IDENTIFICATION AND THE CHARACTERIZATION OF SMALL MOLECULES WITH POTENTIAL ANTICANCER ACTIVITY AGAINST SOLID TUMORS

A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
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THE DEGREE OF MASTER OF SCIENCE

BY
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AUGUST 2011

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

IDENTIFICATION AND THE CHARACTERIZATION OF SMALL MOLECULES WITH POTENTIAL ANTICANCER ACTIVITY AGAINST SOLID TUMORS

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MSc. in Molecular Biology and Genetics
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The ultimate goal of our project was to investigate candiate small molecules with a potential anticancer activity and characterize their mode of action.

Cardiac glycosides are important group of molecules for both their treating properties in heart failure and their potential effects in cancer therapy. We investigated the cardiac glycosides that are extracted from *Digitalis Ferruginea* which can be frequently found in Turkey. These glycosides are Lanatoside A, Lanatoside C and Glucogitorosid. Our results showed that they constitute high cytotoxicity effect against liver cancer cell lines. In addition they cause G2/M cell cycle arrest and thereby induce apoptosis.

For the synthetic molecules, we first tested a set of molecules that are synthesized as derivatives of kinase inhibitors. There are some commercial drugs such as imatinib or erlotinib that are used frequently for cancer treatment. Thus we wanted to investigate if these molecules comprise cytotoxic activities. Our data revealed that especially one of the molecules out of 16 display high cytotoxicity and high kinase inhibitory effect in liver cancer cell lines.

The final group of molecules we tested was composed of thiazolidine ring. In this group of molecules, only one molecule, the one with alkyne terminal precursor, caused cytotoxicity against cancer cell lines. Besides, we have shown that it induces SubG1/G1 cell cycle arrest in cancer cell lines.

ÖZET

POTANSİYEL ANTİKANSER ÖZELLİKLERE SAHİP OLABİLECEK KÜÇÜK MOLEKÜL İNHİBİTÖRLERİNİN TEŞHİS EDİLMESİ VE NİTELENDİRİLMESİ

İrem Durmaz
Moleküler Biyoloji ve Genetik Yüksek Lisansı
Tez Yöneticisi: Doç. Dr. Rengül Atalay
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Bizim bu projedeki amacımız, anti kanser özelliğe sahip olabilecek molekülleri test edip nitelendirmektir.

İncelediğimiz ilk grup, yüksük otundan elde edilen kalp glikosidleriydi. Bu moleküller, gerek kalp hastalığı tedavisinde, gerekse de kanser tedavisindeki potansiyel etkileri sebebiyle oldukça araştırılmakta olan moleküllerdir. Bizim araştırmalarımız sonucunda da bu maddelerin karaciğer kanseri hücrelerine sitotoksik etkileri olduğunu göstermiş bulunmaktayız. Ayrıca bu moleküller, hücre bölünme sürecinde G2/M fazında durmaya sebep vererek apoptos indüklenmesine neden oldukları deney sonuçları da gösterilmiştir.

İkinci grup moleküllerimiz sentetik kinaz inhibitör türevleridir. Günümüzde kinaz inhibitörü olan imatinib, erlotanib gibi birçok madde kanser tedavisinde kullanılmaktadır. Biz de yaptığımız araştırmalar sonucunda gruptaki 16 molekülden özellikle bir molekülün oldukça yüksek citotoksik etki ve kinas inhibisyon gücünün varlığını göstermiş olduk.

Son grup moleküller ise tiyazolidin yapısındaki moleküllerden oluşmaktadır. Bu grupta da özellikle bir molekülün öne çıktığını gösterdik. Alkin içeren bu molekül oldukça yüksek hücre ölümlerine neden oluyor kanser hücre ortamına verildiğinde. Ayrıca bu molekülün de hücre bölünme döngüsünde SubG1/G1 fazında durmaya sebep olduğunu deney sonuçlarımızda gösterdik.

TO MY PARENTS TÜRKAN DURMAZ and HÜSEYİN DURMAZ and TO MY BELOWED KARAMEL

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ATP   Adenosine Tri-phosphate

AP-1   Activator protein 1

BSA   Bovine Serum Albumin

$CaCl_2$ Calcium Chloride

CO_2 Carbon dioxide

CPT   Camptothecin

DAPK1   death-associated protein kinase-1

DMEM   Dulbecco's Modified Eagle's Medium

ddH2O   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

FGF-2   Fibroblast Growth Factor 2

g	Gram
HCC	Hepatocellular Carcinoma
HER2	Human Epidermal growth factor Receptor 2
HRP	Horse Radish Peroxidase
IC50	Inhibitory Concentration 50
K	Potassium
kDa	kilo Dalton
MKK	Mitogen Activated Protein Kinase Kinase
MAPK	Mitogen Activated Protein Kinase
mg	Milligram
$MgCl_2$	Magnesium Chloride
μg	Microgram
μl	Microliter
Na	Sodium
NaOH	Sodium Hydroxide
NaCl	Sodium Chloride
NEAA	Non-essential Amino Acid
ng	Nanogram
nm	Nanometer
nM	Nanomolar
NRTK	non-receptor tyrosine kinase
PARP	Poly(ADP-ribose) Polymerase

PI3K	Phosphotidylinositol-3-kinase
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween-20
PhosStop	Phosphotase Inhibitor Cocktail
PIC	Protease Inhibitor Cocktail
PMSF	Phenylmethylsulphonylfluoride
RNaseA	Ribonuclease A
RTK	Receptor tyrosine kinase
ROS	Reactive Oxygen Species
SRB	Sulphorhodamine B
SDS	Sodium Dodecyl Sulfate
STS	Staurosporine
TBS	Tris-buffered Saline
TBS-T	Tris-buffered Saline with Tween-20
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TRAIL	Apo21/TNF-related apoptosis-inducing ligand

CHAPTER 1. INTRODUCTION

1.1 Small Molecule Inhibitors

In most of the biological processes, protein-protein interactions play a very significant and fundamental role. Therefore, development and characterization of small molecules for therapeutic agents, that have the potential to interfere with enzyme activities or ion channel activities, attracts the attention of most scientists. The ultimate effect of such small molecules is their inhibitory effect on downstream protein-protein interactions in cell signaling cascades (Arkin et al, 2004). The reason that small molecules provide a very big potential for targeted anticancer research is that on the contrary to most of the genetic tools, small molecule inhibitors are reversible most of the time. In addition, they can produce responses inside the cells that are graded and finally they can easily cross the cellular membrane and penetrate inside the cell (Brisson et al, 2004). Novel targets that are aimed to be disrupted by small molecule inhibitors include proteins that are involved in various biological pathways such as regulation of gene expression, signal transduction, DNA repair, cell growth…etc. In most of the cases small molecules compete for ATP-binding with other molecules or directly disrupts protein-protein interaction and thus inhibit their function (London, C. 2007)

1.2. Cardiac Glycosides

Cardiac glycosides are steroid-like compounds and include commonly known therapeutics such as digitoxin, digoxin and ouabain (Figure 1.1). Digitalis is the most widely cardiac glycoside and consists of two components: digitoxin and digoxin (Newman et al, 2008).

Usage of plant extracts containing cardiac glycosides for medical occasions trace back to more than 1500 years ago. In the ancient times, they were used as heart

tonics, diuretics or arrow poisons. Latter in time, they were shown to be therapeutic for heart diseases and even now medical doctors use them in treatment of congestive heart failure. In 1975 it was observed that a patient with congestive heart failure recovered after treated with foxglove which is the extract taken from Digitalis purpurea L (Huxtable et al, 2001). This extract produced from foxglove contains cardenolides that was shown to increase contractility of the cardiac muscles and regulate atrial fibrillation by functioning like antiarrythmic compounds (Gheorghiade et al, 2006; Hamad et al, 2007). Mechanism underlying for their function as therapeutic for heart failure is inhibition of Na^+,K^+-ATPase (energy transducing ion pump) and thus causing an increase in the intracellular concentration of calcium and sodium ions. It also leads to a decrease in the concentration of potassium ions
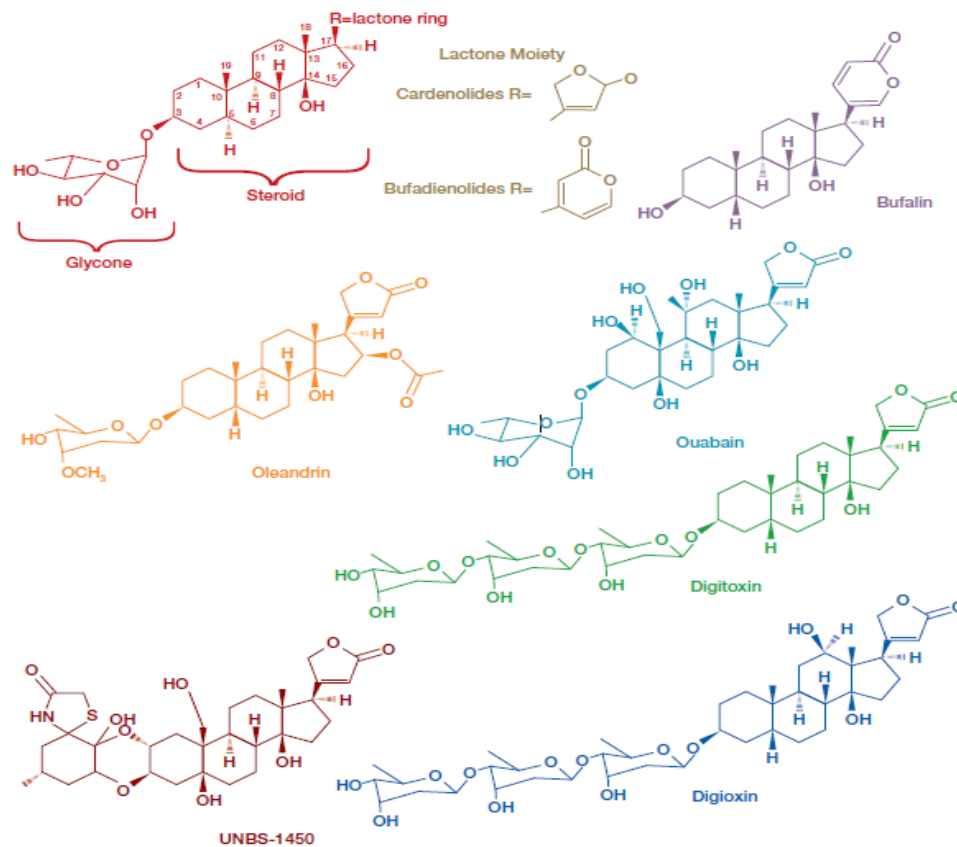
(Kaplan JH, 2002; Newman et al, 2008).

Figure 1.1: Structural representation some cardiac glycosides (Newman et al, 2008)

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1.2.1 Cardiac glycosides and Cancer

Back in 1960s, it has been reported that in vitro cardiac glycosides can inhibit malignant cell formation thus since then various researches has been establish for possible anticancer activity of cardiac glycosides (Shiratori et al, 1967; Winnicka et al, 2006). In breast cancer it has been shown that, five years after mastectomy, the rate of recurrence was 9.6 times fewer in cardiac glycoside taking patients (Stenkvist et al, 1982). Also in another study it was shown that survival rate was higher in patients taking cardiac glycosides (Stenkvist et al, 1999). Moreover, a more recent study revealed that ouabin can induce activation of Src/EGFR which causes activation of ERK1/2 and thus increase in p21/Cip1 levels and finally cause growth arrest. Digitoxin and digoxin also are shown to have similar properties and consequences (Kometiani et al, 2006). Furthermore, in another study it has been proposed that Na^+,K^+-ATPase translocation to the nucleus is responsible for the activation/transactivation of Src/EGFR. Then this activation, as previously reported, cause Ras activation and thereby MEK and ERK1/2 activation followed by ROS production that will either be followed by apoptosis or autophagy (Figure 1.2) (Newman et al, 2008; Xie and Cai, 2003; Schoner and Scheiner-Bobis, 2007). There are many other studies suggesting other consequences of cardiac glycoside application such as inhibition of activator protein-1 (AP-1), Akt and phosphoinositide-3 kinase (PI3K) related protein as well as death-receptor regulated apoptotic pathway inhibition (Figure 1.2) (Newman et al, 2008; Schoner and Scheiner-Bobie, 2007; Chen et al, 2006).

Fourty years ago, usage of cardiac glycosides was prohibited due to possibility of high toxicity. Nonetheless, recent studies revealed that cardiac glycosides such as digitoxin and oleandrin are not toxic to tumor cells derived from rodent. On the other hand, they inhibit proliferation of human and monkey derived tumor cells when applied in nanomolar concentrations (Erdmann and Schoner, 1973; Pathak et al, 2000; Gupta et al 1986).

Thus these findings bring in mind the possibility of tissue specificity. Further studies confirmed that cardiac glycosides in fact have tissue specificity such that they

are not toxic towards normal cells but malignant cell population (Sreenivasan et al, 2003). Moreover, cardiac glycosides were shown to induce tumor cells become more sensitive towards irradiation (Verheye-Dua and Bohm, 1998).

1.2.2 Apoptosis and Cardiac glycosides

Apoptosis, which is the regulatory factor for cell number determination and damaged cell elimination, is key factor in many biological processes such as embryogenesis, immune system regulation or carcinogenesis. It is also called as "programmed cell death" that occurs in multicellular organisms. 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 phagocytosed the apoptotic cell (Wyllie AH).

In some in vitro studies it has been shown that non-toxic concentrations of cardiac glycosides, including digitalis, apoptosis induction is observed. (Haux et al, 1999; Daniel et al, 2003) Morphological changes caused by cytotoxicity of cardiac steroids are very similar to that of apoptosis. For instance phosphatidylserine externalization, DNA fragmentation and disruption of mitochondrial membrane potential are common morphological changes (Daniel et al, 2003). It has been proposed that apoptosis induction property of cardiac glycosides cannot be just through inhibition of Na^+,K^+-ATPase (Winnicka et al 2006).

Calcium ion chelators, calcium channel blockers and antagonists of calmodulin have the potential to delay or totally inhibit apoptosis. Therefore, it was proposed that deregulation of intracellular calcium ion homeostasis by cardiac glycosides through inhibition of Na^+,K^+-ATPase may induce apoptosis in healthy cells as well as cancer cell lines. And this might explain the potential anti-cancer activity of cardiac glycosides (McConkey et al, 2000). Moreover, some research groups points out that in prostate cancer cell lines, when given in drugable

concentrations, cardiac glycosides causes proliferative arrest and apoptosis induction (Johansson et al, 2001; Haux et al, 2001).

Consequently, the alteration of calcium ion concentration might have the potential consequence of apoptotic induction in human cancer cell lines producing a new area for therapeutic approach (Winnicka et al 2006).

In another study it has been shown that malignant cells show more susceptibility towards cardiac glycosides than of healthy cells. A possible explanation for this might be that activity of Na^+,K^+-ATPase is altered in tumor cells (Racker et al, 1983; Kasarov et al, 1974). For instance, bufalin, type of a cardiac glycoside was shown to induce apoptosis in HL60 and ML1 leukemia cell lines but no induction was observed in normal cells. The signal transduction for apoptosis induction is suggested to be through Ras-Raf1-MAPK pathway (Kometiani et al, 2006). In another study it has been shown that inhibition of the decrease in potassium levels prevents activation of caspases and therefore apoptosis cannot be induced (Hashimoto et al, 1997). Furthermore, it was also proposed that in androgen-independent prostate cancer, three cardiac glycosides, oleandrin, ouabain and digoxin induces apoptosis through cytochrome c release (McConkey et al, 2000; Ramiez et al, 2007). In some other studies, cardiac glycosides have been proposed to increase Fas and tumor necrosis factor receptor (TNFR1) which thus raises the induction of apoptosis in tumor cells but not in healthy cells (Newman et al, 2008; Sreenivasan et al, 2006). It was reported by another group that in non-small-cell-lung cancer cell lines, cardiac glycosides oleandrin, digoxin and digitoxin induces apoptosis via activating death receptor pathway (death receptors 4&5) and thus via Apo21/TNF-related apoptosis-inducing ligand (TRAIL) pathway (Frese et al, 2006). However, the induction of apoptosis by cardiac glycosides is a controversary issue and suggested to be tissue specific since in another study of vascular smooth muscle cells, apoptosis was shown to be inhibited by cardiac glycosides (Moss, 1998; Winnicka et al 2006).
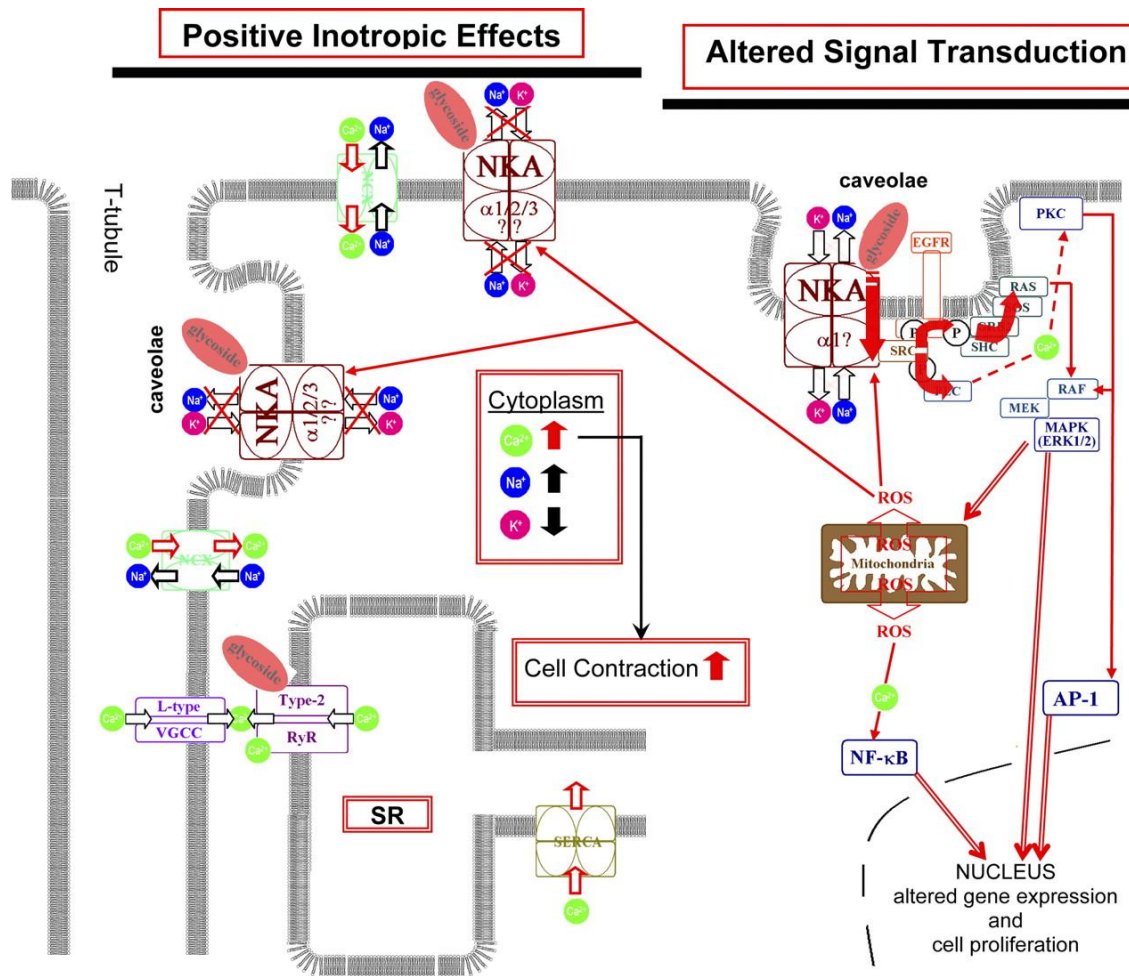
Figure 1.2: Na$^+$,K$^+$-ATPase Signaling cascade (Wasserstromet al, 2005)

1.2.3 Angiogenesis and Cardiac Glycosides

The growth of new capillary blood vessels is called as angiogenesis. It is a vital process for tumor formation and progression and also for metastasis. Cardiac glycosides have been suggested to regulate activation of fibroblast-growth factor-2 (FGF-2) which is an angiogenesis inducing molecule. In addition, in the same study, NF-kB, which prevents apoptosis induction and induce drug resistance, was shown to be inhibited by cardiac glycosides (Cronauer et al, 1997; Oh et al, 1998; Johnson et

al, 2002; Wang et al, 1996; Nakshatri et al, 1997). Regulation of FGF-2 was by cardiac glycosides was suggested to be through the inhibition of Na^+,K^+-ATPase which thus cause the prevention of export of FGF-2 (Smith et al, 2001). On the other hand Na^+,K^+-ATPase pump was shown to play critical role in membrane transport mechanism of some proteins necessary for the growth of the tumor (Florkiewicz et al, 1998).

1.2.4 Digitalis Ferruginea

Digitalis genus, which is suggested to be the main source of cardiac glycosides, is composed of over 20 species. In the literature, there is not much study done on the cardiac glycosides derived from *Digitalis Ferruginea* (also known as "rusty foxglove") but mostly from lanata. Using TLC and PC methodology, the cardenolide ingredients of some of the Digitalis species have been analyzed and Digitalis Ferruginea is one of these species. Previously, Lanatoside A, Lanatoside C, Glucogitorosid, Lugrandoside, Ferruginoside A and Ferruginoside B was isolated from digitalis ferruginea (Çalış et al, 1999; Davie et al, 1978; Çalış et al, 1999

1.3 Protein Kinases

Kinase family of proteins is mostly composed of enzymes with regulatory functions in the activation/inhibition of other proteins inside the cell. They function by adding phosphate groups to amino acid side chains of other proteins. This phosphate addition causes structural change of the protein which will cause the change in the activity of the protein. This is a reversible action and can be removed by protein phosphatases. Kinases are vital component of the cell and have important functions in most intracellular pathways such as growth and cell division, cellular metabolism or DNA damage (Becker et al, 2003; Novak C, 2004).

In structural view, protein kinases have two binding sites. One of them is the catalytic subunit to where ATP binds. The other binding site is for the target protein.

Depending on where the phosphate transferred by the kinase, protein kinases are divided into sub groups such as Serine/Threonine kinases, and Tyrosine kinases (Hanks et al, 1988). Even though Tyrosine kinases are less in number when compared to Serine/Threonine kinases, they have more critical roles in key regulatory cascades. Receptor tyrosine kinases (RTKs) and non-receptor Tyrosine kinases (NRTKs) are two subgroups of Tyrosine kinases. RTKs contain extracellular ligand-binding domains and intracellular kinase domains and EGFR is an example for this group. NRTKs involved in intracellular pathways 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 (Arena S et al, 2005).

1.3.1 Deregulation of kinase family of proteins in cancer

Phosphorylation is the basis of the regulatory circuit of the cell which mediates the proper functioning of the proteins acting in cell proliferation, gene expression, cellular differentiation, cellular metabolism, protein turnover and cell death such as apoptosis (Figure 1.3). In case of abnormalities in phosphorylation of proteins mostly tumorigenesis occurrence is observed (Tsuboi et al, 2004, Venture et al, 2006).

In most of the cancer cells, activation of protein kinases and receptor kinases is found to be altered (Table 1.1). For instance mitogen activated protein kinase (MAPK), phosphotidylinositol-3-kinase (PI3K), AKT, and the epidermal growth factor receptor (EGFR) are found to be hyper-activated in cancer cells. Kinases can have both oncogenic or tumor suppressive effects in cancer. However, most of the time tumor suppressive kinases are found to be serine/threonine or dual-functional kinases whereas most proto-oncogene kinases are found to be tyrosine kinases. For example, SRC and ABL which are NRTKs, function as proto-oncogene.

On the other hand, death-associated protein kinase-1 (DAPK1), which is a serine/threonine kinase, is shown to be tumor suppressive which is down regulated in several cancer types including breast and renal carcinoma (Kissil JL et al, 1997, Tend DH et al, 1997). Therefore, in several studies protein kinases are therapeutically targeted with small molecules or antibodies which has potential to inhibit the interaction between the kinase and its substrate or these inhibitors in some cases target and inhibit ATP binding site of kinases (Novak C, 2004).
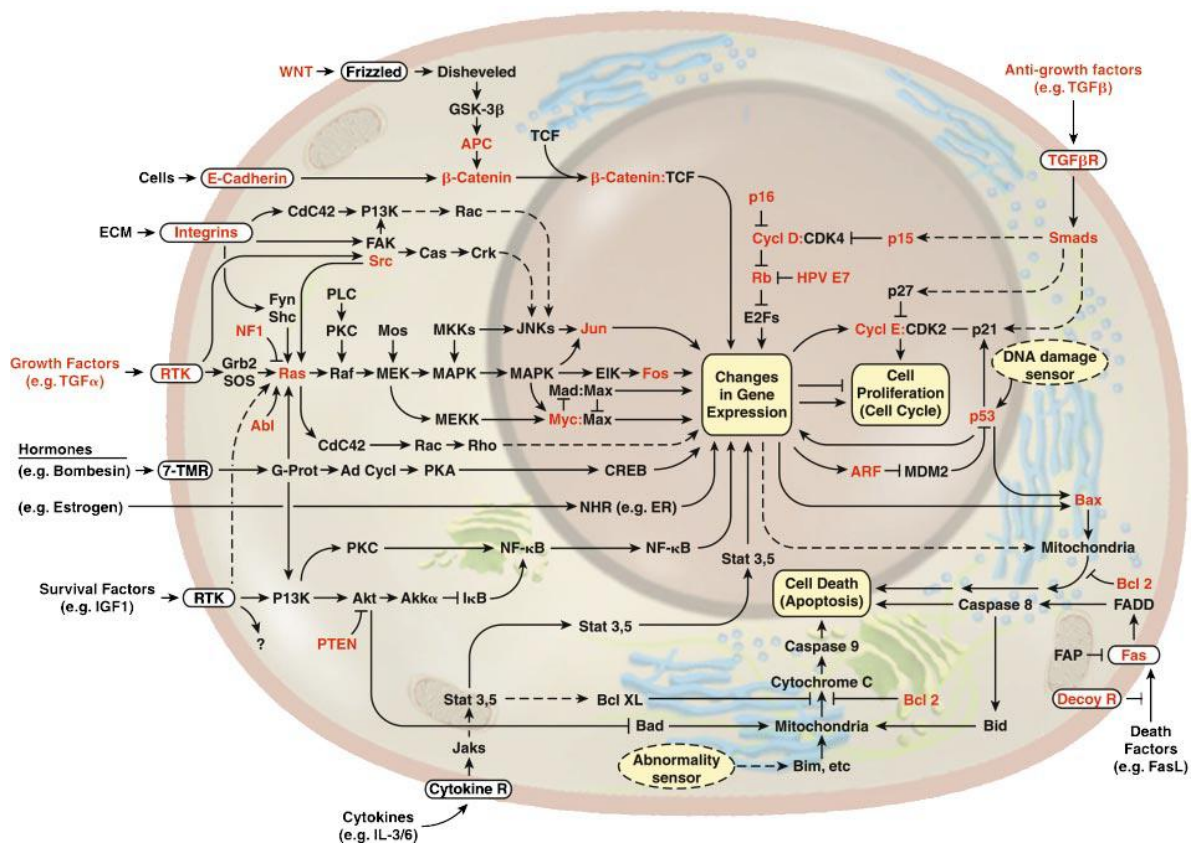
Figure 1.3: Circuit of the cell: Proteins with abnormal functionalities are highlighted in red (Hanahan D, Weinberg RA, 2000).

Table 1.1: Kinases and Disease: Alterations in the activation of some kinases are shown together with diseases that occur. (modified from Fabian et al. 2005 and Cell Signaling http://www.cellsignal.com/reference/kinase_disease.html)

Name	Molecular Basis	Group	Disease Type
ABL1	Translocation	TK	Cancer
ACTR2B	Mutation	TKL	Development
AKT1	Amplified, Overexpression, and Activated	AGC	Cancer
AKT2	Amplified, Overexpression, and Mutation	AGC	Cancer and Diabetes
ALK	Translocation	TK	Cancer
ALK1 (ACVRL1)	Mutation	TKL	Cardiovascular
ANPalpha (NPR1)	SNP and Expression	RGC	Hypertension
ARG (ABL2)	Translocation and Expression	TK	Cancer
ATM	Loss-of-function mutation	Atypical	CNS and Cancer
ATR	Mutation and Splice-Change	Atypical	Cancer,Development, andVirology
AurA	Amplified, Overexpression, and SNP	Other	Cancer
AurB	Overexpression	Other	Cancer
Axl	Overexpression	TK	Cancer
B-RAF	Gain-of-function mutation and Loss-of-function mutation	TKL	Cancer
BCR	Translocation	Atypical	Cancer
BMPR1A	Loss-of-function mutation	TKL	Cancer
BMPR2	Loss-of-function mutation	TKL	Cardiopulmonary
BRD4	Translocation	Atypical	Cancer and Virology
BRK	Overexpression	TK	Cancer
BTK	Loss-of-function mutation	TK	Immunity andCancer
BUB1	Loss-of-function mutation	Other	Cancer
BUBR1 (BUB1B)	Mutation and Overexpression	Other	Cancer
CDC2 (CDK1)	Activated and Splice-Change	CMGC	Cancer
CDK2		CMGC	Cancer

CDK4	Activated, Gain-of-function Mutation, Amplified, and Methylation	CMGC	Cancer
CDK6	Over expression and Translocation	CMGC	Cancer
CDK9	Expression	CMGC	Viral infection andCardiovascular
CHK1	Mutation	CAMK	Cancer
CHK2	Mutation	CAMK	Cancer
CK1epsilon (CSNK1epsilon)	SNP, Mutation, and Loss-of-heterozygosity	CK1	Behavior andCancer
CK2alpha1 and CK2alpha2 (CSNK2alpha1/2)	Over expressionand Activated	Other	Cancer,Neurodegeneration, and Circadian Rhythm
COT/TPL2	Over expression,Amplified, andMutation	STE	Cancer andInflammation
CTK/MATK	Overexpression	TK	Cancer
CYGD (GUCY2D)	Mutation	RGC	Vision
CYGF (GUCY2F)	Mutation	RGC	Cancer
DAPK1	Methylation andExpression	CAMK	Cancer and Epilepsy
DMPK1	Mutation	AGC	Neurodegeneration
DNAPK	Mutation	Atypical	Cancer
EGFR	Amplified,Overexpression, and Gain-of-function Mutation	TK	Cancer
EphA1	Expression	TK	Cancer
EphA2	Overexpression	TK	Cancer
EphA3 (HEK)	Mutation	TK	Cancer
EphB2	Overexpressionand Mutation	TK	Cancer
EphB4 (HTK)	Overexpression	TK	Cancer
Erk5 (BMK1)	Expression	CMGC	Cancer andCardiovascular
FAK (PTK2)	Overexpression,Amplified, andActivated	TK	Cancer
FER	Expression	TK	Cancer
FES	Mutation andTranslocation	TK	Cancer

FGFR1	Mutation andTranslocation	TK	Development andCancer
FGFR2	Mutation andAmplified	TK	Development andCancer
FGFR3	Gain-of-function Mutation andTranslocation	TK	Development andCancer
FGFR4	SNP	TK	Cancer
FGR	Amplified	TK	Cancer
FLT1 (VEGFR1)	Methylation andOverexpression	TK	Cancer
FLT4 (VEGFR3)	Activated andLoss-of-function Mutation	TK	Lymphangiogenesis
FMS (CSF1R)	Mutation	TK	Cancer
GSK3alpha and GSK3beta	SNP andActivated	CMGC	Neurodegeneration,Diabetes,Cardiovascular, andCNS
HGK (ZC1)	Overexpression	STE	Cancer
HIPK1	Overexpression	CMGC	Cancer
HIPK2	Loss-of-heterozygosityand Expression	CMGC	Cancer
Her2 (ErbB2)	Amplified andOverexpression	TK	Cancer
Her3 (ErbB3)	Overexpression	TK	Cancer
Her4 (ErbB4)	Expression	TK	Cancer
IGF1R	Mutation, SNP, andOverexpression	TK	Cancer, Growth, and Longevity
ILK	Overexpression	TKL	Cancer
IRAK2	Mutation	TKL	Cancer andInflammation
IRAK4	Mutation	TKL	Infection
JAK1	Mutation andActivated	TK	Cancer
JAK2	Translocation	TK	Cancer
JAK3	Loss-of-function Mutation	TK	Immunity
JNK3	Expression	CMGC	Cancer and CNS
KDR (FLK1, VEGFR2)	Mutation	TK	Cancer
KIT	Gain-of-function Mutation, Loss-of-function Mutation, andActivated	TK	Cancer andDepigmentation

LATS1	Methylation,Expression, andMutation	AGC	Cancer
LATS2	Expression	AGC	Cancer
LKB1 (STK11)	Loss-of-function Mutation	CAMK	Cancer
LYN	Activated	TK	Cancer
MER	Loss-of-function Mutation	TK	Vision
MET	Gain-of-function Mutation,Overexpression, and Translocation	TK	Cancer
MISR2 (AMHR2)	Mutation	TKL	Reproduction
MKK3 (MAP2K3)	Mutation andDeleted	STE	Cancer
MKK4 (MAP2K4)	Loss-of-function Mutation andDeleted	STE	Cancer
MLK4	Mutation	TKL	Cancer
MST4	Overexpression	STE	Cancer
MYO3A	Mutation	STE	Sensory
NEK2	Overexpression	Other	Cancer
NEK8	Overexpression	Other	Cancer and Renal
PAK3	Loss-of-function Mutation	STE	Cognition
PAK4	Overexpression	STE	Cancer
PDGFRalpha	Translocation,Deleted, andMutation	TK	Cancer andDevelopment
PDGFRbeta	Translocation andOverexpression	TK	Cancer
PEK (PERK)	Loss-of-function Mutation	Other	Diabetes
PHKgamma2	Loss-of-function Mutation	CAMK	Metabolism
PIM1	Translocation,Overexpression, and Mutation	CAMK	Cancer
PINK1	Mutation andExpression	Other	Neurodegenerationand Cancer
PKCalpha	Mutation,Deleted,Overexpression, and Activated	AGC	Cardiovascular andCancer
PKCbeta	SNP	AGC	Diabetes andCancer
PKCdelta	Expression	AGC	Cancer,Cardiovascular, andCNS
PKCepsilon	Amplified andMutation	AGC	Cancer,Cardiovascula

			r, andCNS
PKCgamma	Mutation	AGC	Neurodegenerationand Pain
PKR (PRKR)	Mutation,Expression, andActivated	Other	Cancer,Neurodegeneration, and Virology
PLK1	Expression	Other	Cancer
PRKX	Translocation	AGC	Reproduction
PRKY	Translocation	AGC	Reproduction
RAF1 (c-Raf)	Amplified	TKL	Cancer
RET	Loss-of-function Mutation, Gain-of-function Mutation, andTranslocation	TK	Cancer andDevelopment
RHOK	Loss-of-function Mutation	AGC	Vision
RNAseL	Mutation	Other	Cancer and Virology
RON	Overexpressionand Splice-Change	TK	Cancer
ROR2	Mutation	TK	Development
ROS	Translocation	TK	Cancer
RSK2 (RPS6KA3)	Loss-of-function Mutation	AGC	Development andCNS
SGK1	Expression	AGC	Diabetes, Cancer, and Cognition
SRC	Mutation,Overexpression, and Activated	TK	Cancer
SYK	Methylation andSplice-Change	TK	Cancer and Allergy
TGFbetaR1 (ALK5)	SNP	TKL	Cancer
TGFbetaR2	Loss-of-function Mutation	TKL	Cancer,Development, andFibrosis
TIE2 (TEK)	Mutation andOverexpression	TK	Angiogenesis andCancer
TRKA (NTRK1)	Mutation andTranslocation	TK	Cancer and Sensory
TRKB (NTRK2)	Mutation	TK	Cancer
TRKC (NTRK3)	Translocation andMutation	TK	Cancer
TYRO3 (SKY)	Overexpression	TK	Cancer
WNK1	Intronic Mutation	Other	Hypertension
WNK4	Mutation	Other	Hypertension

eEF2K (CaMKIII)	Overexpressionand Activated	Atypical	Cancer
p38		CMGC	Cancer andInflammation
p70S6K (RPS6KB1)	Overexpressionand Amplified	AGC	Cancer andDiabetes

1.3.2 Kinase Inhibitors

Therapeutic agents that are molecularly targeted have been used since 1990s in clinical practice. Majority of these therapeutic agents target receptor tyrosine kinases (RTKs) such as but not limited to lapatinib, erlotinib, imatinib that inhibit HER2, EGFR and KIT respectively (Table 1.2) (Slamon et al, 2001; Paez et al, 2004; Lynch et al, 2004; Demetri et al, 2002; Kataoka et al, 2001). Kinase inhibitors have a very important advantage when compared to cytotoxic drugs having DNA synthesis inhibition activity. Kinase inhibitors have low side effects and produce very low levels of toxicity (exception trastuzumab) (Novak C, 2004). Therefore, there are ongoing studies for the characterization and development of new novel kinase inhibitor derivatives against new targets, with higher activity and efficacy.

Table 1.2: Some of the clinically used or candidate protein kinase inhibitors
(taken from Fabbro et al, 2002)

Inhibitor	Company	Target kinase(s)	Status
Flavopiridol	Aventis	Cdks	Phase II
E7070	Eisai	Cdks	Phase I
Cyc202	Cyclacel	Cdks	Phase I
Bryostatin-I	Bristol Myers Squibb	PKC	Phase I-II
PKC412	Novartis	PKC, Flt-3	Phase I/IIA
UCN-01	NCI	PKC, chk1	Phase I
ISIS 3521	ISIS	PKC-α	Phase I/II
GEM231 (antisense)	Hybridon	PKA	Phase I
SCIO-469	Scios	p38	Phase I
ISIS 5132 (antisense)	ISIS	c-RAF	Phase I
STI571, Glivec®, Gleevec™	Novartis	Abl (c-Kit, PDGF-R)	Phase IV (p.o.); FDA approval 5/01
ZD 1839 (Iressa)	AstraZeneca	EGF-R	Phase III (p.o.)
OSI-774	Roche/Genentech/OSI	EGF-R	Phase III (p.o.)
PKI166	Novartis	EGF-R/HER-2	Phase I (p.o.)
CI-1033	Pfizer/Warner-Lambert	EGF-R/HER-2	Phase I (p.o.)
EKB-569	Wyeth-Ayerst	EGF-R/HER-2	Phase I (p.o.)
GW-2016	GlaxoSmithKline	EGF-R/HER-2	Phase I (p.o.)
SU5416	SUGEN	KDR	Phase III (i.v.)
PTK787/ZK222584	Novartis/Schering AG	KDR	Phase I (p.o.)
SU6668	SUGEN	KDR (PDGF-R, FGF-R)	Phase I (p.o.)
ZD6474	AstraZeneca	KDR (EGF-R)	Phase I (p.o.)
CEP2583	Cephalon	Trk	Phase II

There is a significant obstacle for the development of effective therapeutic agents for kinase inhibition: selectivity and target site. Producing small-molecule inhibitors that disrupt ligand binding (for RTKs) or protein-substrate interaction was shown to be ineffective (Burke et al, 1997; Sharma et al, 2002; Fabbro et al, 2002). Therefore, best potential strategy for drug discovery is to target the catalytic site by inhibitory molecules that are ATP competitive. Still there are two important problems to solve: membrane penetration and selectivity. In most of the molecules developed, penetration is shown to be prevail however, selectivity still remains to be hard to overcome (Davies et al, 2000; Fabbro et al, 2002).

1.4. Thiazolidine Compounds

With the improvements in proteomics in recent years, huge interest has been emerged for the development of anticancer drugs (Li et al, 2011). This huge interest caused the establishment of chemogenomics which is a screening field for libraries of small-molecules in order to investigate functionalities of proteins and find candidates for drugs (Li et al, 2007). In a previous study, a set of anticancer drug candidates was produced. The aim was to create an agent with potential to induce apoptosis in human cancer cell lines.

Some previous studies showed that molecules with thiazolidine have cytotoxic activity in various cancer cells compared to normal cells (Li et al, 2011; Li et al, 2007; Gududuru et al, 2005). Thiazolidines belong to the group of heterocyclic organic compounds. They contain 5-membered ring that is saturated and there is a thioether group inside this ring at position 1 together with an amine group at position 3. There are many commercial drugs that contain thiazolidine being the most famous as penicillin. On the other hand, in another study some thiazolidine-diones were shown to be inhibitors of protein tyrosine kinases such as EGFR or Src (Geissler et al, 1990).

CHAPTER 2. OBJECTIVES AND RATIONALE

According to the statistical analysis, in 2007, 13% of the human deaths all around the world was caused by cancer and every day the rate of cancer caused death is rising (Jemal et al, 2011). Therefore, scientists make a lot of effort to the field of cancer research. There are many aspects of cancer research however one of the ultimate goals is the development of anti-cancer therapeutic agents with high efficacy but low side-effects.

Due to the fact that protein-protein interactions are vital members of biological processes, development of molecules that can inhibit abnormal protein interactions is of ultimate importance for anti-cancer studies (Arkin et al, 2004). The main aim of this thesis is to identify anti-cancer small molecules with cytotocxic activities targetting specific cellar pathways.

The first group of molecules which were studied during this thesis was plant-derived small molecules from digitalis ferruginea provided by Prof. Dr. Ihsan Calis. As previously stated cardiac glycosides are steroid-like compounds and include commonly known therapeutics such as digitoxin, digoxin and ouabain (Figure 1.1). In previous experiments, it was shown that digitoxin, digoxin or oubain induce apoptosis in cancer cell lines and they are non-toxic against normal cells (Racker et al, 1983; McConkey et al, 2000; Ramiez et al, 2007). Most of the previous experiments were conducted on fragments derived from Digitalis lanata or davisiana, however there are no studies on molecules derived from digitalis ferruginea. Thus our objective was to examine pure molecules obtained by fractional extraction of of digitalis ferruginea and find the ingredient that has the cytotoxic activity in cancer cell lines and induce apoptosis in these cells.

Most of the therapeutic agents developed and in use right now are targeted against protein tyrosine kinases (Slamon et al, 2001; Paez et al, 2004; Lynch et al, 2004; Demetri et al, 2002; Kataoka et al, 2001). There are two reasons for this, first, protein kinases play inevitably significant role in cancer formation and progression. Second, kinase inhibitors have fairly low toxicity to normal cells and low side effects (exception trastuzumab) (Novak C, 2004). Therefore, one of our aims in this project

was to examine the kinase inhibitor derivatives synthesized by Prof. Dr. Birsen Tozkoparan. Our ultimate aim was to find the molecules with highest cytotoxicity with drugable IC50 values and to confirm that they are actiong on protein kinases. Then we showed their mechanism of cell death and the pathway that they interfere with. Finally, we would have small-molecule tyrosine kinase inhibitors with anticancer activity and these molecules would be candidates for anti-cancer therapeutics.

On the other hand, we had small molecules with thiazolidine ring. In previous studies, it was shown that molecules with thiazolidine ring have anti-cancer activities (Li et al, 2011; Li et al, 2007). Thus, in addition to the kinase inhibitor derivatives, we tested the cytotoxic activity of some molecules with thiazolidine ring. Our expectation was to find some molecules with cytotoxic activity in cancer cell lines and that would induce apoptotic cell death.

CHAPTER 3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1 General reagents

Most of the reagents and chemicals used including but not limited to ethanol and methanol, bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, USA) and MERC (Darmstadt, Germany). Bradford reagent for protein quantification was obtained from Sigma (St. Louis, MO, USA). Ponceau S and Dimethyl sulfoxide (DMSO) were from AppliChem Biochemica (Darmstadt, Germany). Trichloroacetic acid is purchased from MERC (Schucdarf, Germany). ECL kit, used for western blot chemiluminesence analysis, was obtained from Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK). Sulforhodamine B (SRB) sodium salt was purchased from Sigma Aldrich. Tris used for the preparation of 10mM TBS was obtained from Amresco (USA). Hoechst stain was purchased from Sigma Aldrich. Phosphatase inhibitor cocktail (PhosStop) and Protease inhibitor cocktail tablet (complete EDTA free) were obtained from Roche Applied Sciences. RNaseA used in FACS was purchased from Fermentas and propidium iodide was from Sigma Aldrich. Software used for FACS analysis, CellQuest was purchased from Becton Dickinson.

3.1.2 Tissue Culture Reagents and Materials:

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), non-essential amino acid (NEAA), penicillin/streptomycin solution, tyripsin and 1xPBS ($CaCl_2$, $MgCl_2$ free) were purchased from GIBCO (Invitrogen, Carlsbad, CA, USA). Flasks, petri-dishes, cell plates, serological pipettes that were used in cell culture were supplied from Corning Life Sciences

3.1.3 Antibodies:

Antibodies were purchased from different companies, which are all listed in Table 3.1. Their working dilutions are also indicated in the Table 3.1.

Table 3.1: Antibody list: All antibodies used are listed together with their working dilutions and incubation periods.

Name	working dilution	Incubation period and type	company name
α-actin (goat polyclonal)	1:500	2 hours, RT	Santa Cruz
Anti-goat HRP	1:5000	1 hour, RT	Santa Cruz
Anti-rabbit HRP	1:5000	1 hour, RT	Sigma Aldrich
PARP (rabbit monoclonal)	1:200	2 hours, RT	Cell Signaling

3.1.4 Western Blot, Electrophoresis, Kinase assay, Spectrophotometer and Microplate Reader for SRB Assay

Spectrometer used for Bradford Assay was Beckman Du640, purchased from Beckman Instruments Inc. (CA, USA). Kinase Assay reagents (Kinase-Glo Plus Luminescent Kinase Assay) were purchased from Promega (USA). The Reporter Microplate Luminometer Reader used for Kinase Assay was obtained from Turner

Bio-Systems (Sunnyvale, USA). Microplate reader used for SRB assay was obtained from Beckman Instruments.

Western Blot reagents such as NuPAGE pre-cast 12% and 10% Bis-Tris mini gels, running buffers (MOPS,MES), transfer buffer, electrophoresis tank, transfer equipment, 4X sample loading buffer, 10X denaturing agent and antioxidant were from Invitrogen. Power supplies Power-PAC200 and Power-PAC300 were from Bio-Rad Laboratories (CA, USA).

3.2 SOLUTIONS AND MEDIA

3.2.1 Cell culture solutions

DMEM growth medium working solution   10% FBS, 1% penicillin/streptomycin, 1% non-essential amino acid. Stored at 4°C.

3.2.2 Western blot and Protein lysis solutions and buffers

NP-40 lysis buffer   150 mM NaCl, 50 mM TrisHCl (pH 7.6), 1% NP-40, 0.1% SDS, 1x protease inhibitor cocktail, 1x phosphatase inhibitor cocktail in double-distilled water.

10X Tris buffered saline (TBS)	12.2 g Trisma base, 87.8 g NaCl in 1 liter ddH$_2$O, pH 7.4.
TBS-tween (TBS-T)	0.1% Tween-20 was dissolved in 1x TBS.
Ponceau S	0.1% (w/v) Ponceau, 5 % (v/v) acetic acid in double-distilled water.
Blocking solution	5% (w/v) non-fat dry milk/bovine serum albumin in 0.2% TBS-T.

3.2.3 Cell cycle analysis solutions

Propidium Iodide staining solution	50 μg/mL propidium iodide, 0.1 mg/mL RNaseA and 0.05% TritonX-100 in 1X PBS.

3.2.4 Kinase Assay solutions

Kinase-Glo reaction buffer	40mM Tris-HCl pH7.6, 20mM MgCl$_2$, 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 50 μl sample solution in Kinase-Glo reaction buffer.

3.2.5 Sulphorhodamine B assay solutions

SRB stain solution	0.04gr SRB stain in 10ml 1% acetic acid solution.
10% TCA solution	100% TCA was diluted into 10% in cold ddH$_2$O.
10mM TBS	0.6gr Tris was dissolved in 1000ml cold ddH$_2$O.

3.2.6 Immunofluorescence staining:

Hoechst stain stock solution	300μg/ml Hoechst dissolved in ddH$_2$O and stored at +4°C.
Hoechst stain working solution	1μg/ml Hoechst diluted from 300μg/ml stock in 1x PBS.

3.3 METHODS:

3.3.1 Cell lines and growth conditions

Human liver cancer cell lines (Huh7, Mahlavu, Focus, HepG2, Hep3B), human breast cancer cell line (MCF7) and human colon cancer cell line (HCT116) were cultivated in complete growth medium composed of DMEM, with 10% FBS, 1% NEAA and 1% P/S. All cell lines were incubated in 37 °C, 5% CO_2, 95% air containing incubators. For the sub culturing of the cells, when they reached %75-%80 confluence, they were washed twice with 1xPBS after aspirating the growth medium and then trypsin-EDTA solution was applied. Then the cells were resuspended in fresh growth medium and aliquots of appropriate concentration (sub culturing ratio between 1:6 to 1:10 depending on the cell type) was transferred into new culture dishes with fresh complete growth media.

For the experiments that require a defined number of cells, after trypsinization and resuspension of the cells in fresh growth medium, hemocytometer was used to count the cell number.

Before usage, all solutions and media were warmed to $37^{o}C$ from $4^{o}C$.

3.3.2 Thawing of the cell lines:

All cell lines used are stored in liquid nitrogen. Before usage, one vial of the cell line of interest is taken out of the tank and directly put into $37^{o}C$ water bath for 1-2 minutes so that the cell solution starts to thaw. When the outer layer of the solution is thawed, the whole cell suspension is resuspended in 5ml warm fresh growth medium, moved into a 15ml falcon tube and centrifugated at 1200rpm, $+4^{o}C$, for 3 minutes. After the supernatant is aspirated, cell pellet is resuspended in 10ml warmed fresh growth medium and transferred into 100mm culture dish. Then the media of the cells are refreshed after 24hr incubation in 37 °C, 5% CO_2, 95% air containing incubators.

3.3.3 Storage of the cell lines:

After cells were harvested by trypsinization, trypsin was neutralized with growth medium, which contains FBS. Then the cells were centrifugated at 1500rpm for 5 min, +4°C. The cell pellet was then resuspended in freezing medium of 10%DMSO, 20%FBS and 70% complete DMEM in a concentration of $4x10^6$ cells/ml. Finally, 1 ml of the cell solution was transferred into 1ml screw capped-cryotubes. They are left in -20°C for 1 hr and then transferred into -80°C. 24hr later, they were located into liquid nitrogen tank.

3.3.4 Crude total protein extraction:

Cancer cell lines of defined cell count (from 100.000 to 250.000) were cultured into 100mm culture dishes. After 24hr incubation, growth medium was aspirated and the cells were washed twice with 1xPBS. Then new growth medium containing the corresponding small molecule inhibitor (or DMSO as control) concentration was applied on the cells. Then in a time dependent manner (12hr, 24hr, 36hr, 48hr time periods), the inhibitor containing medium (or DMSO as control) was aspirated and ice-cold PBS was applied. Finally cells were scraped using rubber scrapers. Cell suspensions were transferred into 15ml falcon tubes and centrifuged for 1200rpm 5min +4°C. Then supernatant was aspirated and cells were resuspended in 2ml ice-cold PBS and centrifuged for 1200rpm 5min +4°C once again. Finally, the supernatant was discarded and cell pellets were frozen in liquid nitrogen and stored in -80°C.

For the production of protein lysates, lysis buffer (150mM NaCl, 50mM Tris-HCl pH=7.6, 1% NP-40, 0.1% SDS, 1x Protease inhibitor cocktail and 1x PhosStop) is applied to the cell pellets in twice the amount of the pellet. After mixing the pellets by vortex, they were incubated on ice for 45min (mixing by vortex every 5 min). In case of viscosity, samples are sonicated (if no viscosity, this step is skipped). After centrifugation, the samples at 13,000rpm for 15-20min at +4°C, the supernatant was transferred into new eppendorphs and stored at -20°C.

3.3.5 Western Blotting:

The quantification of the protein in lysates, the Bradford protein assay was conducted. BSA in solutions with known concentrations was used as reference for standard curve of OD_{595}. The protein concentrations of cell lysates were calculated with respect to the standard curve. Then equal amounts of cell lysates (20ng-50ng of protein) were solubilized with 1x loading dye, SRA (or DTT) and then water is used to fill the total solution volume to 15ul or 25ul. Then the lysates were denatured for 10 min in 70°C following the incubation of lysates on ice for 2 min. Then the samples were spinned down and loaded into the gel.

NuPAGE NOVEX pre-cast gel system was used for all western blot analysis procedures according to the manufacturer's protocol. 12%, 10% Bis-Tris gels were used. Depending on the protein length MOPS or MES running buffer was used. In correspondence with the manufacturer's protocol, 10%methanol was added to the transfer buffer.

After electrophoresis and the transfer of the proteins from the gel to nitrocellulose membrane (30V, 90min) the membrane was incubated overnight in blocking solution (%5milk powder in 1xTBS-T(0.1%tween)) at +4°C. Then the membrane was treated with primary antibody (1hr-2hr) at room temperature. Washing off the membrane 4 times (for 5min, 10min, 10min,and 5min) with 1xTBS-T(0.1%tween) solution was followed by the treatment of the membrane with secondary antibody (1hr-90min) at room temperature. Finally after washing the membrane once more, chemiluminesence was performed with ECL+ kit according to the manufacturer's protocol. The emmition of chemiluminesence was captured on X-ray film with different exposure times.

3.3.6 Kinase Assay:

Kinase-Glo plus luminescence kinase activity assay (Promega) was performed according to manufacturer's protocol.

First the Kinase-Glo reaction buffer (40mM Tris-HCl pH7.6, 20mM $MgCl_2$, 0.1mg/ml BSA) was placed into the wells of a 96-well Elisa plate. Then lysates (25μg protein concentration) (protein quantification was done by Bradford assay) were placed into the wells. The total volume of lysate and kinase reaction buffer is 50μl. Then 50μl Kinase-Glo reagent (Kinase-Glo plus substrate+Kinase-Glo plus Buffer) was applied. After 10min dark incubation at room temperature, the luminescence was detected in luminometer. If kinase activity is diminished, ATP concentration increase and so will the luminescence .

3.3.7 NCI-60 Sulphorhodamine B (SRB) assay:

Human cancer cell lines of breast, colon and breast (range of 2000cell/well to 5000cell/well) were plated into 96-well plates in 200μl of media. After 24hr incubation period of cell lines in 37 °C incubators containing 5% CO_2, 95% air, one plate for each cell line was fixed using 50μl 10% ice-cold trichloroacetic acid (TCA) (time-zero plate representing the behavior of the cells at the time of drug treatment) and in the same time compounds of interest (compounds are solubilized in DMSO) were applied to the cells in desired concentrations and diluted by serial dilution. Following drug treatment, the cells were incubated in 37 °C incubators for 24hours, 36hours or 72hours. In order to terminate the incubation period, cells were fixed with 50μl 10% ice-cold TCA and incubated 1hr in dark at $+4^oC$. After washing TCA with ddH$_2$O repeatedly, the plates were left to dry. Finally, 50μl 0.4%sulphorhodamine B (SRB) solution in 1% acetic acid was used to stain the plates. Following the 10min dark incubation period, 1% acetic acid is used to discard excess dye. For the quantification of the bound stain, SRB is solubilized using 200μl of 10mM Tris-Base. Finally, the OD values were obtained at 515nm wavelength by using Elisa reader.

3.3.8 Real time cell growth surveillance by cell electronic sensing (xCelligence):

In order to eliminate background noise, into each well of the 96-well E-plate (Roche Applied Sciences) 50μl growth media was applied and the absorbance was read. Then, human cancer cell lines (range from 1000cell/well to 5000cell/well in 150μl) were plated into E-plate. In real-time cell electronic sensing RT-CES (xCelligence) the proliferation curve of the cells were obtained. In the first 24hour period, in every 30min, the cell index values were detected (9). After 24hr incubation, growth medium is aspirated and 100μl fresh growth medium was applied. Then the cells were treated with the compounds of interest in the indicated concentrations. In the first 24hr of drug treatment, cell index values were detected for every 10 min (fast-drug response) then it changes to every 30 min (long-term drug response). Detected cell index values symbolizes the impedance measurements. By calculating CI_{drug}/CI_{dmso}, the inhibitory effect of the compounds on the cell lines were calculated in response to DMSO control (10).

3.3.9 Immunofluorescence staining:

Autoclaved coverslips were placed into 6-well plates and human cancer cell lines were inoculated into these 6-well plates (range 30.000-100.000 cell/well) in 2ml growth medium. 24hr later, growth medium was aspirated and 1.5ml fresh growth medium that contains the compounds of interest at indicated concentrations was applied to cells. After 24hr, 48hr or 72hr incubation period, the cells were washed once with ice-cold 1xPBS and fixed with 100% ice-cold methanol for 10min. Then excess methanol was discarded by washing once with ice-cold 1xPBS. Then 1μg/ml Hoechst stain (dissolved in PBS) was applied to the cells and left for dark incubation 5min. Finally destaining was established in ddH_2O for 10 min.

After the staining process, coverslips were mounted onto slides with glycerol and cells were observed under fluorescent microscope and pictures were taken.

3.3.10 Fluorescence-activated cell sorting (FACS) analysis:

Human cancer cell lines of interest were inoculated into 100mm culture dish (100.000-200.000 cell/dish) in 6ml growth medium. 24hours later, growth medium was replaced by the starvation medium (1%FBS, 1%P/S, 1%NEAA in DMEM). After cells were incubated in starvation medium for one more day, drug treatment was applied in growth medium. At the same time, time zero plates were fixed.

For fixation of the cells, first of all swimming cells were collected in 15ml falcon tubes. Then remaining cells inside the culture dish were washed once with 1xPBS and trypsinized. These trypsinized cells were then harvested together with previously collected swimming cells. The cell suspension was then centrifuged for 5min, at 1200rpm. Supernatant was discarded and cell pellet was resuspended in 5ml 1xPBS. Once again the cell suspension was centrifuged for 5min, at 1200rpm and supernatant was discarded. Finally pellet was resuspended in 1ml ice-cold 1xPBS and fixed by addition of 2.5ml, 100% EtOH (final concentration was 70%) drop wise while mixing by vortex meanwhile. Then the samples were stored at +4°C.

Before analyzing the samples with FACS machine, cell pellet was resuspended in 500μl Propidium iodide (PI) solution (25μl PI, 5μl RNaseA (10mg/ml stock), 0.25μl Tritron-X-100, 469.75μl ice cold 1xPBS) and incubated at 37°C (dark) for 40min. Afterwards, 3ml 1xPBS was added onto the samples and centrifuged at 150rpm for 5min, +4°C. Supernatant was discarded and pellet was resuspended in 500μl 1xPBS and loaded to the machine for FACS analysis.

CHAPTER 4. RESULTS

4.1 Digitalis Ferruginea

4.1.1 Cytotoxic activity analysis of all 6 ingredients of Digitalis Ferruginea

Initially, the cytotoxic activity of the Digitalis Ferruginea ingredients Lanatoside A, Lanatoside C, Glucogitorosid, Ferruginoside A, Lugrandoside and Ferruginoside B was analyzed using NCI-60 SRB assay as described in the methods part. Molecules were tested on liver (HUH7, MV, HepG2, Focus) cancer cell lines. Lanatoside A, Lanatoside C and Glucogitorosid showed significant cytotoxic effects on liver cancer cell lines whereas the other three agents weren't effective (Figure 4.1).
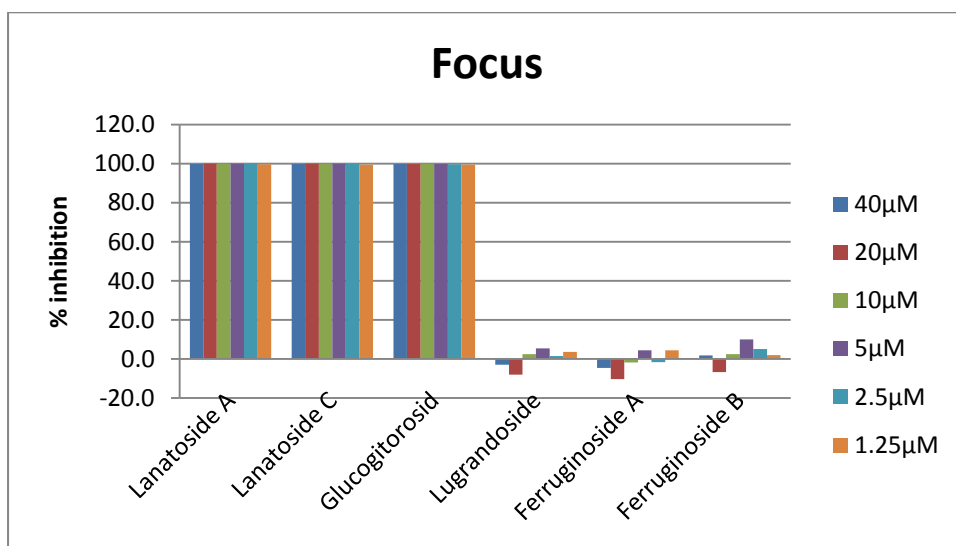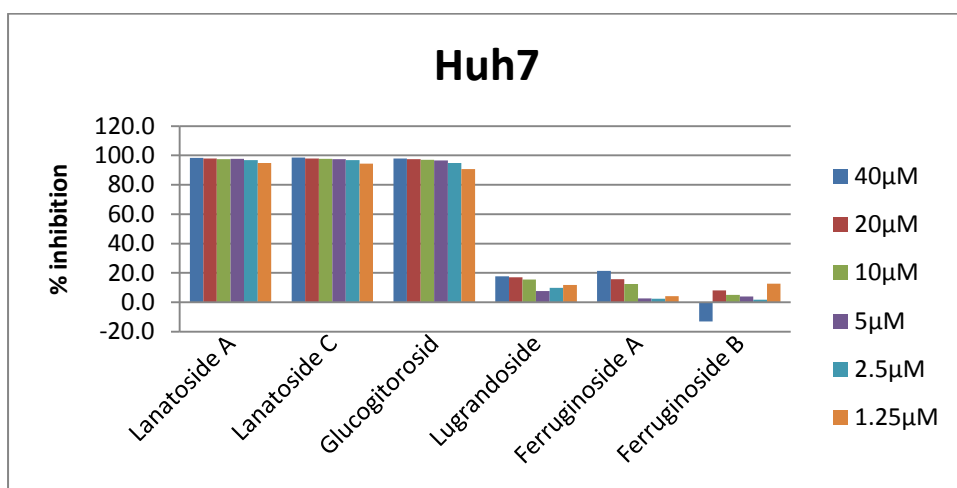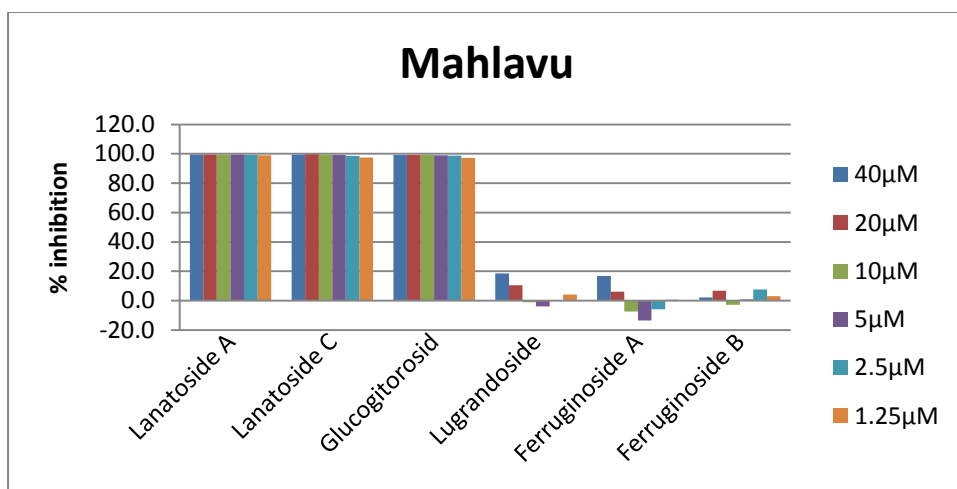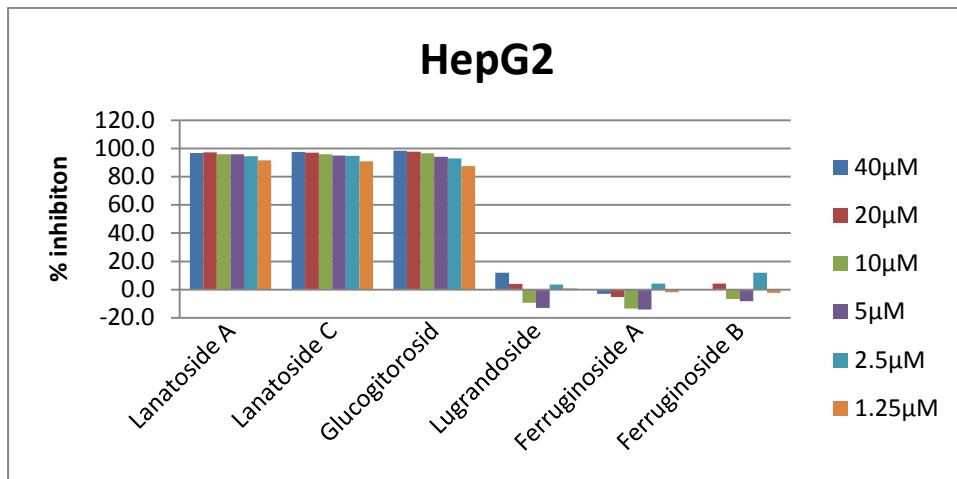
Figure 4.1: Percent cell death inhibition: Liver cancer cell lines Huh7, HepG2, Mahlavu and Focus were treated with Lanatoside A, Lanatoside C, Glucogitorosid, Ferruginoside A, Lugrandoside and Ferruginoside B for 72h. NCI60 SRB analysis was applied as explained in methods.
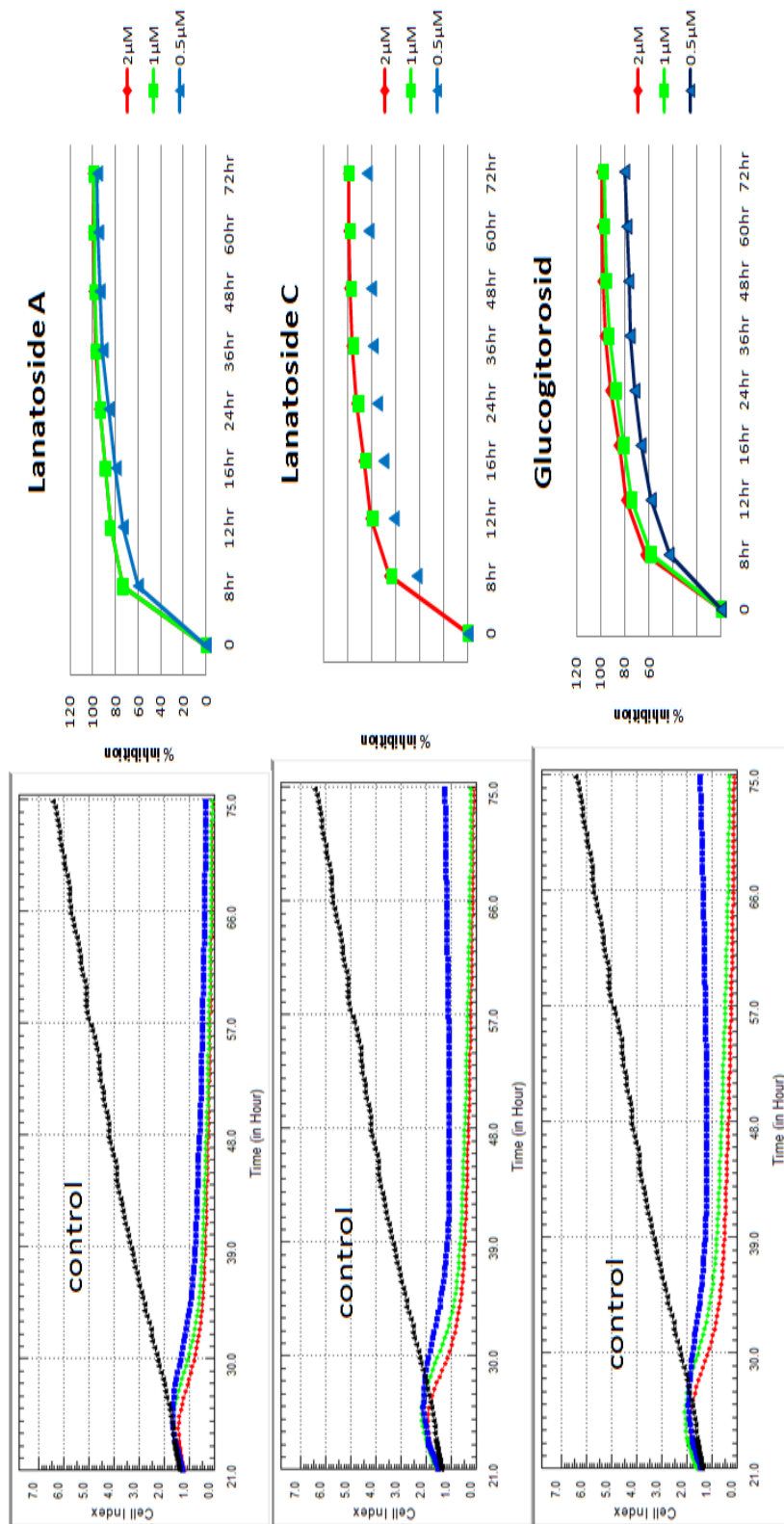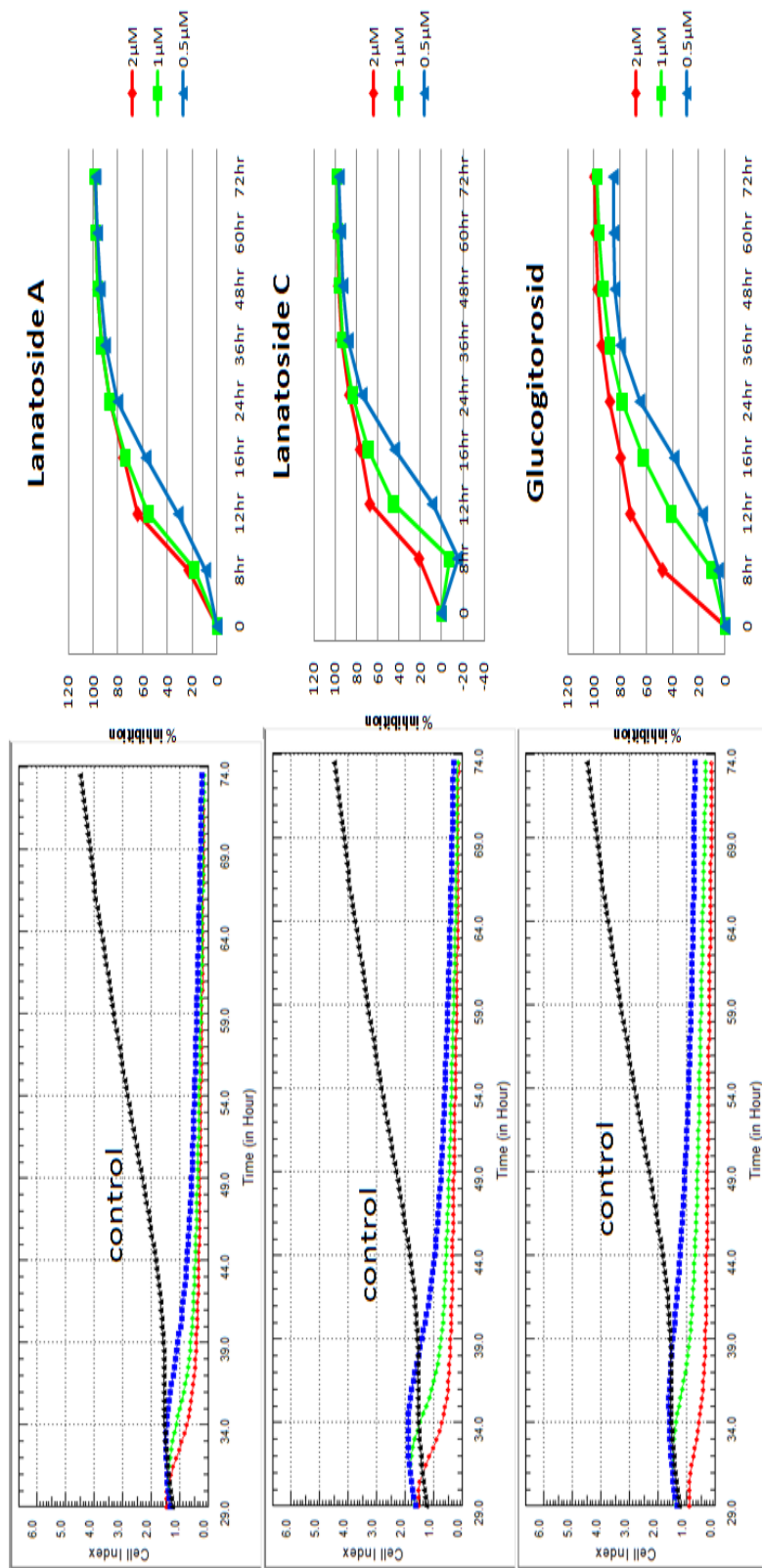
Figure 4.2: Real-time cell growth analysis of the compounds on Mahlavu cell lines:
Compound Lanatoside A,Lanatoside C and Glucogitorosid were applied on mahlavu cell line in indicated concentrations and observed for 72 hours. Cell-impedance based xCelligence system was used to observe the growth inhibitory effect in real-time as explained in the methods part. The graphs represent time-dependent effect of the compounds on cell index and cell growth. DMSO treated cells were used as control (BLACK) and cell growth in compound treated was normalized to the growth in DMSO treated cells. Further normalization was performed according to time-zero values.

Figure 4.3: Real-time cell growth analysis of the compounds on Huh7 cell lines: Compound Lanatoside A, Lanatoside C and Glucogitorosid were applied on Huh7 cell line in indicated concentrations and observed for 72 hours. Cell-impedance based xCelligence system was used to observe the growth inhibitory effect in real-time as explained in the methods part. The graphs represent time-dependent effect of the compounds on cell index and cell growth. DMSO treated cells were used as control (BLACK) and cell growth in compound treated was normalized to the growth in DMSO treated cells. Further normalization was performed according to time-zero values.

Furthermore, the time-dependent cytotoxic activities of three compounds Lanatoside A, Lanatoside C and Glucogitorosid were investigated using the cell electronic assay xCELLigence in real-time. As seen in Figure 4.2 and 4.3, all three compounds have irreversible growth inhibitory effect on both liver cancer cell lines Huh7 and Mahlavu for 72 hrs. The cytotoxic effects of the compounds are highest in 8hr incubation period and it decreases as the incubation time increases to 72 hours. On the other hand, the inhibitory effect of the compounds in Huh7 is relatively lower than Mahlavu at same concentrations. This also confirms the SRB assay result that IC50 value of Huh7 is higher than mahlavu and percent inhibition of the compounds at constant concentration is higher in mahlavu (Figure 4.1, 4.2 and 4.3).

4.1.2 Morphological effect of the compounds

After showing that three compounds of the total 6 have high cytotoxic activity on cancer cell lines, we wanted to investigate the death mechanism that they trigger. Previous data show that cardiac glycosides induce apoptosis in a tissue-specific manner (Moss, 1998; Winnicka et al 2006). On the other hand, in some other study it was mentioned that cardiac glycosides are also activators of autophagy pathway (Hundeshagen et al, 2011). Therefore, in order to investigate the death mechanism the compounds induce, Huh7 and Mahlavu cells were treated with Lanatoside A, Lanatoside C and Glucogitorosid and observed under inverted microscope.
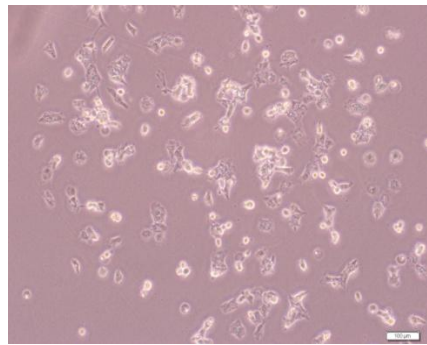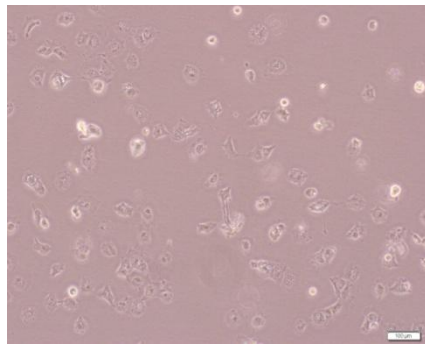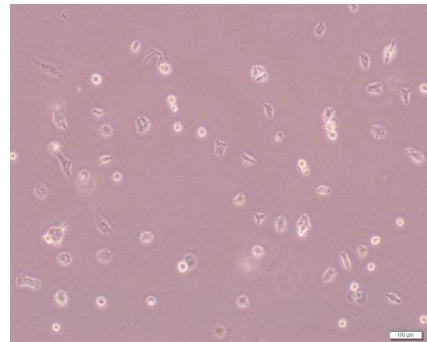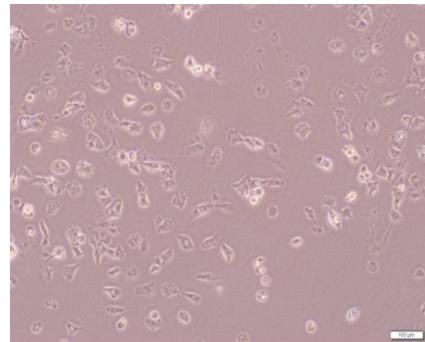
Mahlavu

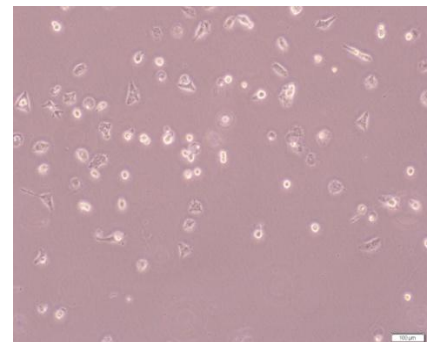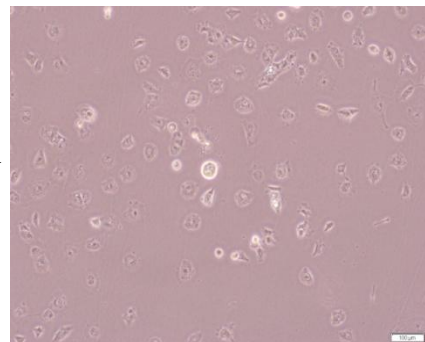24 hours   48 hours

DMSO
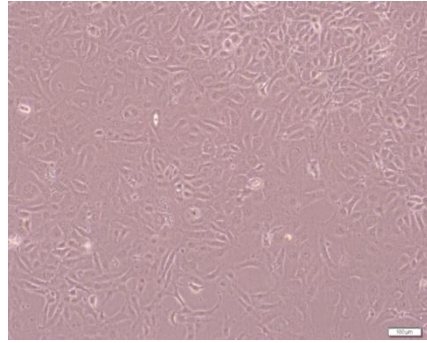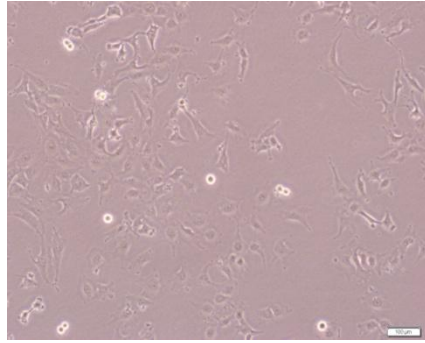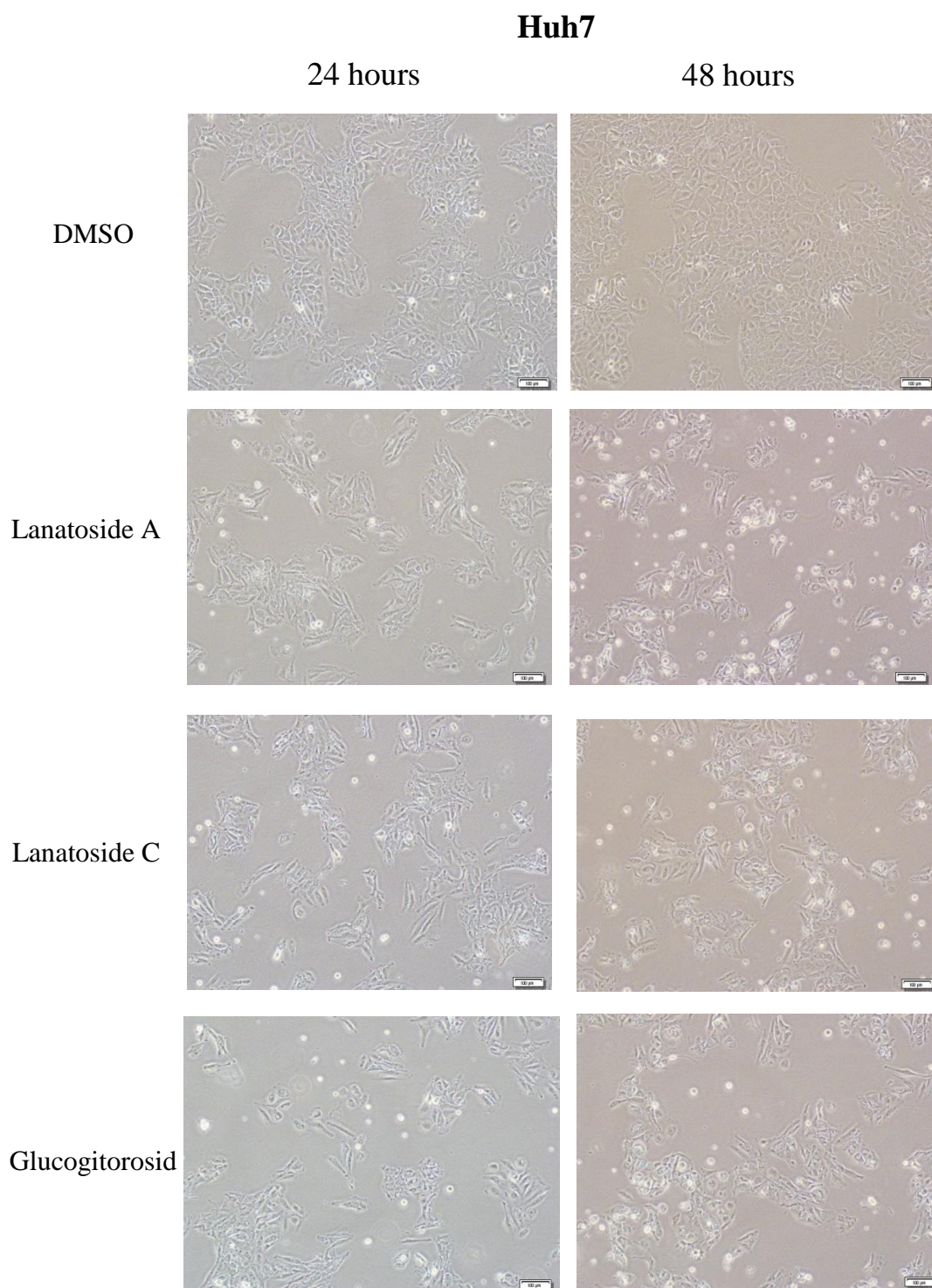
Lanatoside A

Lanatoside C

Glucogitorosid

Figure 4.4: Morphological effect of Cardiac glycosides Lanatoside A, Lanatoside C and Glucogitorosid on Huh7 and Mahlavu human liver cancer cell lines (10x). Cancer cell lines were treated with 2μM of the corresponding molecule. Then photographs of the cells were taken with inverted microscope after 24 and 48hours of incubation. DMSO treated cells were used as control.

As seen in Figure 4.4, cells treated with all three compounds showed apoptotic morphological changes. Shrinking cells were observed as well as cells in "horse-shoe" structure. Most of the cells observed were in a circulated structure.
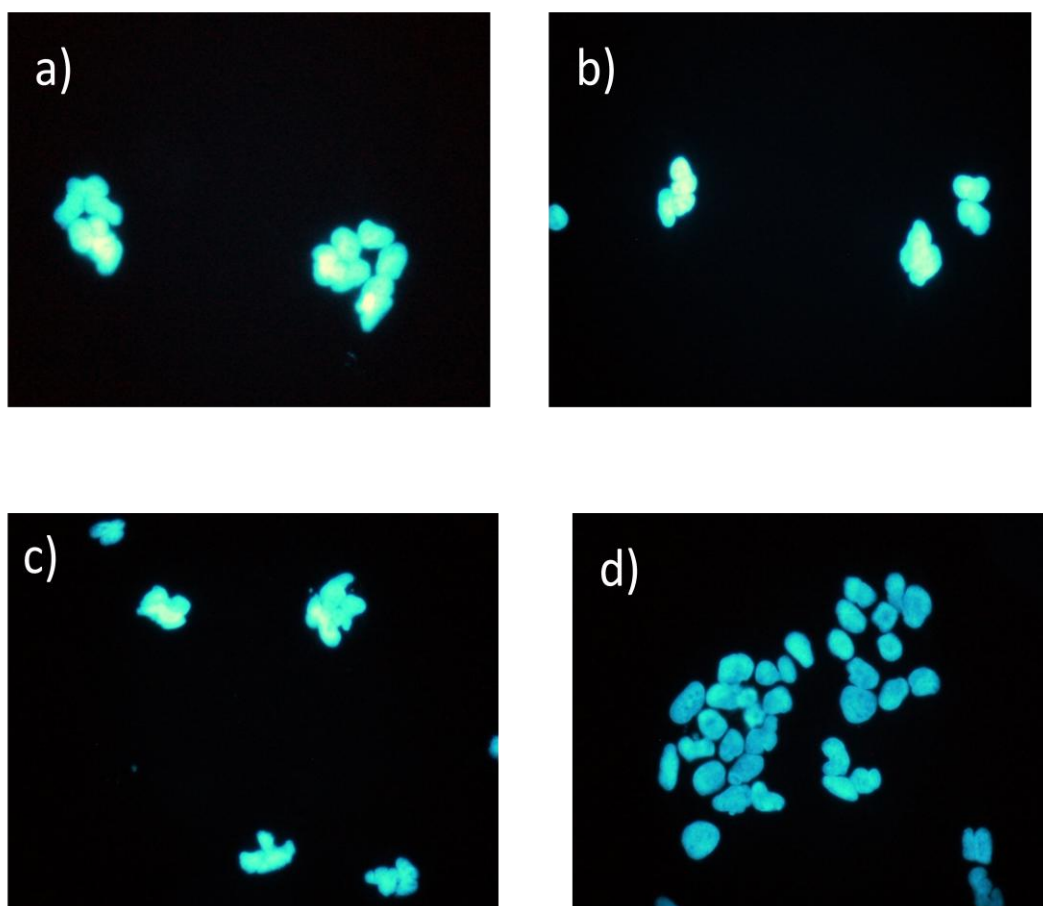

Figure 4.5: Hoechst staining of Huh7 cell lines: Morphological effect of a) Lanatoside A, b) Lanatoside C, c) Glucogitorosid d) DMSO at 2μM concentrations were observed on Huh7 cell lines. Human cancer cell lines were inoculated on coverslips in the confluency of 60-70% and incubated 24hr at 37°C. Then Huh7 cells were treated with corresponding molecules for24hr and Hoechst staining was applied.

After observing the cells under inverted microscope, we wanted to confirm the presence of apoptotic induction by Hoechst staining. For this purpose, human liver cancer cell line Huh7 was treated with three compounds and stained with Hoechst. Then cells were observed under fluorescent microscope. As seen in Figure 4.5, Hoechst staining showed condensed nuclei that indicated apoptotic cells in treated samples. Whereas in Huh7 cell group treated with DMSO, no apoptotic cells were present. This also confirmed that Lanatoside A, Lanatoside C and Glucogitorosid induce apoptosis in liver cancer cell lines.

4.1.3 Cell cycle arrest caused by Lanatoside A, Lanatoside C and Glucogitorosid

Decrease in the proliferation of the cells and induction of cell death points the presence of cell cycle arrest. Therefore, we used propidium iodide labeling, which is a fluorescence molecule that interacts with DNA (Senturk et al, 2010). The progression of cell cycle was observed in propidium iodide labeled Huh7 and Mahlavu cell lines treated with corresponding compounds using flow cytometry. As seen in Figures 4.6 and 4.7, cells treated with Lanatoside A, Lanatoside C and Glucogitorosid all showed a slight increase in G2/M phase when compared to DMSO control.

Normally, healthy well-differentiated HCC cell lines would be 60-70% in G1 phase, 20% in S phase, and 20% in G2-M phase. However, in treated cells, G2/M phase increased to 30-35% and G1 phase decreased to 40-50% (Figure 4.6). Mahlavu, which is a poorly differentiated HCC cell line, also showed similar results in which G2/M phase cells showed an increase when compared to DMSO control (Figure 4.7).
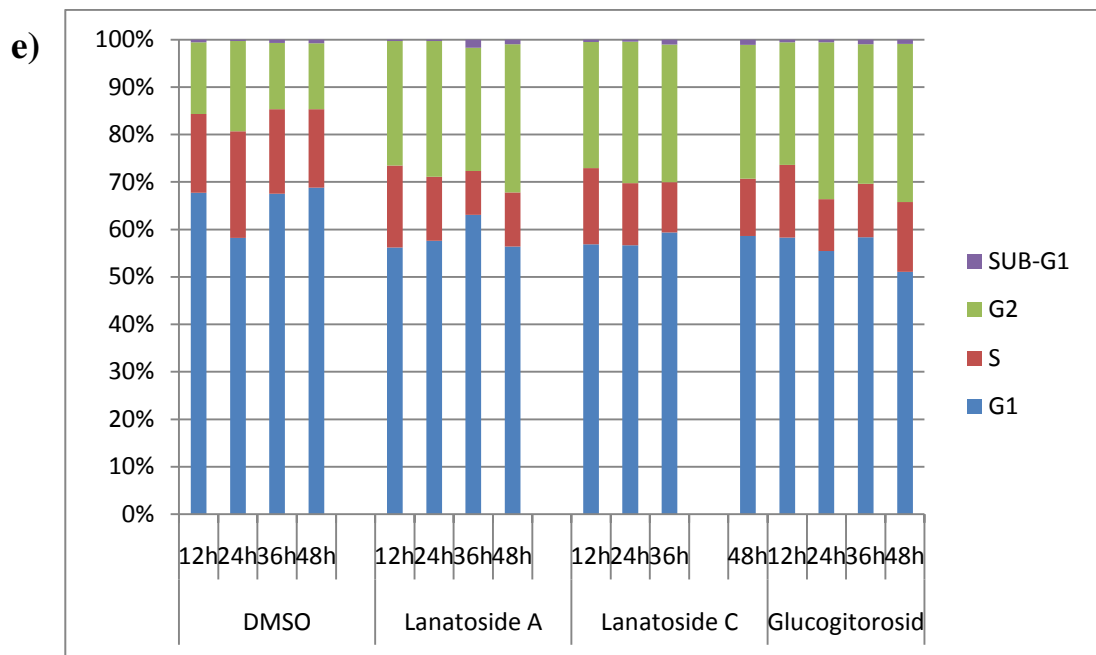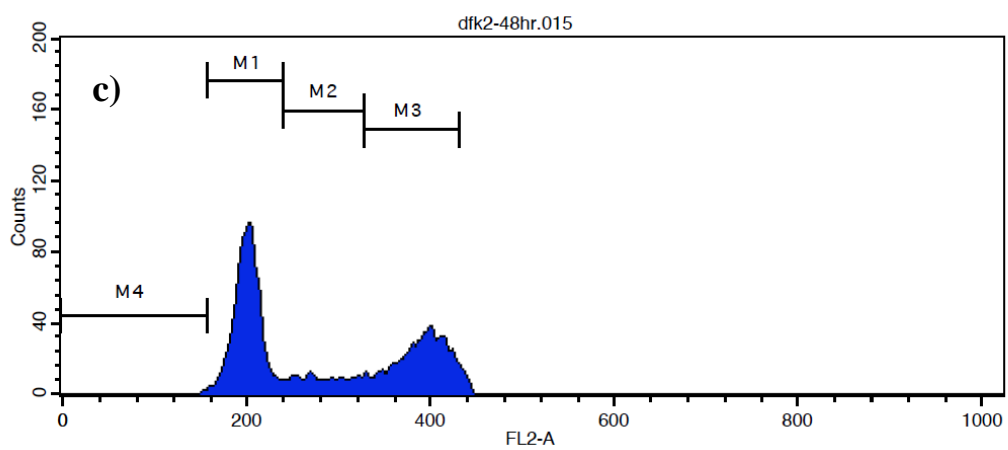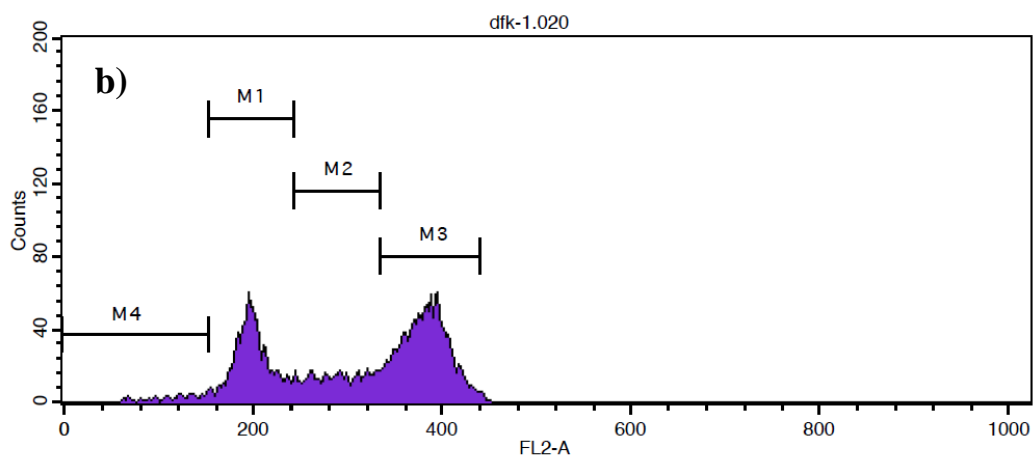
e)
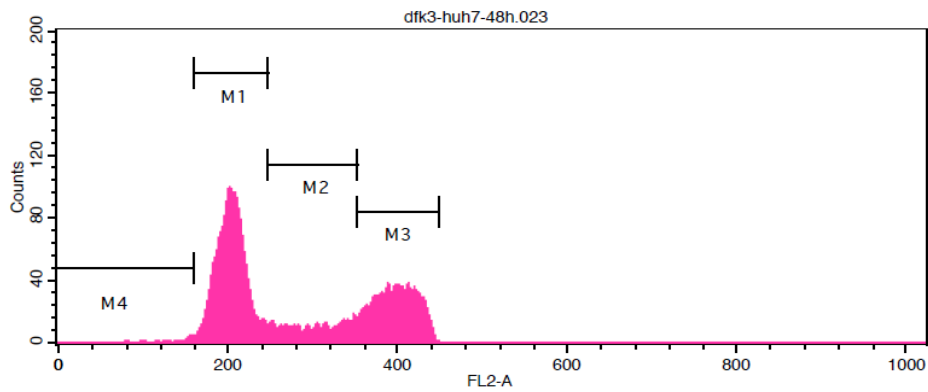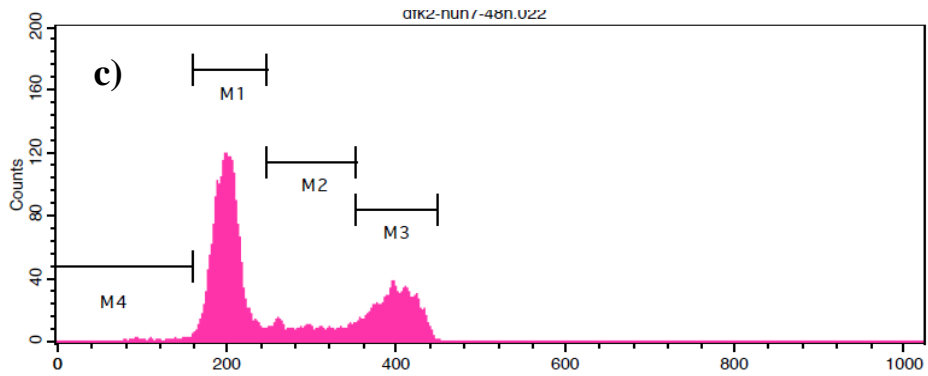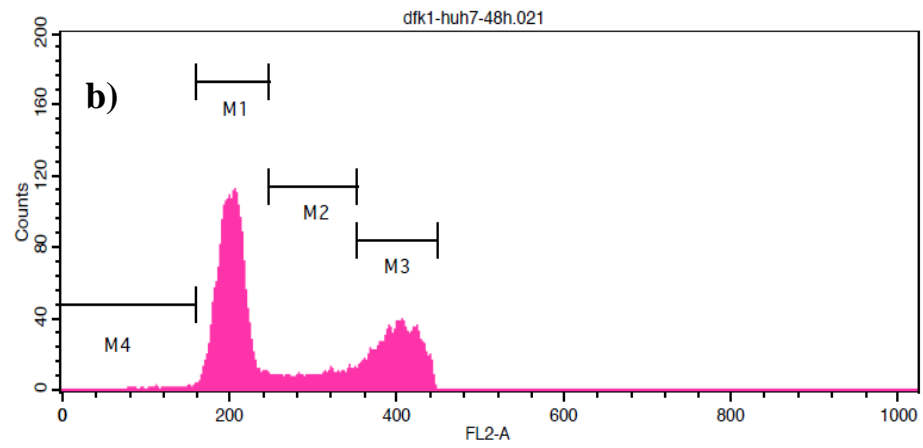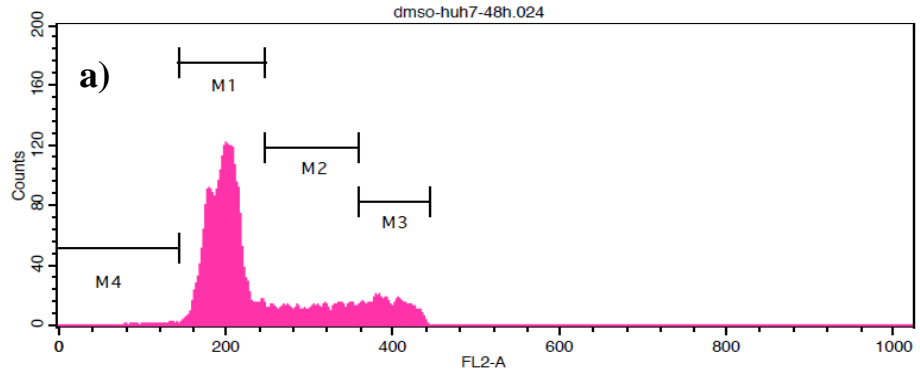
Figure 4.6: Cell cycle analysis after treatment with a) DMSO, b) Lanatoside A, c) Lanatoside C, d) Glucogitorosid, in Huh7 cell lines. In part e) the graphical analysis of cell cylce is shown. In parts a,b ,c and d, the peak at 200 FL2-A represents 2N cells (G1) and the peak at 400 represents 4N cells (G2). The peak in between represents S-phase cells. During gating >4N cells were excluded since those didn't show any variation between control and treated cell groups. FACS analysis was done as explained in the methods part. All drugs are applied at 2μM concentration.
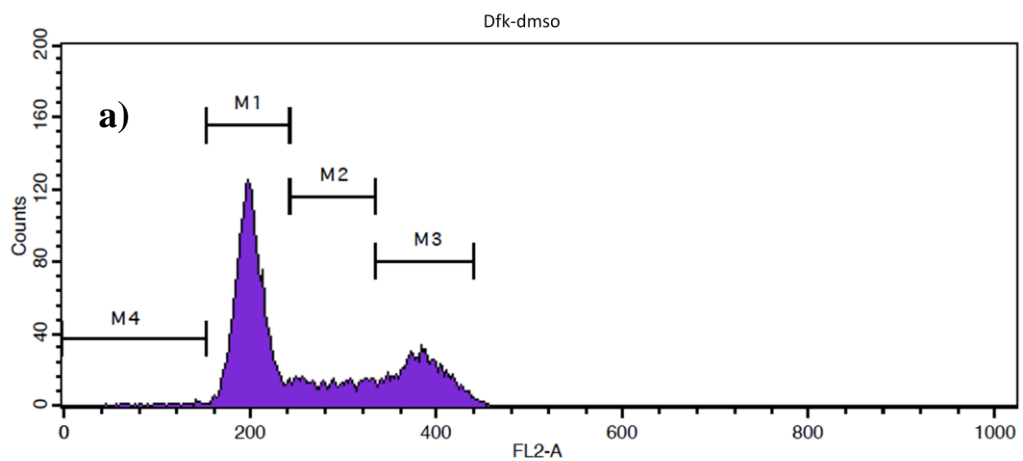
d)

e)

f)

g)

Figure 4.7: Cell cycle analysis after treatment with a) DMSO (for Lanatoside A), b) Lanatoside A, c) Lanatoside C, d) Glucogitorosid, e) DMSO (for Lanatoside C and Glucogitorosid) in Mahlavu cell lines. In part f) and g) the graphical analysis of cell cycle is shown. In parts a,b ,c and d, the peak at 200 FL2-A represents 2N cells (G1) and the peak at 400 represents 4N cells (G2). The peak in between represents S-phase cells. During gating >4N cells were excluded since those didn't show any variation between control and treated cell groups. FACS analysis was done as explained in the methods part. All drugs are applied at 2μM concentration.
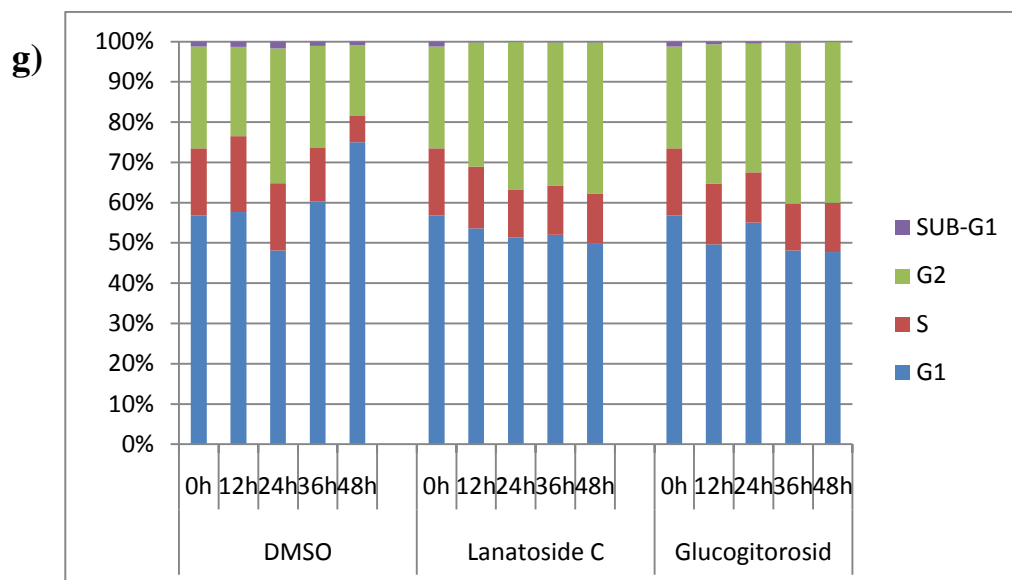
4.1.4 Apoptosis induction by Lanatoside A, Lanatoside C and Glucogitorosid

In the previous parts, it was shown that all three compounds causes morphological changes in cancer cells that represent apoptosis. Finally, we wanted to confirm apoptosis induction on the protein level. Thus we investigate Poly(ADP-ribose) Polymerase (PARP) cleavage in cells treated with the compounds in response to DMSO control. Previously it was shown that PARP cleavage which is catalyzed by caspase 3 is required for apoptosis progression (Boulares et al, 1999). Therefore, we treated Huh7 and Mahlavu liver cancer cell lines with Lanatoside A, Lanatoside C and Glucogitorosid and investigated the presence of PARP cleavage by Western Blot analysis.
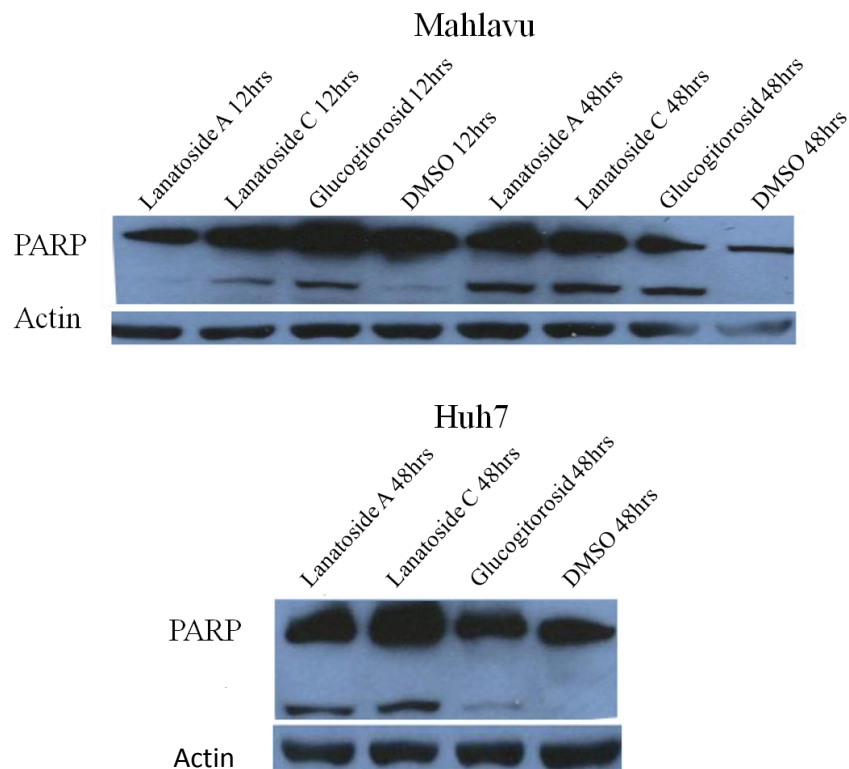
Figure 4.8: PARP cleavage in Huh7 and Mahlavu cells treated with corresponding compounds: Both fragments of PARP are recognized with this antibody so in case of apoptosis two bands are seen. Actin was used for equal loading of the proteins. Western blot procedure was performed as mentioned in the methods part. All drugs are applied at 2μM concentration.

In Figure 4.8, it can be clearly seen that Lanatoside A induces PARP cleavage and thus apoptosis in both Huh7 and Mahlavu cell lines in 48hr. However in 12hr incubation of Mahlavu cell line, PARP cleavage is hardly seen. On the other hand, Lanatoside C promotes PARP cleavage both in 12hr and 48hr in Mahlavu cell line. In addition, it promotes PARP cleavage in Huh7 as well. Finally, Glucogitorosid can induce PARP cleavage strongly in Mahlavu cell lines in both incubation periods however in Huh7 cell line even in 48hr incubation cleavage is very low.

Hence all these experiments show that cardiac glycosides Lanatoside A, Lanatoside C and Glucogitorosid all induces caspase-related apoptosis and cell cycle arrest at G2/M-phase in liver cancer cell lines.

4.2 Kinase Inhibitor Derivatives

4.2.1 Cytotoxic activity analysis

Initially, the cytotoxic activities of all the candidates were analyzed using NCI60 SRB assay in triplicate as described in the methods part. Molecules were tested initially on liver (HUH7), breast (MCF7) and colon (HCT116) cancer cell lines. The best cytotoxic activity was observed in IBUTRI group candidates (Table 4.1). Due to these promising results, number of carcinoma cell lines tested was increased in liver cancer (HepG2, Hep3B and Mahlavu). As seen in Table 4.2, IBUTRI group candidates showed high cytotoxic activity in micromolar concentrations in these liver cancer cell lines as well.

Table 4.1: Inhibitory concentration 50 (IC50) values of the compounds: All compounds were tested for cytotoxic activity by means of SRB assay in triplicates for 72hours as explained in the methods part.

	HUH7	MCF7	HCT116
Molecule Name	IC-50 (μM)	IC-50 (μM)	IC-50(μM)
IBUTRI	33.5	17.8	39.7
NAPTRI	43.4	124.2	20.8
IBUTRI-nTDZ	8.1	6.6	18.6
IBUTRI-ClTDZ	15.2	10.4	17.4
IBUTRI-FTDZ	8.5	4.2	8.9
NAPTRI-nTDZ	13.2	15.4	18.6
NAPTRI-ClTDZ	9.3	9.8	8.4
NAPTRI-FTDZ	13.7	11.9	10.8981
4-CH$_3$FOXAT	no inhibiton	no inhibiton	no inhibiton
4-CH$_3$FOXPT	no inhibiton	no inhibiton	no inhibiton
4-CH$_3$FOXAT-nTDZ	45.4	22.6	54.5
4-CH$_3$FOXAT-ClTDZ	39.9	27.8	51.6
4-CH$_3$FOXAT-FTDZ	18.8	32.7	65.1
4-CH$_3$FOXPT-nTDZ	19.6	14.5	18.6
4-CH$_3$FOXPT-ClTDZ	11.9	13.7	29.2
4-CH$_3$FOXPT-FTDZ	21.9	16.3	27.2

Table 4.2: Inhibitory concentration 50 (IC50) values of the compounds: All compounds were tested for cytotoxic activity by means of SRB assay in triplicates for 72hours as explained in the methods part. Huh7 cell treatment was repeated as a confirmatory experiment.

	HUH7 IC50(μM)	MV IC50(μM)	HEPG2 IC50(μM)	HEP3B IC50(μM)
IBUTRI	16.4	49.4	13.1	4.3
IBUTRI-nTDZ	4.7	5.2	8.0	6.6
IBUTRI-ClTDZ	9.2	9.2	8.3	0.1
IBUTRI-FTDZ	8.8	6.5	8.3	6.7
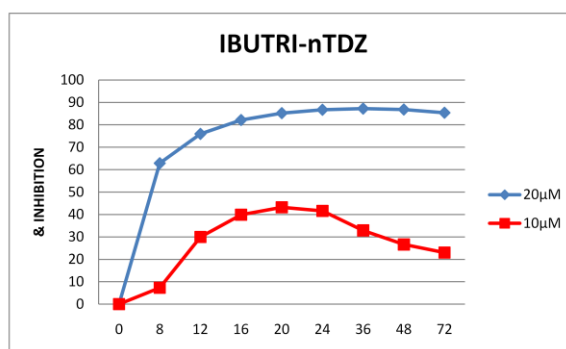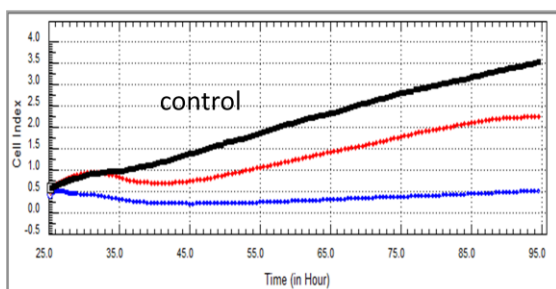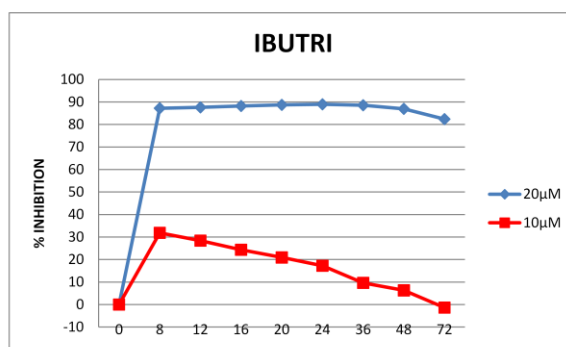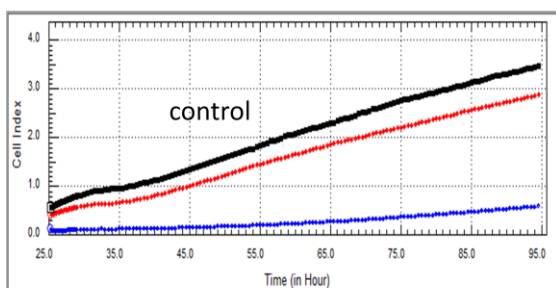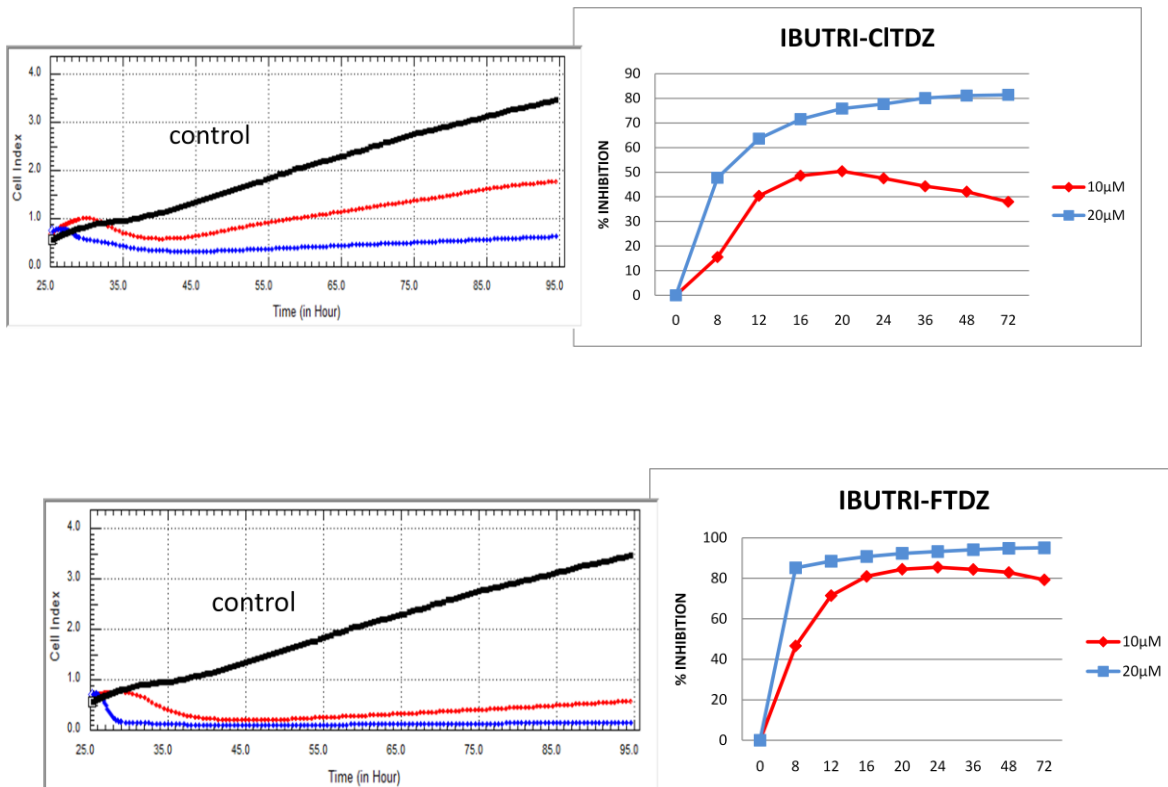
Figure 4.9: Real-time cell growth analysis of IBUTRI compounds on Huh7 cell lines: Compound IBUTRI, IBUTRI-Ntdz, IBUTRI-ClTDZ and IBUTRI-FTDZ were applied on Huh7 cell line in 20µM and 10µM concentrations and observed for 72 hours. Cell-impedance based xCelligence system was used to observe the growth inhibitory effect in real-time as explained in the methods part. The graphs represent time-dependent effect of the compounds on both cell index and cell-growth. DMSO treated control cells (BLACK) were used as control and cell growth in compound treated was normalized to the growth in DMSO treated cells. Further normalization was performed according to time-zero values.

The time-dependent cytotoxic activity surveillance was performed for IBUTRI group compounds using xCelligence for Huh7 cell line. As seen in Figure 4.9, IBUTRI shows a reversible cytotoxic effect. On the other hand, other three compounds have an irreversible growth inhibitory effect on Huh7 cell line during the time of analysis.

4.2.2 Kinase Inhibitory Activity

Kinases use ATP to phosphorylate proteins (Hanks et al, 1988). In other words, kinases cause decrease in the amount of ATP and increase in the amount of ADPs. Thus in the presence of a kinase inhibitor ATP concentration in the environment will not decrease since the kinase will not be able to phosphorylate any protein meaning that it will not use ATP. By using a Kinase Assay, which is luminescence based, we can determine if the molecule of interest inhibits kinase activation or not. The luminescence is bound to ATP so if the molecule is a kinase inhibitor, luminescence signal will increase.

After choosing the IBUTRI group molecules as the most promising candidates, their kinase inhibitory effect had to be investigated. For this purpose, we used Promega KinaseGlo Assay Kit as explained in the methods part and according to the manufacturer's protocol.
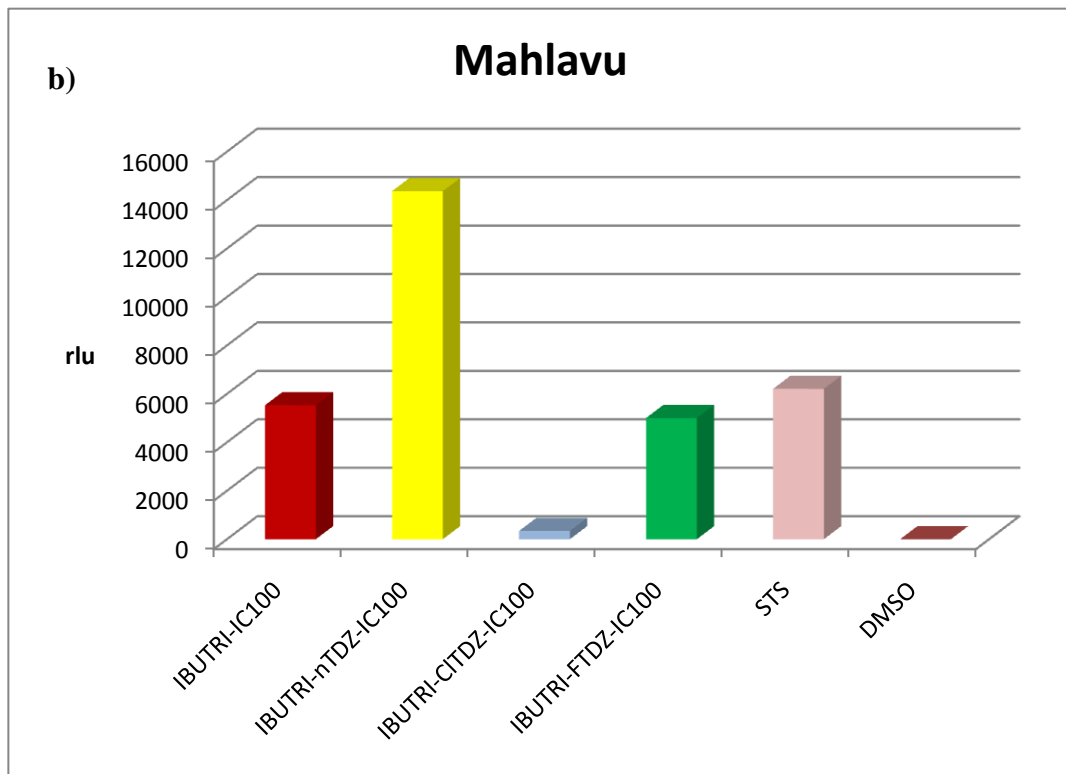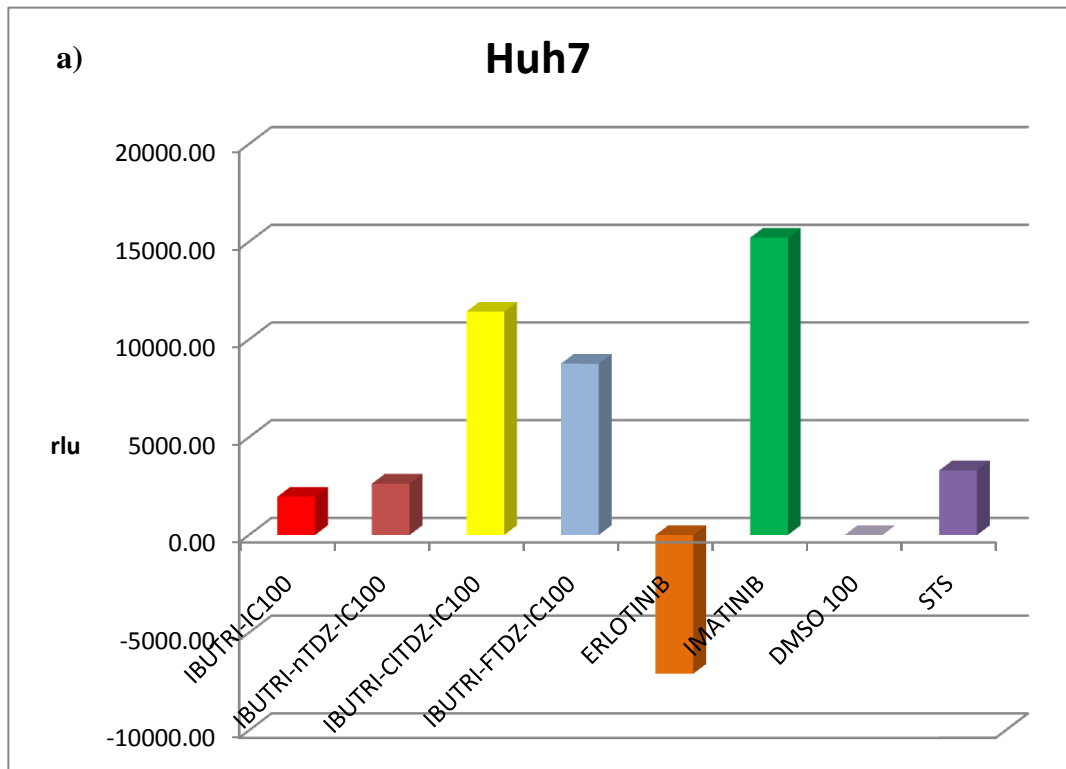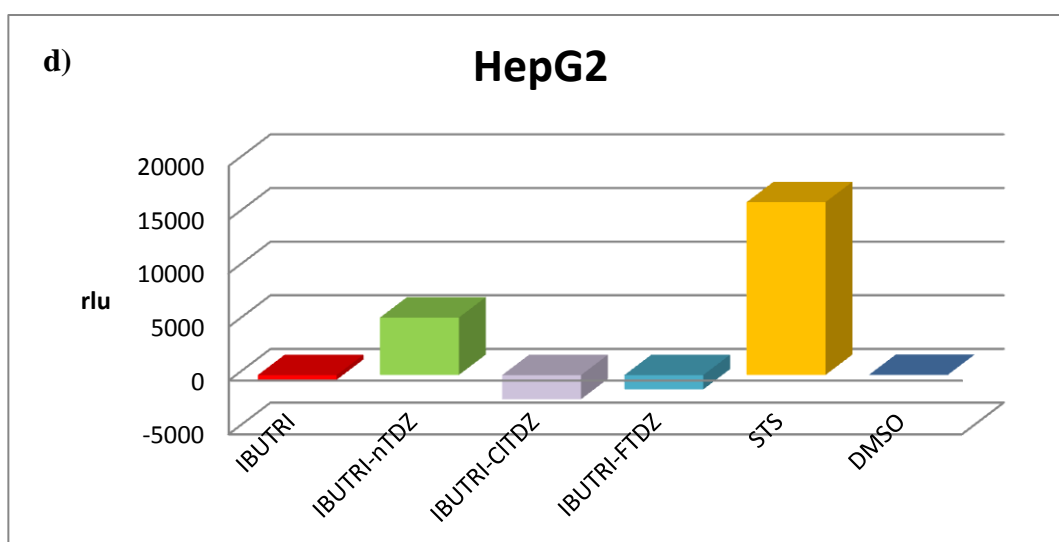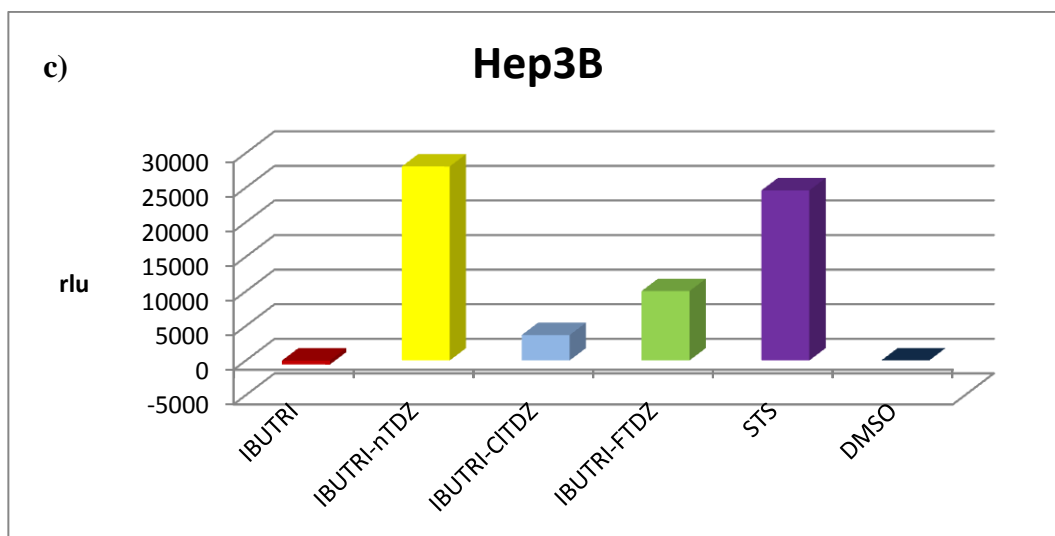
a) **Huh7**

b) **Mahlavu**

Figure 4.10: **Kinase Inhibitory effect of IBUTRI group molecules**: The kinase activity of a) Huh7, b) Mahlavu, c) Hep3B and d) HepG2 when treated with IBUTRI group candidate molecules is shown in the graphs. Drug concentrations of IBUTRI, IBUTRI-nTDZ, IBUTRI-ClTDZ, and IBUTRI-FCL in Huh7 are as follows respectively: 32μM, 10 μM, 20μM, 16 μM. Drug concentrations of IBUTRI, IBUTRI-nTDZ, IBUTRI-ClTDZ, and IBUTRI-FCL in Mahlavu are as follows respectively: 100μM, 10 μM, 18μM, 15 μM. Drug concentrations of IBUTRI, IBUTRI-nTDZ, IBUTRI-ClTDZ, and IBUTRI-FCL in HepG2 are as follows respectively: 26μM, 16 μM, 16μM, 16 μM. Drug concentrations of IBUTRI, IBUTRI-nTDZ, IBUTRI-ClTDZ, and IBUTRI-FCL in Hep3B are as follows respectively: 8μM, 14 μM, 0.2μM, 7 μM Staurosphorine (STS) is used as a positive control (0.5μM) together with Erlotinib (14μM) and Imatinib (10μM) and DMSO as a negative control. Bar graphs represent luminescence signal emitted by the cells. The higher the luminescence signal, the lower the kinase activity. Kinase Glo Assay was performed as explained in the methods part.

This experiment showed that IBUTRI-nTDZ has the highest kinase inhibitory effect on human liver cancer lines and IBUTRI has the lowest inhibitory effect (Figure 4.10).

4.3 Acivity of Thiazolidine ring containing ALC 67 on cancer cell lines

4.3.1 Cytotoxicity analysis

In a previous study set of molecules with thiazolidine core was synthesized by Dr. Esra Onen. There were three groups of molecules: pyrimidic base containing, benzoyl moiety containing and N-acetylated triazoles (Esra et al, 2008). From these molecules a selected set of anticancer drug candidates containing thiazolidine core was synthesized and their cytotoxic activity was denermined by SRB method. In Figure 4.11, it was shown that the compounds did not have significant cytotoxic activity except compound ALC67, which showed a dramatic cytotoxic activity (Table 4.3). ALC67 is a terminal alkyne precursor of triazoles.

After observing the high cytotoxicity of ALC67 in Huh7, MCF7 and HCT116 cell lines, the number of cell lines analysed were increased (Table 4.3). The growth inhibitory effect of ALC67 also continued in these cell lines as well.

Then, to confirm these date, we performed time-dependent cytotoxic activity surveillance using xCELLigence for liver (Huh7, Mahlavu, Focus), breast (Mcf7, Cama-1, T47D) cell lines. As seen in Figure 4.12, highest cytotoxic effect was observed in Huh7. Growth inhibition was almost 100% even in IC25. On the other hand, results of all other cell lines correlate with SRB results. IC50 concentrations show almost 50% inhibitions on growth of the rest of the cell lines. The only problematic result was in Cama-1, all concentrations caused 25% inhibition, even IC100.

Table 4.3: IC50 values of ALC67 in human cancer cell lines: Human cancer cell lines of liver, breast, gastric and endometrial cancer were treated with ALC67 for 72hr in triplicates and IC50 values were determined by means of NCI-60 SRB analysis as explained in methods part.

TISSUE	CELL LINE	IC50
Liver	HEPG2	10
	HUH7	5
	MV	0.41
	FOCUS	5.47
Breast	T47D	7.62
	MCF7	5.04
	BT20	1.6
	CAMA-1	0.01
Gastric	KATO-3	0.0004
Endometrial	MFE-296	0.0027
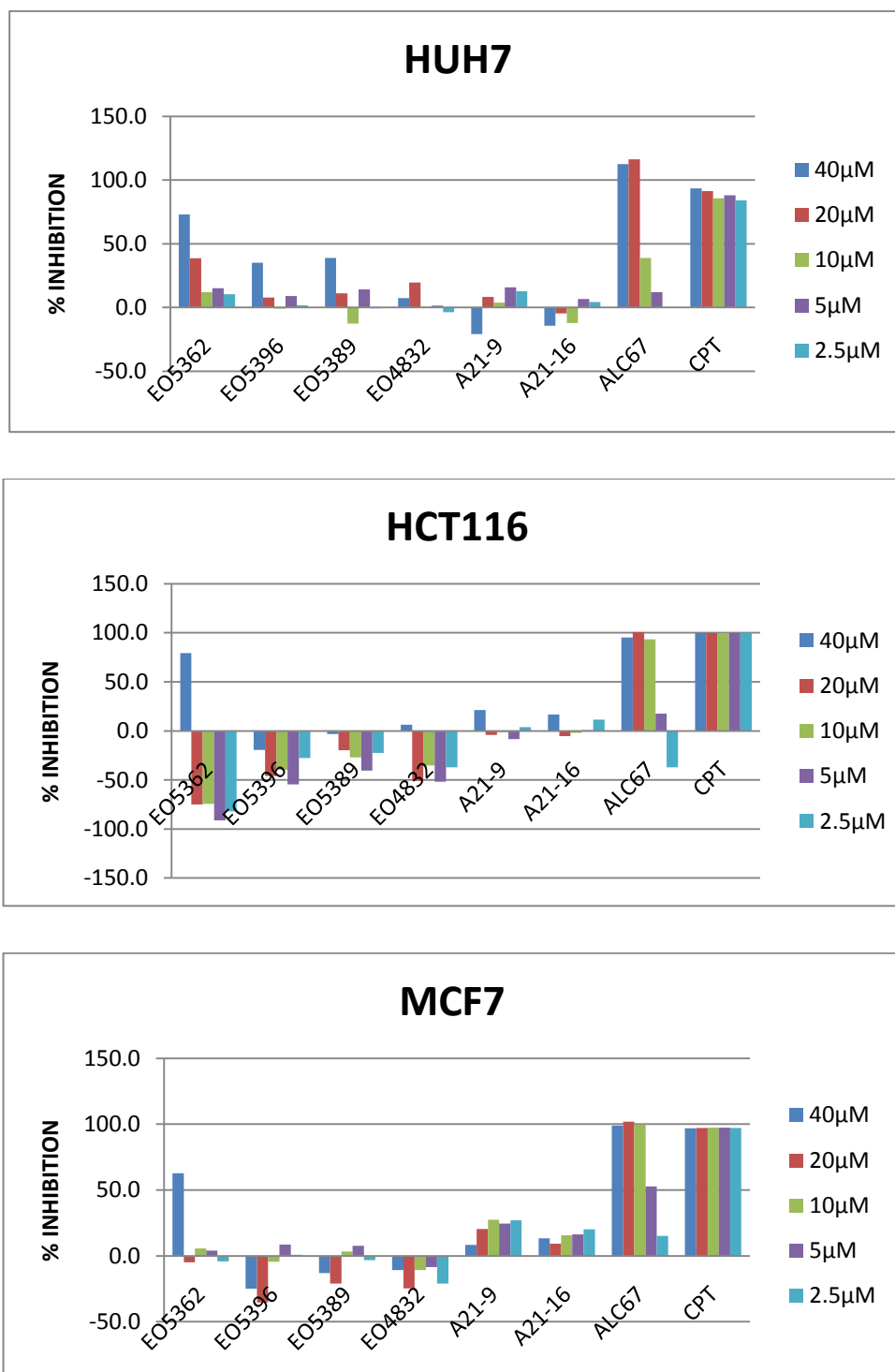
Figure 4.11: Percent cell death inhibition graphs: Preliminary screen was performed on breast (MCF7), colon (HCT116) and liver (HUH7) cancer cell lines. Cell lines were treated with corresponding compounds for 72h. NCI60 SRB analysis was applied as explained in methods. Camptothecin was used as a positive control.
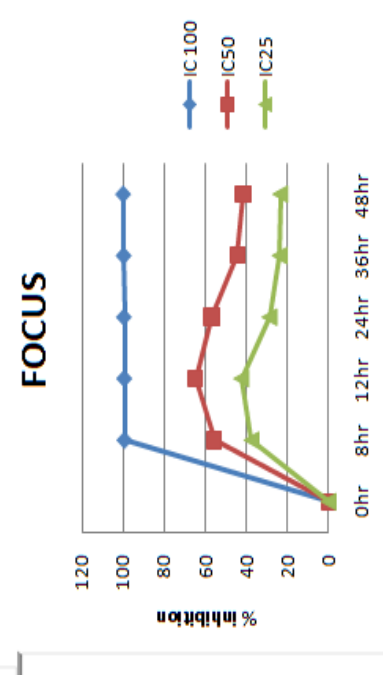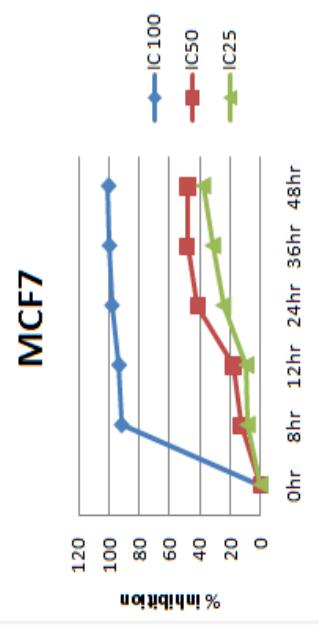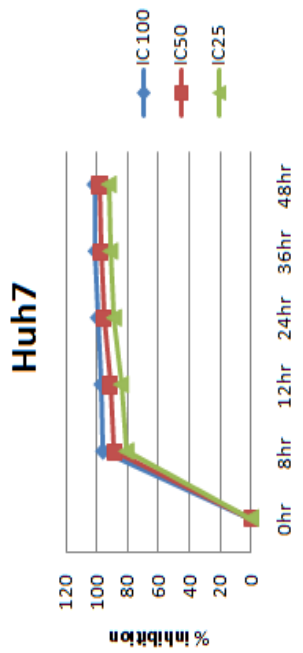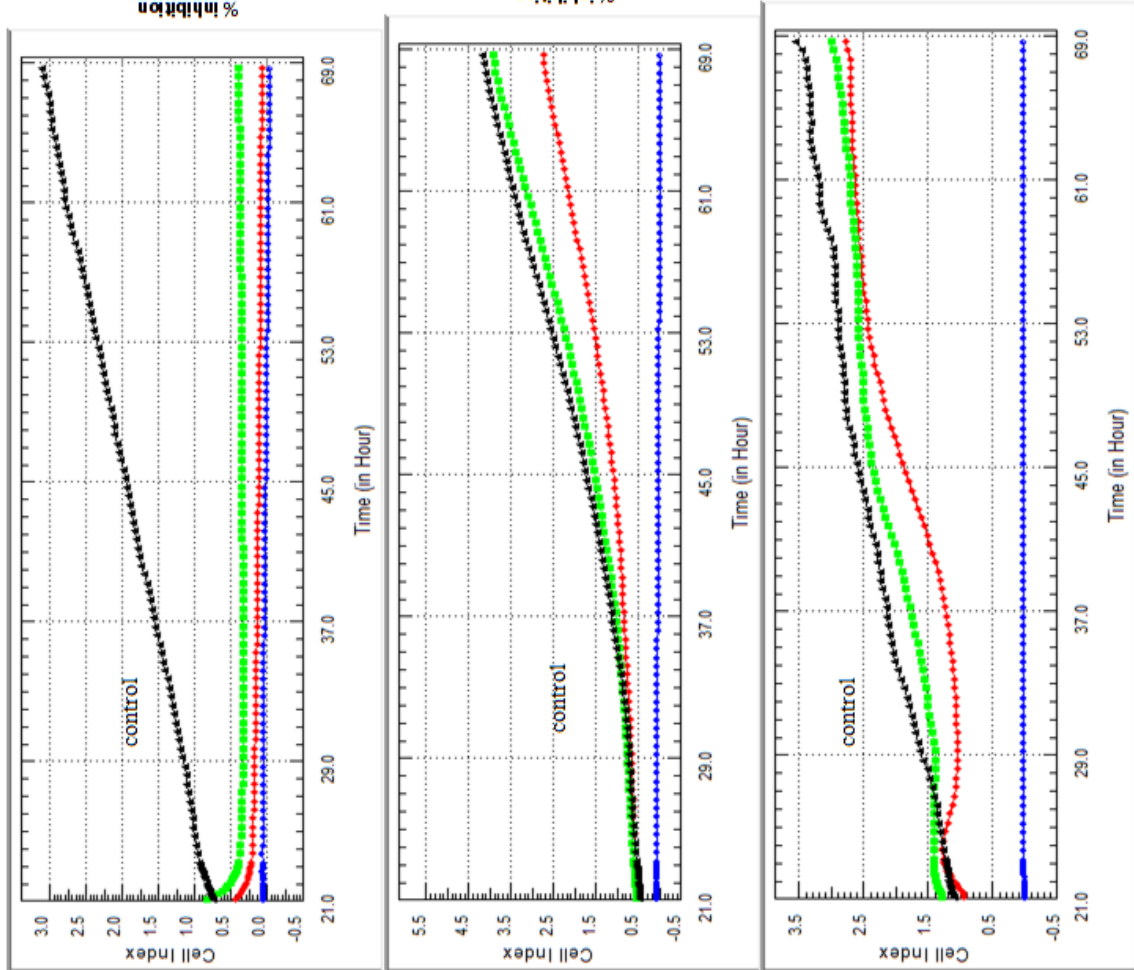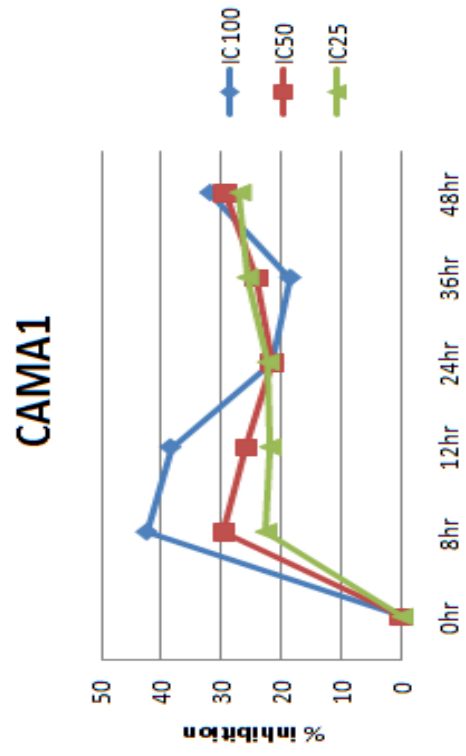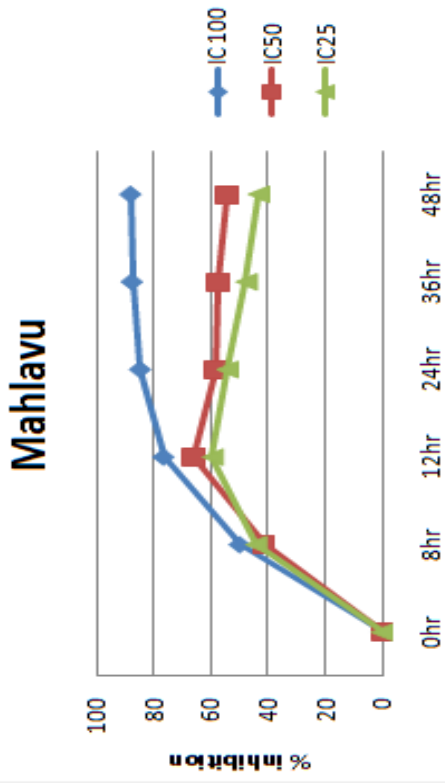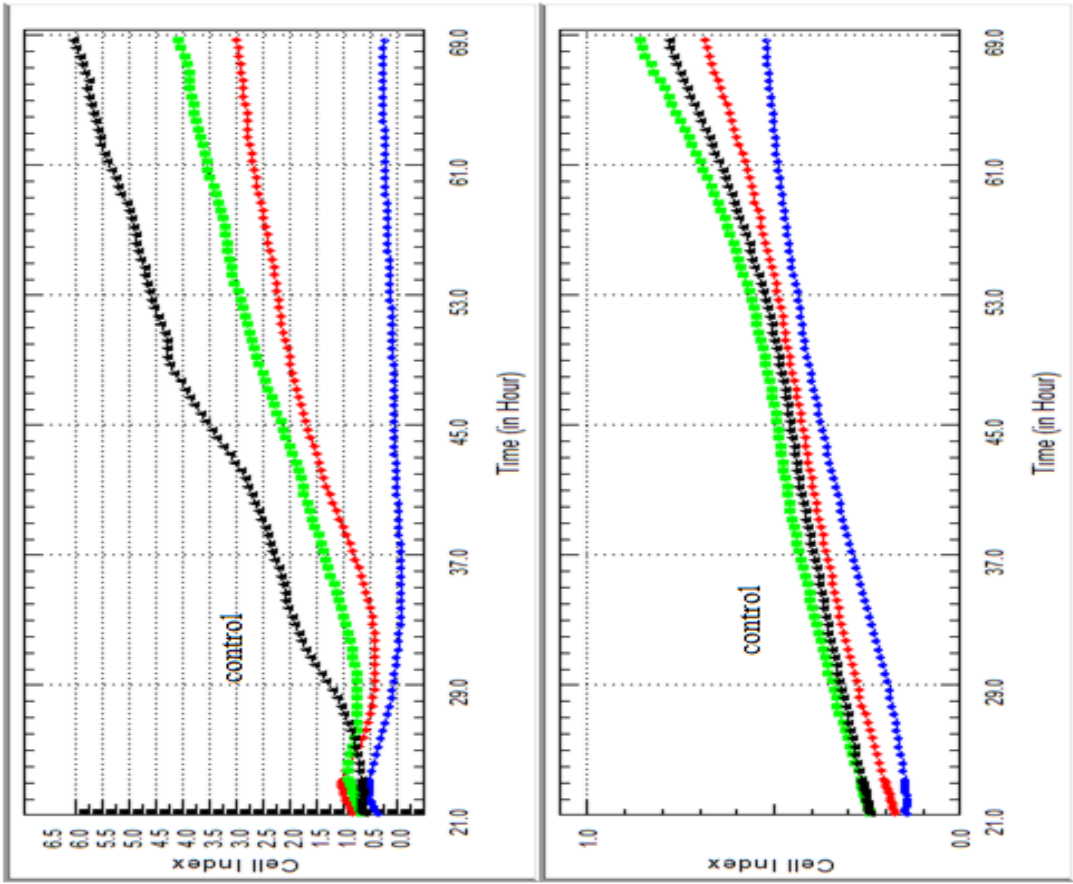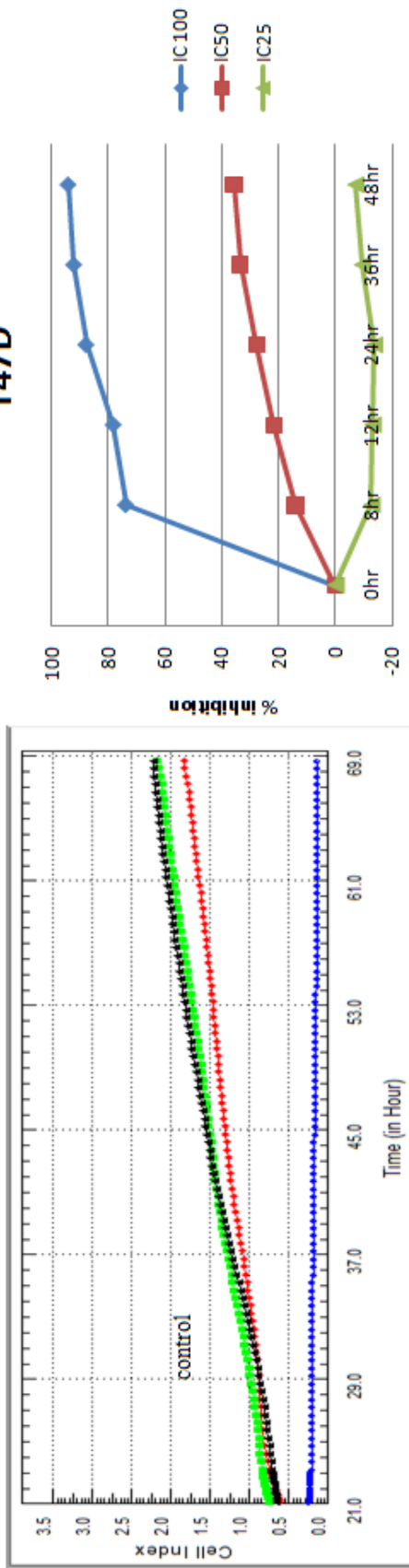
Figure 4.12: Real-time cell growth analysis of ALC67: Human cancer cell lines Huh7, MCF7, FOCUS, Mahlavu, Cama-1, T47D were treated with ALC67 in IC100, IC50 and IC25 concentrations for 72 hours. (IC50 value of ALC67 for MCF7: 5μM, Focus: 5.5μM, Mahlavu: 0.41μM, Cama-1: 0.01μM, T47D: 7.6μM, Huh7:5μM) Cell-impedance based xCelligence system was used to observe the growth inhibitory effect in real-time as explained in the methods part. The graphs represent time-dependent effect of the compounds. DMSO (BLACK) treated cells were used as control and cell growth in compound treated was normalized to the growth in DMSO treated cells. Further normalization was performed according to time-zero values.

4.3.2 Cell cycle arrest induced by ALC67

Furthermore, in order to investigate the consequences of ALC67 treatment in molecular and cellular level, we wanted to investigate the cell cycle and examine if it causes an arrest in cell cycle. Fluorescence-activated cell sorting analysis with propidium iodide revealed cell cycle arrest in ALC67 treated Huh7 cells compared to DMSO treated controls.

As stated above, normally, healthy well-differentiated HCC cell lines would be 60-70% in G1 phase, 20% in S phase, and 20% in G2-M phase. In our data, it can be observed that there is an obvious increase in SubG1/G1 cells indicating an arrest at this point of cell cycle (Figure 4.13).
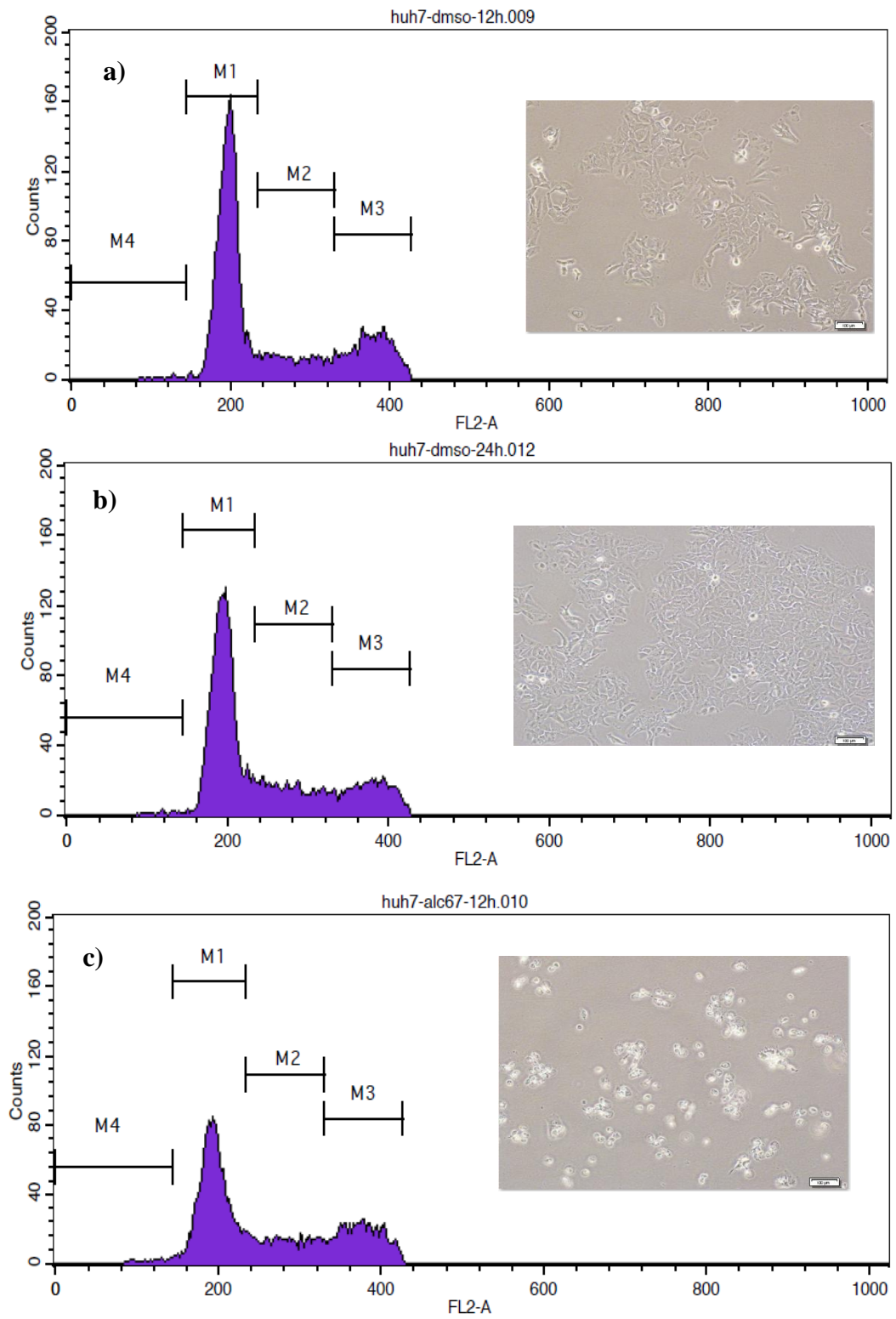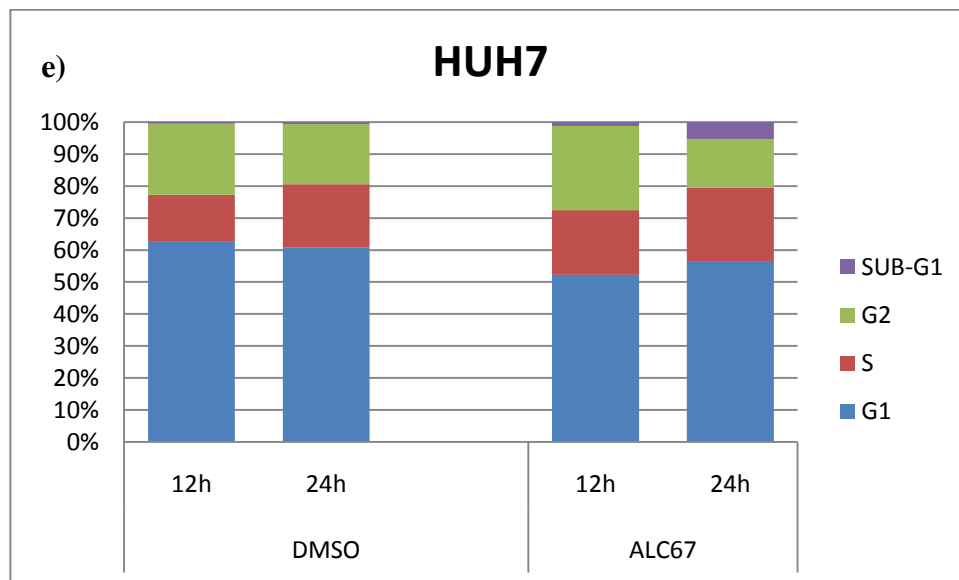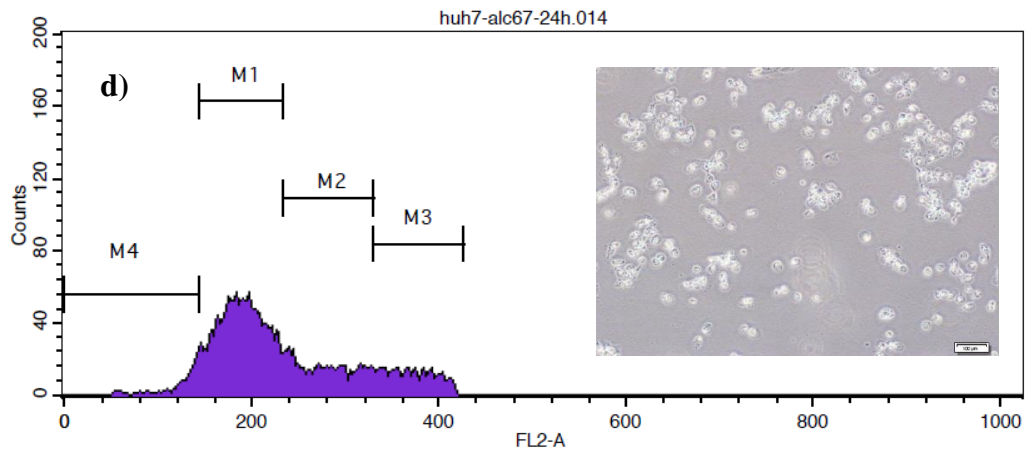
Figure 4.13: Cell cycle distribution analysis: Huh7 cells were treated either with DMSO for a) 12hr and b) 24h or with ALC67 for c)12hr and d) 24hr. e) is the graphical representation of cell cycle. Then cell cycle analysis was performed with FACS analysis as explained in methods part. The peak at 200 FL2-A represents 2N cells (G1) and the peak at 400 represents 4N cells (G2). The peak in-between represents S-phase cells. During gating >4N cells were excluded since those didn't show any variation between control and treated cell groups. Drug concentrations were 5μM.

CHAPTER 5. DISCUSSION AND CONCLUSION

One of the hot topics in cancer research is the development of molecules that can alter protein-protein interactions that are abnormally functioning in cancer cells (Arkin et al, 2004).

In this study, our main focus was to identify and characterize small-molecule inhibitors that have the potential to be anti-cancer therapeutic agent against solid human cancers. For this purpose, we investigated three groups of molecules. First group of molecules were plant-derived cardiac glycosides, and the other two were synthetic molecules which were designed to synthesize kinase inhibitor derivatives and thiazolidine containing molecules.

Steroid-like compounds, Cardiac glycosides have been in use widely for the treatment of heart failures since many years (Gheorghiade et al, 2006; Hamad et al, 2007). It has been shown that they have inhibitory action on the activity of Na+,K+-ATPase and thereby regulate the intracellular concentration of potassium, sodium directly and calcium ions indirectly (Kaplan JH, 2002; Newman et al, 2008;Wasserstromet al, 2005).

In previous studies cardiac glycosides extracted from digitalis lanata were shown to inhibit malignant cell proliferation at nanomolar concentrations (Shiratori et al, 1967; Winnicka et al, 2006; Erdmann and Schoner, 1973; Pathak et al, 2000; Gupta et al 1986). Most of the established studies were done on the extracts of digitalis lanata and davisiana but not of digitalis ferrugiana. In our project, our aim was to investigate the effects of cardiac glycosides extracted from Digitalis Ferrugiana. Initially, we started by analyzing the cytotoxic activity of all Digitalis Ferrugiana extracts obtained from Prof. Dr. Ihsan Calis. As shown in Figure 4.1, three of the six extracts displayed high cytotoxicity to human cancer cell lines at μM concentations. Lanatoside A, Lanatoside C and Glucogitorosid were the cardiac glycoside agents of Digitalis Ferrugiana so it was not surprising to observe their high cytotoxicity against cancer cells. The three extracts that didn not show any cytotoxicity weren't cardiac glycosides but Phenylpropanoid Glycosides. So this result correlates with the previous findings that cardiac glycosides alter cell proliferation (Erdmann and Schoner, 1973; Pathak et al, 2000; Gupta et al 1986). Moreover, we demonstrated that, even when applied in 0.5μM, it was enough to kill all cancer cells at the end of 72hr (Figures 4.2 and 4.3). This data also confirms their immediate and strong cytotoxicity. Thus these compounds are good candidates for anti-cancer therapeutics since they can be applied at nanomolar

concentrations, which are considered as "druggable concentration". Moreover, in the literature, it was reported that cardiac glycosides cause activation of Src/EGFR and consequently lead to apoptosis or autophagy induced cell death (Newman et al, 2008; Xie and Cai, 2003; Schoner and Scheiner-Bobis, 2007). It was also reported that when given in drugable concentrations, cardiac glycosides causes proliferative arrest and apoptosis induction prostate cancer cell lines (Johansson et al, 2001; Haux et al, 2001).

In order to investigate the death pathway they induce, we first examined the cells treated with these molecules under inverted microscope and then observed by Hoechst staining. In Figures 4.4 and 4.5 we can clearly demonstrate that Digitalis Ferugiana extracted glycosides induce apoptosis. In order to confirm the induction of apoptosis, we further analyzed treated cells with western blot against PARP antibody and showed the cleaved PARP in treated cells but not in DMSO controls Figure 4.8. It can be clearly seen that in 48hr, Lanatoside A can induce cleavage of PARP in both cell lines. However, in Mahlavu cell line, PARP cleavage is not seen in 12hr incubation period. Lanatoside C has the ability to induce apoptosis both in Huh7 and Mahlavu even in 12hr incubation. Finally Glucogitorosid induced PARP cleavage strongly in Mahlavu cell lines in both incubation periods. However in Huh7 cell line, even in 48hr incubation, cleavage was not significantly observed. Still this data proves that cardiac glycosides extracted from Digitalis Ferrugiana can induce apoptosis in human liver cancer cell lines.

In the final step of our research on cardiac glycosides extarcts from digitalis ferrugiana, cell cycle analysis by these molecules on cancer cell lines was tested. We demonstrated that, cardiac glycosides Lanatoside A, Lanatoside C and Glucogitorosid induces G2/M arrest in cell cycles of both Huh7 and Mahlavu liver cancer cell lines (In Figure 4.7 and 4.8,). Previous studies also pointed out the relation of cardiac glycosides with Akt pathway as well (Newman et al, 2008; Schoner and Scheiner-Bobie, 2007; Chen et al, 2006). Thus our findings of G2/M arrest and apoptosis induction may also correlate with the idea that possibly Lanatoside A, Lanatoside C and Glucogitorosid induces G2/M arrest, that eventually causes apoptosis, through down-regulation of Akt pathway (Figure 5.1).
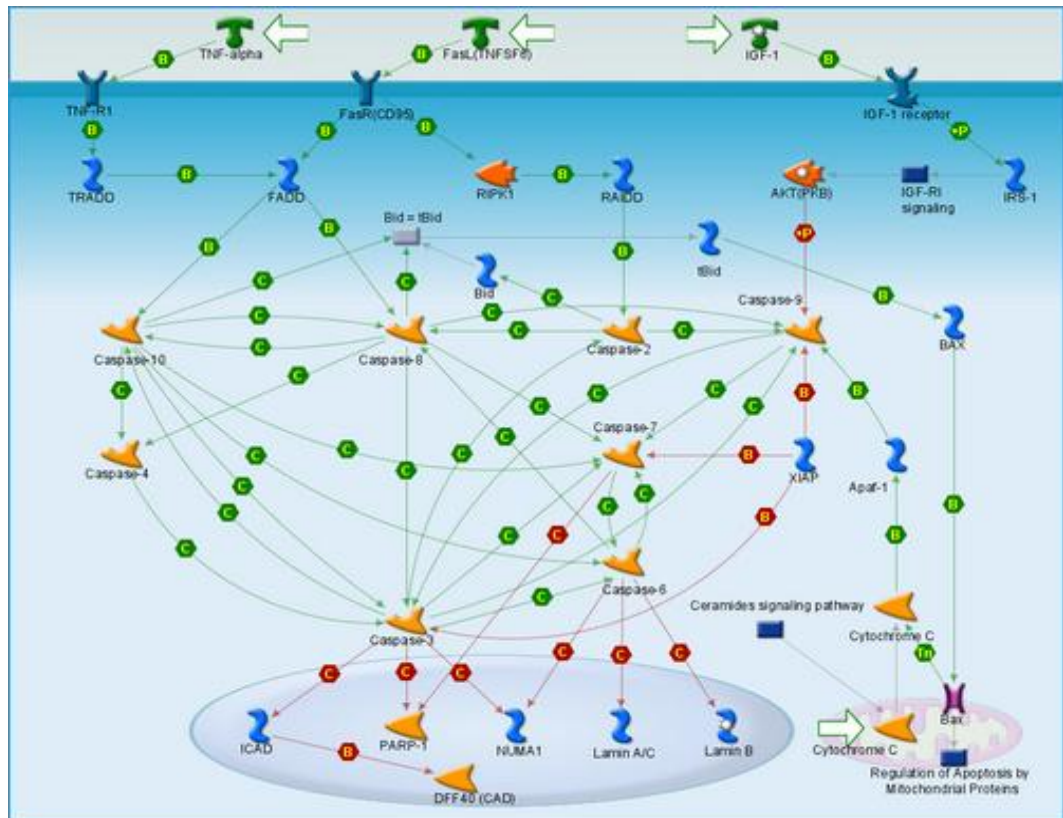
Figure 5.1: Caspase dependent apoptosis signaling cascade (Thomson Reuters http://www.genego.com/map_373.php)

We also checked the presence of autophagy marker LC3B in cells treated with Lanatoside A, Lanatoside C and Glucogitorosid. Normally, LC3B is found inside the cytoplasm. However, it is lipidated and cleaved during autophagy. LC3B has two isoforms at 18kDa and 16kDa. In normal cells, the 18kDa fragment is frequently found. In case of atuophagy, amount of 18kDa fragment decreases and 16kDa increases. In our case, in all samples treated with cardiac glycosides the amount of 18kDa fragment was same with DMSO control. Besides, there was very few 16kDa fragment (Data not shown). This data also confirmed that the cardiac glycosides we tested induces apoptosis, not autophagy in human liver cancer cell lines in the concentrations we used.

Thus, these findings indicate that cardiac glycosides, extracted from Digitalis Ferrugiana, comprise high cytotoxicity against tumor cells and they induce G2/M arrest that leads to apoptosis induction.

In the second part of my thesis research, our aim was to investigate the characteristics of a set of molecules synthesized by Birsen Tozkoparan. These molecules were designed as derivatives of kinase inhibitors having similar sturctures to Imatinib and erlotininib (Figure5.2) . In the recent years, there has been a lot of emphasis on the importance of kinase inhibitors in anticancer therapeutics. The most important advantage of these molecules is their low side effects and relatively low toxicity against normal cells (Novak C, 2004).

Figure 5.2: Schematic representation of Imatinib, Erlotinib, IBUTRI, IBUTRI-nTDZ, IBUTRI-FTDZ, IBUTRI-ClTDZ (http://www.rxlist.com/tarceva-drug.htm, http://www.answers.com/topic/imatinib)

Initilally, cytotoxic activities of the molecules were examined on breast (MCF7), colon (HCT 116) and liver cancer (Huh7) cell lines. The group of molecules with lowest IC50 values was IBUTRI group. Therefore, we investigated this group of molecules in more detail. As shown in Table 4.1 and 4.2 IBUTRI had the highest IC50 within the group and IBUTRI-nTDZ had the lowest 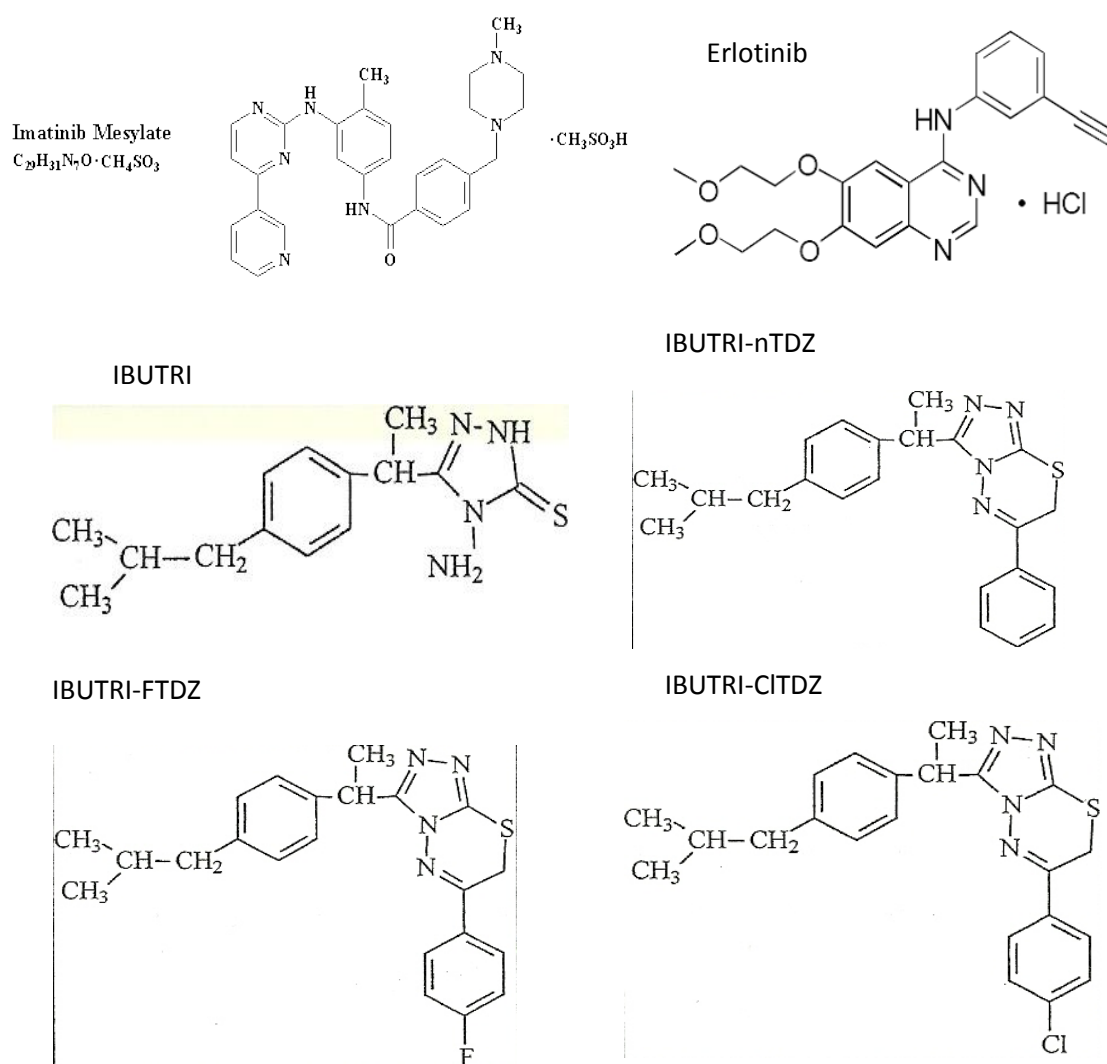IC50 value. This data was also confirmed by the results of the real-time cell growth surveillance analysis by xCelligence (Figure 4.9). The reason for IBUTRI having the highest IC50 compared to other group members might be the difference in penetration through the cell membrane. In other words, nTDZ arm as well as F or Cl addition might ease the penetration of the molecule inside the cell that makes them more cytotoxic.

Furthermore, we confirmed the kinase inhibitory function of these molecules. It can be seen that IBUTRI-nTDZ has the highest kinase inhibitory effect (Figure 4.9). Erlotinib was used as a positive control as well as STS and Imatinib. Imatinib and STS both had high luciferase signal dependent on intarcellular ATP concentration since they inhibit kinase activity and lead to less ATP consumption in the intarcellular environment. On the other hand, even though erlotinib is also classified as a kinase inhibitor, we could get no luciferase signal. The possible reason for this might be the high cytotoxicity of the molecule thus not enough protein would be available to analyze.

The last part of the project was about molecules with thiazolidine arm. Previous studies showed that molecules with thiazolidine have cytotoxic activity in various cancer cells against normal cells (Li et al, 2011; Li et al, 2007; Gududuru et al, 2005). In the first screen of cytotoxicity of the molecules, only one of them showed high cytotoxiciy against cancer cell lines (Figure 4.11 and Table 4.3). The molecule with high cytotoxicty against cancer cell lines was ALC67, which contains a terminal alkyne precursor. Alkynes contain triple bond between the two carbon atoms and they belong to the family of hydrocarbons. They are also called as acetylenes (Kenneth et al, 1963).

The cytotoxic activity of ALC67 (Figure 5.3) was also verified by xCelligence analysis for real-time cell growth surveillance. Only the result of Cama-1 was not satisfactory. Even though, the IC100 value was applied only 25% inhibition was observed. One possible reason is that cell number wasn't confluence enough to distinguish between DMSO control and drug treated cells. The second possible reason is that cells were so confluent in SRB analysis that even a small amount of drug caused the cells die therefore the IC50 value calculated was too low.
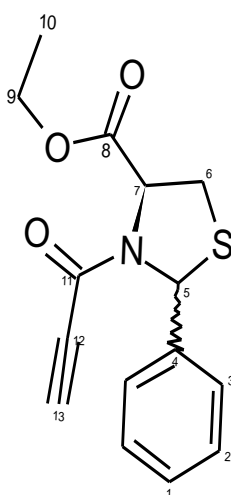
Figure 5.3: Schematic representation of the active compound ALC67

The cytotoxic activity of ALC67 was also verified by xCELLigence analysis for real-time cell growth surveillance. In Figure 4.12, it is obvious that ALC67 has the highest effect on Huh7. In addition, In MCF7, Focus, Mahlavu and T47D, cell inhibition of ALC67 was shown to be as predicted by SRB analysis. Also it is clear that cell growth of all these five cell lines was effected dramatically by the interference of ALC67 when compared to DMSO controls.

Finally we aimed to investigate the cell cycle arrest the compound ALC67 caused in liver cancer cell Huh7. Our data points out SubG1/G1 arrest in cell lines treated with ALC67 compared to DMSO. This result supports the inverted microscope images in a sense that the cell death ALC67 induces is apoptosis. Moreover, Dr. Esra Önen had conducted a previous experiment on intrinsic apoptotic pathway with caspase 9 activity and the extrinsic apoptotic pathway with caspase 8 activity in cells treated with ALC67. She showed that its activity was dependent on the caspase 9 therefore intrinsic apoptotic pathway. This data also confirms our findings that ALC67 induces SubG1/G1 arrest which would then cause apoptosis induction.

CHAPTER 6. FUTURE PERSPECTIVES

Our results demonstrate that all three groups of compounds that we tested can be considered as candidate anti-cancer therapeutic agents. Cardiac glycosides extracted from Digitalis Ferruginea, IBUTRI group molecules especially IBUTRI-nTDZ and ALC67 which is a thiazolidine derivative with alkyne terminal precursor comprises high cytotoxic activity against cancer cell lines.

Future experiments on further characterization and investigation of these molecules are listed below:

Digitalis Ferruginea/Cardiac Glycosides Lanatoside A, Lanatoside C and Glucogitorosid:

- Apoptosis can be further verified through TUNEL Assay to detect DNA fragmentation and Apoptotic DNA ladder kit or Annexin5 staining.

- In order to investigate if cardiac glycosides induce mitotic chatastrophy, phosphorylation of Histon4Ser10 and Histon3Ser10 can be investigated using specific antibodies.

- The data here reveals G2/M arrest. In order to determine if it is an M arrest or G2, cells will be synchronized with Nocodazole at M phase. Then Nocodazole will be removed and some cells will be treated with the cardiac glycosides, the others with DMSO. If M arrest persists in treated cells compared to DMSO treated cells, the conclusion will be that cardiac glycosides induce M-phase cell cycl arrest.

- Then the apoptotic pathway that they induce must be clarified. In our results we showed the cleavage of PARP indicating the induction of caspase cascade. However, the caspase-related apoptosis must be verified by caspase assays such as Caspase 3 activity assay.

- Furthermore, the consequences of the application of these cardiac glycosides must be investigated in the protein level. Previous studies showed the interaction of them with proteins such as but not limited to Src/EGFR, Akt, Erk, TRAIL and

Ras (Newman et al, 2008; Xie and Cai, 2003; Schoner and Scheiner-Bobis, 2007; Kometiani et al, 2006) Therefore, these proteins and their active forms (phosphorylated) must be investigated by western blotting experiments.

- In a study, cardiac glycosides were also related with ROS production and thereby apoptosis induction (Newman et al, 2008; Xie and Cai, 2003; Schoner and Scheiner-Bobis, 2007). Therefore, ROS assay should be performed in cells treated with these molecules in order to investigate the production of ROS.

- Finally, mammalian in vivo experiments need to be performed. After generation of xenogarft tumors in nude-mice, they should be treated with Lanatoside A, Lanatoside C or Glucogitorosid. Then the progression of tumor should be observed.

    IBUTRI-nTDZ:

- The cell cycle arrest can be furher investigated propidium iodide labeled FACS analysis.

- Then the death pathway it induces must be determined. Since we are hypotizing the induction of apoptosis, western blot against PARP cleavage or Annexin V assay can be performed.

- Also, to confirm the induction of apoptosis, TUNEL assay will be performed to investigate DNA fragmentation.

- We have showed that the molecule inhibits kinases however which group of kinases are inhibited must be investigated using kinase assay kits specific for different groups of kinases.

    ALC67:

- FACS analysis with BrdU incorporation will be performed to confirm and investigate SubG1/G1 arrest.

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