DISCOVERY OF NOVEL AGENTS FOR LIVER CANCER THERAPEUTICS AND CHARACTERIZATION OF THEIR BIOACTIVITIES ON CELLULAR PATHWAYS

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DISCOVERY OF NOVEL AGENTS FOR LIVER CANCER THERAPEUTICS AND CHARACTERIZATION OF THEIR BIOACTIVITIES ON CELLULAR PATHWAYS

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DECEMBER, 2015

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ABSTRACT

DISCOVERY OF NOVEL AGENTS FOR LIVER CANCER THERAPEUTICS AND CHARACTERIZATION OF THEIR BIOACTIVITIES ON CELLULAR PATHWAYS

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Hepatocellular carcinoma is the second deadliest and fifth most common cancer type worldwide. Due to the limited therapy options, it is crucial to develop novel targeted therapeutic agents that provide better prognosis and enhance life quality of patients. The specific aim of this thesis was to identify and characterize novel compounds with anticancer properties in liver cancer.

Three groups of molecules were investigated. First group were cardiac glycosides extracted and purified from *Digitalis Ferruginea*. Extensive analysis Glycoside Lanatoside C revealed that these molecules induced ROS accumulation in liver cancer cells with differential downstream targets in mesenchymal-like PTEN-deficient drug-resistant Mahlavu and epithelial-like PTEN-adequate drug-sensitive Huh7 liver cancer cells. Xenograft models on nude mice also confirmed the anti-cancer activities of Lanatoside C *in vivo* with decreased tumor volume and weight.

The second group of compounds were novel molecules that contains triazolothiadiazine and triazolothiadiazole scaffold, derived from known NSAIDs (ibuprofen, naproxen and flurbiprofen). Results indicated that SubG1/G1 cell cycle arrest is induced in treated cells. In addition, extensive molecular analysis disclosed oxidative stress induction and COX activity inhibition leading to ASK1 activation

and Akt inhibition. The levels of downstream elements GSK3β, β-catenin and CyclinD1 were also altered. Apoptosis was characterized as the cell death mechanism that is triggered by these molecules in liver cancer cells.

Novel nucleobase/nucleoside analogues were the third group of molecules explored in this study. 24 of 127 investigated compounds showed significant cytotoxicity during initial screening. 6 molecules were selected for further molecular analysis upon real-time cytotoxicity assay. It was observed that the molecules induced SubG1/G1 cell cycle arrest through Src pathway inhibition. CyclinE-cdk2 complex formation was prevented then the inhibition of Rb leading to a decrease in cell growth and proliferation and induction of apoptosis in liver cancer cells.

This thesis disclosed the mode of action of three groups molecules, glycosides are pure examples of drug repurposing. NSAID represent the modified small molecule compounds for novel targets and finally nucleobase analogs are novel compounds as anti metabolites.

Keywords: Hepatocellular carcinoma, cardiac glycoside, NSAIDs, apoptosis, nucleobase, nucleoside, triazol, thiadiazine, drug repurposing, anticancer

ÖZET

KARACİĞER KANSERİ TEDAVİSİ İÇİN YENİ MADDELERİN KEŞFEDİLMESİ VE HÜCRESEL SİNYAL AĞLARI ÜZERİNDEKİ BİYOAKTİVİTELERİNİN KARAKTERİZASYONU

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Karaciğer kanseri, dünyada görülme sıklığı açısından beşinci, öldürücülük açısından ise ikinci sırada yer almaktadır. Bu kanser, klinik olarak kullanılmakta olan birçok kemoterapötik ve radyoterapötik ajanlara dirençli olduğu için tedavi yöntemleri çok kısıtlıdır. Bu sebeple karaciğer kanseri tedavisinde kullanılabilecek, hem hasta ömrünü uzatıcı hem de yaşam standardını yükseltici yeni aday moleküllerin araştırılıp geliştirilmesi büyük önem taşımaktadır. Bu tezdeki amacımız, karaciğer kanserinde anti-kanser etkiye sahip yeni moleküllerin geliştirilip üretilmesi ve bu moleküllerin etki mekanizmalarının aydınlatılmasıdır.

Bu proje kapsamında üç grup molekül test edilmiştir. İlk grup molekül yüksük otu ailesinden olan *Digitalis Ferruginea* bitkisinden elde edilen ve saflaştırılan kardiyak glikozitleridir. Yapılan araştırmalar göstermiştir ki bu moleküller karaciğer kanser hücrelerinde ROS birikmesine sebep olmaktadırlar. İlaca dirençli PTEN-eksik mezenkimal yapıdaki Mahlavu ve epitel yapıdaki ilaca duyarlı Huh7 hücrelerinde farklı mekanizmaları tetikleyerek apoptotik hücre ölümünü aktif hale geçirmektedirler. Tüysüz farelere Mahlavu karaciğer kanseri hücreleri verilerek oluşturulan ksenograft modelleri de Lanatoside C glikozitinin tümör hacmi ve ağırlığında ciddi azalmaya sebep verdiğini göstermektedir.

Proje kapsamındaki ikinci molekül grubu ise bilinen steroid-olmayan anti-enflamatuar ilaçlardan (ibuprofen, naproxen and flurbiprofen) uyarlanarak sentezlenen ve bünyesinde triazol grubu bulunduran moleküllerdir. Bu moleküllerin hücre döngüsünü SubG1/G1 fazında durdurdukları gözlemlendi. Ayrıca moleküller, karaciğer kanseri hücrelerinde oksidatif stres tetikleyerek ve COX aktivitesini azaltarak apoptotik hücre ölüm mekanizmasını tetiklemektedirler. Protein seviyesinde ise bu moleküller ASK1 proteinin aktive ederek ve Akt proteinin inhibe ederek, GSK3β, β-catenin and CyclinD1 proteinlerini etkilemekte ve hücre ölümüne sebep vermektedirler.

Nukleosit/nukleobaz analogları test edilen son grup moleküllerdir. Toplamda test edilen 127 molekülün 10μM altı IC_{50} değere sahip 24ü ileri sitotoksisite analizinde incelendi ve 6 tanesi detaylı moleküler incelemeler için seçildi. Sonuçlar göstermektedir ki bu moleküller hücre döngüsünü SubG1/G1 fazında duraklatmakta ve apoptotik hücre ölüm mekanizmasını tetiklemektedirler. Western Blot analizi sonucunda da bu moleküllerle muamele edilen karaciğer kanseri hücrelerinde Src hücre yolağı üzerinde etkileri olduğu gözlemlendi. CyclinE-cdk2 kompleksinin oluşumu engellendi ve bunun sonucunda Rb molekülü inhibe edilerek hücre büyümesi ve proliferasyonunda düşüş meydana geldi, sonucunda da karaciğer kanseri hücrelerinde apoptos hücre olumu tetiklenmiş oldu.

Bu tez kapsamında 3 grup molekülün aksiyon mekanizması belirlendi. Glikozitler ‘drug repurposing’ alanı için birer örnek teşkil etmektedirler. Steroid-olmayan anti-enflamatuar türevleri ise modifiye edilmiş yeni küçük molekül bileşikleri temsil etmektedirler. Son olarak nukleobaz analogları ise anti-metabolit özellikli benzersiz bileşiklerdir.

Anahtar kelimeler: Hepatosellüler karsinom, kardiyak glikozit, NSAIDs, apoptos, nucleobaz, nucleosit, triazol, thiadiazine, ilaç, antikanser

TO MY FAMILY

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ABBREVIATIONS

5-FU	5-Fluorouracil
ADP	Adenosine Diphosphate
ATP	Adenosine Tri-phosphate
AP-1	Activator protein 1
BSA	Bovine Serum Albumin
CaCl2	Calcium Chloride
CDK	Cyclin Dependent Kinase
CO2	Carbon dioxide
CPT	Camptothecin
DAPK1	death-associated protein kinase-1
DMEM	Dulbecco's Modified Eagle's Medium
ddH_2O	Double Distilled Water
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FDA	Food & Drug Administration
FGF-2	Fibroblast Growth Factor 2

g	Gram
G1	Gap1
G1	Gap2
GSK3-α/β	Glycogen Synthase Kinase-3 α/β
HBV	Hepatitis B
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C
HER2	Human Epidermal growth factor Receptor 2
HRP	Horse Radish Peroxidase
IC50	Inhibitory Concentration 50
K	Potassium
kDa	kilo Dalton
M	Mitosis
MKK	Mitogen Activated Protein Kinase Kinase
MAPK	Mitogen Activated Protein Kinase
MES	2-(N-morpholino)ethanesulfonic acid.
MOPS	3-(N-morpholino)propanesulfonic acid
mg	Milligram
MgCl2	Magnesium Chloride
MRI	Magnetic Resonance Imaging
µg	Microgram
µl	Microliter
µM	Micromolar
Na	Sodium
NaOH	Sodium Hydroxide

NaCl	Sodium Chloride
NCI	National Cancer Institute
NEAA	Non-essential Amino Acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NI	No Inhibition
ng	nanogram
nm	Nanometer
nM	Nanomolar
NSAID	Nonsteroidal anti-inflammatory drugs
NRTK	non-receptor tyrosine kinase
PAGE	Polyacrylamide Gel Electrophoresis
P/S	Penicillin/Streptomycin Solution
PARP	Poly(ADP-ribose) Polymerase
PI3K	Phosphotidylinositol-3-kinase
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween-20
PhosStop	Phosphatase Inhibitor Cocktail
PI	Propidium Iodide
PIC	Protease Inhibitor Cocktail
PMSF	Phenylmethylsulphonylfluoride
RNaseA	Ribonuclease A
RT-CES	Real-Time Cell Electronic Sensing
RTK	Receptor tyrosine kinase

ROS	Reactive Oxygen Species
S	Synthesis
SRB	Sulphorhodamine B
SDS	Sodium Dodecyl Sulfate
Ser	Serine
Src	Rous Sarcoma Virus
STS	Staurosporine
TBS	Tris-buffered Saline
TBS-T	Tris-buffered Saline with Tween-20
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
Thr	Threonine
Tyr	Tyrosin

CHAPTER 1. INTRODUCTION

1.1. Hepatocellular Carcinoma

Development of Hepatocellular carcinoma is a chronic multi-step process resulting in death of thousands of individuals every year. It is the fifth common and one of the most deadly cancer types all around the world [1] (Figure1.1). Major epidemiological factors for HCC are HCV, HBV infection or alcohol abuse which result in chronic liver disease and cirrhosis [2, 3] (Figure1.2). HBV and HCV are both viral infections transmitting via body fluids such as blood, which can result in acute or chronic liver disease. Since 1982, HBV can be prevented by the developed vaccine [4], however, there is no vaccine yet for HCV. Significant amount of people infected with HBV or HCV develop liver cirrhosis or cancer [5]. Cirrhosis can be defined as continues scarring of the tissue, due to chronic damage of liver. Liver cirrhosis is on of the major risk factors of HCC. Cirrhosis is characterized by impaired functioning of hepatocyte, hypertension of the portal, or formation of ascites [6].

1.2. Molecular pathogenesis of Hepatocarcinogenesis

The pathogenesis of HCC requires a multistep mechanism (Figure 1.2). Upon the injury of the hepatic tissue by viral infection or alcohol abuse or any other factors such as obesity, chronic liver disease occurs. Then, dysplastic nodules are formed via the reactivation of telomerase and genomic instability occurs resulting in malignant phenotype formation. HCC development is induced by several factors such as epigenetic regulations, genetic alterations, inhibition of tumor suppressors (such as TP53) or activation of proto-oncogenes (such as Ras) [7].

Hepatocellular Carcinoma is extremely resistant to conventional chemotherapy and radiotherapy. The five-year survival rate is only about seven percent with very high recurrence [8, 9]. If the patient can be diagnosed at the early stage, surgery or interventional radiotherapy can be used for treatment strategy. However, in most of the cases, patients are diagnosed at the late stages of HCC. In that case, for the treatment of advanced HCC is usually chemotherapy, which have low rate of

response from the patients. There is only one FDA approved drug targeting advanced HCC, Sorafenib (Nexavar, BAY43-9006). Sorafenib is a multi-kinase inhibitor (inhibiting VFGFR, PDGFR, FGF and RAF)which can only prolong survival around 3-5 months [10]. Therefore, it is essential to develop novel preventive or therapeutic approaches towards HCC.

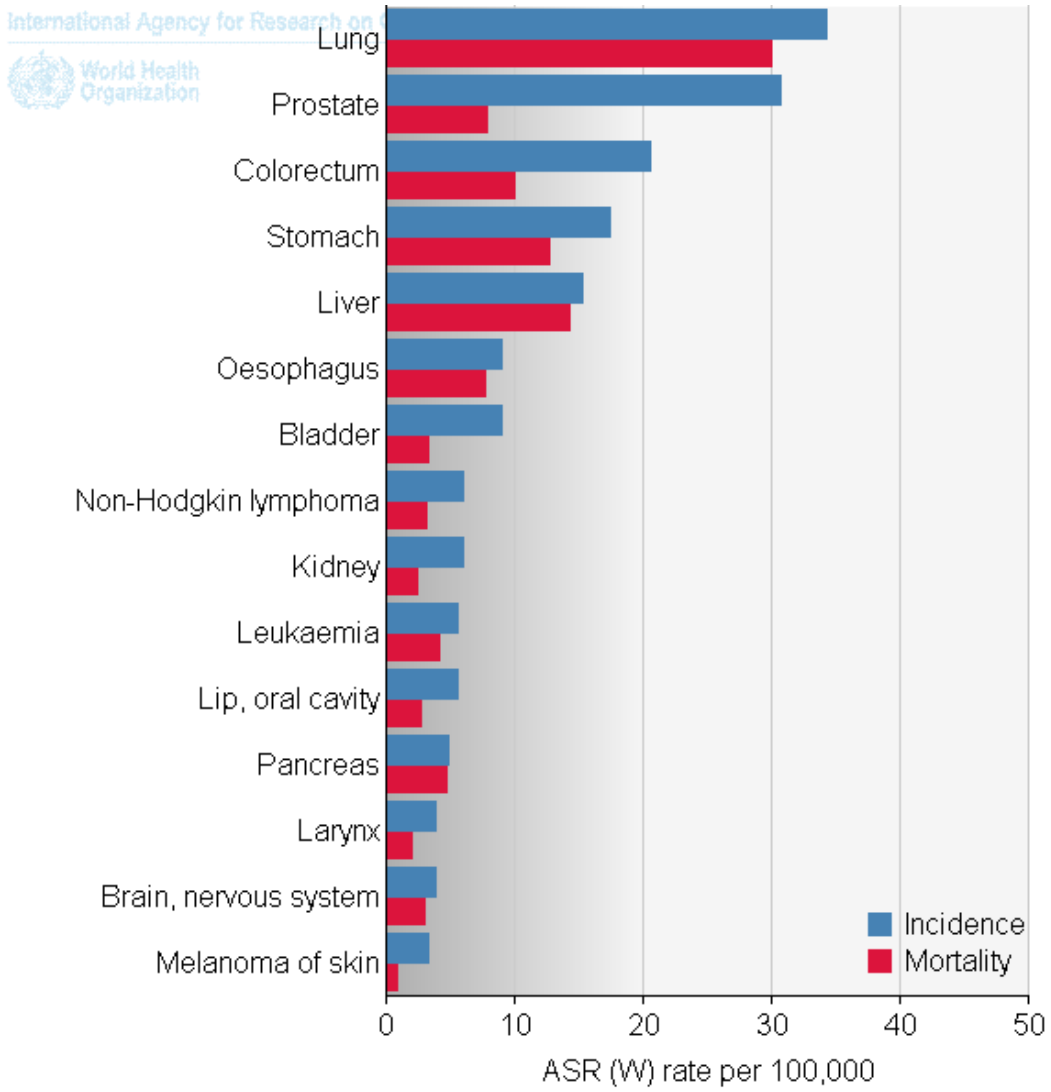
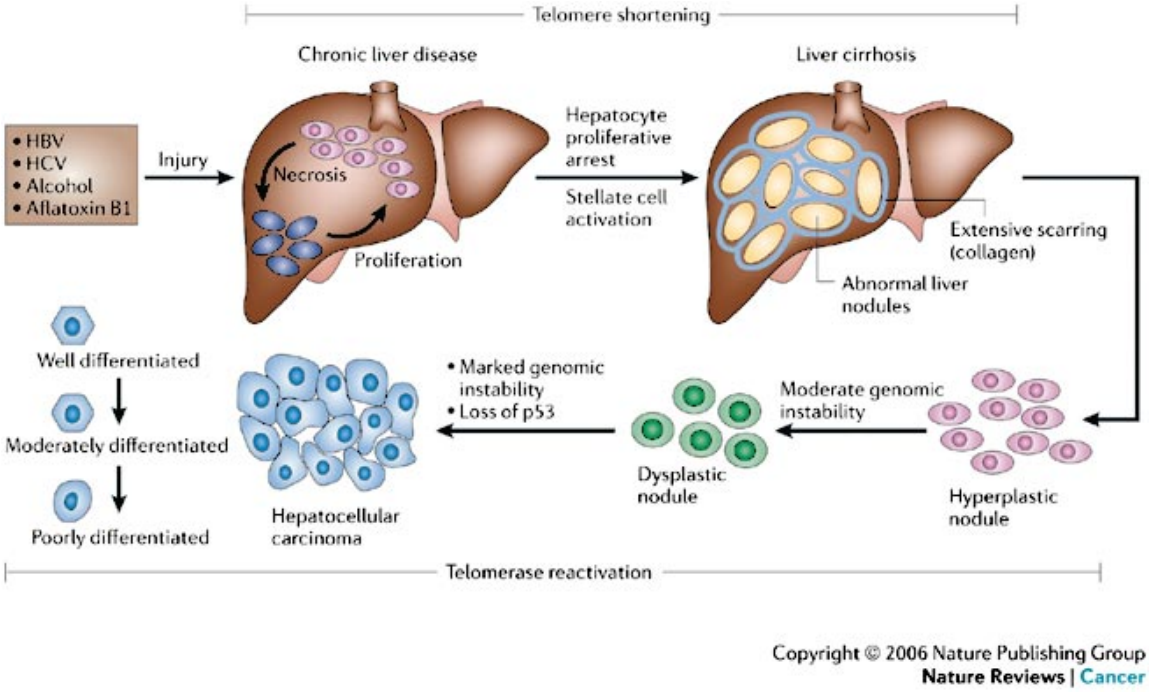


Figure 1.1. The rates of cancer incidence and mortality worldwide © IARC 2015 [1]**.**



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Figure 1.2. The etiological factors of HCC [7]**.**

In the recent years, identification of some key signaling pathways of HCC pathogenesis resulted in new possibilities for targeted therapies for the treatment of advanced HCC[10]. There are on-going clinical trials with compounds targeting different members of these different pathways (Figure 1.3 and Table 1.1). Some of the significant pathways can be summarized as:

Epidermal growth factor receptor (EGFR) pathway: This pathway was reported to be involved in induction of inflammation in HCC by effecting DNA synthesis, regeneration and tumor growth. EGFR was suggested to induce HCC tumor cells to produce factors of inflammation (such as IL-8, IL-1…etc.) [11].

Insulin-like growth factor receptor (IGFR) pathway: The members of this pathway regulate several cellular mechanisms such as cell growth, proliferation or apoptosis…etc. The pathway is activated through its ligands and has an important role in metastasis [12].

Vascular endothelial growth factor (VEGF) pathway: VEGF pathway is the major regulator of angiogenesis during HCC development. In liver cancer cells, VEGF expression levels were shown to be highly increased [10].

PI3K/Akt/mTOR pathway: This pathway that is activated through specific receptor-tyrosine kinases is significant in cell survival, growth and proliferation. PI3K activates mTOR and results in growth and proliferation. mTOR can also be directly stimulated by Akt. The deletion of PTEN, a member of this pathway, is also highly related with aggressiveness of the tumor [10].

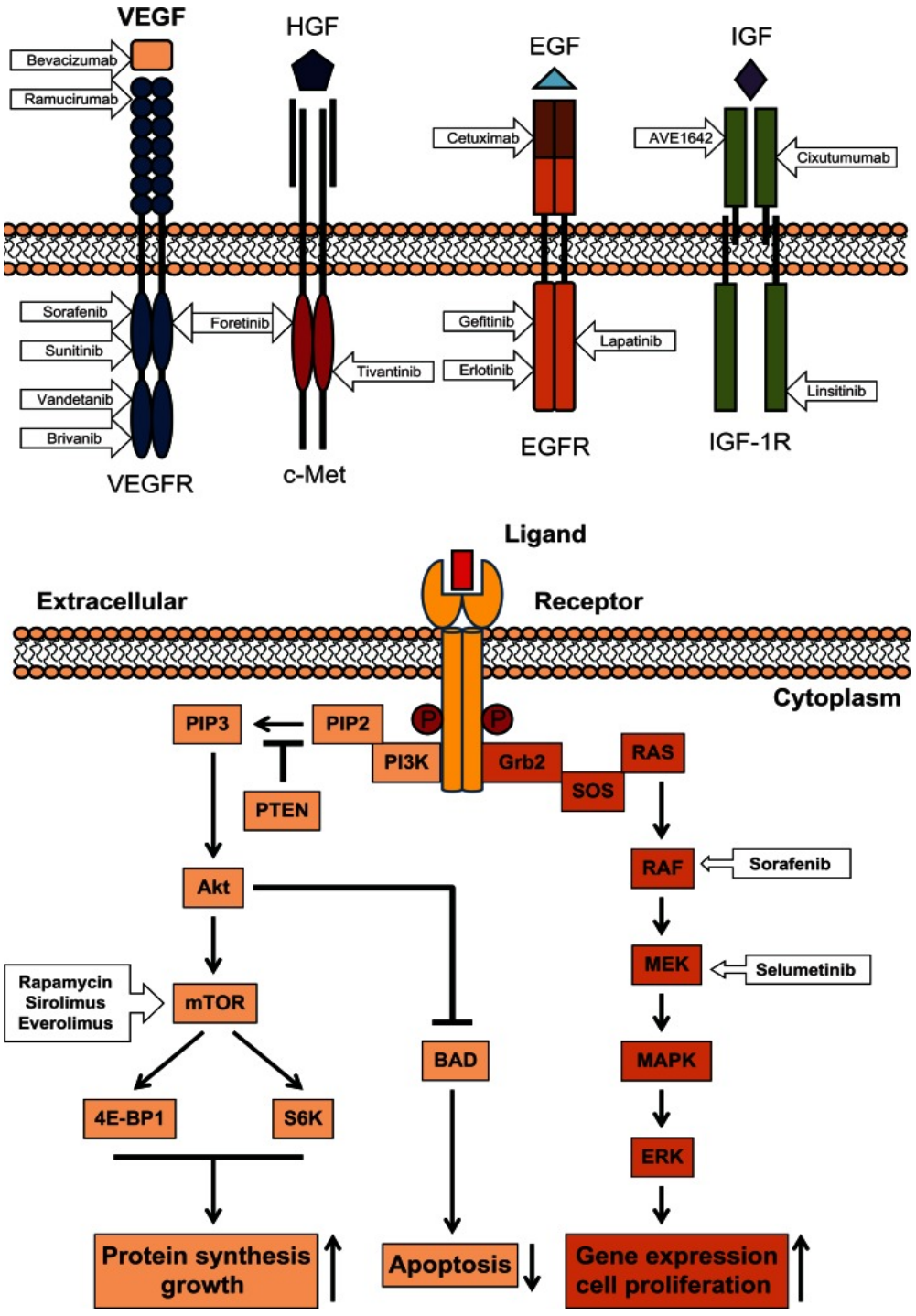


Figure 1.3. Molecular mechanisms of agents targeting different members of the signaling pathways involved in HCC. (Copyright © 2013 the author(s), publisher and licensee Libertas Academica Ltd. This is an open access article published under the Creative Commons CC-BY-NC 3.0 license) [10].

Table 1.1. List of compounds in clinical trials for targeted HCC therapy

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Agent	Classification	Target	Stage	Trial Identifier
Sorafenib	Small molecule compound	VEGFR, PDGFR, RAF	Approved	
Vandetanib	Small molecule compound	VEGFR, PDGFR	Phase I/II	NCT00496509 NCT00508001
Brivanib	Small molecule compound	VEGFR, PDGFR	Phase III	NCT00858871 NCT00825955
Sunitinib	Small molecule compound	VEGFR, PDGFR	Phase III	NCT00699374
Bevacizumab	Monoclonal antibody	VEGF	PhaseII	NCT00162669
Ramucirumab	Monoclonal antibody	VEGFR	Phase II/III	NCT00627042 NCT01140347
Foreetinib	Small molecule compound	c-Met, VEGFR	Phase I/II	NCT00920192
Tivantinib (ARQ197)	Small molecule compound	c-Met	Phase I/II	NCT00802555 NCT00988741
Gefitinib	Small molecule compound	EGFR	Phase II	NCT0071994 NCT00282100
Erlotinib	Small molecule compound	EGFR	Phase I/II	NCT00047346 NCT00047333
Lapatinib	Small molecule compound	EGFR	Phase II	NCT00107536 NCT00101036
Cetuximab	Monoclonal antibody	EGFR	Phase II	NCT00142428
Linsitinib (OSI-906)	Small molecule compound	IGF-IR	Phase II	NCT01101906

AVE1642	**Monoclonal antibody**	**IGF-IR**	**Phase I/II**	**NCT00791544**
Cixutumumab	**Monoclonal antibody**	**IGF-IR**	**Phase II**	**NCT00639509**
Sirolimus	**Small molecule compound**	**mTOR**	**Phase II**	**NCT01374750**
Everolimus	**Small molecule compound**	**mTOR**	**Phase I/II**	**NCT00390195**
Selumetinib (AZD6244)	**Small molecule compound**	**MEK**	**Phase I/II**	**NCT00550719 NCT00604721**
Belinostat	**Small molecule compound**	**HDAC**	**Phase II**	**NCT003211594**

1.3. Cell death mechanisms

Cell death mechanisms that are classified according to the morphological, biochemical or molecular changes they induced.

Apoptosis, is also called as "programmed cell death" that occurs in multicellular organisms. It is the regulatory process for cell number determination and damaged cell elimination, and also key event in many biological processes such as embryogenesis, immune system regulation or carcinogenesis [13]. *Necrosis* is an other type of cell death with self-degradation. Biggest difference between apoptosis and necrosis is that cell debris does not disrupt the surrounding cells. There are some surface proteins that when undergoes apoptotic morphological changes induce the surrounding cells to phagocytized the apoptotic cell. *Autophagy*, in other words "self-eating", is the most evolutionarily conserved type of cell death. It happens by the formation of autophagic vacuolization in the cytoplasm. Autophagy in same cases may link with apoptosis [14]. *Senescence* can also be considered under the concept of cell death. Replicative senescence is described as irreversible arrest of cell cycle.

These cells cannot enter S phase . Biggest difference of senescence from apoptosis is that cells in this stage remain metabolically active if growth medium is applicable [15].

Apoptosis in itself can also be characterized as extrinsic or intrinsic as well. In the extrinsic apoptotic pathway, the presence of death ligands induces death receptors resulting in the formation of the Death Inducing Signaling Complex (DISC) and activation of caspase 8 [16]. Active caspase 8 then activates Caspase 3 and results in apoptosis induction [17]. In case of intrinsic pathway, upon intracellular stress, cytochrome c is released from mitochondria initiates formation of apoptosome (caspase9 and Apaf-1) and activates caspase 9. Caspase 9 then activates caspase 3 and apoptosis. Two pathways are not independent but can be linked by BID which is a BH3 only protein which is cleavage by caspase 8 and then permabilize into mitochondria to initiate membrane permeabilization resulting in activation of intrinsic pathway [18].

Tumor cells encounter genetic alterations that makes them resistance to cell death mechanism. The chemotherapeutic agents try to stimulate these cell death pathways to induce cytotoxicity inside the tumor cells [19].

1.4. Small molecule inhibitors

The development and characterization of small molecules with potential anti-cancer abilities emerged high attraction due to the fact that protein-protein interactions are fundamental processes underlying various biological processes in recent years (Table 1.1).

The ultimate goal of such small molecules is their inhibitory effect on downstream protein-protein interactions in cell signaling cascades [20]. The main advantage of small molecules bearing high potential for targeted anticancer research is that they are reversible most of the time on the contrary to most of the genetic tools and they can penetrate inside the cells easily.

1.5. Cardiac glycosides

Despite all the expenses and time spent, drug discovery process often results in failure or low efficacy and therefore drug industry changes its perspective to new approaches. One of these new approaches is called “drug repurposing/repositioning” in which the aim is to identify and discover the new unknown properties of existing drugs in other words new use for old drugs [21, 22]. Cardiac glycosides used in this research is an example for “drug repositioning/repurposing”.

Cardiac glycosides are known as steroid-like compounds and are used for the treatment of congestive heart failure. The cardiac glycosides digitoxin, digoxin and ouabain are some of the known commercial therapeutics (Figure 1.4). These glycosides function by inhibiting Na^+,K^+/ATPase and thereby increase intracellular calcium and sodium ion concentrations [23].

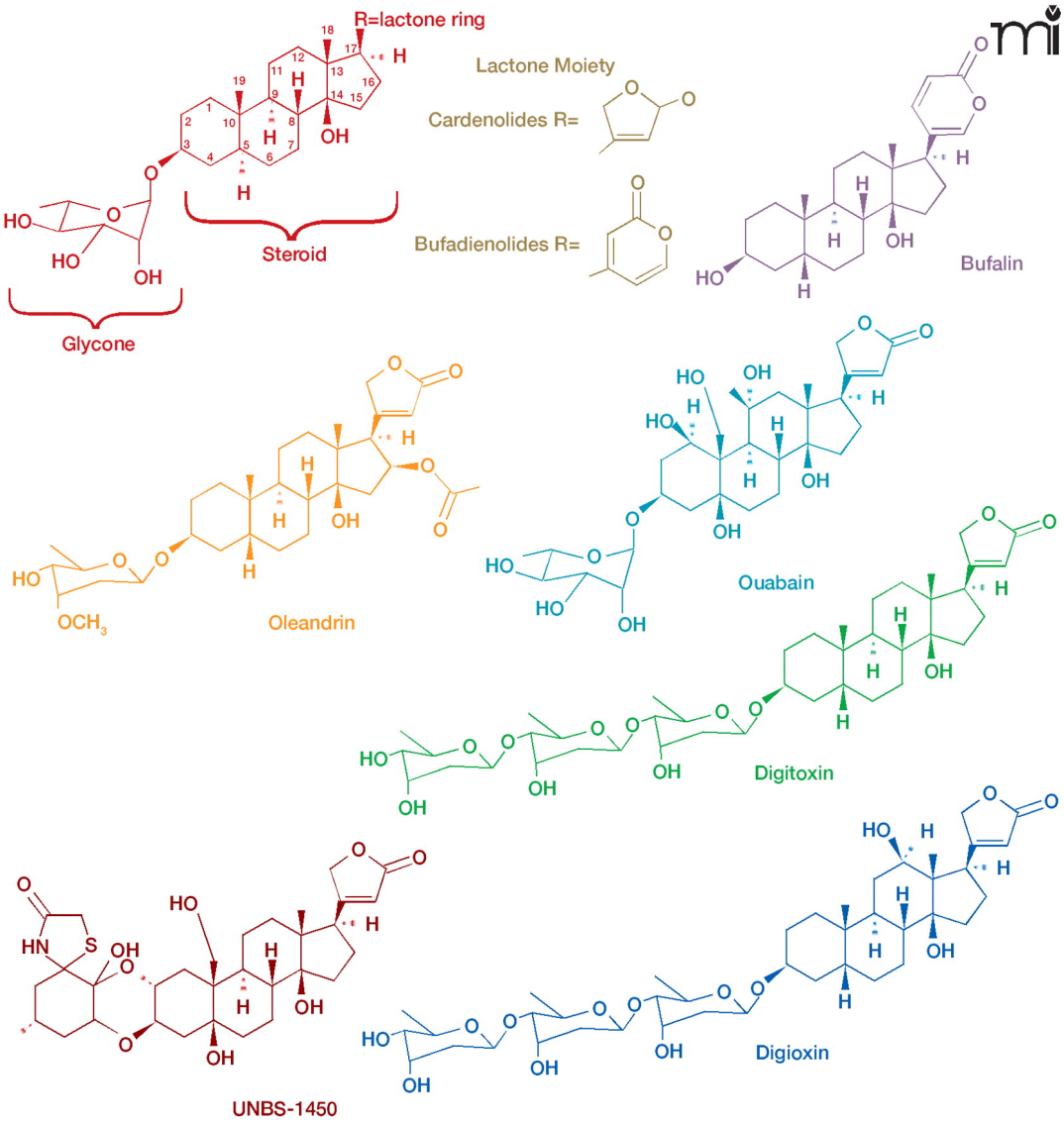


Figure 1.4: Structural representation some cardiac glycosides. Copyright © 2011 by the American Society for Pharmacology and Experimental Therapeutics [23].

1.5.1. Cardiac glycosides and cancer

Cardiac glycosides was shown to inhibit malignancy cell growth [24]. In breast cancer patients, the recurrence rate five years after mastectomy was ~9.6 times less in patients using cardiac glycosides and the survival rate was significantly higher[25]. In addition, an other study revealed that ouabain induce growth arrest by activation of Src/EGFR and ERK1/2 pathways [26]. Moreover, an other cardiac glycoside bufalin was shown to have inhibitory affects against multidrug resistant liver cancer cells by inducing G2/M arrest and mitochondria associated apoptosis [27]. The cardiac glycoside oleandrin was also reported to be cytotoxic against human melanoma cells through activation of reactive oxygen species (ROS) [23]. Thus, there are many data suggesting the therapeutic role of cardiac glycosides in cancer through various biological processes.

1.5.2. Apoptosis and Cardiac glycosides

Cardiac glycosides have been shown to trigger apoptosis in non-toxic concentrations [28, 29]. It was proposed that the mechanism of action of apoptotic induction by cardiac glycosides is to be through the inhibition of Na^+,K^+-ATPase [30]. Some extensive research in prostate cancer cell lines revealed the induction of apoptosis together with proliferative arrest by cardiac glycosides in concentrations which can be administered to the patients [28, 31]. Thus, the alterations in calcium ion concentrations may be the underlying mechanism of apoptotic induction in human cancer cell lines producing a new area for therapeutic approach [30].

Moreover, it was also suggested that cell death susceptibility towards cardiac glycosides is much higher in human cancer cells than healthy cells. This might be due to the altered activity of Na^+,K^+-ATPase in tumor cells [32]. In leukemia cells, bufalin induced apoptotic cellular death but not in normal cells. In this study, bufalin was suggested to induce apoptosis through Ras-Raf-MAPK pathway [23]. Furtermore, cardiac glycosides such as ouabain or digoxin was proposed to induce apoptosis through cytochrome c release in androgen-independent prostate cancer cells [33]. In addition, there are other studies suggesting the increase in Fas and tumor necrosis factor receptor (TNFR1) in tumor cells treated with cardiac

glycosides but not in healthy cells, resulting in apoptotic induction [23, 34]. In studies performed on non-small-cell-lung cancer cells, the cardiac glycosides were shown to induce apoptosis by activation of Apo21/TNF-related apoptosis-inducing ligand (TRAIL) pathway [35]. Consequently, in different cancer types, cardiac glycosides were shown to effect different cellular pathways but the common point is the induction of apoptosis.

1.5.3. Digitalis Ferruginea

Cardiac glycosides are natural products obtained from Digitalis genus, which is composed of 20 species. Prof. Ihsan Calis purified the cardiac glycosides used in this study from *Digitalis ferruginea* Sm (Scrophulariaceae) (also known as 'rusty foxglove') (Figure 1.5) using TLC and PC methodology [36, 37].



Figure 1.5: Rusty foxglove (D. Ferruginea) **(obtained from archive of Dr. Hayri Duman)**

1.6. Link between inflammation and cancer

Cancer is a multistep process which arise with the changes in the genetic background of the cells. The hallmarks of cancer include features such as “self-sufficient growth” or resistance towards anti-proliferative signals or to death inducing signals [38, 39]. Many of these genetic changes inside the cells, such as activation of oncogenes or inhibition of tumor suppressor genes, are not enough on their own for tumor progression but need environmental factors as well such as inflammation [39]. The relation between inflammation and cancer progression has long been explored by many scientists [40, 41]. Normally, inflammation results in host defense against an external infection. The epidemiological data suggests that about 15% of cancer incidence worldwide is closely correlated with microbial infections suggesting a strong connection between cancer development and inflammation [42]. In addition, the presence of virus originated cancers (human papilloma virus leading to cervical caner or Hepatitis infection causing HCC) also contributes to the link between cancer and inflammation [43]. It was suggested that inflammation may cause cancer by creating environments that adopt tumor irritation. The underlying mechanism of inflammation resulting in cancer progression was proposed to be through several pathways such as Wnt/β-catenin, COX1/2 or TGF-β (Figure 1.6) [44–51]. Moreover, it was claimed that the mediators of inflammation (for instance cytokines or chemokines) may induce the proliferation of untransformed cells but also tumor cells. Consequently, the presence of inflammation due to massive cellular death or tissue injury can result in stimulation of tissue repair dependent proliferation response that will then cause promotion of tumor growth [43, 52, 53].

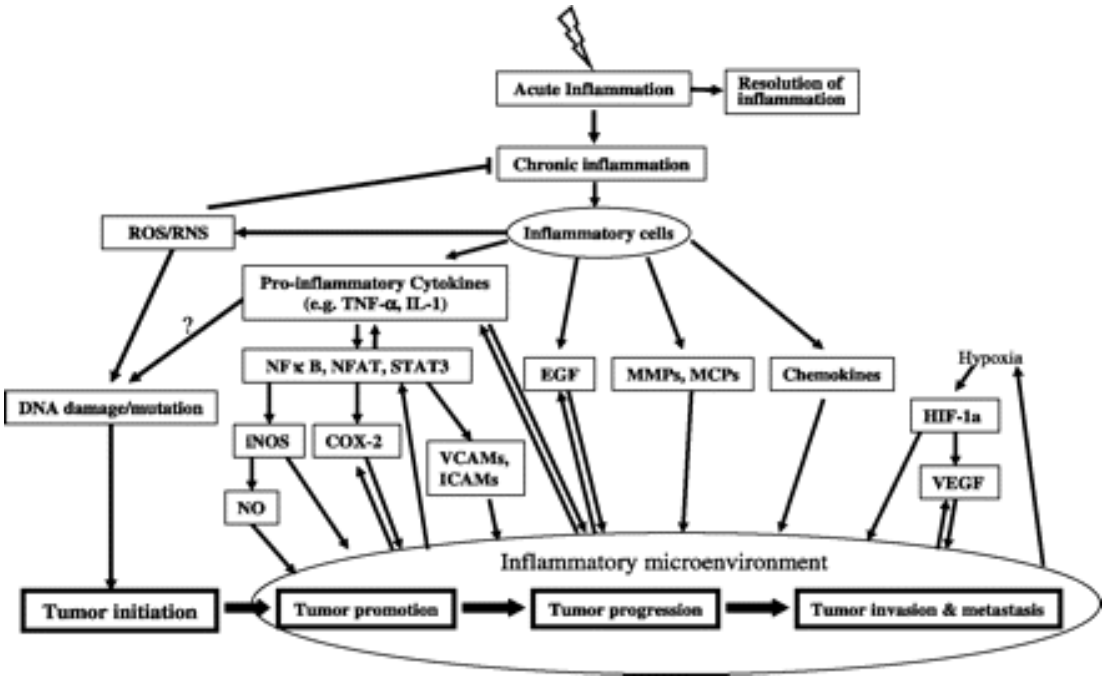


Figure 1.6: Pathways involved in the association between inflammation and cancer Copyright © 2015 American Association for Cancer Research [51]**.**

1.6.1. Non-steroid anti-inflammatory drugs

Non-steroid anti-inflammatory drugs (NSAIDs) are commercial drugs, including Aspirin or Ibuprofen, which are used for several occasions such as reduction of pain or fever. NSAIDs function by inhibiting COX enzymes COX-1 and COX-2 enzymes that are responsible for producing prostaglandins that promote inflammation or pain. Different NSAIDs can act in different kinetics on COX enzymes. For instance: ibuprofen rapidly functions through competitive inhibition of arachidonic acid binding to active site of COX enzyme. On the other hand, flurbiprofen inhibits the arachidonic acid to COX binding in a time dependent manner, which is a slow process. Moreover, aspirin makes a weak ionic bond with COX active site through Arg120 resulting in transacetylation in Ser530 inducing the complete inhibition of COX enzyme [54, 55].

1.6.2. Non-steroid anti-inflammatory drugs in cancer therapeutics

As an innovative approach, NSAIDs have been investigated in the area of drug repositioning. There are significant data claiming the usage of NSAIDs as anticancer agents [56, 57]. There are clinical studies showing that individuals taking regular NSAIDs for a long term possess reduced cancer risk and incidence. For instance, Aspirin, a commercial drug widely used to cardio protective and antithrombotic purposes [58, 59], was shown to have anticancer activities in various cancer types such as breast, lung, prostate or skin cancer [57, 60, 61]. It was also suggested that Aspirin has both chemopreventive and chemotherapeutic functions [62–64]. It was shown that Aspirin could induce apoptosis, cause cell cycle arrest at SubG1/G1, prevent angiogenesis in cancer cells and reduce *in vivo* tumor growth [65–69]. However, today, Aspirin is not clinically used in cancer thereby due to high risk of bleeding [70].

Ibuprofen, another NSAID, was recently shown to inhibit activation of nuclear β-catenin and induce GSK-3 β phosphorylation in colon cancer even though it couldn't reduce the size of the tumor or the degree of cancer [71]. In case of bladder cancer, data suggest that NSAIDs, especially Ibuprofen, is associated with reduced risk [72].

Moreover, an other example for the anticancer activity of NSAIDs is celecoxib, which was shown to inhibit angiogenesis both *in vitro* and *in vivo* [66].

1.6.3. Triazoles in cancer

In recent literature, there are studies showing the cytotoxic affects of triazoles and their condensed derivatives [73–76]. Moreover, some condensed triazoles that carry triazolotriazin and triazolopiridazin were suggested to have anticancer activities [77]. Today, a group of aromatase inhibitors vorozol, letrozol and anastrozol that contains 1,2,4-triazol ring are used clinically in treatment of breast cancer [78, 79].

1.7. Protein Kinases

The part of the human genome encoding for the protein kinases is called the human kinome and encodes more than 500 protein kinases [80]. Protein kinases function by adding phosphate groups to amino acids of proteins inside the cells and thereby activating or inhibiting them. This addition of phosphate group is a reversible effect and it initiates a structural change in the target protein resulting in the change in its activity or localization. Binding site or the protein kinase is the site that it binds to the target protein and the catalytic site is where ATP binds. Protein kinases are essential members of the cells that regulates various cellular pathways such as cell growth or division, metabolism, or DNA damage [81, 82] .

The protein kinases are classified into two groups, according to where they add the phosphate: Serine/Threonine kinases, and Tyrosine kinases [83]. DNA damage, or chemical signals such as calmodulin levels can be regulators of serine/threonine kinases. Tyrosine kinases are divided into two groups: Receptor Tyrosine Kinases (RTKs) and non-receptor tyrosine Kinases (NRTKs). RTKs are transmembrane receptor proteins that are important in various cellular processes such as cell division, or differentiation [84]. The extracellular domain of the protein is responsible for the binding to the ligand molecule. The intracellular domain is the kinase domain. Upon ligand binding to the extracellular domain, dimerization occurs resulting in auto phosphorylation. Then, ATP binds to the catalytic domain and the protein kinase become active [85]. EGFR belongs to RTK group of kinases [86]. On the other hand, NRTKs are cytoplasmic protein kinases such as SRC, ABL…etc. Some kinases have dual properties meaning that they can add a phosphate group to both Serine-Threonine and Tyrosine hydroxyl side chains. Mitogen-activated protein kinase kinases (MKKs) are example of dual-functional kinases [84].

1.7.1. Protein Kinases in Cancer

Uncontrolled proliferation, survival, metastasis and invasion are the fundamental characteristics of cancer cells and appear from dysregulation of signal transduction in the cell. Pathways which involves protein kinases as frequently deregulated in cancer cells [87]. Mainly the epidermal growth factor receptor (EGFR), the mitogen activated

protein kinase (MAPK), AKT, and phosphatidylinositol-3-kinase (PI3K) are the kinase pathways that are reported to be frequently hyperactive in cancer. This oncogenic activation of protein kinases can be either direct mutation or can appear as a secondary event. Kinases not only have oncogenic abilities but can also be tumor-suppressors (mostly tyrosine kinases) [88].

1.7.2. Protein Kinase Inhibitors

In signal transduction, protein kinase family of proteins are essential components in the survival process [80]. Deregulations of the activity of protein kinases have been reported in various disorders including but not limited to cancer, metabolic disorders or inflammation [89–91]. Therefore, protein kinases have been the interest of many scientists in the field of therapeutic discovery. The main obstacle for the design of protein kinase inhibitors is to develop a molecule with selectivity towards a specific group of kinases within the huge family of protein kinases. In addition, they all bind to ATP meaning that if a designed molecule targets the ATP-binding pocket, it will have a very low selectivity. Thus, in addition to targeting the ATP-binding site, scientists also try other mechanisms such as allosteric inhibition or inhibition of substrate binding [92]. Protein kinase inhibitors can be classified in three groups: *Type1 inhibitors* compete with ATP and bind to active form of the protein, thus target the ATP-binding domain. *Type2 inhibitors* are also targeting the ATP binding domain by binding and stabilizing the inactive form of the protein. *Type3 inhibitors* are allosteric inhibitors and have high selectivity [90, 93–95]. Imatinib is one of the first tyrosine kinase inhibitor approved by FDA against leukemia. In 95% leukemia patients, the Philadelphia chromosome (fusion of Bcr and Abl) is present but none in healthy cells. Imatinib targets Bcr-Abl tyrosine kinase in a targeted fashion. In clinical studies, patients at all stages of the disease gave response to Imatinib [87]. This drug demonstrates the importance of selectively in targeting protein kinases for cancer treatment. On the other hand, sorafenib is the only FDA approved drug for the targeted therapy in advanced liver cancer. However, it is a multikinase inhibitor and can only prolong survival for 2.8 mounts [91, 96]. One of the major challenges in kinase inhibitors is the resistance phenomenon. In order to overcome this problem, combinatorial approaches are considered.

1.8. Nucleobase and Nucleoside analogs in cancer

Purine and pyrimidine nucleobase and purine nucleoside analogues have been used in cancer treatments. Nucleotides and their derivatives are fundamentals of various cellular processes such as cell growth, proliferation or division [97]. Thus they are significantly used in cancer treatment. These compounds are antimetabolites since the targeted molecules; nucleobases and nucleosides are the precursors of cell nucleic acid metabolism. Various nucleoside analogues have been used for treatment of different cancers. These analogues inhibit the ribonucleotide reductase enzyme and thereby impair the cellular DNA synthesis and induce apoptotic cell death or senescence in cancer cells [98–100]. For instance, in hematological malignancies, Fludarabine, Cladribine and Pentostatine have been in use clinically [101]. Another example for the success studies can be Gemcitabine for lung cancer. This drug can be taken inside the cell by nucleoside transporters and inside it is activated by deoxycytidine kinase and then got incorporated inside DNA or RNA. In combination with other factors, the drug is effective in many other cancers as well such as in ovarian cancer (in combination with triapine or hydroxyurea) [102]. 5-Fluorouracil (5-FU) is another example that is widely used for cancer therapeutics. Over the past years, many improvements have been made in its anticancer activity however; drug resistance is still a big obstacle for this molecule. Different strategies such as DNA microarray experiments may contribute for the identification of the genes involved in 5-FU resistance [103].

CHAPTER 2. OBJECTIVES AND RATIONALE

Cancer is one of the primary causes of both mortality and morbidity worldwide according to the latest WHO reports. It is suspected that the incidence rate of this deadly disease will increase around 70% in the next two decades [104]. Therefore, there is a significant increase in the number of studies attempting to discover new perspectives in the area of prevention, treatment and progress cancer.

Liver cancer is the second deadliest cancer type worldwide [104]. HCC is resistant to conventional chemotherapy and radiotherapy due to underlying chronic liver disease leading to changes in the genomes of hepatocytes [8, 9]. Early-stage HCC is often treated with partial hepatectomy, liver transplantation or in few cases with tumor ablation, however the recurrence rate is very high. Survival rate for early stage HCC is only 7%. Sorafenib (Nexavar, BAY43-9006) is the only FDA approved targeted drug for HCC. Multi-kinase inhibitor, Sorafenib is widely used for the treatment of advanced liver cancers, which are metastatic and therefore cannot be surgically removed. Sorafenib can only prolong mean survival by 2.8 months [10, 105]. Sorafenib is a "dual-action" drug that functions by inhibiting the formation of new blood vessels (angiogenesis) around tumors and by targeting protein kinases (RAF/MEK/ERK pathway) that induce cell growth [10, 106]. Reduced 5-year survival rate, high recurrence rate and the absence of effective targeted therapy highlighting the significance of development and characterization of new strategies and agents in both prevention and treatment of the liver cancer. Thus, in this research our ultimate goal was to identify and characterize new agents with possible anti-cancer activities which can be contemplated in liver cancer therapeutics.

Three groups of molecules were investigated in this thesis. *The first group* of molecules are cardiac glycosides extracted and purified from *Digitalis ferruginea* by Prof. Dr. Ihsan Calis. Cardiac glycosides are steroid-like compounds clinically used in treatment of cardiovascular diseases. The role of cardiac glycosides in cancer studies were previously characterized in breast and prostate cancers. It was observed that recurrence rate in breast cancer patients taking cardiac glycosides was significantly lower when compared to non-taking ones [33]. In addition, previously in my Master's Thesis, we showed that these cardiac glycosides obtained from

Digitalis Ferruginea had significant cytotoxic activity on liver cancer cells [107]. Thus, in this study we investigated the molecular mechanism underlying the cytotoxic activity of these molecules *in vitro* and demonstrated *in vivo* anti cancer activities of Lanatoside C.

The second group of molecules tested are molecules derived from NSAIDs bearing triazolothiadiazine and triazolothiadiazole rings, synthesized by Prof. Birsen Tozkoparan. In literature there are various studies regarding the repurposing of conventional NSAIDs (aspirin, ibuprofen, naproxen…etc) for anticancer purposes [56, 71]. Many studies suggest that these molecules encounter anti-cancer activity due to their inhibitory action on COX properties [71]. In the context of this research, novel triazolothiadiazines and triazolothiadiazoles were derived from known NSAIDs (ibuprofen, flurbiprofen, and naproxen) and investigated on liver cancer cells.

The third group of molecules that were studies in this thesis are purine and pyrimidine nucleobase analogues and purine nucleoside analogues developed and synthesized by Prof. Meral Tuncbilek. Previous studies revealed anti-cancer activity of nucleobase or nucleoside analogues due to their important functions in DNA replication [97, 98, 100]. Thus, in this study, we investigated the bioactivities of the novel compounds on liver cancer cells with the aim of describing the mechanism of action.

The putative anticancer activities of these molecules were explored together with the death mechanism induced and pathways targeted.

CHAPTER 3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Cell culture reagents and materials

NAME	Company	Cat. Number
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	31885
RPMI 1640	Gibco	31870
Fetal bovine serum (FBS)	Gibco	102700
Non-essential amino acid (NEAA)	Lonza	BE13-114E
Penicillin/streptomycin (P/S)	Gibco	15140
0.25% Trypsin-EDTA 1x	Gibco	25200
L-Glutamine 200nM 100x		25030
1x Phosphate Buffer Saline (1xPBS) ($CaCl_2$, $MgCl_2$ free)	Gibco	14190
Dimethyl Sulphoxide (DMSO)	AppliChem Biochemica	A3672
100mm petri dish	Corning	430167
150mm petri dish	Greiner	639160
5ml serological pipette	Corning	4487
10ml serological pipette	Corning	4482
25ml serological pipette	Corning	4489
50ml serological pipette	Corning	4490
6-well plate	Corning	3516
96-well plate	Corning	3599

E-96 plate	ACEA	05232368001
Cryotube	Corning	430289
1000µl tip	Greiner	740288
300µl tip	Gilson	DF300ST
100µl tip	Greiner	772288
20µl tip	Greiner	774288
10µl tip	Greiner	721288
Reagent reservoir	Heathrow Scientific	9409666
Pipeto Pasteur grad 3ml sterile	Deltalab	200062
CO2 incubator	ESCO	170T-8M
Falcon Tubes (15ml, 50ml)	Greiner	188271, 227261
Pipetting aid	Gilson	
Pipettes	Gilson	
Liquid nitrogen tank	Locator	
Water bath (thermostat)	Yellowline basic	
Centrifuge	Hettich Universal 320	
Inverted microscope	Olympus CKX41	
Fluorescence microscope	Nikon ECLIPSE 50i	
Autoclave machine	Hirayama	HV25L
Biological Safety Cabinet	Nuaire	425-600E

3.1.2. Cytotoxicity screening reagents and materials

NAME	Company	Cat. Number
Camptothecin (CPT)	Sigma	C9911
Fludarabine	Santa Cruz	Sc-204755
Cladribine	Santa Cruz	Sc-202399
Pentostatine	Santa Cruz	Sc-204177
Staurosporine	Calbiochem	569397
5-Fluorouracil	Kocak Farma	8699828770237
1xPBS		
NaCl	MERCK	1.06404.1000
KCl	Carlo Erba	471177
$Na_2HPO_4.2H_2O$	Carlo Erba	480227
KH_2PO4	MERCK	1.04873
Trichloroacetic acid (TCA)	MERCK	1.00810.1000
Sulphorhodamine B (SRB)	Sigma	S1402
Acetic Acid	Riedel-de Haen	27225
Tris	Amresco	0826
RT-CES plate reader (xCelligence, RTCA)	ACEA	V380
Microplate reader for SRB	Beckman Instruments	
Robot	Genetix- Clone fill	

3.1.3. DNA Staining reagents and materials

NAME	Company	Cat. Number
Hoechst 33258, bis-benzimide	Sigma	861405
Glycerol	Carlo Erba	346165
Cover slips (22x22mm)	ISOLAB	
Microscope slides	ISOLAB	

3.1.4. Cell lysis reagents and materials

NAME	Company	Cat. Number
NP-40 lysis buffer		
NaCl	MERCK	1.06404.1000
Tris	Amresco	0826
NonidetP-40	AppliChem	
SDS	Sigma	L5750
EDTA	MERCK	1.08421
Protease Inhibitor Cocktail	Roche	11 873 580 001
PhosSTOP	Roche	04 906 837 001
Albumin from bovine serum (BSA)	Sigma	A7906
Bradford reagent	Sigma	B6916
Spectrophotometer	Beckman Du640	

3.1.5. Western blot reagents and materials

NAME	Company	Cat. Number
DTT	Sigma	D9779
Nu-PAGE LDS Sample Buffer 4x	Invitrogen	NP0007
Nu-PAGE Bis-Tris 10% gels (15well)	Invitrogen	NP0303
Nu-PAGE Bis-Tris 12% gels (15well)	Invitrogen	NP0343
Nu-PAGE MOPS SDS running buffer 20x	Invitrogen	NP0001
Nu-PAGE MES SDS running buffer 20x	Invitrogen	NP0002
Nu-PAGE Transfer buffer 20x	Invitrogen	NP0006-1
PageRuler^{TM} Prestained Protein Ladder	Thermo Scientific	26616
PageRuler^{TM} Prestained Protein Ladder	Fermentas	SM1811
Methanol	Sigma	24229
Hybond ECL Nitrocellulose Membrane (0.2mm pore size)	Amersham	RPN3032D
Whatman paper	GE healthcare	3030917
Non-fat dry milk	SUTAS	
Ponceau-S	Sigma	P-3504
1xTBS-T (0.1%)		
NaCl	MERCK	1.06404.1000
Tris	Amersco	0826
Tween 20	MERCK	822184

ECL+	Amersham	RPN2132
XCell SureLock™ Mini-Cell & XCell II™ Blot Module	Invitrogen	EI0002
Sponge pads	Invitrogen	E19052
Medical X-ray Film, 18x24 cm	KODAK	8143059
Power supply	Bio-Rad	Pac200

3.1.6. Antibodies

Primary/Secondary Antibody	Company and catalog number	Western Blot (tested dilution)
Phospho-SAPK/JNK (Thre183/Tyr185)	Cell Signaling, 9251	1:100 in 5%BSA-TBS-T(0.1%), 2hr, RT
SAPK/JNK	Cell signaling, 9252	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
c-Jun (N)	Santa Cruz, sc45	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
p-c-Jun (KM-1)	Santa Cruz, sc822	1:100 in 5%BSA-TBS-T(0.1%), 2hr, RT
Akt	Cell Signaling, 9272S	1:300 in 5%BSA-TBS-T(0.1%), 2hr, RT
Phosphor-Akt (Ser473)	Cell signaling, 9271L	1:100 in 5%BSA-TBS-T(0.1%), 2hr, RT
ERK1/2 (MK1)	Santa Cruz, sc-135900	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
Phospho-ERK1/2 (Thr202/Tyr204))	Santa Cruz, sc-16982	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
Cleaved Caspase-3 (Asp 175)	Cell Signaling, 9662	1:500 in 5%BSA-TBS-T(0.1%), O/N, +4^{o}C
Cleaved caspase-9	Santa Cruz, sc-22182	1:500 in 5%BSA-TBS-T(0.1%), O/N, +4^{o}C
Cleaved caspase-8	Cell Signaling, 9746S	1:500 in 5%BSA-TBS-T(0.1%), O/N, +4^{o}C

Na,K-ATPase antibody α1 subunit	Cell Signaling, 3010	1:500 in 5%BSA-TBS-T(0.1%), O/N, +4^{o}C
Phospho-GSK3-a/ß (Ser21/9)	Cell Signaling, 9331L	1:100 in 5%BSA-TBS-T(0.1%), 2hr, RT
GSK3-a/ß	Santa Cruz, sc-7291	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
a-cyclin D1 (HD11)	Santa Cruz, Sc246	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
a-cyclin E	Calbiochem, CC05	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
Phospho-ASK1 (S83)	Abcam, ab47304	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
anti-B catenin CLONE 7F7.2	Millipore, 04-958	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
p-β-catenin Antibody (BC-22)	Santa Cruz, sc57535	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
p-Src (Tyr416) antibody	Santa Cruz, sc101802	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
Src antibody	Santa Cruz, sc19	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
p-ASK1 (Ser966)	GenScript, A00340	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
a-Phospho-Rb (Ser807/811)	Cell Signaling, 9308S	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
a-Rb (IF8)	Santa Cruz, sc102	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
a-Cdk2 (D-12)	Santa Cruz, sc6248	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
Tubulin	Calbiochem, CP06	1:5000 in 5%milk-TBS-T(0.1%), 2hr, RT
Calnexin	Sigma, C4731	1:5000 in 5%BSA-TBS-T(0.1%), 2hr, RT
Actin	Santa Cruz, sc1616	1:5000 in 5%milk-TBS-T(0.1%), 2hr, RT
PARP	Cell Signaling, 9532	1:200 in 5%BSA-TBS-T(0.1%), 2hr, RT
Anti-mouse-HRP	Sigma, A0168	1:5000 in 5%milk-TBS-T(0.1%), 2hr, RT

Anti-goat-HRP	Sigma, 8919	1:5000 in 5%milk-TBS-T(0.1%), 2hr, RT
Anti-rabbit-HRP	Sigma, 6154	1:5000 in 5%milk-TBS-T(0.1%), 2hr, RT

3.1.7. Cell cycle analysis (FACS) solutions and materials

NAME	Company	Cat. Number
Ethanol	Sigma	32221
Propidium iodide (PI)	Sigma	D4863
RNaseA	Fermentas	EN0531
Tritron-X-100	Sigma	T8787
BD FACSCalibur		
MUSE CELL ANALYZER	Millipore	
MUSE Cell cycle assay kit	Millipore	MCH100106

3.1.8. Oxidative Stress and ROS measurement analysis solutions and materials

NAME	Company	Cat. Number
DCFH-DA containing ROS assay solution		
HEPES	Sigma	H-1016
KOH	Sigma	P1767
D-Glucose	Amresco	0188
2',7'-Dichlorofluoresceindiacetated (DCFH-DA)	Sigma	D6883
1xPBS	GIBCO	14190
MUSE Oxidative Stress Kit	Millipore	MCH100111

3.1.9. Kinase assay reagents and materials

NAME	Company	Cat. Number
Kinase reaction buffer		
Tris	Amresco	0826
$MgCl_2.6H_2O$	MERCK	A406233
Albumin, from bovine serum (BSA)	Sigma-Aldrich	A7906
Kinase-Glo Luminescent Kinase Assay	Promega	V6712
White 96-well polypropylene assay plate	Corning	07-200-761
the Reporter Microplate Luminometer	Turner Designs	

3.1.10. COX Activity Assay reagents

NAME	Company	Cat. Number
COX Activity Kit	BioVision	K549-100

3.1.11 *In vivo* animal experiment reagents and materials

NAME	Company	Cat. Number
Nude mice	Bilkent Animal Facility	
Simple Syrup		
Sugar in ddH_2O		
Reusable stainless feeding needle	Braintree Scientific	
Vernier caliper		
Class-2 Safety Cabinet		
HEPA-filter top covered cage		
MRI	UMRAM	

3.2. SOLUTIONS AND MEDIA

3.2.1. Cell culture solutions

DMEM growth medium	**10% FBS, 1% P/S, 1% NEAA in DMEM**
RPMI-1640 growth medium	10% FBS, 1% P/S, 1% NEAA, 1%L-glutamine in RPMI
Freezing medium	10% DMSO, 25% FBS in growth medium
Serum (-) medium	0.1% FBS, 1% P/S, 1% NEAA in DMEM

3.2.2 Cytotoxicity screening solutions

10% TCA solution	**Diluted from 100% to 10% TCA in cold ddH_2O**
SRB stain solution	(w/v) 0.4% SRB in 1% acetic acid solution
1% acetic acid	(v/v) 1% acetic acid in ddH_2O
10mM TBS	0.6gr Tris in 1000ml cold ddH_2O
10x PBS	1.5M NaCl, 30mM KCl, 80mM Na_2HPO_4, 20mM KH_2PO_4 (pH 7.4)

3.2.3 DNA Staining reagents and materials

Hoechst 33258 stain stock solution	**300µg/ml Hoechst dissolved in ddH_2O**
Hoechst 33258 stain stock solution	1µg/ml Hoechst diluted from stock solution in 1xPBS

3.2.4 Cell lysis solutions

NP-40 lysis buffer	**150mM NaCl, 50mM Tris-HCl (pH 7.6), 1% NP-40, 0.1% SDS, 0.2mM EDTA, 1xPIC, 1xPhosSTOP in ddH_2O.**
BSA (1mg/ml)	1mg BSA in 1ml ddH_2O

3.2.5 Western blot solutions

Ponceau S	**0.1% (w/v) Ponceau, 5 % (v/v) acetic acid in double-distilled water.**
10x Tris buffered saline (TBS)	12.2 g Trisma base, 87.8 g NaCl in 1-liter ddH2O, pH 7.4.
TBS-tween (TBS-T)	0.1% Tween-20 was dissolved in 1x TBS.
Milk-Blocking solution	5% (w/v) non-fat dry milk/bovine serum albumin in 0.2% TBS-T.
BSA-Blocking solution	5% (w/v) non-fat dry milk/bovine serum albumin in 0.2% TBS-T.
DTT	0.5M DTT in ddH_2O

3.2.6 Cell cycle analysis (FACS) solutions

Ethanol	**70% ethanol in ddH_2O**
PI staining solution	50 µg/mL Propidium iodide, 0.1mg/mL RNaseA and 0.05% Triton-X-100 in 1 x PBS.
MUSE Cell cycle kit solutions	According to manufacturer's protocol

3.2.7 Oxidative stress and ROS measurement assay solutions

DCFH-DA containing ROS assay solution	**10mM HEPES buffer, 10mM glucose, 1µM DCFH-DA(dissolved in methanol) in 1xPBS**
HEPES buffer	1M HEPES in ddH_2O (pH=7.5 with KOH)
Muse Oxidative stress kit solutions	According to manufacturer's protocol

3.2.8 Kinase assay solutions

Kinase-Glo reaction buffer	**40mM Tris-HCl pH7.6, 20mM MgCl2, 0.1mg/ml BSA in double-distilled water.**
Kinase-Glo plus reagent buffer	Mix 1 vial Kinase-Glo plus Substrate and 10ml Kinase-Glo plus buffer than take 1ml aliquots. For each reaction 50µl of this buffer is mixed with 50µl sample solution in Kinase-Glo reaction buffer.

3.2.9 *In vivo* animal experiment solutions

Simple syrup	**16g sugar was dissolved and boiled in 9g ddH2O**

3.3. METHODS

3.3.1 Cell culture methods

3.3.1.1 Growth and sub-culturing of cells

Human liver (Huh7, HepG2, Hep3B, PLC, Mahlavu, FOCUS, Snu475, Snu182, Snu387, Snu398, Snu423, Snu449), breast (MCF7) and colon (HCT116) cancer cells were used in this study.. All cell lines except Snu cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Snu cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium. Growth mediums were both containing penicillin/streptomycin, trypsin-EDTA, fetal bovine serum (FBS) and L-glutamine were from GIBCO (Invitrogen, Carlsbad, CA, USA). As the cells reached 70-70% confluency, they were passaged. In order to subculture cells lines, growth medium was aspirated and cells were washed with 1xPBS. Trypsin was used to detach cells from surface. As the cells were seen to start detaching from the surface, they were collected in fresh growth medium and seeded onto new culture dishes. Cells were placed in 37^{o}C humidified 5% CO_2, 95% air conditioning incubators.

3.3.1.2. Cryopreservation of cells

When cells reach log phase in growth medium, they were collected by trypsinization. Cell suspension was then centrifuged 1500rpm 5 min. Cell pellet was then re-suspended in 1ml/cryo freezing medium and placed into crayon tubes. Cell samples were then stored at -20^{o}C for 1 hour and then placed at -80oC. After at least 24h, samples were transferred to liquid nitrogen.

3.3.1.3. Thawing of frozen cells

one vial of cell line was taken our from liquid nitrogen and placed directly into 37oC water until ¾ of it melts. Cell solution was mixed with 5ml fresh growth medium. In order to get rid of DMSO that is present in freezing medium, cell suspension was centrifuged 1200rpm 5min. Cell pellet was re-suspended in fresh growth medium and transferred into cell culture dish.

3.3.2. Sulphorhodamine B (SRB) cytotoxicity assay

Huh7, Mcf7, Hep3B, Snu182, Snu 387, Snu398, Snu423, Snu449 cells were plated 2000cells/well. Snu475, Mahlavu and FOCUS cells were inoculated 1000cells/well. HepG2 and HCT116 were seeded 3000cells/well into 96well plated in 150µl growth medium. After 24h incubation, growth medium was changed and with 150µl fresh medium. Into the first wells, extra 150µl fresh growth medium was added. Then highest desired concentration was applied to the first well and ½ serial dilution was performed using a multichannel pipette. Each and every drug treatment was performed in triplicate. Since all the compounds used in this research was dissolved in DMSO, negative control treatments were performed using DMSO. One of the plates was not treated with any compounds but instead fixed at the time of drug treatment to be considered as time zero plate to assess the initial cell density. The other plates were incubated for desired time intervals (First screenings were always done for 72h incubation period). In order to fix the cells, medium was discarded and plates were washed twice with 1xPBS. Then 50µl 10%TCA solution was applied to each well and dark-incubated at +4^{o}C for 1hr. In order to remove TCA, cells were then washed with ddH_2O about 4-5 times and left air-dry at room temperature. 50µl 0.4% SRB solution in 1%acetic acid was placed into each well in order to stain the cells and incubated in dark at room temperature for 10min. Finally excess dye was discarded by washing off by 1%acetic acid 4-5 times until no dye comes out and left air dry at room temperature. Lastly, 100µl 1omM cold TBS solution was put on to each well and SRB was solubilized. Absorbance values were measured at 515nm with microplate reader. In order to calculate IC50 values, OD values of compounds were normalized to DMSO controls and time-zero values[108].

%growth inhibition+ (1-(AVARAGE(OD_{Drug})/Avarage(OD_{DMSO})))*100

y-int=INDEX(LINEST(C6:K6,LN(C5:K5),TRUE,FALSE),2)

slope=INDEX(LINEST(C6:K6,LN(C5:K5),TRUE,FALSE),1)

R^2=INDEX(LINEST(C6:K6,LN(C5:K5),TRUE,FALSE),3)

IC_{50}=EXP((50-y-int)/slope

3.3.3. Real-time cell electronic sensing (RT-CES) system for cell growth and cytotoxicity analysis

First, in order to eliminate background noise, 50µl growth medium was put into each well of 96-well E-plate. Plate was then placed into the machine and background reading (1min) was performed. Then Huh7 (2000cell/well) and Mahlavu (1000cells/well) cells were seeded into E-plate in 100µl growth medium. For 24h, in every 30 min, proliferation was monitored and cell index (CI) values were collected. At the end of 24h, old medium was discarded and fresh 100µl growth medium was placed. Additional 100µl growth medium was added to the first wells and molecules at highest indicated concentrations were applied to the first wells then ½ serial dilution was performed using multichannel pipette as in SRB assay. For the first 4h, CI values were detected every 10 min to assess fast drug response. Then data were collected in every 30 min to observe the long-term drug response. CI values are depicted from impedance measurements and reflect cell growth. As the cells adhere at the bottom of the wells that contains electrodes, electricity is insulated and therefore CI values increase as cell growth. The growth inhibitory effect of the molecules were calculated as (CI_{Drug}/CI_{DMSO})

3.3.4. Crude total protein extraction from cultured cells

Huh7 (300,000cell/100mm dish) and Mahlavu (100,000cell/100mm dish) liver cancer cell lines were inoculated into 100mm dishes. 24h later, medium was aspirated and the cells were washed with 1xPBS twice. Then 6ml fresh growth medium was placed into dishes together with the desired concentration of the selected compound or DMSO control. After incubation for a desired time period, treatment was terminated by first collecting the medium that contains swimming cells into a falcon tube. Then cells were washed with 1xPBS (PBS was also collected in the same falcon with medium). Then trypsinization was performed to detach the adherent cells left in the plate. Cells detaching from the plate were then collected with fresh medium and placed into the same falcon tube. If a protein such as a membrane protein will be analyzed, trypsinization was not performed since it can disrupt the protein but instead the cells were collected using scrapers. In case of scraping, after collecting the growth medium containing swimming cells in falcon,

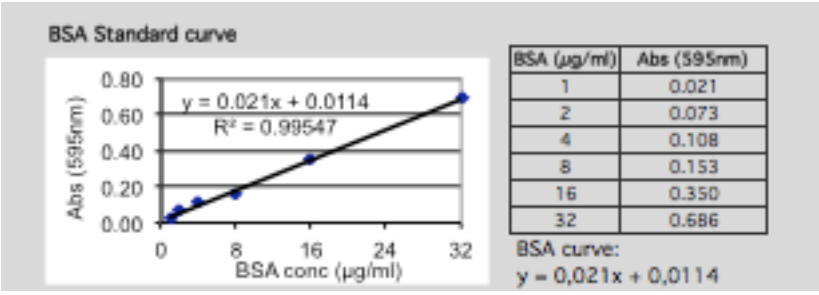
1x ice cold PBS was placed on the adherent cells. Then using the scraper, cells were detached from the plate surface and collected in the same falcon. Then both tyrpsinized and scraped samples were centrifuged at 2000rpm 8 min +4^{o}C. Supernatant was discarded and cell pellet was re-suspended in 5ml ice-cold 1xPBS and centrifuged again at 2000rpm 8 min +4^{o}C. Again supernatant was discarded and cell pellets were frozen in liquid nitrogen and stored at -80^{o}C.

For the lysis of the cell pellets, lysis buffer was placed on the samples twice the amount of the pellet volume and incubated on ice for 30min by vortexing in every 5min (At the end of 30min, if samples were viscous, they were sonicated in order to shear DNA). Samples were then centrifuged at 13000rpm for 20min at +4^{o}C. Proteins were in supernatant and therefore the supernatants were transferred into new eppendorfs and stored at -20^{o}C.

3.3.5. Western Blotting

Bradford assay was used in order to assess the protein concentration in the samples. Bovine serum albumin (BSA) with known concentrations as stated below was used first in order to produce standard curve.

	Blank						
BSA (1mg/mL)	-	1µL	2 µL	4 µL	8 µL	16 µL	32 µL
ddH_2O	100 µL	99 µL	98 µL	96 µL	92 µL	84 µL	68 µL
Bradford Reagent	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL



In order to calculate the protein concentrations of the samples, 2µl of each sample was mixed with 98µl ddH_2O. Finally 900µl Bradford reagent was added and absorbance was measured at 595nm with spectrophotometer. Then the protein concentrations of the samples were normalized according to the standard curve.

Protein samples were arranged by mixing 25µg of protein with 4xNuPAGE loading dye and 25mM DTT in ddH_2O to a final volume of 12µl. Prepared samples were than heated at 70^oC for 10 min in order to denature the proteins and then incubated on ice for 30sec. Then the samples were spinned down and loaded into the gels.

Novex NuPAGE Bis-Tris Electrophoresis system was used for the western blot experiments according to the manufacturer's protocol. The percentage of the pre-cast gel and the running buffer used (MOPS or MES) was determined according to the size of the protein (kDa) that will be investigated in the experiment. After electrophoresis, proteins were then transferred to nitrocellulose membrane by using XCell II^{TM} Blot nodule by applying 30V for 90min. Ponceau staining was then performed for the confirmation of transfer efficiency. Then, the membrane was blocked with either 5%milk or 5%BSA in 1xTBS-T(0.1%) overnight at +4^oC on a shaker. Then the membrane was incubated with desired antibodies for either 2hours at room temperature or overnight at +4^oC on shaker. Then membrane was washed with 1xTBS-T(0.1%) for 30min (5min, 10min, 10min, 5min intervals) in order to remove unbound antibody. Then secondary antibody was applied for 1hour at room temperature on shaker. Membrane was once more washed with 1xTBS-T(0.1%) for 30min (5min, 10min, 10min, 5min intervals) and ECL prime kit was used for visualization with chemiluminescence according to the manufacturer's protocol. X-ray film was used for detection.

3.3.6. Cell cycle distribution analysis with flow cytometry

Huh7 and Mahlavu cell lines were cultured into 100mm culture dishes. 23h later, growth medium was aspirated and cells were washed twice with 1xPBS and fresh medium containing 1xFBS was placed in order to achieve starvation conditions to synchronize the cells. After an additional 24h, fresh growth medium containing desired molecule or DMSO control was replaced. After a desired time period,

medium containing swimming cells was gathered in falcon tube. Adherent cells left were first washed with 1xPBS and then collected in the same falcon tube by trypsinization. Cell suspension was then centrifuged at 2000rpm 8 min. Supernatant was discarded and cell pellet was re-suspended in 5ml 1xPBS. Samples were again centrifuged at 2000rpm 8 min. Finally, supernatant was discarded and 50µl 1xPBS was added per every $1x10^6$ cells. Then 1ml ice cold 70% ethanol was added while vortexing at medium speed and samples were kept at least 3hours at -20^oC. Then the samples were stained with propidium iodide according to manufacturer's protocol of MUSE Cell cycle kit (MCH100106, Millipore) and analyzed.

3.3.7. Immunofluorescence

Huh7 (70,000cell/well) and Mahlavu (30,000cell/well) liver cancer cells were cultured in 6-well plates on coverslips. 23h later, growth medium was replaced with fresh medium containing the desired molecule or DMSO control at desired concentration. After 72h of incubation, cells were washed twice with ice-cold 1xPBS. In order to permabilize the cells, 100% ice cold methanol was used for 10min. Then samples were washed once with ice-cold 1xPBS and stained with Hoechst stain for 5min, dark. Then excess stain was discarded by washing with ddH_2O for 10min on shaker. Coverslips containing the samples were than transferred onto slides by glycerol as mounting medium. Stained cells were then observed under fluorescence microscope and pictures were captured using ZEISS AxioCam MRc5 camera.

3.3.8. Oxidative Stress detection assays

3.3.8.1. DCFH-DA staining procedure

Huh7 and Mahlavu liver cancer cells were cultured into 6-well plates. One day later, growth medium was discarded and cells were washed twice with 1x PBS. Then fresh growth medium containing the molecule of interest or DMSO control at desired concentration was administered. For positive control, selenium deficient serum free medium was administrated in to the corresponding wells [109]. After a determined period of time, wells were washed three times with 1xPBS and incubated with 1ml

ROS assay solution for 15min in humidified 37^{o}C incubator. Then, DCFH-DA containing ROS assay solution was discarded and wells were washes twice with 1xPBS. DCFH-DA is a cell permeable and non-fluorescent probe but then oxidizes to DCF, which is fluorescent in case of ROS production and oxidative stress induction [110]. Therefore, cells were observed under fluorescent microscope in order to detect presence of fluorescence.

3.3.8.2. MUSE Oxidative Stress detection kit

Huh7 and Mahlavu cells (100,000-300,000 cells/dish) were cultured into 100mm culture dish for 24hr. Samples were washed twice with 1xPBS and then desired compounds or DMSO control was administered in fresh growth medium. In addition, one group of cells was grown in selenium deficient serum-free medium as positive control. After desired time periods, cells were collected by trypsinization and prepared according to MUSE Oxidative Stress Kit (MCH100111, Millipore) and analyzed.

3.3.9. Kinase Assay

According to Bradford assay, the determined amount of cell lysate were determined (25µg) and mixed with kinase reaction buffer (final volume will be 50µl) in 96-well polypropylene assay plate. Then, 50µl of Kinase GLO plus reagent was added to the solution and mixed (Any bubbles must be eliminated at this step since they will disrupt the absorbance detection). The sample mixture was then incubated at room temperature, dark for 10 min. Luminescence was then detected by luminometer. If kinase activity is diminished, ATP concentration will increase and so will the luminescence. Staurosporine was used as positive control and DMSO as negative control.

3.3.10. COX Activity Assay

Huh7 and Mahlavu human cancer cells wre treated with compounds and incubated for 48hours. Cell pellet was collected at the end of 48hours and lysis procedure was performed. 30ng protein was used per well. Cyclogenase Activity Assay Kit (Fluorometric) (BioVision) was used according to the manufacturer's protocol.

3.3.11. In vivo tumor xenografts

Mahlavu cells were prepared as 10,000,000 cells in 200µl DMEM and injected subcutaneously into nude mice. Mice were divided into two major and four minor groups. Major groups were chemoprevention and chemotherapy groups and within each group there is one drug taking and one control. In chemoprevention group, 6mg/kg Lanatoside C in 100µl simple syrup (3 mice) or only simple syrup for negative control (2 mice) was orally administered synchronously as the time of Mahlavu injection. Then drug therapy was repeated twice a week for 90days. Tumor volume was measured twice a week with vernier caliper. In chemotherapy group, Mahlavu cells were injected. When the tumor volume reached ~150mm3, drug treatment was initiated. Control group mice (5 mice) received 100µl simple syrup whereas drug-taking mice (8 mice) received 6mg/kg Lanatoside C in 100µl simple syrup 5 days/week for 21 days. Tumor dimensions were again measured twice a week. At the end of 21 days, mice were taken to MRI (acquired with 3-TESLA Siemens MAGNETOM Trio, UMRAM Center, Bilkent University).

CHAPTER 4. RESULTS

4.1. Cardiac glycosides

4.1.1. Previous data on cardiac glycosides extracted from Digitalis Ferruginea

Six glycosides purified from *Digitalis Ferruginea* by Prof. Ihsan Calis were previously examined and three molecules were shown to have high cytotoxic activities on liver cancer cells [107]. While cardiac glycosides Lanatoside A, Lanatoside C, and Glucogitoroside were bioactive, Phenylpropanoid glycosides had no cytotoxic effect. In that thesis, the effects of cardiac glycosides were confirmed with RT-CES assay. Then Hoechst staining and PARP cleavage analysis revealed cell death induction by these three molecules on liver cancer cells. Finally FACS analysis revealed that G2/M cell cycle arrest was established by these cardiac glycosides [107].

Among these three cardiac glycosides, Lanatoside C is preferably used in clinics. Therefore, detailed molecular mode of action analyses performed using Lanatoside C for the sake of versatility during PhD thesis study.

4.1.2. Comparison of cytotoxicities of commercial and purified Lanatoside C

In order to conduct detailed mechanistic and *in vivo* experiments, the required amount of Lanatoside C was more than the amount of Lanatoside C purified from *D. Ferruginea*. Therefore, we decided to continue with commercially available Lanatoside C.

We first compared the cytotoxic activities of the Lanatoside C with the purified from *D. Ferruginea* [36, 37]. The commercial Lanatoside C was found to be more cytotoxic than the purified Lanatoside C from *D. Ferruginea* (Figure 4.1). Thus, we proceeded our experiments with the commercial Lanatoside C.

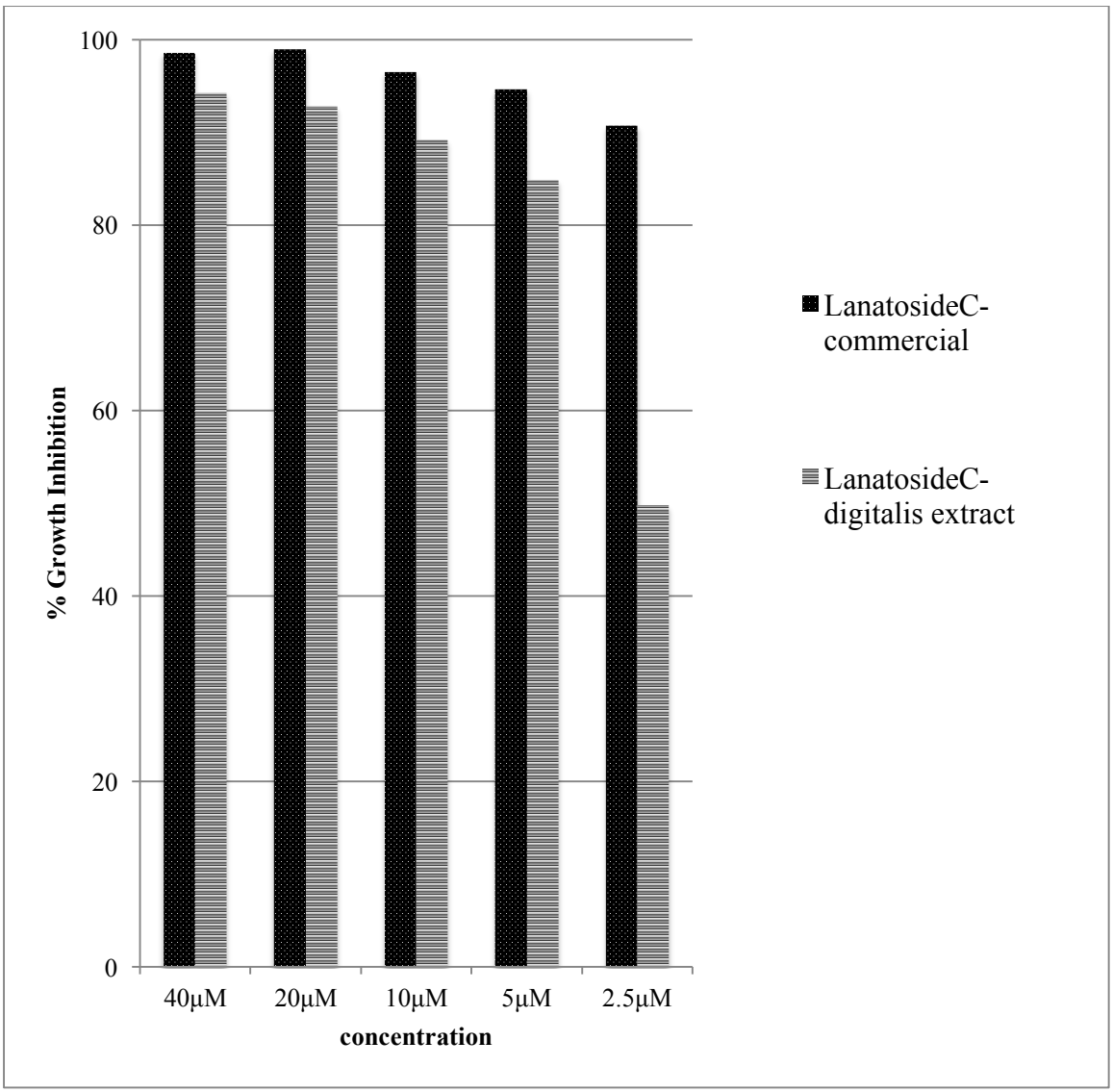


Figure 4.1: Cytotoxic effect of commercial Lanatoside C (Sigma, Aldrich) was compared with Lanatoside C from *D. ferruginea* on human liver cancer (Mahlavu) cells by RT-CES assay. IC_{50} of commercial Lanatoside C was observed to be higher at various time points.

4.1.3. Oxidative stress induction by Lanatoside C

Bearing in mind the previous findings in the literature that cardiac glycosides induce oxidative stress [111] together with the fact that ROS accumulation in oxidative stress can result in G2/M arrest and eventually apoptosis [112], we investigated whether Lanatoside C caused oxidative induction and ROS accumulation in liver cancer cells. First, MUSE Oxidative Stress Kit as used to detect ROS (+) cell concentration in liver cancer (Huh7 and Mahlavu) cell solutions treated with 2μM Lanatoside C or DMSO controls for 6 and 12 hours. During this experiment some liver cancer cells was grown in selenium deficient serum free medium for positive control [113]. Lanatoside C showed 40% ROS (+) cell accumulation similar to the positive control, whereas in DMSO controls ROS (+) cell percentage was very low (Figure 4.2A). Moreover, as a confirmation for this assay, DCFH-DA method was used. Huh7 and Mahlavu cells were treated again with 2μM Lanatoside C or DMSO controls for 6 and 12 hours. Also serum-free selenium deficient medium was used as a positive control. At the end of the incubation times, DCFH-DA dye was applied to the cells. In the presence of oxidative stress, DCFH-DA non-fluorescent dye is oxidized to DF, which is a green fluorescent. Fluorescent microscopy images confirmed that Lanatoside C induced oxidative stress in human liver cancer cells (Figure 4.2B)

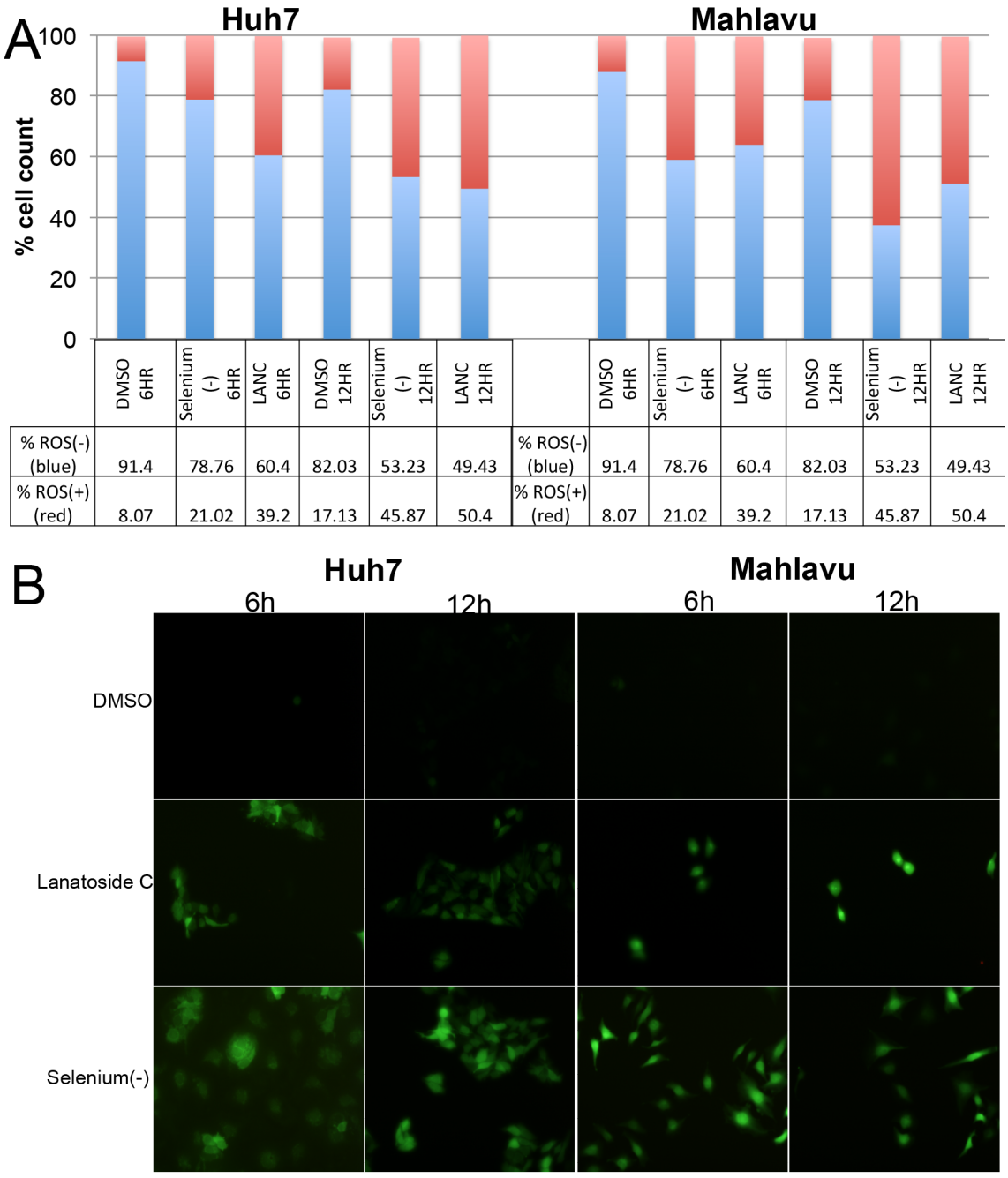


Figure 4.2: Lanatoside C induced oxidative stress in human liver cancer cells. Huh7 and Mahlavu cells were either treated with 2µM Lanatoside C or DMSO or grown in selenium deficient serum free medium for 6 and 12 hours. At the termination time the cell samples were (A) prepared according to the MUSE Oxidative Stress Kit protocols and solutions or (B) stained with DCFH-DA dye and observed under fluorescent microscope (20X).

4.1.4. Cellular Pathways targeted by Lanatoside C

4.1.4.1 Lanatoside C stimulates c-Jun N-terminal kinase phosphorylation

Initially we investigated the molecular mechanism of action of Lanatoside C, the presence of its well-described target, Na^+/K^+,ATPase in liver cancer cells Huh7 and Mahlavu. It was observed that the protein was expressed in both of the cell lines (Figure 4.3 and 4.4).

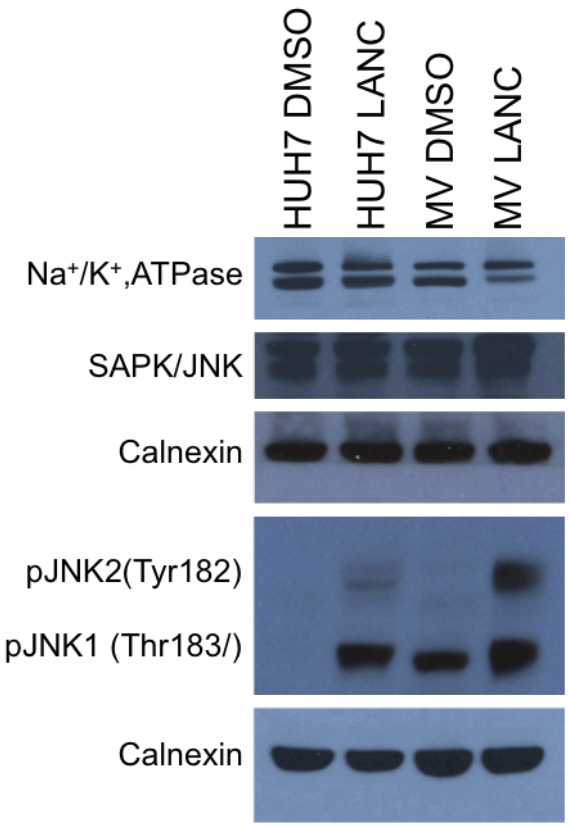


Figure 4.3. Lanatoside C induces phosphorylation of JNK. Huh7 and Mahlavu cells were treated with 2μM Lanatoside C or DMSO for 24hours. Western blot method was applied to investigate the change in the protein levels. Calnexin was used as equal loading.

Previous studies described that c-Jun N-terminal kinase (JNK) and extracellular signal-related kinase (ERK) pathways had players of oxidative stress [114, 115]. On the other hand, phosphorylation of JNK had been shown to provide a critical role in G2/M arrest [116]. Thus, we investigated the changes of the levels of both phosphorylated and unphosphorylated forms of JNK2/JNK1 proteins. PTEN adequate epithelial like Huh7 and PTEN deficient mesenchymal like Mahlavu cells were treated with 2μM Lanatoside C or DMSO control for 24 hours and change in protein levels were investigated by western blot. Lanatoside C treatment resulted in significant increase in phosphorylation of only JNK1 in Huh7 whereas in Mahlavu both JNK1 and JNK2 phosphorylation levels were dramatically increased (Figure 4.3).

4.1.4.2 Lanatoside C increased ERK1/2 phosphorylation

Since ERK pathway was also shown to be involved in oxidative stress, we also examined the levels of ERK1/2 and phospho-ERK1/2 (Thr202, Tyr204). Lanatoside C resulted in significant increase in the levels of phosphorylated ERK1/2 in Huh7 cells. On the other hand in Mahlavu cells, there was no significant change in phosphorylation levels due to the existing high levels of intrinsic pERK1/2 even in DMSO controls (Figure 4.5A).

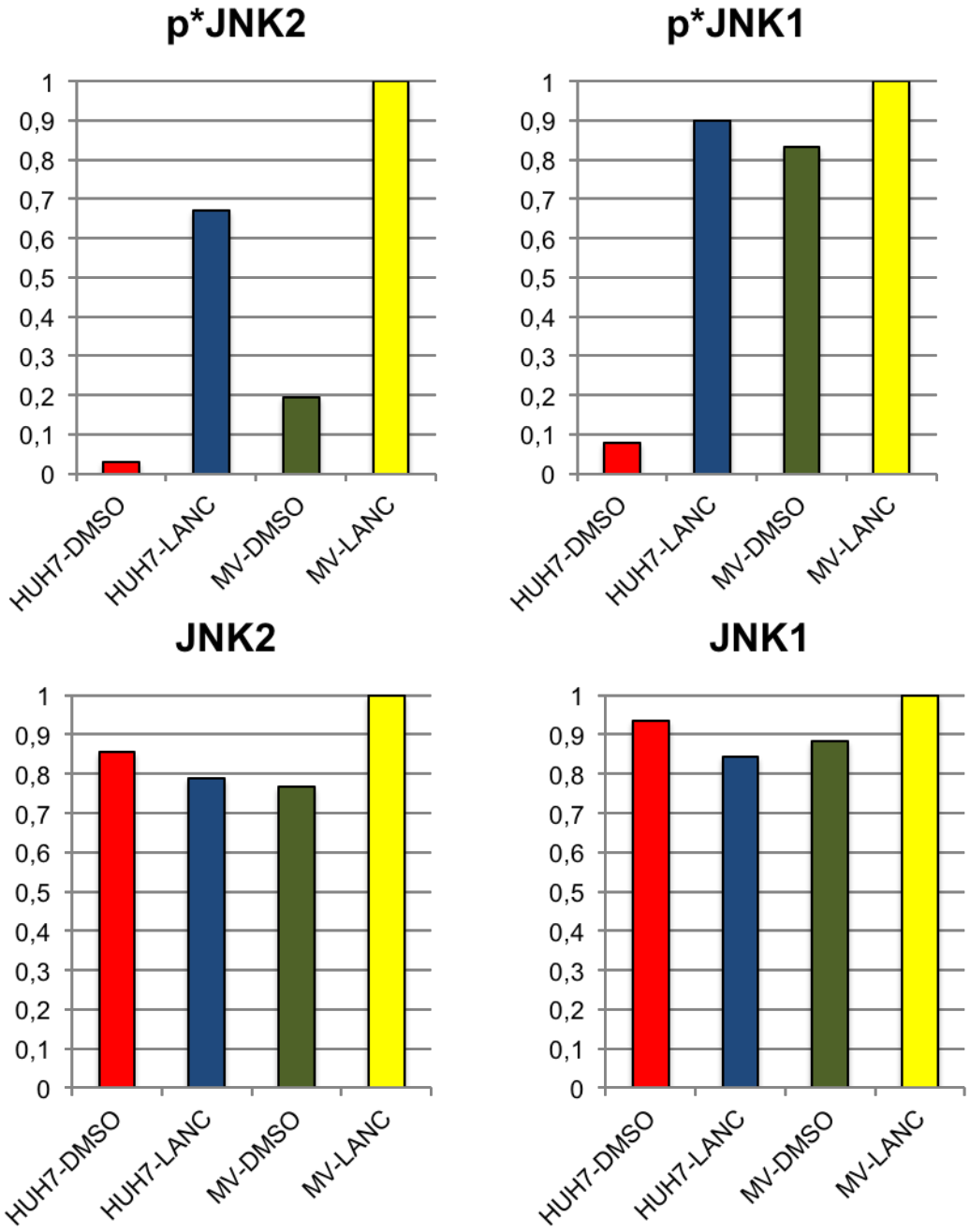


Figure 4.4. Quantification of Western Blot Analysis. Band intensities were measured using ImageJ software. The intensities were normalized to equal loading control Calnexin and then to '1'.

4.1.4.3 Differential effect of Lanatoside C on GSKα/β and Akt pathways differently in two different cells

ROS induction was previously shown to result in inhibition of Akt protein [114, 117]. It was also suggested that this activated pathways can result in increase in GSKα/β phosphorylation [118, 119]. Therefore, as a next step, the levels of GSKα/β and Akt proteins were investigated with their phosphorylation status in the presence of Lanatoside C. In PTEN deficient Mahlavu cells, Akt was shown to be hyperactive [105, 120]. Thus, we also observed high levels of pAkt in DMSO control of Mahlavu cells. On the other hand no band was observed in case of Huh7 (Figure 4.5B and 4.6). Administration of Lanatoside C into Mahlavu liver cancer cells resulted in inactivation of Akt by decreasing its phosphorylation (Figure 4.5B and 4.6). In addition, GSKα/β phosphorylation, the downstream elemen of Akt pathway, was decreased resulting in activation of this protein (Figure 4.5A and 4.6). Instead, in Huh7 cells, Lanatoside C treatment resulted in increase in the phosphorylation of GSKα/β phosphorylation and thereby its inactivation (Figure 4.5A, 4.6 and 4.7).

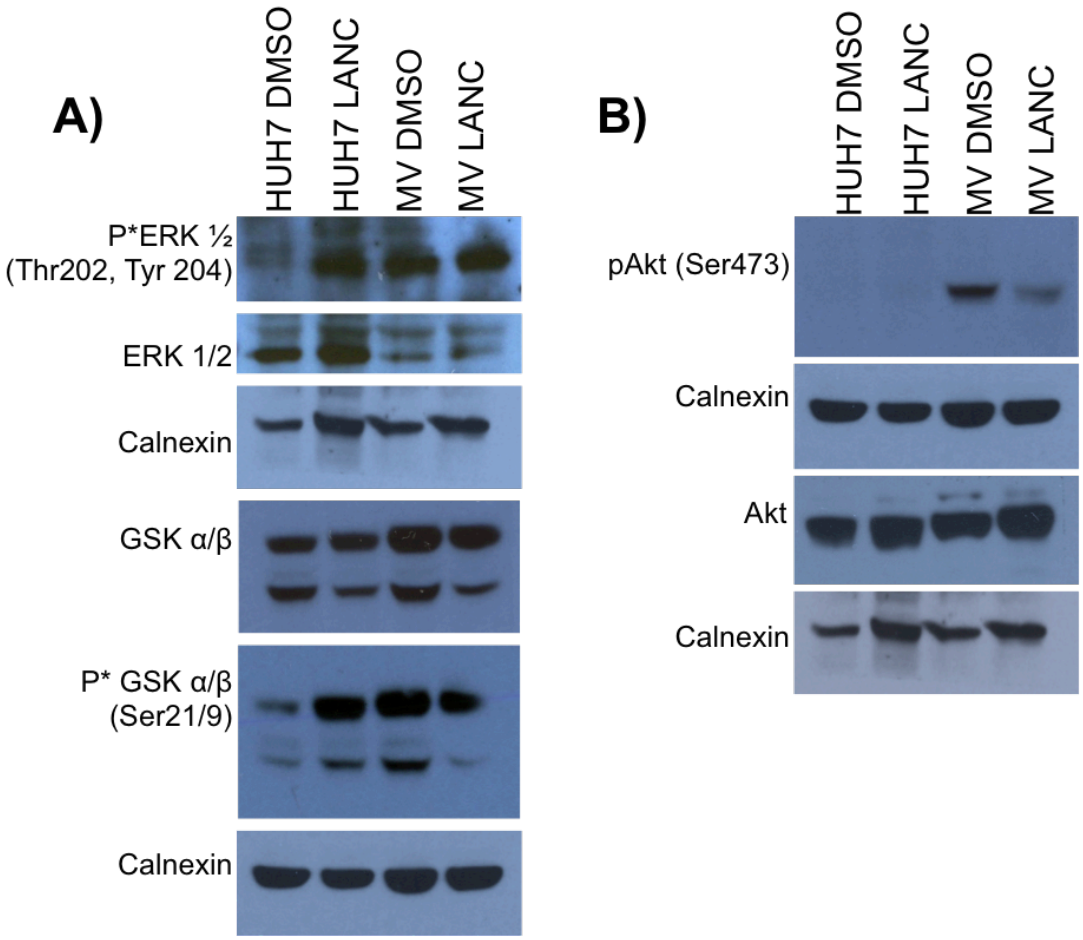


Figure 4.5. Effects of Lanatoside C on ERK, GSKα/β and Akt phosphorylation. Huh7 and Mahlavu cells were treated with 2μM Lanatoside C or DMSO for 24hours. Lanatoside C differentially altered ERK, GSKα/β and Akt proteins. Calnexin was used as equal loading.

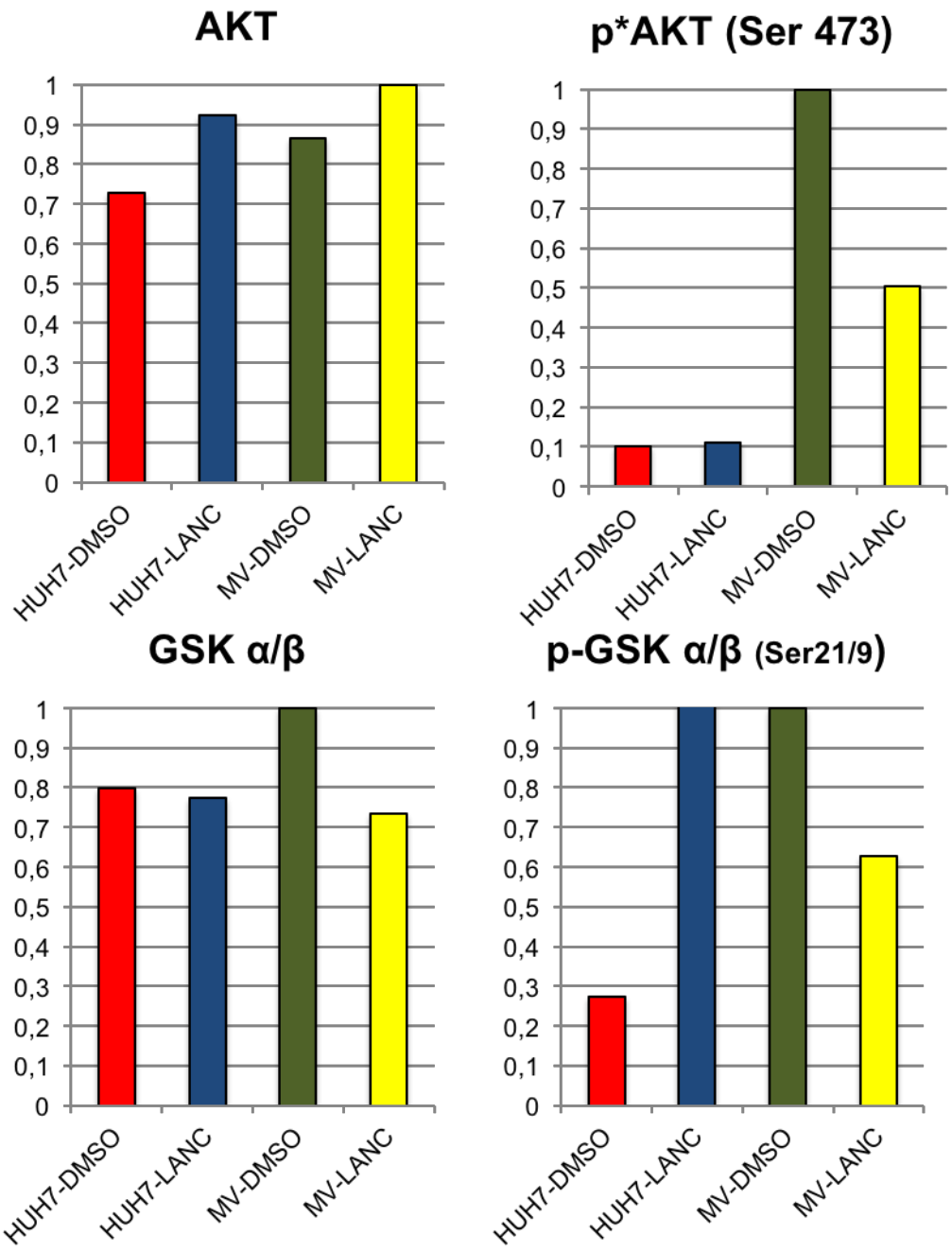


Figure 4.6. Quantification of Western Blot Analysis. Band intensities were measured using ImageJ software. The intensities were normalized to equal loading control Calnexin and then to '1'.

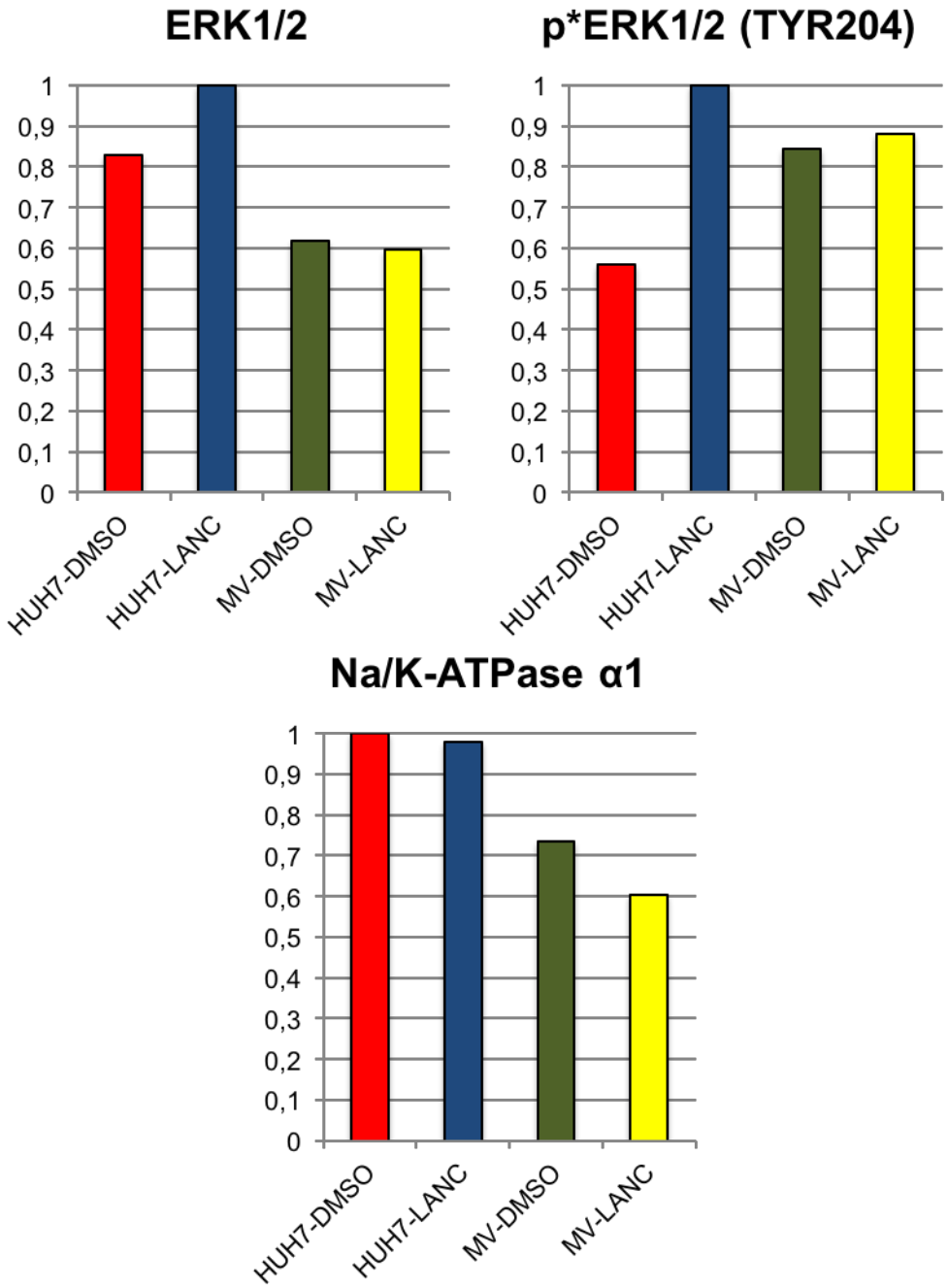


Figure 4.7. Quantification of Western Blot Analysis. Band intensities were measured using ImageJ software. The intensities were normalized to equal loading control Calnexin and then to '1'.

4.1.4.4 Lanatoside C induced extrinsic apoptotic pathway

Cardiac glycosides induced apoptosis was shown in our previous report, however, we did not examine which apoptotic pathway was induced, therefore, in this study we investigated whether extrinsic or intrinsic apoptotic pathway in the presence of Lanatoside C. Western blot analysis revealed that along with the increase in cleaved Poly-ADP-ribosyl-polymerase (PARP) fragments (indicator of apoptosis), Lanatoside C activate cleavage of caspase-3 and caspase-8 (Figure 4.8 and 4.9) but not caspase-9. This observation indicated that Lanatoside C induces apoptosis through extrinsic pathway.

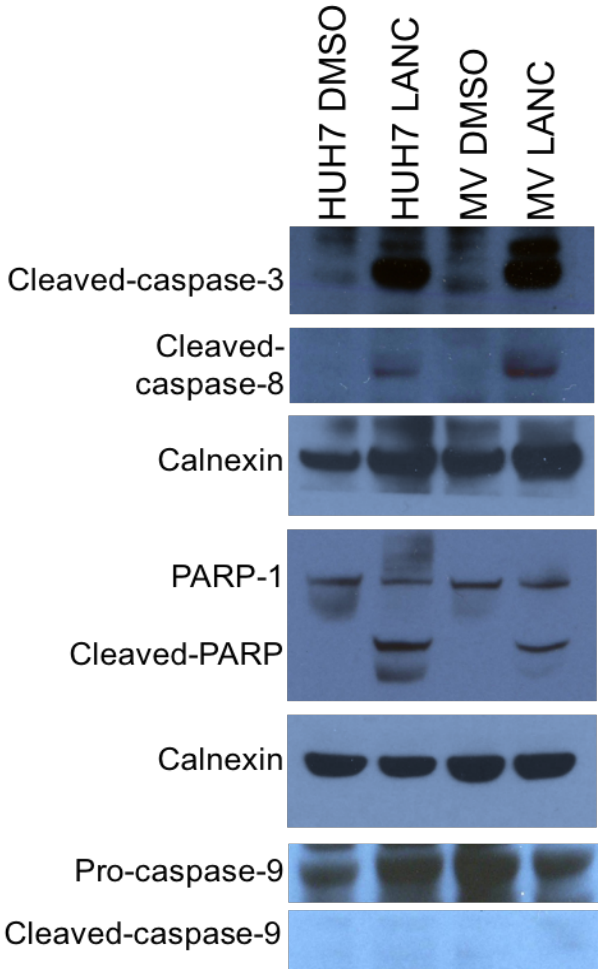


Figure 4.8. Extrinsic apoptotic pathway induced by Lanatoside C. Huh7 and Mahlavu cells were treated with 2µM Lanatoside C or DMSO for 24hours. Lanatoside C resulted in cleavage of PARP, caspase-3 and caspase-8. Calnexin was used as equal loading.

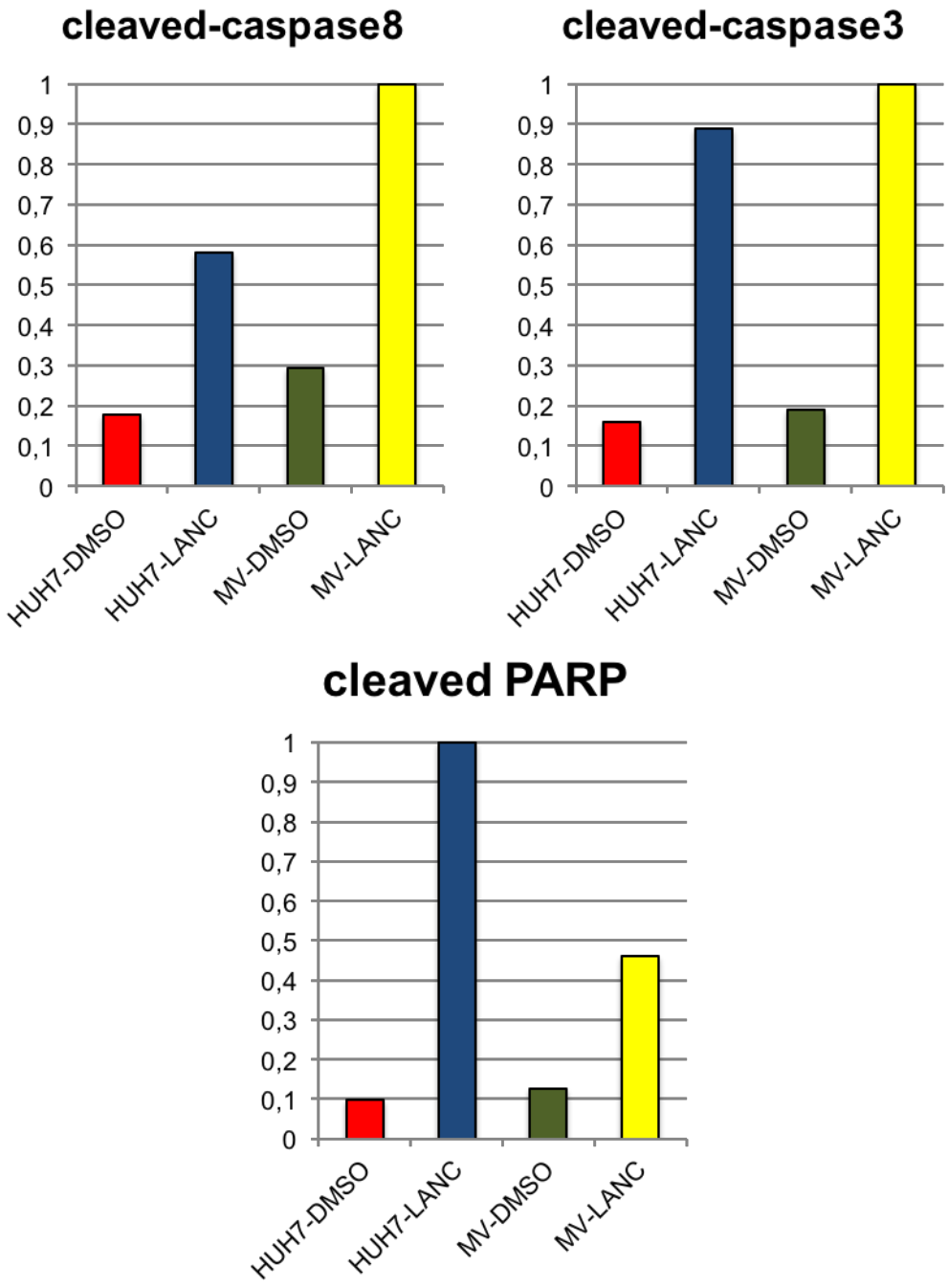


Figure 4.9. Quantification of Western Blot Analysis. Band intensities were measured using ImageJ software. The intensities were normalized to equal loading control Calnexin and then to '1'.

4.1.5 In vivo anti-tumor effects of Lanatoside C on Mahlavu mice xenografts models

Our *in vitro* experiments showed that Lanatoside C induces oxidative stress induced JNK pathway activation which results in extrinsic apoptotic pathway on HCC. In order to investigate the anti-tumor activity of Lanatoside C *in vivo*, immune-deficient athymic nude mice were injected subcutaneously with Mahlavu cells (10,000,000 cells) which are poorly-differentiated and highly metastatic mesenchymal-like liver cancer cells. When the tumor size reached to around 150mm^3, mice were treated with 6mg/kg Lanatoside C orally for 5 days a week for 3 weeks. The tumor size measurements were followed twice a week. At the end of three weeks mice were taken to Bilkent Umram Fascility and, MRI images were acquired. Both the tumor size measurements and MRI images showed reduction tumor size and weight in Lanatoside C taking mice compared to simple syrup control (Figure 4.10). The mean volume detected in control mice was 1581± 266 mm^3 which is around three times the mean tumor volume detected in Lanatoside C taking mice (522±172 mm^3). It was also significant from the MRI images that tumor in vehicle controls is much larger than the Lanatoside C taking mice.

Moreover, data showing the chemotherapeutic activity of Lanatoside C prompt us to investigate the chemopreventive activity of Lanatoside C on nude mice with early treatment. Therefore, in a group of mice, as they received subcutaneous injection of Mahlavu cells, mice were also administered with Lanatoside C or vehicle control synchronously at day zero. Then these mice orally took Lanatoside C twice a week for 90days. Tumor volume was measured twice a week. Observation of mice for 90days revealed that mice Lanatoside C resulted in either reactive tissue response or minor tumor growth in nude mice when compared to vehicle control (Figure 4.11). While the tumor volume reached 1400mm^3 in vehicle control mice whereas the volume of tumors in Lanatoside C taking chemoprevention group stayed around 200-400mm^3 (Figure 4.11).

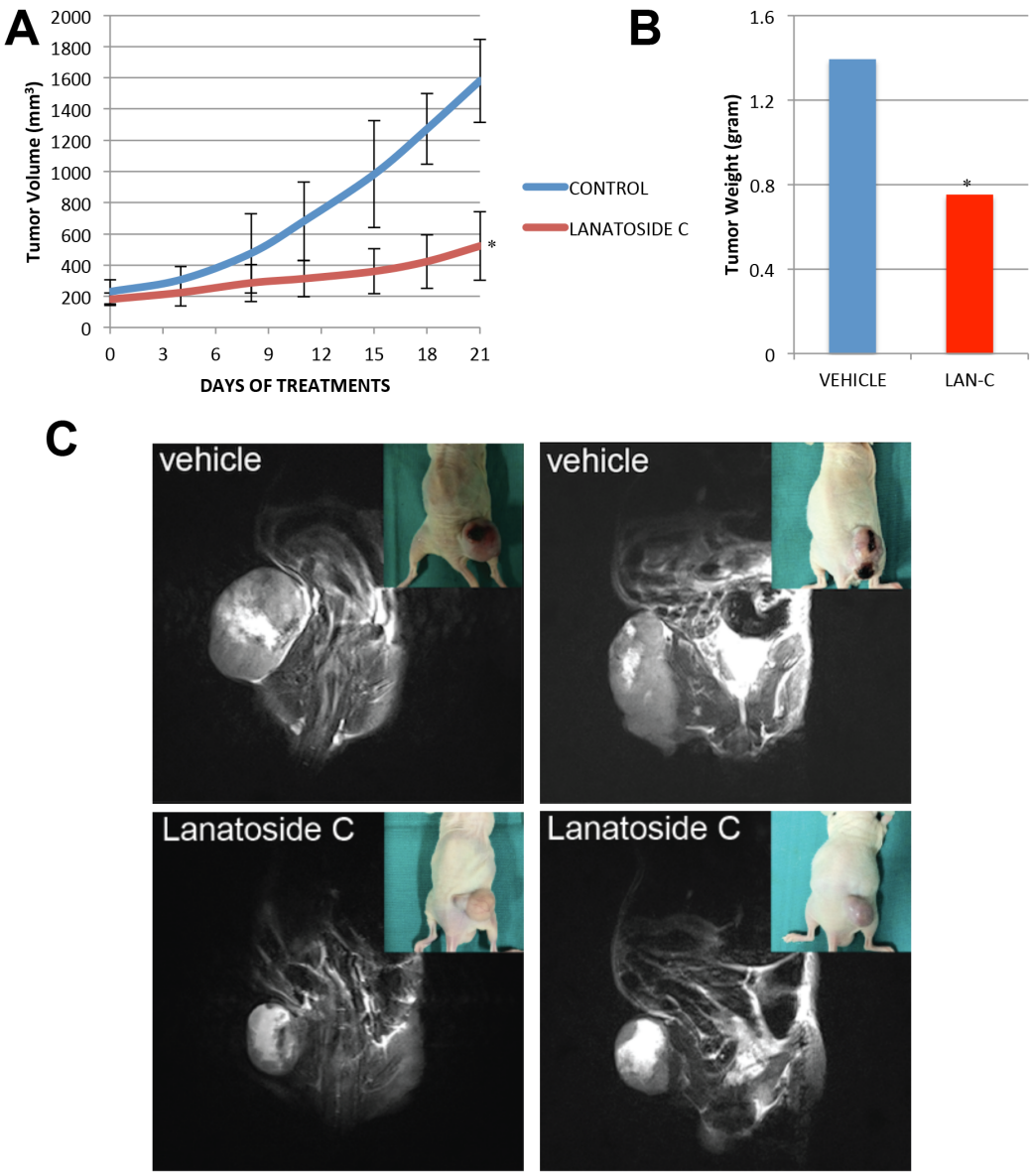


Figure 4.10. *In vivo* anti-tumor activity of Lanatoside C. Nude mice were injected with Mahlavu cells and they were treated with (blue) vehicle control or (red) 6mg/kg Lanatoside C. (A) the average tumor volume calculated from 5 vehicle controls and 8 drug taking mice. (B) Bar-graphs shows average tumor weight (* $p < 0.005$). (C) The MR images.

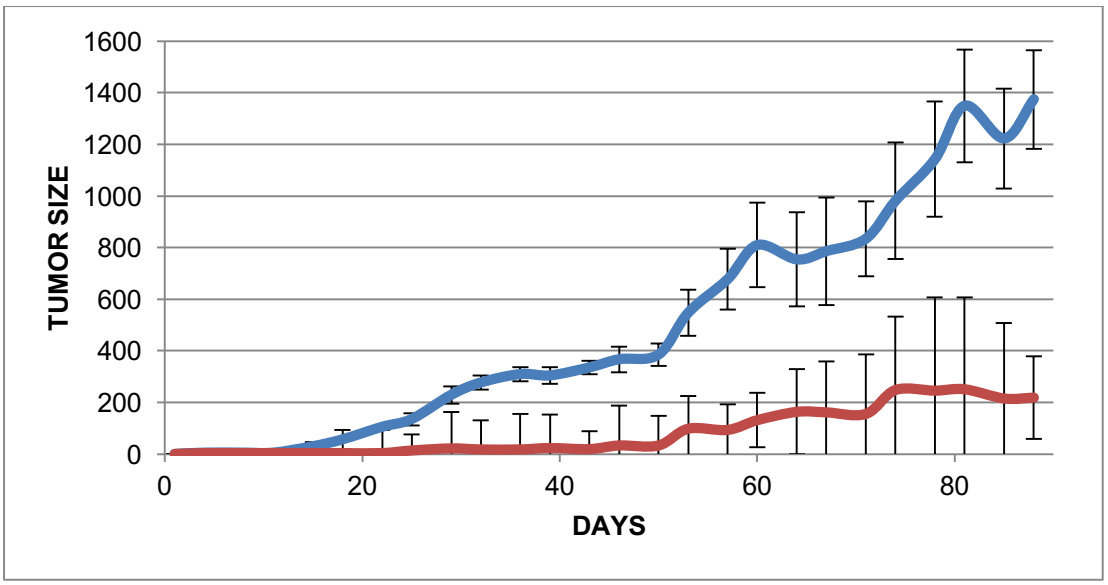


Figure 4.11. In vivo chemopreventive effect of Lanatoside C. Nude mice were injected subcutaneously with Mahlavu cells and treated with Lanatoside C (red) or vehicle control (blue) orally at the same time starting from day zero.

4.1.6 Sorafenib and Lanatoside C has no synergistic effect

Sorafenib is the only FDA drug for advanced hepatocellular cancer treatment[121, 122]. Therefore we questioned if Lanatoside C has a synergistic effect with Sorafenib. RT-CES result together with SRB result showed no combinatorial effect of Lanatoside C and Sorafenib (Table 4.1) .

Table 4.1: Growth inhibition percentages for Lanatoside C and Sprafenib combinational treatment.

		Lanatoside *C*			
		20nM	**10nM**	**5nM**	**0**
Sorafenib	**20µM**	96.58	97.16	96.07	100.00
	10µM	94.91	94.25	94.76	94.69
	5µM	52.18	39.08	41.56	46.29
	2.5µM	5.60	12.23	-8.95	18.92
	0	10.55	14.16	0.00	0.00

4.2. Novel triazolothiadiazine and triazolothiadiazole compounds

The novel triazolothiadiazine and triazolothiadiazole compounds, which were originated from traditional non-steroidal analgesic anti-inflammatory drugs (NSAIDs) (ibuprofen, naproxen, and flurbiprofen), were produced by Birsen Tozkoparan and her student Peri S. Aytac from Hacettepe University Department of Pharmaceutical Chemistry.

4.2.1. Exploration of bioactivities of newly synthesized novel thiadiazine compounds

64 molecules, synthesized by Birsen Tozkoparan, were analyzed for their bioactivities against cancer cells. For the preliminary screening, liver (Huh7), colon (HCT116) and breast (MCF7) cells were treated with 64 molecules in decreasing concentrations from 40μM to 2.5μM for 72hr. NCI-60 SRB assay was performed to assess the cytotoxicity levels of the compounds. The experiments were conducted in duplicates. CPT was used as a general positive control. Moreover, commercially obtained ibuprofen, naproxen and flurbiprofen were used as negative controls. IBUTRI, NAPTRI and FLUTRI are aminomercaptotriazoles obtained from NSAIDs. It was observed that no molecules from naproxen group had significant cytotoxic activity. Therefore, we did not perform any further experiments using any molecules from that group (Table4.3). On the other hand, 4 molecules from ibuprofen derivatives and 6 molecules from flurbiprofen group showed IC_{50} values less than 5.5μM in Huh7 and high cytotoxicity in other cancer types as well (Tables 4.2 and 4.4).

Table 4.2: IC_{50} values of novel compounds derived from Ibuprofen

	HUH7		MCF7		HCT116	
ORIGINAL NAME	**IC_{50}**	R^2	**IC_{50}**	R^2	**IC_{50}**	R^2
IBUTRI-OCF3-TDZ	3.6	0.8	1.1	0.8	3	0.9
IBUTRI-Br-TDZ	4.93	0.96	9.44	0.94	7.11	0.91
IBUTRI-CH3-TDZ	5	0.9	6.3	0.9	4	0.9
IBUOCH3TDZ	5.3	0.9	5.2	0.9	4.3	0.9
IBUTRI-Cl-TDZ	5.65	0.95	8.81	0.93	6.74	0.96
IBUTRI-n-TDZ	6.09	0.82	9.65	0.70	7.56	0.97
IBUTRI-2,4-DICl-TDZ	7.49	0.85	11.77	0.76	11.08	0.84
IBUTRI-F-TDZ	7.76	0.77	8.88	0.78	12.43	0.94
IBUTRI-OCH3-TDZ	8	0.9	7.3	0.9	7.6	0.9
IBUCF3TDZ	8.4	0.9	4.2	0.9	15.2	0.9
IBUTRI-n-TDL	10	0.9	12.2	0.9	18	0.8
IBUTRİ-F-TDL	11.4	0.9	17.2	0.8	28.7	0.8
IBUTRİ-2,4-DİCl-TDL	18.6	0.8	30.9	0.6	44	0.9
IBUTRİ-NO2-TDL	24.30	0.70	9.30	0.80	23.50	0.60
IBUTRİ-Cl-TDL	31.00	0.60	33.70	0.60	74.30	0.70
IBUTRİ-Br-TDL	31.30	0.70	32.90	0.60	NO INH	
IBUTRI-NO2-TDZ	NO INH*		NO INH		NO INH	
IBUTRI-CH3-TDL	10.90	0.90	19.90	0.80	16.50	0.90
IBUTRI-O-CH3-TDL	7.40	0.90	10.50	0.90	8.00	0.90
IBUTRI-CF3-TDL	25.70	0.80	65.30	0.60	NO İNH	
IBUTRI-OCF3-TDL	133.40	0.80	88.40	0.70	43.00	0.90
IBUTRI	67.90	0.21	16.12	0.65	27.39	0.79
ibuprofen	37.2	0.7	52.8	0.8	41.8	0.7
CPT	0.1		<1		<1	

*NO INH: No Inhibition

Table 4.3: IC_{50} values of novel compounds derived from Naproxen

	HUH7		MCF7		HCT116	
ORIGINAL NAME	**IC_{50}**	R^2	**IC_{50}**	R^2	**IC_{50}**	R^2
NAPTRI-CH3-TDZ	8.2	0.9	11	0.9	10.2	0.9
NAPTRI-OCF3-TDZ	16	0.8	8.8	0.9	11.7	0.9
NAPCF3TDZ	14.8	0.9	6.9	0.9	10.5	0.9
NAPOCH3TDZ	8.5	0.9	6.6	0.9	6.5	0.9
NAPTRİ-2,4-DİCl-TDL	53.8	0.5	57.1	0.9	NO INH*	
NAPTRİ-Br-TDL	7.5	1	14.5	1	33.5	0.7
NAPTRI-CF3-TDL	17.3	0.9	21.3	1	22.3	0.7
NAPTRI-CH3-TDL	7.1	0.9	7.6	0.9	6	0.8
NAPTRİ-Cl-TDL	9.6	1	20.9	1	56.7	1
NAPTRİ-F-TDL	10	1	23.9	0.9	NO INH	
NAPTRİ-NO2-TDL	6	0.9	19.4	0.9	51.9	1
NAPTRI-n-TDL	10.4	0.9	12.7	1	NO INH	
NAPTRI-OCF3-TDL	9.1	0.9	17.3	0.9	10.7	0.9
NAPTRI-OCH3-TDL	6.5	0.9	5.8	0.8	11.6	0.8
NAPTRI-n-TDZ	5.7	0.9	11.7	0.9	8.9	0.9
NAPTRI-F-TDZ	7.0	0.8	15.8	0.8	13.1	0.8
NAPTRI-Cl-TDZ	5.6	0.9	10.1	1.0	8.8	0.9
NAPTRI-2,4-DICl-TDZ	10.7	0.5	14.6	0.8	14.0	0.9
NAPTRI-Br-TDZ	9.3	1.0	11.5	0.9	11.7	1.0
NAPTRI-NO2-TDZ	14.7	0.8	18.8	0.9	15.1	0.9
NAPTRI	40.8	0.3	22.3	0.6	20.2	0.8
Naproxen	71	0.7	54.1	0.9	34.6	0.6
CPT	0.1		<1		<1	

*NO INH: No Inhibition

Table 4.4: IC_{50} values of novel compounds derived from flurbiprofen

	HUH7		MCF7		HCT116	
ORIGINAL NAME	**IC_{50}**	R^2	**IC_{50}**	R^2	**IC_{50}**	R^2
FLUTRI-2,4-DICl-TDZ	3.7	0.9	7.6	1.0	6.9	0.9
FLUTRI-Cl-TDZ	3.8	0.9	8.7	1.0	7.8	0.8
FLUTRI-NO2-TDZ	4.3	0.9	7.8	0.9	7.1	0.9
FLUOCH3TDZ	4.3	0.8	2.6	0.7	4.4	0.9
FLUTRI-OCH3-TDL	4.4	1	5.9	1	11.8	1
FLUTRI-F-TDZ	4.9	1.0	8.1	0.9	10.5	0.9
FLUTRI-Br-TDZ	5.0	0.9	7.4	1.0	7.1	1.0
FLUTRİ-NO2-TDL	5	1	18	0.9	7.5	1
FLUTRI-n-TDZ	6.7	1.0	7.4	0.9	9.7	0.9
FLUTRI-CH3-TDZ	7.2	0.9	5	0.8	4.8	0.9
FLUTRI-OCF3-TDZ	8.4	0.9	4.8	0.9	5.4	0.9
FLUCF3TDZ	9.4	0.9	4.3	0.9	6.9	0.9
FLUTRI-CF3-TDL	10.3	0.9	5	1	7.8	0.9
FLUTRI-OCF3-TDL	11.7	1	9.4	0.9	11.1	1
FLUTRI-CH3-TDL	29.2	0.9	26.9	0.7	47.8	0.8
FLUTRİ-2,4-DİCl-TDL	33.6	0.8	NO INH		NO INH	
FLUTRİ-Br-TDL	41	0.8	73.1	0.7	NO INH	
FLUTRI-n-TDL	NO INH		27.3	0.9	NO INH	
FLUTRİ-F-TDL	NO INH		40.3	0.9	NO INH	
FLUTRİ-Cl-TDL	NO INH		39.8	1	NO INH	
FLUTRI	NO INH		NO INH		NO INH	
Flurbiprofen	NO INH		NO INH		NO INH	
CPT	0.1		<1		<1	

*NO INH: No Inhibition

11 molecules had significant cytotoxicity, therefore we explored their activities in an enlarged panel of liver cancer cells (Huh7, HepG2, Hep3B, Mahlavu, FOCUS, SNU475). Liver cancer cells were treated with selected compounds (40μM-2.5μM) for 72hr in triplicates and investigated using SRB assay (Table 4.5). The cytotoxic activities of these compounds were further confirmed with RT-CES analysis (Figure 4.12).

Table 4.5: IC_{50} values of novel compounds in enlarged HCC panel

NAME	Huh7	HepG2	Hep3B	MV	FOCUS	SNU475
IBUTRI-OCF3-TDZ	9.5	9.7	6.4	9.1	8.8	6.8
IBUTRI-Br-TDZ	9.4	6.4	8.3	8.9	5.9	10.1
IBUTRI-CH3-TDZ	6.2	7.3	5.3	8.5	6.8	8.7
IBUOCH3TDZ	3.7	3.8	6.5	13.6	2.4	8.5
FLUTRI-2,4-DICl-TDZ	7.3	8.6	4.2	6.6	5.4	4.7
FLUTRI-Cl-TDZ	6.4	6.7	3.8	6.1	5	3.8
FLUTRI-NO2-TDZ	7.1	4.3	2.9	9.4	3.5	7.1
FLUOCH3TDZ	4.5	4.2	3.5	5.8	4.4	6.7
FLUTRI-F-TDZ	6.3	5.1	2.6	7.4	3.5	6.6
FLUTRI-OCH3-TDL	2.8	26.8	4	4	6.7	1.2
FLUTRI-Br-TDZ	8.7	3.7	1.5	6.2	8.5	7
IBUTRI	23	20.6	13.1	16.2	24.8	11.7
FLUTRI	43.1	21.4	89.1	35.8	82.8	146.9

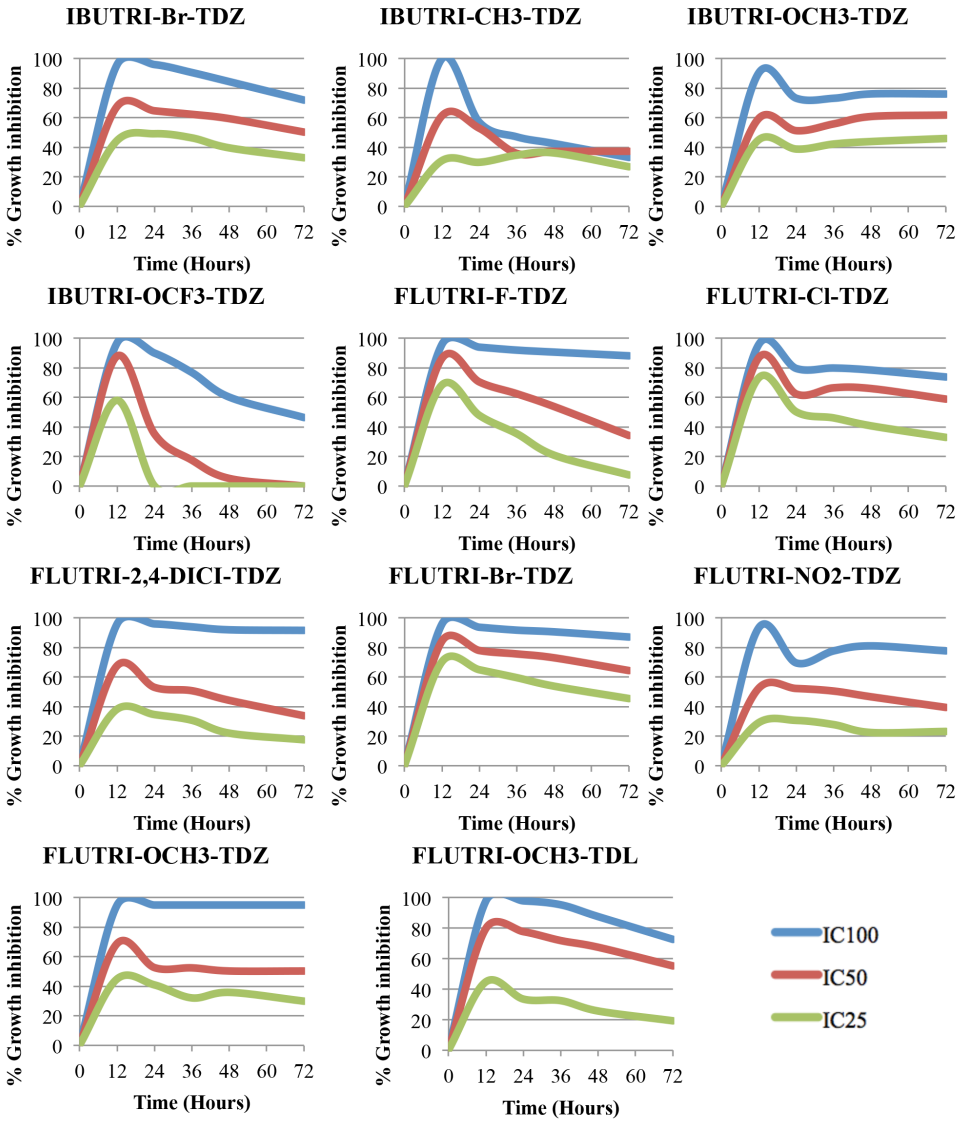


Figure 4.12. Real-time cell growth inhibition. Huh7 cells were treated with the selected compounds or DMSO controls for 72h in increasing concentrations (IC100-blue, IC50-red and IC25-green concentrations from Table4.5). Real-time cell electronic sensing assay was performed in order to confirm the bioactivities of the compounds in real-time. Experiment was performed in triplicate.

4.2.2. Novel thiadiazine derivatives induced nuclear condensation

As a first step in characterization of cell death mechanism, liver cancer cells were treated with 10μM of the selected molecules or DMSO controls for 48hr. Hoechst 33258 stain was performed in order to investigate the changes in the nuclei of the liver cancer cells. When compared to DMSO controls, in some of samples treated with the compounds (IBUTRI-Br-TDZ, IBUTRI-CH3-TDZ, IBUTRI-OCH3-TDZ, IBUTRI-OCF3-TDZ and FLUTRI-Cl-TDZ and FLUTRI-OCH3-TDZ) nuclear condensation and horseshoe structures were observed (Figure 4.13).

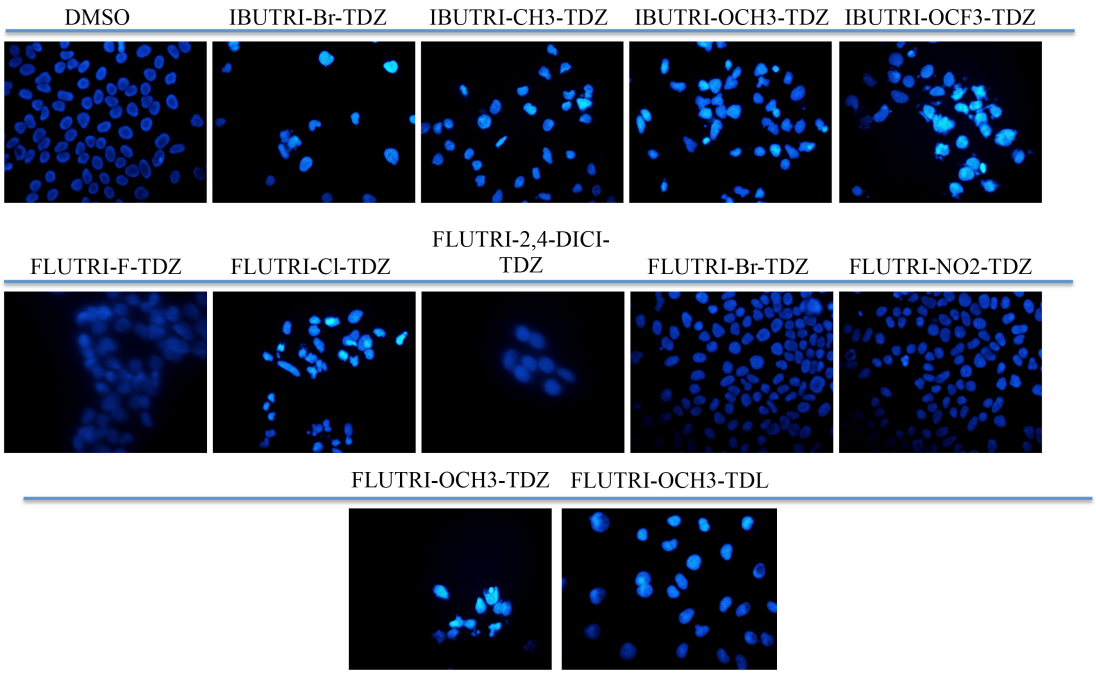


Figure 4.13. Fluorescence nuclei images of liver cancer cells. Huh7 cells were plated on coverslips and treated with 10μM of desired compounds or DMSO control for 48h. Nuclear Hoechst 33258 stain (Sigma) was used to stain the cells. Pictures were taken at 40X objectives.

4.2.3. SubG1/G1 cell cycle arrest is initiated in the presence of novel thiadiazine derivatives

The cell cycle distribution of liver cancer cells (Huh7) in the presence of selected 10 compounds was performed using FACs analysis with propidium iodide stain. Compounds (10µM) or DMSO were applied to Huh7 cells for 24 and 48h periods and stained with DNA-specific fluorescent dye propidium iodide. In 24h, IBUTRI-CH3-TDZ, IBUTRI-OCH3-TDZ, FLUTRI-Cl-TDZ and FUTRI-OCH3-TDZ resulted in significant SubG1/G1 cell cycle arrest. After 48h, IBUTRI-Br-TDZ also resulted in SubG1/G1 cell cycle arrest in liver cancer cells (Figures 4.14 and 4.15).

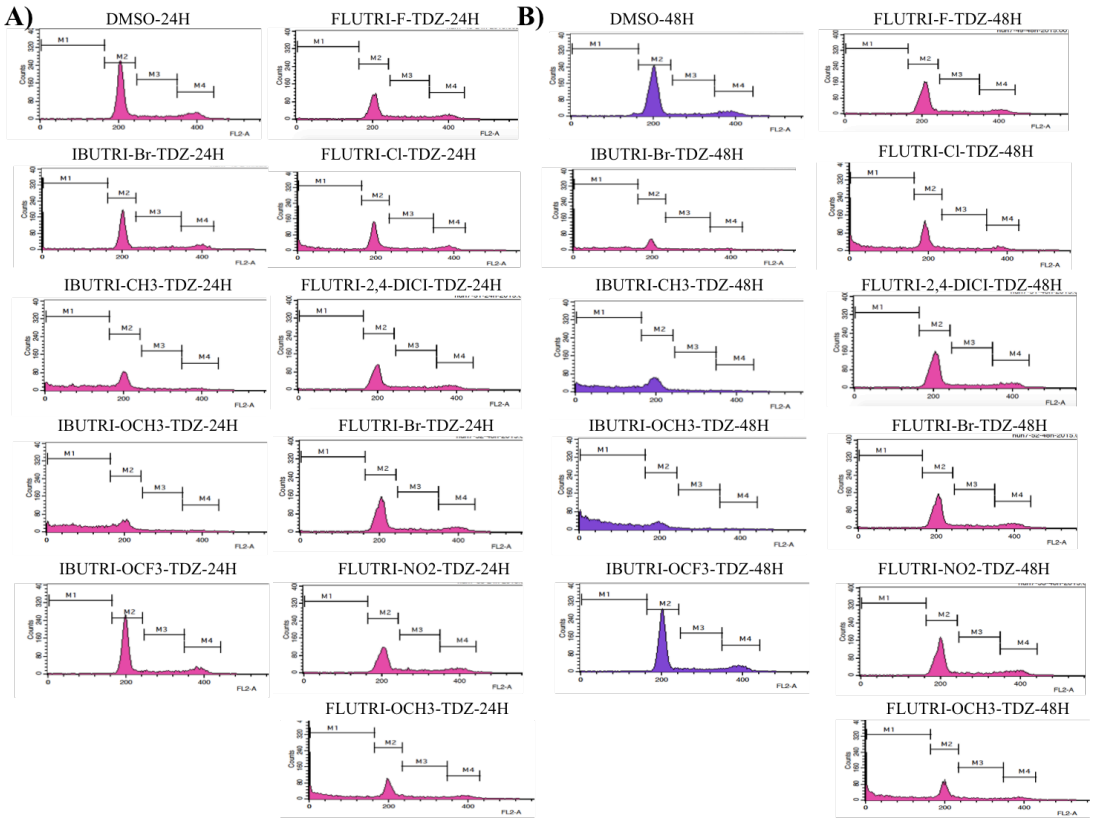


Figure 4.14. Cell cycle distribution analysis. Huh7 cells were treated with the compounds (10µM) or DMSO controls for A) 24h or B) 48h. M1 represents SubG1 phase. The peak at 200FL2-A (M2) represents G1 phase (2N cells). M4, which is the peak at 400 represents 4N cells (G2-phase) and phase in-between is S-phase.

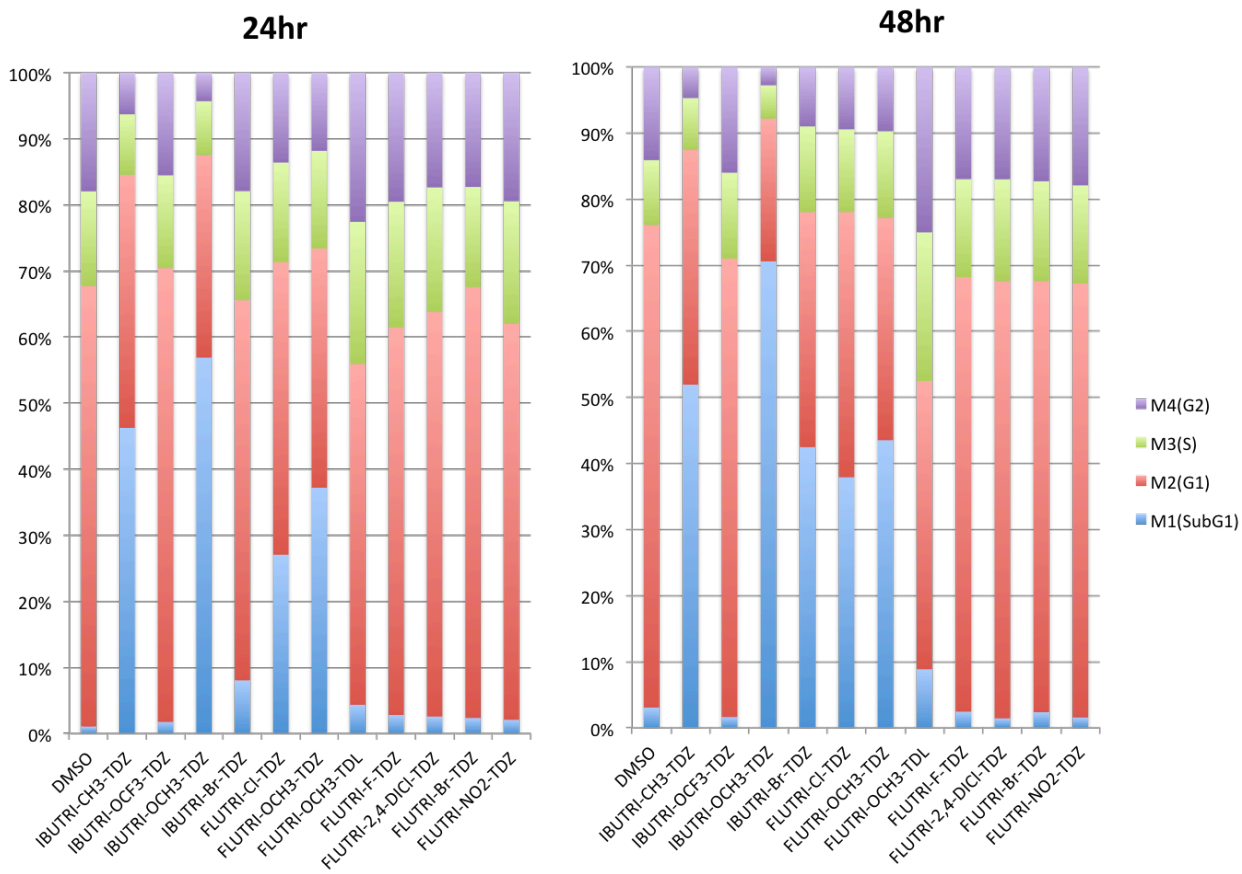


Figure 4.15. Cell cycle distribution analysis.

4.2.4. Thiadiazine Derivatives induced oxidative stress

Our results demonstrated that compounds IBUTRI-OCH3-TDZ and IBUTRI-CH3-TDZ showed the most dramatic effects on liver cancer cells and therefore they were chosen for further research. Bearing in mind the material in literature showing that non-steroid anti-inflammatory drugs induced ROS production and thus oxidative stress [123], we checked the ROS presence in cells treated with the compounds compared to DMSO controls by two different experiments. First, MUSE Oxidative Stress Assay Kit revealed that treatment with the two selected compounds resulted in increase in ROS positive cells similar to the effect of positive control (cells grown in selenium deficient serum free medium) (Figure 4.16). Furthermore, as a confirmation, cells treated with the compounds were stained with DCFH-DA

solution and observed under the fluorescent microscope. This assay also confirmed the induction of oxidative stress by these novel thiadiazine derivatives (Figure 4.17).

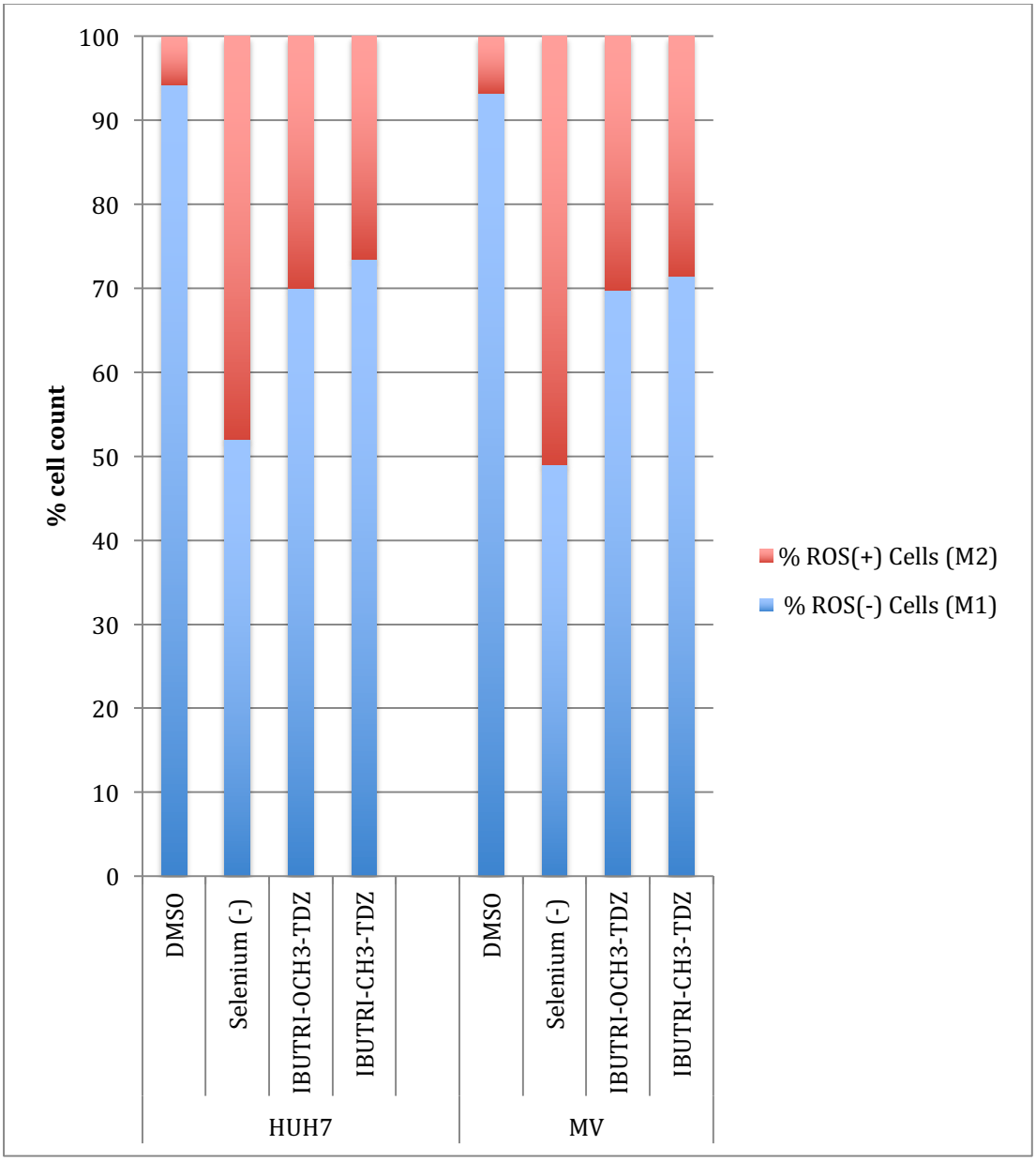


Figure 4.16. Compounds induced increase in ROS (+) cells. Human liver cancer cells (Huh7 and Mahlavu) were treated with 10µM of the selected two compounds or positive (Selenium (-)) and negative (DMSO) controls for 48h then analyzed with MUSE Oxidative Stress Kit.

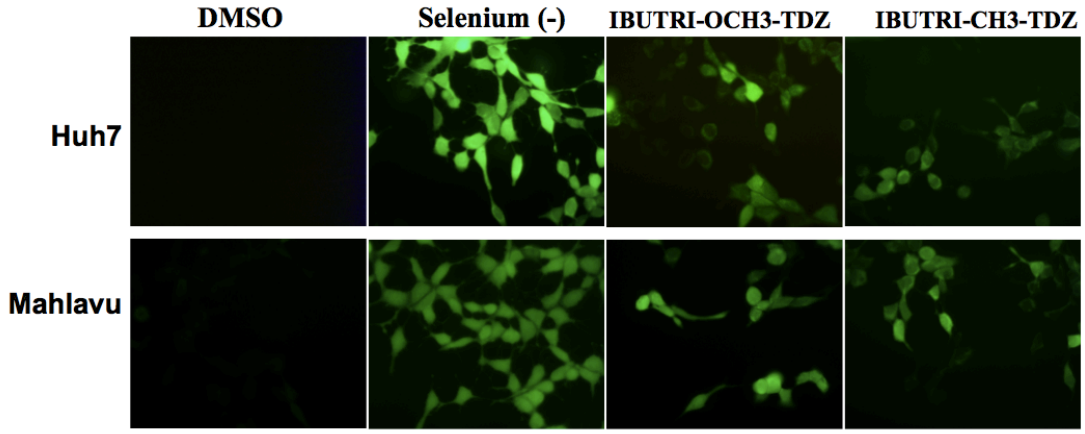


Figure 4.17. DCFH-DA stain of cells treated with thiadiazine derivatives. Liver cancer cells were treated with 10μM of the compounds for 48h and then stained with DCFH-DA. Images were taken under fluorescent microscope (40X objective).

4.2.5. Treatment with the molecules induce phosphorylation of B-catenin and induces apotosis

Considering the fact that ibuprofen acting on β-catenin, we chose to investigate the effect of our compounds on β-catenin and its upstream and downstream components [71, 124]. Our data showed that compounds IBUTRI-OCH3-TDZ and IBUTRI-CH3-TDZ induced increase in levels of phosphorylated β-catenin which means that the proteins induce the degradation of β-catenin. Moreover, the levels of Cyclin E and cdk2 were decreased in treated cells (Figure 4.18). In addition, p-Akt (active form) and p-GSK3β (inhibited form) levels were significantly decreased together with Cyclin D1 (Figure 4.18).

Our previous experiments showed the induction of oxidative stress by these two compounds, but we also confirmed this action at the protein levels as well. Therefore, we investigated the differences in the key factors of oxidative stress pathway: ASK-1 and JNK protein levels. The compounds caused decrease in both

inhibited forms of ASK-1 (phosphorylated at S83 and S966), meaning that ASK-1 protein is activated. Moreover, compounds induced activation of SAPK/JNK (Figure 4.18). Both confirms the activation of oxidative stress. Finally, the presence of PARP cleavage in liver cancer cells treated with IBUTRI-OCH3-TDZ and IBUTRI-CH3-TDZ suggests the induction of apoptosis (Figure 4.18).

4.2.6. COX Activity is reduced after treatment with the molecules

Previous studies suggested that the possible anticancer activity of NSAIDs can be through their COX inhibiting properties. Therefore, here the levels of COX activity were measure using COX Activity kit in cells treated with selected novel molecules for 48h. Results indicated that treatment with IBUTRI-OCH3-TDZ and IBUTRI-CH3-TDZ resulted in around 50% COX inhibition. In positive control experiment groups, in which cells were treated with Ibuprofen, naproxen and flurbiprofen, inhibition was more than 70% (Figure 4.19).

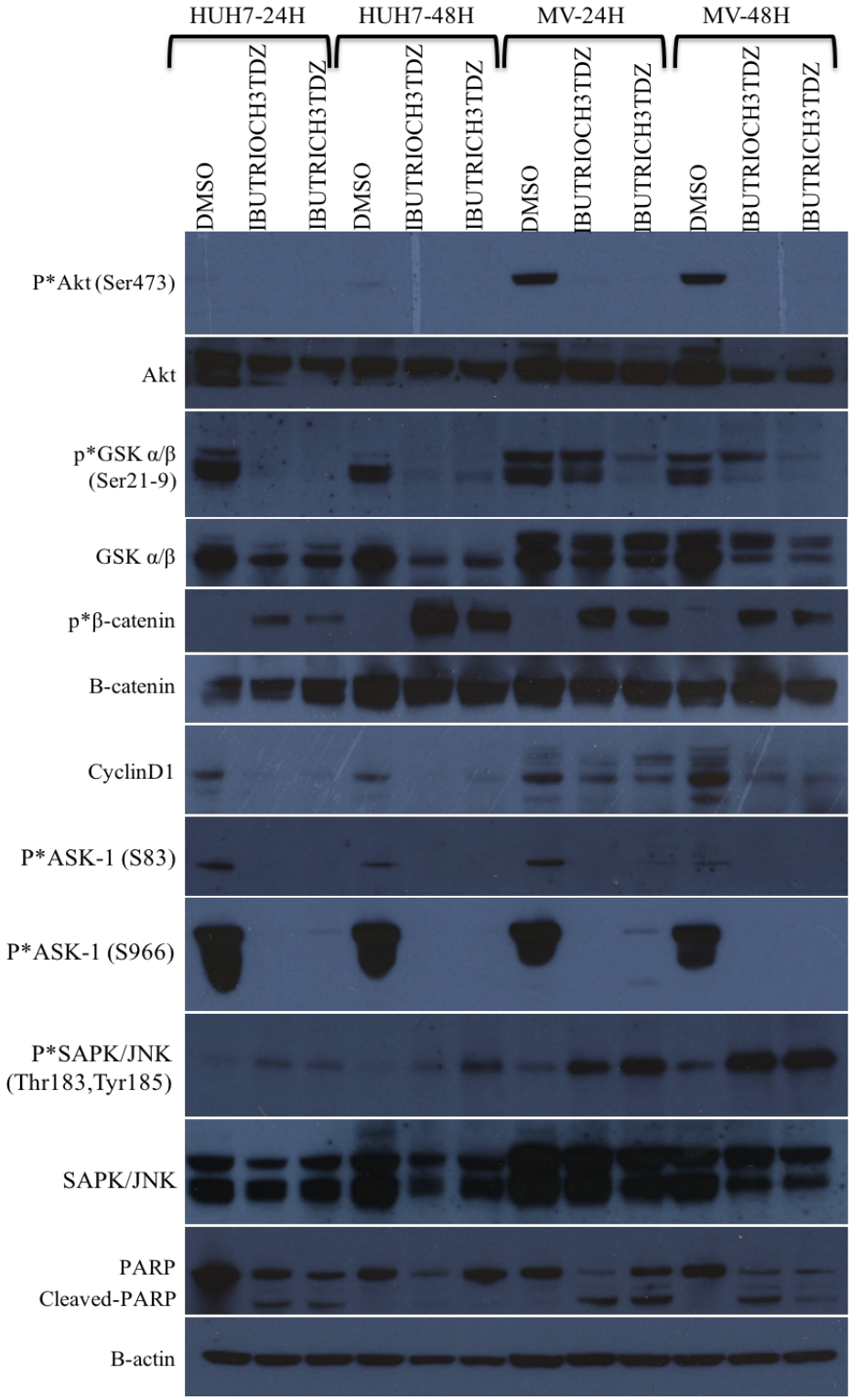


Figure 4.18. Mechanism of action of the compounds on protein level. Huh7 and Mahlavu cells were treated with 10µM of the compounds for 24 and 48h. Western blot analysis was performed using specific antibodies.

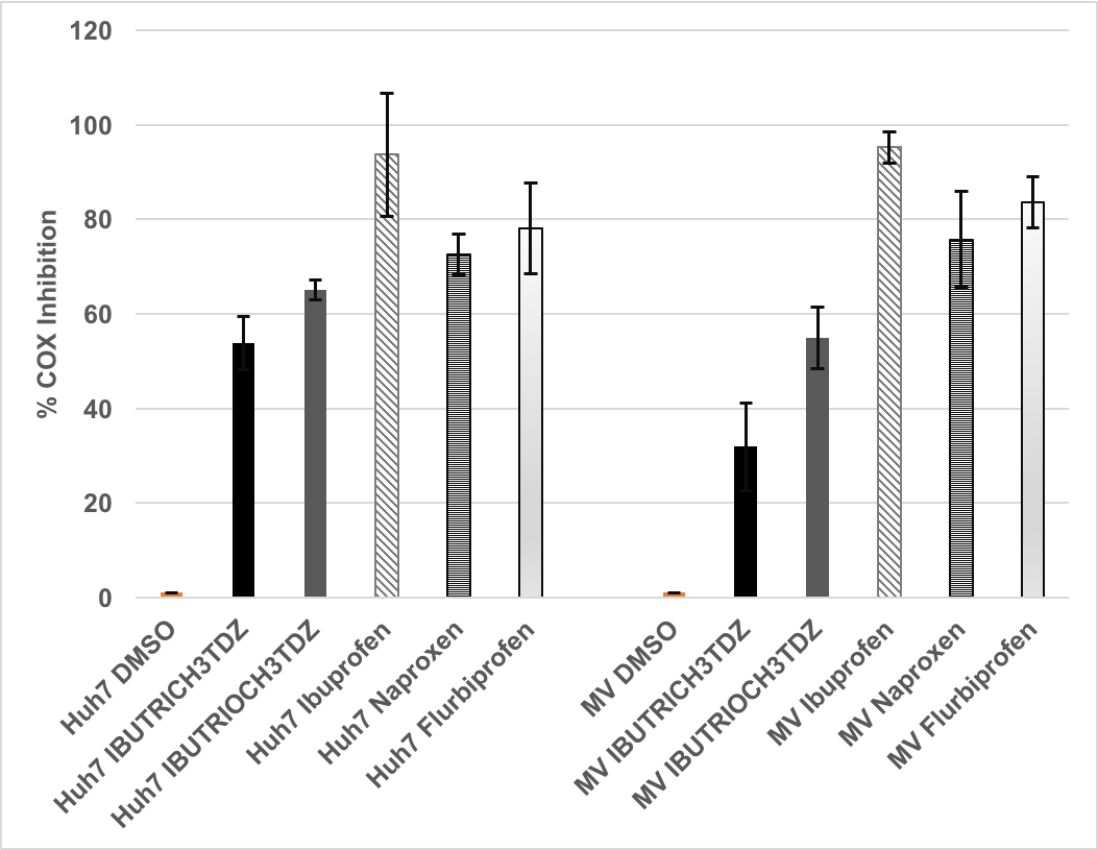


Figure 4.19. COX inhibition by novel molecules. Huh7 and Mahlavu cells were treated with 10μM of the compounds for 48h. COX Activity Assay Kit was used for the assessment of COX activity levels in cells.

4.3. Nucleobase and nucleoside analogues

The novel small molecules that are nucleobase or nucleoside analogues were synthesized by Meral Tuncbilek, Ankara University.

4.3.1. Cytotoxicity screening of novel, substituted purinee nucleobase and pyrimidine and purinee nucleoside analogues

All the molecules obtained were dissolved in DMSO to prepare the stock solutions. Then cytotoxic activities of the molecules were initially analyzed using SRB assay on liver (Huh7), colon (HCT116) and breast (MCF7) liver cancer cells. Molecules were applied in five concentrations, starting from 40μM to 2.5μM for 72h. Campthotecin (CPT), 5-fluorauracil (5-FU), Cladribine, Pentostatine and Fludarabine were used as positive controls. ME5,7,8,9,10,11,12,13,16,23,34,35,36,46,47,51,58, 102, 103, 111, 114, 116, 118, and 125 were shown to have IC_{50} values less than 10μM (Table 4.6, Supplementary Figures1-3). Therefore, these molecules were selected to be analyzed further in an enlarged panel of HCC cell lines.

Table 4.6: IC50 values of small molecules for 72h

	HUH7		HCT116		MCF7	
	IC_{50}(μM)	R_2	IC_{50}(μM)	R_2	IC_{50}(μM)	R_2
ME1	13.1	1.0	12.0	0.9	11.2	0.8
ME2	49.0	0.7	196.6	0.6	NI	
ME3	14.7	1.0	11.5	1.0	17.2	0.9
ME4	12.3	0.9	32.4	0.8	12.7	0.9
ME5	9.0	0.9	12.0	0.9	5.2	1.0
ME6	34.4	0.8	44.8	0.8	10.5	0.9
ME7	8.0	0.9	8.4	1.0	6.5	0.7
ME8	7.1	1.0	10.4	1.0	8.6	0.8
ME9	1.0	0.8	2.1	0.9	0.1	0.7
ME10	1.4	1.0	1.9	0.9	6.4	0.9
ME11	1.6	0.8	1.6	0.9	9.1	0.9

ME12	0.0	1.0	0.0	0.4	0.2	0.7
ME13	3.8	0.9	5.0	1.0	1.0	0.8
ME14	12.8	0.9	13.9	0.7	15.7	0.8
ME15	23.8	0.9	22.4	0.9	17.7	0.9
ME16	6.3	0.8	6.6	0.9	12.6	1.0
ME17	29.8	0.9	6.0	0.9	20.3	0.9
ME18	6.7	0.9	6.1	1.0	15.6	1.0
ME19	15.6	1.0	NI		NI	
ME20	16.0	1.0	NI		NI	
ME21	10.6	1.0	39.0	0.6	19.5	0.9
ME22	15.1	1.0	26.3	0.9	24.0	1.0
ME23	1.2	0.9	0.8	0.8	2.8	0.9
ME24	25.9	0.7	13.1	1.0	24.6	0.9
ME25	29.9	0.9	25.1	0.6	69.8	0.6
ME26	36.0	1.0	5453.0	0.8	119.6	0.8
ME27	51.4	0.9	147.0	0.9	129.0	0.9
ME28	44.4	0.9	165.7	0.8	56.8	0.9
ME29	53.0	0.3	1593.8	0.8	84.1	0.9
ME30	65.0	0.6	NI		124.7	1.0
ME31	17.0	0.6	20.4	0.8	11.7	0.9
ME32	7.6	1.0	25.3	0.8	25.9	0.8
ME33	16.1	1.0	46.2	0.7	30.1	0.9
ME34	9.3	0.9	10.8	0.9	6.1	0.8
ME35	8.6	0.9	24.0	0.9	5.2	0.9
ME36	7.8	0.9	15.7	0.9	17.0	0.9
ME37	61.4	0.6	657.0	0.6	57.9	0.9
ME38	35.1	0.6	135.0	0.7	7.2	0.9
ME39	11.1	0.9	28.8	0.7	15.0	0.8
ME40	28.4	0.9	52.3	0.8	24.8	0.8
ME41	58.1	0.8	58.5	0.8	17.4	0.9
ME42	16.8	0.8	26.8	0.9	3.9	0.9
ME43	59.3	1.0	41.3	0.7	0.5	0.8

ME44	380.0	0.7	134.3	0.8	51.6	0.7
ME45	322.0	0.9	257.7	0.7	60.7	0.9
ME46	0.1	1.0	0.1	0.4	2.5	1.0
ME47	0.1	1.0	0.6	1.0	4.1	0.9
ME48	57.1	1.0	42.3	1.0	NI	
ME49	NI		NI		58.4	0.7
ME50	9.8	0.9	NI		NI	
ME51	2.9	0.7	30.7	0.9	47.7	1.0
ME52	5.9	0.8	15.7	0.9	NI	
ME53	5.5	0.9	30.9	0.8	69.8	0.5
ME54	7.4	1.0	33.6	0.8	30.6	0.9
ME55	11.4	1.0	13.7	0.9	68.2	0.8
ME56	20.8	0.9	26.1	0.8	101.1	0.7
ME57	11.4	0.7	13.5	0.9	32.4	0.8
ME58	8.3	1.0	13.0	1.0	22.5	0.9
ME59	10.3	1.0	20.4	0.9	26.9	0.7
ME60	20.2	1.0	51.4	0.9	103.6	0.4
ME61	30.0	1.0	50.6	0.9	NI	
ME62	23.2	0.8	5.8	0.7	19.8	0.9
ME63	15.7	1.0	6.5	0.7	21.6	0.9
ME64	19.4	0.8	151.4	0.7	30.3	0.9
ME65	24.5	0.8	33.6	0.9	22.9	0.9
ME66	26.3	1.0	193.2	0.7	28.4	1.0
ME67	39.0	0.9	64.9	0.9	35.8	0.9
ME68	58.7	1.0	NI		128.7	0.9
ME69	17.9	0.9	17.2	0.8	39.6	1.0
ME70	14.2	0.8	13.7	0.8	41.7	0.9
ME71	41.5	0.7	21.8	0.8	NI	
ME72	23.6	0.9	30.4	0.8	NI	
ME73	80.1	0.9	19.5	0.9	69.2	0.8
ME74	NI		17.6	0.9	NI	
ME75	NI		48.2	0.5	NI	
ME76	12.9	1.0	18.8	0.9	21.4	0.9

ME77	17.4	1.0	33.4	0.9	47.5	0.9
ME78	13.1	1.0	34.3	0.9	26.2	0.9
ME79	10.7	0.9	19.7	0.9	37.6	1.0
ME80	18.1	0.8	53.3	0.9	167.0	0.9
ME81	61.8	1.0	NI		NI	
ME82	165.1	0.9	NI		NI	
ME83	24.5	0.5	32.5	0.7	24.8	0.6
ME84	15.7	0.9	23.8	0.7	20.3	0.9
ME85	35.6	0.7	NI		17.9	0.7
ME86	14	1	10.7	0.9	18.9	1
ME87	18.9	0.7	18.2	0.6	14.9	0.7
ME88	11.6	1	13.2	0.9	13.3	0.9
ME89	16.7	0.6	25.9	0.7	14.3	0.9
ME90	12.9	0.9	13	0.9	8.8	0.9
ME91	30.9	0.9	47.7	0.5	42.1	0.9
ME92	19.9	1	22.3	0.8	50.4	0.8
ME93	22.1	1	18.9	0.8	14.1	0.9
ME94	47.5	0.8	52.7	0.6	16.5	0.9
ME95	18.3	0.9	155.9	0.3	16.5	0.8
ME96	13.6	0.9	34.9	0.6	20.5	0.6
ME97	19.4	1	47.1	0.5	12.5	0.9
ME98	17.9	0.9	27.9	0.6	12.2	0.7
ME99	14.4	0.9	18.3	0.9	11.6	1
ME100	14.1	0.8	13.1	0.9	11.2	0.8
ME101	14.2	0.9	15.3	0.9	19.9	0.8
ME102	2.7	0.7	7.7	0.9	5	0.8
ME103	1.2	0.7	10.5	1	1	0.7
ME104	12.7	1	16.8	0.7	11.8	0.8
ME105	21.9	1	147	0.5	11.5	0.7
ME106	34.3	0.9	11.4	0.9	31.4	0.6
ME107	24.4	0.9	88.8	0.9	66.7	0.5
ME108	13.5	1	16.6	0.9	19.5	0.9
ME109	11.4	0.9	13.6	1	12.2	1

ME110	12.1	0.8	22.3	0.8	14.8	0.9
ME111	3.2	0.9	13.6	1	12.8	0.9
ME112	11	0.9	24.5	0.7	12.8	0.7
ME113	26.6	0.9	465.9	0.6	21.4	0.7
ME114	3.2	0.8	4.6	0.8	5	0.9
ME115	23.6	0.9	126.2	0.9	21.1	0.8
ME116	4.5	0.9	324.4	0.8	36.9	0.6
ME117	11.1	0.8	80.1	0.7	38.5	0.4
ME118	1.6	0.8	263.5	0.7	4.2	0.9
ME119	28.7	1	181.8	0.6	45.2	0.7
ME120	15.2	0.9	NI		11.1	0.8
ME121	21.1	0.7	NI		25	0.6
ME122	173.6	0.7	NI		35	0.6
ME123	20.8	0.9	35.1	0.6	13.5	0.6
ME124	16.4	0.8	77.3	0.5	20.3	0.9
ME125	1.5	1	30.8	0.9	7	0.9
ME126	15.9	1	NI		14.5	0.8
ME127	40.8	0.9	33.3	0.9	12	0.9
CPT	<1		<1		<1	
5-FU	30.7	1	18.7	0.9	3.5	0.7
CLADRIBINE	1.3	1.0	<0.1		2.0	1.0
PENTOSTATINE	NI		NI		NI	
FLUDARABINE	29.9	0.7	8.3	0.9	15.2	0.9

NI: No Inhibition

According to the first screen, the selected 24 molecules were further tested on enlarged HCC panel consisting of 12 cell lines (Huh7, Hep3B, HepG2, PLC, Mahlavu, FOCUS, SNU475, SNU182, SNU387, SNU398, SNU423, SNU449 with SRB assay in triplicate (Table 4.7 and Figure 4.20).

Furthermore, RT-CES equipment was used for the monitoring of the bioactivities of the compounds in real time (Figure 4.21). Especially, molecules ME9,11,13,23,46 and 47 showed high cytotoxicity on enlarged HCC panel and RT-CES experiment. Therefore, for further detailed molecular assays, these 6 molecules were selected. During the selection process, the first criteria were to have IC_{50} less than 5μM. and that the bioactivity of the compounds should be more in poor-differentiated cells compared to well-differentiated HCC cell lines.

Table 4.7: IC50 values of small molecules on enlarged HCC panel for 72h.

	HUH7	HEP3B	HEPG2	PLC	MV	FOCUS	SNU 475	SNU 182	SNU 387	SNU 398	SNU 423	SNU 449
ME5	9	6.1	4.8	15.4	11.4	12.6	21.6	47.9	11.1	8	9.5	16.3
ME7	9.1	4.3	6.3	9.2	20.5	15.9	9	60.6	15.9	4	10.5	8.5
ME8	17	71.8	2.7	84.6	9	10.1	57.5	NI	15.8	6.9	6.8	20
ME9	3	<0.1	2.4	4	0.2	0.6	5.8	6	13	1.2	11	2
ME10	2.9	3.1	0.9	20.7	4.2	5	17.7	61	88	1.9	17.5	17
ME13	1.3	0.3	0.3	9.3	2.7	2.8	81	57	31	3.2	13.7	7.3
ME11	1.5	4.3	0.5	6	0.6	2.6	13	13	38	0.3	15	2
ME12	0.4	0.03	0.02	7	7	1.5	7	41	26	1	16	3.3
ME16	8.1	6.7	2.7	3.4	4	2	12.3	12.2	2	1.8	4	6.9
ME23	0.7	0.1	0.002	6.5	0.1	0.5	2.5	65	20	0.2	9.9	<0.1
ME34	20.3	10.3	6.6	99.1	19.8	4.5	17	16.4	98.7	19	24.9	60.6
ME35	20.3	32	5.1	NI	NI	16.1	54.8	39.5	NI	10.3	68.3	NI
ME36	26.2	8.4	5.9	NI	34	10.7	47.5	81.9	NI	11.1	45.1	36.5
ME46	0.2	0.01	0.01	NI	38.8	0.6	72.9	NI	NI	0.4	NI	26.2
ME47	1.7	0.4	0.01	19.3	9.9	1.8	14.4	35.8	30.6	1.4	36	10.3
ME51	13.1	17.8	15.1	NI	41.7	13.6	53.3	54.9	NI	11.6	NI	71.5
ME58	15.1	8.9	6.7	NI	83.9	11.2	NI	NI	NI	10.6	NI	43.7
ME102	9.7	6.5	7	12.5	23.4	7.6	15.6	6	19.2	6.9	15.9	10
ME103	7.3	5	5.4	8.9	22.4	4	15	2.9	23.6	4.9	16.4	10.8
ME111	17.4	11.3	13	40.7	NI	20.4	54.5	12.3	NI	17.4	63.8	44.6
ME114	7.8	7.2	5.6	16.5	29.4	8	22	9.9	44.7	6	13.4	12.3
ME116	32	47.6	35.4	81.1	78	31.6	30	18.6	39.4	NI	14	NI
ME118	5.8	9.5	8.3	29	33	9.6	45.4	11.4	52.7	6.6	19.6	17.3
ME125	24.2	15.5	37.2	46.3	40	17.2	40.7	NI	NI	34	22.5	NI
CLAD*	0.6	0.1	0.4	9.1	<0.1	<0.1	2.5	23.3	7.1	<0.1	6.7	37.2
FLU*	24.4	27.8	17	41.7	14.2	13.7	41.5	37.2	33.8	0.2	32.6	25.1
PEN*	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

***NI=No Inhibition, CLAD: Cladribine, FLU: Fludarabine, PEN: Pentostatine**

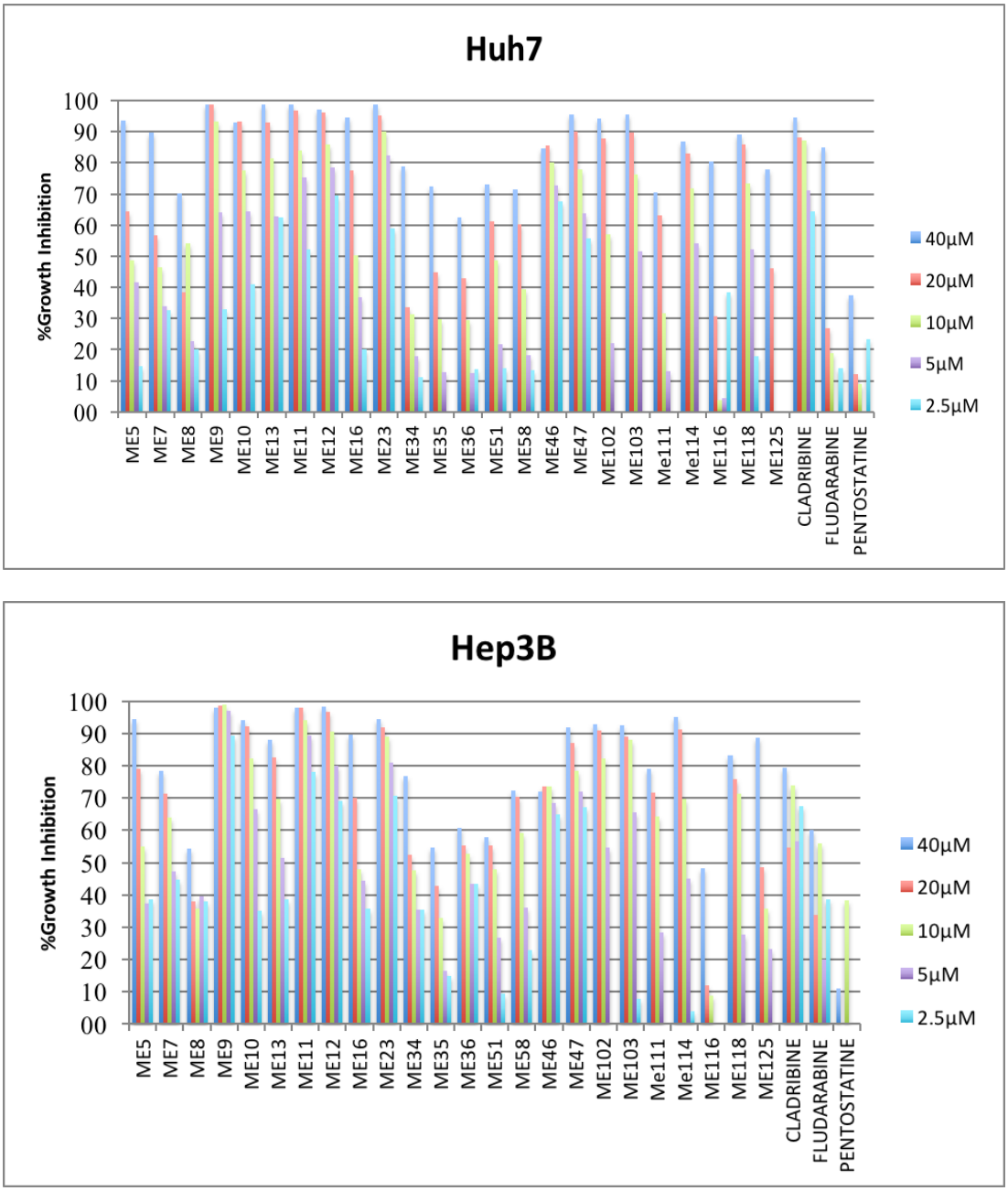


Figure 4.20. Growth inhibition rates of selected molecules on HCC cell lines. 12 liver cancer cells were treated with the compounds for 72h and analyzed with NCI-SRB assay.

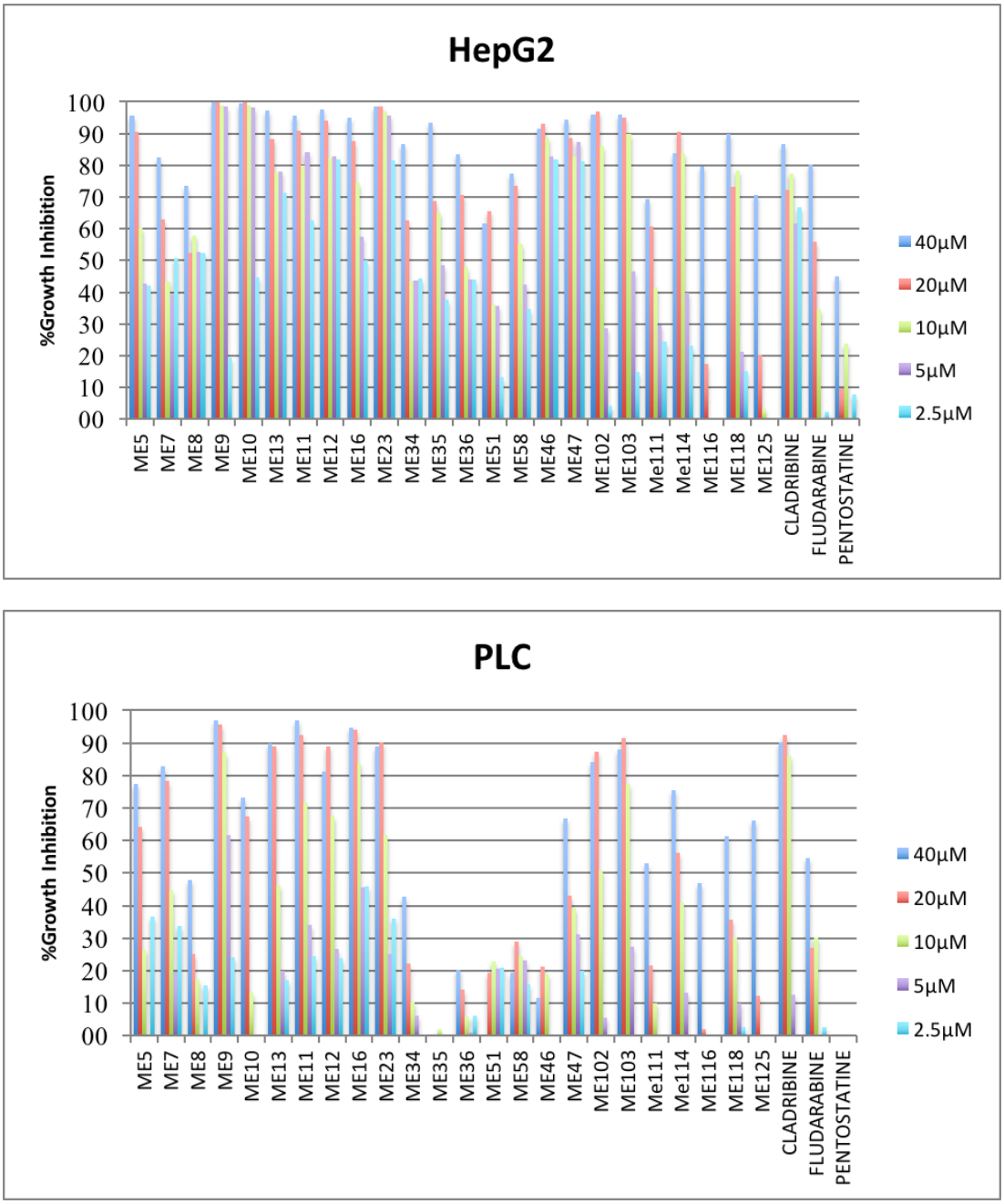
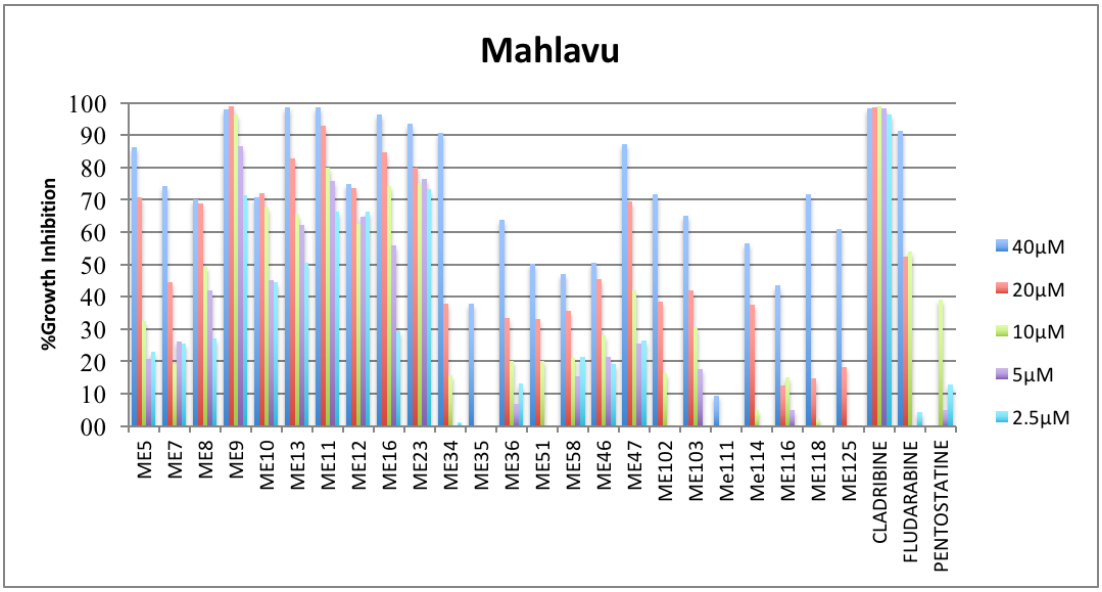


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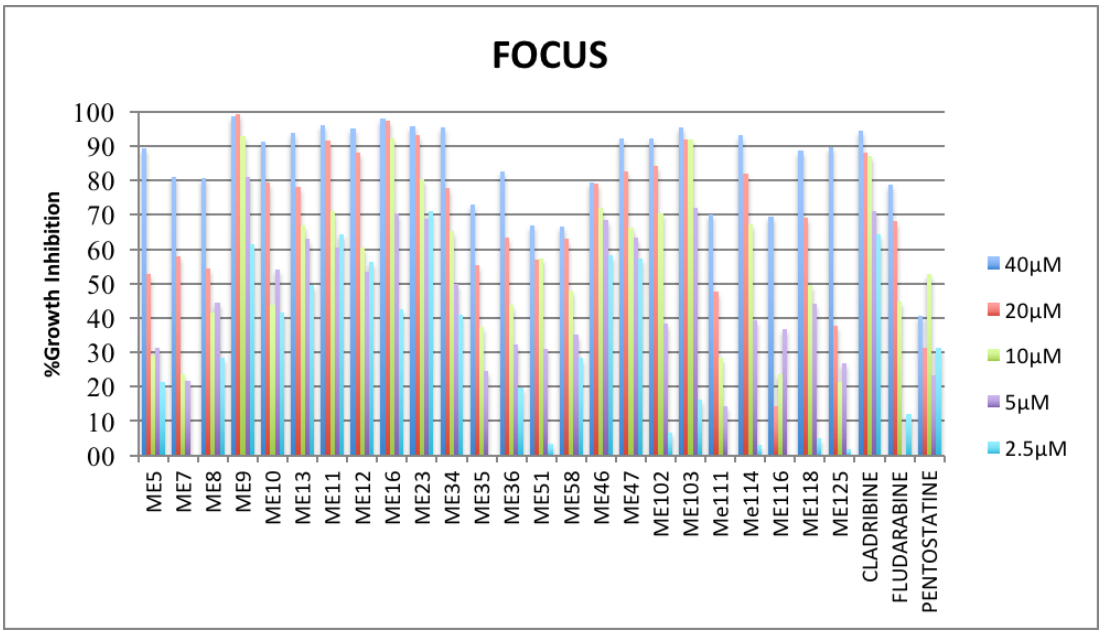
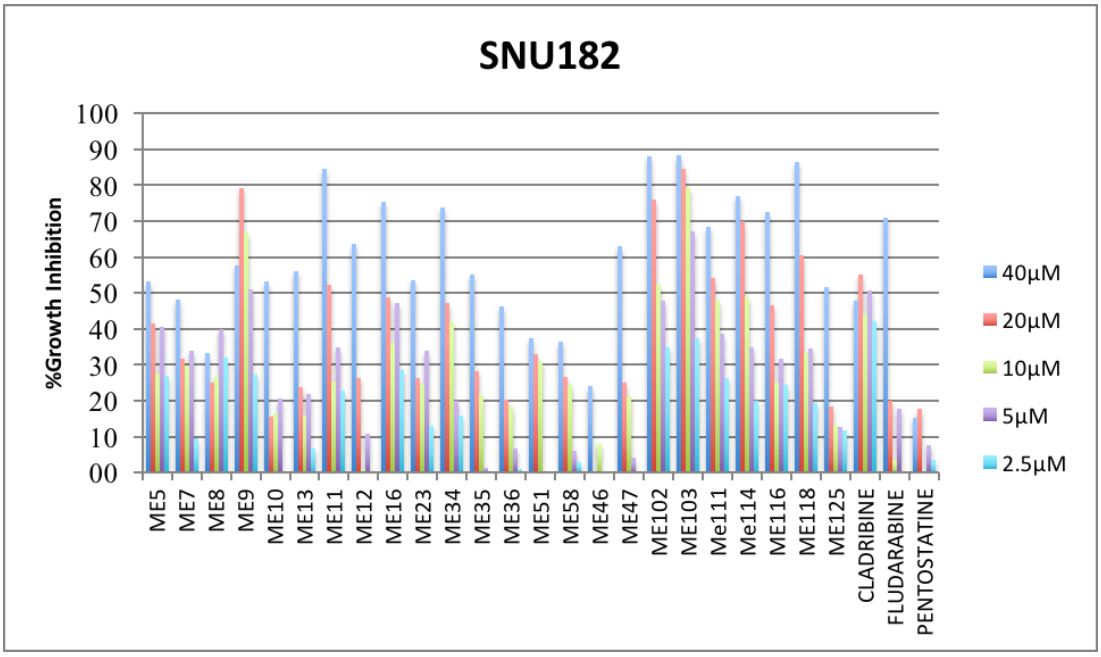


Figure 4.20. Growth inhibition rates of selected molecules on HCC cell lines. 12 liver cancer cells were treated with the compounds for 72h and analyzed with NCI-SRB assay.



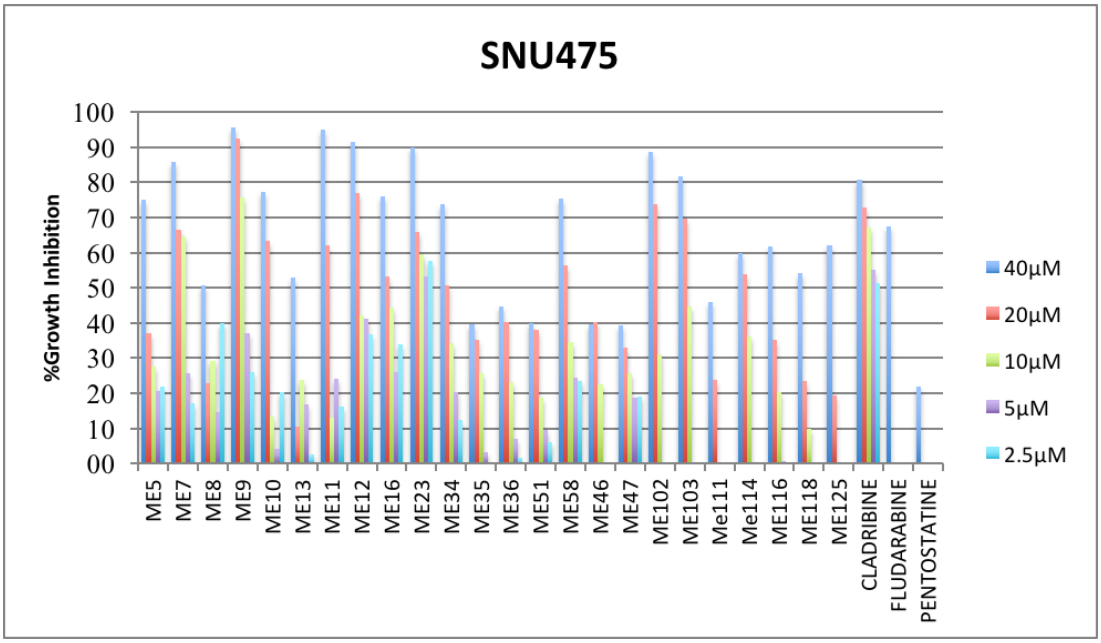


Figure 4.20. Growth inhibition rates of selected molecules on HCC cell lines. 12 liver cancer cells were treated with the compounds for 72h and analyzed with NCI-SRB assay.

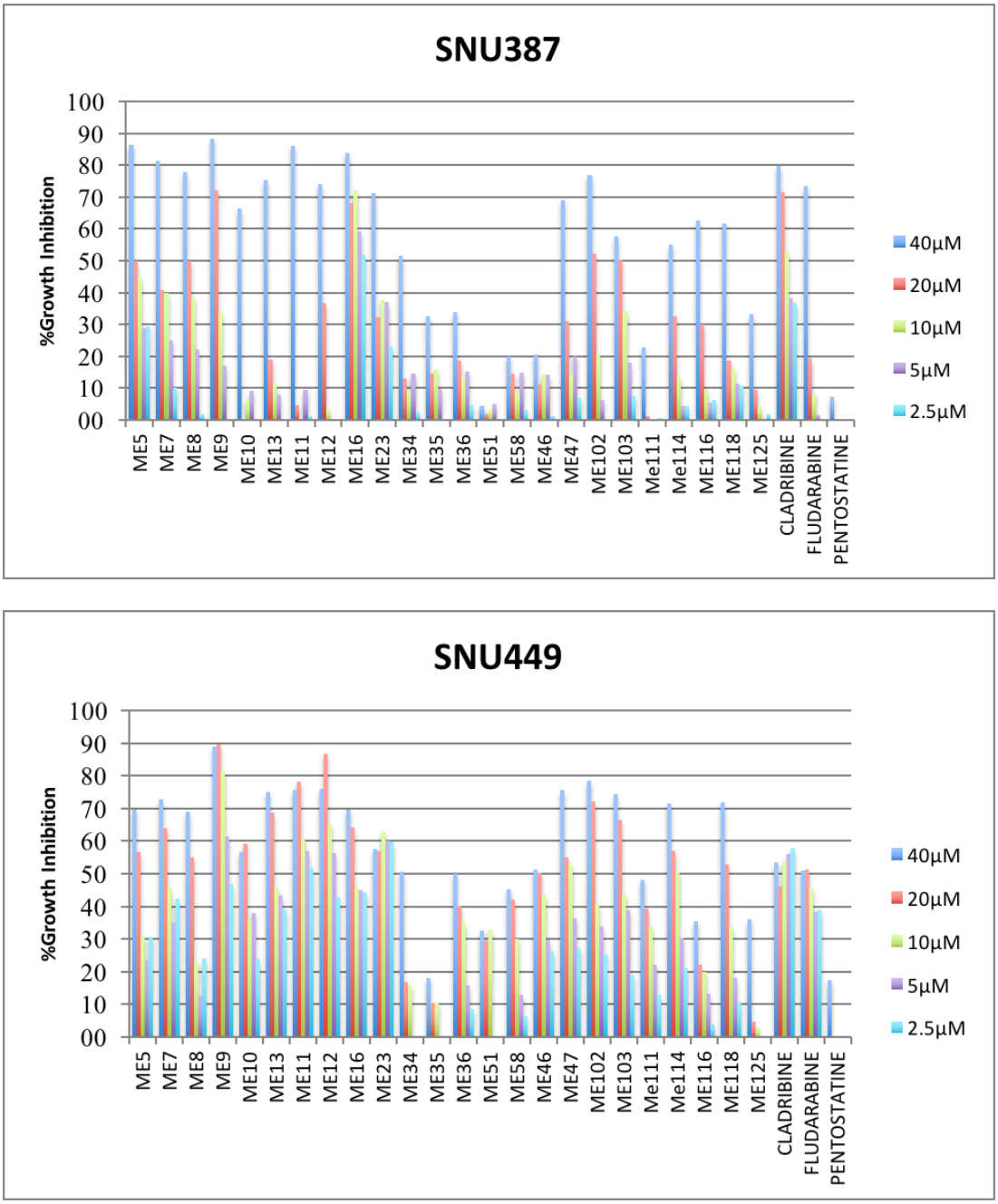


Figure 4.20. Growth inhibition rates of selected molecules on HCC cell lines. 12 liver cancer cells were treated with the compounds for 72h and analyzed with NCI-SRB assay.

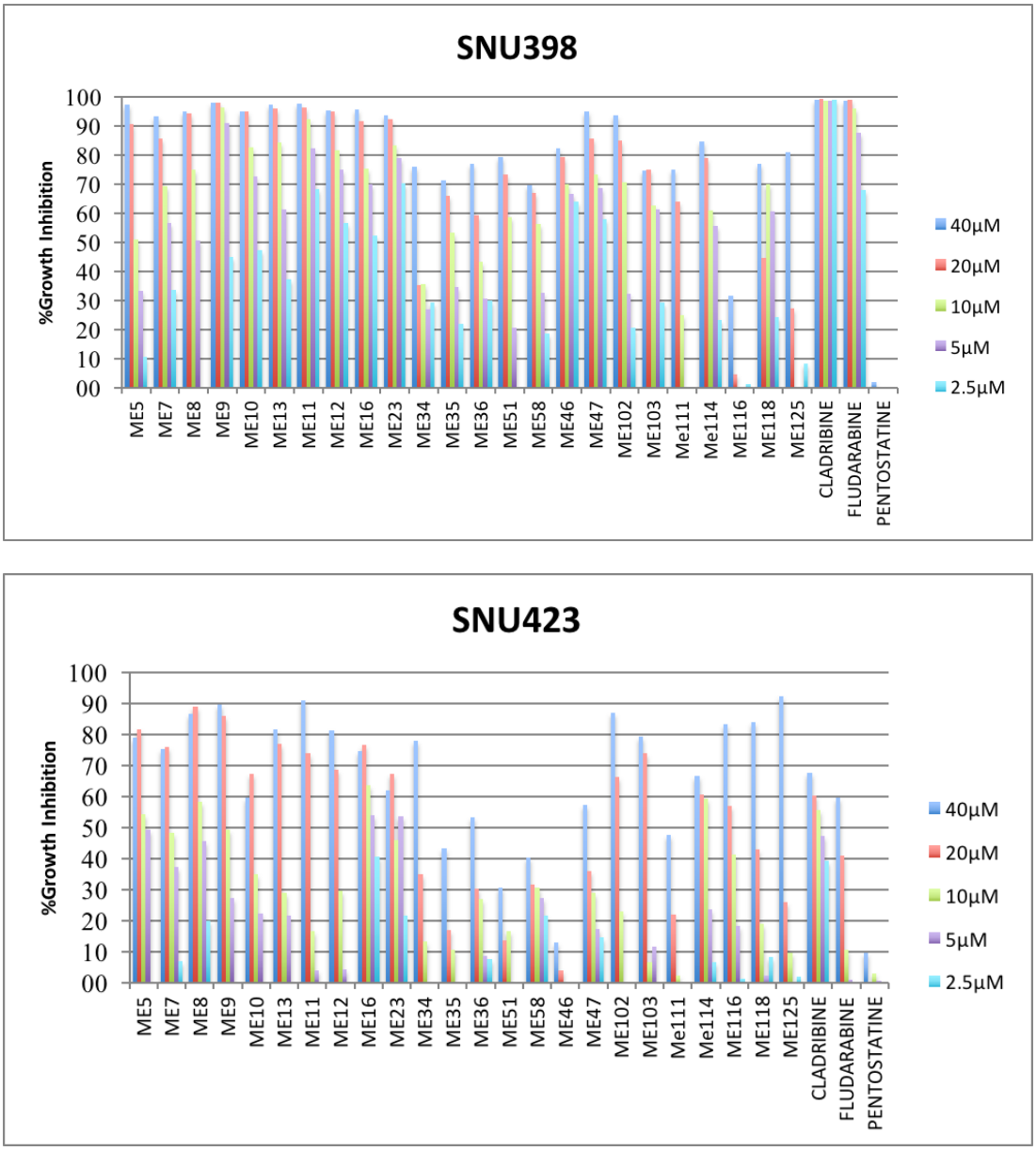


Figure 4.20. Growth inhibition rates of selected molecules on HCC cell lines. 12 liver cancer cells were treated with the compounds for 72h and analyzed with NCI-SRB assay.

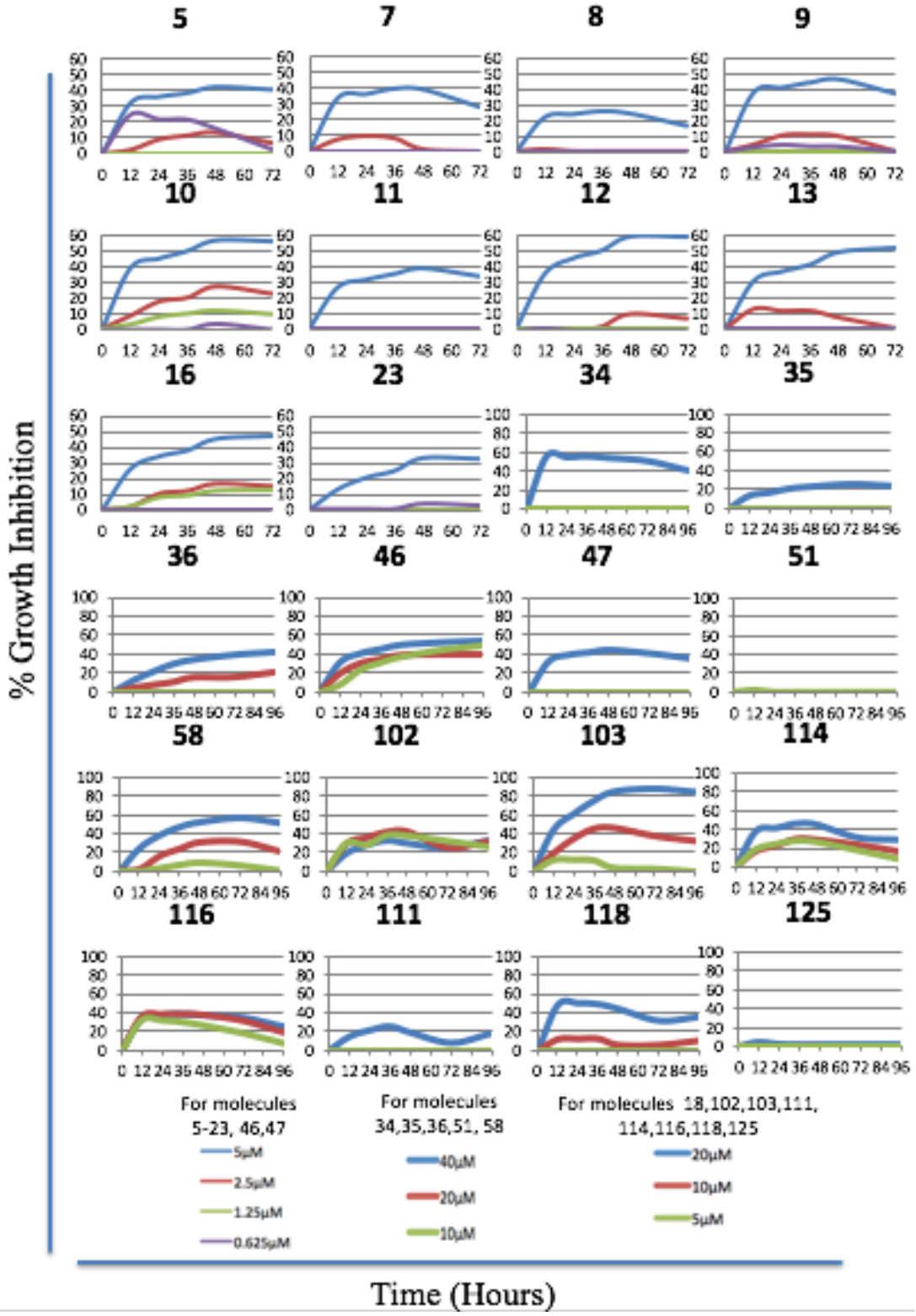


Figure 4.21. Real-time cell growth assay on Huh7 liver cancer cell line. Huh7 liver cancer cells were treated with the compounds in the indicated concentrations for 72h or 96h. RT-CES software was used for monitoring.

4.3.2. Morphological changes induced by novel nucleobase and nucleoside analogues

In order to investigate the changes in the morphological changes of the cells, we used Hoechst 33258 stain that binds to DNA. Liver cancer cells were treated with 6 selected compounds (ME9,11,13,23,46 and 47) for 72h according to their IC50 values. At the end of the incubation time, methanol fixation was performed and cells were stained with Hoechst 33258dye. Observation under fluorescent microscope revealed condensed nuclei and DNA fragmentation together with appearance of horseshoe-like structures and shrinkage of cells bearing apoptosis (Figure 4.22).

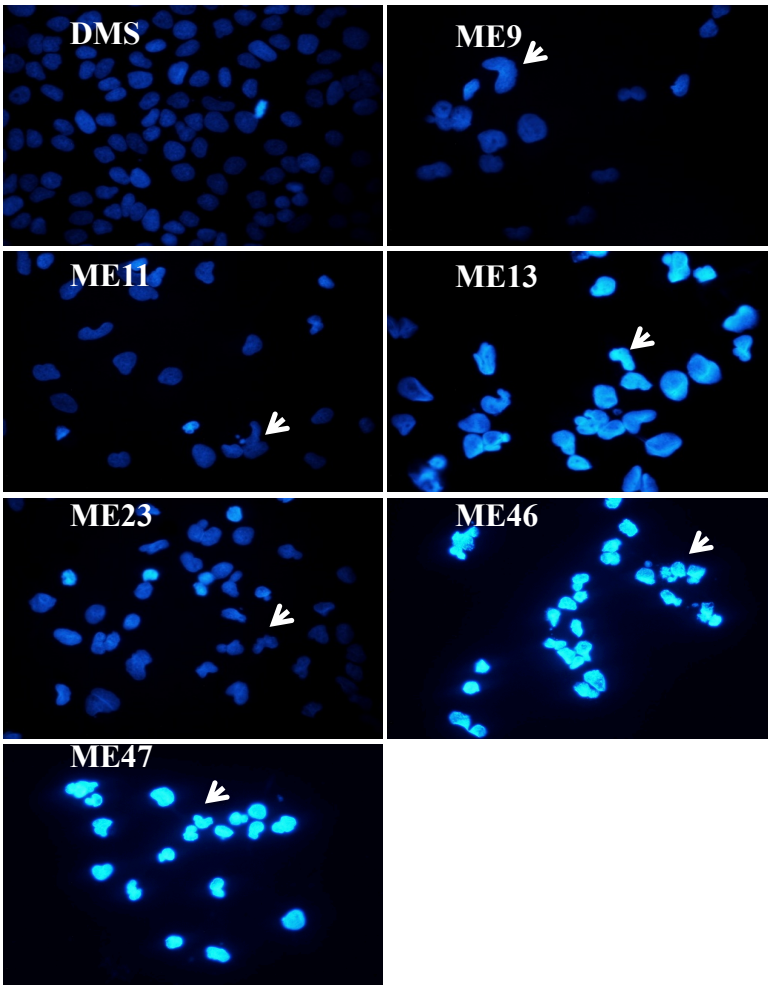


Figure 4.22. Apoptotic morphologies induced on Huh7 liver cancer cell line. Huh7 liver cancer cells were treated with the compounds for 72h and stained with DNA-specific Hoechst 33258. DMSO was used as negative control and cell images were captured under 40x objective.

4.3.3. SubG1 cell cycle arrest is induced by newly synthesized novel nucleobase and nucleoside analogues

Bearing in mind that compounds induced apoptotic like morphological changes in cancer cells, we further analyzed the effect of the compounds on cell cycle. For this purpose, we used propidium iodide, which is a DNA-intercalating fluorescent dye, frequently used for cell cycle analysis [125] .

Cells that are in G1 phase of cell cycle or at quiescent state (G_0) contain one DNA copy, whereas cells at G2/M phase have double copies of DNA. Moreover, if the cells are in S phase of the cell cycle, they have varying copy number. In healthy well-differentiated liver cancer cells would have about 60-70% of the cells to be in G1 phase, 20%in S phase. Moreover, G2/m phase cells would comprise 20% of the total cell population.

In order to assess the cell cycle data, liver cancer cells (Huh7 and Mahlavu) were treated with 5μM of the selected 6 compounds for 72h then fixed with ethanol and stained with propidium iodide. FACS analysis revealed that the molecules induced SubG1/G1 arrest, which is an indicator of apoptotic induction (Figure 4.23 and Figure 4.24)

4.3.4. Potential kinase inhibitory activity of the compounds

The newly synthesized nucleobase and nucleoside analogues were suspected to have kinase inhibitory activity based on similar previous findings in the literature. Therefore, we performed Kinase Assay on these molecules. Human liver cancer (Huh7 and Mahlavu) cells were treated 5μM of the compounds for 72h. Then using Lonza Kinase Glo Plus luminescent kit, the effect of the compounds on ATP levels were detected. Multikinase inhibitor Staurospourine was used as positive control.

Results revealed that compounds except ME9 had no affect on the intracellular ATP levels indicating that they do not have kinase inhibitory effects (Figure 4.25). Only ME9 showed relative increase in luminescence indicating high ATP levels which indirectly may suggest that this compound might be inhibiting intracellular kinases.

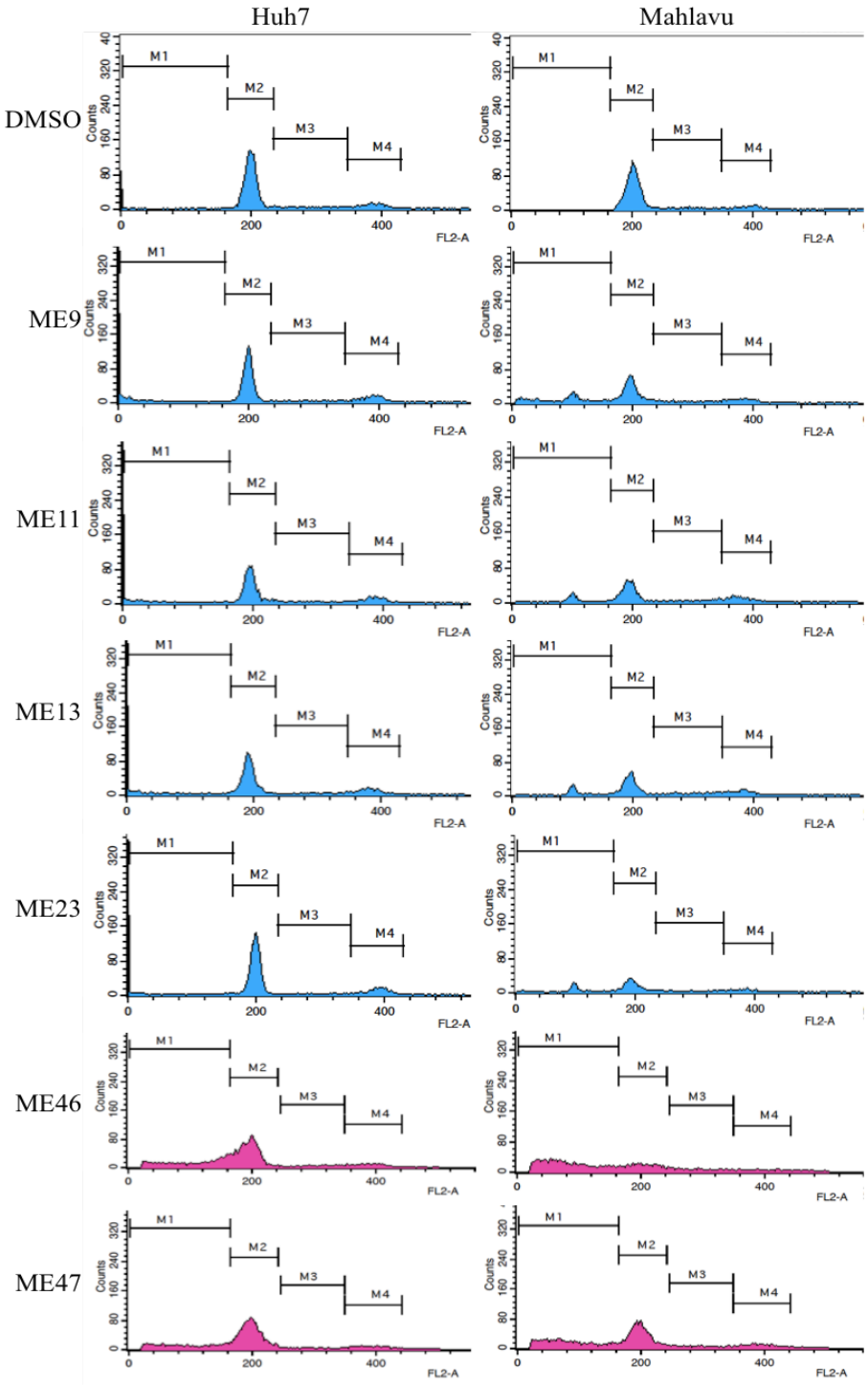


Figure 4.23. Cell cycle distribution pattern of liver cancer cells treated with selected compounds. Huh7 and Mahlavu cells were treated with 5µM of compounds or DMSO control for 72h. The peak at 200 FL2-A represents 2 N cells (G1-phase) and the peak at 400 represents 4 N cells (G2-phase). The peak in-between represents S-phase cells.

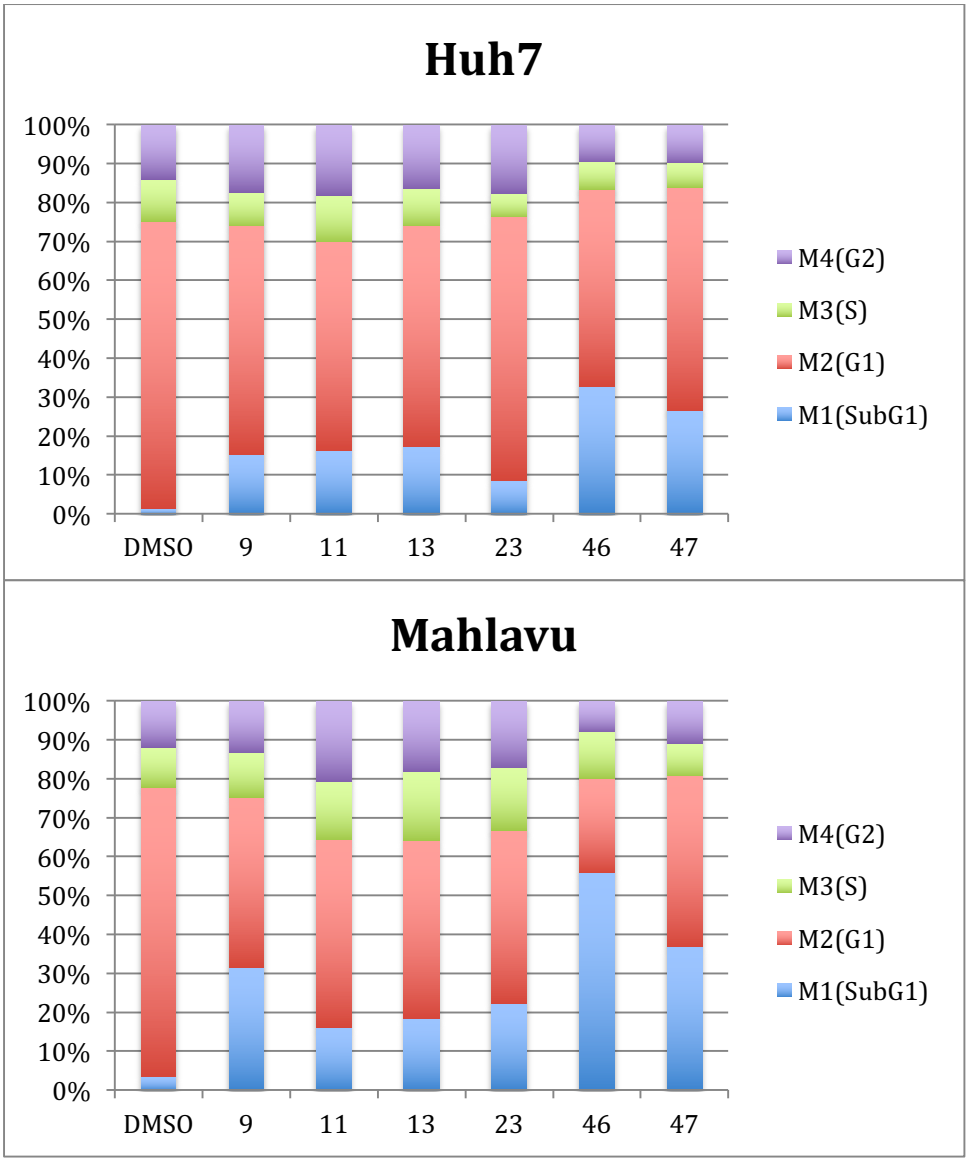


Figure 4.24. Cell cycle analysis in the presence of nuclobase analogs

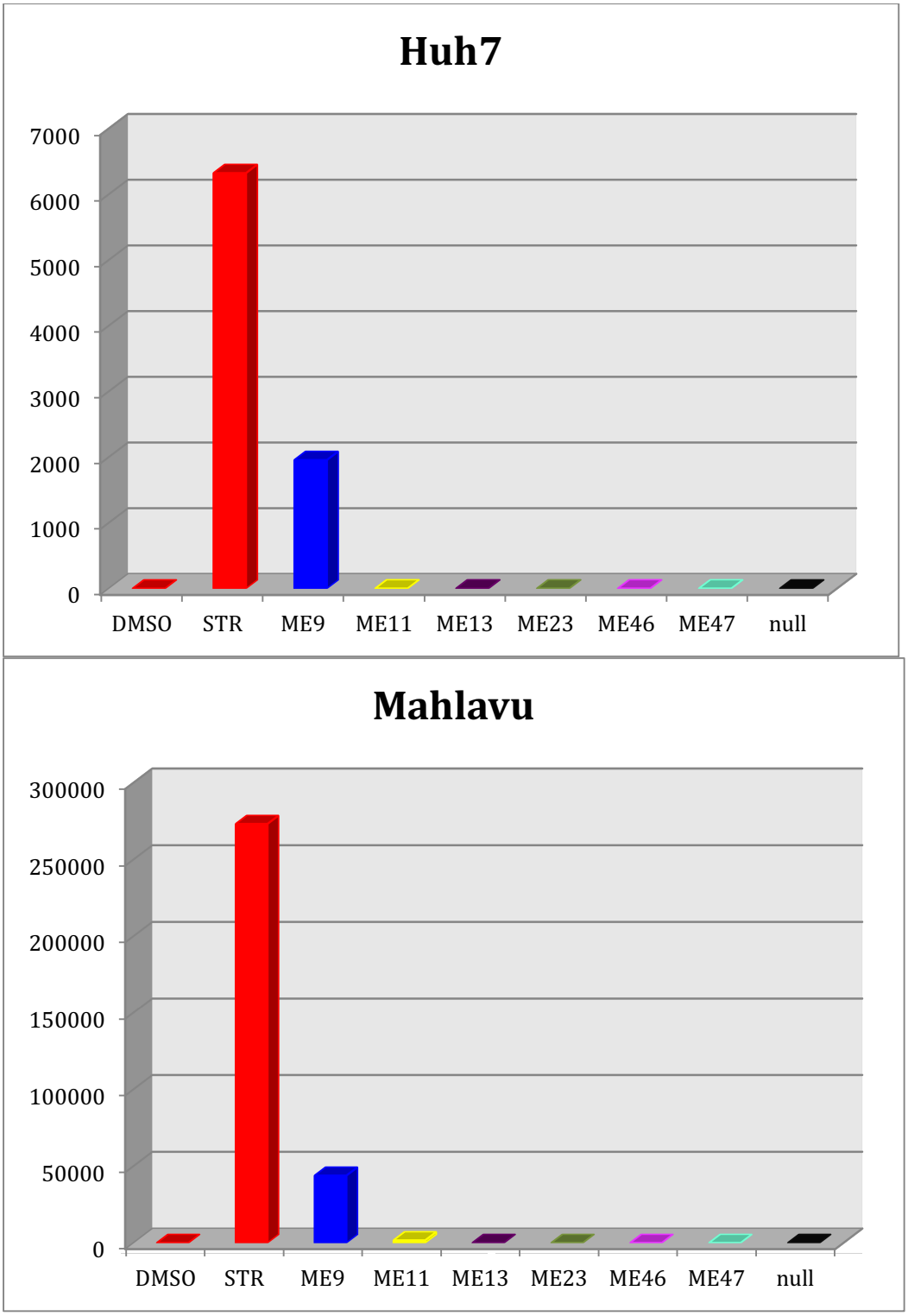


Figure 4.25. Kinase inhibition potential of the selected compounds. Huh7 and Mahlavu cells were treated with 5µM of compounds or DMSO control. Then Lonza Kinase Glo luminescent kit was applied to observe the kinase activity present in the cell samples after treated with the compounds.

4.3.5. Molecular Mechanism of Action

Finally, our aim was to identify the pathways targeted by these novel molecules. Bearing in mind the observation of apoptotic nuclear morphologies observed together with the cell cycle results, first we investigated presence of cleaved PARP, which is an apoptotic marker [126], in presence of the compounds. Results pointed out that all six compounds induced PARP cleavage confirming induction of programmed cell death mechanism in liver cancer cells (Figure 4.26).

In literature, it was shown that a group of novel purine derivatives can inhibit c-Src kinase [127]. Therefore the activity levels of c-Src kinases were first to be investigated in order to find the mechanism of action of our novel nucleobase and nucleoside analogues. For this purpose, Huh7 cells were treated with 5µM of the six compounds for 72h and analyzed with western blot using specific antibodies for c-Src kinase and its phosphorylated active form. Our data demonstrated that treatment with ME9, 46 and 47 resulted in significant decrease in the levels of p-Src meaning that these proteins inhibits the activation of Src protein (Figure 4.27). Moreover, the downstream elements of Src pathway was investigated [128]. We observed that, ME9 and 46 compounds decreased p-Rb levels which means that Rb protein is activated in these samples. Moreover, levels of Cyclin E and cdk2 were also decreased in treated cells compared to controls (Figure 4.28).

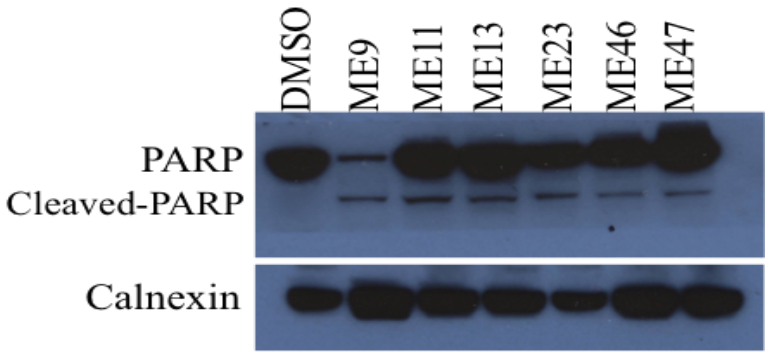


Figure 4.26. PARP cleavage induced by the molecules. Huh7 cells were treated with 5µM of the compounds for 72h. Calnexin was used for equal loading control.

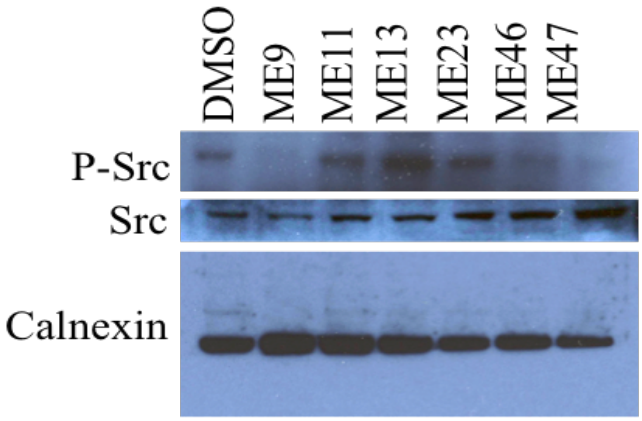


Figure 4.27. Src inhibition by ME9, 46 and 47. Huh7 cells were treated with 5μM of the compounds for 72h. Calnexin was used for equal loading control.

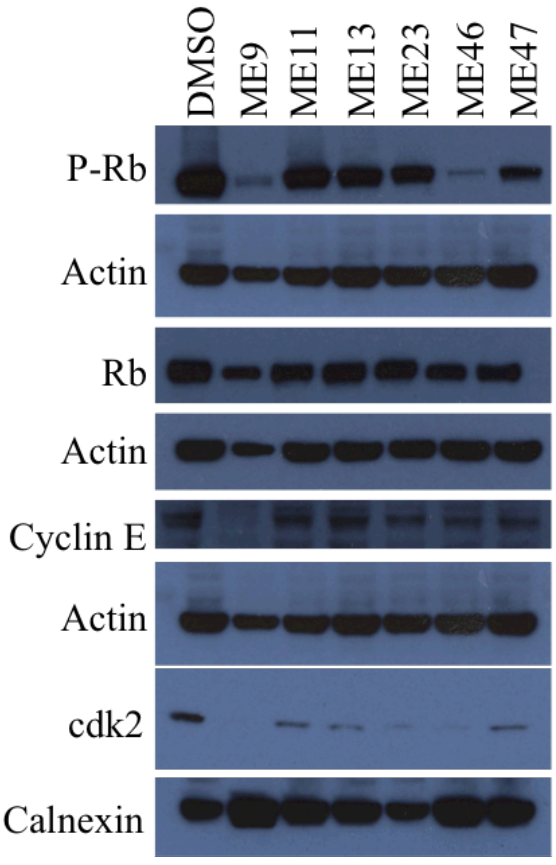


Figure 4.28. Src pathway components affected by ME9, 46 and 47. Huh7 cells were treated with 5μM of the compounds for 72h. Calnexin and actin were used for equal loading control.

CHAPTER 5. DISCUSSION AND CONCLUSION

Current treatment options requires novel chemotherapeutic agents for the cure of Hepatocellular carcinoma which is one of the most deadly cancers and the sixth commonly seen worldwide [1]. Due to various etiological factors, liver cancer has a heterogeneous genetic background, which leads to the alterations of cellular signals. HCC is resistant to conventional therapy strategies and there is only one FDA approved drug Sorafenib that can only prolong survival up to 5 months. Thus it is crucial to develop new strategies for the treatment of this disease.

For centuries, the steroid-like compounds, cardiac glycosides, have been used for the treatment of heart failure [129, 130]. These molecules were shown to inhibit Na^+/K^+-ATPase and indirectly regulate cell signaling [23, 131–133]. During this research study, we investigated in detail the molecular mechanisms of cardiac glycosides from *D. ferruginea* on liver cancer cells. Previously we demonstrated that three cardiac glycosides extracted from *Digitalis Feruginea* by Prof. Ihsan Calis inhibited cell growth of all epithelial like (Huh7 and HepG2) and mesenchymal like (Mahlavu and FOCUS) liver cancer cells. Moreover, they were shown to induce apoptosis and G2/M arrest on liver cancer cells. In this present study, we aimed to explore the death mechanism and targeted cellular pathways in detail. Lanatoside C was selected to further investigate the molecular mechanisms involved in this cell death, because this compound is preferably used in clinics. Previous findings revealed ROS accumulation resulting in oxidative stress was induced by cardiac glycosides[111]. Our results indicated that Lanatoside C stimulated the accumulation of ROS in liver cancer cells (Figure 4.2). Then, we identified the cellular pathways targeted by Lanatoside C in the context of epithelial vs. mesenchymal background. In literature it was proposed that, cardiac glycosides, upon translocation into nucleus, inhibits the function of Na/K^+ATPase, and cause ROS accumulation inside the cells leading to activation of ERK1/2 and JNK proteins [111, 134, 135]. Then the activation of ERK1/2 phosphorylates and inactivates GSKα/β, and hence results in the activation of JNK [119, 136, 137]. Our data suggested that, PTEN adequate epithelial like Huh7 cells treated with Lanatoside C displayed active ERK1/2 and inactive GSK3β

proteins, which results in the activation of JNK1 induced caspase-8 cascade (Figure 4.3-9 and 5.1).

Cell death by apoptosis can be either induced intrinsically or extrinsically by death receptors. Their downstream caspase activation is an indicator of the apoptotic pathway induction type [138]. Caspase-8 cleavage is an indicator of extrinsic pathway. In this study, we showed the cleavage of this caspase and its downstream targets caspase 3 and PARP proteins (Figure 4.8 and 5.1). GSKα/β was shown to both trigger and inhibit apoptotic pathways. Un-phosphorylated form of active GSKα/β is involved in the activation of mitochondria related intrinsic pathway. Additionally, death-receptor initiated extrinsic apoptosis pathway is inhibited by active GSKα/β. Thus our data illustrates that phosphorylation of GSKα/β together with the presence of cleaved caspase8 and 3 suggested that Lanatoside C induced extrinsic apoptotic pathway through JNK1 activation in human liver cancer cells (Figure 4.3-9 and 5.1).

However, in PTEN deficient mesenchymal like Mahlavu cells the story is more complicated. Mahlavu cells did not display a significant increase in the levels of ERK1/2 phosphorylation when compared to DMSO controls. Mahlavu cells have already activated ERK1/2 and inactive pGSKα/β levels in untreated cells (Figure 4.5). It was previously demonstrated by our group that PI3K/Akt pathway is constitutively hyperactive in Mahlavu cell line due to PTEN deficiency[105]. Therefore the ROS-induced ERK1/2 phosphorylation was masked by the hyperactivity of the Akt pathway (Figure 4.5 and 5.1). Moreover, the observed decrease in inactive pGSKα/β levels was due to the decrease in pAkt in Mahlavu cells. Akt pathway hyperactivation normally inhibits GSKα/β resulting in the induction of cellular survival. Thus, by inhibiting Akt and activating GSKα/β, Lanatoside C inhibits cellular survival in Mahlavu cells (Figure 4.3-9 and 5.1). Additionally, active GSKα/β and JNK2 proteins were previously shown to involve together in the prevention of cell survival [139]. Similarly in our study, in Mahlavu cells treated with Lanatoside C, increase in phospho-JNK2 and decrease in phospho-GSKα/β were observed, in favor of the prevention of cell survival (Figure 4.3-9 and 5.1). Thus, our comparative data using drug sensitive epithelial like Huh7 and drug resistant PTEN deficient mesenchymal like Mahlavu liver cancer cells revealed that

cardiac glycoside Lanatoside C may induce cell death by two parallel pathways. The compound can function by inducing ERK1/2 dependent apoptosis while inhibiting Akt dependent cell survival by differential activation of JNK1 and JNK2 respectively (Figure 5.1).

These *in vitro* findings regarding the detailed mechanism of action of Lanatoside C, inspired us to pursue with the exploration of the *in vivo* effects of the compound on nude mice xenografts. Xenograft models developed with Mahlavu cells on nude mice presented shrinkage of tumor size by Lanatoside C treatment (Figure 4.10). In early treatment studies, the compound also caused chemopreventive effects on xenografts models (Figure 4.11).

Consequently, this study revealed that cardiac glycosides, Lanatoside A, Lanatoside C and Glucogitoroside, comprise high cytotoxicity *in vitro* and antitumor effect *in vivo* against liver cancer at nanomolar and even picomolar concentrations. For managing heart disease, daily preventive dosages of these cardiac glycosides are between 0.125 and 0.25 mg, which is about ~2μM for an adult body, and thus non-toxic [140]. Based on the above results, these compounds can be considered as candidates for anti-cancer therapeutics or chemoprevention agents when administered at nanomolar concentrations during chronic liver disease, which often results in liver cancer in the long term.

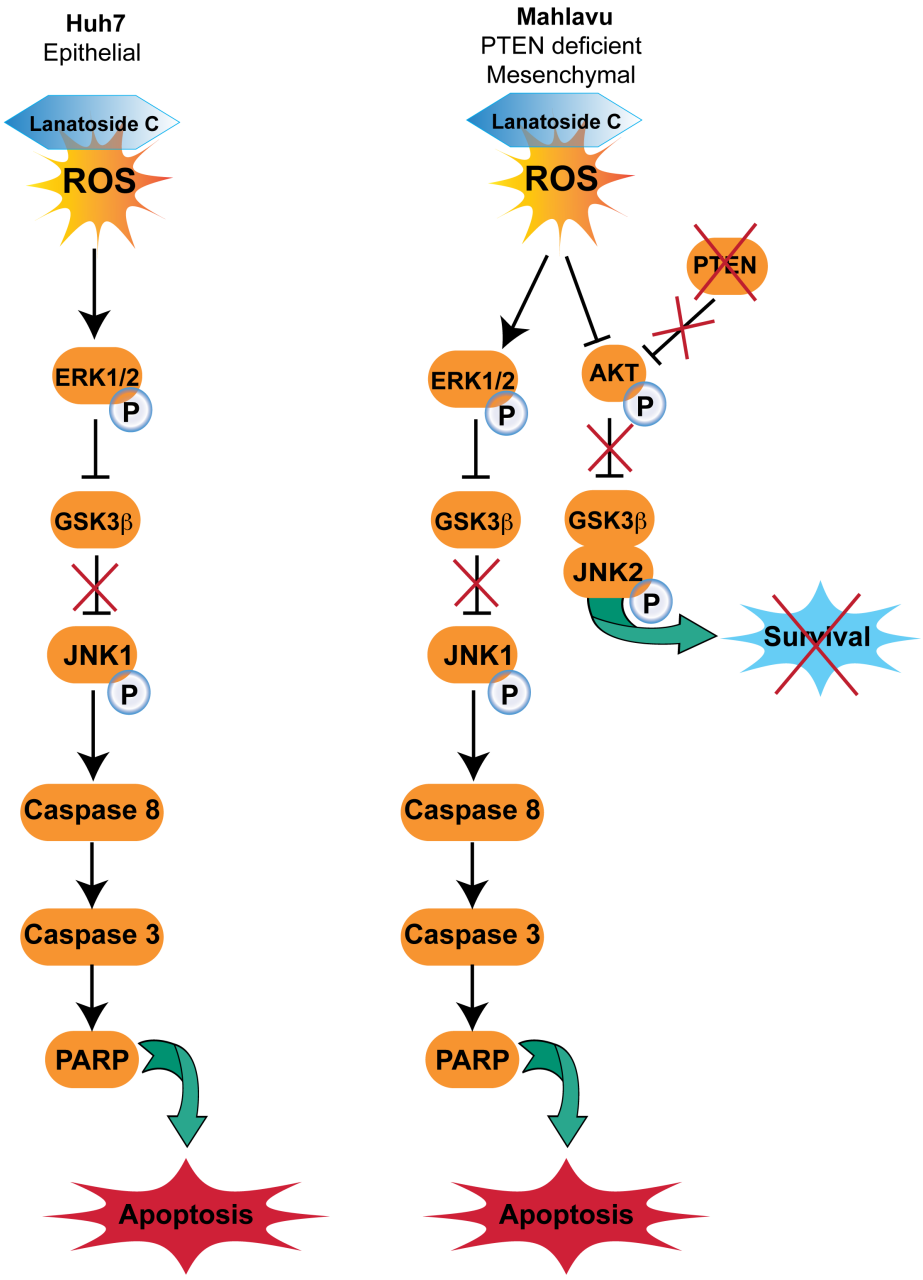


Figure 5.1. Involvement of differentially altered ERK1/2 and Akt pathways in PTEN adequate and deficient liver cancer cells treated with Lanatoside C. Huh7 and Mahlavu cells were treated with 10μM of the compounds for 24 and 48h. Western blot analysis was performed using specific antibodies.

In recent years, the repurposing of the existing drugs for novel purposes attract high attention, and non-steroidal anti-inflammatory drugs (NSAIDs) (such as aspirin, naproxen, etc.) are examples for this field. NSAIDs were reported to have anticancer activity in various cancers through COX-dependent or independent pathways[22, 141, 142]. In a part of this research, we have investigated the effects of newly-synthesized compounds derived from triazolothiadiazine and triazolothiadiazole scaffolds (synthesized by Birsen Tozkoparan and Peri Aytac from Hecettepe University). Initial SRB assay revealed that 11 of the 67 compounds tested had significant growth inhibitory activity against epithelial cancer cells (Tables 4.3, 4.4 and 4.5). Therefore, these 11 compounds were further tested on enlarged panel of HCC cell lines with SRB assay (Table 4.6). Also, the cytotoxic activity of the compounds in real-time was confirmed using RT-CES assay (Figure 4.12). As a first step in determining the cell death type induced, the change in the cell nuclei was observed using Hoechst stain on cells treated with the 11 selected compounds. Data showed apoptosis like morphological changes in cells treated with especially IBUTRI-BR-TDZ, IBUTRI-CH3-TDZ, IBUTRI-OCH3-TDZ, IBUTRI-OCF3-TDZ AND FLUTRI-CL-TDZ and FLUTRI-OCH3-TDZ (Figure 4.13). Results of cell cycle analysis (G2/M arrest) supported that apoptosis was induced by these compounds (Figure 4.14 and 4.15). Based on the results obtained, compounds IBUTRI-OCH3-TDZ and IBUTRI-CH3-TDZ were chosen for the investigation of detailed mechanism of action.

The results about mechanism of action demonstrated that compounds IBUTRI-OCH3-TDZ and IBUTRI-CH3-TDZ caused ROS accumulation, which in turn results in the inhibition of Akt, and the activation of ASK-1 proteins. ASK-1, which is a major component of ROS pathway, then activates JNK protein and result in the activation of apoptosis (Figures 4.16-18 and Figure 5.2). Furthermore, inhibition of Akt protein then stimulates the activation of GSK3βand thus the phosphorylation-dependent degradation of β-catenin. Cyclin D1 levels were also altered by IBUTRI-OCH3-TDZ and IBUTRI-CH3-TDZ as a result of both the inhibition of its transcriptional activator β-catenin and activation of its degradation by GSK3β. Decrease in Cyclin D1 protein levels leads to SubG1 arrest and induction of apoptosis in liver cancer cells treated with IBUTRI-OCH3-TDZ and IBUTRI-CH3-

TDZ (Figures 4.16-18 and Figure 5.2). Moreover, these two molecules possess COX inhibitory properties similar to conventional NSAIDs (Figure 4.19). There are studies showing that COX-2 related dephosphorylation of Akt results in apoptosis induction in HCC [143]. Therefore, these results suggest that IBUTRI-OCH3-TDZ and IBUTRI-CH3-TDZ molecules inhibits Akt pathway through both ASK-1/JNK and COX related pathways. Consequently, these results about the novel synthesized triazolothiadiazines and triazolothiadiazoles (derived from known NSAIDs) indicates that they can be potentially good candidates in liver cancer therapeutics.

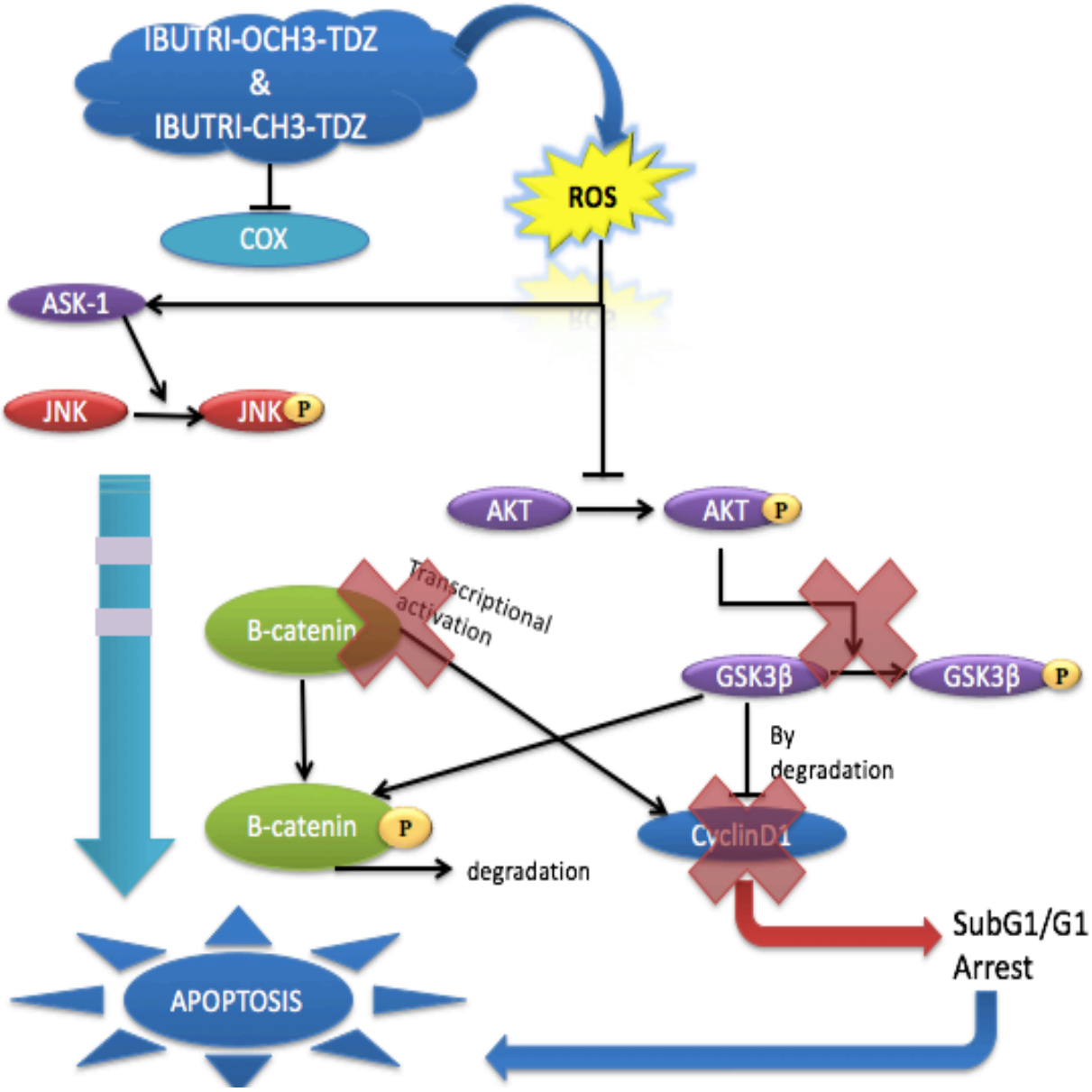


Figure 5.2. The proposed action mechanism of IBUTRI-OCH3-TDZ and IBUTRI-CH3-TDZ. Treatment with the compounds caused the induction of oxidative stress (ROS), the activation of ASK-1 and the inactivation of Akt. ASK-1 in return activates SAPK/JNK. Akt protein inhibition resulted in activation of GSK3β (non-phosphorylated form), which induced phosphorylation of β-catenin and thus its degradation. Finally, molecules induced decrease in CyclinD1 levels resulting in $SubG_1/G_1$ arrest and apoptotic cell death in liver cancer cells.

Due to their fundamental role in various cellular processes such as cell growth or proliferation nucleobase and nucleoside analogues have been used in cancer treatments for a long time.[97]. In this research, nucleobase and nucleoside analogues were synthesized by Meral Tuncbilek's group from Ankara University. 127 novel compounds were analyzed primarily on breast (MCF7), colon (HCT116) and liver (Huh7) cancer cells (Table 4.6). At this step, Camptothecin (CPT), 5-fluorourasil (5-FU) Pentostatine, Fludarabine and Cladribine were used as positive controls. 24 of the compounds showed significant growth inhibitory effects and these molecules had IC50 values less than 10μM (Table 4.6). These 24 compounds were analyzed in enlarged HCC panel by SRB (Table 4.7) and their cytotoxic effects were then confirmed with RT-CES experiment in real-time (Figure 4.21). After these two experiments, 6 of the 24 compounds (ME9-11-13-23-46 and 47) were selected for further molecular analysis on liver cancer cells. Hoechst analysis suggested induction of apoptosis by these compounds due to the presence of apoptosis like morphological changes such as horse-shoe structures or condensed nuclei (Figure 4.22). Furthermore, FACS analysis revealed SubG1/G1 cell cycle arrest by these molecules and especially ME46 (Figure 4.23 and 4.24) which supports apoptotic induction in liver cancer cells by these novel nucleobase/nucleoside analogues. Possible kinase inhibitory effect of these compounds were also investigated but no inhibition was observed (Figure 4.25). In order to confirm apoptosis as the type of cell death that is triggered by ME compounds, PARP cleavage was analyzed by Western Blot analysis. Results were in correlation with FACS and immunofluoresance images. Treatment with ME compounds induced PARP cleavage in liver cancer cells which supports apoptosis induction (Figure 4.26). Due to the fact that in previous studies nucleobase analogues were shown to effect Src pathway, phosphorylation status of Src protein was explored in liver cancer cells treated with ME compounds. In ME9, ME46 and ME47, phospho-Src levels were decreased meaning that Src protein was inhibited (Figure 4.27 and Figure 5.3). Moreover, the effect of the compounds on downstream elements were also investigated. Cyclin E and cdk-2 levels were also decreased in treated cells when compared to DMSO controls as well as p-Rb levels very significantly in ME9 and 46 (Figure 4.28 and Figure 5.3). Consequently, newly synthesized novel nucleobase and nucleoside derivatives transports inside the cell

and inhibits the phosphorylation of Src protein thus disrupts the formation of Cyclin E and cdk2 complex. This complex would normally induce phosphorylation of Rb and inhibits it. Decrease in the phosphorylation of Rb, meaning its activation, was observed in treated cells compared to DMSO controls and thus inhibition of cell cycle (Figure 4.27 and Figure 5.3) in liver cancer cells.

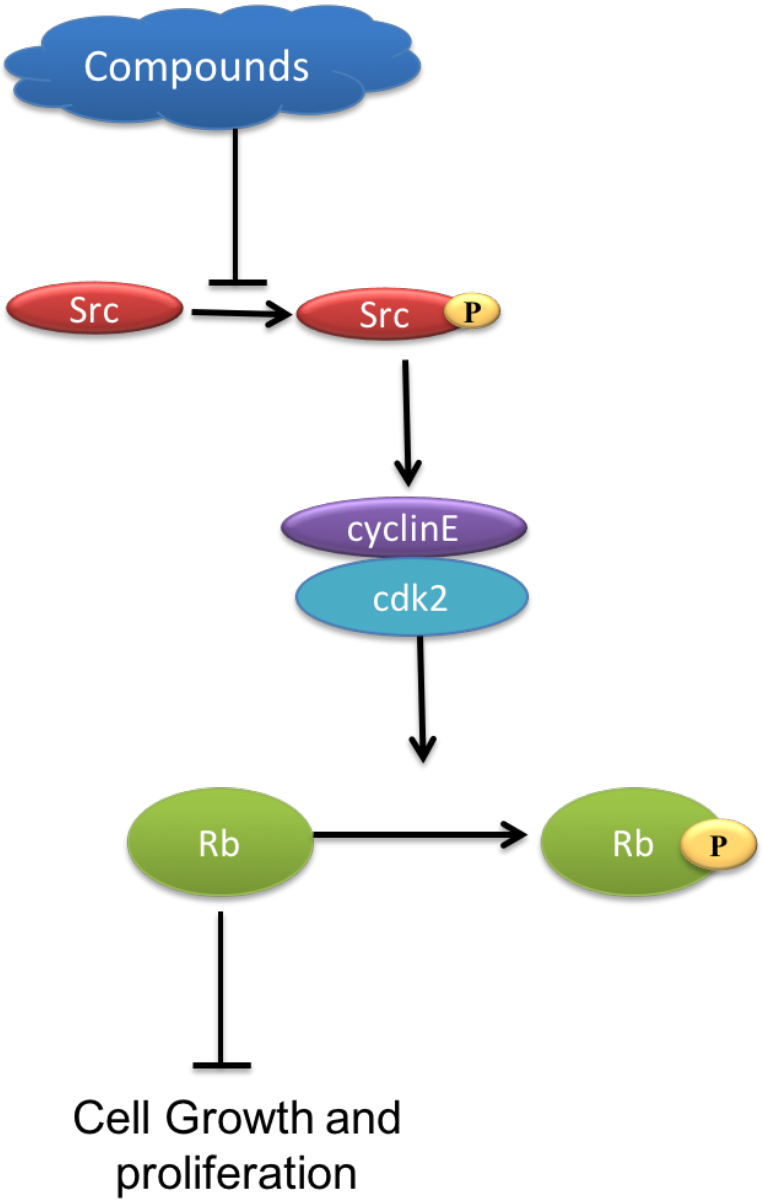


Figure 5.3. The effect of novel nucleobase/nucleoside analogues on Src pathway resulting in inhibition of cellular growth and proliferation in liver cancer cells.

CHAPTER 6. FUTURE PERSPECTIVES

All together our results indicated that all three group of molecules can be considered as potential candidates for anticancer research with different action mechanisms in liver cancer. Cardiac glycosides, specifically Lanatoside C, induced ROS related differentially effected ERK1/2 and Akt pathways in drug-sensitive Huh7 and drug-resistant Mahlavu liver cancer cells, which was investigated both *in vitro* and *in vivo*. Novel nucleobase/nucleoside analogues resulted in alteration of Src pathway. Finally, the third group, novel thiadiazine compounds originated from traditional non-steroidal analgesic anti-inflammatory drugs (NSAIDs) also induced ROS but was effective on B-catenin, CyclinD1 pathway together with ASK1/JNK pathway.

Some future experiments can be efficient and essential in order to further confirm the activity of the molecules but also contribute to their anticancer properties.

Cardiac Glycosides:

- To support our findings further clinical analysis can be done in a large cohort of patients taking cardiac glycosides for their cardiac conditions having liver disease at the same time similar to the previous other cancer studies.
- Cytotoxic activities of the compounds should be analyzed on normal-like cell lines to ensure that the action is cancer specific and that the molecules will not be toxic to healthy cells.
- The *in vivo* experiments mentioned in this research was performed only on xenografts produced with Mahlavu cells. Further *in vivo* experiment on xenografts produced by Huh7 cells should be performed.
- The *in vivo* analysis should be further detailed such that tissue samples will be gathered from the tumor side and will be analyzed using staining experiments and western blot experiments. These experiments will be a confirmation for the in vitro data for the changes in the levels of specific proteins.

Thiadiazine compounds:

- In order to confirm the *in vitro* data, *in vivo* xenograft models on nude mice should be analyzed after treatment with the selected compounds. Also survival assay should be performed with nude mice by Kaplan Meier survival analysis.
- Wound-healing experiment with time-lapse microscopy is necessary in order to explore the effect of the molecules on cell migration.
- RNA-seq, microarray or protein-array experiments can be performed in order to designate the specific target of the molecules
- ROS inhibitor can be used to further investigate the outcome of treatment with these molecules when ROS is inhibited.

Nucleobase/nucleoside analogues:

- *In vivo* experiments should be performed on liver cancer xenograft models with nude mice.
- Synthesized molecules were suspected to have kinase inhibitory activity. During this research, the kinase activity assay kit used was not actually specific to kinase activity but measured the ATP present in the environment. Thus, more extensive and targeted Kinase Assay Kits (For instance KinomeScan) should be used to investigate the possible kinase inhibitory activity.
- Since these compounds are nucleobase and nucleoside analogues, DNA-damage pathway should be explored in cells treated with these compounds. H2AX staining can be performed together with analysis of levels of ATM/ATR proteins in cancer cells treated with these compounds.

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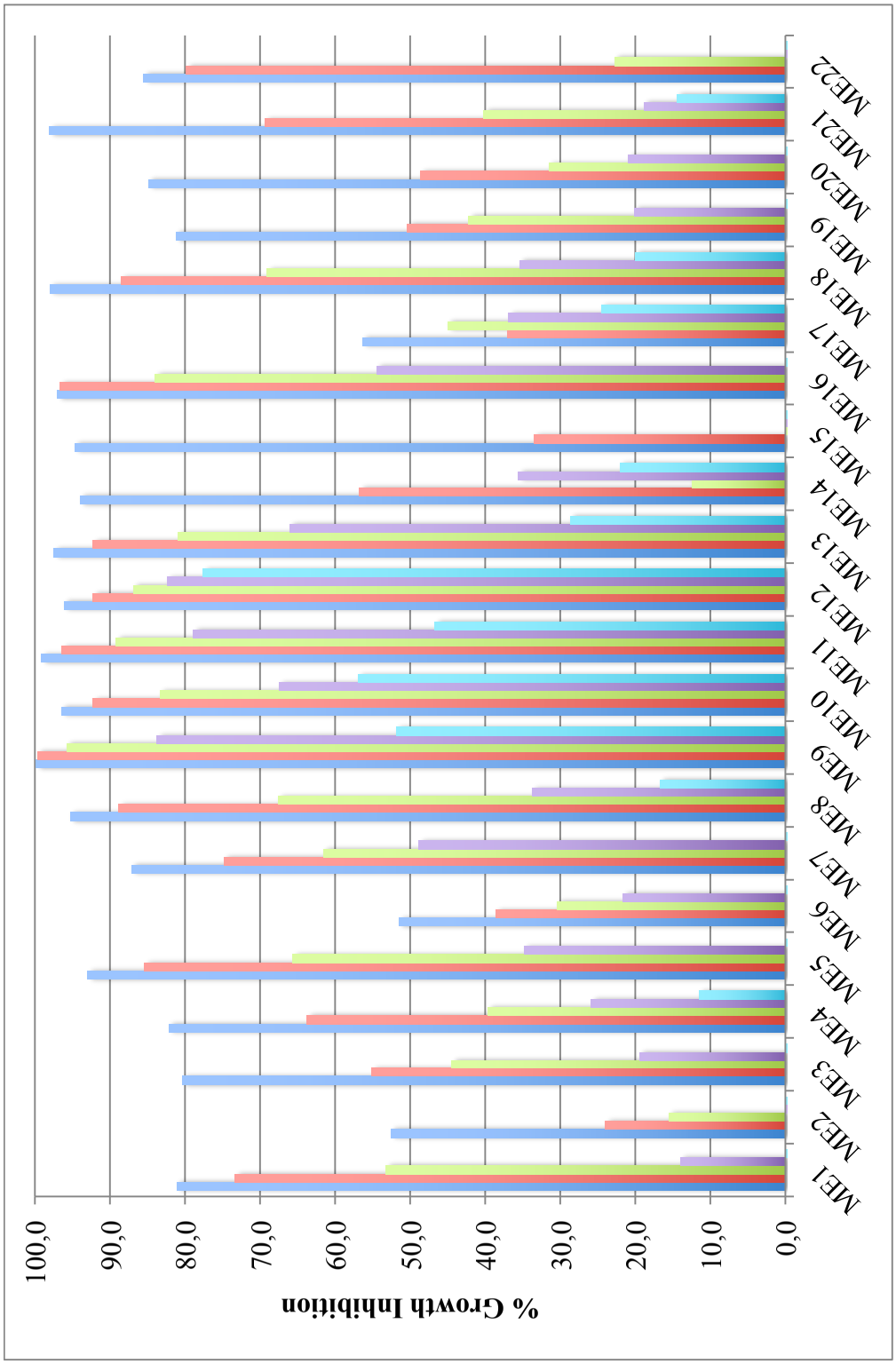
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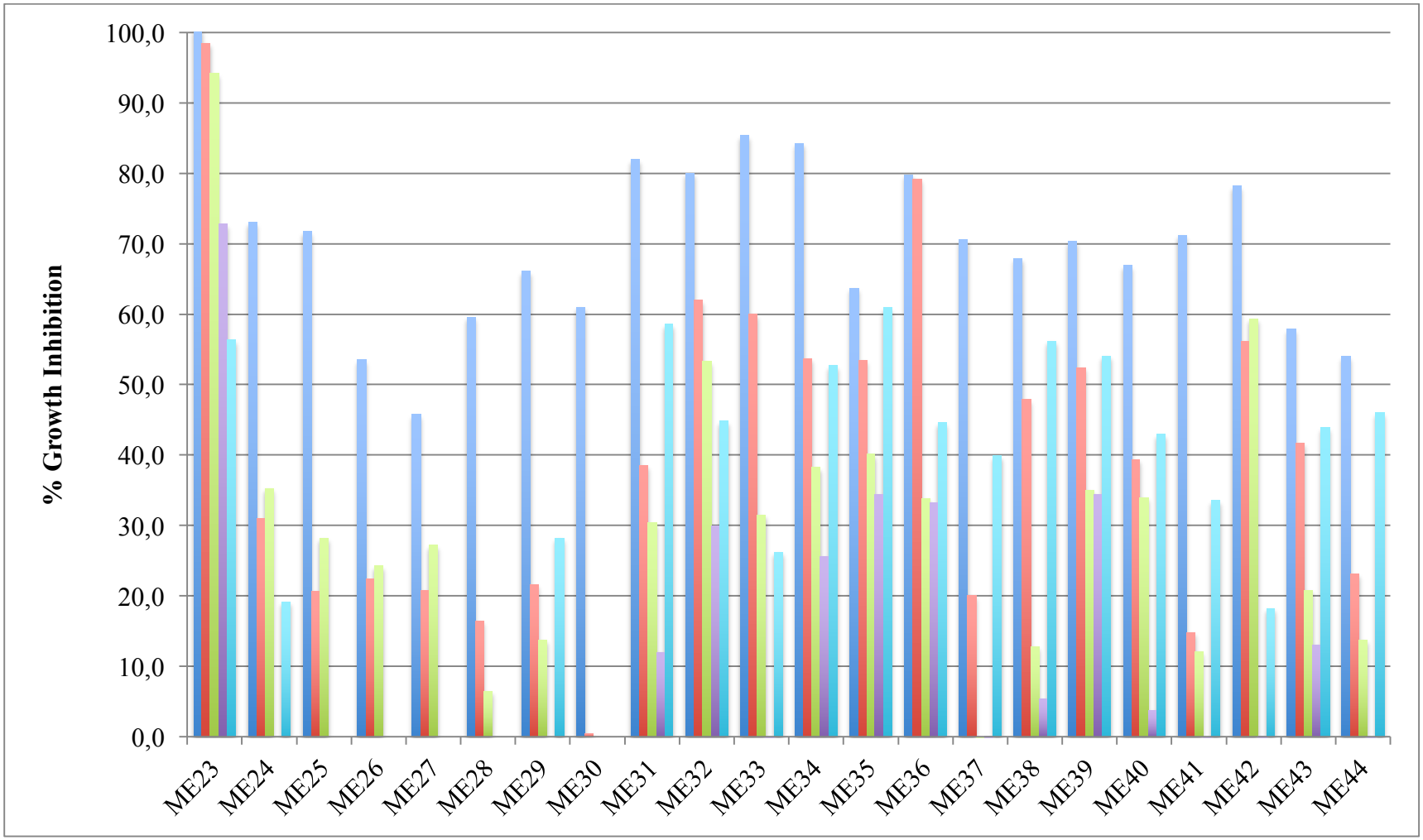
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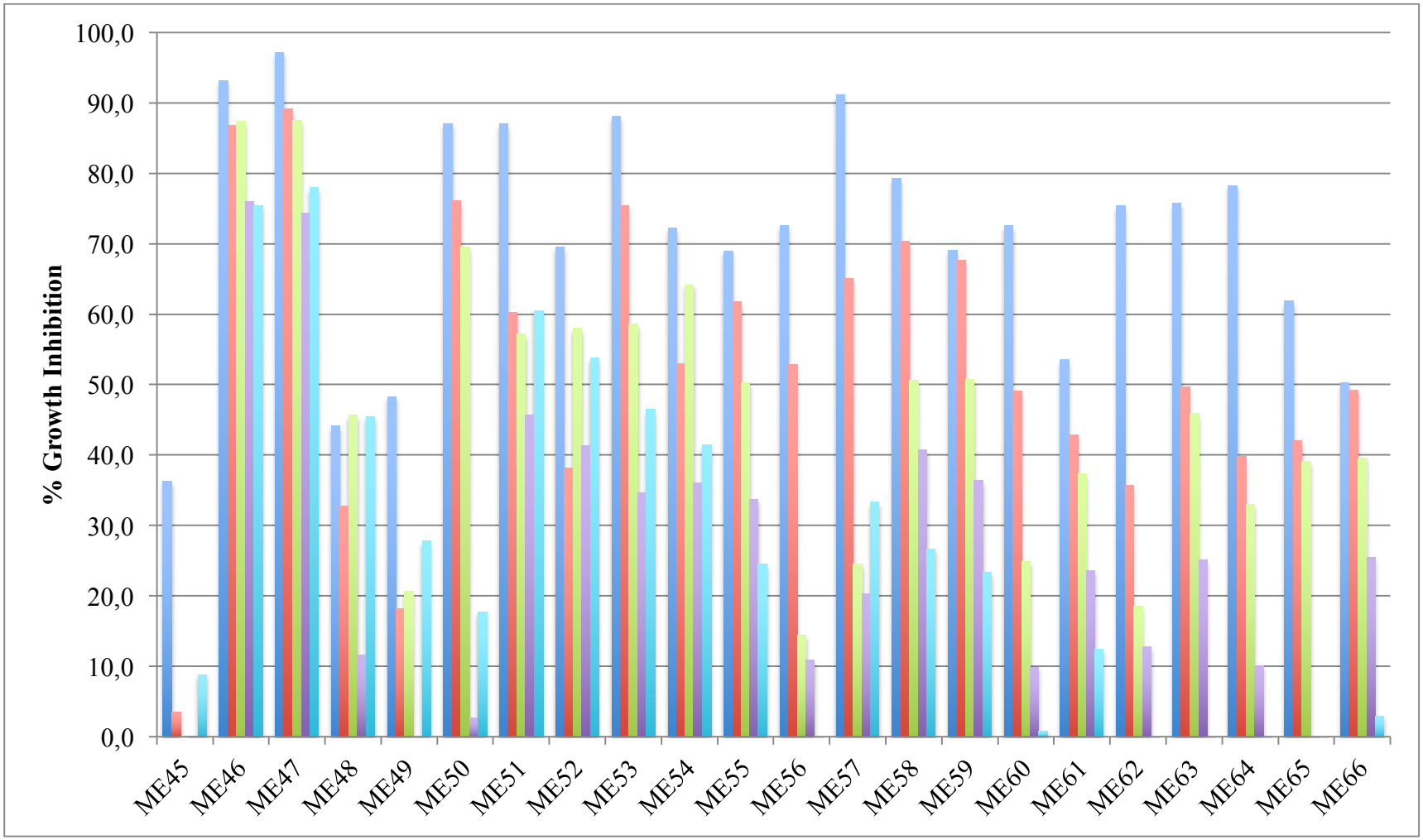
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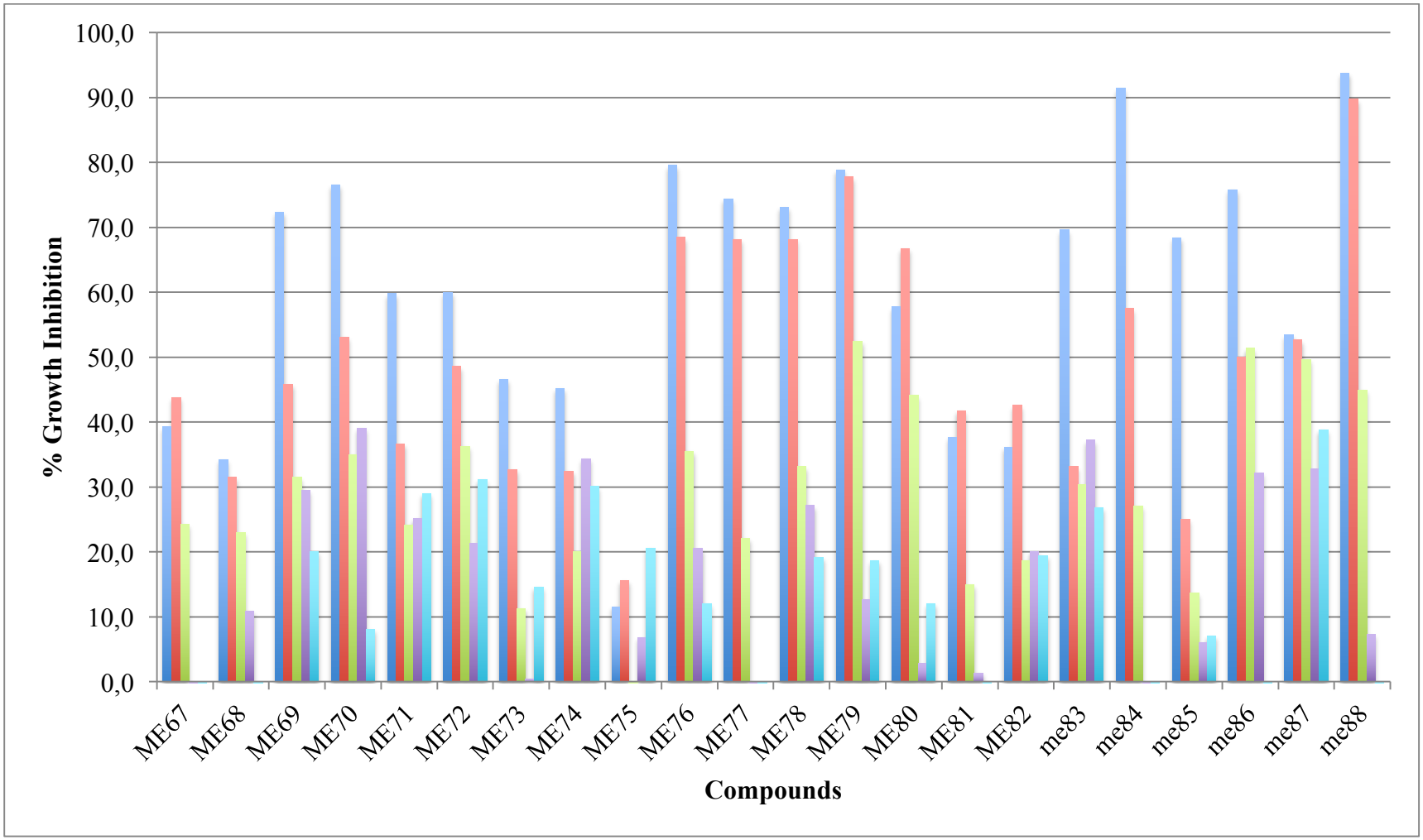
SUPPLEMENTARY

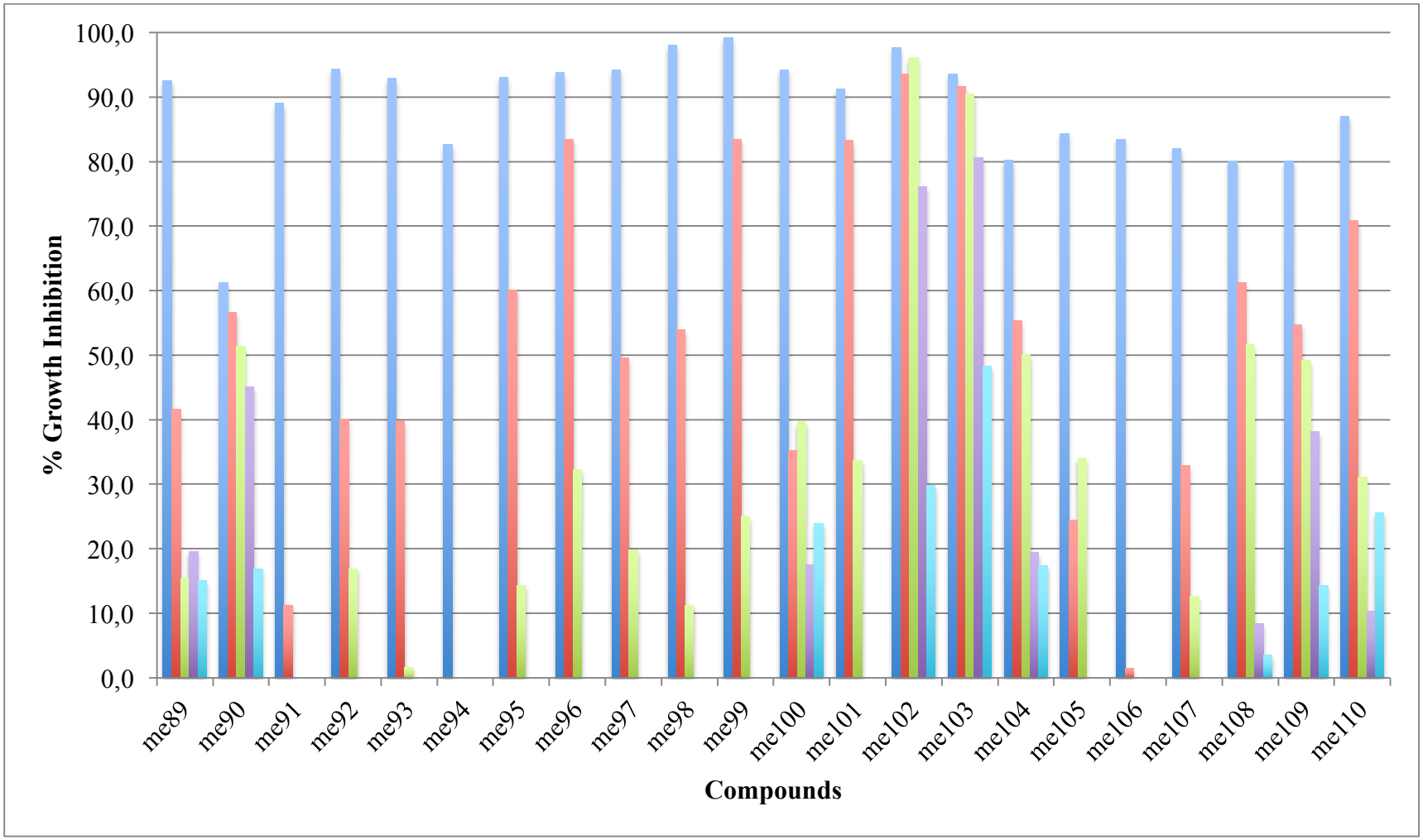
Growth Inhibition Graphs of Novel Nucleobase and Nucleoside Analogues

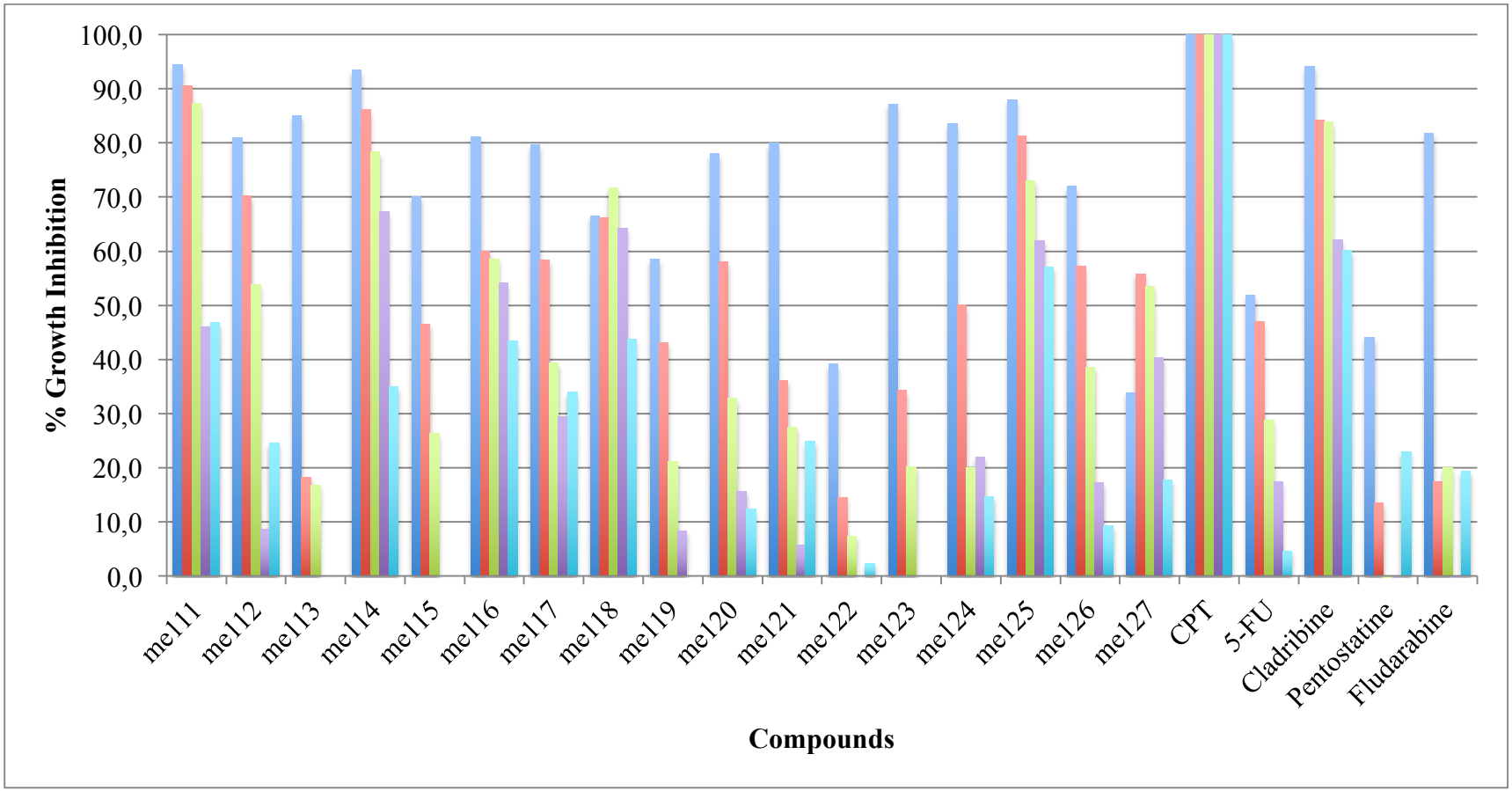



% Growth Inhibition
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
ME23
ME24
ME25
ME26
ME27
ME28
ME29
ME30
ME31
ME32
ME33
ME34
ME35
ME36
ME37
ME38
ME39
ME40
ME41
ME42
ME43
ME44

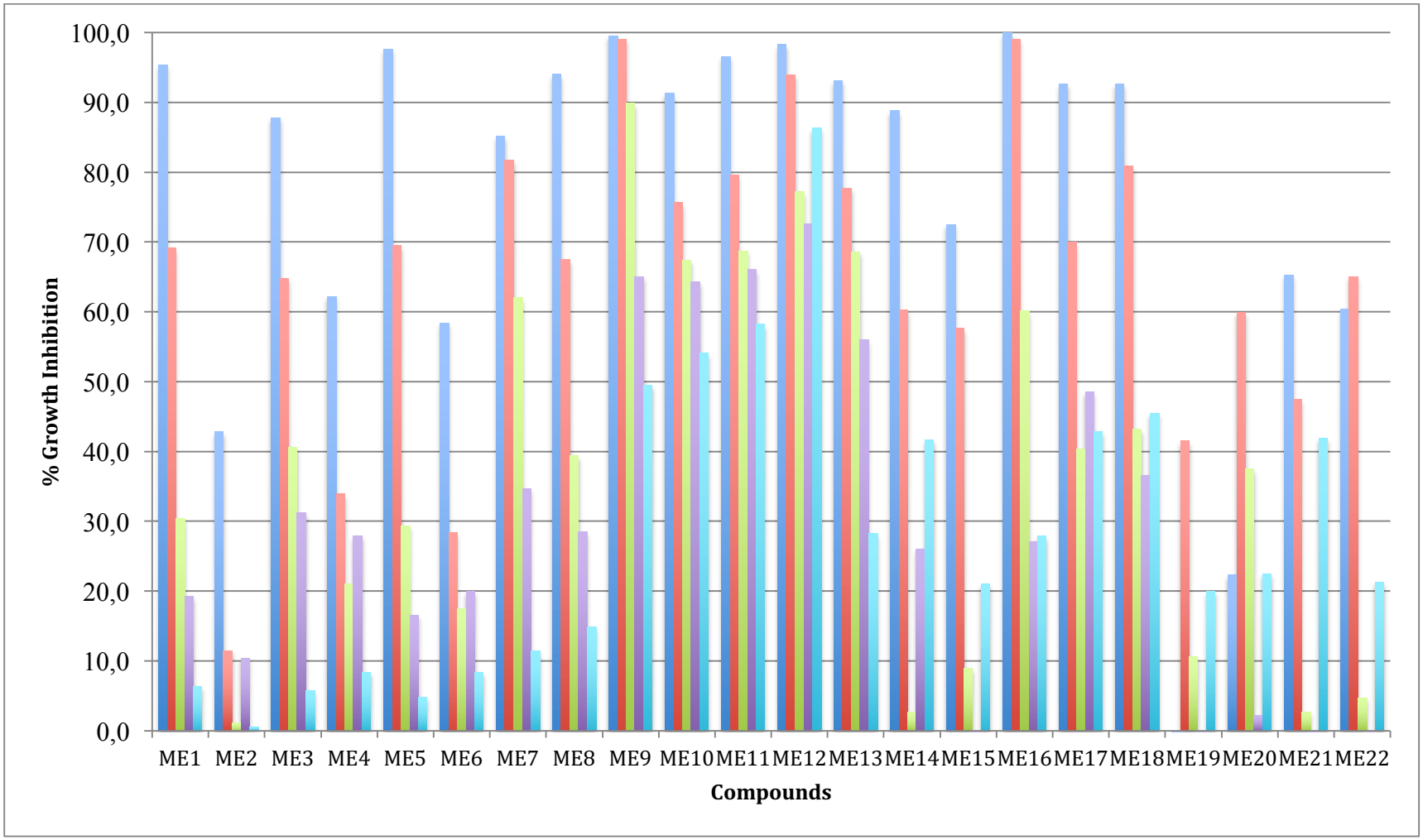

% Growth Inhibition
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
ME45
ME46
ME47
ME48
ME49
ME50
ME51
ME52
ME53
ME54
ME55
ME56
ME57
ME58
ME59
ME60
ME61
ME62
ME63
ME64
ME65
ME66

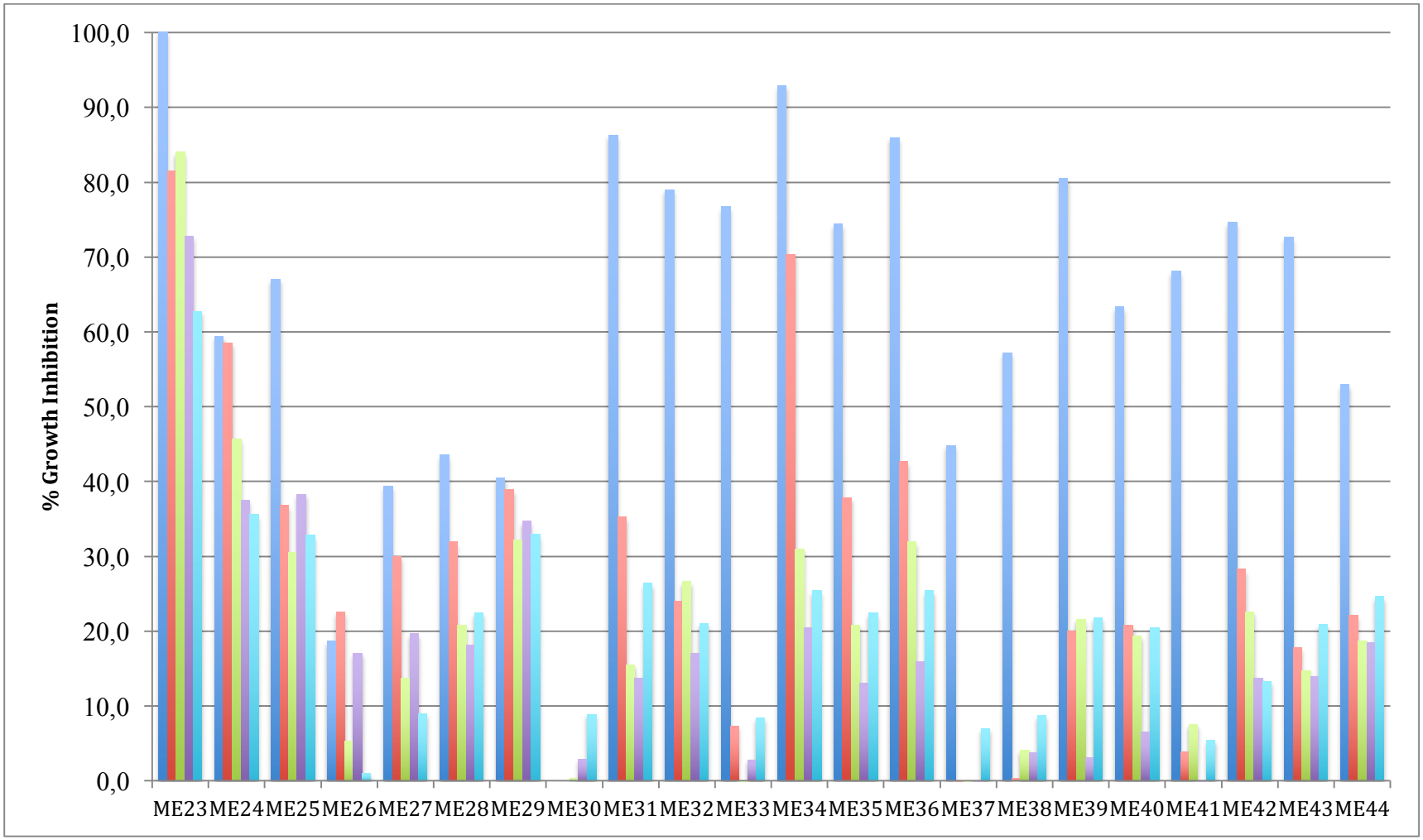

100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% Growth Inhibition
ME67
ME68
ME69
ME70
ME71
ME72
ME73
ME74
ME75
ME76
ME77
ME78
ME79
ME80
ME81
ME82
me83
me84
me85
me86
me87
me88
Compounds

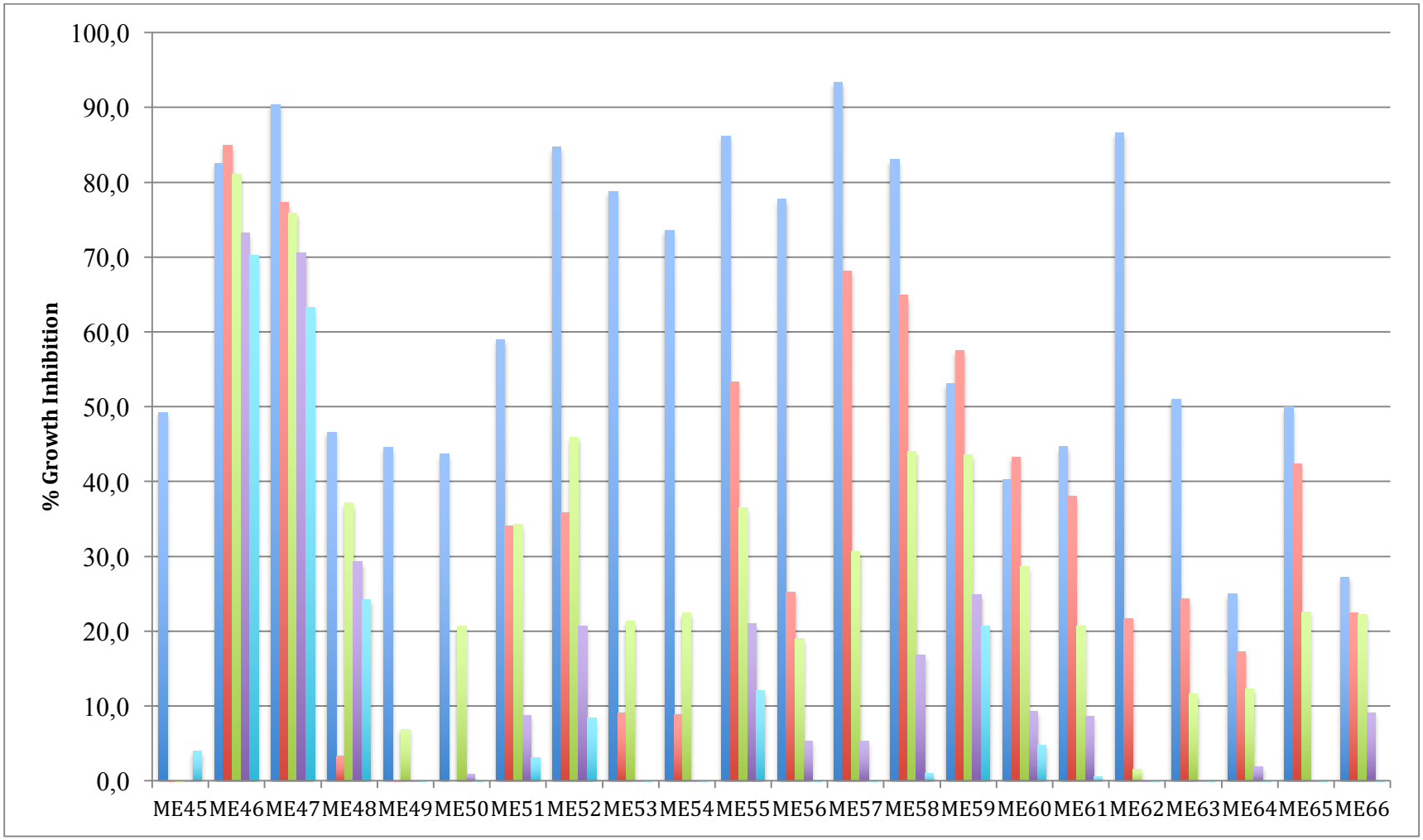

100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% Growth Inhibition
me89
me90
me91
me92
me93
me94
me95
me96
me97
me98
me99
me100
me101
me102
me103
me104
me105
me106
me107
me108
me109
me110
Compounds

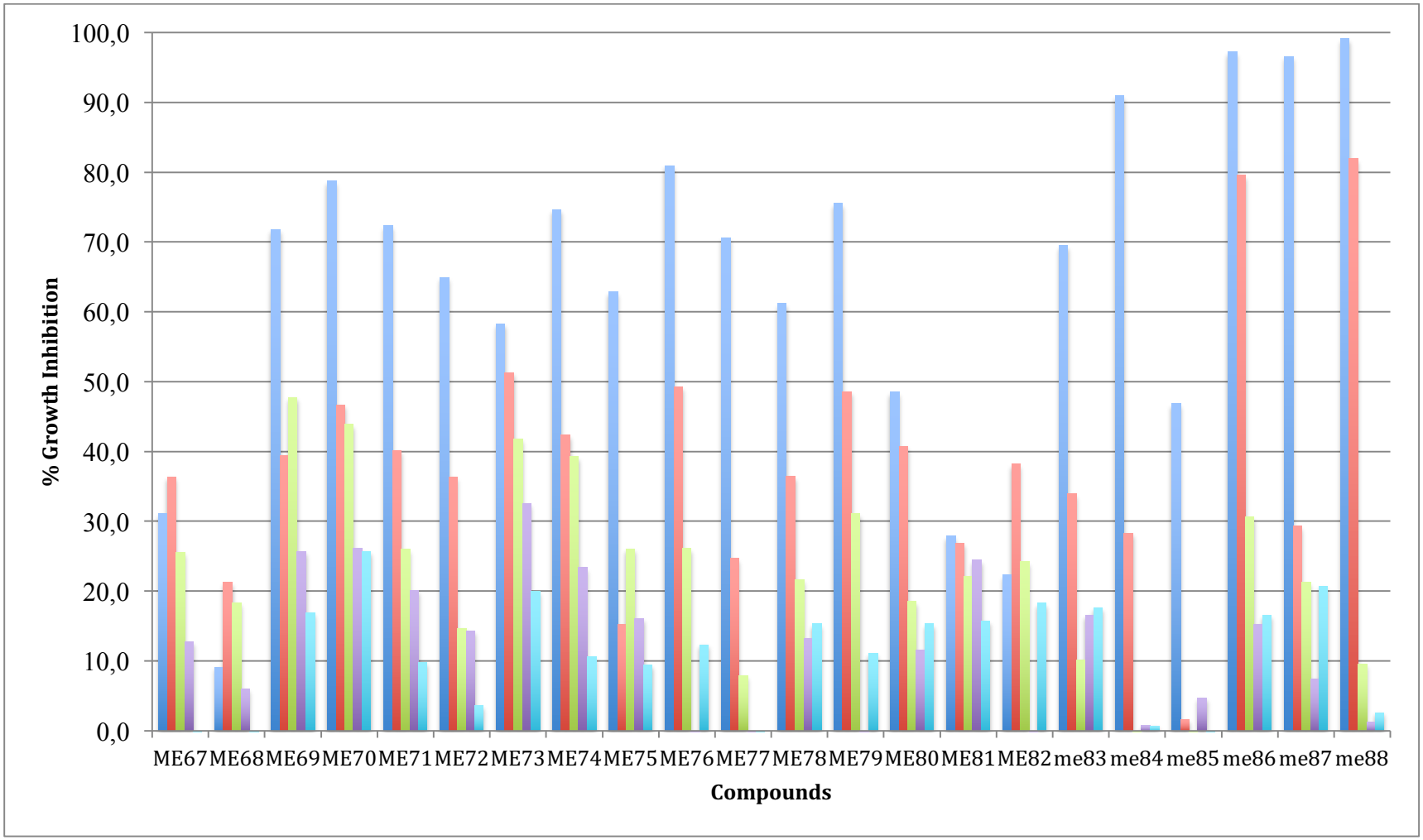


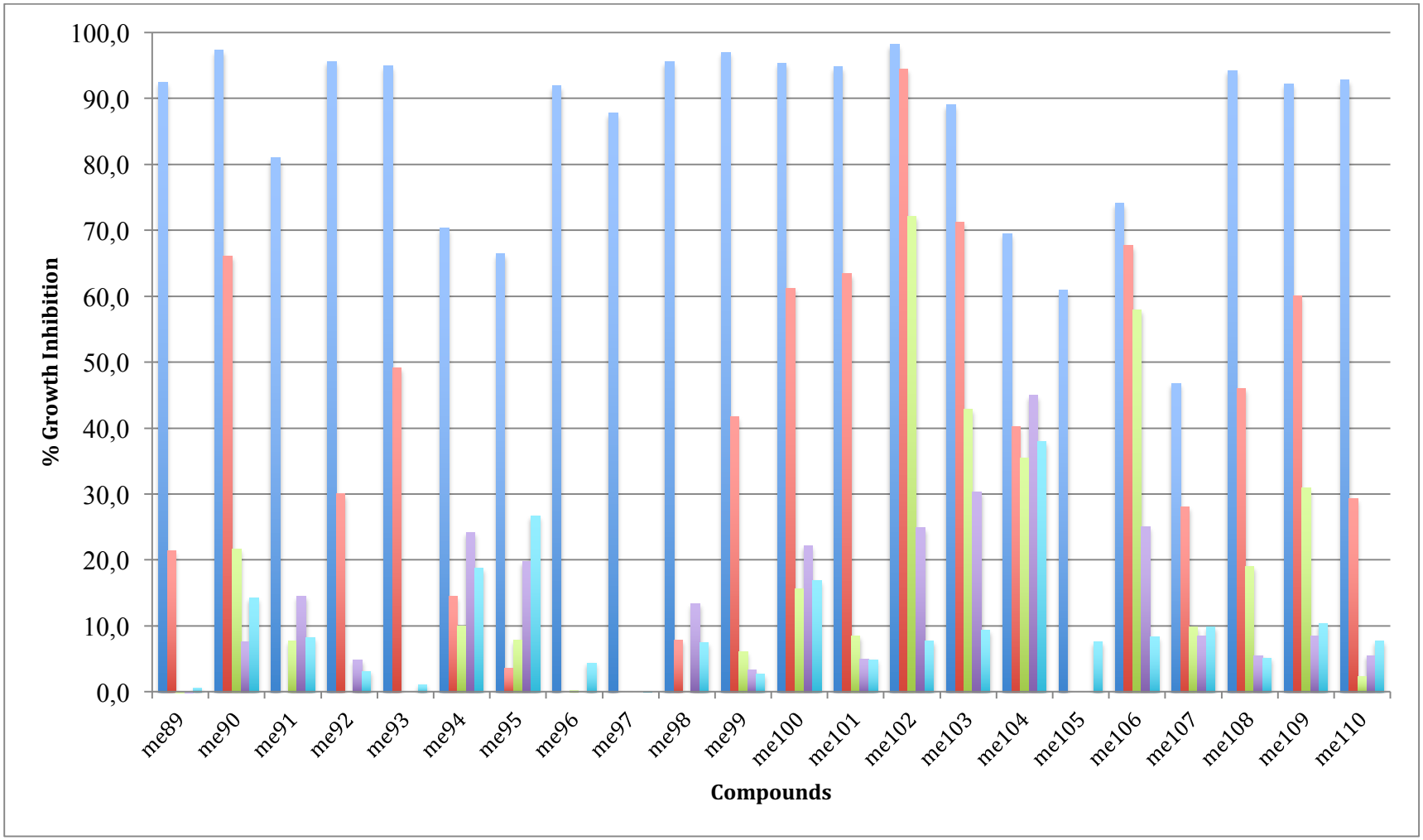
Supplementary Figure 1. Growth Inhibition of novel nucleobase and nucleoside analogues in Huh7 liver cancer cell lines. Liver cancer cells were treated with small molecules (40µM(dark blue), 20µM (red), 10µM (green), 5µM (purple), 2.5µM(light blue))for 72h. NCI60 SRB analysis was applied as explained in methods.

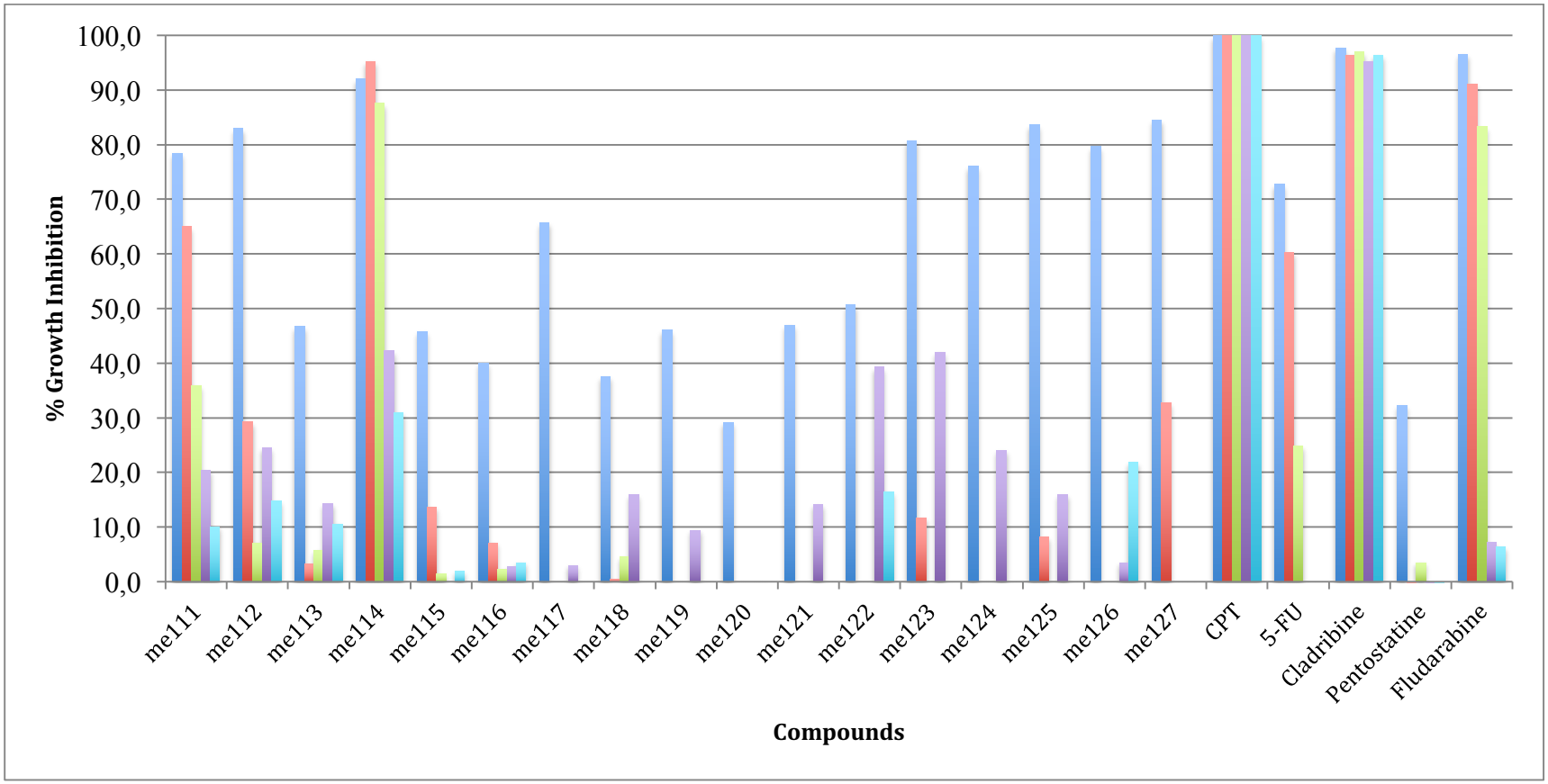

100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% Growth Inhibition
ME1 ME2 ME3 ME4 ME5 ME6 ME7 ME8 ME9 ME10 ME11 ME12 ME13 ME14 ME15 ME16 ME17 ME18 ME19 ME20 ME21 ME22
Compounds


% Growth Inhibition
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
ME23 ME24 ME25 ME26 ME27 ME28 ME29 ME30 ME31 ME32 ME33 ME34 ME35 ME36 ME37 ME38 ME39 ME40 ME41 ME42 ME43 ME44

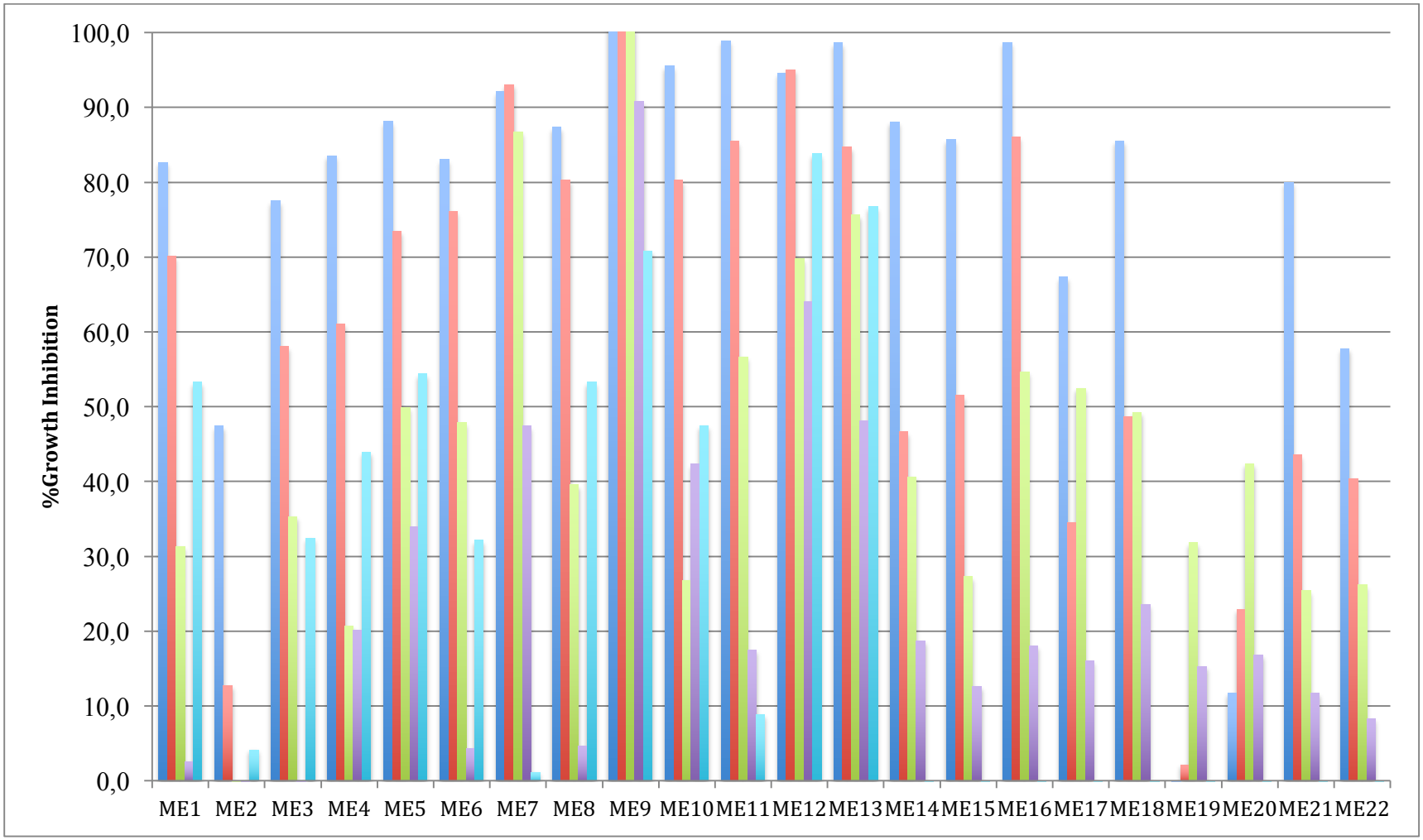

% Growth Inhibition
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
ME45 ME46 ME47 ME48 ME49 ME50 ME51 ME52 ME53 ME54 ME55 ME56 ME57 ME58 ME59 ME60 ME61 ME62 ME63 ME64 ME65 ME66

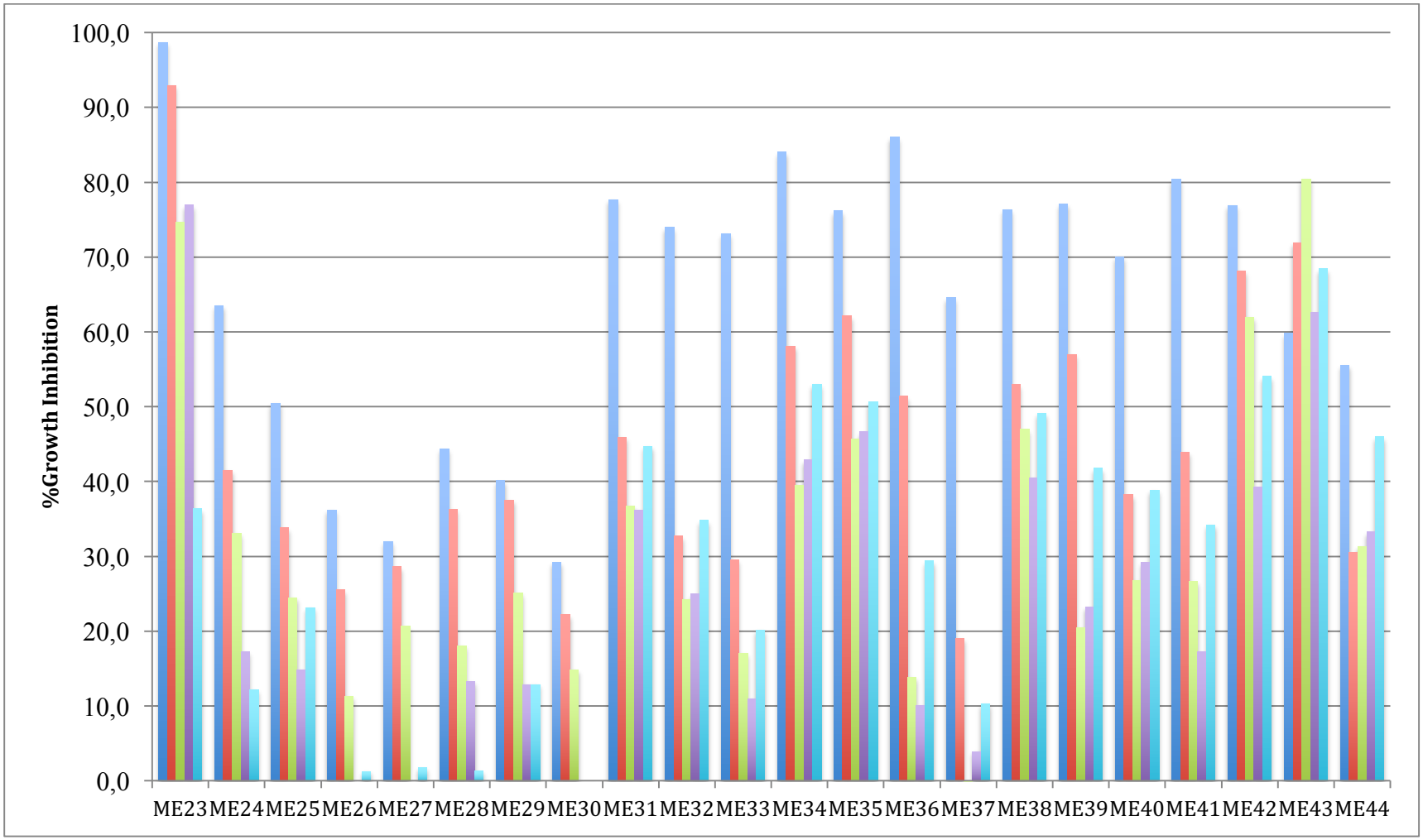

100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% Growth Inhibition
ME67 ME68 ME69 ME70 ME71 ME72 ME73 ME74 ME75 ME76 ME77 ME78 ME79 ME80 ME81 ME82 me83 me84 me85 me86 me87 me88
Compounds

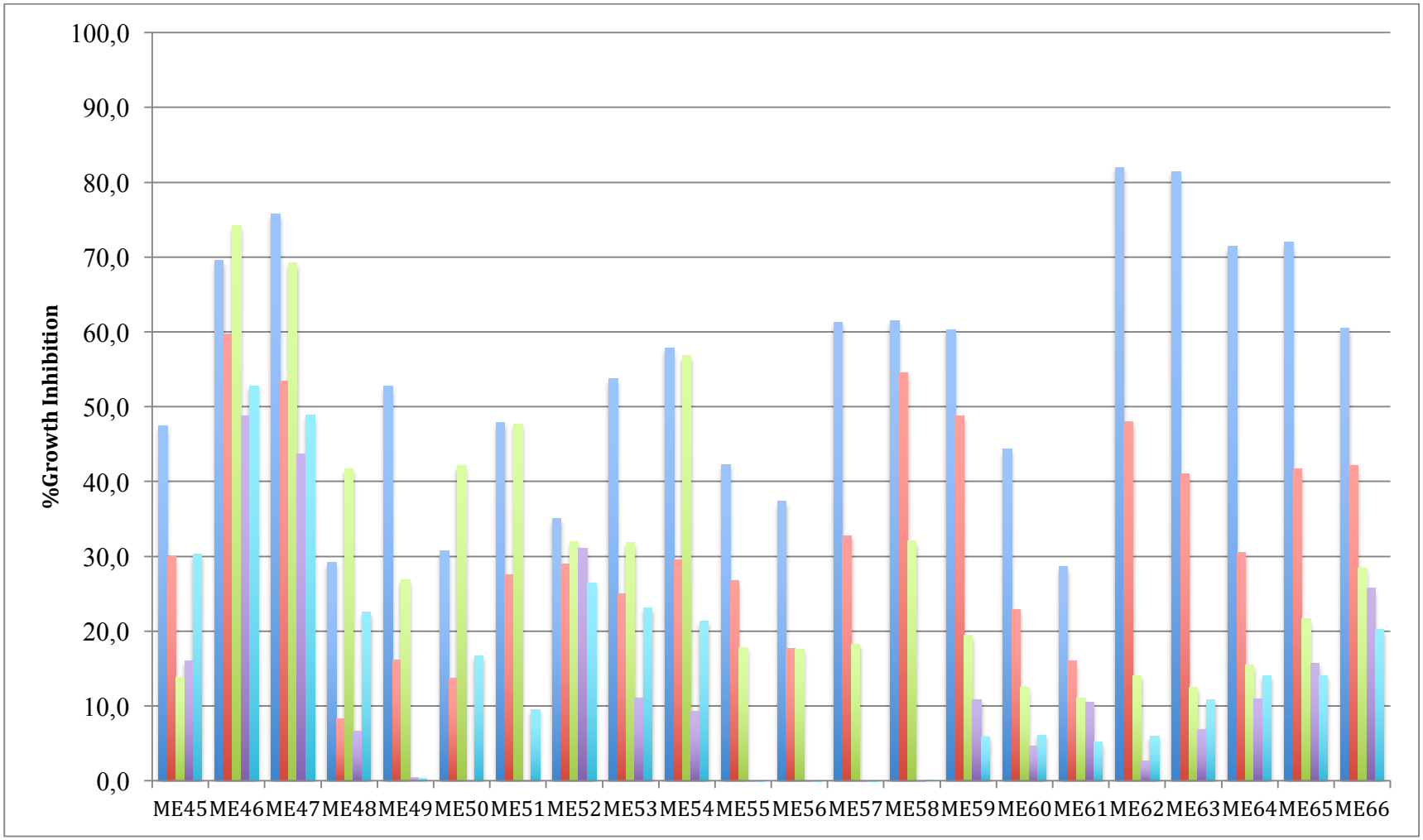

100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% Growth Inhibition
me89
me90
me91
me92
me93
me94
me95
me96
me97
me98
me99
me100
me101
me102
me103
me104
me105
me106
me107
me108
me109
me110
Compounds

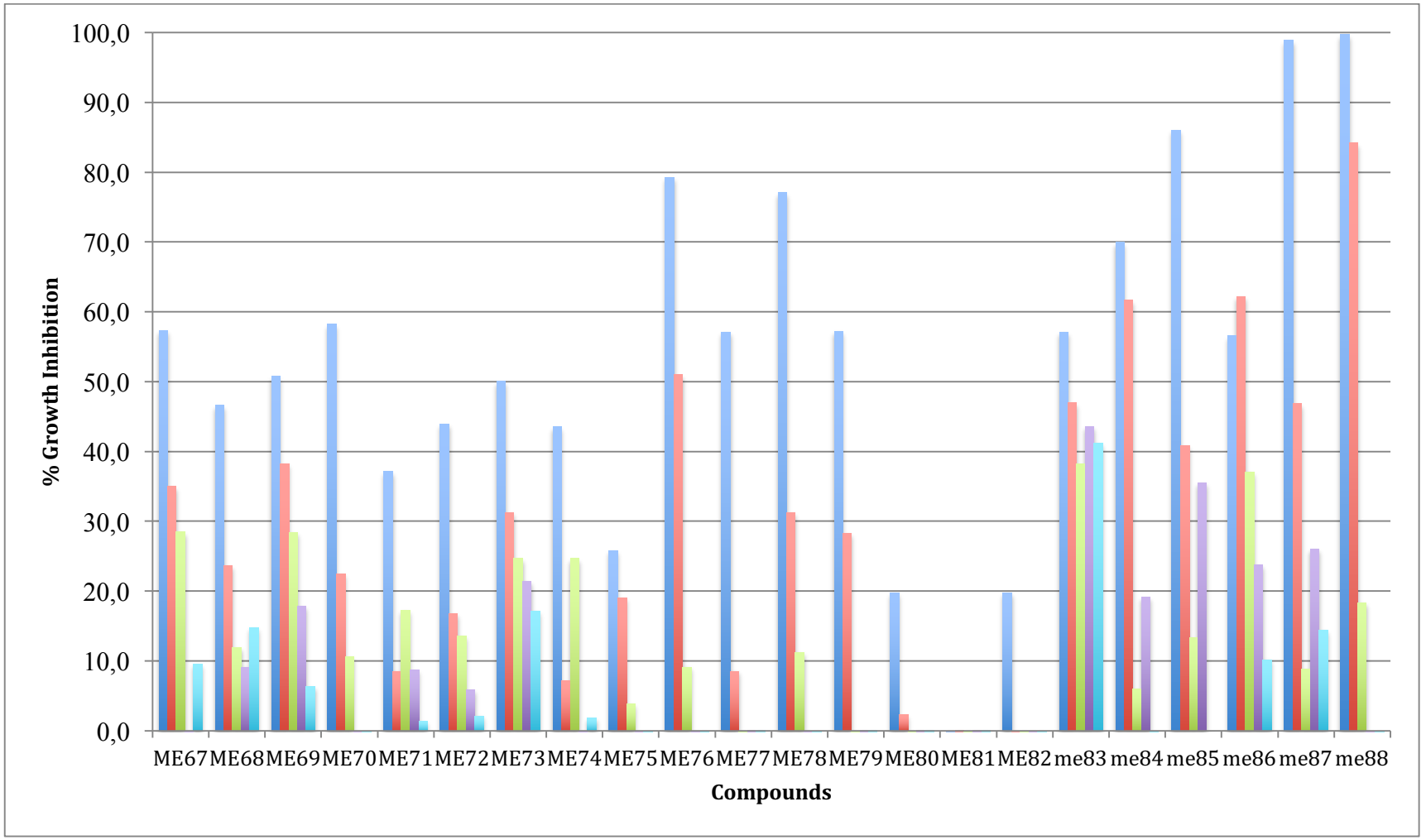


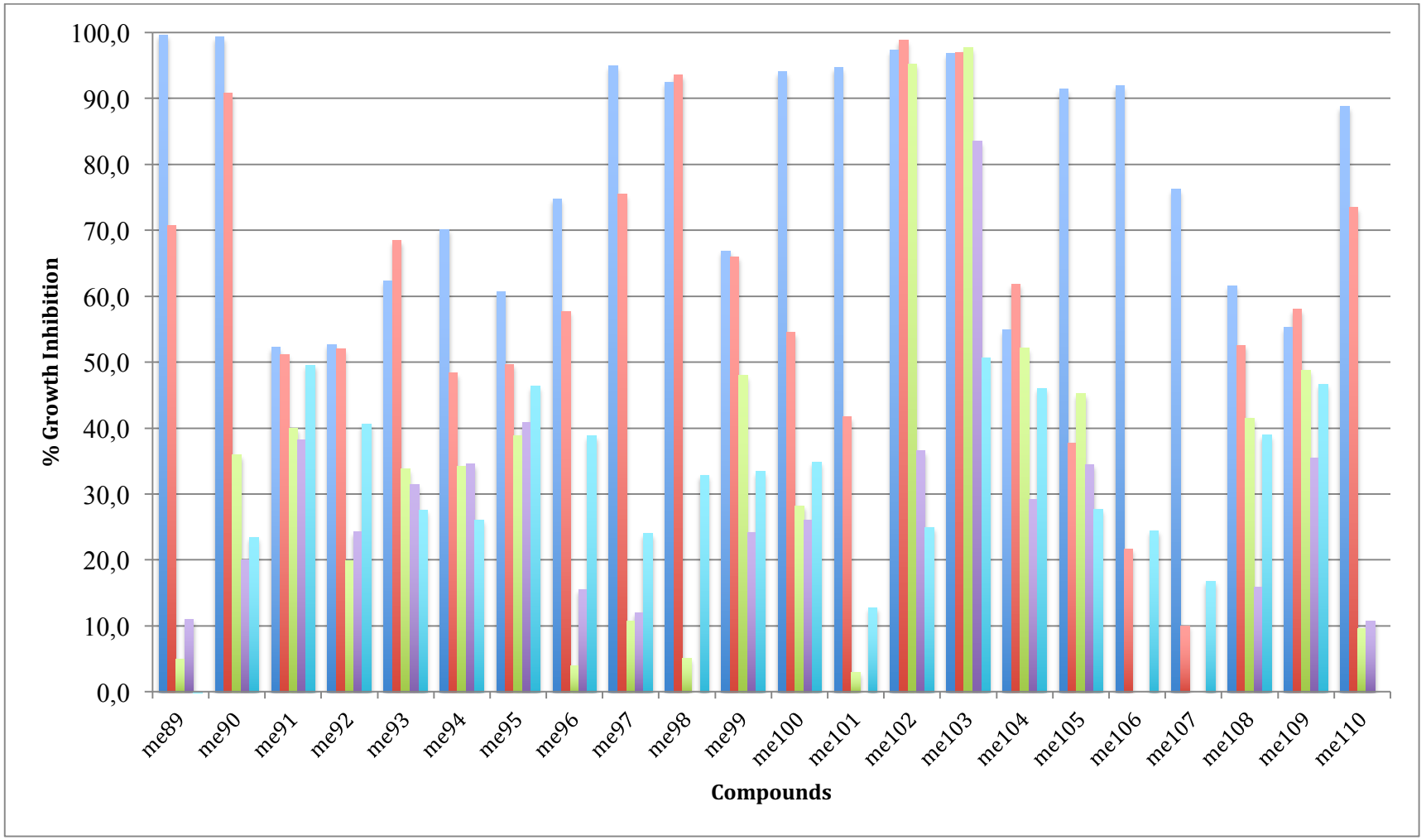
Supplementary Figure 2. Growth Inhibition of novel nucleobase and nucleoside analogues in HCT116 colon cancer cell lines. Colon cancer cells were treated with small molecules (40µM(dark blue), 20µM (red), 10µM (green), 5µM (purple), 2.5µM(light blue))for 72h. NCI60 SRB analysis was applied as explained in methods.

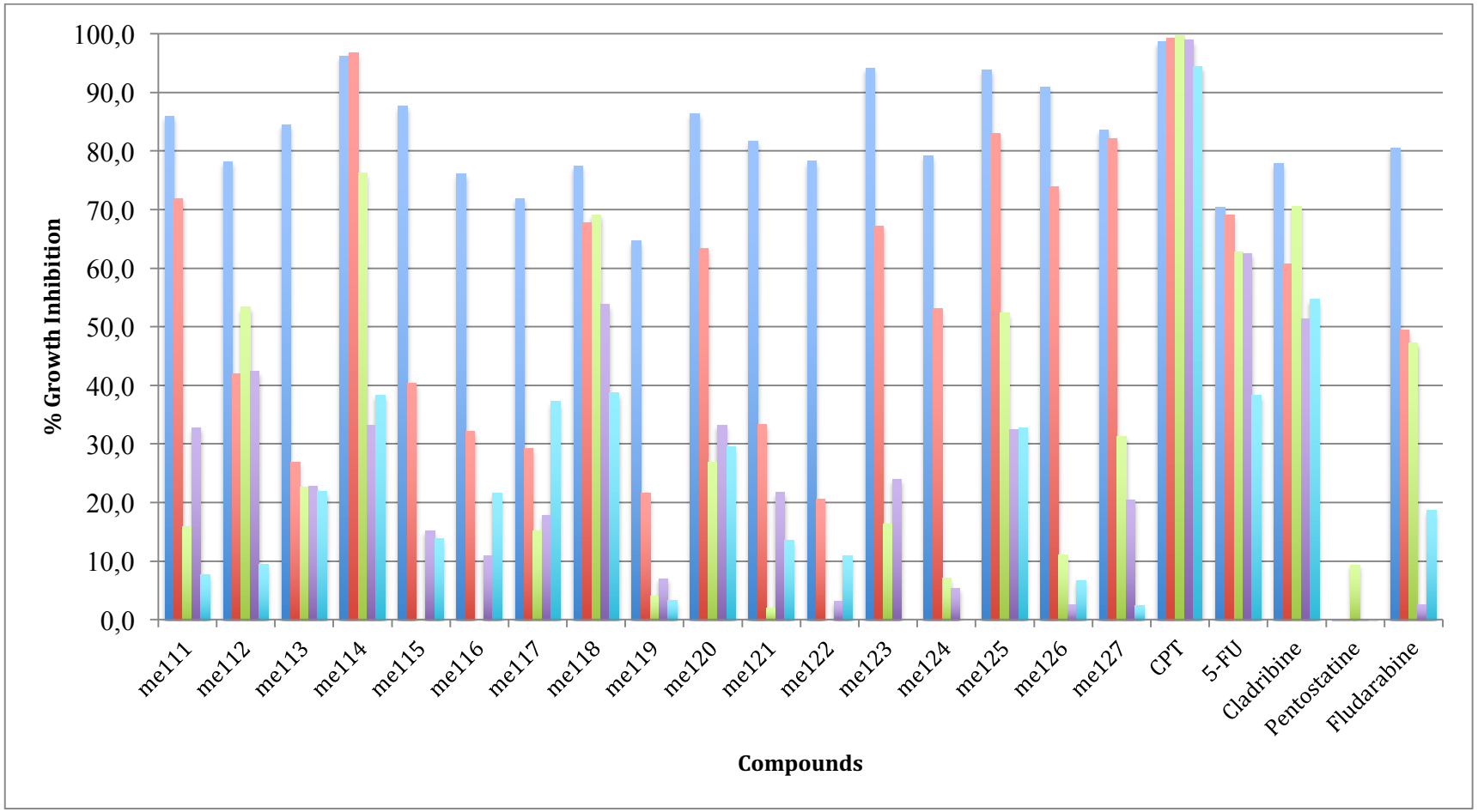

%Growth Inhibition
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
ME1 ME2 ME3 ME4 ME5 ME6 ME7 ME8 ME9 ME10 ME11 ME12 ME13 ME14 ME15 ME16 ME17 ME18 ME19 ME20 ME21 ME22


%Growth Inhibition
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
ME23 ME24 ME25 ME26 ME27 ME28 ME29 ME30 ME31 ME32 ME33 ME34 ME35 ME36 ME37 ME38 ME39 ME40 ME41 ME42 ME43 ME44


%Growth Inhibition
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
ME45 ME46 ME47 ME48 ME49 ME50 ME51 ME52 ME53 ME54 ME55 ME56 ME57 ME58 ME59 ME60 ME61 ME62 ME63 ME64 ME65 ME66


100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% Growth Inhibition
ME67 ME68 ME69 ME70 ME71 ME72 ME73 ME74 ME75 ME76 ME77 ME78 ME79 ME80 ME81 ME82 me83 me84 me85 me86 me87 me88
Compounds


100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% Growth Inhibition
me89
me90
me91
me92
me93
me94
me95
me96
me97
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me99
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me101
me102
me103
me104
me105
me106
me107
me108
me109
me110
Compounds



Supplementary Figure 3. Growth Inhibition of novel nucleobase and nucleoside analogues in MCF7 breast cancer cell lines. Breast cancer cells were treated with small molecules (40µM(dark blue), 20µM (red), 10µM (green), 5µM (purple), 2.5µM(light blue))for 72h. NCI60 SRB analysis was applied as explained in method

APPENDIX

CURRICULUM VITAE

İREM DURMAZ

Gender: Female
Date of Birth: October 20th, 1987
Address: Bilkent 1, Camlik Sitesi, F1 Blok
Kat:4 Daire:15 Cankaya, Ankara, 06800 TURKEY
Mobile Phone: 0090 536 680 48 63
E-mail: durmaz87@yahoo.com
durmaz@bilkent.edu.tr

EDUCATION

Graduate School:

2011- 2015 **Bilkent University, Ankara Turkey**
Molecular Biology and Genetics, PhD
cGPA: 3.90/4.00

2009-2011 **Bilkent University, Ankara Turkey**
Molecular Biology and Genetics, M.S
cGPA: 3.88/4.00

Undergraduate School:

2005- 2009 **Bilkent University, Ankara Turkey**
Molecular Biology and Genetics, B.S.
cGPA: 3.32/4.00

2001-2005 **American Collegiate Institute, İzmir Turkey**
Ranked 4th

RESEARCH EXPERIENCE:

Fall 2011-Fall 2015 Ph.D. THESIS PROJECT**:**
Bilkent University, Faculty of Science, Molecular Biology and Genetics
Drug screening and bioactivity analysis of novel compounds on liver cancer cells and to enlighten the mechanism of action.

Supervisor: Assoc. Prof. Isik Yulug, Ph.D.
Co-supervisor: Assoc. Prof. Rengul C. Atalay, M.D., Ph.D

Fall 2009-Spring 2011 M.Sc. THESIS PROJECT**:**
Bilkent University, Faculty of Science, Molecular Biology and Genetics
Inhibition of Fibroblast growth factor receptor 2 (FGFR2) in human endometrial and gastric cancer cell lines using synthetic small peptide inhibitors
Drug screening and cytotoxicity assays on novel kinase inhibitor derivatives and investigate the target mechanism of the promising candidate kinase inhibitor derivatives

Supervisor: Assoc. Prof. Rengul C. Atalay, M.D., Ph.D

Fall 2008- Spring 2009 GRADUATION PROJECT:
Bilkent University, Faculty of Science, Molecular Biology and Genetics
Investigation of mutations on candidate genes for hepatocellular carcinoma

Supervisor: Assist. Prof. M. Cengiz Yakıcıer, M.D., Ph.D.

June 18- August 10 2007 RESEARCH INTERN:
Georgetown University Medical Center
Lombardi Comprehensive Cancer Center
Department of Oncology Division of Biochemistry and Pharmacology
Observed the localization of Raf and Ras proteins in both in cancer pancreatic cells(PANC-1) and in healthy cells and investigated the compounds that can inhibit the expression of these proteins.

Supervisor: Research Assist. Prof. York Tomita, Ph. D.

July 17-August 29 2008 RESEARCH INTERN:
Ecole Polytechnique Fédérale de Lausanne / Science de la Vie (School of life sciences) / Brain Mind Institute / Functional Neurogenomics Laboratory (LNGF)
Observed the Erk1/2 Dependent Transcriptional and Functional Effects of BDNF

Supervisor: Assist. Prof. Ruth-Luthi Carter, Ph.D.

RESEARCH INTERESTS:

Cell Biology, Cancer, Epigenetics, Signal Transduction, Developmental Biology, Stem Cell, Drug Screening, Cytotoxicity, Animal Xenografts, Molecular Biology

SKILLS AND ABILITIES

Technical Expertise

Protein Purification: Various chromatography, including: Anion Exchange (Mono Q), Cation Exchange (Mono S), Gel Filtration (Superdex 75, S-300, S-200), Hydrophobic Interaction Chromatography (phenol sepharose) and especially fast protein liquid chromatography (FPLC). Knowledge of column packing and preparation of protein affinity columns using NHS based coupling chemistry. Protein precipitation techniques including ammonium sulfate, TCA, and organic and dialysis techniques.

Biochemistry/Protein Analysis: Electrophoresis skills including: Western Blot, SDS-PAGE, Native PAGE, dot blot, ELISA. Skilled in immunoblotting, immunoprecipitations. Experience performing phosphoamino acid analysis.

Molecular Biology: PCR, Agarose electrophoresis, DNA plasmid purification (mini/maxipreps), RNA purification, Northern Blotting, Southern Blotting, ligations, restriction digests, bacterial vector cloning (InFusion cloning kit), bacterial protein expression (GST fusion, His6x fusion), and transformations, Mutagenesis (Stratagene Quick Change Mutagenesis Kit), transfection, electroporation, RT-PCR, Chromatin Immunoprecipitation Assay (ChIP), Fluorescent-activated cell sorting (FACS) with propidium iodide, Oxidative stress assays (such as DCFH-DA staining)

Immunohistochemistry:
Staining procedures such as: Methylene blue stain, Gimsa stain, fluorescent staining using Hoechst stain,

Drug screening:
Cytotoxicity assays: Sulforhodomine B assay (SRB), MTT cell proliferation assay,
Enzymatic activity assays: PKLight HTS Protein Kinase Assay,
Apoptosis detection assays: Caspase activity assay, TUNEL, Annexin V, usage of anti-PARP antibody in Western Blot, Apoptotic DNA ladder kit, Fluorescent-activated cell sorting (FACS)

Cell Culture:
Routine cell culture techniques

Animal experiments:
Xenograft production via subcutaneous injection, gavage application, animal handling, MRI processing

Computer: Windows 7, Windows Vista, Windows XP, Windows 2000, Windows 95/98, Excel, Word, Powerpoint, Java, Matlab, Picture Manager, Perl, Photoshop, BRB tools, R programming.

Languages: Advanced English, Introductory French, Turkish (native speaker)

TEACHING EXPERIENCE:

Fall 2009	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Biochemistry Course, Bilkent University
Spring 2010	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Molecular Basis of Evolution Course, Bilkent University
Fall 2010	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Biochemistry Course, Bilkent University
Spring 2011	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Introduction to Molecular Biology Course, Bilkent University
Fall 2011	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Biochemistry Course, Bilkent University
Spring 2012	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Introduction to Molecular Biology Course, Bilkent University
Fall 2012	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Biochemistry Course, Bilkent University
Spring 2013	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Introduction to Molecular Biology Course, Bilkent University
Fall 2013	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Biochemistry Course, Bilkent University
Spring 2014	Teaching Assistant, Assoc. Prof. Dr. Rengul C. Atalay's Introduction to Molecular Biology Course, Bilkent University
Fall 2014	Teaching Assistant, Assoc. Prof. Dr. Ozgur Sahin, Biochemistry Course, Bilkent University

CONFERENCES ATTENDED

Certificate for attending EACR-AACR-SIC "Special Conference on Anticancer Drug Action and Drug Resistance: from Cancer Biology to the Clinic", 2015, poster presentation
Certificate for attending 3rd International Congress of the Molecular Biology Association, 2014, poster presentation
Certificate for attending International Multidisciplinary Symposium on Drug Research & Development (DRD2013), performed an oral presentation
Certificate for attending 24th EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics", 2012
Certificate for attending EBI Bioinformatics Roadshow, 2010
Certificate for attending workshop on animal testing (the proper utilization of lab animals), 2010
Certificate for participating the 2nd annual Horizons in Molecular Biology and Genetics Symposium, 2009
Certificate for attending Federation of European Neuroscience Societies (FENS)
Forum, 2008

ACHIEVEMENTS AND CERTIFICATES

Bilkent University High Honor Certificate, 2009 (Spring)
Bilkent University High Honor Certificate, 2008 (Fall)
Bilkent University High Honor Certificate, 2008 (Spring)
Bilkent University Honor Certificate, 2007 (Fall)
Bilkent University Honor Certificate, 2006 (Fall)
Bilkent University Honor Certificate, 2005 (Fall)
Certificate of Model United Nations Experience:
TIMUN, 2002(as a Japan delegate), 2003(as the chairman)
THIMUN, 2003(as a Cuba delegate), 2004(as the delegate of PNA)
THIMUN Youth Assembly, 2005(committee on substance abuse)
TIYA, 2005(committee on water), 2006(chairman of committee on MDG)
MUNTR, 2006 (the **Middle Eastern Multilateral Summit)**
Certificate for Attending TUBITAK Physics Project Competition,

HOBBIES AND SOCIAL ACTIVITIES

Active member of Bilkent Genetics society 2005-
Member of the Bilkent Tennis Team, 2005-2008
Member of the BilkentMUN Club, 2005
In high school, Member of:
Model United Nations Club, 2002-2005
Biology Projects Club, 2002-2005
Physics Projects Club, 2004-2005
Student Council, 2002-2003
Class Monitors Committee, 2003-2005

MS Club (visiting, entertaining and helping to people with MS), 2002-2003

Ender's Children Club (entertaining and helping autistic children), 2001-2005

PUBLICATIONS:

Durmaz, I., Guven E.B., Ersahin, T., Ozturk M., Calis, I., Cetin-Atalay, R. Liver cancer cells are sensitive to Lanatoside C induced cell death independent of their PTEN status.. Phytomedicine 2015. doi:10.1016/j.phymed.2015.11.012 (**In Press, Available Online**)

Aytac, P.*, **Durmaz, I**.*, Houston, D.R., Cetin-Atalay, R., Tozkoparan, B. Novel triazolothiadiazines act as potent anticancer agents in liver cancer cells through Akt and ASK-1 proteins. Bioorganic and Medicinal Chemistry, 2015. (* equal contribution) (**Accepted**)

Pelin Çıkla-Süzgün, Neerja Kaushik-Basu, Amartya Basu, Payal Arora, Tanaji T Talele, **Irem Durmaz**, Rengül Çetin-Atalay, Ş Güniz Küçükgüzel. Anti-cancer and anti-hepatitis C virus NS5B polymerase activity of etodolac 1,2,4-triazoles. Journal of Enzyme Inhibition and Medicinal Chemistry 02/2015; DOI:10.3109/14756366.2014.971780 · (I.F=1.50)

Gurdal EE, Buclulgan E, **Durmaz I**, Cetin-Atalay R, Yarim M. Synthesis and Anticancer Activity Evaluation of Some Benzothiazole-Piperazine Derivatives. Anticancer Agents Med Chem. 2014 Dec 16. PubMed PMID: 25511511. (I.F= 2.9)

Jousheed Pennakalathil, Alp Ozgun, **Irem Durmaz**, Rengul Cetin-Atalay, Donus Tuncel. pH-Responsive Near-Infrared Emitting Conjugated Polymer Nanoparticles for Cellular Imaging and Controlled-Drug Delivery . Journal of Polymer Science, Received 2 September 2014; accepted 28 October 2014; published online 17 November 2014 DOI: 10.1002/pola.27458. (I.F=3.8)

Şeyma Cankara Pirol, Burcu Çalışkan, **İrem Durmaz**, Rengül Atalay, Erden Banoglu, Synthesis and preliminary mechanistic evaluation of 5-(p-tolyl)-1-(quinolin-2-yl)pyrazole-3-carboxylic acid amides with potent antiproliferative activity on human cancer cell lines, European Journal of Medicinal Chemistry, Volume 87, 24 November 2014, Pages 140-149, ISSN 0223-5234. (I.F=3.432)

Gurdal EE, **Durmaz I**, Cetin-Atalay R, Yarim M. Cytotoxic activities of some benzothiazole-piperazine derivatives. J Enzyme Inhib Med Chem. 2014 Oct 21:1-6. PubMed PMID: 25333766. (I.F=2.4)

Esra Filiz ONEN BAYRAM, Kerem Buran, **Irem Durmaz**, Barkin Berk and Rengul Cetin-Atalay. 3-propyonyl-thiazoldine-4-carboxylic acid

ethyl esters: a Family of Antiproliferative Thiazolidines. *Med. Chem. Commun.*, 2014, Accepted Manuscript DOI: 10.1039/C4MD00306C. Received 14 Jul 2014, Accepted 14 Sep 2014 First published online 15 Sep 2014 (I.F=2.626)

Ö.Gezici, **I. Durmaz**, E.Bilget Güven, Ö. Ünal, A. Ozgün, R. Cetin-Atalay, D. Tuncel. Dual Functionality of Conjugated Polymer Nanoparticles as an Anticancer Drug Carrier and a Fluorescent Probe for Cell Imaging. RSC Adv., 2013, DOI: 10.1039/C3RA45120H. (I.F=2.562)

Gurdal EE, Yarim M, **Durmaz I**, Cetin-Atalay R. Cytotoxic Activities of some Novel Benzhydrylpiperazine Derivatives. Drug Res (Stuttg). 2013 Mar;63(3):121-8. PubMed PMID: 23447090 (I.F=0.6).

Gurdal EE, **Durmaz I**, Cetin-Atalay R, Yarim M. Synthesis and cytotoxicity studies of novel benzhydrylpiperazine carboxamide and thioamide derivatives. J. Enzyme Inhib Med Chem. 2013 Feb 8. PMID: 23391169 (I.F=1.62).

Koyuncu CF, Arslan S, **Durmaz I**, Cetin-Atalay R, Gunduz-Demir C (2012) Smart Markers for Watershed-Based Cell Segmentation. PLoS ONE 7(11):e48664. doi:10.1371/ (I.F= 4.09)

Onen-Bayram FE*, **Durmaz I***, Scherman D, Herscovici J, Cetin-Atalay R. A novel thiazolidine compound induces caspase-9 dependent apoptosis in cancer cells. Bioorg Med Chem. 2012 Sep 1;20(17):5094-102. Epub 2012 Jul 20. PubMed PMID: 22867707. (I.F= 3.157) (* equal contribution)

Yarim M, Koksal M, **Durmaz I**, Atalay R. Cancer Cell Cytotoxicities of 1-(4-Substitutedbenzoyl)-4-(4-chlorobenzhydryl) piperazine Derivatives. Int J Mol Sci. 2012;13(7):8071-85. Epub 2012 Jun 28. PubMed PMID: 22942690; PubMed Central PMCID: PMC3430221. (I.F= 2.6)

Koksal M, Yarim M, **Durmaz I**, Cetin-Atalay R. Synthesis and Cytotoxic Activity of Novel 3-methyl-1-[(4-substitutedpiperazin-1-yl)methyl]- 1H-indole Derivatives. Arzneimittelforschung. 2012 Jun 29. [Epub ahead of print] PubMed PMID: 22753155. (I.F= 0.6)

Meriç Köksal Akkoç, Mine Yarım Yüksel, **İrem Durmaz**, Rengül Çetin Atalay. Design, synthesis, and biological evaluation of indole-based 1,4-disubstituted piperazines as cytotoxic agents. Turk. J. Chem., 36, (2012), 515-525. (I.F= 0.9)