

**IDENTIFICATION OF
PUTATIVE PROTEIN KINASE INHIBITORS
ACTING ON LIVER CANCER CELLS**

**A THESIS SUBMITTED TO
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THE DEGREE OF DOCTOR OF PHILOSOPHY**

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AUGUST 2012**

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ABSTRACT

IDENTIFICATION OF PUTATIVE PROTEIN KINASE INHIBITORS ACTING ON LIVER CANCER CELLS

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Hepatocellular carcinoma (HCC) as a, heterogeneous, multi-step, slow-progressing disease, has very limited treatment options due to its chemoresistant nature and late diagnosis. According to World Health Organization (WHO) reports HCC is a major public health problem. Each year, 748,000 new cases appear and 696,000 people lose their lives, all due to liver cancer alone. Sorafenib, the multi-tyrosine kinase inhibitor, is still the only (Food and Drug Administration) FDA-approved drug available for treatment. Therefore, there is an urgent need for novel, target-specific drugs based on the underlying molecular mechanisms of liver carcinogenesis.

In this Ph.D. dissertation, synthetic purine, purine nucleoside analogs, aminotriazole and thiadiazine derivatives were evaluated for their cytotoxic activities and mechanisms of action against liver cancer. These novel molecules were selected because of their potential kinase inhibitory activity. Protein kinases, involved in signaling pathways, are the main enzymes of target-specific drug discovery; therefore, discovery of novel, putative protein kinase inhibitors as drug candidates can be promising for the treatment of primary liver cancer.

Initially, sulforhodamine B (SRB) assay was used to screen the novel small-molecules for their cytotoxic activities against breast, colon and liver cancer cell lines. Active molecules, then, were further exploited on a panel of HCC cell lines. The differential IC₅₀ (half-maximal inhibitory concentration) values obtained, might indicate that these small-molecules interfere with cell signaling; since, these

cell lines have individual characteristics of cell signaling activities. Further investigations are envisaged for the identification of the molecular mechanisms that these putative kinase inhibitors are involved in.

Among the 228 newly synthesized putative kinase inhibitors, 3 small-molecules were identified as promising anti-cancer agents against liver cancer with druggable cytotoxic activities and remarkable kinase inhibition potentials. The purine analogue, AUM32, and the purine nucleoside analogue, AUM42, were revealed as pro-senescence therapeutic agents in liver cancer. Both drug candidates were shown to initiate senescence-induced cell death and the underlying mechanism was confirmed for AUM42 as the induction of p15(INK4b) and the correlated decrease in Rb phosphorylation.

Synthetic 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine derivative, CIAT-TM, is the other effective small-molecule with kinase inhibition potential. Upon CIAT-TM treatment, liver cancer cells experience a growth inhibition accompanying with dramatic morphological changes. Rounded, swollen and eventually detached cells were shown to be arrested in the G2/M stage of the cell cycle and eventually undergo apoptosis. Moreover, ROS (reactive oxygen species) accumulation and the activation of JNK signaling pathway were found as associated with the mechanism of action CIAT-TM cytotoxicity.

ÖZET

POTANSİYEL PROTEİN KİNAZ İNHİBİTÖRLERİNİN KARACİĞER KANSERİNE KARŞI İLAÇ ADAYI OLARAK TANIMLANMASI

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Hepatosellüler karsinom (HSK), heterojen, çok aşamalı, yavaş gelişen bir hastalık olup, kemoterapiye dirençli yapısı ve sıklıkla geç teşhis edilmesi nedeniyle tedavi seçenekleri sınırlıdır. Dünya Sağlık Örgütü'nün raporlarına göre HSK önemli bir toplum sağlığı sorunudur. Her yıl, dünya genelinde 748,000 yeni kanser vakası ve 696,000 ölüm sadece karaciğer kanserine bağlı olarak gelişmektedir. Çoklu-tirozin kinaz inhibitörü olan Sorafenib, ileri seviyede HSK için hala FDA onayı olan tek ilaçtır. Bu nedenlerle, karaciğer kanser gelişiminin altında yatan moleküler mekanizmaları hedef alan, yeni ilaç adaylarının tespit edilmesine acil olarak gereksinim vardır.

Bu doktora tez çalışmasında, sentetik pürin ve pürin nükleozid analogları ile aminomerkaptotriazol ve triazolotiyadiazin türevleri, karaciğer kanseri üzerindeki sitotoksik etkileri ve hareket mekanizmaları açısından değerlendirilmiştir. Bu yeni moleküller, potansiyel kinaz inhibisyon aktiviteleri nedeniyle seçilmişlerdir. Hücre sinyal yollarında yer alan protein kinazlar, hedef-odaklı ilaç keşfinde temel enzimler olup, yeni keşfedilecek potansiyel kinaz inhibitörleri, HSK tedavisinde umut veren ilaç adaylarıdır.

Öncelikle, yeni sentezlenen küçük-moleküllerin sitotoksik etkileri, sülforadamin B (SRB) yöntemi kullanılarak meme, kolon ve karaciğer kanseri hücrelerinde incelenmiştir. Aktif olduğu tespit edilen moleküller, daha sonra HSK hücre hatlarına karşı değerlendirilmiştir. Farklı kanser hücre hatlarında, farklı IC50 değerlerine ulaşılması ve kanser hücrelerinde sinyalizasyonun hücre hattına

özgü, ayırt edici özellikler taşıdığıının bilinmesi, bu küçük-moleküllerin sinyal yollarına müdahale ettiklerini düşündürmüştür. Potansiyel protein kinaz inhibitörlerinin moleküler mekanizmalarını tanımlayacak ileri analizler öngörülmüştür.

Bu doktora tez çalışması kapsamında, yeni sentezlenen 228 adet potansiyel protein kinaz inhibitöründen 3 küçük-molekül, düşük dozlarda gösterdikleri sitotoksik etki ve dikkate değer kinaz inhibisyon potansiyelleri ile kuvvetli ilaç adayları olarak saptanmıştır. Nükleozid analoglarından AUM32 ve AUM42, karaciğer kanserinde erken-yaşlanmaya yol açan terapötik ajan olarak gösterilmişlerdir. Her iki ilaç adayının da yaşlanmanın tetiklediği hücre ölümüne yol açtığı, hatta AUM42 molekülünün, p15(INK4b) artışına bağlı olarak düşen fosforile-Rb üzerinden etki ettiği ortaya konmuştur

Sentetik 1,2,4-triazol[3,4-b]-1,3,4-tiyadiazin türevi, CIAT-TM, bir diğer etkin, potansiyel protein kinaz inhibitörüdür. Karaciğer kanser hücreleri, CIAT-TM varlığında, yapısal bozulmaların yanında büyümede de gerileme göstermiştir. Yuvarlaklaşarak şişen ve nihayetinde kültür ortamından kopan hücreler, apoptoza girmeden önce hücre döngüsünün G2/M aşamasında tutulma yaşamaktadırlar. Üstelik ROS (reaktif oksijen türleri) birikmesi ve JNK sinyal yolağının aktif hale geçmesi, CIAT-TM molekülünün etki mekanizmasıyla ilişkilendirilmiştir.

To my lovely daughter, Kayra

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ABBREVIATIONS

5-FU	5-Fluorouracil
ADP	Adenosine diphosphate
AGC	Containing PKA, PKG, PKC families
AhR	Aryl hydrocarbon Receptor
AP1	Activating Protein
Apaf-1	Apoptosis protease activating factor-1
APC	Adenomatous Polyposis Coli
ASK1	Apoptosis Signal-Regulating Kinase 1
Atg13/FIP200/ULK	Autophagy-Related Gene/Focal Adhesion Kinase Family Interacting Protein of 200 kDa/UNC-51-like-kinase
ATM	Ataxia-Telangiectasia-Mutated
ATP	Adenosine Tri-Phosphate
ATR	ATM and Rad3-Related
BrdU	5-Bromo-2-Deoxyuridine
BSA	Bovine Serum Albumin
CAMK	Calcium/Calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CDK	Cyclin Dependent Kinase
CI	Cell Index
CK1	Casein kinase 1
CKI	Cyclin-dependent Kinase Inhibitors
CMGC	Containing CDK, MAPK, GSK3, CLK families
CPT	Camptothecin
DCFH-DA	2', 7'-Dichlorofluorescein diacetate
DDR	DNA Damage Response
DEB	Doxorubicin Eluting Beads
DFG	Asp-Phe-Gly
DISC	Death Initiating Signaling Complex

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSB	Double Strand Breaks
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine-Tetra-Acetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum
ERK1/2	Extracellular Signal-Regulated Kinase
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food & Drug Administration
FZD7	Frizzled
G1	Gap1
G2	Gap2
GSK3	Glycogen Synthase Kinase-3
GSK3- β	Glycogen Synthase Kinase-3 β
HBV	Hepatitis B
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C
HDAC	Histone deacetylase
HDV	Hepatitis D
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTP	Highthroughput
IAP	Inhibitor of Apoptosis
IARC	International Agency for Research on Cancer
IC50	Inhibitory Concentration 50
IFN- α	Interferon-Alpha
IGF	Insulin-like Growth Factor

IGFR	Insulin-like Growth Factor Receptor
IKK	I κ B Kinase
IL-1	Interleukin-1
IRA	Insulin Receptor A
IRB	Insulin Receptor B
IRS	Insulin-Receptor Substrates
kDa	kilo Dalton
M	Mitosis
MAPK	Mitogen-Activated Protein Kinase
MC	Mitotic Catastrophe
MES	2-(N-morpholino)ethanesulfonic acid.
MOPS	3-(N-morpholino)propanesulfonic acid
MPPs	Matrix metalloproteinases
MRI	Magnetic Resonance Imaging
mTOR	Mammalian Target of Rapamycin
NAD	Nicotinamide Adenine Dinucleotide
NBS1	Nijmegen Breakage Syndrome 1
NCI	National Cancer Institute
NEAA	Non-Essential Amino Acid
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NI	No Inhibition
NSAID	Nonsteroidal anti-inflammatory drugs
OIS	Oncogene-induced Senescence
P/S	Penicillin/Streptomycin Solution
PARP-1	Poly-(ADP-Ribose) Polymerase
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDK	Phosphatidylinositol-Dependent Kinase
Ph	Phenyl
PI	Presidium Iodide
PI3	Phosphatidylinositol-3

PI3K	Phosphatidylinositol-3-K
PICS	PTEN loss-induced cellular senescence
PIP2	Phosphotidylinositol-3,4-Biphosphate
PIP3	Phosphotidylinositol-3,4,5-Triphosphate
PK	Protein Kinase
PKA	cAMP-Dependent Protein Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PKG	Protein Kinase G
PKI	Protein Kinase Inhibitor
PLADO	Cisplatin/Doxorubicin
PMSF	Phenylmethanesulphonylfluoride
PtdIns(4)P	Phosphotidylinositol-4-Phosphate
PtdIns(4,5)P ₂	Phosphotidylinositol-4,5-Biphosphate
PTEN	Phosphatase and Tensin Homolog Deleted on Chromosome 10
rlu	Relative Light Unit
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RR	Ribonucleotide Reductase
RT-CES	Real-Time Cell Electronic Sensing
RT-PCR	Reverse Transcriptase PCR
S	Synthesis
SAPK1/JNK1/2/3	Stress-Activated Protein Kinase/c-Jun N-Terminal Kinase
SARA	Smad Anchor for Receptor Activation
SASP	Senescence Associated Secretory Phenotype
Sa β -gal	Senescence Associated- β -gal
SC	Subcutaneous
SDS	Sodium Dodecyl Sulphate
Ser	Serine
SKP2	S Phase Kinase Associated Protein-2

Smac/DIABLO	Second Mitochondria-derived Activator of Caspases / Direct IAP-Binding Protein with a Low Isoelectric Point
SOD	Superoxide Dismutase
SRB	Sulforhodamine B
src	Rous Sarcoma Virus
STAT	Signal Transducer and Activator of Transcription
STE	Homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases
STS	Saturosporine
TACE	Transarterial Chemoembolization
TBS-T	Tris-Buffered Saline with Tween-20
TCA	Trichloroacetic Acid
TGF- α	Transforming Growth Factor- α
TGF- β	Transforming Growth Factor- β
Thr	Threonine
Thyr	Tyrosine
TK	Tyrosine Kinase
TKL	Tyrosine Kinase-Like
TNF- α	Tumor Necrosis Factor-Alpha
T β RI	TGF- β Receptor I
T β RII	TGF- β Receptor II
VEGF	Vascular Endothelial Growth Factor
Vps34	Vesicular Protein Sorting 34
XBP-1	X-box Binding Protein-1

CHAPTER 1. INTRODUCTION

1.1 Protein phosphorylation:

The form of life we know gives privilege to phosphate group not only to link the nucleosides of DNA and RNA but also to be part of the primary cellular energy source, ATP. These privileges provide phosphates a vital role in living organisms. Phosphorus atom uses its 5 valence electrons to form bonds with oxygen atoms and yields phosphate group ($-\text{PO}_4$). Oxygens offer phosphate group a hydrated ionic shell and high solubility in water. Moreover, a phosphate has 3 ionizable groups which facilitate preserving nucleic acids within cell membrane. Phosphate group is reactive at physiological temperature which is another cellular advantage. The question Weistheimer asked 25 years ago, “Why nature chose phosphates?” highlighted a developing research field (Weistheimer FH, 1987).

Protein phosphorylation, a common post-translational modification, was first discovered when Levene showed vitelline, an egg yolk protein, contains phosphate (Levene PA and Alsberg CL, 1906). Subsequently, Levene had identified phosphoserine in 1932 and achieved to synthesize phosphotyrosine in 1933 (Lipmann FA and Levene PA, 1932; Levene PA and Schormüller A, 1933). After that, Burnett and Kennedy described the first protein kinase activity when they discovered an enzyme capable of catalyzing the transfer of phosphate from ATP to protein in 1954 (Burnett G and Kennedy EP, 1954). Edwin Krebs and Edmond Fischer were able to demonstrate that γ -phosphate of ATP was incorporated into a particular serine residue of phosphorylase b producing the activated, phosphorylase a, form (Krebs EG et al., 1959). Today it is well-described that, through chemically adding the γ -phosphate of ATP to target proteins, many cellular processes such as cell growth, proliferation, division, differentiation, adhesion, metabolism and apoptosis are regulated (Figure 1.1).

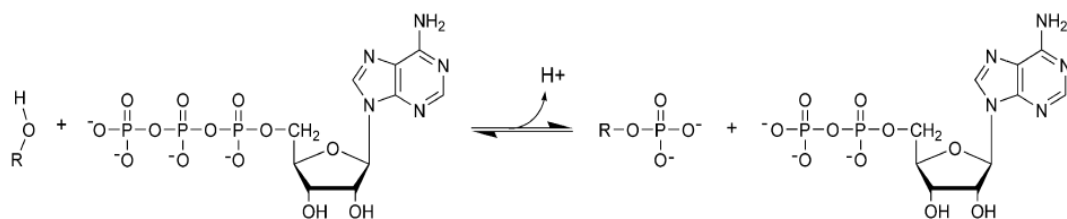


Figure 1.1 The transfer of γ -phosphate of ATP to the hydroxyl group of an amino acid residue: (R: Ser/Thr/Tyr) (Schwartz PA and Murray BW, 2011)

1.1.1 Protein kinases:

Protein kinases (PK), as being responsible for the post-translational phosphorylation of various proteins, are one of the largest gene families sharing the same molecular action. The protein kinase complement of the human genome, namely the human kinome, encodes more than 500 protein kinases corresponding to almost 2% of the entire genome (Manning G et al., 2002). Seven main groups are labeled and positioned on the kinome tree by comparing the sequence of their catalytic domains: the spatial distance between two kinases represents the divergence between their sequences (Figure 1.2).

PKs can be broadly classified according to their substrate specificity: serine/threonine (Ser/Thr) kinases and tyrosine (Tyr) kinases. There are some dual-specific PKs able to phosphorylate both hydroxyl groups of (Ser/Thr) and (Tyr) residues (Bogoyevitch MA and Fairlie DP, 2007). The (Ser/Thr) kinases are four-fold of (Tyr) kinases in number while the latter are involved in key signaling mechanisms (Arena S et al., 2005). Although cAMP-dependent protein kinase (PKA) is the second discovered, it is the first sequenced PK (Shoji S et al., 1981). Then, the transforming protein from Rous sarcoma virus, Src was shown to be related to the catalytic chain of PKA. Moreover, it was shown that Src is a Tyr kinase while PKA is a Ser/Thr kinase (Barker WC and Dayhoff MO, 1982). Further analysis on the sequences of PKA and Src demonstrated that these two share a common precursor. The conserved subdomains, each with a unique motif, have been accepted in time as a definition of PK core (Hanks SK et al., 1988).

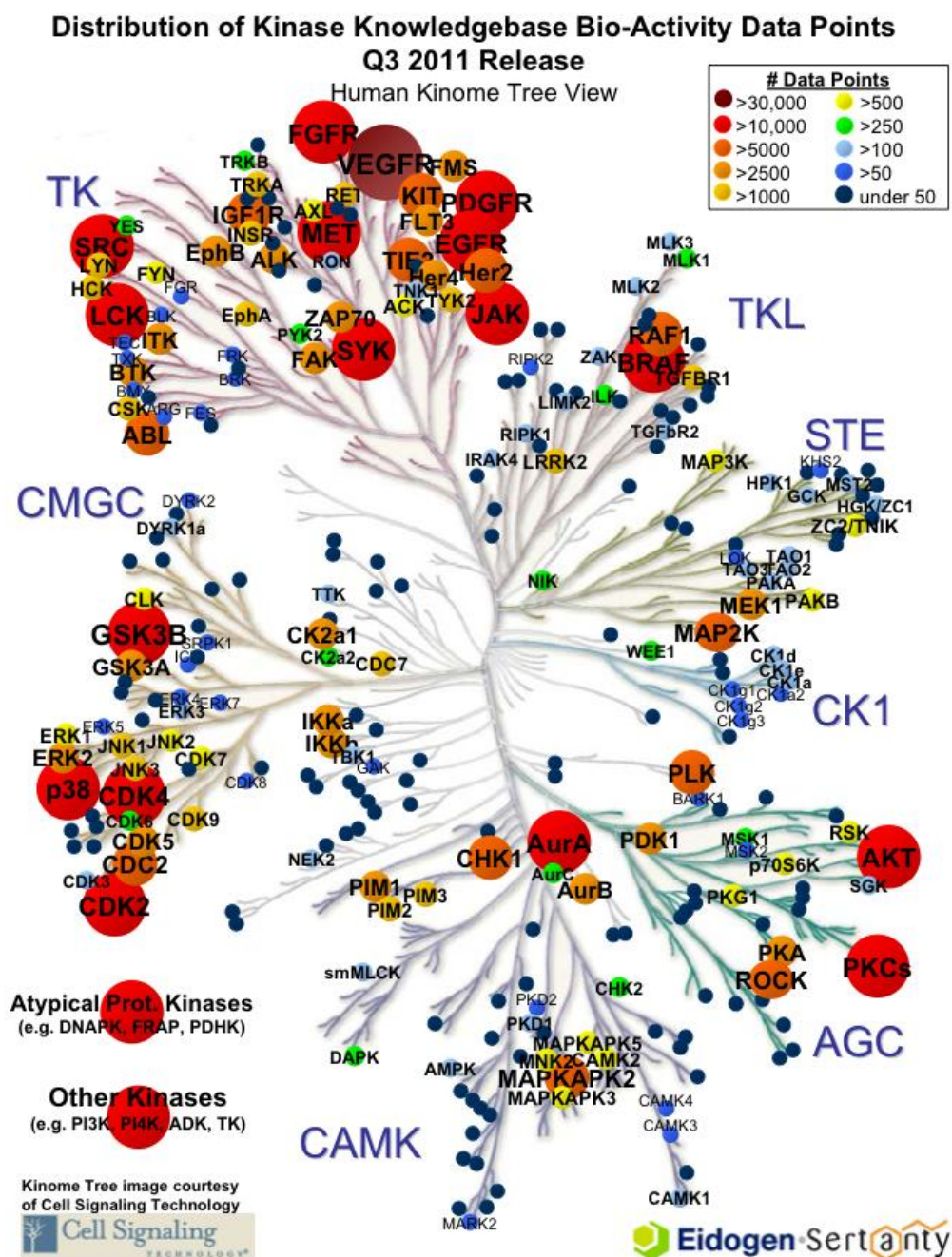


Figure 1.2 The human kinome tree: Groups are **AGC** Containing PKA, PKG, PKC families; **CAMK** Calcium/calmodulin-dependent protein kinase; **CK1** Casein kinase 1; **CMGC** Containing CDK, MAPK, GSK3, CLK families; **STE** Homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases; **TK** Tyrosine kinase; **TKL** Tyrosine kinase-like. The Kinase Knowledgebase is Eidogen-Sertanty's database of biological activity data, structure-activity relationships (SAR), and chemical synthesis data focused on protein kinases. (www.eidogen-sertanty.com)

The conserved catalytic core of PKs is composed of two lobes: N-terminal domain and C-terminal domain which are associated with ATP binding and peptide binding respectively (Figure 1.3). Protein kinases are molecular switches regulating numerous cellular processes; even protein kinases themselves are under the control of other upstream kinases. In such a system, the essential feature of PK activation is the spatial assembly of its domains (Kornev AP et al., 2006).

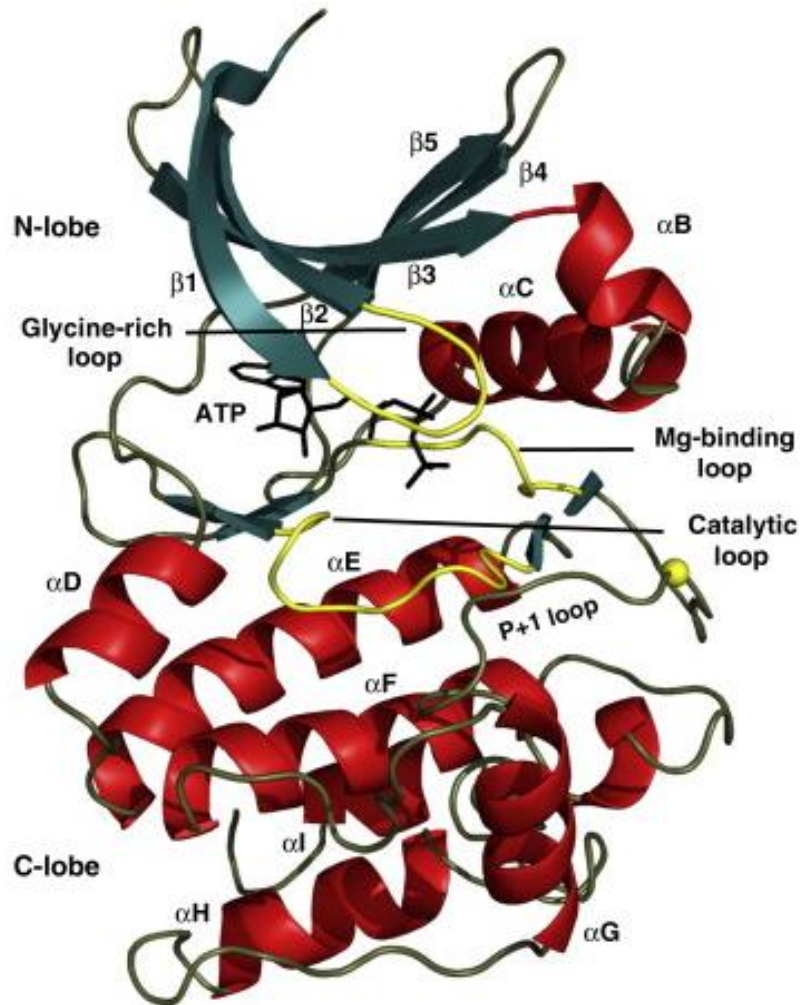


Figure 1.3 Structure of the conserved protein kinase core: Protein kinases (PK) are composed of two domains. The N-terminal domain has Glycine-rich loop, 5 β strands (green) and the α C-helix. C-terminal domain is generally helical (red). Catalytically important loops are colored yellow. Glycine-rich loop coordinates the phosphates. The P-loop accommodates the residues of their peptide substrate (Taylor SS and Kornev AP, 2011).

The mostly helical C-terminal domain is more stable when compared to N-terminal domain and accommodates the substrate proteins. The activation fragment has a Mg-binding loop at its N-terminal beginning with Asp-Phe-Gly (DFG) motif, followed by the most diverse fragment: the activation loop. The phosphorylation site is protected by the “P-loop” which forms a hydrophobic pocket in order to accommodate the residue of the substrate protein positioned just after the residue (Ser/Thr/Tyr) to be phosphorylated. (Kornev AP and Taylor SS, 2010; Taylor SS and Kornev AP, 2011).

1.1.2 Protein kinase inhibitors:

Dysregulation of protein kinase activity rise as a major mechanism in disease conditions, especially in cancer. To date, amplification, translocation, missense mutation and deletion of certain PK genes have been associated with numerous cancers. Analysis on the human kinome showed that 244 of the catalogued 518 putative protein kinase genes are mapped to disease loci or cancer amplicons (Manning G et al., 2002). Thus, human kinome offers an important opportunity to the drug discovery field.

Type I inhibitors: This group of “first generation” of small molecule kinase inhibitors are ATP-competitive compounds targeting the active DFG motifs of PKs (Zuccotto F et al., 2010). They typically include a heterocyclic ring system in order to be able to imitate the interactions of the adenine moiety and bind to the kinase via H-bonds (Zhang J et al., 2009). The region occupied by type I inhibitors is composed of subregions as illustrated in Figure 1.4. Some of the type I kinase inhibitors prefer the inactive conformation of the PK and there are significant structural differences of the type I inhibitor-kinase complex depending on the characteristics of the inhibitor. Gefitinib, erlotinib, dasatinib, sunitinib, and most notably staurosporine are the typical examples for this type I inhibitors (Fabbro D et al., 2012).

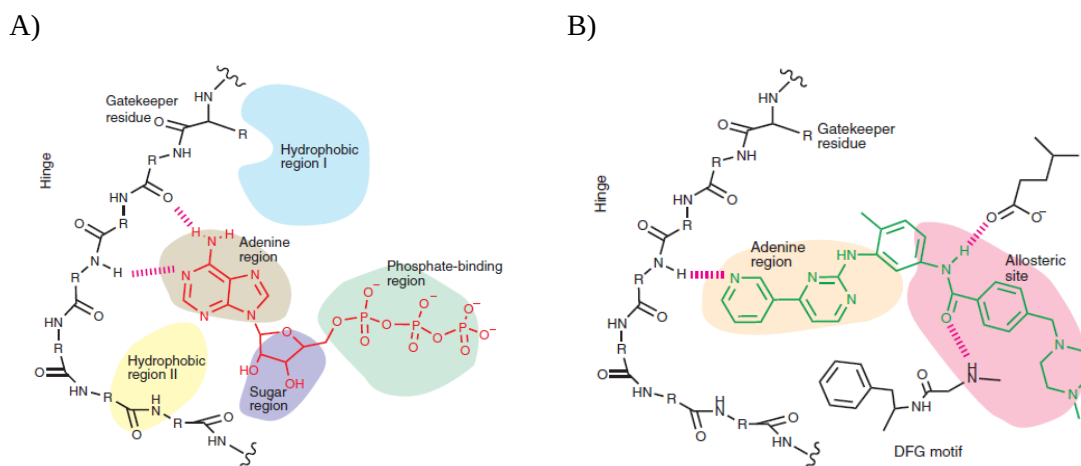


Figure 1.4 The binding modes of protein kinase inhibitors: A) The subregions of the ATP binding site. H-bonds are represented by dashed lines B) Allosteric binding site (Liu Y and Gray NS, 2006).

Type II inhibitors: This group of inhibitors targets the DFG motif projected-out conformation to occupy the hydrophobic region that is adjacent to ATP binding site, in other words, they are designed to recognize the inactive form of the kinase (Zuccotto F et al., 2010). The first (Food and Drug Administration) FDA approved small molecule kinase inhibitor, imatinib, and others nilotinib, sorafenib and vatalinib are the well-known examples of type II inhibitors (Fabbro D et al., 2012).

Type III inhibitors: This group of compounds can be named as non-ATP-competitive or allosteric kinase inhibitors since they do not interact with ATP binding domain (Figure 1.4.). Considering that the binding site exploited is relatively kinase-specific, type III inhibitors show the highest degree of selectivity. The number of type III inhibitors discovered so far is very limited. The most well-defined allosteric kinase inhibitor (of MEK) is CI-1040 and some others are rapamycin analogs and Abl, IKK, Akt, Chek1, MEK, Src, IGF1R inhibitors (Zhang J et al., 2009).

1.1.3 Protein kinase drug discovery:

Cancer cells sustain their malignant phenotype by extensively tangled signaling pathways. Therefore, the identification of the critical and functional nodes of this network is the key step to a successful therapy. FDA approved (Table 1.1) or in preclinical / clinical development chemicals target all stages of signal transduction.

Table 1.1 FDA approved protein kinase inhibitors

Compound	Kinase Target	Cancer target	Company	Approval date
Imatinib (Glivec, Gleevec, STI571)	Abl 1-2, PDGFR, Kit	CML, Ph+ B-ALL, NML, CEL, GIST	Novartis	05 / 2001
Gefitinib (Iressa, ZD1839)	EGFR	NSCLC	AstraZeneca	05 / 2003
Erlotinib (Tarceva, OSI-774)	EGFR	NSCLC, pancreatic cancer	OSI, Genentech Inc, Roche	11 / 2004
Lapatinib (Tykerb, GW2016)	EGFR, ErbB2	Breast cancer	Glaxo Smith Kline	07 / 2007
Dasatinib (Sprycel, BM-354825)	Abl 1-2, PDGFR, Kit, Src	CML	Bristol Myers	06 / 2006
Nilotinib (Tasigna, AMN107)	Abl 1-2, PDGFR, Kit	CML	Novartis	10 / 2007
Sunitinib (Sutent, SU11248)	VEGFR1-3, Kit, PDGFR, Ret, CSFIR, Flt3	RCC, GIST	Pfizer	01 / 2006
Sorafenib (Nexavar, Bay 43-9006)	VEGFR2, Kit, PDGFR, Braf, Flt3	RCC, HCC	Onyx and Bayer Pharmaceuticals	12 / 2005
Pazopanib (Votrient, GW-786034)	VEGFR1-3, PDGFR, Kit	RCC	Glaxo Smith Kline	04 / 2012
Everolimus (Afinitor, Rad001)	mTOR	RCC	Novartis	03 / 2009
Temsirolimus (Torisel, CCI-779)	mTOR	RCC	Wyeth	05 / 2007
Axitinib (Inlyta, AG013736)	VEGF	RCC	Pfizer	01 / 2012

The complexity of the rewired pathways of cancer cells cause them to become more dependent on the activity of a specific oncogene since during carcinogenesis they have lost the function of another gene that normally performs a similar function (Weinstein IB and Joe AK, 2006). Targeting one of the synthetic lethal genes, known as their mutations lead to death if they exist simultaneously, is an elegant strategy for small molecule kinase inhibitors (Kaelin WG, 2005).

Another major challenge for kinase drug discovery is the rising resistance to kinase inhibition. A combinatorial drug treatment approach could be considered as a strategy to improve the therapy. In oncogenic resistance, although the drug fits into its target, inhibitors of apoptosis block the cell death. In order to overcome this resistance, the block should be released or the apoptotic pathways downstream of the block should be taken as a supplementary target (Figure 1.5) (Blagosklonny MV, 2004).

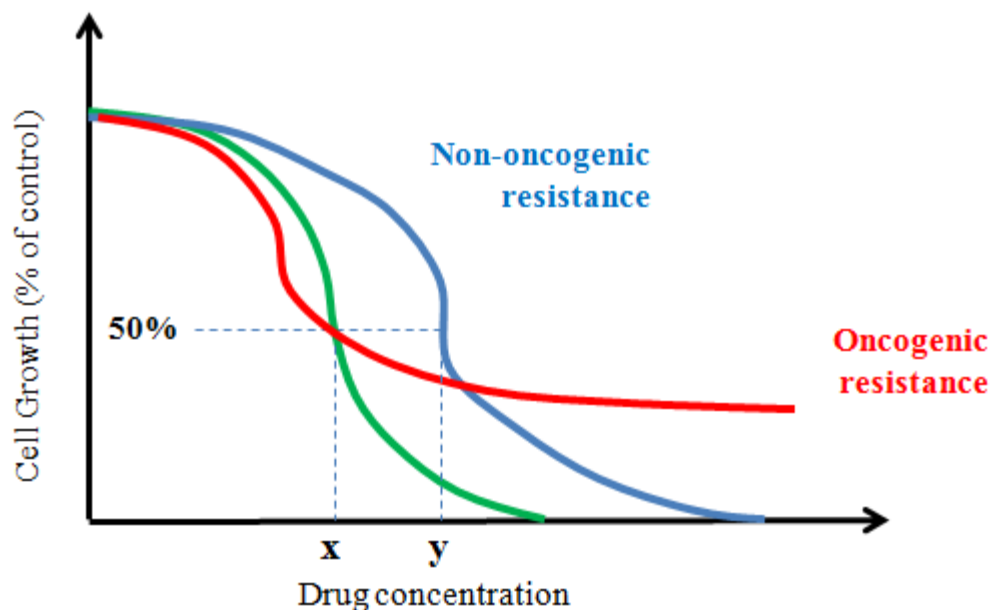


Figure 1.5 Non-oncogenic and oncogenic drug resistance: Inhibitory concentration 50 (IC₅₀) is the drug concentration leading to 50% decrease in cell growth. In case of non-oncogenic drug resistance, dose-response curve shifts to the right with an increase in the IC₅₀ value. If the resistance is oncogenic, the IC₅₀ value does not change; but, since the dose-response curve reaches a plateau,

the drug does not kill the cells although it interacts with its target. Therefore, inhibitory concentrations corresponding below the plateau cannot be achieved. (adapted from Blagosklonny MV, 2004)

1.2 Cell death:

Cancer cells show resistance to cell death due to the defects in signaling pathways developed during tumorigenesis. Chemotherapies stimulate these pathways as an attempt to create cytotoxicity with an ultimate aim to lead to cancer cell death. In addition to apoptosis, non-apoptotic mechanisms such as necrosis, autophagy, mitotic catastrophe and senescence are the cellular activities that anti-cancer drugs initiate (Okada H and Mak TW, 2004).

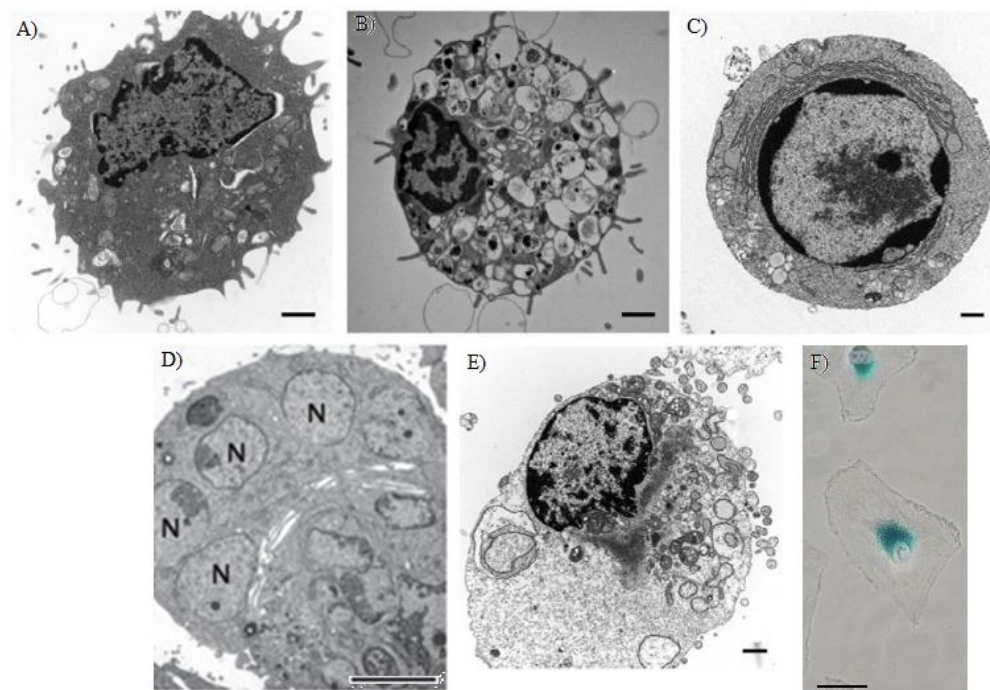


Figure 1.6 Morphological features of cells confronting apoptotic and non-apoptotic cell deaths: A) normal, B) autophagic, C) apoptotic, D) mitotic catastrophic, E) necrotic and F) senescent cells. The scale bars represent 1 μ m for A), B), C), E); 5 μ m for D) and 50 μ m for F). (Edinger AL and Thompson CB, 2004; Leontieva OV and Blagosklonny MV, 2010; Vitale I et al., 2011).

Apoptosis is strictly regulated, “programmed” cell death and can be initiated by either intracellular signals or extracellular ligands. In early apoptosis, cells round up, lose contact to their neighbors and shrink with blebbing cell membranes (Figure 1.6). The chromatin condenses and DNA fragmentation starts. The nucleus becomes tangled, disintegrates into encapsulated, compact fragments called “apoptotic bodies” (Lawen A, 2003). Signals originating from death receptors initiate the Death Inducing Signaling Complex (DISC) which activates caspase-8 (Gewies A, 2003; de Bruin EC and Medema JP, 2008). Cysteineaspartate proteinases, caspases, are inactive enzyme precursors involved in the cleavage of Asp residue containing substrates (Lavrik IN et al., 2005; Kumar S, 2007). Apoptotic stimuli trigger the initiator group of caspases (caspase-8, -9, -10) which consequently activates the executioner group of caspases (caspase-3, -6, -7) (Kurokawa M and Kornbluth S, 2009).

This direct caspase cascade which is sufficient to start apoptosis is called the extrinsic pathway. If the apoptotic signal from DISC needs to be amplified, caspase-8 activates BH3-only protein Bid which subsequently induces the mitochondria to release cytochrome c (Gewies A, 2003; Ricci MS and Zong WX, 2006). This small, mobile molecule, known as an electron carrier, associates with the Apaf-1 (apoptosis protease activating factor-1) and forms apoptosome (Goodsell DS, 2004). The heptameric apoptosome activates caspase-9, another initiator caspase, able to activate caspase cascade through caspase-3 (Gewies A, 2003). Another proapoptotic factor released from mitochondria is Smac/DIABLO (second mitochondria-derived activator of caspases / direct IAP-binding protein with a low isoelectric point) which mainly targets the inhibitor of apoptosis (IAP) to prevent the blockage of caspase cascade (Herr I and Debatin KM, 2010).

Bcl-2 family members, both pro- and anti-apoptotic Bcl-2 proteins, are located upstream of mitochondria and play an essential role in determining whether the cell will live or die (Gross A et al., 1999). The ratio between the anti-apoptotic Bcl-2 members (Bcl-2, Bcl-xL) and the pro-apoptotic ones (Bid, Bax, Bak, Puma, Noxa) helps to decide the cell's fate. Puma (p53 upregulated modulator of apoptosis) and Noxa are involved in p53-mediated apoptosis. DNA

damage as a consequence of irradiation, drugs or other external stress activates the tumor suppressor protein, p53, which performs in favor of apoptosis (Elmore S, 2007)

Cell death by autophagy is a self-degradation process, in which double-membrane vesicles sequester the organelles with cytoplasm to form autophagosomes that fuse with lysosomes (Figure 1.6). Eventually, vesicles breakdown and the contents are hydrolytically digested (Edinger AL and Thompson CB, 2004; Coates JM et al., 2009). Autophagy, “self-eating” is a ubiquitous, evolutionarily conserved process, which is activated in normal tissue, in order to maintain not only cellular homeostasis but also ATP levels during starvation (Jin S and White E, 2007; Coates JM et al., 2009). Although in normal cells autophagy has a tumor suppressive role, in cancer cells its function is complex and context dependent (Cheong H et al., 2012).

Autophagy starts with the formation of phagophore which is regulated by numerous signaling pathways. In mammalian cells phagophore membranes are originated from endoplasmic reticulum (ER) and expand to sequester the organelles with cytoplasm to form autophagosomes (Glick D, 2010). After inhibiting the negative regulator of autophagy, energy-sensing mTOR (mammalian target of rapamycin) kinase, Atg13/FIP200/ULK (Autophagy-related gene/focal adhesion kinase family interacting protein of 200 kDa/UNC-51-like kinase) complex is stimulated and this initiates phagophore formation. Phosphatidylinositol 3 (PI3) kinase complex, composed of Vps34 (vesicular protein sorting 34), Atg14L, Becn1, regulates the vesicle nucleation step (Cheong H et al., 2012).

In contrast to apoptosis and autophagy, necrosis is an unrestrained form of cell death. Necrotic cells swell, lose the membrane integrity and morphologically recognized by vacuolation of their cytoplasm (Figure 1.6) (de Bruin EC and Medema JP, 2008). Necrosis, which has been considered as an uncontrolled cell death, is weakly characterized at the molecular level (Portugal J, 2010). Until recently, necrotic cells were distinguished by the swollen organelles, randomly

degraded nuclear DNA, lacking chromatin condensation, ruptured plasma membrane and eventually total cell collapse (Zhivotovsky B, 2004). Earlier studies define necrosis as a traumatic cellular response to severe toxicity without a stimulating signaling cascade (Okada H and Mak TW, 2004). On the contrary, the following studies evidently showed that depending on the context, necrosis is a tightly controlled interaction of signaling pathways, originated from various stimuli (Duprez L et al., 2009).

Poly-(ADP-ribose) polymerase (PARP-1) is an 113kDa nuclear enzyme. Under normal circumstances, PARP-1 detects and repairs DNA damages; but, massive DNA damage hyperactivates PARP-1, reduces NAD⁺ levels, leads to ATP shortage and eventually causes necrosis (Duprez L et al., 2009; Chaitanya GV et al., 2010).

Table 1.2 Proteolytic fragments of PARP-1 (Chaitanya GV et al., 2010)

Caspase-3 and -7	24kDa N-terminal fragment
	89kDa C-terminal fragment
Calpain-1	≈ 70 – 40 kDa N-terminal fragments
Cathepsin-B and -D	89, 74, 62 kDa N-terminal fragments
	55, 44 kDa C-terminal fragments
Cathepsin-G	74, 62 kDa N-terminal fragments
	55, 44 kDa C-terminal fragments
Granzyme-A	55 kDa N-terminal fragment
	≈ 55 kDa C-terminal fragment
Granzyme-B	64, 61 kDa N-terminal fragments
	54, 42 kDa C-terminal fragments
MMP-2	<48 kDa fragment
	66 kDa fragment

Caspases, calpains, cathepsins, granzymes and matrix metalloproteinases (MPPs), in other words suicide proteases, create PARP-1 fragments specific to the type of cell death (Table 1.2). Cleavage of PARP-1 by means of caspases occurs during apoptosis and therefore of 89kDa cleaved-PARP is considered as an

apoptosis marker (Chaitanya GV et al., 2010). On the other hand, the major fragment obtained during necrosis is 50 kDa (Gobeil S et al., 2001).

Mitotic catastrophe (MC) is occasionally accepted as an aberrant mitosis eventually leading cell irreversibly to death rather than a form of death (Ricci MS and Zong WX, 2011). Abnormal mitosis drives cells to mitotic catastrophe (MC) through several different pathways but finally the enlarged, multi-nucleated cells share a common, characteristic morphology. They lack the nuclear envelope, have fragmented nuclei as in apoptotic cells but contain uncondensed chromatin (Figure 1.6) (Roninson IB et al., 2002). Additionally, MC has been evaluated as a distinctive subset of apoptosis due to the common biochemical features both apoptosis and MC share, such as caspase activation and the permeabilization of mitochondrial membrane (Castedo M et al., 2004). There are also accumulated data describing MC as a survival mechanism that the tumor cells exploit. Another currently supported opinion is that MC is a preliminary process leading to apoptosis or necrosis (Vakifahmetoglu et al., 2008).

Mitotic catastrophe does not occur in a thoroughly established homogenous manner; instead, depending on the duration between mitotic arrest and the cell death, MC can happen in various modes. Recently, three different fates for cells experiencing mitotic arrest have been described. First, the cells can undergo mitotic death; in other words, they can die during mitosis following mitotic arrest. Second, the cell death can happen during the interphase of the subsequent cell cycle. Third, instead of cell death, the cells can be subjected to senescence during the interphase of the next cell cycle (Vitale I et al., 2011).

1.2.1 Senescence:

Senescence was described long ago after the observation of the limited life span of normal human embryonic fibroblasts in culture dish. Senescent cells are large, flattened and multi-nucleated with granular cytoplasm. A commonly used senescence biomarker is the increased senescence associated- β -gal (SA- β -gal)

activity, which can be detected by X-gal, forming a blue precipitate at pH 6.0 (Figure 1.6) (Dimri GP et al., 1995; Dimri GP 2005).

The phenomenon named as “replicative senescence” and defined as irreversible cell cycle arrest (Hayflick L and Moorhead PS, 1961; Hayflick L, 1965). Different from quiescent cells, senescent cells do not enter S phase in the presence of mitogenic stimuli. Unlike apoptotic cells, they remain alive if fresh culture medium is supplied; namely senescence provides them resistance to apoptosis (Zhang H, 2007). Additionally, they develop a senescence associated secretory phenotype (SASP) including various cytokines, growth factors and proteases containing strong autocrine and paracrine effects (Rodier F and Campisi J, 2011).

1.2.1.1 Replicative senescence:

The underlying molecular mechanism for cultured cells halting replication after a finite number of passaging is explained by telomere shortening (Harley C et al., 1990). The repetitive DNA sequence (TTAGGG) and its specific proteins are called telomeres. These DNA-protein complexes cap and therefore protect the ends of human chromosomes. Since most of the human cells lack telomerase, responsible for the *de novo* synthesis of telomeric DNA, telomeres shorten after each cell cycle. When the telomere size reaches to a threshold, cells stop replicating permanently; in other words, they undergo senescence due to damaged DNA at the ends of the chromosomes (Campisi J, 2001; Campisi J and d’Adda di Fagagna F, 2007). Not only telomere shortening but also oncogene activation, DNA-damage, oxidative stress, PTEN (phosphatase and tensin homolog deleted on chromosome 10) loss and cytotoxic drugs can lead to senescence (Collado M and Serrano M, 2006; Kuilman T et al., 2010).

1.2.1.2 Oncogene-induced senescence:

Oncogene-induced senescence (OIS) emerges as a tumor-suppressive response to hyper-replication of oncogene proteins such as myc and H-ras (Lee S

et al., 2011) Oncogenic ras-dependent transformation of normal cells to tumor cells requires the cooperation of an additional oncogene or the lack of tumor suppressor genes. Otherwise, with the accumulation of p53 and/or p16, oncogenic ras drives cells to G1 arrest and eventually senescence (Serrano M et al., 1997).

The expression levels of the oncogene determine the future of senescence. Surprisingly, OIS requires overexpressed oncogene; whereas, basal levels are capable of inducing neither senescence nor tumorigenesis. In other words, when the tumor have already been installed but have not yet been completely malignant, senescence can be induced. This phenomenon makes oncogene-induced senescence markers available to detect tumorigenesis at early stages (Collado M and Serrano M, 2006).

1.2.1.3 DNA damage-induced senescence:

DNA damage-induced senescent cells follow the same pathways of replicative senescence (Shay JW and Roninson IB, 2004). They are continuously cell cycle arrested, secrete inflammatory cytokines and accommodate permanent nuclear foci including DNA damage response (DDR) related proteins (Rodier F, 2011). Double strand breaks (DSB) on DNA strands, ruin the integrity of the genome and should be immediately fixed by means of the interacting pathways generally called DDR (Mah L et al., 2010). DDR signaling is vital to provoke senescence (Rodier F et al., 2011). Therefore, the inactivation of the cell cycle checkpoint kinases (e.g. ATM, Chk2), blocks the p53-dependent senescence; moreover, impedes p53-independent secretion of inflammatory cytokines which compose the SASP (Rodier F et al., 2009; Rodier F et al., 2011).

1.2.1.4 ROS-induced cellular senescence:

Oxygen concentration, which is directly related to the formation of reactive oxygen species (ROS), limits cellular lifespan (Lu T and Finkel T, 2008). The large fraction of ROS production (90%) takes place at mitochondria (Irmak MB et al., 2003). Mitochondrial ROS generation initiates with an oxygen molecule (O₂)

which can be turned into singlet oxygen ($^1\text{O}_2$) or by a single electron (e^-) reduction to superoxide anion ($\text{O}_2^{\cdot-}$). Either superoxide dismutase (SOD) provides another single e^- or spontaneously, superoxide anion ($\text{O}_2^{\cdot-}$) can be further reduced and form hydrogen peroxide (H_2O_2), a more stable molecule which can pass through the lipid membranes of cells. H_2O_2 can oxidize ferrous iron to ferric iron in a Fenton reaction and consequently get reduced to hydroxyl radical ($\text{OH}^{\cdot}$). ROS can be neutralized and form water, oxygen and hypochlorous acid. Mitochondria, intends to increase the energy output to its maximum level while trying to minimize the excess ROS in a mediating redox state (Figure 1.7) (Aon MA et al., 2010; Chen L et al., 2012).

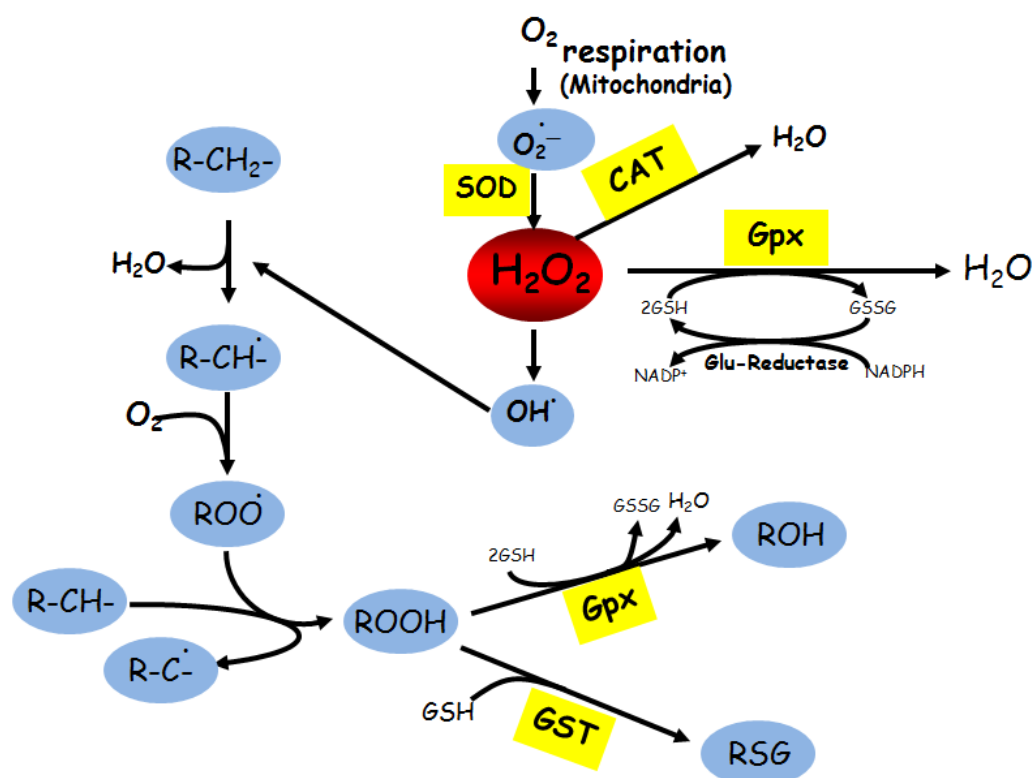


Figure 1.7 ROS (reactive oxygen species) generation

The hindered senescence process in the presence of antioxidants and the early initiation of senescence upon inhibiting the cellular oxidant scavengers, clearly demonstrates the contribution of ROS to induce senescence (Chen Q et al., 1995; Yuan H et al., 1995). Recently, it was shown that, induction of senescence requires a dynamic feedback loop, between p21 and ROS, triggered by a DDR (Passos JF et al., 2010). Significantly, ROS have been detected as a key

intermediary between replicative and oncogene-induced senescence. High O₂ concentrations lead cells to replicative senescence; whereas under low O₂ conditions their lifespan expands (Ozturk M et al., 2008).

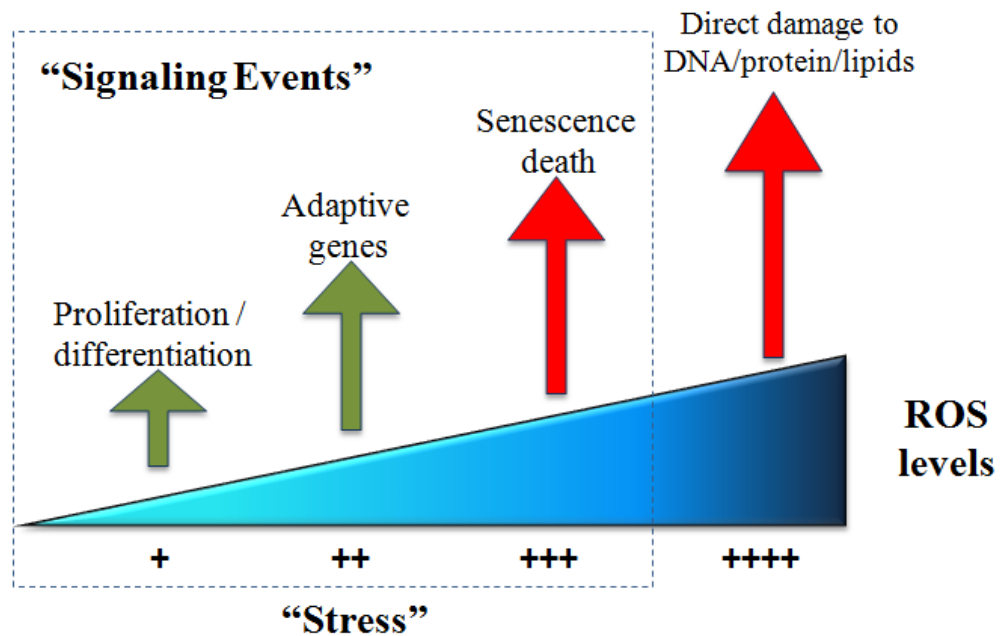


Figure 1.8 Mitochondrial ROS levels and the cell's fate (modified from Hamanaka RB and Chandel NS, 2010)

ROS, as a key player of signaling pathways, influence the cell's fate according to its cellular levels. While low levels of ROS involve in proliferation and differentiation, increasing ROS production first upregulates antioxidant genes, then induce senescence and finally cause cell death. With extreme ROS levels, cellular contents experience permanent damage beyond the signaling interactions (Figure 1.8) (Hamanaka RB and Chandel NS, 2010).

1.2.1.5 PTEN loss-induced cellular senescence:

In premalignancies, loss of a tumor suppressor gene leads to senescence. In this telomere-independent form of senescence, in addition to the absence of hyperreplication, as seen in OIS, DNA damage response is also missing (Nardella C et al., 2011). Due to its distinctive features, this form of senescence

was named as PTEN loss-induced cellular senescence (PICS); since, it was first observed rapidly after PTEN inactivation (Alimonti A et al., 2010). PI3K/Akt pathway, known to be one of the major survival pathways cancer cells exploit, becomes constitutively active in the absence of PTEN (Song MS et al., 2012). In addition to PTEN, loss of other tumor suppressor genes, such as NF1, VHL and Rb, also induce senescence (Acosta JC and Gil J, 2012). Resembling OIS, PICS is associated with amplified p53 translation (Alimonti A et al., 2010). Nevertheless, in PICS mTOR mediates the p53 upregulation; whereas, p53 activation is promoted by the phosphorylation of DDR and ARF in OIS (Nardella C et al., 2011; Acosta JC and Gil J, 2012).

1.2.1.6 Drug-induced cellular senescence:

Senescence is a potent barrier to tumor development which recently started to be considered as a promising approach for cancer therapy (Ewald JA et al., 2010). Recent findings reveal that cancer cells can also undergo senescence. Thus, inducing senescence in tumors; namely, pro-senescence therapy, is a new strategy in the fight against cancer. Senescence can prevent immortalization; thus senescence-inducing drugs emerge rapidly (Cairney CJ et al., 2012). Small-molecule inhibitors dominate the market of pro-senescence drugs and can be categorized according to their therapeutic approaches. p53-enhancing approaches include both inhibiting the MDM2-p53 interaction and restoring the mutant p53. Therapeutic modulation of cell cycle machinery approach can be achieved by inhibiting either the S phase kinase associated protein-2 (SKP2) or cyclin dependent kinase 2 (CDK2). Oncogene addiction approach exploits the inhibition of the oncogene, known that the tumor is addicted to, which induces senescence. The approach for the compounds enhancing PICS is to inhibit PTEN and activate p53 indirectly. Utilizing telomerase inhibitors, in order to let the telomeres of cancer cells get shorter, is also another approach guiding to senescence (Nardella C et al., 2011)

1.2.1.7 Senescence pathways :

Replicative senescence and telomere-independent forms of senescence use one of the two major tumor suppressor pathways, p53 and p16. Activation of p53, including not only its induction but also the post-translational modifications p53 encounter, is essential in senescence (Caino MC et al., 2009). Despite the unchanged p53 mRNA and protein levels, during senescence p53 gets phosphorylated and activated (Zhang H, 2007). Throughout the pathway initiated with p53, one of the downstream activated proteins is p21, a member of the Cip/Kip family of CDK inhibitors (Sherr CJ and Roberts JM, 1999). It is demonstrated that, independent from p53 activation, p21 can induce senescence; moreover, p21 deletion delays senescence (Larsson O, 2005).

In senescence pathways, p53 cooperates with Rb, which represses E2F-DP transcription factors; therefore, keeps cells away from entering S phase of cell cycle. To inactivate Rb; in other words, to phosphorylate Rb, CDK activity is required. In senescent cells p16, a member of the INK4 family of CDK inhibitors, is mainly responsible for the hypophosphorylated status of Rb (Wei W et al., 2003). Other than p16, p21 upregulation can directly prevent Rb phosphorylation and this finding supports the idea of a linear p53-Rb pathway (Figure 1.9) (Ben-Porath I and Weinberg RA, 2005).

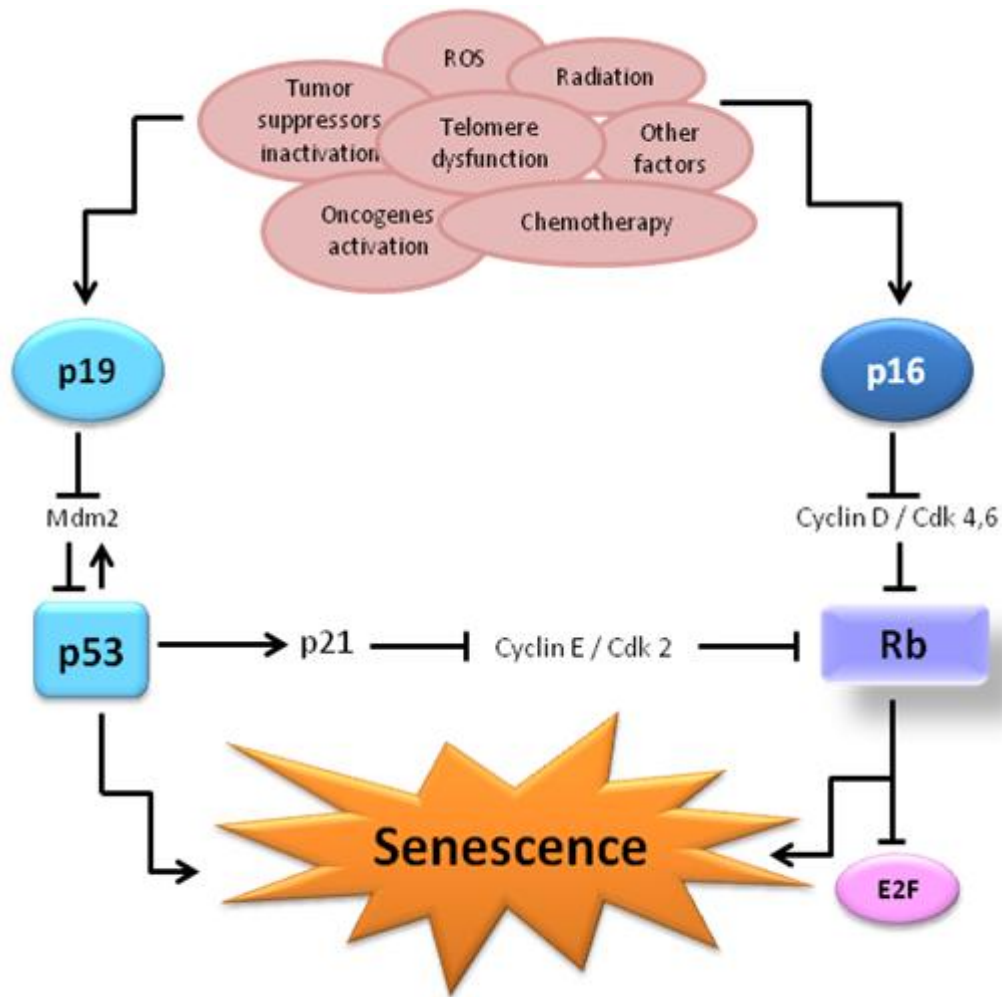


Figure 1.9 Senescence pathways

(modified Ben-Porath I and Weinberg RA, 2005)

1.2.1.8 The cell cycle and its regulation:

Healthy cells traverse the cell cycle in strictly-regulated phases of two main stages: mitosis (M) and interphase, the interval between two consecutive M phases (Vermeulen K et al., 2003). Mitosis is composed of karyokinesis, the process in which the nucleus divides into two, and cytokinesis, the further division of the cytoplasm. Karyokinesis also has ensuing stages: prophase, prometaphase, metaphase, anaphase and telophase. As soon as M-phase has been accomplished, G1 (Gap1) phase initiates the interphase. Cellular contents other than chromosomes are duplicated during G1, where the cells commit to enter another

cell cycle (Ma HT and Poon RYC, 2011). DNA replication occurs in the S (synthesis) phase preceding G2 (Gap2) phase (Vermeulen K et al., 2003).

The quality control mechanisms of the cell cycle are located all through the process. DNA damage checkpoints take place before entering the S phase (G1-S checkpoint), after the duplication of DNA (G2-M checkpoint) and also during S and M phases (Dipaola RS, 2002; Vermeulen K et al., 2003). M phase checkpoints detect malformed spindle assemblies and incorrect spindle alignments (Okada H and Mak TW, 2004). For the induction of S phase arrest, ataxia-telangiectasia-mutated (ATM)-mediated phosphorylation of Nijmegen breakage syndrome 1 (NBS1) is required (Lim DS et al., 2000). If the genes involved in the induction of mitotic catastrophe are faulty, tumor formation can be accelerated. These genes encode the protein kinases regulating the cell cycle (Okada H and Mak TW, 2004). In response to DNA damage, ATM and ATM-and Rad3-related (ATR) get activated in order to initiate a kinase cascade. Finally, CDK1, the chief conductor of M phase, become inactivated leading to G2/M arrest (Dipaola RS, 2002).

Another important regulator of the cell cycle is the retinoblastoma gene product (Rb). In its active form, Rb, the tumor suppressor gene, is hypophosphorylated and forms an inhibitory complex with transcription factor, E2F-DP (E2F-1, E2F-2, and E2F-3). Hyperphosphorylation of Rb by CDK2, CDK4 and CDK6 inactivates Rb and releases E2F-DP. Initiation of the transcription of various S phase proteins facilitates the G1-S transition (Schwartz GK and Shah MA, 2005).

1.2.1.9 Cyclin-dependent kinases (CDKs):

Cyclin-dependent kinases (CDKs) are fundamental regulators of cell cycle. They belong to the Ser/Thr kinase family and are active during the cell cycle if associated with cyclins. Although CDK levels remain constant, their activating proteins, cyclins, fluctuate as the cell cycle proceeds (Vermeulen K et al., 2003). Cell cycle phase-specific assembly of CDK-cyclin complex depends on the

accurate completion of the preceding step; namely, cell cycle checkpoints (Knockaert M et al., 2002).

1.2.1.10 Cyclin-dependent kinase inhibitors (CKIs):

In addition to cyclins, two distinct families of CKIs also regulate the activity of CDKs. The INK4 family members p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d) negatively regulates the G1-specific CDKs, CDK4 and CDK6. The Cip/Kip family includes p21 (Waf1, Cip1), p27 (Cip2) and p57 (Kip2), general inhibitors of CDK-cyclin complex (Denicourt C and Dowdy SF, 2004; Cánepa ET et al., 2007).

1.3 Hepatocellular carcinoma (HCC):

Liver is the second largest organ after skin and that makes it the largest internal organ in human. It has a distinguishing blood supply coming directly not only from the heart but also from the digestive tract. Corresponding to its superior physical features, liver is a vital organ responsible homeostasis of human body. The majority of the cells in liver are hepatocytes, macrophages (Kupffer cells) and stellate cells (fat-storing cells). In a healthy liver stellate cells store vitamin A, preserve the liver membrane; most importantly stay inactive. In case of liver injury, they become activated; thus, use their vitamin A stocks to produce fibrous scar tissue. The accumulation of the scar tissue, leads to liver diseases depending on its severity (Kolios G et al., 2006). Although it was a controversial issue formerly, now it is accepted that liver contains progenitor stem cells. In cases of chronic and severe liver injuries, a subgroup of liver cells (oval cells) is induced not only to proliferate but also to differentiate according to conditions (Qin AL et al., 2004).

Benign growths in liver, which do not transform into cancer, are a common issue. They can be treated with or without surgery successfully; whereas, liver cancer treatment options are limited and mostly palliative (Lau WY and Lai EC, 2008). The major type of primary liver cancer, Hepatocellular carcinoma (HCC),

is the sixth most common and the third lethal cancer (Forner A et al., 2012). HCCs are characterized as heterogeneous tumors both phenotypically and genetically (Blum HE and Spangenberg HC, 2007). Generally, HCC is a tough cancer type with poor prognosis and low overall survival rate.

1.3.1 Hepatocarcinogenesis:

Hepatocarcinogenesis is a slow, multi-step process which involves various genetic and epigenetic alterations, chromosomal abnormalities, pathway dysregulations and mutations (Alves RC et al., 2011). The major etiologies of HCC include chronic hepatitis B (HBV), C (HCV) and D (HDV), alcohol abuse, aflatoxin-B1-intoxication, hereditary metabolic liver diseases and obesity (Farazi PA and DePinho RA, 2006; Blum HE and Spangenberg HC, 2007).

Following the hepatic injury initiated by any one of these etiologies, a closed-loop system composed of hepatocyte-necrosis and -proliferation takes place. This continuous destructive-regenerative cycles, prepare the liver for cirrhosis, which is characterized by structurally abnormal nodules surrounded by scarring tissue. As a further step in hepatocarcinogenesis, hyperplastic nodules are observed which transform into dysplastic nodules with moderate genomic instability. Loss of p53 and increase in the level of genomic instability leads to HCC (Bressac B et al., 1990; Farazi PA and DePinho RA, 2006). While telomere shortening has been considered as a marker of hyperproliferative liver disease, the reactivation of telomerase has been associated with hepatocarcinogenesis (Ozturk M et al., 2008).

1.3.2 Cell signaling pathways involved in HCC:

The principal signaling pathways implicated in the pathogenesis of HCC can be classified according to the biological process they have been involved in (Figure 1.10). The molecular abnormalities responsible from the dysregulation of signaling pathways (i.e. protein kinases) accommodate the key targets for HCC therapy (Llovet JM and Bruix J, 2008).

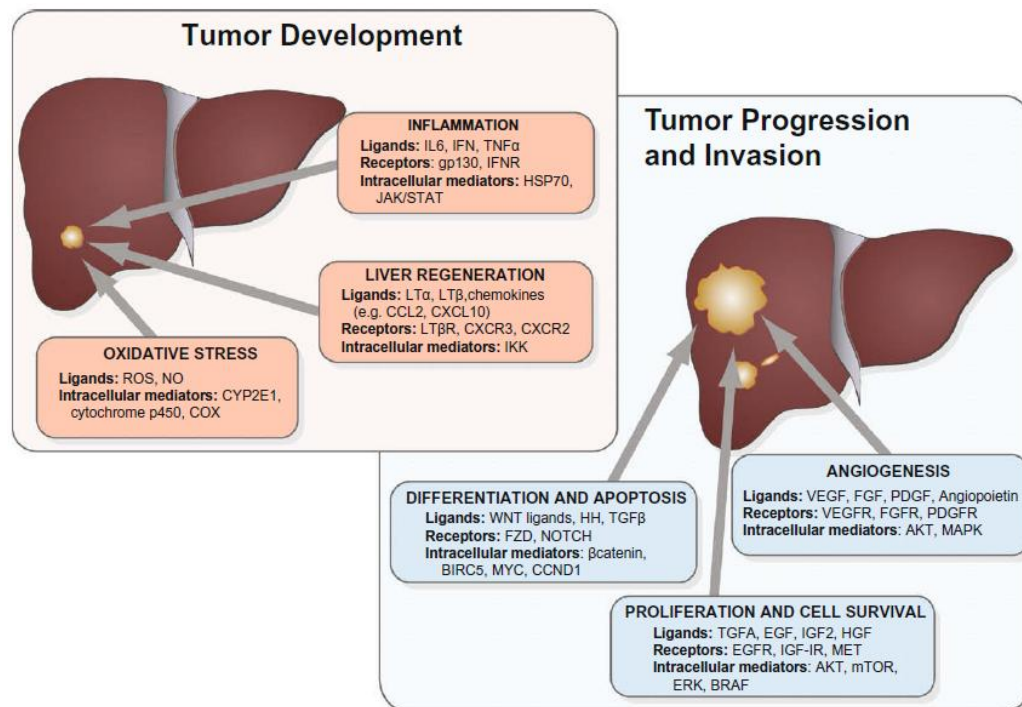


Figure 1.10 Dysregulated signaling pathways in HCC (Zender L et al., 2010)

1.3.2.1 EGF/EGFR pathway:

Epidermal growth factor receptor (EGFR; Her1/ErbB1) belongs to a family of four (Her2/Neu, ErbB2, ErbB4) receptor tyrosine kinases (RTKs). All have an extracellular ligand-binding domain, single-pass transmembrane domain and an intracellular domain with a TK activity (Cervello M et al., 2012). EGFR is highly expressed in HCC with two major ligands, TGF- α (transforming growth factor-alpha) and EGF, stimulating its activation (Harada K et al., 1999). Ligand binding stimulates the homo- or hetero- dimerization of EGFR, which results in auto-phosphorylation of specific tyrosine residues in the C-terminal (Tao RH and Maruyama IN, 2008). These phospho-tyrosines serve as docking sites for intracellular mediators which eventually activate several signaling pathways such as, Ras/MAPK, PI3K/Akt, PKC and STATs pathways (Brand TM et al., 2011).

1.3.2.2 Ras/Raf/MEK/ERK pathway:

Mitogen-activated protein kinases (MAPKs) have at least five distinct subgroups: ERK1/2 (extracellular signal-regulated kinase), ERK5 (also known as the big MAPK-1 or BMK-1), SAPK1/JNK1/2/3 (stress-activated protein kinase/c-Jun N-terminal kinase), and SAPK2 homologs: α, β, δ (p38 $\alpha/\beta/\delta$) and ERK6 (p38 γ) (Pimienta G and Pascual J, 2007).

ERK1 and ERK2 are homologous Ser/Thr kinases with 88% similarity in protein sequence (Min L et al., 2011). ERK1/2 can be activated by RTKs (e.g. EGFR). The ligand stimulated RTK undergoes dimerization and by auto-phosphorylation generates binding sites for adaptor proteins and activates Ras. Activated Ras, Ras:GTP, recruits Raf to the membrane in order to activate for further and subsequent phosphorylations of MEK1/2 and ERK1/2 (Steelman LS et al., 2011). Activated ERK1 and ERK2 phosphorylate more than 160 substrates which are known to be related with cell proliferation. Moreover, ERK activity also suppresses apoptosis and supports survival (Min L et al., 2010).

1.3.2.3 PI3K/PTEN/Akt/mTOR pathway:

Phosphatidylinositol-3-kinase (PI3K) is a lipid kinase, consisting of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit, is recruited to the membrane after growth factor stimulation. The activated catalytic subunit of PI3K, phosphorylates membrane phospholipids, phosphatidylinositol-4-phosphate (PtdIns(4)P) and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂); generates phosphatidylinositol-3,4-bisphosphate (PIP₂) and phosphatidylinositol-3,4,5-trisphosphate (PIP₃). PIP₂ and PIP₃ serve as second messengers and activate phosphatidylinositol-dependent kinases (PDKs). PDKs are responsible from the activation of Akt, a Ser/Thr kinase, which is also known as protein kinase B, PKB (Franke TF, 2008; Liu P et al., 2009).

Following its activation, Akt departs from the membrane and phosphorylates its intracellular substrates in order to promote cell survival. The

deficiency of PTEN, the negative regulator of Akt, supports its constitutive activation (Buontempo F et al., 2010). Moreover, activated Akt is a positive regulator of mTOR which phosphorylates p70S6 kinase (40S ribosomal protein kinase) and 4E-BP1 (the translation repressor eukaryotic initiation factor 4E-binding protein-1) in order to promote the translation of significant factors involved not only in cell proliferation but also in angiogenesis (Cervello M et al., 2012).

1.3.2.4 IGF/IGFR pathway:

The insulin-like growth factor family consists of two circulating ligands, IGF-1 and IGF-2, both are vital regulators of cell growth, proliferation, differentiation, development and apoptosis (Pollak MN et al., 2004). While the insulin receptor have two isoforms (IRA, IRB), IGF-1R has only one and it is a heterotetramer with two ligand-binding extracellular domains and two TK intracellular domains. Half of each receptor dimerize with each other as they finally form six receptor species with altering ligand-binding affinities. Cancer cells and tissues which are neither insulin-target nor IGF1-R-responsive express all the six types of receptors (Pollak MN, 2012). After the stimulation of receptors, the recruitment of insulin-receptor substrates (IRS) precedes the activation of PI3K/Akt/mTOR and Ras/Raf/MEK/ERK pathways (Cervello M et al., 2012).

1.3.2.5 TGF- β pathway:

Transforming growth factor-beta (TGF- β) superfamily, consisting of structurally related dimeric proteins, involve in cell proliferation, differentiation, motility, adhesion, development and cell death (Massagué J, 1998). Seven of the twelve members of TGF- β receptors are type I (T β RI) and the rest is type II (T β RII) and all are Ser/Thr kinases. The signaling pathway is initiated with ligand binding to T β RII, on the cell membrane, which recruits T β RI and phosphorylates Ser/Thr residues in the TTSGSGSG motif of T β RI. Prior to this, an inhibitory protein associated with T β RI, keeps it inactive. Following its activation, T β RI,

recruits Smad2/3 in the presence of an anchoring protein, SARA (Smad anchor for receptor activation). Activated (i.e. phosphorylated) Smad2/3 associates with Smad4 to form a heterocomplex, which translocates into the nucleus to promote the transcription of target genes (Huang F and Chen YG, 2012). It is known that, TGF- β signaling pathway suppresses hepatocarcinogenesis (Gupta GP and Massagué J, 2006; Baek HJ et al., 2008). Paradoxically, a recent study reveals that TGF- β signaling promotes liver tumor formation in the lack of p53 (Morris SH et al., 2012).

1.3.2.6 Wnt/ β -catenin pathway:

Wnt/ β -catenin signaling pathway is crucial not only in hepatocarcinogenesis but also in normal liver tissue development and maintenance, during embryogenesis and adulthood (Dahmani R et al., 2011). The receptors for the Wnts, which are lipid-modified morphogens, are seven-pass transmembrane proteins (Janda CY et al., 2012). In the absence Wnt signaling, a degradation complex including APC (adenomatous polyposis coli), GSK-3 β (glycogen synthase kinase-3 β) and axin, maintains β -catenin inactive (i.e. phosphorylated). β -catenin is the key player of the pathway and its phosphorylation causes its degradation through ubiquitylation (Giles RH et al., 2003). When the Wnt ligands bind to their receptor, Frizzled (FZD7), a series of events initiate to inactivate the degradation complex (APC-GSK-3 β -axin) and keep β -catenin away from degradation. Translocation of β -catenin to the nucleus follows its accumulation in the cytoplasm. Eventually, β -catenin stimulates the transcription of Wnt-target genes (Dahmani R et al., 2011). Upregulated FZD7 gene expression is the most common abnormality of Wnt pathway associated with hepatocarcinogenesis. Besides, β -catenin gene mutations, which keep it constitutively active, were demonstrated in 20% - 40% of the HCC patients (Cervello M et al., 2012).

1.4 c-Jun-N-terminal kinases and liver:

c-Jun-N-terminal kinases (JNKs) constitute one of the sub-groups of MAPK family (Weston CR and Davis RJ, 2007). They are Ser/Thr kinases with a dual phosphorylation Thr-Pro-Tyr motif conserved between the domains seven and eight (out of 11 PK subdomains) for full kinase activation. JNKs have a common docking domain for their substrates in the C-terminal and a glutamate/aspartate domain in the N-terminal, in order to mediate their interactions with other players. There are three JNK isoforms, JNK1, JNK2 and JNK3 (encoded by MAPK8, MAPK9 and MAPK10 respectively) corresponding to ten alternative splicing variants (Figure 1.11) (Manning AM and Davis RJ, 2003; Seki E et al., 2012).

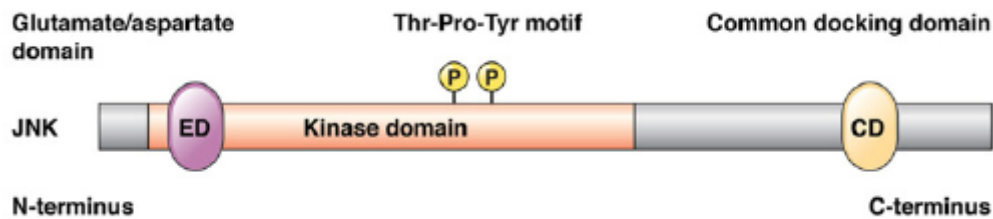


Figure 1.11 c-Jun-N-terminal kinases (JNKs) (Seki E et al., 2012)

1.4.1 JNK pathway :

The long list of diverse stimuli, consisting of growth factors (e.g. TGF β , PDGF, EGF), cytokines (e.g. TNF- α , IL-1), ROS, pathogens, toxins, ER-stress, drugs and metabolic changes), initiates the JNK signaling pathway (Figure 1.12). JNKs locate on the lowest level of a three levels signaling cascade. There are at least fourteen MAP3Ks (including ASK1, DLK, LZK, MEKK1, MEKK2, MEKK4, MLK1, MLK2, MLK3, MLK4, TAK1, TAO1, TAO2, ZAK) confronting the stimuli first, and activate MAP2Ks next. MAP2Ks, MKK4 and MKK7, are dual-specificity PKs (Seki E et al., 2012). The initiative stimulus determines which MAP2K activate JNKs; in other words, MKK4 generally

responds to environmental stress and MKK7 feedback to cytokines (Min L et al., 2010). Moreover, MKK4 phosphorylates both JNKs and p38 homologs but MKK7 phosphorylates only JNKs (Haeusgen W et al., 2011). Among the approximately 50 substrates of JNKs, c-Jun is the representative target which forms the activating protein (AP-1), a dimeric transcription factor, with JunB, JunD or Fos (Shaulian E, 2010).

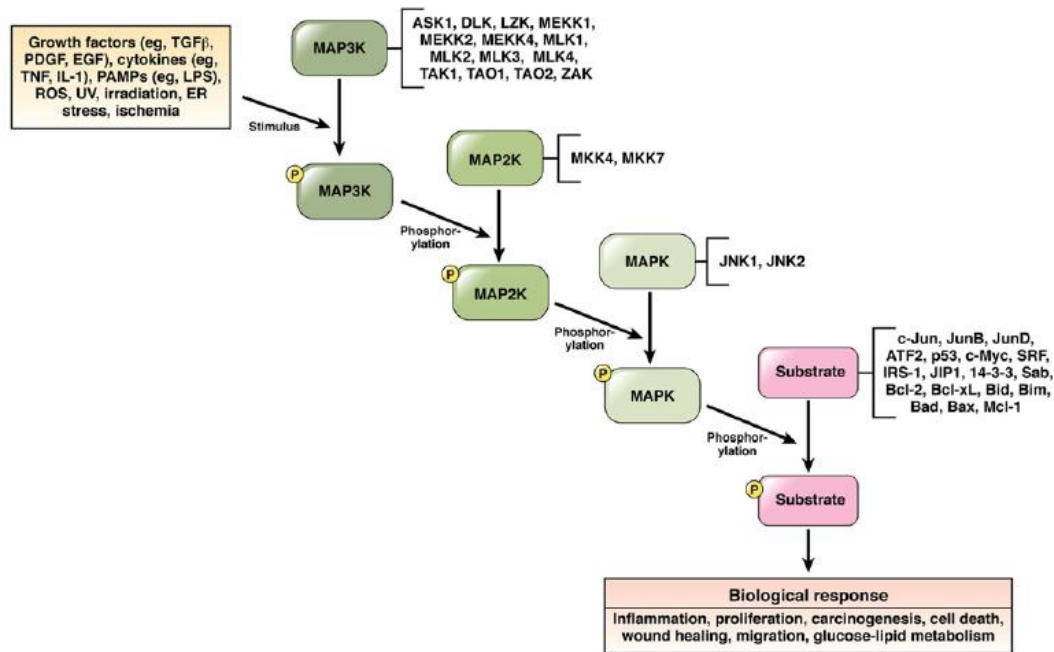


Figure 1.12 JNK signaling pathway (Seki E et al., 2012)

NF- κ B is a transcription factor in a dimeric structure, associated with its inhibitor protein, I κ B. After the phosphorylation of I κ B by activated IKK (I κ B kinase) complex, first I κ B degrades and second NF- κ B gets activated. (Hayden MS and Ghosh S, 2008). TNF- α (tumor necrosis factor-alpha), is the common denominator of the two intricately interacting pathways: JNK and NF- κ B. Furthermore, TNF- α is a double-crosser which can be either survival or apoptotic depending on the context (Wang X and Lin Y, 2008). In addition to all, TNF- α can activate caspase 8, NF- κ B and JNK, simultaneously (Deng Y et al., 2003). Inhibited NF- κ B, allows TNF- α to activate JNK permanently and this prolonged

JNK activation leads the cell to apoptosis (Tang G et al., 2001 ; De Smaele E et al., 2001). TNF- α initiates the transient JNK activation; but, blocking NF- κ B is required to upgrade JNK activation (Bubici C et al., 2004). Whether JNK acts pro-survival or pro-apoptotic depends on cell type, stimulus and mostly the duration time of its activation (Liu J and Lin A, 2005).

1.4.2 ROS mediates crosstalk between JNK and NF- κ B:

Prolonged JNK activation is TRAF-independent but depends on the ROS generation. ROS induces JNK activation and JNK induces ROS accumulation (Nakano H, 2004). A mutual antagonism was previously shown between NF- κ B signaling pathway and the aryl hydrocarbon receptor (AhR) (Tian Y et al., 1999; Ke S et al., 2001). Inhibition of NF- κ B, blocks this antagonism and consequently upregulates the p450 family members, which promote the ROS generation. The accumulated ROS prolongs the JNK activation which leads cell to apoptosis (Zhang Y and Chen F, 2004). ROS also inhibits MAPK phosphatases in order to promote TNF- α induced apoptosis associated with prolonged activation of JNK (Kamata H et al., 2005).

1.5 Targeted therapy of hepatocellular carcinoma:

Hepatocellular carcinoma (HCC), the fifth most common cancer worldwide, has one and only FDA approved drug, sorafenib. Less than 30-40% of the HCC patients are suitable for curative therapies while the rest receive only palliative ones. Systemic chemotherapies become the best option despite the high probability of collateral damage they may cause (Cao H et al., 2012). Sorafenib, the first small molecule multikinase inhibitor approved by the FDA, prolongs median survival and the time to progression by nearly 3 months in patients with advanced HCC (Galle PR, 2008). Consequently, it is essential to discover drug candidates for HCC. In the last years, sorafenib was also combined with agents, previously used for advanced HCC, such as PR-104, a pre-prodrug of the bioreductive prodrug PR-104A, (Abou-Alfa GK et al., 2011), interferon-alpha (IFN- α), (Wang L et al., 2012), panobinostat, pan-HDAC inhibitor, (Lachenmayer

A et al., 2012), transarterial chemoembolization (TACE) with doxorubicin-eluting beads (DEB), (Pawlik TM et al., 2011), cisplatin/doxorubicin (PLADO) (Schmid I et al., 2012).

Since the 1950s, conventional chemotherapy has been considered controversial due to the harm they cause in order to heal. Cytotoxic chemotherapy drugs are deadly not only for cancer cells but also for normal, healthy cells surrounding the tumor. Therefore, severe side effects accompany the benefits of standard chemotherapy drugs. Among the known cancer types, hepatocellular carcinoma is the third most common cause of cancer death due to its late diagnosis (Tanaka S and Arii S, 2010). Moreover, the tolerability of HCC patients to chemotherapy is significantly low because of their chronic liver diseases (Chan SL and Yeo W, 2012). Chemotherapy drugs can be classified as :

Antimetabolites : Drugs interfering with the usual metabolic processes of a cell. This group of drugs eventually interrupts DNA replication (Kaye SB, 1998). Nucleoside analogs, which are effective for the treatment of solid tumors and hematological malignancies, comprise the major sub-group of the antimetabolites (Karran P, 2006).

Alkylating drugs : Drugs adding an alkyl group to some or all of the proteins which bond together to form the double helix structure of a DNA molecule. This leads to breakage of the DNA strands which precedes the cancer cell death (Ben-Efraim S, 2001) .

Antitumor antibiotics : Drugs which bind and uncoil DNA in order to stop RNA synthesis; thus prevent the synthesis of essential proteins for survival (De Beer EL, 2001).

Plant alkaloids : These plant-derived drugs act either throughout the cell cycle or are effective during the S and M phases of cell cycle, in order to prevent cell division (Kim J and Park EJ, 2002).

Steroid hormones : Drugs which change the three-dimensional shape of a cell receptor and consequently prevent the ligand-binding to that receptor eventually inactivates the receptor (Howell Sj et al., 2004).

On the contrary, targeted cancer therapies include drugs which interfere with the key members of aberrant molecular pathways involved in tumorigenesis. The distinctive features of healthy and cancer cells allow for the development of targeted therapies. The hallmarks of cancer, suggested as six acquired capabilities of cancer cells, are self-sufficiency in growth signaling, insensitivity to anti-growth signaling, evading apoptosis, activating tissue invasion and metastasis, gaining replicative immortality and sustaining angiogenesis (Hanahan D and Weinberg RA, 2000). The rapidly increasing data creates new hallmarks which drug discovery can exploit (Figure 1.13).

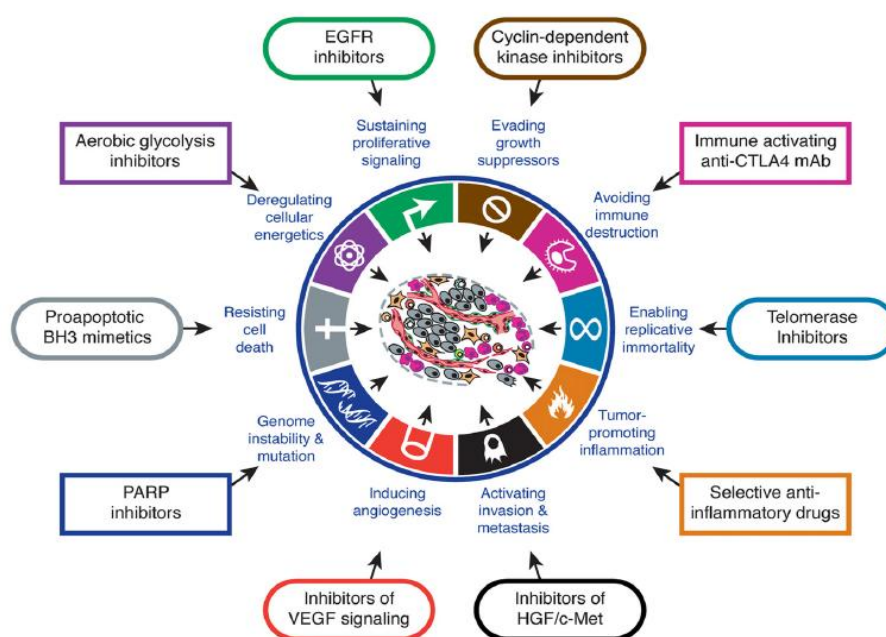


Figure 1.13 Therapeutic targets for drug candidates

(Hanahan D and Weinberg RA, 2011)

Identifying the target is the first step of developing innovative cancer therapies. Next step is discovering agents that can exploit these targets. Monoclonal antibodies and small molecule kinase inhibitors constitute the

majority of drug candidates currently in preclinical and clinical trials (Ma WW and Adjei AA, 2009; Huynh H, 2010). Sorafenib is the one and the only drug with an FDA approval for the treatment of advanced HCC. Sorafenib is a tyrosine kinase inhibitor which involves not only Raf/MEK/ERK pathway but also VEGF/VEGFR (vascular endothelial growth factor receptor) pathway (Cao H et al., 2012). Monotherapy of Sorafenib prolongs median survival almost three months in advanced HCC patients(Llovet JM et al., 2008). Therefore, combined therapies of Sorafenib with various conventional chemotherapy drugs and novel targeted agents have been initiated to enhance its therapeutic effect (Cao H et al., 2012). As a result, identification of putative kinase inhibitors acting on liver cancer cells is a promising research field needs to be explored (Figure 1.14 and Figure 1.15).

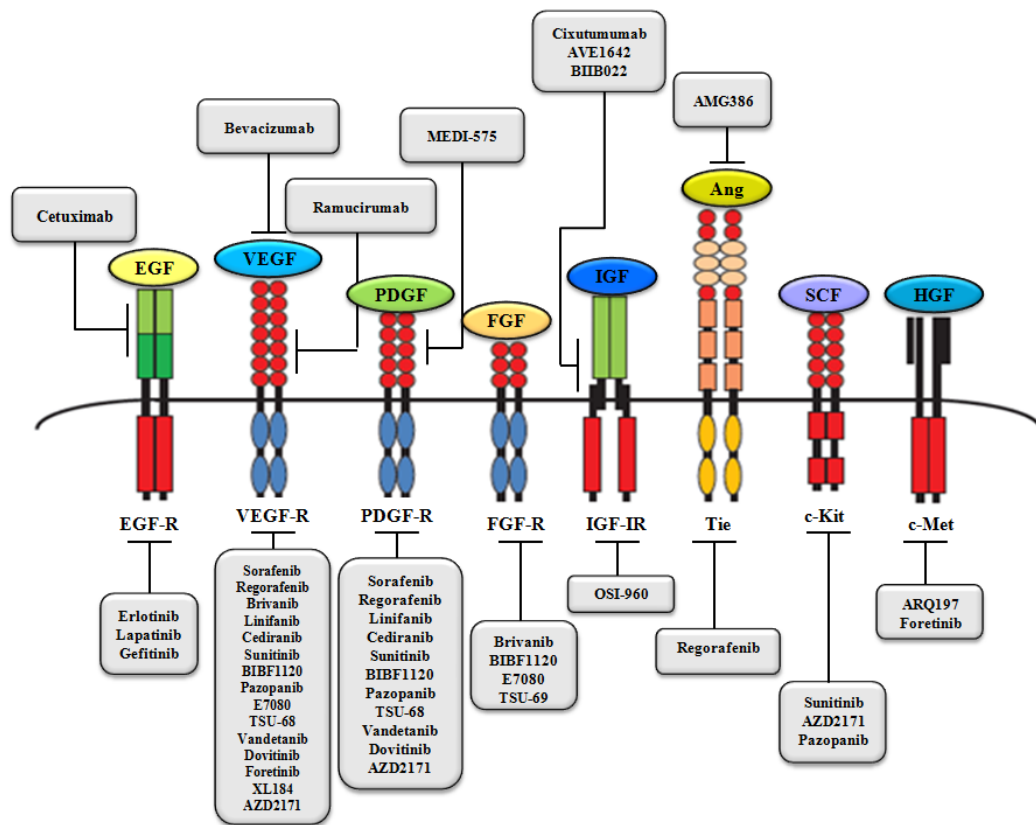


Figure 1.14 The molecular targeted agents currently tested in preclinical and clinical HCC trials (Cervello Met al., 2012)

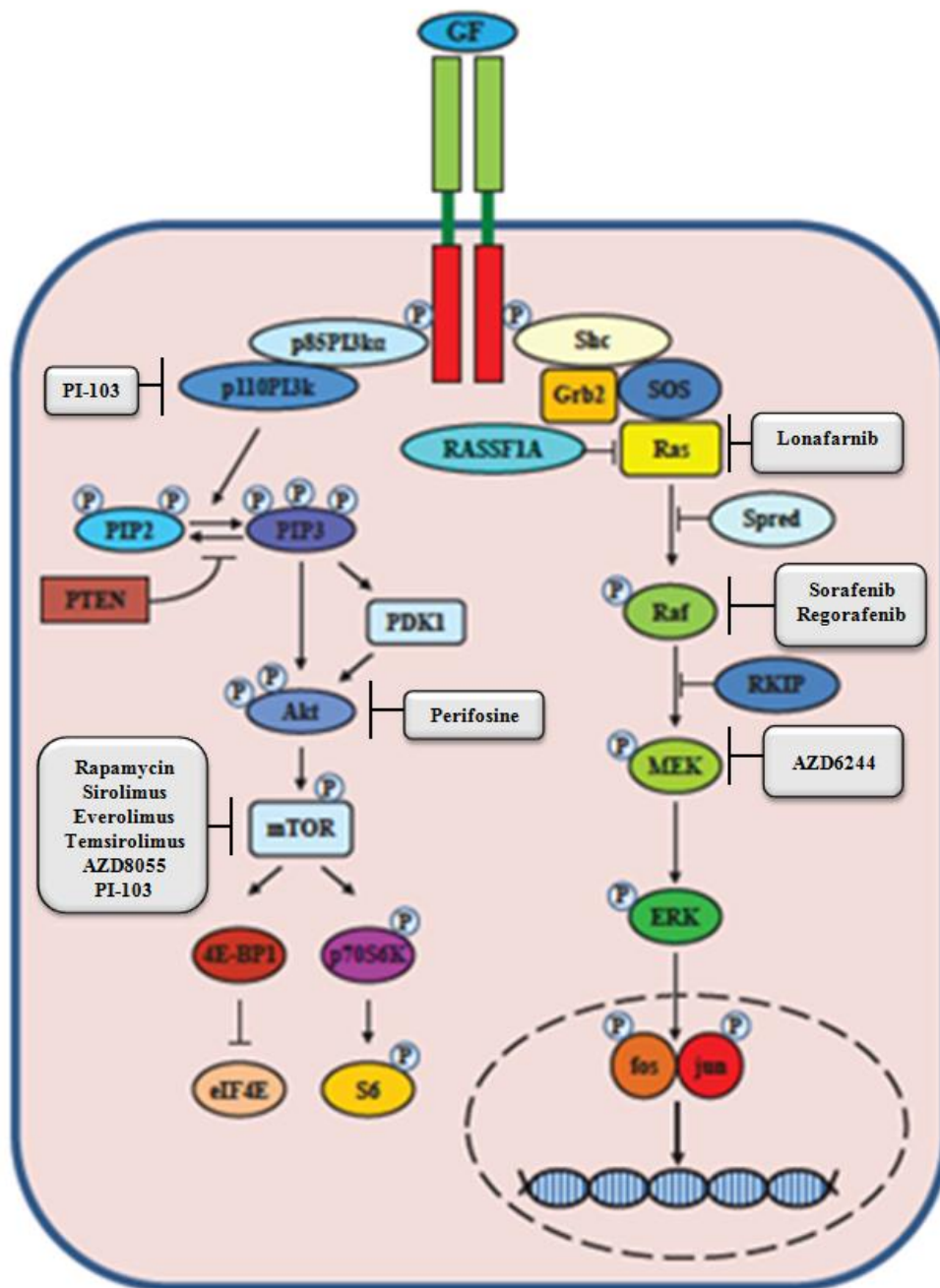


Figure 1.15 PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways and the molecular targeted agents involved (Cervello M et al., 2012)

CHAPTER 2. MATERIALS AND METHODS

2.1 REAGENTS

2.1.1 Cell lines:

Liver cancer cell lines	Colon cancer cell lines	Breast cancer cell lines
Huh7	HCT116	T47D
HepG2		MCF7
Hep3B		
PLC		
SK-Hep1		
Mahlavu		
FOCUS		
Snu182		
Snu387		
Snu475		

2.1.2 Cell culture reagents:

Dulbecco's Modified Eagle Medium (DMEM)	31885	GIBCO
RPMI 1640	31870	GIBCO
fetal bovine serum (FBS)	102700	GIBCO
non-essential amino acid (NEAA)	BE13-114E	Lonza
penicillin/streptomycin solution (P/S)	15140	GIBCO
0.25% Trypsin-EDTA 1x	25200	GIBCO
L-Glutamine 200mM 100x	25030	GIBCO
1xPhosphate Buffer Saline (1xPBS) (CaCl ₂ , MgCl ₂ free)	14190	GIBCO
Dimethyl sulfoxide (DMSO)	A3672	AppliChem Biochemica

2.1.3 Drug screening reagents:

Dimethyl sulfoxide (DMSO)	A3672	AppliChem Biochemica
(S)-(+)-Camptothecin	C9911	Sigma
5-Fluorouracil	8699828770237	Kocak Farma
<u>1xPBS</u> NaCl KCl Na ₂ HPO ₄ ·2H ₂ O KH ₂ PO ₄	1.06404.1000 471177 480227 1.04873	MERCK Carlo Erba Carlo Erba MERCK
Trichloroacetic acid (TCA)	1.00810.1000	MERCK
Sulforhodamine B (SRB) sodium salt	S1402	Sigma
Acetic Acid	27225	Riedel-de Haen
Tris	0826	Amresco

2.1.4 Nuclear staining reagents:

Hoechst 33258, pentahydrate (bis-benzimide)	861405	Sigma
<u>1xPBS</u>		
NaCl	1.06404.1000	MERCK
KCl	471177	Carlo Erba
Na ₂ HPO ₄ ·2H ₂ O	480227	Carlo Erba
KH ₂ PO ₄	1.04873	MERCK
Formaldehyde	310356	Carlo Erba
Glycerol	346165	Carlo Erba

2.1.5 Cell lysis reagents:

<u>1xPBS</u>		
NaCl	1.06404.1000	MERCK
KCl	471177	Carlo Erba
Na ₂ HPO ₄ ·2H ₂ O	480227	Carlo Erba
KH ₂ PO ₄	1.04873	MERCK
<u>NP-40 lysis buffer</u>		
NaCl	1.06404.1000	MERCK
Tris	0826	Amresco
NonidetP-40		Applichem
SDS	L5750	Sigma
Protease Inhibitor Cocktail	11 873 580 001	Roche
PhosSTOP	04 906 837 001	Roche
<u>Phospho lysis buffer</u>		
HEPES	H1016	Sigma
Tetrasodium pyrophosphate	P8010	Sigma
EDTA	1.08421	Merck
NaF	S7920	Sigma
Triton X-100	T8787	Sigma
Na ₃ VO ₄	S6808	Sigma
PMSF	0754	Amresco
Protease Inhibitor Cocktail	11 873 580 001	Roche
PhosSTOP	04 906 837 001	Roche
Albumin, from bovine serum (BSA)	A7906	Sigma-Aldrich
Bradford Reagent	B6916	Sigma

2.1.6 Kinase assay reagents:

<u>Kinase reaction buffer</u>		
Tris	0826	Amresco
MgCl ₂ ·6H ₂ O	A406233	MERCK
Albumin, from bovine serum (BSA)	A7906	Sigma-Aldrich
PKLight [®] Assay Kit	LT07-500	Lonza
Kinase-Glo Luminescent Kinase Assay	V6712	Promega
MeSAdo	260585	Calbiochem

Staurosporine	569397	Calbiochem
Aminopurvalanol-A	164640	Calbiochem

2.1.7 Western-blot reagents:

DTT	D9779	Sigma
Nu-PAGE LDS Sample Buffer 4x	NP0007	Invitrogen
Nu-PAGE Bis-Tris 4-12% gels	NP0335	Invitrogen
Nu-PAGE Bis-Tris 10% gels	NP0303	Invitrogen
Nu-PAGE Bis-Tris 12% gels	NP0343	Invitrogen
Nu-PAGE MOPS SDS running buffer 20x	NP00001	Invitrogen
Nu-PAGE MES SDS running buffer 20x	NP00002	Invitrogen
Nu-PAGE Transfer buffer 20x	NP0006-1	Invitrogen
PageRuler™ Prestained Protein Ladder	SM0671	Fermentas
PageRuler™ Prestained Protein Ladder	26616	Thermo Scientific
PageRuler™ Plus Prestained Protein Ladder	SM1811	Fermentas
Nu-PAGE Antioxidant	NP0005	Invitrogen
Methanol	24229	Sigma-Aldrich
Hybond ECL Nitrocellulose Membrane (0.2mm pore size)	RPN3032D	Amersham, GE Healthcare
Whatman Paper	3030917	GE Healthcare
Non-fat dry milk		SUTAS
Albumin, from bovine serum	A7906	Sigma-Aldrich
Ponceau-S	P-3504	Sigma
Acetic acid	27225	Riedel-de Haen
1xTBS-T (0.1%) NaCl Tris Tween 20	1.06404.1000 0826 822184	MERCK Amresco MERCK
ECL+	RPN2132	Amersham, GE Healthcare

2.1.8 Antibodies:

Antibody	Company & Catalog Number	Western Blot (immunoblotting details)
Bcl-2	Santa Cruz sc-7382	1:200 (2 hours; RT)
α-Cytochrome-c	Santa Cruz sc-7159	1:200 (2 hours; RT)
p15 (C-20)	Santa Cruz sc-612	1:200 (overnight; +4°C)
Purified mouse Anti-Human Retinoblastoma Protein	BD Pharmingen 554136	1:300 (2 hours; RT)
Phospho-Rb (Ser807/811)	Cell Signaling #9308	1:200 ((2 hours; RT)

α -p21	BD-556430	1:200 ((2 hours; RT)
α -phospho H2AX	Millipore 05-636	1:200 ((2 hours; RT)
PARP-1 (F-2)	Santa Cruz sc8007	1:200 (2 hours; RT)
JNK	Cell Signaling #9252	1:300 (2 hours; RT)
Phospho-SAPK/JNK (Thr183/Tyr185)	Cell Signaling #9251	1:100 (2 hours; RT)
α -c-Jun (N)	Santa Cruz sc-45	1:300 (2 hours; RT)
p-c-Jun (KM-1)	Santa Cruz sc-822	1:100 (2 hours; RT)
MEK4 antibody [8A8]	Abcam ab90092	1:300 (2 hours; RT)
MEK4 (Phospho S257+T261)	Abcam ab4760	1:100 (overnight; +4°C)
MEK7 antibody [EP1455Y]	Abcam ab52618	1:300 (2 hours; RT)
Phospho-MKK7 (Ser271/Thr275)	Cell Signaling #4171	1:100 (overnight; +4°C)
ASK1 (Phospho S83)	Abcam ab47304	1:100 (overnight; +4°C)
Akt	Cell Signaling #9272	1:300 (2 hours; RT)
Phospho-Akt (Ser475)	Cell Signaling #9271	1:100 (2 hours; RT)
JKK (H-60)	Santa Cruz sc-134588	1:100 (overnight; +4°C)
Calnexin	Sigma C4731	1:5000 (2 hours; RT)
Actin I-19	Santa Cruz sc1616	1:5000 (2 hours; RT)
anti-goat HRP	Sigma A8919	1:5000 (2 hours; RT)
anti-mouse HRP	Sigma A0168	1:5000 (2 hours; RT)
anti-rabbit HRP	Sigma A6154	1:5000 (2 hours; RT)

2.1.9 Senescence Associated- β -gal (SA- β -gal) assay and BrdU proliferation

co-staining reagents:

BrdU (5-bromo-2-deoxyuridine)	M0744	DAKO
Formaldehyde	310356	Sigma
<u>SA-β-gal assay buffer</u>		
Citric acid (anhydrous)	C0759	Sigma
Na ₂ HPO ₄ ·2H ₂ O	480227	Carlo Erba
NaH ₂ PO ₄	S-0751	Sigma
MgCl ₂ ·6H ₂ O	A406233	MERCK
NaCl	K36100804	MERCK
K ₄ Fe(CN) ₆ ·3H ₂ O	P-9387	Sigma
K ₃ Fe(CN) ₆	P-8131	Sigma
X-gal	X-gal	X-gal
<u>Ethanol</u>	32221	Sigma
<u>1xPBS</u>		
NaCl	1.06404.1000	MERCK
KCl	471177	Carlo Erba
Na ₂ HPO ₄ ·2H ₂ O	480227	Carlo Erba
KH ₂ PO ₄	1.04873	MERCK
HCl	403872	Carlo Erba
Albumin, from bovine serum (BSA)	A7906	Sigma-Aldrich

Tween 20	822184	MERCK
Envision™ + Dual Link System-HRP	K4063	Dako
Liquid DAB + Substrate Chromogen System	K3468	Dako
Glycerol	346165	Carlo Erba
Doxorubicine	7AA015	DABUR

2.1.10 Cell Cycle Analysis with Fluorescence-Activated Cell Sorting (FACS) – Propidium Iodide (PI) reagents:

1xPhosphate Buffer Saline (1xPBS) (CaCl ₂ , MgCl ₂ free)	14190	GIBCO
Ethanol	32221	Sigma
Propidium Iodide (PI)	D4864	Sigma
RNaseA	EN0531	Fermentas
Triton-X-100	T8787	Sigma

2.1.11 ER (Endoplasmic Reticulum) stress detection assay reagents:

(RNA extraction, cDNA synthesis, polymerase chain reaction (PCR) and agarose gel electrophoresis reagents)

NucleoSpin RNA II Kit	740 955.50	Macherey-Nagel
Fermentas RevertAid First Strand cDNA synthesis kit	K1622	Fermentas
<u>ER stress detection assay</u> Human XBP1 (X box-binding protein 1) F:TTACGAGAGAAAACATCATGGCC R:GGGTCCAAGTTGTCCAGAATGC GAPDH F:GGCTGAGAACGGGAAGCTTGTCAT R:CAGCCTTCTCCATGGTGGTGAAGA PCR reagents	EP0405	Iontek Iontek Fermentas
<u>Agarose gel electrophoresis</u> Agarose (BASICA LE) Tris Acetic acid EDTA Ethidium Bromide 6x gel loading dye solution gene ruler (100bp DNA ladder)	0826 27225 1.08421 E7637 R0611 SM0241	Prona Amresco .Riedel-de Haen Merck Sigma Fermentas Fermentas

2.1.12 Reactive Oxygen Species (ROS) detection assay reagents:

1xPhosphate Buffer Saline (1xPBS) (CaCl ₂ , MgCl ₂ free)	14190	GIBCO
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ROS-assay		
HEPES	H-1016	Sigma
KOH	P1767	Sigma
D-Glucose	0188	Amresco
2',7'-Dichlorofluoresceindiacetate (DCFH-DA)	D6883	Sigma

2.1.13 in vivo drug treatment assay reagents:

Nude mice		Kobay AS
Simple syrup		
sugar		
ddH ₂ O		

2.2 MATERIALS

Cell-culture plates (100mm) (150mm)	(633171) (639160)	Greiner
6-well plates	657160	Greiner
96-well plate	3599	Corning
E-96 plate	05 232 368 001	Roche
White 96-well polypropylene assay plate	07-200-761	Corning
Serological pipettes (5mL; 10mL; 25mL)	606180 607180 760180	Greiner
Pipetting aid		Gilson
Pipettes		Gilson
Filter Tips		
Falcon tubes (15mL); (50mL)	188271; 227261	Greiner
Cryotubes	430289	Corning
hemocytometer		
Microscope slides (76x26mm)		ISOLAB
Coverslips (22x22mm)		
Cell lifter	3008	Corning
Waterbath (thermostat)		yellowline basic
Vacuum pump		
CO ₂ air-jacketed incubator		Nuaire
Biological Safety Cabinet		Nuaire
Centrifuge		Hettich Universal 320
xCELLigence		Roche
Inverted phase microscope		Olympus CKX41
Fluorescence microscope		Nikon ECLIPSE 50i
Precision balance		Denver Instrument

Microplate reader		Biotek
Multi-purpose rotator		Bafristead / Lab-Line
Spectrophotometer		Biotek
the Reporter Microplate Luminometer		Turner Designs
Medical X-ray Film, 18x24 cm	8143059	Kodak
XCell SureLock™ Mini-Cell & XCell II™ Blot Module	EI0002	Invitrogen
Sponge pads	EI9052	Invitrogen
Power supply		Bio-Rad, Power pac200
Hyperprocessor		Amersham Life Science
BD FACSCalibur		
Inverted phase microscope		Olympus CKX41
Class II Safety Cabinet		Metisafe
HEPA-filter top covered cage		
Vernier caliper		
U-100 insulin syringe	1014	Ayset
Venipuncture Set, 23G	4056353	Braun

2.3 SOLUTIONS

2.3.1 Cell culture solutions:

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

2.3.2 Drug screening solutions:

10 x PBS	1.5M NaCl, 30mM KCl, 80mM Na ₂ HPO ₄ , 20mM KH ₂ PO ₄ (pH 7.4)
10% TCA	(v/v) 10% TCA in ddH ₂ O
SRB dye	(w/v) 0.4 % SRB in 1% acetic acid solution
1% acetic acid	(v/v) 1% acetic acid in ddH ₂ O
10mM TBS	10mM Tris in ddH ₂ O

2.3.3 Nuclear staining solutions:

Hoechst 33258 stock solution	300µg/ml Hoechst dissolved in ddH ₂ O
Hoechst 33258 working solution	1µg/ml Hoechst diluted from 300µg/ml stock in 1x PBS
3% formaldehyde	(v/v) 3% formaldehyde in ddH ₂ O

2.3.4 Cell lysis solutions:

NP-40 lysis buffer	150mM NaCl, 50mM Tris-HCl (pH 7.6), 1% NP-40, 0.1% SDS, 1xPI, 1xPhosSTOP in ddH ₂ O.
Phospho lysis buffer	50mM HEPES, 100mM NaCl, 4mM NaPP, 10mM EDTA, 10mM NaF, (v/v) 1% Triton-X, 2mM Na ₃ VO ₄ , 1mM PMSF, 1xPI, 1xPhosSTOP in ddH ₂ O.
BSA (1mg/mL)	1mg BSA in 1mL ddH ₂ O.

2.3.5 Kinase assay solutions:

Kinase reaction buffer	40mM Tris-HCl (pH 7.6), 20mM MgCl ₂ , 0.1mg/mL BSA in ddH ₂ O
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2.3.6 Western blot solutions:

DTT	0.5M DTT in ddH ₂ O
10 x TBS (pH 7.6)	0.1M Tris, 1.5M NaCl in ddH ₂ O
1 x TBS-T (0.1%)	(v/v) 0.1% Tween-20 dissolved in 1x PBS
Ponceau-S solution	(w/v) 0.1% Ponceau-S, (v/v) 5% acetic acid in ddH ₂ O
Blocking Solution	(w/v) 5% non-fat dry milk / BSA in 1 x TBS-T (0.1%)

2.3.7 Senescence Associated-β-gal (SA-β-gal) assay and BrdU proliferation co-staining solutions:

SA-β-gal assay buffer (pH 6.0)	200mM citric acid, 1M Na-P buffer, 100mM K-Ferro-CN, 100mM K-Ferri-CN, 2M NaCl, 100mM MgCl ₂ , 40mg/mL X-gal in ddH ₂ O
Na-P buffer (pH 6.0)	(1M NaH ₂ PO ₄) and (1M Na ₂ HPO ₄) mixture in ddH ₂ O
HCl	2N HCl in ddH ₂ O

2.3.8 ER (Endoplasmic Reticulum) stress detection assay solutions:

(RNA extraction, cDNA synthesis, polymerase chain reaction (PCR) and agarose gel electrophoresis reagents)

50X Tris-acetic acid-EDTA (TAE)	2 M Tris-acetate, 50 mM EDTA in ddH ₂ O (pH 8.5) 1X for working solution.
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2.3.9 Reactive Oxygen Species (ROS) detection assay solutions:

ROS assay solution	10mM HEPES buffer, 10mM glucose, 1μM DCFH-DA in 1 x PBS
HEPES buffer (pH 7.5)	1M HEPES in ddH ₂ O (adjust the pH with KOH solution)

2.3.10 Cell Cycle Analysis with Fluorescence-Activated Cell Sorting (FACS) – PI assay solutions:

Ethanol	(v/v) 70% ethanol in ddH ₂ O
PI staining solution	50 μg/mL propidium iodide, 0.1mg/mL RNaseA and 0.05% Triton-X-100 in 1 x PBS.

2.3.11 in vivo drug treatment assay solutions:

Simple syrup	16g sugar was dissolved and boiled in 9g ddH ₂ O
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2.4 METHODS

2.4.1 Cell culture protocols:

2.4.1.1 Maintenance of cell lines:

Human liver cancer cell lines Snu182, Snu387 and Snu475 were maintained in RPMI-1640 growth medium while the other cell lines (Huh7, HepG2, Hep3B, PLC, SK-Hep1, Mahlavu, Focus, HCT116, T47D and MCF7) were cultivated in DMEM growth medium. Each cell line has its own splitting agenda according to

its proliferation rate. All the cell lines mentioned above are adherent cells growing attached to the surface of cell culture dishes. To subculture these cells, trypsin (protein degrading enzyme) was used to bring them into cell suspension through breaking the protein bridges between the cancer cell and the surface of the dish. Prior to trypsinization, the cells were washed twice with 1xPBS to get rid of the swimming (i.e. dead) cells and the cell growth medium, containing FBS. Since FBS inactivates trypsin, growth medium was re-added just after the cells were detached. Depending on the splitting ratio of the cell line, the required amount of cell suspension was inoculated to a new cell culture dish with fresh cell growth medium. The cell culture dishes were kept at 37 °C in humidified 5% CO₂, 95% air containing incubators.

2.4.1.2 Freezing of cell lines:

First, cells should be grown to log phase in their regular growth medium. After that the cell suspension obtained via trypsinization was centrifuged at 1200rpm for 5 minutes and the supernatant was discarded. Next, the cell pellet was resuspended in cell line-specific freezing medium. Finally, this suspension was allocated into screw-capped cryotubes (1mL per each tube). The cryotubes were kept at -20°C and -80°C for 1 hour and 24 hours respectively before immersing them into liquid nitrogen tank.

2.4.1.3 Thawing of cell lines:

One vial of frozen cell line was taken out of the liquid nitrogen tank and placed in 37°C water until only a small frozen bit remains. Sufficiently wiped (with 70% ethanol) cryotube was taken rapidly to cell culture hood and transferred (dropwise) into 5 mL fresh, cell line-specific growth medium. In order to discard the DMSO used as cryoprotectant, the cell suspension was centrifuged at 1200rpm for 5 minutes and the supernatant was aspirated off. The cell pellet was resuspended in 10ml fresh growth medium, transferred into 100mm culture dish and kept at 37 °C in a humidified 5% CO₂, 95% air containing incubator.

2.4.2 Drug screening protocols:

2.4.2.1 NCI-60 Sulphorhodamine B (SRB) assay:

Cancer cell lines of interest were inoculated (2000cell/well to 10,000cell/well in 200 μ L/well) to 96-well plates and left for proliferation at 37°C in a humidified 5% CO₂ and 95% air incubator. Next day, the media of the wells were refreshed and the molecules to be tested which were already dissolved in DMSO were applied directly to the first wells in calculated volumes to obtain the decided highest drug concentration. Cancer cells were also treated with DMSO-only simultaneously to avoid its solitary effect. Following the drug treatment, plates were incubated again at 37°C in a humidified 5% CO₂ and 95% air incubator for different time periods. At the 24th, 48th and 72th hour of drug treatment, cancer cells were fixed with ice-cold TCA (50 μ L/well) and kept at +4°C in dark for exactly 1 hour. TCA fixation was terminated by washing the wells with ddH₂O for 5 times. Air-dried plates were stained with SRB dye solution for 10 minutes in dark and at room temperature. The plates which were quickly rinsed with 1% acetic acid solution to get rid of the unbound SRB dye were left to air dry. The protein-bound and dried SRB dye is then solubilized with 10mM Tris-Base solution (200 μ L/well) and homogenized on a shaker for 5-10 minutes. The absorbance values were obtained at 515nm by means of a microplate reader.

2.4.2.2 Real-Time Cell Electronic Sensing (RT-CES) method for cytotoxicity profiling (xCELLigence):

The method utilizes the microelectronic plates (E-plates, 96-well) covered with gold microelectrodes at the bottom of wells. An electric field forms between these electrodes after a low voltage application. Addition of adherent cancer cells causes changes in electrical impedance (Z) directly proportional to their numbers. These changes are displayed as Cell Index (CI) values reflecting us the adhesion, morphology and quantitative status of the cells in the electrode-surrounded well.

Prior to cell inoculation, 50µL medium was distributed to each well of the E-96 plate to obtain background readings for the elimination of any possible background noise. Cell suspensions of interest were added (150µL/well) and incubated at room temperature for 30 minutes under a laminar flow hood to let the cells settle before placing the E-96 plate on the RT-CES station in the humidified 5% CO₂ and 95% air incubator at 37°C. The CI values were recorded at every 30 minutes for the first 24 hours. Next day, 150µL medium from each well was replaced with 100µL fresh medium and the molecules to be tested and DMSO-only as control were administered to wells in decided concentrations. The drug-treated E-96 plate was again placed on the RT-CES station in the humidified 5% CO₂ and 95% air incubator at 37°C. The CI values were recorded at every 10 minutes for the first 4 hours and then at every 30 minutes to monitor the fast drug response and long-term drug response respectively.

2.4.3 Nuclear staining with Hoechst 33258 protocol:

Cancer cell lines of interest were inoculated onto coverslips placed in 6-well plates in 1.5 mL growth medium. The day after, cells were treated with the decided concentrations of molecules to be tested and their controls and incubated at 37°C in 5% CO₂. At the end of determined incubation period, the wells were washed with ice-cold 1xPBS and fixed with 3% formaldehyde before stained 5 minutes with Hoechst 33258 working solution at room temperature. The wells were destained with ddH₂O for 10 minutes. The nuclear morphology was examined under a fluorescent microscope after mounting the coverslips onto slides with glycerol.

2.4.4 Cell lysis protocol and Bradford assay:

Cancer cell lines of interest were inoculated in 100mm culture dishes with 10mL growth medium. The day after, cells were washed with 1xPBS and incubated at 37°C in 5% CO₂ with fresh growth medium containing the decided concentrations of molecules to be tested and their controls. At the end of

determined incubation period, the medium of cells were collected into falcon tubes and the remained cells (attached to the surface) were scraped gently (on ice) with ice-cold 1xPBS and combined with the previously collected medium. The cell suspension was centrifuged at 1500rpm for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 5mL, ice-cold 1xPBS, again centrifuged at 1500rpm for 5 minutes and the supernatant was discarded. *(The cell pellets can be stored at -80°C after immersing them into liquid nitrogen until cell lysis.)*

Either NP-40 lysis buffer or phospho-lysis buffer (approximately three times the cell pellet volume) was added on top of cell pellet. The mixture was and incubated on ice for 30 minutes with vortexing every 5 minutes. Next, the mixture was centrifuged at 13,000rpm for 30 minutes at +4°C. The supernatant (cell lysate) was transferred into a new tube and the pellet (cell debris) was discarded.

	Blank	Tube #1	Tube #2	Tube #3	Tube #4	Tube #5	Tube #6
BSA (1mg/mL)	-	1μL	2μL	4μL	8μL	16μL	32μL
ddH ₂ O	100μL	99μL	98μL	96μL	92μL	84μL	68μL
Bradford Reagent	900μL	900μL	900μL	900μL	900μL	900μL	900μL

To estimate the total protein concentration of cell lysate Bradford assay was used. Initially, sequential doubling concentrations of BSA were used (as given above) and a standard curve was plotted with the absorbance values obtained at 595nm with each BSA concentration. Next, the unknown protein concentration of the diluted cell lysate was calculated according to the standard curve equation, after obtaining its absorbance at 595nm.

2.4.5 Kinase assay:

The calculated amount of cell lysate (according to Bradford assay results) was mixed with kinase reaction buffer to give a final volume of 40 μ L in a white, 96-well, polypropylene assay plate. Then 20 μ L Lonza reagent or 40 μ L Kinase-GLO reagent was added. After 10 minutes dark incubation at room temperature, the luminescence was detected through a luminometer. This assay measures the consumed ATP by measuring the bioluminescence caused by the remained ATP in the well after kinase activity. The enzyme luciferase generates light from luciferin and ATP. The intensity of the stable light signal emitted is linearly proportional to the concentration of ATP. Hence, high levels of bioluminescence depicts the inhibition of protein kinase activity.

2.4.6 Western blotting:

Novex[®] NuPAGE[®] Bis-Tris Electrophoresis system was used according to the manufacturer's protocol for all the western blotting analysis. The pre-cast gel percentage and the running buffer type were chosen according to the size (kDa) of the protein under investigation. In order to load the samples to each well equally their quantified protein contents were taken into consideration. The final volume of the samples were decided in accordance with the capacity of the wells. The samples were prepared by adding 4x loading dye, 10% 0.5M DTT were added to cell lysates and ddH₂O was used to complete the total volume and then, denatured at 70°C for 10 minutes. Following electrophoresis, the proteins were transferred to nitrocellulose membrane via XCell II[™] Blot Module at 30V for 90-150 minutes depending on the size (kDa) of the protein under investigation. After checking the transfer efficiency with Ponceau staining, the membrane was blocked with either 5% milk or 5% BSA (both in 1xTBS-T(0.1%)) for 1 hour at room temperature or overnight at +4°C (both on a shaker) prior to immunoblotting steps. Next, the membrane was incubated with the 1^o antibody for 2 hours at room temperature (on a bench) or overnight at +4°C (on a shaker). The unbound antibody was washed off from the membrane with 1xTBS-T (0.1%) for 5 times (5min, 10min, 10min,

10min, and 5min). Following the incubation with the 2^o antibody for 1 hour at room temperature (on a bench) another 40 minutes (5min, 10min, 10min, 10min, and 5min) were spent to wash the membrane with 1xTBS-T (0.1%). Eventually, the expression of the protein levels were visualized via ECL+ kit that is used according to the manufacturer's recommendations. X-ray film was used to capture chemiluminescence with different exposure times. All the 1^o antibody-specific immunoblotting details were given at section 3.1.8 (pages 39 and 40).

2.4.7 Senescence Associated- β -gal (SA- β -gal) assay and BrdU proliferation co-staining:

Huh7 cells were inoculated onto coverslips in 2 identical 6-well plates and kept at 37°C in a humidified 5% CO₂ and 95% air incubator. Next day, the wells were washed twice with 1xPBS and the small molecules of interest and their corresponding DMSO controls were applied simultaneously to both 6-well plates. Following the drug treatment, plates were incubated again at 37°C in a humidified 5% CO₂ and 95% air incubator. At the 48th hour of incubation, mediums (both the small molecules of interest and their corresponding DMSO control containing) were refreshed. For one of the 6-well plates, the freshly prepared mediums were also containing 30 μ M BrdU (5-bromo-2-deoxyuridine). Both 6-well plates were again placed in a humidified 5% CO₂ and 95% air incubator at 37°C. After another 24 hours of incubation, the 6-well plate with the BrdU containing mediums was fixed with 3% formaldehyde initially and left at 37°C with 2 mL SA- β -gal assay buffer per well. At the end of 12 hour incubation, the SA- β -gal assay buffer was aspirated off and the wells were washed with 1xPBS. After that, the cells were fixed with ice-cold 70% ethanol and washed gently with 1xPBS for 20 minutes on a shaker. Next, the 6-well plate was incubated with 2mL, 2N HCl per well for 20 minutes at room temperature. Washing the wells with 1xPBS three times on a shaker for 15 minutes is followed by blocking the wells with 3% BSA in 1xPBS-T on a shaker for an hour. Blocked coverslips were incubated with anti-BrdU antibody diluted in 1xPBS (1:1000) for 1 hour at room temperature. Dako Envision+Dual Link System-HRP and Dako Liquid DAB+Substrate Chromogen

System were used according to the manufacturer's directions for the rest of the steps. Photos taken from the coverslips mounted on to the slides with glycerol. For the other 6-well plate, at the 120th hour of incubation (both the small molecules of interest and their corresponding DMSO control containing) the BrdU proliferation and SA- β -gal assay co-staining performed exactly as done to the former 6-well plate.

2.4.8 Cell Cycle Analysis with Fluorescence-Activated Cell Sorting (FACS) – PI assay:

Cancer cell lines of interest were inoculated in 100mm culture dishes with 10mL growth medium. The day after, cells were washed with 1xPBS and their mediums were refreshed with 1%FBS containing starvation mediums. After another 24 hours at 37°C in a humidified 5% CO₂ and 95% air incubator, the small molecule of interest and its DMSO control were administered to the cancer cells in 6mL growth medium per dish. At the end of determined incubation time, the swimming cells were collected into falcon tubes and the attached cells were washed with 1xPBS once, trypsinized and harvested with the previously collected (swimming cells containing) medium. The cell suspension was centrifuged at 1500rpm for 5 minutes. After discarding the supernatant, the cell pellet was resuspended in 5mL 1xPBS and re-centrifuged at 1500rpm for 5 minutes. Next, the cell pellet was resuspended in 1mL, ice-cold 1xPBS and fixed by adding 2.5mL, 70% ice-cold ethanol dropwise on vortex. (*The fixed cells can be stored at +4°C for one week.*)

Prior to FACS-Propidium Iodide (PI) analysis, you should re-centrifuge the ethanol-fixed cell suspension and discard the supernatant. Next, the cell pellet was resuspended in 500 μ L PI solution and incubated for at 37°C for 40 minutes at dark. After that, 3mL 1xPBS added (PI solution incubated) cell suspension was centrifuged at 1500rpm for 5 minutes. The PI stained cell pellet was resuspended in 500 μ L – 1mL 1xPBS (according to the amount of cell pellet) and transferred into FACS analysis tubes. BD FACSCalibur and CellQuest Software was used for the further cell cycle analysis.

2.4.9 ER (Endoplasmic Reticulum) stress detection assay:

Total RNAs were isolated from cancer cells, treated as planned, via the Nucleospin RNA II kit according to the manufacturer's protocol. From these isolated total RNAs first strand cDNA synthesis was performed using RevertAid First Strand cDNA synthesis kit. All first strand cDNA samples (1 μ L) were used for PCR amplification of glyceraldehydes-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, transcript in order to normalize the PCR products.

The spliced isoform of XBP1 (X box-binding protein 1) is accepted as an indicator of ER stress. In order to detect the presence of this spliced variant, semi-quantitative reverse transcriptase PCR (RT-PCR) assay was performed using XBP1 specific primers. The PCR was run for 30 cycles (30 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C) and the products were separated on 3% agarose gel. Agarose was dissolved in 1xTAE electrophoresis buffer in microwave oven and ethidium bromide was added to have 30 μ g/mL final concentration prior to pour it into the gel mold. 100bp DNA ladder and the PCR products mixed with 6x loading dye were loaded onto the gel which was run in 1xTAE buffer at 70V for 120 minutes.

2.4.10 Reactive Oxygen Species (ROS) detection assay:

Cancer cell lines were inoculated onto coverslips in 6-well plates. On the next day, wells were 1xPBS washed and the small molecule of interest and its DMSO control was administered to the cells. After the determined time of incubation, wells were washed thoroughly with 1xPBS three times and incubated with 1mL ROS assay solution for 15 minutes at 37°C in a humidified 5% CO₂ and 95% air incubator. Aspirating off the DCFH-DA containing ROS assay solution terminates the incubation. After washing wells twice with 1xPBS the coverslips were mounted on slides with glycerol. This assay utilizes DCFH-DA, a cell permeable, non-fluorescent probe which oxidizes to a fluorescent molecule, DCF.

2.4.11 *in vivo* drug treatment assay:

Mahlavu liver cancer cells were grown in 150mm culture dishes to 70-75% confluency. Following thoroughly washing the cells with 1xPBS twice, minimum amount of trypsin was used to harvest the cells. Upon counting the cells through hemocytometer, Mahlavu suspensions were prepared as each contains 10,000,000 cells. In order to get rid of the trypsin used, Mahlavu suspensions were centrifuged at 1200rpm for 5 minutes and the supernatants were aspirated off. Mahlavu pellets were washed with 1xPBS twice and then each was suspended in 100 μ L DMEM growth medium prior to subcutaneous (SC) injection to the flank of nude mice. The nude mice were 8-16 weeks old and spent a week upon arrival as acclimatization period. After the SC injection of Mahlavu cells, the nude mice were monitorized for tumor growth. Twice a week tumor dimensions were measured with vernier caliper and recorded. When the tumors had reached approximately 150mm³ volume, the drug treatment had started. Drug was suspended in simple syrup (100mg/kg) and given orally by means of venipuncture set, 23G inserted U-100 insulin syringe (without the needle). Drug treatments were done twice a week for 3 weeks and for the subsequent 3 weeks nude mice were non-treated. At the end of 3 rounds of chemotherapy the nude mice (both drug and control groups) were taken to magnetic resonance imaging (MRI).

CHAPTER 3. AIM and STRATEGY

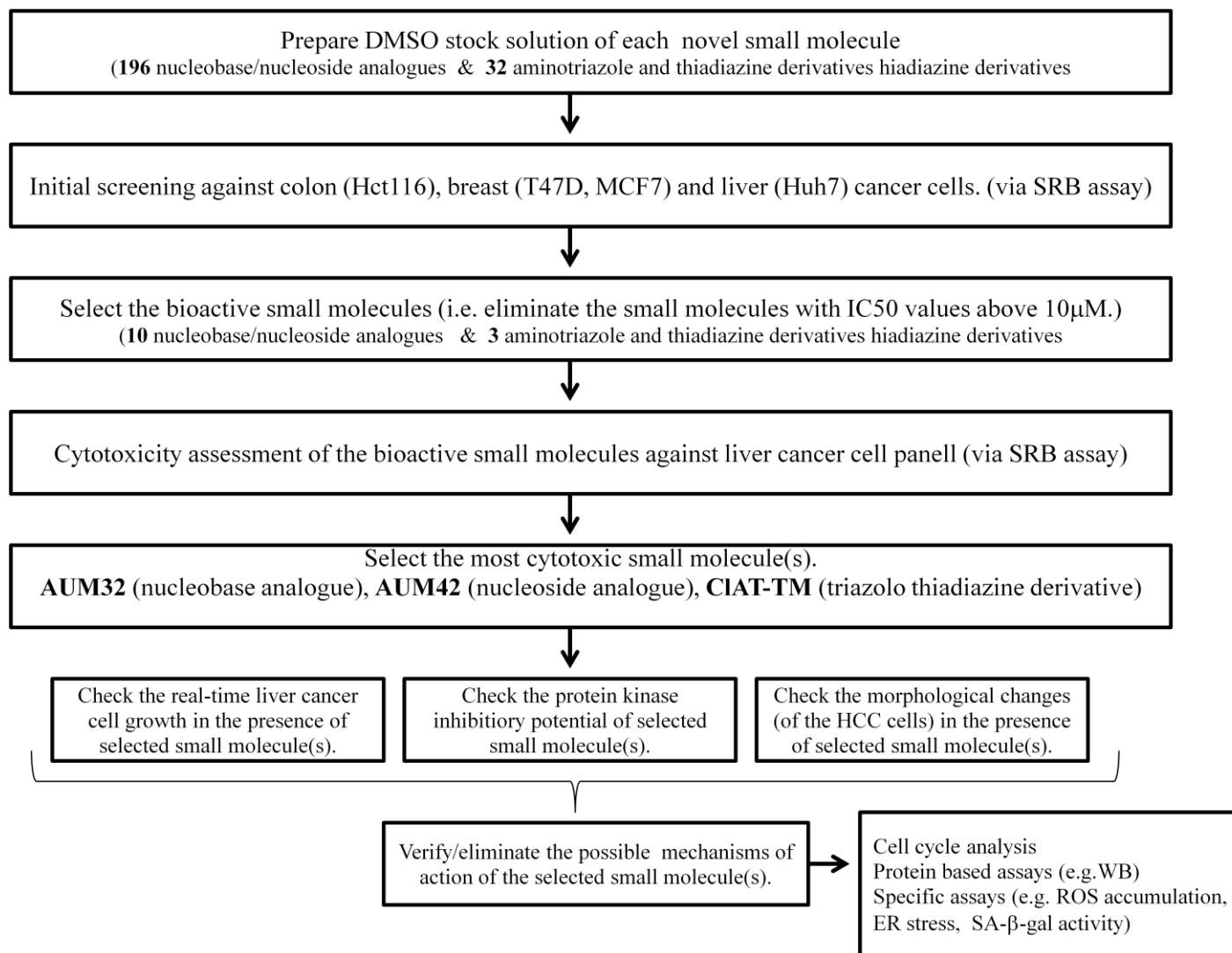
Cancer is one of the ongoing health problems, briefly defined as disordered growth and expansion of aberrant cells, which can eventually result in death. American Cancer Society's report estimates that by the end of 2012, about 28,720 new cases will be diagnosed for primary liver cancer and 20,550 people will die from (<http://www.cancer.org>). Hepatocellular carcinoma is a serious global public health problem with a slow-growing multi-step setup, in which treatment options diminish as time passes for detection. Each year, 748,000 new HCC cases and 696,000 deaths from HCC arise according to the estimations of International Agency for Research on Cancer (IARC) (Ferlay J et al., 2010). Chronic hepatitis B, hepatitis C, alcohol abuse is among the major risk factors and the 5-year survival rate is approximately 10% for patients diagnosed for advanced HCC (Altekruse SF et al., 2009). Patients suffering with early-stage HCC, can benefit from surgical resection, liver transplantation, ablation, embolization, chemotherapy; but at an advanced stage of HCC, alternatives are very limited (Ye SL et al., 2010).

Targeted therapy, which focuses on the inhibition of specific mediators involved in the altered signaling pathways, is a promising approach for the treatment of HCC (Chua CWL and Choo SP, 2011). Sorafenib is the only targeted therapy agent approved for the treatment of advanced HCC, which can prolong survival almost three months (Cao H et al., 2012). Regarding to the accumulated data on targeted therapy of HCC, small molecules can successfully inhibit the abnormal protein-protein interactions leading a cell to cancer (White AW et al., 2008). In such a highly-challenging field, the main aim of this thesis is to identify novel, putative protein kinase inhibitors acting on liver cancer cells. The small molecules in this thesis, which were investigated for this ultimate aim, were designed and synthesized by two separate collaborating groups. The first group of small molecules was novel nucleoside analogues obtained from Prof. Dr. Meral Tuncbilek and the other group of aminotriazole and thiadiazine derivatives was obtained from Prof. Dr. Birsen Tozkoparan.

The newly synthesized small molecules were first tested for their anti-cancer activities against human breast, colon and liver cancer cell lines. The pre-screening approach, which was introduced by National Cancer Institute (NCI), was the strategy we applied (Shoemaker RH, 2006). Following the evaluation of cytotoxicities of these small molecules, the promising drug candidates were chosen according to the druggability of their half-maximal inhibitory concentration (IC₅₀) values. Subsequent drug screening assays were used to analyze the cytotoxicity profile of these compounds against a panel of HCC cell lines. The distinctive features of the cell lines included to the panel, such as known mutations, gave us hints about the possible mechanisms of action which the small molecules exploit. Besides, the protein kinase inhibitory activities of these small molecules were demonstrated by a luminescence based, ATP detecting assay.

The ultimate aim of this Ph.D. dissertation was not only to detect promising anti-cancer agents, but also to characterize the form of death they induce and to identify the underlying mechanism of action. To achieve this goal, the selected, novel, promising small-molecules were further analyzed. Morphological analysis (e.g. light microscopy; Hoechst 33258 staining), protein based experiments, cell cycle analysis, specific assays (e.g. ROS accumulation, SA- β -gal), and eventually *in vivo* drug treatment assays were accomplished not only to define the type of induced-cell death, but also to figure out the mediators involved in this mechanism. Both, verifying the assumed mechanism for the drug candidate and reasoning the eliminated mechanisms were also included within the strategy we used.

STUDY PROTOCOL



CHAPTER 4. RESULTS

The small molecules analyzed in this thesis are classified into two main parts as shown in Table 4.1. The nucleobase and nucleoside analogues composing the first part can be further divided into three subgroups as purine analogues (36 small-molecules), purine ribonucleoside analogues (5 small-molecules) and substituted purine and purine nucleoside analogues (155 small-molecules). The novel aminotriazole and thiadiazine derivatives (32 small molecules) were taken into consideration as the second part of results we obtained and presented in this thesis.

Table 4.1: The small molecules analyzed in this thesis

	# of small-molecules
PART 1. Nucleobase and nucleoside analogues	
PART 1a. Purine analogues	36
PART 1b. Purine ribonucleoside analogues	5
PART 1c. Substituted purine and purine nucleoside analogues	155
PART 2. Aminotriazole and thiadiazine derivatives	32

PART 1

4.1 Nucleobase and nucleoside analogues

The newly synthesized small molecules we obtained from Prof. Dr. Meral Tunçbilek, Ankara University were either nucleobase or nucleoside analogues. The structural modifications on the heterocyclic bases and/or on the sugar moiety of natural nucleosides are required for analogue development. Although nucleoside analogues are structurally similar antimetabolites, they show diverse activities generally affecting the structural integrity of DNA (Ewald B et al., 2008; Kumar V et al., 2010).

PART 1a

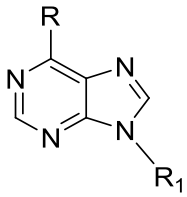
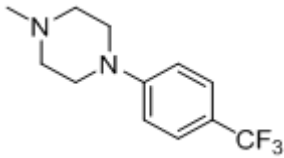
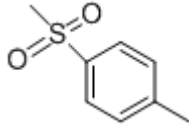
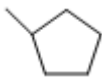
4.1.1 Anti-cancer drug screening of novel, substituted purine analogues

Novel purine analogues in the small molecule pool coded AUM were received in powdered (solid) form (Supplementary Table 1). Following the preparation of their stock solutions in DMSO, each compound was pre-screened against three different cancer cell types. Colon cancer cells (HCT116), breast cancer cells (T47D) and liver cancer cells (Huh7) were chosen to evaluate the cytotoxic activity of these small molecules. Starting from 40 μ M, 5 serially diluted concentrations of each molecule were administered to the cancer cell-inoculated wells. After 72 hours-long incubation period, via Sulforhodamine B (SRB) assay as described in the chapter of Materials and Methods (page 45), the percent cytotoxicity of these small molecules were calculated by comparing the cell growth in small molecule treated wells to control (DMSO-only treated) wells. The percent cytotoxicities of each small molecule were given as a chart (Supplementary Figure 1). The half-maximal inhibitory concentration (IC₅₀) value of a compound is the required amount of that compound to cause 50% cytotoxicity. The IC₅₀ values of these novel purine analogues were calculated according to the dose-response curves obtained from the percent cytotoxicity charts given in Supplementary Figure 1 and given in Supplementary Table 1.

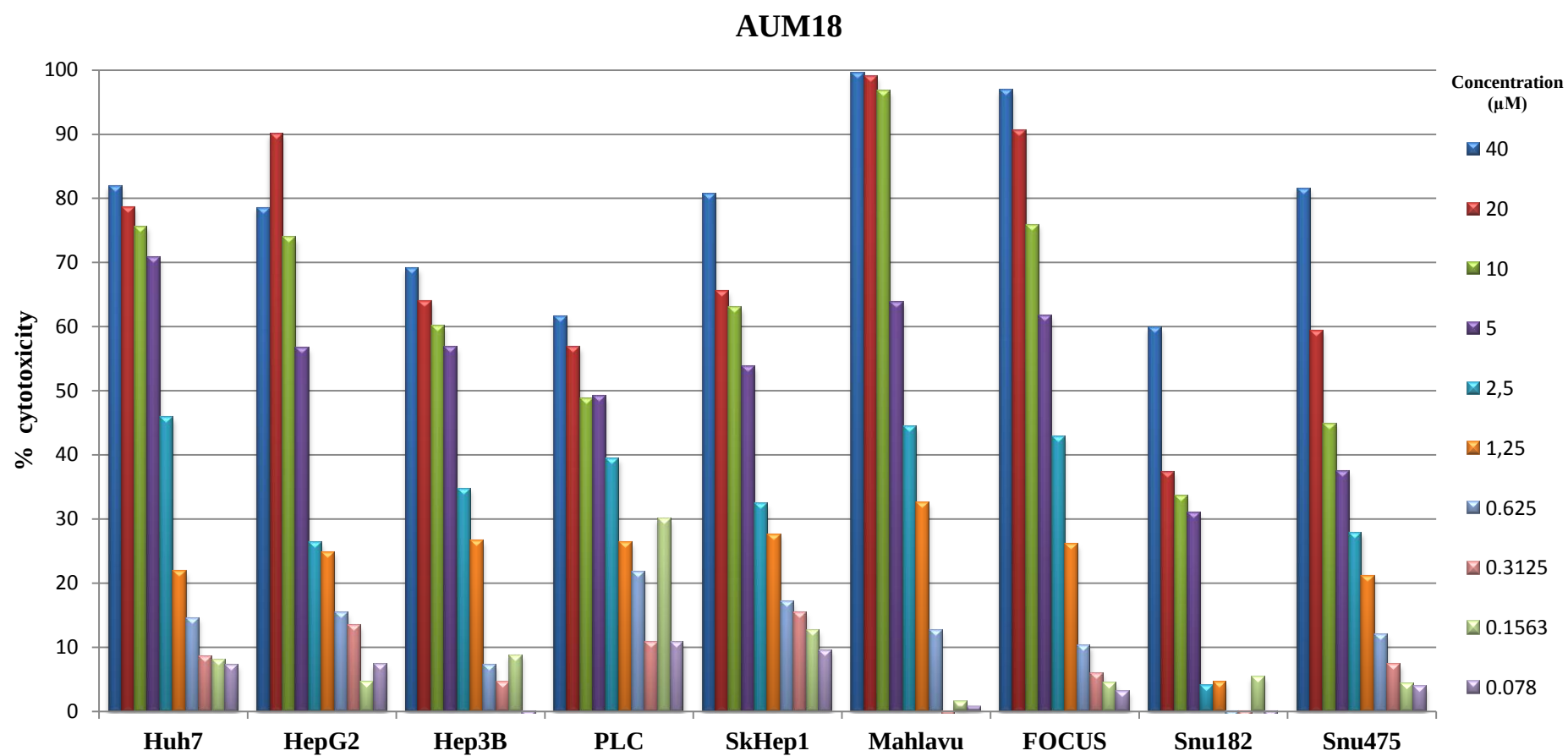
Among the 36 newly synthesized small molecules, AUM18, AUM23 and AUM32 exhibit significantly promising cytotoxicity profiles against Huh7, HCT116 and T47D cancer cells. Interestingly these three analogues were sharing 4-(4-Trifluoromethylphenyl)piperazine common as a substituent at the 6th-position of the purine moiety (N⁶) and their substituent at the 9th-position were creating the difference in their activities (Table 4.2). AUM18, AUM23 and AUM32 have shown cytotoxic percentages corresponding to IC₅₀ values below 10 μ M (except AUM18 and AUM23 have IC₅₀ values 39.4 and 42.7 respectively, for breast (T47D) cancer cell line) (Supplementary Table 1). Not only due to their promising cytotoxic profile, but also because they are structurally very closely related we chose these three purine analogues to further investigate. These three 6- and/or 9-

substituted novel purine analogues (AUM18, AUM23 and AUM32) were further analyzed for their anti-cancer activity on a HCC panel consisting of Huh7, HepG2, Hep3B, PLC, SK-Hep1, Mahlavu, FOCUS, Snu182, Snu475 cancer cell lines. The cancer cells inoculated into 96-well plates were treated with AUM18, AUM23, AUM32 and their DMSO controls in triplicates within 10 different concentrations. The cytotoxicity percentages and the IC₅₀ values calculated accordingly demonstrate that these 3 small molecules are bioactive against HCC cell line panel composing of Huh7, HepG2, Hep3B, PLC, SkHep1, Mahlavu, FOCUS, Snu182 and Snu475 cells (Supplementary Figure 1 and Table 4.2). The IC₅₀ values were in micromolar range, varying between 0.1 μ M to 10 μ M and among these 3 purine analogues, AUM32 is the most cytotoxic analogue with IC₅₀ values below 1 μ M for almost all cell lines in the HCC panel. However, we did not observe significant cytotoxic effect on Snu182 cells with AUM18 purine derivative and observed no inhibition on Snu475 with AUM32. The differential inhibition that we observe, can be considered as an indication of the specificity of these inhibitors against their target.

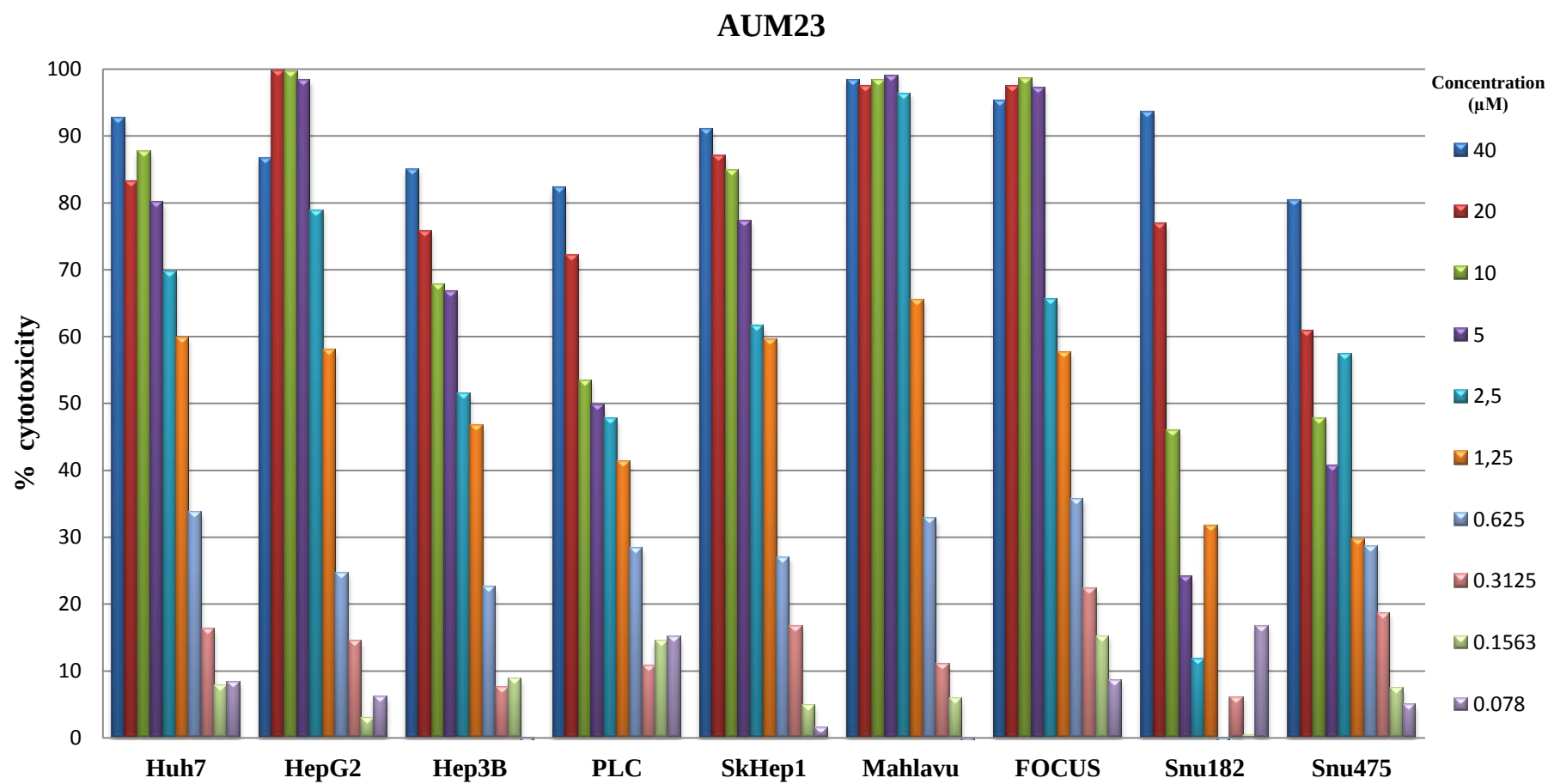
Table 4.2: IC₅₀ values of AUM18, AUM23 and AUM32 against HCC cell line panel: The liver cancer cells were incubated with each molecule for 72 hours and the IC₅₀ values are in μM range. NI stands for “no inhibition”.

	 R :		
	R₁		
	AUM18	AUM23	AUM32
	H		
HCC cell line:	IC₅₀ values (μM)		
Huh7	3.2 ± 0.07	1.4 ± 0.01	0.2 ± 0.04
HepG2	3.7 ± 0.25	1.2 ± 0.2	0.3 ± 0.01
Hep3B	6.7 ± 0.20	2.9 ± 0.63	0.8 ± 0.45
PLC	10.5 ± 0.40	3.8 ± 0.46	6.4 ± 1.19
SK-Hep1	4.9 ± 0.29	1.7 ± 0.17	0.2 ± 0.01
Mahlavu	2.3 ± 0.06	1.0 ± 0.09	0.1 ± 0.01
FOCUS	2.9 ± 0.27	1.0 ± 0.02	0.2 ± 0.06
Snu182	51.0 ± 4.7	8.4 ± 0.26	9.8 ± 2.48
Snu475	9.6 ± 0.20	5.4 ± 0.49	NI

A)



B)



C)

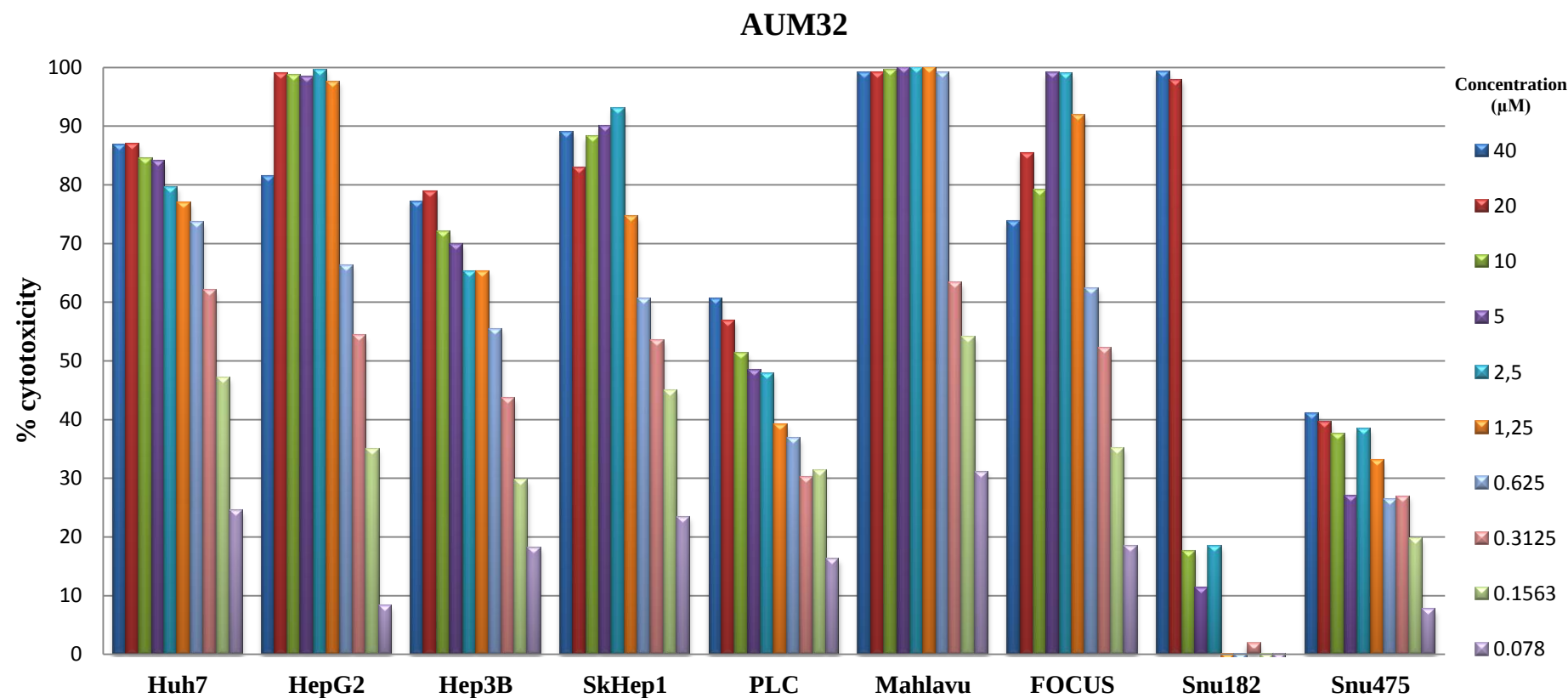


Figure 4.1: Percent cytotoxicity in the presence of AUM18 (A), AUM23 (B) and AUM32 (C) purine analogues: AUM18 (A), AUM23 (B) and AUM32 (C) and their DMSO controls were administered to the HCC panel cancer cells, inoculated in 96-well plates, with ten different concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

4.1.2 AUM18, AUM23 and AUM32 trigger nuclear condensation and DNA fragmentation but do not induce apoptosis

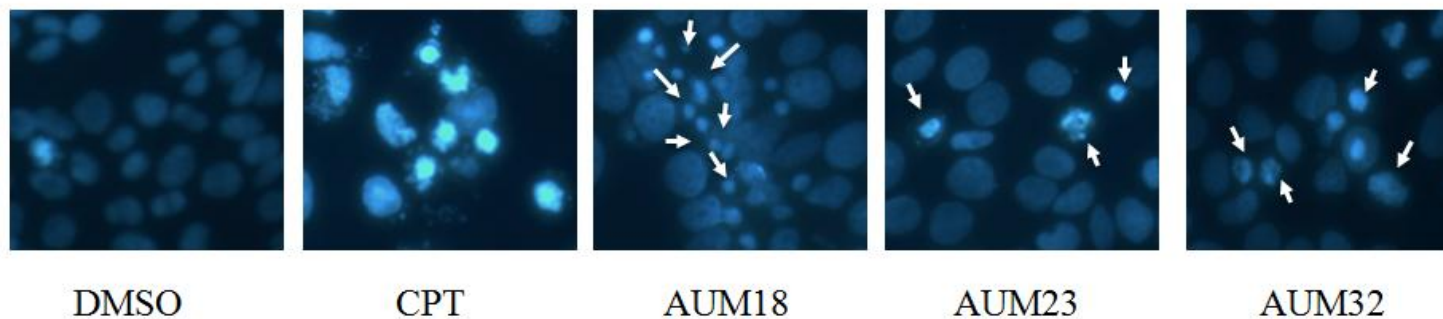
To further clarify the cytotoxic effect emerged after the cancer cells were treated with these 3 novel purine derivatives, we used Hoechst 33258, a DNA-specific dye, to reveal apoptotic morphology. The condensed DNA observed as horseshoe-like structures and shrunk cells following drug treatment most likely indicates apoptosis is induced (Figure 4.2).

After detecting apoptotic bodies in the presence of AUM18, AUM23 and AUM32 by Hoechst 33258 staining, we wanted to confirm apoptosis through inspecting the expression levels of certain proteins known as apoptosis markers. Staurosporine was used as positive control at its apoptosis-inducing dose. No significant decrease in the expression levels of the anti-apoptotic protein Bcl-2 and total Cytochrome-c levels was observed in FOCUS cells treated with these novel small molecules (Figure 4.3-A).

Furthermore, the poly (ADP-ribose) polymerase (PARP-1), a 113 kDa nuclear protein, known to be cleaved not only into fragments of 89kDa and 24kDa fragments during apoptosis, but also into a main fragment of 55kDa if the cell is necrotic, was checked. The HCC cells treated with AUM18, AUM23 and AUM32 at their cell-line-specific IC₅₀ values for 72 hours were assessed for their PARP-1 cleavage via western blot in order to characterize the cell death type. Neither 89kDa and 24kDa apoptotic fragments, nor the 55kDa necrotic fragment were obtained, instead PARP-1 protein remained intact (Figure 4.3-B,C). In other words, we could not name the underlying mechanism of action of these three purine analogues as apoptosis.

A)

Huh7



B)

FOCUS

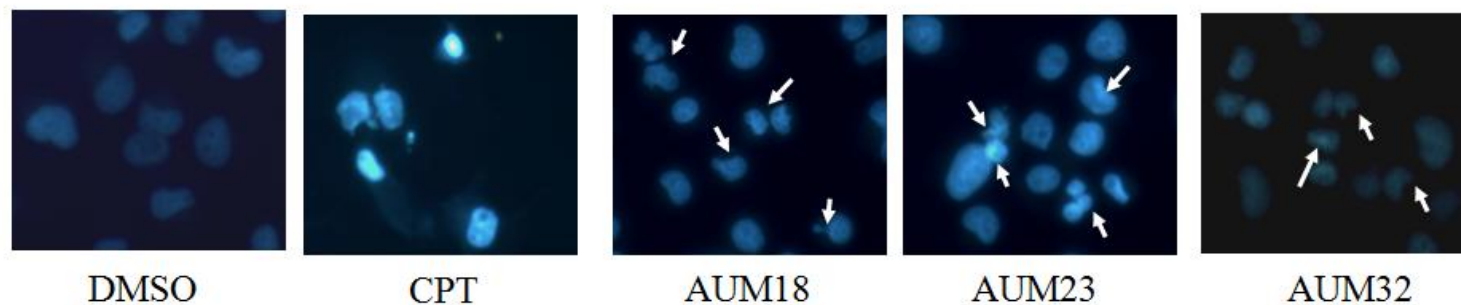


Figure 4.2: Hoechst 33258 nuclear staining of purine analogues treated FOCUS (A) and Huh7 (B) cells. Each AUM small molecule was administered to the liver cancer cells plated on coverslips at their calculated cell line-specific IC₅₀ values for 72 hours. CPT; camptothecin was used as positive control. Cells were visualized with 40x objective.

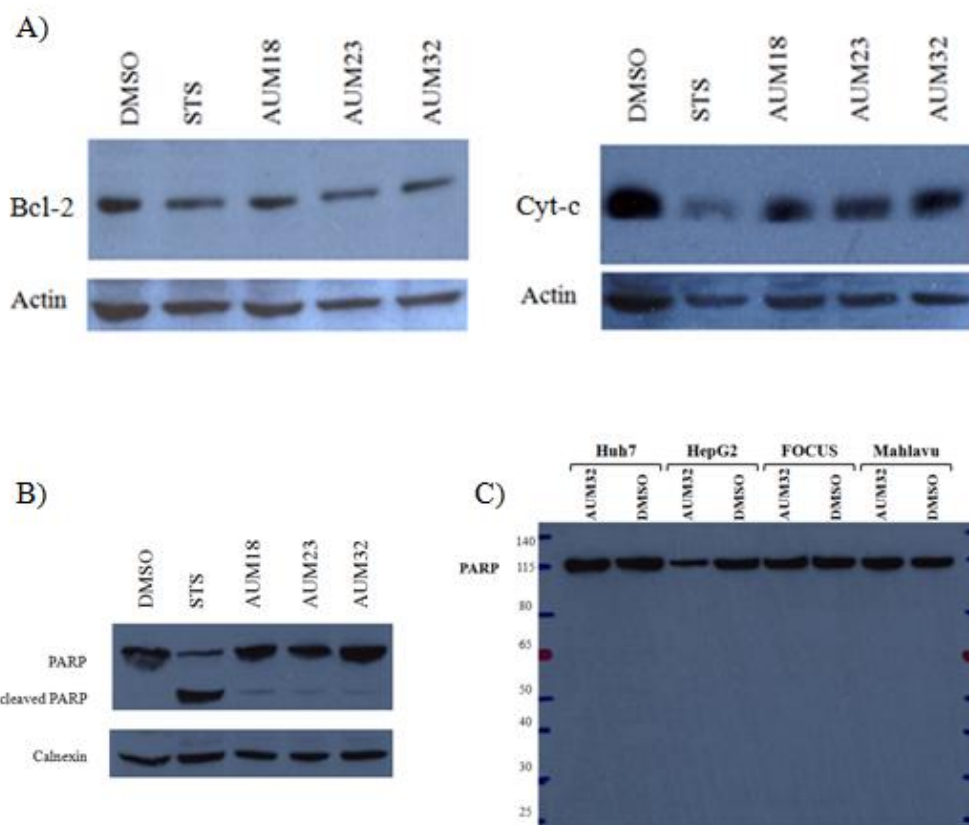


Figure 4.3: (A) Bcl-2 and Cyt-c protein expression levels of FOCUS cells treated with purine analogues Each AUM small molecule was applied to FOCUS cells at their calculated IC₅₀ values for 24 hours. STS; staurosporine was used as positive control. Actin was used as equal loading. **PARP cleavage analysis via western blot for AUM18, AUM23 and AUM32 treated Mahlavu cells (B) and only AUM32 treated Huh7, HepG2, FOCUS and Mahlavu cells (C).** Liver cancer cell(s) were treated with indicated purine analogue(s) at their calculated IC₅₀ values for 72 hours. STS; staurosporine was used as positive control. Calnexin was used as equal loading.

4.1.3 Kinase inhibitory potential of AUM18, AUM23 and AUM32

The growth inhibitory effects that we observed with these three purine derivatives were comparable to those of the known anticancer agents camptothecin (IC₅₀ ~ 10 nM) and 5-fluorouracil (IC₅₀ ~10 μ M). The differential (cell-line-dependent) cytotoxic activity of each molecule can be considered as an indication of the specificity of these inhibitors against their target. They might

interfere with the activity of certain kinases instead of acting as a multi-kinase inhibitor. Furthermore, we evaluated the protein kinase inhibitory activity of these small molecules. Therefore, the kinase assay based on the detection of the amount of ATP in the reaction mixture through bioluminescence (Figure 4.4) was performed with Huh7 and Mahlavu cells (Figure 4.5) (Lundin A, 1981; Singh P et al., 2004).

The liver cancer cells incubated with these putative kinase inhibitors and staurosporine (STS), a multi-kinase inhibitor (used as a positive control) for 24 hours. The blockage of ATP consumption, in other words increase in the light intensity, in the presence of these three purine analogues was more evident in Huh7 cells comparing to Mahlavu cells (Figure 4.5). Based on the results we obtained, the newly synthesized purine derivatives, AUM18, AUM23 and AUM32 may be suggested as putative protein kinase inhibitors, which must be further analyzed at the molecular level.

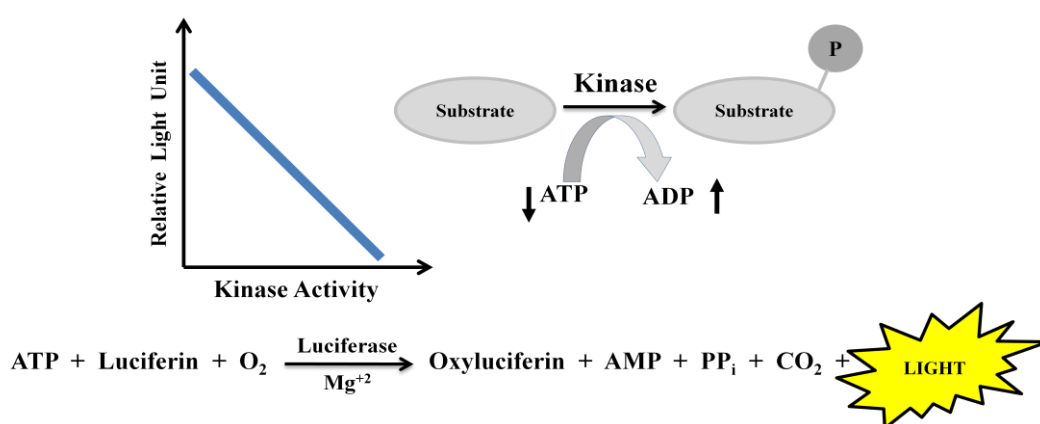


Figure 4.4: Schematic kinase assay principle: The enzyme luciferase generates light from luciferin and ATP-remained after the kinase activity. The intensity of the light is linearly proportional to the concentration of ATP. Hence, high levels of bioluminescence depict the inhibition of protein kinase activity.

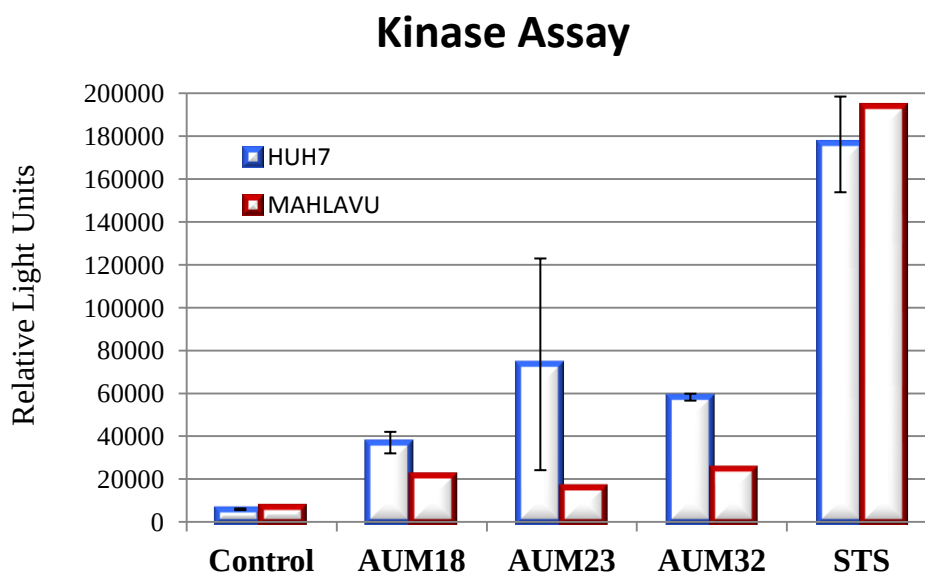


Figure 4.5: Kinase inhibitory potential of AUM18, AUM23 and AUM32: The relative light units measured after the kinase assay was performed in duplicate for the Huh7 and Mahlavu cells treated with the purine derivatives at their given IC₅₀ values. STS; staurosporine (0.25 μ M) was used as positive control. Lonza PKLight™ assay kit was used to test the ATP content of 20 μ g protein containing cell lysates.

4.1.4 Time-dependent cytotoxicity and the real-time cell growth analysis in the presence of AUM32

To thoroughly explain the mechanism behind the cytotoxic activity of AUM32 on cancer cells, we established a time- and dose- dependent assay with HCC cell lines including Huh7, HepG2, Mahlavu and FOCUS. The cancer cells inoculated into 96-well plates were treated with AUM32 and its DMSO control in triplicates within 10 different concentrations and incubated for 24, 48 and 72 hours. Time-dependent cytotoxicity percentages and the IC₅₀ values of AUM32 were given in Figure 4.6 and Table 4.3 respectively. Although the AUM32 induced-cytotoxicity was similar for all the cell lines at 72h, the 24h incubation with AUM32 was not effective in Mahlavu and FOCUS cells as it is in Huh7 and HepG2 cells (Figure 4.6). The time dependent IC₅₀ values corresponding to the cytotoxicity percentage chart of AUM32 demonstrate that this novel purine

analogue induces cell-line-dependent “short term” cytotoxicity; while, the “long term” AUM32 response is comparable in all chosen liver cancer cells (Table 4.3).

Table 4.3: Time-dependent IC50 values of AUM32: AUM32 was applied in triplicates to liver cancer cells inoculated into 96-well plates and incubated for 24, 48 and 72 hours. The IC50 values are in μM range.

	IC50 (μM)		
	24h	48h	72h
Huh7	2.7 ± 0.31	1.4 ± 0.30	0.6 ± 0.04
HepG2	1.2 ± 0.25	0.8 ± 0.04	0.2 ± 0.04
Mahlavu	50.1 ± 11.66	0.3 ± 0.03	0.2 ± 0.01
FOCUS	16.7 ± 4.1	0.3 ± 0.02	0.1 ± 0.04

Moreover, we performed the real-time, label-free cytotoxic activity of AUM32 on Huh7, HepG2 and Mahlavu cancer cells via real-time cell electronic sensing (RT-CES) experiment. The microelectronic sensors of the E-Plate 96, the wells we inoculated the cancer cells into, utilizes the dynamic monitoring of the logarithmic growth of these cells and correlates with cell index (CI). The data recorded clearly demonstrates that AUM32 suppresses the cell growth; in other words, AUM32 treated wells experience a decrease in their CI values after approximately 72h while DMSO-only treated wells (shown in black) display a proliferating profile (Figure 4.7).

4.1.5 The novel, purine analogue AUM32 induces cellular senescence

Both the time-dependent SRB assays and the real-time cellular response of liver cancer cells with AUM32 clearly display that the cytotoxic activity of AUM32 is a “long-term” arising response. This fact indicates that the cell death type AUM32 inducing might be cellular senescence. In order to identify this possibility, we used SA- β -gal assay and BrdU incorporation assays simultaneously. Huh7 cells plated with coverslips to 6-well plates (5000 cells/well) were incubated with IC50 and IC100 values of AUM32, doxorubicin and DMSO-only for 3 days and 6 days. Doxorubicin was used as a positive control at its senescence-inducing dose (25ng/mL) (Senturk S et al., 2010). BrdU (30 μ M) was administered to test its incorporation into cellular DNA of Huh7 cells 24h prior to the end of 3 days and 6 days long incubations. AUM32 treated Huh7 cells showed senescence associated morphology (blue arrows) and also the blue-stained (SA- β -gal positive) cells were negative for BrdU incorporation, whereas DMSO-only applied Huh7 cells were BrdU positive (brown arrows), proliferating cells (Figure 4.8).

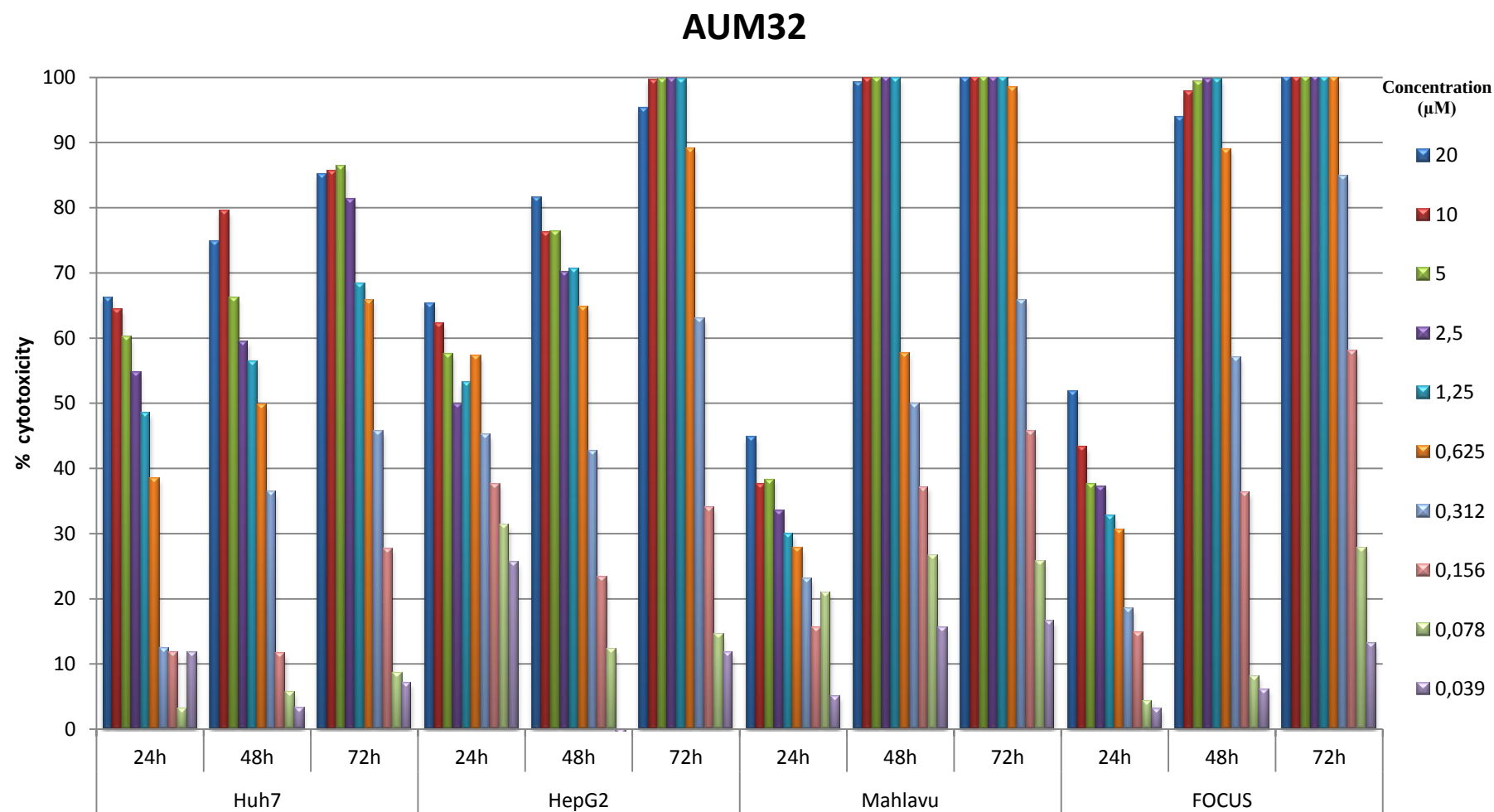


Figure 4.6: Time- and dose- dependent percent cytotoxicity in the presence of AUM32: AUM32 and its DMSO control were administered to the liver cancer cells, inoculated in 96-well plates, in triplicate with ten different concentrations for 24, 48 and 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

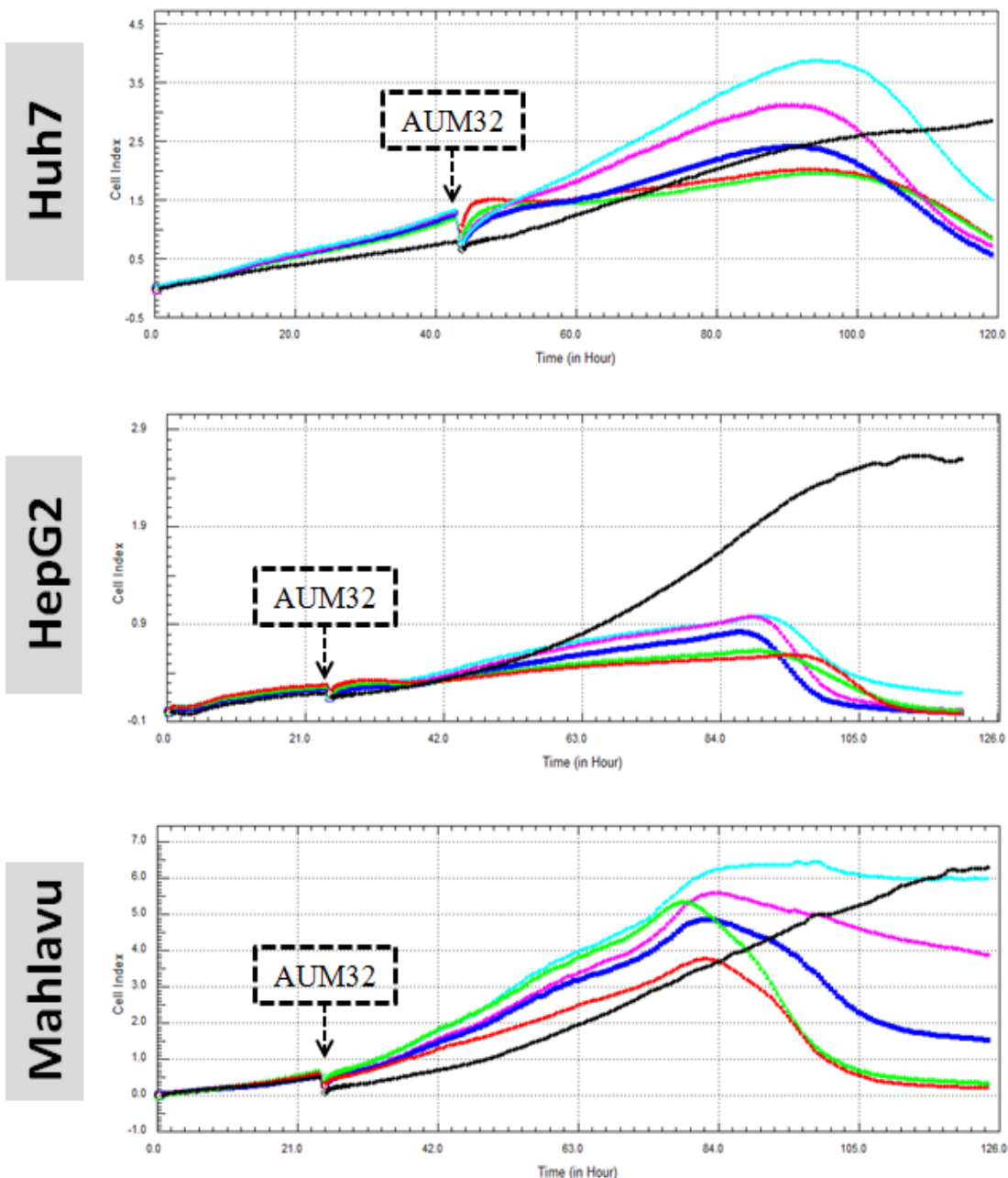


Figure 4.7: Monitored real-time cell growth of Huh7, HepG2 and Mahlavu cells in the presence of AUM32: AUM32 and its DMSO control were administered to the liver cancer cells, inoculated in E-Plate 96, in triplicates. The cell growth index was monitored every 30 minutes in the presence of the five different concentrations of AUM32 (40 μ M-red, 20 μ M-green, 10 μ M-blue, 5 μ M-pink, 2.5 μ M-cyan and DMSO control-black).

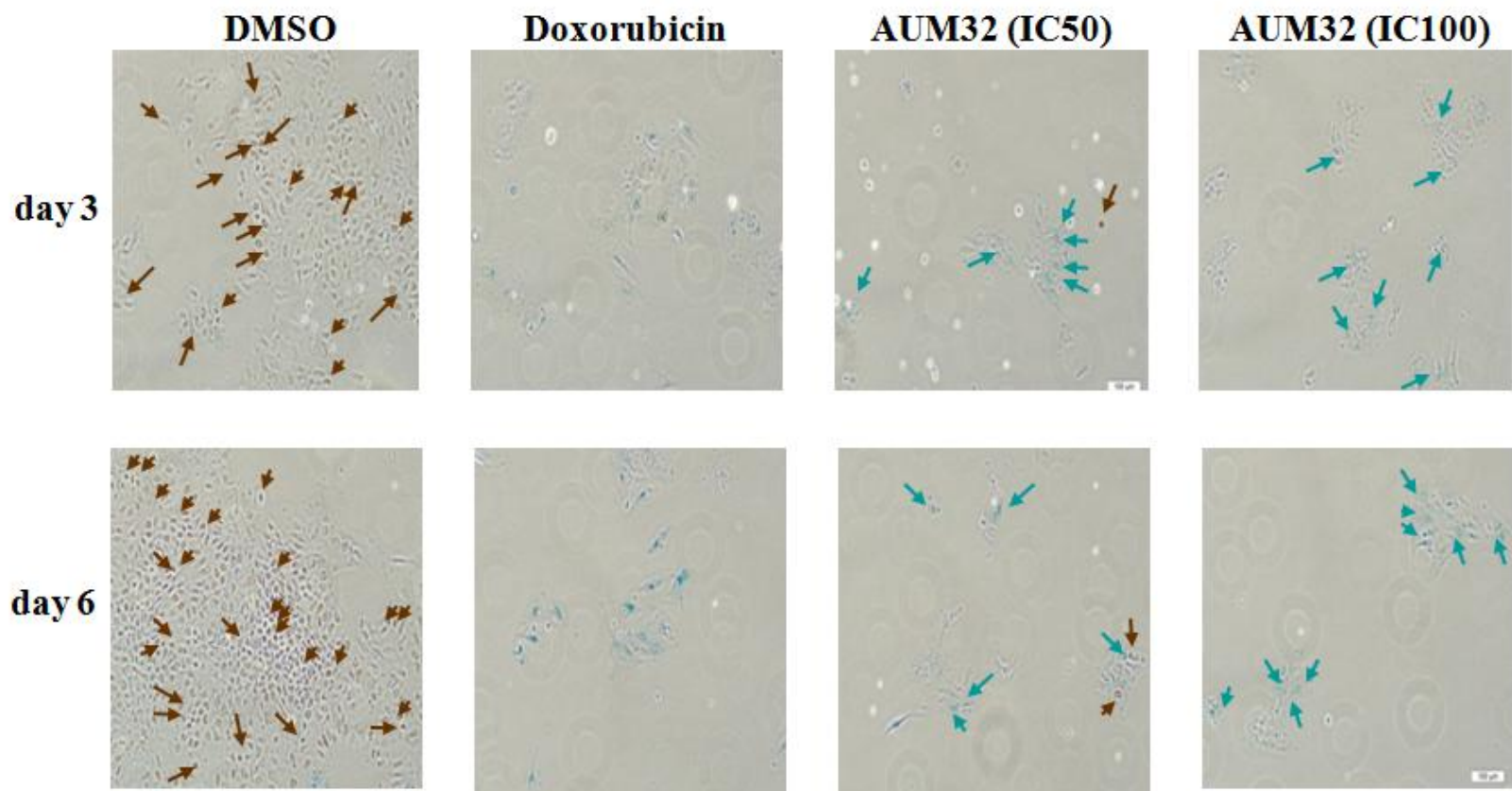


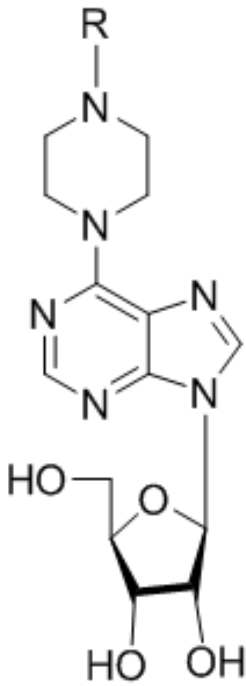
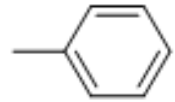
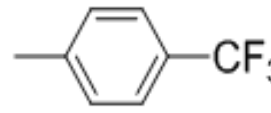
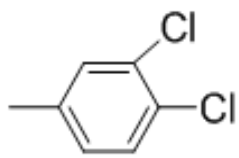
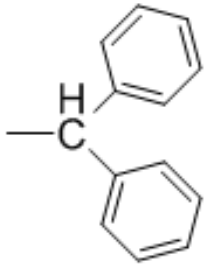
Figure 4.8: SA- β -gal and BrdU incorporation assays in the presence of AUM32: Huh7 cells (5000cells/well) inoculated into 6-well plates with coverslips were incubated with IC50 and IC100 values of AUM32, Doxorubicin (25ng/mL) and DMSO-only for 3 days and 6 days. SA- β -gal and BrdU incorporation assays were performed in parallel. BrdU (30 μ M) was administered to the cells 24 hours prior to the end of 3rd and 6th days of incubation.

PART 1b

4.1.6 Anti-cancer drug screening of novel purine ribonucleoside analogues

Novel purine ribonucleoside analogues were received in powdered form. In Table 4.4, not only the structures of these derivatives but also the published, corresponding compound codes were given. Following the preparation of their stock solutions in DMSO, all were evaluated for their cytotoxicity against liver (Huh7, HepG2, Mahlavu and FOCUS), colon (HCT116) and breast (MCF7) cancer cells, by the previously explained SRB assay, in a dose- and time-dependent manner. The cancer cells inoculated into 96-well plates were treated with AUM40-44 and their DMSO controls in triplicates within 5 different concentrations prior to incubation for 24, 48 and 72 hours. Time-dependent cell death percentages and the IC₅₀ values of AUM40-44 were given in Supplementary Figure 2 and Table 4.5 respectively. Although AUM42 and AUM43 both exhibit promising cytotoxic effects on liver (Huh7, HepG2, FOCUS, Mahlavu), colon (Hct116) and breast (MCF7) cancer cell lines, we decided to choose AUM42 for further analysis because its cytotoxicity is slightly more distinguished (Table 4.5). Therefore we performed assays to characterize the cytotoxicity induced by AUM42 and identify the underlying mechanism of action.

Table 4.4: Novel 6-(4-substituted piperazine-1-yl)-9-(β -D-ribofuranosyl) purine analogues

		Compound Code*	R
	AUM40	9	
	AUM41	10	
	AUM42	11	
	AUM43	12	
	AUM44	13	

*Tuncbilek M, Bilget Guven E, Onder T, Cetin-Atalay R. 2012. Synthesis of novel 6-(4-substituted piperazine-1-yl)-9-(β -D-ribofuranosyl)purine derivatives, which lead to senescence-induced cell death in liver cancer cells. J Med Chem. Apr 12;55(7):3058-65.

Table 4.5: Time-dependent IC50 values of purine ribonucleoside analogues: AUM40-44 were applied in triplicates to the cancer cells inoculated into 96-well plates and incubated for 24, 48 and 72 hours. The IC50 values are in μM range. NI stands for “no inhibition”. ND stands for “not determined”.

		IC50 (μM)						
		AUM40	AUM41	AUM42	AUM43	AUM44	5-FU	CPT
Huh7	24h	NI	NI	2.5 $\pm$ 0.1	26.9 $\pm$ 3.1	NI	ND	< 0.1
	48h	NI	NI	9.0 $\pm$ 0.7	10.1 $\pm$ 0.6	NI	ND	< 0.1
	72h	NI	NI	7.8 $\pm$ 0.3	7.2 $\pm$ 1.2	NI	30.7	< 0.1
HepG2	24h	NI	NI	12.1 $\pm$ 2.6	18.3 $\pm$ 5.4	NI	ND	< 0.1
	48h	NI	NI	7.2 $\pm$ 0.8	6.8 $\pm$ 0.5	NI	ND	< 0.1
	72h	NI	NI	5.7 $\pm$ 0.7	6.1 $\pm$ 0.3	NI	5.0	< 0.1
Mahlavu	24h	NI	NI	14.4 $\pm$ 0.3	31.7 $\pm$ 3.2	NI	ND	< 0.1
	48h	NI	NI	8.6 $\pm$ 0.5	13.9 $\pm$ 1.8	NI	ND	< 0.1
	72h	NI	NI	9.2 $\pm$ 0.4	7.0 $\pm$ 0.5	NI	10.0	< 0.1
FOCUS	24h	NI	NI	10.8 $\pm$ 0.6	26.0 $\pm$ 1.6	NI	ND	< 0.1
	48h	NI	NI	4.9 $\pm$ 0.5	8.7 $\pm$ 1.0	NI	ND	< 0.1
	72h	NI	NI	5.2 $\pm$ 0.7	5.5 $\pm$ 0.1	NI	7.6	< 0.1
HCT116	24h	39.6 $\pm$ 7.5	NI	11.6 $\pm$ 0.7	20.8 $\pm$ 6.5	NI	ND	< 0.1
	48h	NI	NI	6.3 $\pm$ 0.7	8.2 $\pm$ 2.0	40.3 $\pm$ 7.9	ND	< 0.1
	72h	NI	NI	6.7 $\pm$ 0.7	7.8 $\pm$ 1.4	44.9 $\pm$ 5.5	6.0	< 0.1
MCF7	24h	NI	NI	12.6 $\pm$ 1.7	18.8 $\pm$ 3.5	NI	ND	< 0.1
	48h	NI	NI	8.7 $\pm$ 1.5	12.8 $\pm$ 2.1	43.3 $\pm$ 5.5	ND	< 0.1
	72h	NI	NI	7.4 $\pm$ 0.5	9.5 $\pm$ 0.4	42.2 $\pm$ 5.6	3.5	< 0.1

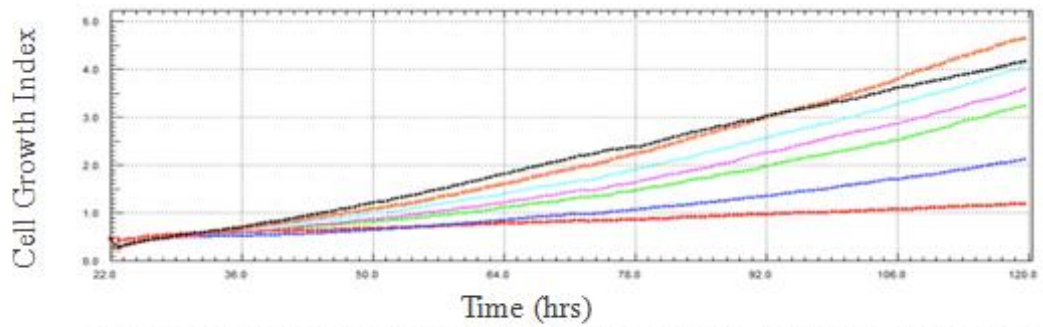
4.1.7 Real-time cellular response of liver cancer cells in the presence of AUM42

Real-time Cell Electronic Sensing (RT-CES) system was used to analyze the AUM42 mediated cytotoxicity on Huh7, HepG2, Mahlavu and FOCUS hepatocellular carcinoma cells in triplicate. The real-time dynamic monitoring of the electrode impedance is indicated as Cell Index (CI) correlating with cell growth. AUM42, applied in 6 different concentrations (30-5 μ M), triggered a time- and dose- dependent decrease in CI for all cancer cells (Figure 4.9). The AUM42-induced suppression of the cell growth was cell-line-dependent; in other words, Mahlavu cells were more resistant to AUM42-induced cytotoxicity which is correlative with the previous SRB data. IC₅₀ values of AUM42 is highest for Mahlavu cells in the HCC panel. This observation might be indicating that AUM42 is an interfering molecule as a putative kinase inhibitor.

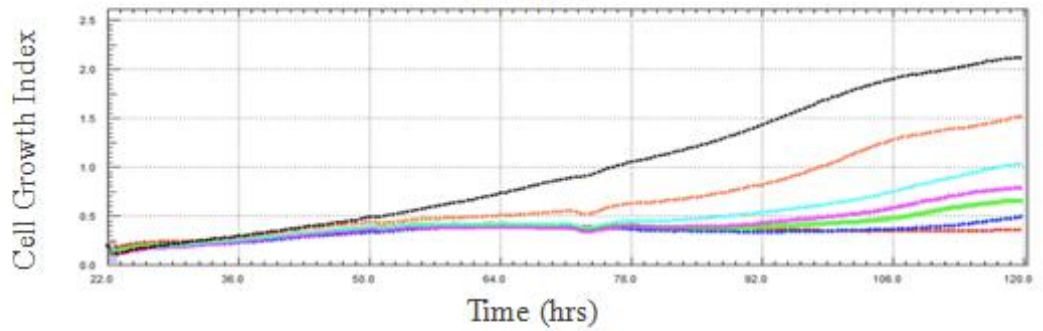
4.1.8 Kinase inhibitory potential of AUM42

Cellular response profiles exhibiting differences between cell lines bring to mind that AUM42 might be interfering with the cell signaling. To clarify if AUM42 possesses a kinase inhibition potential or not, we used a luminescent ATP-detection assay as explained previously (Figure 4.4). Luminescence (relative light unit), correlates with the amount of ATP in the milieu; therefore, an increase in the luminescence can be considered as a sign of the existence of a protein kinase inhibitor. We used staurosporine (STS), a potent multi-kinase inhibitor, and MeSAdo (5'-Deoxy-5'-methylthioadenosine), which has an open structure resembling that of AUM42 molecule, as positive controls of the assay. Huh7 cells incubated with IC₅₀ and IC₁₀₀ values of AUM42, 5 μ M MeSAdo and 0.5 μ M STS for 72h were then tested. The dose dependent ATP increase in the presence of AUM42, suggests that AUM42 might be a putative kinase inhibitor (Figure 4.10). High rlu obtained with the STS treated Huh7 cells is consistent with both the principle of the assay and the potential of STS as a multi-kinase inhibitor.

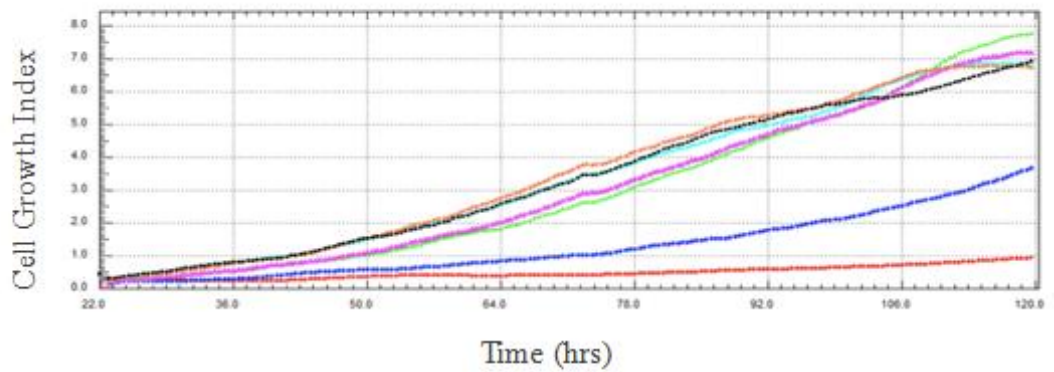
Huh7



HepG2



Mahlavu



FOCUS

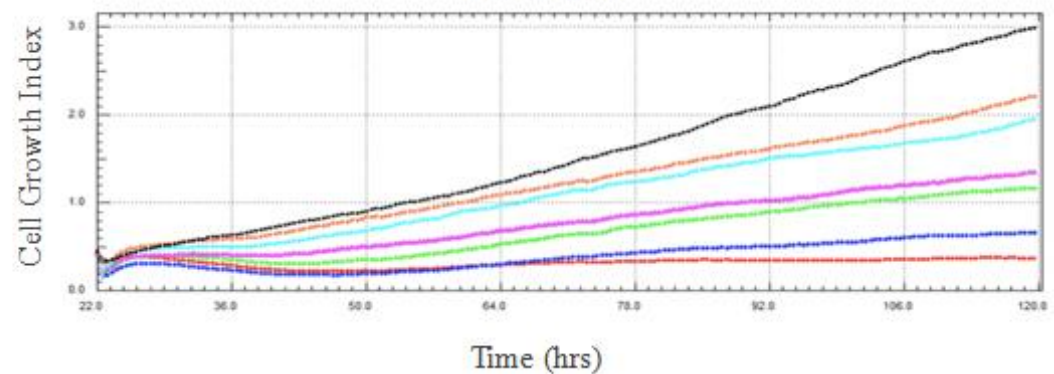


Figure 4.9: Monitored real-time cell growth of Huh7, HepG2, Mahlavu and FOCUS cells in the presence of AUM42: AUM42 and its DMSO control were administered to the cancer cells, inoculated in E-Plate 96, in triplicates. The cell growth index was monitored every 30 minutes in the presence of the six different concentrations of AUM42 (30µM-red, 20µM-blue, 15µM-green, 10µM-pink, 7.5µM-cyan, 5µM-coral and DMSO control-black).

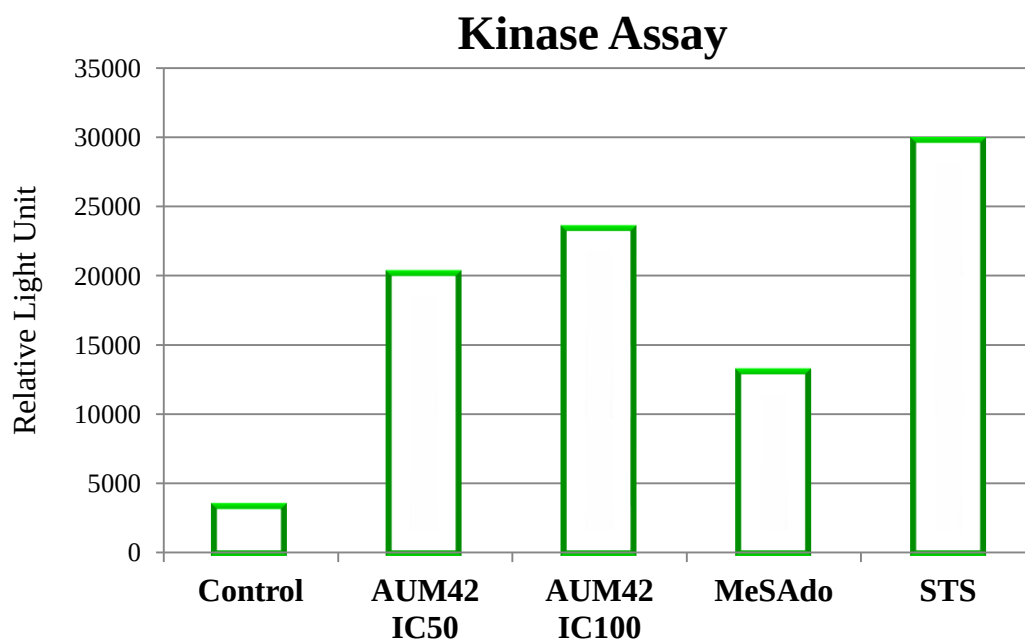


Figure 4.10: Kinase inhibitory potential of AUM42: The relative light units measured after the kinase assay performed for the Huh7 cells treated with the IC50 and IC100 values of AUM42, STS (staurosporine, 0.5 μ M), MeSAdo (5 μ M) and their corresponding DMSO controls for 72 hours. Kinase-GLO assay kit was used to test the ATP content of 80000 cells.

4.1.9 The cytotoxic activity of AUM42 is neither apoptosis nor necrosis but it induces cellular senescence

The indicator of an activated apoptotic pathway, Poly ADP-ribosyl polymerase (PARP) cleavage, was checked for AUM42 treated Huh7, HepG2, Mahlavu and FOCUS cells for 72 hours at its cell-line-specific IC50 values. Neither apoptotic fragments (89kDa and 24kDa) nor the necrotic fragment (55kDa) were obtained; instead PARP protein remained intact, 113kDa (Figure 4.11).

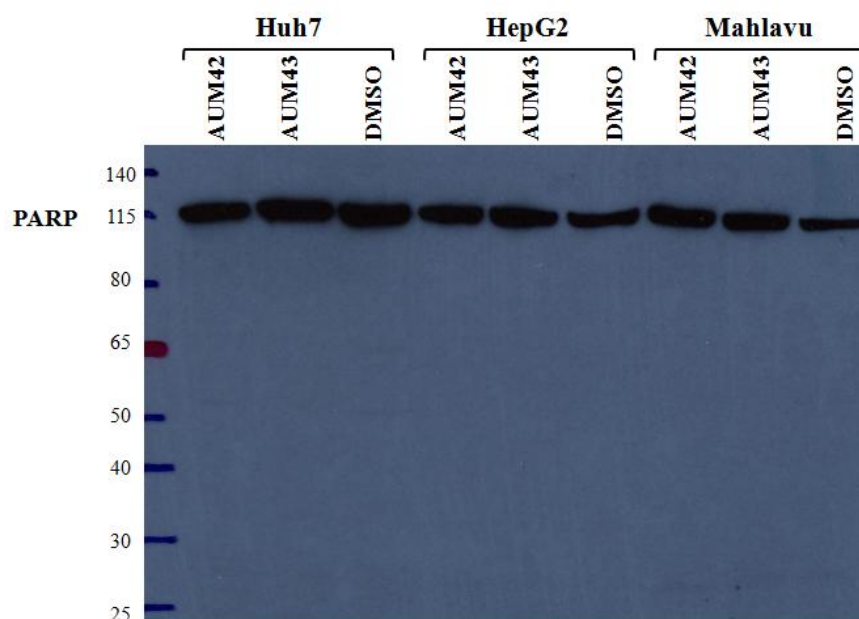
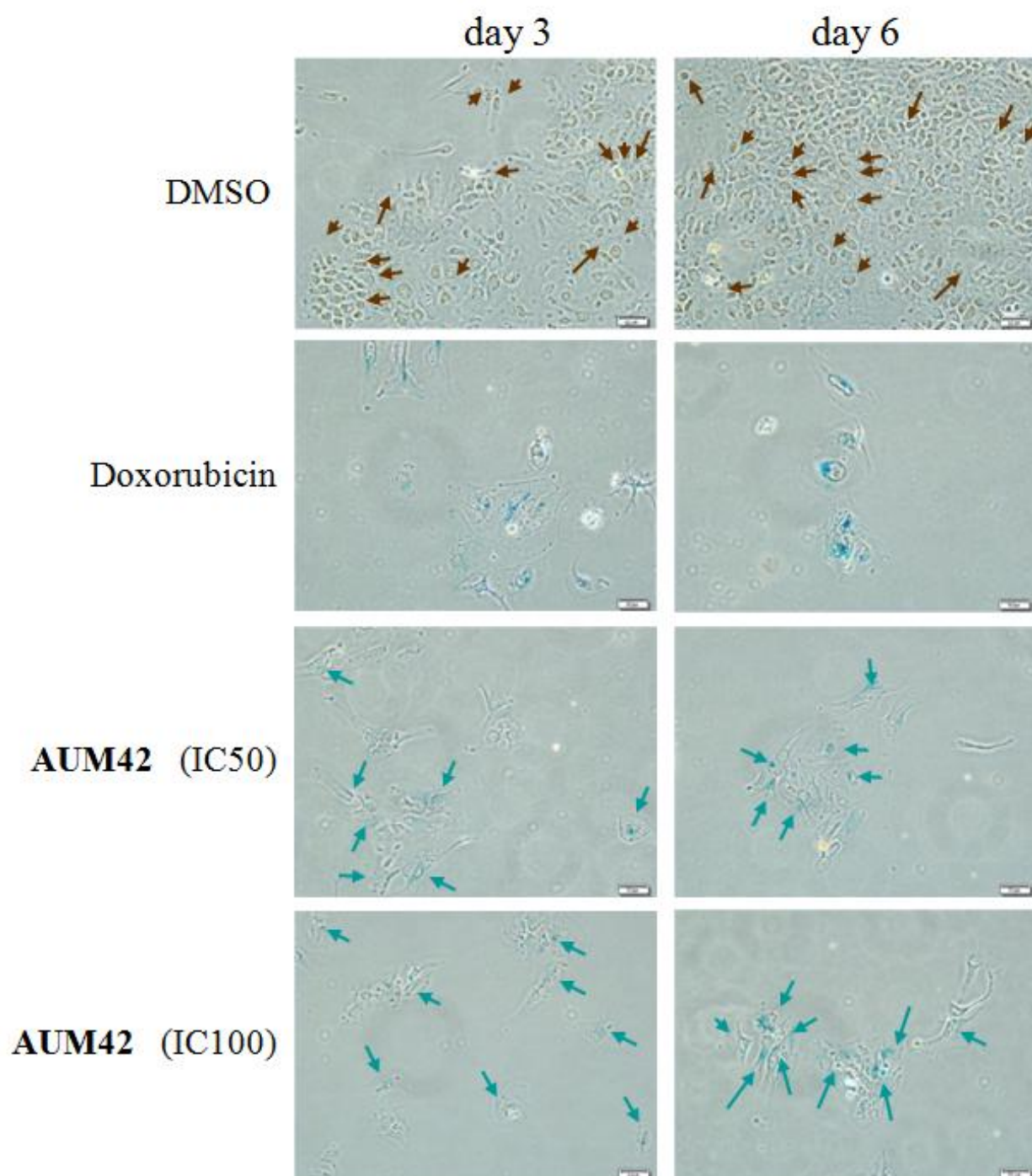


Figure 4.11: PARP cleavage analysis via western blot. Huh7, HepG2 and Mahlavu cells were treated with AUM42 and AUM43 at their calculated IC₅₀ values for 72 hours. No cleaved PARP fragment was detected.

To identify if the proliferative arrest generated by AUM42 is due to senescence or not, we performed SA- β -gal and BrdU incorporation assays in parallel. Huh7 cells plated with coverslips to 6-well plates were incubated with IC₅₀ and IC₁₀₀ values of AUM42, Doxorubicin and DMSO-only for 3 days and 6 days. Doxorubicin was used as a positive control at its senescence-inducing dose (25ng/mL) (Senturk S et al., 2010). BrdU (30 μ M) was administered to test its incorporation into cellular DNA of Huh7 cells 24h prior to the end of 3 days and 6 days long incubations. SA- β -gal assay positive, large, blue stained cells were obtained when treated with AUM42 or doxorubicin. These senescent cells were also negative for BrdU incorporation where BrdU positive proliferating cells were excessive in the DMSO-only treated wells (Figure 4.12).

A)



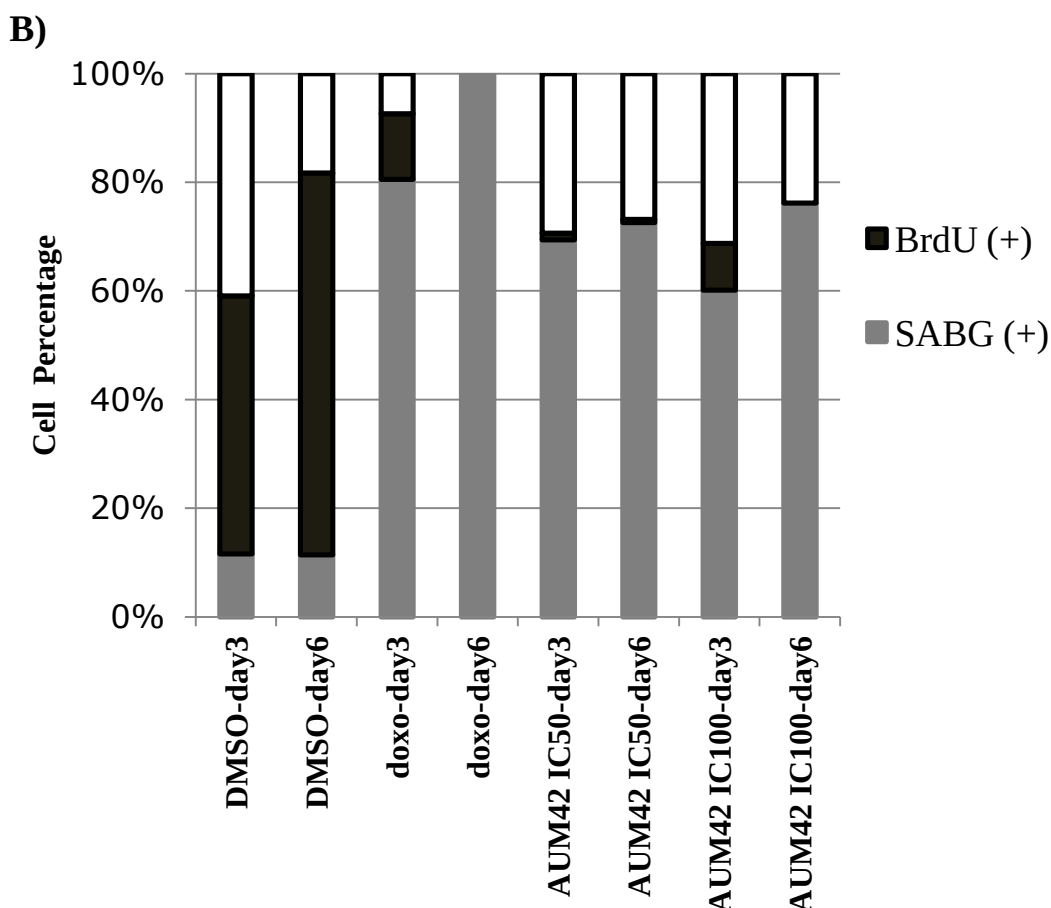


Figure 4.12: SA- β -gal and BrdU incorporation assays in the presence of AUM42: (A) Huh7 cells inoculated into 6-well plates with coverslips (5000cells/well) were incubated with IC50 and IC100 values of AUM42, doxorubicin (25ng/mL) and DMSO-only for 3 days and 6 days. SA- β -gal and BrdU incorporation assays were performed in parallel. BrdU (30 μ M) was administered to the cells 24 hours prior to the end of 3rd and 6th days of incubation. (B) For each treatment condition, cells were counted and the SA- β -gal and BrdU positive cell percentages were given.

In addition to the most commonly used marker of senescence, SA- β -gal assay, we aimed to check p15(INK4b) levels as another senescence-associated marker, which is known to be increased in senescent cells. Total protein lysates were obtained from Huh7 cells treated with IC50 and IC100 values of AUM42, Doxorubicin (25ng/mL) and their DMSO controls and incubated for 3 and 6 days; afterwards, western blot analysis was done. For the AUM42 treated Huh7 cells,

the increase in the protein expression levels of p15(INK4b) was both time- and dose- dependent (Figure 4.13-A). In order to verify this increase, next, we decided to check the downstream effect of p15(INK4b) on the Rb protein. It is known that p15(INK4b) activates Rb protein; in other words, p15(INK4b) inhibits the phosphorylation of Rb by inhibiting CyclinD1/CCDK4CD. The decrease observed in the phosphorylated form of Rb protein correlates with the AUM42-induced accumulation of p p15(INK4b) (Figure 4.13-B,C).

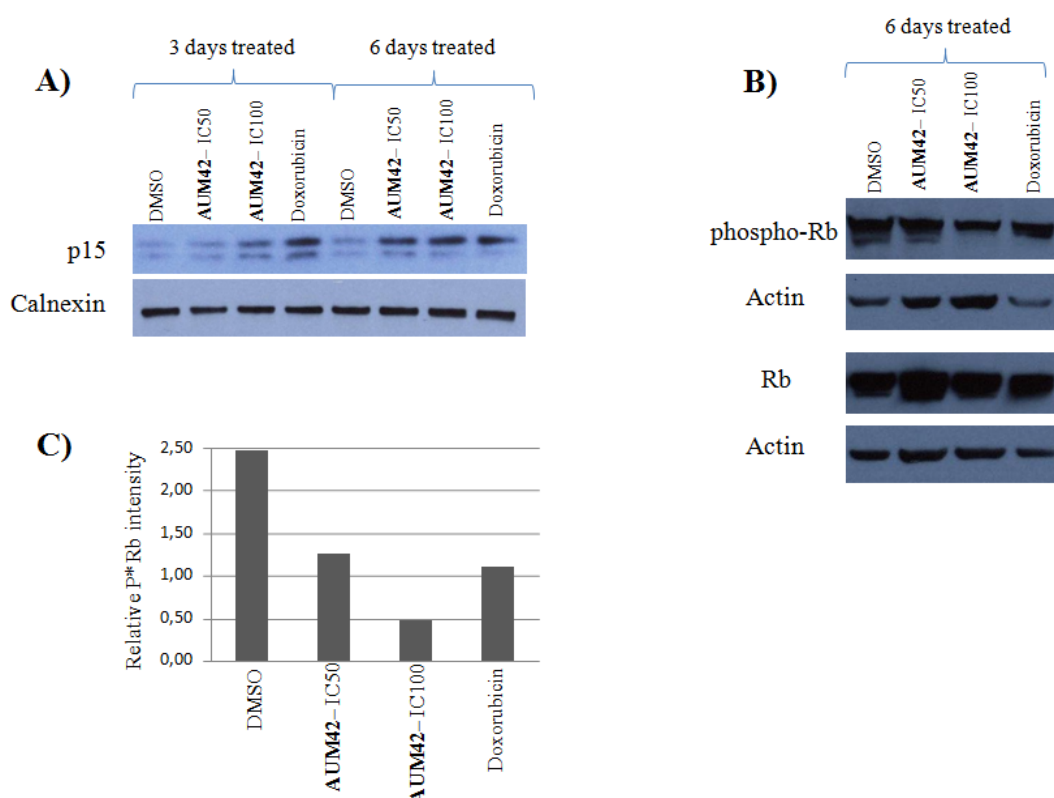


Figure 4.13: Senescence-associated proteins p15(INK4b) and Rb in the presence of AUM42: Huh7 cells were treated with IC50 and IC100 values of AUM42, Doxorubicin (25ng/mL) and their DMSO controls for 3 days and/or 6 days. Total cell lysates were obtained and the protein expression levels of p15(INK4b) (A), Rb and phospho-Rb (B) were checked. Doxorubicin at its senescence-inducing dose was used as positive control. Calnexin and actin were used to control equal loading. The relative phospho-Rb (P*Rb) intensities of each treatment (C) confirms senescence-induced cytotoxicity of AUM42.

Besides, when we checked the expression level of p21 in order to identify the action mechanism of senescence-inducing analogue, AUM42, we observed no significant change. Moreover, phosphorylated-H2AX, indicating DNA damage, was also not detectable (Figure 4.14). Therefore, we concluded that AUM42 is inducing senescence through the activation of Rb protein depending on the increased levels of p15(INK4b).

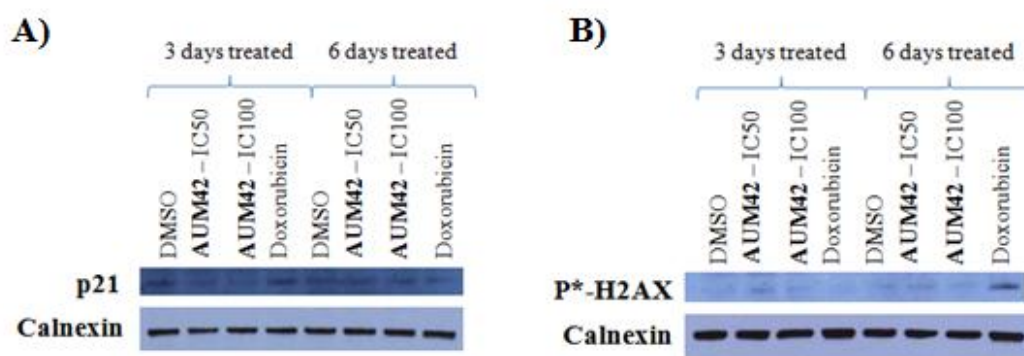


Figure 4.14: p21 and phospho-H2AX (P*-H2AX) expression levels in the presence of AUM42: Huh7 cells were treated with IC50 and IC100 values of AUM42, Doxorubicin (25ng/mL) and their DMSO controls for 3 days and/or 6 days. Total cell lysates were obtained and the protein expression levels of p21 (A), P*-H2AX (B) were checked. Doxorubicin at its senescence-inducing dose was used as positive control. Calnexin was used to control equal loading.

PART 1c

4.1.10 Anti-cancer drug screening of novel, substituted purine and purine nucleoside analogues

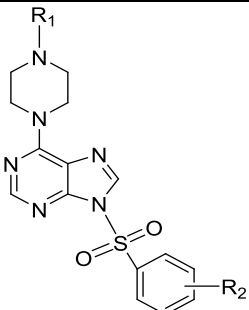
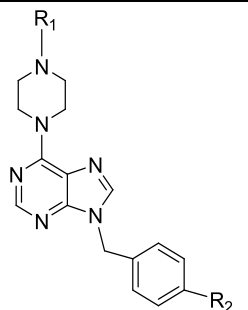
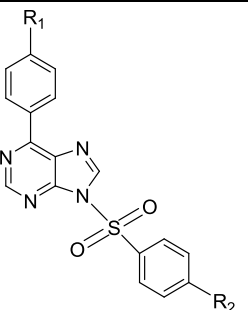
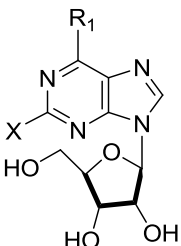
Novel purine and purine nucleoside analogues in the small molecule pool of AUM were received in powdered form (Table 4.6). Following the preparation of their stock solutions in DMSO, each and every compound was prescreened against three different cancer cell types. Colon cancer cells (HCT116), breast cancer cells (MCF7) and liver cancer cells (Huh7) were chosen to evaluate the cytotoxic potential of these small molecules via Sulforhodamine B (SRB) assay as described previously. The half-maximal inhibitory concentration (IC50) values of these molecules were given in Supplementary Tables 2-5. The IC50 values were

calculated according to the dose-response curves obtained from the percent cytotoxicity charts given in Supplementary Figures 3-5.

The initial screening of these substituted purine and purine nucleoside analogues against liver, colon and breast cancer cell lines resulted with a high number of promising small molecules with anti-cancer activities. The percent cytotoxicity charts were used to calculate the IC₅₀ values; but for some of the analogues (AUM 54, AUM53 and AUM168) the dose-response curves were extrapolating the IC₅₀ value to a false negative region due to the high cytotoxicity percentages obtained with lower concentrations of the small molecules (Supplementary Figure 4). In other words, the IC₅₀ values were “not applicable” which means that the starting concentration of the serial dilution should be decreased (Supplementary Table 3).

Among the 155 newly synthesized purine derivatives, the small molecules, with IC₅₀ values less than 20 μM against at least one of the cancer cell lines, were further analyzed for their anti-cancer activity against a HCC panel consisting of Huh7, HepG2, Mahlavu and FOCUS cancer cell lines (Table 4.6). The cancer cells inoculated into 96-well plates were treated with the promising purine derivatives and their DMSO controls in triplicates within five different concentrations. The cell death percentages and the IC₅₀ values, calculated accordingly, exhibit the cytotoxic potential of these small molecules (Supplementary Tables 6-8, Supplementary Figures 6-8). Different from the initial screening conditions, the starting concentration for each small molecule was determined independently. Although 5 different serially diluted concentrations were applied for each novel small molecule, varying starting concentrations, chosen according to the first screening data obtained, were applied. Therefore the IC₅₀ values were calculated by interpolating these 5 concentrations. For the compounds in PART 3, the calculated IC₅₀ values of 37 analogues were lower than 10 μM for all four liver cancer cell lines and 14 small molecules were lower than 10 μM for all but Mahlavu cancer cells. Interestingly the 33 analogues out of 37 are 6-(substituted piperazine) -9-(substituted benzyl) purine derivatives (Table 4.6).

Table 4.6: Substituted purine and purine nucleoside analogues

	6-(substituted piperazine) - 9-(substituted phenyl) sulfonyl purine derivatives	6-(substituted piperazine) - 9-(substituted benzyl) purine derivatives	6-(4-substituted phenyl) - 9-(4-substituted phenyl) sulfonyl purine derivatives	2-amino/2-chloro/ 6-(4-substituted piperazine) / 6-(4-substituted phenyl amino) / 6-(2-substituted ethylamino) – 9-(β-D-ribofuranosyl) purine derivatives
				
# of small-molecules	59	64	16	16
# of bioactive molecules IC50 < 20μM	32	56	6	8
# of bioactive molecules IC50 < 10μM	12	39	0	1

4.1.11 Kinase inhibitory potential of AUM52, AUM53, AUM54, AUM56 and AUM57

The newly synthesized small molecules, AUM52, AUM53, AUM54, AUM56 and AUM57 are structurally very similar even among the members of the subgroup they belong. Moreover, their IC₅₀ values are below 5 μ M. In order to evaluate their protein kinase inhibition potential we performed the kinase assay based on the detection of the amount of ATP in the reaction mixture through bioluminescence, previously explained in Figure 4.4 with Huh7 and Mahlavu cells (Figure 4.15). The liver cancer cells were incubated with these putative kinase inhibitors and staurosporine (STS), a multi-kinase inhibitor (used as a positive control) for 72 hours. AUM54 and AUM57 inhibit the ATP consumption only in Huh7 cells while the other purine analogues were able to block the utilization of ATP in both Huh7 and Mahlavu cell lines. AUM56 is the small molecule with the highest kinase inhibitory potential in this group (Figure 4.15).

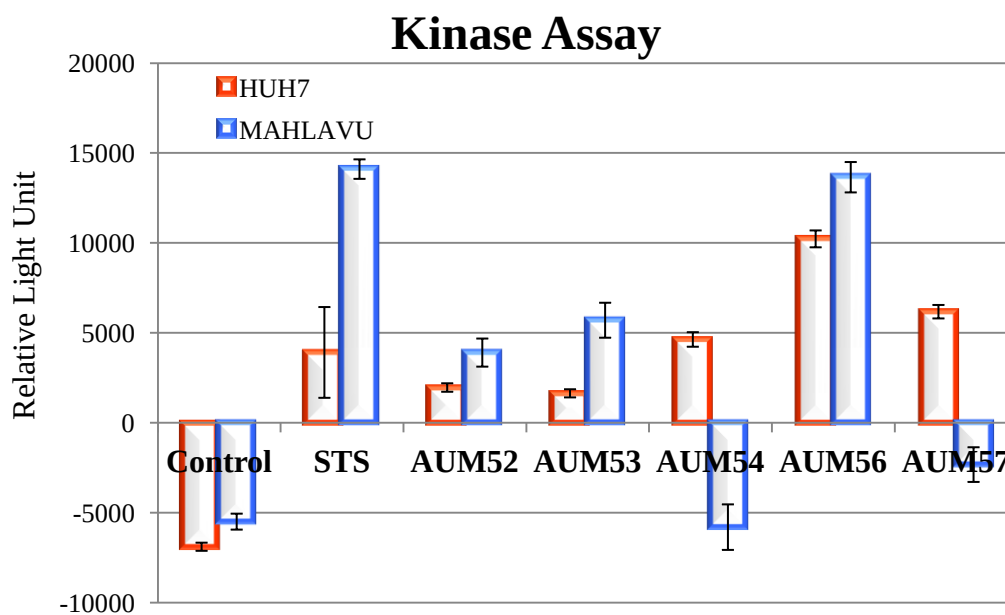


Figure 4.15: Kinase inhibitory activity of AUM52, AUM53, AUM54, AUM56 and AUM57: The relative light units measured after the kinase assay performed in duplicate for the Huh7 and Mahlavu cells treated with the purine derivatives at their given IC₁₀₀ values. STS; staurosporine (0.25 μ M) was used as positive control. Kinase GLO[®] assay kit was used to test the ATP content of 25 μ g protein containing cell lysates.

PART 2

4.2 Novel aminotriazole and thiadiazine derivatives

4.2.1 Anti-cancer drug screening of novel 4-amino-3-substituted-1,2,4-triazole-5-thiones and 3,6-disubstituted-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazines:

Novel aminotriazole and thiadiazine derivatives, coded as HUBT, were received in solid form. Following the preparation of their stock solutions in DMSO, each and every compound was initially screened against three different cancer cell types. Colon cancer cells (HCT116), breast cancer cells (T47D) and liver cancer cells (Huh7) were chosen to evaluate the cytotoxic potential of these small molecules via Sulforhodamine B (SRB) assay as described in the chapter of Materials and Methods (page 45). The half-maximal inhibitory concentration (IC₅₀) values of these molecules were given in Supplementary Table 9. The IC₅₀ values were calculated according to the dose-response curves obtained from the percent cell-death charts given in Supplementary Figure 9. Among the 32 newly synthesized small molecules, FAT-TM, ClAT-TM and n-AT-TM performed promising cytotoxic activities within μ M concentrations against Huh7 (HCC), HCT116 (colon) and T47D (breast) cancer cell lines. These three 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine derivatives were further tested for their anti-cancer activities on a HCC panel composed of Huh7, HepG2, Hep3B, PLC, SK-Hep1, Mahlavu, FOCUS, Snu182, Snu475 cancer cell lines. The cancer cells inoculated into 96-well plates were treated with FAT-TM, ClAT-TM, n-AT-TM and their DMSO controls in quadruplicate within at least 5 different concentrations for 72 hours. The cytotoxicity percentages demonstrate the bioactivity of these 3 small molecules, FAT-TM, ClAT-TM, n-AT-TM, against liver cancer cells (Supplementary Figure 10). ClAT-TM displays a promising cytotoxic profile against liver cancer cells. After 72 hour incubation, 1 μ M ClAT-TM cause more than 50% cytotoxicity in all HCC cells.

Afterwards, in order to inspect the time-dependent cytotoxic activities of these 3 small molecules, the same HCC panel was used. FAT-TM, ClAT-TM, n-AT-TM and their DMSO controls were applied to these liver cancer cells in

triplicate within 5 different concentrations for 24 and 48 hours (Supplementary Figure 11). The cytotoxicity percentages for all time points were used to calculate the time-dependent, cell-line-specific IC₅₀ values of FAT-TM, CIAT-TM and n-AT-TM (Table 4.7). Comparing to the other derivatives, n-AT-TM and FAT-TM, the Cl- substituted small molecule, CIAT-TM has a very promising cytotoxicity profile with the lowest IC₅₀ values of its group (Table 4.7).

Table 4.7: IC₅₀ values of FAT-TM, CIAT-TMN and n-AT-TM against HCC cell lines panel: Each molecule was tested in triplicate/quadruplicate. The IC₅₀ values are in μ M range and NI stands for “no inhibition”.

		IC ₅₀ (μ M)		
		FAT-TM	CIAT-TM	n-AT-TM
Huh7	24h	33.9 $\pm$ 18.4	< 0.1	4.4 $\pm$ 1.0
	48h	10.6 $\pm$ 6.0	< 0.1	1.6 $\pm$ 0.02
	72h	16.5 $\pm$ 1.1	0.3 $\pm$ 0.02	8.6 $\pm$ 0.5
HepG2	24h	NI	0.5 $\pm$ 0.4	10.0 $\pm$ 3.75
	48h	14.0 $\pm$ 8.8	< 0.1	3.6 $\pm$ 1.0
	72h	7.8 $\pm$ 2.2	0.2 $\pm$ 0.06	5.6 $\pm$ 0.7
Hep3B	24h	14.7 $\pm$ 7.2	1.2 $\pm$ 1.1	24.9 $\pm$ 16.2
	48h	NI	0.1 $\pm$ 0.04	13.7 $\pm$ 5.5
	72h	30.6 $\pm$ 3.6	0.8 $\pm$ 0.04	14.8 $\pm$ 2.0
PLC	24h	NI	2.1 $\pm$ 0.6	NI
	48h	NI	0.3 $\pm$ 0.1	NI
	72h	46.6 $\pm$ 8.0	1.7 $\pm$ 0.13	24.2 $\pm$ 4.1
SK-Hep1	24h	NI	5.3 $\pm$ 3.31	NI
	48h	NI	0.1 $\pm$ 0.02	42.9 $\pm$ 17.4
	72h	NI	6.1 $\pm$ 1.4	24.6 $\pm$ 3.5
Mahlavu	24h	27.6 $\pm$ 0.4	< 0.1	35.8 $\pm$ 11.8
	48h	NI	< 0.1	20.7 $\pm$ 3.1
	72h	NI	1.0 $\pm$ 0.05	21.4 $\pm$ 2.4
FOCUS	24h	NI	0.4 $\pm$ 0.7	33.9 $\pm$ 39.9
	48h	33.2 $\pm$ 6.7	< 0.1	12.8 $\pm$ 5.2
	72h	41.7 $\pm$ 3.3	0.6 $\pm$ 0.04	13.7 $\pm$ 0.4
Snu182	24h	NI	2.3 $\pm$ 1.2	NI
	48h	NI	< 0.1	NI
	72h	18.2 $\pm$ 9.7	1.1 $\pm$ 0.43	12.4 $\pm$ 3.9
Snu475	24h	NI	0.5 $\pm$ 0.2	NI
	48h	NI	< 0.1	15.5 $\pm$ 1.0
	72h	26.6 $\pm$ 4.8	0.5 $\pm$ 0.1	15.1 $\pm$ 3.2

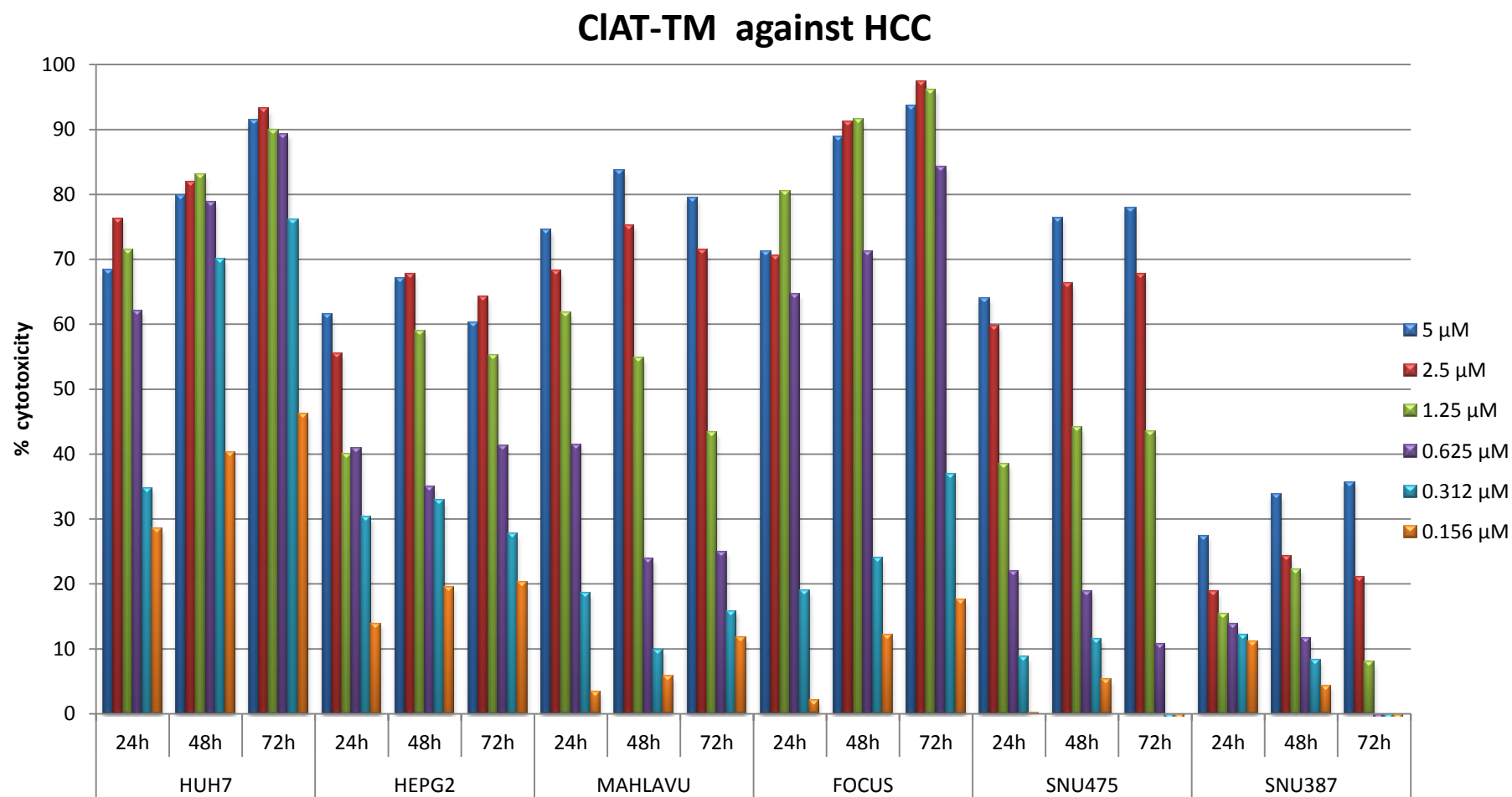


Figure 4.16: The time- and dose- dependent cytotoxicity of CIAT-TM on Huh7, HepG2, Mahlavu, FOCUS, Snu475 and Snu387: Liver cancer cells were inoculated in 96-well plates and then, 6 different concentrations of CIAT-TM and their DMSO controls were administered to the cells in triplicates for 24, 48 and 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

Table 4.8: IC₅₀ values of CIAT-TM against liver cancer cells: were calculated from the cytotoxicity percentages (Figure 4.16) obtained with 6 different concentrations of CIAT-TM incubated for 24, 48 and 72 hours. The IC₅₀ values are in μM range.

		IC ₅₀ (μM)
Huh7	24h	0.7 ± 0.2
	48h	0.2 ± 0.1
	72h	0.2 ± 0.0
HepG2	24h	1.9 ± 0.8
	48h	1.1 ± 0.2
	72h	1.3 ± 0.3
Mahlavu	24h	1.3 ± 0.3
	48h	1.2 ± 0.1
	72h	1.3 ± 0.2
FOCUS	24h	0.8 ± 0.1
	48h	0.5 ± 0.1
	72h	0.4 ± 0.0
Snu475	24h	2.1 ± 0.2
	48h	1.6 ± 0.2
	72h	1.7 ± 0.2
Snu387	24h	4.7 ± 1.0
	48h	1.3 ± 0.2
	72h	2.5 ± 0.4

4.2.2 The kinase inhibitory potential of CIAT-TM

Protein kinases, as being responsible for the phosphorylation of various proteins, play one of the major roles in cell signaling networks. Through chemically adding phosphate groups to proteins, they manage many cellular processes such as cell growth, proliferation, division, differentiation and apoptosis. It is known that, synthetic 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine derivative CIAT-TM was designed as a putative protein kinase inhibitor. The differential IC₅₀ values it has against HCC panel might be a consequence of its interference with cell signaling, since these cell lines have individual characteristics of cell signaling activities. Taking all these into consideration, we performed a kinase assay, based

on the detection of the amount of ATP in the reaction mixture through bioluminescence (Figure 4.4), to assess the protein kinase inhibitory potential of CIAT-TM. The liver cancer cells were incubated with CIAT-TM at the calculated cell-line-specific IC₅₀ and IC₁₀₀ values, staurosporine (STS) and aminopurvalanol-A for 24 hours (Figure 4.17). STS is a potent, unselective, multi-kinase inhibitor which stimulates both caspase dependent and independent apoptosis while aminopurvalanol-A, a purvalanol derivative, reversibly inhibits cyclin dependent kinases, causes cell cycle arrest in G2/M phase but does not induce massive apoptosis. These two commercially available protein kinase inhibitors were chosen as positive controls because of their distinctive kinase inhibitory potentials. The results we obtained suggests that CIAT-TM might be a putative protein kinase inhibitor acting on liver cancer cells.

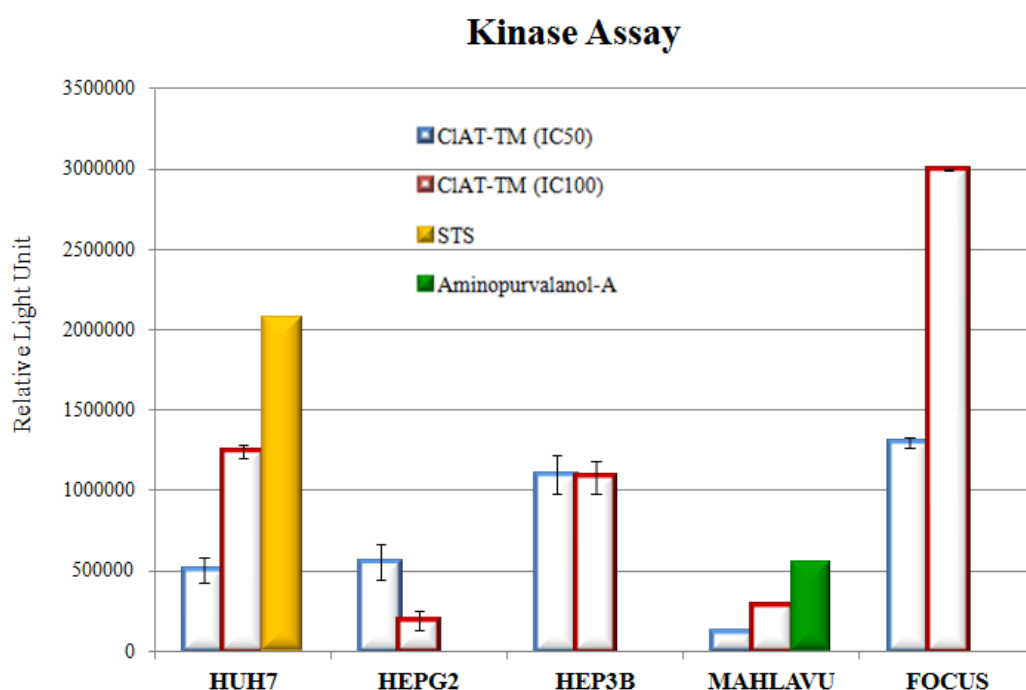
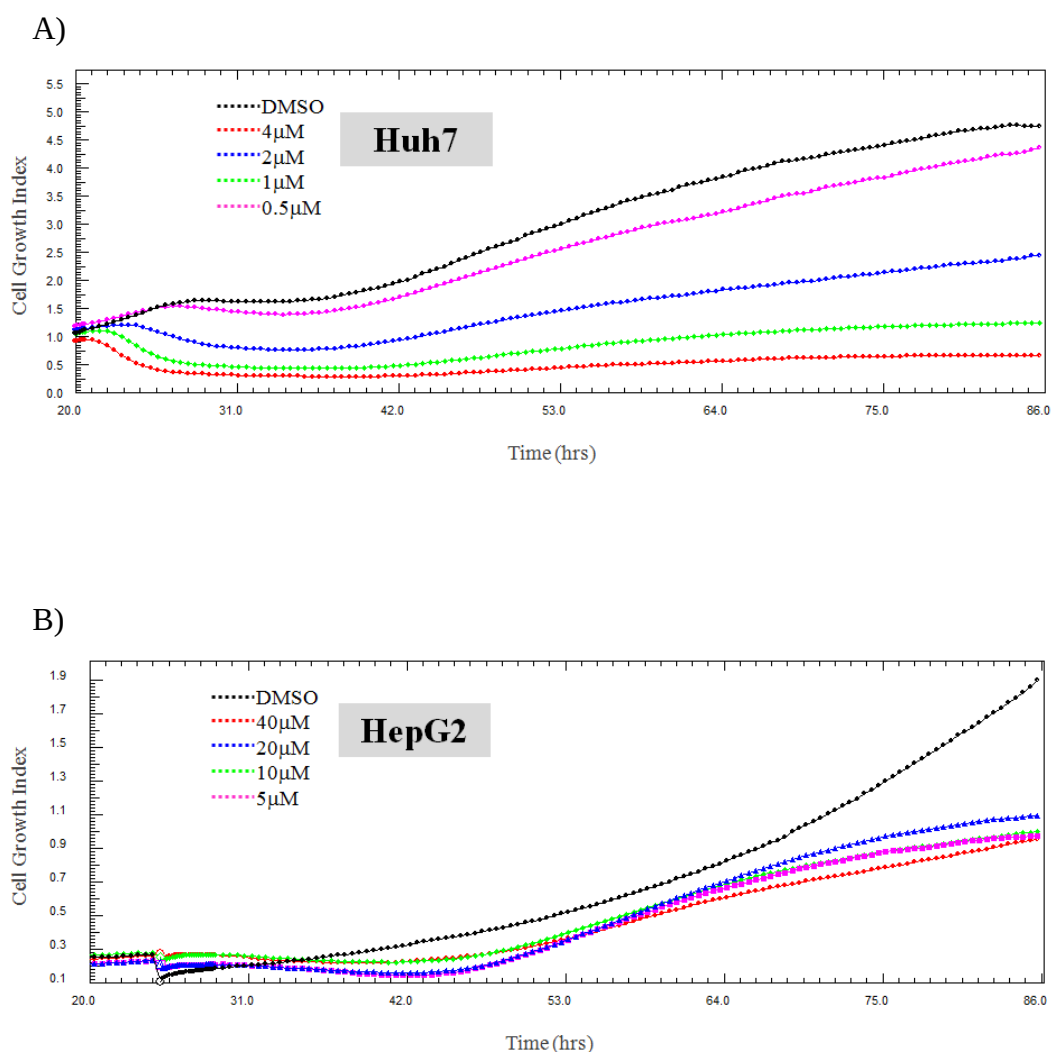


Figure 4.17: Kinase inhibitory potential of CIAT-TM against HCC cell lines:

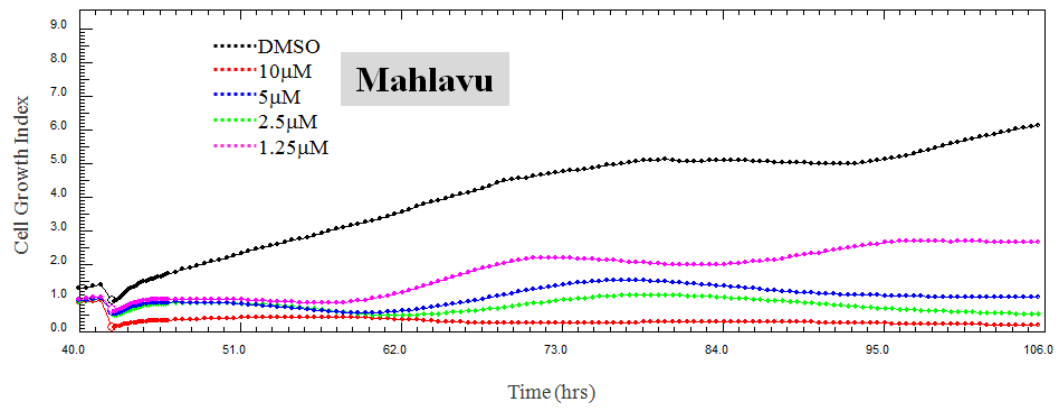
The relative light units were measured after the kinase assay performed in duplicate for the Huh7, HepG2, Hep3B, Mahlavu and FOCUS cells treated with CIAT-TM at their calculated IC₅₀ and IC₁₀₀ values. STS; staurosporine (1 μ M) and aminopurvalanol-A (2.5 μ M) were used as positive controls for Huh7 and Mahlavu cells respectively. Lonza PKLightTM assay kit was used to test the ATP content of 10 μ g protein containing cell lysates.

4.2.3 Real-time cell growth analysis in the presence of CIAT-TM

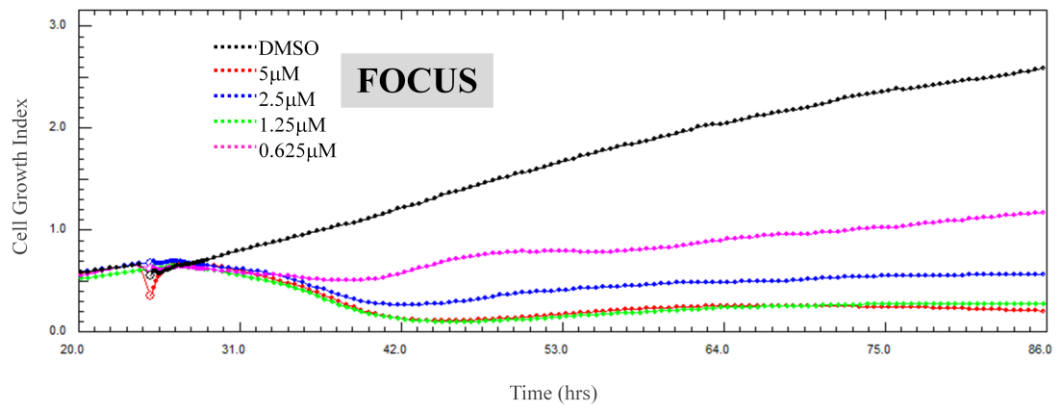
To figure out the real-time cytotoxic effect of CIAT-TM on a panel of liver cancer cells, we used the label-free, real-time cell electronic sensing (RT-CES) system which utilizes an impedance read-out to quantify the cell growth. Dynamic monitoring of the logarithmic growth of these cells that correlates with cell index (CI), requires these cell lines to be adherent. Cell growth and proliferation directly correlate with the strength of cell attachment. Huh7, HepG2, Mahlavu, Focus, Snu475 and Snu387 cancer cells were inoculated into these E-Plate 96 and the data recorded demonstrates that CIAT-TM clearly suppresses the cell growth index values in a dose- and cell-line- dependent manner (Figure 4.18).



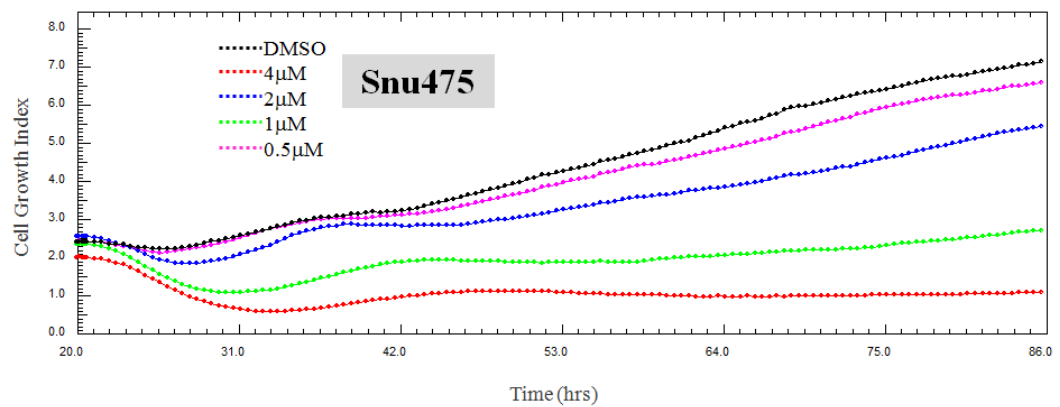
C)



D)



E)



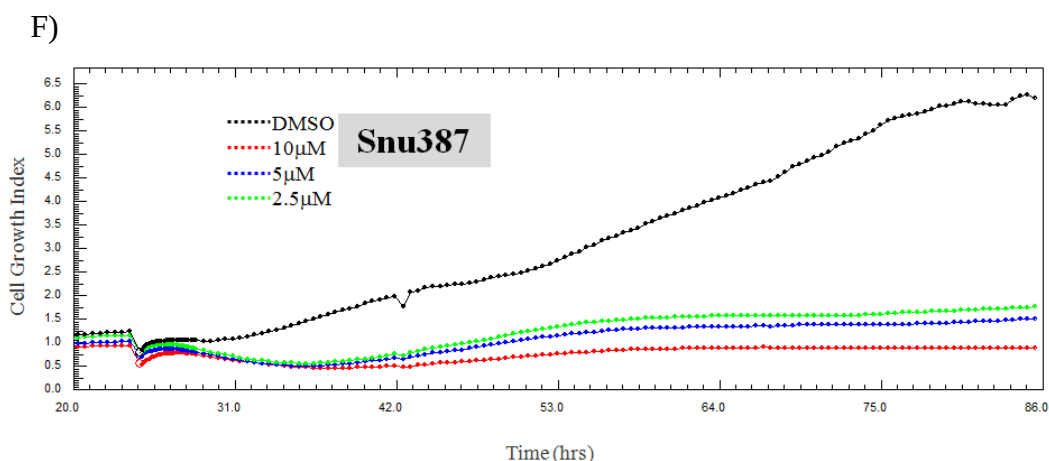


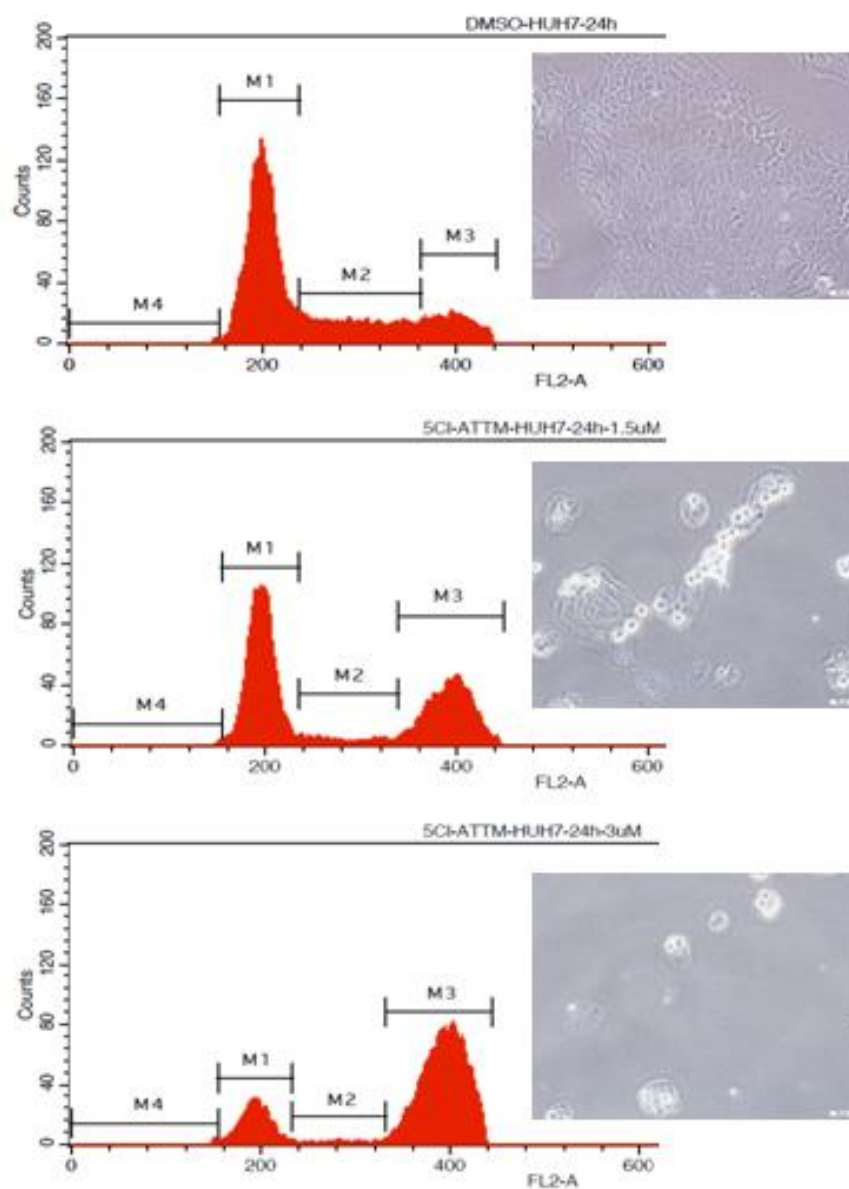
Figure 4.18: Dynamic monitoring of ClAT-TM treated liver cancer cells: ClAT-TM and its DMSO control were administered to Huh7 (A), HepG2 (B), Mahlavu (C), FOCUS (D), Snv475 (E) and Snv387 (F) cells, inoculated in E-Plate 96, in triplicates. The cell growth index was monitored every 30 minutes in the presence of different concentrations of ClAT-TM.

4.2.4 ClAT-TM induces G2/M cell-cycle arrest

All the preliminary and further analysis results confirm that ClAT-TM triggers cytotoxicity in a dose- and time- dependent manner. In order to enlighten the triggered mechanism beneath this cell death, we checked the distribution of the cells in cell-cycle stages in the presence of ClAT-TM. Hence, we used propidium iodide (PI), a DNA-intercalating, fluorescent agent, commonly used to identify the cell-cycle distribution patterns of a cell suspension. PI labeling of the nuclei of cells in suspension allows correlating the fluorescence intensity with the cell-cycle stages. While quiescent (G0) and G1 cells have one copy of DNA, G2/M cells double their DNA. The cells in S phase have varying numbers of DNA since they synthesize DNA in the meantime. Cell-cycle analysis of ClAT-TM treated HCC cells demonstrated that this novel, putative kinase inhibitor induces time- (data not shown) and dose- dependent G2/M arrest (Figure 4.19). Flow cytometric determination of DNA content for each condition was analyzed and the percentages of cells in each cell cycle stage were given as an overall chart (Figure 4.19-G).

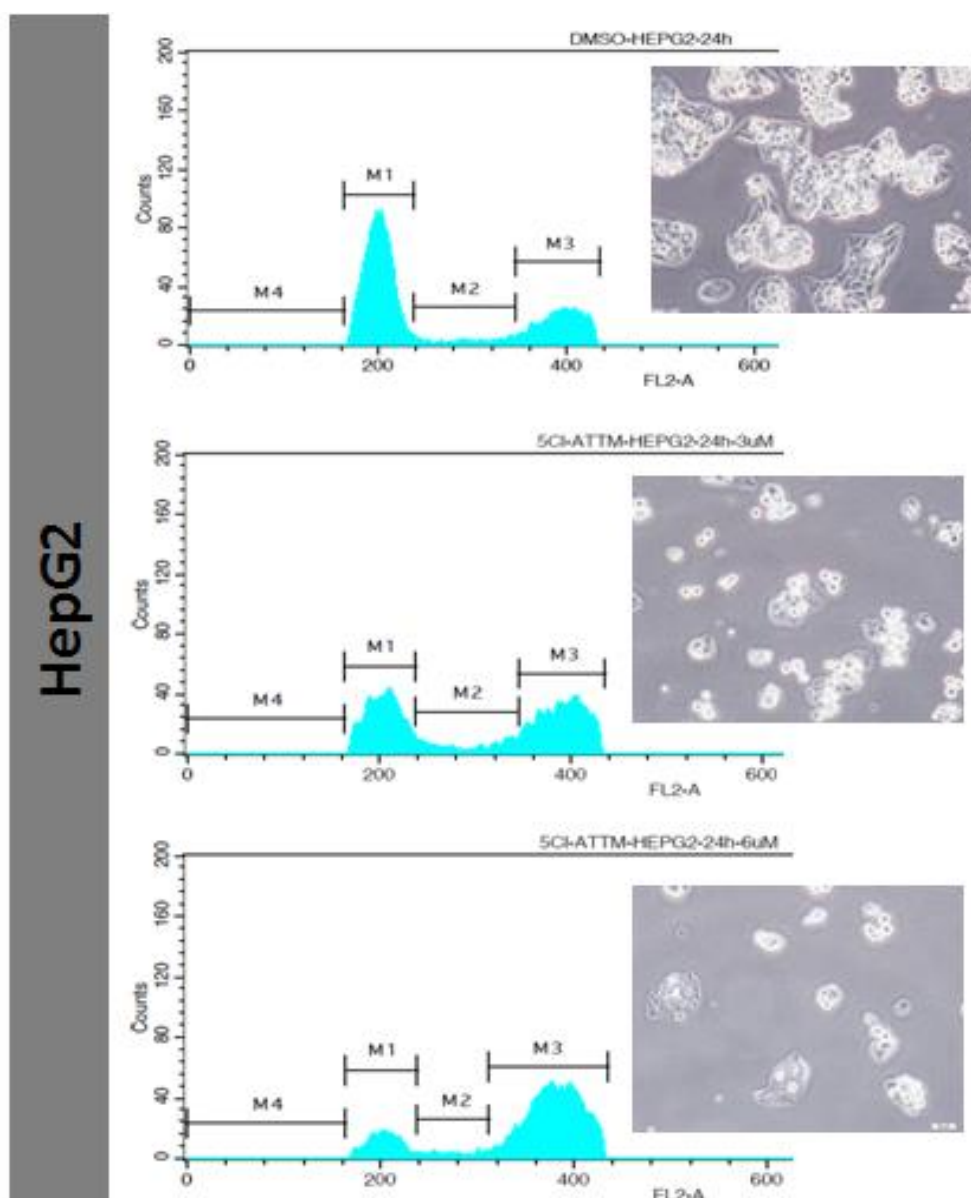
A)

Huh7



Huh7	DMSO	CIAT-TM (IC50)	CIAT-TM (IC100)
G1	62,26	55,24	18,52
S	23,58	6,04	3,18
G2/M	14,51	38,75	78,08
sub-G1	0,23	0,21	0,38

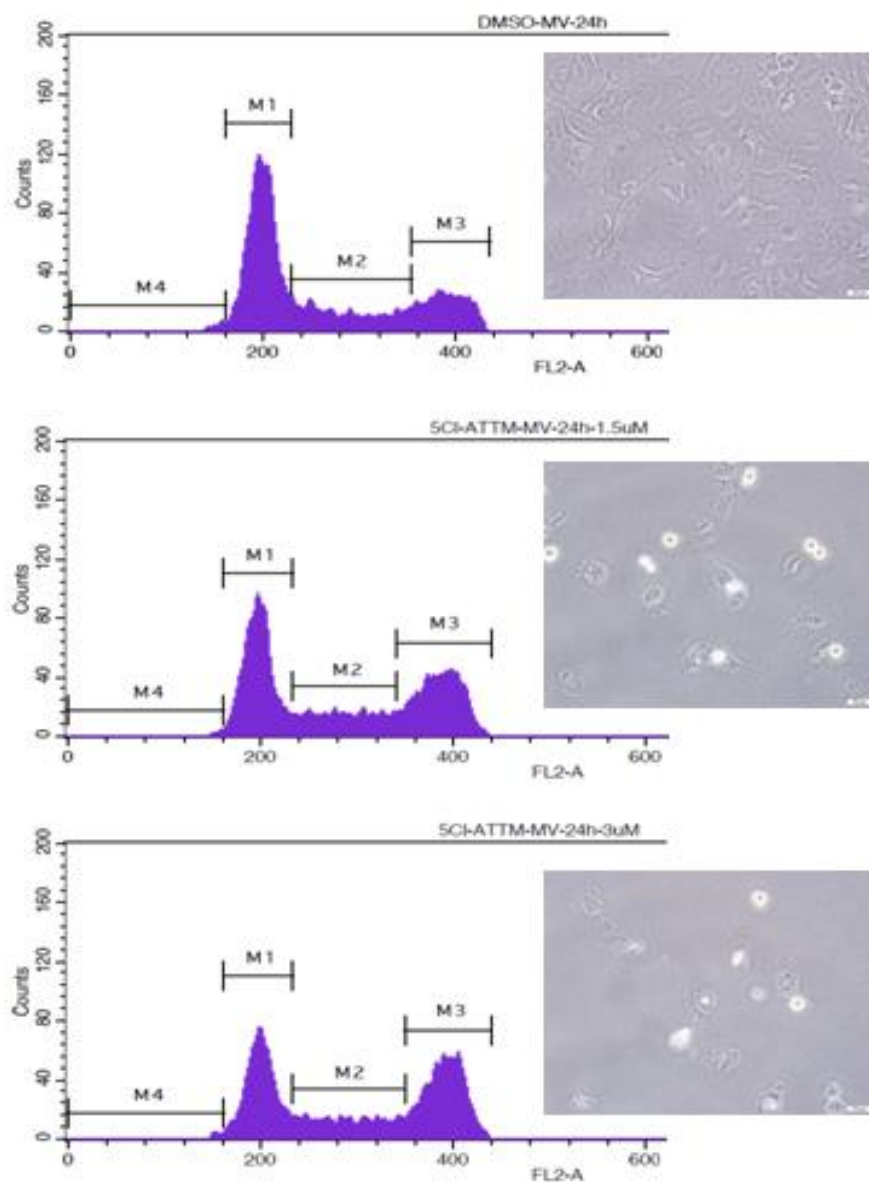
B)



HepG2	DMSO	CIAT-TM (IC50)	CIAT-TM (IC100)
G1	65,93	40,86	15,95
S	5,88	11,56	4,9
G2/M	28,42	48,12	79,27
sub-G1	0	0	0

C)

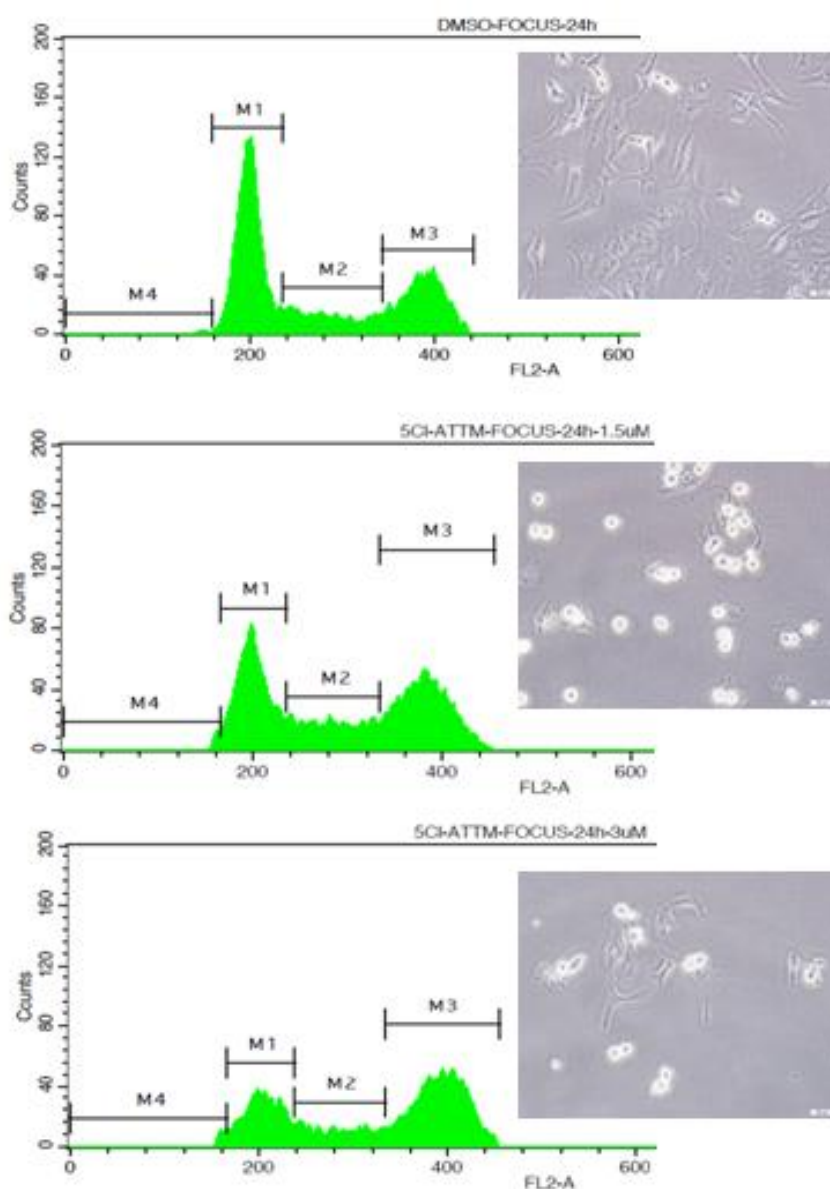
Mahlavu



Mahlavu	DMSO	CIAT-TM (IC50)	CIAT-TM (IC100)
G1	55,89	43,38	36,98
S	22,5	21,03	21,65
G2/M	21,24	35,57	41,17
sub-G1	0,97	0,42	0,6

D)

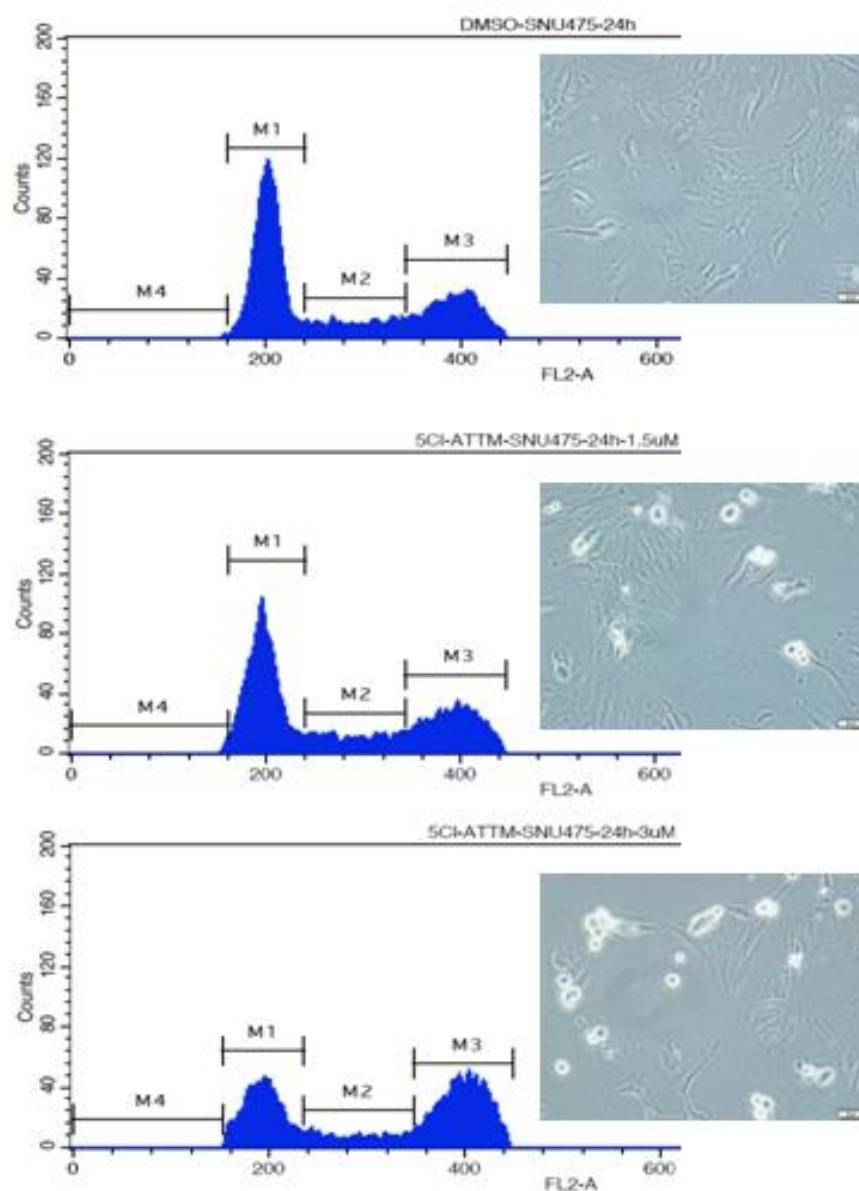
FOCUS



FOCUS	DMSO	CIAT-TM (IC50)	CIAT-TM (IC100)
G1	55,24	37,12	28,01
S	15,59	21,43	16,54
G2/M	29,28	40,94	54,56
sub-G1	0,2	1,07	1,59

E)

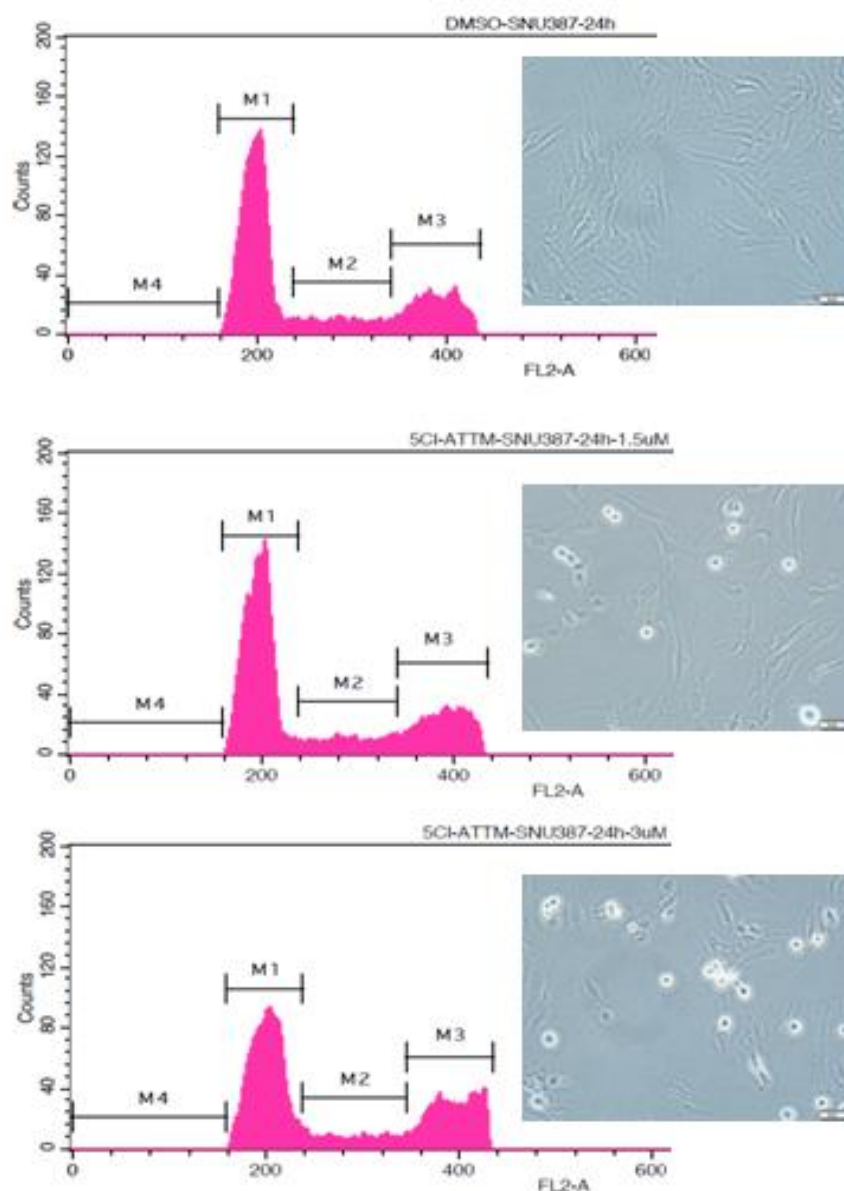
Snu475



Snu475	DMSO	CIAT-TM (IC50)	CIAT-TM (IC100)
G1	55,24	51,34	36,61
S	15,59	16,57	14,15
G2/M	29,28	32,02	49,46
sub-G1	0,2	0,58	0

F)

SNU 387



Snu 387	DMSO	CIAT-TM (IC50)	CIAT-TM (IC100)
G1	62,21	61,66	55,86
S	13,12	12,92	12,15
G2/M	25,09	25,75	32,28
sub-G1	0	0	0

G)

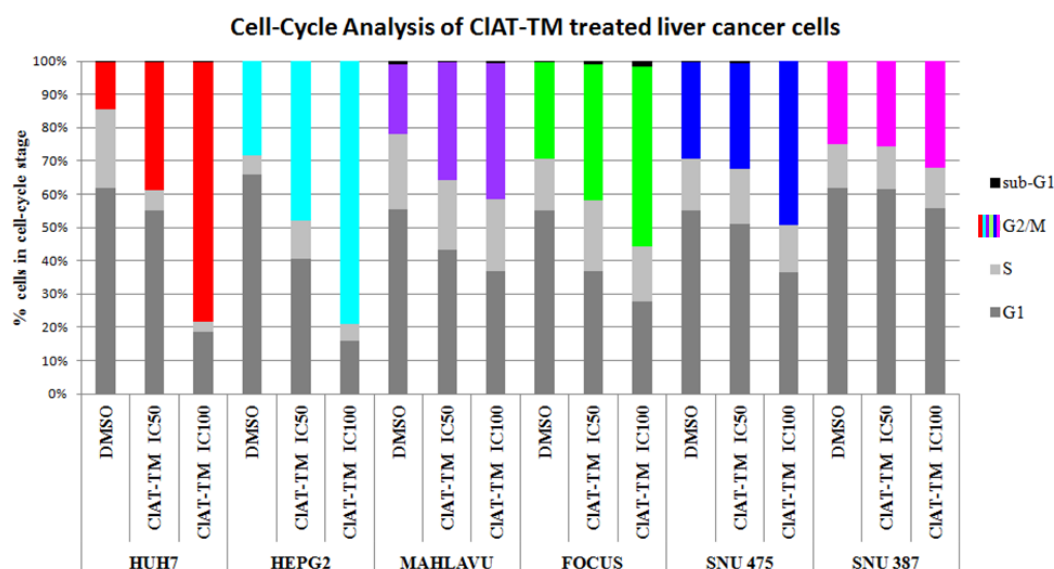
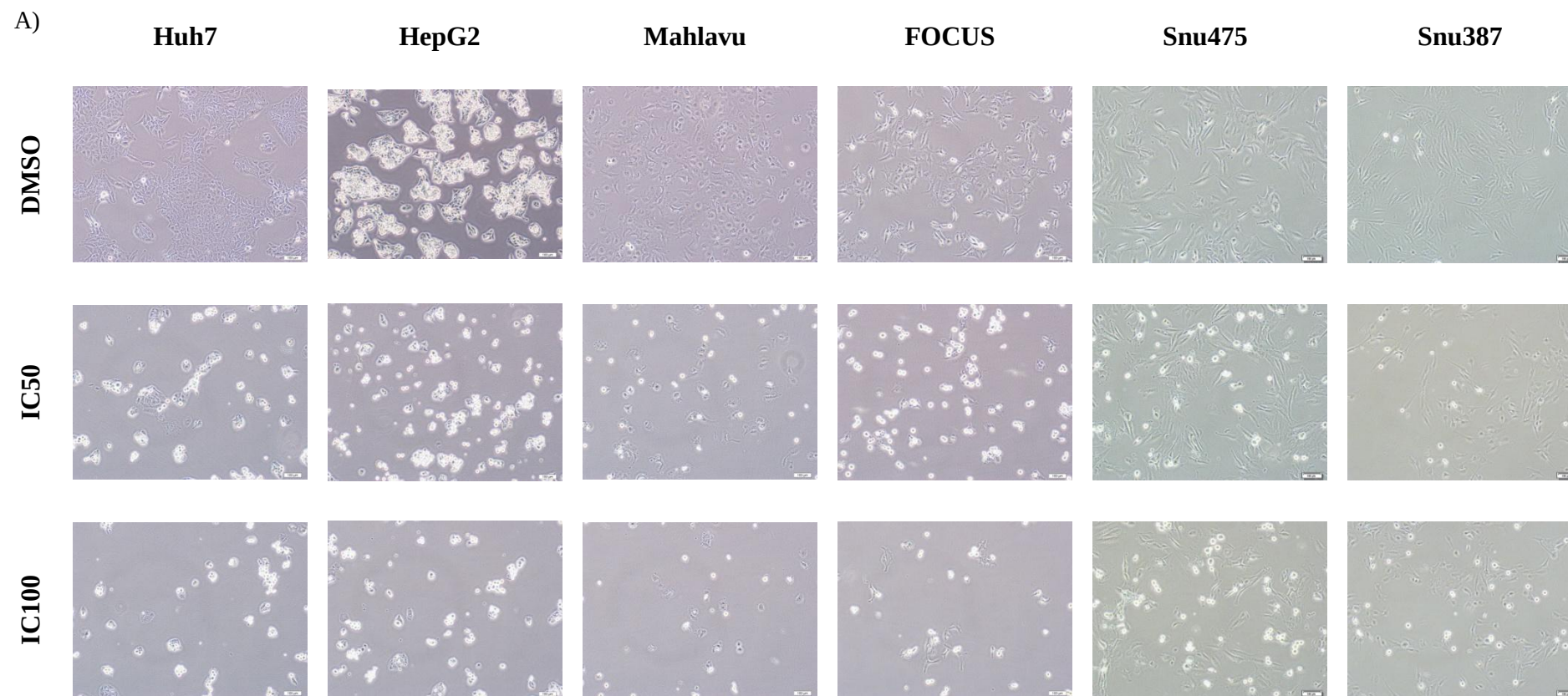


Figure 4.19: Cell-cycle analysis of CIAT-TM treated HCC cells: Huh7 (A), HepG2 (B), Mahlavu (C), Focus (D), Snu475 (E) and Snu387 (F) cancer cells were treated with IC50 and IC100 values of CIAT-TM and their DMSO controls for 24 hours. Next, fixed cells were stained with propidium iodide (PI) prior to flow cytometry analysis. M1, M2, M3 and M4 stands for G1, S, G2/M and sub-G1 (apoptotic cells) respectively. The peaks at 200 and 400 FL2-A signifies 2N cells (G1) and 4N cells (G2) respectively. Percentage of cells in cell-cycle stage was analyzed by CELLQuest and given as an overall chart (G). Cell-line special colors Huh7 (red), HepG2 (cyan), Mahlavu (purple), Focus (green), Snu475 (blue) and Snu387 (pink) are representing G2/M stage, while G1 (dark gray), S (light gray), sub-G1 (black) are shown in same colors for all cell lines. The images were taken with 20x objective.

CIAT- TM induced cytotoxicity also changed the morphology of the cancer cells obviously. Rounded, swollen and eventually detached cells were accumulated. (Figure 4.20). The cell morphology in the presence of CIAT-TM is distinctive when compared to DMSO treated cells. It is clear that upon the application of CIAT-TM, cell proliferation ceases and depending on the dose and incubation time blebbing cells and apoptotic bodies appear. The morphology of the CIAT-TM treated liver cancer cells is correlative with the G2/M arrest they experience upon treatment (Figure 4.19 and 4.20).



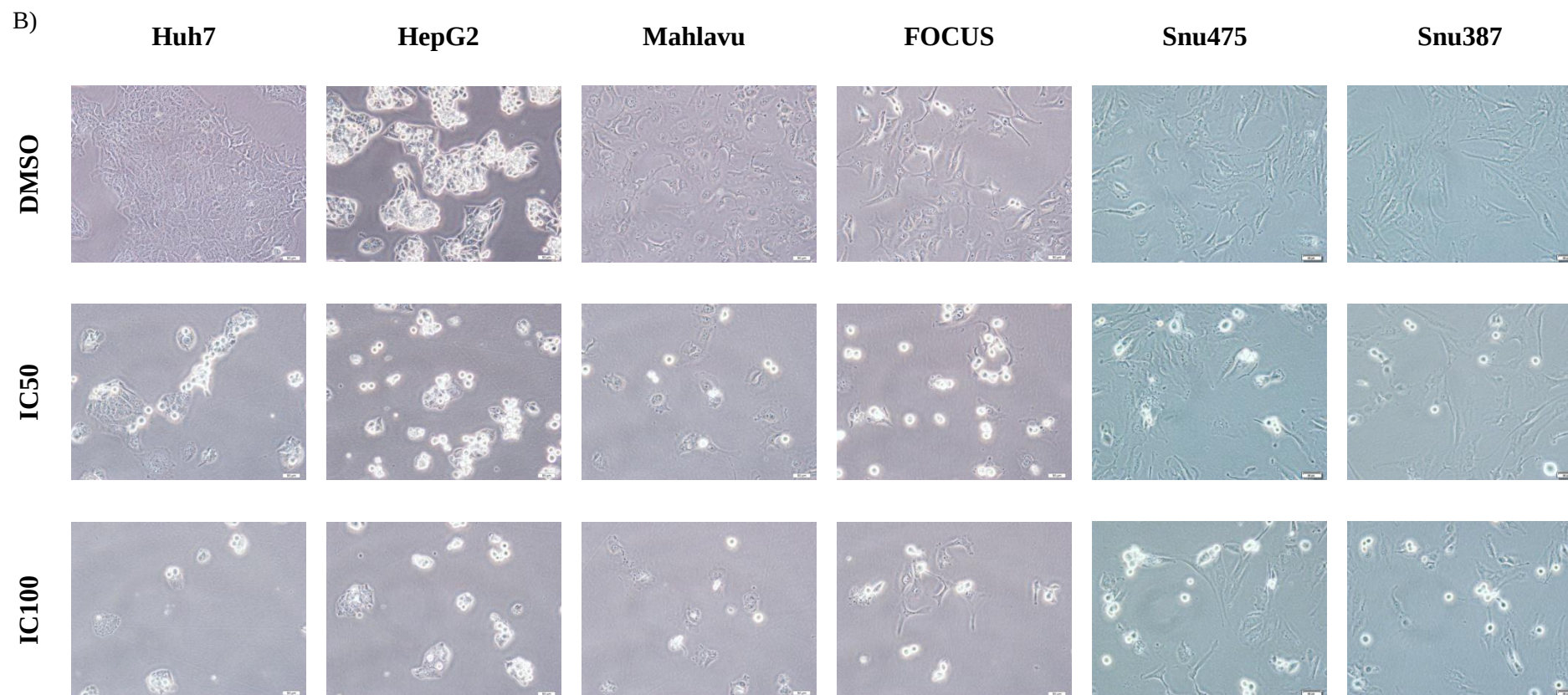


Figure 4.20 : Morphological effect of CIAT-TM on liver cancer cell lines : Cancer cell lines were incubated with DMSO and CIAT-TM at its IC50 and IC100 values of the corresponding cell line for 24 hours. The images were taken with both 10x (A) and 20x (B) objectives.

4.2.5 CIAT-TM induces apoptosis

In addition to the dramatic changes in the morphology of CIAT-TM treated cells, we performed a western blot analysis to verify the CIAT-TM induced apoptosis in the protein level. Unlike the drug treatments done so far, this time we incubated the cells with 2.5 μ M CIAT-TM (not with cell-line-specific IC50/IC100 values of) for 24 hours. The activated-apoptotic pathway indicator, cleaved Poly-ADP-ribosyl-polymerase (PARP) fragments, were detectable in most of the HCC cells treated with CIAT-TM (Figure 4.21).

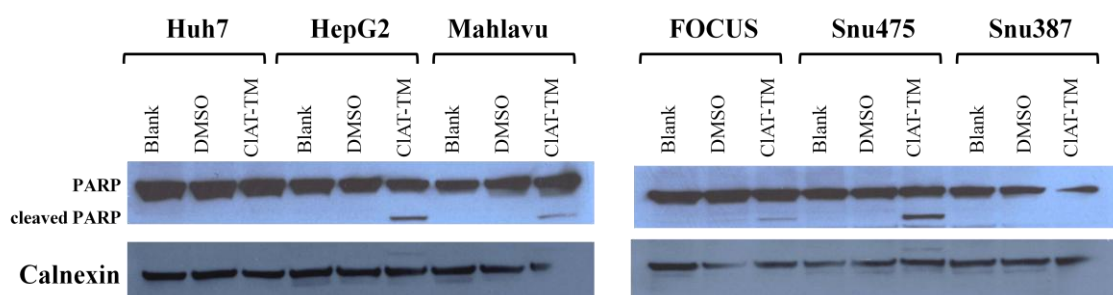


Figure 4.21 : Western blot analysis of CIAT-TM treated HCC cells for cleaved PARP:

All HCC cell lines were treated with 2.5 μ M CIAT-TM, its corresponding DMSO and left untreated for 24 hours. Cleaved PARP's expression levels were detected. Calnexin protein was used as an equal loading control.

Although PARP cleavage was detectable in CIAT-TM treated liver cancer cells, CIAT-TM did not induce sub-G1 accumulation, which is characteristic for the “typical” apoptosis pattern (Figure 4.19 and 4.21). “Atypical” apoptosis pattern has been reported to occur in cells treated with acetylsalicylate or aspirin like non-steroidal anti-inflammatory drugs (NSAID) (Shiff SJ et al., 1995; Smith ML et al., 2000; Reinacher-Schick A et al., 2003). It was previously demonstrated that, synthetic 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine derivative CIAT-TM is a NSAID with analgesic and anti-inflammatory activities (Tozkoparan B et al., 2009).

4.2.6 Not the endoplasmic reticulum (ER), but the oxidative stress is the underlying mechanism of ClAT-TM induced cell death

ER-stress and the accumulation of reactive oxygen species (ROS) are the most typical underlying mechanisms triggering cells to G2/M arrest and eventually to apoptosis (Bouruugaa K et al, 2010; Wu SH et al, 2010; Macip S et al, 2006). One of the alternative methods to detect ER-stress is to trace the XBP-1 splicing via semi-quantitative PCR (Samali A et al., 2010). Huh7 cells were treated with ClAT-TM at its IC₅₀ and IC₁₀₀ values, DMSO and 500ng/mL tunicamycin (TN) as positive control for 24 hours. Tunicamycin was used as positive control at ER stress-inducing dose (Ilieva EV et al., 2007). Although spliced XBP-1 mRNA could be detected with TN treated cells, ClAT-TM did not induce ER-stress-specific XBP-1 splicing (Figure 4.22).

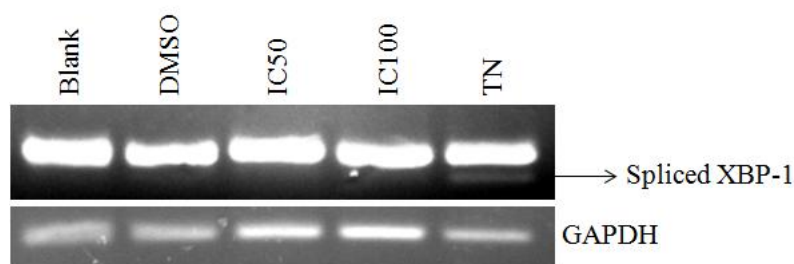
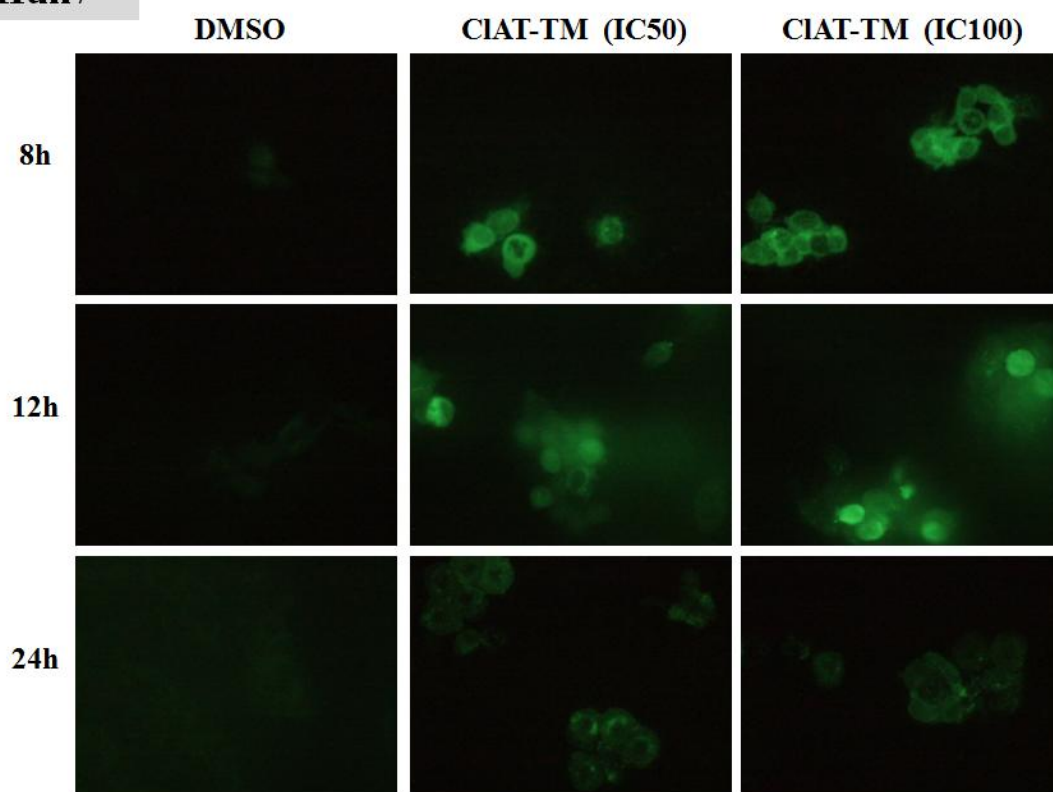


Figure 4.22 : Endoplasmic reticulum (ER) stress detection assay with ClAT-TM treated Huh7 cells: Huh7 cells untreated and treated with ClAT-TM at its IC₅₀ and IC₁₀₀ values, the corresponding DMSO, and the positive control tunicamycin (TN) were checked for splicing variants of XBP-1 as an indicator of ER stress.

ROS, the major players of oxidative stress, is known to induce G2/M arrest and apoptosis (Lee et al, 2009). We aimed to detect the accumulation of ROS, if the triggered mechanism upon ClAT-TM treatment is oxidative stress. Huh7 and Mahlavu liver cancer cells were treated with the IC₅₀ and IC₁₀₀ values of ClAT-TM and DMSO for 8, 12 and 24 hours. At the termination of each incubation period, oxidant-sensitive, non-fluorescent dye DCFH-DA was administered to the cells. Oxidation of DCFH-DA to DCF, which gives green fluorescent signals, visualizes oxidative stress (Figure 4.23).

Huh7



Mahlavu

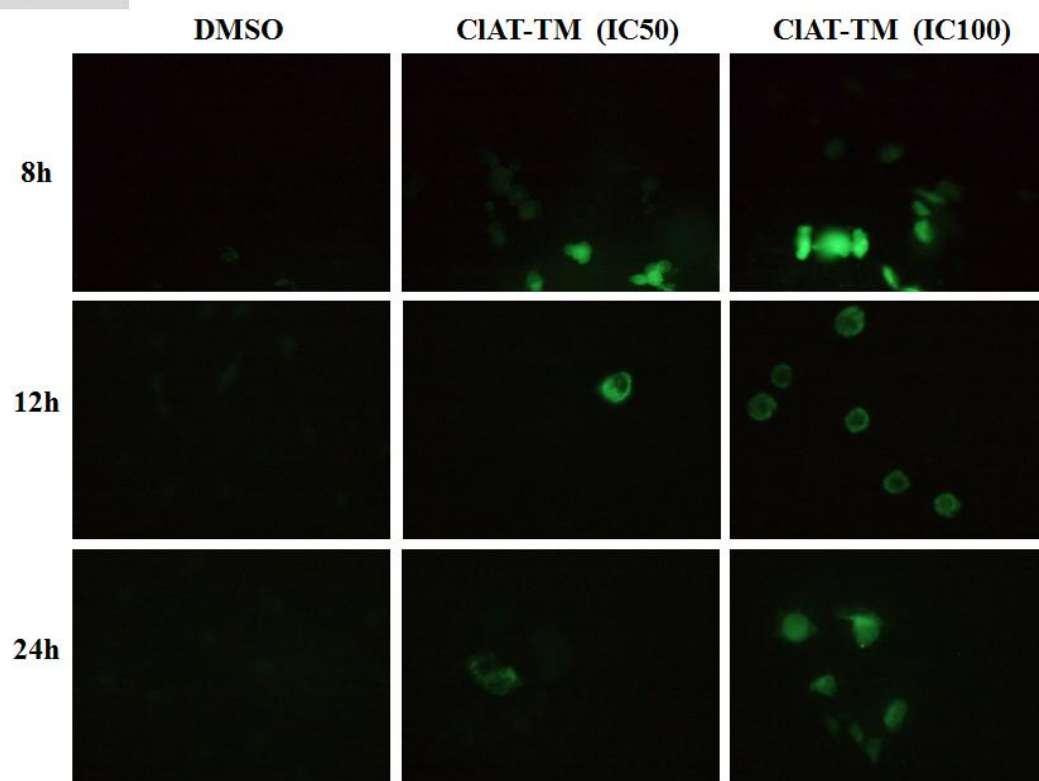


Figure 4.23: ROS assay images of CIAT-TM treated liver cancer cells : Both Huh7 (A) and Mahlavu (B) HCC cells CIAT-TM (IC50 and IC100) and corresponding DMSO for 8, 12 and 24 hours. Microscopic images were taken with 20x objectives following DCFH-DA staining.

4.2.7 CIAT-TM induces the phosphorylation of c-Jun N-terminal kinase

Accumulation of ROS has long been correlated with permanent c-Jun N-terminal kinase (JNK) activation (Zhang Y and Chen F, 2004). Moreover it is reported that JNK activation is vital for the induction of G2/M arrest (Cho SD et al, 2009 ; Yamamoto K et al, 1999). To find out the influence of CIAT-TM on JNK activation we checked the phosphorylated-JNK (P-JNK) protein levels by means of western blotting. Huh7, HepG2, Mahlavu and FOCUS liver cancer cells were treated with IC100 values of CIAT-TM, JNKInhV at its cell-line-specific IC50 values (Huh7: 2 μ M, HepG2: 2 μ M, Mahlavu: 5 μ M, FOCUS: 0.5 μ M) and corresponding DMSO for 24 hours. CIAT-TM increased the phosphorylated-JNK protein levels in both cell-line- and JNK isoform- dependent manner (Figure 4.24).

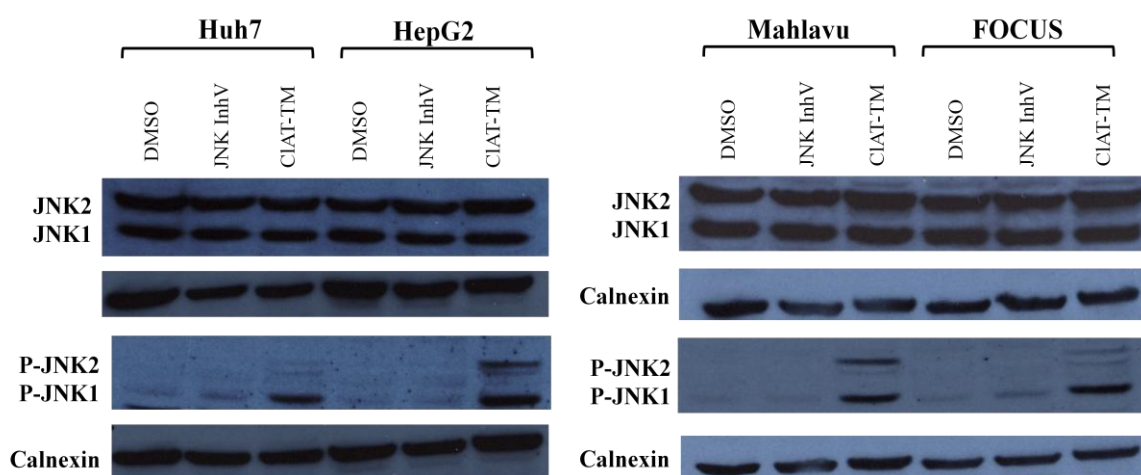


Figure 4.24: Effect of CIAT-TM on the expression and activation of c-Jun N-terminal kinase: Huh7, HepG2, Mahlavu and FOCUS cells were treated with CIAT-TM (with IC100 values), JNKInhV (with cell line specific IC50) and corresponding DMSO for 24 hours. Calnexin was used as equal loading control.

Activated JNK1 and JNK2 isoforms translocate to the nucleus and there phosphorylate transcription factor c-Jun. Therefore, we verified the ClAT-TM induced JNK activation by evaluating the phosphorylated-c-Jun (P-c-Jun) protein levels. We treated Huh7, HepG2, Mahlavu, FOCUS, Snu475 and Snu387 cells, with 2.5 μ M ClAT-TM and corresponding DMSO for 24 hours. The western blotting analysis performed subsequently clearly demonstrated the ClAT-TM induced phosphorylation of c-Jun (Figure 4.25).

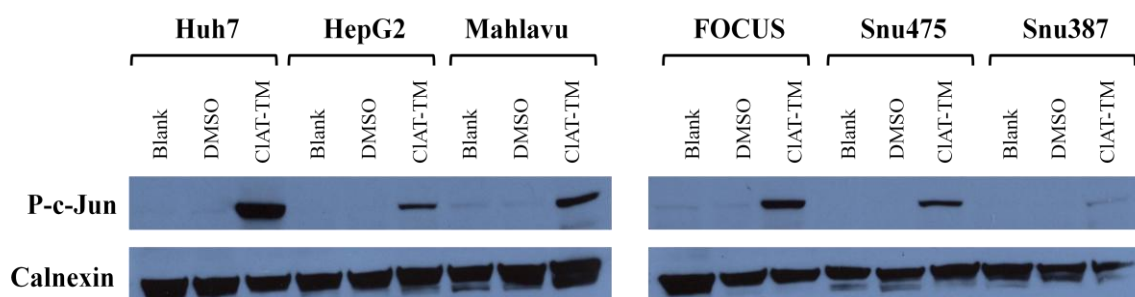


Figure 4.25: The phosphorylated-c-Jun protein levels upon ClAT-TM treatment: Huh7, HepG2, Mahlavu, FOCUS, Snu475 and Snu387 cells were treated with ClAT-TM (2.5 μ M), corresponding DMSO and left un-treated for 24 hours. Calnexin was used as equal loading control.

4.2.8 The upstream components of JNK signaling pathway in the presence of ClAT-TM

To further investigate how JNK signaling is activated, we checked the upstream components of JNK pathway. It has been reported that JNKs are phosphorylated at their Thr and Tyr residues which are conserved in a dual phosphorylation Thr-Pro-Tyr motif of their activation loop (Kallunki T et al., 1996). MAP2Ks, in other words MKK4 and MKK7, are the activators of JNKs (Figure 1.18). TNF α mostly stimulate MKK7 which phosphorylates only JNKs; whereas, other environmental stress factors activate MKK4 which in turn phosphorylates both JNKs and p38 (Min L et al., 2010; Haeusgen W et al., 2011). To find out the activator of JNK in the presence of ClAT-TM, we checked both the phosphorylated-MKK7 (P-MKK7) and phosphorylated-MKK4 (P-MKK4) protein levels by means of western blotting. Huh7, HepG2, Mahlavu and FOCUS liver cancer cells were treated with cell-line-specific IC₅₀ and IC₁₀₀ values of ClAT-TM and corresponding DMSO for 24 hours. ClAT-

TM increased the P-MKK7 protein levels in a cell-line-dependent manner; despite, no difference was observed in the P-MKK4 protein levels (Figure 4.26).

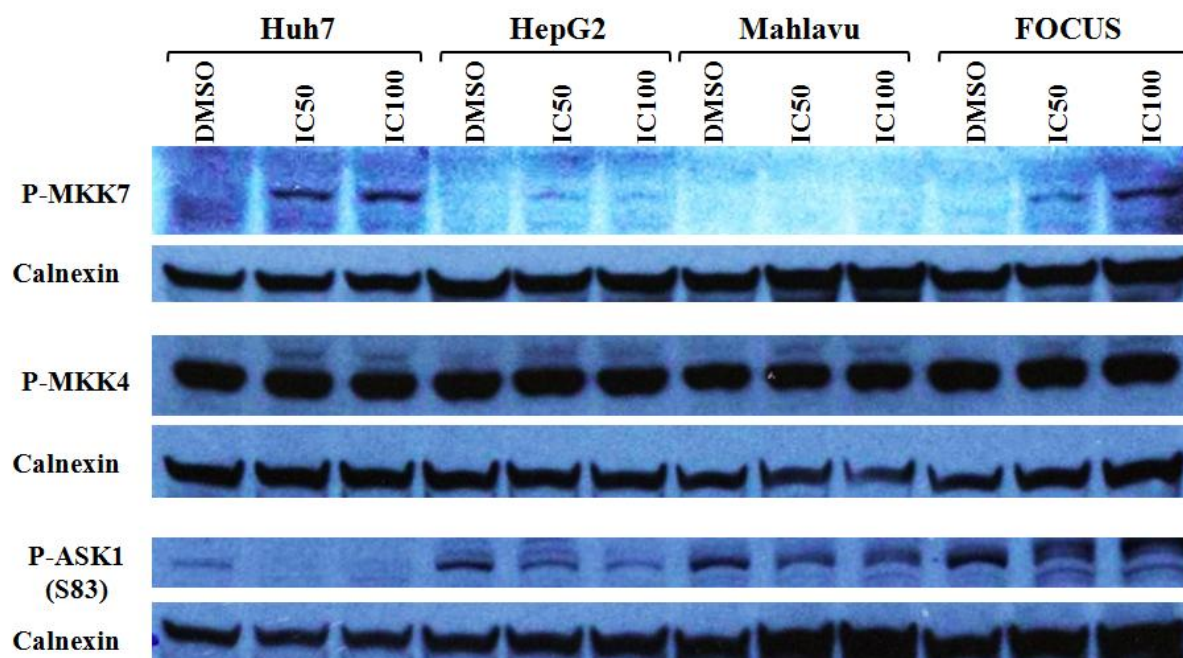


Figure 4.26: The protein levels of phosphorylated-MKK7, phosphorylated-MKK4 and phosphorylated-ASK1(S83) upon ClAT-TM treatment: Huh7, HepG2, Mahlavu and FOCUS cells were treated with ClAT-TM (with IC50 and IC100 values) and corresponding DMSO for 24 hours. Calnexin was used as equal loading control.

One of the MAP3Ks, ASK1, might be the upstream activator of MKK7 which has been confirmed as the activator of JNK upon ClAT-TM treatment. ASK1 is composed of an internal kinase domain, a regulatory domain and an inhibitory domain with three separate phosphorylation sites. Thus, pro-survival factors intend to keep ASK1 in an inactive status while pro-apoptotic ones do the opposite. In order to activate ASK1, phosphorylation at Thr845 is required, whereas phosphorylation at either Ser967 or Ser83 maintains ASK1 inactive (Zhang R et al., 2005). We checked the protein levels of phosphorylated-ASK1 at Ser83 (P-ASK1 at S83) through western blotting. Huh7, HepG2, Mahlavu and FOCUS liver cancer cells were treated with cell-line-specific IC50 and IC100 values of ClAT-TM and corresponding DMSO for 24 hours. ClAT-TM decreased the protein levels of P- ASK1 at S83 in a cell-line-dependent manner (Figure 4.26).

Furthermore, it has been demonstrated that Akt phosphorylates ASK1 at Ser83 in order to negatively regulate apoptosis (Kim AH et al., 2001). With respect to this data, we hypothesized that ClAT-TM might have inhibitory effect on Akt which in turn prevent the phosphorylation of ASK1 at Ser83. Hence, we checked the protein levels of phosphorylated-Akt (P-Akt) at Ser473 through western blotting. Huh7, HepG2, Mahlavu and FOCUS liver cancer cells were treated with cell-line-specific IC50 and IC100 values of ClAT-TM and corresponding DMSO for 24 hours. Indeed, ClAT-TM decreased the levels of P-Akt (Figure 4.27). Hyperactivation of Akt in Mahlavu cancer cells has been associated with PTEN loss (Buontempo F et al., 2010). Therefore, in order to catch the cell line-dependent differences in the P-Akt levels, different exposure times were presented collectively.

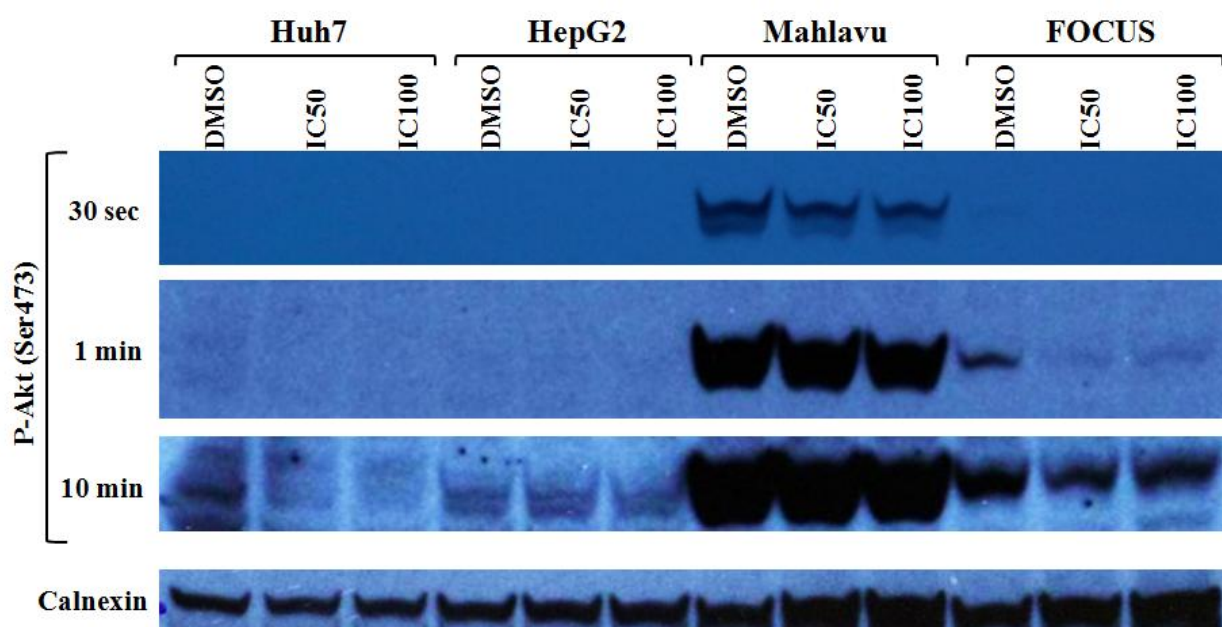


Figure 4.27: The phosphorylated-Akt (P-Akt) (Ser473) protein levels upon ClAT-TM treatment: Huh7, HepG2, Mahlavu and FOCUS cells were treated with ClAT-TM (with IC50 and IC100 values) and corresponding DMSO for 24 hours. Calnexin was used as equal loading control.

4.2.9 KINOMEScanTM Profiling of ClAT-TM

The experiments have done so far demonstrated that ClAT-TM is involved in the JNK signaling pathway. To further clarify any existing direct interaction of ClAT-TM with the

human kinome, we preferred KINOMEScanTM Profiling which is a highthroughput (HTP) approach based on active site-directed competition binding of the sample to be tested and the selected list of human kinases. The primary or secondary activators of JNK signaling pathway constituted our list of selected possible protein kinases. The screen report from KINOMEScanTM Profiling Service found only inefficient interaction between ClAT-TM and the protein kinase candidates in our list (Figure 4.28). With respect to this report, ClAT-TM-interacting protein kinase should be further searched in an extended list of kinases or the ClAT-TM interacting proteins other than the kinases, might be investigated for their involvement in the JNK signaling pathway.

4.2.10 *in vivo* anti-tumor activity of ClAT-TM in Mahlavu xenografts

The *in vivo* anti-tumor effects of ClAT-TM were tested on Mahlavu xenografts in nude (immunodeficient) mice. Considering the results of previously done *in vitro* experiments, we chose Mahlavu cell line not only due to its significant response to ClAT-TM, but also because it is known as one of the poorly-differentiated; highly metastatic HCC cell lines. Following the SC injection of Mahlavu cells, the nude mice were monitorized until the tumors reach 150mm³ volume. From that time on, ClAT-TM, suspended in simple syrup (100mg/kg), was given orally (as described in Materials and Methods-2.4.11) to nude mice. Drug treatments were twice a week for 3 weeks and for the following 3 weeks nude mice were un-treated. At the end of 3 rounds of chemotherapy, the nude mice (both drug and control groups) were taken to magnetic resonance imaging (MRI). The recorded tumor sizes and the MRI data were given in Figure 4.29.

ClAT-TM was able to increase the overall survival of nude mice; moreover, the longest dimension of tumors in un-treated nude mice (#2 and #4 in Figure 4.29) was almost twice of the ClAT-TM treated nude mice (#1 and #3 in Figure 4.29). Besides, tumor volumes were dramatically decreased if the nude mice are in the ClAT-TM treated group. Collectively, our *in vivo* findings demonstrate that ClAT-TM is a novel, potent drug candidate against liver cancer which is also orally tolerable in nude mice.

CIAT-TM @ 100nM
84 Assays Tested
0 Interactions Mapped

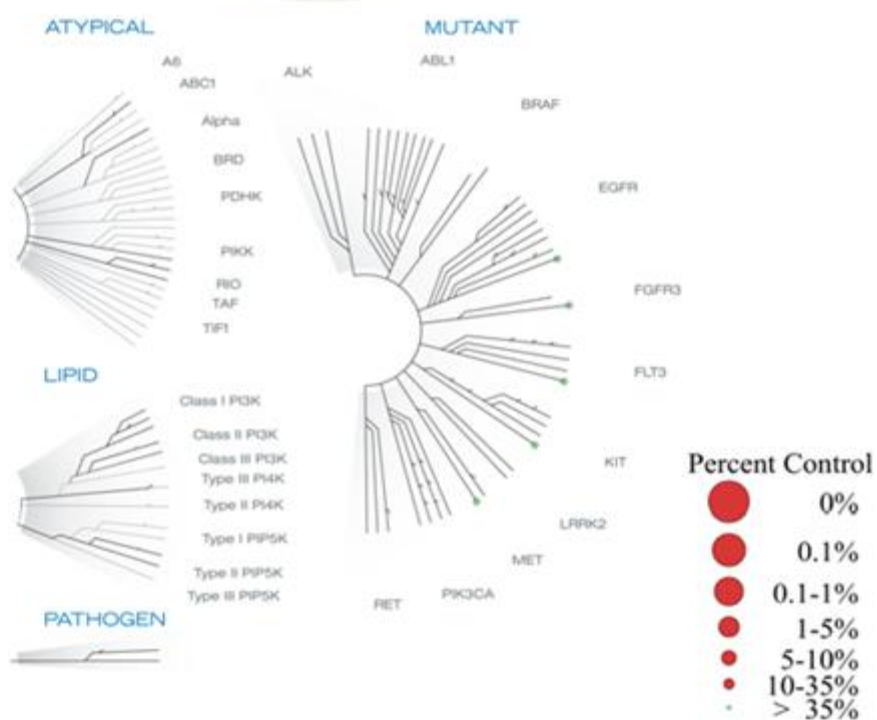
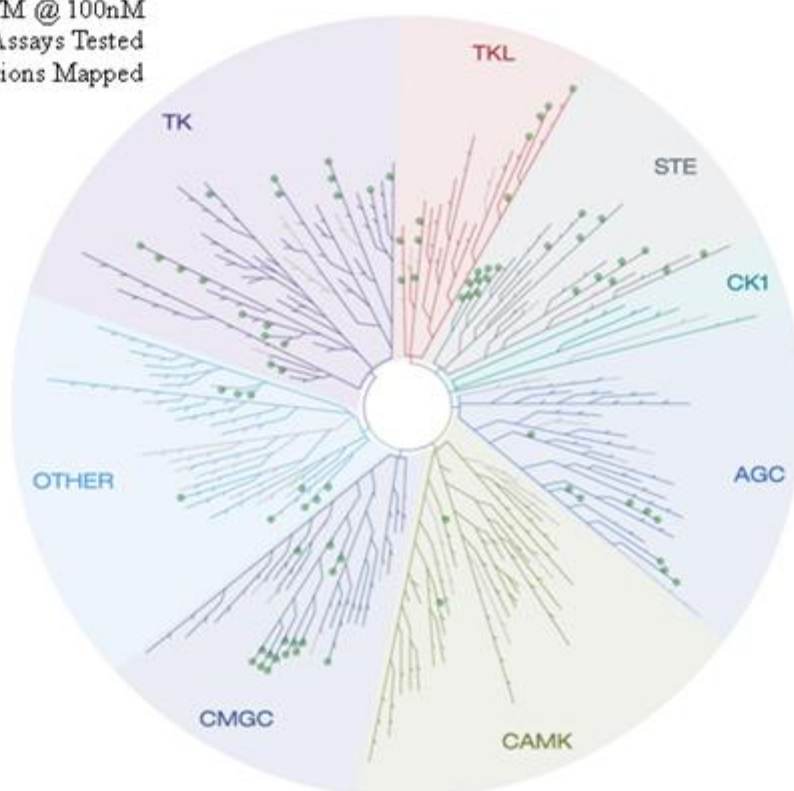
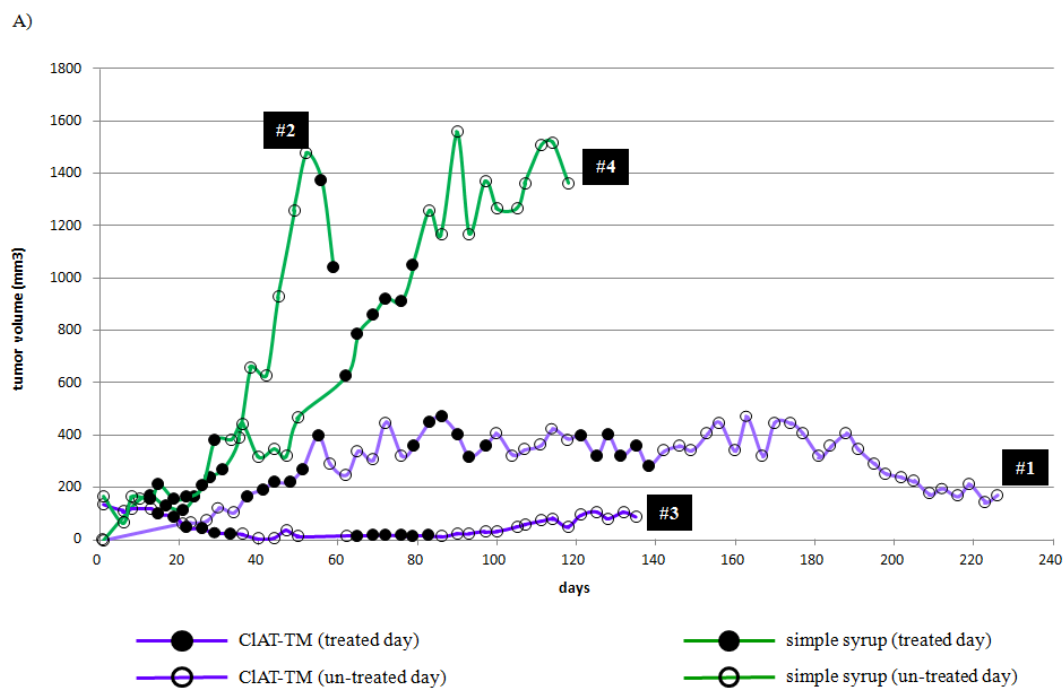


Figure 4.28: TREEspot™ Kinase Interaction Mapping for CIAT-TM



B)

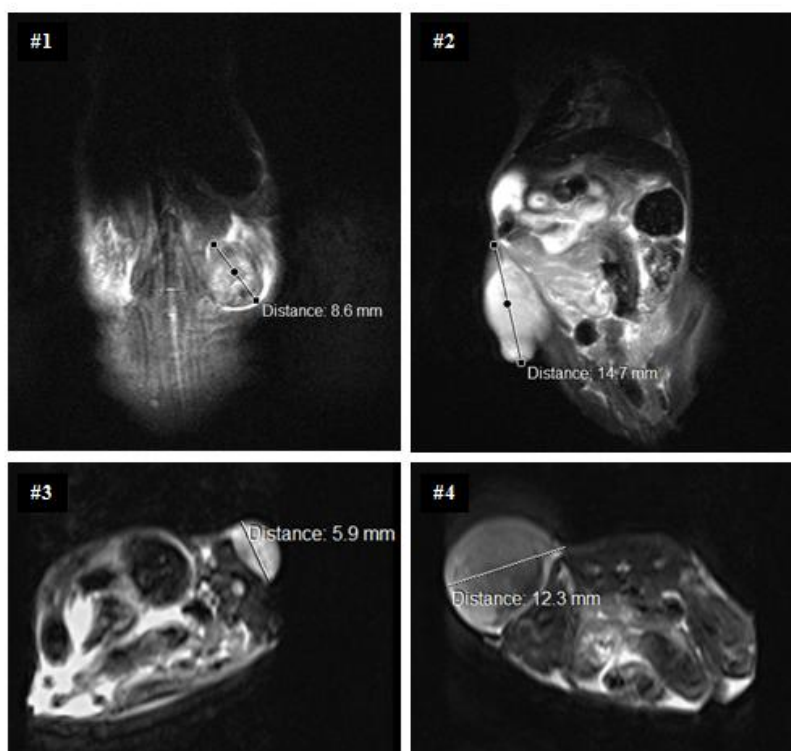


Figure 4.29: *in vivo* anti-tumor activity of CIAT-TM in Mahlavu xenografts: A) Mahlavu xenografted nude mice were treated with (100mg/kg) CIAT-TM suspended in simple syrup or simple syrup-only twice a week and the tumor sizes were recorded accordingly. B) The MRI images were taken at the 181st day for #1, 60th day for #2, 135th day for #3 and 105th day for #4. (#1 and #3 are CIAT-TM treated; #2 and #4 are controls)

CHAPTER 5. DISCUSSION

The process of developing novel molecular-targeted therapeutic agents, from gene to drug, requires cooperation between chemical biology and structural biology both using the small molecules. The small-molecules we obtained from our collaborators were either nucleobase and nucleoside analogues (from Prof. Dr. Meral Tunçbilek, Ankara University) or triazole and thiadiazine derivatives (from Prof. Dr. Birsen Tozkoparan, Hacettepe University).

Nucleobase and nucleoside analogues are structurally, metabolically and pharmacodynamically similar drugs and all aim to destroy the integrity of DNA; but, they show diverse biological activities (Sampath D et al., 2003). Nucleobase analogue, 5-fluorouracil is the first antimetabolite chemotherapeutic agent on cancer cells (Kuhn JG, 2001). Later, pyrimidine analogues such as arabinofuranosyl cytidine (Ara-C) and gemcitabine were investigated in cancer treatments (Sampath D et al., 2003). Purine nucleoside analogues such as cladribine, fludarabine and pentostatin are the currently used group of antimetabolites against hematological malignancies (Lech-Maranda E et al., 2006).

Nucleobase and nucleoside analogues, analyzed in this thesis can be classified into three sub-groups: adenine and purine derivatives, purine ribonucleoside derivatives and substituted purine and purine nucleoside derivatives were tested for their bioactivities initially on human breast, colon and liver cancer lines; the calculated IC₅₀ values were used to select the promising ones. Eventually, the selected small molecules through the initial screening were further analyzed for their anti-cancer activities against a panel of HCC cell lines. Among the newly synthesized 196 nucleobase and nucleoside analogues AUM18, AUM23, AUM32, AUM42 and AUM43 were the selected ones with IC₅₀ values less than 10 μ M.

The cell-line-dependent differences between the cytotoxicities of these selected novel nucleoside analogues, might be indicating that they are small molecule putative kinase inhibitors and interfering with cell signaling pathways. Therefore, their kinase inhibition potentials were assessed. Among them, AUM42 and AUM56 were the analogues with kinase inhibition potentials comparable to the positive controls used in the assays.

In spite of the nuclear condensation and DNA fragmentation observed in the presence of AUM18, AUM23 and AUM32 (in Huh7 and FOCUS cells), the further data we obtained; namely, no significant decrease in Bcl-2 and Cyt-C protein expression levels and the absence of PARP cleavage, prevented us to name the underlying mechanism of the cytotoxicity, induced by these small molecules, as apoptosis. Moreover, the cytotoxicity analysis demonstrated that the long term (72 hour) drug response of liver cancer cells is considerably more effective comparing to short term (24 hour) drug response. Our results indicated that AUM32 induces senescence-associated cytotoxicity; in other words, AUM32 treatment causes cancer cells to stop proliferation and initiates senescence in a dose- and time- dependent manner, which has been considered as a therapeutic target of small molecule inhibitors recently (Nardella C et al., 2011). The similar mechanism of action was also verified for AUM42 nucleoside analogue.

Cellular senescence is accompanied by the activation of the cyclin-dependent kinase inhibitors. The INK4b-ARF-INK4a locus, which is also known as senescence promoting locus, has an extraordinary gene structure encoding both INK4 family of cyclin-dependent kinase inhibitors, p15(INK4b) and p16(INK4a) and a completely unrelated protein, ARF (Gil J and Peters G., 2006). When overexpressed, p15 (INK4b), which is a closely related protein to p16 (INK4a), engages the Rb pathway in order to promote a permanent senescent state which involves the stable silencing of E2F target genes (Serrano M et al., 1997; Erickson S et al., 1998; Kuilman T et al., 2010) .

Additionally, p15 (INK4b) levels as another senescence-associated marker, which is known to be increased in senescent cells, were also shown to be increased in AUM42 treated liver cancer cells. Moreover, the associated decrease in the phosphorylated form of Rb protein was correlative with the AUM42-induced accumulation of p15 (INK4b). In AUM42 treated Huh7 cells, we could not observe a significant change in the p21 protein expression levels; therefore, we did not involve p21 to the action mechanism of AUM42. Ultimately, we were able to identify the small molecule, purine ribonucleoside analogue, AUM42, acting on liver cancer cells with an unraveled mechanism of action (Figure 5.1).

Nucleoside analogs block nucleotide mechanism by incorporating into DNA and by inhibiting ribonucleotide reductase (RR). Inhibition of the enzyme RR leads to a decrease in dNTP pools which ends in impaired DNA synthesis (Wilson P et al., 2004). Therefore, the most commonly observed cell death induced by nucleoside analogues is apoptosis (Sampath D et al., 2003). However, significant decrease in the RR levels and changes in the dNTP pools have been reported for the senescence in diploid fibroblasts (Dick J and Wright J, 1985; Nakamura J et al., 2010). Therefore, nucleoside analogues may induce senescence; such as toyocamycin and decitabine (Kurihara Y et al., 2002; Amatori S et al., 2011).

Besides the reported nucleoside analogs, which were known to induce senescence, AUM32 and AUM42 are the two newly synthesized, small molecules, both containing a 4-substituted piperazine in the substituent at N⁶, were shown to induce senescence-associated cytotoxicity against liver cancer cells. Moreover, the activation of the Rb protein by AUM42-induced p15 (INK4b) overexpression also confirmed the mechanism of action we identified for this novel purine ribonucleoside analogue. Senescence is an effective barrier to tumor development which recently started to be considered as a promising approach for cancer therapy (Ewald JA et al., 2010). Recent findings disclose that cancer cells can also undergo senescence. Thus, inducing senescence in tumors; namely, pro-senescence therapy is a new strategy in the fight against cancer (Nardella C et al., 2011). Senescence, the irreversible arrest of proliferation, can prevent

immortalization; thus senescence-inducing drugs emerge rapidly (Cairney CJ et al., 2012).

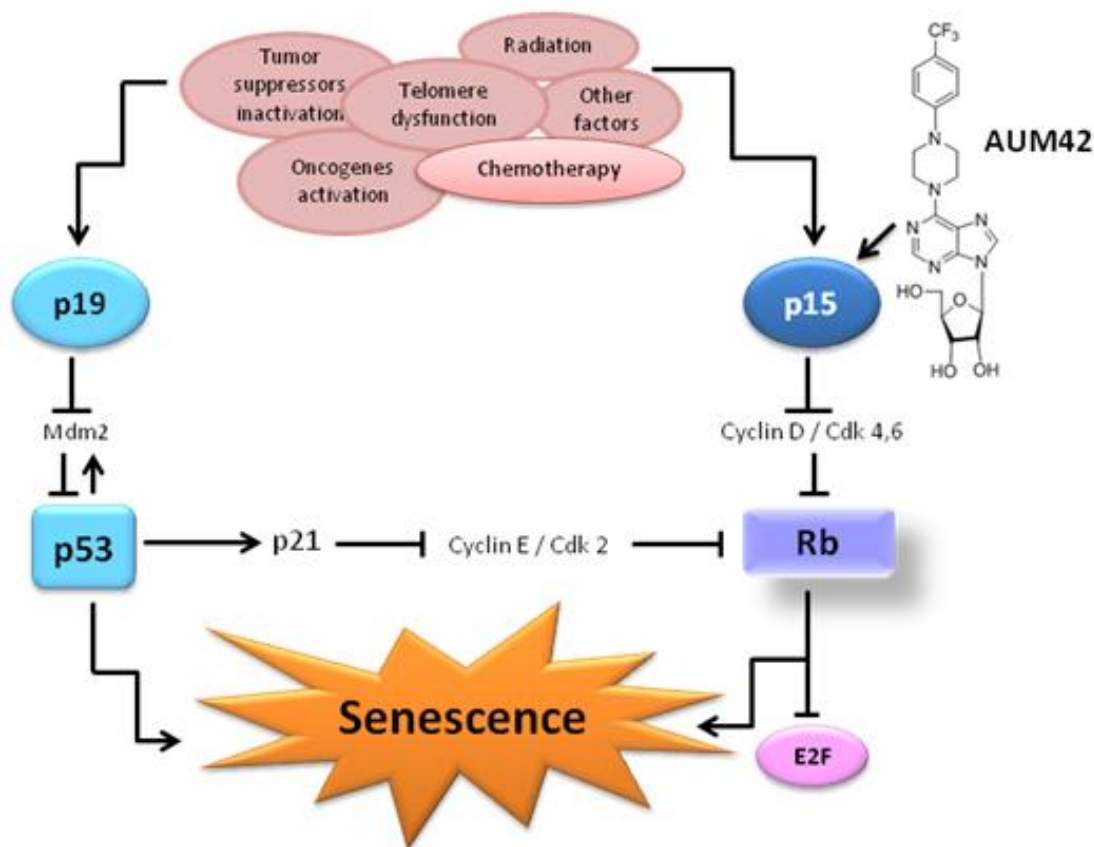


Figure 5.1. Novel purine ribonucleoside analogue, AUM42, leads to senescence-induced cell death in liver cancer cells (Tuncbilek M et al., 2012).

Aminotriazole and thiadiazine derivatives, composing the second group of small molecules (also analyzed in this thesis), were initially screened against human breast, colon and liver cancer cell lines and then, the calculated IC₅₀ values were used to select the promising ones. Among this group of compounds, three small molecules (FAT-TM, CIAT-TMN and n-AT-TM) were further analyzed for their anti-cancer activities against a panel of HCC cell lines. Time dependent IC₅₀ values calculated for CIAT-TM demonstrated that this small molecule was able to create a cytotoxic response even in 24 hours within micromolar concentrations.

It was previously demonstrated that, synthetic 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine derivative, CIAT-TM was designed as a putative protein kinase inhibitor with analgesic and anti-inflammatory activities (Tozkoparan B et al., 2009). Besides, it has differential IC₅₀ values against HCC panel, which might be a consequence of its interference with cell signaling. Thus, we performed a kinase assay to assess the protein kinase inhibitory potential of CIAT-TM. It revealed a cell line-specific profile; moreover, except HepG2, a dose-dependent protein kinase inhibitory potential in all cell lines.

CIAT-TM induced-cytotoxicity changed the morphology of the cancer cells dramatically. It is clear that upon the administration of CIAT-TM, cell proliferation stopped and depending on the dose and incubation time, first, rounded, swollen and eventually detached cells were accumulated and next, blebbing cells and apoptotic bodies were appeared.

In addition to the distinctive changes in the morphology of CIAT-TM treated cells, we performed a western blot analysis to verify the CIAT-TM induced apoptosis in the protein level. In spite of the detectable cleaved PARP in CIAT-TM treated liver cancer cells, CIAT-TM did not induce sub-G1 accumulation, a well-known indicator of “typical” apoptosis, during cell cycle analysis. “Atypical” apoptosis, lacking the characteristic features of apoptosis, such as an increase in the sub-G1 fraction, has been reported to occur in cells treated with acetylsalicylate or aspirin like non-steroidal anti-inflammatory drugs (NSAID) (Shiff SJ et al., 1995; Smith ML et al., 1999; Reinacher-Schick A et al., 2003). It was previously demonstrated that, synthetic 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine derivative CIAT-TM is a NSAID with analgesic and anti-inflammatory activities (Tozkoparan B et al., 2009). Therefore, the apparently conflicting results we obtained, are actually correlative.

Cell-cycle analysis in the presence of CIAT-TM demonstrated time- and dose- dependent G2/M arrest. The most typical triggering mechanisms of G2/M

arrest are either ER-stress or the accumulation of reactive oxygen species (ROS) (Macip S et al., 2006; Bouruugaa K et al, 2010; Wu SH et al, 2010). After eliminating the possibility of ER-stress, we were able to show the ROS accumulation upon CIAT-TM treatment.

The cell line specific IC₅₀ values and the kinase assay results indicating the kinase inhibition potential of 5CI-ATTM reinforced the idea that this small-molecule interferes with cell signaling. Among the signaling pathways involved in hepatocarcinogenesis, we analyzed first the stress-activated protein kinase/c-Jun NH₂-terminal kinase (SAPK/JNK), due to the evident oxidative stress induced by CIAT-TM. It is known that prolonged JNK activation depends on the ROS generation. ROS induces JNK activation, and JNK induces ROS accumulation (Nakano H, 2004). The accumulated ROS prolongs the JNK activation which leads cell to apoptosis (Zhang Y and Chen F, 2004).

In fact, phosphorylated-JNK (P-JNK), and in parallel (its downstream target) phosphorylated-c-Jun (P-c-Jun) were both shown to be increased. Indeed c-Jun N-terminal kinase (JNK) activation upon ROS accumulation was shown to be vital for the induction of G2/M arrest (Yamamoto K et al., 1999; Zhang Y and Chen F, 2004; Cho SD et al., 2009). JNK is regulated by two upstream kinases, MAP2Ks (MKK4 and MKK7). We checked the protein expression levels and the phosphorylation status of (MKK4/P-MKK4) and (MKK7/P-MKK7) and detected a cell line-specific activation of MKK7 after CIAT-TM treatment, while the other JNK activator MKK4 was unaltered.

One of the MAP3Ks activating JNK signaling pathway is ASK1 (apoptosis signal-regulating kinase 1) and its regulation is highly intricate. ASK1 has three phosphorylation sites and among them Ser83 is the inhibitory site. Akt phosphorylates ASK1 on Ser83 and promotes cell survival (Kim et al., 2001). It is reported that Ser83 of ASK1 becomes dephosphorylated upon cellular stress in order to restore ASK1 activity which eventually induces apoptosis (Zhang et al.,

2005). Indeed, we detected a decrease not only in the levels of P-ASK1 (Ser83) but also P-Akt (Ser473) upon CIAT-TM treatment.

In order to identify any existing direct interaction of CIAT-TM with one of the mediators of JNK signaling pathway, we screened a large number of proteins with KINOMEscanTM Profiling Service. However we could not find any direct interaction between CIAT-TM and the “protein kinases” in our list. It has been shown that ROS also inhibits MAPK phosphatases in order to promote TNF- α induced apoptosis associated with prolonged activation of JNK (Kamata H et al., 2005). Therefore, all these findings indicate that CIAT-TM causes cell death through activating JNK pathway by inducing ROS accumulation in these cells. (Fig 5.2)

Following the *in vitro* cytotoxicity experiments, *in vivo* anti-tumor effects of CIAT-TM were tested on Mahlavu xenografts in nude mice. The increased overall survival and the decrease in the tumor size upon CIAT-TM treatment clearly strengthen our data revealing that CIAT-TM is a novel small molecule acting on liver cancer cells.

In this thesis, we found that the novel small-molecule, CIAT-TM sensitized the liver cancer cells to growth inhibition in a time- and dose- dependent manner. Exposure to CIAT-TM resulted in dramatic morphological changes in HCC cell lines and the triggered underlying molecular mechanism forced cancer cells first, to G2/M stage cell cycle arrest and finally, to apoptosis. Throughout this period, CIAT-TM treatment increased the protein levels of not only phospho-JNK but also phospho-c-Jun, a downstream target of JNK pathway. The accumulated data provided a molecular mechanism for CIAT-TM as a chemopreventive agent in human HCC cell lines (Figure 5.2). Further investigations are required to identify the roles of the other members of the JNK pathway in the CIAT-TM induced G2/M arrest and apoptosis.

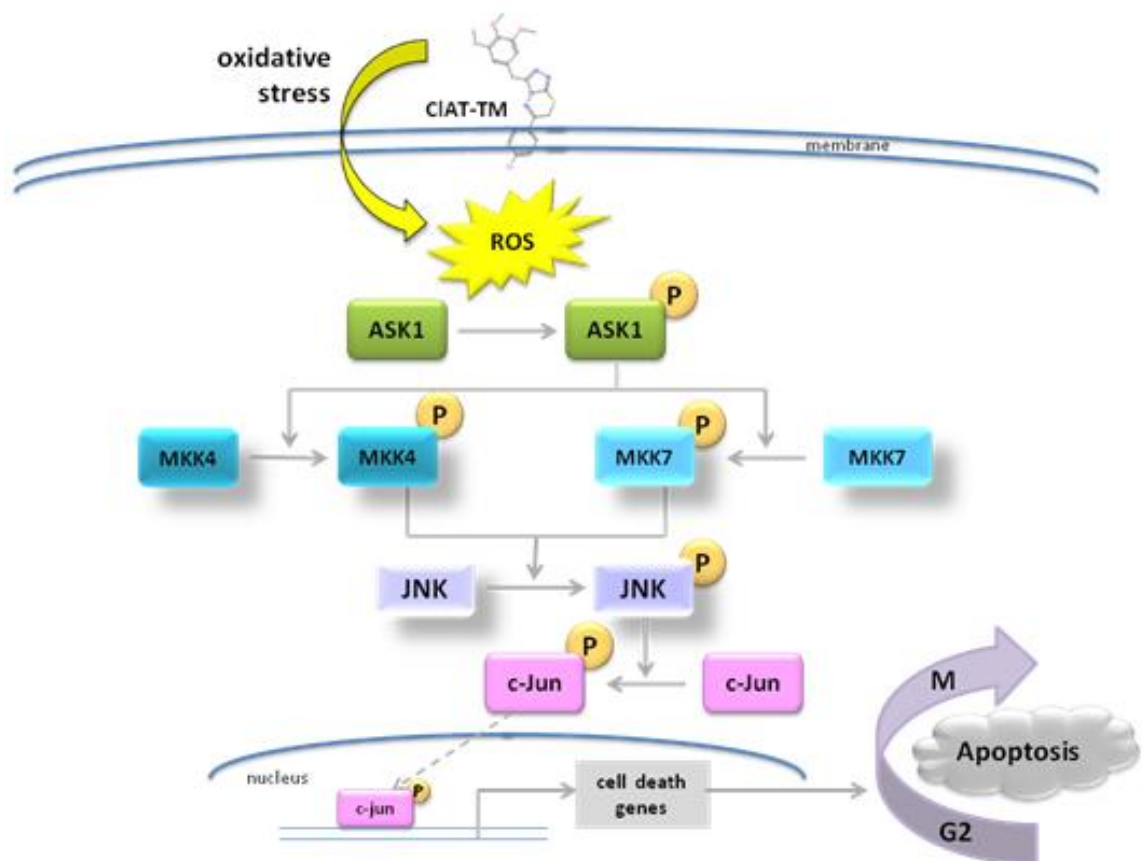


Figure 5.2. The molecular mechanism that CIAT-TM is involved in.

CHAPTER 6. FUTURE PERSPECTIVES

Drug candidates, nucleobase and nucleoside analogues (AUM series) and aminotriazole and thiadiazine derivatives (HUBT series) were designed as putative protein kinase inhibitors. The results of this thesis demonstrated that AUM32 and AUM42 lead liver cancer cells to senescence-induced cell death; while, ClAT-TM is another small-molecule with kinase inhibition potential, able to force liver cancer cells initially to G2/M stage cell cycle arrest and eventually to apoptosis.

From the first group of putative kinase inhibitors, substituted purine and purine nucleoside derivatives (Supplementary Tables 2-5) were evaluated for their anti-cancer activity and protein kinase inhibition potentials. Further analysis, in order to identify their mechanism of action, is required. The type of cell death induced by these groups of small-molecules should be characterized.

Senescence is known to control not only the cell proliferation but also the invasion of malignant cells, as a tumor suppressor mechanism (Kraus S et al., 2011). Therefore, the effect of both AUM32 and AUM42 on cell motility can be evaluated by wound healing assay. These two senescence-inducing small-molecules might be able to prevent migration and invasion.

DNA damage response upon treatment of nucleobase and nucleoside analogues can be further investigated. The phosphorylation status of ATM and ATR can be checked.

From the other group of molecules, ClAT-TM is a powerful anti-cancer agent and its mechanism of action is mostly clarified. ROS accumulation is the initiative response of cancer cells in the presence of ClAT-TM. We were able to figure out that ClAT-TM both leads to G2/M arrest and ROS accumulation. Furthermore, ROS levels can be checked throughout the cell cycle by flow cytometry.

The phosphorylation status of ASK1 upon ClAT-TM treatment was checked only for Ser83. There are two more phosphorylation sites for ASK1: Ser967 and Thr845. ASK1 associates with 14-3-3 protein and promotes cell survival when gets phosphorylated at Ser 967 (Zhang L et al., 1999). Moreover, oxidative stress is known to induce not only the de-phosphorylation of ASK1 at Ser967 but also the phosphorylation of ASK1 at Thr845 (Goldman EH et al., 2004). In addition to the western blot analysis which have done to check the phosphorylation status of ASK1 at Ser83, these two other phosphorylation sites can be further investigated.

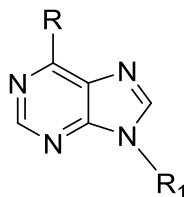
In order to identify any existing direct interaction of ClAT-TM with the human kinome, an extended list of protein kinases can be used for the report request from KINOMEscanTM Profiling Service. The ClAT-TM interacting proteins other than the kinases might be investigated for their involvement in the JNK signaling pathway.

The results obtained in the *in vivo* evaluation of the anti-cancer activity of ClAT-TM are encouraging. Both, the dose and the application conditions chosen are well suited. Nevertheless, it may be worth to increase the number of the nude mice. Moreover, after MRI images are taken, the nude mice can be sacrificed and the tumor tissue samples, taken from both ClAT-TM treated and un-treated nude mice, can be analyzed through immunohistochemistry.

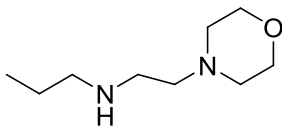
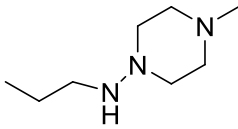
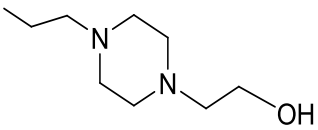
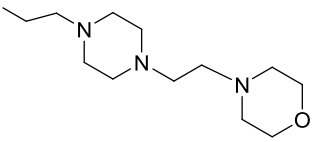
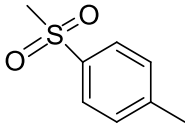
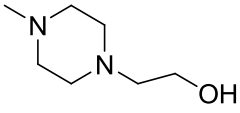
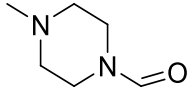
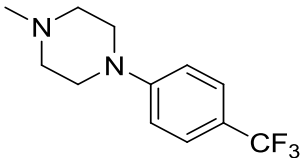
As another approach to the identification of newly synthesized, promising anti-cancer drug candidates, microarray analysis can be envisaged. The ultimate aim of this analysis is to focus on the genes that are up- or down- regulated upon drug treatment. The related and responsible genes that change expression profile would disclose the mechanism of action that the drug candidate is involved in. Therefore, the microarray analysis of AUM32, AUM42 and ClAT-TM treated liver cancer cells, with comparison to their DMSO controls, would be informative.

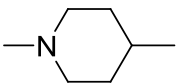
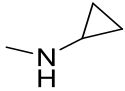
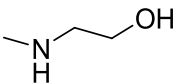
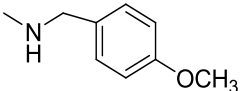
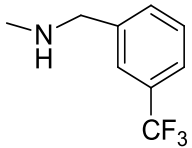
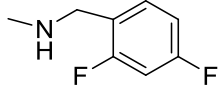
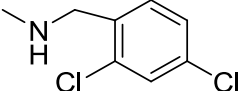
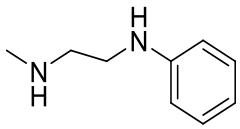
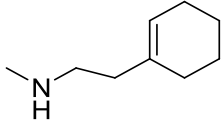
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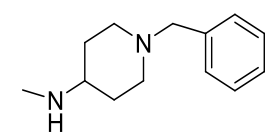
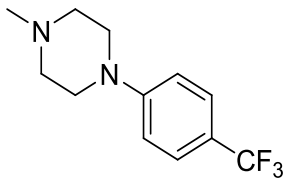
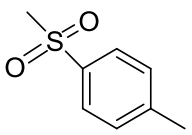
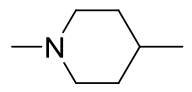
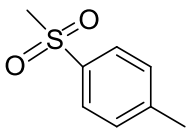
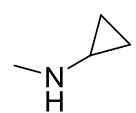
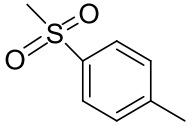
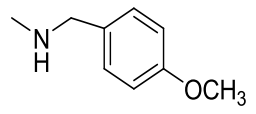
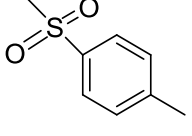
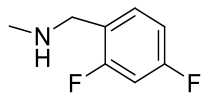
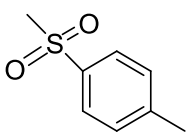
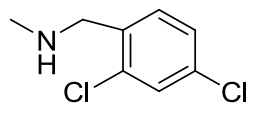
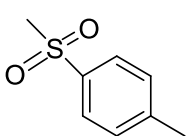
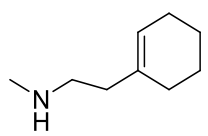
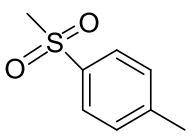
Supplementary Table 1: IC50 values of AUM purine analogues: All molecules were tested in duplicate and incubated for 72 hours. The IC50 values are in μM range. NI stands for “no inhibition”.

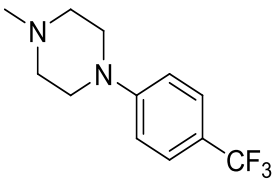
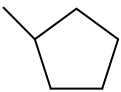
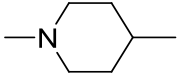
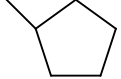
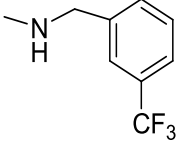
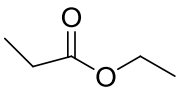
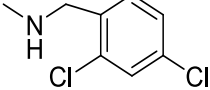
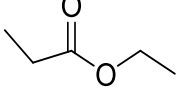
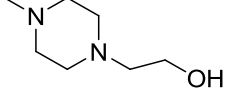
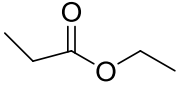
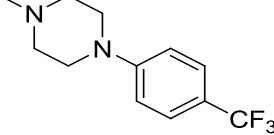
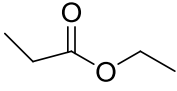


AUM code	R	R ₁	IC50 (μM)		
			Huh7	HCT116	T47D
AUM5	NH ₂		NI	NI	NI
AUM6	NH ₂		NI	NI	NI
AUM7	NH ₂		NI	NI	NI
AUM8	NH ₂		NI	NI	NI
AUM12	NH ₂		NI	NI	NI

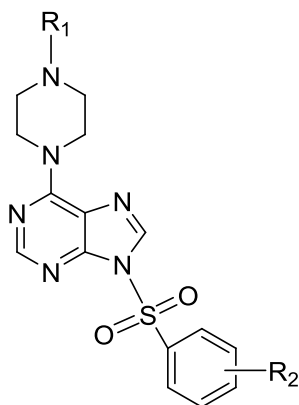
AUM13	NH ₂		NI	NI	NI
AUM9	NH ₂		NI	NI	NI
AUM10	NH ₂		NI	NI	NI
AUM11	NH ₂		NI	NI	NI
AUM34	Cl		20.8	22.8	13.9
AUM26		H	NI	NI	NI
AUM35		H	NI	NI	NI
AUM18		H	3.2	5.1	39.4

AUBMC13		H	NI	NI	NI
AUM31		H	NI	NI	NI
AUM30		H	NI	NI	NI
AUM25		H	NI	86.6	NI
AUM16		H	68.3	102.1	NI
AUM14		H	NI	NI	NI
AUM15		H	NI	NI	146.8
AUM19		H	NI	131.6	NI
AUBMC10		H	14.2	12.9	40.3

AUM17		H	NI	NI	NI
AUM23			6.6	4.5	42.7
AUM37			17.8	14.6	22.5
AUM38			NI	NI	NI
AUM22			NI	NI	NI
AUM21			NI	NI	116.4
AUM28			NI	NI	NI
AUM36			9.4	8.7	34.5

AUM32			< 2.5	< 2.5	< 2.5
AUBMC30			17.4	16.6	21.0
AUM39			NI	NI	NI
AUM20			NI	NI	99.9
AUM33			NI	NI	NI
AUM29			53.3	72.4	NI
5-FU			15.6	10.1	7.9
CPT			< 1	< 1	< 1

Supplementary Table 2: IC50 values of 6-(substituted piperazine)-9-(substituted phenyl) sulfonyl purine analogues: All molecules were tested in duplicate and incubated for 72 hours and the IC50 values are in μM range. NI stands for “no inhibition”.

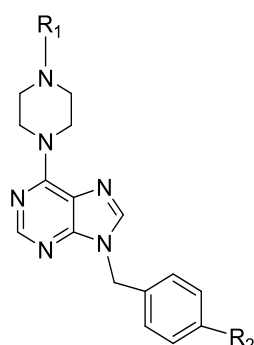


AUM codes	R ₁	R ₂	IC50 (μM)		
			Huh7	HCT116	MCF7
AUM122	2-pyrimidinyl	H	NI	NI	NI
AUM123	Ph	H	22.9	54.1	NI
AUM124	4-CH ₃ -Ph	H	11.0	17.7	16.6
AUM125	4-CF ₃ -Ph	H	1.6	2.4	0.8
AUM126	4-F-Ph	H	20.9	22.7	NI
AUM127	3,4-diCl-Ph	H	5.5	3.9	5.5
AUM128	(Ph) ₂ CH	H	94.8	NI	NI
AUM82	2-pyrimidinyl	4-CH ₃	NI	NI	52.9
AUM48	Ph	4-CH ₃	NI	NI	NI
AUM62	4-CH ₃ -Ph	4-CH ₃	22.9	41.4	36.9
AUM50	4-F-Ph	4-CH ₃	54.3	NI	NI
AUM83	2,4-diF-Ph	4-CH ₃	16.8	15.0	NI
AUM137	3,4-diCl-Ph	4-CH ₃	4.3	4.2	4.1
AUM51	(Ph) ₂ CH	4-CH ₃	NI	NI	NI
AUM87	C ₆ H ₁₁	4-OCH ₃	NI	NI	NI
AUM88	2-pyrimidinyl	4-OCH ₃	87.4	NI	54.6
AUM84	Ph	4-OCH ₃	14.6	14.5	74.5
AUM89	4-CH ₃ -Ph	4-OCH ₃	8.4	5.2	75.5
AUM85	4-CF ₃ -Ph	4-OCH ₃	<1.0	<0.1	32.7
AUM63	4-F-Ph	4-OCH ₃	26.4	12.1	15.6

AUM90	2,4-diF-Ph	4-OCH ₃	12.2	9.7	76.9
AUM138	3,4-diCl-Ph	4-OCH ₃	0.2	2.3	2.8
AUM86	(Ph) ₂ CH	4-OCH ₃	NI	NI	NI
AUM111	C ₆ H ₁₁	4-F	77.1	NI	88.6
AUM112	2-pyrimidinyl	4-F	39.7	NI	34.2
AUM108	Ph	4-F	12.2	14.6	17.7
AUM113	4-CH ₃ -Ph	4-F	10.2	11.5	16.0
AUM110	4-CF ₃ -Ph	4-F	1.7	0.4	9.2
AUM109	4-F-Ph	4-F	16.5	18.1	40.0
AUM114	2,4-diF-Ph	4-F	36.4	NI	34.1
AUM139	3,4-diCl-Ph	4-F	3.1	4.7	6.0
AUM107	(Ph) ₂ CH	4-F	NI	NI	NI
AUM49	4-F-Ph	3-CF ₃	28.4	38.1	NI
AUM103	C ₆ H ₁₁	4-Cl	32.5	NI	55.4
AUM104	2-pyrimidinyl	4-Cl	15.5	25.5	22.8
AUM100	Ph	4-Cl	20.5	17.1	36.4
AUM105	4-CH ₃ -Ph	4-Cl	10.7	9.8	15.1
AUM102	4-CF ₃ -Ph	4-Cl	2.9	1.0	12.23
AUM101	4-F-Ph	4-Cl	24.5	34.5	72.9
AUM106	2,4-diF-Ph	4-Cl	15.5	18.3	17.1
AUM140	3,4-diCl-Ph	4-Cl	4.8	6.2	7.5
AUM99	(Ph) ₂ CH	4-Cl	NI	NI	NI
AUM129	C ₆ H ₁₁	4-Br	NI	NI	NI
AUM130	2-pyrimidinyl	4-Br	15.0	24.3	46.3
AUM131	Ph	4-Br	13.6	28.2	NI
AUM132	4-CH ₃ -Ph	4-Br	13.5	25.5	NI
AUM133	4-CF ₃ -Ph	4-Br	2.1	3.7	1.6
AUM134	4-F-Ph	4-Br	17.9	25.1	37.1
AUM135	2,4-diF-Ph	4-Br	8.2	17.9	16.0
AUM136	3,4-diCl-Ph	4-Br	3.9	4.6	5.3
AUM95	C ₆ H ₁₁	2-Cl-4-F	20.4	7.8	NI
AUM96	2-pyrimidinyl	2-Cl-4-F	NI	NI	NI
AUM92	Ph	2-Cl-4-F	23.1	23.2	NI
AUM97	4-CH ₃ -Ph	2-Cl-4-F	21.8	33.3	45.2
AUM94	4-CF ₃ -Ph	2-Cl-4-F	1.1	0.5	20.7
AUM93	4-F-Ph	2-Cl-4-F	21.4	18.5	NI
AUM98	2,4-diF-Ph	2-Cl-4-F	41.9	39.9	NI
AUM141	3,4-diCl-Ph	2-Cl-4-F	2.9	5.6	6.7

AUM91	C ₆ H ₁₁	2-Cl-4-F	NI	NI	NI
CPT			<0.1	<0.1	<0.1
5-FU			30.7	6.0	3.5

Supplementary Table 3: IC₅₀ values of 6-(substituted piperazine)-9-(substituted benzyl) purine analogues: All molecules were tested in duplicate and incubated for 72 hours and the IC₅₀ values are in μ M range. NI stands for “no inhibition”. NA stands for “not applicable”.

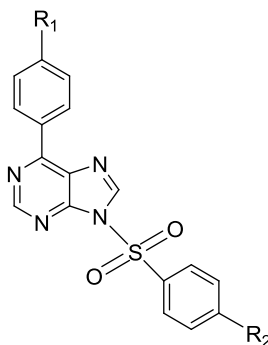


AUM codes	R ₁	R ₂	IC ₅₀ (μ M)		
			Huh7	HCT116	MCF7
AUM157	2-pyrimidinyl	H	17.2	16.5	22.5
AUM158	Ph	H	3.9	2.7	0.9
AUM159	4-CH ₃ -Ph	H	5.9	NI	NI
AUM160	4-CF ₃ -Ph	H	0.3	<1.0	<1.0
AUM161	4-F-Ph	H	53.0	1.3	3.4
AUM162	3,4-diCl-Ph	H	0.2	1.2	<1.0
AUM163	(Ph) ₂ CH	H	9.5	14.3	15.4
AUM174	HOCH ₂ CH ₂	4-CH ₃	36.1	43.8	NI
AUM175	2-pyrimidinyl	4-CH ₃	5.2	9.7	11.2
AUM176	Ph	4-CH ₃	2.8	2.8	0.8
AUM177	4-CH ₃ -Ph	4-CH ₃	<1.0	5.1	1.1
AUM178	4-CF ₃ -Ph	4-CH ₃	0.1	<1.0	2.6
AUM179	4-F-Ph	4-CH ₃	0.1	0.2	<1.0
AUM180	2,4-diF-Ph	4-CH ₃	1.4	5.3	88.0
AUM181	3,4-diCl-Ph	4-CH ₃	<1.0	<1.0	<1.0

AUM182	(Ph) ₂ CH	4-CH ₃	NI	NI	13.2
AUM147	HOCH ₂ CH ₂	4-F	79.4	NI	NI
AUM148	cyclohexyl	4-F	9.0	13.1	15.3
AUM149	2-pyrimidinyl	4-F	70.7	NI	NI
AUM52	Ph	4-F	5.0	2.5	0.7
AUM150	4-CH ₃ -Ph	4-F	29.8	24.3	NI
AUM54	4-CF ₃ -Ph	4-F	NA	NA	NA
AUM53	4-F-Ph	4-F	NA	1.1	NA
AUM151	3,4-diCl-Ph	4-F	<1.0	<1.0	4.7
AUM55	(Ph) ₂ CH	4-F	19.1	18.7	22.1
AUM173	HOCH ₂ CH ₂	4-Br	12.8	15.8	30.3
AUM164	Cyclohexyl	4-Br	3.6	6.0	7.0
AUM165	2-pyrimidinyl	4-Br	10.6	23.9	NI
AUM166	Ph	4-Br	0.7	0.7	0.8
AUM167	4-CH ₃ -Ph	4-Br	<1.0	3.0	1.5
AUM168	4-CF ₃ -Ph	4-Br	<1.0	<1.0	NA
AUM169	4-F-Ph	4-Br	0.1	<0.1	8.0
AUM170	2,4-diF-Ph	4-Br	13.9	71.7	NI
AUM171	3,4-diCl-Ph	4-Br	9.4	44.5	1.8
AUM172	(Ph) ₂ CH	4-Br	11.2	14.8	16.6
AUM72	HOCH ₂ CH ₂	2,4-diCl	10.8	9	9.2
AUM142	Cyclohexyl	2,4-diCl	5.1	11.8	NI
AUM143	2-pyrimidinyl	2,4-diCl	4.0	3.3	7.3
AUM56	Ph	2,4-diCl	2.9	<1.0	0.4
AUM144	4-CH ₃ -Ph	2,4-diCl	1.8	4.7	8.1
AUM57	4-CF ₃ -Ph	2,4-diCl	<1.0	<1.0	<1.0
AUM145	2,4-diF-Ph	2,4-diCl	8.4	7.2	61.2
AUM146	3,4-diCl-Ph	2,4-diCl	0.1	0.9	<1.0
AUM73	(Ph) ₂ CH	2,4-diCl	20.4	19.5	18.1
AUM78	HOCH ₂ CH ₂	4-CF ₃	18.1	19.1	17.9
AUM79	Cyclohexyl	4-CF ₃	7.8	7.8	6.5
AUM154	2-pyrimidinyl	4-CF ₃	9.6	11.2	17.9
AUM74	Ph	4-CF ₃	<1.0	<1.0	<1.0
AUM155	4-CH ₃ -Ph	4-CF ₃	<1.0	<1.0	<1.0
AUM76	4-CF ₃ -Ph	4-CF ₃	0.1	0.4	0.4
AUM75	4-F-Ph	4-CF ₃	1.5	1.3	1.1
AUM80	2,4-diF-Ph	4-CF ₃	5.0	5.5	4.3
AUM156	3,4-diCl-Ph	4-CF ₃	0.6	0.7	<1.0

AUM77	(Ph) ₂ CH	4-CF ₃	15.4	13	12.2
AUM68	HOCH ₂ CH ₂	4-Cl	21.7	19	21.1
AUM69	Cyclohexyl	4-Cl	9.3	8.9	8.6
AUM71	2-pyrimidinyl	4-Cl	37.7	56.4	93.9
AUM64	Ph	4-Cl	<1.0	<1.0	0.4
AUM70	4-CH ₃ -Ph	4-Cl	<1.0	<1.0	0.5
AUM66	4-CF ₃ -Ph	4-Cl	0.1	<1.0	0.05
AUM65	4-F-Ph	4-Cl	0.6	<1.0	1.4
AUM152	2,4-diF-Ph	4-Cl	10.0	NI	NI
AUM153	3,4-diCl-Ph	4-Cl	0.3	<1.0	<1.0
AUM67	(Ph) ₂ CH	4-Cl	18.8	13.2	13.9
CPT			<0.1	<0.1	<0.1
5-FU			30.7	6.0	3.5

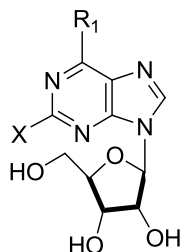
Supplementary Table 4: IC₅₀ values of 6-(4-substituted phenyl)-9-(4-substituted phenyl) sulfonyl purine analogues: All molecules were tested in duplicate and incubated for 72 hours and the IC₅₀ values are in μ M range. NI stands for “no inhibition”.



AUM codes	R ₁	R ₂	IC ₅₀ μ M		
			Huh7	HCT116	MCF7
AUM197	H	F	NI	68.3	NI
AUM198	H	CF ₃	19.8	NI	54.7
AUM199	H	C(CH ₃) ₃	NI	NI	NI
AUM200	F	F	NI	59.7	NI
AUM201	F	CF ₃	NI	58.1	NI
AUM202	F	C(CH ₃) ₃	NI	NI	NI

AUM203	Cl	F	NI	59.1	NI
AUM204	Br	F	NI	NI	NI
AUM205	CF ₃	F	NI	NI	NI
AUM206	C	CF ₃	NI	NI	NI
AUM207	CF ₃	C(CH ₃) ₃	NI	NI	NI
AUM208	C(CH ₃) ₃	F	28.3	NI	NI
AUM209	C(CH ₃) ₃	CF ₃	12.8	30.1	27.1
AUM210	OPh	F	13.7	14.7	22.7
AUM211	OPh	CF ₃	13.4	12.7	16.9
AUM212	OPh	C(CH ₃) ₃	8.6	18.41	21.1
CPT			<0.1	<0.1	<0.1
5-FU			30.7	6.0	3.5

Supplementary Table 5: IC₅₀ values of 2-amino/2-chloro/6-(4-substituted piperazine)/6-(4-substituted phenyl amino)/6-(2-substituted ethylamino)-9-(β-D-ribofuranosyl) purine analogues: All molecules were tested in duplicate and incubated for 72 hours and the IC₅₀ values are in μM range. NI stands for “no inhibition”.



AUM codes	X	R ₁	IC ₅₀ μM		
			Huh7	HCT116	MCF7
AUM213	H	4-cyclohexylpiperazine	NI	NI	NI
AUM214	H	4-(2-pyrimidinyl)piperazine	NI	NI	43.1
AUM215	H	4-methylphenylpiperazine	78.1	64.7	NI
AUM225	NH ₂	2-pyrimidinylpiperazine	NI	NI	NI
AUM223	NH ₂	Phenylpiperazine	46.6	33.4	NI
AUM226	NH ₂	4-methylphenylpiperazine	NI	NI	NI
AUM224	NH ₂	4-fluorophenylpiperazine	93.5	28.1	NI
AUM222	NH ₂	diphenylmethylpiperazine	22.0	16.3	16.4
AUM217	H	4-(methylthiophenyl)amino	NI	NI	NI

AUM218	H	4-(dimethylaminophenyl) amino	NI	36,9	32.4
AUM216	H	4-(isopropylphenyl) amino	23.2	14.6	47.4
AUM219	H	4-(morpholinophenyl) amino	NI	NI	66.6
AUM220	H	(2-cyclohexenylethyl) amino	NI	6.6	2.6
AUM221	H	(2-phenylaminoethyl) amino	NI	30.2	83.5
AUM228	Cl	4-(4-trifluoromethylphenyl) piperazine	2.1	1.1	3.7
AUM229	Cl	(2- cyclohexenylethyl) amino	20.7	62.3	28.0
CPT			<0.1	<0.1	<0.1
5-FU			30.7	6.0	3.5

Supplementary Table 6: IC50 values of 6-(substituted piperazine)-9-(substituted phenyl) sulfonyl purine analogues: All molecules were tested in triplicate and incubated for 72 hours and the IC50 values are in μM range. NI stands for “no inhibition”.

AUM codes	IC50 (μM)			
	Huh7	HepG2	MAHLAVU	FOCUS
AUM123	12.3	25.4	17.0	17.0
AUM124	9.7	16.3	32.0	19.0
AUM125	0.4	< 0.1	< 1.0	1.0
AUM126	17.2	69.0	1.7	NI
AUM127	1.5	< 1.0	12.9	3.6
AUM83	12.4	15.2	25.83	30.0
AUM137	1.8	6.7	20.5	6.4
AUM88	38.3	54.5	NI	NI
AUM84	12.0	22.1	NI	NI
AUM89	15.2	9.1	5.8	NI
AUM85	0.3	0.2	< 0.1	< 0.1
AUM63	15.0	12.4	34.8	14.2
AUM90	11.0	13.8	15.0	17.4
AUM138	< 1.0	2.9	64.5	3.3
AUM113	9.1	10.9	11.8	13.7
AUM110	1.9	1.1	0.3	0.5
AUM139	2.7	7.7	33.9	7.9
AUM105	9.3	14.9	11.8	12.7
AUM102	2.7	2.4	1.2	1.2

AUM106	12.9	15.4	27.9	21.8
AUM140	4.6	8.9	15.2	8.0
AUM130	19.2	37.4	20.5	25.0
AUM131	21.0	23.3	26.2	30.2
AUM132	11.0	13.4	18.4	17.3
AUM133	1.8	2.7	13.6	3.8
AUM134	25.7	22.1	79.5	78.7
AUM135	5.3	17.3	14.2	10.2
AUM136	3.0	7.5	17.3	7.0
AUM92	14.2	23.0	NI	NI
AUM94	2.0	1.7	0.5	1.2
AUM93	15.6	26.0	33.4	68.8
AUM141	4.3	9.5	19.8	7.1
5-FU	5.4	0.8	10.1	3.7
CPT	< 0.1	< 0.1	< 0.1	< 0.1

Supplementary Table 7: IC50 values of 6-(substituted piperazine)-9-(substituted benzyl) purine analogues: All molecules were tested in triplicate and incubated for 72 hours and the IC50 values are in μM range. NI stands for “no inhibition”.

AUM codes	IC50 (μM)			
	Huh7	HepG2	MAHLAVU	FOCUS
AUM157	7.2	14.1	13.2	24.3
AUM158	3.1	3.3	1.0	4.9
AUM159	1.6	< 1.0	35.0	9.6
AUM160	< 0.1	< 0.1	11.7	1.0
AUM162	0.2	< 0.1	77.2	2.2
AUM163	9.9	7.4	17.9	8.7
AUM174	24.7	44.2	29.2	49.8
AUM175	5.0	7.1	7.4	9.4
AUM176	1.6	1.3	0.3	3.4
AUM177	< 1.0	< 1.0	51.5	2.2
AUM178	0.1	< 0.1	8.3	1.4
AUM179	0.5	< 0.1	5.9	2.4
AUM180	1.4	< 1.0	11.8	4.4
AUM181	0.1	< 0.1	6.3	2.5

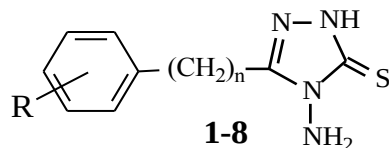
AUM148	8.5	6.6	11.8	9.4
AUM52	4.4	0.8	5.0	< 1.0
AUM150	12.7	26.9	14.4	19.0
AUM54	1.3	0.7	0.5	0.4
AUM53	2.7	2.3	3.4	2.4
AUM151	< 1.0	< 1.0	2.9	< 1.0
AUM173	10.5	11.1	11.5	22.1
AUM164	4.0	2.5	6.3	4.3
AUM165	7.7	15.5	9.1	NI
AUM166	0.6	1.0	< 0.1	0.1
AUM167	< 1.0	< 1.0	9.3	1.5
AUM168	< 0.1	< 0.1	8.0	1.2
AUM169	0.1	< 0.1	8.1	3.1
AUM170	2.5	5.4	11.2	29.9
AUM172	< 1.0	13.6	12.7	8.2
AUM72	13.0	7.1	45.7	7.7
AUM142	10.1	17.2	20.6	17.5
AUM143	5.4	4.8	9.5	8.2
AUM56	1.4	10.9	3.5	1.6
AUM144	2.6	2.2	2.2	1.0
AUM57	0.5	1.2	4.6	0.6
AUM145	1.1	0.7	0.5	0.3
AUM146	3.5	3.2	5.9	6.2
AUM73	0.1	< 0.1	5.7	1.6
AUM78	25.6	29.0	40.3	15.5
AUM79	28.3	24.8	NI	NI
AUM154	5.4	6.4	8.0	6.4
AUM74	3.3	4.3	6.0	7.1
AUM155	1.4	1.4	1.9	1.5
AUM76	0.2	< 1.0	94.4	0.5
AUM75	0.1	< 0.1	< 0.1	< 0.1
AUM80	2.1	1.6	2.3	1.9
AUM156	5.5	3.8	4.8	5.1
AUM77	0.2	< 0.1	8.1	1.4
AUM68	19.2	24.6	10.8	11.6
AUM69	23.9	61.3	80.3	83.3
AUM64	6.7	7.1	13.0	9.1
AUM66	1.9	1.5	2.3	< 1.0

AUM65	0.2	< 0.1	< 0.1	< 0.1
AUM152	2.3	188	3.2	4.5
AUM153	2.2	1.2	10.8	5.0
AUM67	< 0.1	< 0.1	5.6	1.2
5-FU	5.4	0.8	10.1	3.7
CPT	< 0.1	< 0.1	< 0.1	< 0.1

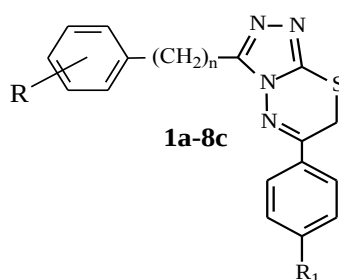
Supplementary Table 8: IC₅₀ values of 6-(4-substituted phenyl)-9-(4-substituted phenyl) sulfonyl and 2-amino/2-chloro/6-(4-substituted piperazine)/6-(4-substituted phenyl amino)/6-(2-substituted ethylamino)-9-(β -D-ribofuranosyl) purine analogues: All molecules were tested in triplicate and incubated for 72 hours and the IC₅₀ values are in μ M range. NI stands for “no inhibition”.

AUM codes	IC ₅₀ (μ M)			
	Huh7	HepG2	MAHLAVU	FOCUS
AUM198	41.2	NI	89.9	82.2
AUM208	40.4	NI	29.6	33.3
AUM209	16.0	48.6	17.3	NI
AUM210	14.4	32.8	16.5	17.3
AUM211	13.6	23.4	21.0	26.3
AUM212	11.0	14.6	23.4	22.3
AUM215	NI	NI	93,2	39,6
AUM223	78,4	NI	NI	9,1
AUM222	11,5	19,0	7,5	17,0
AUM218	50,9	28,4	27,2	22,2
AUM216	39,4	40,4	37,4	21,2
AUM220	NI	< 1,0	NI	NI
AUM228	2,1	0,1	5,1	23,7
AUM229	20,7	46,6	30,2	45,2
5-FU	5.4	0.8	10.1	3.7
CPT	< 0.1	< 0.1	< 0.1	< 0.1

Supplementary Table 9: IC50 values of HUBT coded 4-amino-3-substituted-1,2,4-triazole-5-thiones (1-8) and 3,6-disubstituted-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazines (1a-8c): All molecules were tested in duplicate and incubated for 72 hours. The IC50 values are in μM range. NI stands for “no inhibition”.



					IC50 (μM)		
	HUBT Code	R	R ₁	n	Huh7	HCT116	T47D
1	AA-2M	2-OCH ₃	-	1	NI	NI	NI
2	PA-2M	2- OCH ₃	-	2	NI	NI	NI
3	AA-3M	3- OCH ₃	-	1	NI	99.9	NI
4	PA-3M	3- OCH ₃	-	2	NI	NI	NI
5	AA-4M	4- OCH ₃	-	1	NI	89.7	NI
6	PA-4M	4- OCH ₃	-	2	NI	97.8	NI
7	AA-TM	3,4,5- OCH ₃	-	1	NI	NI	NI
8	PA-TM	3,4,5- OCH ₃	-	2	NI	NI	NI

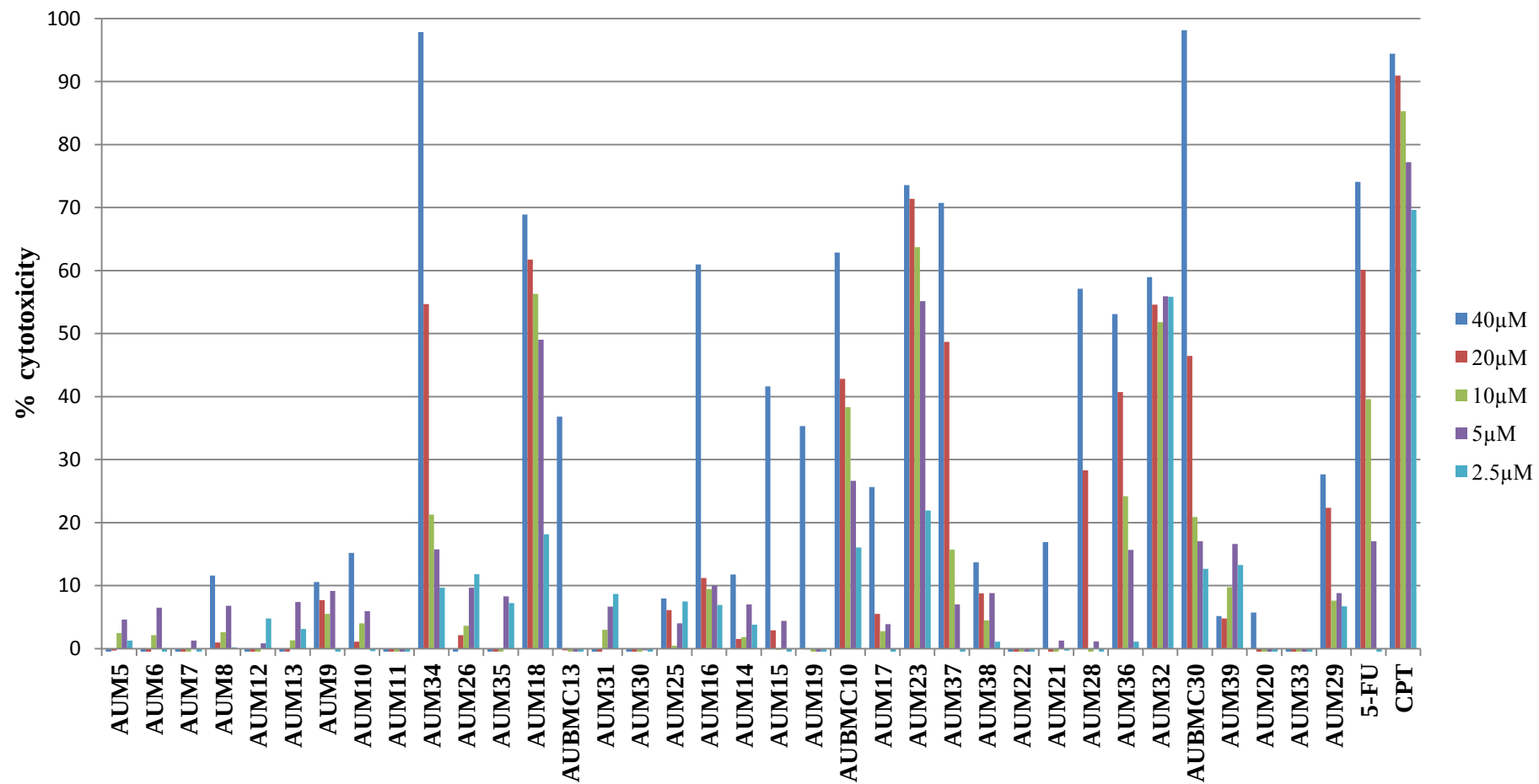


					IC50 (μM)		
	HUBT Code	R	R ₁	n	Huh7	HCT116	T47D
1a	n-AT-2M	2-OCH ₃	H	1	37.6	48.1	NI
1b	CIAT-2M	2-OCH ₃	Cl	1	NI	NI	32.5
1c	FAT-2M	2-OCH ₃	F	1	90.3	NI	33.2

2a	n-PT-2M	2-OCH ₃	H	2	NI	NI	NI
2b	CIPT-2M	2-OCH ₃	Cl	2	23.0	NI	10.4
2c	FPT-2M	2-OCH ₃	F	2	44.6	NI	95.9
3a	n-AT-3M	3-OCH ₃	H	1	24.7	30.9	23.7
3b	CIAT-3M	3-OCH ₃	Cl	1	56.1	NI	76.2
3c	FAT-3M	3-OCH ₃	F	1	NI	NI	NI
4a	n-PT-3M	3-OCH ₃	H	2	78.4	NI	NI
4b	CIPT-3M	3-OCH ₃	Cl	2	43.9	NI	NI
4c	FPT-3M	3-OCH ₃	F	2	NI	NI	NI
5a	n-AT-4M	4-OCH ₃	H	1	64.8	73.6	35.5
5b	CIAT-4M	4-OCH ₃	Cl	1	72.4	NI	50.2
5c	FAT-4M	4-OCH ₃	F	1	28.6	NI	77.6
6a	n-PT-4M	4-OCH ₃	H	2	18.8	35.4	26.2
6b	CIPT-4M	4-OCH ₃	Cl	2	30.1	47.6	95.8
6c	FPT-4M	4-OCH ₃	F	2	NI	NI	61.0
7a	n-AT-TM	3,4,5-OCH ₃	H	1	5.0	15.0	14.3
7b	CIAT-TM	3,4,5-OCH ₃	Cl	1	< 1.0	< 1.0	< 1.0
7c	FAT-TM	3,4,5-OCH ₃	F	1	5.3	17.2	13.0
8a	n-PT-TM	3,4,5-OCH ₃	H	2	27.8	NI	NI
8b	CIPT-TM	3,4,5-OCH ₃	Cl	2	13.7	41.9	NI
8c	FPT-TM	3,4,5-OCH ₃	F	2	40.9	NI	NI

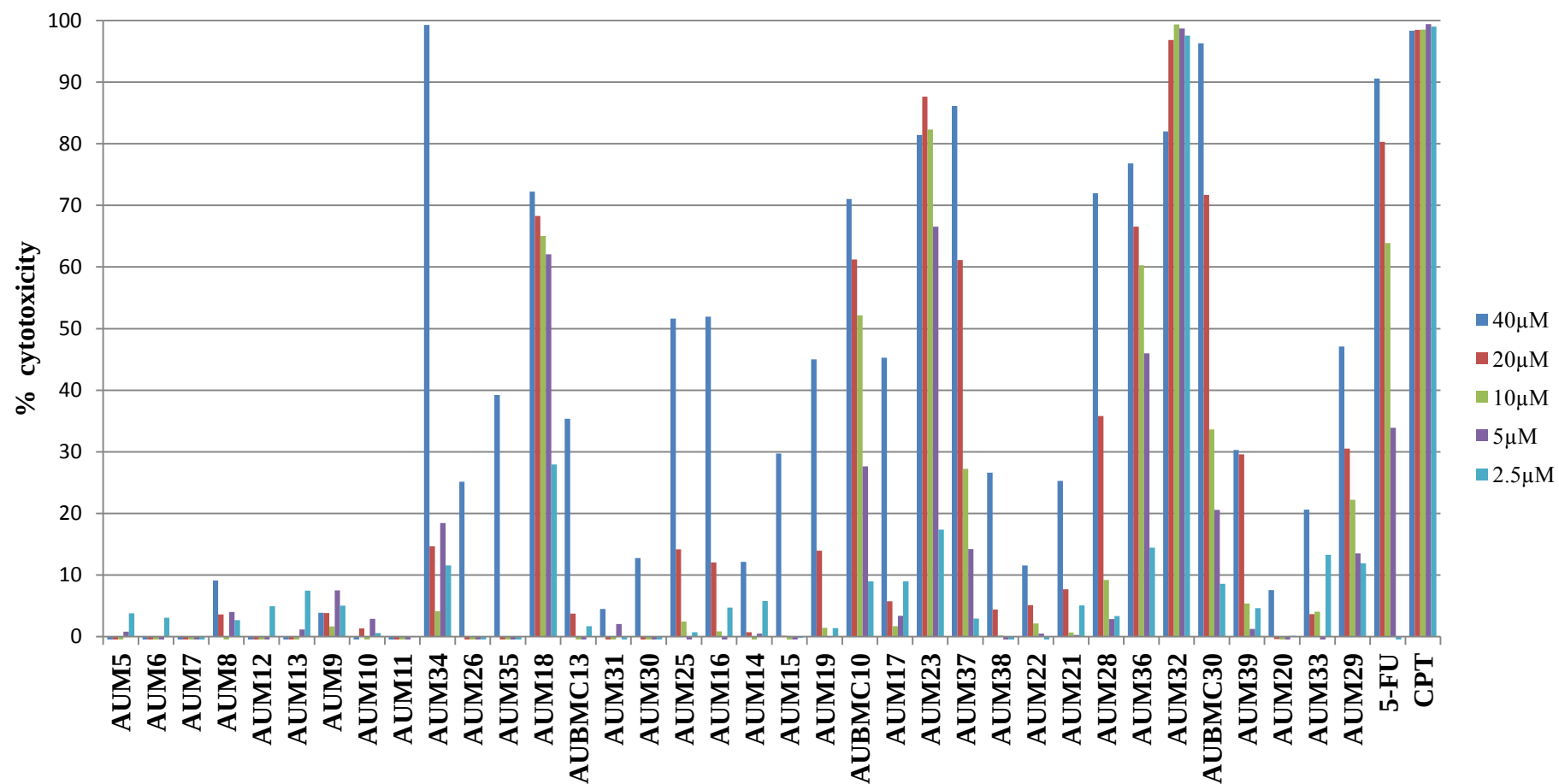
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HUH7



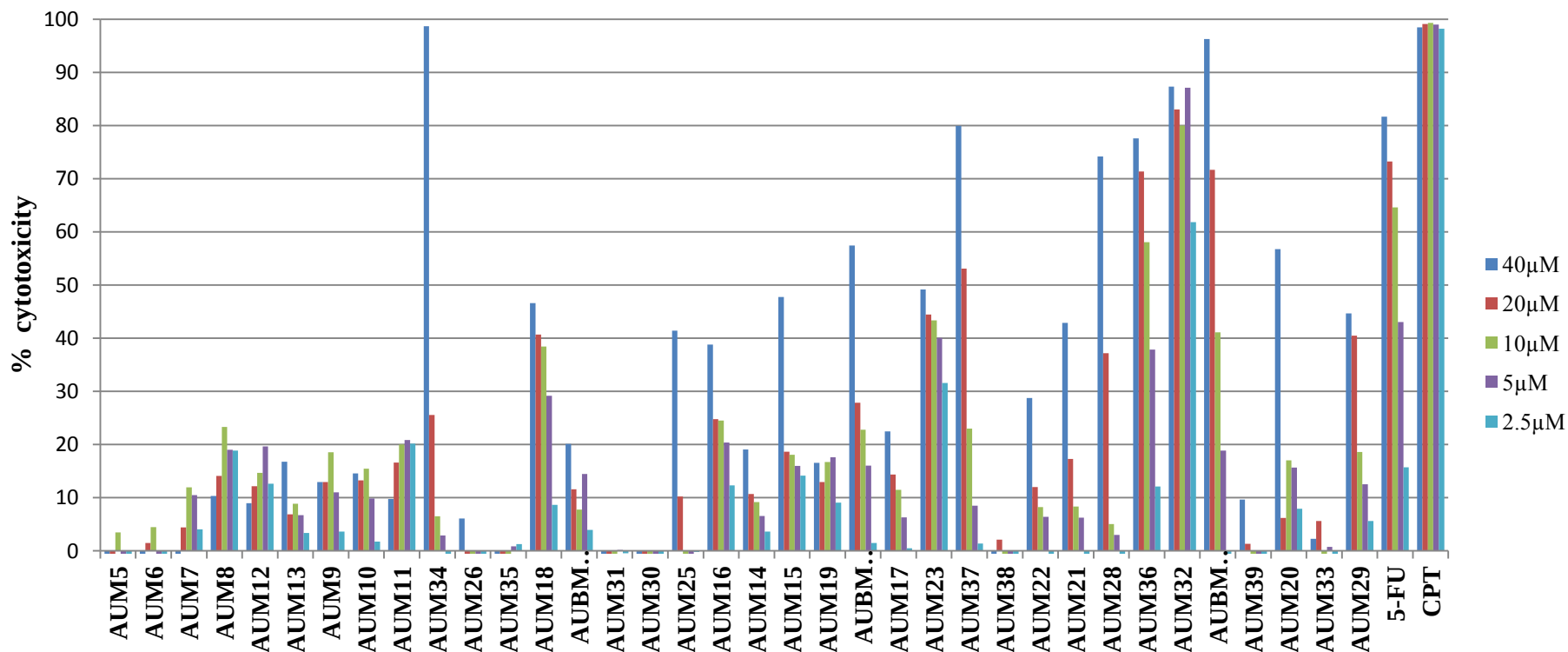
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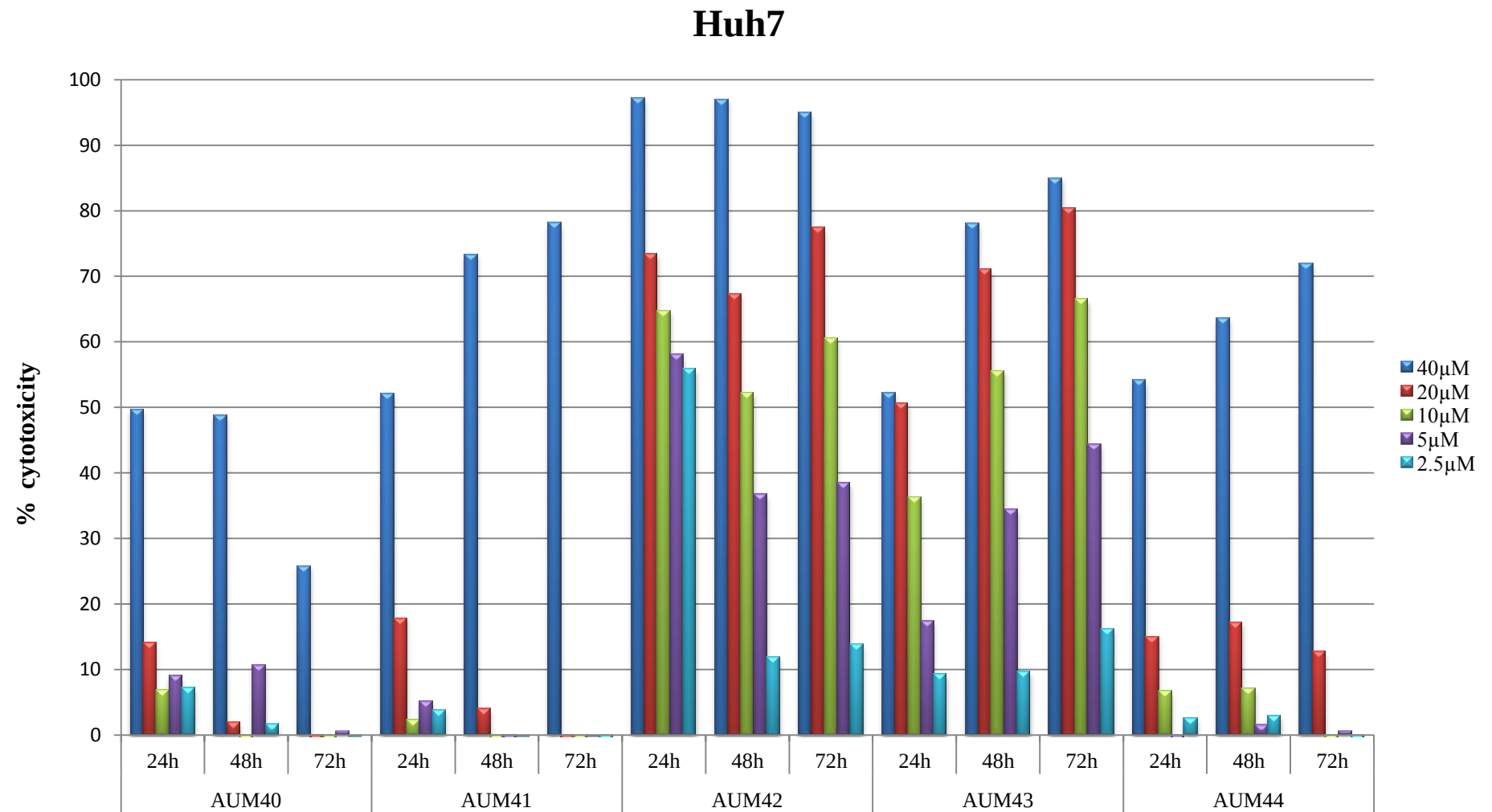
C)

T47D

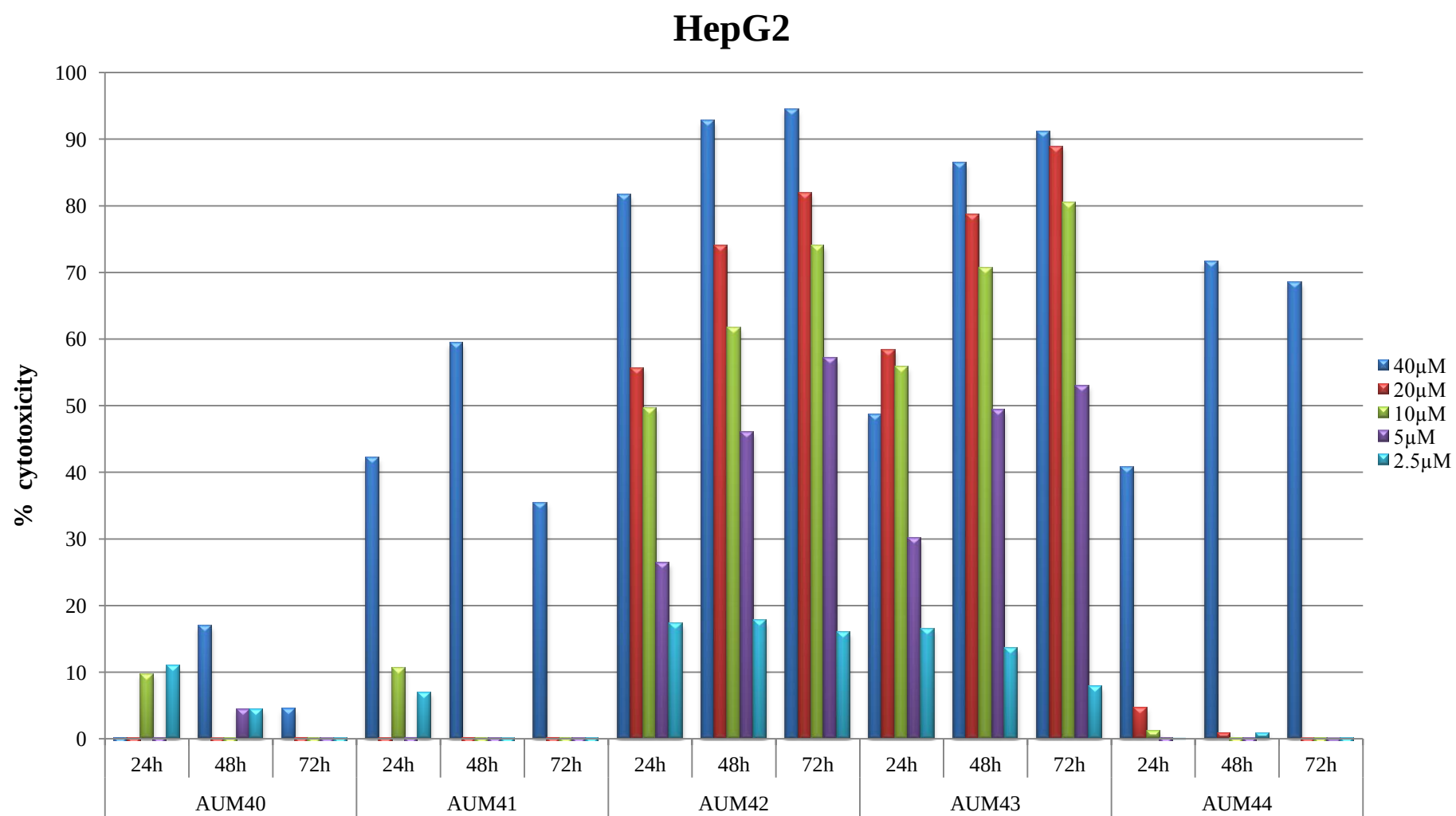


Supplementary Figure 1: Percent cytotoxicity in the presence of novel substituted purine analogues: Huh7 (A), HCT116 (B) and T47D (C) cells, inoculated in 96-well plates, were treated with these small molecules and their DMSO controls in duplicate with five different concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

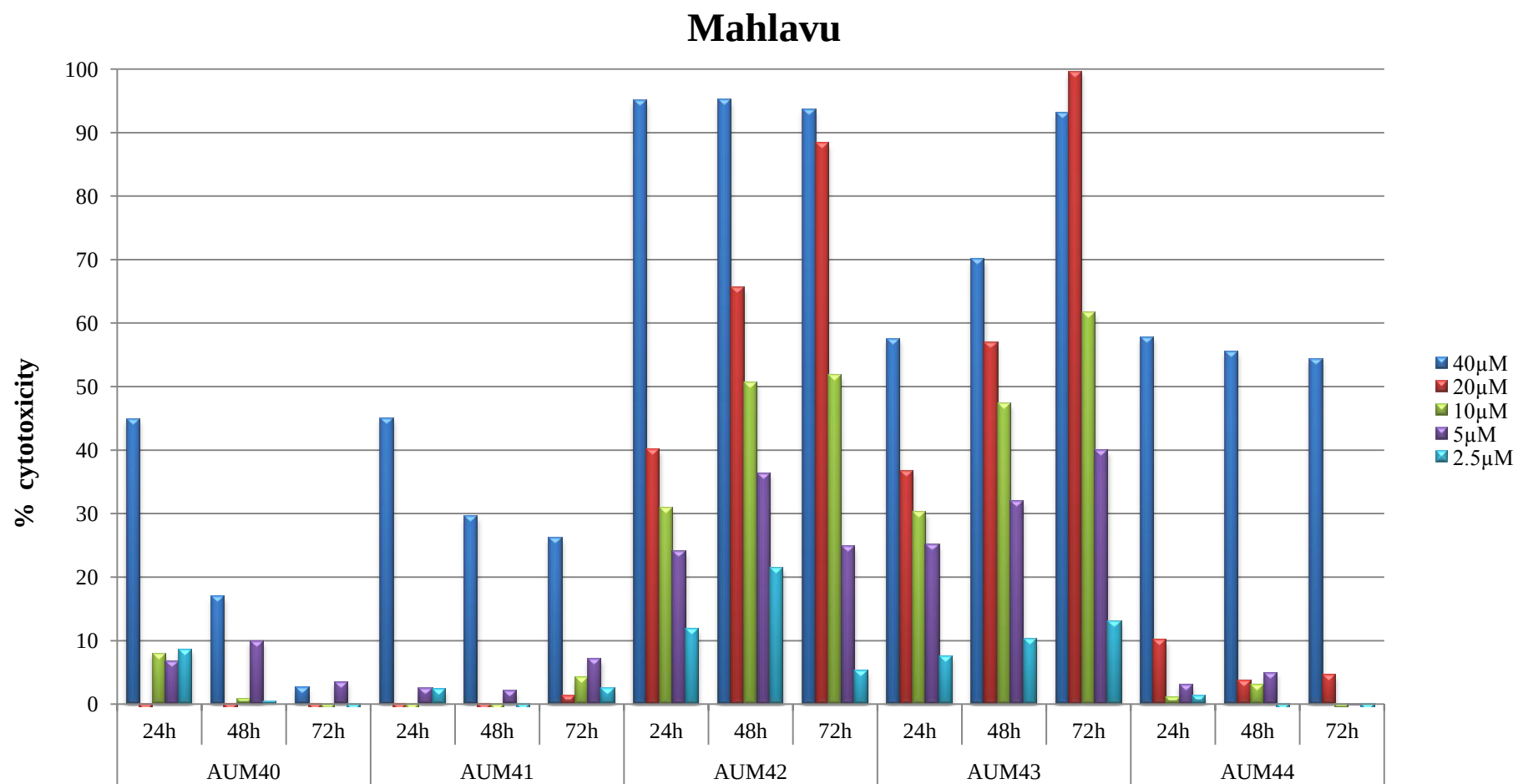
A)



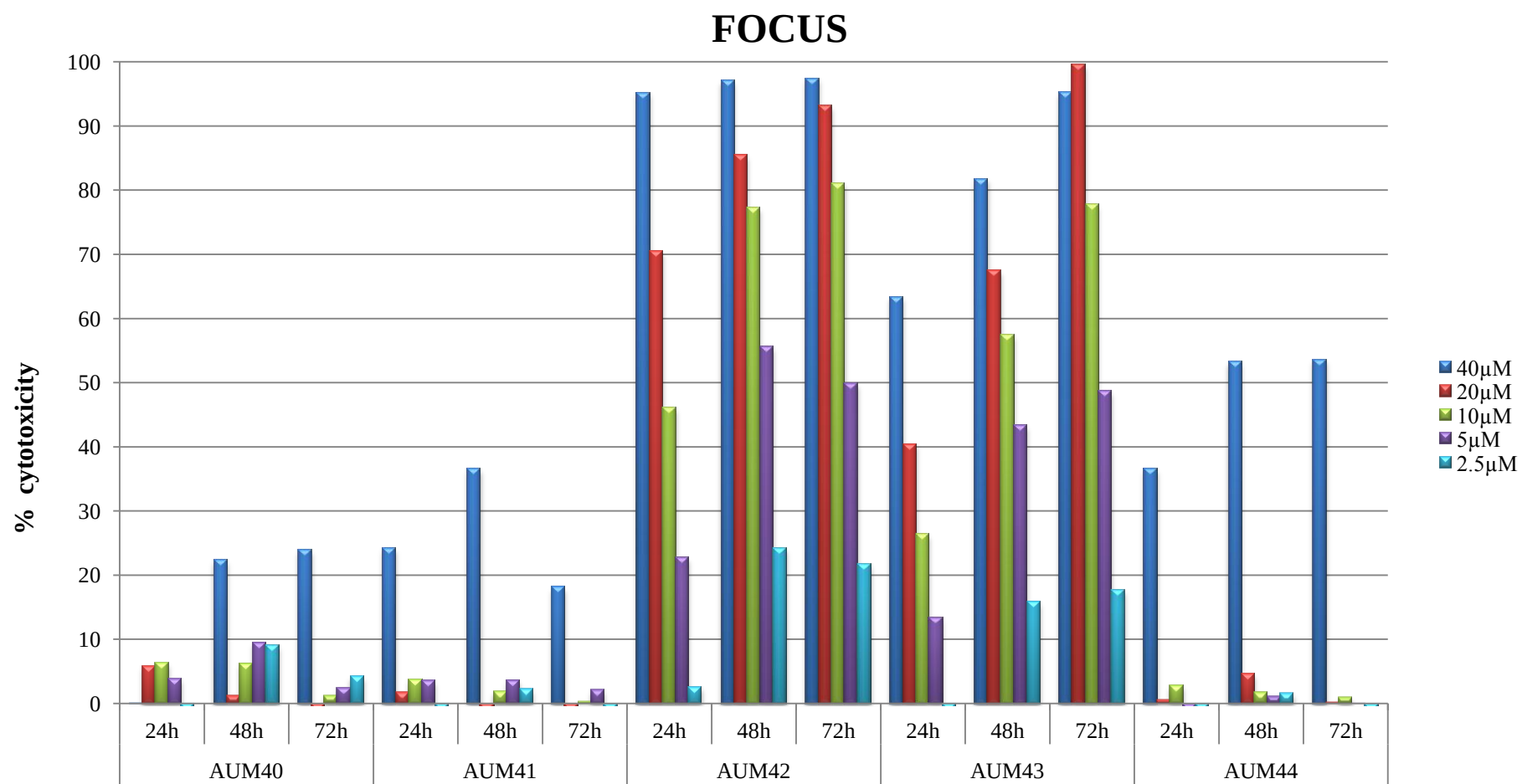
B)



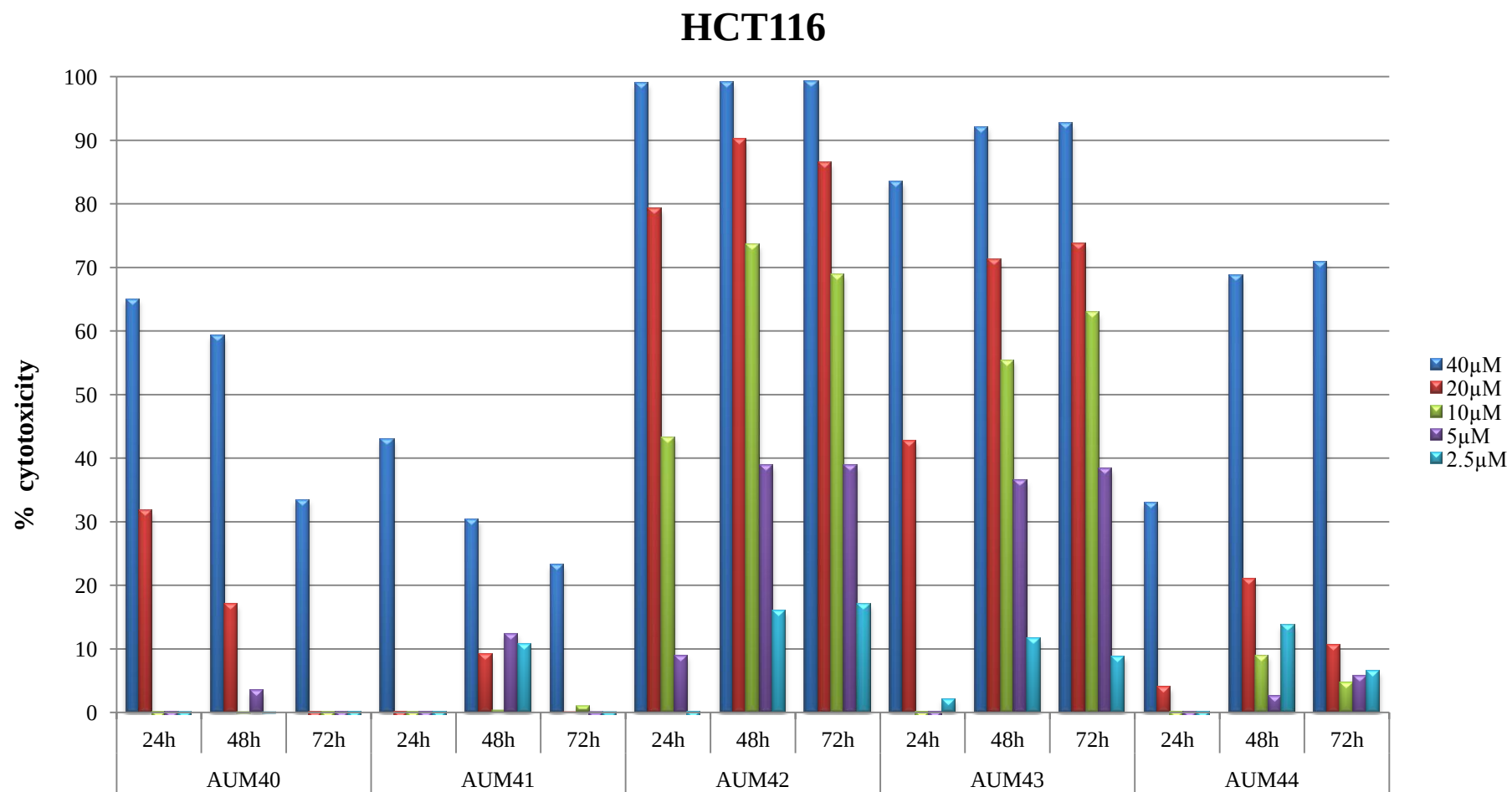
C)



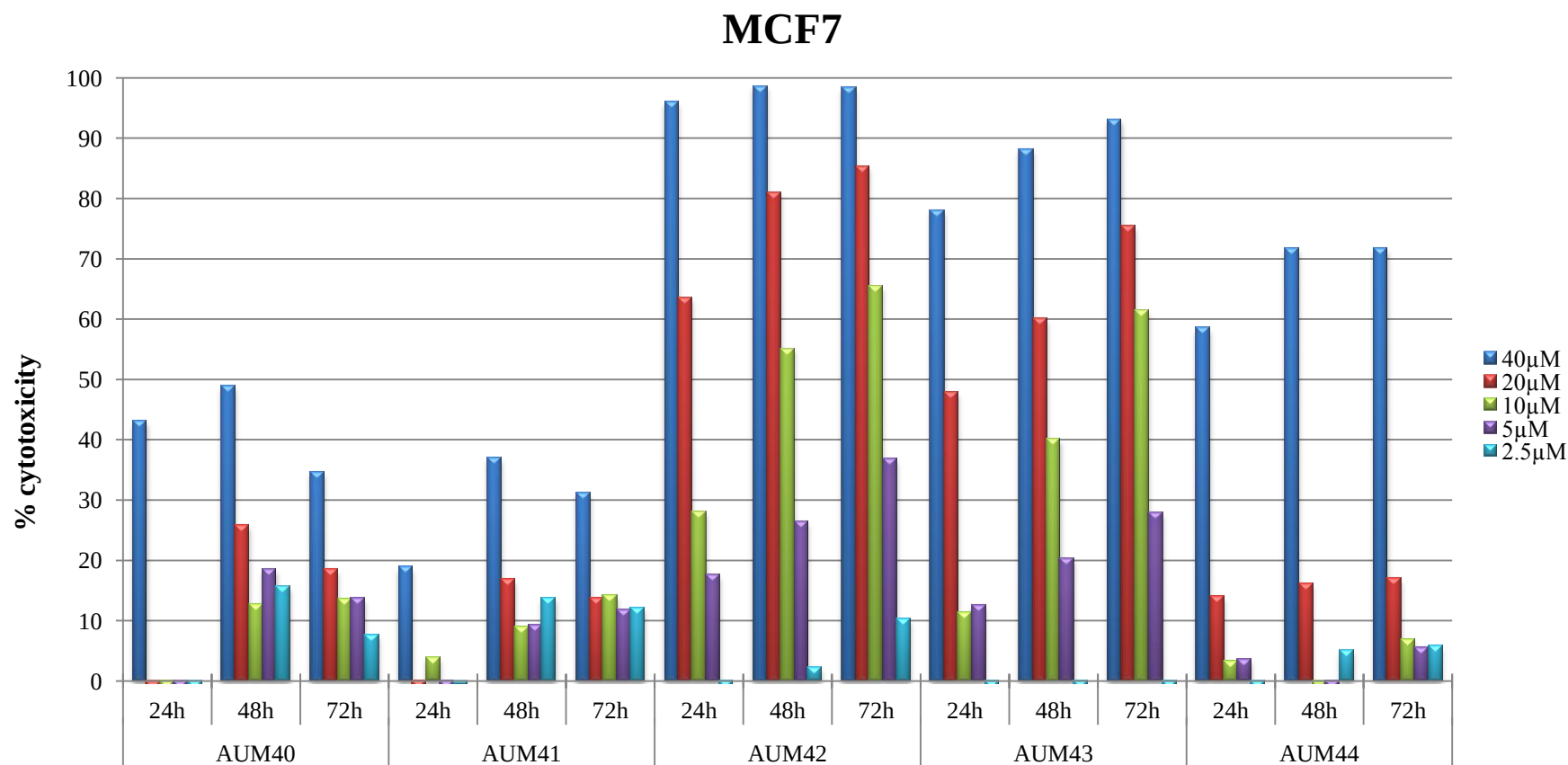
D)



E)



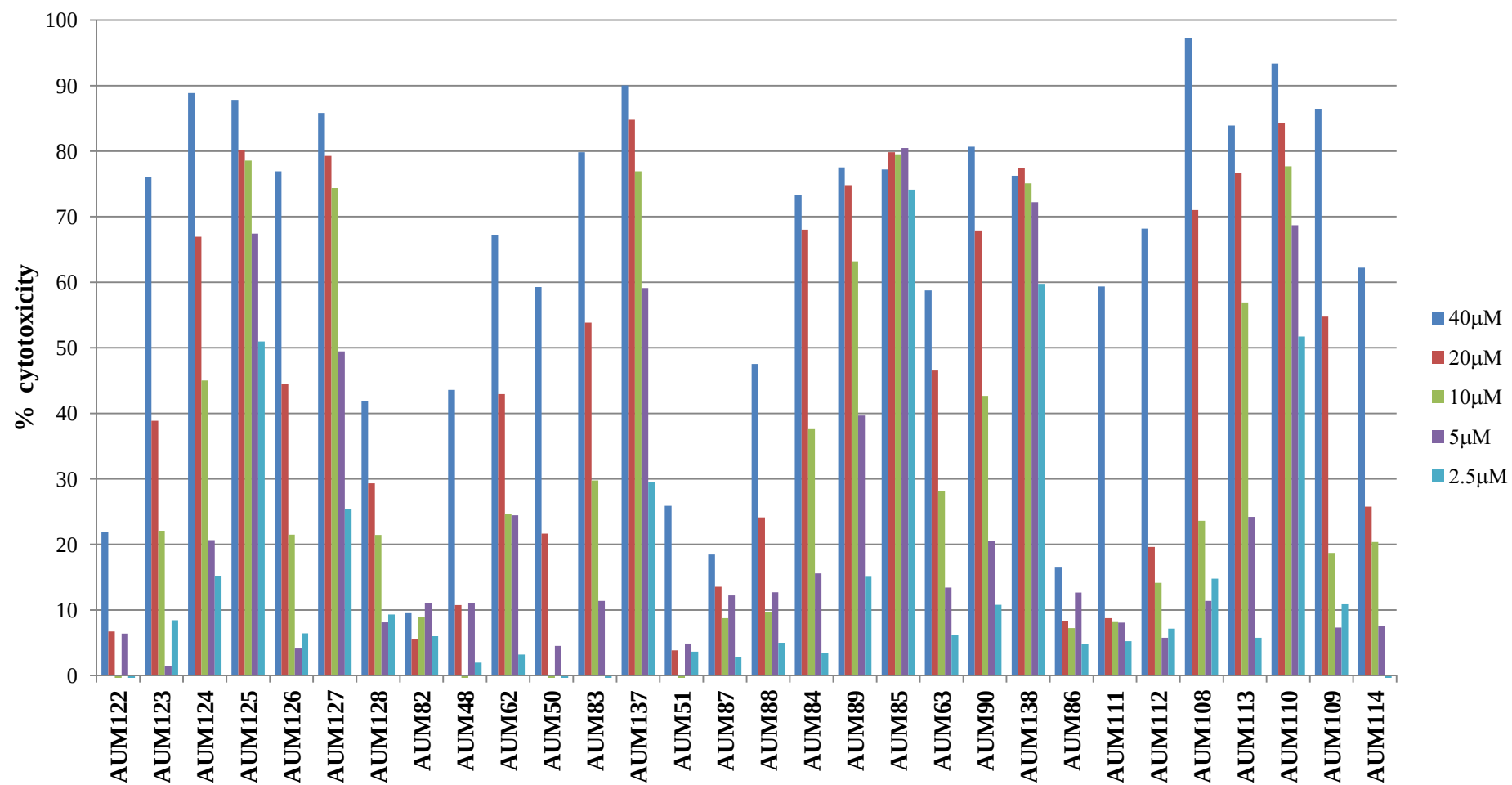
F)



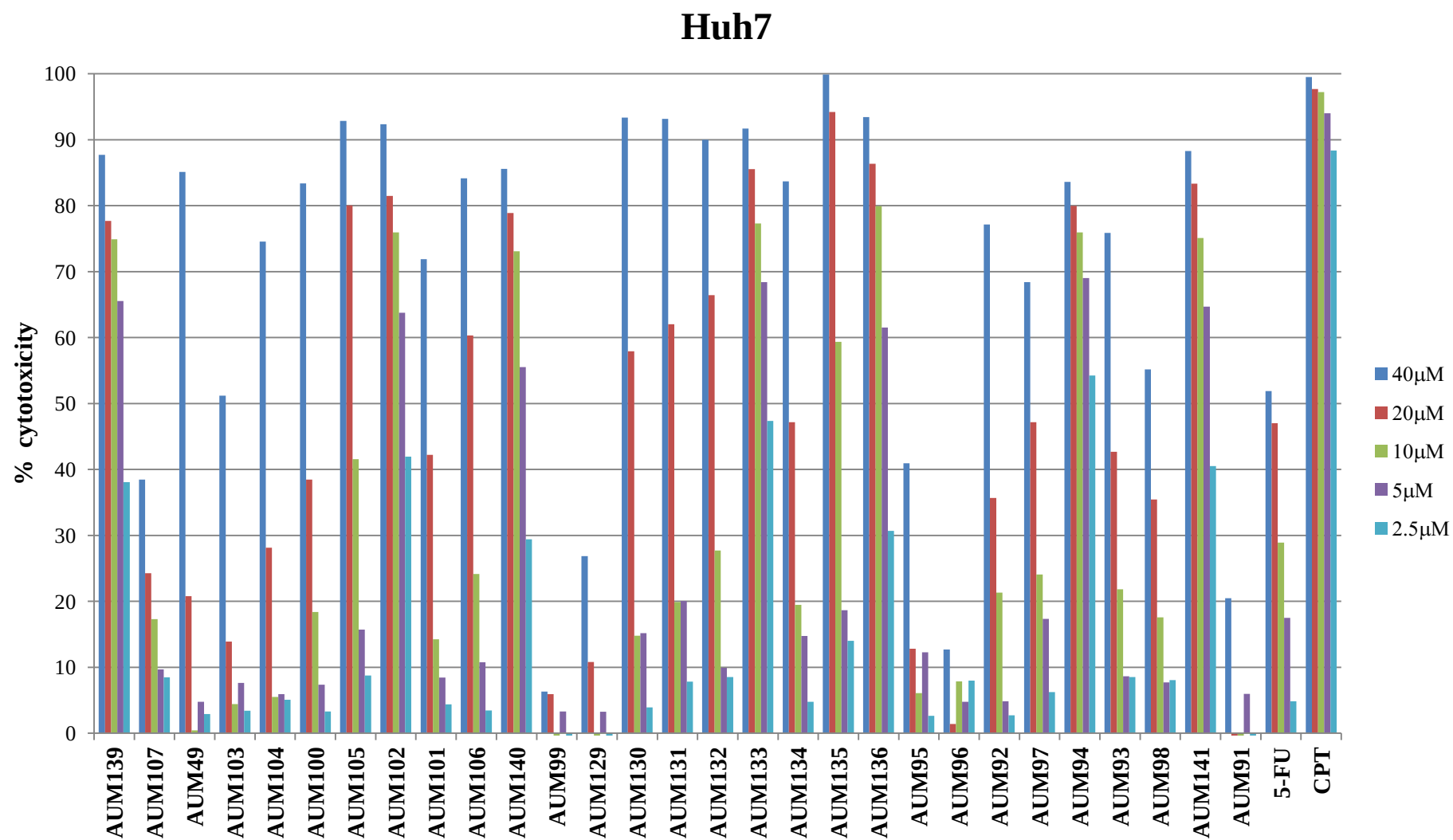
Supplementary Figure 2: Time- and dose- dependent percent cytotoxicity in the presence of novel purine ribonucleoside analogues: Huh7 (A), HepG2 (B), Mahlavu (C), FOCUS (D), HCT116 (E) and MCF7 (F) cells were inoculated in 96-well plates. All small molecules and their DMSO controls were applied to the cells in triplicate with five different concentrations for 24, 48 and 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison with DMSO-only treated wells.

A)

Huh7

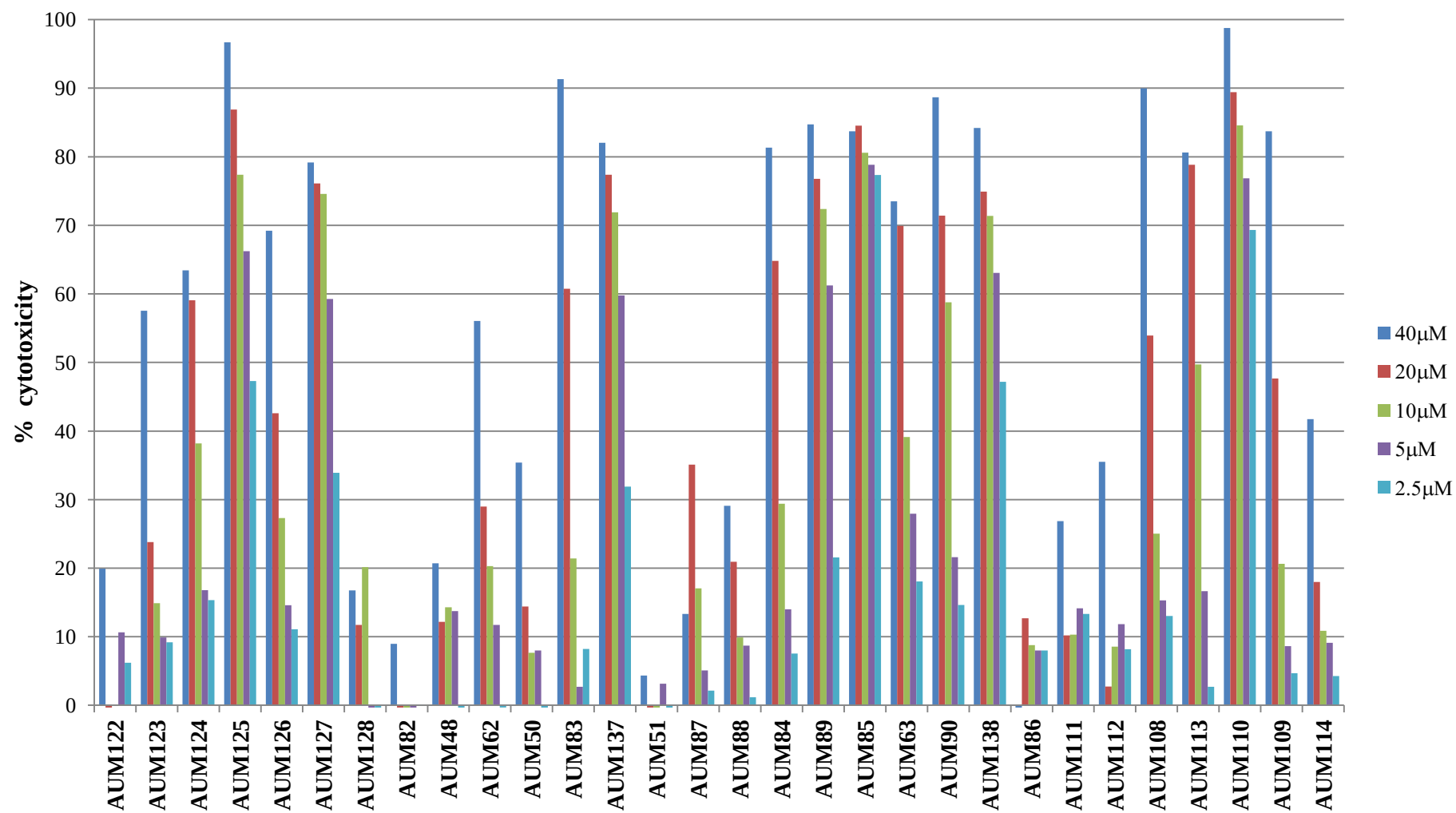


B)



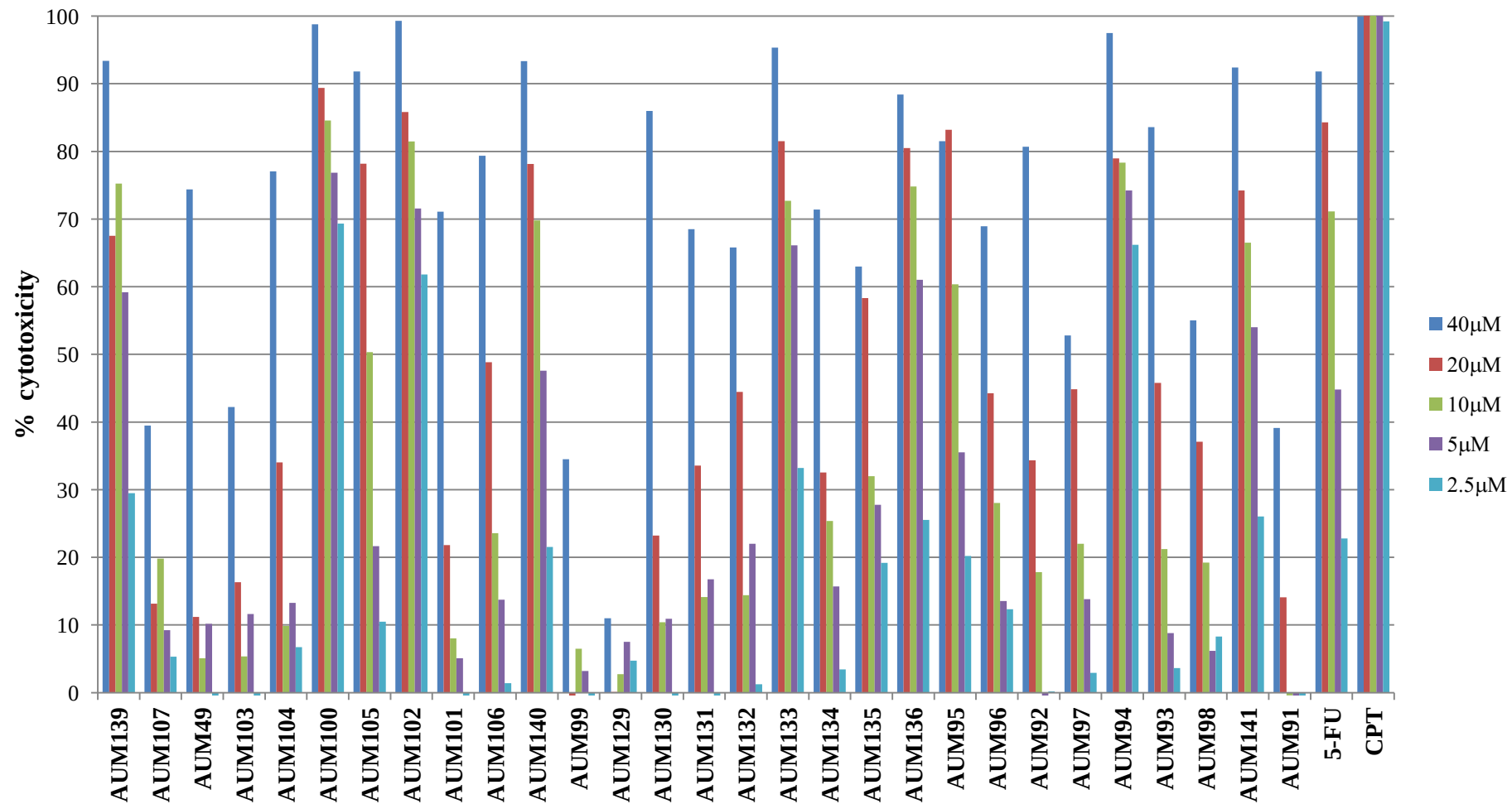
C)

HCT116



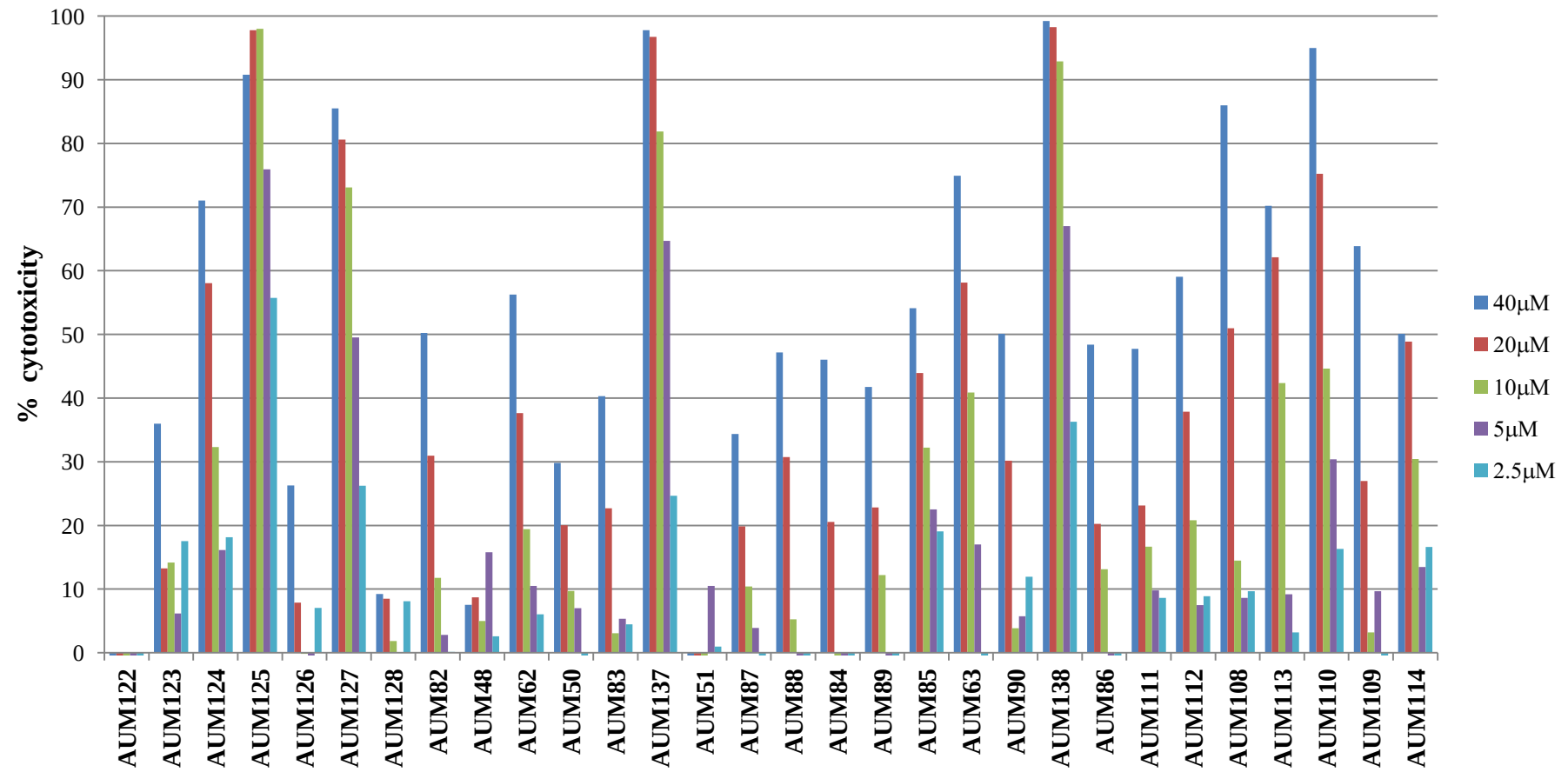
D)

HCT116

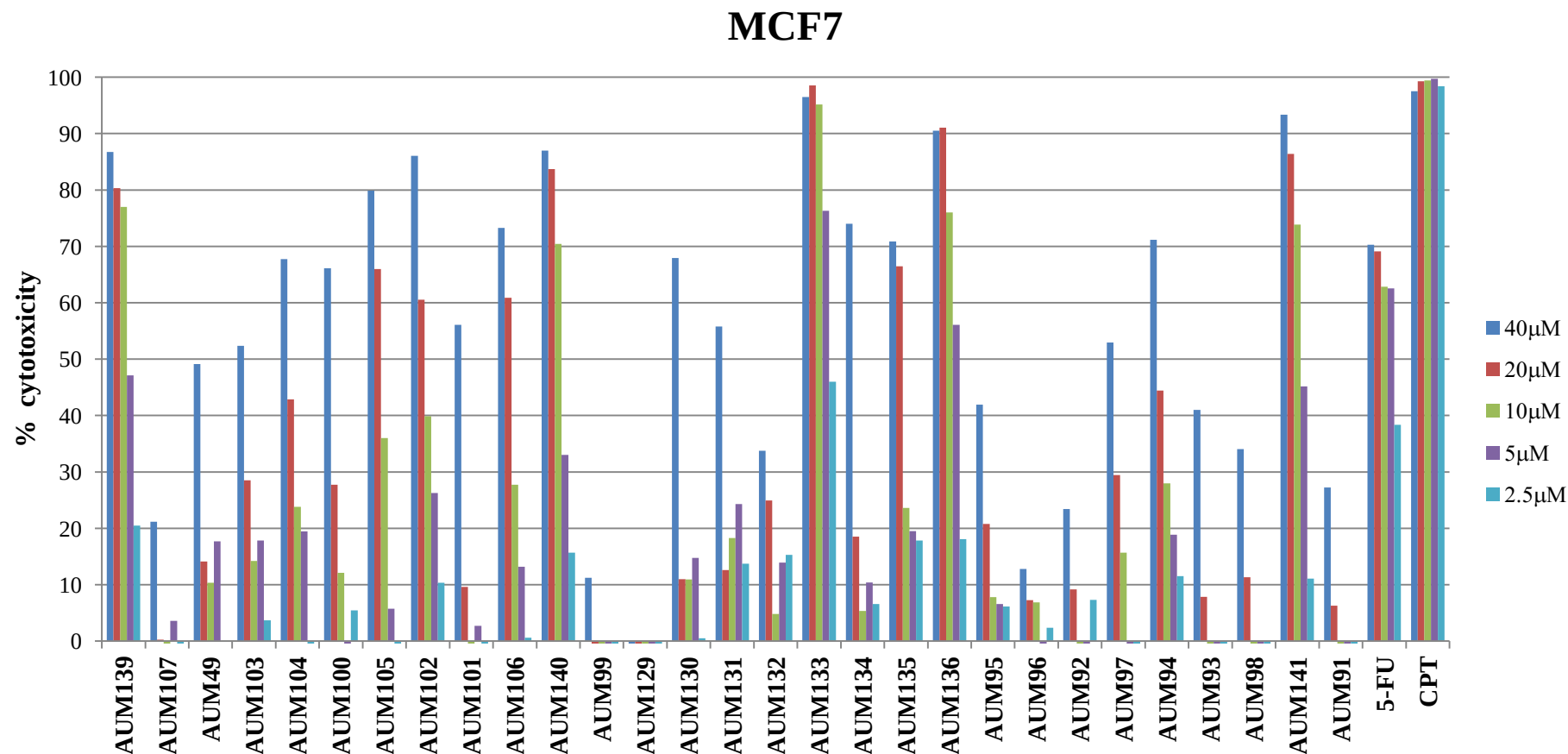


E)

MCF7



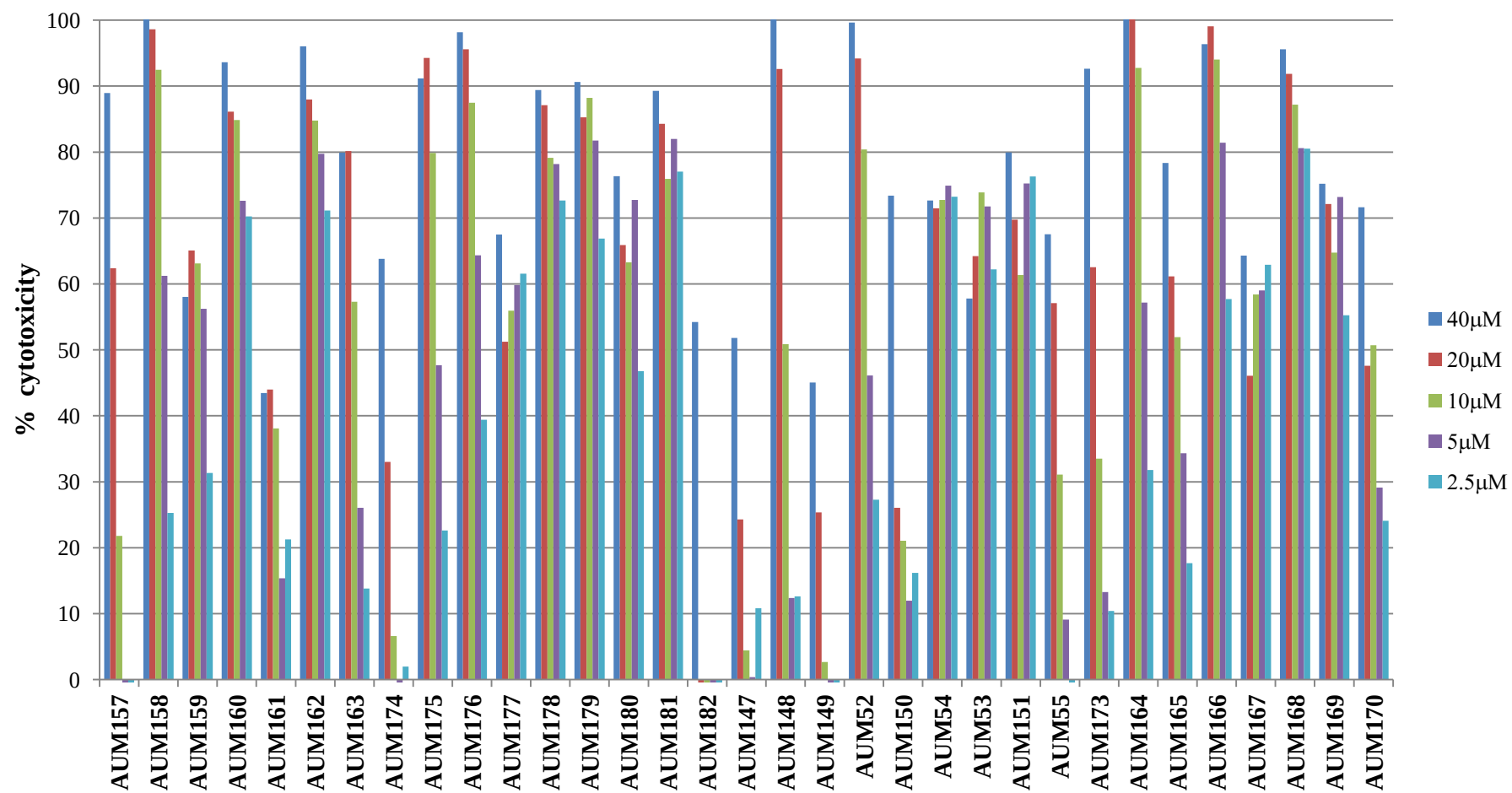
F)



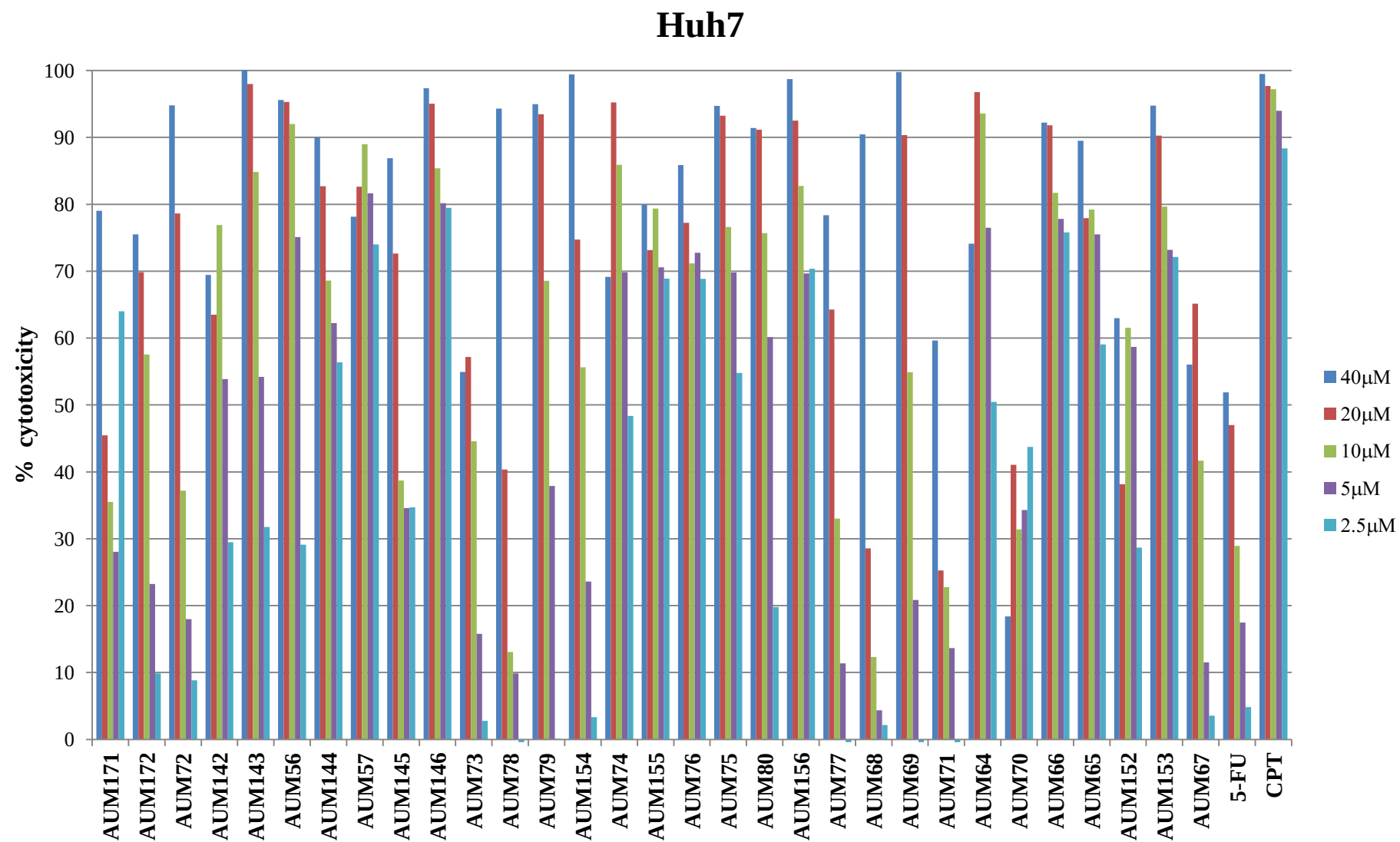
Supplementary Figure 3: Percent cytotoxicity in the presence of novel 6-(substituted piperazine)-9-(substituted phenyl) sulfonyl purine analogues given in Supplementary Table 2: Huh7 (A, B), HCT116 (C,D) and MCF7 (E,F) cells, inoculated in 96-well plates, were treated with these small molecules and their DMSO controls in duplicate with five different concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

A)

Huh7

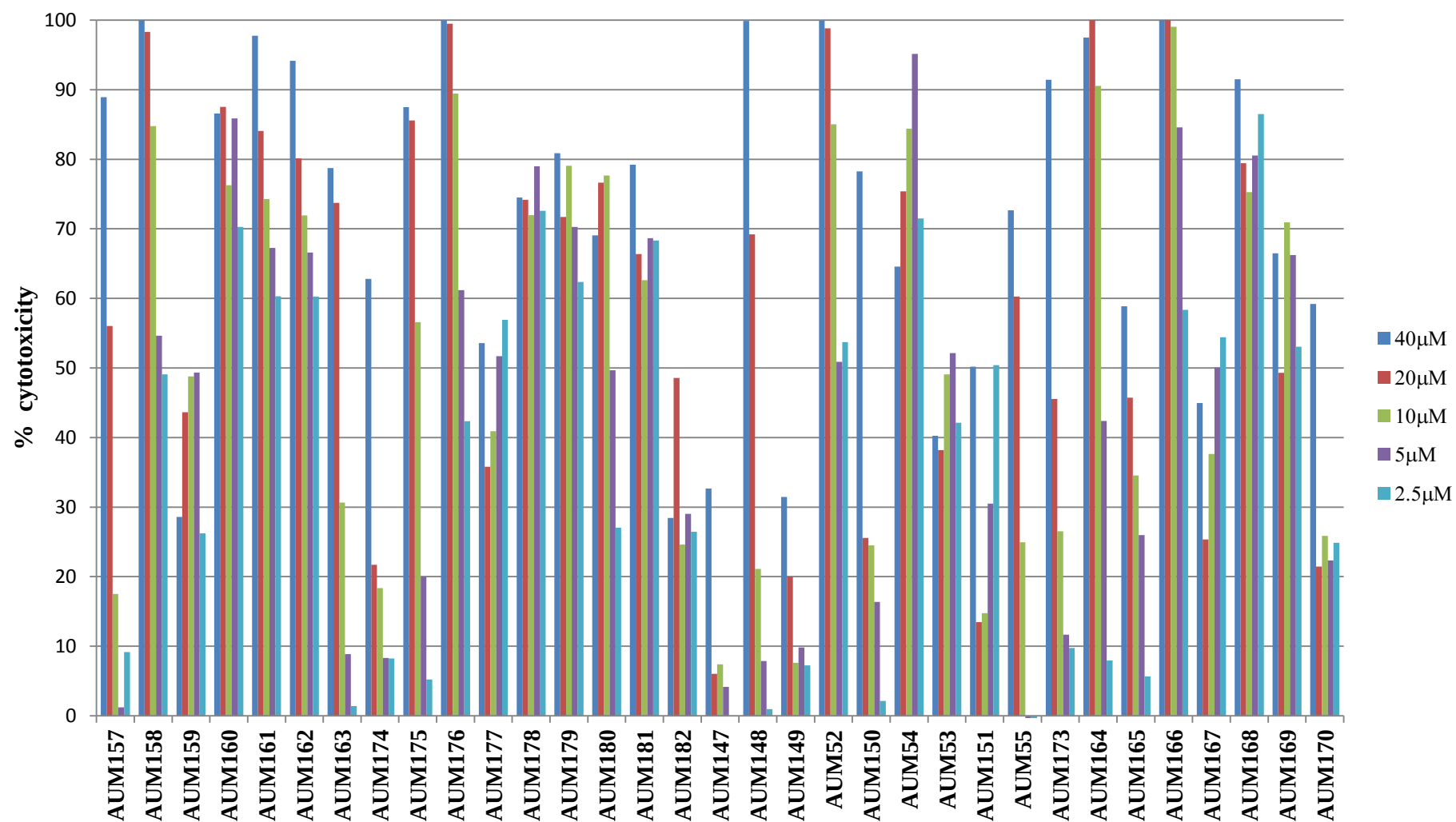


B)

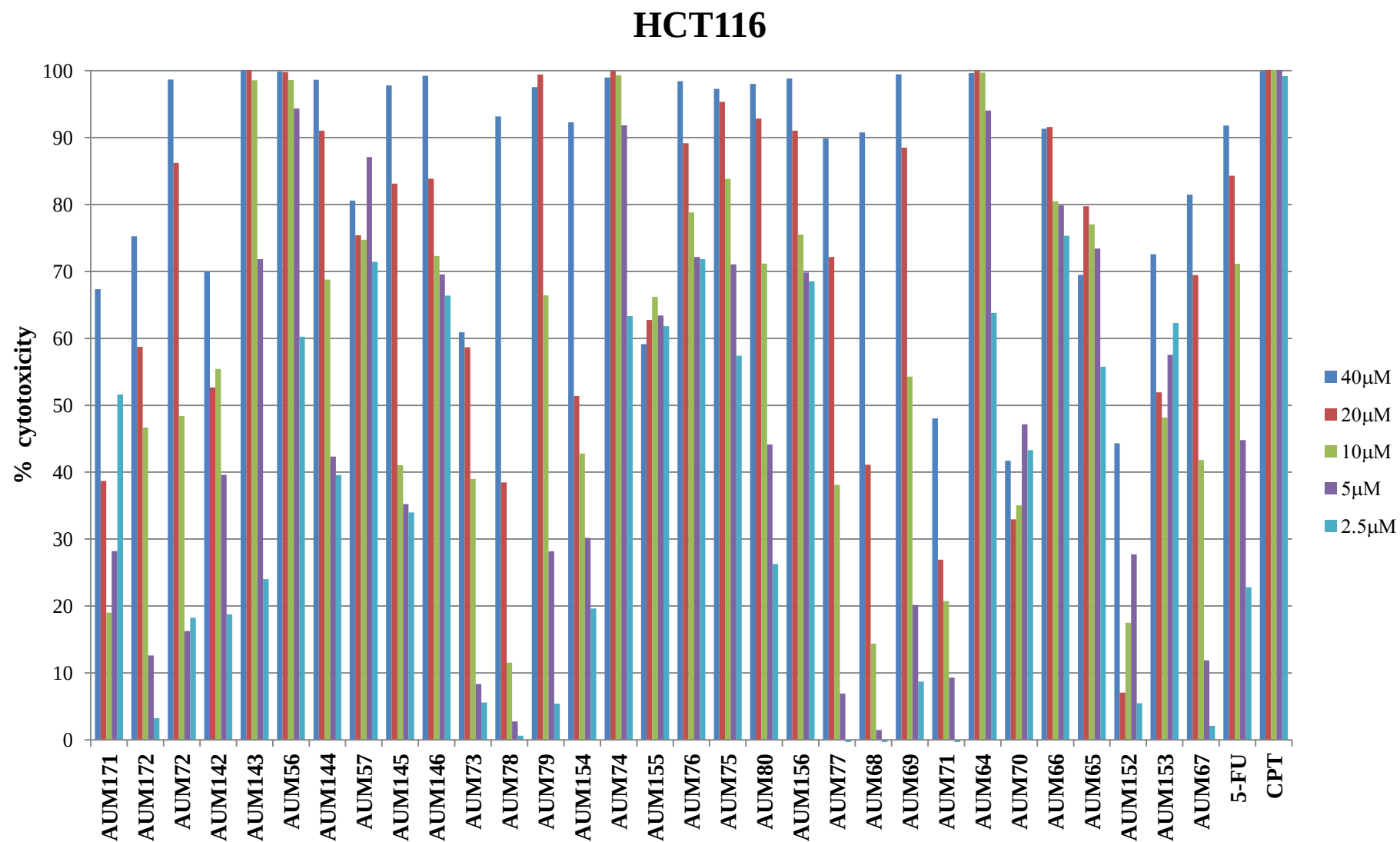


C)

HCT116

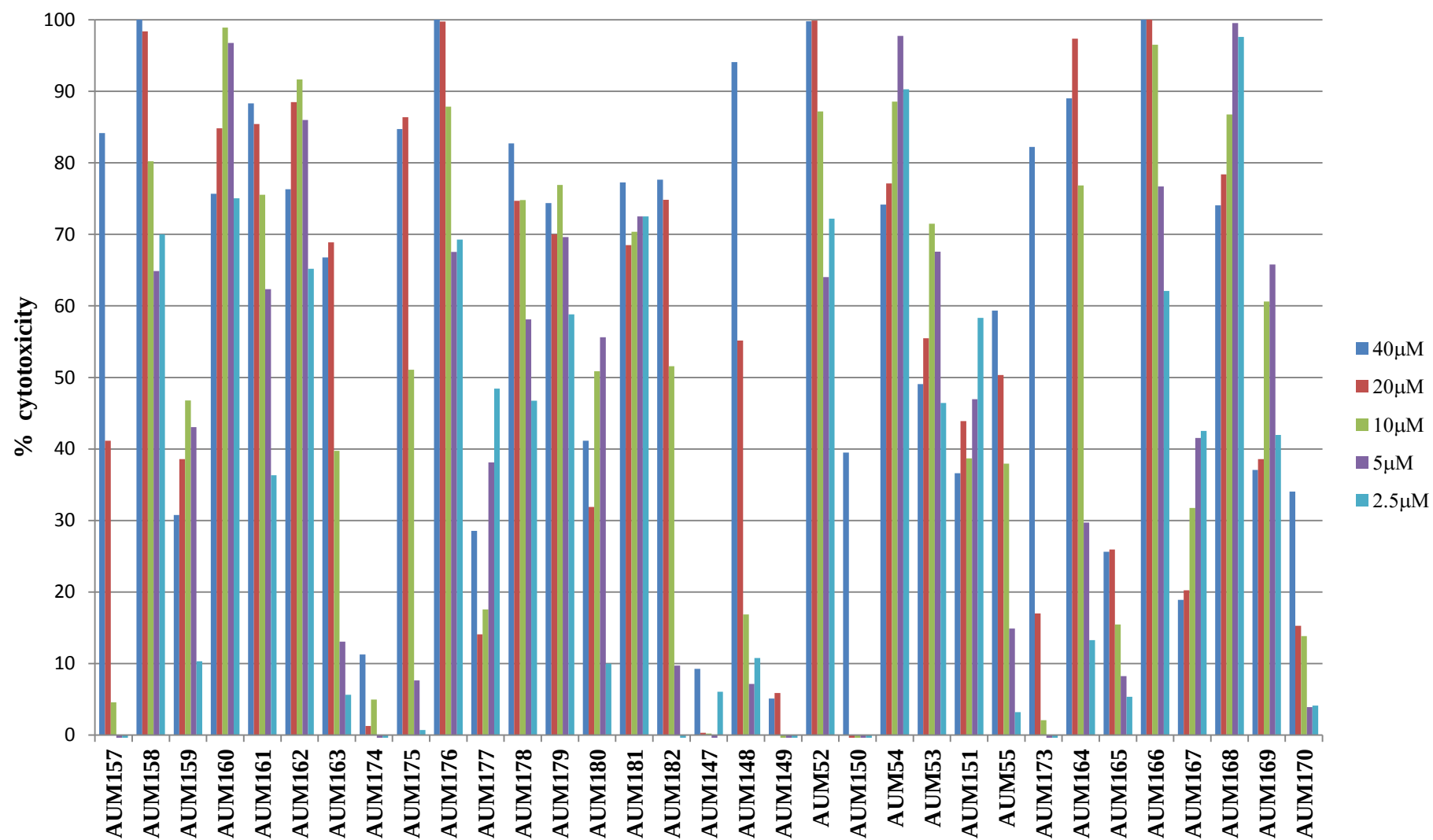


D)

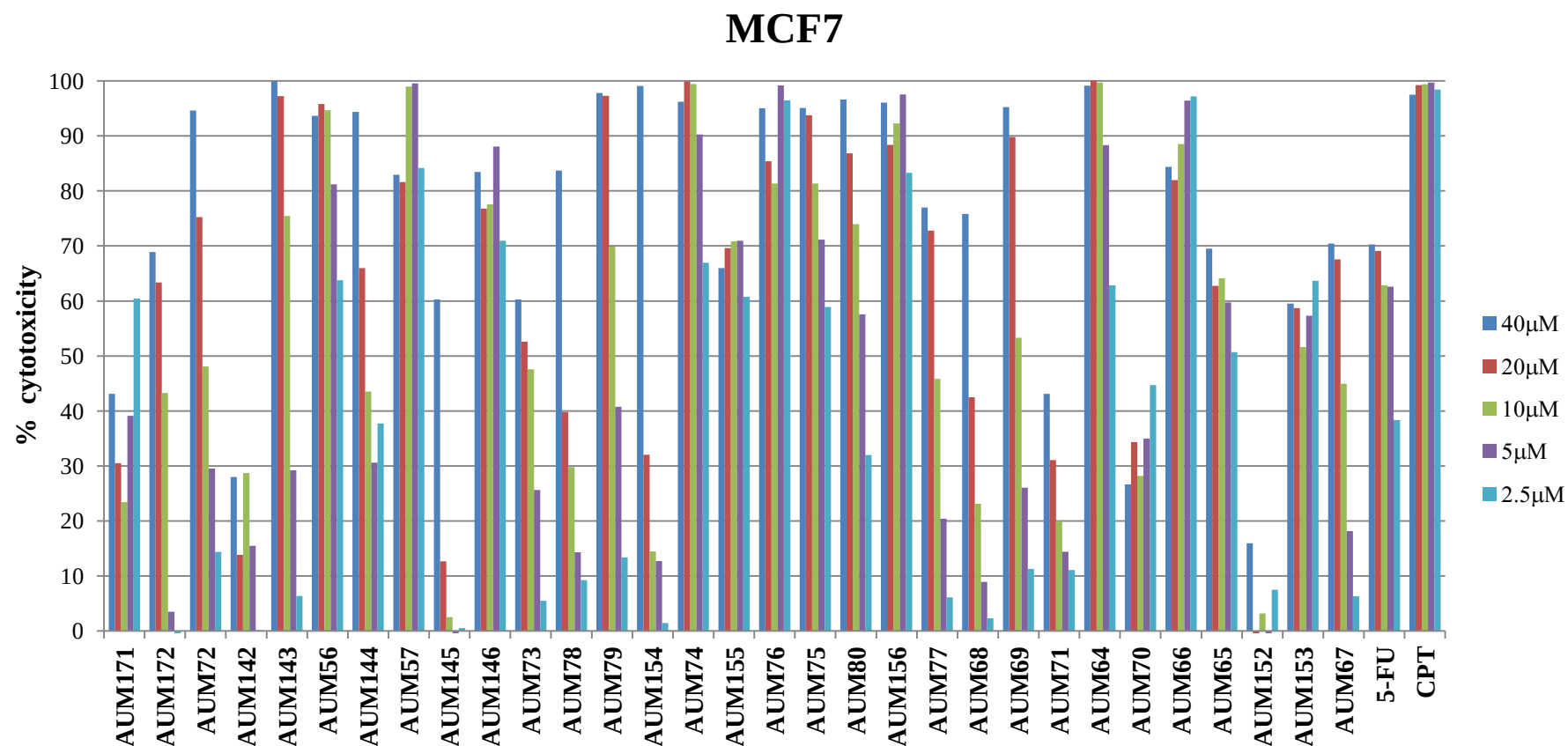


E)

MCF7

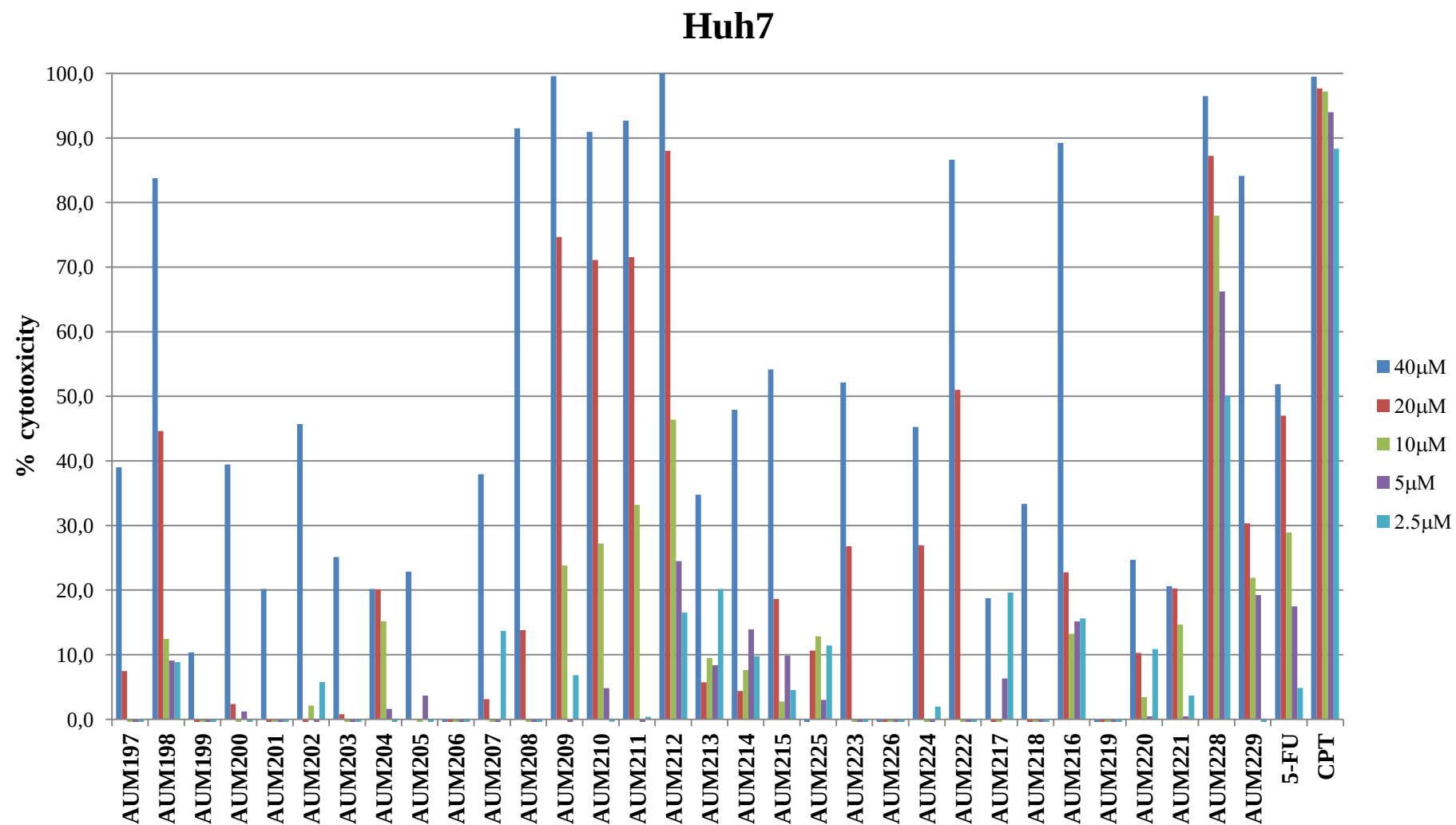


F)

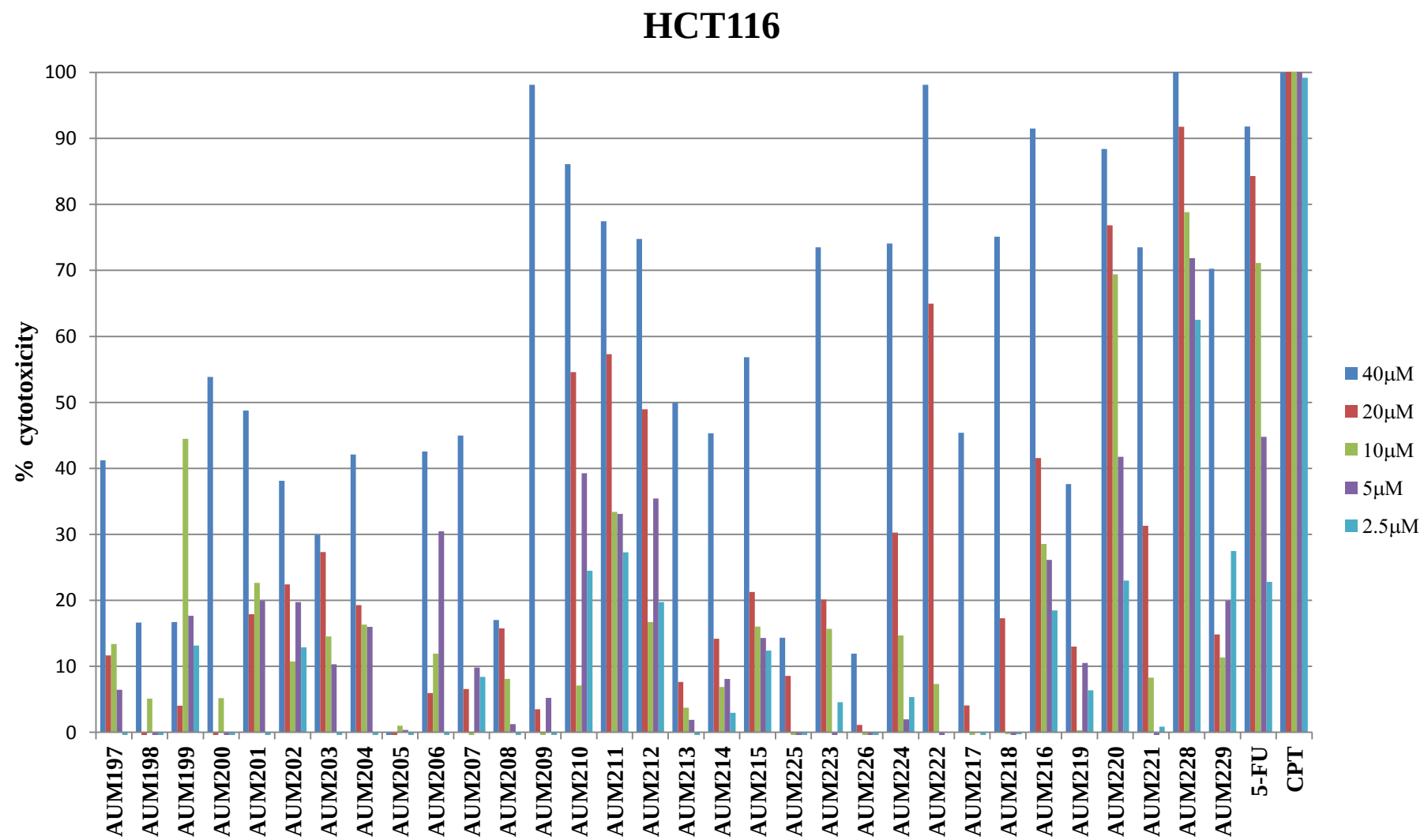


Supplementary Figure 4: Percent cytotoxicity in the presence of novel 6-(substituted piperazine)-9-(substituted benzyl) purine analogues given in Supplementary Table 3: Huh7 (A, B), HCT116 (C,D) and MCF7 (E,F) cells, inoculated in 96-well plates, were treated with these small molecules and their DMSO controls in duplicate with five different concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

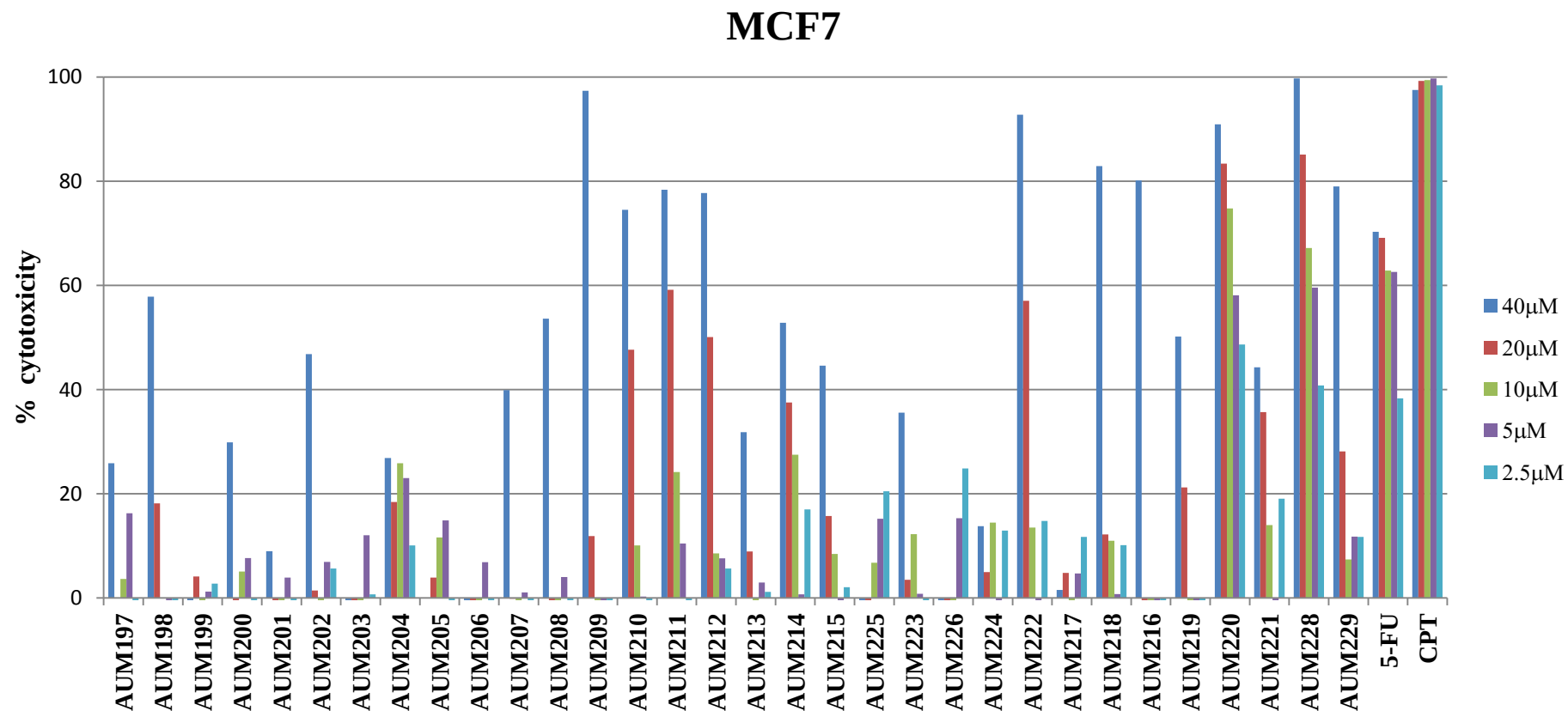
A)



B)

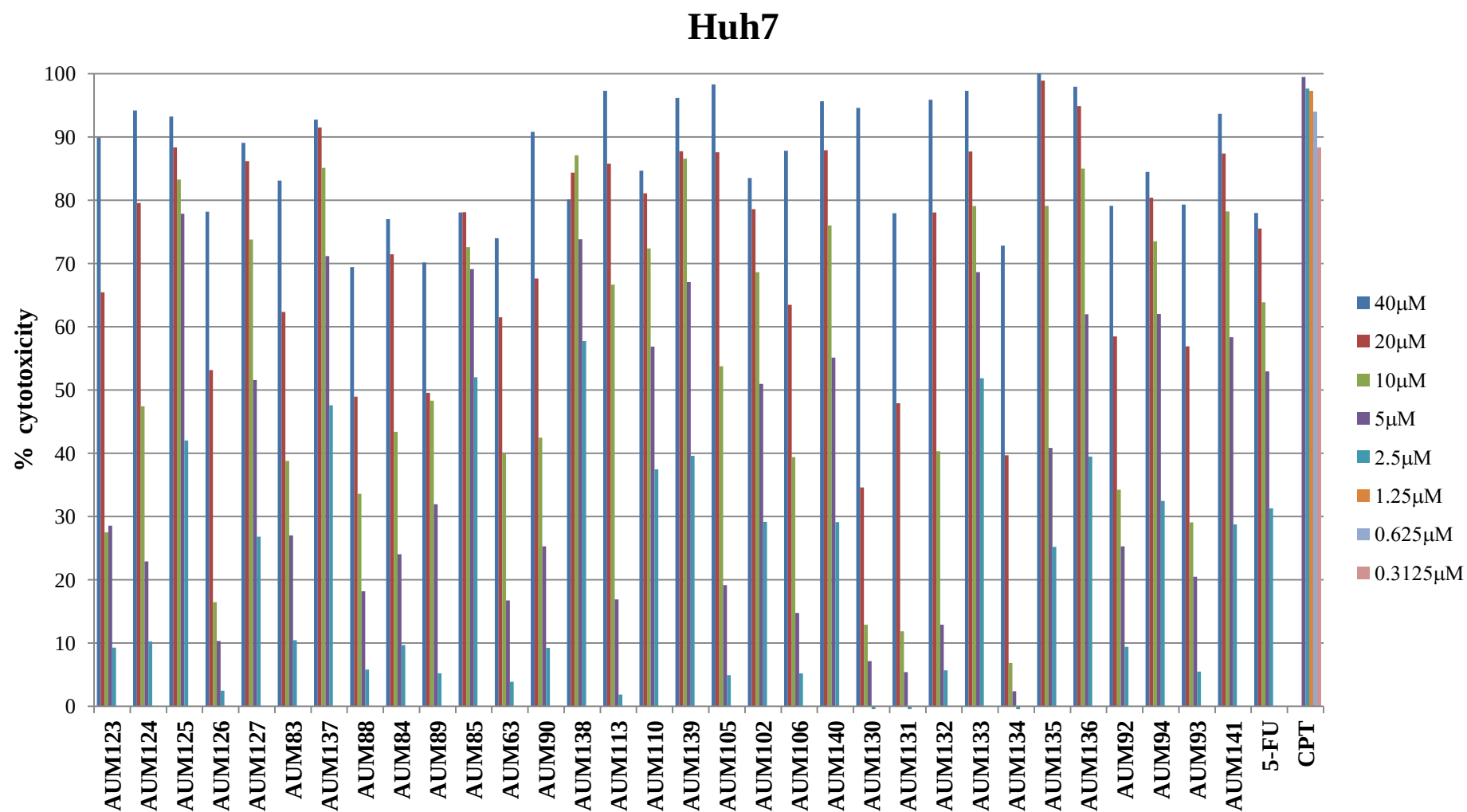


C)



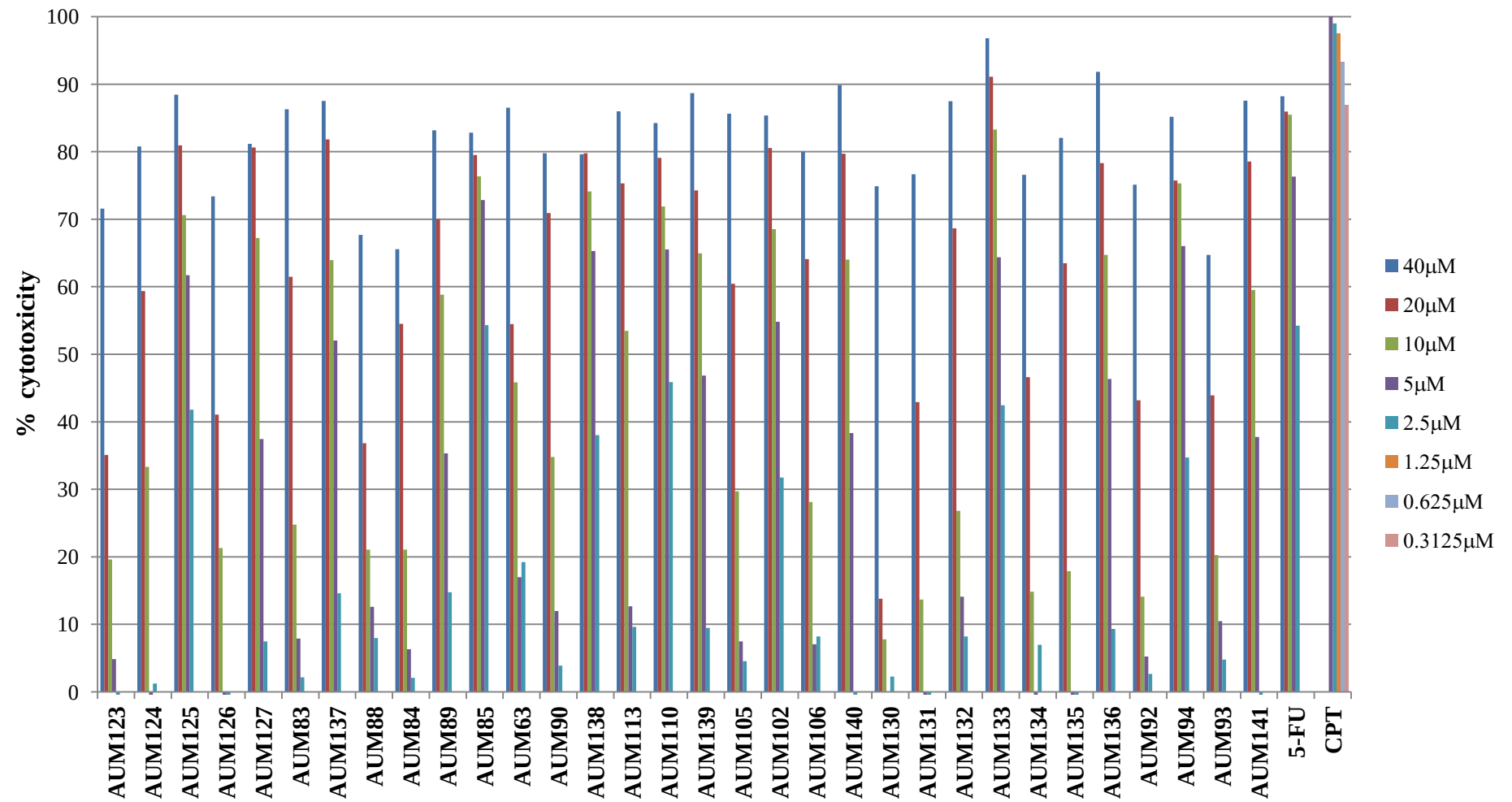
Supplementary Figure 5: Percent cytotoxicity in the presence of novel 6-(4-substituted phenyl)-9-(4-substituted phenyl) sulfonyl purine analogues given in Supplementary Table 4 and 2-amino/2-chloro/6-(4-substituted piperazine)/6-(4-substituted phenyl amino)/6-(2-substituted ethylamino)-9-(β -D-ribofuranosyl) purine analogues given in Supplementary Table 5: Huh7 (A), HCT116 (B) and MCF7 (C) cells, inoculated in 96-well plates, were treated with these small molecules and their DMSO controls in duplicate with five different concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

A)

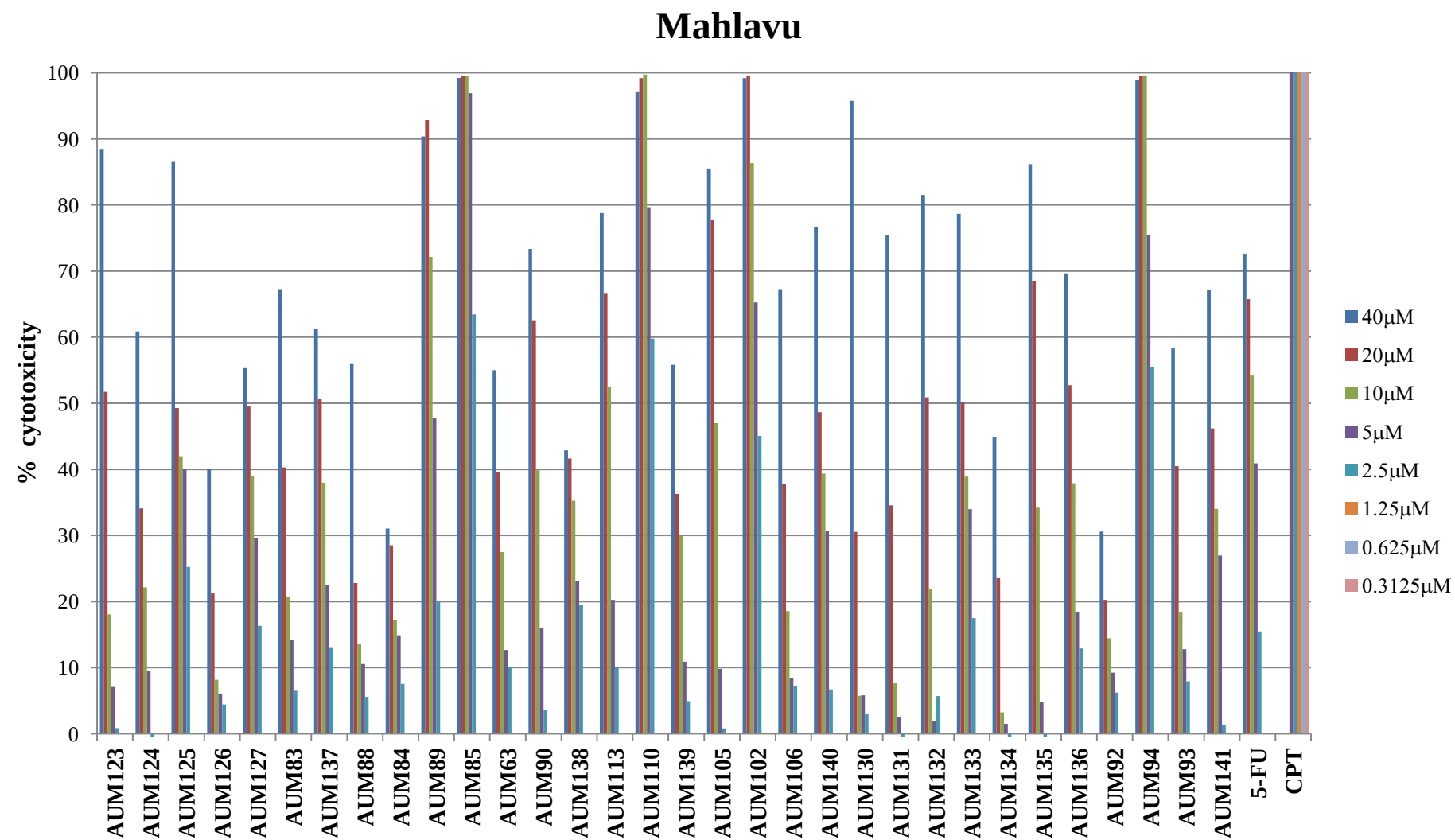


B)

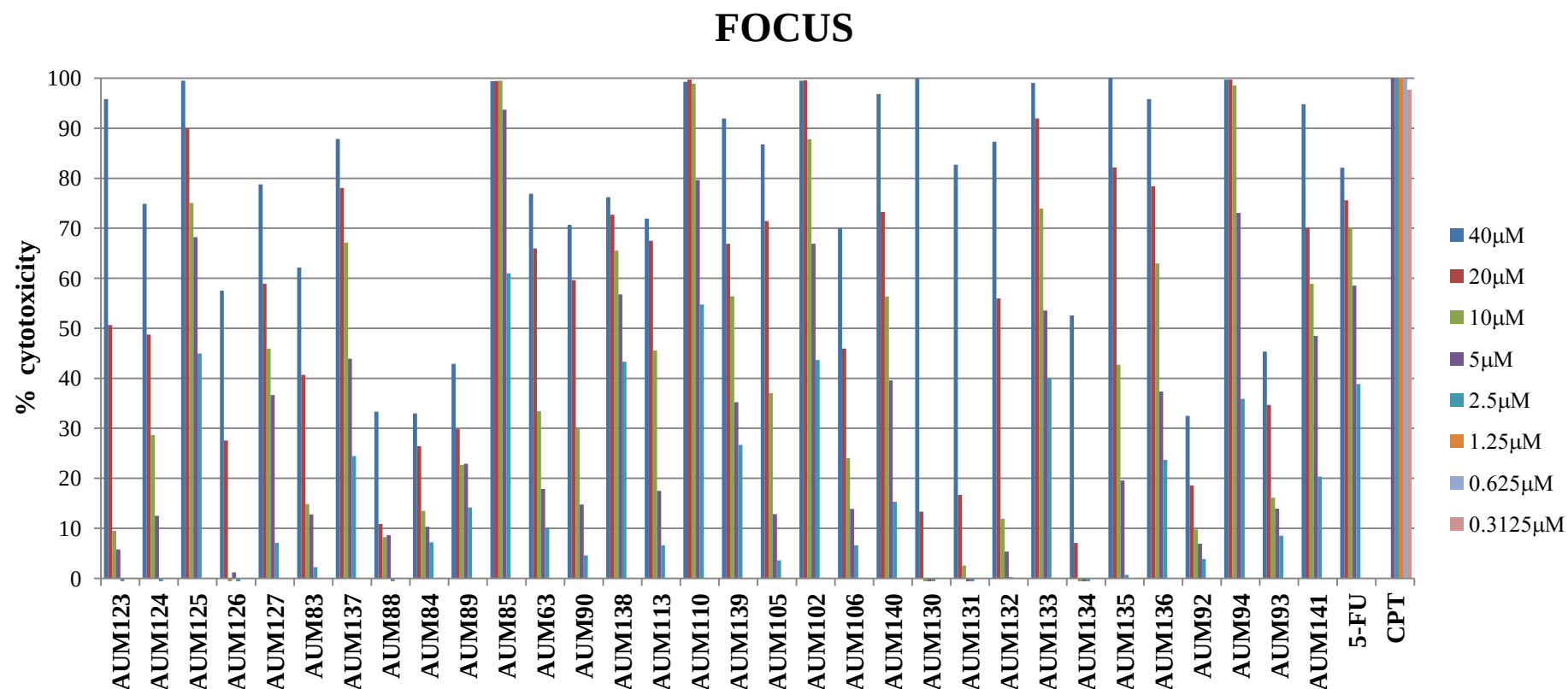
HepG2



C)



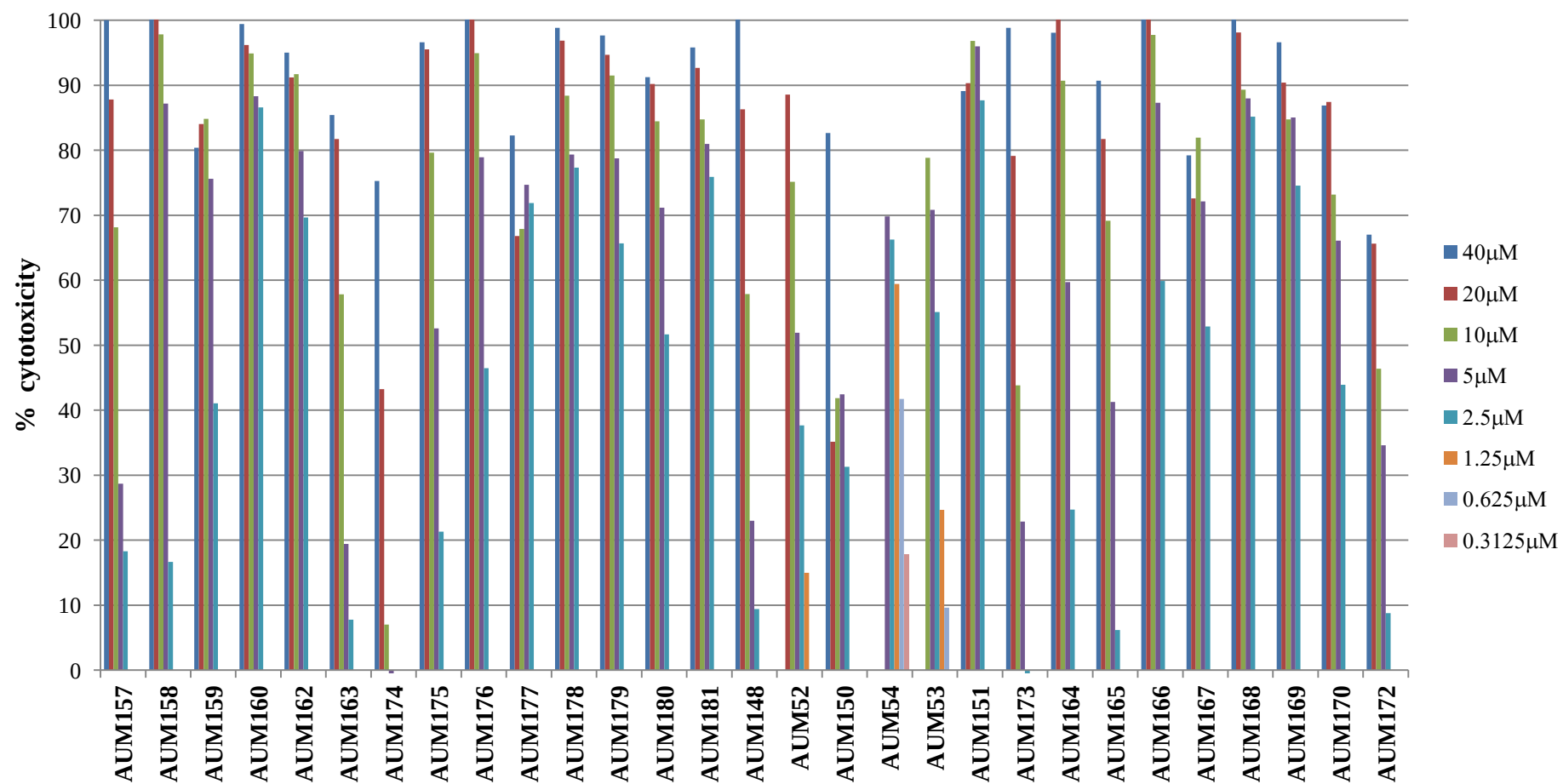
D)



Supplementary Figure 6: Percent cytotoxicity of the further analyzed novel 6-(substituted piperazine)-9-(substituted phenyl) sulfonyl purine analogues given in Supplementary Table 6: Huh7 (A), HepG2 (B), Mahlavu (C) and FOCUS (D) cells, inoculated in 96-well plates, were treated with these small molecules and their DMSO controls in triplicate with five different concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

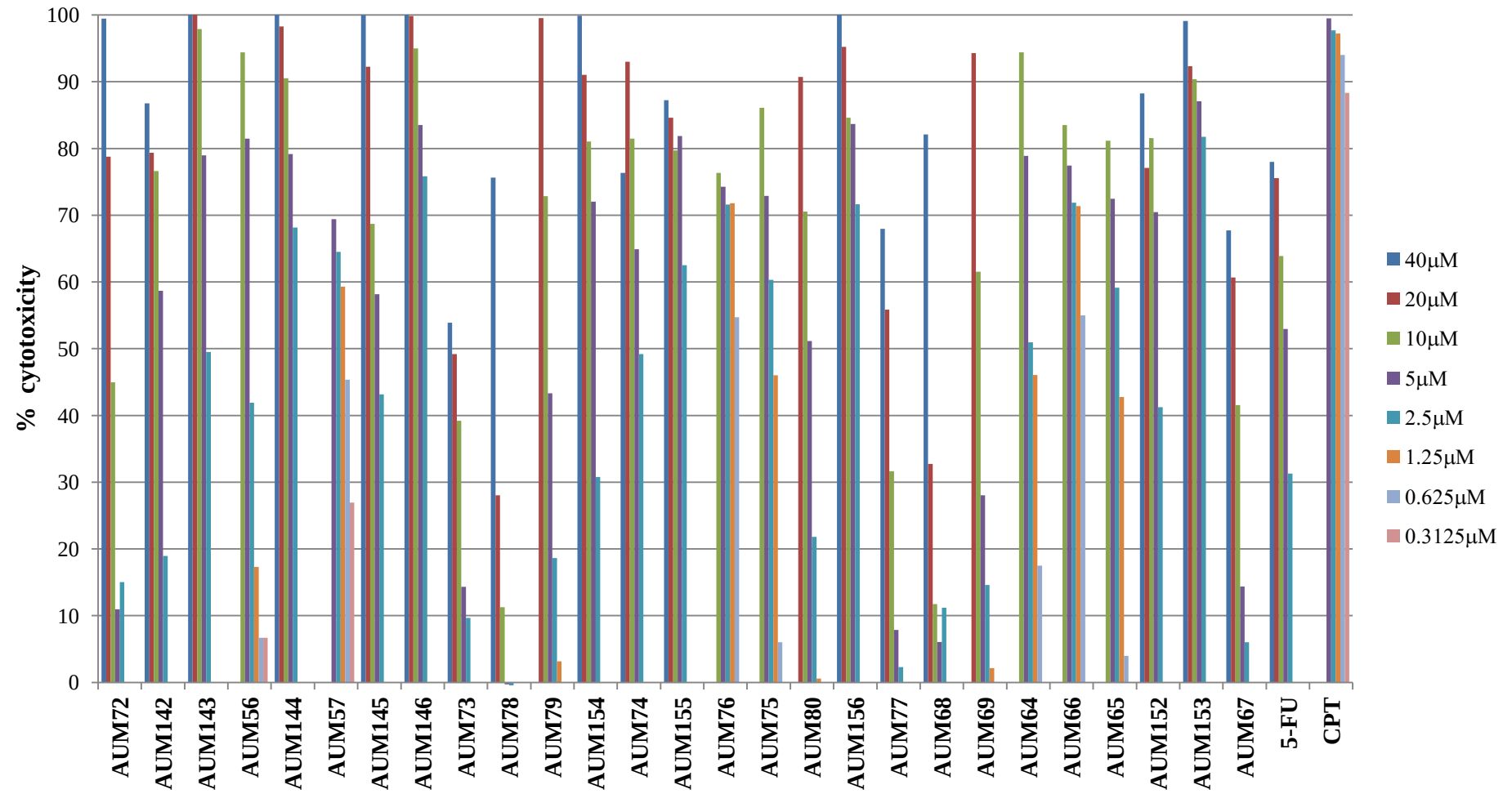
A)

Huh7

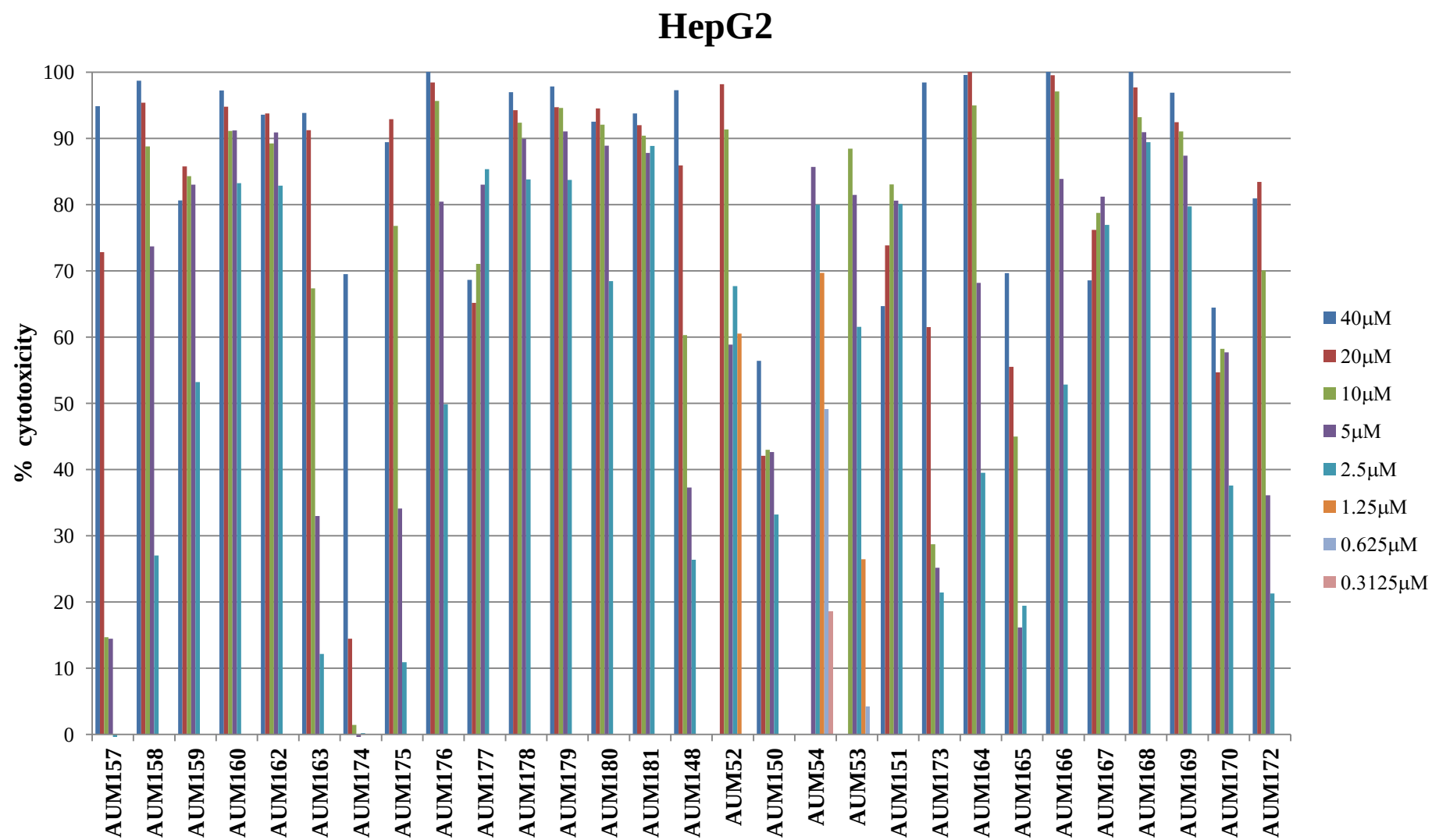


B)

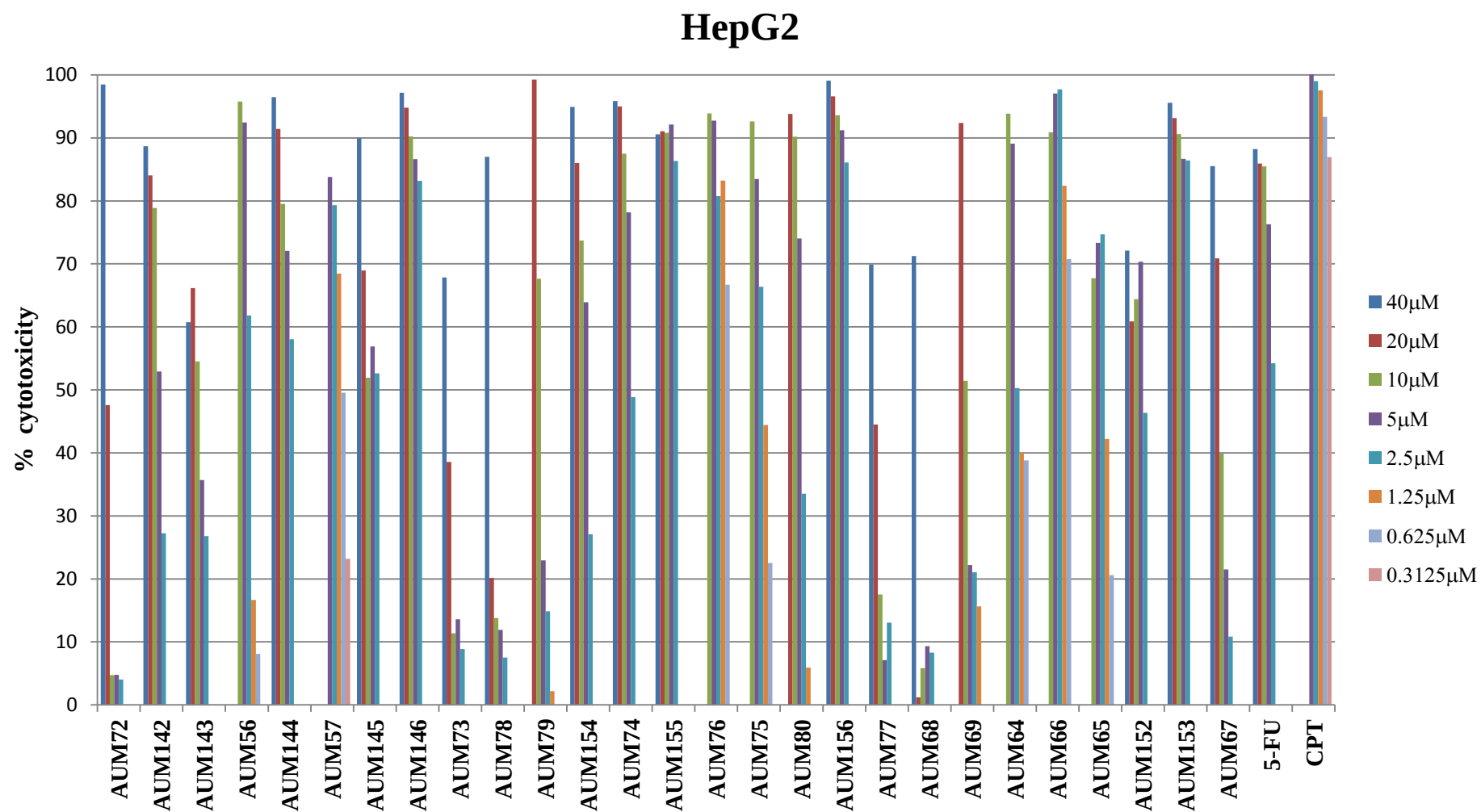
Huh7



C)

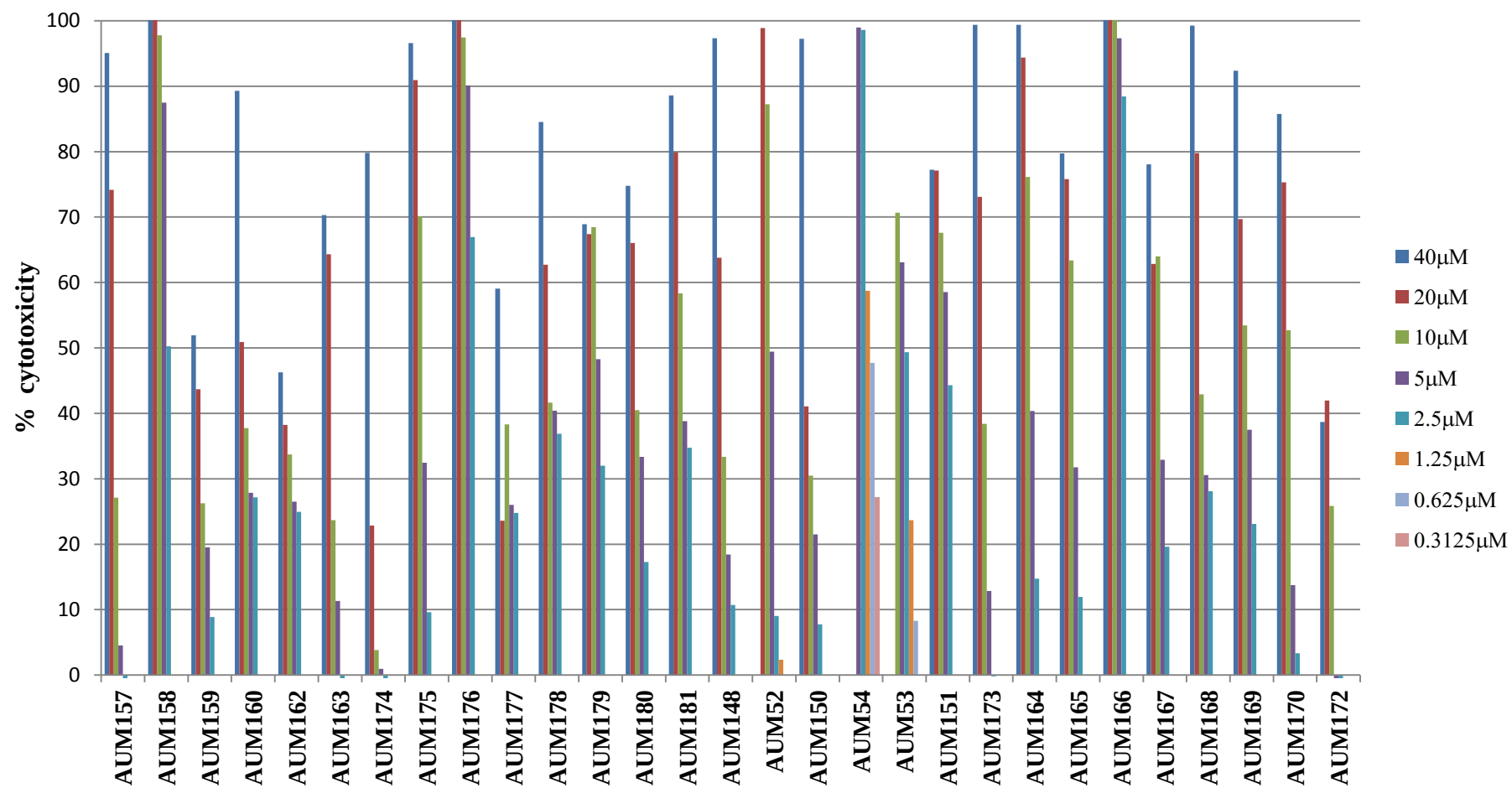


D)



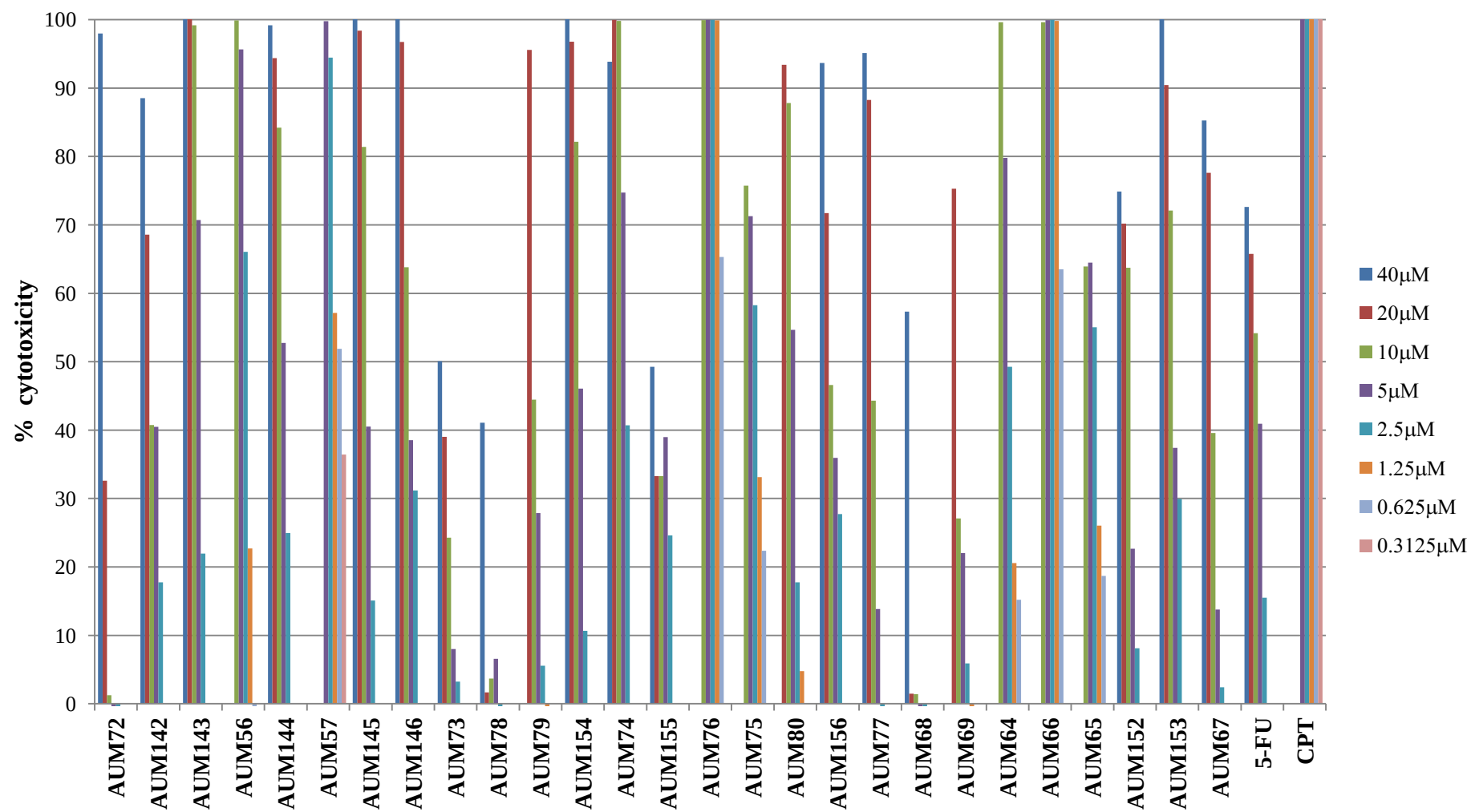
E)

Mahlavu



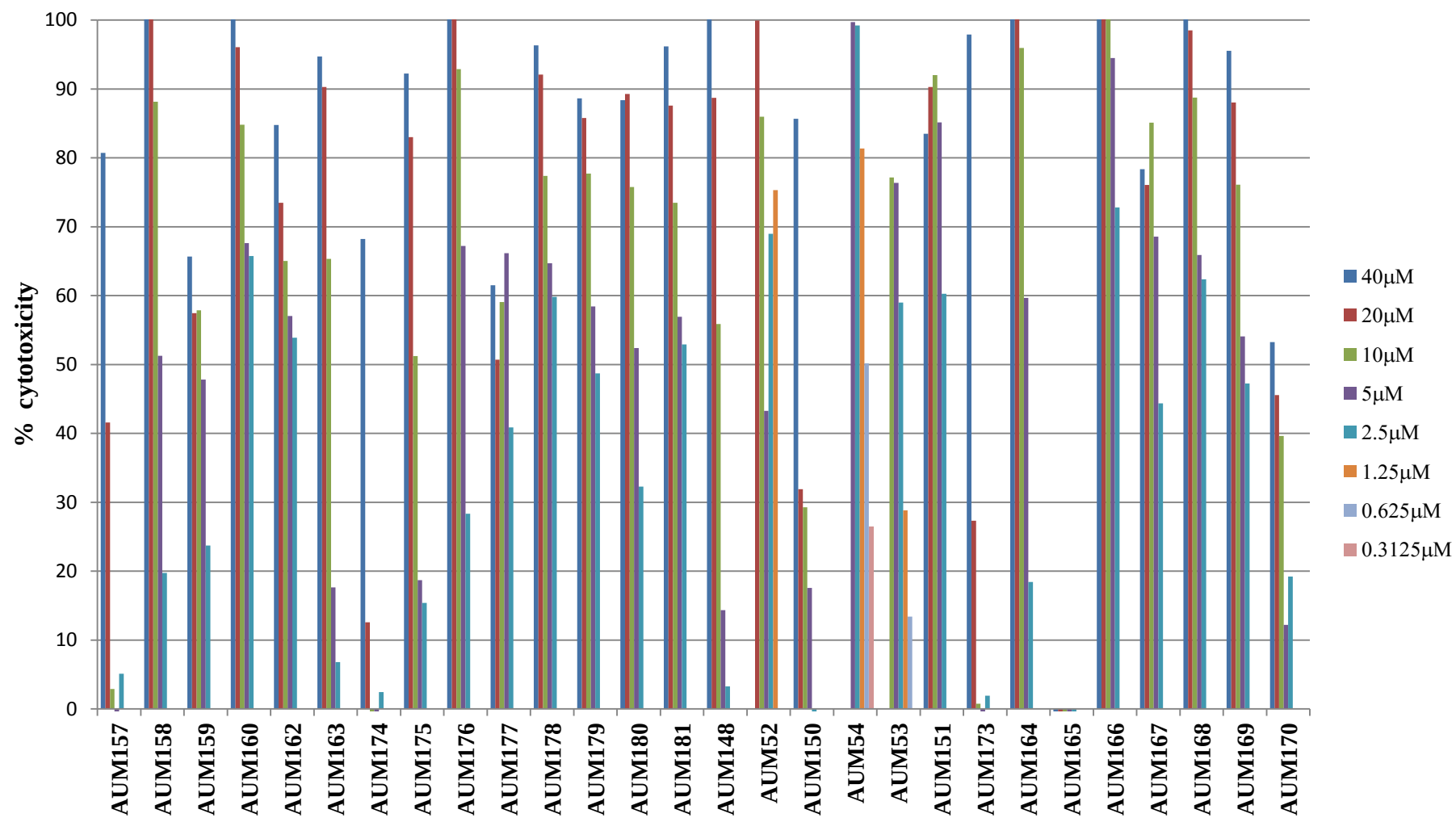
F)

Mahlavu

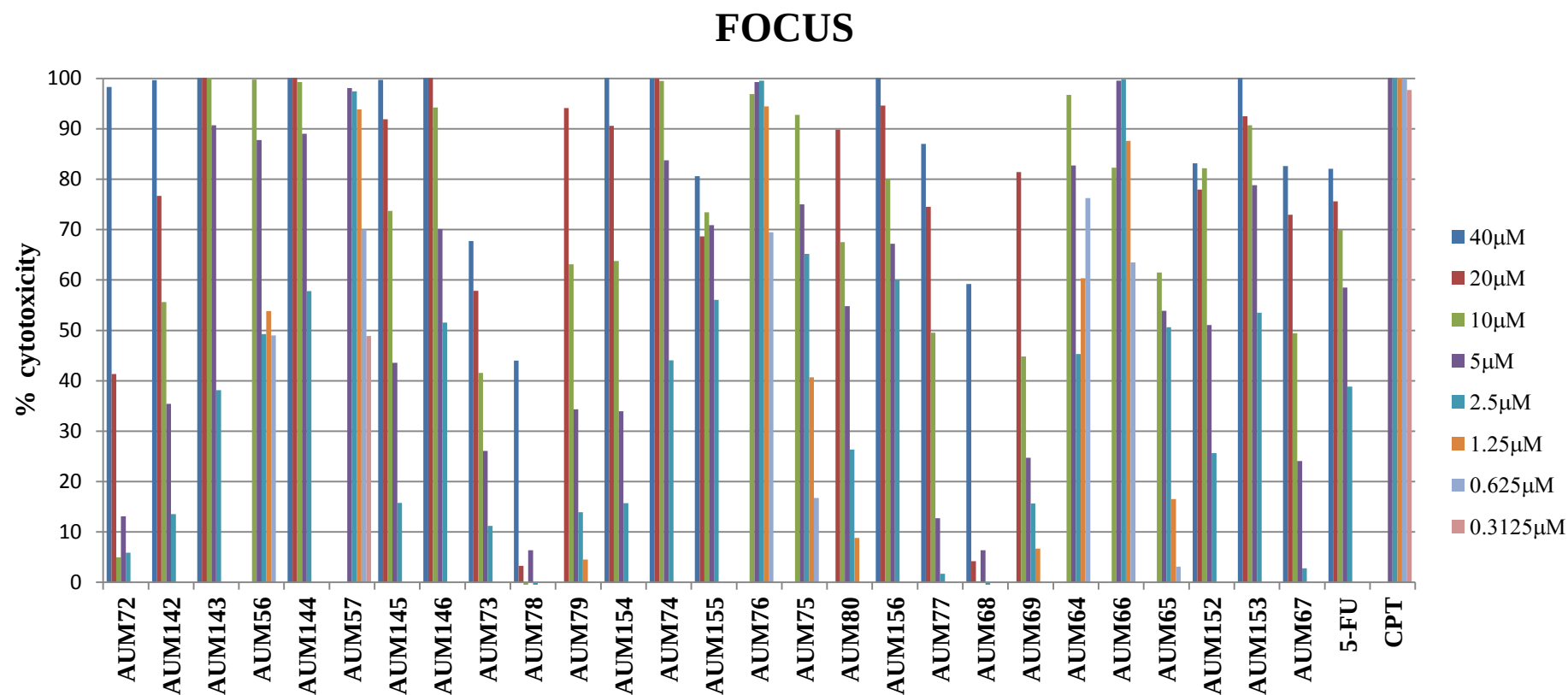


G)

FOCUS

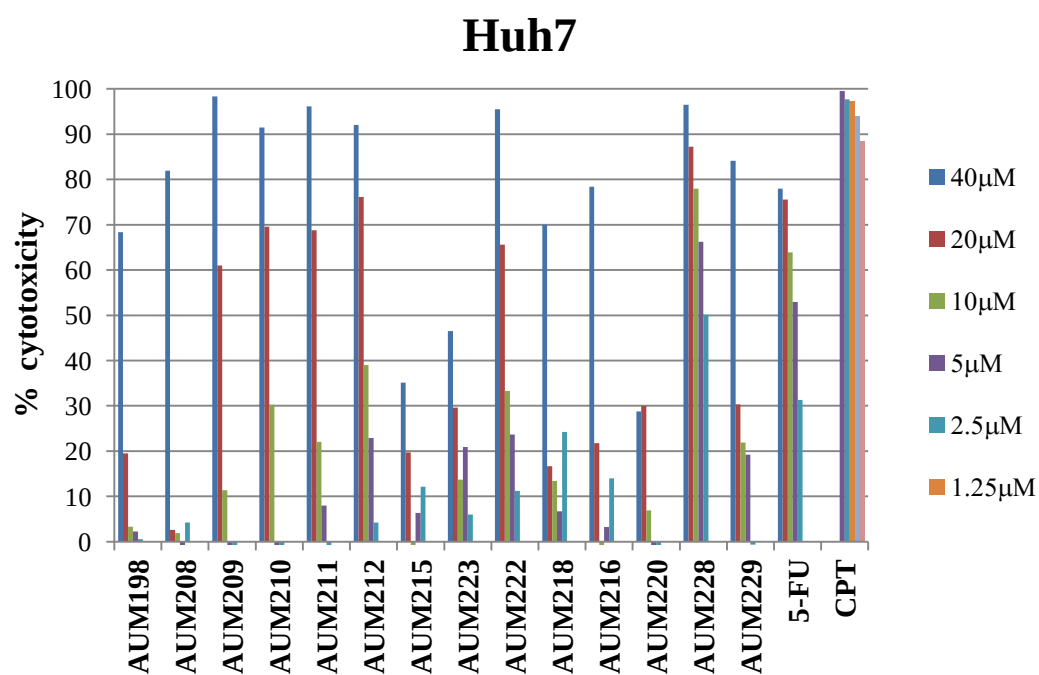


H)

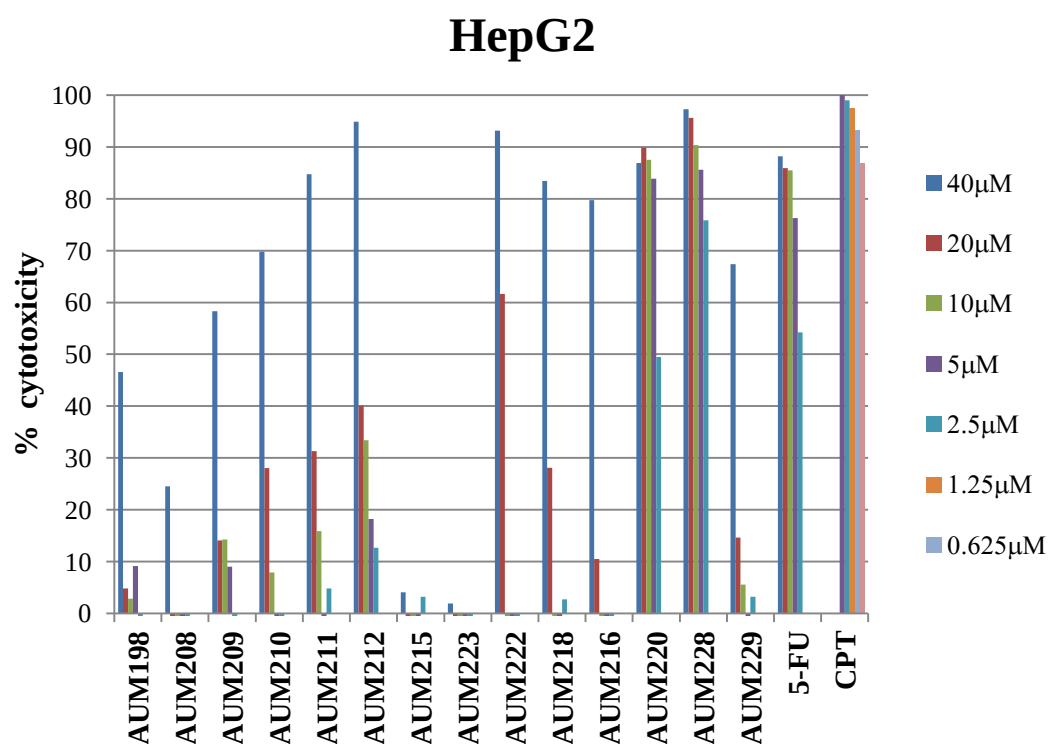


Supplementary Figure 7: Percent cytotoxicity of the further analyzed novel 6-(substituted piperazine)-9-(substituted benzyl) purine analogues given in Supplementary Table 7: Huh7 (A, B), HepG2 (C, D), Mahlavu (E, F) and FOCUS (G, H) cells, inoculated in 96-well plates, were treated with these small molecules and their DMSO controls in triplicate with five different concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

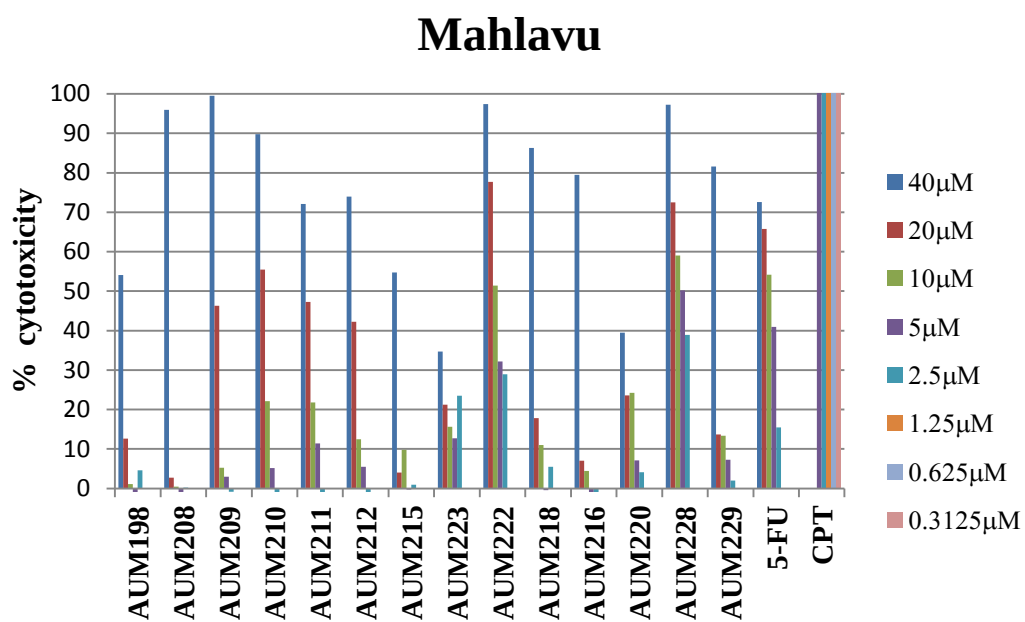
A)



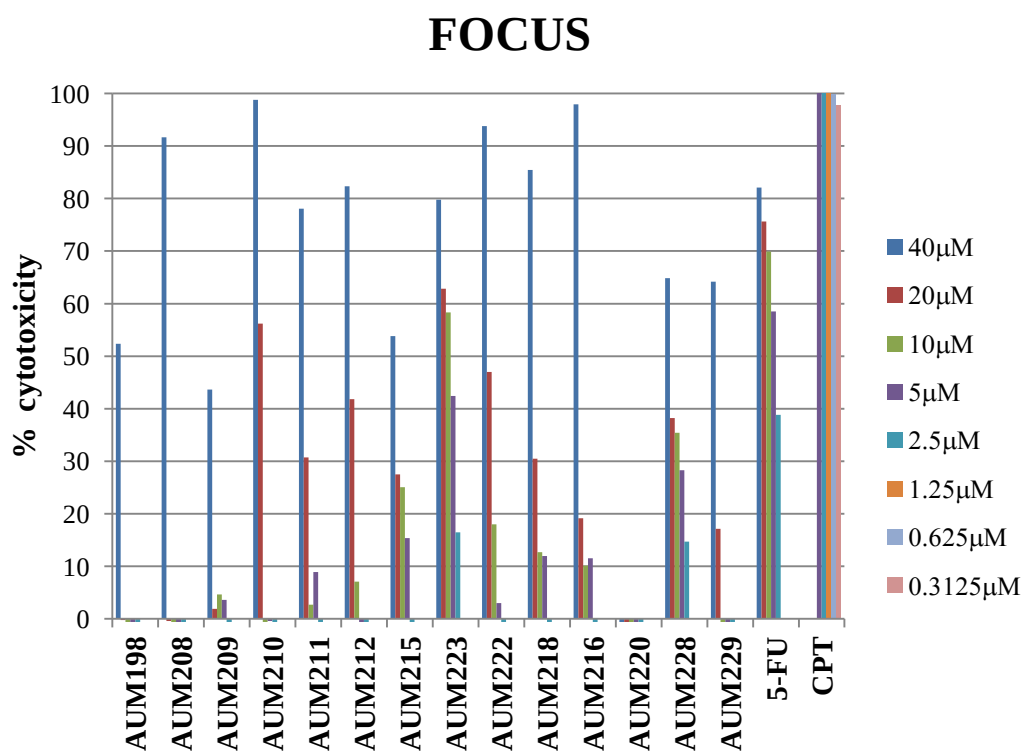
B)



C)



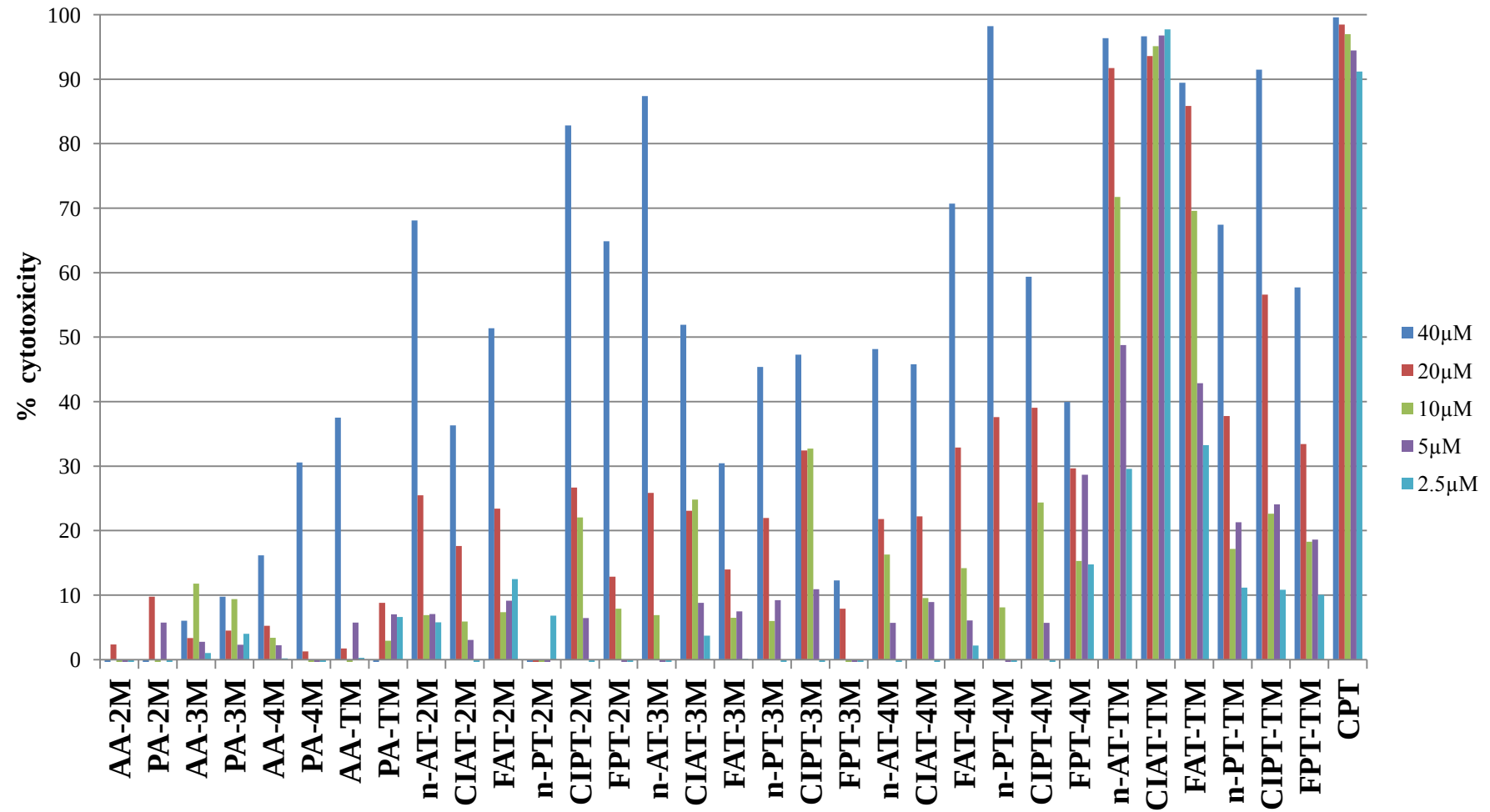
D)



Supplementary Figure 8: Percent cytotoxicity of the further analyzed novel 6-(4-substituted phenyl)-9-(4-substituted phenyl) sulfonyl AND 2-amino/2-chloro/6-(4-substituted piperazine)/6-(4-substituted phenyl amino)/6-(2-substituted ethylamino)-9-(β -D-ribofuranosyl) purine analogues given in Supplementary Table 8: Huh7 (A), HepG2 (B), Mahlavu (C) and FOCUS (D) cells, inoculated in 96-well plates, were treated with these small molecules and their DMSO controls in triplicate with five different concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

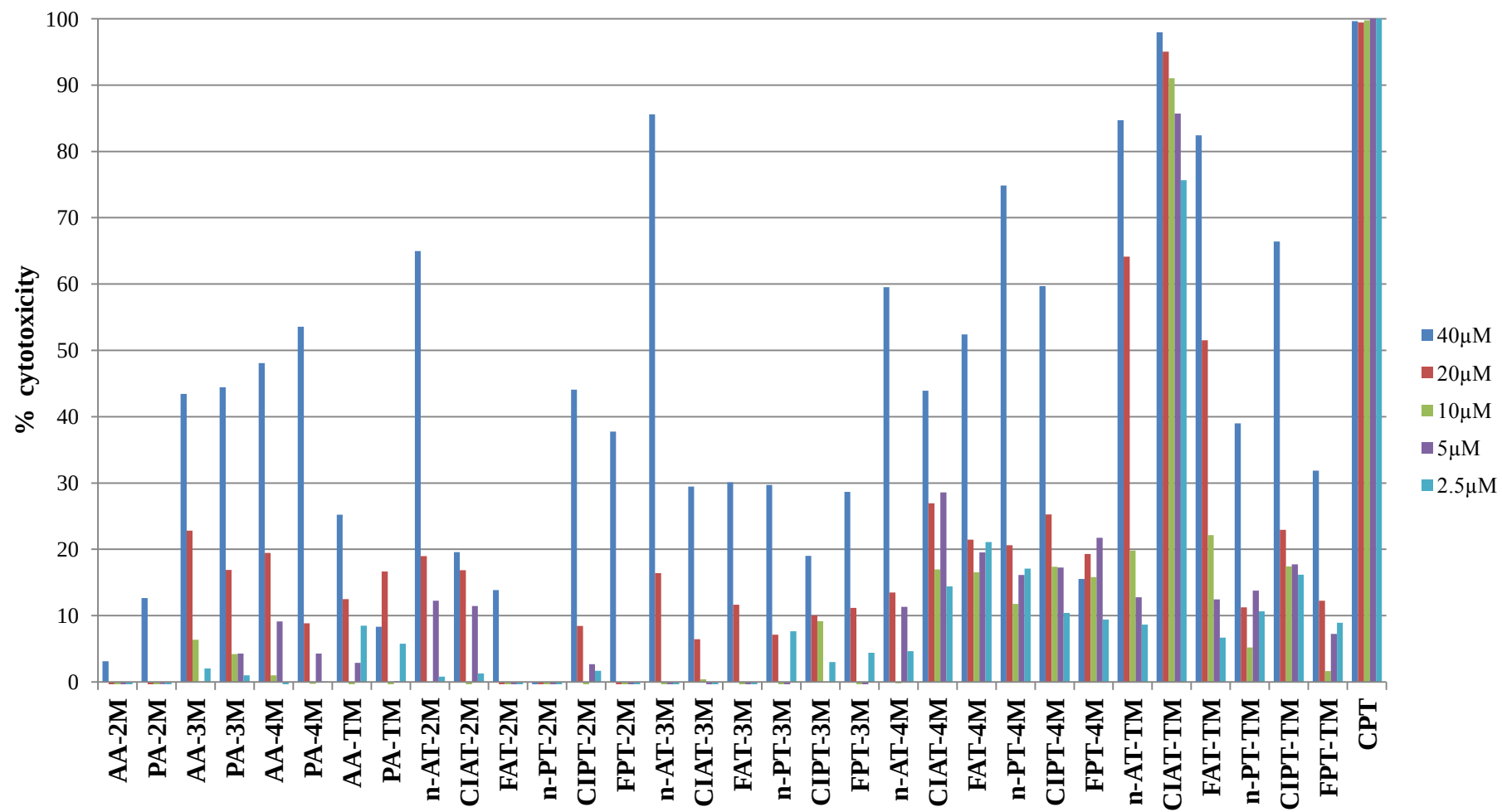
A)

Huh7



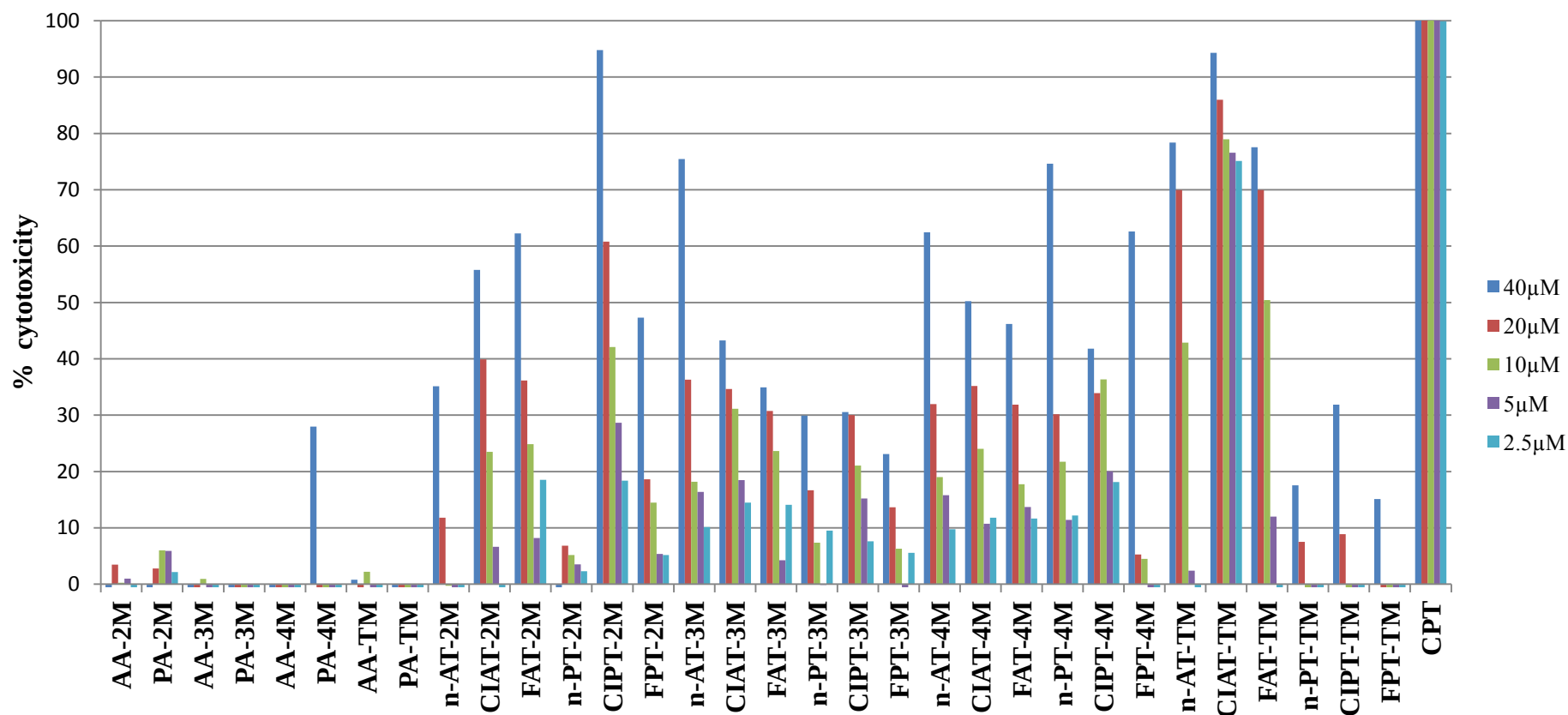
B)

HCT116



C)

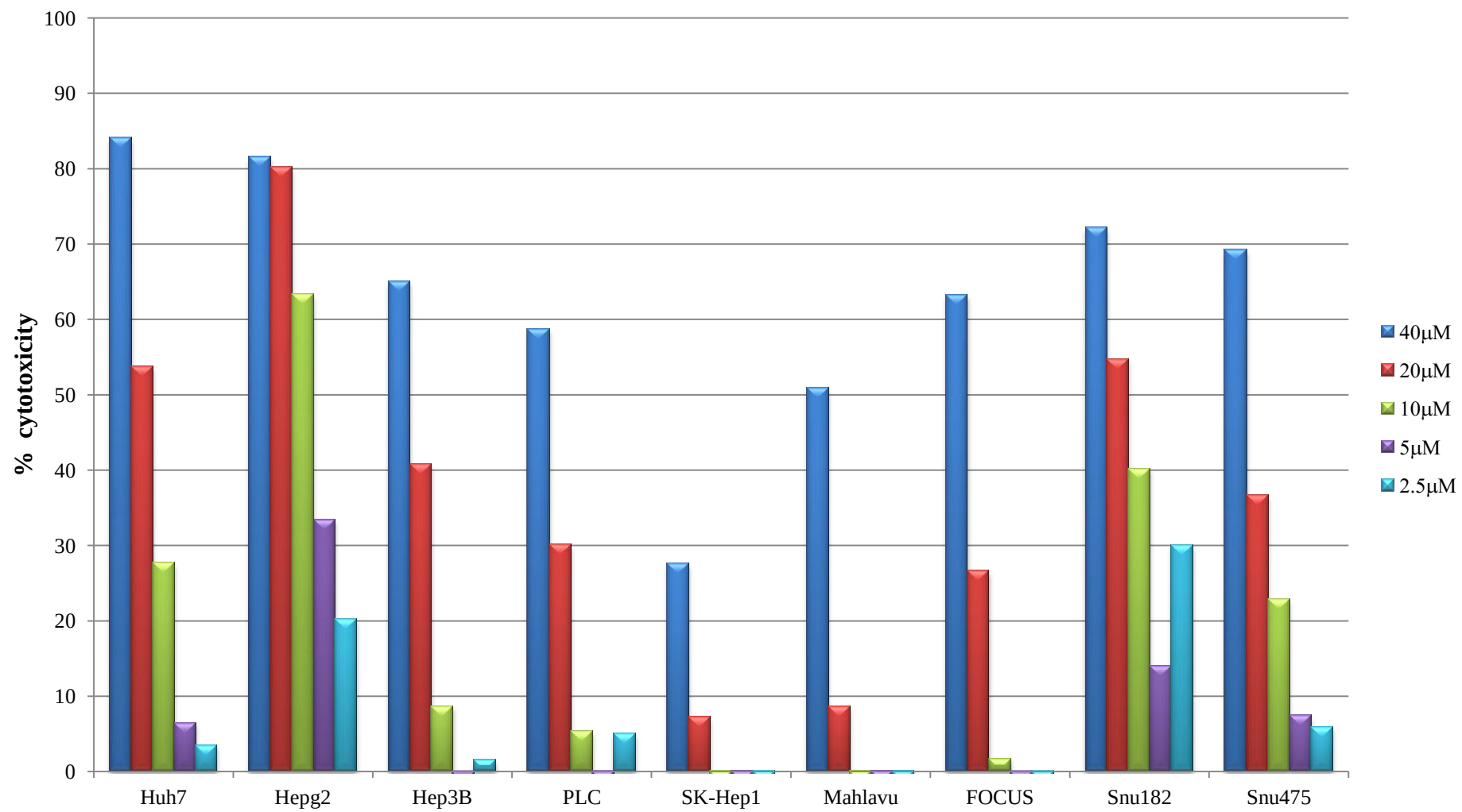
T47D



Supplementary Figure 9: Percent cytotoxicity in the presence of HUBT coded novel aminotriazole and thiadiazine derivatives: Huh7 (A), HCT116 (B) and T47D (C) cells, inoculated in 96-well plates, were treated with these small molecules and their DMSO controls in duplicate with five different concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

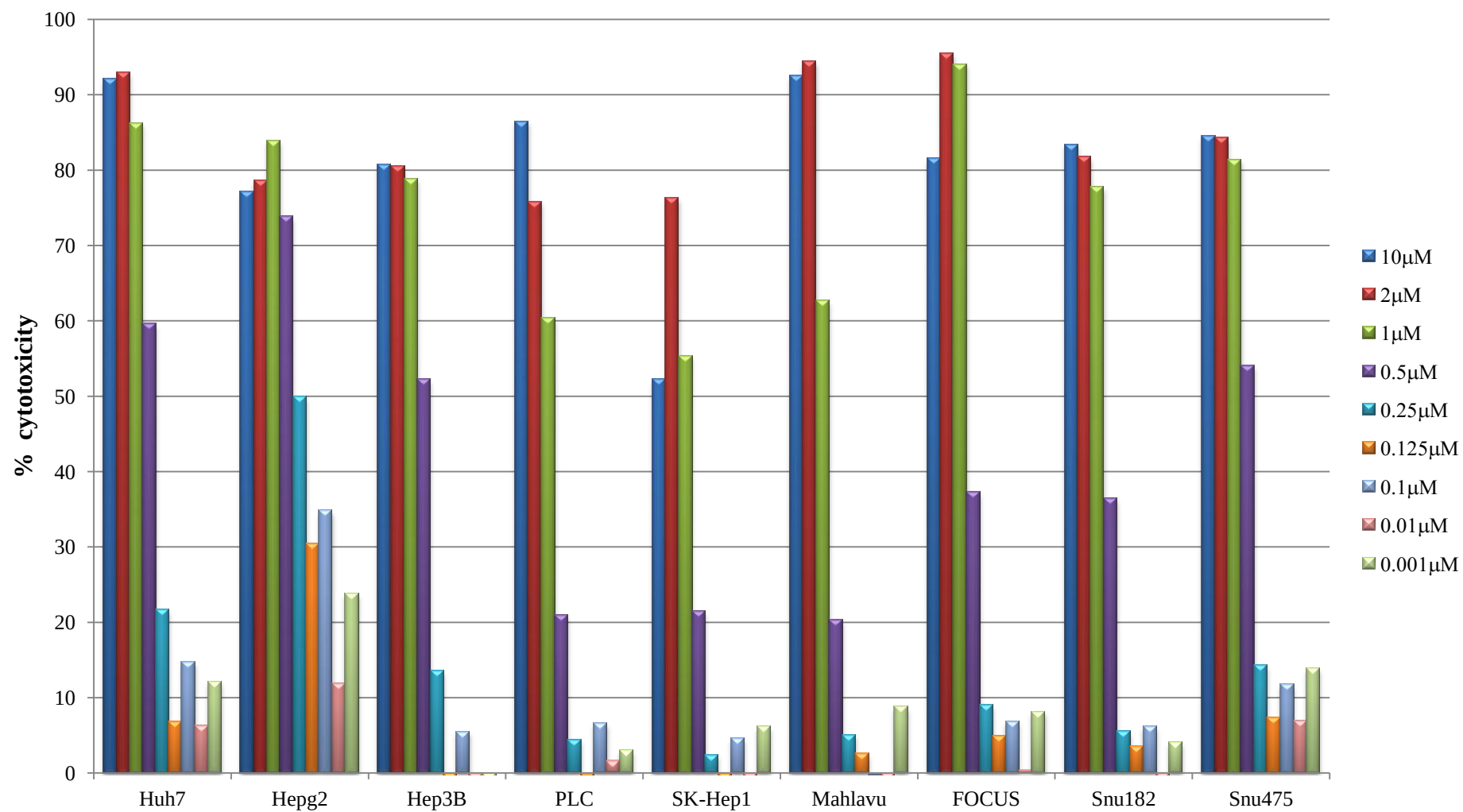
A)

FAT-TM

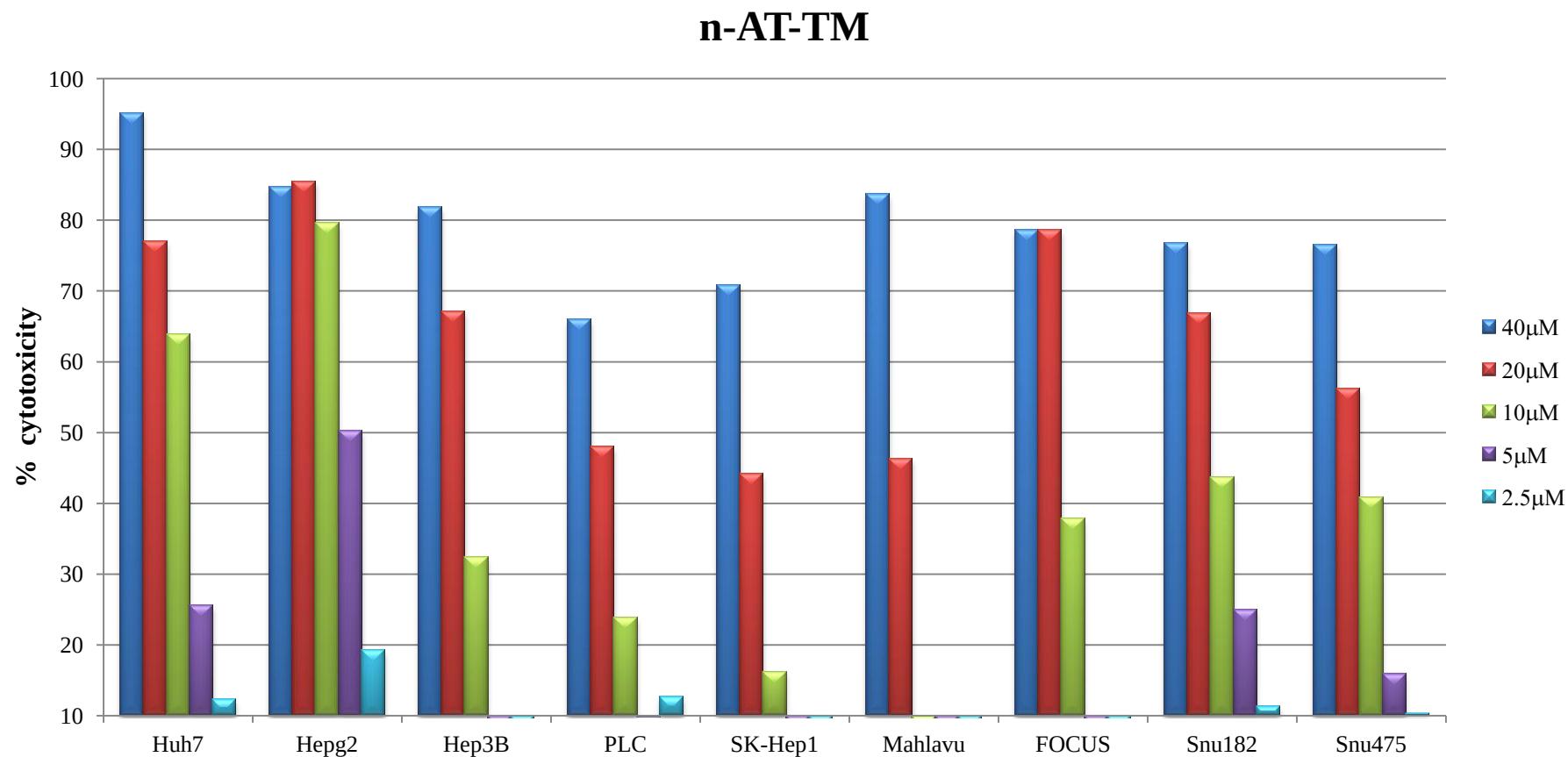


B)

CIAT-TM

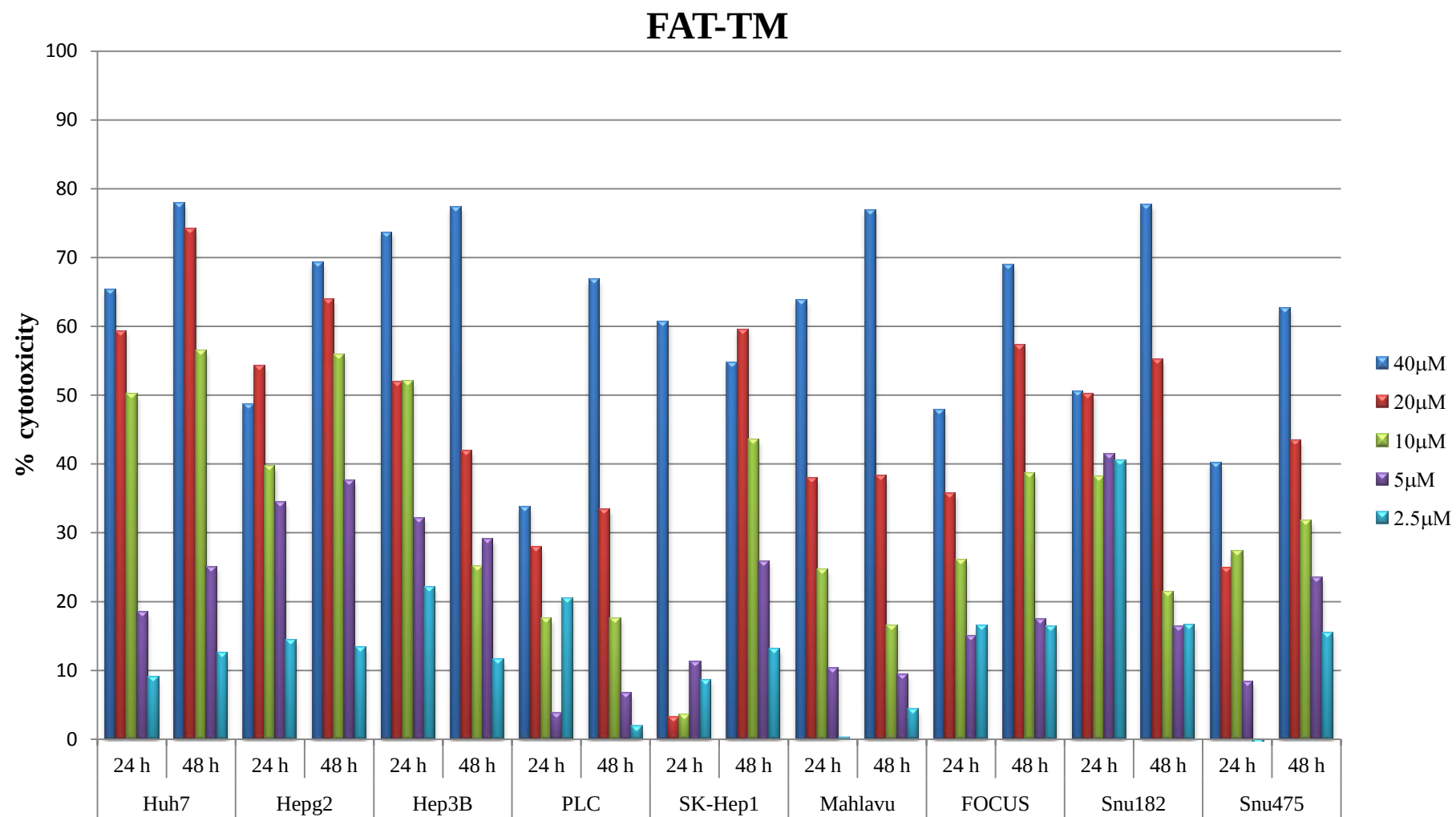


C)



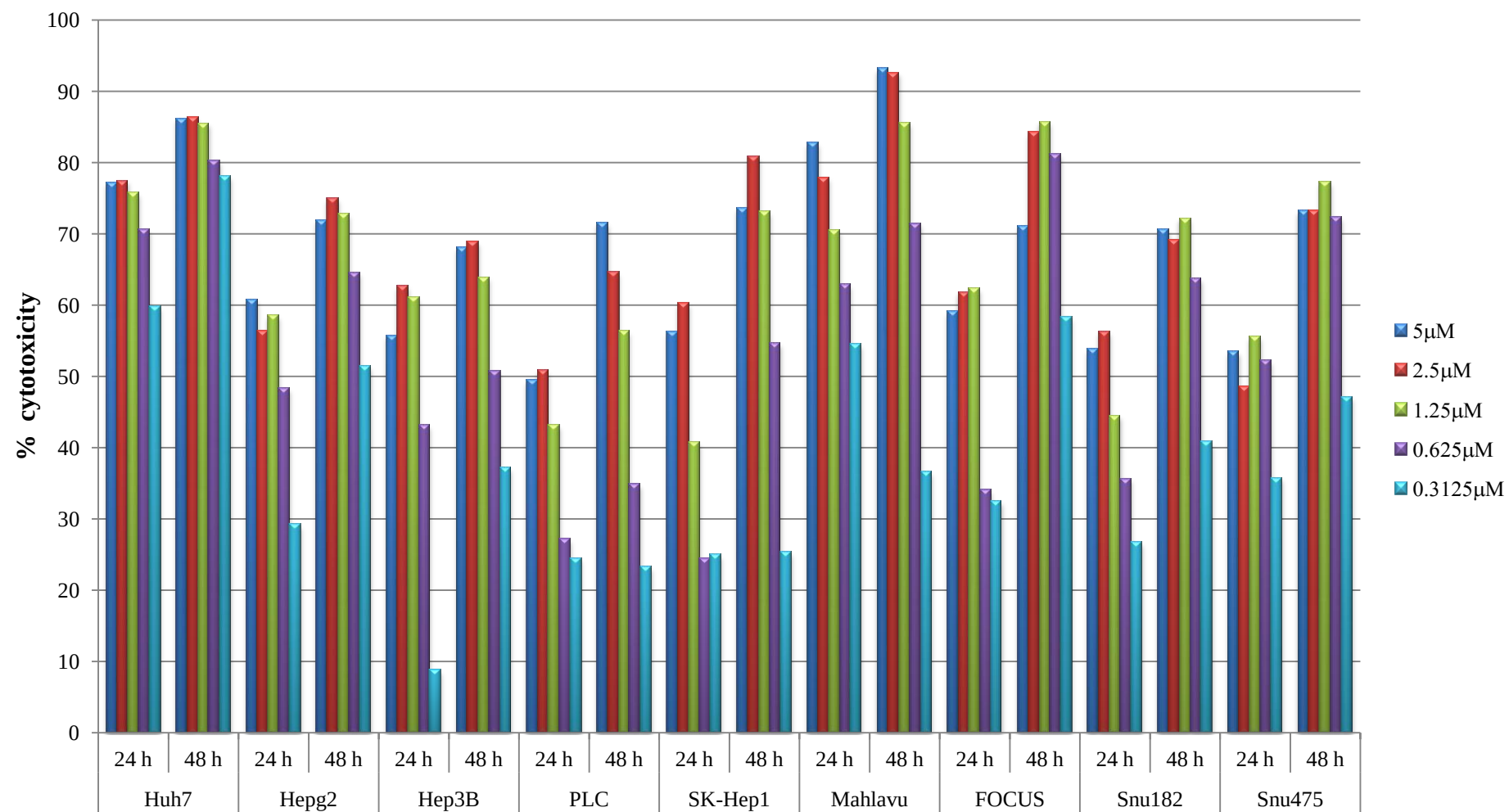
Supplementary Figure 10: Percent cytotoxicity in the presence of novel 1,2,4 triazolo[3,4-b]-1,3,4-thiadiazine derivatives (72hour): FAT-TM (A), CIAT-TM (B), n-AT-TM (C) derivatives and their DMSO controls were applied to the liver cancer cells, inoculated in 96-well plates, in quadruplicate with indicated concentrations for 72 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

A)

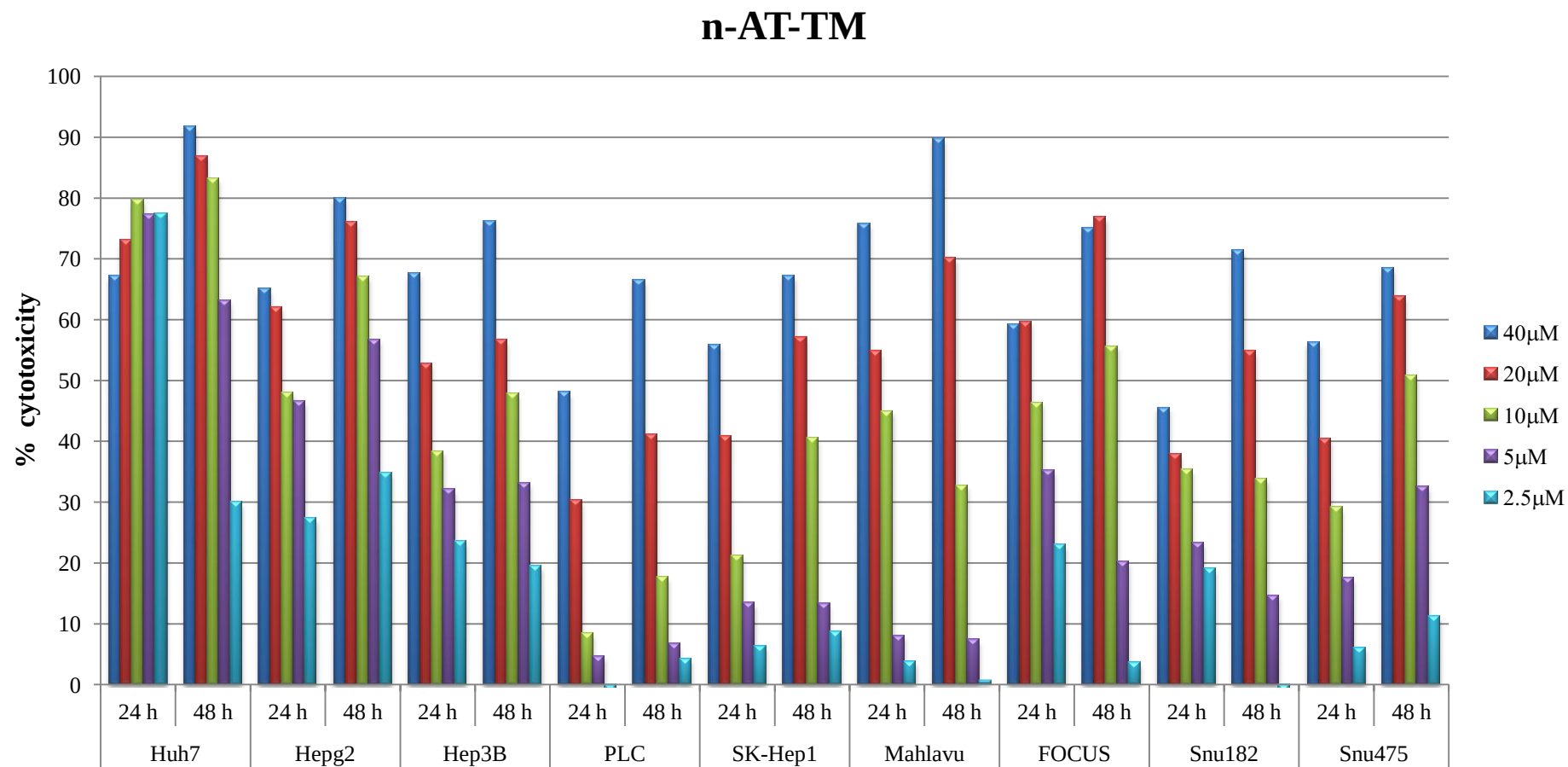


B)

CIAT-TM



C)



Supplementary Figure 11: Percent cytotoxicity in the presence of novel 1,2,4 triazolo[3,4-b]-1,3,4-thiadiazine derivatives (24hour and 48 hour): Huh7 (A), HCT116 (B) and T47D (C) cells, inoculated in 96-well plates, were treated with these small molecules and their DMSO controls in quadruplicate with five different concentrations for 24 and 48 hours. Following the SRB assay, the cytotoxicity percentages were calculated in comparison to DMSO-only treated wells.

REFERENCES

- Abou-Alfa GK, Chan SL, Lin CC, Chiorean EG, Holcombe RF, Mulcahy MF, Carter WD, Patel K, Wilson WR, Melink TJ, Gutheil JC, Tsao CJ. 2011. PR-104 plus sorafenib in patients with advanced hepatocellular carcinoma. *Cancer Chemother Pharmacol*. Aug;68(2):539-45.
- Acosta JC and Gril J. 2012. Senescence: a new weapon for cancer therapy. *Trends Cell Biol*. Apr; 22(4):211-9.
- Alimonti A, Nardella C, Chen Z, Clohessy JG, Carracedo A, Trotman LC, Cheng K, Varmeh S, Kozma SC, Thomas G, Rosivatz E, Woscholski R, Cignetti F, Scher HI, Pandolfi PP. 2010. A novel type of cellular senescence that can be enhanced in mouse models and human tumor xenografts to suppress prostate tumorigenesis. *J Clin Invest*. Mar;120(3):681-93.
- Alves RC, Alves D, Guz B, Matos C, Viana M, Hariz M, Terrabuio D, Kondo M, Gampel O, Polletti P. 2011. Advanced hepatocellular carcinoma. Review of targeted molecular drugs. *Ann Hepatol*. Jan-Mar;10(1):21-7.
- Altekruse SF, McGlynn KA, Reichman ME. 2009. Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. *J Clin Oncol*. Mar 20;27(9):1485-91.
- Amatori S, Bagaloni I, Viti D, Fanelli M. 2011. Premature senescence induced by DNA demethylating agent (Decitabine) as therapeutic option for malignant pleural mesothelioma. *Lung Cancer*. Jan;71(1):113-5.
- Aon MA, Cortassa S, O'Rourke B. 2010. Redox-optimized ROS balance: a unifying hypothesis. *Biochem Biophys Acta*. Jun-Jul;1797(6-7):865-77.
- Arena S, Benvenuti S, Bardelli A. 2005. Genetic analysis of the kinome and phosphatome in cancer. *Cell Mol Life Sci*. Sept;62(18):2092-9.
- Baek HJ, Lim SC, Kitisin K, Jogunoori W, Tang Y, Marshall MB, Mishra B, Kim TH, Cho KH, Kim SS, Mishra L. 2008. Hepatocellular cancer arises from loss of transforming growth factor beta signaling adaptor protein embryonic liver fodrin through abnormal angiogenesis. *Hepatology*. Oct;48(4):1128-37.
- Barker WC and Dayhoff MO. 1982. Viral src gene products are related to the catalytic chain of mammalian cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A*. May;79(9):2836-9.

- Ben-Efraim S. 2001. Immunomodulating anticancer alkylating drugs: targets and mechanisms of activity. *Curr Drug Targets*. Jun;2(2):197-212.
- Ben-Porath I and Weinberg RA. 2005. The signals and pathways activating cellular senescence. *Int J Biochem Cell Biol*. May;37(5):961-76.
- Blagosklonny MV. 2004. Prospective strategies to enforce selectively cell death in cancer cells. *Oncogene* 23, 2967–2975
- Blum HE and Spangenberg HC. 2007. Hepatocellular carcinoma: an update. *Arch Iran Med*. Jul;10(3):361-71.
- Bogoyevitch MA and Fairlie DP. 2007. A new paradigm for protein kinase inhibition: blocking phosphorylation without directly targeting ATP binding. *Drug Discov Today*. Aug;12(15-16):622-33.
- Bouruugaa K, Naski N, Boularan C, Mlynarczyk C, Candeias MM, Marullo S, Fåhræus R. 2010. Endoplasmic reticulum stress induces G2 cell-cycle arrest via mRNA translation of the p53 isoform p53/47. *Mol Cell*. Apr 9;38(1):78-88.
- Brand TM, Lida M, Li C, Wheeler DL. 2011. The nuclear epidermal growth factor receptor signaling network and its role in cancer. *Discov Med*. Nov;12(66):419-32.
- Bressac B, Galvin KM, Liang TJ, Isselbacher KJ, Wands JR, Ozturk M. 1990. Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. *Proc Natl Acad Sci U S A*. Mar;87(5):1973-7.
- Bubici C, Papa S, Pham CG, Zazzeroni F, Franzoso G. 2004. NF-kappaB and JNK: an intricate affair. *Cell Cycle*. Dec;3(12):1524-9.
- Buontempo F, Ersahin T, Missiroli S, Senturk S, Etro D, Ozturk M, Capitani S, Cetin-Atalay R, Neri ML. 2010. Inhibition of Akt signaling in hepatoma cells induces apoptotic cell death independent of Akt activation status. *Invest New Drugs*. Dec;29(6):1303-13
- Burnett G and Kennedy EP. 1954. The enzymatic phosphorylation of proteins. *J Biol Chem*. Dec;211(2):969-80.
- Cairney CJ, Bilsland AE, Evans TR, Roffey J, Bennett DC, Narita M, Torrance CJ, Keith WN. 2012. Cancer cell senescence: a new frontier in drug development. *Drug Discov Today*. Mar;17(5-6):269-76.
- Caino MC, Meshki J, Kazanietz MG. 2009. Hallmarks for senescence in carcinogenesis: novel signaling players. *Apoptosis*. Apr;14(4):392-408.

Campisi J. 2001. Cellular senescence as a tumor-suppressor mechanism. *Trends Cell Biol.* Nov;11(11):S27-31.

Campisi J and d'Adda di Fagagna F. 2007. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol.* Sep;8(9):729-40.

Cánepa ET, Scassa ME, Ceruti JM, Marazita MC, Carcagno AL, Sirkin PF, Ogara MF. 2007. INK4 proteins, a family of mammalian CDK inhibitors with novel biological functions. *IUBMB Life.* Jul;59(7):419-26.

Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. 2004. Cell death by mitotic catastrophe: a molecular definition. *Oncogene.* Apr 12;23(16):2825-37.

Cao H, Phan H, Yang LX. 2012. Improved chemotherapy for hepatocellular carcinoma. *Anticancer Res.* Apr;32(4):1379-86.

Cervello M, McCubrey JA, Cusimano A, Lampiasi N, Azzolina A, Montalto G. 2012. Targeted therapy for hepatocellular carcinoma: novel agents on the horizon. *Oncotarget.*;3(3):236-60.

Chaitanya GV, Steven AJ, Babu PP. 2010. PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. *Cell Commun Signal.* Dec 22;8:31.

Chan SL and Yeo W. 2012. Targeted therapy of hepatocellular carcinoma: present and future. *J Gastroenterol Hepatol.* May;27(5):862-72.

Cao H, Phan H, Yang LX. 2012. Improved chemotherapy for hepatocellular carcinoma. *Anticancer Res.* Apr;32(4):1379-86.

Chen L, Jia H, Tian Q, Du L, Gao Y, Miao X, Liu Y. 2012. Protecting effect of phosphorylation on oxidative damage of D1 protein by down-regulating the production of superoxide anion in photosystem II membranes under high light. *Photosynth Res.* Jun;112(2):141-8.

Chen Q, Olashaw N, Wu J. 1995. Participation of reactive oxygen species in the lysophosphatidic acid-stimulated mitogen-activated protein kinase kinase activation pathway. *J Biol Chem.* Dec 1;270(48):28499-502.

Cheong H, Lu C, Lindsten T, Thompson CB. 2012. Therapeutic targets in cancer cell metabolism and autophagy. *Nat Biotechnol.* Jul 10;30(7):671-678

Cho SD, Li G, Hu H, Jiang C, Kang KS, Lee YS, Kim SH, Lu J. 2005. Involvement of c-Jun N-terminal kinase in G2/M arrest and caspase-mediated apoptosis induced by sulforaphane in DU145 prostate cancer cells. *Nutr Cancer.* 52(2):213-24.

- Chua CWL and Choo SP. 2011. Targeted therapy in hepatocellular carcinoma. *Int J Hepatol.*348297
- Coates JM, Galante JM, Bold RJ. 2009. Cancer therapy beyond apoptosis: autophagy and anoikis as mechanisms of cell death. *J Surg Res.* Dec;164(2):301-8.
- Collado M and Serrano M. 2006. The power and the promise of oncogene induced senescence markers. *Nat Rev Cancer.* Jun;6(6):472-6.
- Dahmani R, Just PA, Perret C., 2011. Wnt/ β -catenin pathway as a therapeutic target in human hepatocellular carcinoma. *Clin Res Hepatol Gastroenterol.*Nov;35(11):709-13.
- de Bruin EC and Medema JP. 2008. Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treat Rev.* Dec;34(8):737-49.
- De Beer EL, Bottone AE, Voest EE. 2001. Doxorubicin and mechanical performance of cardiac trabeculae after acute and chronic treatment: a review. *Eur J Pharmacol.* Mar 9;415(1):1-11.
- De Smaele E, Zazzeroni F, Papa S, Nguyen DU, Jin R, Jones J, Cong R, Franzoso G.2001. Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. *Nature.* Nov 15;414(6861):308-13.
- Deng Y, Ren X, Yang L, Lin Y, Wu X. 2003. A JNK-dependent pathway is required for TNFalpha-induced apoptosis. *Cell.* Oct 3;115(1):61-70.
- Denicourt C and Dowdy SF. 2004. Cip/Kip proteins: more than just CDKs inhibitors. *Genes Dev.* Apr 15;18(8):851-5.
- Dick JE and Wright JA. 1985. On the importance of deoxyribonucleotide pools in the senescence of cultured human diploid fibroblasts. *FEBS Lett.* Jan 1;179(1):21-4.
- Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi J. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A.* Sep 26;92(20):9363-7.
- Dimri GP. 2005. What has senescence got to do with cancer? *Cancer Cell.* Jun;7(6):505-12.
- Dipaola RS. 2002. To arrest or not to G(2)-M Cell-cycle arrest : commentary re: A. K. Tyagi et al., Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G(2)-M arrest, and apoptosis. *Clin. cancer Res.* 8: 3512-3519

- Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P. 2009. Major cell death pathways at a glance. *Microbes Infect.* Nov;11(13):1050-62.
- Edinger AL and Thompson CB. 2004. Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol.* Dec;16(6):663-9.
- Elmore S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol.* Jun;35(4):495-516.
- Erickson S, Sangfelt O, Heyman M, Castro J, Einhorn S, Grander D. 1998. Involvement of the Ink4 proteins p16 and p15 in T-lymphocyte senescence. *Oncogene.* Aug 6;17(5):595-602.
- Ewald B, Sampath D, Plunkett W. 2008. Nucleoside analogs: molecular mechanisms signaling cell death. *Oncogene.* Oct 27;27(50):6522-37.
- Ewald JA, Desotelle JA, Wilding G, Jarrard DF. 2010. Therapy-induced senescence in cancer. *J Natl Cancer Inst.* Oct 20;102(20):1536-46.
- Fabbro D. 2012. BCR-ABL signaling: A new STATus in CML. *Nat Chem Biol.* Feb 15;8(3):228-9
- Farazi PA and DePinho RA. 2006. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer.* Sep;6(9):674-87.
- Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer.* Dec 15;127(12):2893-917.
- Forner A, Llovet JM, Bruix J. 2012. Hepatocellular carcinoma. *Lancet.* Mar 31;379(9822):1245-55.
- Franke TF, 2008. PI3K/Akt: getting it right matters. *Oncogene.* Oct 27;27(50):6473-88.
- Galle PR. 2008. Sorafenib in advanced hepatocellular carcinoma - We have won a battle not the war. *J Hepatol.* Nov;49(5):871-3.
- Gewies A. 2003. ApoReview - introduction to Apoptosis. (<http://www.celldeath.de/encyclo/aporev/apointro.pdf>)
- Gil J and Peters G. 2006. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat Rev Mol Cell Biol.* Sep;7(9):667-77.

Giles RH, van Es JH, Clevers H. 2003. Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta*. Jun 5;1653(1):1-24.

Gobeil S, Boucher CC, Nadeau D, Poirier GG. 2001. Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases. *Cell Death Differ*. Jun;8(6):588-94.

Goldman EH, Chen L, Fu H. 2004. Activation of apoptosis signal-regulating kinase 1 by reactive oxygen species through dephosphorylation at serine 967 and 14-3-3 dissociation. *J Biol Chem*. Mar 12;279(11):10442-9.

Goodsell DS. 2004. The Molecular Perspective: Cytochrome c and Apoptosis. *Stem Cells*. 22(3):428-9.

Gross A, McDonnell JM and Korsmeyer SJ. 1999. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev*. 13: 1899-1911.

Gupta GP and Massagué J. 2006. Cancer metastasis: building a framework. *Cell*. Nov 17;127(4):679-95.

Haeusgen W, Herdegen T, Waetzig V. 2011. The bottleneck of JNK signaling: molecular and functional characteristics of MKK4 and MKK7. *Eur J Cell Biol*. Jun-Jul;90(6-7):536-44.

Hamanaka RB and Chandel NS. 2010. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends Biochem Sci*. Sep;35(9):505-13.

Hanahan D and Weinberg RA. 2000. The hallmarks of cancer. *Cell*. Jan 7;100(1):57-70.

Hanahan D and Weinberg RA, 2011. Hallmarks of cancer: the next generation. *Cell*. Mar 4;144(5):646-74.

Hanks SK, Quinn AM, Hunter T. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*. Jul 1;241(4861):42-52.

Harada K, Shiota G, Kawasaki H. 1999. Transforming growth factor-alpha and epidermal growth factor receptor in chronic liver disease and hepatocellular carcinoma. *Liver*. Aug;19(4):318-25.

Harley C, Futcher AB, Greider CW. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature*. May 31;345(6274):458-60.

Hayden MS and Ghosh S. 2008. Shared principles in NF-kappaB signaling. *Feb* 8;132(3):344-62.

Hayflick L and Moorhead PS. 1961. The serial cultivation of human diploid cell strains. *Exp Cell Res.* Dec;25:585-621.

Hayflick L. 1965. The Limited In Vitro Lifetime Of Human Diploid Cell Strains. *Exp Cell Res.* Mar;37:614-36.

Herr I and Debatin KM. 2010. Cellular stress response and apoptosis in cancer therapy. *Blood.* Nov 1;98(9):2603-14.

Howell Sj, Johnston SR, Howell A. 2004. The use of selective estrogen receptor modulators and selective estrogen receptor down-regulators in breast cancer. *Best Pract Res Clin Endocrinol Metab.* Mar;18(1):47-66.

Huang F and Chen YG, 2012. Regulation of TGF- β receptor activity. *Cell BioSci.* Mar 15;2:9.

Huynh H, 2010. Molecularly targeted therapy in hepatocellular carcinoma. *Biochem Pharmacol.* Sep 1;80(5):550-60.

Ilieva EV, Ayala V, Jove M, Dalfo E, Cacabelos D, Povedano M, Bellmunt MJ, Ferrer I, Pamplona R, Portero-Otin M. 2007. Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis. *Brain.* Dec; 130(Pt 12):3111-23.

Irmak MB, Ince G, Ozturk M, Cetin-Atalay R. 2003. Acquired tolerance of hepatocellular carcinoma cells to selenium deficiency: a selective survival mechanism?. *Cancer Res.* Oct 15;63(20):6707-15.

Janda CY, Waghray D, Levin AM, Thomas C, Garcia KC. 2012. Structural basis of Wnt recognition by Frizzled. *Science.* Jul 6;337(6090):59-64.

Jin S and White E, 2007. Role of autophagy in cancer: management of metabolic stress. *Autophagy.* Jan-Feb;3(1):28-31.

Kaelin WG. 2005. The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer.* Sep;5(9):689-98.

Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. 2005. Reactive oxygen species promote TNF α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell.* Mar 11;120(5):649-61.

Karran P. 2006. Thiopurines, DNA damage, DNA repair and therapy-related cancer. *Br Med Bull.* 79-80:153-70.

Kaye SB. 1998. Multidrug resistance: clinical relevance in solid tumours and strategies for circumvention. *Curr Opin Oncol.* Aug;10 Suppl 1:S15-9.

- Ke S, Rabson AB, Germino JF, Gallo MA, Tian Y. 2001. Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor- α and lipopolysaccharide. *J Biol Chem.* Oct 26;276(43):39638-44.
- Kim AH, Khursigara G, Sun X, Franke TF, Chao MV. 2001. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol Cell Biol.* Feb;21(3):893-901.
- Kim J and Park EJ. 2002. Cytotoxic anticancer candidates from natural resources. *Curr Med Chem Anticancer Agents.* Jul;2(4):485-537.
- Knockaert M, Greengard P, Meijer L. 2002. Pharmacological inhibitors of cyclin-dependent kinases. *Trends Pharmacol Sci.* Sep;23(9):417-25.
- Kolios G, Valatas V, Kouroumalis E. 2006. Role of Kupffer cells in the pathogenesis of liver disease. *World J Gastroenterol.* Dec 14;12(46):7413-20.
- Kornev AP, Haste NM, Taylor SS, Eyck LF. 2006. Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *Proc Natl Acad Sci U S A.* Nov 21;103(47):17783-8.
- Kornev AP and Taylor SS, 2010. Defining the conserved internal architecture of a protein kinase. *Biochim Biophys Acta.* Mar;1804(3):440-4.
- Kraus S, Bunsen T, Schuster S, Cichon MA, Tacke M, Reinheckel T, Sommerhoff CP, Jochum M, Nagler DK. 2011. Cellular senescence induced by cathepsin X downregulation. *Eur J Cell Biol.* Aug;90(8):678-86.
- Krebs EG, Graves DJ, Fischer EH. 1959. Factors affecting the activity of muscle phosphorylase b kinase. *J Biol Chem.* Nov;234:2867-73.
- Kuhn JG. 2001. Fluorouracil and the new oral fluorinated pyrimidines. *Ann Pharmacother.* Feb;35(2):217-27.
- Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. 2010. The essence of senescence. *Genes Dev.* Nov 15;24(22):2463-79.
- Kumar S. 2007. Caspase function in programmed cell death. *Cell Death Differ.* Jan;14(1):32-43.
- Kumar V, Parmar SV, and Malhotra SV. 2010. Structural modifications of nucleosides in ionic liquids. *Biochimie.* Sep; 92(9):1260-5.

Kurihara Y, Eqawa K, Kunimoto S, Takeuchi T, Nose K. 2002. Induction of p16/INK4a gene expression and cellular senescence by toyocamycin. *Biol Pharm Bull.* Oct;25(10):1272-6.

Kurokawa M and Kornbluth S. 2009. Caspases and kinases in a death grip. *Cell.* Sep 4;138(5):838-54.

Lavrik IN, Golks A, Krammer PH. 2005. Caspases: pharmacological manipulation of cell death. *J Clin Invest.* Oct;115(10):2665-72.

Lawen A. 2003. Apoptosis-an introduction. *Bioessays.* Sep;25(9):888-96.

Lech-Maranda E, Korycka A, Robak T. 2006. Meta-analysis of randomised trials comparing thiopurines in childhood acute lymphoblastic leukaemia. *Mini-Rev Med Chem.* 6,575-581.

Leontieva OV and Blagosklonny MV. 2010. DNA damaging agents and p53 do not cause senescence in quiescent cells, while consecutive re-activation of mTOR is associated with conversion to senescence. *Aging (Albany NY).* Dec;2(12):924-35.

Levene PA and Alsberg CL. 1906. The Cleavage Products of Vitellin. *J. Biol. Chem.* 2: 127-133.

Levene PA and Schormüller A. 1933. The Synthesis of TyrosinePhosphoric Acid. *J Biol Chem.* 100 (2): 583.

Liu Y and Gray NS, 2006. Rational design of inhibitors that bind to inactive kinase conformations. *Nat Chem Biol.* Jul;2(7):358-64.

Lachenmayer A, Alsinet C, Savic R, Cabellos L, Toffanin S, Hoshida Y, Villanueva A, Minguez B, Newell P, Tsai HW, Barretina J, Thung S, Ward SC, Bruix J, Mazzaferro V, Schwartz M, Friedman SL, Llovet JM. 2012. Wnt-pathway activation in two molecular classes of hepatocellular carcinoma and experimental modulation by sorafenib. *Clin Cancer Res.* Jul 18.

Larsson O. 2005. Cellular senescence—an integrated perspective. *Cancer Therapy* Vol 3, 495-510.

Lau WY and Lai EC, 2008. Hepatocellular carcinoma: current management and recent advances. *Hepatobiliary Pancreat Dis Int.* Jun;7(3):237-57.

Lee S, Schmitt CA, Reimann M. 2011. The Myc/macrophage tango: oncogene-induced senescence, Myc style. *Semin Cancer Biol.* Dec;21(6):377-84.

- Lee HZ, Liu WZ, Hsieh WT, Tang FY, Chung JG, Leung HW. 2009. Oxidative stress involvement in *Physalis angulata*-induced apoptosis in human oral cancer cells. *Food Chem Toxicol.* Mar;47(3):561-70.
- Llovet JM, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, Sherman M, Schwartz M, Lotze M, Talwalkar J, Gores GJ. 2008. Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst.* May 1;100(10):698-711.
- Llovet JM, Bruix J. 2008. Molecular targeted therapies in hepatocellular carcinoma. *Hepatology.* Oct;48(4):1312-27.
- Lim DS, Kim ST, Xu B, Maser RS, Lin J, Petrini JH, Kastan MB. 2000. ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature.* Apr 6;404(6778):613-7.
- Liu J and Lin A. 2005. Role of JNK activation in apoptosis: a double-edged sword. *Cell Res.* Jan;15(1):36-42.
- Liu P, Cheng H, Roberts TM, Zhao JJ. 2009 Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov.* Aug;8(8):627-44.
- Lu T and Finkel T, 2008. Free radicals and senescence. *Exp Cell Res.* Jun 10;314(9):1918-22.
- Lundin A. 1981. Applications of firefly luminescence. In *Bioluminescence and Chemiluminescence: Basic Chemistry and Analytical Applications* (M. Deluca and W. McElroy, eds.), pp.187-196, Academic Press, New York.
- Ma HT, Poon RY. 2011. How protein kinases co-ordinate mitosis in animal cells. *Biochem J.* Apr 1;435(1):17-31.
- Ma WW and Adjei AA, 2009. Novel agents on the horizon for cancer therapy. *CA Cance J Clin.* Mar-Apr;59(2):111-37.
- Macip S, Kosoy A, Lee SW, O'Connell MJ, Aaronson SA. 2006. Oxidative stress induces a prolonged but reversible arrest in p53-null cancer cells, involving a Chk1-dependent G2 checkpoint. *Oncogene.* Oct 5;25(45):6037-47.
- Mah LJ, El-Osta A, Karagiannis TC. 2010. GammaH2AX as a molecular marker of aging and disease. *Epigenetics.* Feb 16;5(2):129-36.
- Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. 2002. The protein kinase complement of the human genome. *Science.* Dec 6;298(5600):1912-34.

Manning AM and Davis RJ. 2003. Targeting JNK for therapeutic benefit: from junk to gold?. *Nat Rev Drug Discov.* Jul;2(7):554-65.

Massagué J. 1998. TGF-beta signal transduction. *Annu Rev Biochem.* 67:753-91.

Min L, He B, Hui L. 2011. Mitogen-activated protein kinases in hepatocellular carcinoma development. *Semin Cancer Biol.* Feb;21(1):10-20.

Morris SM, Baek JY, Koszarek A, Kannurn S, Knoblaugh SE, Grady WM. 2012. Transforming growth factor-beta signaling promotes hepatocarcinogenesis induced by p53 loss. *Hepatology.* Jan;55(1):121-31.

Nakamura J, Kohya N, Kai K, Ohtaka K, Hashiquchi K, Hiraki M, Kitajima Y, Tokunaga O, Noshiro H, Miyazaki K. 2010. Ribonucleotide reductase subunit M1 assessed by quantitative double-fluorescence immunohistochemistry predicts the efficacy of gemcitabine in biliary tract carcinoma. *Int J Oncol.* Oct;37(4):845-52.

Nakano H. 2004. Signaling crosstalk between NF-kappaB and JNK. *Trends Immunol.* Aug;25(8):402-5.

Nardella C, Clohessy JG, Alimonti A, Pandolfi PP. 2011. *Nat Rev Cancer.* Jun 24;11(7):503-11.

Okada H and Mak TW. 2004. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer.* Aug;4(8):592-603.

Ozturk M, Arslan-Ergul A, Bagislar S, Senturk S, Yuzugullu H. 2009. Senescence and immortality in hepatocellular carcinoma. *Cancer Lett.* Dec 1;286(1):103-13.

Passos JF, Nelson G, Wang C, Richter T, Simillion C, Proctor CJ, Miwa S, Olijslagers S, Hallinan J, Wipat A, Saretzki G, Rudolph KL, Kirkwood TB, von Zglinicki T. 2010. Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Mol Syst Biol.*;6:347.

Pawlik TM, Reyes DK, Cosgrove D, Kamel IR, Bhagat N, Geschwind JF. 2011. Phase II trial of sorafenib combined with concurrent transarterial chemoembolization with drug-eluting beads for hepatocellular carcinoma. *J Clin Oncol.* Oct 20;29(30):3960-7.

Pimienta G and Pascual J. 2007. Canonical and alternative MAPK signaling. *Cell cycle.* Nov 1;6(21):2628-32.

Pollak M. 2012. The insulin and insulin-like growth factor receptor family in neoplasia: an update. *Nat Rev Cancer.* Feb 16;12(3):159-69.

Pollak MN, Schernhammer ES, Hankinson SE. 2004. Insulin-like growth factors and neoplasia. *Nat Rev Cancer*. Jul;4(7):505-18.

Qin AL, Zhou XQ, Zhang W, Yu H, Xie Q. 2004. Characterization and enrichment of hepatic progenitor cells in adult rat liver. *World J Gastroenterol*. May 15;10(10):1480-6.

Reinacher-Schick A, Schoeneck A, Graeven U, Schwarte-Waldhoff I, Schmiegel W. 2003. Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells. *Carcinogenesis*. Mar;24(3):443-51.

Ricci MS and Zong WX. 2006. Chemotherapeutic approaches for targeting cell death pathways. *Oncologist*. Apr;11(4):342-57.

Ricci MS and Zong WX. 2006. Chemotherapeutic approaches for targeting cell death pathways. *Oncologist*. Apr;11(4):342-57.

Rodier F, Coppé JP, Patil CK, Hoeijmakers WA, Muñoz DP, Raza SR, Freund A, Campeau E, Davalos AR, Campisi J. 2009. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol*. Aug;11(8):973-9.

Rodier F, Muñoz DP, Teachenor R, Chu V, Le O, Bhaumik D, Coppé JP, Campeau E, Beauséjour CM, Kim SH, Davalos AR, Campisi J. 2011. DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *J Cell Sci*. Jan 1;124(Pt 1):68-81.

Rodier F, Campisi J. 2011. Four faces of cellular senescence. *J Cell Biol*. Feb 21;192(4):547-56.

Roninson IB. 2002. Tumor senescence as a determinant of drug response in vivo. *Drug Resist Updat*. Oct;5(5):204-8.

Samali A, FitzGerald U, Deegan S, and Gupta S. 2010. Methods for monitoring endoplasmic reticulum stress and the unfolded protein response. *Int J of Cell Biol*. 830307

Sampath D, Rao VA, Plunkett W. 2003. Mechanisms of apoptosis induction by nucleoside analogs. *Oncogene*. Dec 8;22(56):9063-74.

Schmid I, Häberle B, Albert MH, Corbacioglu S, Fröhlich B, Graf N, Kammer B, Kontny U, Leuschner I, Scheel-Walter HG, Scheurlen W, Werner S, Wiesel T, von Schweinitz D. 2012. Sorafenib and cisplatin/doxorubicin (PLADO) in pediatric hepatocellular carcinoma. *Pediatr Blood Cancer*. Apr;58(4):539-44.

Schwartz GK and Shah MA, 2005. Targeting the cell cycle: a new approach to cancer therapy. *J Clin Oncol*. Dec 20;23(36):9408-21.

Schwartz PA and Murray BW. 2011. Protein kinase biochemistry and drug discovery. *Bioorg Chem.* Dec;39(5-6):192-210.

Seki E, Brenner DA, Karin M. 2012. A Liver Full of JNK: Signaling in Regulation of Cell Function and Disease Pathogenesis, and Clinical Approaches . *Gastroenterology.* Aug;143(2):307-20.

Senturk S., Mumcuoglu M., Gursay-Yuzugullu O., Cingoz B., Akcali KC., Ozturk M. 2010. Transforming Growth Factor-Beta Induces Senescence Arrest in Hepatocellular Carcinoma Cells and Inhibits Tumor Growth. *Hepatology.* Sep;52(3):966-74.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW.1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell.* Mar 7;88(5):593-602.

Shaulian E. 2010. AP-1--The Jun proteins: Oncogenes or tumor suppressors in disguise?. *Cell Signal.* Jun;22(6):894-9.

Shay JW and Roninson IB. 2004. Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene.* Apr 12;23(16):2919-33.

Sherr CJ and Roberts JM. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* Jun 15;13(12):1501-12.

Shiff SJ, Qiao L, Tsai LL, Rigas B. 1995. Sulindac sulfide, an aspirin-like compound, inhibits proliferation, causes cell cycle quiescence, and induces apoptosis in HT-29 colon adenocarcinoma cells. *J Clin Invest.* Jul;96(1):491-503.

Shoemaker RH. 2006. The NCI60 human tumour cell line anticancer drug screen. *Nat Rev Cancer.* Oct;6(10):813-23.

Shoji S, Parmelee DC, Wade RD, Kumar S, Ericsson LH, Walsh KA, Neurath H, Long GL, Demaille JG, Fischer EH, Titani K. 1981. Complete amino acid sequence of the catalytic subunit of bovine cardiac muscle cyclic AMP-dependent protein kinase. *Proc Natl Acad Sci U S A.* Feb;78(2):848-51.

Singh P, Harden BJ, Lillywhite BJ, Broad PM. 2004. Identification of kinase inhibitors by an ATP depletion method. *Assay Drug Dev Technol.* 2:161-9.

Smith ML, Hawcroft G, Hull MA. 2000. The effect of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: evidence of different mechanisms of action. *Eur J Cancer.* Mar;36(5):664-74.

Song MS, Salmena L, Pandolfi PP. 2012. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol.* Apr 4;13(5):283-96.

Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, Maksimovic-Ivanic D, Stivala F, Mazzarino MC, Donia M, Fagone P, Malaponte G, Nicoletti F, Libra M, Milella M, Tafuri A, Bonati A, Bäsecke J, Cocco L, Evangelisti C, Martelli AM, Montalto G, Cervello M, McCubrey JA. 2011. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging (Albany NY)*. Mar;3(3):192-222.

Tanaka S and Arii S. 2010. Current status of molecularly targeted therapy for hepatocellular carcinoma: basic science. *Int J Clin Oncol*. Jun;15(3):235-41.

Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M, Lin A. 2001. Inhibition of JNK activation through NF-kappaB target genes. *Nov 15;414(6861):313-7*.

Tao RH and Maruyama IN. 2008. All EGF(ErbB) receptors have preformed homo- and heterodimeric structures in living cells. *J Cell Sci*. Oct 1;121(Pt 19):3207-17.

Taylor SS and Kornev AP. 2011. Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem Sci*. Feb;36(2):65-77.

Tian Y, Ke S, Denison MS, Rabson AB, Gallo MA. 1999. Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity. *J Biol Chem*. Jan 1;274(1):510-5.

Tozkoparan B, Aytaç SP, Aktay G. 2009. Novel 3,6-disubstituted 7H-1,2,4-triazolo[3,4-b][1,3,4]thiadiazines: synthesis, characterization, and evaluation of analgesic/anti-inflammatory, antioxidant activities. *Arch Pharm (Weinheim)*. May;342(5):291-8.

Tuncbilek M, Bilget Guven E, Onder T, Cetin-Atalay R. 2012. Synthesis of novel 6-(4-substituted piperazine-1-yl)-9-(β -D-ribofuranosyl)purine derivatives, which lead to senescence-induced cell death in liver cancer cells. *J Med Chem*. Apr 12;55(7):3058-65.

Vakifahmetoglu H, Olsson M, Zhivotovsky B. 2008. Death through a tragedy: mitotic catastrophe. *Cell Death Differ*. Jul;15(7):1153-62.

Vermeulen K Van Bockstaele DR, Berneman ZN. 2003. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif*. Jun;36(3):131-49.

Vitale I, Galluzzi L, Castedo M, Kroemer G. 2011. Mitotic catastrophe: a mechanism for avoiding genomic instability. *Nat Rev Mol Cell Biol*. Jun; 12(6):385-92.

Wang L, Jia D, Duan F, Sun Z, Liu X, Zhou L, Sun L, Ren S, Ruan Y, Gu J. 2012. Combined anti-tumor effects of IFN- α and sorafenib on hepatocellular carcinoma in vitro and in vivo. *Biochem Biophys Res Commun*. Jun 15;422(4):687-92.

Wang X and Lin Y. 2008. Tumor necrosis factor and cancer, buddies or foes?. *Acta Pharmacol Sin*. November; 29(11): 1275-1288.

Wei W, Herbig U, Wei S, Dutriaux A, Sedivy JM. 2003. Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts. *EMBO Rep.* Nov;4(11):1061-6.

Weinstein IB and Joe AK, 2006. Mechanisms of disease: Oncogene addiction--a rationale for molecular targeting in cancer therapy. *Nat Clin Pract Oncol.* Aug;3(8):448-57.

Weistheimer FH. 1987. Why nature chose phosphates. *Science.* Mar 6;235(4793):1173-8.
Weston CR and Davis RJ. 2007. The JNK signal transduction pathway. *Curr Opin Cell Biol.* Apr;19(2):142-9.

White AW, Westwell AD, Brahemi G. 2008. Protein-protein interactions as targets for small-molecule therapeutics in cancer. *Expert Rev Mol Med.* Mar 19;10:e8.

Wilson PK, Mulligan SP, Christopherson RI. 2004. Metabolic response patterns of nucleotides in B-cell chronic lymphocytic leukaemias to cladribine, fludarabine and deoxycoformycin. *Leuk Res.* Jul;28(7):725-31.

Wu SH, Hang LW, Yang JS, Chen HY, Lin HY, Chiang JH, Lu CC, Yang JL, Lai TY, Ko YC, Chung JG. 2010. Curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells through ER stress and caspase cascade- and mitochondria-dependent pathways. *Anticancer Res.* Jun;30(6):2125-33.

Yamamoto K, Ichijo H, Korsmeyer SJ. 1999. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. *Mol Cell Biol.* Dec;19(12):8469-78.

Yuan H, Kaneko T, Matsuo M. 1995. Relevance of oxidative stress to the limited replicative capacity of cultured human diploid cells: the limit of cumulative population doublings increases under low concentrations of oxygen and decreases in response to aminotriazole. *Mech Ageing Dev.* Jul 14;81(2-3):159-68.

Ye SL, Takayama T, Geschwind J, Marrero JA, Bronowicki JP. 2010. Current approaches to the treatment of early hepatocellular carcinoma. *Oncologist.*;15 Suppl 4:34-41.

Zender L, Villanueva A, Tovar V, Sia D, Chiang DY, Llovet JM. 2010. Cancer gene discovery in hepatocellular carcinoma. *J Hepatol.* Jun;52(6):921-9.

Zhang J, Yang PL, Gray NS. 2009. Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer.* Jan;9(1):28-39.

Zhang H. 2007. Molecular signaling and genetic pathways of senescence: Its role in tumorigenesis and aging. *J Cell Physiol.* Mar;210(3):567-74.

Zhang L, Chen J, Fu H. 1999. Suppression of apoptosis signal-regulating kinase 1-induced cell death by 14-3-3 proteins. *Proc Natl Acad Sci U S A*. Jul 20; 96(15):8511-5.

Zhang Y and Chen F. 2004. Reactive oxygen species (ROS), troublemakers between nuclear factor-kappaB (NF-kappaB) and c-Jun NH(2)-terminal kinase (JNK). *Cancer Res*. Mar 15;64(6):1902-5.

Zhang R, Luo D, Miao R, Bai L, Ge Q, Sessa WC, Min W. 2005. Hsp90-Akt phosphorylates ASK1 and inhibits ASK1-mediated apoptosis. *Oncogene*. Jun 2;24(24):3954-63.

Zhivotovsky B. 2004. Apoptosis, necrosis and between. *Cell Cycle*. Jan;3(1):64-6.

Zuccotto F, Ardini E, Casale E, Angiolini M. 2010. Through the "gatekeeper door": exploiting the active kinase conformation. *J Med Chem*. Apr 8;53(7):2681-94.

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DNA damaging agents and p53 do not cause senescence in quiescent cells, while consecutive re-activation of mTOR is associated with conversion to senescence

[Olga V. Leontieva](#) and [Mikhail V. Blagosklonny](#)

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Targeted therapy for hepatocellular carcinoma: novel agents on the horizon

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Synthesis of Novel 6-(4-Substituted piperazine-1-yl)-9-(β -D-ribofuranosyl)purine Derivatives, Which Lead to Senescence-Induced Cell Death in Liver Cancer Cells

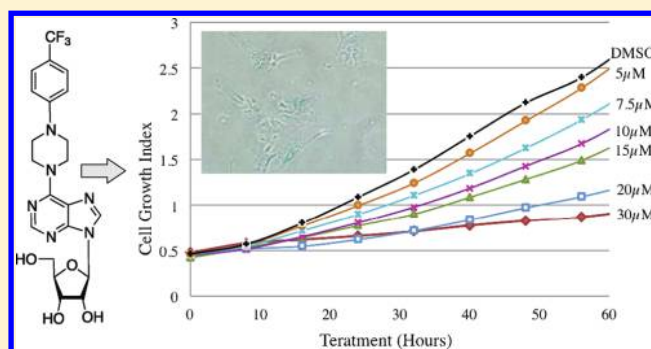
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ABSTRACT: Novel purine ribonucleoside analogues (9–13) containing a 4-substituted piperazine in the substituent at N⁶ were synthesized and evaluated for their cytotoxicity on Huh7, HepG2, FOCUS, Mahlavu liver, MCF7 breast, and HCT116 colon carcinoma cell lines. The purine nucleoside analogues were analyzed initially by an anticancer drug-screening method based on a sulforhodamine B assay. Two nucleoside derivatives with promising cytotoxic activities (11 and 12) were further analyzed on the hepatoma cells. The N⁶-(4-Trifluoromethylphenyl)piperazine analogue 11 displayed the best antitumor activity, with IC₅₀ values between 5.2 and 9.2 μ M. Similar to previously described nucleoside analogues, compound 11 also interferes with cellular ATP reserves, possibly through influencing cellular kinase activities. Furthermore, the novel nucleoside analogue 11 was shown to induce senescence-associated cell death, as demonstrated by the SA β -gal assay. The senescence-dependent cytotoxic effect of 11 was also confirmed through phosphorylation of the Rb protein by p15^{INK4b} overexpression in the presence of this compound.



INTRODUCTION

Nucleobase analogues and nucleoside analogues are significant drugs used in chemotherapy for the treatment of solid tumors and hematological malignancies.¹ These groups of compounds are considered antimetabolites because nucleobases and nucleosides are the metabolic precursors of nucleotides. Nucleotides and their derivatives are involved in a large number of cellular processes, including cell growth and division, and for this reason nucleobase and nucleoside analogues have been exploited as anticancer agents.² Initially, nucleobase analogues such as fluorinated pyrimidines were investigated as antimetabolite chemotherapeutic agents on cancer cells. Later, pyrimidine analogues Ara-C and Gemcitabine were used in cancer therapy.³ Success with pyrimidine nucleoside analogues in cancer therapy led to the discovery of purine nucleoside analogues. For more than six decades, 6-mercaptapurine and 6-thioguanine have been used as inhibitors of nucleic acid metabolism in pediatric acute lymphoblastic leukemia.⁴ Currently, the purine nucleoside analogues fludarabine, cladribine, and pentostatin are used for treating hematological malignancies.⁵ The synthetic nucleoside analogues fludarabine and cladribine are synthesized into the dATP analogues, and the natural substance pentostatin leads to an increase in the dATP levels in the cell. Nucleoside analogues create an imbalance in the cellular dNTP reserve by inhibiting the ribonucleotide reductase enzyme, which in turn leads to

impaired DNA synthesis.⁶ For this reason, nucleoside analogues often cause apoptosis-induced cell death.³ Recently, expression levels of ribonucleotide reductase subunits have been proposed as molecular markers for nucleoside analogue-induced cell death in cancer therapy response; nevertheless, it was previously shown that reduced ribonucleotide reductase and altered dNTP pools have been associated with cellular senescence in diploid fibroblasts.^{7–9} Hence, nucleoside analogues too, may induce senescence-associated cell death.

Apoptosis and necrosis are the most-studied chemotherapeutic-induced cell death mechanisms. However, in the past decade, senescence, autophagy, and mitotic catastrophe have been shown to be induced by cytotoxic agents.¹⁰ Senescence-associated growth arrest is a significant cellular event in tumor development and progression. Initially, replicative senescence was reported to be due to telomere shortening during replication.¹¹ Later, Serrano et al. showed that premature senescence was associated with cancer.¹² Studies on the molecular analysis of senescence in cancer revealed oncogene-induced senescence (OIS) and tumor-suppressor-dependent senescence (PICS).^{13,14} Therefore, senescence-induced cell death through pro-senescence therapy is currently the target of small-molecule inhibitors.¹⁴

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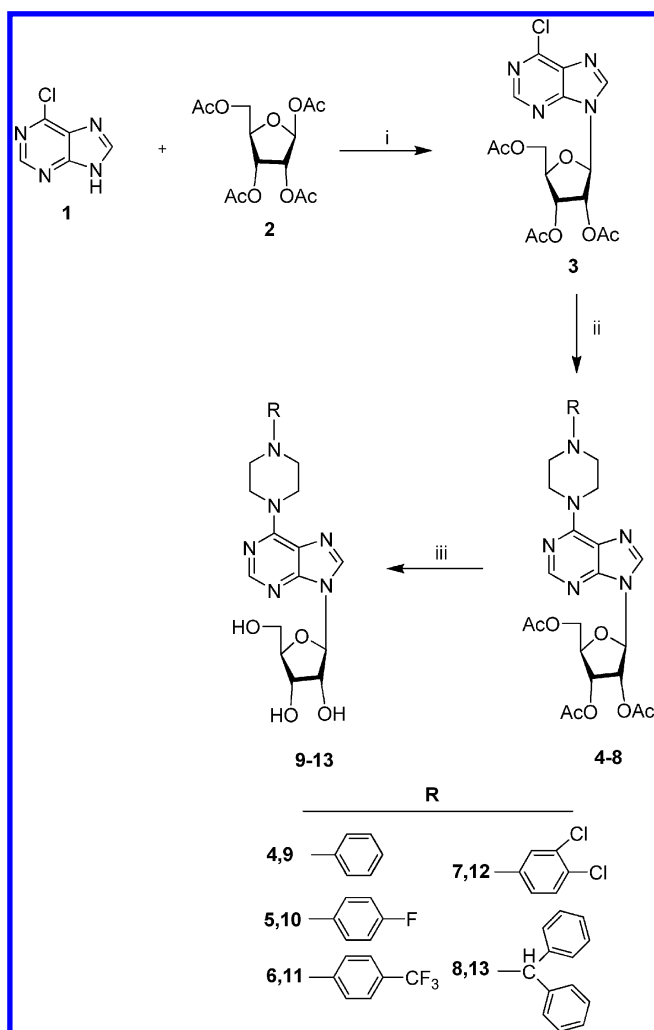
Recently, studies focusing on evading senescence in murine premalignant hepatocytes have revealed a mechanism called senescence surveillance during hepatocarcinogenesis.¹⁵ Furthermore, replacement of the tumor suppressor p53 in murine liver cancer models has led to senescence and therefore to regression of these tumors. Primary liver cancer, hepatocellular carcinoma (HCC), is the fourth most common cause of cancer mortality and the third most common malignancy in human cancers. Chronic liver injury is due to viral diseases, exposure to chemicals, and other environmental or autoimmune conditions that are the risk factors for HCC. These factors induce an acquired tolerance to genotoxic stress, but ultimately a cancerous state that does not respond to the cellular death mechanisms.¹⁶ Recently, Sorafenib, a multikinase inhibitor, was approved by the FDA and the EU for hepatocellular carcinoma treatment.¹⁷ Sorafenib prolongs median survival and the time to progression by nearly three months in patients with advanced hepatocellular carcinoma. Therefore, there is a need for new liver-cancer-specific drugs based on the molecular mechanisms involved in liver carcinogenesis. In this study, we synthesized novel purine ribonucleoside analogues (9–13) containing a 4-substituted piperazine in the substituent at N⁶ as putative cytotoxic agents. The newly obtained compounds were then characterized for their anticancer senescence-inducing activity in liver cancer cells.

RESULTS AND DISCUSSION

Chemistry. The 6-(4-substituted piperazine-1-yl)-9-(β -D-ribofuranosyl)purine derivatives (9–13) were synthesized as shown in Scheme 1. 6-Chloropurine (1) was condensed with the sugar 1,2,3,5-tetra-*O*-acetyl- β -D-ribofuranose (2) under microwave irradiation (30 min) to obtain 6-chloro-9-(2,3,5-tri-*O*-acetyl- β -D-ribofuranosyl)-9*H*-purine (3) as a yellowish foam in good yield of 75.7%. This reaction gave significantly higher yields than the previously published method.¹⁸ Displacement of the 6-chloro was accomplished by nucleophilic substitution with appropriate N-substituted piperazines. Removal of the acetyl-protecting groups was performed with NaOMe in MeOH to produce nucleosides 9–13.

Biological Evaluation and Discussion. The newly synthesized compounds 9–13 were first evaluated for their antitumor activities against human liver (Huh7), colon (HCT116), and breast (MCF7) carcinoma cell lines (Figure 1A). The IC₅₀ values were in micromolar concentrations with N⁶-(substituted phenyl)piperazine purine nucleoside derivatives (Figure 1A and Table 1). We then tested the cytotoxic effect of these molecules on additional hepatocellular carcinoma (HCC) cell lines: HepG2, Mahlavu, and FOCUS (Figure 1B). We observed strong cell growth inhibition in the presence of the novel nucleosides 11 and 12. Time-dependent IC₅₀ values for each molecule were also calculated in comparison with the nucleobase analogue 5-fluorouracil (5-FU) and DNA topoisomerase inhibitor camptothecin (CPT) (Table 1). N⁶-(4-Trifluoromethylphenyl)piperazine derivative 11 displayed the best cytotoxic activity, with IC₅₀ values of 5.2–9.2 μ M (Table 1). The (3,4-dichlorophenyl)piperazine derivative 12 was also very active (IC₅₀ values in the range of 5.5–9.7 μ M) against all tested cell lines. When there was a larger substituent at the 4-position of piperazine moiety (diphenylmethyl group, 13), cytotoxic activity was decreased. On the other hand, compound 9, which has no substitution at the phenyl ring, did not show any significant cytotoxic activity; the compound 10, with 4-fluorophenyl, had some cytotoxicity (Table 1). Nucleosides 11

Scheme 1^a



^aReagents: (i) silica gel 60, EtOAc, microwave irradiation; (ii) the appropriate piperazine, TEA, EtOH; (iii) NaOMe, MeOH.

and 12 demonstrated significant cytotoxicity for all the cell lines tested. When we compared their IC₅₀ values with the known cell growth inhibitors CPT and 5-FU, we observed that our compounds 11 and 12 had showed lower values in micromolar concentrations. Compounds 11 and 12 had a better cytotoxic activity on Huh7 cells (7.8 and 7.1 vs 30.7 μ M for 5-FU).

Considering the cytotoxic activity of our novel nucleosides 9–13 on hepatoma cell lines, we further analyzed the cellular activity of the most potent inhibitor (11) on these cell lines as a promising candidate anticancer agent.

Real-Time Cellular Response of Hepatocellular Carcinoma Cells with Compound 11 Treatment. Real-time cell electronic sensing (RT-CES) was used to evaluate compound 11's mediated cytotoxicity on Huh7, HepG2, Mahlavu, and FOCUS hepatoma cells in triplicate (Figure 2). Real-time dynamic monitoring of the electrode impedance indicates a cell index (CI) that correlates with cell growth. Compound 11 triggered a time- and dose-dependent decrease in CI cell growth indexes in all hepatoma cells (Figure 2). A cell growth index with 30–5 μ M of compound 11 treatment clearly demonstrates the potent inhibitor action of compound 11, which correlates with our initial observation with the NCI-SRB assay. The PTEN-deficient cell line Mahlavu was the least

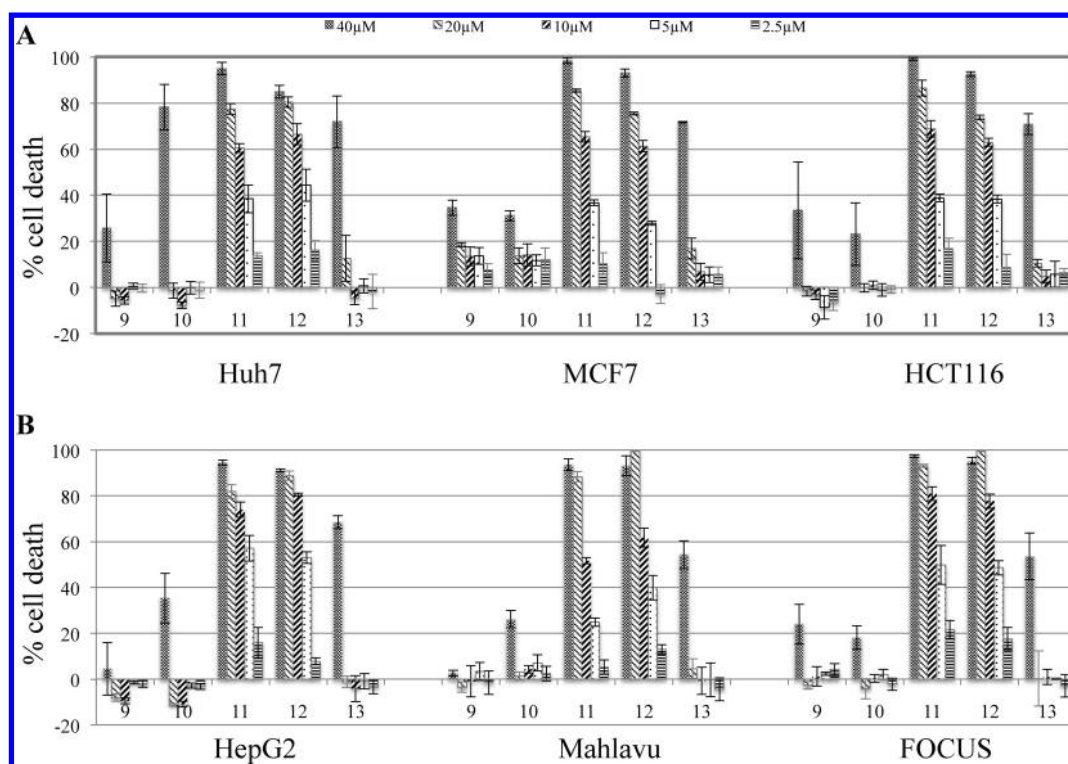


Figure 1. Percent cell death in the presence of compounds 9–13. Huh7, HCT116, MCF7 (A) and HepG2, Mahlavu and FOCUS (B) cells were inoculated in 96-well plates. All molecules and their DMSO controls were administered to the cells in triplicate with five different concentrations: 40, 20, 10, 5, and 2.5 μ M. After 72 h of incubation, SRB assays were generated and the cell death percentages were calculated in comparison with DMSO-treated wells.

Table 1. IC₅₀ Values in μ M Concentrations for 9–13 with 72 h of Treatment^a

	9	10	11	12	13	5-FU	CPT
Huh7	>100	49.7	7.8	7.1	44.4	30.7	<0.1
HepG2	>100	>100	5.7	6.1	63.5	5.0	<0.1
Mahlavu	>100	>100	9.2	7.0	92.7	10.0	<0.1
FOCUS	>100	>100	5.2	5.5	>100	7.6	<0.1
HCT116	>100	>100	6.7	8.4	48.5	6.0	<0.1
MCF7	>100	>100	7.5	9.7	40.1	3.5	<0.1

^aIC₅₀ values were calculated from the cell growth inhibition percentages obtained with five different concentrations.

affected by **11** (Mahlavu cells have a hyperactive PI3K/Akt pathway due to PTEN deficiency).¹⁹ Higher nucleoside–**11** concentrations were needed for the cytotoxicity on Mahlavu cells. This observation indicated that compound–**11** might be a putative kinase–protein interfering molecule. For that reason, we tested a nontargeted broad-spectrum kinase assay with the aim of detecting cellular ATP levels affected by the presence of **11**.

Nucleoside Analogue 11 Possesses Kinase-Inhibitor Potential. With the aim of elucidating the possible kinase-interfering activity of **11**, we used a luminescent ATP-detection assay. Luminescence correlates with the amount of ATP in the milieu, therefore an increase in the luminescence might indicate the presence of a protein kinase inhibitor. Because of their established kinase-inhibition potentials, we used staurosporine (STS), a multikinase inhibitor, and the nucleoside analogue 5'-deoxy-5'-methylthioadenosine (MeSAdo) as positive controls for the experiment. Huh7 cells were incubated with IC₅₀ and IC₁₀₀ values of **11** (Table 1), 5 μ M MeSAdo, and 0.5 μ M STS

for 72 h. A luminescent ATP-detection assay was then achieved. The luminescence (measured as relative light units (*rlu*)) indicated a dose-dependent ATP amount in the presence of **11** similar with MeSAdo (Figure 3) with the same cell count. High *rlu* values obtained with the STS-treated Huh7 cells are consistent with both the principle of the assay and the molecular mechanism of STS as a multikinase inhibitor.

The Cytotoxic Activity of 11 Is Neither Apoptosis nor Necrosis. We then characterized the cytotoxic pathways involved in the molecular action of **11**. The apoptotic pathway activation indicator Poly-ADP-ribosyl-polymerase (PARP)'s protein cleavage was assessed on Huh7, HepG2, Mahlavu, and FOCUS cells in the presence of **11**. For each cell line, **11** was used as its cell-line-specific IC₅₀ value for 72 h (Table 1). The endogenous PARP protein has an atomic mass of 113 kDa. During apoptosis, PARP is cleaved into 89 kDa and 24 kDa fragments, and when the cytotoxic effect is due to necrosis, the cleaved PARP is detected as a 50 kDa fragment band in Western blot analysis.²⁰ Seventy-two hours of treatment with **11** did not induce cleavage of the PARP protein in all treated liver cancer cell lines (data not shown). This cleavage analysis demonstrated that the cytotoxic activity of **11** was neither apoptosis nor necrosis.

Compound 11 Induces Cellular Senescence. Replicative senescence has long been characterized as proliferative arrest that occurs in normal cells after a limited number of population doublings. Recently, premature senescence has been associated with cancer cells. INK4a and INK4b proteins inhibit CyclinD1/CCDK4, leading to pRB activation and therefore induction of senescence. For this reason, higher expression of these proteins is among the premature senescence markers (in addition to

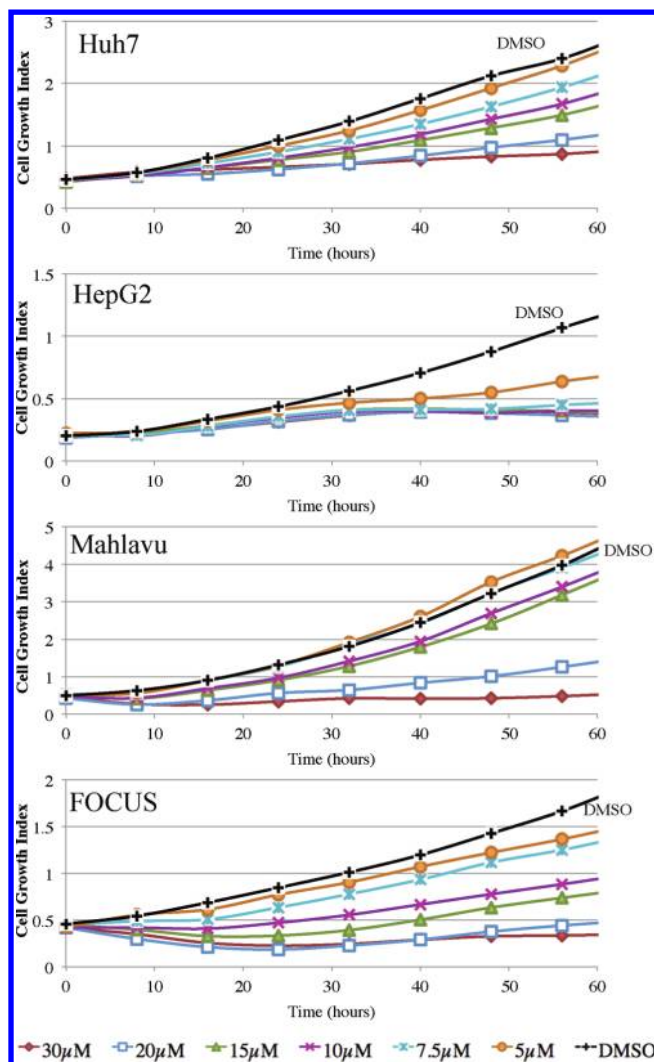


Figure 2. Real-time cell growth of Huh7, HepG2, Mahlavu, and FOCUS cells in the presence of compound **11**. Cells were inoculated in triplicate in E-plates. The cell growth index was monitored every 30 min in the presence of the different concentrations of compound **11** (30 μ M, red; 20 μ M, blue; 15 μ M, green; 10 μ M, magenta; 7.5 μ M, cyan; 5 μ M, coral; DMSO control, black).

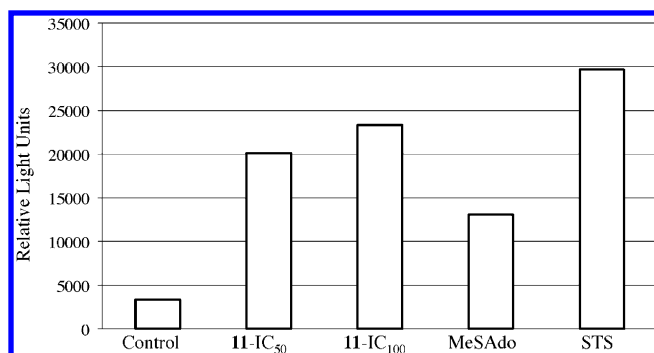


Figure 3. Whole cell protein kinase activity in the presence of **11**. Huh7 cells were incubated with IC_{50} and IC_{100} values of **11** (Table 1), staurosporine (STS, 0.5 μ M), MeSAAdo (0.5 μ M), and their corresponding DMSO controls for 72 h. Then, using the Kinase-Glo assay kit, 80000 were cells tested for their ATP content by chemoluminescence.

senescence-associated β -galactosidase (SA β -gal) activity at pH 6.0 due to increased lysosomal activity).

With the aim of identifying the possible senescence-involved cytotoxic activity of **11**, we performed a SA β -gal assay and BrdU incorporation assays in parallel. Huh7 cells were plated in six-well plates on coverslips at low density for the logarithmic phase growth. The next day, Huh7 cells were treated with **11** at its IC_{50} and IC_{100} values both for three or six days. Doxorubicin (25 ng/mL) was used as a positive control for senescence-inducing agent, and DMSO was used as the negative control. Twenty-four hours prior to the end of the incubation with the compound, BrdU was administered to test its incorporation into the cellular DNA. The large blue-stained senescent (SA β -gal-assay-positive) cells were negative for BrdU incorporation for compound **11** and doxorubicin (Figure 4A,B) when

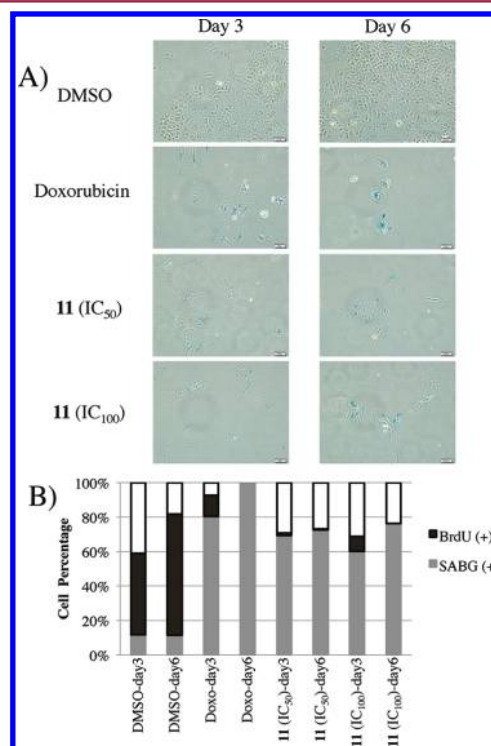


Figure 4. SA β -gal and BrdU assays with compound **11**. Huh7 cells were plated on coverslips in six-well plates (5000 cells/well). (A) Huh7 cells were incubated with IC_{50} and IC_{100} values of **11**, doxorubicin, and DMSO only controls for three and six days. Doxorubicin was used as a positive control at its senescence-inducing dose (25 ng/mL). BrdU (30 μ M) was administered to the cells 24 h prior to the end of three and six days of incubation. (B) Cells were counted and the percent distribution between SA β -gal and BrdU positive was presented.

compared to the DMSO control. However, BrdU-positive proliferating cells were marked visible in DMSO-treated wells only.

Compound 11-Induced Senescence Is Associated with the Induction of p15^{INK4b} and a Decrease in Rb Phosphorylation. In addition to testing for the most widely used and accepted marker of senescent cells (an increase in SA β -gal activity), we tested another senescence-associated marker (p15^{INK4b} levels) in **11**-treated Huh7 cells. Huh7 cells were treated in the presence of IC_{50} and IC_{100} concentrations of **11** both for three and six days, then Western blot analysis was realized. Indeed, we observed an increase in the protein

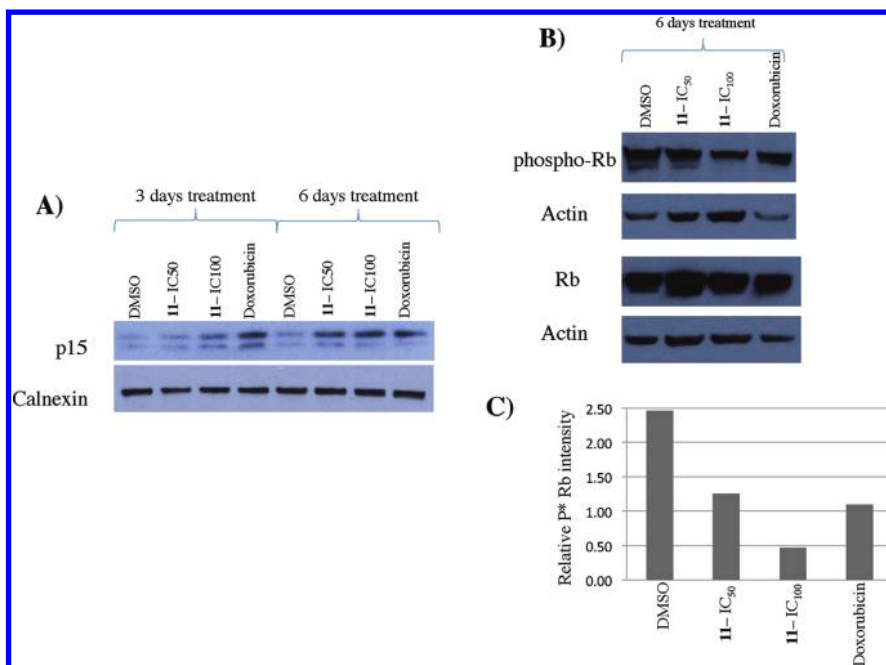


Figure 5. Senescence-associated proteins p15^{INK4b} and pRb proteins in the presence 11. Huh7 cells were treated with IC₅₀ and IC₁₀₀ values of 11, doxorubicin, and DMSO for three and six days. Doxorubicin was used as a positive control at its senescence-inducing dose (25 ng/mL). (A) p15^{INK4b} protein expression by Western blotting after three and six days of treatment in comparison with positive control doxorubicin and negative control DMSO. Treatment of Huh7 cells with 11 induced the accumulation of p15^{INK4b} in a dose- and time-dependent manner. (B) Rb activity is detected with anti-Rb and antiphospho-Rb antibodies. (C) Comparative Rb phosphorylation levels confirmed senescence-induced cytotoxicity by compound 11. Calnexin protein was used as an equal loading control.

expression levels of p15^{INK4b} in a dose- and time-dependent manner (Figure 5A). Next, we determined the downstream effect of p15^{INK4b} on the phosphorylation of the Rb protein. It is known that p15^{INK4b} activates the Rb protein by inhibiting CyclinD1/CCDK4CD and therefore inhibits the phosphorylation of Rb. An observed decrease in the phosphorylated-form of the Rb protein correlates with the 11-induced accumulation of p15^{INK4b} (Figure 5A,B). This observation thus also confirmed the senescence-induced cytotoxic activity of compound 11.

CONCLUSION

We synthesized a novel group of nucleoside analogues (N⁶-substituted piperazine derivatives) as putative anticancer agents. We identified their cytotoxic activity and determined the minimum required concentration for their action. Two molecules, 11 and 12, were promising as candidate chemotherapeutic agents and had IC₅₀ values less than 10 μM. We selected the most active compound (11) to pursue further experiments on with the aim of analyzing its molecular cytotoxic action on hepatoma cells. Our results indicated that the novel candidate chemotherapeutic agent 11 induces senescence-associated cell death through the inhibition of some kinase proteins (Figures 3 and 4). In addition our analysis with p15^{INK4b} protein levels in 11-treated cells indicates that the target kinases could be upstream of this protein; this must be further investigated in detail.

Recent studies on the involvement of senescence-associated cell death in cancer have established the concept stress or aberrant signaling-induced senescence (STASIS), which is telomere independent.²¹ Reprogramming senescence in cancer cells was extensively discussed as one of the hallmarks of cancer.²² Targeting replicative immortality and inducing

senescence has also been proposed for mechanism-based drug discovery. For this reason, induction of irreversible cell cycle arrest by senescence with novel candidate chemotherapeutic agents has become an important strategy against cancer.

EXPERIMENTAL SECTION

Chemistry. Melting points were recorded with a capillary melting point apparatus (Electrothermal 9100) and are uncorrected. NMR spectra were recorded on a VARIAN Mercury 400 FT-NMR spectrometer (400 for ¹H, 100.6 MHz for ¹³C). TMS was used as internal standard for the ¹H and ¹³C NMR spectra; values are given in δ (ppm) and *J* values are in Hz. High resolution mass spectra data (HRMS) were collected in-house using a Waters LCT Premier XE mass spectrometer (high sensitivity orthogonal acceleration time-of-flight instrument) operating in ESI (+) method, also coupled with an AQUITY Ultra Performance liquid chromatography system (Waters Corporation, Milford, MA, USA). All compounds were of >95% purity. Elemental analyses (C, H, N) were determined on a Leco CHNS 932 instrument and gave values within ±0.4% of the theoretical values. Microwave reactions were carried out using a domestic microwave oven (White Westinghouse SG-KM97VL, 50 Hz, 1400 W). Column chromatography was accomplished on silica gel 60 (40–63 mm particle size). The chemical reagents used in synthesis were purchased from E. Merck, Fluka, Sigma, and Aldrich.

6-Chloro-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-9H-purine (3). 6-Chloropurine (1) (154 mg, 1 mmol) and 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (2) were dissolved in EtOAc, and then 500 mg of silica gel 60 (200–400 mesh) was added. The mixture was concentrated in vacuo, and the dry residue was irradiated for 30 min in a White Westinghouse SG-KM97VL domestic microwave oven (50 Hz, 1400 W). The residue was purified by flash chromatography on silica gel (EtOAc–hexane, 3:1) to yield 3 as yellowish viscous oil (312.3 mg, 75.72%). ¹H NMR (CDCl₃) δ 2.07 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.14 (s, 3H, OAc), 4.34–4.48 (m, 3H, H-4', H-5'), 5.63 (t, *J* = 5.2 Hz, 1H, H-3'), 5.93 (t, *J* = 5.6 Hz, 1H, H-2'), 6.21 (d, *J* = 5.2 Hz, 1H, H-1'), 8.28 (s, 1H, H-8), 8.76 (s, 1H, H-2). ¹³C NMR (CDCl₃) δ

20.59, 20.74, 20.97 ($3 \times \text{CH}_3$), 63.09 ($\text{CH}_2\text{-S}'$), 70.67 ($\text{CH-4}'$), 73.32 ($\text{CH-3}'$), 80.74 ($\text{CH-2}'$), 87.08 ($\text{CH-1}'$), 132.57 (C-5), 143.79 (C-8), 151.44 (C-6), 151.87 (C-4), 152.54 (C-2), 169.56, 169.78, 170.48 ($3 \times \text{CO}$). HRMS (ESI+) m/z calcd for $\text{C}_{16}\text{H}_{18}\text{ClN}_4\text{O}_7$ ($\text{M} + \text{H}$)⁺ 413.0864, found 413.0859. Anal. Calcd for $\text{C}_{16}\text{H}_{17}\text{ClN}_4\text{O}_7 \cdot 0.6\text{EtOAc}$: C, 47.46; H, 4.71; N, 12.03. Found C, 47.84; H, 4.59; N, 11.89.

General Procedure for the Synthesis of Compounds 4–8. 6-Chloro-9-(2,3,5-tri-*O*-acetyl- β -D-ribofuranosyl)-9*H*-purine (3) was dissolved in 10 mL of absolute EtOH, and then 1-substituted piperazines and (Et)₃N (3 equiv) were added. The mixture was refluxed for 3–8 h. The reaction mixture was concentrated in vacuo, and the residue was purified by column chromatography.

6-(4-Phenylpiperazine-1-yl)-9-(2,3,5-tri-*O*-acetyl- β -D-ribofuranosyl)-9*H*-purine (4). The compound was prepared from (3) (174.4 mg, 0.4 mmol) and 1-phenylpiperazine (0.13 mL, 0.8 mmol) at reflux for 4 h according to the general procedure and was purified by column chromatography (EtOAc–hexane, 1:1) to yield 4 (209 mg; 92%); mp 62–64 °C. ¹H NMR (CDCl_3) δ 2.08 (s, 3H, OAc), 2.14 (s, 6H, OAc), 3.32 (t, 4H, piperazine CH_2), 4.34–4.50 (m, 7H, H-4', H-5', piperazine CH_2), 5.66 (t, $J = 5.6$ Hz, 1H, H-3'), 5.91 (t, $J = 5.2$ Hz, 1H, H-2'), 6.22 (d, $J = 5.6$ Hz, 1H, H-1'), 6.91 (t, 1H, $J = 7.2$ Hz, H-4 in phenyl), 6.98 (d, 2H, $J = 7.6$ Hz, H-2,6 in phenyl), 7.30 (t, 2H, $J = 7.6$ Hz, H-3,5 in phenyl), 7.92 (s, 1H, H-8), 8.37 (s, 1H, H-2). ¹³C NMR (CDCl_3) δ 20.65, 20.78, 21.03 ($3 \times \text{CH}_3$), 45.24, 49.85 (CH_2 in piperazine), 63.41 ($\text{CH}_2\text{-S}'$), 70.95 ($\text{CH-3}'$), 73.32 ($\text{CH-2}'$), 80.40 ($\text{CH-4}'$), 86.12 ($\text{CH-1}'$), 116.78, 120.63, 129.45 (C in phenyl), 136.71 (C-5), 150.99 (C-8), 151.37 (C-6), 152.98 (C-2), 154.01 (C-4), 169.63, 169.85, 170.57 ($3 \times \text{CO}$). HRMS (ESI+) m/z calcd for $\text{C}_{26}\text{H}_{31}\text{N}_6\text{O}_7$ ($\text{M} + \text{H}$)⁺ 539.2254, found 539.2253. Anal. Calcd for $\text{C}_{26}\text{H}_{30}\text{N}_6\text{O}_7 \cdot 0.5\text{H}_2\text{O}$: C, 57.03; H, 5.70; N, 15.34. Found C, 57.35; H, 5.58; N, 14.97.

6-[4-(4-Fluorophenyl)piperazine-1-yl]-9-(2,3,5-tri-*O*-acetyl- β -D-ribofuranosyl)-9*H*-purine (5). The compound was prepared from (3) (140.1 mg, 0.3 mmol) and 1-(4-fluorophenyl)piperazine (61.1 mg, 0.3 mmol) at reflux for 7 h according to the general procedure and was purified by column chromatography (EtOAc–hexane, 1:1) to yield 5 (151 mg; 80.3%); mp 57–59 °C. ¹H NMR (CDCl_3) δ 2.08 (s, 3H, OAc), 2.14 (s, 6H, OAc), 3.22 (t, 4H, piperazine CH_2), 4.35–4.47 (m, 6H, H-4', H-5', piperazine CH_2), 5.66 (t, $J = 4.8$ Hz, 1H, H-3'), 5.91 (t, $J = 5.2$ Hz, 1H, H-2'), 6.22 (d, $J = 5.6$ Hz, 1H, H-1'), 6.91–7.02 (m, 4H, H-2,3,5,6 in phenyl), 7.92 (s, 1H, H-8), 8.37 (s, 1H, H-2). ¹³C NMR (CDCl_3) δ 20.64, 20.77, 21.02 ($3 \times \text{CH}_3$), 45.29, 50.88 (CH_2 in piperazine), 63.39 ($\text{CH}_2\text{-S}'$), 70.92 ($\text{CH-3}'$), 73.32 ($\text{CH-2}'$), 80.39 ($\text{CH-4}'$), 86.16 ($\text{CH-1}'$), 115.77, 115.99, 118.67 (2), 120.63 (C in phenyl), 136.75 (C-5), 148.02 (C-8), 150.99 (C-6), 152.95 (C-2), 153.98 (C-4), 169.62, 169.84, 170.56 ($3 \times \text{CO}$). HRMS (ESI+) m/z calcd for $\text{C}_{26}\text{H}_{30}\text{FN}_6\text{O}_7$ ($\text{M} + \text{H}$)⁺ 557.2160, found 557.2163. Anal. Calcd for $\text{C}_{26}\text{H}_{29}\text{FN}_6\text{O}_7 \cdot 1.3\text{EtOAc}$: C, 55.84; H, 5.91; N, 12.52. Found C, 56.10; H, 5.87; N, 12.14.

6-[4-(4-Trifluoromethylphenyl)piperazine-1-yl]-9-(2,3,5-tri-*O*-acetyl- β -D-ribofuranosyl)-9*H*-purine (6). The compound was prepared from (3) (92.8 mg, 0.23 mmol) and 1-(α,α,α -trifluoro-*p*-tolyl)-piperazine (52.1 mg, 0.23 mmol) at reflux for 2.5 h according to the general procedure and was purified by column chromatography (EtOAc–hexane, 1:1) to yield 6 (106.4 mg; 78.23%); mp 68–70 °C. ¹H NMR ($\text{DMSO-}d_6$) δ 2.04 (d, 6H, OAc), 2.13 (s, 3H, OAc), 3.45 (br s, 4H, piperazine CH_2), 4.25–4.43 (m, 7H, H-4', H-5', piperazine CH_2), 5.63 (t, 1H, H-3'), 6.03 (t, 1H, H-2'), 6.26 (d, $J = 5.6$ Hz, 1H, H-1'), 6.13 (d, $J_0 = 8.8$ Hz, 2H, H-2,6 in phenyl), 7.54 (d, $J_0 = 8.4$ Hz, 2H, H-3,5 in phenyl), 8.33 (s, 1H, H-8), 8.46 (s, 1H, H-2). ¹³C NMR ($\text{DMSO-}d_6$) δ 20.87, 21.03, 21.17 ($3 \times \text{CH}_3$), 44.50, 47.61 (CH_2 in piperazine), 63.47 ($\text{CH}_2\text{-S}'$), 70.73 ($\text{CH-3}'$), 72.58 ($\text{CH-2}'$), 80.11 ($\text{CH-4}'$), 86.24 ($\text{CH-1}'$), 115.05, 118.78 (q), 120.23, 124.29 (C in phenyl), 126.87 (q) (CF_3), 139.91 (C-5), 150.93 (C-8), 152.89 (C-6), 153.76 (C-2), 153.81 (C-4), 169.96, 170.14, 170.72 ($3 \times \text{CO}$). HRMS (ESI+) m/z calcd for $\text{C}_{27}\text{H}_{30}\text{F}_3\text{N}_6\text{O}_7$ ($\text{M} + \text{H}$)⁺ 607.2128, found 607.2115. Anal. Calcd for $\text{C}_{27}\text{H}_{29}\text{F}_3\text{N}_6\text{O}_7 \cdot 0.3\text{EtOAc}$: C, 53.51; H, 5.00; N, 13.28. Found C, 53.88; H, 5.04; N, 13.03.

6-[4-(3,4-Dichlorophenyl)piperazine-1-yl]-9-(2,3,5-tri-*O*-acetyl- β -D-ribofuranosyl)-9*H*-purine (7). The compound was prepared from

(3) (241.5 mg, 0.58 mmol) and 1-(3,4-dichlorophenyl)piperazine (135.1 mg, 0.58 mmol) at reflux for 8 h according to the general procedure and was purified by column chromatography (EtOAc–hexane, 1:1) to yield 7 (166.2 mg; 46.69%); mp 67–69 °C. ¹H NMR (CDCl_3) δ 2.08 (s, 3H, OAc), 2.15 (s, 6H, OAc), 3.29 (t, 4H, piperazine CH_2), 4.34–4.48 (m, 7H, H-4', H-5', piperazine CH_2), 5.67 (t, $J = 4.8$ Hz, 1H, H-3'), 5.92 (t, $J = 5.2$ Hz, 1H, H-2'), 6.22 (d, $J = 5.6$ Hz, 1H, H-1'), 6.79 (dd, $J_0 = 8.8$ Hz, $J_m = 2.8$ Hz, 1H, H-6 in phenyl), 7.0 (d, $J_m = 2.8$ Hz, 1H, H-2 in phenyl), 7.29 (d, $J_0 = 9.2$ Hz, 1H, H-5 in phenyl), 7.93 (s, 1H, H-8), 8.37 (s, 1H, H-2). ¹³C NMR (CDCl_3) δ 20.64, 20.77, 21.02 ($3 \times \text{CH}_3$), 44.94, 49.21 (CH_2 in piperazine), 63.37 ($\text{CH}_2\text{-S}'$), 70.91 ($\text{CH-3}'$), 73.33 ($\text{CH-2}'$), 80.39 ($\text{CH-4}'$), 86.23 ($\text{CH-1}'$), 115.92 (CH in phenyl), 117.86 (CH in phenyl), 120.66 (CH in phenyl), 122.98 (C in phenyl), 130.76 (C in phenyl), 133.09 (C in phenyl), 136.91 (C-5), 150.76 (C-8), 150.99 (C-6), 152.92 (C-2), 153.91 (C-4), 169.62, 169.83, 170.55 ($3 \times \text{CO}$). HRMS (ESI+) m/z calcd for $\text{C}_{26}\text{H}_{28}\text{Cl}_2\text{N}_6\text{O}_7$ ($\text{M} + \text{H}$)⁺ 607.1475, found 607.1475. Anal. Calcd for $\text{C}_{26}\text{H}_{28}\text{Cl}_2\text{N}_6\text{O}_7 \cdot 0.2\text{EtOAc}$: C, 51.50; H, 4.77; N, 13.44. Found C, 51.43; H, 4.65; N, 13.08.

6-(4-(Diphenylmethyl)piperazine-1-yl)-9-(2,3,5-tri-*O*-acetyl- β -D-ribofuranosyl)-9*H*-purine (8). The compound was prepared from (3) (312 mg, 0.7 mmol) and 1-(diphenylmethyl)piperazine (190.9 mg, 0.7 mmol) at reflux for 5 h according to the general procedure and was purified by column chromatography (EtOAc–hexane, 1:1) to yield 8 (166.8 mg; 35.1%); mp 81–83 °C. ¹H NMR (CDCl_3) δ 2.06 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.13 (s, 3H, OAc), 2.52 (t, 4H, piperazine CH_2), 4.19–4.44 (m, 8H, CH, H-4', H-5', piperazine CH_2), 5.63 (t, $J = 4.8$ Hz, 1H, H-3'), 5.87 (t, $J = 5.2$ Hz, 1H, H-2'), 6.20 (d, $J = 5.2$ Hz, 1H, H-1'), 7.19 (t, 2H, $J_0 = 7.6$ Hz, H-4 in phenyl), 7.29 (t, 4H, $J_0 = 7.6$ Hz, H-3,5 in phenyl), 7.44 (d, 4H, $J_0 = 7.2$ Hz, H-2,6 in phenyl), 7.84 (s, 1H, H-8), 8.30 (s, 1H, H-2). ¹³C NMR (CDCl_3) δ 20.65, 20.78, 21.02 ($3 \times \text{CH}_3$), 45.46 (CH_2 in piperazine), 52.26 (CH_2 in piperazine), 63.39 (CH), 70.93 ($\text{CH}_2\text{-S}'$), 73.30 ($\text{CH-3}'$), 76.35 ($\text{CH-2}'$), 80.34 ($\text{CH-4}'$), 85.96 ($\text{CH-1}'$), 120.52 (CH in phenyl), 127.31 (CH in phenyl), 128.19 (CH in phenyl), 128.78 (C in phenyl), 136.32 (C-5), 142.47 (C-8), 150.88 (C-6), 152.99 (C-2), 154.03 (C-4), 169.61, 169.85, 170.57 ($3 \times \text{CO}$). HRMS (ESI+) m/z calcd for $\text{C}_{33}\text{H}_{37}\text{N}_6\text{O}_7$ ($\text{M} + \text{H}$)⁺ 629.2724, found 629.2719. Anal. Calcd for $\text{C}_{33}\text{H}_{36}\text{N}_6\text{O}_7 \cdot 0.7\text{H}_2\text{O}$: C, 61.80; H, 5.87; N, 13.10. Found C, 61.57; H, 5.89; N, 13.07.

General Procedure for the Deacetylation of the Protected Nucleosides 9–13. The protected nucleosides (4–8) were dissolved in 10 mL of absolute MeOH, and then NaOMe (30% in MeOH) (2 equiv) was added and stirred at room temperature for 1–12 h. The reaction mixture was concentrated in vacuo. The residue was dissolved with CH_2Cl_2 :MeOH and purified by column chromatography.

6-(4-Phenylpiperazine-1-yl)-9-(β -D-ribofuranosyl)-9*H*-purine (9). The compound was prepared from (4) (209.1 mg, 0.388 mmol) at room temperature for 1 h according to general procedure and was purified by column chromatography (EtOAc–hexane, 3:1) to yield 9 (27 mg; 16.3%); mp 99–100 °C. ¹H NMR ($\text{DMSO-}d_6$) δ 3.26 (t, 4H, piperazine CH_2), 3.53–3.70 and 3.65–3.73 (2m, 2H, $\text{CH}_2\text{-S}'$), 3.96–4.16 (m, 2H, H-2',3'), 4.38 (br s, 4H, piperazine CH_2), 4.58 (q, 1H, H-4'), 5.21 (d, 1H, 3'-OH), 5.33 (t, 1H, 5'-OH), 5.48 (d, 1H, 2'-OH), 5.93 (d, 1H, 1'-H), 6.81 (t, 1H, $J = 7.2$ Hz, H-4 in phenyl), 7.0 (d, 2H, $J = 8.4$ Hz, H-2,6 in phenyl), 7.24 (t, 2H, $J = 8.4$ Hz, H-3,5 in phenyl), 8.28 (s, 1H, H-8), 8.45 (s, 1H, H-2). ¹³C NMR ($\text{DMSO-}d_6$) δ 49.15 (CH_2 in piperazine), 62.14 ($\text{CH}_2\text{-S}'$), 71.13 ($\text{CH-3}'$), 74.20 ($\text{CH-2}'$), 86.42 ($\text{CH-4}'$), 88.44 ($\text{CH-1}'$), 116.51, 119.97, 120.32, 129.65 (C in phenyl), 139.66 (C-5), 150.99 (C-8), 151.61 (C-6), 152.47 (C-2), 153.81 (C-4). HRMS (ESI+) m/z calcd for $\text{C}_{20}\text{H}_{25}\text{N}_6\text{O}_4$ ($\text{M} + \text{H}$)⁺ 413.1937, found 413.1938. Anal. Calcd for $\text{C}_{20}\text{H}_{24}\text{N}_6\text{O}_4 \cdot 1.2\text{H}_2\text{O}$: C, 55.34; H, 6.13; N, 19.36. Found C, 54.97; H, 6.07; N, 18.98.

6-[4-(4-Fluorophenyl)piperazine-1-yl]-9-(β -D-ribofuranosyl)-9*H*-purine (10). The compound was prepared from (5) (151 mg, 0.27 mmol) at room temperature for 5 h according to the general procedure and was purified by column chromatography (EtOAc–hexane, 4:1 and then EtOAc) to yield 10 (42.4 mg; 36.5%); mp 189–191 °C. ¹H NMR ($\text{DMSO-}d_6$) δ 3.21 (t, 4H, piperazine CH_2), 3.56 and 3.68 (2 $\times$ dd, 2H, $\text{CH}_2\text{-S}'$), 3.97 (q, 1H, H-4'), 4.16 (t, 1H, H-2'),

4.38 (br s, 4H, piperazine CH₂), 4.59 (t, 1H, H-3'), 5.23 (br s, 1H, 3'-OH), 5.48 (br s, 1H, 5'-OH), 5.94 (d, 1H, 1'-H), 7.01–7.10 (m, 4H, H-2,3,5,6 in phenyl), 8.28 (s, 1H, H-8), 8.45 (s, 1H, H-2). ¹³C NMR (DMSO-*d*₆) δ 45.16, 49.96 (CH₂ in piperazine), 62.15 (CH₂-5'), 71.14 (CH-3'), 74.22 (CH-2'), 86.42 (CH-4'), 88.45 (CH-1'), 115.91, 116.13, 118.37 (2), 120.33 (C in phenyl), 139.67 (C-5), 148.51 (C-8), 150.99 (C-6), 152.47 (C-2), 153.79 (C-4). HRMS (ESI+) *m/z* calcd for C₂₀H₂₄FN₆O₄ (M + H)⁺ 431.1843, found 431.1846. Anal. Calcd for C₂₀H₂₃FN₆O₄·0.5H₂O: C, 54.66; H, 5.50; N, 19.12. Found C, 54.62; H, 5.32; N, 19.25.

6-[4-(4-Trifluoromethylphenyl)piperazine-1-yl]-9-(β-D-ribofuranosyl)-9H-purine (11). The compound was prepared from (6) (106.4 mg, 0.17 mmol) at room temperature for 1 h according to the general procedure and was purified by column chromatography (EtOAc) to yield **11** (15 mg; 17.8%); mp 108–110 °C. ¹H NMR (DMSO-*d*₆) δ 3.45 (t, 4H, piperazine CH₂), 3.52–3.60 and 3.64–3.72 (2m, 2H, CH₂-5'), 3.96–4.17 (m, 2H, H-2', 3'), 4.38 (br s, 4H, piperazine CH₂), 4.59 (q, 1H, H-4'), 5.21 (d, 1H, 3'-OH), 5.33 (t, 1H, 5'-OH), 5.48 (d, 1H, 2'-OH), 5.93 (d, 1H, 1'-H), 7.14 (d, J_o = 8.8 Hz, 2H, H-2,6), 7.54 (d, J_o = 8.8 Hz, 2H, H-3,5), 8.29 (s, 1H, H-8), 8.46 (s, 1H, H-2). ¹³C NMR (DMSO-*d*₆) δ 44.87, 47.65 (CH₂ in piperazine), 62.15 (CH₂-5'), 71.14 (CH-3'), 74.24 (CH-2'), 86.42 (CH-4'), 88.46 (CH-1'), 115.06, 118.79 (q), 120.37, 124.29 (C in phenyl), 126.87 (q) (CF₃), 139.73 (C-5), 151.01 (C-8), 152.48 (C-6), 153.79 (C-2), 153.82 (C-4). HRMS (ESI+) *m/z* calcd for C₂₁H₂₄F₃N₆O₄ (M + H)⁺ 481.1811, found 481.1810. Anal. Calcd for C₂₁H₂₃F₃N₆O₄·0.6H₂O: C, 51.34; H, 4.96; N, 17.10. Found C, 51.65; H, 4.74; N, 16.73.

6-[4-(3,4-Dichlorophenyl)piperazine-1-yl]-9-(β-D-ribofuranosyl)-9H-purine (12). The compound was prepared from (7) (166.2 mg, 0.27 mmol) at room temperature for 12 h according to the general procedure and was purified by column chromatography (EtOAc–hexane, 4:1 and then EtOAc) to yield **12** (90 mg; 68.7%); mp 211 °C. ¹H NMR (DMSO-*d*₆) δ 3.34 (t, 4H, piperazine CH₂), 3.51–3.60 and 3.65–3.73 (2m, 2H, CH₂-5'), 3.92–4.63 (m, 7H, H-2',3',4', piperazine CH₂), 5.21 (d, 1H, 3'-OH), 5.33 (t, 1H, 5'-OH), 5.47 (d, 1H, 2'-OH), 5.94 (d, J = 5.6 Hz, 1H, 1'-H), 7.00 (d, J_o = 7.2 Hz, 1H, H-6 in phenyl), 7.21 (s, 1H, H-2 in phenyl), 7.29 (d, J = 9.2 Hz, 1H, H-5 in phenyl), 8.29 (s, 1H, H-8), 8.45 (s, 1H, H-2). ¹³C NMR (DMSO-*d*₆) δ 29.69 (CH₂ in piperazine), 48.30 (CH₂ in piperazine), 62.18 (CH₂-5'), 71.16 (CH-3'), 74.25 (CH-2'), 86.44 (CH-4'), 88.47 (CH-1'), 116.26 (CH in phenyl), 117.25 (CH in phenyl), 120.37 (CH in phenyl), 120.60 (C in phenyl), 131.21 (C in phenyl), 132.24 (C in phenyl), 139.76 (C-5), 151.03 (C-8), 151.31 (C-6), 152.50 (C-2), 153.81 (C-4). HRMS (ESI+) *m/z* calcd for C₂₀H₂₃Cl₂N₆O₄ (M)⁺ 481.1158, found 481.1156. Anal. Calcd for C₂₀H₂₂Cl₂N₆O₄·0.2C₆H₁₄: C, 51.07; H, 5.01; N, 16.86. Found C, 51.46; H, 4.88; N, 16.56.

6-(4-(Diphenylmethyl)piperazine-1-yl)-9-(β-D-ribofuranosyl)-9H-purine (13). The compound was prepared from (8) (166.8 mg, 0.26 mmol) at room temperature for 8 h according to the general procedure and was purified by column chromatography (EtOAc and then EtOAc–MeOH, 6:1) to yield **13** (60.5 mg; 45.5%); mp 117–119 °C. ¹H NMR (DMSO-*d*₆) δ 2.43 (br s, 4H, piperazine CH₂), 3.50–3.58 ve 3.62–3.69 (2m, 2H, CH₂-5'), 3.92–4.38 (m, 7H, piperazine CH₂, CH, H-2', 3'), 4.55 (q, 1H, H-4'), 5.19 (d, J = 4.4 Hz, 1H, 3'-OH), 5.31 (t, J = 5.2 Hz, 1H, 5'-OH), 5.46 (d, J = 6 Hz, 1H, 2'-OH), 5.90 (d, J = 5.6 Hz, 1H, H-1'), 7.21 (t, J_o = 7.2 Hz, 2H, H-4 in phenyl), 7.32 (t, J_o = 7.2 Hz, 4H, H-3,5 in phenyl), 7.45 (d, J_o = 8 Hz, 4H, H-2,6 in phenyl), 8.22 (s, 1H, H-8), 8.39 (s, 1H, H-2). ¹³C NMR (DMSO-*d*₆) δ 52.18 (CH₂ in piperazine), 62.14 (CH), 71.13 (CH₂-5'), 74.20 (CH-3'), 75.48 (CH-2'), 86.39 (CH-4'), 88.42 (CH-1'), 120.27 (CH in phenyl), 127.65 (CH in phenyl), 128.35 (CH in phenyl), 129.26 (C in phenyl), 139.54 (C-5), 143.12 (C-8), 150.93 (C-6), 152.41 (C-2), 153.82 (C-4). HRMS (ESI+) *m/z* calcd for C₂₇H₃₁N₆O₄ (M + H)⁺ 503.2407, found 503.2406. Anal. Calcd for C₂₇H₃₀N₆O₄·0.2EtOAc·1.0H₂O: C, 62.03; H, 6.29; N, 15.61. Found C, 61.72; H, 6.05; N, 15.21.

Cells and Culture. The human primary liver cancer cell lines (Huh7, HepG2, Mahlavu, and FOCUS) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen GIBCO), with 10% fetal bovine serum (FBS) (Invitrogen GIBCO), nonessential amino

acids, and 1% penicillin (Biochrome). It was incubated at 37 °C with 5% CO₂. DMSO (Sigma) was used as a solvent for the compounds. The concentration of DMSO was always less than 1% in the cell culture medium. The cytotoxic drugs (Camptothecin, SFU, doxorubicin, and MeSAO) used as positive controls were from Calbiochem.

Sulforhodamine B (SRB) Assay for Cytotoxicity Screening. Huh7, HCT116, MCF7, HepG2, Mahlavu, and FOCUS cells were inoculated (2000–10000 cells/well in 200 μL) in 96-well plates. The next day, the media were refreshed and the compounds dissolved in DMSO were applied in concentrations between 1 and 40 μM in parallel with DMSO-only treated cells as negative controls. At the 72nd hour of treatment with compounds **9**–**13** and the other drugs, the cancer cells were fixed with 100 μL of 10% (w/v) trichloroacetic acid (TCA) and kept at +4 °C in the dark for one hour. TCA fixation was terminated by washing the wells with ddH₂O five times. Air-dried plates were stained with 0.4% sulphorhodamine B (SRB) dissolved in 1% acetic acid solution for 10 min in the dark and at room temperature. The protein-bound and dried SRB dye was then solubilized with 10 mM Tris-Base pH 8. The absorbance values were obtained at 515 nm in a microplate reader. The data normalized against DMSO only treated wells, which were used as controls in serial dilutions. In all experiments, a linear response was observed, with serial dilutions of the compounds and the drugs; R₂ ≥ 0.9.

Real-Time Cell Electronic Sensing (RT-CES) for Cytotoxicity Profiling. The proliferation of primary liver cancer cell lines was monitored in real-time cell electronic sensing (RT-CES) (xCELLigence-Roche Applied Science), and the cell index (CI) was measured every 30 min for 96 h. Huh7, HepG2, Mahlavu, and FOCUS cells were inoculated (2000 cells/well in 200 μL) in the 96 well E-plate on the xCELLigence station in 5% CO₂ at 37 °C. The CI values were recorded every 30 min. The next day, 150 μL of medium from each well was replaced with 100 μL of fresh medium containing compound **11** applied in the indicated concentrations. The CI values were recorded every 30 min to monitor real-time drug response. DMSO-only and medium-only wells were also included in the monitoring to account for their possible solitary effects on cancer cells.

Kinase Assay. Kinase-Glo-Plus luminescence kinase activity assay (Promega) was performed according to the manufacturer's protocol. First, the Kinase-Glo reaction buffer (40 mM Tris-HCl pH7.6, 20 mM MgCl₂, 0.1 mg/mL BSA) was placed in the wells of a 96-well Elisa plate. Then, Huh7 cells treated with **11**, Staurosporin (STS), and MeSAO for 72 h. Next, lysates from 80000 cells were placed into the wells. DMSO used as negative control. The total volume of the lysates and the kinase reaction buffer was 50 μL. Then, 50 μL of Kinase-Glo reagent (Kinase-Glo plus substrate + Kinase-Glo-Plus buffer) was applied. After 10 min of dark incubation at room temperature, the luminescence was detected with a luminometer. If kinase activity is diminished, ATP concentration will increase and so will luminescence.

Senescence Associated-β-gal Assay and BrdU Proliferation Co-staining. Huh7 cells (5000cell) were inoculated in two identical six-well plates on coverslips. The next day, compound **11** and doxorubicin at the indicated concentrations, and their corresponding DMSO controls were applied to the plates. On day three and the day six, the senescence-associated-β-gal (SAβgal) assay and BrdU costaining were performed as described previously.^{23,24}

Western Blot Analysis. Proteins from Huh7 cells treated with compound **11** and doxorubicin for three and six days were separated on a 10% SDS-PAGE, transferred onto nitrocellulose membranes, and visualized, as described previously.¹⁹ The total homogenates from the cells were resuspended in 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS with a protease-inhibitor cocktail and phosphatase inhibitors. p15, Rb and pRb were detected by Western blotting. Actin protein was used for equal loading control.

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Karran, P. Thiopurines, DNA damage, DNA repair and therapy-related cancer. *Br. Med. Bull.* **2006**, 79–80, 153–170.
- (2) Bosch, L.; Harbers, E.; Heidelberger, C. Studies on fluorinated pyrimidines. V. Effects on nucleic acid metabolism in vitro. *Cancer Res.* **1958**, 18, 335–343.
- (3) Sampath, D.; Rao, V. A.; Plunkett, W. Mechanisms of apoptosis induction by nucleoside analogs. *Oncogene* **2003**, 22, 9063–9074.
- (4) Escherich, G.; Richards, S.; Stork, L. C.; Vora, A. J. Childhood Acute Lymphoblastic Leukaemia Collaborative Group (CALLCG). Meta-analysis of randomised trials comparing thiopurines in childhood acute lymphoblastic leukaemia. *Leukemia* **2011**, 25, 953–959.
- (5) Lech-Maranda, E.; Korycka, A.; Robak, T. Meta-analysis of randomised trials comparing thiopurines in childhood acute lymphoblastic leukaemia. *Mini-Rev. Med. Chem.* **2006**, 6, 575–581.
- (6) Wilson, P. K.; Mulligan, S. P.; Christopherson, R. I. Metabolic response patterns of nucleotides in B-cell chronic lymphocytic leukaemias to cladribine, fludarabine and deoxycytidine. *Leukemia Res.* **2004**, 28, 725–731.
- (7) Nakamura, J.; Kohya, N.; Kai, K.; Ohtaka, K.; Hashiguchi, K.; Hiraki, M.; Kitajima, Y.; Tokunaga, O.; Noshiro, H.; Miyazaki, K. Ribonucleotide reductase subunit M1 assessed by quantitative double-fluorescence immunohistochemistry predicts the efficacy of gemcitabine in biliary tract carcinoma. *Int. J. Oncol.* **2010**, 37, 845–852.
- (8) Simon, G. R.; Schell, M. J.; Begum, M.; Kim, J.; Chiappori, A.; Haura, E.; Antonia, S.; Bepko, G. Preliminary indication of survival benefit from ERCC1 and RRM1-tailored chemotherapy in patients with advanced nonsmall cell lung cancer: Evidence from an individual patient analysis. *Cancer* **2011**, Oct 25, Epub (DOI: 10.1002/cncr.26522).
- (9) Dick, J.; Wright, J. On the importance of deoxyribonucleotide pools in the senescence of cultured human diploid fibroblasts. *FEBS Lett.* **1985**, 179, 21–24.
- (10) Ricci, M. S.; Zong, W.-X. Chemotherapeutic approaches for targeting cell death pathways. *Oncologist* **2006**, 11, 342–357.
- (11) Harley, C. B.; Futcher, A. B.; Greider, C. W. Telomeres shorten during ageing of human fibroblasts. *Nature* **1990**, 345, 458–460.
- (12) Collado, M.; Serrano, M. The power and the promise of oncogene-induced senescence markers. *Nature Rev. Cancer* **2006**, 6, 472–476.
- (13) Serrano, M.; Lin, A. W.; McCurrach, M. E.; Beach, D.; Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **1997**, 88, 593–602.
- (14) Nardella, C.; Clohessy, J. G.; Alimonti, A.; Pandolfi, P. P. Pro-senescence therapy for cancer treatment. *Nature Rev. Cancer* **2011**, 11, 503–511.
- (15) Kang, T.-W.; Yevsa, T.; Woller, N.; Hoenicke, L.; Wuestefeld, T.; Dauch, D.; Hohmeyer, A.; Gereke, M.; Rudalska, R.; Potapova, A.; Iken, M.; Vucur, M.; Weiss, S.; Heikenwalder, M.; Khan, S.; Gil, J.; Bruder, D.; Manns, M.; Schirmacher, P.; Tacke, F.; Ott, M.; Luedde, T.; Longerich, T.; Kubicka, S.; Zender, L. Pro-senescence therapy for cancer treatment. *Nature* **2011**, 479, 547–551.
- (16) Irmak, M. B.; Ince, G.; Ozturk, M.; Cetin-Atalay, R. Acquired tolerance of hepatocellular carcinoma cells to selenium deficiency: a selective survival mechanism? *Cancer Res.* **2003**, 63, 6707–6715.
- (17) Finn, R. S. Sorafenib use while waiting for liver transplant: We still need to wait. *J. Hepatol.* **2012**, 56, 723–725.
- (18) Andrzejewska, M.; Kamiński, J.; Kazimierzczuk, Z. Microwave induced synthesis of ribonucleosides on solid support. *Nucleosides, Nucleotides, Nucleic Acids* **2002**, 21, 73–78.
- (19) Buontempo, F.; Ersahin, T.; Missiroli, S.; Senturk, S.; Etro, D.; Ozturk, M.; Capitani, S.; Cetin-Atalay, R.; Neri, M. L. Inhibition of Akt signaling in hepatoma cells induces apoptotic cell death independent of Akt activation status. *Invest. New Drugs* **2011**, 29, 1303–1313.
- (20) Gobeil, S.; Boucher, C. C.; Nadeau, D.; Poirier, G. G. Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases. *Cell Death Differ.* **2001**, 8, 588–594.
- (21) Shay, J. W.; Roninson, I. B. Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene* **2004**, 23, 2919–2933.
- (22) Hanahan, D.; Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **2011**, 144, 646–674.
- (23) Dimri, G. P.; Lee, X.; Basile, G.; Acosta, M.; Scott, G.; Roskelley, C.; Medrano, E. E.; Linskens, M.; Rubelj, I.; Pereira-Smith, O. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, 92, 9363–9367.
- (24) Ozturk, N.; Erdal, E.; Mumcuoglu, M.; Akcali, K. C.; Yalcin, O.; Senturk, S.; Arslan-Ergul, A.; Gur, B.; Yulug, I.; Cetin-Atalay, R.; Yakicier, C.; Yagci, T.; Tez, M.; Ozturk, M. Reprogramming of replicative senescence in hepatocellular carcinoma-derived cells. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, 103, 2178–2183.