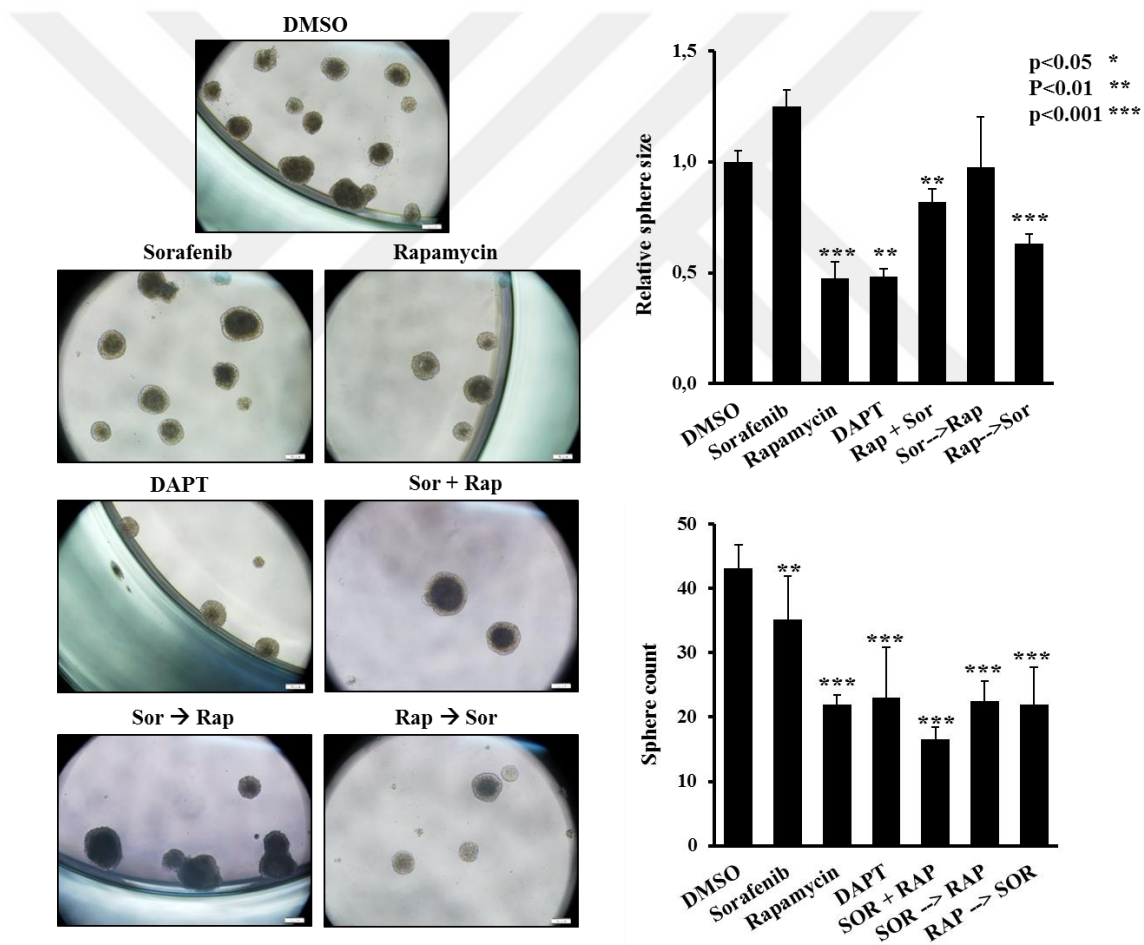


Figure 4.10. Sphere formation capacity of Huh7 cells treated following sequential treatment strategy B. Representative microscopy images represent spheres formed after 6 days by Huh7 cells treated with either a single inhibitor (IC₅₀ conc. for 72h) or in combination (corresponding IC₅₀ conc. for each inhibitor are used for 72h at the same time (Sor+Rap) or sequentially (Rap → Sor or Sor → Rap)) Bar graphs represent sphere

size and sphere count from at least 12 wells at day 6 for each treatment relative to DMSO treated group.

Together with these functional assays, it was concluded that sequential treatment strategy B, which involves treatment of cells with Rapamycin prior to Sorafenib, was promising in terms of inhibiting enrichment of LCSCs compared to Sorafenib treatment alone.

4.1.6. Large scale gene expression analysis of Huh7 cells treated with Rapamycin, Sorafenib, their combinations or DAPT

By taking into consideration, the potential of Rapamycin to attenuate the LCSC enrichment with Sorafenib, a large scale gene expression analysis using the Nanostring nCounter Technology was performed to compare the effects of each treatment group on cancer-related pathways, as well as stemness-related genes of Huh7 cells.

Initially, clustering of 3 different treatment groups was done using the differential expression data to demonstrate differentially regulated cancer pathways (Figure 4.11). This analysis has provided important information about the possible mechanisms involved in regulation of stemness-related pathways of Rapamycin treated cells, where Wnt and Notch pathways were regulated similar to cells treated with DAPT.

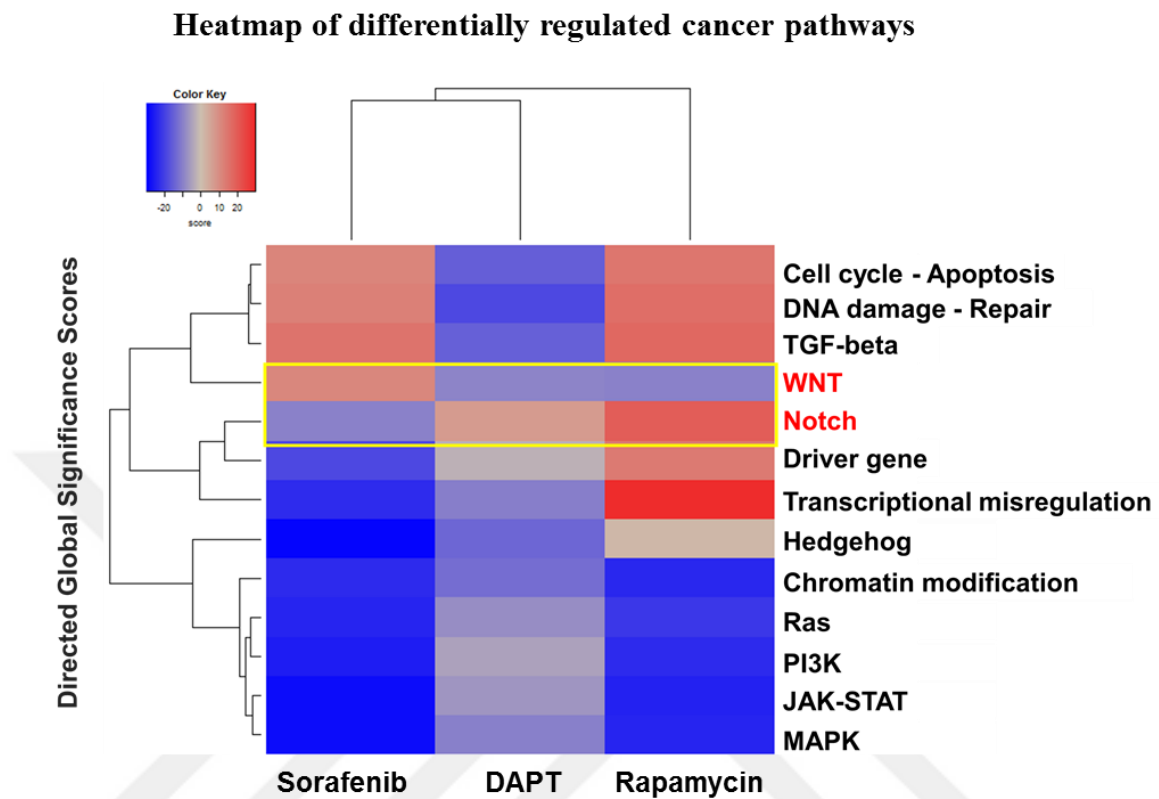


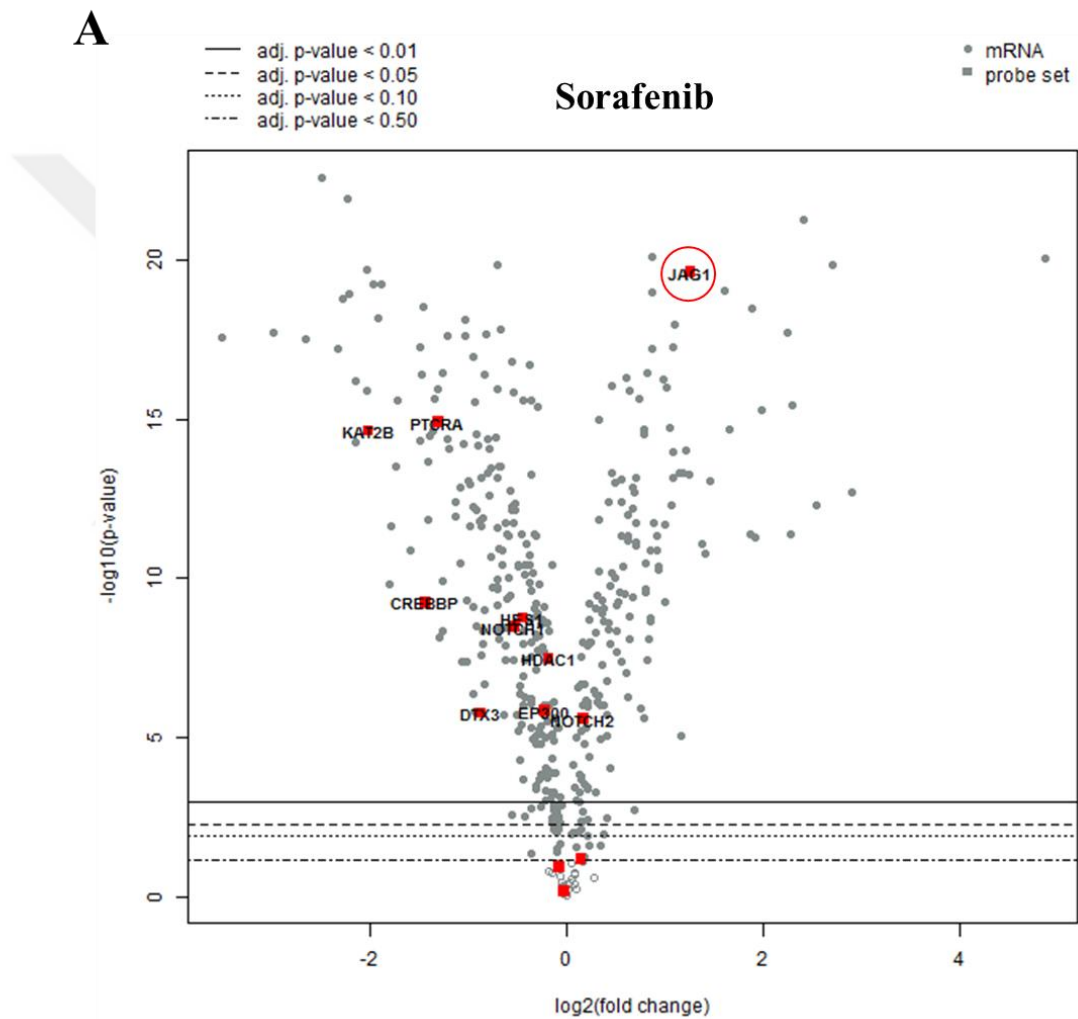
Figure 4.11. Heatmap of differentially regulated cancer pathways. Figure representing the clustering of 3 different treatment groups with respect to differentially regulated pathways involved in cancer.

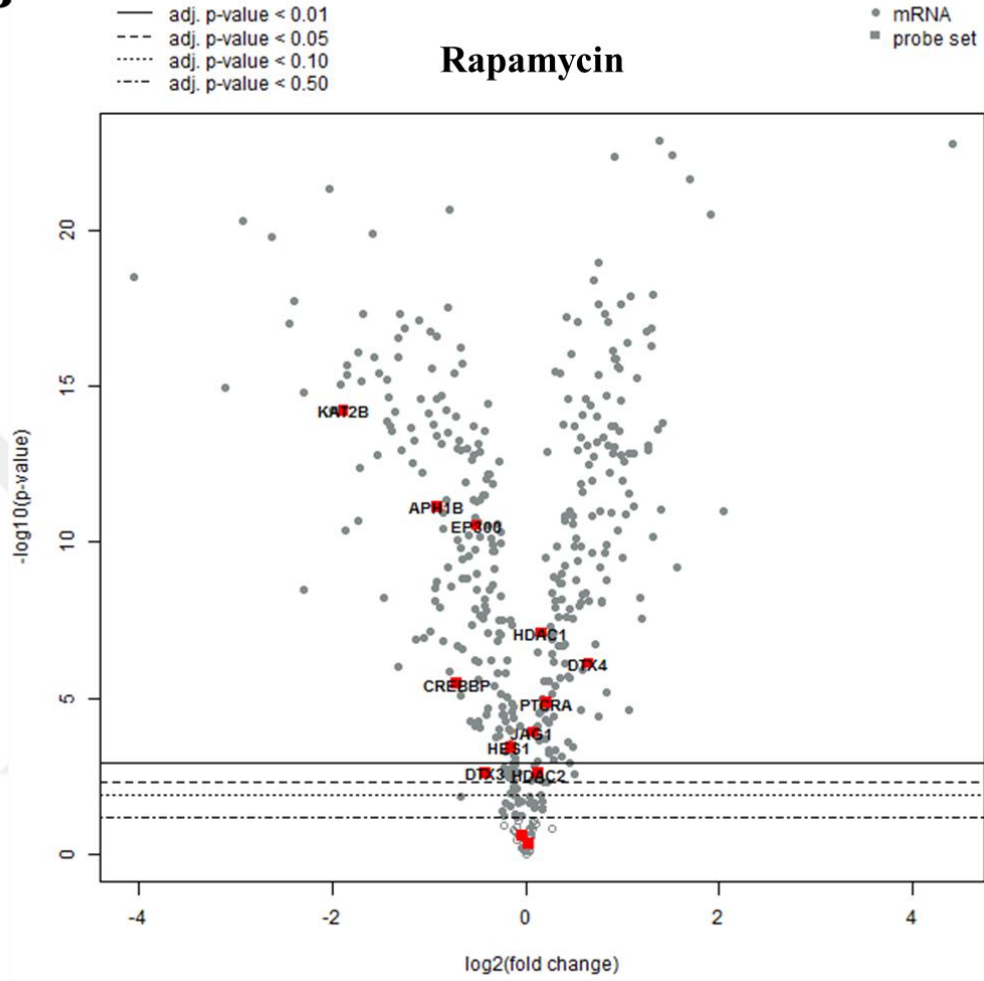
On the contrary, these pathways were found to be differentially regulated once cells were treated with Sorafenib. Other than this information, it has been shown that other than stemness related pathways, genes involved in transcriptional misregulation, driver genes, and hedgehog pathway genes were also differentially regulated in Rapamycin treated cells, compared to other treatment groups.

4.1.6.1. Identification of differentially expressed genes in each treatment group

Once the differential expression of the genes was analyzed, it was revealed that JAG1 expression, which encodes for Jagged-1, a Notch receptor ligand responsible for

enhancing cancer development and angiogenesis in many cancers, was found to be upregulated significantly in cells treated with Sorafenib only. This effect was not observed in any of the other two groups (DAPT or Rapamycin) (Figure 4.12)



B

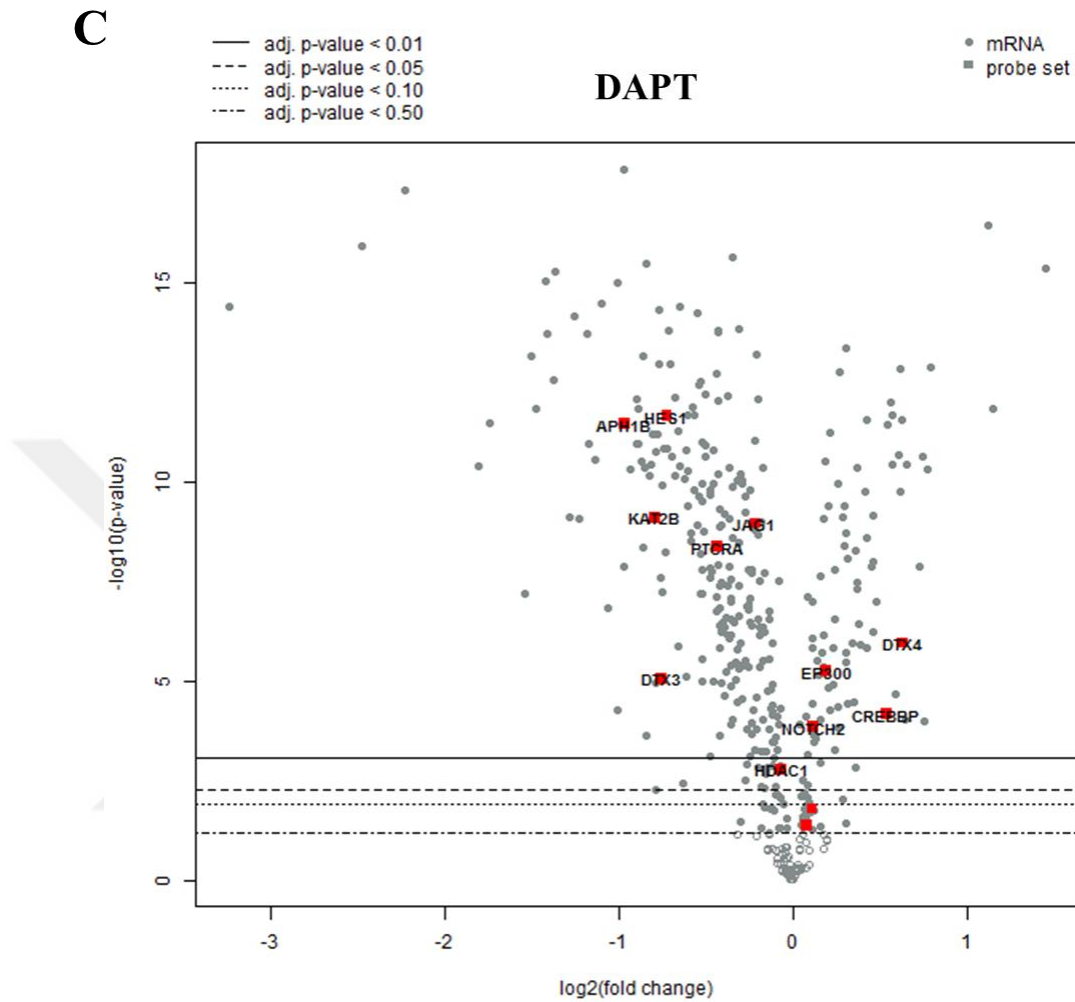


Figure 4.12. Differential expression analysis of genes involved in Notch pathway. Volcano plots representing regulation of genes in **A.** Sorafenib, **B.** Rapamycin, and **C.** DAPT treated Huh7 cells.

When the top 15 differentially expressed genes were analyzed, expression of IL-8, along with FLNA was found to be strongly upregulated upon Sorafenib treatment (~5 fold, log₂). On the contrary, these genes were downregulated upon treatment with DAPT (~-3 fold, log₂), or not changed when treated with Rapamycin (~0.18 fold, log₂). Further analysis was done with qRT-PCR, where gene expression levels of IL-8 were found to be in parallel with the results obtained from the data obtained from the nCounter system

(Figure 4.13). Detailed lists and volcano plots representing gene expression changes are also available in Table A1, Figure A1, A2, and A3).

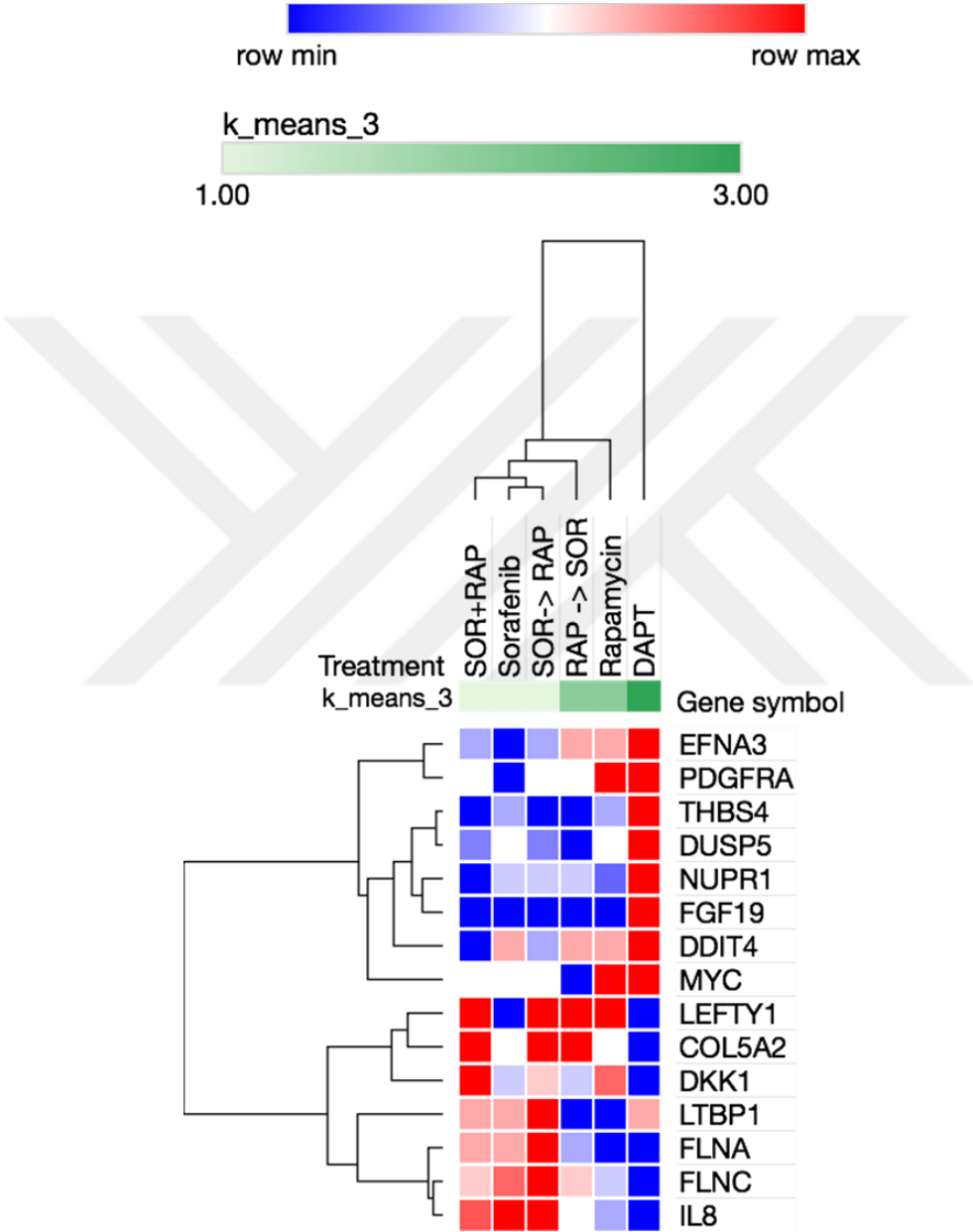


Figure 4.13. Heatmap representing k-means-clustering of top 15 differentially expressed genes.

Additionally, changes in the expression of NANOG was also determined for all treatment groups to find out if there is a correlation between IL-8 and NANOG expression (Figure 4.14). Results have shown that IL-8 and NANOG expression were correlated in almost all experimental groups. These results further supported the idea that IL-8 might be involved in regulation of cancer stem cell survival/enrichment.

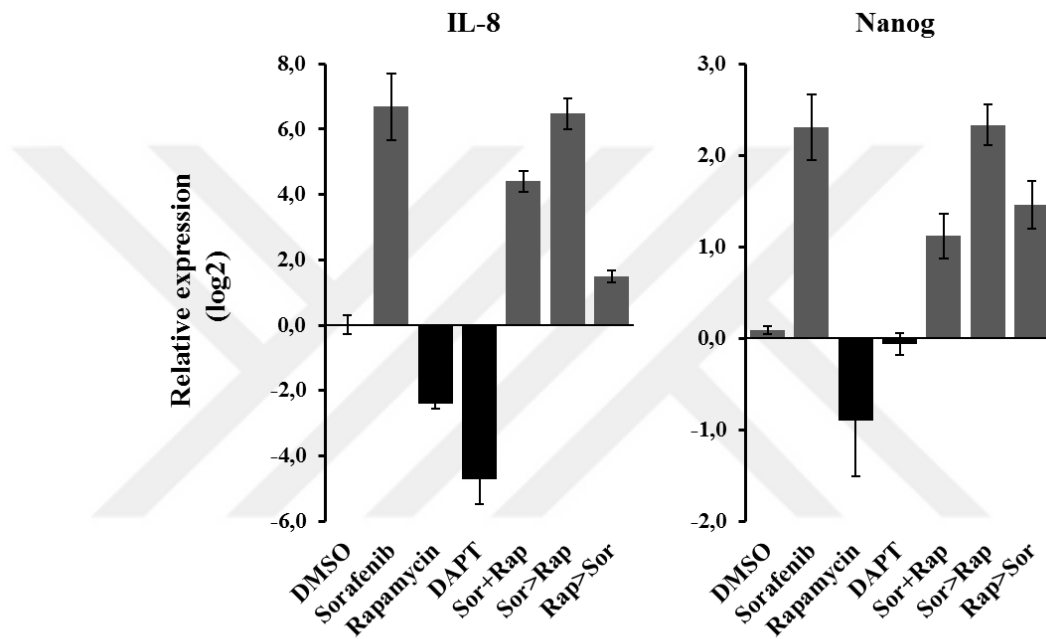


Figure 4.14. Relative expression of IL-8 and NANOG obtained by qRT-PCR.

Altogether, these results have provided information on possible mechanisms involved in the enrichment of LCSCs upon Sorafenib treatment as well as ideas on how Rapamycin might be regulating stemness-related pathways and causing inhibition of LCSC enrichment.

4.1.6.2. Execution of “pathway scoring application” and evaluation of the activity of signaling pathways involved in cancer

The differential expression data obtained from the Nanostring nCounter platform was further used to execute a signal transduction score flow algorithm (“Pathway scoring application”) developed by our lab, in order to verify these results through network-level signaling analysis. This method imitates protein-level signal flow of a cell and specifies a score for each gene involved in “pathways in cancer” KEGG database. The steps followed to adapt the differential gene expression data into this algorithm using Cytoscape platform were described in the methods section and summarized in Figure 4.1. Results showed that IL-8 gained significantly enriched scores, further emphasizing the potential of IL-8 as a target in HCC. (Figure 4.15)

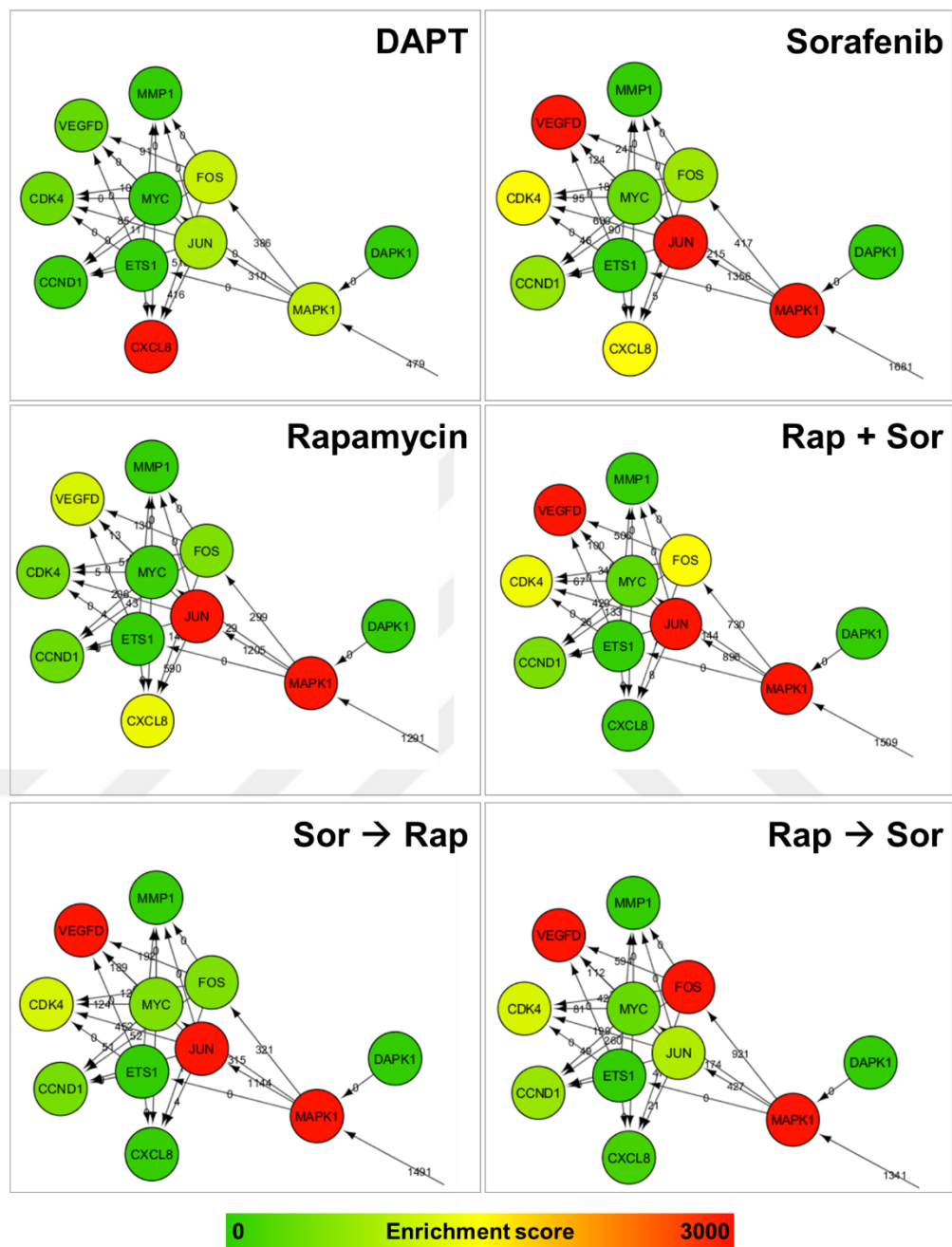


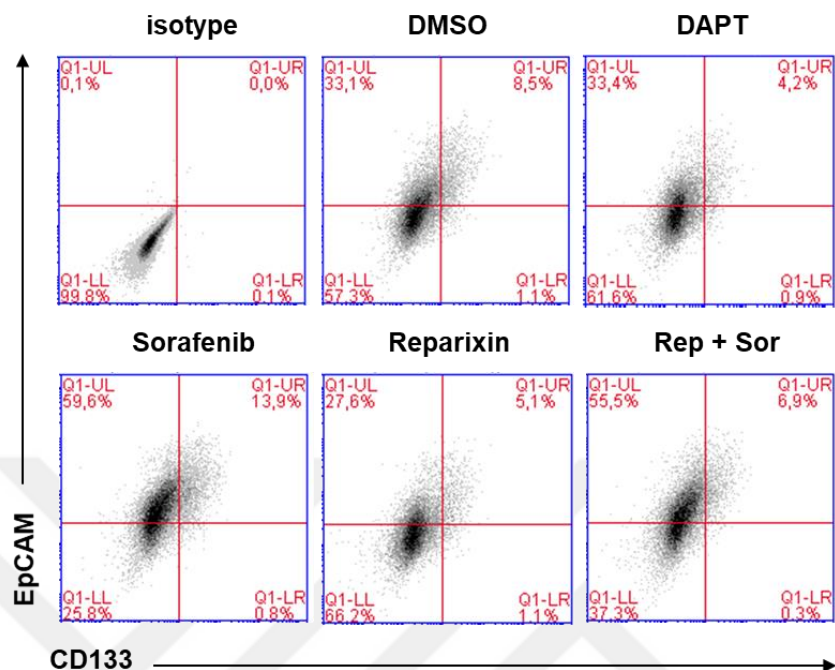
Figure 4.15. Cytoscape representation of differential regulation of IL-8 in Huh7 cells treated with indicated inhibitors.

4.1.7. Effects of IL-8 inhibition on enrichment of LCSCs

The results obtained from both differential gene expression and pathway scoring analysis indicated the possible role of IL-8 signaling in regulation of cancer stem cell enrichment. To test this hypothesis, IL-8 signaling of Huh7 cells was blocked by Reparixin (inhibitor of IL-8), and changes in the expression of LCSC markers and stemness markers along with sphere formation capacity were evaluated. The inhibitory concentration (IC₅₀ value) of Reparixin identified through NCI-60 SRB assay was 50 μ M.

4.1.7.1. Flow cytometry analysis of cells treated with Reparixin only and in combination with Sorafenib

Flow cytometry analysis has shown that inhibition of IL-8 resulted in significant reduction in positivity of CD133/EpCAM markers in Huh7 cells. In addition, when cells are treated with both Reparixin and Sorafenib, the percentage of CD133+/EpCAM+ population decreased as well. (Figure 4.16, Figure A5).



LCSC positivity (%)				
DMSO	DAPT	Sorafenib	Reparixin	Rep + Sor
8.5	4.2	13.9	5.1	6.9

Figure 4.16. Expression of LCSC markers after treatment with Reparixin. Flow cytometry analysis representing positivity of CD133 and EpCAM markers in Huh7 cells treated with IC₅₀ concentrations of indicated inhibitors for 72h.

4.1.7.2. Sphere formation assay of cells treated with Reparixin only and in combination with Sorafenib

Sphere formation assay showed that Huh7 cells treated with Reparixin for 72h lose their capacity to form dense and confluent spheres. Combination of Reparixin and Sorafenib were also as effective as Reparixin alone in terms of decreasing sphere formation capacity of cells. (Figure 4.17)

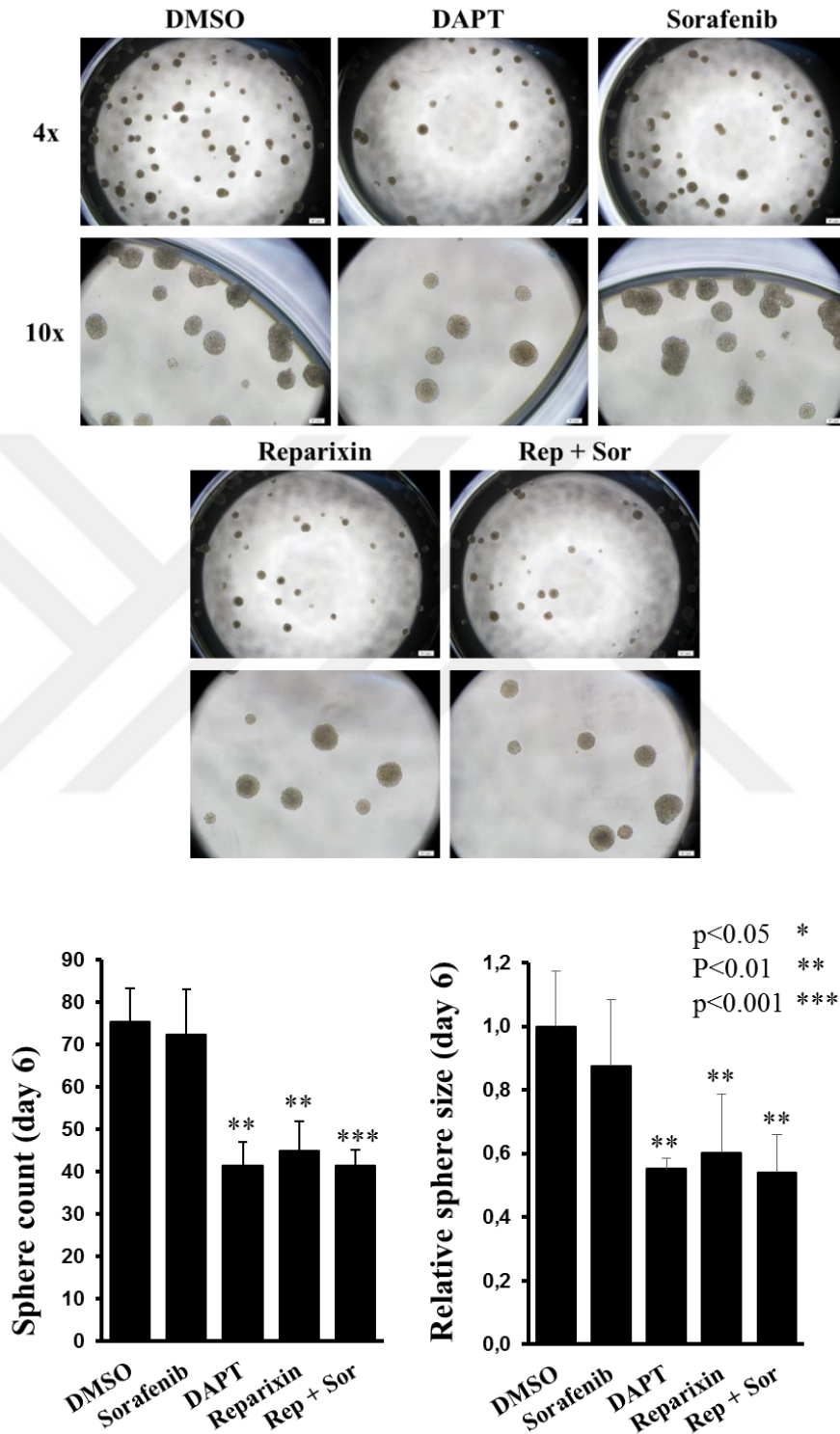


Figure 4.17. Sphere formation capacity of Huh7 cells treated with Reparixin. Representative images of spheres at day 6 formed by Huh7 cells after treatment with indicated inhibitors for 72h. Bar graphs show number and size of spheres.

4.1.7.3. Western blot analysis of SOX2 and Nanog proteins upon treatment with Reparixin and in combination with Sorafenib

Western blot analysis of the spheres collected from the previous step showed that both Nanog and SOX2 levels were decreased in spheres formed by Huh7 cells treated with DAPT, Reparixin. Spheres formed by Sorafenib treated cells had increased levels of both stemness markers. However, the combination of Sorafenib and Reparixin resulted in lower levels of SOX2 and Nanog proteins. (Figure 4.18)

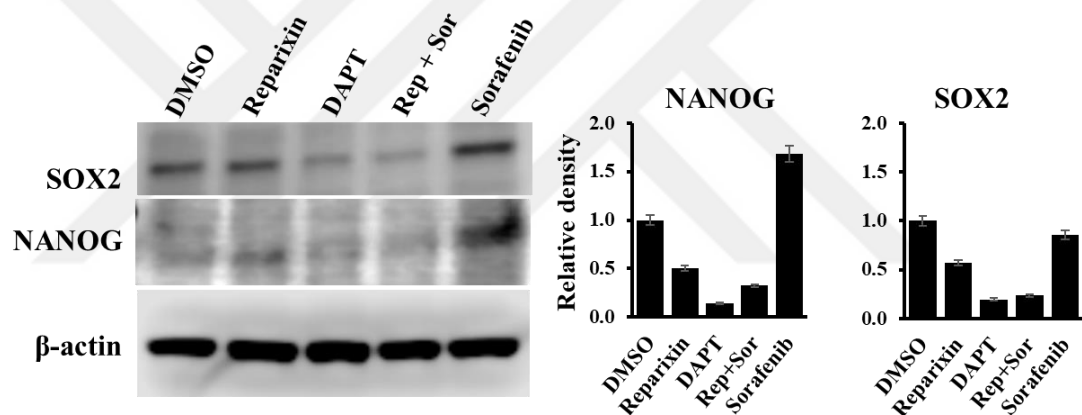


Figure 4.18. Western blot analysis of SOX2 and Nanog in Reparixin treated cells. Representative images of SOX2 and Nanog protein levels by western blot analysis in spheres formed after treatment with indicated inhibitors.

Altogether these results supported the idea that IL-8 signaling is crucial in regulation of LCSC enrichment process and that targeting this pathway could be promising in combination with Sorafenib for the treatment of HCC.

4.2. PART-II: Influence of VEGFR2 TKIs and quinones on the fate of HCC and its cancer stem cells

In this part of the study, efficacies of newly synthesized VEGFR2 TKIs and quinone derivatives against HCC cells and liver cancer stem cells were evaluated. The synthesis of the compounds was done by Gilles Hanquet from Université de Strasbourg, Laboratoire de Stéréochimie and Andrej Bohac from Univerzita Komenskeho v Bratislave, Natural Sciences; Biomagi Ltd. The structures of the tested quinoides and VEGFR2 TKIs are given in Figure 4.19.

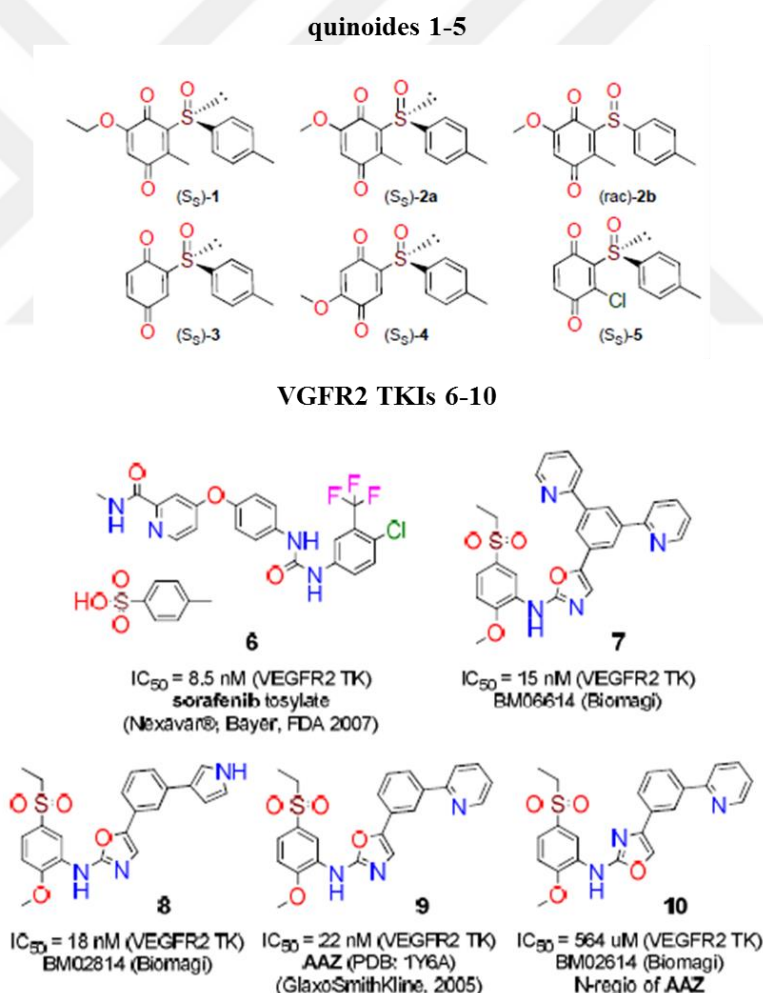


Figure 4.19. Chemical structures of quinoids (1-5) and VEGFR2 TKIs (6-10) tested in this study.

4.2.1. Cytotoxic activities of quinoids and VEGFR2 TKIs on HCC cell lines

The bioactivities of compounds 1-10 together with DAPT on Huh7, Hep3B and Mahlavu cell lines (as CSC inhibitor) were evaluated by NCI-60 SRB assay. Results have shown that IC₅₀ values of quinoids were higher in general (4.3-31.3 μ M) compared to IC₅₀ values of VEGFR2 TKIs (0.4-3.0 μ M) in all cell lines, whereas compound 5 had no inhibitory activity on any of the cells tested (Table 4.2).

Table 4.2. IC₅₀ values of quinoides and VEGFR2 TKIs. Each treatment was done in triplicates, and the standard deviations were calculated accordingly.

Compound	IC ₅₀ (μ M)		
	Huh7	Hep3B	Mahlavu
1	7.0 $\pm$ 0.8	12.0 $\pm$ 1.7	18.3 $\pm$ 0.8
2a	8.0 $\pm$ 0.9	5.5 $\pm$ 1.8	14.5 $\pm$ 1.3
2b	5.5 $\pm$ 0.4	4.3 $\pm$ 0.7	11.6 $\pm$ 0.2
3	20.0 $\pm$ 1.7	17.4 $\pm$ 2.1	31.3 $\pm$ 0.3
4	10.6 $\pm$ 2.0	9.9 $\pm$ 2.3	11.3 $\pm$ 0.6
5	Ni	Ni	Ni
6	1.6 $\pm$ 0.8	1.5 $\pm$ 0.3	0.7 $\pm$ 0.4
7	2.8 $\pm$ 1.3	2.0 $\pm$ 0.9	3.0 $\pm$ 0.5
8	1.7 $\pm$ 0.7	2.1 $\pm$ 0.3	0.8 $\pm$ 0.5
9	1.5 $\pm$ 0.7	0.7 $\pm$ 0.1	1.2 $\pm$ 0.4
10	0.4 $\pm$ 0.3	0.9 $\pm$ 0.3	1.1 $\pm$ 0.7
DAPT	5.0 $\pm$ 1.2	5.0 $\pm$ 2.0	5.0 $\pm$ 1.5

Ni: no inhibition.

4.2.2. Effects of VEGFR2 TKIs on enrichment of LCSCs

Initially, compounds 1-10 were studied to determine their efficacy to inhibit LCSC enrichment in Huh7 cells. The cells were treated with the IC₅₀ concentrations of the compounds, and the cells that remained were stained for CD133 and EpCAM to analyze by flow cytometry. Interestingly, some of the compounds were able to reduce the percentage of LCSC, while some enriched this population. In general, the VEGFR2-TKIs

were not effective on LCSC inhibition, whereas some of the quinoides were able to attenuate the side population of Huh7 cells (Figure 4.20).

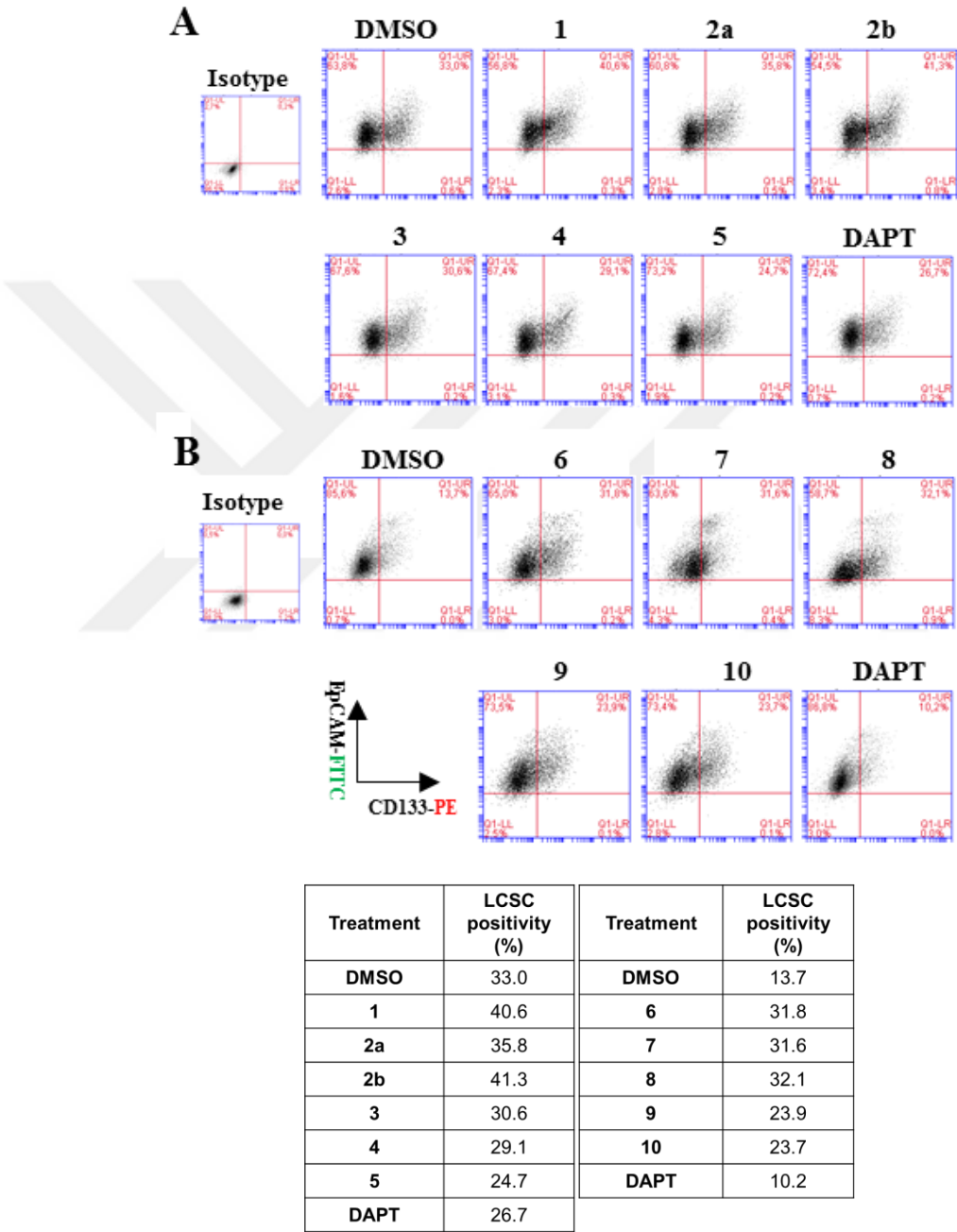


Figure 4.20. Expression of LCSC markers after treatment with quinoids or VEGFR2 TKIs. Flow cytometry results of Huh7 cells treated with **A.** quinoids 1-5 and **B.** VEGFR2 TKIs 6-10 and DAPT. Tables show the percentage of CD133+/EpCAM+ cells after each treatment.

Bioactivities of quinoids were also evaluated for SNU475. The IC_{50} values were 5.5 ± 1.4 , 10.5 ± 1.5 , 6.1 ± 2.0 , 0.3 ± 1.2 , 9.0 ± 2.1 , and 0.6 ± 1.1 μ M for compounds **1**, **2a**, **2b**, **3**, **4**, and DAPT, respectively. No growth inhibition was observed for compound **5**.

Moreover, the alterations in the percentage of CSC population of Hep3B, Mahlavu, and SNU475 after treatment with quinoids were determined to test whether similar effects would be observed on other HCC cell lines. Among all compounds tested, **3** was able to decrease the CSC percentage, especially in mesenchymal-like cells, exceeding the efficacy of DAPT (Figure 4.21).

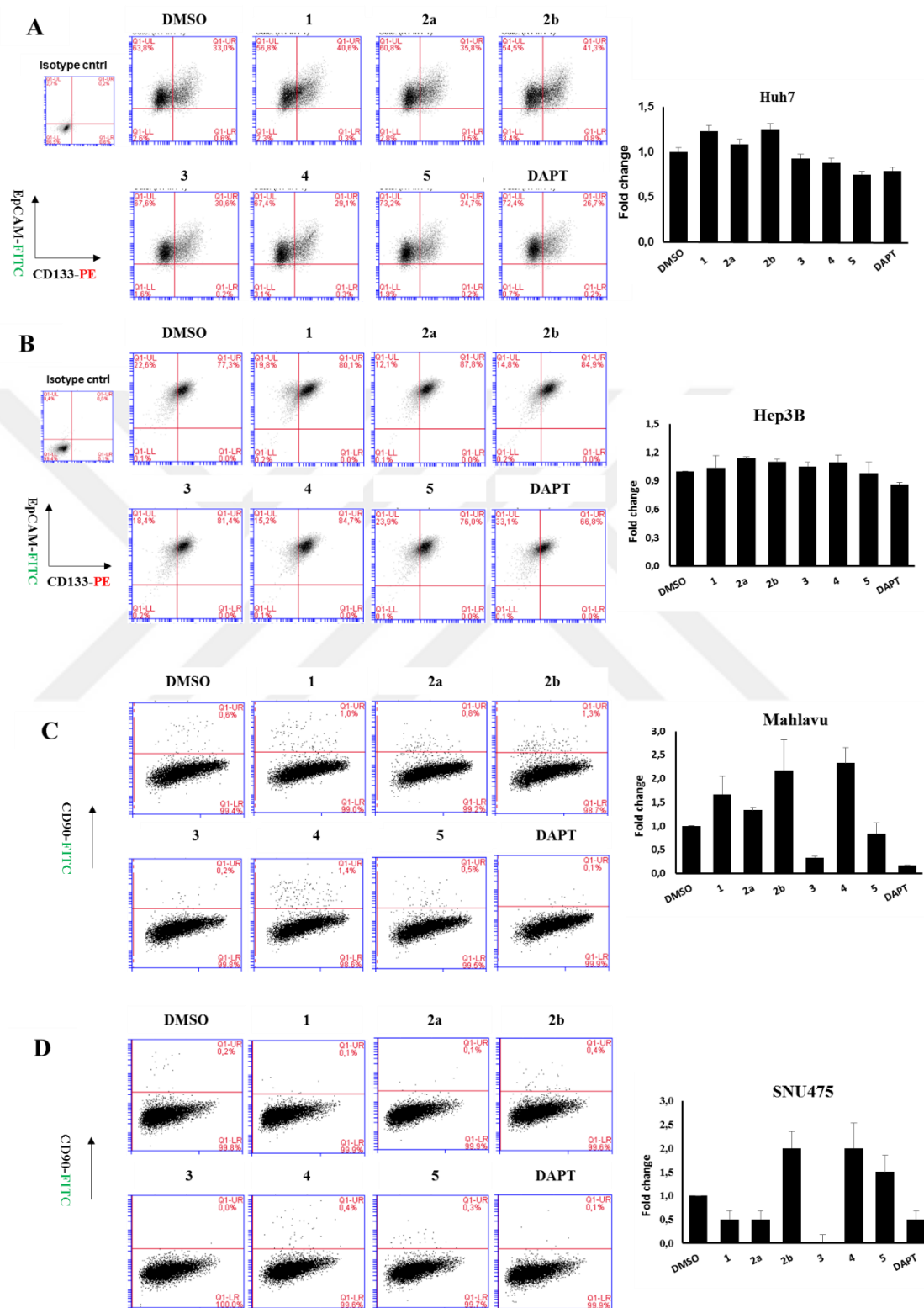


Figure 4.21. Expression of LCSC markers in HCC cells after treatment with quinoids. Flow cytometry analysis of HCC cell lines after 72 hours of treatment with

quinoides 1-5. A. Huh7, B. Hep3B, C. Mahlavu, D. SNU475. Cancer stem cells were defined by their positivity for CSC markers; CD133 together with EpCAM (for Huh7 and Hep3B), or CD90 (for Mahlavu and SNU475). Each treatment was compared to its corresponding DMSO control to define the changes in percentage of double positive population. Bar graphs indicate the fold change in positivity for CSC markers relative to DMSO control groups.

4.2.3. Evaluation of VEGFR2 TKIs and quinones for their ability to inhibit enrichment of LCSCs

After defining the effects of both quinoids and VEGFR2 TKIs, on both liver cancer cells and liver cancer stem cells, each compound was given a value representing its potential to eliminate or enhance cancer stem cell survival. This value was defined as “aggressiveness factor (A_F)” and calculated based on the cancer stem cell density that remains alive after treatment vs. before treatment. Such that, A_F value for a compound = [(total number of CSCs after exp. cmpd. treatment) / (total number of CSCs before treatment)]. It reflects the fold increase of CSCs that survived the treatment independent of shrinkage of the tumor itself. Therefore, the lower the A_F value, the lower the ability of the drug candidate to enhance more aggressive tumors or drug resistance. (Table 4.3) Thus, compounds with A_F values that are above 1 signifies molecules that enrich CSC population, whereas values that remain below 1 signifies compounds that are able to attenuate cancer stem cell enrichment.

Table 4.3. IC₅₀ values and AF factors of quinoides and VEGFR2-TKIs on HCC cell lines. A. Huh7 and Hep3B epithelial cells and, B. Mahlavu and SNU475 mesenchymal cells.

A	IC₅₀ (μM)		A_F	
	Huh7	Hep3B	Huh7	Hep3B
1	7.0	12.0	1.2	1.0
2a	8.0	5.5	1.1	1.1
2b	5.5	4.3	1.3	1.1
3	20.0	17.4	0.9	1.1
4	10.6	9.9	0.9	1.1
5	Ni	Ni	0.7	1.0
6	1.6	1.5	2.3	2.3
7	2.8	2.0	2.3	2.9
8	1.7	2.1	2.3	2.1
9	1.5	0.7	1.7	1.7
10	0.4	0.9	1.7	2.6
DAPT	5.0	5.0	0.8	0.7

B	IC₅₀ (μM)		A_F	
	MV	SNU-475	MV	SNU-475
1	18.3	5.5	1.7	0.5
2a	14.5	10.5	1.3	0.5
2b	11.6	6.1	2.2	2.0
3	31.3	0.3	0.3	0.0
4	11.3	9.0	2.3	2.0
5	Ni	Ni	0.8	1.5
DAPT	5.0	0.6	0.2	0.5

Ni: no inhibition

Altogether, these results have demonstrated that, unlike VEGFR TKIs such as Sorafenib, some quinoids have the potential to attenuate the survival of liver cancer stem cell population within the heterogeneous cancer cells independent of their cytotoxic potential (IC₅₀ values).

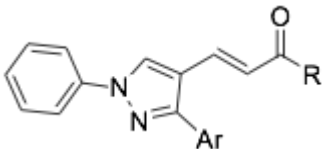
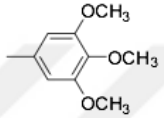
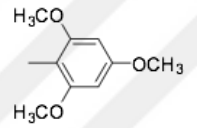
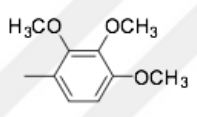
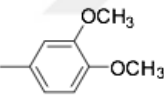
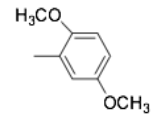
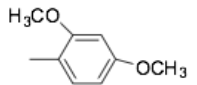
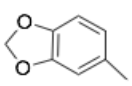
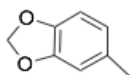
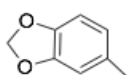
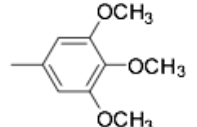
4.3. PART-III: Biological Evaluation of Novel Pyrazolic Chalcone Derivatives as Novel HCC therapeutics

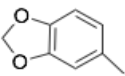
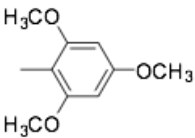
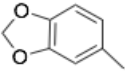
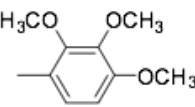
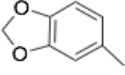
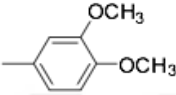
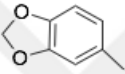
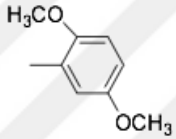
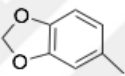
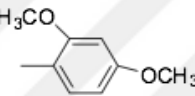
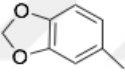
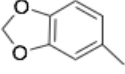
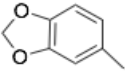
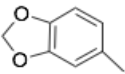
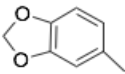
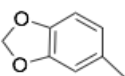
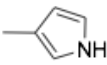
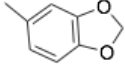
In this part of the study, biological evaluation of newly synthesized pyrazolic chalcone derivatives against HCC cell lines was demonstrated. The design and synthesis of the molecules were done by Prof. Dr. Sultan Baytaş from Department of Pharmaceutical Chemistry of Gazi University.

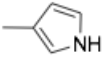
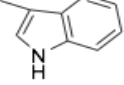
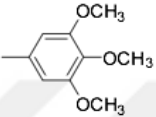
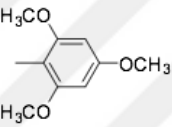
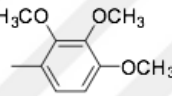
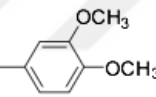
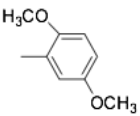
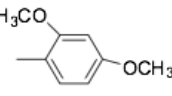
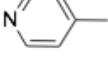
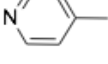
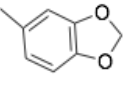
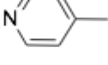
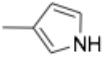
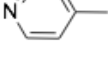
4.3.1. Cytotoxic activities of pyrazole chalcone derivatives on cancer cells

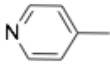
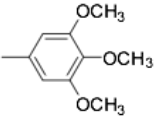
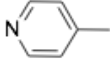
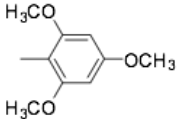
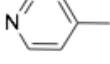
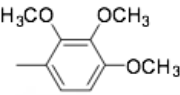
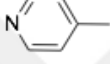
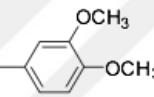
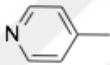
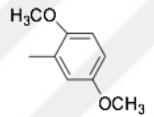
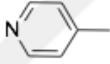
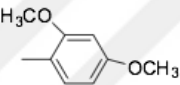
41 different pyrazolic chalcone derivatives (12-53) were screened against Huh7 (HCC), MCF7 (breast cancer), and HCT116 (colon cancer) cells by NCI-60 SRB assay and IC_{50} values after 72 hours of treatment were evaluated for each compound. Bioactivities of 2 well-known chemotherapeutic agents, nucleobase analog 5-fluorouracil (5-FU) and nucleoside analog cladribine were also determined to compare the results to that of the novel compounds. Results have shown that the IC_{50} values were between 0.3-29 μ M (Table 4.4). Among all compounds, 39, 42, 49, and 52 were found to have very low IC_{50} values (0.3-2.6 μ M) in all cell lines, They were further tested in a panel of HCC cell lines (Huh7, HepG2, Mahlavu, and SNU475), and normal-like cells MCF12A (normal breast epithelial cells), and MRC5 (human fetal lung fibroblast cells). In addition, when compared to the activity of known chemotherapeutic agents, it was shown that IC_{50} values of these compounds were lower than 5-FU and comparable to that of cladribine.

Table 4.4. Chemical structures and IC₅₀ values of compounds 12-53.

 IC₅₀ (μM)					
Compound	Ar	R	Huh7	MCF7	HCT116
12			4.7±0.74	1.9±0.30	5.1±0.55
13			3.6±0.58	4.9±0.01	3.2±0.65
14			6.1±0.03	2.7±0.54	5.2±1.47
15			4.2±0.47	7.4±0.59	5.8±1.57
16			2.9±0.54	2.8±0.51	1.6±0.32
17			2.1±1.37	0.4±0.25	3.4±0.48
18			3.0±0.54	9.0±1.05	2.2±0.43
19			22.6±0.87	5.3±0.22	12.2±0.78
20			10.5±0.33	11.1±0.59	1.16±0.01
21			11.7±0.53	10.9±1.25	8.3±1.07

Compound	Ar	R	Huh7	MCF7	HCT116
22			6.7±0.21	11.5±0.32	10.8±0.98
23			10.8±0.8	7.4±0.17	3.2±0.54
24			15.4±0.9	11.7±0.37	11.4±0.01
25			6.1±0.11	5.8±0.82	1.3±0.36
26			19.0±1.01	14.5±0.6	8.6±0.9
27			3.2±0.44	4.9±0.04	8.9±0.71
28			3.4±0.71	4.1±0.04	6.7±0.47
29			3.4±0.9	2.2±0.55	3.8±0.20
30			9.9±0.77	17.5±0.7	27.2±1.0
31			9.2±0.95	9.9±0.27	20.3±0.56
32			1.7±0.51	0.5±0.04	1.3±0.9
33			6.4±0.8	4.2±0.57	2.1±0.35
34			6.7±0.31	5.6±0.77	1.8±0.67

Compound	Ar	R	Huh7	MCF7	HCT116
35			3.3±0.78	6.9±0.05	7.4±1.02
36			3.2±1.07	2.6±0.00	6.4±0.24
37			2.3±0.49	4.1±0.05	2.0±0.71
38			1.9±0.53	14.5±0.06	8.6±0.35
39			0.5±0.17	1.0±0.50	0.3±0.15
40			2.3±1.43	1.4±0.14	1.1±0.46
41			3.1±0.32	1.7±0.84	4.3±1.20
42			1.3±0.16	1.5±0.52	1.0±0.50
43			2.4±0.56	2.3±0.02	1.3±0.75
44			2.2±0.5	1.5±0.58	1.1±0.60
45			0.4±0.14	0.8±0.02	5.3±0.90
46			28.8±1.85	13.6±1.47	15.7±0.66
47			1.8±0.51	3.4±0.44	3.1±0.88

Compound	Ar	R	Huh7	MCF7	HCT116
48			3.5±0.07	3.5±0.04	0.3±0.29
49			2.6±0.58	1.6±0.66	0.6±0.18
50			3.7±0.03	3.3±0.04	1.3±0.86
51			2.0±0.25	4.3±0.07	1.2±0.32
52			1.7±0.21	1.4±0.38	0.9±0.23
53			4.4±0.52	8.8±0.18	4.4±0.52
5-FU			21.0±0.75	1.4±0.26	18.4±1.1
Cladrabine			1.8±0.43	2.0±0.11	0.3±0.15

Results have shown that selected pyrazolic chalcone derivatives were cytotoxic against all HCC cells tested, yet they were less cytotoxic against normal-like epithelial cells. In addition, compound 49 had no cytotoxic effect on MRC5 cells. (Table 4.5)

Table 4.5. IC₅₀ values of compounds 39, 42, 49 and 52 on HCC cell lines. (Huh7, HepG2, Mahlavu and SNU475) and epithelial cells (MCF12A and MRC-5).

Compound	IC ₅₀ Values (μM)					
	Huh7	HepG2	Mahlavu	SNU-475	MCF12A	MRC-5
39	0.5 ± 0.2	1.4 ± 0.2	1.1 ± 0.2	0.8 ± 0.1	1.2 ± 0.5	1.3 ± 0.6
42	1.3 ± 0.2	3.4 ± 0.8	2.0 ± 0.3	1.9 ± 0.1	2.4 ± 0.8	2.7 ± 0.9
49	2.6 ± 0.6	1.7 ± 0.1	1.1 ± 0.1	1.4 ± 0.3	8.2 ± 0.6	NI
52	1.7 ± 0.9	4.8 ± 0.3	1.6 ± 0.2	2.0 ± 0.3	3.0 ± 0.6	3.1 ± 0.9

NI: no inhibition

4.3.2. Real-time cellular response of HCC treated with selected compounds

In order to determine the time-dependent bioactivities of the selected pyrazolic chalcone derivatives on HCC cell lines, real-time cell electronic sensing (RT-CES) technology was used. The cells were monitored for 48 hours under the influence of compounds 39, 42, 49 and 52 and the growth curves were compared to that of DMSO treated cells. The results have shown that, all compounds were able to reduce the growth rate of HCC cells (Figure 4.22). This effect was predicted to be an effect related to an arrest in cell cycle progression, since the cells are neither proliferating nor dying as opposed to DMSO control group, where cells continue to proliferate and reach confluence [116].

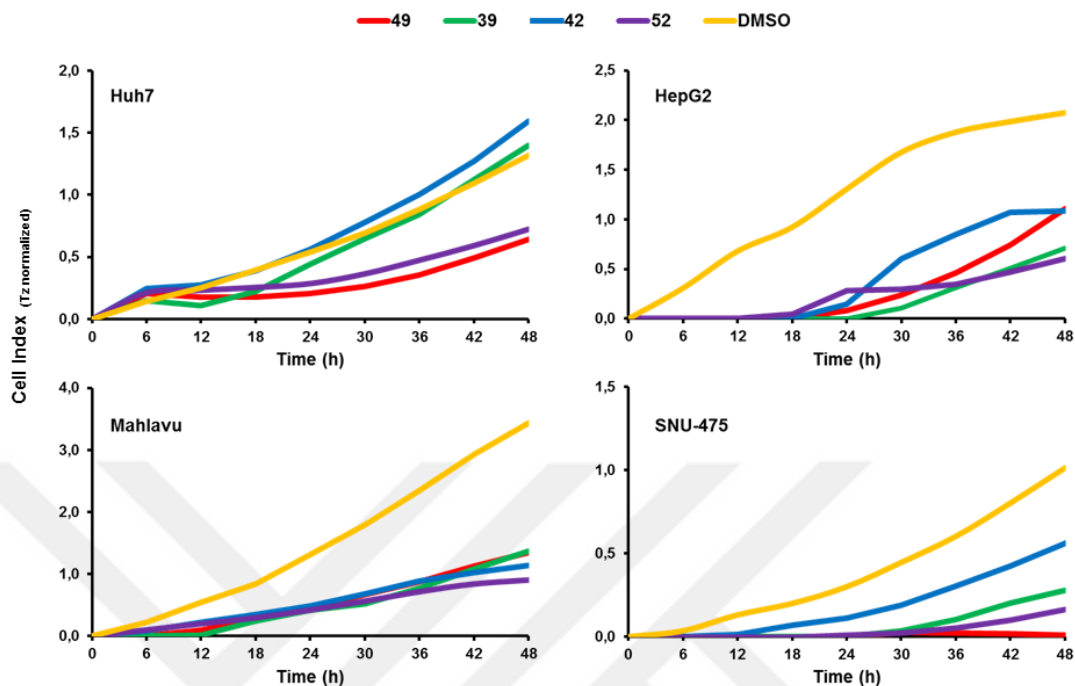


Figure 4.22. Growth curve of HCC cell lines after treatment with selected compounds. RT-CES analysis of HCC cell lines treated with IC₁₀₀ concentrations of selected compounds 39, 42, 49, and 52 for 48 hours. Each treatment was done in triplicates, and DMSO control did not exceed 0.1% of the total volume.

For the following experiments, HepG2 and SNU475 cells were used representative of epithelial-like and mesenchymal-like cells.

4.3.3. Cell death mechanisms induced by pyrazolic chalcone derivatives

To determine the mechanism by which the selected pyrazolic chalcone derivatives cause cell death, Annexin V staining was done, and cells were visualized under fluorescence microscopy. Live images of cells have shown that after 48 hours of treatment with the compounds, cells started losing their capacity to attach to the surface of cell culture dishes (Figure 4.23).

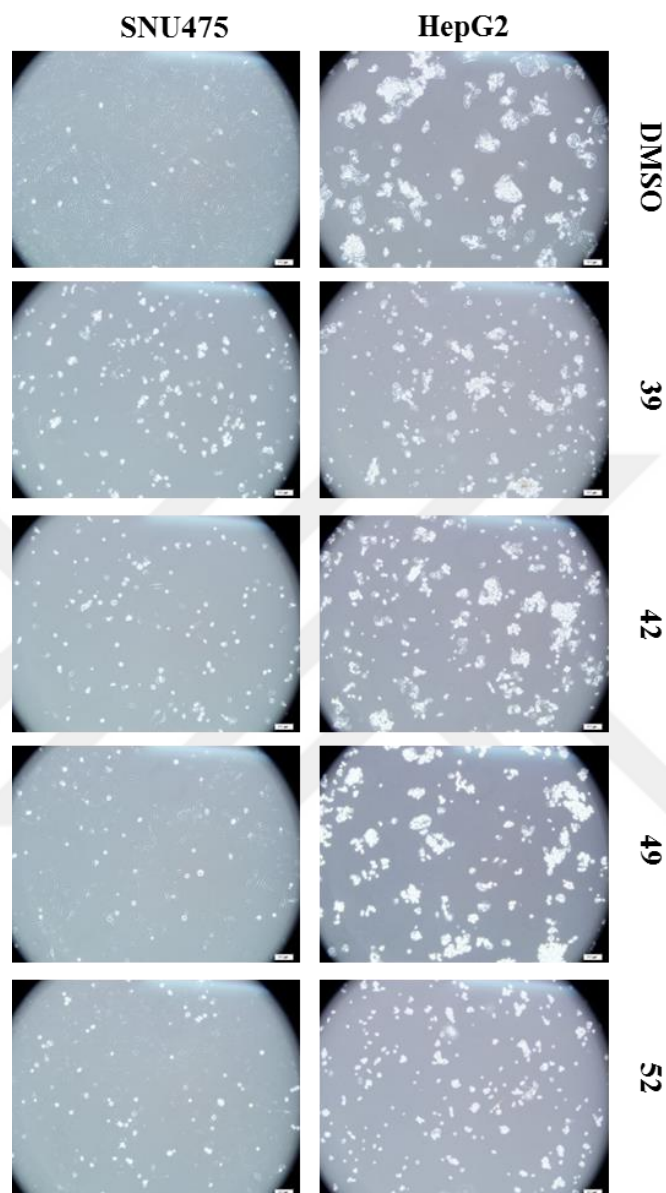
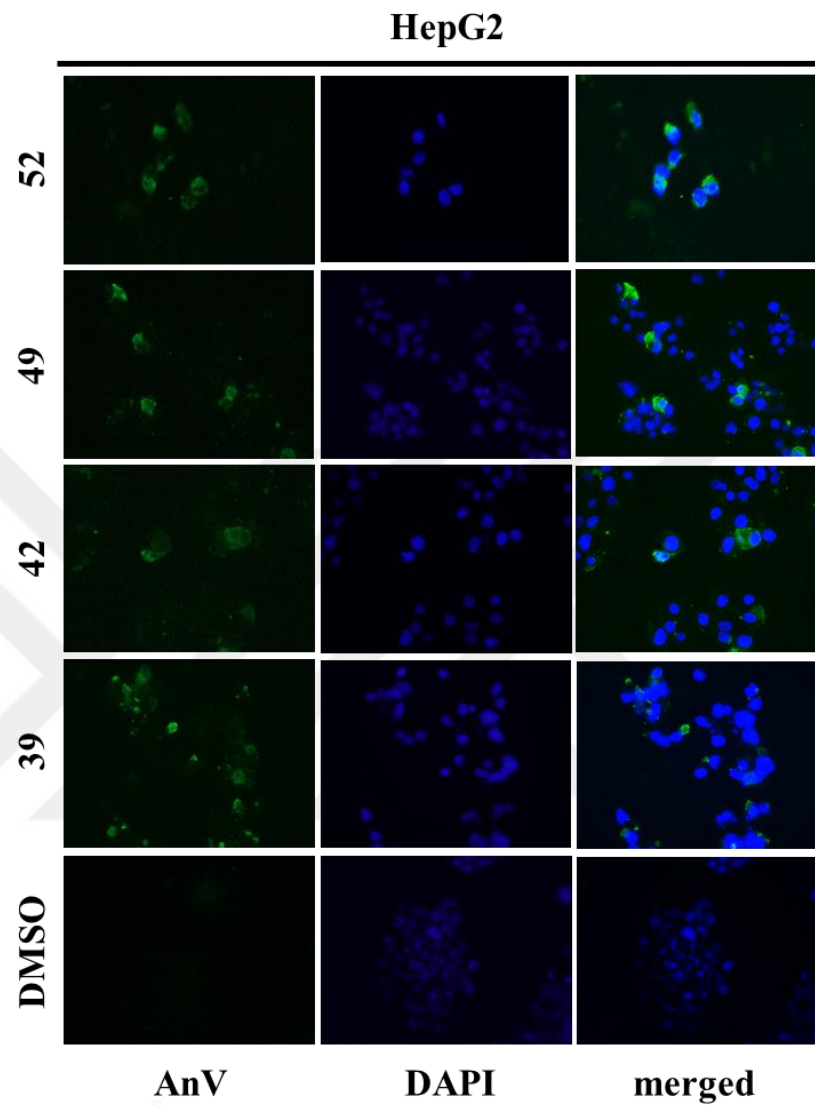


Figure 4.23. Live images of HepG2 and SNU475 cells after treatment with selected compounds. Treatments were done with IC₅₀ concentrations of selected compounds **39**, **42**, **49** and **52** for 48h.

Fluorescence microscopy results have shown that cells were positive for Annexin V and had condensed nuclei which indicated that these compounds were causing apoptotic cell death (Figure 4.24). In addition, after 24 hours of treatment, PARP cleavage was detected by western blot analysis, especially in SNU475 cells (Figure 4.25).



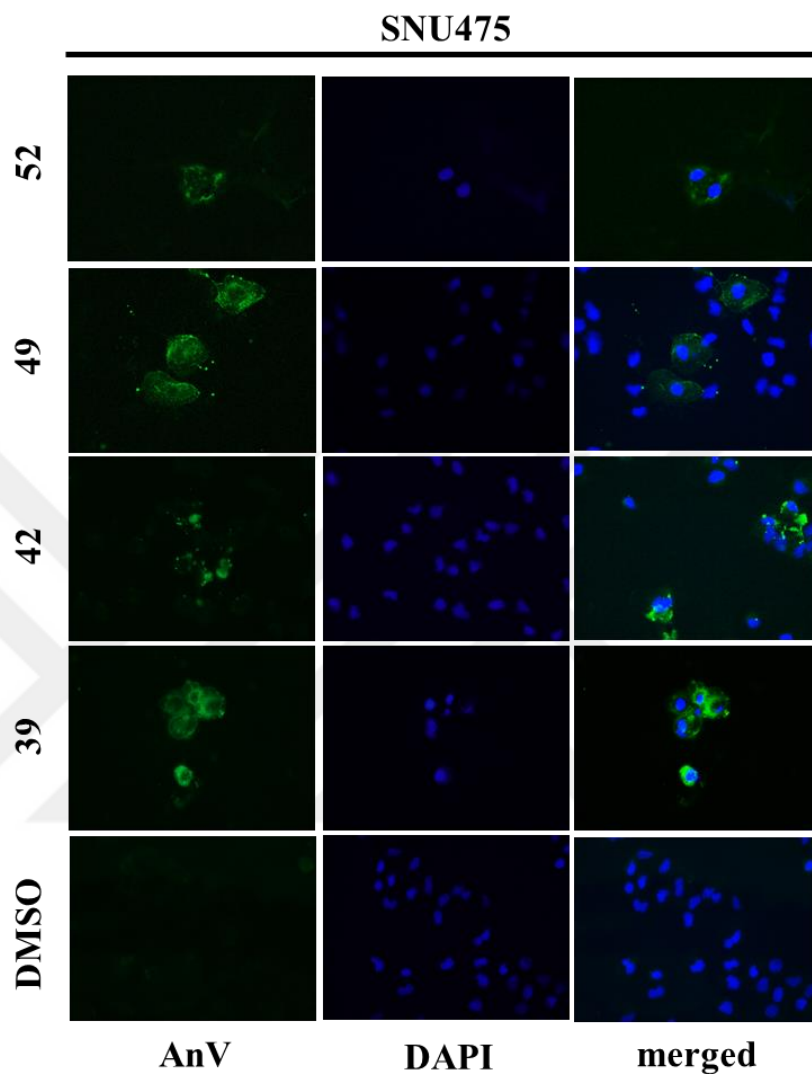


Figure 4.24. Detection of apoptotic cell death after treatment of HCC cells with selected compounds. Representative fluorescence microscopy images of HepG2 and SNU475 cells treated with IC₅₀ concentrations of selected inhibitors for 48h. Condensed nuclei appear in bright blue (DAPI) which is co-localized with membrane stained with Annexin V (green).

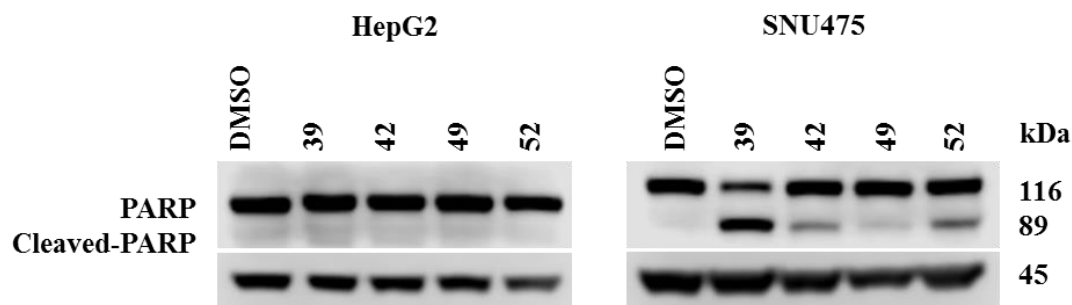


Figure 4.25. PARP cleavage induced by selected inhibitors. Western blot images of PARP protein (116 kDa) and cleaved-PARP (89 kDa) from whole lysates of HepG2 and SNU475 cells treated with IC₁₀₀ concentrations of selected inhibitors for 24 hours.

Altogether these results have revealed that selected pyrazolic chalcone derivatives cause apoptotic cell death, especially in mesenchymal-like cells (SNU475).

4.3.4. Induction of cell cycle arrest in G2/M phase of HCC cells by pyrazolic chalcone derivatives

Cell cycle analysis of HepG2 and SNU475 cells were performed, since the growth pattern of cells in RT-CES analysis suggested a possible arrest in the progression of the cell cycle. Following treatment with IC₁₀₀ concentrations of each compound for 24, 48 and 72 hours, PI-stained cells were analyzed using flow cytometry. Results have shown that percentage of cells in G2/M phase increased upon treatment with compounds **39**, **42** and **52** which lead to subsequent entry into sub-G1 phase afterward (Figure 4.26), especially in SNU475 cells. These results indicated that pyrazolic chalcone derivatives induce cell cycle arrest at the G2/M phase followed by apoptotic cell death in HCC cells.

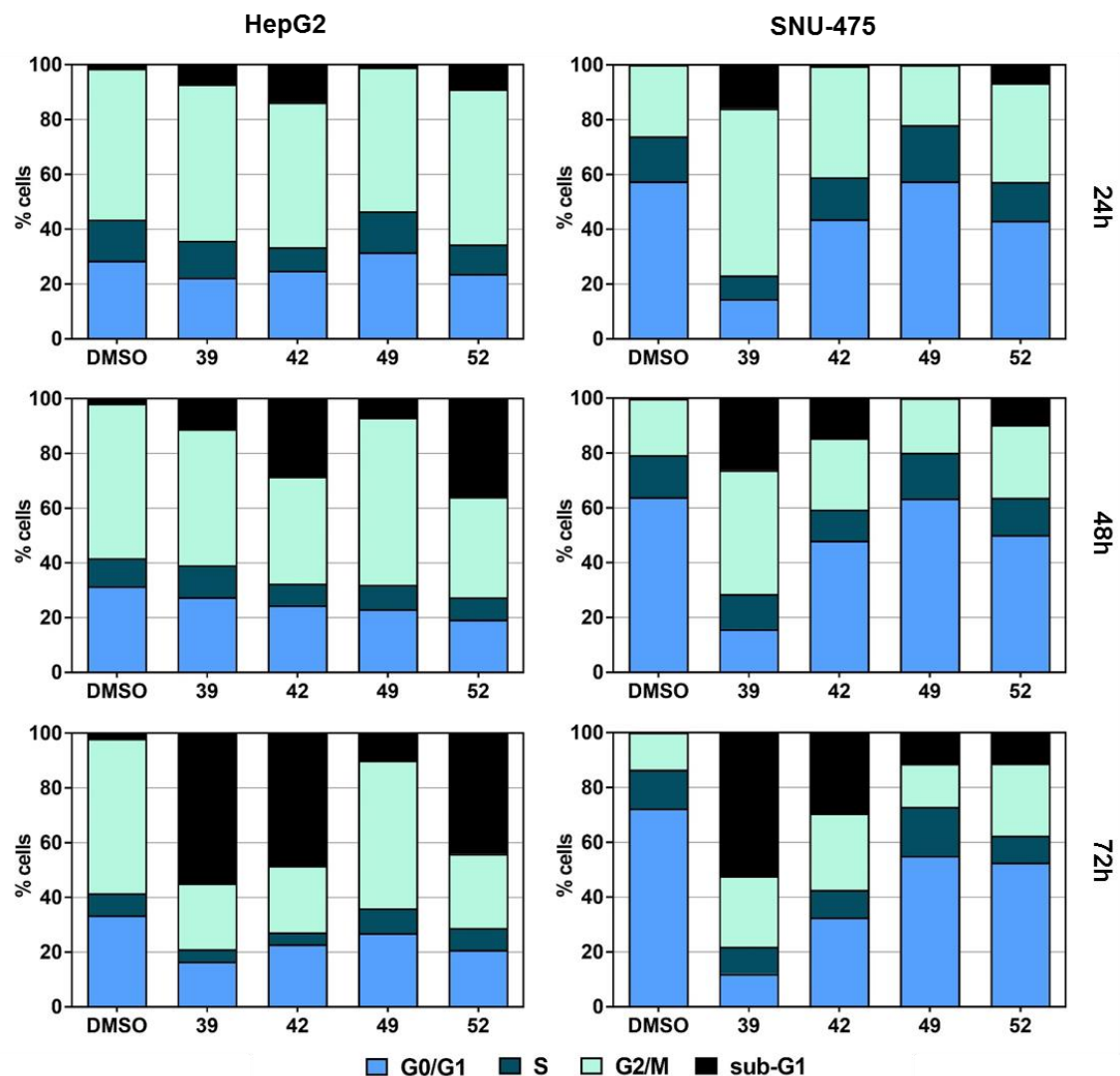
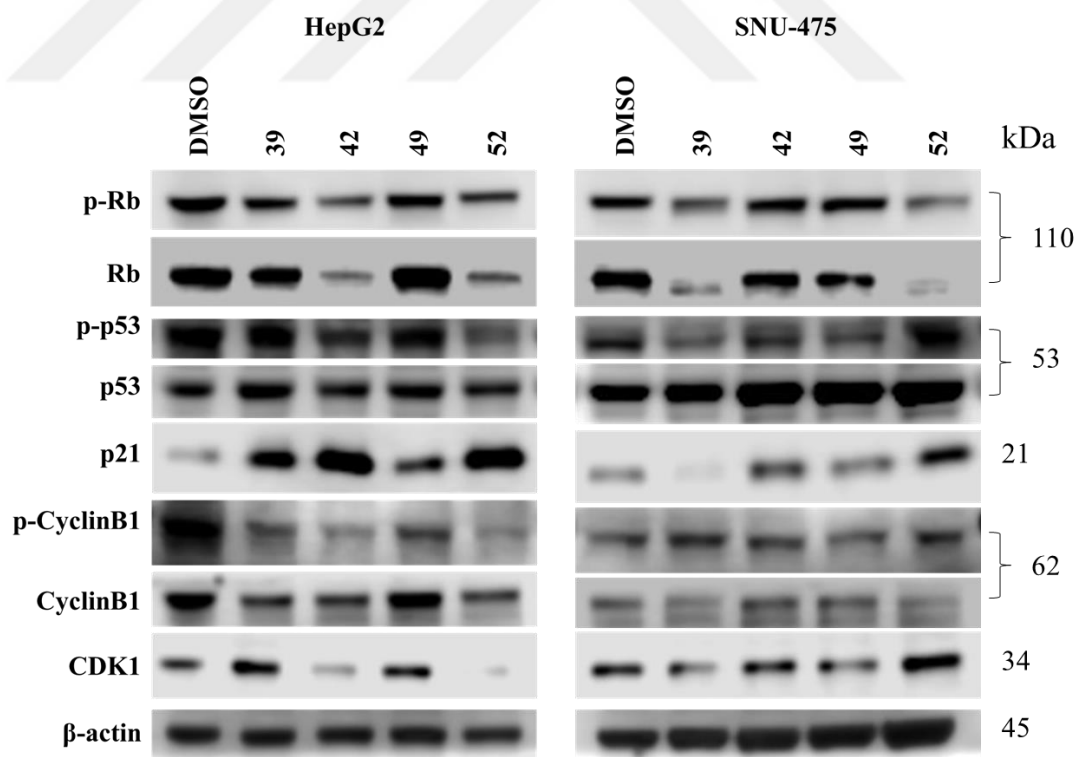


Figure 4.26. Cell cycle analysis of HepG2 and SNU475 cells after treatment with selected compounds. Treatments were done with IC₁₀₀ conc. of selected compounds for 24, 48 and 72h. Bar graphs show cell cycle distribution of cells (G0/G1, S, G2/M, and sub-G1) in percentages.

4.3.5. Cellular pathways altered by pyrazolic chalcone derivatives

In order to determine the alterations in protein levels in HCC cells upon treatment with selected compounds, western blot analysis was performed (Figure 4.27). For this, especially cell cycle-related proteins involved in G2/M phase (CyclinB1 and CDK1) were analyzed. It was shown that upon treatment with compounds **42** and **52**, CDK1 levels

were shown to decrease in both cell lines. However, this alteration was more apparent in HepG2 cells whereas a minor change was observed in SNU475 cells. In addition, phospho-CyclinB1 levels were also shown to decrease by half in HepG2 cells. Since phosphorylation of Ser147 is necessary for the nuclear translocation of CyclinB1 during prophase, these results could be indicative of pyrazole chalcone derivatives to be involved in a mechanism that causes the inhibition of CyclinB1 phosphorylation and consequently decreased amount of CyclinB1 translocating into the nuclei. Additionally, levels of p21, which is the inhibitor of CyclinB1/CDK1 complex, were shown to increase by 5 to 6 fold in HepG2 cells and nearly 2 fold in SNU475 upon treatment with compounds **42** and **52**. However, this change in p21 levels were found to be independent of p53. The levels of phosphorylated Rb levels were also found to be decreased, which may be contributing to the impairment of cell cycle machinery as a secondary effect of pyrazolic chalcone derivatives on cell cycle progression.



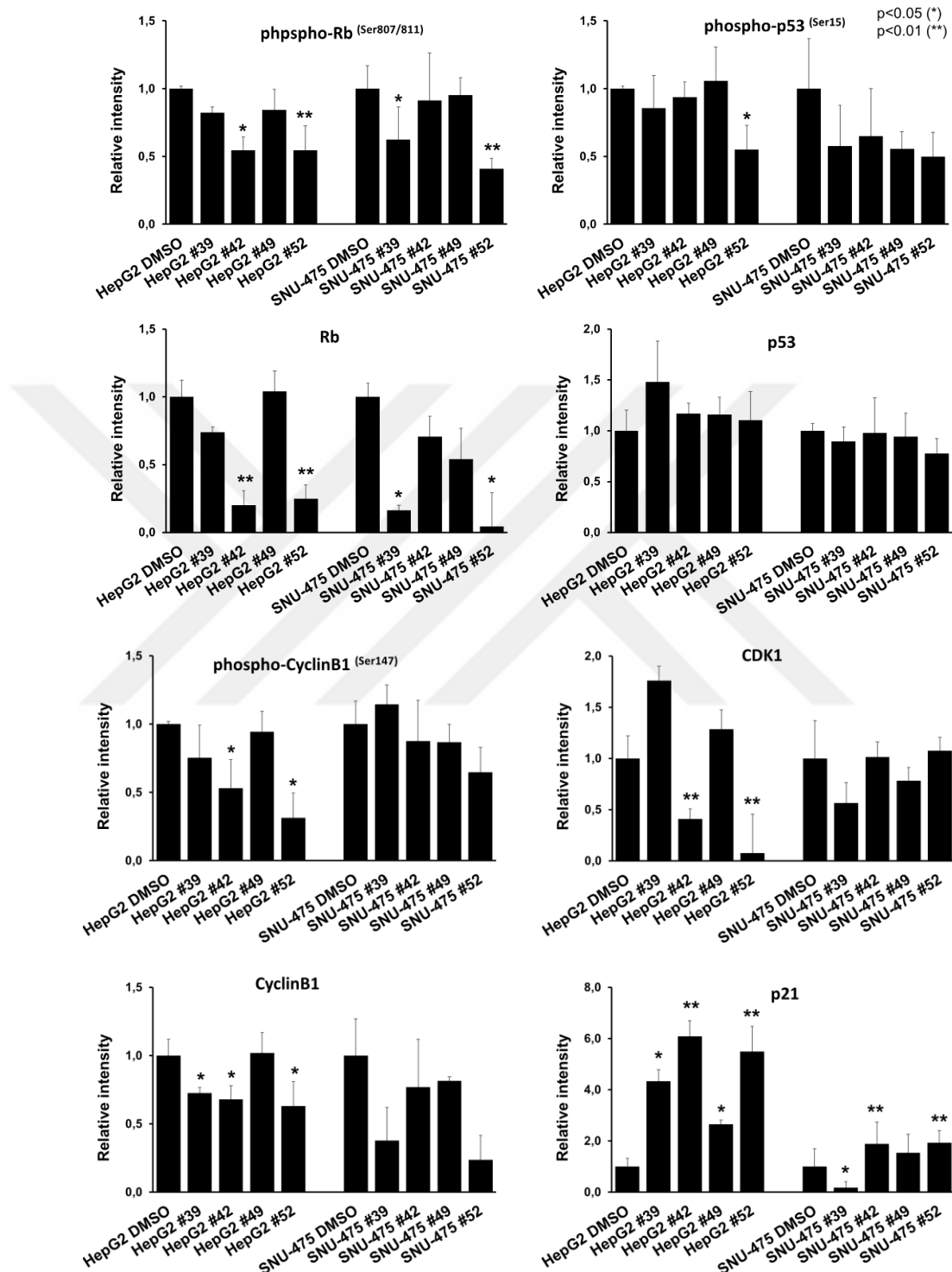


Figure 4.27. Western blot analysis of cell cycle proteins in HCC cells treated with selected compounds. Representative images of three independent western blot experiments demonstrating alterations in levels of cell cycle-related proteins in HepG2

and SNU475 cells treated with IC₁₀₀ concentrations of selected pyrazolic chalcone derivatives for 24h. Bar graphs represent band intensities normalized to β -actin (loading control) levels relative to DMSO controls.





Chapter 5

Discussion and Conclusion

5.1. PART-I

Due to its highly heterogeneous structure, Hepatocellular carcinoma is one of the most resistant and malignant cancer worldwide. Resistance of HCC to conventional chemotherapeutic agents, as well as small molecule inhibitor Sorafenib, stems from activation of compensatory pathways such as PI3K/Akt pathway, EMT pathways as well as enrichment of liver cancer stem cells. In addition to these mechanisms, deregulation of other processes such as metabolism, inflammation or immune response are in close interaction with these compensatory pathways. Tumor-promoting pro-inflammatory

signals (chemokines and cytokines) are also known to activate PI3K/Akt/mTOR and Raf/MEK/ERK pathways. Perhaps one of the new concepts in therapy resistance is the presence of tumor-initiating (cancer stem) cells which by themselves are found responsible for chemo-resistance, tumor relapse, and metastasis. Therefore, therapies targeting not only the compensatory pathways but also liver cancer stem cells are necessary for HCC.

Here we provide evidence for therapeutic potential of combining Sorafenib with mTOR inhibitor Rapamycin to overcome the enrichment of LCSCs. In addition, alterations in the expression of cancer-related pathways triggered by Sorafenib or Rapamycin are presented, which helped define additional targets for the attenuation of LCSC enrichment.

Since, there are ongoing debates on the efficiency of cancer stem cell markers to identify these cells, characterization of CD133/EpCAM markers positive HCC cells were done. Both the results obtained from the sphere formation assay and the expression analysis of well-known stemness factor proteins (Nanog and OCT4) confirmed that these markers are efficient to identify and isolate cancer stem cells of HCC. Supportive to our results, literature has also discussed the association between the PI3K/Akt/mTOR pathway and LCSCs markers CD133 and EpCAM by analyzing the tumor and non-tumor tissues from HCC patients and concluded that p-Akt and p-mTOR expression was positively correlated with CD133, EpCAM and CD90 expression [117]. Furthermore, expression of PTEN in combination with CD133 or EpCAM was suggested to serve as a screening tool to monitor recurrence and predict prognosis in HCC [117].

Although Sorafenib and other small molecule kinase inhibitors targeting PI3K/Akt or Ras/Raf pathways are well known for their anti-cancer activities, in recent years their unstable efficacy in HCC patients became a great concern. This is due to the high-level heterogeneity of the disease. With regard to these problems, many studies suggest combinatory treatment strategies that target both Akt/mTOR and Ras/MAK pathways to ensure better clinical outcomes [118]. However, a recently completed phase II clinical trial [119] which compares everolimus (targeting PI3K/Akt/mTOR pathway) combined

with Sorafenib, and a phase III trial [120] of Sorafenib and erlotinib (an EGFR inhibitor) showed no improvement on survival compared to Sorafenib alone. Given the fact that not all PI3K/Akt/mTOR pathway inhibitors are effective on HCC, a group of small molecule inhibitors was investigated for their efficacy against LCSCs of HCC cell lines in comparison with Sorafenib and clinically used chemotherapeutics (DNA intercalators). In parallel with the literature, each inhibitor had differential effects on CD133+/EpCAM+ population. While Sorafenib, DNA intercalators and some PI3K/Akt pathway inhibitors (ZSTK and NVP-BEZ235) enriched the ratio of LCSCs, very few achieved to decrease the LCSC population significantly (Rapamycin and LY294002). For this reason, we claim that PI3K/Akt/mTOR pathway inhibitors may alter hepatic cancer stem cell composition and gene expression in favor or to the detriment of cancer stem cell survival. Therefore, composition and the timing of the combined treatment with Sorafenib can be in favor of the patient survival or constitute a risk for tumor relapse.

To elucidate the combinatorial potential of effective inhibitors Rapamycin and LY294002 together with Sorafenib, different strategies were followed. Our findings showed that there is a great difference between “strategy A” and “strategy B” in terms of inhibitory effects on LCSCs. When cells were treated initially with Sorafenib or a DNA intercalator, and then with Rapamycin or LY294002, LCSCs were more likely to enrich, even more than cells treated with Sorafenib or Doxorubicin only. Interestingly, when the order of administration of drugs was changed (strategy B), completely different effects were observed, where CSCs were less likely to enrich compared to cells treated with Sorafenib alone. These results have led to the assumption that, pre-treatment of cells with Rapamycin caused cellular events that somehow regulate the stemness-related pathways, and sensitized the LCSCs to the preceding treatment with Sorafenib. In fact, literature has supportive information about the potential of mTOR pathway inhibitors to block stemness-related pathways. A recent study introduced a novel mTOR inhibitor Anthracimycin, which was capable of suppressing proliferation of CSCs and non-CSCs equally in Huh7 cells [121].

In order to trace the alterations in the expression of genes related with cancer pathways, and to identify potential targets with significant roles in LCSC survival mechanisms, a panel consisting a total of 770 cancer-related genes were analyzed using Nanostring nCounter technology. Differential expression analysis has provided possible answers to several questions asked in this study. First was the effects of Sorafenib treatment on the expression of genes related to stem cell survival. As expected downregulation of Ras/MAPK pathway-related genes (PDGFRA, EFNA1, EFNA1, VEGFA, RASA4) were observed in Sorafenib treated cells. Nevertheless, the distinct gene expression pattern, where genes involved in stemness regulating pathways, such as Notch and Wnt, (JAG1 and DKK1) were differentially regulated compared to DAPT or Rapamycin treated cells, further emphasized the potential of Sorafenib to enhance LCSC survival. Second, was the regulation of IL-8 gene, where IL-8 expression was found to be strongly correlated with the effects of inhibitors on LCSC enrichment (Figure 5.1).

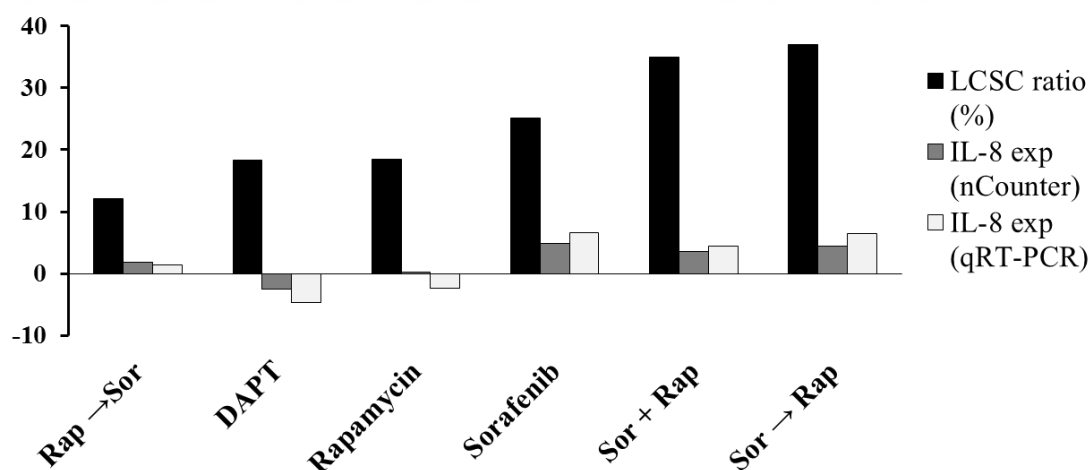


Figure 5.1. Correlation of IL-8 expression and LCSC enrichment. Bar graph represents the LCSCs ratio and IL-8 expression (both Nanostring and qRT-PCR) after treatment with indicated inhibitors for 72h.

Treatment of cells with Sorafenib was found to upregulate IL-8 expression, whereas DAPT resulted in downregulation of this gene. Interestingly, similar to the effect of DAPT, Rapamycin was able to downregulate IL-8 expression and treatment of cells with Rapamycin prior to Sorafenib, prevented upregulation of IL-8 unlike other combination strategies (summarized in Figure 5.2). Such regulation of IL-8 expression drew our attention since IL-8 signaling in cancer was studied in many cancers, especially in breast cancer, where the knockout of IL-8 lead to poor survival of CSCs [122-124]. In addition, integrating of the differential expression data to execute the pathway scoring application helped evaluate the activity of signaling pathways. IL-8 gene gained significantly enriched scores which suggested that Rapamycin could be inducing events that do not allow IL-8 expression to elevate, yet inhibit survival of LCSCs.

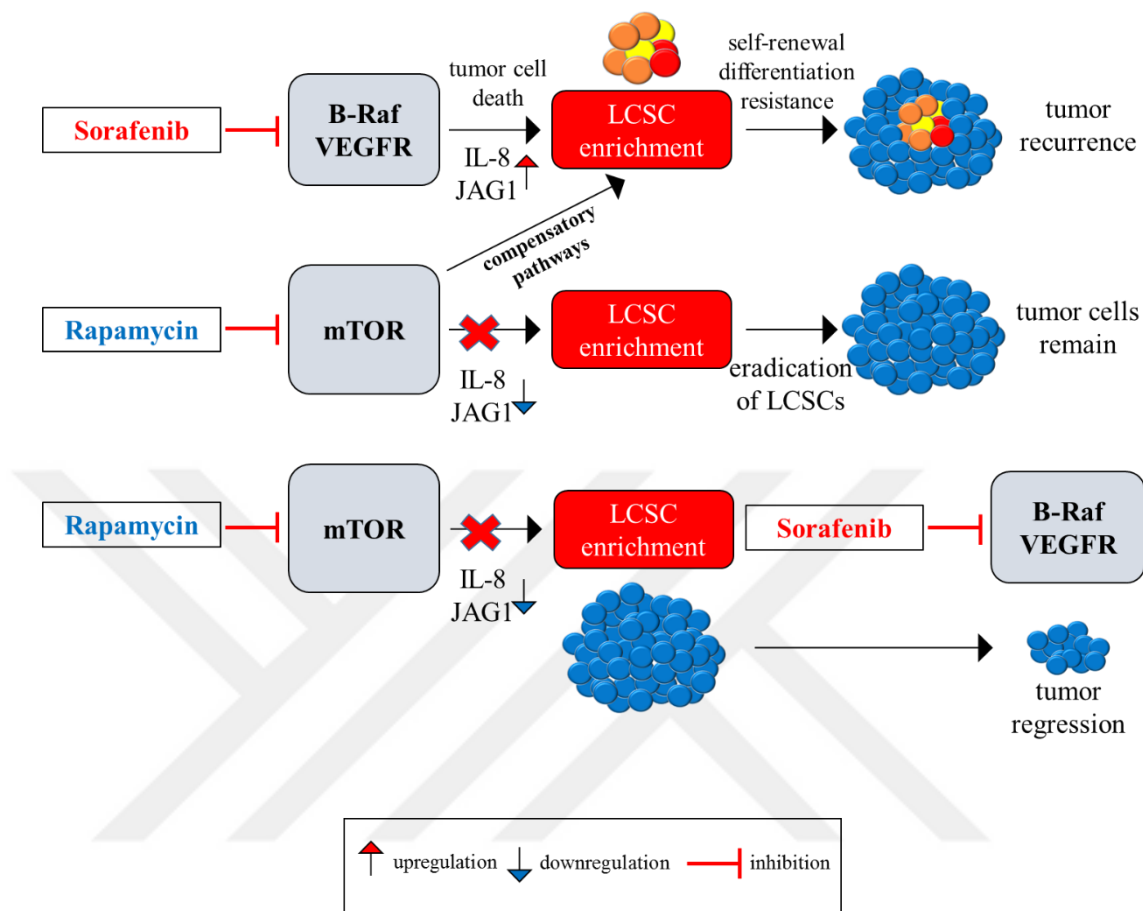


Figure 5.2. Effects of Sorafenib and Rapamycin treatments on LCSCs and tumor progression.

In fact, inhibition of IL-8 by treating cells with Reparixin (allosteric inhibitor of CXCR1) was informative for the hypothesis stated above. Upon Reparixin treatment both the cancer stem cell marker expression and the sphere formation capacity of Huh7 cells were reduced significantly (summarized in Figure 5.3).

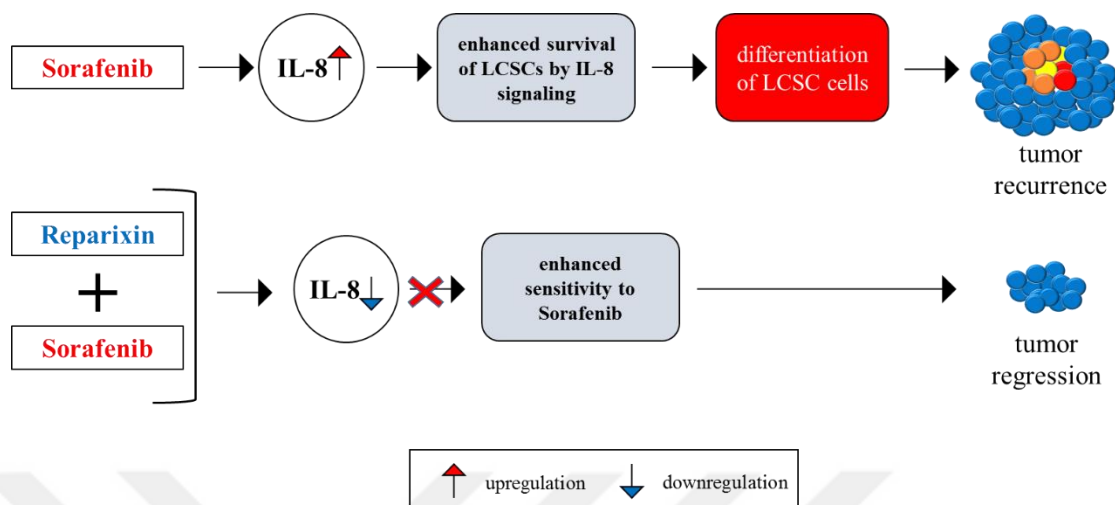


Figure 5.3. Schematic representation of effects of IL-8 inhibition on LCSC enrichment.

Altogether, this thesis study provided a better understanding of alterations in distribution of stem/non-stem cells in HCC upon treatment with different PI3K/Akt/mTOR pathway inhibitors and possible mechanisms involved in regulation of LCSC survival.

5.2. PART-II

In this part of the thesis, the bioactivities of newly synthesized VEGFR2 TKIs and quinoids against liver cancer cells and liver cancer stem cells were evaluated and a new concept concerning the potential of the effect of a compound on aggressiveness of cancer, so-called “A_F factor” was introduced. (Summarized in Figure 5.4)

Initially, the inhibitory effects of each compound were defined by NCI-60 SRB assay. The results have shown that VEGFR2 TKIs can exert their inhibitory effects in lower concentrations (IC₅₀ values ranging from 0.4 to 3 μM), whereas quinoids were effective in higher concentrations (4-30 μM).

It is crucial to identify compounds against HCCs and LCSCs since patients suffer from relapse or incomplete recovery after treatment if the treatment fails to eradicate LCSCs.

The effects of quinoids on cancer stem cells are not studied, yet there is only one study describing the macrocyclic benzoquinone herbimycin A [CAS: 70563-58-5, BRN: 4834067] that was shown to inhibit the human bone marrow mesenchymal stem cells at 1-5 μ M concentration [125]. Here, we describe the potential of quinoids in inhibition of LCSCs as well as liver cancer cells. Among all benzoquinone compounds tested, compound **3** was found to be promising against LCSCs in mesenchymal-like cells (Mahlavu and SNU475). When the structure of this compound was compared to the other quinoids tested, it was noticed that quinoid **3** was the one that misses most of the substitutions on its benzoquinone skeleton. Considering the potential of quinones in selective activity in hypoxic conditions, and since hypoxia is one of the major mechanisms involved in drug resistance, the potential of quinone compounds to inhibit cancer stem cells is of vital importance.

On the other hand, none of the VEGFR TKIs, tested in this study including Sorafenib was effective against liver cancer stem cells, even though their inhibitory concentrations were much lower to that of quinoids. As discussed in the first part of this thesis, Sorafenib and VEGFR2 TKIs are highly prone to cause resistance in cancer patients due to the activation of compensatory pathways. Once again, it has been shown that the potential of VEGFR2 TKIs in overcoming cancer stem cell enrichment is very unlikely.

Here we introduced a new concept called “aggressiveness factor, (A_F)” to demonstrate the differential action of compounds on cancer cells and cancer stem cells. A_F allows selection of promising compounds that have a lower probability to reconstitute aggressive tumors and can serve as a simple method to evaluate this probability for novel compounds in comparison to a particular drug well known for its clinical behavior.

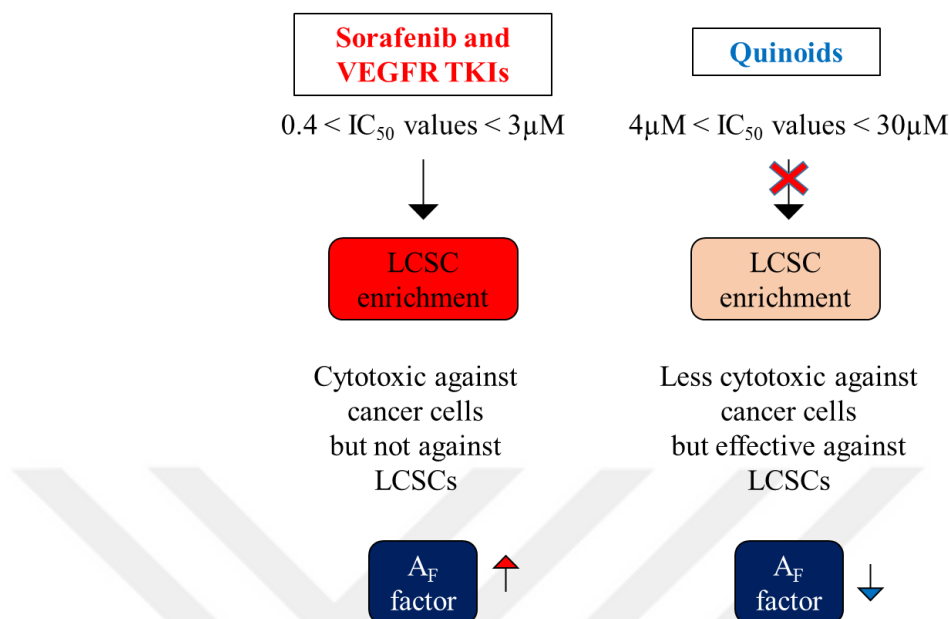


Figure 5.4. Schematic explanation of comparative effects of VEGFR2 TKIs and quinoids on liver cancer cells and LCSCs.

5.3. PART-III

Chalcones and pyrazolic structures are well known for their anticancer activities as well as anti-inflammatory effects. The selectivity of pyrazolic structures towards their biological targets depends on the type of peripheral substituents. Here, the bioactivities of pyrazolic chalcone derivatives were investigated on human cancer cell lines (HCC, breast and colon cancer) in comparison with clinically used chemotherapeutics. The IC₅₀ values of novel compounds **12-52** ranged from 0.3 μM to 29 μM in the initial screening, and these values were lower or comparable to that of 5-FU and cladribine. Compounds that displayed significant bioactivities at low concentrations on cancer cells were selected (**39,42,49,52**) to be tested against a panel of HCC cell lines (Huh7, HepG2, Mahlavu and SNU475) as well as normal-like cells (MCF12A and MRC5) to evaluate if the compounds exhibit selective cytotoxic activity towards cancer cells. Results have shown that these compounds were cytotoxic to HCC cells in lower concentrations (IC₅₀ < 5 μM), but less cytotoxic to normal-like cells, such that compound **49** was not even bioactive in MRC5 cells.

Further analysis of cell cycle progression and detection of cell death have revealed that compound **42** and **52** caused cell cycle arrest at the G2/M phase and induced apoptotic cell death. When proteins involved in cell cycle progression was analyzed, levels of p21, active CyclinB1 (phospho-CyclinB1^{Ser147}) and CDK1 were shown to decrease independent of p53 levels (summarized in Figure 5.5). Phosphorylation of Cyclin B1 at Ser147 is known to be necessary for the nuclear translocation of Cyclin B1 during prophase [126]. This could be indicating the possibility that pyrazolic chalcone derivatives are interfering with this mechanism and causing decreased amount of active CyclinB1 translocating into the nuclei.

Recent studies address the role of chalcone derivatives as tubulin binding and depolymerizing agents [127] For instance chalcone analogs of natural product combretastatin were shown to inhibit the assembly of tubulin and exhibit anticancer activity [128] and are currently involved in preclinical studies [129, 130]. In this respect, the effects of compounds **12-53** on tubulin polymerization were analyzed, yet no inhibitory effect on tubulin polymerization was found (data not shown).

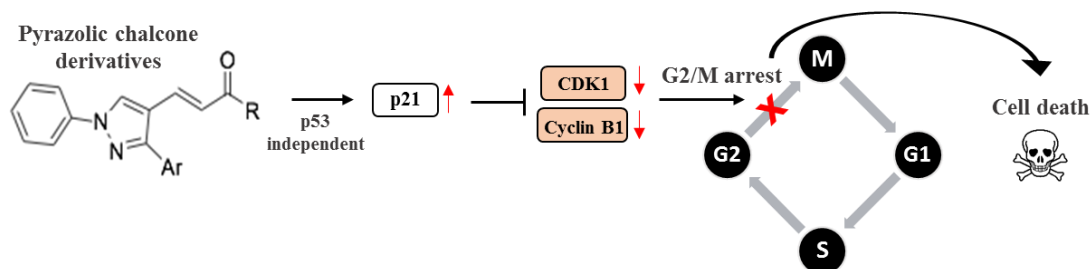


Figure 5.5. Effects of pyrazolic chalcone derivatives on cell cycle progression.



Chapter 6

Future Perspectives

Today curing cancer by targeting proliferation of tumor cells is not sufficient enough since, in most of the cases with aggressive cancers, drug resistance or tumor relapse is inevitable. In HCC, primarily the activation of PI3K/Akt pathway and the potential of liver cancer stem cells to escape the effects of chemotherapeutic agents due to their advanced DNA repair machinery constitute the major mechanisms involved in drug resistance and low success rate of therapy in clinics. This study provides valuable information on the possible molecular patterns altered upon treatment of HCC cells with different PI3K/Akt/mTOR inhibitors as well as Sorafenib and DNA intercalators, and

suggested that mTOR pathway and IL8 signaling are related with conservation of stemness features of LCSCs through functional and gene expression analysis. However, how these factors are associated with the regulation of LCSC survival remains to be studied further *in vitro* and *in vivo*. Knock-out experiments targeting potential effectors (IL-8, mTOR) found in this study could provide a clearer understanding of the downstream processes in HCC cells. Since most of the experiments are performed using Huh7 and Hep3B cells, an extended HCC panel should be studied to provide comprehensive results taking into consideration of the genetic features/mutations of different cell lines. In addition, *in vivo* experiments (tumor xenografts) are necessary to validate the combinatorial treatment strategies discussed in this study.

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The second part of this thesis focuses on the identification of bioactivities of the VEGFR2 TKIs and quinoids on liver cancer cells as well as their potential to eliminate the liver cancer stem cells that reside inside the heterogeneous population of HCC cells as a small entity. It was shown that although newly synthesized VEGFR2 TKIs were cytotoxic in very low concentrations, these compounds were not effective on LCSCs and even caused their enrichment. On the other hand, quinoids were more potent in inhibition LCSCs but had higher IC₅₀ values compared to VEGFR2 TKIs. Yet, additional *in vitro* experiments are needed to verify this effect through functional assays, such as sphere formation assay, *in vivo* xenograft assays. In addition, in order to solve the molecular mechanism behind the effectiveness of quinoids against LCSCs, gene expression analysis using microarray or its counterparts (Nanostring).

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The third part of this thesis focuses on the effects of newly synthesized 41 novel pyrazolic chalcone derivatives on HCC cell lines. Although the selected compounds were found to affect the cell cycle progression and the protein complexes involved in G2/M phase, the exact mechanism by which these compounds exhibit such activity remains to be solved. Future studies that involve detailed cellular networks that are effected through next-

generation sequencing, or transcriptome analysis in the presence of pyrazolic chalcone derivatives. Additionally, biotinylated forms of compounds can be synthesized and immunoprecipitated to identify their targets. In vivo xenograft experiments are also necessary to verify the anti-cancer properties of these compounds.





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Appendix

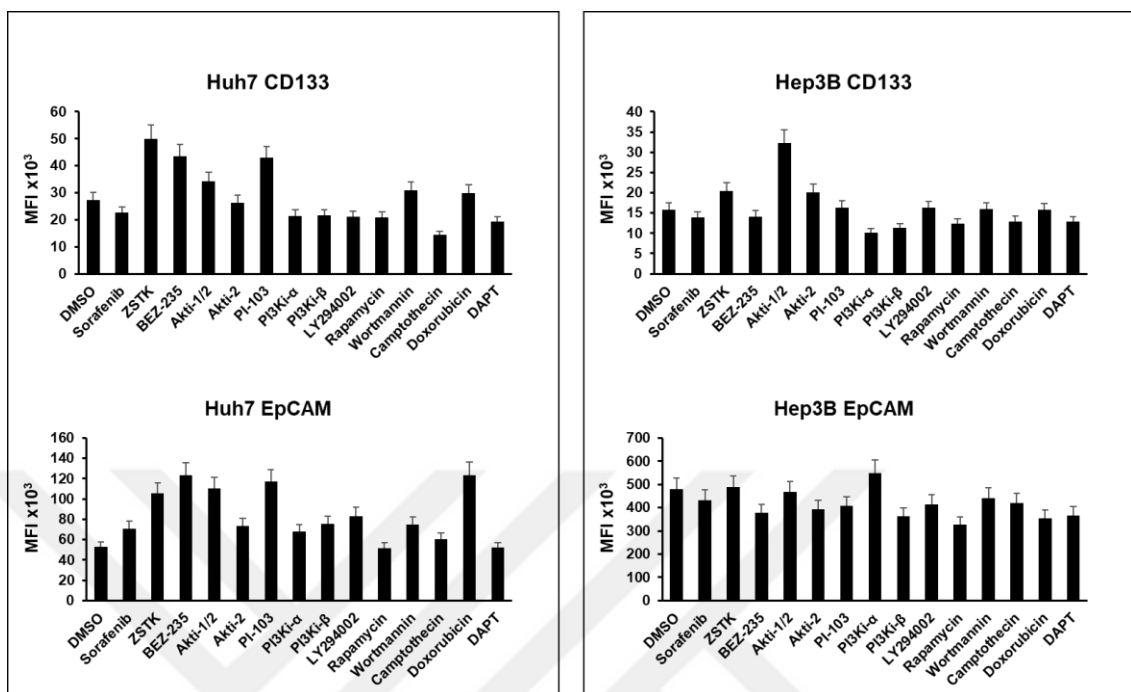


Figure A1. Mean fluorescence intensity values (MFI) of CD133 and EpCAM staining of Huh7 and Hep3B cells treated with small molecule inhibitors.

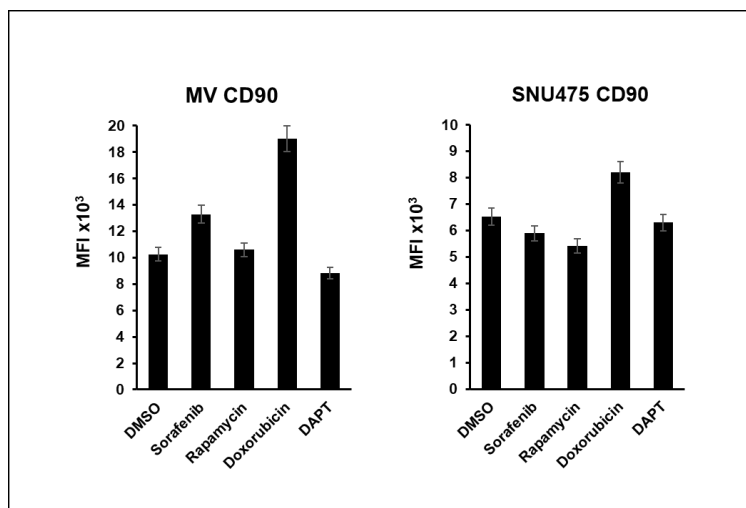


Figure A2. Mean fluorescence intensity values (MFI) of CD90 staining of Mahlavu and SNU475 cells with selected small molecule inhibitors.

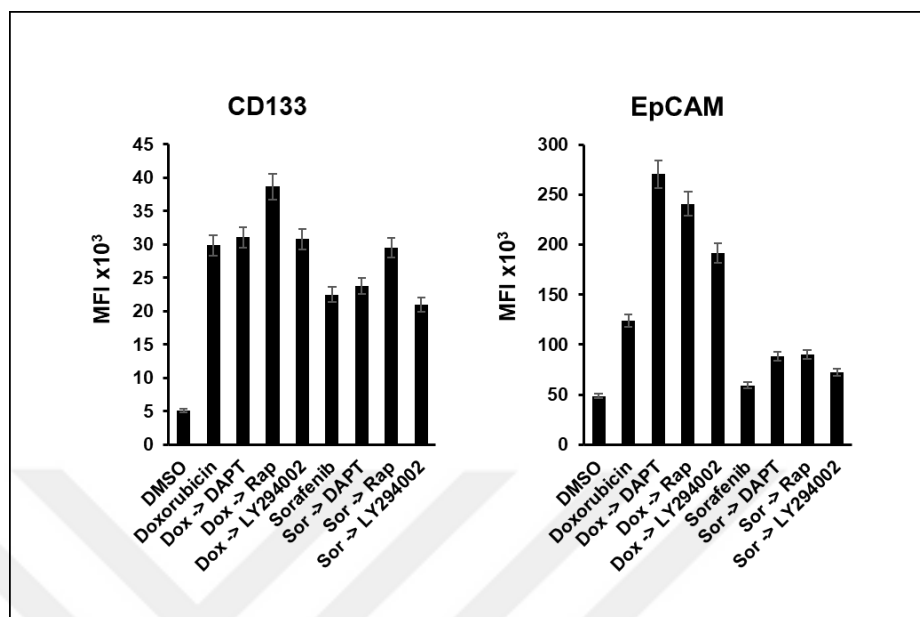


Figure A3. Mean fluorescence intensity values (MFI) of CD133 and EpCAM staining of Huh7 cells treated with sequential treatment strategy A.

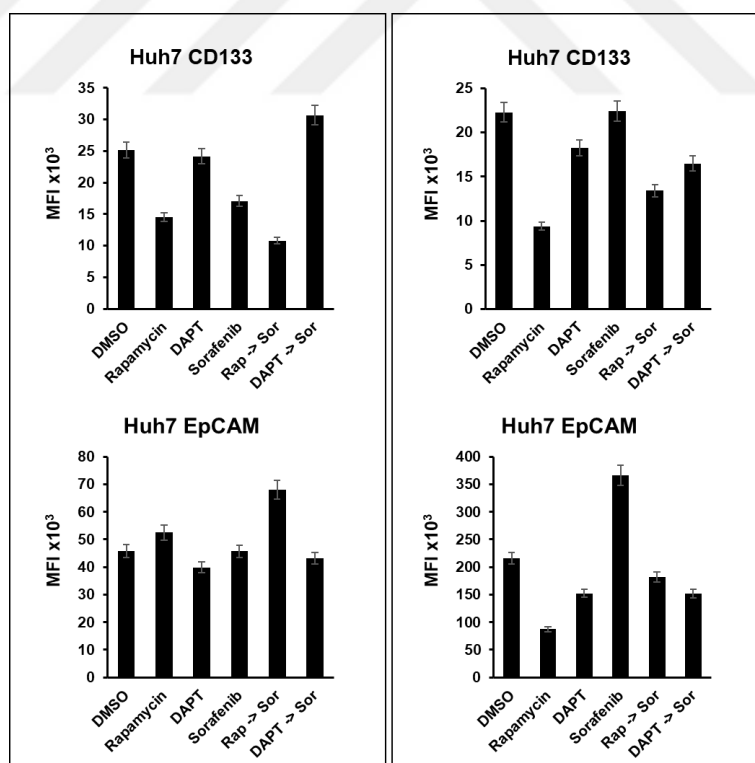


Figure A4. Mean intensity fluorescence values (MFI) of CD133 and EpCAM staining of Huh7 and Hep3B cells treated with sequential treatment strategy B.

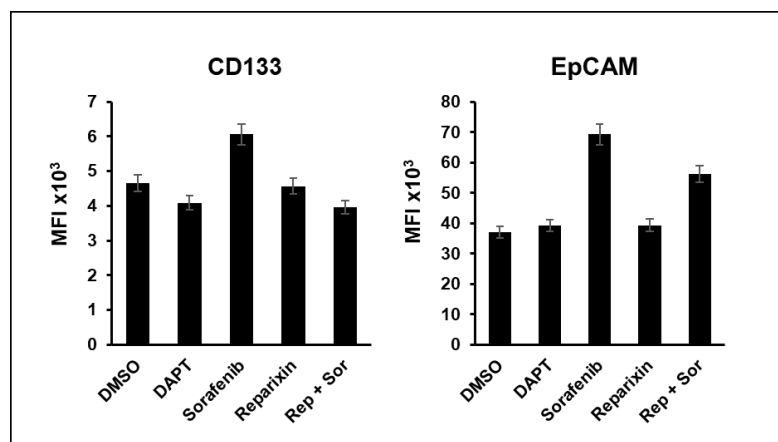


Figure A5. Mean fluorescence intensity values (MFI) of CD133 and EpCAM staining of Huh7 cells treated with Reparixin alone and in combination with Sorafenib.

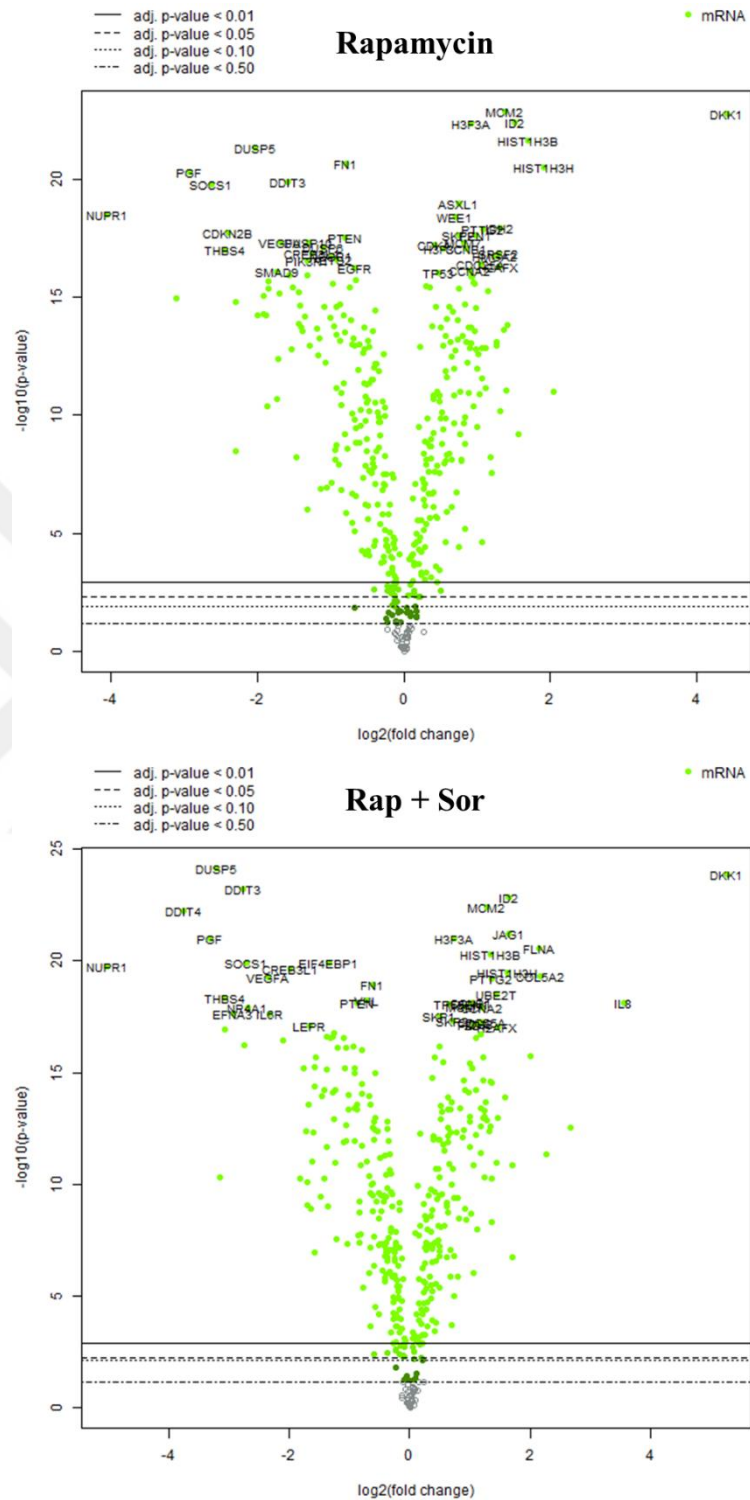


Figure A7. Volcano plots showing gene expression in Rapamycin or Rap + Sor treated cells. y-axis indicates significance and x-axis indicates fold change.

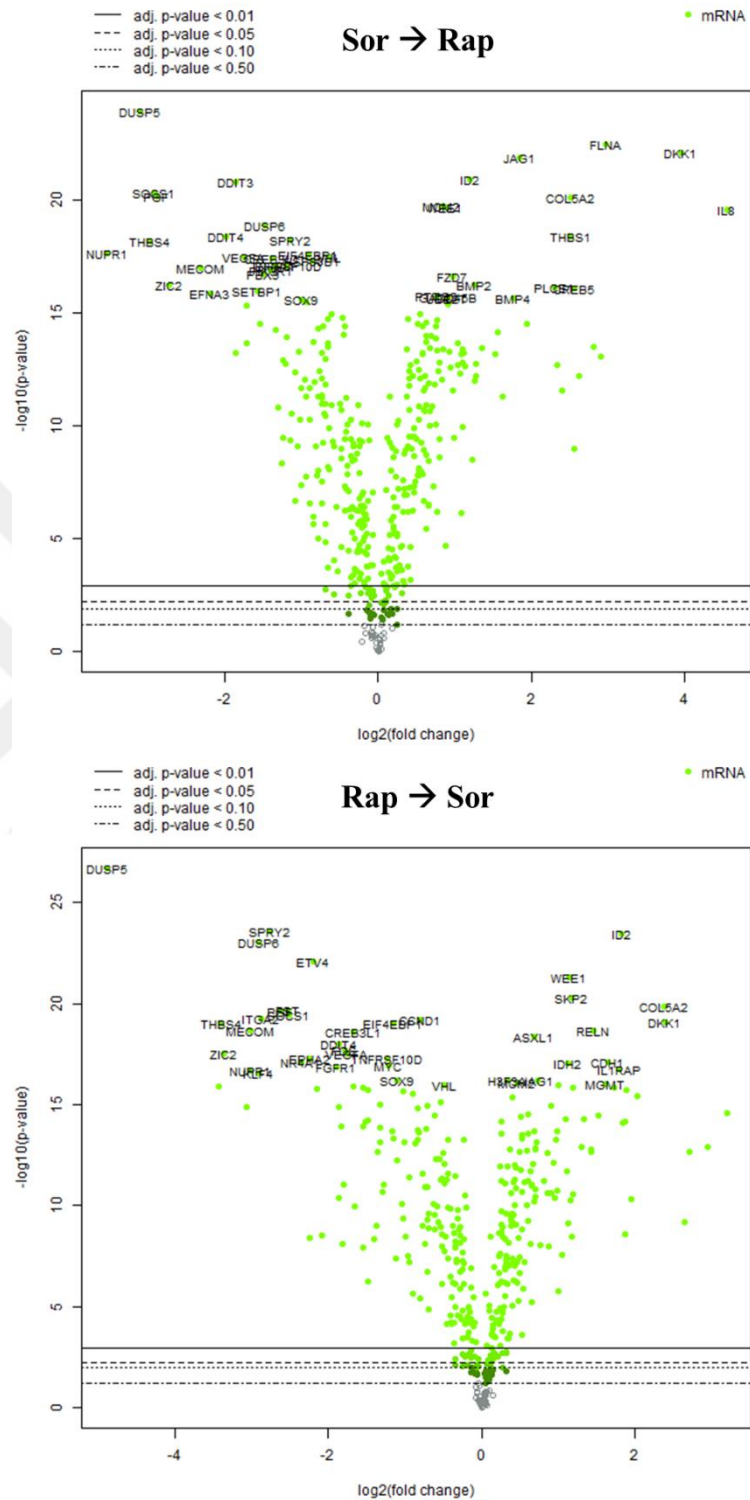


Figure A8. Volcano plots showing gene expression in Sor → Rap or Rap → Sor treated cells. y-axis indicates significance and x-axis indicates fold change.

Table A1. Differential expression of top 30 genes (log2) and the pathways involved.

differential expression of top30 genes (log2)							Gene.sets
Gene	SOR	DAPT	RAP	SOR + RAP	SOR -> RAP	RAP -> SOR	
IL8	4.87	-2.47	0.18	3.56	4.54	1.86	Transcriptional Misregulation
FLNC	1.93	-3.24	-1.74	0.13	2.81	-0.95	
DKK1	2.71	0.20	4.41	5.26	3.94	2.39	Wnt
DUSP5	-2.48	-0.27	-2.03	-3.21	-3.11	-4.89	MAPK
DDIT4	-1.91	0.80	-1.32	-3.76	-1.99	-1.86	PI3K
NUPR1	-3.51	-0.73	-4.06	-5.03	-3.54	-3.01	Transcriptional Misregulation
FLNA	2.41	-0.76	0.12	2.15	2.96	1.00	
CAPN2	1.87	-0.09	-0.47	1.71	2.33	3.19	Cell Cycle - Apoptosis
SPRY4	0.22	-0.09	-0.89	-0.85	-1.14	-3.43	JAK-STAT
ZIC2	-1.73	-0.02	-1.53	-3.07	-2.73	-3.37	Hedgehog
BDNF	0.69	-0.63	0.28	1.70	2.56	2.65	MAPK
PGF	-2.27	-0.02	-2.92	-3.30	-2.88	-2.63	PI3K, Ras
PLA1A	0.11	-1.01	-2.29	-3.14	-0.68	-2.25	Ras
SOCS2	2.90	-0.21	-0.25	1.37	2.39	1.94	JAK-STAT
SOCS3	2.55	-0.07	2.05	2.67	2.90	2.70	JAK-STAT
FGF12	2.28	0.20	1.57	2.26	2.61	2.94	MAPK, PI3K, Ras
EFNA3	-2.98	-0.33	-1.91	-2.92	-2.19	-1.87	PI3K, Ras
THBS4	-2.65	-0.75	-2.44	-3.06	-2.98	-3.40	PI3K
PDGFRA	-2.32	0.30	-0.68	-1.41	-1.71	-1.33	Driver Gene, MAPK, PI3K, Ras
LEFTY1	-1.06	-1.80	-0.80	0.79	0.18	-0.34	
LTBP1	-1.78	-1.74	-3.10	-1.41	-0.73	-3.07	TGF-beta
COL5A2	1.89	0.13	0.99	2.17	2.50	2.38	PI3K
MYC	-0.28	1.12	0.97	-0.17	-0.14	-1.22	Cell Cycle - Apoptosis, JAK-STAT, MAPK, PI3K, TGF-beta, Transcriptional Misregulation, Wnt
CDKN2B	-0.12	-2.23	-2.40	-1.58	-0.34	-1.15	
FGF19	-0.44	1.45	-0.43	-0.79	-0.69	0.10	Cell Cycle - Apoptosis, TGF-beta
SOCS1	-1.34	-1.10	-2.63	-2.70	-2.91	-2.51	MAPK, PI3K, Ras
PTCH1	-0.97	0.77	0.41	-0.27	-0.26	-0.16	Driver Gene, JAK-STAT
HIST1H3H	0.57	0.30	1.92	1.62	0.58	0.61	Driver Gene, Hedgehog
HIST1H3B	0.33	0.16	1.69	1.35	0.52	0.51	Transcriptional Misregulation
PRDM1	0.05	1.15	0.07	0.22	-0.13	1.11	Driver Gene, Transcriptional Misregulation
							Driver Gene

Curriculum Vitae and Publications

PERSONAL INFORMATION

Name Surname Deniz Cansen Kahraman
Date of Birth 16. Aug. 1989
Nationality Turkish
Languages spoken Turkish (Mother tongue)
English (Fluent)
Address Molecular Biology and Genetics, Bilkent University, Ankara, Turkey
Phone +90-536-482-3937
E-mail deniz.yildirim@bilkent.edu.tr, denizcansen89@gmail.com

EDUCATION

2011- **Bilkent University**, Department of Molecular Biology and Genetics, Ankara, Turkey (PhD Degree)
2007-2011 **Bilkent University**, Department of Molecular Biology and Genetics, Ankara, Turkey (B.Sc. Degree)

PUBLICATIONS

Article Publications

1. **Deniz Cansen Kahraman**, Tamer Kahraman, Rengul Cetin Atalay; Differential alteration of IL-8 in liver cancer cells in response to Sorafenib and PI3K/Akt/mTOR inhibitors and to LCSC enrichment. (*in preparation for Hepatology*)
2. Mohammed M.A. Hawash*, **Deniz Cansen Kahraman***, Rengul Cetin-Atalay, Ernest Hamel, Sultan Nacak Baytas; Induction of Apoptosis in Hepatocellular Carcinoma Cell Lines by Novel Indolylacrylamide Derivatives: Synthesis and Biological Evaluation. *European Journal of Medicinal Chemistry (submitted)* *Equal contribution
3. Mohammed M.A. Hawash*, **Deniz Cansen Kahraman***, Fikriye Eren, Rengul Cetin Atalay, Sultan Nacak Baytas: Synthesis and biological evaluation of novel pyrazolic chalcone derivatives as novel hepatocellular carcinoma therapeutics. *European Journal of Medicinal Chemistry* 02/2017; 129., DOI:10.1016/j.ejmech.2017.02.002 *Equal contribution

4. **Deniz Cansen Kahraman**, Gilles Hanquet, Loïc Jeanmart, Steve Lanners, Peter Šramel, Andrej Boháč, Rengul Cetin-Atalay: Quinoides and VEGFR2 TKIs influence the fate of hepatocellular carcinoma and its cancer stem cells. *Medicinal Chemistry Communication* 01/2016; 8(1)., DOI:10.1039/C6MD00392C
5. Margaréta Vojtičková, Juraj Dobiaš, Gilles Hanquet, Gabriela Addová, Rengul Cetin-Atalay, **Deniz Cansen Yildirim**, Andrej Boháč: Ynamide Click chemistry in development of triazole VEGFR2 TK modulators. *European Journal of Medicinal Chemistry* 09/2015; 103:105-122., DOI:10.1016/j.ejmech.2015.08.012
6. Mattia Stucchi, Silvia Cairati, Rengul Cetin-Atalay, Michael S Christodoulou, Giovanni Grazioso, Gennaro Pescitelli, Alessandra Silvani, **Deniz Cansen Yildirim**, Giordano Lesma: Application of the Ugi reaction with multiple amino acid-derived components: Synthesis and conformational evaluation of piperazine-based minimalist peptidomimetics. *Organic & Biomolecular Chemistry* 03/2015; 13(17)., DOI:10.1039/c5ob00218d
7. Mohammad Tofighi, Onur Yorulmaz, Kivanc Kose, **Deniz Cansen Yildirim**, Rengul Cetin Atalay, A. Enis Cetin: Phase and TV Based Convex Sets for Blind Deconvolution of Microscopic Images. *IEEE Journal of Selected Topics in Signal Processing* 03/2015; 10(1):1-1., DOI:10.1109/JSTSP.2015.2502541
8. Ilke Simsek Turan, Fatma Pir Cakmak, **Deniz Cansen Yildirim**, Rengul Cetin-Atalay, Engin U. Akkaya: Near-IR Absorbing BODIPY Derivatives as Glutathione-Activated Photosensitizers for Selective Photodynamic Action. *Chemistry - A European Journal* 12/2014; 20(49)., DOI:10.1002/chem.201405450

Selected Oral Presentations

1. **Deniz Cansen Kahraman, Rengul Cetin-Atalay**. "Potency of Interleukin 8 Blockade in Prevention of Liver Cancer Stem Cell Enrichment". *5th International BAU Drug Design Congress*, 19-21 October, 2017, İstanbul, Turkey.
2. **Deniz Cansen Kahraman**. "Recent Advances in Cancer Stem Cell Research". *COST Action CM1106 4th Workshop* 10 - 11 March, 2016, Chioggia-Venice, Italy.
3. **Deniz Cansen Yildirim, Rengul Cetin-Atalay**. "Bioactivities of small molecule kinase inhibitors in liver cancer stem cells". *COST Action CM1106 3rd Working Group Meeting*, 26 - 27 March, 2015, Athens, Greece.

Selected Poster Presentations

1. **Deniz Cansen Yildirim, Rengul Cetin-Atalay**. "Differential alteration of IL-8 in response to Sorafenib and PI3K/Akt/mTOR inhibitors in liver cancer cells and in liver cancer stem cells". *AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics*, 26-30 October, 2017, Philadelphia, USA.
2. **Deniz Cansen Kahraman, Rengul Cetin-Atalay**. "Potency of Interleukin 8 Blockade in Prevention of Liver Cancer Stem Cell Enrichment". *5th International BAU Drug Design Congress*, 19-21 October, 2017, İstanbul, Turkey.
3. **Deniz Cansen Kahraman**, Tamer Kahraman, Rengul Cetin Atalay "Sequential treatment of HCC cells with mTOR pathway inhibitors prior to Sorafenib attenuates cancer stem cell enrichment" 24th Biennial Congress of the European Association for Cancer Research (EACR24), 9-12 July, 2016, Manchester, UK.

4. **Deniz Cansen Yildirim, Rengul Cetin-Atalay**. “Bioactivities of small molecule kinase inhibitors in liver cancer stem cells”. *COST Action CM1106 3rd Working Group Meeting*, 26 - 27 March, 2015, Athens, Greece.
5. **Deniz Cansen Yildirim, Rengul Cetin-Atalay**. “Bioactivities of small molecule kinase inhibitors in liver cancer stem cells”. *4th International Congress of Molecular Biology Association of Turkey*. 27-29 November, 2015, Ankara, Turkey.
6. **Deniz Cansen Yildirim, Fikriye Eren, Mohammad A. Hawwash, Sultan Nacak Baytas, Rengul Cetin-Atalay**. “Biological Evaluation of New Pyrazole Derivatives for Novel Therapeutic Opportunities of Hepatocellular Carcinoma”. *MolBiyoKon 2014 - 3rd International Congress of the Molecular Biology Association of Turkey*. 10-12 September, 2014, İzmir, Turkey.
7. **Deniz Cansen Yildirim, Rengul Cetin-Atalay**. “Identification of Novel Small Molecule Kinase Inhibitors Against Liver Cancer Stem Cells”. *EACR-Sponsored 2nd Anticancer Agents Congress*, 23-25 April, 2014, Bodrum, Turkey.
8. **Deniz Cansen Yildirim, Ebru Bilget-Guven, Peri Aytac, Birsen Tozkoparan, Rengul-Cetin-Atalay**. “Novel small molecule kinase inhibitors against liver cancer and liver cancer stem cells”. *COST CM1106 2nd Working Group Meeting*, 27 - 28 March 2014 Budapest – Eötvös Loránd University (ELTE).
9. **Deniz Cansen Yildirim, Peri S. Aytac, Birsen Tozkoparan, Rengul-Cetin Atalay**. “Enrichment of cancer stem cell characteristics of liver cancer cells by prolonged drug selection and identification of cytotoxic activity of new drug candidates”. *International Multidisciplinary Symposium on Drug Research and Development*, 28-30 November, 2013, Antalya, Turkey.

SCHOLARSHIPS and AWARDS

- The Best Poster Award, 5th International Drug Design Congress , 19-21 October 2017, Istanbul, Turkey
- Bilkent University, Department of Molecular Biology and Genetics, PhD Program Full Scholarship

SOCIETY MEMBERSHIPS

- American Association for Cancer Research (AACR)
- European Association for Cancer Research (EACR)
- Moleküler Kanser Araştırma Derneği (MOKAD)

RESEARCH ARTICLE

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Quinoides and VEGFR2 TKIs influence the fate of hepatocellular carcinoma and its cancer stem cells†‡

 Deniz Cansen Kahraman,^a Gilles Hanquet,^b Loïc Jeanmart,^c Steve Lanners,^c
Peter Šramel,^{de} Andrej Boháč^{*de} and Rengul Cetin-Atalay^{*f}

Bioactivities of quinoides 1–5 and VEGFR2 TKIs 6–10 in hepatocellular cancer (HCC) and cancer stem cells (HCSCs) were studied. The compounds exhibited IC₅₀ values in μ M concentrations in HCC cells. Quinoid 3 was able to eradicate cancer stem cells, similar to the action of the stem cell inhibitor DAPT. However, the more cytotoxic VEGFR TKIs (IC₅₀: 0.4–3.0 μ M) including sorafenib, which is the only FDA approved drug for the treatment of HCC, enriched the hepatocellular cancer stem cell population by 2–3 fold after treatment. An aggressiveness factor (A_F) was proposed to quantify the characteristics of drug candidates for their ability to eradicate the CSC subpopulation. Considering the tumour heterogeneity and marker positive cancer stem cell like subpopulation enrichment upon treatments in patients, this study emphasises the importance of the chemotherapeutic agent choice acting differentially on all the subpopulations including marker-positive CSCs.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent and second most deadly cancer worldwide.¹ HCC patients are resistant to chemotherapy and radiotherapy, because conventional therapies can only reduce the bulk of the tumour mass but are unable to restrain tumour regrowth and relapse. HCC is a highly heterogeneous tumour in terms of morphology and clinical outcome.² The enrichment of cancer stem cells is one of the main reasons behind failure in treatment of HCC patients. Sorafenib (6), a multi-kinase inhibitor acting on VEGFR2-TK and also the only FDA approved drug for the treatment of HCC patients, inhibits proliferation and migration of tumour cells and angiogenesis. Recently, it has been reported that many patients develop resistance to sorafenib. This was explained by the enrichment of cancer stem cells that have the capacity to self-renew, differentiate into cancer

cells and acquire resistance to chemotherapy in most of the cancer types.^{3,4} Hence, it is crucial to develop novel drugs against the differentiated cancer cells as well as liver cancer stem cells in order to successfully eradicate liver cancer. Liver cancer stem cells could be identified and isolated by several surface markers including CD133, CD90, CD44, CD13, EpCAM, OV-6, CD24, DLK1 and ICAM-1.⁵ Cells that carry one or two of these markers were shown to possess CSC features.

The epithelial–mesenchymal transition (EMT) is a critical step for stemness. In this study, we have selected liver cancer cell lines with different phenotypic properties based on their genotypes. Huh7 and Hep3B cells are known to have epithelial features (well-differentiated), whereas Mahlavu and SNU-475 cells were characterized as mesenchymal-like (poorly-differentiated) cells.⁶ Huh7 and Hep3B cells express genes associated with epithelial like properties. These cells express the HCC marker AFP along with the epithelial marker E-cadherin, while Mahlavu and SNU-475 cells don't express AFP and E-cadherin genes while they express high levels of vimentin protein.⁶ Furthermore, Huh7 and Hep3B cells have normal migratory properties, but Mahlavu and SNU-475 cells have high migratory properties which may be due to their differential PI3K/Akt pathway activities. It was shown that Mahlavu and SNU-475 cells have constitutively active Akt protein due to loss of the PTEN tumor suppressor.⁷ Current studies and clinical trials focus on the anti-CSC compounds targeting extracellular mediators or cell surface molecules as well as molecules involved in EMT and metastasis.⁸ Thus, it is crucial to define which marker is efficient to detect and

^a Dept. of Mol. Biol. and Gen., Bilkent University, Ankara, 06800, Turkey

^b Laboratoire Syncat, UMR CNRS 7509, ECPM, Université de Strasbourg, 25 rue Becquerel, 67087 Strasbourg, France

^c Department of Chemistry and Namur Medicine & Drug Innovation Center (NAMEDIC), University of Namur, 61 rue de Bruxelles, 5000 Namur, Belgium

^d Faculty of Natural Sciences, Comenius University, Ilkovičova 6, Mlynská dolina 842 15 Bratislava (PŠ, AB), Slovakia

^e Biomagi Ltd., Mamatyova 26, 851 04 Bratislava, Slovakia

^f Cancer Systems Biology Laboratory, Graduate School of Informatics, ODTU, Ankara, 06800, Turkey. E-mail: rengul@metu.edu.tr

† The authors declare no competing interests.

‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c6md00392c

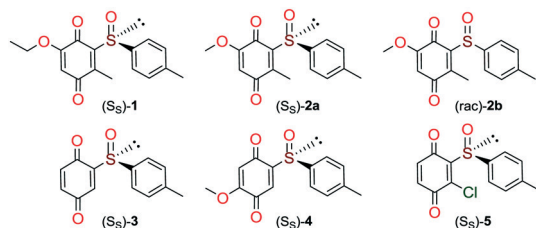


Fig. 1 The structures of tested quinoides 1–5.

analyse cancer stem cell markers in each cell line, which we studied by flow cytometry.

Quinones and quinone-like compounds seem to be promising candidates against cancer stem cells; however, they are mainly organotoxic. It has been reported that minor changes in the side chains of the quinone structure can lead to a strong variation in the biological toxicity;^{9–11} thus, modelization of various quinoid derivatives with strong inhibitory activity and low toxicity is conceivable. Therefore, we prepared a series of quinoides, 1–5 (Fig. 1), and evaluated their activities in different HCC cell lines and their cancer stem cell (HCSC) population (Tables 1, 2A and B and Fig. 5). Furthermore, VEGFR2 TKIs 6–10 (Fig. 2) were also screened in HCC cell lines (Tables 1 and 2A). Compound 6 is sorafenib tosylate (Nexavar®), and compound 9 was developed by GlaxoSmithKline as a drug candidate to inhibit VEGFR2 TK. Compounds 7, 8 and 10 were designed by Biomagi, Ltd. to modulate VEGFR2 TK activity.

Results and discussion

Chemistry

Compounds 1, 2a and 2b were prepared according to our previous work starting from commercially available 2-methylhydroquinone (S1) (Scheme 1).¹²

Compounds 3 and 5 were prepared (Scheme 2) according to Carreño's procedure with slight modifications (see the ESI†).^{13–15}

Table 1 Screening of quinoides 1–5 and VEGFR2-TKIs 6–10 in HCC cell lines

Compound	IC ₅₀ (μM) ^a		
	Huh7	Hep3B	Mahlavu
1	7.0 ± 0.8	12.0 ± 1.7	18.3 ± 0.8
2a	8.0 ± 0.9	5.5 ± 1.8	14.5 ± 1.3
2b	5.5 ± 0.4	4.3 ± 0.7	11.6 ± 0.2
3	20.0 ± 1.7	17.4 ± 2.1	31.3 ± 0.3
4	10.6 ± 2.0	9.9 ± 2.3	11.3 ± 0.6
5	Ni	Ni	Ni
6	1.6 ± 0.8	1.5 ± 0.3	0.7 ± 0.4
7	2.8 ± 1.3	2.0 ± 0.9	3.0 ± 0.5
8	1.7 ± 0.7	2.1 ± 0.3	0.8 ± 0.5
9	1.5 ± 0.7	0.7 ± 0.1	1.2 ± 0.4
10	0.4 ± 0.3	0.9 ± 0.3	1.1 ± 0.7
DAPT	5.0 ± 1.2	5.0 ± 2.0	5.0 ± 1.5

^a Experiments were performed in triplicate; Ni: no growth inhibition.

Table 2 IC₅₀ values and A_F factors of quinoides 1–5 and VEGFR2-TKIs 6–10 in (A) Huh7 and Hep3B epithelial cells and of quinoides 1–5 in (B) Mahlavu (MV) and SNU-475 (S-475) mesenchymal cells

A	IC ₅₀		A _F	
	Huh7	Hep3B	Huh7	Hep3B
1	7.0	12.0	1.2	1.0
2a	8.0	5.5	1.1	1.1
2b	5.5	4.3	1.3	1.1
3	20.0	17.4	0.9	1.1
4	10.6	9.9	0.9	1.1
5	Ni	Ni	0.7	1.0
6	1.6	1.5	2.3	2.3
7	2.8	2.0	2.3	2.9
8	1.7	2.1	2.3	2.1
9	1.5	0.7	1.7	1.7
10	0.4	0.9	1.7	2.6
DAPT	5.0	5.0	0.8	0.7

B	IC ₅₀		A _F	
	MV	S-475	MV	S-475
1	18.3	5.5	1.7	0.5
2a	14.5	10.5	1.3	0.5
2b	11.6	6.1	2.2	2.0
3	31.3	0.3	0.3	0.0
4	11.3	9.0	2.3	2.0
5	Ni	Ni	0.8	1.5
DAPT	5.0	0.6	0.2	0.5

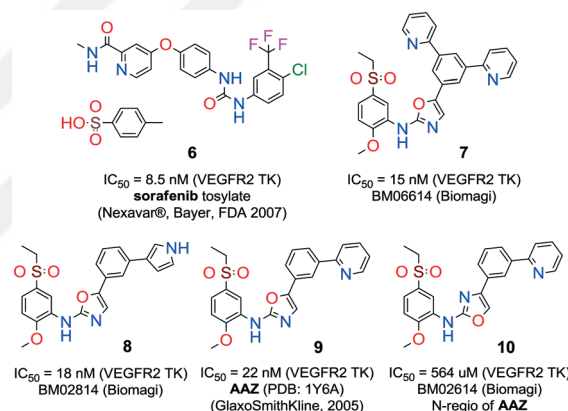


Fig. 2 VEGFR2 TKIs 6–10 used in this study and their origin and VEGFR2 IC₅₀ activities that were obtained through a commercial service in Germany (www.proqinase.com).

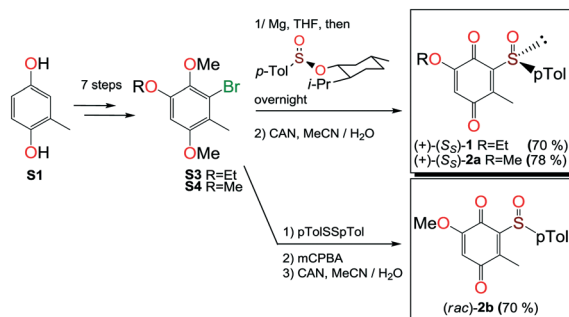
The new quinoid 4 was obtained from vanillin (S7) in very good overall yield using the sequence depicted in Scheme 3.

VEGFR2 TKI 6 was obtained from Bayer Inc. Synthesis of 7 was described by Lintnerová *et al.*¹⁶ Compound 9 was prepared according the procedure described by Harris *et al.*¹⁷ Novel compounds 8 and 10 were synthesized using the sequence depicted in Scheme 4 (see also the ESI†).

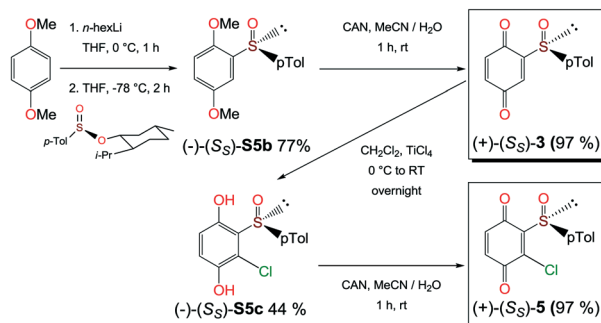
Biological evaluation

Bioactivities of quinoides and VEGFR2-TKs in HCC cells. NCI-SRB assay was initially performed to define the inhibitory concentrations (IC₅₀ values) of compounds in HCC cell lines Huh7, Hep3B, and Mahlavu. Results have shown that almost all compounds (except for 5) had IC₅₀ values in μM concentrations for all cell lines (Table 1). DAPT (γ-secretase inhibitor) was used as a control for CSC inhibition.

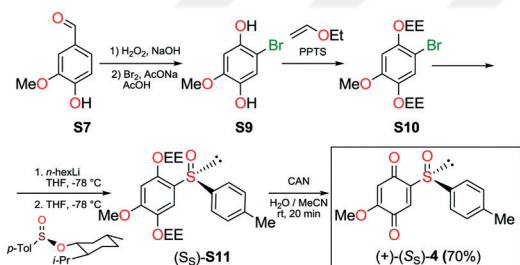
Expression of cancer stem cell markers in HCC cell lines. CSC marker expression varies among cell lines of HCC, since



Scheme 1 Preparation of quinones 1, 2a and 2b from 2-methylhydroquinone (S1).



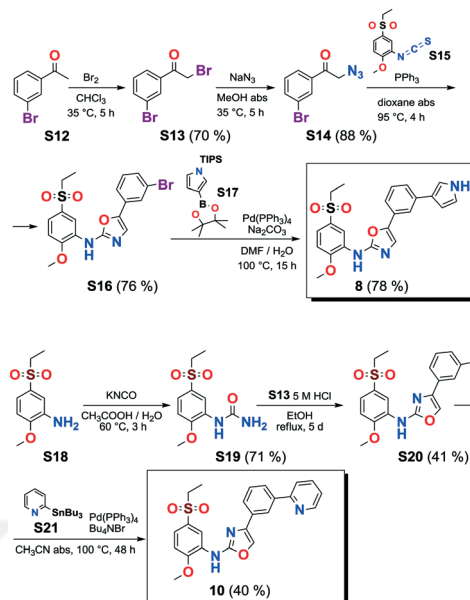
Scheme 2 Syntheses of quinones 3 and 5 from 1,4-dimethoxybenzene.



Scheme 3 Synthesis of compound 4.

liver CSCs exist heterogeneously and these features determine the characteristics of the cancer (tumourigenicity and metastatic tendency). It has previously been reported that the epithelial cell adhesion molecule (EpCAM) and CD133 expressing cells are more likely to execute epithelial features, whereas CD90 expressing cells are more likely to be mesenchymal.^{18,19} Expression of these markers in HCC cell lines was analyzed using flow cytometry. It was shown that CSCs of Huh7 cells could be identified by CD133 and EpCAM positivity (20–30%). CSCs of Mahlavu and SNU-475 could be identified by CD90 positivity (0.2–0.6%) (Fig. 3).

As stated above, the EMT is critical for cancer cell stemness. Huh7 and Hep3B cells carry epithelial features, whereas Mahlavu and SNU-475 cells carry migratory mesenchymal-like properties.^{6,7} Furthermore, Huh7 and Hep3B cells differ in their p53 gene status: Huh7 cells express p53(Y220S), but Hep3B cells are null for the TP53 gene. There-



Scheme 4 Syntheses of 8 and 10.

fore, the expression of mutant p53 causes differential activation of the β -catenin protein and its downstream mesenchymal proteins such as E-cadherin, vimentin, snail and slug. The significantly higher expression of β -catenin²⁰ due to the lack of p53 protein in Hep3B cells correlates with the higher expression of EpCAM and CD133 markers in this cell (Fig. 3).

Effects of quinoides and VEGFR2-TKIs on hepatocellular cancer stem cells (HCSCs). It is essential to discover novel compounds that were able to alter the enrichment of the CSC

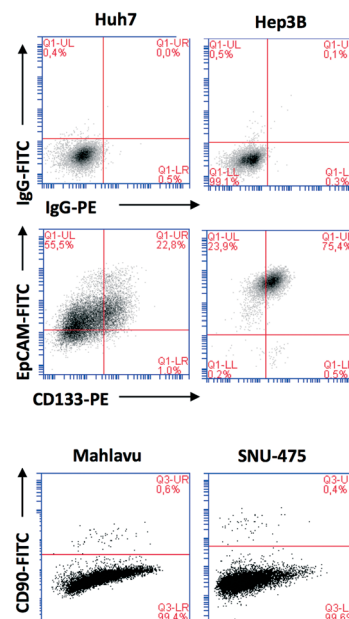


Fig. 3 Flow cytometry analysis of HCC cell lines indicating the positivity of HCC cell lines for cancer stem cell markers CD133, EpCAM and CD90. Top row: IgG isotype controls, middle row: cells stained for CD133 and EpCAM markers, bottom row: cells stained for CD90.

population as well as the cancer cells. For this reason, compounds 1–10 that were initially tested against HCC cell lines were further studied to determine the changes in the CSC marker positivity of Huh7 cells. Cells that were killed by the treatment were discarded, and the remaining cells were used in flow cytometry analyses. Interestingly, some of the compounds were able to reduce the percentage of CSC population, while some had the adverse effect on Huh7 cells that enriched the CSC subpopulation (Fig. 4). It was observed that unlike VEGFR2-TKIs, some of the quinoides were able to attenuate the CSC side population in Huh7 cells.

Due to the distinct dispersion of CD90 cells, we decided to include SNU-475 cells for testing the anti-HCSC activities of quinoides. Therefore, we also determined the bioactivities of quinoides in SNU475 cells. The IC_{50} values were 5.5 ± 1.4 , 10.5 ± 1.5 , 6.1 ± 2.0 , 0.3 ± 1.2 , 9.0 ± 2.1 , and 0.6 ± 1.1 μ M for compounds 1, 2a, 2b, 3, 4, and DAPT, respectively. No growth inhibition was observed for compound 5 (Fig. 5 and Table 2B).

Although compounds 6–10 were stronger HCC inhibiting agents as quinoides 1–5, they caused enrichment of Huh7 HCSCs in the rest of the HCC after treatment. Therefore, the positive cytotoxic effect of 6–10 was discredited by their ability to leave the HCC residue fraction after treatment enriched with aggressive HCSC cells that are highly susceptible to disease relapse and that acquired drug resistance frequently observed after HCC treatment in clinics.

Further analyses were carried out on Hep3B, Mahlavu and SNU-475 cells to test whether the same effect could be observed in other HCC cell lines as well. Among all compounds tested, 3 was able to attenuate the CSCs especially in mesenchymal-like cells, surpassing the efficacy of the stem

cell inhibitor, DAPT (Fig. 5). Altogether, the results have shown that compound 3 is potentially capable of impairing the cancer stem cells in HCC cells with a mechanism not yet discovered.

On the other hand, treatments of cells with VEGFR2-TKIs 6–10 were shown to enrich the cancer stem cell population of Huh7 and Hep3B cells at about 1.7–2.9 times as opposed to the effect of DAPT (Fig. 4 and Table 2A).

Conclusions

There is only one literature report describing macrocyclic benzoquinone herbimycin A [CAS: 70563-58-5, BRN: 4834067] that inhibits (61–78%) human bone marrow mesenchymal stem cells at 1–5 μ M concentration.²¹ We present here for the first time the screening of benzoquinone compounds in CSCs. One of the achievements of our pivotal study is identification of quinoid 3 as an exceptional compound to treat HCSCs. Although its mechanism is not yet known, we hypothesize different behaviours of screened quinoides 1–5 based on their structural differences, benzoquinone reactivity and general medicinal chemistry knowledge. All quinoides 1–5 possess a chemically reactive benzoquinone fragment that can likely be an irreversible inhibitor binding to a biological target *e.g.* by the Michael reaction with a cysteine amino acid residue. The most active quinoid 3 in Mahlavu and SNU-475 is the one that misses most of substitutions on its benzoquinone skeleton compared to the other tested quinoides. The steric and electronic properties of 3 could be a reason why quinoid 3 is the most active one included (Fig. 5). In the case of the SNU-475 tumour, there are three active quinoides (1, 2a and 3); therefore, other mechanism(s) can also be included. As could be seen, all VEGFR2 TKIs 6–10 are cytotoxic to HCC cell lines (IC_{50} = 3.0–0.4 μ M, Table 1), and at the same time, they are less toxic towards HCSCs since it was observed that cancer stem cells were enriched up to 3 times after treatment (Fig. 5 and Table 2A). The most synergic case would be to identify cytotoxic compounds for both HCCs and HCSCs. The results obtained by screening of VEGFR2 TKIs can deliver two positive consequences: i) VEGFR2 TKIs can be used to enrich the % of stem cells in the final fraction before stem cell isolation for research purposes. Moreover, such stem cells can remember VEGFR2 TKI treatment and can be used for the development of the next generation of inhibitors. ii) It is obvious that experimental compounds can be differently cytotoxic towards heterogeneous tumour cells. Their different influence on CSCs should be quantified separately because of its practical meaning.

In cancer therapeutics, it is important to identify treatment regimens against cancer stem cells as well as tumor cells, because patients suffer from relapse or incomplete recovery after treatment if it fails to eliminate CSCs. The percentage of DMSO treated cells in flow cytometry analysis represents the initial CSC marker positivity of the cells before treatment (Fig. 4). Thus, we suggest that once the cells were treated with a compound, the number of cells with marker

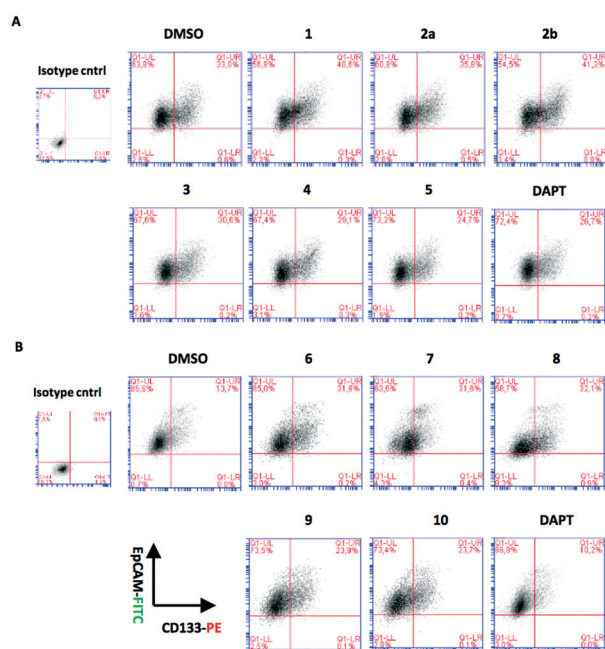


Fig. 4 Expression of stem cell markers CD133 and EpCAM in Huh7 cells treated with (A) quinoides 1–5 and (B) VEGFR2-TKIs 6–10 as shown by flow cytometry analysis.

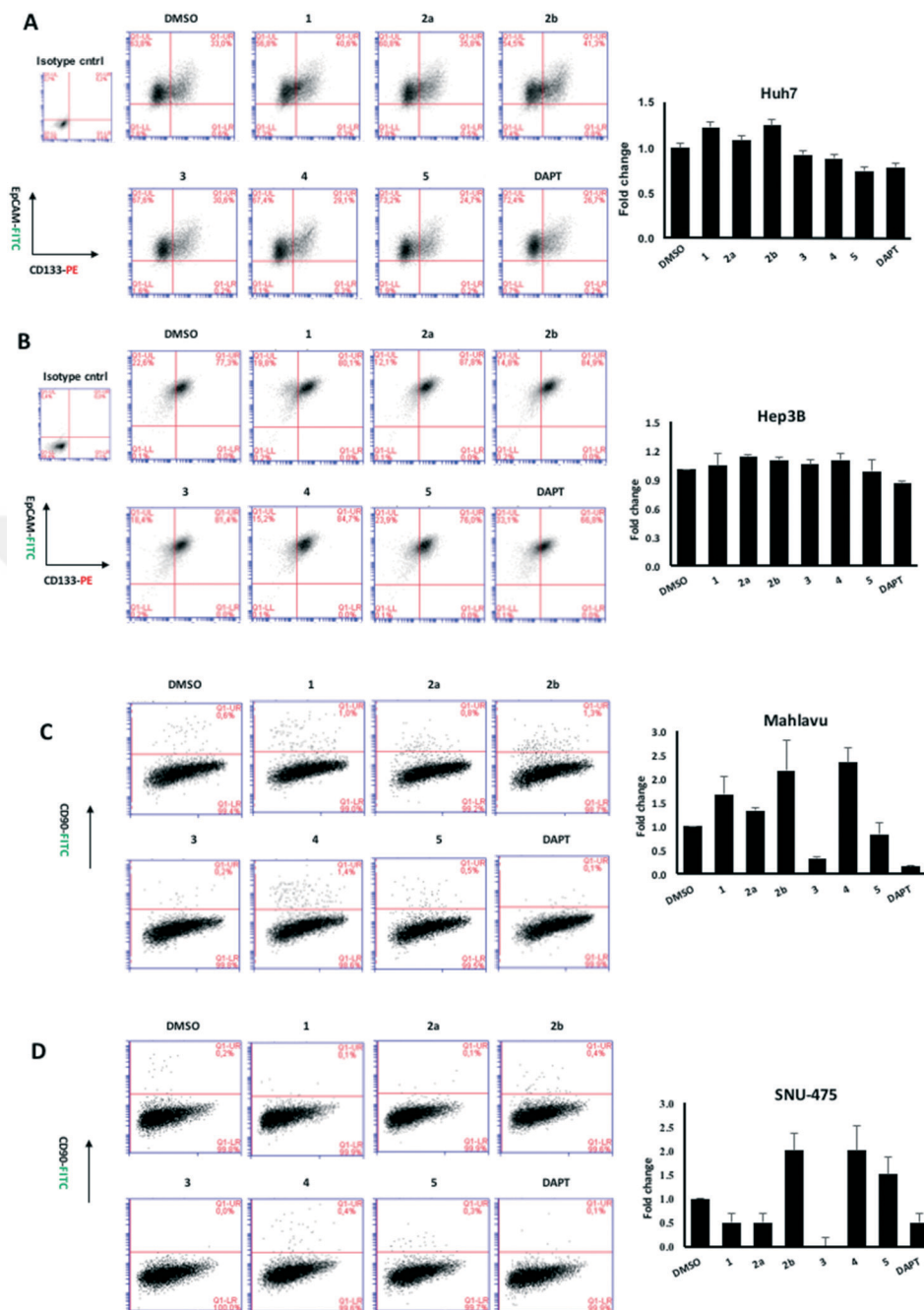


Fig. 5 Flow cytometry analysis of HCC cell lines after 72 hours of treatment with quinoides 1–5. (A) Huh7, (B) Hep3B, (C) Mahlavu, and (D) SNU-475. Cancer stem cells were defined by their positivity for CSC markers, CD133 together with EpCAM, or CD90. Each treatment was compared to its corresponding DMSO control to define the changes in percentage of double positive population. DAPT is used as positive control for CSC inhibition. The bar graphs indicate the fold change in positivity for markers between DMSO control and experimental groups.

positivity can be compared with the DMSO control in order to define the fold change in the percentage of cancer stem cells. Since only the cells that stay alive after treatment are analyzed (see methods), it is important to emphasize the density of the cancer stem cells before and after treatment. Therefore, the compounds that cause a decrease or enrichment in cancer stem cell population can be quantified simply by normalizing the treated cell population by DMSO controls.

Here, we suggest the aggressiveness factor (A_F), a new characteristic quantifying the risk for certain compounds to be able to develop a more aggressive disease: A_F (exp. compd.) = [(total number of CSCs after exp. compd. treatment)/(total number of CSCs before treatment)].

The low A_F value is an indication for the quality of a drug candidate toward cancer cells. Such molecules don't possess drug resistance or induce more aggressive tumours (Table 2). A_F

reflects the fold increase of CSCs that survived the compound treatment independently of the shrinkage of the tumour itself.

The compounds with A_F values below 1 can be regarded as molecules which reduce the cancer stem cell population, whereas A_F values above 1 signify molecules that enrich the cancer stem cell population. Thus, the usage of A_F values defines the success or failure of the compounds in affecting cancer cells toward cancer stem cell population.

Cancer stem cell-like subpopulations carry behaviors such as higher tumor-forming and metastasis capacities along with resistance to antitumor drugs which allows tumors to survive and relapse.⁸ The analysis of the action of the chemotherapeutics that deplete the CSC-like population by the following surface markers may enumerate the qualities of the compounds and may allow one to assess their differential action. Our results represent parallel findings with sorafenib (6) and other compounds and allow comparative analysis of the compounds toward cancer stem cell marker positive cell populations. The A_F concept introduced in this study clearly demonstrates the differential action of the compounds.

It is known that patients used to develop resistance towards sorafenib (6). This is consistent with our data with sorafenib (6) having an A_F value that is 2 fold higher compared to that of the DMSO control while maintaining good cytotoxicity through the low IC_{50} values (Table 2A). However, the known CSC inhibitor (DAPT) has low A_F values and its IC_{50} is much higher than that for sorafenib (6). Furthermore, while quinoide 5 does not possess cytotoxic actions (for each HCC line) and 3 has high IC_{50} values against Huh7, Hep3B or MV (Mahlavu) cells, they both significantly reduce the CSC marker positive subpopulations in mesenchymal Mahlavu and SNU-475 cells (Tables 2A and B).

The normalized aggressiveness factor allows selection of promising experimental compounds possessing lower probability to form aggressive tumours in comparison to a particular drug with known clinical behaviour. The methodology can serve as a simple and valuable tool for pre-clinical screening.

Experimental section

Syntheses of compounds 1–10

The syntheses and physicochemical properties of prepared organic compounds 1–10 can be found in the ESI.†

Cell culture

Huh7 and Mahlavu, human hepatocellular carcinoma (HCC) cell lines, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen/GIBCO), supplemented with 10% fetal bovine serum (FBS) (Invitrogen/GIBCO) and 0.1 mM nonessential amino acid, whereas SNU-475 cells were maintained in RPMI (Invitrogen/GIBCO), supplemented 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Both media contained 100 units per mL penicillin and 100 units per mL streptomycin. Cells were grown at 37 °C in a humidified incubator under 5% CO_2 .

NCI-60 sulforhodamine B (SRB) cytotoxicity assay

Huh7 and Hep3B (2000 cells per well), SNU-475 (1000 cells per well) and Mahlavu (1000 cells per well) cells were inoculated into 96-well plates (150 μ l per well). After 24 hours, molecules of interest and DMSO control were applied in concentrations of 40 μ M to 2.5 μ M in serial dilutions. After 72 h of treatment, cells were fixed with cold 10% (w/v) trichloroacetic acid (MERCK) for an hour. Then, the wells were washed with ddH₂O and dried. 50 μ l of 0.4% SRB dye (Sigma-Aldrich) was applied to each well and incubated at room temperature for 10 min. Each well was washed with 1% acetic acid and left for air-drying. SRB dye was solubilised using 100 μ l per well 10 mM Tris-Base solution, and the absorbance was measured at 515 nm. The experiment was performed in triplicate, and the absorbance values were normalized to DMSO controls.

Flow cytometry

HCC cells are inoculated into 100 mm² culture dishes (100 000–200 000 cells). 24 hours later, cells were treated with the compounds (IC_{100} conc.) for 72 hours. Dead cells that no longer remained attached to the surface of the culture plates were discarded through vacuum aspiration and cells that remained attached were collected to be fixed with 4% para-formaldehyde for 30 minutes. Huh7 and Hep3B cells were stained for cancer stem cell markers using anti-CD133/1 (AC133)–biotin (Miltenyi, 130-090-664), anti-biotin-PE (Miltenyi, 130-090-756), and anti-EpCAM-FITC (Miltenyi, 130-080-301), whereas Mahlavu and SNU-475 cells were stained using anti-CD90-FITC (Miltenyi, 130-095-403). For isotype controls, mouse IgG1 isotype control-FITC conjugate (Miltenyi, 130-092-213) and mouse IgG1 isotype control antibody-biotin conjugate (Miltenyi, 130-093-018) were used. Staining of cells was performed according to the manufacturer's protocol. Cells were analyzed using the BD Accuri C6 Flow Cytometer and Software (BD Biosciences). The same staining procedure was applied for the analysis of HCC cells in order to determine the CSC marker positivity.

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Research paper

Synthesis and biological evaluation of novel pyrazolic chalcone derivatives as novel hepatocellular carcinoma therapeutics



Mohammed M.A. Hawash ^{a,1}, Deniz Cansen Kahraman ^{b,1}, Fikriye Eren ^a,
Rengul Cetin Atalay ^{c,**}, Sultan Nacak Baytas ^{a,*}

^a Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, 06330, Ankara, Turkey

^b Department of Molecular Biology and Genetics, Bilkent University, 06800, Ankara, Turkey

^c Cancer Systems Biology Laboratory, Graduate School of Informatics, METU, 06800, Ankara, Turkey

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ABSTRACT

Despite having the second highest mortality associated with cancer, currently Sorafenib is the only FDA-approved chemotherapeutic agent available for liver cancer patients which can only improve survival for few months. In this study, various pyrazolic chalcone analogous compounds were synthesized and evaluated as potential chemotherapeutic agents for the treatment of hepatocellular carcinoma (HCC). Modifying the central pyrazole ring at the C(3)-position with different heteroaryl rings and substituting the C(4)-position of pyrazole with differently substituted chalcone moiety produced fourty two variant compounds. For all these compounds, cytotoxicity was evaluated using sulforhodamine B assay and real time cell growth tracking, respectively. Based on 50% inhibitory concentration (IC₅₀) values, compounds **39**, **42**, **49**, and **52** were shown to exhibit potent cytotoxic activity against all the cancer cell lines tested, and had better cytotoxic activities than the well-known chemotherapeutic drug 5-FU. Therefore, these compounds were chosen to be further evaluated in a panel of HCC cell lines. Flow cytometric analysis of HCC cells treated with compounds **39**, **42**, **49**, and **52** demonstrated that these compounds caused cell cycle arrest at G2/M phase followed by the apoptotic cell death and impaired cell growth as shown by real-time cell growth surveillance. Consistent with these results, western blotting of HCC cells treated with the compounds resulted in molecular changes for cell cycle proteins, where p21 levels were increased independent of p53 and the levels of the key initiators of mitosis Cyclin B1 and CDK1 were shown to decrease upon treatment. In conclusion, chalcone derivatives **42** and **52** show potent bio-activities by modulating the expression of cell-cycle related proteins and resulting in cell-cycle arrest in the HCC cell lines tested here, indicating that the compounds can be considered as preclinical candidates.

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1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second most frequent cause of cancer-related mortality [1]. Each year, around five hundred thousand new cases are diagnosed world-wide and nearly 85% of all cases are from developing countries. The 5 year survival rate of HCC patients are low and the incidence of HCC continues to rise (3 fold increase in 20 years). The etiological factors associated with HCC are chronic

infection by Hepatitis B virus (HBV) and Hepatitis C virus (HCV), aflatoxin exposure, chronic alcohol intake, and obesity [2,3]. Currently, Sorafenib, a multikinase inhibitor, is the only drug approved by FDA for the treatment of HCC which can only prolong patient survival for a short period of time (2–3 months) [4]. Since liver cancer cells display inherent resistance to conventional chemotherapy and radiotherapy, it is critical to develop novel therapeutic drugs against HCC.

Dysregulation of cell cycle control and cell division is highly correlated with cancer development, growth and progression. Liver cancer develops during chronic liver injury which involves immune response along with cytokines and chemokine activation. Hence during chronic liver disease, cycles of hepatocyte cell death and regeneration are induced which engage the activation of cell cycle proteins [5]. Cyclin-dependent kinases (CDKs) are one of the key

* Corresponding author.

** Corresponding author.

E-mail addresses: rengul@metu.edu.tr (R. Cetin Atalay), baytas@gazi.edu.tr (S.N. Baytas).

¹ These authors contributed equally.

cellular kinases involved in cell cycle regulation in liver [6]. They act together with their cyclin partners to phosphorylate the Rb protein which helps the release of transcription factors to ensure cell cycle transition. Alterations in the activities of CDKs lead to the transformation of normal cells toward cancerous state [7–12]. Therefore, design and synthesis of therapeutic agents targeting CDKs have been the subject of recent drug discovery research studies and a number of CDK inhibitors have been considered in several clinical trials [13–16]. Hence Palbociclib, which is a selective inhibitor of CDK4 and CDK6, was approved by the FDA to be used in combination with letrozole for the treatment of postmenopausal women with HER2-negative advanced breast cancer [17–20].

Chalcones, 1,3-diaryl-2-propen-1-ones, are naturally occurring intermediates of flavonoid compounds [21]. Chalcones have shown broad range of biological activities such as antioxidant, antibacterial, antifungal, anti-HIV, antileishmanial, antitubercular, anti-inflammatory, and anticancer properties [22,23]. It has been determined that many chalcone derivatives exhibit cytotoxic activity in various cancer cell lines through different molecular mechanisms [24,25].

Pyrazoles with their distinctive scaffold possess wide range of anticancer bioactivities [26–31]. Though more widely known for their use in analgesic, antipyretic and anti-inflammatory actions, pyrazoles can also have antileukemic [32,33], antitumor [34,35] and anti-proliferative [36]. Although the pyrazole skeleton is an important building block in mediating its biological effects, the type of peripheral substituents are crucial for their selectivity toward their targets. Studies concerning structure–activity relationships have shown that the cytotoxic potency of the compounds has been highly dependent on the substitution types and patterns on the aryl ring. Thus, 3,4-disubstituted pyrazole analogues, and 3-

(imidazol-2-yl)-4-[2-(pyridin-3-yl)-vinyl]-pyrazoles (1), have been reported as cyclin-dependent kinase (CDK) inhibitors and have been shown to inhibit *in vitro* cellular proliferation in various human cancer cells [37]. In another study, Huang et al. showed that *N*-((1,3-diphenyl-1*H*-pyrazol-4-yl)methyl)aniline derivative **2** [38] had inhibitory activity on MCF-7 and B16-F10 cell lines with IC_{50} values of 1.88 and 2.12 μ M, respectively. Compound **2** inhibits the CDK2/cyclin E holoenzyme activities with IC_{50} of 0.98 μ M (Fig. 1). Recently, *N*-((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)-1,3-diphenyl-1*H*-pyrazole-4-carboxamide derivatives were reported as CDK1/Cdc2 inhibitors [39].

In our previous studies, we reported 1,3-diarylpyrazole derivatives containing thiophene and pyridine heterocycles as significant antiproliferative agents against MCF7, MDA-MB-231, HeLa, Raji and HL60 human cancer cell lines (**3** in Fig. 1) [40,41]. Therefore considering the limited availability of targeted therapies in liver cancer, in this study, the cytotoxicities of pyrazolic chalcone derivatives are investigated in order to identify the potential bioactivities as drug candidates in treatment of HCC. Here we report the synthesis and the biological activities of a new series of 1,3,4-trisubstituted pyrazoles **12–53** in which; *i*) a heteroaryl moiety (3-thienyl, 3-(benzo[d][1,3]dioxol-5-yl, 3-pyridyl, 4-pyridyl) is attached to the central pyrazole ring at position 3; *ii*) phenyl is attached to the pyrazole ring at position 1 and *iii*) chalcone moiety is attached to the central pyrazole ring at position 4.

2. Results and discussion

2.1. Chemistry

The 1,3-diarylpyrazole chalcone derivatives (**12–53**) were

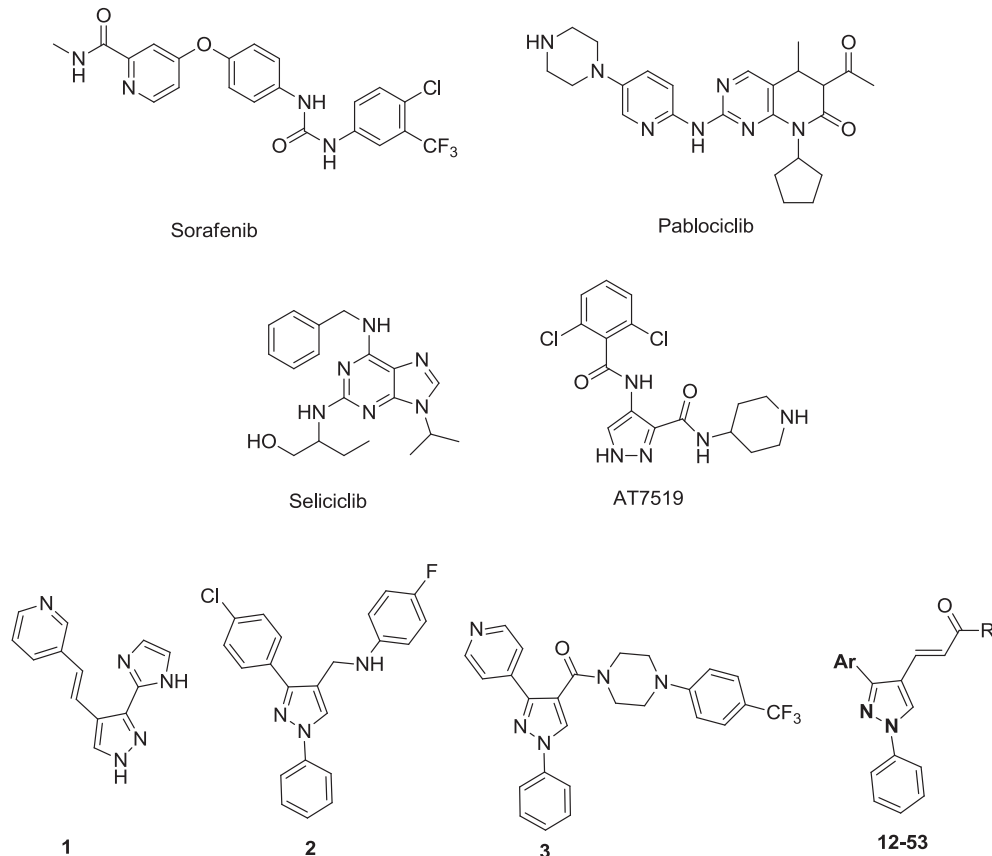


Fig. 1. Structure of hepatotoxic agents and some CDK inhibitors which are already approved or have reached clinical trials for the treatment of cancer; lead CDK inhibitors and title compounds.

synthesized as outlined in Scheme 1. The hydrazone derivatives **4–7** were generated by condensing appropriate methyl ketone and phenylhydrazine in the presence of acetic acid in refluxing ethanol. The IR spectra of hydrazone **4–7** showed disappearance of the carbonyl peak that belonged to acetyl group of methyl ketone derivatives. This hydrazone derivative was then reacted with POCl₃ and DMF resulting in 1,3-diaryl pyrazoles **8–11** with aldehyde group at 4 position. 1,3-Diarylpyrazole chalcone analogs **12–53** were prepared by condensation of 1-phenyl-3-heteroaryl-1*H*-pyrazole-4-carboxaldehyde derivatives **8–11** and corresponding methyl ketones in ethanol solution, applying 40% aqueous NaOH as a catalyst.

All compounds were purified either by automated flash chromatography or recrystallization; or automated flash chromatography then recrystallization and checked for purity by elemental analysis or UPLC (purity was >97%) before being tested in biological assays. The structures of these compounds were confirmed by high-resolution mass spectrometry (HRMS), IR, ¹³C NMR and ¹H NMR spectral data. Elemental analysis results were also provided for eighteen of the title compounds.

2.2. Biological evaluations

2.2.1. Cytotoxicities of the pyrazolic chalcone derivatives in liver cancer cells

Pyrazolic chalcone derivatives (**12–53**) were initially screened against Hepatocellular (Huh7), breast (MCF7) and colon carcinoma (HCT116) cells. Bioactivities of each compound were evaluated by sulforhodamine B (SRB) assay. The IC₅₀ values after 72 h of treatment with each molecule were calculated in comparison with the well known chemotherapeutic agents such as, nucleobase analogue 5-fluorouracil (5-FU) and nucleoside analogue cladribine. IC₅₀ values were in between 0.3 and 29 μM as shown in Table 1.

In general, compounds having thenyl moiety at position 3 on the pyrazole ring (**12–19**) displayed great antiproliferative potency on all cancer cell lines with the exception of the benzo[d][1,3]dioxol-5-yl chalcone derivative **19**. Compounds having methoxy substituent at positions 3 and 4 or at 2 and 5 on the phenyl chalconic moiety of the molecules (**16, 17**) showed IC₅₀ values of 0.4–3.4 μM in Huh7, MCF7 and HCT116 cell lines.

Replacing the thenyl moiety (**12–19**) with benzo[d][1,3]dioxol-5-yl ring (**20–31**) resulted in a significant decrease in the cytotoxic activity of the compounds which bears methoxy groups on the phenyl chalconic moiety especially against Huh7 (6.1–19.0 μM) and MCF7 (5.8–14.5 μM). Compounds having benzo[d][1,3]dioxol-5-yl moiety at position 3 on the pyrazole ring and heterocyclic rings such as 3-thenyl, 3-/2-pyridyl at the chalconic part (**27–29**) showed notable antiproliferative activity against Huh7 (3.2–3.4 μM), MCF7

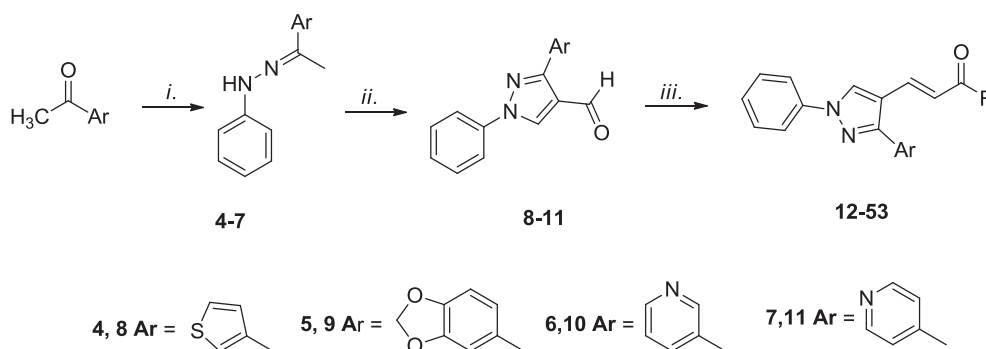
(2.2–4.9 μM) and HCT116 (3.8–8.9 μM) cell lines. Derivatives **30** (having benzo[d][1,3]dioxol-5-yl moiety at both on the pyrazole and chalcone part) and **31** (bearing benzo[d][1,3]dioxol-5-yl moiety at position 3 on the pyrazole and 3-pyridyl moiety at chalcone part) had weak activity for all cell lines used in this study (9.2–27.2 μM).

The 3-/4-pyridyl derivatives at 3 position of pyrazole ring (**32–53**) displayed very significant antiproliferative activity on all cancer cell lines (IC₅₀ values of below 7 μM) with the exception of compound **46** which bears 3-pyridyl moiety at chalcone part. Introduction of heterocyclic rings at chalcone part as in compounds **33, 34, 35, 36, 46** did not result in any advantage toward the activity of the compounds. In 3-pyridyl series, regarding the effect of substitution on the phenyl ring (R) at chalcone side, the 2,4,6-trimethoxy derivative **39** has remarkable cytotoxic activity against all tested human cancer cells, especially to Huh7 (Table 1). Compound **42**, the 2,5-dimethoxy derivative were also showed significant activity against all test cell lines (IC₅₀ values of 1.3 μM Huh7; 1.5 μM MCF7; 1 μM HCT116). Their analogs in 4-pyridyl series, compounds **49** and **52** were also very active, with IC₅₀ values in the range of 0.6–2.6 μM against all tested cell lines following 72 h of treatment. When we compared the IC₅₀ values of these compounds to well-known chemotherapeutic drugs, we observed that they were lower than 5-FU and comparable to that of cladribine. Therefore, the compounds which displayed significant cytotoxic effects (**39, 42, 49, 52**) were chosen to be further evaluated in a panel of HCC cell lines composed of Huh7, HepG2, Mahlavu and SNU-475 cells, MCF12A, normal human breast epithelial cells and MRC5, human fetal lung fibroblast cells. Our results showed that pyrazolic chalcone derivatives were cytotoxic to HCC cells, however they were less cytotoxic to MCF12A and MRC-5 cells. (Table 2). Additionally, compound **49** had no cytotoxicity on MRC5 cells.

Recent studies have shown that some chalcone derivatives inhibit cell growth of several human cancer cell lines as tubulin binding and depolymerizing agents [34]. The chalcone analogs of some natural products such as combretastatin have been synthesized and developed by Ducki et al. [42]. These chalcone analogs show potent inhibition of tubulin assembly and possess promising anticancer activity, and are currently under preclinical evaluation [43,44]. Based on this information, we analyzed the effect of compounds **12–53** on tubulin polymerization, however no inhibitory effect on tubulin polymerization was observed (data not shown).

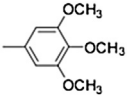
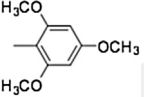
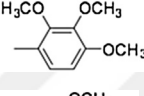
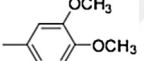
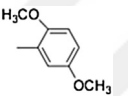
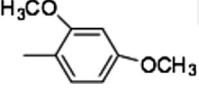
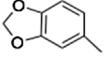
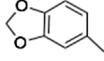
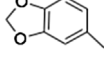
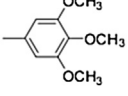
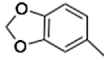
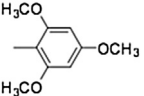
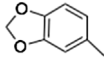
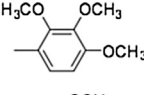
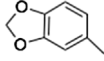
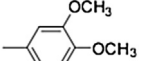
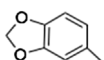
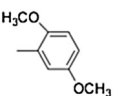
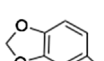
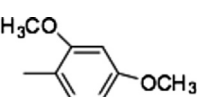
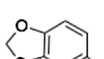
2.2.2. Real-time cellular response of hepatocellular carcinoma cells with **39, 42, 49** and **52** treatment

Time-dependent cytotoxic activities of pyrazole chalcone derivatives were performed with real time cell electronic sensing (RT-CES) on Huh7, HepG2, Mahlavu and SNU-475 cells. It was shown that all compounds reduced the growth rate of cells compared to



Scheme 1. Synthesis of 1,3-diarylpyrazole chalcone derivatives. Reagents and conditions: i.) Phenyl hydrazine, acetic acid, ethanol, 2 h reflux. ii.) Dimethyl formamide, POCl₃, 50 °C, 4 h. iii.) methyl ketone derivatives, NaOH, EtOH.

Table 1Cytotoxicity of compounds **12–53** and nucleoside analogs 5-FU and cladribine assessed in different human cancer cells.

Compound	Ar	R	IC ₅₀ (μM)		
			Huh7	MCF7	HCT116
12			4.7 ± 0.74	1.9 ± 0.30	5.1 ± 0.55
13			3.6 ± 0.58	4.9 ± 0.01	3.2 ± 0.65
14			6.1 ± 0.03	2.7 ± 0.54	5.2 ± 1.47
15			4.2 ± 0.47	7.4 ± 0.59	5.8 ± 1.57
16			2.9 ± 0.54	2.8 ± 0.51	1.6 ± 0.32
17			2.1 ± 1.37	0.4 ± 0.25	3.4 ± 0.48
18			3.0 ± 0.54	9.0 ± 1.05	2.2 ± 0.43
19			22.6 ± 0.87	5.3 ± 0.22	12.2 ± 0.78
20			10.5 ± 0.33	11.1 ± 0.59	1.16 ± 0.01
21			11.7 ± 0.53	10.9 ± 1.25	8.3 ± 1.07
22			6.7 ± 0.21	11.5 ± 0.32	10.8 ± 0.98
23			10.8 ± 0.8	7.4 ± 0.17	3.2 ± 0.54
24			15.4 ± 0.9	11.7 ± 0.37	11.4 ± 0.01
25			6.1 ± 0.11	5.8 ± 0.82	1.3 ± 0.36
26			19.0 ± 1.01	14.5 ± 0.6	8.6 ± 0.9
27			3.2 ± 0.44	4.9 ± 0.04	8.9 ± 0.71

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Table 1 (continued)

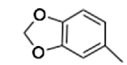
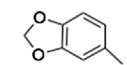
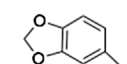
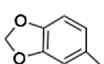
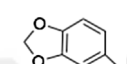
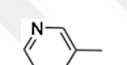
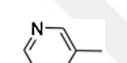
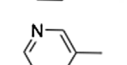
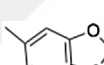
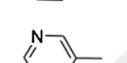
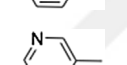
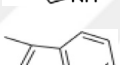
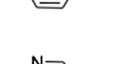
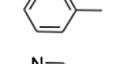
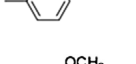
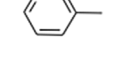
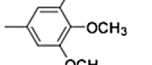
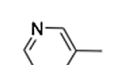
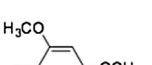
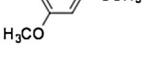
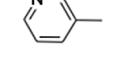
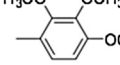
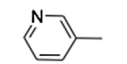
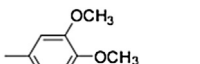
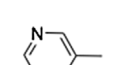
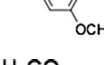
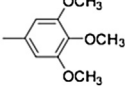
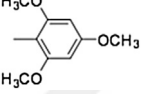
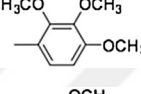
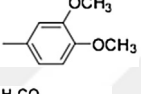
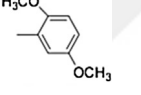
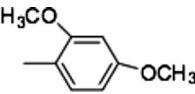
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			Huh7	MCF7	HCT116
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29			3.4 ± 0.9	2.2 ± 0.55	3.8 ± 0.20
30			9.9 ± 0.77	17.5 ± 0.7	27.2 ± 1.0
31			9.2 ± 0.95	9.9 ± 0.27	20.3 ± 0.56
32			1.7 ± 0.51	0.5 ± 0.04	1.3 ± 0.9
33			6.4 ± 0.8	4.2 ± 0.57	2.1 ± 0.35
34			6.7 ± 0.31	5.6 ± 0.77	1.8 ± 0.67
35			3.3 ± 0.78	6.9 ± 0.05	7.4 ± 1.02
36			3.2 ± 1.07	2.6 ± 0.00	6.4 ± 0.24
37			2.3 ± 0.49	4.1 ± 0.05	2.0 ± 0.71
38			1.9 ± 0.53	14.5 ± 0.06	8.6 ± 0.35
39			0.5 ± 0.17	1.0 ± 0.50	0.3 ± 0.15
40			2.3 ± 1.43	1.4 ± 0.14	1.1 ± 0.46
41			3.1 ± 0.32	1.7 ± 0.84	4.3 ± 1.20
42			1.3 ± 0.16	1.5 ± 0.52	1.0 ± 0.50
43			2.4 ± 0.56	2.3 ± 0.02	1.3 ± 0.75
44			2.2 ± 0.5	1.5 ± 0.58	1.1 ± 0.60
45			0.4 ± 0.14	0.8 ± 0.02	5.3 ± 0.90

Table 1 (continued)

Compound	Ar	R	IC ₅₀ (μM)		
			Huh7	MCF7	HCT116
46			28.8 ± 1.85	13.6 ± 1.47	15.7 ± 0.66
47			1.8 ± 0.51	3.4 ± 0.44	3.1 ± 0.88
48			3.5 ± 0.07	3.5 ± 0.04	0.3 ± 0.29
49			2.6 ± 0.58	1.6 ± 0.66	0.6 ± 0.18
50			3.7 ± 0.03	3.3 ± 0.04	1.3 ± 0.86
51			2.0 ± 0.25	4.3 ± 0.07	1.2 ± 0.32
52			1.7 ± 0.21	1.4 ± 0.38	0.9 ± 0.23
53			4.4 ± 0.52	8.8 ± 0.18	4.4 ± 0.52
5-FU			21.0 ± 0.75	1.4 ± 0.26	18.4 ± 1.1
Cladribine			1.8 ± 0.43	2.0 ± 0.11	0.3 ± 0.15

NI: No inhibition.

Table 2

IC₅₀ values of compounds **39**, **42**, **49** and **52** for HCC cell lines (Huh7, HepG2, Mahlavu and SNU-475) and in epithelial cells (MCF12A and MRC-5).

Compound	IC ₅₀ Values (μM)					
	Huh7	HepG2	Mahlavu	SNU-475	MCF12A	MRC-5
39	0.5 ± 0.2	1.4 ± 0.2	1.1 ± 0.2	0.8 ± 0.1	1.2 ± 0.5	1.3 ± 0.6
42	1.3 ± 0.2	3.4 ± 0.8	2.0 ± 0.3	1.9 ± 0.1	2.4 ± 0.8	2.7 ± 0.9
49	2.6 ± 0.6	1.7 ± 0.1	1.1 ± 0.1	1.4 ± 0.3	8.2 ± 0.6	NI
52	1.7 ± 0.9	4.8 ± 0.3	1.6 ± 0.2	2.0 ± 0.3	3.0 ± 0.6	3.1 ± 0.9

NI: No inhibition in cell growth.

the DMSO control (Fig. 2). This real-time growth pattern suggested that the growth inhibition was due to cell cycle arrest, where the cells were neither proliferating nor dying, whereas the cells treated with DMSO continued to proliferate until they reach confluence [45].

2.2.3. Characterization of cell death mechanism induced by pyrazolic chalcone derivatives

HepG2 and SNU475 cells were treated with the compounds for 48 h. In order to determine the death mechanism induced by pyrazolic chalcone derivatives, fluorescent microscopy using Annexin V staining was employed. Compared to DMSO controls, cells treated with all four of the chosen compounds were detached from the

surface (Suppl. Fig. 5) and cells with condensed nuclei were positive for Annexin V staining, which indicates apoptotic cell death (Fig. 3a). After 24 h of treatment, PARP cleavage was observed in poorly-differentiated SNU475 cells by western blot analysis compared to well-differentiated HepG2 cells (Fig. 3b) [46].

2.2.4. Induction of G2/M cell cycle arrest by the pyrazolic chalcone derivatives

Since the pattern of cell growth observed from the real-time cell analysis suggested the induction of cell cycle arrest flow cytometry analysis using propidium iodide (PI) staining of DNA was then performed. Following 48hrs of treatment entry to G2/M phase was observed to be increased, which subsequently caused entry into sub-G1 phase at 72 h, especially with compounds **39**, **42** and **52** (Fig. 4). Altogether, our results indicate that pyrazolic chalcone derivatives induce cell cycle arrest at the G2/M phase which was followed by apoptotic cell death in hepatocellular carcinoma cell lines.

2.2.5. Analysis of targeted cellular pathways

Cell cycle-related proteins more specifically those that are involved in the G2/M phase were analyzed by western blotting (Fig. 5). CyclinB1 and CDK1 complex was previously shown to be critical for the progression of cells in and out of M phase of the cell

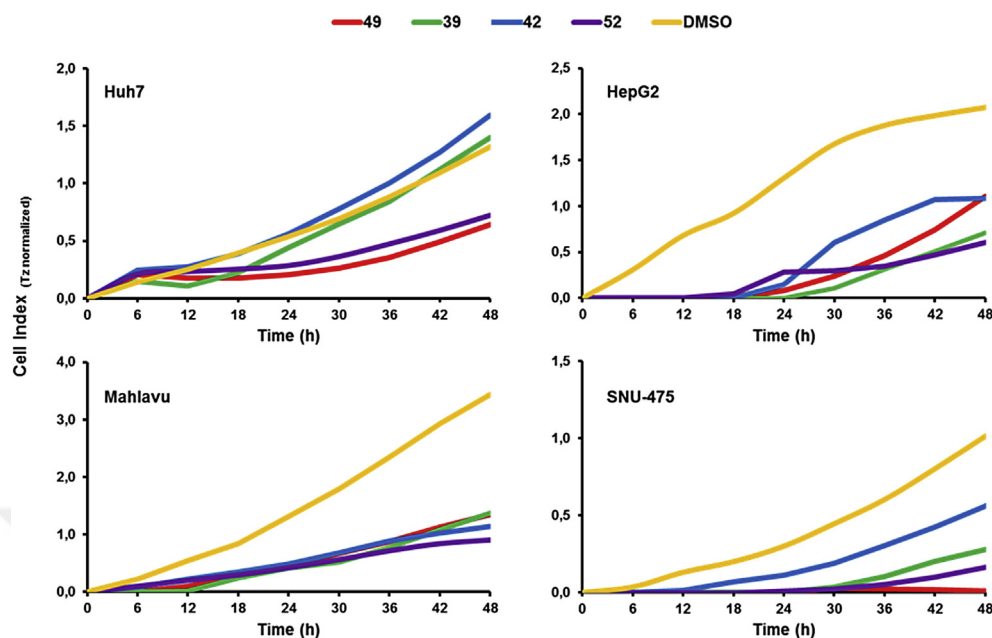


Fig. 2. RT-CES analysis of HCC cell lines treated with compounds **39**, **42**, **49** and **52** and with DMSO control (0.1%) at IC_{100} concentrations for 48 h.

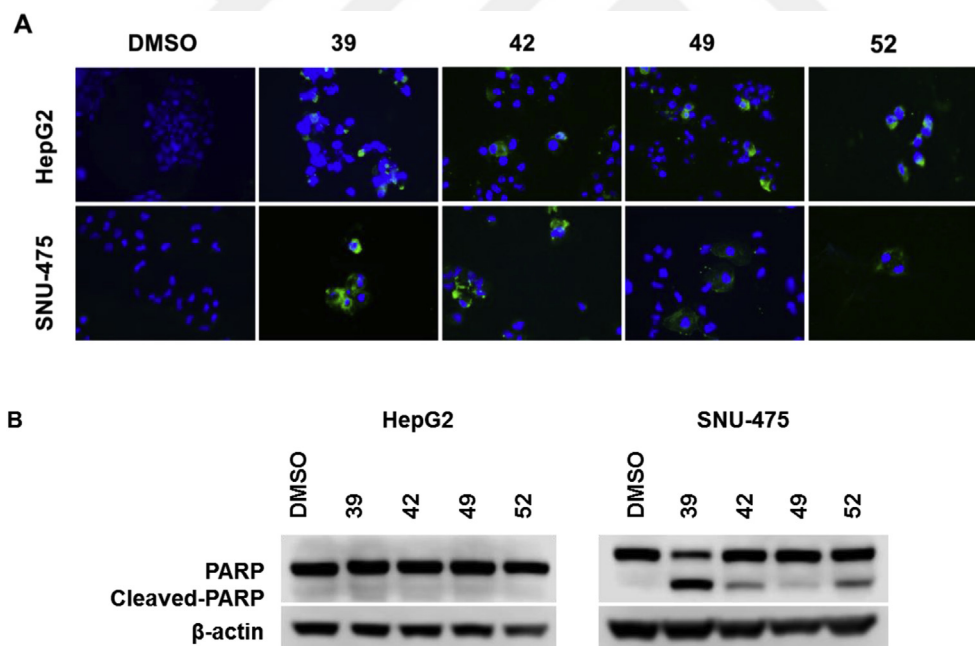


Fig. 3. Detection of apoptosis using fluorescent microscopy and western blotting. **A.** Annexin V staining of HepG2 and SNU-475 cells treated with compounds **39**, **42**, **49** and **52** for 48 h with IC_{100} concentrations. Condensed nuclei appear blue DAPI staining co-localize with Annexin V stained cells appear green. **B.** PARP cleavage after 24 h of treatment as shown by western blot analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cycle. Upon treatment with pyrazolic chalcone derivatives **42** and **52**, levels of CDK1 were shown to decrease by half in HepG2 cells, whereas a minor change was observed for SNU475 cells. Phospho-Cyclin B1 levels were shown to decrease by half in HepG2 cells, although there was a slight reduction in Cyclin B1 protein levels. It is well known that phosphorylation of Ser147 is necessary for the nuclear translocation of Cyclin B1 during prophase. Therefore, our results indicate that pyrazole chalcone derivatives could be involved in a mechanism inducing inhibition of Cyclin B1 phosphorylation, which results in a decreased amount of active Cyclin

B1, translocating into the nuclei. In addition to this, levels of p21, known to inhibit the activation of CyclinB1/CDK1 complex, were shown to increase by 5–6 fold in HepG2 cells and nearly 2 fold in SNU475 cells upon treatment with compounds **42** and **52**. It was further explored that this change in p21 levels were independent of p53 levels. The phospho-Rb levels were also found to be decreased, further impairing the cell cycle machinery, which could be the implication of secondary effects of pyrazolic chalcone derivatives on cell cycle.

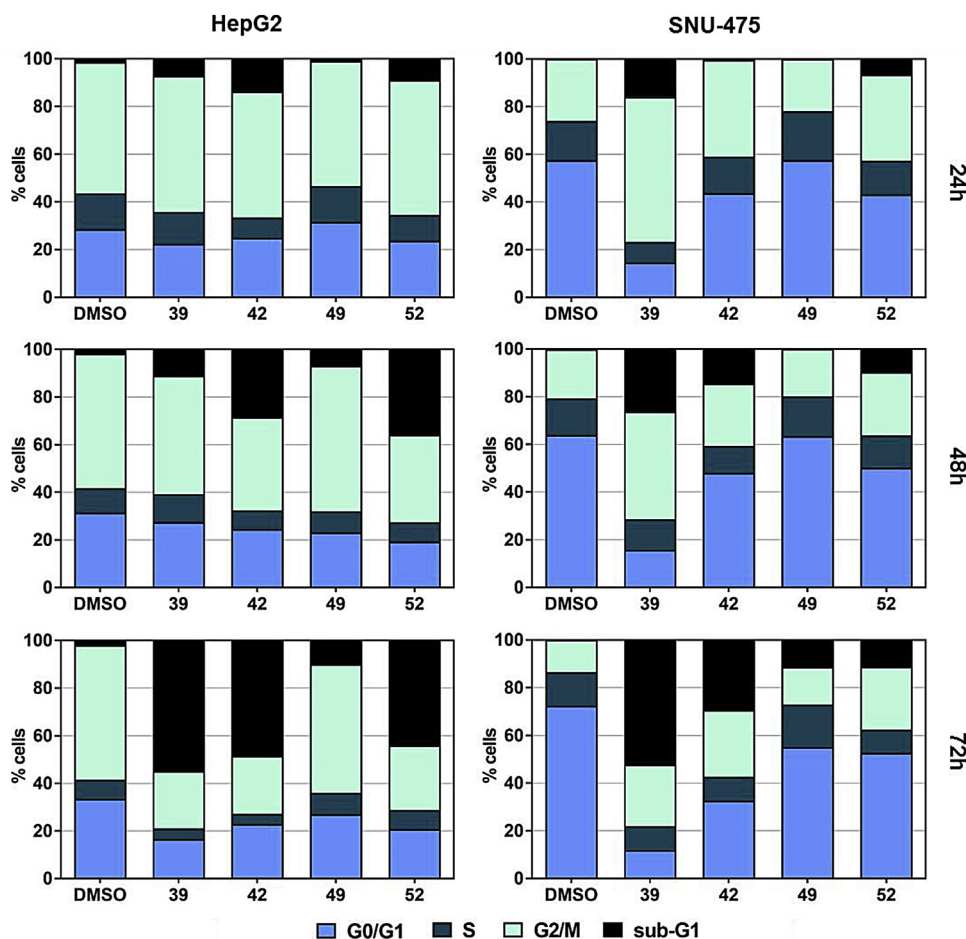


Fig. 4. Cell cycle analysis of HepG2 and SNU475 cells after treatment with compounds **39**, **42**, **49** and **52** and DMSO controls following 24 h, 48 h, and 72 h of treatment.

3. Conclusion

In this study, we synthesized a series of pyrazolic chalcone derivatives and evaluated their antiproliferative activities against human cancer cell lines in comparison with clinically used chemotherapeutics such as 5-FU and Cladribine. The preliminary structure bioactivity relations in these compounds have been established and discussed in results section in detail. The central pyrazole ring was modified at the C(3)-position with different heteroaryl rings and substituted at the C(4)-position with differently substituted chalcone moiety. Based on preliminary screenings the benzo[d][1,3]dioxol-5-yl ring (**20–31**) was not tolerated and resulted in mostly inactive or weak inhibitory compounds. Modifications of C(3)-position of central pyrazole ring with 3-/4-pyridyl moiety improved the bioactivity bearing more potent cytotoxic compounds. Therefore compounds **39**, **42**, **49**, and **52** were the most effective derivatives which displayed antiproliferative activity with IC_{50} values smaller than $5 \mu M$ against HCC cells used here. Moreover, these compounds were less active on transformed and normal cells (MCF12A and MRC-5). By further investigating their molecular effectors, we showed that compounds **42** and **52** caused cell cycle arrest at the G2/M phase and induced apoptotic cell death (as summarized in Fig. 6). Moreover, decrease in levels of phosphocyclin B1 at the Ser147 residue upon treatment is worth exploring, since it could provide further information about the mechanism of action of the compounds on cancer cells. Future studies can evaluate the detailed cellular networks that are affected by the use of

high throughput genomic screening methods such as transcriptome analysis with next-generation sequencing in the presence of selected compounds. This may allow to identify molecular targets involved in cell cycle for eventual drug design and development in cancer.

4. Experimental

4.1. Chemistry

Melting points were determined with an SMP-II Digital Melting Point Apparatus and are uncorrected (Schorpp Geaetetechnik, Germany). IR spectra were obtained using a Perkin Elmer Spectrum 400 FTIR/FTNIR spectrometer equipped with a Universal ATR Sampling Accessory. ^1H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ or $DMSO-d_6$ on a Varian Mercury 400 MHz High Performance Digital FT-NMR spectrometer at the NMR facility of the Faculty of Pharmacy, Ankara University or on a Varian Mercury 300 MHz FT-NMR spectrometer at the NMR facility of FARGEM (Pharmaceutical Research and Development Center) Inc. using tetramethylsilane as the internal standard. All chemical shifts were recorded as δ (ppm). High resolution mass spectra data (HRMS) were collected using a Waters LCT Premier XE Mass Spectrometer (high sensitivity orthogonal acceleration time-of-flight instrument) using ESI (+) method. The instrument was coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA). Elemental analyses were performed with a LECO-

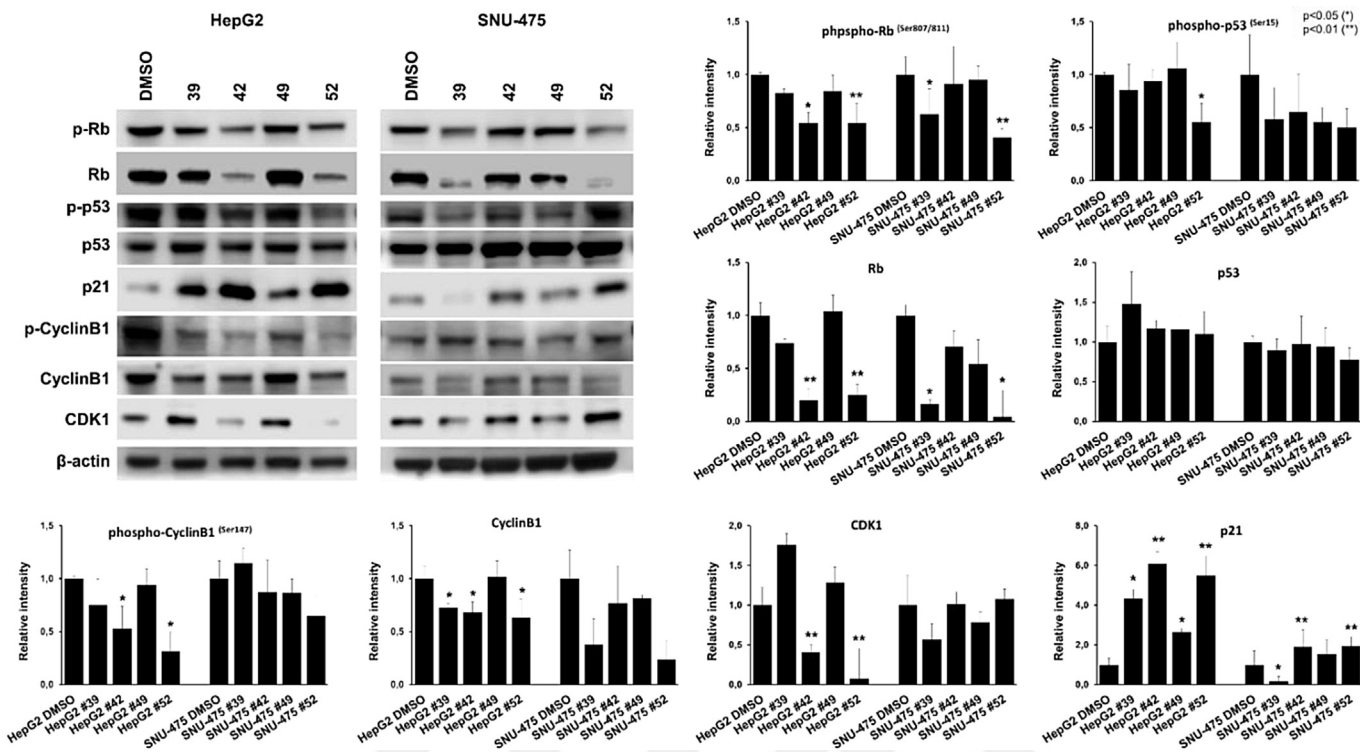


Fig. 5. Representative images from three independent western blot analysis of G2/M arrest proteins in whole lysates of HepG2 and SNU-475 cells treated with compounds **39**, **42**, **49** and **52** in IC₁₀₀ concentrations for 24 h. Graphs demonstrate comparative analysis of protein levels in the presence of compounds normalized to DMSO controls.

932 (C, H, N, S-Elemental Analyzer) at the Faculty of Pharmacy, Ankara University. Flash chromatography was performed with a Combiflash®Rf automated flash chromatography system with RediSep columns (Teledyne-Isco, Lincoln, NE, USA) using DCM-MeOH, hexane-EtOAc or MeOH-EtOAc solvent gradients.

4.1.1. General procedure for 1,3-diarylpyrazole chalcone synthesis

To a stirred solution of 1,3-diaryl-1H-pyrazole-4-carboxaldehyde (1 mmol) and corresponding methyl ketone (1.05 mmol) in ethanol (5 ml), an aqueous solution of 40% NaOH (2 ml) was added. The resulting solution was heated to 60 °C for 4 h and then allowed to stand overnight at room temperature with continuous stirring. The reaction mixture was poured into water and precipitate was collected by filtration, washed with 50% ethanol, dried, and purified by flash chromatography or recrystallization.

4.1.2. (E)-1-phenyl-3-(1-phenyl-3-(thiophen-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (CAS No: 303773-77-5) (**12**)

Purified by column flash chromatography (0% → 50% ethyl acetate in hexane). Yield 76%, mp 138–139 °C; IR (FTIR/FTNIR-ATR): 1657 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 300 MHz) δ: 8.27 (1H, s), 7.94–7.93 (2H, m), 7.90 (1H, d, J = 15.6 Hz), 7.71 (2H, d, J = 9.9 Hz), 7.59–7.58 (1H, m), 7.54–7.37 (7H, m), 7.33 (1H, d, J = 15.9 Hz), 7.27 (1H, d, J = 7.5 Hz). ¹³C NMR (DMSO-d₆, 400 MHz) δ: 188.68, 148.72, 138.89, 137.67, 134.08, 133.01, 132.64, 129.66, 128.77, 128.57, 128.19, 127.51, 127.23, 127.10, 124.69, 121.39, 118.60, 117.89. HRMS (m/z): [M+H]⁺ calcd. for C₂₂H₁₇N₂O₂S 357.1062, found 357.1064.

4.1.3. (E)-3-(1-phenyl-3-(thiophen-3-yl)-1H-pyrazol-4-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (**13**)

Purified by column flash chromatography (0% → 50% ethyl acetate in hexane). Yield 21%, mp 167–168 °C; IR (FTIR/FTNIR-ATR):

1651 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 300 MHz) δ: 8.25 (1H, s), 7.89 (1H, d, J = 15.6 Hz), 7.71 (2H, d, J = 8.7 Hz), 7.60–7.58 (1H, m), 7.46–7.38 (4H, m), 7.31 (1H, s), 7.25 (1H, d, J = 15.6 Hz), 7.19 (2H, s), 3.86 (9H, s). ¹³C NMR (DMSO-d₆, 400 MHz) δ: 187.46, 152.91, 148.68, 141.97, 138.89, 133.89, 133.06, 132.72, 129.70, 128.72, 127.57, 127.27, 127.17, 124.77, 121.26, 118.77, 117.89, 106.01, 60.21, 56.23. HRMS (m/z): [M+H]⁺ calcd. for C₂₅H₂₃N₂O₄S 447.1379, found 447.1384.

4.1.4. (E)-3-(1-phenyl-3-(thiophen-3-yl)-1H-pyrazol-4-yl)-1-(2,4,6-trimethoxyphenyl)prop-2-en-1-one (**14**)

Purified by column flash chromatography (0% → 50% ethyl acetate in hexane). Yield 81%, mp 162.5–163.5 °C; IR (FTIR/FTNIR-ATR): 1626 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 300 MHz) δ: 8.14 (1H, s), 7.67 (2H, d, J = 7.8 Hz), 7.48–7.26 (6H, m), 7.19 (1H, d, J = 5.1 Hz), 6.73 (1H, d, J = 16.2 Hz), 6.09 (2H, s), 3.78 (3H, s), 3.72 (6H, s). ¹³C NMR (DMSO-d₆, 400 MHz) δ: 193.13, 161.89, 157.85, 148.11, 138.89, 134.47, 132.70, 129.61, 128.72, 128.31, 127.28, 127.16, 127.02, 123.99, 118.62, 117.21, 110.80, 90.91, 55.77, 55.47. HRMS (m/z): [M+H]⁺ calcd. for C₂₅H₂₃N₂O₄S 447.1379, found 447.1360.

4.1.5. (E)-3-(1-phenyl-3-(thiophen-3-yl)-1H-pyrazol-4-yl)-1-(2,3,4-trimethoxyphenyl)prop-2-en-1-one (**15**)

Purified by recrystallization of ethyl acetate and hexane. Yield 23%, mp 90.5–91 °C; IR (FTIR/FTNIR-ATR): 1649 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 300 MHz) δ: 8.22 (1H, s), 7.78–7.69 (3H, m), 7.59–7.58 (1H, m), 7.46–7.35 (5H, m), 7.30–7.24 (2H, m), 6.71–6.68 (1H, d, J = 8.7 Hz), 3.86 (6H, s), 3.82 (3H, s). ¹³C NMR (DMSO-d₆, 400 MHz) δ: 190.05, 156.39, 152.60, 148.50, 141.64, 138.90, 133.22, 132.71, 129.60, 128.30, 127.47, 127.20, 127.04, 126.32, 125.93, 124.94, 124.57, 118.70, 117.66, 107.88, 61.80, 60.53, 56.07. HRMS (m/z): [M+H]⁺ calcd. for C₂₅H₂₃N₂O₄S 447.1379, found 447.1362.

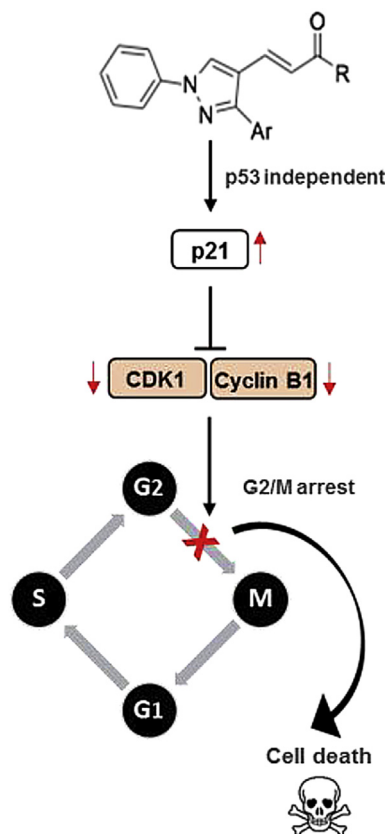


Fig. 6. Representation of molecular mechanisms initiated by pyrazolic chalcone derivatives (**42** and **52**). Upon treatment activation of p21 is initiated independent of p53, which then inhibits CDK1 and Cyclin B1 to form a complex resulting in cell cycle arrest at G2/M phase followed by cell death.

4.1.6. (*E*)-1-(3,4-dimethoxyphenyl)-3-(1-phenyl-3-(thiophen-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (16**)**

Purified by recrystallization of ethyl acetate and hexane. Yield 62%, mp 151–152 °C; IR (FTIR/FTNIR-ATR): 1650 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.26 (1H, s), 7.88 (1H, d, J = 15.6 Hz), 7.72 (2H, d, J = 8.1 Hz), 7.60–7.53 (3H, m), 7.46–7.36 (3H, m), 7.31–7.26 (2H, m), 7.19 (1H, s), 6.85 (1H, d, J = 8.1 Hz), 3.90 (6H, s). ^{13}C NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 186.89, 153.17, 148.82, 148.59, 138.94, 133.03, 132.77, 130.58, 129.68, 128.46, 127.54, 127.23, 127.07, 124.64, 122.91, 121.35, 118.65, 118.01, 110.87, 110.66, 55.78, 55.59. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{24}\text{H}_{21}\text{N}_2\text{O}_3\text{S}$ 417.1273, found 417.1273.

4.1.7. (*E*)-1-(2,5-dimethoxyphenyl)-3-(1-phenyl-3-(thiophen-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (17**)**

Purified by recrystallization of ethyl acetate and hexane. Yield 73%, mp 105.5–106 °C; IR (FTIR/FTNIR-ATR): 1649 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.18 (1H, s), 7.74 (1H, s), 7.71–7.68 (2H, m), 7.55–7.53 (2H, m), 7.45–7.34 (1H, m), 7.27 (2H, t, J = 7.5 Hz), 7.19 (1H, d, J = 5.1 Hz), 7.15 (1H, s), 7.09 (1H, d, J = 3 Hz), 6.96 (1H, d, J = 3.3 Hz), 6.93 (1H, d, J = 3 Hz), 3.77 (3H, s), 3.73 (3H, s). ^{13}C NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 191.78, 153.00, 151.49, 148.49, 138.88, 133.63, 132.69, 129.61, 129.44, 128.50, 127.44, 127.19, 127.05, 126.29, 124.52, 118.69, 118.02, 117.58, 113.97, 113.64, 56.30, 55.54. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{24}\text{H}_{21}\text{N}_2\text{O}_3\text{S}$ 417.1273, found 417.1256.

4.1.8. (*E*)-1-(2,4-dimethoxyphenyl)-3-(1-phenyl-3-(thiophen-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (18**)**

Purified by column flash chromatography (0% → 50% ethyl acetate in hexane). Yield 60%, mp 180.5–181 °C; IR (FTIR/FTNIR-ATR):

1642 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.17 (1H, s), 7.78–7.66 (4H, m), 7.58–7.56 (1H, m), 7.46–7.24 (6H, m), 6.50 (1H, d, J = 10.5 Hz), 6.42 (1H, s), 3.80 (6H, s). ^{13}C NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 189.28, 163.74, 159.95, 148.40, 138.93, 132.81, 131.87, 131.75, 129.60, 128.28, 127.52, 127.14, 126.98, 126.55, 124.56, 121.45, 118.70, 117.85, 105.85, 98.53, 55.92, 55.57. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{24}\text{H}_{21}\text{N}_2\text{O}_3\text{S}$ 417.1273, found 417.1267.

4.1.9. (*E*)-1-(benzo[d][1,3]dioxol-5-yl)-3-(1-phenyl-3-(thiophen-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (19**)**

Purified by column flash chromatography (0% → 50% ethyl acetate in hexane). Yield 81% as a colorless oil; IR (FTIR/FTNIR-ATR): 1646 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 400 MHz) δ : 8.31 (1H, s), 7.93 (1H, d, J = 15.6 Hz), 7.78 (2H, d, J = 8 Hz), 7.65–7.64 (1H, m), 7.59 (1H, dd, J = 2 Hz, J = 8 Hz), 7.52–7.44 (5H, m), 7.36–7.32 (2H, m), 6.88 (1H, d, J = 8.4 Hz), 6.06 (2H, s). ^{13}C NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 186.43, 151.47, 148.62, 148.01, 138.92, 133.35, 132.70, 132.32, 129.67, 128.42, 127.49, 127.22, 127.08, 124.61, 124.57, 121.27, 118.59, 117.99, 108.15, 107.65, 102.09. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{23}\text{H}_{17}\text{N}_2\text{O}_3\text{S}$ 401.0960, found 401.0954.

4.1.10. (*E*)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-phenylprop-2-en-1-one (20**)**

Purified by column flash chromatography (0% → 30% ethyl acetate in hexane). Yield 95%, mp 163.5–164 °C; IR (FTIR/FTNIR-ATR): 1662 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.27 (1H, s), 7.90 (2H, d, J = 8.7 Hz), 7.79 (1H, d, J = 15.6 Hz), 7.72 (2H, d, J = 9.6 Hz), 7.51–7.40 (5H, m), 7.34–7.28 (2H, m), 7.14–7.07 (2H, m), 6.86 (1H, d, J = 8.1 Hz), 5.96 (2H, s). ^{13}C NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 189.22, 153.16, 148.28, 148.21, 139.40, 138.15, 134.80, 133.50, 130.14, 129.24, 129.18, 128.66, 127.57, 126.17, 122.91, 121.74, 119.10, 118.10, 109.14, 108.97, 101.86. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{25}\text{H}_{19}\text{N}_2\text{O}_3$ 395.1396, found 395.1399.

4.1.11. (*E*)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (21**)**

Purified by column flash chromatography (0% → 30% ethyl acetate in hexane). Yield 53%, mp 163.5–164 °C; IR (FTIR/FTNIR-ATR): 1656 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.32 (1H, s), 7.89–7.77 (3H, m), 7.52–7.14 (8H, m), 6.93 (1H, d, J = 8.1 Hz), 6.02 (2H, s), 3.93 (9H, s). ^{13}C NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 187.52, 152.90, 152.65, 147.80, 147.72, 141.96, 138.92, 134.11, 133.06, 129.68, 128.80, 127.13, 125.79, 122.47, 121.12, 118.77, 117.60, 108.66, 108.54, 106.00, 101.40, 60.20, 56.21. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{28}\text{H}_{25}\text{N}_2\text{O}_6$ 485.1713, found 485.1716.

4.1.12. (*E*)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(2,4,6-trimethoxyphenyl)prop-2-en-1-one (22**)**

Purified by column flash chromatography (0% → 50% ethyl acetate in hexane). Yield 40%, mp 205–205.5 °C; IR (FTIR/FTNIR-ATR): 1650 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.15 (1H, s), 7.67 (2H, d, J = 7.8 Hz), 7.44–7.35 (3H, m), 7.29–7.24 (1H, m), 7.05–7.02 (2H, m), 6.81–6.67 (2H, m), 6.07 (2H, s), 5.94 (2H, s), 3.77 (3H, s), 3.73 (6H, s). ^{13}C NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 193.40, 161.81, 157.81, 151.95, 147.72, 147.71, 138.92, 135.15, 129.61, 128.60, 128.46, 127.01, 125.59, 122.02, 118.64, 116.84, 110.53, 108.56, 108.16, 101.40, 90.84, 55.73, 55.44. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{28}\text{H}_{25}\text{N}_2\text{O}_6$ 485.1713, found 485.1712.

4.1.13. (*E*)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(2,3,4-trimethoxyphenyl)prop-2-en-1-one (23**)**

Purified by recrystallization of ethyl acetate and hexane. Yield 27%, mp 144.5–145 °C; IR (FTIR/FTNIR-ATR): 1657 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.22 (1H, s), 7.72–7.65 (3H, m), 7.45–7.38 (3H, m), 7.29–7.22 (2H, m), 7.13–7.07 (2H, m), 6.85 (1H, d,

$J = 7.8$ Hz), 6.69 (1H, d, $J = 8.7$ Hz), 5.95 (2H, s), 3.85 (6H, s), 3.82 (3H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 190.03, 156.38, 152.63, 152.45, 147.75, 147.69, 141.64, 138.93, 133.46, 129.60, 128.49, 127.01, 126.30, 125.77, 124.94, 122.40, 118.71, 117.37, 108.61, 108.50, 107.83, 101.37, 61.80, 60.53, 56.06. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{28}\text{H}_{25}\text{N}_2\text{O}_6$ 485.1713, found 485.1711.

4.1.14. (E)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(3,4-dimethoxyphenyl)prop-2-en-1-one (24)

Purified by column flash chromatography (0% $\rightarrow$ 30% ethyl acetate in hexane). Yield 45%, mp 158.5–159 °C; IR (FTIR/FTNIR-ATR): 1649 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.26 (1H, s), 7.79 (1H, d, $J = 15.3$ Hz), 7.71 (2H, d, $J = 7.8$ Hz), 7.45–7.40 (2H, m), 7.56–7.52 (2H, m), 7.33 (1H, d, $J = 15.6$ Hz), 7.27 (1H, d, $J = 7.5$ Hz), 7.14 (1H, s), 7.10 (1H, d, $J = 9.6$ Hz), 6.88 (1H, s), 6.84 (1H, d, $J = 8.4$ Hz), 5.96 (2H, s), 3.89 (6H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 186.93, 153.15, 152.53, 148.81, 147.76, 147.72, 138.96, 133.25, 130.56, 129.66, 128.56, 127.04, 125.81, 122.89, 122.41, 121.19, 118.64, 117.71, 110.86, 110.66, 108.65, 108.50, 101.38, 55.77, 55.57. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{27}\text{H}_{23}\text{N}_2\text{O}_5$ 455.1607, found 455.1606.

4.1.15. (E)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(2,5-dimethoxyphenyl)prop-2-en-1-one (25)

Purified by column flash chromatography (0% $\rightarrow$ 50% ethyl acetate in hexane). Yield 28%, mp 125–126 °C; IR (FTIR/FTNIR-ATR): 1652 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.18 (1H, s), 7.71–7.68 (2H, d, $J = 9.9$ Hz), 7.60 (1H, d, $J = 16.2$ Hz), 7.44–7.39 (2H, m), 7.29–7.24 (1H, m), 7.16 (1H, s), 7.11 (1H, m), 7.08–7.05 (2H, m), 6.94 (1H, d, $J = 3.3$ Hz), 6.86–6.82 (2H, m), 5.96 (2H, s), 3.77 (3H, s), 3.73 (3H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 192.04, 153.00, 152.42, 151.37, 147.76, 147.69, 138.91, 134.15, 129.60, 129.41, 128.64, 127.04, 126.16, 125.71, 122.33, 118.71, 117.92, 117.24, 113.92, 113.55, 108.56, 108.44, 101.39, 56.26, 55.54. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{27}\text{H}_{23}\text{N}_2\text{O}_5$ 455.1607, found 455.1608.

4.1.16. (E)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(2,4-dimethoxyphenyl)prop-2-en-1-one (26)

Purified by recrystallization of ethyl acetate and hexane. Yield 28%, mp 154.8–155.3 °C; IR (FTIR/FTNIR-ATR): 1645 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.17 (1H, s), 7.71–7.62 (4H, m), 7.44–7.39 (2H, m), 7.29–7.22 (2H, m), 7.13–7.07 (2H, m), 6.84 (1H, d, $J = 8.1$ Hz), 6.49 (1H, d, $J = 10.8$ Hz), 6.14 (1H, s), 5.95 (2H, s), 3.08 (6H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 189.51, 163.68, 159.87, 152.33, 147.70, 147.65, 138.96, 132.19, 131.84, 129.57, 128.39, 126.94, 126.41, 125.89, 122.37, 121.43, 118.70, 117.53, 108.60, 108.50, 105.81, 101.36, 98.51, 55.87, 55.54. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{27}\text{H}_{23}\text{N}_2\text{O}_5$ 455.1607, found 455.1607.

4.1.17. (E)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(thiophen-3-yl)prop-2-en-1-one (27)

Purified by recrystallization of ethyl acetate and hexane. Yield 56%, mp 138.5–139.5 °C; IR (FTIR/FTNIR-ATR): 1659 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.25 (1H, s), 8.01–8.02 (1H, m), 7.78 (1H, d, $J = 15.6$ Hz), 7.70 (2H, d, $J = 8.4$ Hz), 7.56 (1H, d, $J = 6$ Hz), 7.45–7.39 (2H, m), 7.30–7.06 (5H, m), 6.86 (1H, d, $J = 7.8$ Hz), 5.96 (2H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 182.77, 152.57, 147.78, 147.72, 142.90, 138.93, 133.31, 133.18, 129.68, 128.46, 127.75, 127.10, 126.93, 125.72, 122.65, 122.39, 118.65, 117.58, 108.66, 108.46, 101.38. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{23}\text{H}_{17}\text{N}_2\text{O}_3\text{S}$ 401.0960, found 401.0964.

4.1.18. (E)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(pyridin-2-yl)prop-2-en-1-one (28)

Purified by recrystallization of ethyl acetate and hexane. Yield 83%, mp 188.5–189.2 °C; IR (FTIR/FTNIR-ATR): 1650 cm^{-1} (C=O).

^1H NMR (CDCl_3 , 300 MHz) δ : 8.88 (1H, d, $J = 4.4$ Hz), 8.42 (1H, s), 8.14–7.72 (6H, m), 7.46–7.28 (4H, m), 7.16 (1H, s), 7.12 (1H, d, $J = 9.9$ Hz), 6.88 (1H, d, $J = 7.8$ Hz), 5.97 (2H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 188.58, 153.57, 152.91, 149.01, 147.84, 147.75, 138.95, 137.71, 134.72, 129.59, 128.71, 127.50, 127.04, 125.69, 122.56, 122.48, 120.20, 118.75, 117.63, 108.68, 108.61, 101.41. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{24}\text{H}_{18}\text{N}_3\text{O}_3$ 396.1348, found 396.1344.

4.1.19. (E)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(pyridin-3-yl)prop-2-en-1-one (29)

Purified by column flash chromatography (0% $\rightarrow$ 50% ethyl acetate in hexane). Yield 10%, mp 174–176.5 °C; IR (FTIR/FTNIR-ATR): 1660 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 400 MHz) δ : 9.17 (1H, s), 8.78 (1H, dd, $J = 1.6$ Hz, $J = 4.8$ Hz), 8.36 (1H, s), 8.25 (1H, dt, $J = 2$ Hz, $J = 8$ Hz), 7.93–7.89 (1H, d, $J = 15.6$ Hz), 7.79 (2H, d, $J = 7.6$ Hz), 7.52–7.48 (2H, m), 7.46–7.42 (1H, m), 7.38–7.32 (2H, m), 7.19 (1H, s), 7.14 (1H, dd, $J = 1.6$ Hz, $J = 7.6$ Hz), 6.94 (1H, d, $J = 8$ Hz), 5.97 (2H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 187.82, 153.28, 152.86, 149.40, 147.86, 147.77, 138.88, 135.62, 135.01, 132.90, 129.72, 128.92, 127.20, 125.58, 123.99, 122.46, 120.95, 118.65, 117.53, 108.69, 108.49, 101.41. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{24}\text{H}_{18}\text{N}_3\text{O}_3$ 396.1348, found 396.1330.

4.1.20. (E)-1-(benzo[d][1,3]dioxol-5-yl)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)prop-2-en-1-one (30)

Purified by recrystallization of ethyl acetate and hexane. Yield 51%, mp 169.5–170 °C; IR (FTIR/FTNIR-ATR): 1644 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.25 (1H, s), 7.78 (1H, d, $J = 16.5$ Hz), 7.71 (2H, d, $J = 7.8$ Hz), 7.53 (1H, d, $J = 9.9$ Hz), 7.45–7.40 (3H, m), 7.30–7.07 (4H, m), 6.84 (1H, qd, $J = 1.6$ Hz, $J = 9.3$ Hz), 6.00 (2H, s), 5.96 (2H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 186.44, 152.56, 151.44, 147.99, 147.76, 147.71, 138.94, 133.55, 132.32, 129.65, 128.53, 127.04, 125.75, 124.54, 122.38, 121.09, 118.58, 117.71, 108.63, 108.46, 108.27, 107.65, 102.08, 101.37. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{26}\text{H}_{19}\text{N}_2\text{O}_5$ 439.1294, found 439.1304.

4.1.21. (E)-3-(3-(benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-pyrazol-4-yl)-1-(1H-pyrrol-3-yl)prop-2-en-1-one (31)

Purified by column flash chromatography (0% $\rightarrow$ 50% ethyl acetate in hexane). Yield 11%, mp 200–203 °C; IR (FTIR/FTNIR-ATR): 1638 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 400 MHz) δ : 9.50 (1H, s), 8.30 (1H, s), 7.84 (1H, d, $J = 15.6$ Hz), 7.78 (2H, d, $J = 7.6$ Hz), 7.50–7.47 (2H, m), 7.36–7.32 (1H, m), 7.21–7.16 (3H, m), 7.08 (1H, s), 6.95–6.92 (3H, m), 6.34–6.32 (1H, m), 6.03 (2H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 177.61, 152.27, 147.69, 139.01, 132.99, 130.71, 130.53, 129.66, 128.16, 126.97, 126.24, 125.92, 122.72, 122.38, 118.58, 117.75, 116.63, 110.09, 108.66, 108.50, 101.36. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{23}\text{H}_{18}\text{N}_3\text{O}_3$ 384.1348, found 384.1347.

4.1.22. (E)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)-1-(thiophen-3-yl)prop-2-en-1-one (32)

Purified by column flash chromatography (0% $\rightarrow$ 50% ethyl acetate in hexane). Yield 82%, mp 172–173 °C; IR (FTIR/FTNIR-ATR): 1655 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 400 MHz) δ : 8.99 (1H, d, $J = 1.6$ Hz), 8.69 (1H, dd, $J = 1.6$ Hz, $J = 5.2$ Hz), 8.37 (1H, s), 8.07 (1H, dd, $J = 1.2$ Hz, $J = 2.8$ Hz), 8.01 (1H, dt, $J = 2$ Hz, $J = 8$ Hz), 7.84–7.79 (3H, m), 7.61 (1H, dd, $J = 1.2$ Hz, $J = 4.8$ Hz), 7.54–7.50 (2H, m), 7.45–7.35 (3H, m), 7.23 (1H, d, $J = 16.4$ Hz). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 182.69, 149.93, 149.68, 148.73, 142.81, 138.84, 135.77, 133.47, 132.40, 129.74, 128.88, 127.91, 127.82, 127.39, 126.92, 123.94, 123.30, 118.81, 118.14. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{21}\text{H}_{16}\text{N}_3\text{OS}$ 358.1014, found 358.1004.

4.1.23. (*E*)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)-1-(pyridin-2-yl)prop-2-en-1-one (**33**)

Purified by column flash chromatography (0% → 40% ethyl acetate in hexane) then by recrystallization of ethyl acetate and hexane. Yield 11%, mp 187.5–188 °C; IR (FTIR/FTNIR-ATR): 1664 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 400 MHz) δ: 9.00 (1H, d, *J* = 1.2 Hz), 8.72 (1H, d, *J* = 4 Hz), 8.69 (1H, dd, *J* = 1.6 Hz, *J* = 5.2 Hz), 8.52 (1H, s), 8.18 (1H, d, *J* = 8 Hz), 8.14 (1H, d, *J* = 16 Hz), 8.03 (1H, d, *J* = 7.6 Hz), 7.98–7.94 (1H, m), 7.90–7.86 (1H, m), 7.83–7.77 (1H, m), 7.54–7.34 (6H, m). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 188.50, 153.43, 150.24, 149.71, 149.01, 148.84, 138.83, 137.73, 135.92, 133.89, 129.63, 129.15, 127.90, 127.55, 127.31, 123.93, 122.49, 120.80, 118.87, 118.14. HRMS (*m/z*): [M+H]⁺ calcd. for C₂₂H₁₇N₄O 353.1402, found 353.1398.

4.1.24. (*E*)-1-(benzo[d][1,3]dioxol-5-yl)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (**34**)

Purified by column flash chromatography (0% → 50% ethyl acetate in hexane). Yield 82%, mp 204–204.6 °C; IR (FTIR/FTNIR-ATR): 1663 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 400 MHz) δ: 8.99 (1H, d, *J* = 1.6 Hz), 8.69 (1H, dd, *J* = 1.6 Hz, *J* = 5 Hz), 8.37 (1H, s), 8.01 (1H, dt, *J* = 2 Hz, *J* = 5 Hz), 7.83–7.79 (3H, m), 7.56 (1H, dd, *J* = 2 Hz, *J* = 8.4 Hz), 7.54–7.48 (3H, m), 7.44–7.35 (2H, m), 7.31 (1H, s), 6.87 (1H, d, *J* = 8.4 Hz), 6.06 (2H, s). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 186.40, 151.53, 149.93, 149.65, 148.74, 148.02, 138.85, 135.78, 132.76, 132.22, 129.73, 128.93, 127.94, 127.36, 124.64, 123.93, 121.78, 118.76, 118.27, 108.16, 107.67, 102.12. HRMS (*m/z*): [M+H]⁺ calcd. for C₂₄H₁₈N₃O₃ 396.1348, found 396.1344.

4.1.25. (*E*)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)-1-(1H-pyrrrol-3-yl)prop-2-en-1-one (**35**)

Purified by column flash chromatography (0% → 2% methanol in ethyl acetate). Yield 15%, mp 211.8–212.5 °C; IR (FTIR/FTNIR-ATR): 1644 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 400 MHz) δ: 9.55 (1H, s), 9.00 (1H, s), 8.69 (1H, d, *J* = 4.8 Hz), 8.36 (1H, s), 8.01 (1H, dd, *J* = 2 Hz, *J* = 8 Hz), 7.83–7.79 (3H, m), 7.53–7.49 (2H, m), 7.44–7.35 (2H, m), 7.18 (1H, d, *J* = 15.6 Hz), 7.09–7.08 (1H, m), 6.95–6.93 (1H, m), 6.34–6.32 (1H, m). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 177.48, 149.61, 148.76, 138.92, 135.81, 132.95, 129.79, 129.73, 128.60, 128.13, 127.28, 126.40, 123.96, 123.37, 118.75, 118.31, 116.78, 110.07. HRMS (*m/z*): [M+H]⁺ calcd. for C₂₁H₁₇N₄O 341.1402, found 341.1389.

4.1.26. (*E*)-1-(1H-indol-3-yl)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (**36**)

Purified by column flash chromatography (0% → 30% ethyl acetate in hexane). Yield 17%, mp 267.5–268 °C; IR (FTIR/FTNIR-ATR): 1646 cm⁻¹ (C=O). ¹H NMR (DMSO-*d*₆, 400 MHz) δ: 12.14 (1H, s), 9.33 (1H, s), 8.90 (1H, d, *J* = 1.6 Hz), 8.71 (1H, dd, *J* = 1.6, *J* = 4.8 Hz), 8.51 (1H, s), 8.30 (1H, d, *J* = 6.8 Hz), 8.11 (1H, dt, *J* = 2, *J* = 8 Hz), 7.96 (2H, d, *J* = 7.6 Hz), 7.78 (1H, d, *J* = 15.2 Hz), 7.63–7.41 (6H, m), 7.27–7.20 (2H, m). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 183.26, 149.55, 149.52, 148.70, 138.98, 136.87, 135.70, 134.02, 129.75, 128.62, 128.19, 128.15, 127.26, 125.76, 125.08, 123.94, 123.13, 121.85, 121.66, 118.75, 118.56, 117.64, 112.24. HRMS (*m/z*): [M+H]⁺ calcd. for C₂₅H₁₉N₄O 391.1559, found 391.1566.

4.1.27. (*E*)-1-phenyl-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-on [47] (**37**)

Purified by flash column chromatography (0% → 25% EtOAc in Hexane). Yield 30%, mp 161–161.8 °C; IR (FTIR/FTNIR-ATR): 1664 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 400 MHz) δ: 8.99 (1H, d, *J* = 1.2 Hz), 8.69 (1H, dd, *J* = 1.6 Hz, *J* = 5.2 Hz), 8.39 (1H, s), 8.02 (1H, dt, *J* = 2 Hz, *J* = 8 Hz), 7.97–7.95 (2H, m), 7.83 (1H, d, *J* = 15.6 Hz), 7.79 (2H, m), 7.59–7.38 (7H, m), 7.37 (1H, d, *J* = 15.6 Hz). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 188.68, 150.05, 149.70, 148.76, 138.84,

137.57, 135.82, 133.52, 133.14, 129.74, 129.11, 128.80, 128.22, 127.90, 127.39, 123.95, 121.90, 118.79, 118.17. Anal. calcd for C₂₃H₁₇N₃O C, 78.61; H, 4.88; N, 11.96. found: C, 78.77; H, 4.85; N, 11.58. HRMS (*m/z*): [M+H]⁺ calcd. for C₂₃H₁₈N₃O 352.1450; found 352.1443.

4.1.28. (*E*)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-on (CAS No: 1287408-93-8) (**38**)

Purified by flash column chromatography (0% → 25% EtOAc in Hexane). Yield 21%, mp 115–116 °C; IR (FTIR/FTNIR-ATR): 1646 cm⁻¹ (C=O). ¹H NMR (CDCl₃) δ: 8.99 (1H, d, *J* = 1.2 Hz), 8.69 (1H, dd, *J* = 1.6 Hz, *J* = 5.2 Hz), 8.36 (1H, s), 8.02 (1H, dt, *J* = 2 Hz, *J* = 8 Hz), 7.84 (1H, d, *J* = 15.6 Hz), 7.54–7.34 (6H, m), 7.29 (1H, d, *J* = 15.6 Hz), 7.19 (2H, s), 3.92 (6H, s), 3.86 (3H, s). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 187.51, 152.91, 149.99, 149.72, 148.81, 142.02, 138.84, 135.88, 133.36, 132.95, 129.75, 129.24, 128.01, 127.44, 123.96, 121.82, 118.94, 118.22, 118.14, 106.03, 60.21, 56.23. Anal. calcd for C₂₆H₂₃N₃O₄ C, 70.73; H, 5.25; N, 9.52; found: C, 70.95; H, 5.53; N, 9.12. HRMS (*m/z*): [M+H]⁺ calcd. for C₂₆H₂₄N₃O₄ 442.1767; found 442.1777.

4.1.29. (*E*)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)-1-(2,4,6-trimethoxyphenyl)prop-2-en-1-on (**39**)

Purified by flash column chromatography (0% → 20% EtOAc in Hexane). Yield 22%, mp 206–207 °C; IR (FTIR/FTNIR-ATR): 1625 cm⁻¹ (C=O). ¹H NMR (DMSO-*d*₆, 400 MHz) δ: 9.26 (1H, s), 8.73 (1H, d, *J* = 2 Hz), 8.67 (1H, dd, *J* = 1.6 Hz, *J* = 4.8 Hz), 7.96–7.93 (3H, m), 7.59–7.54 (3H, m), 7.42–7.38 (1H, m), 7.16 (1H, d, *J* = 16 Hz), 6.86 (1H, d, *J* = 16 Hz), 6.29 (2H, s), 3.81 (3H, s), 3.75 (6H, s). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 193.08, 161.90, 157.89, 149.63, 149.36, 148.53, 138.83, 135.46, 133.98, 129.66, 129.18, 128.92, 127.84, 127.29, 123.85, 118.78, 117.46, 110.57, 90.86, 55.76, 55.44. Anal. calcd for C₂₆H₂₃N₃O₄ C, 70.73; H, 5.25; N, 9.52; found: C, 70.92; H, 5.38; N, 9.48. HRMS (*m/z*): [M+H]⁺ calcd. for C₂₆H₂₄N₃O₄ 442.1767; found 442.1762.

4.1.30. (*E*)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)-1-(2,3,4-trimethoxyphenyl)prop-2-en-1-on (**40**)

Purified by flash column chromatography (0% → 25% EtOAc in Hexane). Yield 28%, mp 91–92 °C; IR (FTIR/FTNIR-ATR): 1648 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 400 MHz) δ: 8.98 (1H, d, *J* = 1.6 Hz), 8.67 (1H, dd, *J* = 1.6 Hz, *J* = 5.2 Hz), 8.33 (1H, s), 8.02 (1H, dt, *J* = 1.6 Hz, *J* = 8 Hz), 7.79 (2H, m), 7.70 (1H, d, *J* = 15.6 Hz), 7.53–7.37 (5H, m), 7.33 (1H, d, *J* = 15.6 Hz), 6.75 (1H, d, *J* = 8.8 Hz), 3.92 (3H, s), 3.91 (3H, s), 3.87 (3H, s). Anal. calcd. for C₂₆H₂₃N₃O₄ C, 70.73; H, 5.25; N, 9.52; found: C, 70.92; H, 5.17; N, 9.52. HRMS (*m/z*): [M+H]⁺ calcd. for C₂₆H₂₄N₃O₄ 442.1767; found 442.1765.

4.1.31. (*E*)-1-(3,4-Dimethoxyphenyl)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-on (CAS No: 1002609-04-2) (**41**)

Purified by flash column chromatography (0% → 25% EtOAc in Hexane). Yield 36%, mp 162–163 °C; IR (FTIR/FTNIR-ATR): 1655 cm⁻¹ (C=O). ¹H NMR (CDCl₃, 300 MHz) δ: 8.97 (1H, s), 8.65 (1H, d, *J* = 3.6 Hz), 8.34 (1H, s), 8.03 (1H, d, *J* = 7.8 Hz), 7.79–7.77 (2H, m), 7.76 (1H, d, *J* = 15.9 Hz), 7.55–7.33 (7H, m), 6.85 (1H, d, *J* = 7.8 Hz), 3.91 (6H, s). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 186.85, 153.22, 149.88, 149.66, 148.82, 148.77, 138.87, 135.81, 132.46, 130.46, 129.73, 128.98, 128.02, 127.34, 123.93, 122.94, 121.85, 118.81, 118.26, 110.87, 110.64, 55.78, 55.57. Anal. calcd. for C₂₅H₂₁N₃O₃ C, 72.98; H, 5.14; N, 10.21; found: C, 72.49; H, 5.05; N, 10.03. HRMS (*m/z*): [M+H]⁺ calcd. for C₂₅H₂₂N₃O₃ 412.1661; found 412.1650.

4.1.32. (*E*)-1-(2,5-Dimethoxyphenyl)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-on (**42**)

Purified by recrystallization from hexane-ethyl acetate mixture.

Yield 57%, mp 129–130°C; IR (FTIR/FTNIR-ATR): 1656 cm^{-1} (C=O). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 9.29 (1H, s), 8.82 (1H, d, J = 1.6 Hz), 8.69 (1H, d, J = 4.8 Hz), 8.04 (1H, d, J = 8.4 Hz), 7.96 (2H, d, J = 8 Hz), 7.59–7.39 (5H, m), 7.28 (1H, d, J = 16 Hz), 7.13–7.07 (2H, m), 7.02 (1H, s), 3.80 (3H, s), 3.74 (3H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 191.72, 152.99, 151.50, 149.74, 149.68, 148.72, 138.81, 135.77, 133.13, 129.66, 129.21, 129.18, 127.98, 127.32, 126.64, 123.86, 118.85, 118.19, 117.80, 113.94, 113.59, 56.25, 55.53. Anal. calcd for $\text{C}_{25}\text{H}_{21}\text{N}_3\text{O}_3$ C, 72.98; H, 5.14; N, 10.21; found: C, 73.31; H, 5.57; N, 10.40. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{25}\text{H}_{22}\text{N}_3\text{O}_3$ 412.1661; found 412.1649.

4.1.33. (*E*)-1-(2,4-Dimethoxyphenyl)-3-(1-phenyl-3-(pyridin-3-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (CAS No: 1002461-21-3) (**43**)

Purified by recrystallization from acetone-water mixture. Yield 34%, mp 130–131°C; IR (FTIR/FTNIR-ATR): 1646 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 9.01 (1H, s), 8.63 (1H, d, J = 3.4 Hz), 8.24 (1H, s), 8.20 (1H, d, J = 7.2 Hz), 7.73–7.68 (2H, m), 7.62 (1H, d, J = 15.6 Hz), 7.55–7.53 (2H, m), 7.46 (1H, t, J = 7.8 Hz), 7.34–7.32 (1H, m), 7.31 (1H, d, J = 15.6 Hz), 6.52 (1H, d, J = 8.7 Hz), 6.43 (1H, d, J = 1.8 Hz), 3.81 (6H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 189.16, 163.83, 159.99, 149.61, 148.78, 138.87, 135.84, 131.96, 131.27, 129.64, 129.00, 128.16, 127.24, 126.88, 123.88, 121.28, 118.87, 118.10, 105.89, 98.50, 55.88, 55.57. Anal. calcd. for $\text{C}_{25}\text{H}_{21}\text{N}_3\text{O}_3$ C, 72.98; H, 5.14; N, 10.21; found: C, 73.33; H, 5.38; N, 10.21. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{25}\text{H}_{22}\text{N}_3\text{O}_3$ 412.1661; found 412.1643.

4.1.34. (*E*)-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)-1-(thiophen-3-yl)prop-2-en-1-one (**44**)

Purified by column flash chromatography (0% → 50% ethyl acetate in hexane) then by recrystallization of ethyl acetate and hexane. Yield 54%, mp 219–220°C; IR (FTIR/FTNIR-ATR): 1655 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 400 MHz) δ : 8.74 (2H, d, J = 6 Hz), 8.36 (1H, s), 8.08 (1H, d, J = 2.8 Hz), 7.90 (1H, d, J = 15.6 Hz), 7.79 (2H, d, J = 7.6 Hz), 7.67–7.62 (3H, m), 7.52 (2H, t, J = 8 Hz), 7.41–7.36 (2H, m), 7.26 (1H, d, J = 15.6 Hz). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 182.68, 150.26, 149.94, 142.76, 139.28, 138.76, 133.55, 132.13, 129.75, 129.15, 127.84, 127.53, 126.91, 123.74, 122.61, 118.88, 118.30. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{21}\text{H}_{16}\text{N}_3\text{O}_3\text{S}$ 358.1014, found 358.1008.

4.1.35. (*E*)-1-(benzo[d][1,3]dioxol-5-yl)-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (**45**)

Purified by column flash chromatography (0% → 50% ethyl acetate in hexane). Yield 60%, mp 148.2–148.8°C; IR (FTIR/FTNIR-ATR): 1656 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 400 MHz) δ : 8.73 (2H, d, J = 5.6 Hz), 8.36 (1H, s), 7.87 (1H, d, J = 15.2 Hz), 7.80 (2H, d, J = 7.6 Hz), 7.66 (2H, d, J = 6 Hz), 7.59–7.50 (4H, m), 7.38–7.34 (2H, m), 6.88 (1H, d, J = 8.4 Hz), 6.06 (2H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 186.37, 151.47, 150.17, 149.85, 147.95, 139.27, 138.73, 132.41, 132.14, 129.63, 129.13, 127.40, 124.57, 122.52, 122.25, 118.79, 118.36, 108.07, 107.60, 102.03. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{24}\text{H}_{18}\text{N}_3\text{O}_3$ 396.1348, found 396.1338.

4.1.36. (*E*)-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)-1-(1H-pyrrol-3-yl)prop-2-en-1-one (**46**)

Purified by column flash chromatography (0% → 6% methanol in dichloromethane). Yield 28%, mp 258–258.6°C; IR (FTIR/FTNIR-ATR): 1645 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 400 MHz) δ : 9.62 (1H, bs), 8.74 (2H, d, J = 4.2 Hz), 8.35 (1H, s), 7.87 (1H, d, J = 15.6 Hz), 7.80 (2H, d, J = 7.6 Hz), 7.67 (2H, d, J = 4.4 Hz), 7.53 (2H, t, J = 8 Hz), 7.40–7.36 (1H, m), 7.20 (1H, d, J = 15.6 Hz), 7.10 (1H, s), 6.96 (1H, m), 6.35–6.33 (1H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 177.44, 150.25, 149.65, 139.51, 138.83, 132.93, 129.72, 129.54, 128.85, 127.41, 126.45, 123.83, 122.64, 118.83, 118.49, 116.89, 110.07. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{21}\text{H}_{17}\text{N}_4\text{O}$ 341.1402, found 341.1400.

4.1.37. (*E*)-1-phenyl-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (CAS No: 1390831-45-4) (**47**)

Purified by flash column chromatography (0% → 25% EtOAc in Hexane). Yield 36%, mp 215–216 °C; IR (FTIR/FTNIR-ATR): 1656 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.70 (2H, d, J = 4.5 Hz), 8.37 (1H, s), 7.96 (2H, d, J = 7.5 Hz), 7.86 (2H, d, J = 15.6 Hz), 7.78–7.69 (2H, m), 7.58–7.36 (3H, m), 7.58–7.36 (4H, m), 7.22–7.20 (1H, m). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 188.66, 150.27, 150.07, 139.27, 138.76, 137.52, 133.24, 133.14, 129.74, 129.35, 128.20, 128.24, 127.52, 122.65, 122.32, 118.85, 118.32. Anal. calcd. for $\text{C}_{23}\text{H}_{17}\text{N}_3\text{O}$ C, 78.61; H, 4.88; N, 11.96. found C, 78.25; H, 4.85; N, 11.82. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{23}\text{H}_{18}\text{N}_3\text{O}$ 352.1450; found 352.1447.

4.1.38. (*E*)-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (CAS No: 1390911-60-0) (**48**)

Purified by flash column chromatography (0% → 25% EtOAc in Hexane). Yield 18%, mp 161–162°C; IR (FTIR/FTNIR-ATR): 1656 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.67 (2H, bs), 8.34 (1H, s), 7.84 (1H, d, J = 15.6 Hz), 7.75–7.70 (4H, m), 7.49–7.36 (3H, m), 7.33 (1H, d, J = 15.6 Hz), 7.16 (2H, s), 3.87 (9H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 187.40, 152.91, 150.27, 150.05, 142.08, 139.40, 138.76, 133.03, 132.90, 129.76, 129.44, 127.56, 122.72, 122.22, 119.02, 118.30, 106.08, 60.21, 59.72, 56.23. Anal. calcd. for $\text{C}_{26}\text{H}_{23}\text{N}_3\text{O}_4$ C, 70.73; H, 5.25; N, 9.52. found C, 70.34; H, 5.63; N, 9.65. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{26}\text{H}_{24}\text{N}_3\text{O}_4$ 442.1767; found 442.1757.

4.1.39. (*E*)-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)-1-(2,4,6-trimethoxyphenyl)prop-2-en-1-one (**49**)

Purified by recrystallization from ethanol-water mixture. Yield 68%, mp 192–193°C; IR (FTIR/FTNIR-ATR): 1636 cm^{-1} (C=O). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 9.26 (1H, s), 8.71 (2H, d, J = 6 Hz), 7.94 (2H, d, J = 8 Hz), 7.57 (2H, t, J = 8 Hz), 7.52 (2H, d, J = 5.6 Hz), 7.43–7.40 (1H, m), 7.21 (1H, d, J = 16 Hz), 6.89 (1H, d, J = 16 Hz), 6.33 (2H, s), 3.83 (3H, s), 3.75 (6H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 193.13, 161.97, 157.89, 150.16, 149.48, 139.21, 138.76, 133.82, 129.68, 129.51, 129.11, 127.43, 122.39, 118.87, 117.66, 110.54, 90.89, 55.79, 55.74. Anal. calcd. for $\text{C}_{26}\text{H}_{23}\text{N}_3\text{O}_4$ C, 70.73; H, 5.25; N, 9.52. found: C, 70.42; H, 5.36; N, 9.41. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{26}\text{H}_{24}\text{N}_3\text{O}_4$ 442.1767; found 442.1758.

4.1.40. (*E*)-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)-1-(2,3,4-trimethoxyphenyl)prop-2-en-1-one (**50**)

Purified by flash column chromatography (0% → 25% EtOAc in Hexane). Yield 30%, mp 160–161°C; IR (FTIR/FTNIR-ATR): 1654 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.66 (2H, d, J = 6 Hz), 8.27 (1H, s), 7.74–7.68 (5H, m), 7.48–7.35 (4H, m), 7.33 (1H, d, J = 15.6 Hz), 6.70 (1H, d, J = 8.7 Hz), 3.87 (3H, s), 3.86 (3H, s), 3.83 (3H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 189.76, 156.56, 152.75, 150.22, 149.87, 141.65, 139.40, 138.77, 132.28, 129.67, 129.15, 127.45, 126.71, 126.11, 125.07, 122.69, 118.95, 118.10, 107.88, 61.83, 60.53, 56.08. Anal. calcd. for $\text{C}_{26}\text{H}_{23}\text{N}_3\text{O}_4$ C, 70.73; H, 5.25; N, 9.52. found: C, 70.47; H, 5.12; N, 9.37. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{26}\text{H}_{24}\text{N}_3\text{O}_4$ 442.1767; found 442.1781.

4.1.41. (*E*)-1-(3,4-Dimethoxyphenyl)-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)prop-2-en-1-one (CAS No: 1147371-58-1) (**51**)

Purified by recrystallization from acetone-water mixture. Yield 44%, mp 190–191°C; IR (FTIR/FTNIR-ATR): 1653 cm^{-1} (C=O). ^1H NMR (CDCl_3 , 300 MHz) δ : 8.68 (2H, bs), 8.34 (1H, s), 7.83 (1H, d, J = 15.6 Hz), 7.76–7.69 (4H, m), 7.55–7.20 (5H, m), 6.87 (1H, d, J = 9 Hz), 3.92 (3H, s), 3.91 (3H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 186.83, 153.26, 150.26, 149.93, 148.84, 139.40, 138.80, 132.18, 130.43,

129.74, 129.24, 127.49, 123.02, 122.67, 122.30, 118.90, 118.43, 110.87, 110.65, 55.80, 55.58. Anal. calcd for $C_{25}H_{21}N_3O_3$ C, 72.98; H, 5.14; N, 10.21. found: C, 72.58; H, 5.12; N, 10.05. HRMS (m/z): $[M+H]^+$ calcd. for $C_{25}H_{22}N_3O_3$ 412.1661; found 412.1651.

4.1.42. (E)-1-(2,5-Dimethoxyphenyl)-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)prop-2-en-1-on (52)

Purified by recrystallization from acetone-water mixture. Yield 32%, mp 136–137°C; IR (FTIR/FTNIR-ATR): 1656 cm^{-1} (C=O). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 9.30 (1H, s), 8.73 (2H, d, $J = 5.2$ Hz), 7.96 (2H, d, $J = 8$ Hz), 7.63 (2H, d, $J = 6$ Hz), 7.60–7.56 (2H, m), 7.50 (1H, d, $J = 16$ Hz), 7.44–7.40 (1H, m), 7.32 (1H, d, $J = 16$ Hz), 7.15–7.09 (2H, m), 7.04 (1H, d, $J = 2.8$ Hz), 3.81 (3H, s), 3.75 (3H, s). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 191.69, 153.01, 151.56, 150.14, 149.86, 139.43, 138.75, 132.83, 129.68, 129.33, 129.21, 127.47, 127.07, 122.68, 118.95, 118.19, 117.98, 114.01, 113.65, 56.30, 55.55. Anal. calcd. for $C_{25}H_{21}N_3O_3$ C, 72.98; H, 5.14; N, 10.21. found: C, 72.87; H, 5.12; N, 9.88. HRMS (m/z): $[M+H]^+$ calcd. for $C_{25}H_{22}N_3O_3$ 412.1661; found 412.1641.

4.1.43. (E)-1-(2,4-Dimethoxyphenyl)-3-(1-phenyl-3-(pyridin-4-yl)-1H-pyrazol-4-yl)prop-2-en-1-on (CAS No: 1147368-37-3) (53)

Purified by recrystallization from acetone-water mixture. Yield 36%, mp 182–183°C; IR (FTIR/FTNIR-ATR): 1644 cm^{-1} (C=O). ^1H NMR (CDCl₃, 300 MHz) δ : 8.66 (2H, d, $J = 4.5$ Hz), 8.22 (1H, s), 7.73–7.67 (6H, m), 7.48–7.43 (2H, m), 7.37–7.32 (2H, m), 6.50 (1H, dd, $J = 2.1$ Hz, $J = 8.7$ Hz), 6.42 (1H, d, $J = 2.4$ Hz), 3.80 (3H, s), 3.79 (3H, s). Anal. calcd for $C_{25}H_{21}N_3O_3$ C, 72.98; H, 5.14; N, 10.21. found: C, 72.67; H, 5.12; N, 9.98. HRMS (m/z): $[M+H]^+$ calcd. for $C_{25}H_{22}N_3O_3$ 412.1661; found 412.1642.

4.2. Biological evaluation

4.2.1. Cells culture

Hepatocellular carcinoma cell lines (Huh7, HepG2 and Mahlavu), human breast carcinoma cells (MCF7) and human colon carcinoma cells (HCT116) were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen GIBCO), 1% non-essential amino acids (GIBCO, Invitrogen) and SNU-475 were grown in RPMI-1640 media (Invitrogen GIBCO) supplemented with 10% FBS, 2 mM L-glutamine. MCF12A, human breast epithelial cells, were grown in DMEM/HAMS F12 media (Invitrogen GIBCO) supplemented with 10% FBS, 1% NEAA, 100 ng/ml EGF, 500 ng/ml hydrocortisone, and 10 μ g/ml insulin. MRC-5, human fetal lung fibroblast cells, were maintained in Minimum Essential Medium (MEM) (Invitrogen GIBCO), with 10% fetal bovine serum (FBS), 2 mM L-glutamine. All media contained 100 units/ml penicillin and streptomycin and cells were maintained at 37 °C in a humidified incubator under 5% CO₂.

4.2.2. NCI-60 sulforhodamine B assay

Cells were plated in 96-well plates (2000–3000 cell/well in 150 μ l/well) and grown for 24 h at 37 °C. Treatment of cells with the compounds (dissolved in DMSO) was done in a concentration range of 40 μ M–0.3 μ M (in triplicates). After 72 h of incubation, cells were fixed with 10% (v/v) trichloroacetic acid (MERCK) for an hour and stained with sulforhodamine B (SRB) solution (50 μ l of a 0.4% (m/v) of SRB in 1% acetic acid solution). For the removal of unbound SRB, cells were washed with 1% acetic acid and left for air-drying. Protein bound SRB was solubilized in 10 mM Tris-base prior to absorbance measurement (515 nm) using 96-well plate reader. Cells treated with DMSO alone were used as controls for percent inhibition and IC₅₀ calculations.

4.2.3. Real-time cell growth surveillance by electronic sensing (RT-CES)

Real-time cell growth was monitored using the xCELLigence System (Roche Applied Sciences). Human cancer cell lines (1000–3000 cells/well) were seeded into 96-well E-plates. The proliferation of cells were monitored every 30 min for 24 h after seeding, (when cells were in log growth phase) treatments with the compounds were done. The cell index (CI) values were recorded every 10 min for the first 24 h and then every 30 min for the remaining 48 h. The cell growth ratios were calculated by CI_{drug}/CI_{DMSO} .

4.2.4. Detection of apoptosis

Cells were seeded onto coverslips in 6-well plates. After 24 h in culture, cells were treated with the compounds with their IC₅₀ concentrations. After 48 h, apoptotic cells were identified by Annexin-V-Fluos (Roche) and DAPI (Santa Cruz) staining according to the manufacturer's recommendations (Roche). Cells were then analyzed using the fluorescence microscope (Nikon Eclipse 50i).

4.2.5. Flow cytometry for cell cycle analysis

Cells were seeded onto 100 mm culture dishes. After 24 h, cells were treated with the compounds with their IC₁₀₀ concentrations. Cells were fixed with ice-cold 70% ethanol for 3 h at –20 °C after 24 h, 48 h and 72 h of incubation. Cell cycle analysis was carried out by PI (Propidium Iodide) staining using MUSE Cell Analyzer according to the manufacturer's recommendations (Millipore).

4.2.6. Western blot analysis

Human cancer cell lines were treated with the compounds (IC₁₀₀ concentrations) or with DMSO control for 24 h. Novex® NuPAGE® Bis-Tris Electrophoresis system was used according to the manufacturer's protocol for all the western blotting analysis. For gel electrophoresis (NuPAGE), 20–50 μ g of protein was used per well. Then the proteins were transferred to nitrocellulose membrane via XCell ITM Blot Module. Cyclin-B1 (#554177, BD), Cdc2/Cdk1 (#PC25, Calbiochem), phospho-Cyclin-B1 (Ser147) (bs-3122R, Bioss), p21/WAF1/Cip1 (#05–345, Millipore), p53 (#05–224, Millipore), phospho-p53 (Ser15) (#9286S, Cell Signaling), Rb (#sc102, Santa Cruz), and phospho-Rb (Ser807/811) (#9308S Cell Signaling) antibodies were used in 1:200 to 1:500 5% BSA-TBS-T. β -actin (#A5441, Sigma) antibody was used for equal loading.

Author contributions

S.N.B designed the compounds and their synthesis. M.M.H and F.E performed the chemical synthesis and characterized the compounds. S.N.B and M.M.H contributed to the writing of the manuscript. R.C.A and D.C.K designed the biological experiments and contributed to the writing of the manuscript. D.C.K performed the experiments for biological evaluation of the compounds on HCC cell lines. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.ejmech.2017.02>.

002. These data include MOL files and InChIKeys of the most important compounds described in this article.

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