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# THERAPEUTIC DRUGS IN CANCER AND RESISTANCE

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy at Virginia Commonwealth University.

by

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### List of abbreviations

4-OH TAM	4-hydroxy tamoxifen
ABCG-2	ATP-binding cassette, sub-family G, member 2
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor 1
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
BAD	BCL2-associated agonist of cell death
BAK	Bcl-2 antagonist/killer
BAX	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xl	Basal cell lymphoma extra-large
BH domain	Bcl-2 homology domain
BID	BH3 interacting domain death agonist
CD95L	CD95 ligand
c-FLIP-l	cellular FLICE-like inhibitory protein long
c-FLIP-s	cellular FLICE-like inhibitory protein short

CI	Combination index in colony formation assay
C <sub>max</sub>	Maximum concentration
CMV	Cytomegalo virus vector/control virus
c-KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
DAPI	4',6-diamidino-2-phenylindole
DcR3	Decoy receptor 3
DISC	Death inducing signaling complex
DMSO	Dimethy sulfoxide
DNA	Deoxyribonucleic acid
dnIκB	Dominant negative IκB
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ER	Estrogen receptor
ERBB	Erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian
ERK	Extracellular signal-regulated kinase
Fa	Fraction affected in colony formation assay
FADD	Fas associated death domain
FasL	Fas ligand

FITC	Fluorescein isothiocyanate
FLIP	Cellular FLICE-like inhibitory protein
FLT3	FMS-like tyrosine kinase 3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Grb2	Growth factor receptor-bound protein 2
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
Gy	Gray
HAT	Histone acetyltransferase
HCl	Hydrogen chloride
HDAC	Histone deacetylase
HDACI	Histone deacetylase inhibitor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRas	Harvey Ras
HSP90	Heat shock protein 90
IAP	Inhibitor of apoptosis
IC <sub>50</sub>	Half maximal inhibitory concentration
ICAD	Inhibitor of caspase activated DNase
IGF-1R	Insulin-like growth factor -1 receptor
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells

	Inhibitor
IKK	I $\kappa$ B kinase
IP	Immunoprecipitation
JNK	c-jun N-terminal kinase
KRas	Kirsten Ras
Lap	Lapatinib
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia sequence-1
MDM2	Murine double minute 2
MDR	Multi-drug resistance
MEK	Mitogen-activated /extracellular-regulated protein kinase
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic acid
MRP-1	Multi-drug resistance protein-1
NaCl	Sodium chloride
NF $\kappa$ B	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NRas	Neuroblastoma Ras
P-Akt	Phosphorylated Akt
PBS	Phosphate buffered saline
PDGFR	Platelet derived growth factor receptor

PDK1	Phosphoinositol-dependent kinase-1
P-ERK	Phosphorylated ERK
Pgp	P-glycoprotein
PH domain	Pleckstrin homology domain
PI	Propidium iodide
PI3K	Phosphoinositide 3 kinase
PIP2	Phosphatidylinositol bisphosphate
PIP3	Phosphatidylinositol trisphosphate
PMSF	Phenylmethanesulphonylfluoride
PP2	Protein phosphatase 2
P-p53	Phosphorylated p53
PPP	Picropodophyllin
pRb	Phosphorylated retinoblastoma protein
PTEN	Phosphatase and tensin homolog
P-Tyr	Phosphorylated tyrosine
ROS	Reactive oxygen species
RPM	Rotations per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S.E.M.	Standard error of mean



SERM	Selective estrogen receptor modulator
SH3 domain	Src homoly 3 domain
SHC	Src homology 2 containing domain
siAIF	siRNA targeted for knock-down of AIF
siBcl-2	siRNA targeted for knock-down of Bcl-2
siBcl-xl	siRNA targeted for knock-down of Bcl-xl
siCD95	siRNA targeted for knock-down of CD95
siMcl-1	siRNA targeted for knock-down of Mcl-1
siRNA	Small-interfering RNA
siSCR	control siRNA ("scrambled")
SOS	Son of sevenless
Src	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
STAT	signal transducer and activator of transcription
STAT3 IP	STAT inhibitory peptide
t-BID	truncated BID
TGF- $\alpha$	Transforming growth factor- $\alpha$
TKI	Tyrosine kinase inhibitor
VEGFR	Vascular endothelial growth factor receptor
VEH	Vehicle
WT	Wild-type HCT 116 colon cancer cells

WT-AD	Lapatinib resistant HCT 116 colon cancer cells
WT-AD10	WT-AD cells cultured in absence of lapatinib for >10 flask passages

## **Abstract**

### **THERAPEUTIC DRUGS IN CANCER AND RESISTANCE**

By Aditi P. Martin, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: Paul Dent  
Professor, Department of Biochemistry and Molecular Biology

We investigated the mechanism of toxicity and resistance development of small molecule tyrosine kinase inhibitor lapatinib in HCT 116 colon cancer cells. Lapatinib mediated cell death in HCT 116 cells was caspase independent and involved cytosolic release of apoptosis inducing factor. Treatment of HCT 116 cells with 10 $\mu$ M Lapatinib lead to the outgrowth of lapatinib resistant HCT 116 cells. Our studies show that alterations in the expression and activation of Bcl-2 family proteins allow lapatinib resistant HCT 116 cells to resist cytotoxic effects of lapatinib as well as of other commonly used chemotherapeutic agents.

In hepatoma and pancreatic cancer cells, the effects of combining multi-kinase inhibitor sorafenib with histone deacetylase inhibitors (HDACIs) namely, vorinostat and sodium

valproate were investigated. It was found that sorafenib synergizes with HDACIs resulting in enhanced cell death compared to death induced by the drugs individually. The mechanism of action of sorafenib and vorinostat combination treatment as well as sorafenib and sodium valproate combined treatment was shown to involve activation of the CD95 death receptor pathway. Alterations in the CD95 pathway can render cancer cells resistant to chemotherapeutic agents. Hence, we combined sorafenib+sodium valproate with a BH-3 domain mimetic named obatoclax (GX-15-070) which resulted in enhanced toxicity to cancer cells. More importantly, knock-down of CD95 (to mimic non-functional CD95 pathway) reduced cell death induced by sorafenib+sodium valproate combined but failed to protect cells from cell death induced by sorafenib+sodium valproate+obatoclax combined. This suggests that combining sorafenib+HDACI with obatoclax may not only enhance toxicity to cancer cells but may also reduce chances of resistance development via alterations in the CD95 pathway. These studies enhance our knowledge of existing treatment strategies for cancer as well as throw light on how current approaches can be improved in order to better diagnose and treat cancer. Understanding mechanisms of drug action as well as resistance development will allow us to combine existing therapies effectively in order best target cancer cells as well as provide us with information that can help us design new cancer treatment strategies.

## GENERAL INTRODUCTION

### A. Hallmarks of cancer:

Cancer is often defined as a disease involving uncontrolled division of abnormal cells.

Cancer is one of the leading causes of death, with one in eight deaths worldwide resulting due to cancer ([www.cancer.org](http://www.cancer.org)(1)). In the United States, cancer is the second leading cause of death and it is estimated that in 2009, 1500 people will die everyday as a result of cancer ([www.cancer.org](http://www.cancer.org)(2)).

It is thought that a normal cell undergoes a series of changes in its genome that can eventually lead to the generation of an aberrant cancer cell. Hanahan and Weinberg identified six hallmarks of a cancer cell that are shared by most cancers (Hanahan D and Weinberg RA, 2000).

- 1) No requirement for external growth signals
- 2) Insensitivity to anti-growth signals
- 3) Evasion of cell death signaling
- 4) Infinite potential to replicate
- 5) Ability to maintain oxygen and nutrient supply via angiogenesis
- 6) Ability to invade tissue and metastasize

There are a variety of growth factor receptors such as epidermal growth factor receptors (EGFR), platelet-derived growth factor receptor (PDGFR), insulin-like growth factor-1 receptor (IGF-1R) and vascular endothelial growth factor receptor (VEGFR). Such

receptors and their ligands as well as a variety of transcription factors and cell death regulators are called oncogenes as mutant forms of these proteins or increased expression of these proteins can lead to transformation of a normal cell into a cancerous cell (Croce CM, 2008).

Normal healthy cells have growth factor receptors that are activated upon binding stimulatory growth factors/ligands. Active receptors can mediate downstream signaling that can stimulate cell growth and proliferation. There are no known normal cells that can proliferate in the absence of such stimulatory signals (Hanahan D and Weinberg RA, 2000). However, cancer cells have been known to be able to proliferate independent of such stimulatory signals. For instance, HCT 116 colon cancer cells are highly malignant and are growth factor independent. HCT 116 cells have one mutant allele of KRas with a Gly13Asp mutation. This mutation in KRas has been shown to be linked to up-regulation of epiregulin (a pan-ERBB ligand) since loss of mutant KRas leads to reduction in levels of epiregulin in HCT 116 cells (Yacoub A, *et. al.* 2006). This mutant KRas mediated up-regulation of epiregulin has been shown to be involved in promoting the tumorigenic potential of HCT 116 cells (Baba I, *et. al.* 2000). Other studies have shown that upon nutrient deprivation induced growth arrest, growth factor-dependent cells require both nutrients and growth factors to recover from growth arrest, whereas HCT 116 cells require only nutrients to re-start DNA synthesis. This is because HCT 116 cells up-regulate transforming growth factor- $\alpha$  (TGF- $\alpha$ ) via enhanced transcription which can activate EGFR and downstream pro-survival signaling even under growth arrest conditions. Hence, HCT 116 cells have a TGF- $\alpha$  autocrine loop that can allow constitutive EGFR activation

even under nutrient-deprivation and this allows them to be self sufficient in growth signals (Awwad RA, *et. al.* 2003). Other cancer cells have also been shown to have increased growth factor/ligand production due to altered gene expression resulting in an autocrine loop and hence aberrant activation of growth factor receptors and downstream signaling (Sizeland AM and Burgess AW. 1992).

Growth factor receptors belonging to the EGFR family are tyrosine kinases and play an important role in cancer progression. They have been known to be over-expressed or mutated such that they are constitutively active in a variety of cancers and this can lead to increased activation of downstream pro-survival signaling resulting in aggressive cancer (Peschard, P. and Park, M. 2003; Sunpaweravong, P. *et. al.* 2005; Hynes NE and Lane HA. 2005). These downstream pro-survival signaling pathways include the mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol-3 kinase pathway (PI3K) (Grant S, *et. al.* 2002).

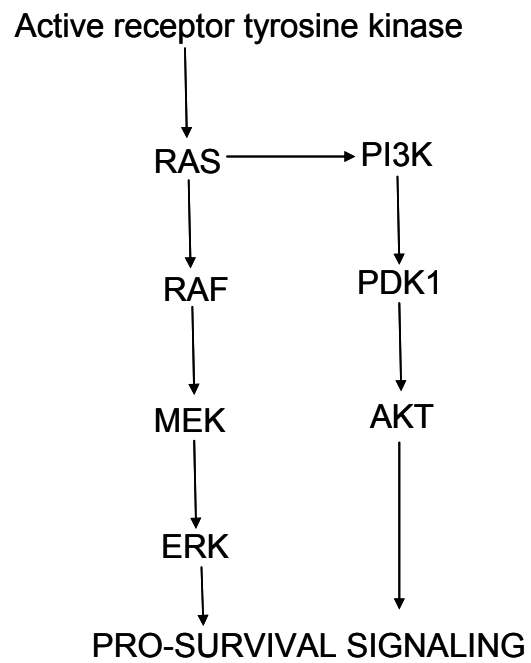
### **B. The MAPK pathway:**

In order for MAPK signaling to occur downstream of EGFR activation, phosphorylated tyrosine residues on active EGFR are recognised by Src homology 2 domain containing scaffolding protein (SHC) which can hold growth factor receptor bound protein 2 (Grb2) in association with a guanine nucleotide exchange factor called son of sevenless (SOS) via its Src homology 3 (SH3) domain. SOS assists activation of Ras protein (that is tethered to the cell membrane via farnesylation or geranylgeranylation) by allowing Ras to release guanosine diphosphate (GDP) in exchange for guanosine triphosphate (GTP) (Seabra MC. 1998). There are three types of Ras proteins known to date – Harvey Ras (HRas), Kirsten

Ras (KRas), Neuroblastoma Ras (NRas) with KRas being the most frequently mutated Ras isoform in cancer (Downward J. 2003). Active Ras is thought to recruit Raf protein, a serine/threonine kinase also called a MAPkinase kinase kinase, to the cell surface. There are three types of Raf proteins known to exist namely, c-Raf-1, B-Raf and A-Raf with B-Raf being the most commonly mutated form (Sridhar SS, *et. al.* 2005; Davies H, *et. al.* 2002). Raf proteins exist in an inactive state, phosphorylated on certain residues and also bound to 14-3-3 protein (Sridhar SS, *et. al.* 2005). The mechanism involving Raf activation is not completely understood but it is thought that Ras-GTP is responsible for bringing Raf protein to the cell surface and interacting with it to alter its conformation assisting in Raf activation (Chong H, *et. al.* 2003). Some studies show that phosphatase enzymes such as protein phosphatase 2 (PP2) are known to dephosphorylate Raf on certain residues and allow it to be further phosphorylated on different residues by a Src family kinase rendering Raf in an active state (Jaumot M and Hancock JF. 2001; Alavi A, *et. al.* 2003). Active Raf can phosphorylate and activate mitogen-activated /extracellular-regulated protein kinase (MEK), also called MAPkinase kinase, which is a dual specificity kinase that can phosphorylate serine/threonine or tyrosine residues (Kyriakis JM, *et. al.* 1992; Dent P, *et. al.* 1992). Active MEK1/2 can activate MAPK called extracellular signal regulated kinase 1/2 (ERK1/2) which is a serine/threonine kinase (Wu J, *et. al.* 1992). ERK1/2 can activate various proteins such as Fos, Myc and Cyclin D-1 that can result in cells entering the cell cycle (Birtwistle MR, *et. al.* 2007). ERK can phosphorylate and activate p90 ribosomal S6 kinase or p90rsk which can phosphorylate transcription factors involved in cell proliferation (Amorino GP, *et. al.* 2002). ERK is also known to translocate into the nucleus



where it can phosphorylate and activate a variety of other proteins such as Elk-1, STAT proteins and the estrogen receptor (Whitmarsh AJ and Davis RJ. 1996; Adachi M, *et. al.* 2000). ERK can phosphorylate and inhibit activation of caspase 9 and pro-apoptotic protein BAD (Allan LA, *et. al.* 2003; Scheid MP, *et. al.* 1999). The MAPK pathway can be aberrantly activated in cancer due to alterations in the upstream receptors (Nair PN, *et. al.* 2001) or due to mutations in upstream Ras or Raf proteins rendering them constitutively active (Bakin RE, *et. al.* 2003; Pfister S, *et. al.* 2008).



**Figure 1: The mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 kinase (PI3K) pathway**

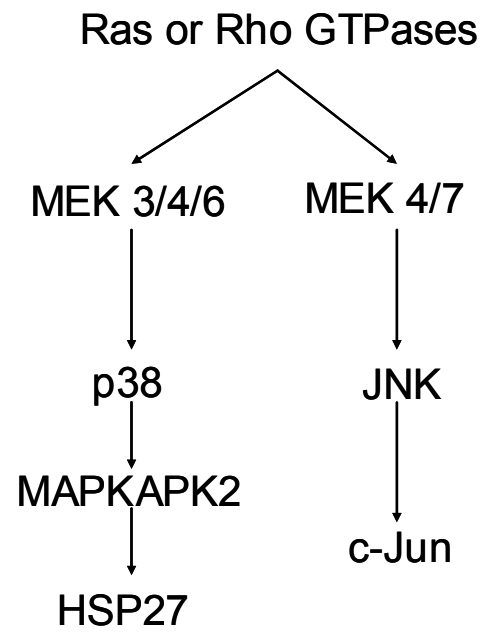
### **C. The p38 and JNK pathways:**

There are other MAP kinases such as p38 and c-Jun N-terminal kinase (JNK) (Davis RJ. 2000). Four isoforms of p38 namely,  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  and three JNK proteins namely JNK 1, 2 and 3 are known to date (Alonso G, *et. al.* 2000; Waetzig V and Herdegen T. 2005). Upon growth factor receptor activation, exposure to UV radiation, cytokines or mitogens, Ras and Rho family GTPases can lead to p38 and or JNK activation downstream (Johnson GL and Nakamura K. 2007). MEK 3 and MEK 6 can activate p38, MEK4 has been known to activate p38 and JNK where as MEK 7 solely activates JNK (Johnson GL and Nakamura K. 2007). Depending on cell type and stimulus, p38 and JNK signaling can promote cell growth and survival or it can enhance cell death signaling (Lamb JA, *et. al.* 2003; Thorton TM and Rincon M. 2009). For instance, depending on cell type and stimulus, p38 activation can influence either the progression of cell cycle transition by phosphorylation of the retinoblastoma protein (pRb) or can lead to phosphorylation of the tumor suppressor p53 (which is often called “guardian of the genome” and is discussed in detail later) which can result in cell cycle arrest (Hoozemans JJ, *et. al.* 2004; Bulavin DV, *et. al.* 1999). p38 activation has also shown to lead to mitogen-activated protein kinase-activated protein kinase 2 activation which can in turn activate HSP27. This pathway has been shown to be involved in cellular invasion in prostate cancer (Xu L, *et. al.* 2006).

It is thought that transient activation of JNK can mediate cell survival but prolonged activation of JNK can lead to cell death (Chen YR, *et. al.* 1996). Also, studies from our laboratory have shown that JNK isoforms may have variable functions. Treatment of hepatocytes with deoxycholic acid lead to activation of JNK1/2 but it was found that under

this treatment condition, JNK1 signaling was cytotoxic where as JNK2 signaling was cytoprotective (Qiao L, *et. al.* 2003).

Activated JNK can translocate to the mitochondria where it has been known to be involved in the release of cytochrome c into the cytosol which is associated with activation of cell death pathways (Tournier C, *et. al.* 2000). Upon activation, JNK can also translocate to the nucleus and transactivate a variety of proteins including c-Jun, other transcription factors and can also promote apoptosis by increase the expression of pro-apoptotic genes (Dhanasekaran DN and Reddy EP). Reports show that in unstressed cells, JNK can lead to p53 ubiquitination and degradation (Fuchs SY, *et. al.* 1998). However, under certain stressful situations (such as upon DNA damage), JNK can phosphorylate and stabilize p53, preventing its degradation, and allowing p53 to initiate cell cycle arrest or cell death pathways (BuschmannT, *et. al.* 2001). Active JNK can phosphorylate pro-apoptotic protein BAD and allow this protein to engage in apoptotic signaling (Donovan N, *et. al.* 2002). The role of JNK in cell death pathways is consistent with JNK having a tumor suppressive role (Kennedy NJ and Davis RJ. 2003). However, other studies show that JNK has a preferential role in proliferation since JNK deficient mice were resistant to the induction of papillomas (Chen N., *et. al.* 2001). Secondly, it has been shown that mice harboring prostate cancer cells when treated with antisense JNK1 and JNK2 inhibited tumor growth and lead to significant tumor regression (Yang YM, *et. al.* 2003). Other reports show that JNK signaling is involved in the malignant transformation of osteoblasts and in the progression of osteosarcomas (Papachristou DJ, *et. al.* 2003). Hence, JNK is often thought to have a pro-oncogenic role.



**Figure 2: p38 and JNK signaling pathways.**

#### **D. The PI3K pathway:**

The PI3K pathway can also mediate pro-survival signaling. PI3K is a dimer consisting of a p85 regulatory subunit and a p110 catalytic subunit (Cantley LC. 2002). Activation of receptor tyrosine kinases can recruit PI3K directly or via adaptor molecules (SHC-GRb2-SOS complex) (Cantley LC. 2002). PI3K can then phosphorylate phosphatidylinositol bisphosphate (PIP2) to generate phosphatidylinositol trisphosphate (PIP3). PIP3 in turn can recruit signaling proteins with pleckstrin homology domain (PH domain) such as serine threonine kinases phosphoinositol-dependent kinase-1 (PDK-1) and Akt (also called protein kinase B) to the membrane (Luo J, *et.al.* 2003). This brings Akt and PDK1 in close proximity allowing them to interact resulting in PDK1 mediated Akt phosphorylation and activation (Lawlor MA and Alessi DR. 2001). Active Akt can then regulate a variety of proteins that control cell proliferation, survival, growth and other processes. For instance, Akt can inhibit the function of death-inducing proteins such as BAD and p53 and it can regulate cell cycle by stabilizing cyclin-D (Luo J, *et.al.* 2003). There are three known mammalian isoforms of Akt namely, Akt 1, 2 and 3 that are generated from distinct genes (Franke TF. 2008). Reports suggest that these isoforms of Akt have diverse functions (Bae SS, *et. al.* 2003; Jiang ZY, *et. al.* 2003). PTEN, a tumor suppressor and a negative regulator of the PI3K-Akt pathway can dephosphorylate PIP3 and convert it back to PIP2 (Cantley LC and Neel BG. 1999). Certain cancers have been shown to have mutant PTEN such that it can no longer inhibit the PI3K pathway and thereby allow constant activation of this pro-survival pathway (Maehama T and Dixon JE. 1999). Other oncogenic changes that can lead to hyperactivity of the PI3K-Akt pathway are alterations in growth factor

receptors and Ras which are both upstream of Akt. Hyperactivity of the Akt pathway is regarded as a negative prognostic marker for disease outcome in cancer patients (LoPiccolo J, *et. al.* 2008). A mutation in the PH domain of Akt1 has also been associated with increased plasma membrane recruitment and activation of the kinase and its downstream pathways (Bleeker FE, *et. al.* 2008). Hence, the PI3K/Akt pathway has been a prime target in developing therapies to treat cancer.

#### **E. The tumor suppressor p53:**

As mentioned previously, p53 is often referred to as the “guardian of the genome”. Murine double minute 2 or MDM2 (HDM2 in humans) protein maintains p53 at low levels in normal, unstressed cells by ubiquitinating p53 and targeting it for degradation by the proteasome (Kubbuttat MH, *et. al.* 1997). Post translational modifications of p53 such as acetylation, phosphorylation and methylation also play a role in stabilizing and activating p53 under stressful situations (Moll UM and Petrenko O. 2003). Several stimuli such as hypoxia and DNA damage can activate enzymes such as ATM and ATR that can phosphorylate p53 thereby increasing its stability and transcriptional ability (Graeber TG, *et. al.* 1994; Siliciano JD, *et. al.* 1997). These enzymes can also rapidly increase p53 levels via phosphorylation of MDM2 that prevents it from interacting with p53 (Maya R, *et. al.* 2001; Shinozaki T, *et. al.* 2003). p53 activation can in turn lead to increase in ARF protein levels which can interact with MDM2 and inhibit its p53-ubiquitin ligase activity there by allowing p53 to stabilize (Palmero I, *et. al.* 1998). On the other hand, p53 activation can increase transcription of the gene encoding for MDM2 which will eventually lead to inhibition of p53 forming a negative feedback loop (Wagner J, *et. al.* 2005). In several

cancers with wild type p53, the MDM2 gene has been shown to be amplified (Oliner JD, *et. al.* 1992). Hence, in spite of bearing wild-type p53, the amplified MDM2 expression may prevent appropriate activation of p53 upon toxic insult to these cells thereby allowing them to evade cell death. In certain cancers, mutations or epigenetic alterations in the gene encoding for ARF have been noted which could also result in lack of appropriate p53 response to prevent unwarranted cell growth and proliferation (Saporita AJ, *et. al.* 2007). Above mentioned alterations in MDM2 and ARF function can prevent appropriate p53 activation there by assisting cancer cells in evading cell death pathways.

Failure to arrest cell cycle progression can allow a cell with damaged DNA to replicate. p53 is known to regulate cell cycle check points in order to maintain the genetic stability of cells. Active p53 is known to mediate cell cycle arrest by inducing the transcription of p21 which is a cyclin dependent kinase inhibitor (Miyashita T and Reed JC. 1995). Once active, p53 is also known to lead to the transcription of a variety of genes that encode proteins involved in cell death pathways such as BAX, Fas, Noxa, Puma, Caspase 1 and 6, Apaf-1, etc. These genes are expressed to similar levels during p53 mediated G1 arrest and apoptosis (Attardi LD, *et. al.* 2000). Depending on cell type and stimuli, activation of p53 can result in not only apoptosis or cell cycle arrest but also in induction of differentiation or senescence (Vousden KH and Lu X. 2002). p53 mediated effects can also occur independent of its transcription activity. In the presence of stress, p53 is thought to initiate cell death pathways by translocating to mitochondria resulting in loss of mitochondrial membrane potential and activation of the caspase cascade (Marchenko ND, *et.al.* 2000; Nemajerova A, *et.al.* 2005). Reports suggest that mitochondrial migration of p53 has a



more important role to play in p53 mediated cell death than cell cycle arrest. Studies show that translocation of p53 to the mitochondria after exposure to stress occurs in cells that undergo cell death but not in cells that undergo cell cycle arrest (Marchenko ND, *et.al.* 2000; Erster S, *et. al.* 2004). About 50% of cancers lack p53 protein expression, bear mutant or inactive p53 (Toledo F and Wahl GM. 2006). Mutation in p53 in germ line cells has also been implicated in Li-Fraumeni syndrome making patients prone to a variety of cancers (Senzner N, *et. al.* 2007). Mutant p53 can play an important role in the transformation of normal cells to cancerous cells and it is also known that tumors bearing wild type p53 are more sensitive to chemotherapy than tumors bearing mutant p53 (Wang W and El-Deiry WS. 2008). Most p53 mutations in cancer cells are missense mutations that occur in its DNA binding domain, precluding p53 from binding to its response elements on DNA and therefore preventing transcription of p53 target genes (Strano S, *et. al.* 2007; Hussain SP and Harris CC. 1998; Prives C and Hall PA. 1999). p53 bears a tetramerization domain and upon activation, this domain allows p53 proteins to tetramerize which is essential for p53 function, enabling p53 to bind DNA and transcribe genes. In cancer cells, mutations have been noted in the tetramerization domain of p53 that can prevent wild type p53 function by inhibiting p53 tetramer formation (Chène P. 2001).

Mutations in tumor suppressor genes are recessive in nature and hence, in order for a cell to become cancerous, both alleles of the tumor suppressor gene need to be mutated. This theory is called “Knudson’s two hit hypothesis” and was suggested by Dr. Alfred Knudson in 1971 (Knudson A. 1971). p53 is an exception to this rule since mutant p53 can exert a “dominant negative” effect such that mutant p53 can inhibit the function of wild type p53

generated from the un-mutated allele by forming non-functional p53 tetramers that can not transcribe genes successfully (Blagosklonny MV. 2000).

Studies have also shown that certain cancer cells have p53 protein with gain of function mutations that allow mutant p53 to exert functions that the wild type protein can not. These mutants may not only behave as dominant negative mutants to inhibit wild type p53 function but also provide a growth and survival advantage to the cancer cell as they have been implicated in increasing expression of genes such as the multi drug resistance gene which can prove to be very beneficial for cancer cells by allowing them to pump out toxic drugs from their systems (van Oijen M and Slootweg PJ. 2000; Chin KV, *et. al.* 1992). Cancers bearing a gain of function mutation in p53 indicate poorer prognosis than cancers with no p53 protein at all (Dittmer D, *et. al.* 1993).

#### **F. Cell death pathways:**

As discussed previously, the presence of cellular stress can lead to activation of cell death pathways. There are various types of cell death pathways such as autophagy, apoptosis and necrosis. Attempts have been made to reach a consensus on the criteria used to differentiate between these forms of death but have been unsuccessful partially due to overlaps in characteristics observed in these form of cell death (Krantic S, *et. al.* 2007).

Autophagy (derived from Greek, “auto” meaning self and “phagy” meaning to eat) is a form of programmed cell death (Yorimitsu T and Klionsky DJ. 2005; Clarke PG. 1990).

Autophagy has a role in protein degradation and cellular turnover and is characterized by sequestration of the cytosol and organelles in a multi-membrane vesicle which is then

degraded by the lysosomal system (Gozuacik D, Kimchi A. 2004). Autophagy usually occurs at a low basal level and is often upregulated during events such as starvation when the nutrient demands of a cell are not met (Kourtis N and Tavernarakis N. 2009). In such situations, autophagy can allow degradation of cytoplasmic components of a cell, thereby providing the nutrient deprived cell with substrates for energy metabolism and protein synthesis in order to survive. However, there is an ongoing debate regarding the role of autophagy since it has not only been observed to be involved in survival processes but it has also been known to cause cell death (Levine B and Klionsky DJ. 2004; Shintani T and Klionsky DJ. 2004; Wang CW and Klionsky DJ. 2003; Bursch W. 2004).

Apoptosis is a type of programmed cell death and cells undergoing apoptosis have a very characteristic morphology that includes membrane blebbing, cell shrinkage, chromatin condensation, nuclear and DNA fragmentation (Savill J, *et. al.* 2003). Necrosis is an un-programmed form of cell death that occurs due to external insults on a cell or due to non-physiological attacks such as ischemia, hypothermia or hypoxia (de Bruin EC and Medema JP. 2008). Necrosis is characterized by loss of membrane integrity, swelling of mitochondria and the cell itself resulting in cell lysis (de Bruin EC and Medema JP. 2008). It is generally accepted that necrosis is a process that does not require energy whereas apoptosis is an ATP-dependent process (Leist M, *et. al.* 1997). Another difference between apoptosis and necrosis is that apoptosis occurs in individual cells whereas necrosis occurs in contiguous cells and also generates an immune response which is absent in apoptotic cell death (Vakkila J and Lotze MT. 2004).

Extrinsic and intrinsic death pathways are apoptotic cascades that can be activated by several chemotherapeutic agents. The extrinsic cell death pathway involves activation of death receptors that are members of the tumor necrosis factor receptor superfamily, known to regulate cell death and survival (Ashkenazi A. 2002). One of the best characterized death receptor is CD95, also called Fas or APO-1 receptor, which binds to CD95/Fas ligand (CD95L/FasL) (Walczak H and Krammer PH. 2000). Upon ligand binding, CD95 death receptors can trimerize and recruit adaptor molecules such as the Fas associated death domain (FADD) (Walczak H and Krammer PH. 2000). FADD can then recruit pro-caspase 8 or pro-caspase 10 to the death receptor which leads to the formation of the death inducing signaling complex or DISC (Kischkel FC, *et. al.* 1995). Caspases are cysteine proteases, synthesized as pro-enzymes and cleaved next to aspartate residues for activation after which they can act as death effector molecules for several cell death pathways (Degterev A, *et. al.* 2003). Once recruited by FADD to the death receptors, pro-caspases can become activated by undergoing cleavage and can further activate downstream caspases resulting in amplification of the caspase cascade (Degterev A, *et. al.* 2003; Scaffidi C, *et. al.* 1998). Once caspases are activated, they can lead to cleavage of various cytosolic and nuclear substrates such as cytoskeletal proteins and nuclear lamins that can lead to loss of cell shape and result in shrinking of the nucleus, respectively (Degterev A, *et. al.* 2003). Active caspases can also cleave the inhibitor of caspase activated DNase (ICAD) leading to polynucleosomal DNA fragmentation resulting in the characteristic oligomeric DNA fragments seen upon caspase activation (Nagata S. 2000). Similar to various other signaling pathways, the extrinsic cell death pathway also can be negatively

regulated by FLIP proteins. There are several splice variants of FLIP known to exist at the mRNA levels but only three isoforms of FLIP protein namely, c-FLIP-s, c-FLIP-r and c-FLIP-l have been identified (Djerbi M, *et. al.* 2001; Rasper DM, *et. al.* 1998; Golks A, *et. al.* 2005). Studies have suggested similar roles of c-FLIP-s and c-FLIP-r in death receptor mediated apoptosis (Golks A, *et. al.* 2005). c-FLIP-s plays an anti-apoptotic role as it can inhibit the activation of pro-caspase 8 and thereby inhibit CD95 induced cell death (Golks A, *et. al.* 2005). These FLIP proteins have sequence homology to caspase 8 but lack their catalytic domain and hence when FLIP is recruited to activated death receptors, it can block activation of the caspase cascade and the cell death cascade (Krueger A, *et. al.* 2001). However, the role of c-FLIP-l in the activation of CD95 pathway remains controversial. Some studies suggest the c-FLIP-l is an anti-apoptotic molecule whereas other studies ascribe pro-apoptotic functions to c-FLIP-l referring to its assistance in pro-caspase 8 activation (Chang DW, *et. al.* 2003; Micheau O, *et. al.* 2002).

Death receptors play an important role in immunity. T-cells can regulate the immune system via production of CD95L which can trigger cell death in neighboring target cells such as lymphocytes by activating the CD95 receptors in these cells (Krammer PH. 2000). However, if the T-cells secrete CD95L resulting in soluble CD95L, this can activate CD95 receptors on their own cell membrane and can lead to their death (Klas C, *et. al.* 1993). Reports suggest that some cancers express CD95L but lose CD95 receptor expression. This can allow them to evade attack from host immune cells by killing tumor-attacking T-cells via activation of the CD95 receptors on these cells (Muschen M, *et. al.* 2000). Studies have shown that certain cancer cells that do express CD95 receptors can also evade attacks from

host immune cells by expressing soluble decoy receptor 3 (DcR3), known to compete with CD95 receptor for CD95L, thereby interfering with CD95 mediated cell death activation in cancer cells (Roth W, *et. al.* 2001). Down-regulation or absence of surface CD95 receptor expression in cancer cells can occur via epigenetic changes such as hypermethylation of gene promoters and can play an important role in mediating resistance to chemotherapeutic agents that require CD95 receptors to induce their toxic effects (van Noesel MM, *et. al.* 2002; Friesen C, *et. al.* 1997). It has been suggested that the CD95 death receptor system has a tumor suppressive function due to the occurrence of CD95 receptor mutations in cancers (Fulda S and Debatin KM. 2006). Elevated FLIP expression has also been found in cancer cells that can render them resistant to chemotherapeutic agents requiring activation of the extrinsic cell death pathway to mediate their toxic effects (Longley DB, *et. al.* 2006).

Activation of the intrinsic cell death pathway depends on the balance between pro-death and pro-survival Bcl-2 family proteins. The Bcl-2 family proteins have up to four Bcl-2 homology (BH) domains (BH1, BH2, BH3 or BH4) (Yip KW and Reed JC. 2008). The pro-survival Bcl-2 proteins (such as Bcl-x1, Mcl-1, Bcl-2, etc.) have all four BH domains whereas the pro-death members can be further divided into two classes: the “multi-domain” proteins that have BH1, BH2 and BH3 domains (such as Bax, Bak and Bok) and the “BH3 only proteins” that, as the name suggests, bear only the BH3 domain (such as Bid, Bim, Bad, Puma, Noxa) (Yip KW and Reed JC. 2008; Reed JC. 2006; Strasser A. 2005). Bcl-2 family members are known to interact with each other via the BH3 domain (Chittenden T, *et. al.* 1995).

The “BH3 only” proteins can interfere with the actions of pro-survival Bcl-2 family proteins and on the other hand, can also interact with Bak and Bax leading to their activation (A. Letai *et. al.* 2002). Studies suggest that oligomerization of pro-death members of the Bcl-2 family such as Bak and Bax can activate the intrinsic cell death pathway by forming pores in the outer mitochondrial membrane leading to mitochondrial outer membrane permeabilization (MOMP) (Chipuk JE and Green DR. 2008; Green DR and Kroemer G. 2004). Cells obtained from mice that do not express Bak and Bax fail to undergo cell death in response to insults that would typically lead to death in cells that express these proteins (Wei MC, *et. al.* 2001). Loss of mitochondrial membrane potential and MOMP can lead to release of various proteins from the inter-membrane space of the mitochondria that can induce cell death. These include proteins that can activate caspases (e.g. cytochrome c), proteins that can interfere with the function of caspase inhibitors (e.g. SMAC and Omi/Htra2 known to interfere with inhibitors of apoptosis or IAPs) and also proteins that are thought to cause cell death independent of caspase activation (e.g. apoptosis inducing factor and endonuclease G that can make their way to the nucleus where they can lead to DNA degradation) (Penninger JM and Kroemer G. 2003; Reed JC. 2002). Cytochrome c release from the mitochondria can lead to caspase activation via formation of the apoptosome which consists of cytochrome c, apoptotic protease activating factor 1 (Apaf-1) and pro-caspase 9 (Cain K, *et. al.* 2000). Binding of cytochrome c to Apaf-1 is thought to facilitate ATP binding to the apoptosome which then allows for caspase 9 activation (Adrain C, *et. al.* 1999). Active caspase 9 can lead to activation of various other caspases including caspase 2, 3, 6, 7, 8 and 10 (Guerrero AD, *et. al.* 2008). It

is important to note that the extrinsic and intrinsic cell death pathways can crosstalk. As mentioned earlier, activated death receptors can lead to caspase 8 activation. Active caspase 8 can cleave Bid (a “BH3 only” protein) which can translocate to the mitochondria and interfere with the pro-survival functions of Bcl-xl and is also thought to play a role in assisting in oligomerization of Bak and/or Bax that leads to pore formation in the mitochondrial membrane and MOMP (Gross A, *et. al.* 1999; Korsmeyer SJ, *et. al.* 2000). Disruption of the intrinsic cell death pathway has been observed in cancer. For instance, over-expression of pro-survival Bcl-2 family proteins can promote oncogenesis (Tsujimoto Y, *et. al.* 1984; Fulda S and Debatin KM. 2006). Defects in the expression of pro-death Bcl-2 family members can also occur in cancer cells. Homozygous deletions and inactivating mutations have been identified in genes encoding for pro-death Bcl-2 family proteins in cancer cells which can prevent activation of cell death pathways and hence prove beneficial for cancer cell survival (Meijerink JP, *et. al.* 1998).

### **G. Chemotherapeutic agents:**

The above mentioned pathways and proteins can play an important role in tumor development and progression. Hence, drugs have been developed to target them in order to treat cancer. As mentioned previously, receptor tyrosine kinases such as EGFR have been implicated in cancer progression as they have been found to be mutated such that they are constitutively activated or over-expressed in cancer (Peschard, P. and Park, M. 2003; Sunpaweravong P, *et. al.* 2005). Hence, several drugs have been developed to target these kinases. The development of small molecule tyrosine kinase inhibitors (TKIs) targeting EGFR family members was based on the observation that mutations in the ATP-binding



site affected receptor kinase function severely (Honegger AM, *et. al.* 1987). TKIs prevent ATP binding on the internal kinase domain of EGFR therefore preventing phosphorylation of key tyrosine residues which is an essential step in receptor activation (Imai K and Takaoka A. 2006). Gefitinib and erlotinib are two TKIs that inhibit ERBB1 receptors (Janne PA. 2008). Lapatinib was developed to target both ERBB1 and ERBB2 receptors of the EGFR family and targeting two tyrosine kinases is advantageous as it can be used to treat cancers that over-express ERBB1 and/or ERBB2 and also reduces the chances of resistance development in cancer cells (Yarden Y and Sliwkowski MX. 2001). Several monoclonal antibodies have been developed that recognize and bind to the extracellular domain of receptor tyrosine kinases thereby preventing ligand binding and downstream signaling (Rowinsky EK. 2004). Binding of monoclonal antibodies to receptors can induces dimerization and internalization of the receptor, eventually leading to receptor degradation and downregulation (Mendelsohn J. 2002). Herceptin/Trastuzumab is a monoclonal antibody that inhibits ERBB2 activation and has proven to be quite successful in treating breast cancers that frequently over-express ERBB2 receptors (Bange J, *et. al.* 2001).

In solid tumors, hypoxia can induce the release of vascular endothelial growth factor (VEGF) by tumor cells and by regions around the tumor called stroma (Das B, *et. al.* 2005). VEGF can bind to its receptors (that are also tyrosine kinases) and can stimulate angiogenesis (Parikh AA and Ellis LM. 2004). Hence, VEGF inhibitors were developed to inhibit the remodelling of the network of blood vessels required by certain solid tumors for growth and survival. Some VEGFR inhibitors are: semaxinib, vatalinib and sutent.

Platelet derived growth factor receptors (PDGFR) are also receptor tyrosine kinases that have been implicated in cancer progression as they can mediate downstream pathways that can regulate cellular growth and proliferation (Sedlacek HH. 2000). PDGFR has been implicated in cellular transformation and has been found to be over-expressed in a variety of cancer cells (Sedlacek HH. 2000). Leflunomide is a PDGFR inhibitor that was developed to inhibit PDGFR mediated signalling in cancer cells (Shawver LK, *et. al.* 1997). As mentioned previously, alterations in Ras oncogene have been implicated in cancer progression (Downward J. 2003). Ras is tethered to cell membranes via farnesylation or geranylgeranylation (Seabra MC. 1998). Hence, inhibitors of farnesyl transferase (e.g. FTI-277) and geranylgeranyl transferase-1 (e.g. GGTI-298) have been developed to prevent the prenylation of Ras protein which is required for its activity (Der CJ and Cox AD. 1991). Active Raf, which is downstream of Ras, can mediate pro-survival signaling and has been frequently observed to be mutated in cancer cells (Davies H, *et. al.* 2002). Hence Raf kinase inhibitors such as sorafenib have been developed. However, it is now known that sorafenib can inhibit Raf as well as a variety of other kinases including VEGFR and PDGFR (Strumberg D, *et. al.* 2005).

In tumor cells, over-expression of MDM2 can inhibit wild-type p53 activation protecting cancer cells from toxic effects of chemotherapeutic agents (Oliner JD, *et. al.* 1992). Hence, drugs such as Nutlins have been developed that can mimic p53 and thereby bind MDM2 (Vassilev LT. 2005). Nutlins can therefore inhibit p53-MDM2 interaction and allowing appropriate activation of the p53 pathway in cancer cells leading to cell cycle arrest, cell

death and also growth inhibition of tumor cells in human tumor xenograft models

(Vassilev LT, *et. al.* 2004).

Histone deacetylase inhibitors (HDACI) are another family of chemotherapeutic agents that are thought to inhibit tumor survival and growth by regulating expression of several genes involved in proliferation and/or apoptosis (Peart MJ, *et. al.* 2005). Histone deacetylase inhibitors (HDACI) represent a class of agents that act by blocking histone deacetylation, thereby modifying chromatin structure and gene transcription. HDAC inhibitors (HDACIs) promote histone acetylation and neutralization of positively charged lysine residues on histone tails, allowing chromatin to assume a more open conformation, which favors transcription (Gregory PD, *et. al.* 2001). However, HDACIs are known to promote acetylation of non-histone proteins as well (Dasmahapatra G, *et. al.* 2007; Marks PA, *et. al.* 2003; Bali P, *et. al.* 2005; Kwon SH, *et. al.* 2002). Most HDACIs are thought to function by binding to the zinc atom in the HDAC catalytic site thereby inhibiting the enzyme's activity (Richon VM. 2006; Taddei A, *et. al.* 2005). HDACI such as vorinostat and sodium valproate are known to cause up-regulation of pro-apoptotic genes, down-regulation of anti-apoptotic genes, growth arrest, differentiation as well as cell death in a variety of cancer cells (Mitsiades CS, *et. al.* 2004; Richon VM, *et. al.* 1998; Kuendgen A, *et. al.* 2007).

Certain cancer cells have been shown to be highly dependent on over-expression of pro-survival Bcl-2 family proteins in order to evade death signals induced by chemotherapeutic agents (Tsujimoto Y, *et. al.* 1984). Hence, BH-3 mimetics were developed that mimic the BH-3 domain of the pro-death Bcl-2 family members and interact with pro-survival Bcl-2

members there by inhibiting their function (Zhang L, *et. al.* 2007). One such agent is ABT-737 that has a high affinity for binding Bcl-xl, Bcl-2 and Bcl-w but since it is unable to effectively inhibit Mcl-1, several cancer cells have been shown to be resistant to this agent (van Delft MF, *et. al.* 2006). Another BH-3 mimetic is (-) Gossypol that is known to inhibit Bcl-2, Bcl-xl and Mcl-1 and is currently in Phase II clinical trials for treatment of prostate cancer (Meng Y, *et. al.* 2008). Gossypol can induce a conformational change in Bcl-2 and Bcl-xl proteins thereby converting their pro-survival function to pro-death resulting in cytochrome c release from the mitochondria that can induce cell death pathways (Lei X, *et. al.* 2006). Obatoclax is another such BH-3 mimetic that is currently in Phase II clinical trials being investigated both as a single agent and in combination with other drugs ([www.geminx.com](http://www.geminx.com)).

Hence, new molecular targets are being discovered and novel drugs as well as drug combinations are being identified that may enhance our ability to treat as well as prevent resistance development in cancer.

## MECHANISM OF LAPATINIB TOXICITY AND RESISTANCE IN HCT 116 CELLS

### A. Introduction:

The EGFR family consists of four members, namely ERBB1, ERBB2, ERBB3 and ERBB4 (Olayioye, *et. al.* 2000; Yarden Y and Sliwkowski MX. 2001). These receptors are present on the cell surface as monomers and upon ligand binding, can homo- or hetero-dimerize thereby increasing their affinity for ATP binding which allows autophosphorylation of specific tyrosine residues on the cytosolic domain of the receptors that serve as docking sites for molecules which can mediate downstream intracellular signaling (Hynes NE and Lane HA. 2005; Lin NU and Winer EP. 2004; Nelson MH and Dolder CR. 2006). A variety of cancers have been shown to have deregulated EGFR signaling via various mechanisms such as constitutive receptor activation, impaired receptor down-regulation and increased receptor stimulation via an autocrine loop leading to constitutive downstream pro-survival signaling resulting in very aggressive tumors (Peschard, P. and Park, M. 2003; Sizeland AM and Burgess AW. 1992; Sunpaweravong P, *et. al.* 2005; Salomon, DS, *et. al.* 1995; Hynes NE and Lane HA. 2005). Hence, studies have been performed to identify mechanisms via which ERBB receptors and their downstream signaling pathways can be inhibited in such tumor cells. Current strategies aimed at inhibiting receptor activation include (i) monoclonal antibodies that prevent ligand binding to the receptors (example: cetuximab), (ii) quinazoline-derived small molecule tyrosine kinase inhibitors (TKIs) that prevent ATP binding in the kinase domain of the receptors thereby inhibiting receptor kinase activity required to activate downstream

signaling (example: lapatinib, erlotinib) and (iii) dominant negative ERBB1 and ERBB2 receptors (CD533 and CD572, respectively) that have truncated C-terminal kinase domains, hence forming non-functional dimers with wild type receptors (Imai K and Takaoka A. 2006; Schmidt-Ullrich RK, *et. al.* 2003).

Lapatinib is a reversible small molecule TKI developed by GlaxoSmithKline that can efficiently inhibit ERBB1 and ERBB2 activation. IC<sub>50</sub> values for purified ERBB1 and ERBB2 inhibition via lapatinib are 10.2 and 9.8 nM, respectively (Wood ER, *et. al.* 2004; Rusnak DW, *et. al.* 2001). Several studies show lapatinib to be a promising therapeutic agent for treating cancer. Lapatinib (Tykerb) was recently approved by the Food and Drug Administration to be used in combination with capecitabine to treat patients with advanced or metastatic breast cancer that over-express ERBB2 and have been previously treated with other drugs (National Cancer Institute). Lapatinib has been studied in herceptin/trastuzumab resistant breast cancer lines and has been shown to induce apoptosis in such cells by inhibiting downstream pro-survival signaling mediated by ERBB1, ERBB2 and also insulin like growth factor –1 receptor (IGF-1R) (Nahta R, *et. al.* 2007). Bcl-2 is an anti-apoptotic protein that has been shown to be over-expressed in certain cancer cells and associated with drug resistance by allowing cells to evade death signals (Reed JC, *et. al.* 1996; McDonnell TJ, *et. al.* 1992). Combination studies involving lapatinib and Bcl-2 inhibitors in certain cancer cells show synergistic anti-tumor effects (Witters LM, *et. al.* 2007). Lapatinib is >300 fold selective for ERBB1 and ERBB2 inhibition compared to its ability to inhibit other kinases commonly found in a cell (Rusnak DW, *et. al.* 2001). This high selectivity may prevent undesirable effects arising

from non-specific inhibition of other molecules by lapatinib. Lapatinib is a particularly efficacious anti-cancer agent because it can inhibit two ERBB receptors simultaneously. This may allow lapatinib to be used to treat a wide variety of cancers that may depend on ERBB1 and/or ERBB2 signaling and also reduce the chances of resistance development in cancer cells.

Usually, cancer patients treated with chemotherapeutic agents respond well initially resulting in the reduction of tumor size and death of cancer cells. However, months or years later, the cancer can reappear as an aggressive and therapeutically refractory malignancy that may be cross-resistant to many other therapeutic drug treatments, making such refractory cancers very difficult to manage (Kobayashi S, *et. al.* 2005). There are several ways in which a cancer cell can become resistant to chemotherapeutic agents.

Resistance to trastuzumab has been suggested to be mediated via the insulin like growth factor –1 receptor (IGF-1R) signaling that can activate downstream pro-survival pathways (Lu Y, *et. al.* 2001; Camirand A, *et. al.* 2002). Src (a tyrosine kinase) and estrogen receptor have also been implicated in mediating resistance to TKIs in certain cancer cells by activating pro-survival signaling pathways (Qin B, *et. al.* 2006; Xia W, *et. al.* 2006).

Resistance to ERBB targeted drugs can also occur due to mutations in ERBB receptors resulting in the inability of drug to inhibit receptor activation (Pao W, *et. al.* 2005; Sok JC, *et. al.* 2006). Multi-drug resistance pumps may also be involved in drug resistance by pumping out toxic drugs from cancer cells (Szakacs G, *et. al.* 2006). As mentioned previously, studies have also shown that certain cancer cells over-express anti-apoptotic molecules belonging to the Bcl-2 family which can protect these cells from cyto-toxic

effects of drugs by allowing them to evade cell death signals and hence render them resistant to chemotherapeutic agents (Raffo AJ, *et. al.* 1995).

Constitutive activity of the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) has also been implicated in drug resistance (Herrmann JL, *et. al.* 1997; Sumitomo M, *et. al.* 1999).

Inhibitor of  $\kappa$ B (I $\kappa$ B) usually sequesters NF- $\kappa$ B in the cytosol. I $\kappa$ B Kinase (IKK) can phosphorylate I $\kappa$ B leading to its degradation. This releases NF- $\kappa$ B which can then translocate to the nucleus where it can initiate transcription of genes involved in survival (Baldwin AS. 2001). In certain cancer cells, constitutive NF- $\kappa$ B activation due to defective I $\kappa$ B activity or persistent IKK activity, allows constant transcription of genes involved in cellular growth (Cabannes E, *et. al.* 1999; Kordes U, *et. al.* 2000).

Resistance to the effects of lapatinib has been demonstrated in BT474 breast cancer cells and was reported to occur via estrogen receptor signaling (Xia W, *et. al.* 2006). As noted by the authors, a combination of lapatinib and estrogen receptor inhibitors may decrease the probability of development of refractory cancers and therefore enhance the chances of patient survival. Lapatinib has not been approved for the treatment of colon cancer yet, although its potential effectiveness has been reported. Various colon cancer cell lines have been reported to express ERBB1 and ERBB2 receptors, including HCT116 cells and lapatinib has been shown to cause inhibition of cell proliferation and apoptosis *in vitro* and *in vivo* in cancer cells of this origin (Cunningham MP, *et. al.* 2006; Zhou Y, *et. al.* 2006).

We have generated lapatinib resistant HCT 116 colon cancer cells and the aim of this project was to understand the mechanism of action of lapatinib as well as lapatinib resistance in HCT116 colon cancer cells. Understanding drug resistance may enable us to



reduce the chances of occurrence of refractory cancers by targeting the pathways utilized by resistant cells for survival and increase the likelihood of patient survival.

## **B. Materials:**

Dulbecco's modified Eagle's medium, penicillin/streptomycin, and 0.25% trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). HCT116 cells were originally purchased from American Type Culture Collection (Manassas, VA) before multiple transfection procedures. Fetal bovine serum was purchased from Hyclone (Logan, UT). Trypan blue dye and crystal violet for colony-formation assays were purchased from Sigma-Aldrich (St. Louis, MO). For Western blot analysis, 8 to 16% Tris-HCl gels were used (Bio-Rad Laboratories, Hercules, CA). CMV control virus, ERBB1-CD533, and ERBB2-CD572 were obtained from Dr. Kristoffer Valerie (Virginia Commonwealth University, Richmond, VA). BCL-XL recombinant adenovirus was obtained from Dr. J. Moltken (University of Cincinnati, Cincinnati, OH). Dominant-negative (dn) dnI $\kappa$ B (S32A) recombinant adenovirus and STAT inhibitory peptide were purchased from Cell Biolabs (Philadelphia, PA) and Calbiochem, respectively. Control siRNA and siRNA to knockdown apoptosis-inducing factor (AIF) (SI02662114, SI02662653), Bcl-xl (SI03025141, SI03068352, SI03112018, SI00023191), Mcl-1 (SI02781205, SI00131768), and BAK (SI00299376, SI02654512) were purchased from QIAGEN (Valencia, CA). Lapatinib was obtained from GlaxoSmithKline (Boston, MA). The IGF-1R inhibitor PPP, the Src family kinase inhibitor PP2, 4-hydroxy tamoxifen, and epidermal growth factor were purchased from Calbiochem (San Diego, CA). Primary antibodies against Mcl-1, Bcl-xl, BAX, BAK, AIF, and cytochrome *c* were purchased from Cell Signaling Technology

Inc. (Danvers, MA). ERBB1 (Ab-5) antibody for fluorescence microscopy, primary antibody for active BAK (Ab-1), caspase 8 inhibitor LEHD, caspase 9 inhibitor IETD, and pan-caspase inhibitor zVAD were purchased from Calbiochem. EGF receptor (Ab-13 cocktail) and c-ERBB2 (Ab-11 cocktail) to immunoprecipitate ERBB1 and ERBB2 were purchased from NeoMarkers (Freemont, CA). Anti-Phospho-Tyr 4G10 antibody was purchased from Millipore Bioscience Research Reagents (Temecula, CA). Primary antibodies for GAPDH, wild-type p53 (FL-393), mutant p53 (Pab 240), ERK2, active BAX (6A7), and protein A/G Plus agarose beads for immunoprecipitation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary mouse antibody (Alexa Fluor 680 goat anti-mouse IgG) was purchased from Invitrogen, and secondary rabbit antibody (anti-rabbit IgG) was purchased from Rockland Immunochemicals (Gilbertsville, PA). UCN-01 was kindly supplied by was provided by the Cancer Treatment and Evaluation Program of the National Cancer Institute. VP-16 was purchased from Sigma.

### **C. Methods:**

**Cell culture:** Parental HCT116 colon cancer cells (WT cells) were cultured in DMEM media containing 5% fetal bovine serum and 1% Penicillin/Streptomycin. Lapatinib resistant HCT116 cells (WT-AD cells) were cultured in DMEM media containing 5% fetal bovine serum, 1% Penicillin/Streptomycin and 2 $\mu$ M Lapatinib. When WT-AD cells were thawed out of the freezer, they were plated in DMEM media containing 5% fetal bovine serum, 1% Penicillin/Streptomycin and 10 $\mu$ M Lapatinib for 3 days to ensure they were Lapatinib resistant.

**Detection of Cell Death by Trypan Blue Assay:** After treatment, medium was removed and cells were washed in 1X PBS. Cells were then harvested by trypsinization with Trypsin/EDTA for ~5 min at 37°C. Because some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1400 RPM for 5 min. The pooled cell pellets were resuspended and mixed with trypan blue dye. Trypan blue stain, in which blue dye-incorporating cells were scored as being dead, was performed by counting of cells using a light microscope and a hemacytometer. The number of dead cells was counted and expressed as a percentage of the total number of cells counted.

**Culture of Cells and Drug Treatments for Colony Formation Assays:** Cells were plated (250–1000 cells/well of a 6-well plate). 12 h after plating medium was removed and serum-free medium was added to the cells for 24 or 48 h as indicated. After this, the serum-free media was carefully removed and fresh media (with serum) was added. Colony formation assays were cultured for an additional 8-10 days, after which the media were removed, cells were fixed with methanol, stained with crystal violet, and counted manually.

**Immunoprecipitation and Western Blotting:** 12 hours after plating cells, they were either infected with CD533 and CD572 or control virus for 24h or serum starved and treated with indicated concentrations of lapatinib or dimethyl sulfoxide (DMSO) for 2h. After either of these treatments, cells were treated with 20ng/ml EGF or vehicle for 10 mins. Cells were then scraped using RIPA buffer (50mM Tris HCl pH 7.4, 150mM NaCl, 1%NP-40, 1% Sodium Deoxycholate, 1mM PMSF, 1mM Sodium orthovanadate, 10mM Sodium Fluoride, 10mM  $\beta$ -glycerophosphate, 0.5mM EGTA, 0.5mM EDTA and protease

inhibitor cocktail purchased from (Roche)) and ERBB1 or ERBB2 was immunoprecipitated as indicated, after which samples were boiled for 10 minutes in whole cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1%  $\beta$ -mercaptoethanol, 0.02% bromophenol blue). 12 hours after plating cells, they were also scraped using a non-denaturing lysis buffer (50mM Tris HCl pH 7.4, 150mM NaCl, 1%NP-40, 0.1% sodium deoxycholate, protease and phosphatase inhibitor cocktails (Roche)) and mutant p53 was immunoprecipitated after which samples were boiled for 10 min in whole cell lysis buffer. Cells were also scraped in CHAPS buffer (10mM HEPES, 140mM NaCl, 1% CHAPS) and then active BAK or active BAX was immunoprecipitated. Samples were boiled for 10 min in whole cell lysis buffer. All samples were then loaded on 8%-16% Criterion pre-cast gels (BIORAD) after normalizing total protein and run for about 2 hours. Proteins were then electrophoretically transferred onto 0.22um nitrocellulose membranes and immunoblotted with various primary antibodies as indicated.

**Virus Infections:** Cells were infected 12h after plating with adenoviruses at an approximate multiplicity of infection of 30 for 4 h with gentle rocking, after which time the media was replaced. Cells were further incubated for 24 h to ensure adequate expression of transduced gene products before drug exposures.

**Transfection of Cells with Small Interfering RNA Molecules:** RNA interference for down-regulating the expression of AIF, Mcl-1, Bcl-xl and BAK was performed using validated target sequences designed by Qiagen. For transfection, 20-40 nM concentration of the annealed siRNA-targeting AIF, Mcl-1, Bcl-xl or BAK, or the negative control (a "scrambled" sequence with no significant homology to any known gene sequences from

mouse, rat, or human cell lines) were used. The siRNA molecules were transfected into cells according to the manufacturer's instructions. Cells were cultured for 24 h after transfection before any additional experimentation.

**Cell Fractionation:** 12h after plating cells, they were serum starved and treated with 2 $\mu$ M lapatinib or DMSO for 36h. This experiment was performed on ice at all times. Medium from plates was then aspirated and cells were scraped in buffer (75mM NaCl+8mMNa<sub>2</sub>HPO<sub>4</sub>+1mMNa<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>+0.5mMEDTA+ 0.5mMEGTA with freshly added 350ug/ml digitonin, 250mM sucrose, protease and phosphatase inhibitor cocktails (Roche)) and passed through a 25 gauge needle 12 times. After 15 to 30 minutes on ice, cells were spun down at 5000RPM for 1.5 minutes at 4°C to remove cell debris. Pellet was discarded and supernatant was transferred to a new tube and spun down at 13000 RPM for 25 minutes at 4°C. The supernatant obtained is the cytosolic fraction where as the pellet is the mitochondrial fraction. Whole cell lysis buffer was added to the supernatant and the pellet, boiled for 10 minutes and then western blot analysis was performed. This protocol was adapted from Leist M, *et. al.* (1998).

**Flow Cytometry:** Flow cytometric analysis of cells was performed after staining by the the ANNEXIN V-FITC kit (Beckman Coulter) according to the manufacturer's instructions and read on Beckton Dickinson FACScan.

**D. Results:**

***Generation of Lapatinib resistant HCT116 cells:*** HCT116 colon cancer cells (henceforth called WT cells) were cultured in the presence of 10 $\mu$ M lapatinib, a concentration that is below the lapatinib  $C_{max}$  in patients (GlaxoSmithKline). Within 72 h of lapatinib treatment, many cells became detached and died from this drug exposure. Cells were cultured in the presence of 10 $\mu$ M lapatinib for a further ~3 months until a homogeneous population of cells grew out from the survivors that were resistant to lapatinib (henceforth called WT-AD cells).

***Confirmation of Lapatinib resistance in WT-AD cells:*** 24 h after plating WT and WT-AD cells, they were serum-starved and exposed to increasing concentrations of lapatinib (0-10  $\mu$ M) for 48 h. Trypan blue analysis showed that at each concentration of lapatinib tested, WT-AD cells showed significantly lower levels of cell death compared to WT cells (Figure 3). The level of cell death observed in WT-AD cells treated with 10  $\mu$ M lapatinib, which was the highest concentration of lapatinib used in this experiment, was comparable to the level of cell death observed in WT cells that were simply serum-starved (i.e. no lapatinib was added). Also, WT-AD cells were significantly protected from serum-starvation induced cell death compared to WT cells. This indicates that compared to WT cells, WT-AD cells are resistant to lapatinib as well as serum-starvation.

In another experiment, WT and WT-AD cells were serum-starved and exposed to vehicle or 2 $\mu$ M lapatinib for varying times (12 h – 48 h) and cell viability was measured by Annexin-PI staining flow cytometric analysis. Results confirmed that WT-AD cells were

resistant to the effects of 2 $\mu$ M lapatinib as well as serum-depletion compared to WT cells at each time point tested (Figure 4).

WT and WT-AD cells were treated with either vehicle or increasing doses of lapatinib (0.1-10 $\mu$ M) without serum-starvation. Annexin-PI flow cytometric analysis showed that WT-AD cells were more resistant to the cyto-toxic effects of lapatinib at all doses tested compared to WT cells even in the presence of serum (Figure 5).

Reinforcing our previous observations, colony formation assays showed that WT-AD cells are more resistant to the effects of serum-starvation than WT cells (Figure 6). Colony formation assays also showed that ionizing radiation reduced survival by comparable levels in both WT and WT-AD cells (Figure 7).

***Culturing WT-AD cells in the absence of lapatinib:*** To investigate whether lapatinib resistant WT-AD cells maintained their resistant phenotype if cultured in the absence of lapatinib, these cells were cultured without lapatinib for >10 flask passages (~2 months) and are labeled WT-AD10. Cell death levels in serum-starved WT-AD10 cells were not significantly different than the levels in serum-starved WT-AD cells under vehicle as well as lapatinib treated conditions. WT-AD and WT-AD10 cells showed much lower cell death levels compared to WT cells under serum starved conditions in the presence of vehicle or 2 $\mu$ M lapatinib. Hence, WT-AD10 cells maintained their resistance to serum-starvation as well as lapatinib in spite of being cultured in the absence of the drug (Figure 8).

***Effect of chemotherapeutic agents on WT and WT-AD cells:*** To determine whether WT-AD cells were cross-resistant to toxicity induced by commonly used chemotherapeutic agents, WT-AD and WT cells were treated with other chemotherapeutic agents namely,

VP-16 (etoposide), UCN-01 (an analogue of staurosporine) and Taxotere. WT-AD cells were significantly more resistant to all of these agents than parental WT cells at all doses tested (Figure 9 and 10).

***Effect of lapatinib on ERBB1 receptor activation:*** Next we wanted to determine whether lapatinib was able to inhibit ERBB1 receptor activation in WT and WT-AD cells.

Increasing concentrations of lapatinib (0-2 $\mu$ M) resulted in increased inhibition of basal and epidermal growth factor (EGF) induced ERBB1 tyrosine phosphorylation in WT and WT-AD cells (Figure 11). This confirmed that lapatinib was able to inhibit ERBB1 receptor activation in both cell lines and that lack of lapatinib uptake in WT-AD cells was not the mode of resistance in these cells. It was also noted that total ERBB1 levels in WT and WT-AD cells were comparable as judged by immunoblotting of whole cell lysates.

***Cell surface ERBB1 receptor level in WT and WT-AD cells:*** Results from the previous experiment showed that basal and EGF stimulated levels of ERBB1 receptor tyrosine phosphorylation in WT-AD cells appeared to be lower compared to WT cells. Also, EGF mediated ERK1/2 phosphorylation in WT-AD cells was observed to be reduced compared to WT cells (Figure 11). These data suggested that there may be fewer cell surface ERBB1 receptors available for activation in WT-AD cells compared to WT cells. To investigate this possibility, immunocytochemistry was performed to look at the cell surface level of ERBB1 receptors in non-permeabilized, serum-starved WT and WT-AD cells. Results indicated that WT-AD cells appeared to have considerably lower plasma membrane associated ERBB1 levels compared to WT cells which could possibly explain why basal



and EGF induced ERBB1 phosphorylation appeared to be lower in WT-AD cells than in WT cells (Figure 12).

***VEGFR and c-KIT levels are comparable in WT and WT-AD cells:*** Over-expression of other growth factor receptors such as VEGFR-1 and c-KIT has been implicated in mediating resistance to chemotherapeutic agents (Bianco R, *et. al.* 2008; Raspollini MR, *et. al.* 2004). Immunoblotting analysis showed that levels of VEGFR-1 and c-KIT receptor were similar in WT and WT-AD cells and hence unlikely to be responsible for mediating lapatinib resistance in WT-AD cells (Figure 13).

***Role of Src, IGF-1R, estrogen receptor (ER), NF- $\kappa$ B or STAT in mediating lapatinib resistance:*** Since previous studies have implicated Src and IGF-1R in mediating drug resistance we wanted to investigate whether these kinases were important in mediating lapatinib resistance in WT-AD cells (Qin B, *et. al.* 2006; Lu Y, *et. al.* 2001). Inhibition of neither Src family kinases using the inhibitor PP2 nor IGF-1R function using the inhibitor PPP restored lapatinib sensitivity in WT-AD cells (Figure 14). It is noteworthy that inhibition of the IGF-1R with PPP caused significant toxicity in parental WT cells that was abolished in WT-AD cells. These data suggested that Src and IGF-1R were not primary mediators on lapatinib resistance in WT-AD cells.

Previous studies have also implicated ER, NF- $\kappa$ B and STAT proteins in mediating drug resistance in cancer cells (Xia W, *et. al.* 2006; Herrmann JL, *et. al.* 1997; Sumitomo M, *et. al.* 1999; Catlett-Falcone R, *et. al.* 1999). Hence, we wanted to determine whether any of these proteins played a role in mediating lapatinib resistance in WT-AD cells. Incubation of WT-AD cells with ER inhibitor 4-hydroxy tamoxifen in phenol-red free media,

inhibition of NF- $\kappa$ B function by over-expression of I $\kappa$ B super-repressor (dominant negative I $\kappa$ B) or inhibition of STAT function by expression of STAT inhibitory peptide did not restore lapatinib sensitivity in WT-AD cells (Figure 15). In control studies, it was noted that expression of super-repressor I $\kappa$ B or STAT inhibitory peptide suppressed reporter construct activity in WT and WT-AD cells and in phenol red-free media, basal estrogen receptor activity was reduced by tamoxifen treatment in both WT and WT-AD cells (Figure 16). These control studies confirmed that super-repressor I $\kappa$ B, STAT inhibitory peptide and 4-hydroxy tamoxifen were functional.

***Role of multi-drug resistance (MDR) pumps in mediating lapatinib resistance:*** Since studies have shown that MDR pumps can mediate drug resistance in cancer cells, immunoblotting was performed to determine whether MDR pumps had a role to play in mediating lapatinib resistance in WT-AD cells (Szakacs G, *et. al.* 2006). Little change was observed in the levels of MRP-1, ABCG-2 and Pgp transporters in WT and WT-AD cells suggesting that these MDR pumps were not major players in mediating lapatinib resistance in WT-AD cells (Figure 17).

***Levels of pro- and anti-apoptotic proteins in WT and WT-AD cells:*** Alterations in the levels of Bcl-2 family members have been shown to mediate drug resistance in cancer cells (Raffo AJ, *et. al.* 1995; Tsujimoto Y, *et. al.* 1984; Fulda S and Debatin KM. 2006; Meijerink JP, *et. al.* 1998). To investigate this possibility in WT-AD cells, immunoblotting studies were performed which indicated that compared to WT cells, WT-AD cells expressed higher levels of p53 as well as anti-apoptotic Bcl-2 family proteins namely, Mcl-1 and Bcl-xl (Figure 18). Over-expression of Bcl-xl abolished lapatinib toxicity on WT

cells (Figure 19). WT-AD cells expressed lower levels of pro-apoptotic protein BAX compared to WT cells (Figure 18). Further, immunoprecipitation studies showed that upon treatment with lapatinib, WT-AD cells have reduced BAK and BAX activation compared to WT cells (Figure 18). This occurred in spite of the observation that total BAK levels appeared to increase in WT-AD cells, post lapatinib treatment.

In WT-AD cells, knockdown of Mcl-1 expression, to a greater extent than that of Bcl-xl, partially reverted lapatinib resistance by ~50% compared to WT cells treated with lapatinib (Figure 20). In WT-AD cells, knockdown of BAK activation significantly reduced the reversion of their resistant phenotype due to Mcl-1 knock-down (Figure 21).

***Role of p53 in lapatinib resistance:*** Previous immunoblotting data indicated that WT-AD cells showed less p53 phosphorylation under vehicle or lapatinib treated conditions compared to WT cells. This is important since phosphorylation has been shown to play an important role in stabilizing and activating p53 so that it can elicit an appropriate response to cellular stress (Jimenez GS, *et. al.* 1999). Also, WT-AD cells have higher total p53 levels than WT cells. It is known that in cancer cells, p53 expression is often elevated when p53 is mutated (Kohler MF, *et al.* 1992). Hence, we wanted to further investigate whether p53 in WT-AD cells was mutated. Native p53 proteins were immunoprecipitated from WT and WT-AD cells using an antibody that specifically recognizes mutant forms of p53, as judged by the recognition of mutant p53 tertiary structure within the DNA binding domain of p53. The p53 proteins were then separated on denaturing SDS-PAGE and immunoblotted. WT-AD cells, but not WT cells, immunoprecipitated “mutant” p53 (Figure 22). Total mRNA was isolated from WT and WT-AD cells, amplified and

sequenced using primers specific for the DNA binding domain of p53. We noted, however, that a mutation in the p53 DNA binding domain was not detected in WT-AD cells. Further experiments will be required to investigate the role of p53 in WT-AD cells.

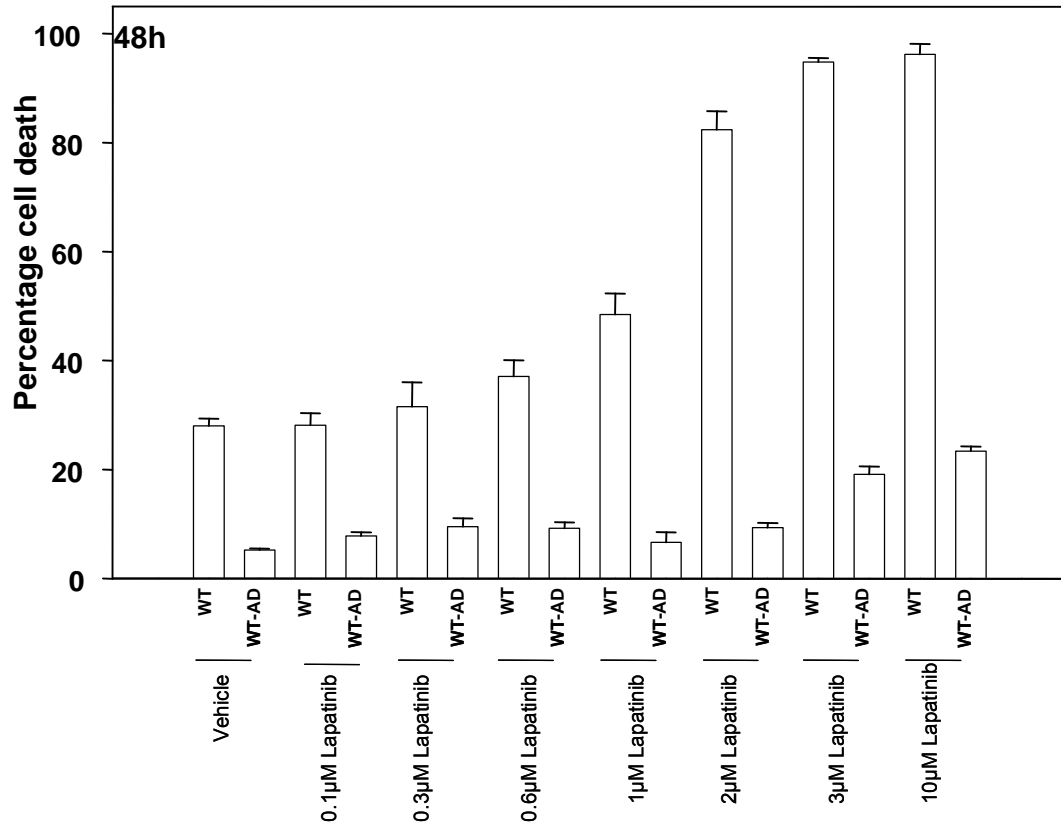
***Co-expression of dominant negative ERBB1 and ERBB2 to mimic lapatinib treatment:***

In order to investigate the mechanism of lapatinib mediated cell death, dominant negative ERBB1 and ERBB2 receptors were expressed in WT and WT-AD cells. Co-expression of dominant negative ERBB1 (CD533) and dominant negative ERBB2 (CD572) proteins inhibited both basal and EGF mediated ERBB1 and ERBB2 tyrosine phosphorylation in WT cells (Figure 23A). In spite of inhibiting ERBB1 and ERBB2 tyrosine phosphorylation similar to lapatinib, trypan blue analysis showed that co-expression of CD533 and CD572 failed to recapitulate the toxic effects of lapatinib in serum-starved WT and WT-AD cells suggesting that lapatinib mediated cell death may involve mechanisms in addition to/other than inhibition of the ERBB receptor system (Figure 23C). Also, in WT cells, the lack of increased cell death in the presence of CD533 and CD572 was not due to the inability of these dominant negative receptors to inhibit survival pathways downstream of ERBB receptors as shown by their capability to inhibit ERK1/2 and AKT phosphorylation (Figure 23B).

***Role of caspases in lapatinib mediated cell death:*** In WT cells, it was noted that inhibition of caspase enzymes resulted in protection from cell death induced by serum starvation (Figure 24). However, inhibition of caspase 8 or caspase 9 or treatment with a pan-caspase inhibitor did not protect WT cells from lapatinib mediated cell death (Figure 25). This indicated that lapatinib mediated cell death was likely caspase independent. As calpains,

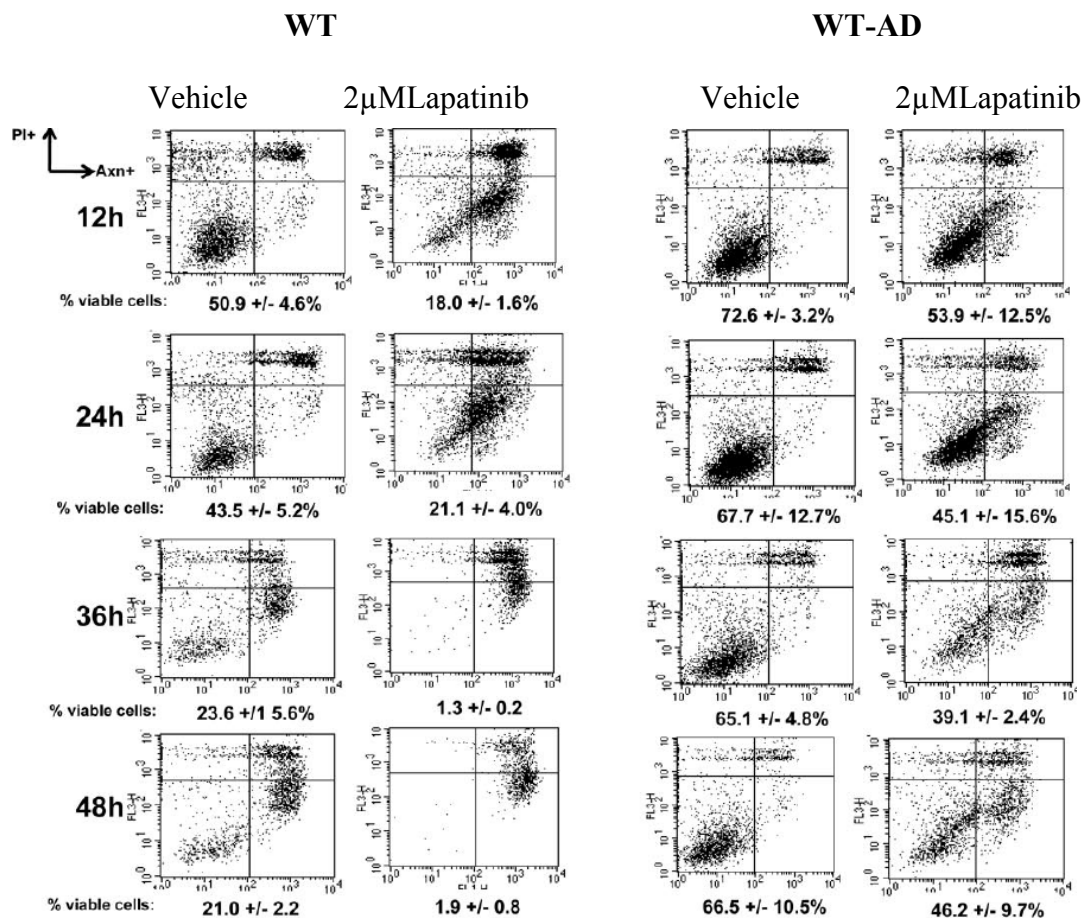
cathepsins and serine proteases can also initiate cell death pathways, we wanted to investigate whether they were involved in lapatinib mediated cell death (Vandenabeele P, *et. al.* 2005; Droga-Mazovec G, *et. al.* 2008; Broker LE, *et. al.* 2004). Inhibition of calpains, cathepsins and serine proteases did not protect WT cells from lapatinib toxicity suggesting that they were not likely involved in lapatinib mediated cell death (Figure 26 and 27).

***Caspase independent mechanism of lapatinib mediated cell death:*** Since previous experiments suggested that lapatinib mediated cell death was likely caspase independent, we wanted to investigate the role of apoptosis inducing factor (AIF) as it is thought to initiate cell death pathways independent of caspase enzymes (Penninger JM and Kroemer G. 2003). Immunoblotting and cell fractionation studies showed that increased cytosolic release of AIF as well as cytochrome c was observed in WT cells treated with lapatinib but not in WT-AD cells under the same conditions (Figure 28). Knockdown of AIF expression reduced lapatinib toxicity in WT cells, and knockdown of AIF expression combined with pan-caspase inhibition further reduced lapatinib toxicity in these cells (Figure 29).



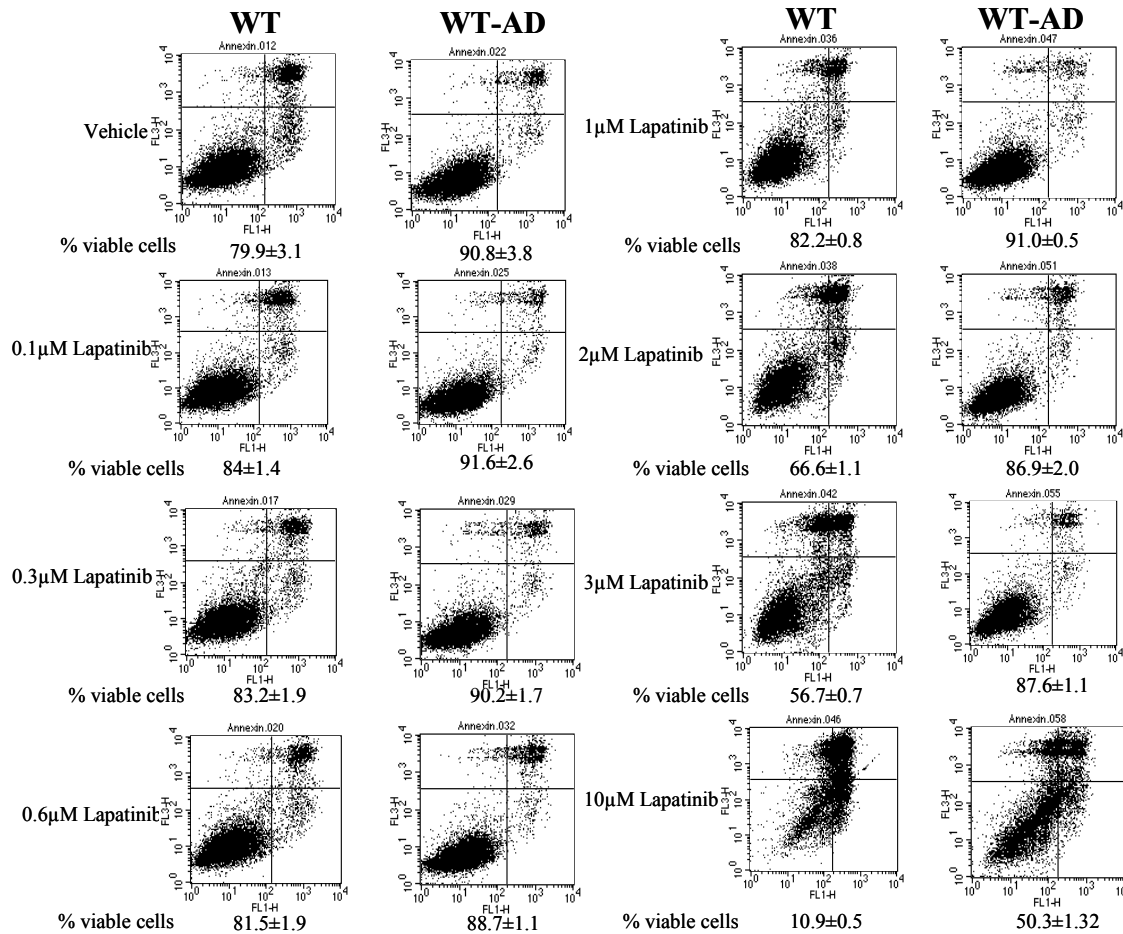
**Figure 3: WT-AD cells are resistant to effects of lapatinib and serum-starvation.**

Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (0–10µM). Cells were isolated 48 h after serum-starvation/lapatinib addition, and cell viability was determined by trypan blue exclusion assay. Significant effects of lapatinib dose and cell type were observed (Two-way ANOVA  $p < 0.0001$ ,  $n=6$ ). Bonferroni's post-test revealed that under vehicle treated conditions and at each concentration of lapatinib tested, there was significantly higher cell death in WT cells compared to WT-AD cells ( $p < 0.0001$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



**Figure 4: WT-AD cells are resistant to effects of lapatinib.**

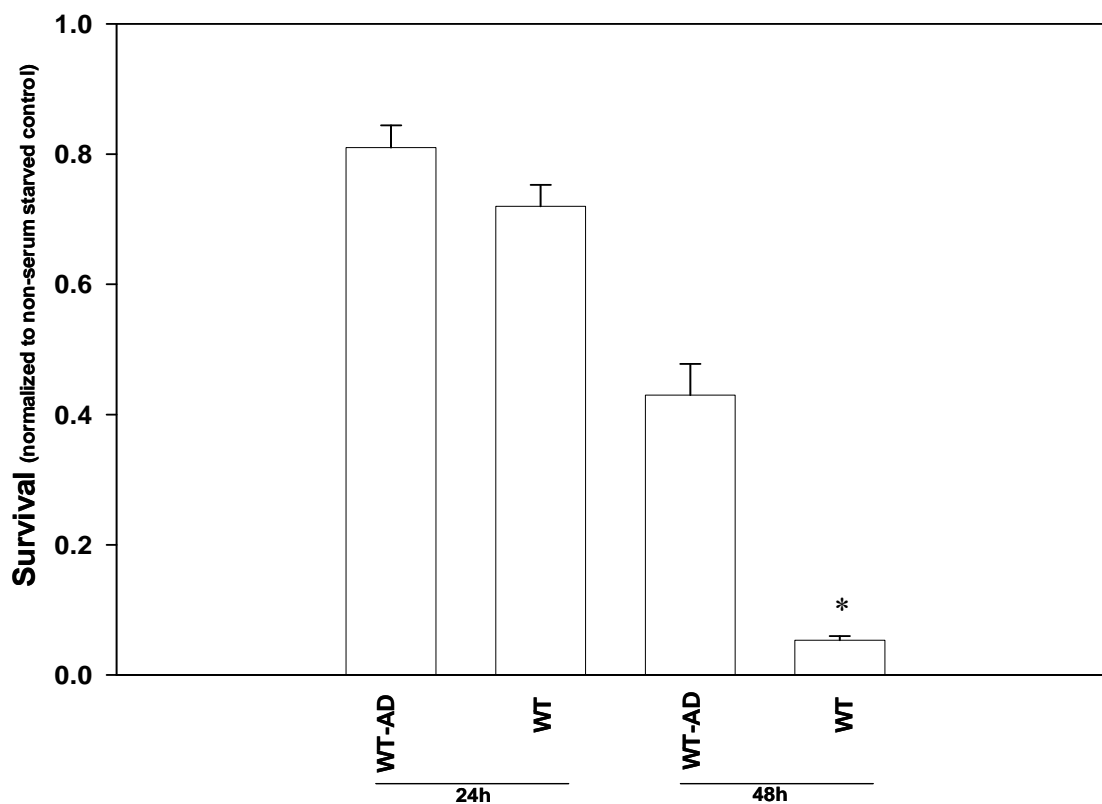
Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or 2μM lapatinib. Cells were isolated at the indicated times, and cell viability (indicated under each panel) was determined by annexin-PI flow cytometric analysis (n=6). Data (mean ± S.E.M.) are from two independent experiments combined.



**Figure 5: WT-AD cells are resistant to effects of lapatinib**

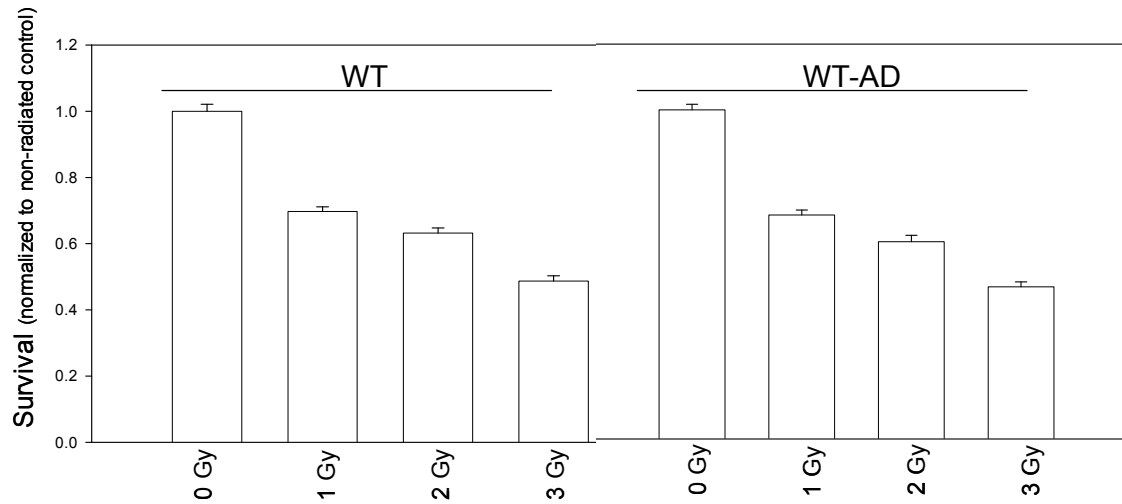
24 h after plating parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD), they were treated with vehicle (DMSO) or increasing concentrations of lapatinib (0.1-10 μM). 48 h later, cells were isolated and cell viability (indicated under each panel) was determined by annexin-PI flow cytometric analysis (n=6). Data (mean ± S.E.M.) are from two independent experiments combined.





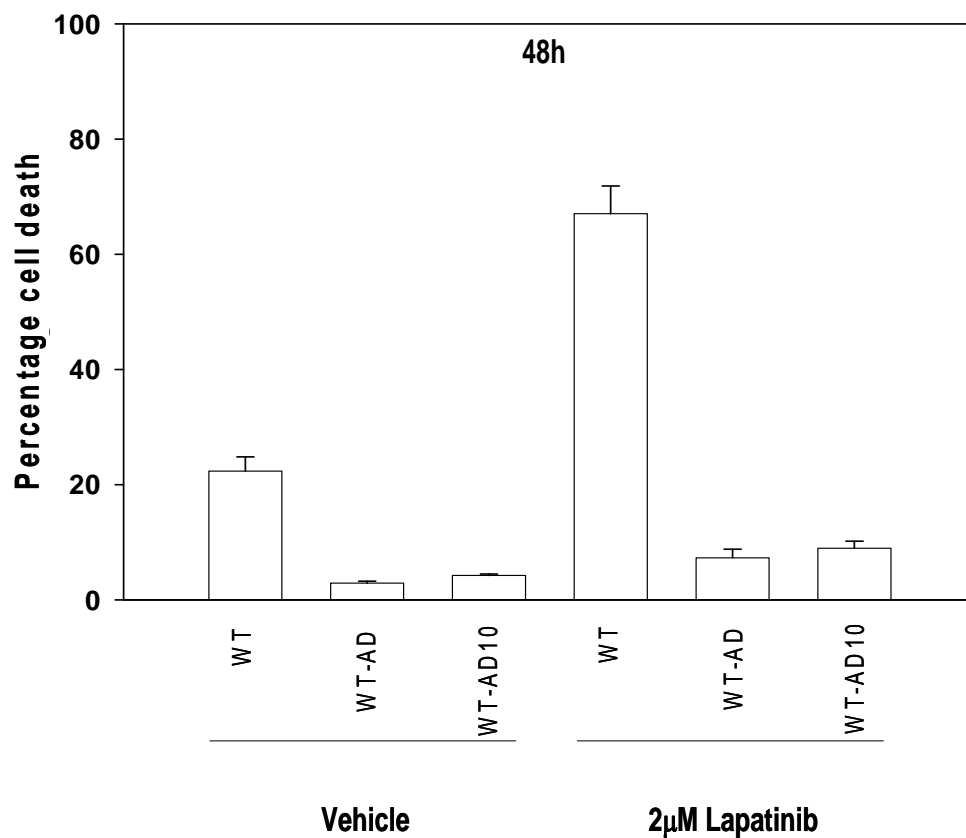
**Figure 6: WT-AD cells are resistant to serum-starvation.**

Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated as single cells and 12 h after plating were placed into serum-depleted medium for either 24 or 48. Colonies were permitted to form over the following 10 to 14 days in fresh media with serum and no lapatinib, after which the media were removed, the colonies fixed and stained with crystal violet, and the colonies of >50 cells were counted. Significant effect of serum starvation duration was observed in both cell lines ( $p < 0.0001$ , Two-way ANOVA,  $n=8$ ) and Bonferroni's post test revealed that 48 h after serum starvation, WT cells showed significantly decreased survival than WT-AD cells ( $p < 0.05$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

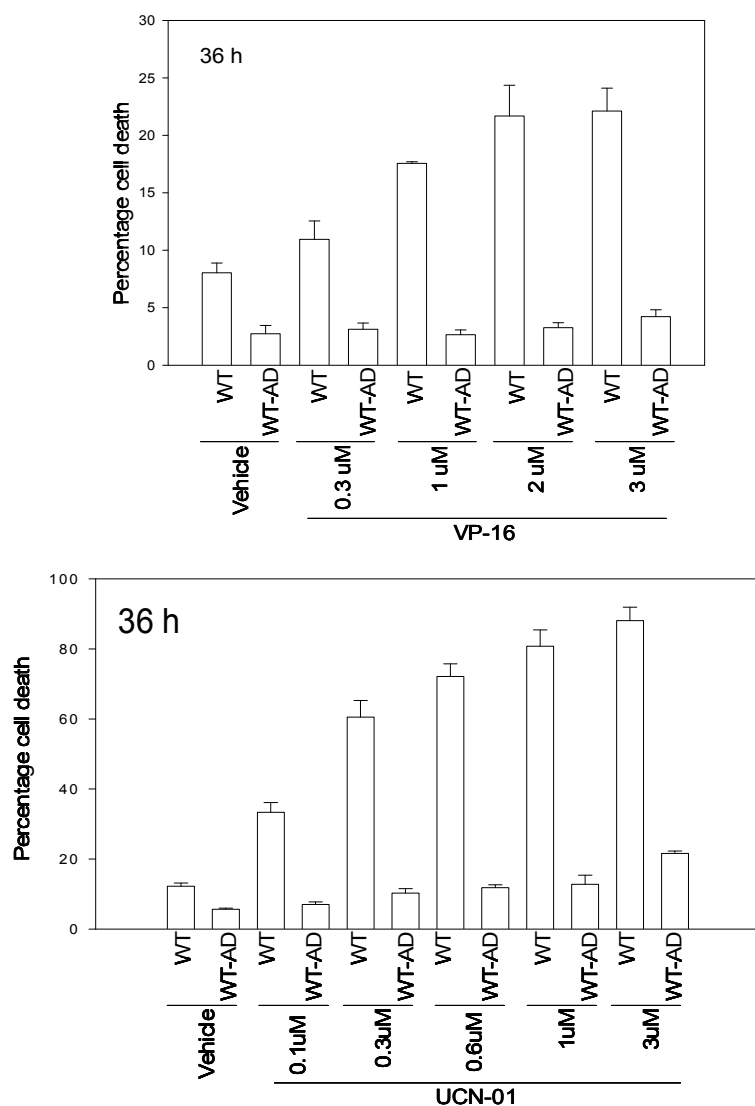


**Figure 7: Ionizing radiation affects WT and WT-AD cell survival.**

Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated as single cells in sextuplicate and 12 h after plating were exposed to ionizing radiation (0-3Gy). Colonies were permitted to form over the following 10 to 14 days, after which the media were removed, the colonies fixed and stained with crystal violet, and the colonies of >50 cells were counted. A significant effect of radiation dose in both cell lines ( $p < 0.0001$ , Two-way ANOVA,  $n=8$ ) but no significant difference in the cell types was observed. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

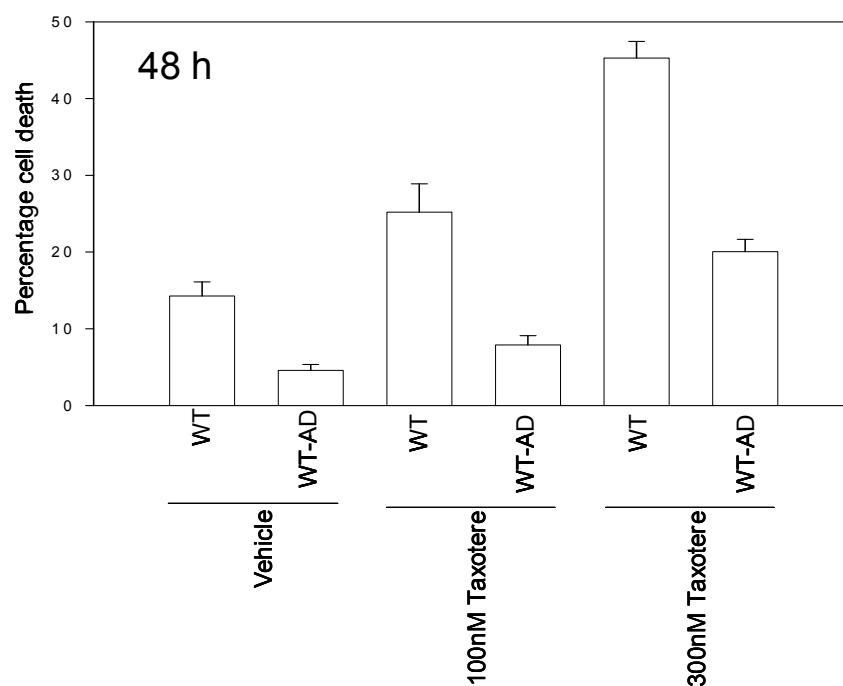


**Figure 8: Absence of lapatinib in culture does not restore lapatinib sensitivity in WT-AD cells.** Parental HCT116 cells (WT), HCT116 lapatinib-resistant cell (WT-AD) and HCT116 lapatinib-resistant cells that had been grown for ~2 months in the absence of lapatinib (WT-AD10) were plated and 24 h after plating were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2µM). Cells were isolated 48 h after serum starvation/lapatinib addition, and cell viability was determined in triplicate by trypan blue exclusion assay. Student's t-test showed that there was no significant difference between cell death levels in WT-AD and WT-AD10 cells under vehicle or lapatinib treated conditions. Data (mean ± S.E.M.) are from two independent experiments combined.



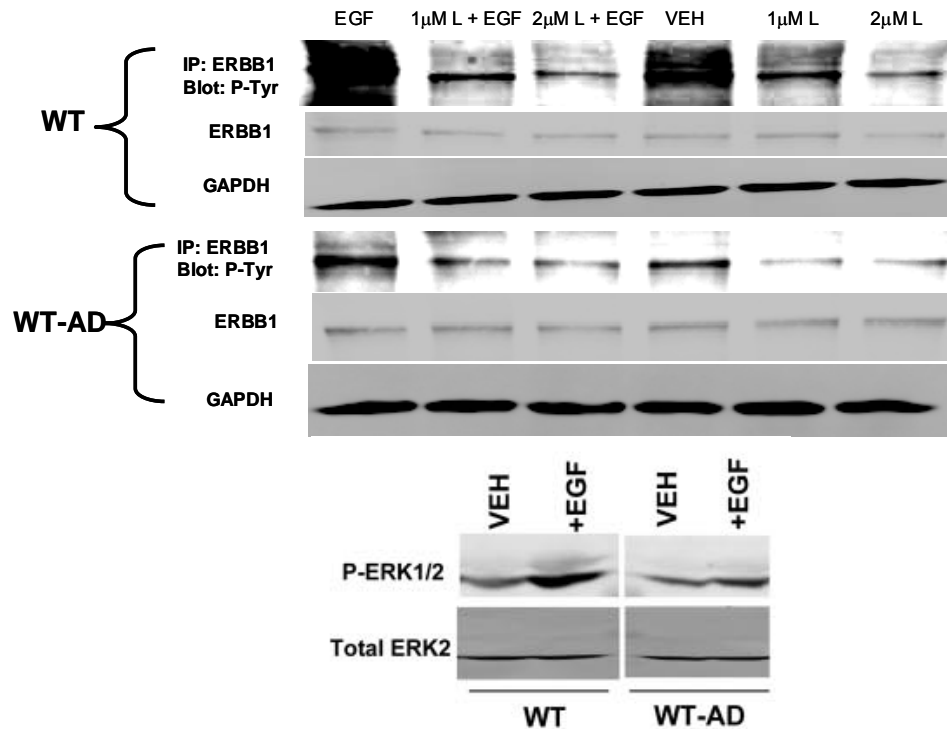
**Figure 9: WT-AD cells are resistant to effects of VP-16 and UCN-01.**

Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were treated with increasing concentrations of VP-16 (0-3 $\mu$ M) or UCN-01 (0-3 $\mu$ M). 36 h later, cell viability was determined by trypan blue exclusion assay. For both experiments effects of dose and cell type were observed (Individual Two-way ANOVA,  $p < 0.0001$ ,  $n=6$ ). Bonferroni's post test revealed higher levels of cell death in WT cells relative to WT-AD cells under vehicle conditions and with VP-16 and UCN-01 treatment at all concentrations tested.

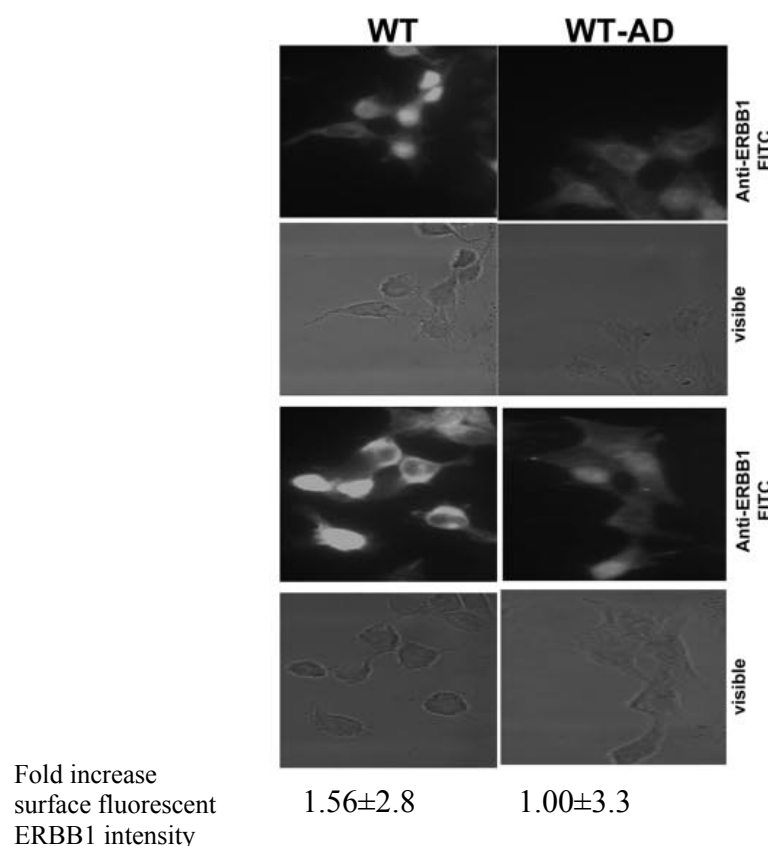


**Figure 10: WT-AD cells are resistant to effects of Taxotere.**

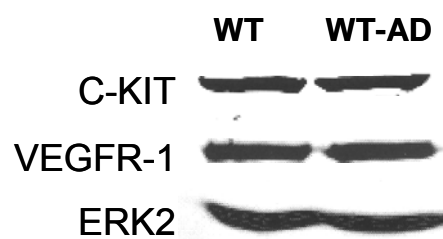
Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were treated with increasing concentrations of Taxotere (0-300nM). 48 h later, cell viability was determined by trypan blue exclusion assay. An effect of dose and an effect of cell type were observed (Two-way ANOVA,  $p < 0.0001$ ,  $n=6$ ). Bonferroni's post test revealed that with increasing concentration of Taxotere, significantly higher level of cell death was observed in WT cells compared to WT-AD cells under each condition. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



**Figure 11: Lapatinib inhibits ERBB1 and ERBB2 phosphorylation in WT and WT-AD cells.** Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium for 2 h followed by treatment with vehicle (DMSO) or lapatinib (L, 0–2  $\mu$ M), as indicated. Thirty minutes after lapatinib exposure, cells were treated as indicated with 20 ng/ml EGF. Ten minutes after EGF addition, cells were harvested and subjected to lysis, and portions of the lysate either were immunoprecipitated to isolate ERBB1 and determine ERBB1 tyrosine phosphorylation or were directly subjected to SDS-PAGE to determine total ERBB1, GAPDH and ERK2 expressions and ERK1/2 phosphorylation. A representative study is shown ( $n = 2$ ).



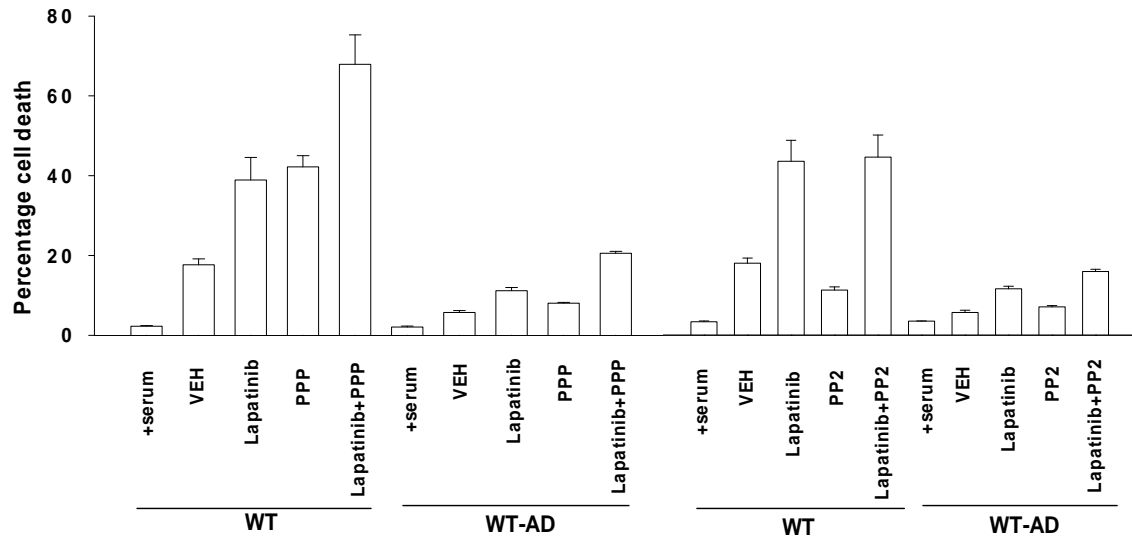
**Figure 12: WT-AD cells appear to have fewer cell surface ERBB1 receptors than WT cells.** Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium for 2 h. Cells were fixed but not permeabilized, and cell surface levels of ERBB1 were determined by immunostaining and examination under fluorescent light microscope. Two representative images from one experiment are shown. Quantitative assessment of increase in fluorescence intensity was determined from fifty cells counted over two independent experiments.



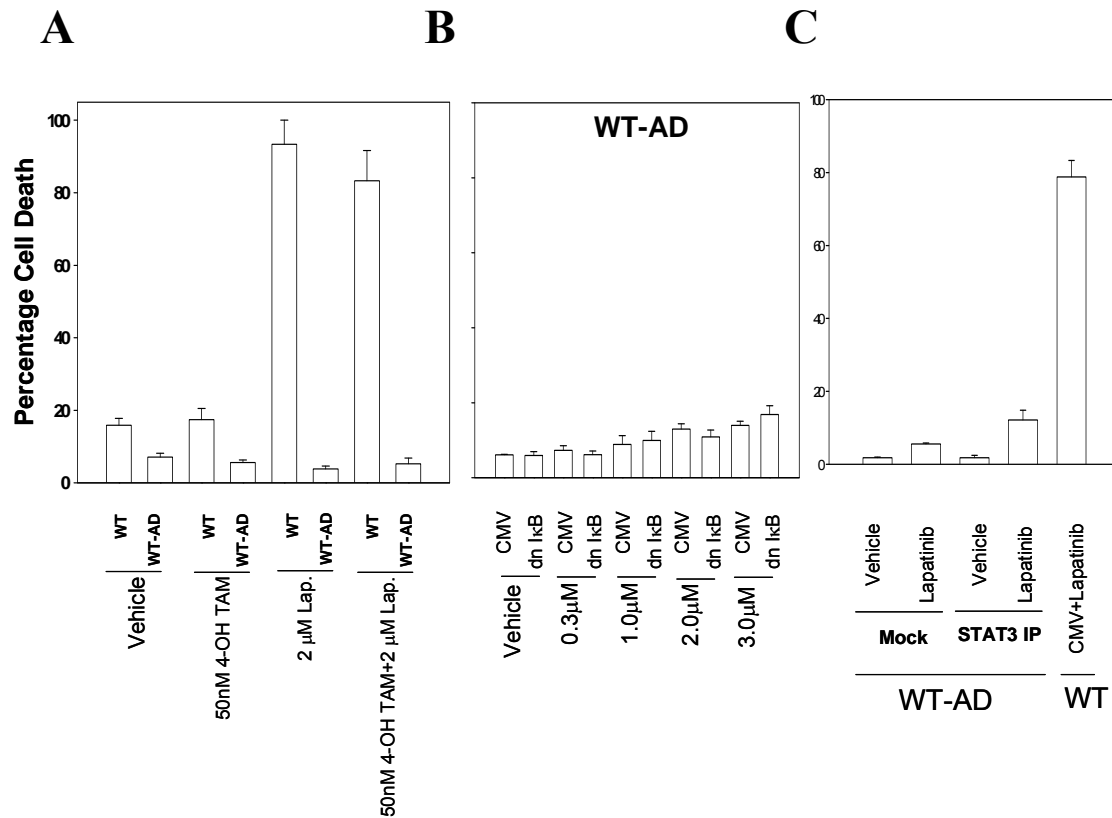
**Figure 13: No change in VEGFR and c-KIT receptor levels in WT-AD cells.**

Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h later were lysed and lysate was subjected to SDS PAGE followed by immunoblotting to determine the expression of c-KIT and VEGFR-1 receptors as well as ERK2 as a loading control.

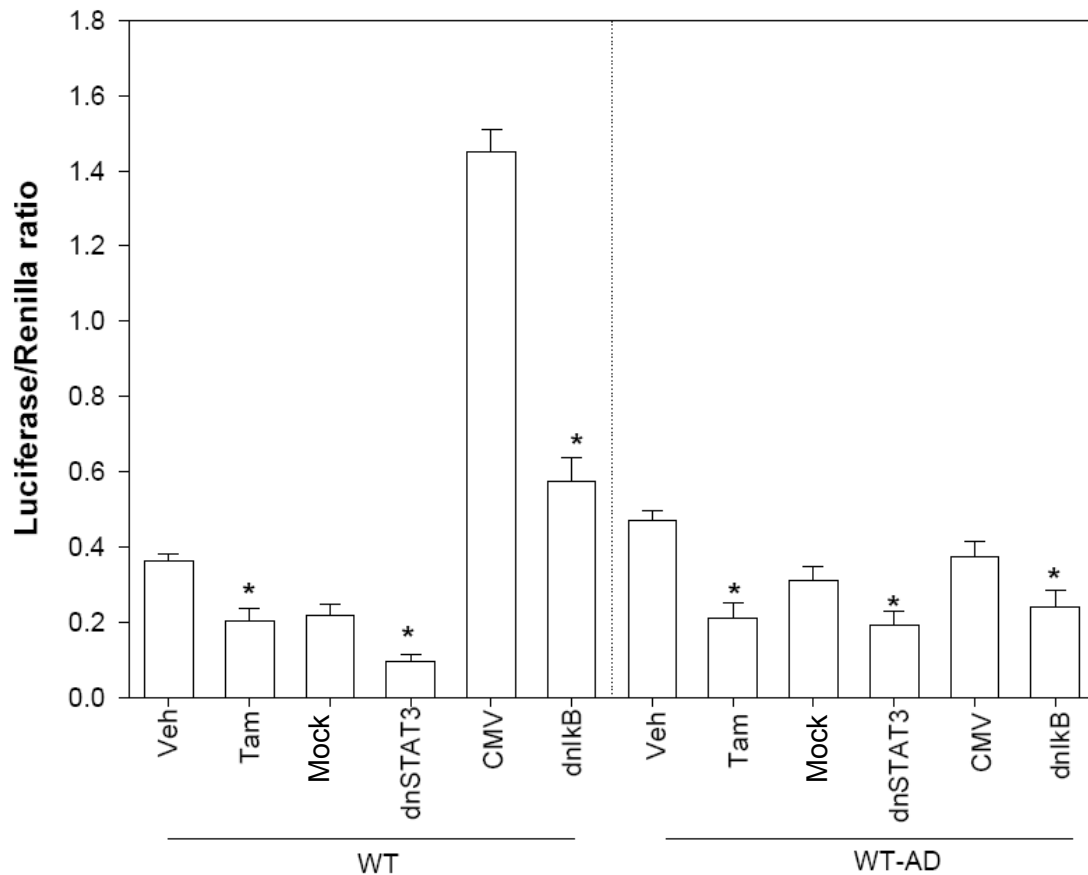




**Figure 14: Lapatinib resistance in WT-AD cells is unlikely to be mediated via Src or IGF-1R.** Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h later were grown in serum-depleted medium with vehicle (DMSO), Lapatinib (2 $\mu$ M), PP2 (10 $\mu$ M), PPP (250nM), or the drug combinations indicated. Cells were isolated 48 h after serum starvation, and cell viability was determined trypan blue exclusion assay. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



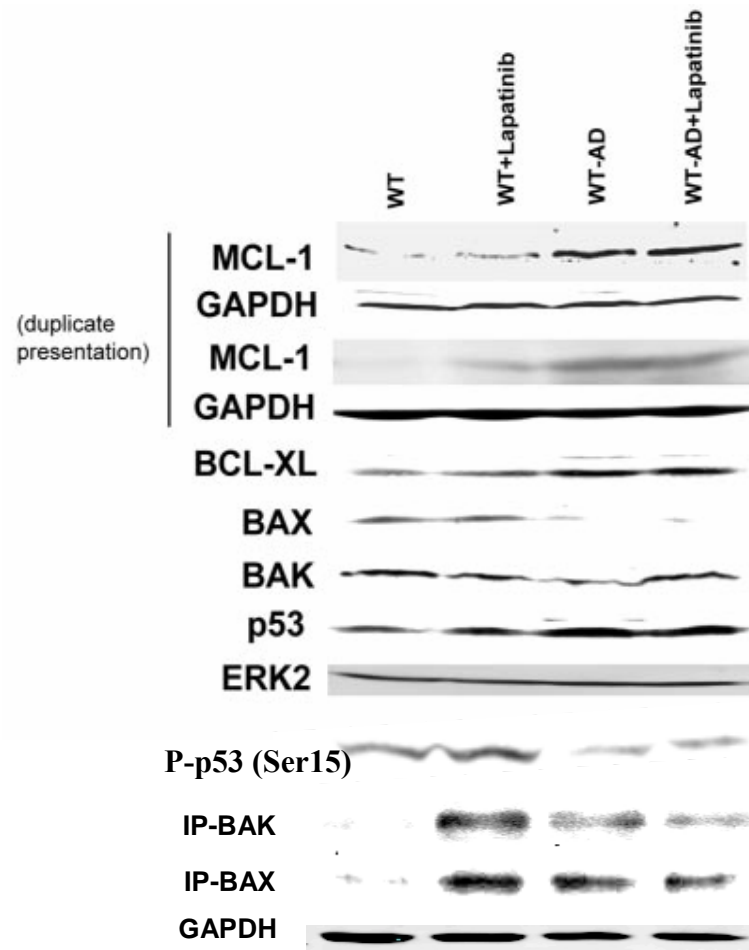
**Figure 15: Lapatinib resistance in WT-AD cells is unlikely to be mediated via estrogen receptor, NF $\kappa$ B or STAT.** A) Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted, phenol red-free medium in the presence or absence of vehicle (DMSO), 4-hydroxytamoxifen (4-OH TAM, 50 nM) or lapatinib (2 $\mu$ M); B) HCT116 lapatinib resistant cells (WT-AD) were plated and 12 h after plating were infected with either a control empty vector adenovirus or a recombinant adenovirus to express dominant-negative IkB (dnIkB). 24 h after virus infection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (0–3 $\mu$ M). Cells were isolated 48 h after drug addition, and cell viability was determined by trypan blue exclusion assay; C) HCT116 lapatinib-resistant cells (WT-AD) were plated and 12 h after plating were infected with either a control empty vector adenovirus or a recombinant adenovirus to express dominant negative STAT3 (dnSTAT3). 24 h after virus infection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (3 $\mu$ M). Cells were isolated 48 h after drug addition, and cell viability was determined by trypan blue exclusion assay. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



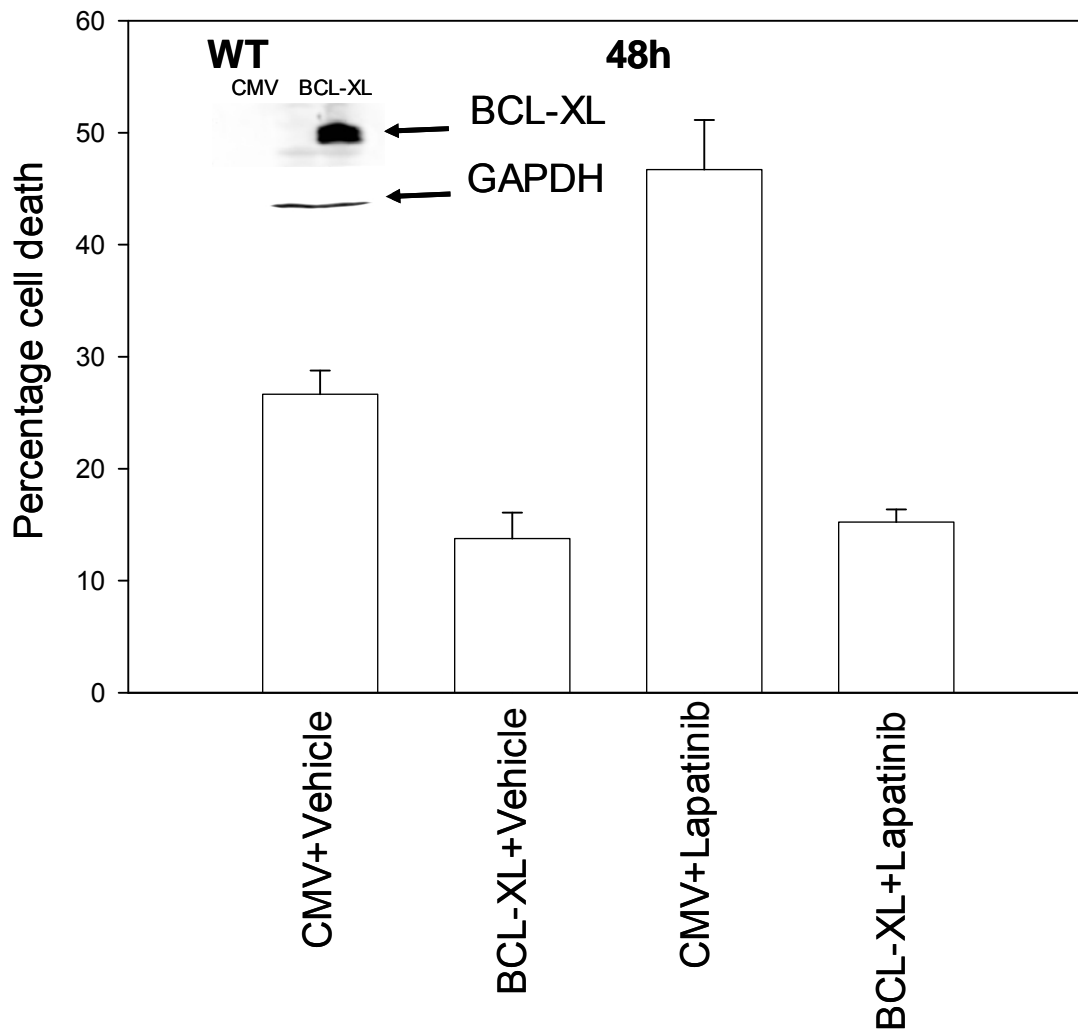
**Figure 16: Tamoxifen, STAT IP and dn IκB inhibit reporter construct activity in WT and WT-AD cells.** 24 h after plating HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) in triplicate, they were infected with vector control virus (CMV), a virus to express dominant negative IκB (dnIκB), or treated with STAT3 inhibitory peptide (STAT IP). 24h later, cells were transfected with plasmids to express firefly luciferase under NFκB or STAT promoter regulation, respectively, together with an internal control renilla luciferase plasmid under constitutive expression. Parallel triplicate sets of plates of WT and WT-AD cells in phenol red free media were plated and 24 h later were transfected to express firefly luciferase under an estrogen responsive element (ERE), together with an internal control renilla luciferase plasmid under constitutive expression. 24 h later, cells were treated with Vehicle (VEH, DMSO) or 4-Hydroxy-tamoxifen (TAM, 50nM). Promoter activity for ERE, STAT and NFκB elements was measured 24 h after transfection/tamoxifen addition and corrected for transfection efficiency using the renilla luciferase plasmid ±S.E.M. (n=3). \*,  $p < 0.05$  less than corresponding vehicle control/vector control value (Student's t-test).



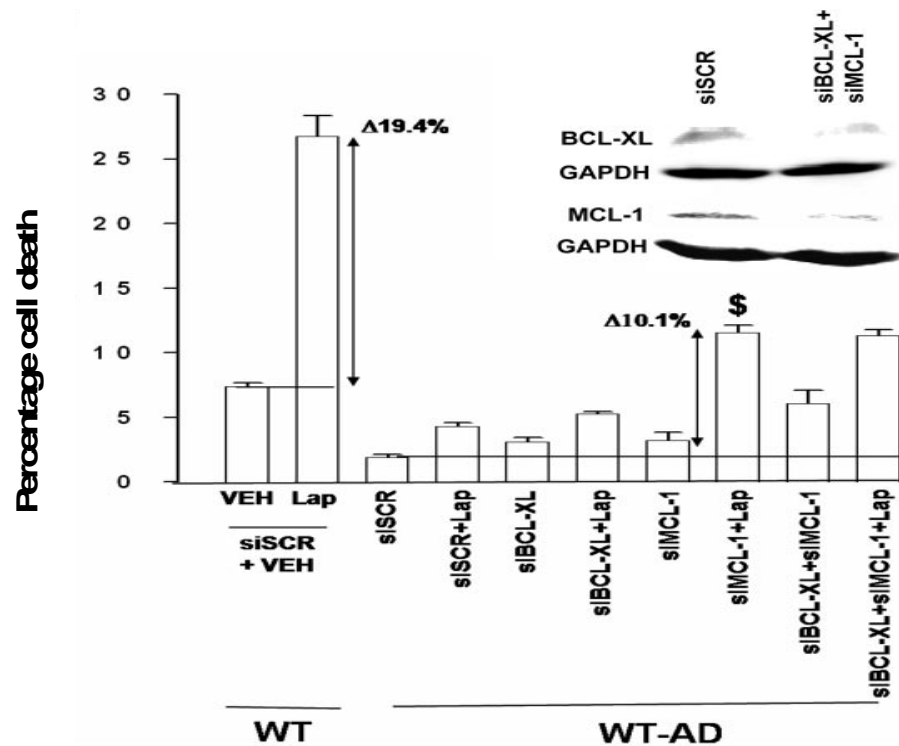
**Figure 17: Lapatinib resistance in WT-AD cells is unlikely to be mediated via MDR pumps.** 24 h after plating HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD), they were serum-starved for 24 h. Cells were then isolated and cell lysates were subjected to SDS PAGE followed by immunoblotting to determine the expression of MRP1, Pgp and ABCG2. Loading controls (GAPDH and EF1) were also measured. As a positive control for Pgp, lysates from doxorubicin resistant SW480 cells was included. Data from representative study (n=2).



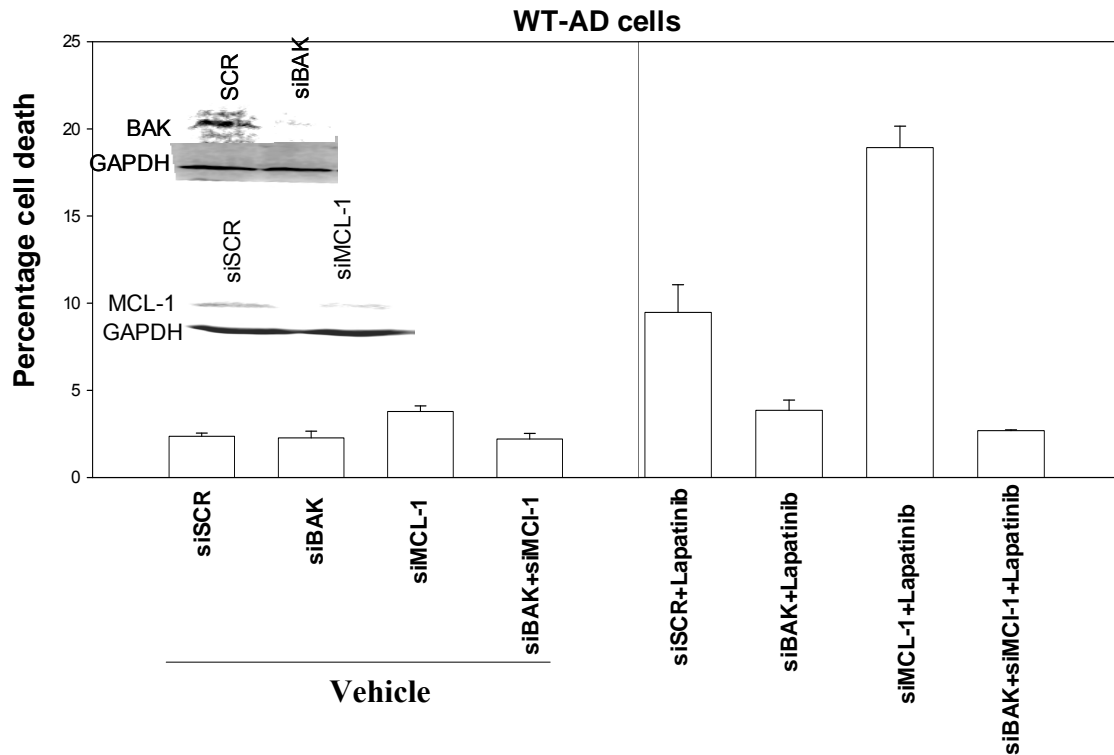
**Figure 18: Alterations in protein levels in WT-AD cells.** Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h later were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 $\mu$ M). Cells were isolated 48 h after lapatinib addition and were subjected to SDS-PAGE to determine the expression of multiple proteins. Or, cell were isolated 36 h after serum depletion/lapatinib addition, cells were isolated for immunoprecipitation to determine the amount of the activated forms of BAX and BAK. Representative images shown (n=2).



**Figure 19: Bcl-xl over-expression protects WT cells from effects of lapatinib.** Parental HCT116 cells (WT) were plated and 12 h after plating were infected with either a control empty vector adenovirus or a recombinant adenovirus to express BCL-XL. 24 h after virus infection, cells were grown in serum depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 $\mu$ M). Cells were isolated 48 h after drug addition, and cell viability was determined by trypan blue exclusion assay. One-way ANOVA (n=6) revealed that lapatinib induced significantly high cell death compared to all other conditions ( $p < 0.0001$ ) and over-expression of BCL-xl significantly protected WT cells from cell death under both basal ( $p < 0.05$ ) and lapatinib treated conditions ( $p < 0.0001$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

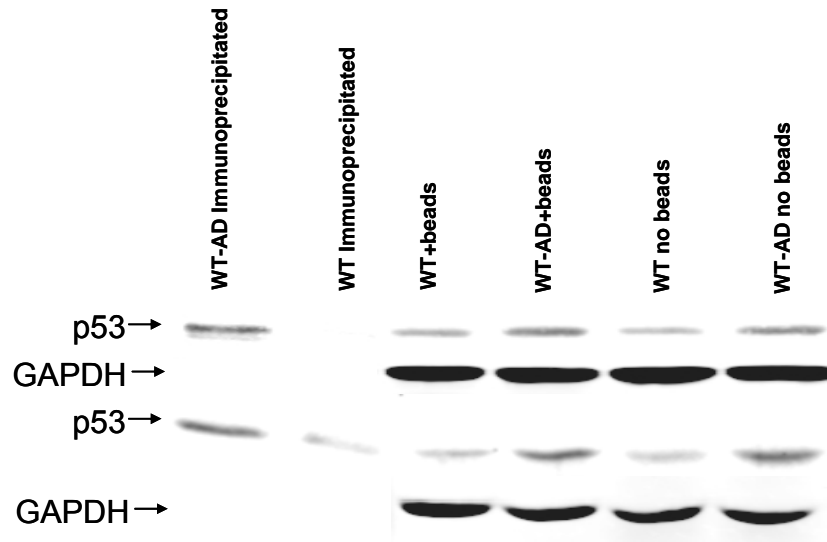


**Figure 20: Knockdown of Mcl-1 partially reverted lapatinib resistance in WT-AD cells.** Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 12 h later as indicated were transfected with siRNA molecules to reduce the expression of nothing/control (siSCR), Bcl-xl (siBcl-xl), or Mcl-1 (siMcl-1). 48 h after transfection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or Lapatinib (2 $\mu$ M) as indicated. Cells were isolated 36 h after serum starvation, and cell viability was determined by trypan blue exclusion assay. \$,  $p < 0.05$  greater than corresponding HCT116 siSCR + lapatinib cell value (Student's t-test,  $n=2$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

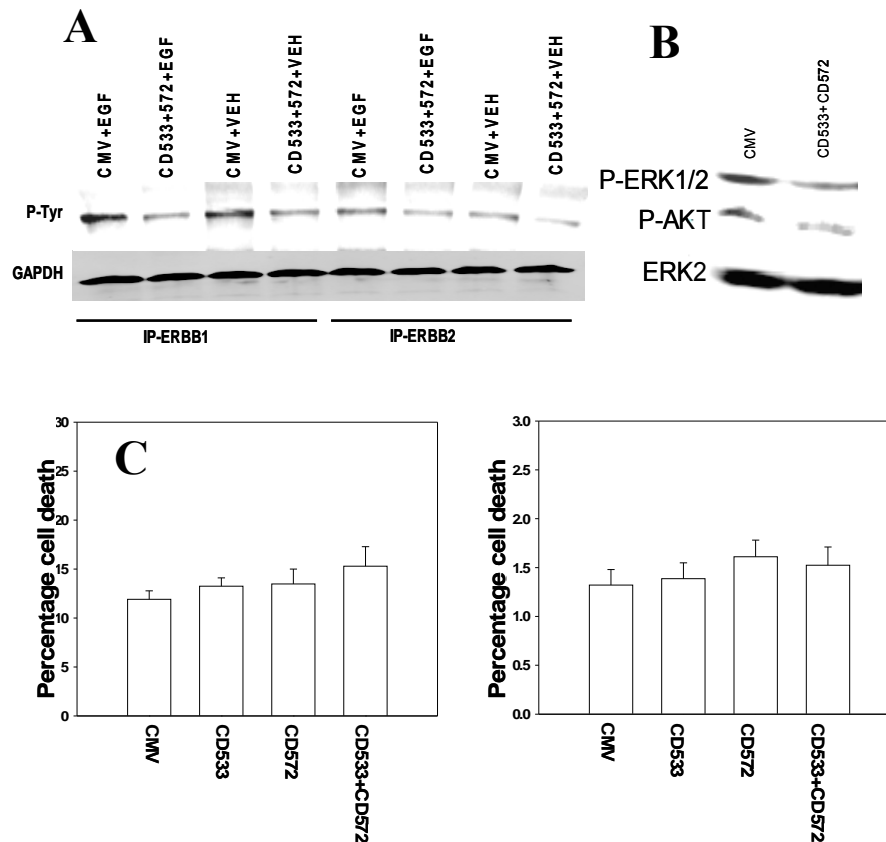


**Figure 21: In WT-AD cells, knockdown of BAK reduced reversion of resistant phenotype due to Mcl-1 knock-down.** HCT116 lapatinib-resistant cells (WT-AD) were plated and 12 h later as indicated were transfected with siRNA molecules to reduce the expression of nothing/control (siSCR), MCL-1 (siMCL-1), BAK (siBAK), or MCL-1 and BAK (siMCL+siBAK). Forty-eight hours after transfection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2  $\mu$ M). Cells were isolated 48 h after serum starvation, and cell viability was determined by trypan blue exclusion assay. One-way ANOVA (n=6) with Bonferroni's post test revealed that lapatinib induced significantly higher level of cell death compared to vehicle treated cells, knock-down of MCL-1 further significantly enhanced lapatinib lethality compared to all other conditions, knock-down of BAK significantly reduced lapatinib mediated cell death ( $p < 0.001$ ) and knock-down of BAK and MCL-1 simultaneously not only prevented lapatinib mediated cell death ( $p < 0.001$ ) but also reverted MCL-1 mediated enhancement of lapatinib induced cell death ( $p < 0.001$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

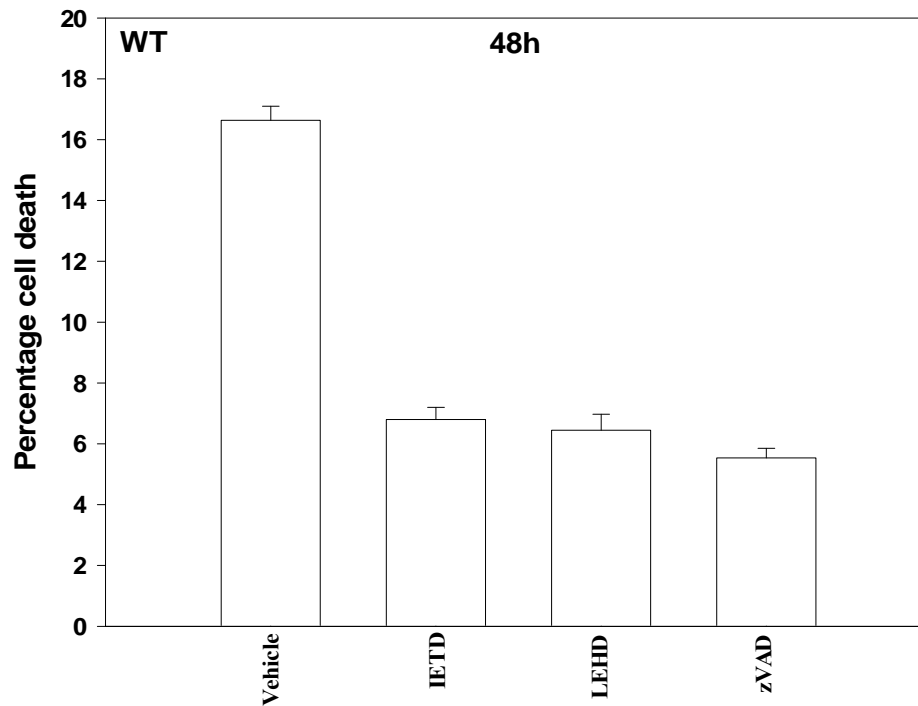




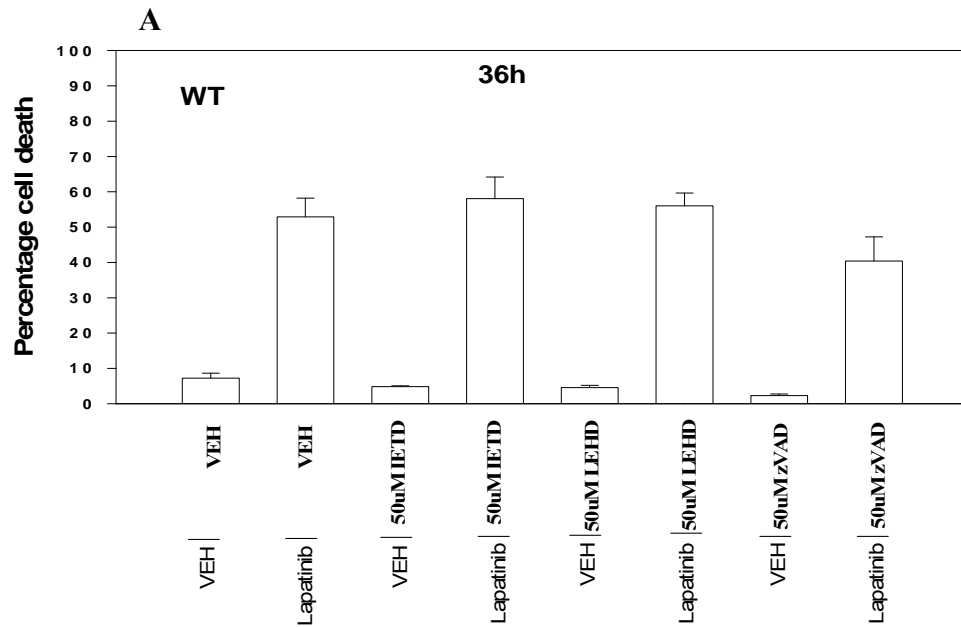
**Figure 22: “Mutant” p-53 immunoprecipitated in WT-AD cells.** Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h after plating were lysed and prepared for immunoprecipitation and pseudoimmunoprecipitation with or without mixing with agarose beads against mutated inactive p53 followed by SDS-PAGE. The mixing with or without agarose beads was to ensure as a control that no spurious effects were observed on the SDS-PAGE mobility of p53 due to agarose bead inclusion in the loading of the gel. The SDS-PAGE was transferred to nitrocellulose and probed with an anti-p53 antibody and an anti-GAPDH antibody. Two representative images shown from three separate studies.



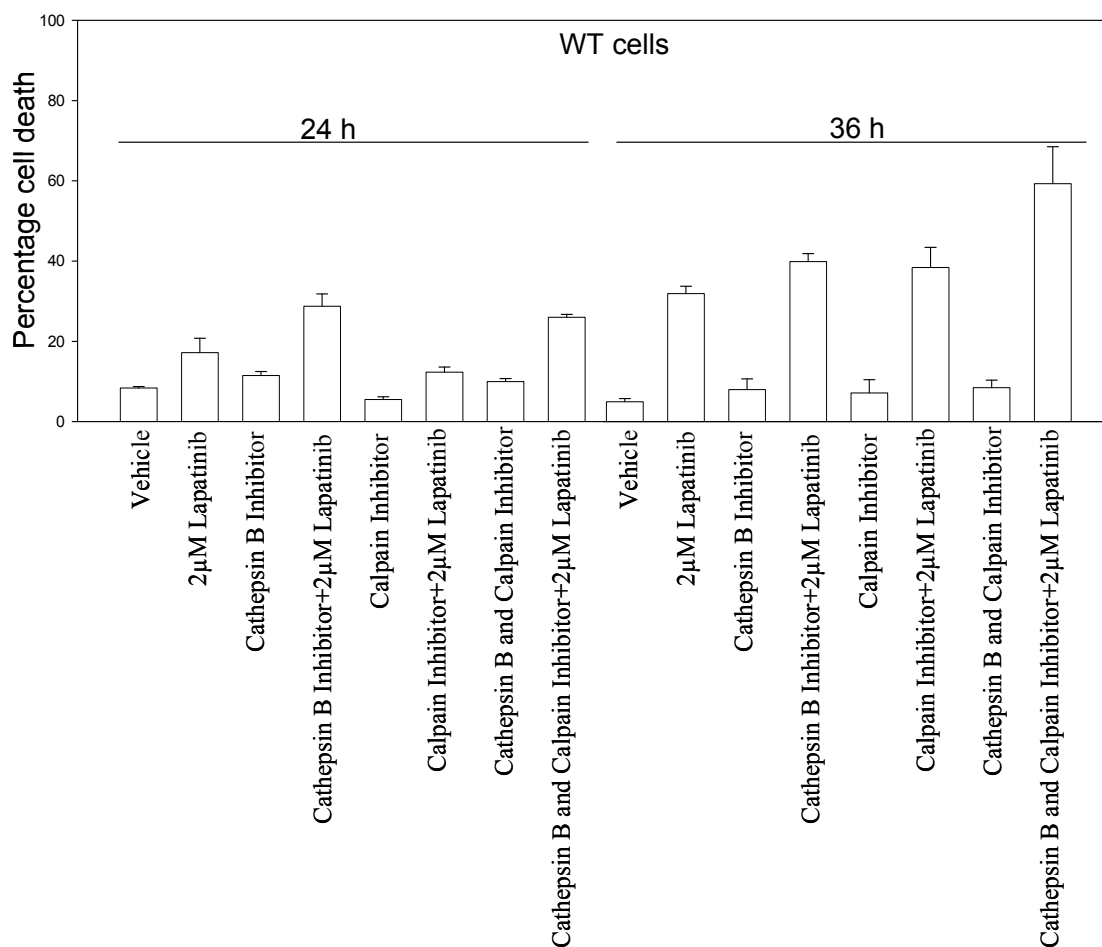
**Figure 23: Dominant negative ERBB1 and ERBB2 fail to produce lapatinib like cell death levels in WT cells.** A) Parental HCT116 cells (WT) were plated and 12 h after plating were infected with either a control empty vector adenovirus (CMV) or recombinant adenoviruses to express dominant-negative ERBB1-CD533 and dominant negative ERBB2-CD572. 24 h later, cells were grown in serum-depleted medium for 2 h and then treated with 20 ng/ml EGF as indicated for 10 min. Cells were isolated and subjected to immunoprecipitation of ERBB1 or ERBB2 followed by SDS-PAGE as indicated followed by anti-phospho-tyrosine blotting to determine the activation of ERBB1/2 proteins. A representative study is shown (n = 3). B) CD533 and CD572 also inhibit basal ERK and AKT phosphorylation. A representative study is shown (n = 2). C) 12 h after plating WT and WT-AD cells, they were infected with CMV, CD533, CD572 or both CD533 and CD572. 24h later, cells were grown in serum-depleted medium. Cells were isolated 48 h after serum starvation, and cell viability was determined by trypan blue exclusion assay. No significant effect on cell death was observed in either cell line under each condition tested (One-way ANOVA). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



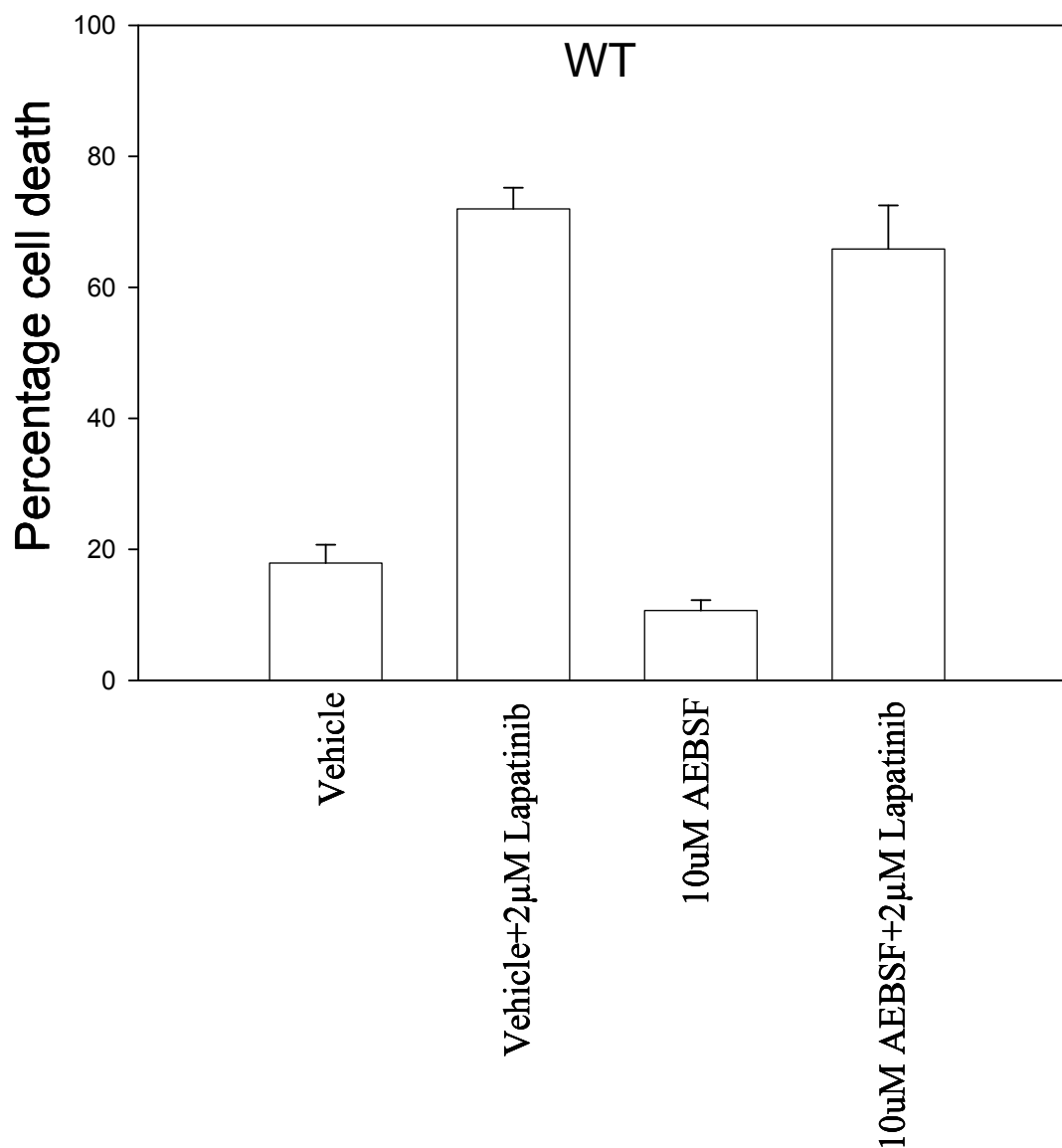
**Figure 24: Inhibition of caspases protects WT cells from serum starvation induced death.** Parental HCT116 cells (WT) were plated and 24 h later were grown in serum-depleted medium. Cells were isolated 48 h after serum-starvation and cell viability was measured by trypan blue exclusion assay. Cells were significantly protected from the effects of serum starvation in the presence of IETD (caspase 8 inhibitor), LEHD (caspase 9 inhibitor) and ZVAD (pan-caspase inhibitor) ( $p < 0.0001$ , One-way ANOVA,  $n=6$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



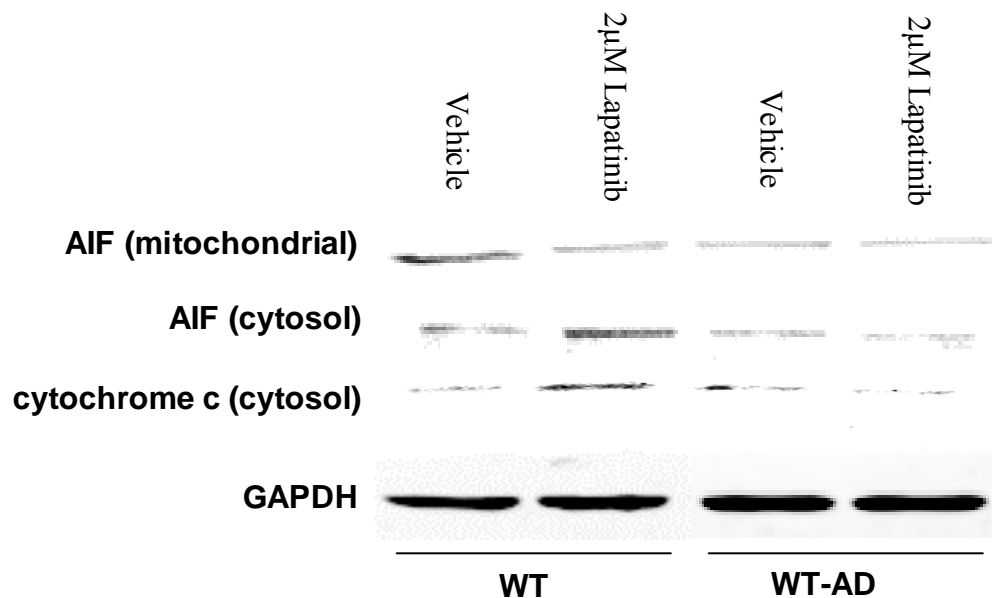
**Figure 25: Inhibition of caspases fails to protect WT cells from effects of lapatinib.** Parental HCT116 cells (WT) were plated and 24 h later were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 $\mu$ M). In parallel, cells were grown in the presence or absence of vehicle (DMSO), the caspase 8 inhibitor IETD (50 $\mu$ M), the caspase 9 inhibitor LEHD (50 $\mu$ M) or the pan-caspase inhibitor zVAD (50 $\mu$ M). Cells were isolated 36 h after serum starvation, and cell viability was determined in triplicate by trypan blue exclusion assay. One-way ANOVA with Bonferroni's post test revealed that WT cells were not protected from lapatinib induced cell death under any condition ( $p > 0.05$ ,  $n=6$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



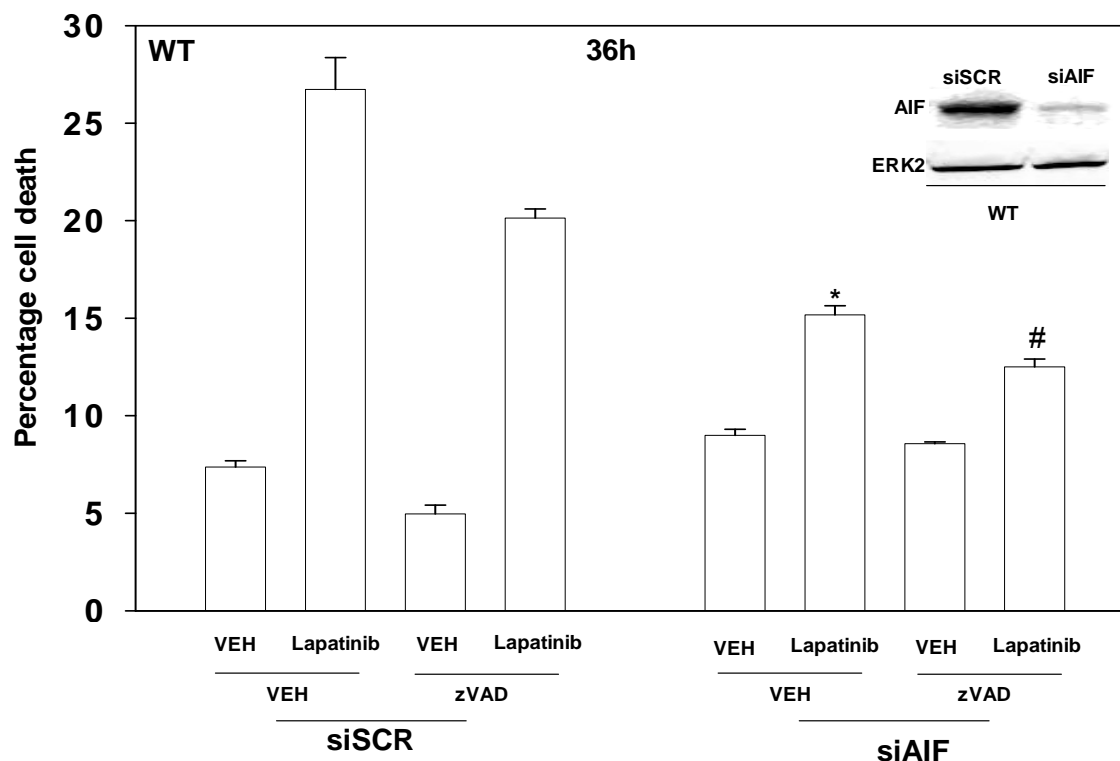
**Figure 26: Inhibition of calpains and/or cathepsins fails to protect WT cells from effects of lapatinib.** HCT116 cells (WT) were plated and treated with vehicle or lapatinib in combination with Cathepsin B and/or Calpain inhibitors. 24 or 36 h after treatment, cell viability was measured using trypan blue exclusion assay (n=6). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



**Figure 27: Inhibition of serine proteases fails to protect WT cells from effects of lapatinib.** HCT116 cells (WT) were plated and treated with vehicle or lapatinib in combination with serine protease inhibitor, AEBSF. 48 h after treatment, cell viability was measured using trypan blue exclusion assay (n=6). Data (mean ± S.E.M.) are from two independent experiments combined.



**Figure 28: Cytosolic AIF and cytochrome c release observed in WT cells treated with lapatinib.** Parental HCT116 cells (WT) and HCT116 lapatinib-resistant cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2μM). 36 h after serum depletion/lapatinib addition, cells were isolated for cell fractionation assay to determine AIF and cytochrome c release into the cytosol. A representative from two separate studies is shown.



**Figure 29: Knock-down of AIF expression in WT cells reduces lapatinib toxicity.**

Parental HCT116 cells (WT) were plated and 12 h later as indicated were transfected with siRNA molecules to reduce the expression of nothing/control (siSCR) or AIF (siAIF). 48 h after transfection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO), lapatinib (2 $\mu$ M), in parallel with or without pan-caspase inhibitor zVAD (50 $\mu$ M), as indicated. Cells were isolated 36 h after serum starvation, and cell viability was determined by trypan blue exclusion assay. \*,  $p < 0.05$  less than corresponding siSCR cell value; #,  $p < 0.05$  less than parallel siSCR value (Student's t-test,  $n=6$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



**E. Discussion:**

Development of resistance to chemotherapeutic agents is a common occurrence in the clinic (Kobayashi S, *et. al.* 2005). This study investigated the mode of lapatinib action as well as the mechanism of lapatinib resistance in HCT 116 colon cancer cells. We have shown that resistance to lapatinib is possible in colon cancer cells using cell viability as well as cell survival assays. These studies show that lapatinib resistant HCT 116 (WT-AD) cells are indeed resistant to lapatinib and also to serum-starvation (Figure 3, 4, 5, 6). It is known that refractory cancers are difficult to manage since often times they are not only resistant to the primary drug that was used to treat the cancer but also to other chemotherapeutic agents (Kobayashi S, *et. al.* 2005). To investigate whether this was true in the case of WT-AD cells, we treated WT (parental HCT116 cells) and WT-AD cells with various chemotherapeutic agents. Results showed that as expected, WT-AD cells were significantly more resistant to other chemotherapeutic agents that we tested, namely, UCN-01, VP-16 and Taxotere compared to WT cells (Figure 9,10). Another important finding was that the presence of lapatinib in the culture medium of WT-AD cells was not essential to maintain their lapatinib resistant phenotype (Figure 7). This shows that removal of lapatinib from the culture media of WT-AD cells does not restore lapatinib sensitivity in these cells.

Studies have shown that one of the mechanisms by which cells can become resistant to ERBB tyrosine kinase inhibitors is by outgrowth of cells that have a mutation in the tyrosine kinase domain such that the inhibitor can no longer bind to the receptor (Pao W, *et. al.* 2005; Sok JC, *et. al.* 2006). Our studies show that lapatinib is able to inhibit basal

and EGF stimulated levels of ERBB1 receptor phosphorylation in both WT and WT-AD cells (Figure 11). This suggests that it is unlikely that a mutation in the ERBB1 receptor, such that it can no longer bind lapatinib, is the mechanism of lapatinib resistance in WT-AD cells. These results also indicate that lapatinib intake is not disrupted in WT-AD cells and hence is not a mode of lapatinib resistance in these cells. It was noted that in spite of total ERBB1 levels in the two cell types being similar, both basal and EGF stimulated levels of ERBB1 receptor phosphorylation in WT-AD cells appeared to be lower compared to WT cells (Figure 11). To investigate the reason behind this observation, immunocytochemistry studies were performed to look at cell surface levels of ERBB1 receptors available for activation in non-permeabilized WT and WT-AD cells under serum-starved conditions. Results showed that WT-AD cells appeared to have a lower cell surface ERBB1 receptor level than WT cells (Figure 12). Hence, this can also explain why EGF treatment resulted in a lower level of ERK1/2 phosphorylation in WT-AD cells compared to WT cells.

Studies have shown that resistance to ERBB receptor inhibitors can occur due to over-expression of other receptors and tyrosine kinases that can also mediate pro-survival signals (Lu Y, *et. al.* 2001; Camirand A; Qin B, *et. al.* 2006; Xia W, *et. al.* 2006). Vascular endothelial growth factor receptors (VEGFR) are known to play a major role in regulating cell cycle in certain cells and VEGFR-1 has been found to be over-expressed in certain cancer cells (Huang Y, *et. al.* 2007; Bianco R, *et. al.* 2008). c-KIT receptors are also known to play a role in cell survival, proliferation and differentiation pathways and increased expression of this receptor in certain cancers has been linked to poor patient

prognosis (Taniguchi M, *et. al.* 1999; McIntyre A, *et. al.* 2005). To determine whether over-expression of VEGFR-1 or c-KIT receptors accounted for resistance to lapatinib in WT-AD cells, receptor expression levels were measured in WT and WT-AD cells. Results showed that there was no obvious difference in the expression levels of VEGFR-1 or c-KIT receptors between WT and WT-AD cells (Figure 13). This suggested that lapatinib resistance was not likely due to altered expression of these receptors.

In some cell types, including colon cancer cells, Src family nonreceptor tyrosine kinases and the insulin-like growth factor-1 receptor tyrosine kinase (IGF-1R) have been linked to drug resistance (Lu Y, *et. al.* 2001; Camirand A, *et. al.* 2002; Xia W, *et. al.* 2006). Hence, we wanted to investigate whether these proteins had a role to play in mediating lapatinib resistance in WT-AD cells. However, we noted that inhibition of neither Src family kinases (using the inhibitor PP2) nor IGF-1 receptor function (using the inhibitor PPP) restored lapatinib sensitivity in WT-AD cells (Figure 14). It is noteworthy that inhibition of the IGF-1 receptor using PPP caused significant toxicity in WT cells but not in WT-AD cells, arguing that WT-AD cells are also cross-resistant to agents that inhibit the function of other receptor tyrosine kinases that are known to compensate for ERBB survival signaling. As mentioned previously, constitutive activation of a variety of transcription factors such as nuclear factor  $\kappa$ B (NF $\kappa$ B) and signal transducers and activators of transcription (STAT) have been implicated in mediating drug resistance (Herrmann JL, *et. al.* 1997; Sumitomo M, *et. al.* 1999; Bewry NN, *et. al.* 2008). In order to investigate whether NF $\kappa$ B was involved in mediating lapatinib resistance, WT and WT-AD cells were infected with adenovirus that over-expressed I $\kappa$ B super-repressor. NF $\kappa$ B is usually sequestered in the

cytosol by inhibitor of  $\kappa$ B (I $\kappa$ B) (Baldwin AS. 2001). I $\kappa$ B kinase (IKK) phosphorylates I $\kappa$ B which leads to its degradation, releasing NF $\kappa$ B to perform its functions (Baldwin AS. 2001). However, the super-repressor I $\kappa$ B (S32A) lacks one of the residues that is required to be phosphorylated by IKK to allow I $\kappa$ B to release NF $\kappa$ B. In the presence of super-repressor I $\kappa$ B (S32A), NF $\kappa$ B activation is constitutively inhibited. Hence, if lapatinib resistance in WT-AD cells was due to constitutive NF $\kappa$ B activation, infection of these cells with super-repressor I $\kappa$ B would inhibit NF $\kappa$ B activation and therefore render WT-AD cells lapatinib sensitive. However, WT-AD cells maintained lapatinib resistance in spite of constitutive NF $\kappa$ B inhibition (Figure 15B). This indicated that NF $\kappa$ B was not likely involved in mediating lapatinib resistance in WT-AD cells. To investigate the involvement of STAT proteins, STAT inhibitory peptide was introduced in WT-AD cells. STAT inhibitory peptide is known to significantly lower the DNA-binding activity of STAT proteins by forming an inactive STAT:peptide complex and reduce the levels of active STAT:STAT dimers that can bind to promoter elements and induce transcription of genes involved in migration, survival and proliferation (EMD Biosciences). It was noted that treatment of WT-AD cells with STAT inhibitory peptide did not make them sensitive to the effects of lapatinib suggesting that STAT proteins were not primary candidates involved in mediating lapatinib resistance in WT-AD cells (Figure 15C).

Previous studies have shown that the estrogen receptor can mediate lapatinib resistance in BT474 breast cancer cells (Xia W, *et. al.* 2006). Some colon cancer cells have been known to express estrogen receptor (Cho NL, *et. al.* 2007; Xia W, *et. al.* 2006). Tamoxifen is a selective estrogen receptor modulator (SERM) and is thought to antagonize the estrogen

receptor in most tissues (Riggs BL and Hartmann LC. 2003). Hence, to investigate whether estrogen receptor was involved in mediating lapatinib resistance in WT-AD cells, these cells were treated with 4-hydroxy tamoxifen (one of the active metabolites of tamoxifen) in phenol-red free media since phenol-red is a weak estrogen mimic and its presence may alter the effect of 4-hydroxy tamoxifen in WT-AD cells (Reddel RR, *et. al.* 1983; Berthois Y, *et. al.* 1986). Results showed that inhibition of estrogen receptor did not revert lapatinib resistance in WT-AD cells indicating that unlike in BT474 breast cancer cells, estrogen receptor did not appear to be involved in mediating lapatinib resistance in WT-AD cells (Figure 15A) (Xia W, *et. al.* 2006). In control studies, we noted that expression of super-repressor I $\kappa$ B, treatment with STAT inhibitory peptide or 4-hydroxy tamoxifen suppressed reporter construct activity in parental and lapatinib resistant cells confirming that these agents were functional (Figure 16).

Multi drug resistance pumps (MDR pumps) have been implicated in mediating drug resistance in a variety of cancer cells as they can pump toxic drugs out of cells (Szakacs G, *et. al.* 2006). In WT-AD cells, drug efflux could be a mechanism of lapatinib resistance, particularly as we observed cross-resistance to multiple cytotoxic therapeutic drugs. Hence, we performed immunoblotting analyses to determine the expression of multidrug-resistant plasma membrane drug transporters that have been commonly implicated in mediating drug resistance in cancer cells. Comparing WT and WT-AD cells, no obvious change in the protein level of membrane drug transporters tested was observed arguing that changes in drug efflux was unlikely to be a major component of the lapatinib-resistance mechanism in WT-AD cells (Figure 17).

Since we were unable to identify candidates responsible for mediating lapatinib resistance in WT-AD cells, we decided to look at other molecules known to modulate cell death pathways. Immunoblotting analyses showed that compared to WT cells, WT-AD cells expressed higher levels of Mcl-1, Bcl-xl, p53 proteins and lower levels of pro-apoptotic protein BAX (Figure 18). Unlike WT-AD cells, lapatinib treatment in WT cells induced BAK and BAX activation (Figure 18). It was noted that total BAK levels increased in lapatinib treated WT-AD cells but no obvious increase in BAK activation was noticed under the same conditions (Figure 18). This may occur because the increased levels of pro-apoptotic proteins Bcl-xl and Mcl-1 may be able to sequester any up-regulated BAK and prevent it from being activated thereby inhibiting activation of cell death pathways. Hence, these alterations in the Bcl-2 family proteins in WT-AD cells are likely to profoundly protect these cells from toxic insults. Along the same lines, it was also noted that over-expression of anti-apoptotic protein Bcl-xl protected WT cells from the toxic effects of lapatinib (Figure 19). Based on the established concept of the so-called “apoptotic rheostat,” in which the anti-apoptotic Bcl-2 family proteins act in a dynamic balance to suppress the pro-apoptotic signals generated by proteins such as BAX and BAK, our data suggest that WT-AD cells could be resistant to lapatinib compared to WT cells because they have increased expression of the mitochondrial protective proteins Bcl-xl and Mcl-1, reduced expression of the mitochondrial toxic protein BAX and reduced activation of pro-apoptotic proteins BAK and BAX upon lapatinib treatment.

Since WT-AD cells had increased expression of anti-apoptotic Bcl-xl and Mcl-1 proteins, we wanted to investigate whether knock-down of expression of one or both of these

proteins would render WT-AD cells sensitive to lapatinib. Results showed that knock-down of Mcl-1 expression partially reverted lapatinib resistance in WT-AD cells to a greater extent than by knock-down of Bcl-xl expression (Figure 20). Recent studies have shown that over-expression of Mcl-1, but not Bcl-2 or Bcl-xl, abrogated BAK activation after exposure to ABT-737 (a Bcl-2/Bcl-xl inhibitor) and roscovitine (a cyclin-dependent kinase inhibitor), arguing that Mcl-1 plays a major role in regulating BAK function (Chen S, *et. al.* 2007). This is consistent with data demonstrating that Mcl-1 binds with greater affinity to BAK compared with Bcl-xl ( $IC_{50} < 10$  versus  $< 100$  nM) (Willis SN, *et al.* 2005). In WT-AD cells, knock-down of BAK activation significantly reduced the reversion of their resistant phenotype by reduced Mcl-1 expression (Figure 21). Thus, the mechanism of lapatinib resistance in WT-AD cells seems to be in part due to the loss of BAX expression and loss of BAK activation primarily due to over-expression of Mcl-1. Along these lines, a recently published study showed that in human H3255 non-small-cell lung cancer cells, Erlotinib (a tyrosine kinase inhibitor of ERBB1) induced mitochondria-mediated apoptosis via loss of mitochondrial membrane potential, cytosolic cytochrome c release and activation of BAK and BAX proteins (Ling YH, *et. al.* 2008).

The tumor suppressor protein p53, known as the “guardian of the genome”, can regulate the cell cycle and prevent its progression upon recognition of DNA damage or other cellular insults (Read AP and Stachan T. 1999; Bhana S and Llyod DR. 2008). p53 can also activate DNA damage repair proteins and can activate cell death cascades if the DNA damage appears to be irreparable (Offer H, *et. al.* 2002). It is known that WT cells express wild-type p53 (Zawacka-Pankau J, *et al.* 2007). In our studies to determine the mechanism

of lapatinib resistance, we noted that p53 was over-expressed in WT-AD cells and that the expression of a transcriptional target of p53, BAX, was lower in these cells compared to WT cells (Figure 18). It is known that in cancer cells, expression of p53 is often elevated when it is mutated (Kohler MF, *et al.* 1992). It was also noted that p53 phosphorylation was reduced in WT-AD cells compared to WT cells under vehicle as well as lapatinib treated conditions (Figure 18). This is important since phosphorylation of p53 has been shown to play an important role in stabilizing and activating p53 so that it can elicit an appropriate response to cellular stress (Jimenez GS, *et. al.* 1999). Hence the lack of p53 phosphorylation may suggest that WT-AD cells may be able to avoid activation of cell death pathways due to lack of appropriate activation of p53. Together, these data suggested that it was possible that HCT 116 cells expressing a wild-type p53 protein may have become lapatinib resistant by developing a p53 mutation or by selection of cells already bearing a p53 mutation. In agreement with this hypothesis, WT-AD cells but not WT cells expressed a p53 protein that could be immunoprecipitated by an antibody that specifically recognizes p53 protein mutated in its DNA binding domain (Figure 22). This antibody differentiates between wild-type and mutant p53 under non-denaturing conditions since a mutation in the DNA binding domain of p53 can alter its tertiary structure such that it allows exposure of an epitope that can be recognized by this antibody (Gannon JV, *et. al.* 1990). This epitope is usually hidden and therefore not exposed to the antibody in the correctly folded structure of wild-type p53 protein. To further identify whether there existed a mutation in p53 in WT-AD cells, gene sequencing was performed. However, upon sequencing the coding regions in the DNA binding domains of p53, no mutations



were noted in WT-AD cells. This suggests that our antibody was recognizing an alteration in p53 tertiary conformation in WT-AD cells unrelated to a p53 DNA binding domain mutation but that was in all likelihood still suppressing p53 function (i.e., reduced BAX expression) or that p53 mutation had occurred in a domain unrelated to the DNA binding domain of p53 but that was affecting the tertiary conformation of the DNA binding domain.

Further studies are required to understand how p53 function, with respect to modulation of all p53 targets has been altered in lapatinib resistant HCT116 cells.

Next, we wanted to determine whether lapatinib mediated toxicity in WT cells depended solely on inhibition of ERBB1 and ERBB2 receptors and their downstream pro-survival pathways. Since lapatinib is thought to mediate its effects by inhibition of ERBB1 and ERBB2 function, we wanted to mimic this effect using adenovirus-mediated expression of dominant negative ERBB1 (CD533) and dominant negative ERBB2 (CD572) proteins (Wood ER, *et. al.* 2004; Rusnak DW, *et. al.* 2001). CD533 and CD572 function as dominant negative ERBB1 and ERBB2 receptors, respectively because they have truncated C-terminal kinase domains, therefore forming non-functional dimers with wild-type receptors (Imai K and Takaoka A. 2006; Schmidt-Ullrich RK, *et. al.* 2003). In spite of being able to inhibit ERBB1 and ERBB2 receptor phosphorylation similar to lapatinib in WT cells, the dominant-negative receptors did not recapitulate the toxic effects of lapatinib in serum-starved WT or WT-AD cells (Figure 23). This suggests that there are possibly other targets that lapatinib inhibits/interacts with that may play an important role in inducing toxic effects mediated by lapatinib.

Since we observed changes in the expression of proteins that act at the mitochondrion to modulate mitochondrial stability, we next determined whether activation of caspase proteases played a role in lapatinib toxicity. Caspases are known to activate cell death pathways by activating pro-apoptotic Bcl-2 family members that can then alter mitochondrial integrity or vice versa (Gross A, *et. al.* 1999). Serum-starvation has been known to induce cell death by activation of caspases (Kilic M, *et. al.* 2002). Inhibition of caspase 8 and caspase 9 using IETD and LEHD (inhibitors for the respective enzymes), as well as using zVAD (a pan-caspase inhibitor), significantly reduced the level of cell death induced by serum-starvation in WT cells (Figure 24). However, inhibition of caspase 8, caspase 9 or pan-inhibition of caspase function did not suppress lapatinib toxicity in serum-starved WT cells (Figure 25). This suggested that lapatinib mediated cell death involves caspase-independent mechanisms. Since other enzymes such as calpains, cathepsins and serine proteases have also been implicated in activating cell death pathways, we wanted to determine whether these factors were involved in lapatinib mediated cell death (Vandenabeele P, *et. al.* 2005; Droga-Mazovec G, *et. al.* 2008; Broker LE, *et. al.* 2004). However, inhibiting these enzymes did not significantly protect WT cells from lapatinib induced cell death, indicating that these enzymes are not prime mediators of lapatinib toxicity in WT cells (Figure 26, 27).

To investigate caspase-independent mechanisms of lapatinib mediated cell death, we decided to focus on apoptosis inducing factor (AIF). AIF is normally present in the inter-membrane space of the mitochondrion, tethered to the inner mitochondrial membrane (Otera H, *et. al.* 2005). In certain stressful situations, AIF is cleaved, released into the

cytosol upon mitochondrial outer membrane permeabilization (MOMP) and it then makes its way to the nucleus where it can initiate DNA fragmentation (Modjtahedi N, *et. al.* 2006; Penninger JM and Kroemer G. 2003). Cell fractionation studies showed that AIF was released into the cytosol of WT cells treated with lapatinib but a similar increase in cytosolic release of AIF was not observed in WT-AD cells under the same treatment conditions (Figure 28). Similar results were observed for cytochrome c although cytochrome c is typically thought to be involved in caspase-dependent cell death pathways (Figure 28). However, it has been suggested that cytochrome c release can occur in caspase independent cell death due to MOMP and this can occur after AIF release because electrostatic interactions with cardiolipin can retain cytochrome c at the inner mitochondrial membrane and hence, delay its release (Uren RT, *et. al.* 2005). Knock-down of AIF expression in WT cells protected them from lapatinib mediated toxicity and knock-down of AIF expression combined with pan-caspase inhibition further reduced cell death induced by lapatinib in these cells (Figure 29). These results suggested that AIF is a key player in lapatinib mediated cell death in WT cells and failure of AIF cytosolic release in lapatinib treated WT-AD cells possibly protected them from cell death.

Our results show that an alteration in the Bcl-2 family proteins has an important role to play in mediating lapatinib resistance in HCT 116 cells. Future studies involving Bcl-2/Bcl-xl/Mcl-1 antagonists such as obatoclax, combined with lapatinib will need to be performed in order to determine whether such drugs will enhance the lethality of lapatinib as well reduce the chances of occurrence of lapatinib resistant cancers. Also, our studies

suggest that the mechanism of lapatinib mediated toxicity in WT cells may involve effects of lapatinib on targets other than ERBB1 and ERBB2.

## **OBATOCLAX ENHANCES SORAFENIB+HDACI TOXICITY AND OVERCOMES BLOCKADE OF CD95 PATHWAY TO FACILITATE KILLING**

### **A. Introduction:**

The Raf-MEK1/2-ERK1/2 pathway is frequently dysregulated in neoplastic transformation (Dent P, *et. al.* 2003; Valerie K, *et. al.* 2007). The MEK1/2-ERK1/2 module comprises, along with c-Jun NH<sub>2</sub>-terminal kinase (JNK1/2) and p38 MAPK, members of the MAPK super-family. These kinases are involved in responses to diverse mitogens and environmental stresses and have also been implicated in cell survival processes. Activation of the ERK1/2 pathway is often associated with promoting cell survival whereas JNK1/2 and p38 MAPK pathway signaling often induces apoptosis. Although the mechanisms by which ERK1/2 activation promote survival are not fully characterized, a number of anti-apoptotic effector proteins have been identified, including increased expression of anti-apoptotic proteins such as c-FLIP, Bcl-xl and Mcl-1 and also direct inactivation of pro-apoptotic proteins such as caspase 9, BAD and BIM (Grant S and Dent P. 2004; Allan LA, *et. al.* 2003; Mori M, *et. al.* 2003; Ley R, *et. al.* 2003; Wang WF, *et. al.* 2007; Qiao L, *et. al.* 2003). In view of the importance of the RAF-MEK1/2-ERK1/2 pathway in neoplastic cell survival, inhibitors have been developed that have entered clinical trials, such as sorafenib (4-[4-[[4-chloro-3-(trifluoromethyl)phenyl] carbamoylamino] phenoxy]-N-methyl-pyridine-2-carboxamide, Bay 43-9006, Nexavar®; a Raf kinase inhibitor) (Li N, *et. al.* 2007). Sorafenib is a multi-kinase inhibitor that was originally developed as an inhibitor of Raf-1 (c-Raf). Sorafenib occupies the ATP-binding domain of Raf-1 and B-Raf, thereby preventing ATP from binding the kinase (Wan P, *et. al.* 2004). Subsequently however,

sorafenib was shown to inhibit multiple other kinases, including platelet-derived growth factor, vascular endothelial growth factor receptors 1 and 2, c-Kit and FLT3 (14).

Sorafenib is also known to inhibit mutant <sup>V559E</sup>B-Raf (Wilhelm S and Chien DS. 2002). It is important to note that sorafenib did not significantly increase cell death in non-transformed cells (Rosato RR, *et. al.* 2007). Anti-tumor effects of sorafenib in renal cell carcinoma and in hepatoma have been ascribed to anti-angiogenic actions of this agent through inhibition of the growth factor receptors (Rini BI. 2006; Gollob JA. 2005).

Sorafenib has also been shown to inhibit tumor cell proliferation and tumor xenograft growth (Wilhelm SM, *et. al.* 2004). Previous studies have shown *in vitro* that sorafenib kills human leukemia cells at concentrations below the maximum achievable dose ( $C_{max}$ ) of 15-20  $\mu$ M, through a mechanism involving down-regulation of the anti-apoptotic Bcl-2 family member Mcl-1 (Rahmani M, *et. al.* 2005; Rahmani M, *et. al.* 2007a). In these studies sorafenib-mediated Mcl-1 down-regulation occurred through a translational rather than a transcriptional or post-translational process that was mediated by endoplasmic reticulum stress signaling (Dasmahapatra G, *et. al.* 2007; Rahmani M, *et. al.* 2007b).

Hence, previously observed anti-tumor effects of sorafenib are mediated by a combination of inhibition of Raf family kinases; receptor tyrosine kinases that signal angiogenesis; and the induction of ER stress signaling.

There are 18 known histone deacetylases (HDACs) in humans and these can be divided into three classes: Class I include HDAC1, HDAC2, HDAC3 and HDAC8; Class II include HDAC4, HDAC5, HDAC7 and HDAC9. HDAC6 and HDAC10 belong to Class IIa and have two catalytic sites. HDAC11 shares characteristics with both Class I and Class

II and hence is often placed in Class IV. Class III HDACs include sirtuins that require  $\text{NAD}^+$  for their activity, lack zinc in their catalytic sites and are not inhibited by commonly used HDACIs such as vorinostat (suberoylanilide hydroxamic acid, SAHA, Zolinza<sup>TM</sup>) (Dokmanovic M, *et. al.* 2007; Marks P, *et. al.* 2001). HDACs, along with histone acetyltransferases (HATs), reciprocally regulate the acetylation status of the positively charged  $\text{NH}_2$ -terminal histone tails of nucleosomes there by regulating transcription of genes (Gregory PD, *et. al.* 2001). HDACs can act not only on histones but on other proteins as well such as p53. Acetylation of p53 can lead to increased sequence specific binding activity and hence increased transcription of p53 target genes (Tang Y, *et. al.* 2008). HDACs can deacetylate p53 which can decrease the ability of p53 to transcribe target genes (Glozak MA, *et. al.* 2005). Alterations in HATs and HDACs have been found in a variety of cancers. Alterations in expression levels of HDACs have been observed in certain cancers; however, structural mutations in HDACs are a rare event in cancer cells (Bolden JE, *et. al.* 2006; Marks P, *et. al.* 2001; Dokmanovic M, *et. al.* 2007). Aberrant activity of HDACs in cancer cells is often associated with HDACs being recruited by oncogenic translocation protein complexes (Wilson AJ, *et. al.* 2006). Histone deacetylase inhibitors (HDACI) represent a class of agents that act by blocking histone de-acetylation, thereby modifying chromatin structure and gene transcription. HDAC inhibitors (HDACIs) promote histone acetylation and neutralization of positively charged lysine residues on histone tails, allowing chromatin to assume a more open conformation, which favors transcription (Gregory PD, *et. al.* 2001). Most HDACIs are thought to function by binding to the zinc atom in the HDAC catalytic site thereby inhibiting the enzyme's activity

(Richon VM. 2006; Taddei A, *et. al.* 2005). However, HDACIs have also been known to have pleiotropic biological consequences, including inhibition of chaperone HSP90 function (thought to be required for survival of oncogenic fusion proteins), induction of oxidative injury and up-regulation of death receptor expression (Dasmahapatra G, *et. al.* 2007; Marks PA, *et. al.* 2003; Bali P, *et. al.* 2005; Kwon SH, *et. al.* 2002).

Vorinostat is a hydroxamic acid HDACI that has shown preliminary pre-clinical evidence of activity in hepatoma and other malignancies with a  $C_{max}$  of  $\sim 9 \mu M$  (Wise LD, *et. al.* 2007; Zhang G, *et. al.* 2008). Vorinostat is a pan-inhibitor of HDACs belonging to classes I and II and has been known to induce growth arrest in a variety of transformed cells (Marks PA and Breslow R. 2007; Mitsiades CS, *et. al.* 2004). Vorinostat was also found to be efficacious in vivo by inhibiting the growth of human cancer xenografts (Marks PA, *et. al.* 2005). Vorinostat is known to increase reactive oxygen species (ROS) in cancer cells but not in normal cells. In addition, vorinostat can increase the levels of thioredoxin binding protein-2 in cancer cells thereby reducing thioredoxin (known to have ROS scavenging function) levels and this may facilitate ROS mediated cell death in cancer cells (Marks PA and Breslow R. 2007). This can explain, at least in part, the relative resistance to vorinostat toxicity in normal cells and the sensitivity of many cancer cells.

Valproic acid is used as an anti-epileptic drug and also as a mood-stabilizer (Robert E, 1991). It has been known to have teratogenic effects that have now been linked to its ability to inhibit HDAC activity which is also thought to be responsible for its ability to inhibit tumor growth, proliferation, angiogenesis, induction of differentiation and radiosensitization in certain cancer cells (Alsdorf R and Wyszynski DF. 2005; Cinatl J. Jr.,



*et. al.* 1997; Duenaz-Gonzalez A, *et. al.* 2008). Valproic acid administered alone or in combination with other agents such as cytosine arabinoside, hydroxyurea, cisplatin and etoposide has been shown to be effective in killing cancer cells (Blaheta RA, *et. al.* 2005; Gupta E, *et. al.* 1997). Valproic acid is a much weaker inhibitor of HDAC activity than vorinostat and is known to inhibit the function of Class I (1, 2, 3) and Class II (4, 5, 7) HDACs (Xu W, *et. al.* 2007; Gurvich N, *et. al.* 2004).

With respect to combinatorial drug studies with a multi-kinase inhibitor such as sorafenib, HDACIs are of interest in that they have potential to down-regulate multiple oncogenic kinases by interfering with HSP90 function, leading to proteasomal degradation of these proteins. Sorafenib and HDACIs target multiple overlapping downstream signaling pathways implicated in tumor cell survival. Hence, if combined, there was a possibility that the two drugs administered together would be more effective than either drug individually and also that lower doses of these agents may be required to observe toxic effects on cancer cells. Previous studies have shown that low doses of sorafenib and vorinostat can act synergistically in a variety of cancer cells (Zhang G, *et. al.* 2008; Park MA, *et. al.* 2008). This drug combination is known to activate the de novo ceramide synthesis pathway which along with the acidic sphingomyelinase ceramide generation pathway initiates CD95 death receptor activation. Hence, according to previous studies, sorafenib and vorinostat mediated cell death is thought to occur via ligand independent but ceramide dependent activation of the CD95 death receptor signaling pathway and this drug combination is now entering Phase I clinical trials (Zhang G, *et. al.* 2008; Park MA, *et. al.* 2008). It is important to note that previous studies have shown that unlike cancer cells,

sorafenib and vorinostat combined failed to cause significant cell death in normal bone marrow cells (Dasmahapatra G, *et. al.* 2007). Studies have shown that sorafenib interacts with other HDACI (such as Trichostatin A and sodium butyrate) as well to markedly increase lethality in cancer cells (Dasmahapatra G, *et. al.* 2007).

This study investigated whether low dose of sorafenib combined with sodium valproate (another HDACI, sodium salt of valproic acid) also acted synergistically to kill cancer cells and whether CD95 death receptor activation had a role to play in mediating cell death under these conditions.

Several cancers are known to lose CD95 expression/function and this can enable cells to become resistant to chemotherapeutic agents that utilize the CD95 signaling pathway to mediate cell death (Ozoren N and El-Deiry WS. 2003; Los M, *et. al.* 1997; Houston A and O'Connell J. 2004). Increased expression of mitochondrial protective proteins such as Mcl-1 and Bcl-xl have also been implicated in mediating drug resistance in cancer cells (Martin AP, *et. al.* 2008; Del Poeta G, *et. al.* 2008). Since previous studies show that sorafenib and HDACI combine synergistically to cause cell death via CD95 signaling, it was possible that in the clinic, resistance to these agents may occur via dysregulation of the CD95 signaling pathway. Hence, it would be logical to combine sorafenib and HDACIs with an agent that facilitated cell death independent of CD95 by targeting the mitochondrial protective proteins and could synergize with the two drugs to result in enhanced cell death. Theoretically, this would reduce the likelihood of tumor cells being inherently resistant as well as potentially circumventing the development of drug resistant cancers.

Obatoclax (GX 15-070) is a small molecule antagonist of the BH-3 binding domain of the Bcl-2 family proteins and is known to mediate its effects in cancer cells independent of the CD95 pathway via inhibition of Mcl-1-BAK interaction, BIM upregulation and cytochrome c release into the cytosol (Trudel S, *et. al.* 2007). Short term toxicity assays showed that obatoclax has minimal effects on normal human peripheral blood lymphocyte viability where as significant reduction in cell viability was observed in obatoclax treated cancer cells (Trudel S, *et. al.* 2007). Hence, we investigated whether obatoclax enhanced the toxicity of sorafenib and sodium valproate and whether this three-drug combination maintained high cell death levels in spite of knock-down of CD95 in cancer cells.

#### **B. Materials:**

Sorafenib tosylate was generously provided by Bayer Inc. Vorinostat was generously provided by Merck & Co., Inc. GX15-070 was generously provided by Gemin X Pharmaceuticals. Trypsin-EDTA, DMEM, RPMI, penicillin-streptomycin were purchased from GIBCOBRL (GIBCOBRL Life Technologies, Grand Island, NY). Sodium valproate was purchased from Sigma. Fetal bovine serum was purchased from hyclone (Logan, UT). Trypan blue dye and crystal violet were purchased from Sigma-Aldrich. For western blot analysis, 8-16% gels were used (BIORAD, Carlsbad, CA). HEPG2, HEP3B and PANC1 cells were purchased from the ATCC. Commercially available validated short hairpin RNA molecules to knock down RNA/protein levels were purchased from Qiagen (Valencia, CA). Primary antibodies recognizing BAX, BAK, BIM, Mcl-1 and cytochrome c were purchased from Cell Signaling (San Diego, CA). Primary antibody for active BAK (Ab-1) was purchased from Calbiochem (San Diego, CA). Primary antibodies for GAPDH,

active BAX (6A7) and protein A/G plus agarose beads for immunoprecipitation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary mouse antibody was purchased from Invitrogen Molecular Probes (Eugene, OR) and secondary rabbit antibody was purchased from Rockland (Gilbertsville, PA). All siRNAs were purchased from Qiagen. C-FLIP-s virus was purchased from Vector Biolabs. DAPI stain was purchased from Vector Laboratories Inc (Burlingame, CA).

### **Methods:**

**Cell Culture:** Panc1 pancreatic cancer cells were cultured in DMEM media containing DMEM media containing 20% fetal bovine serum and 1% Penicillin/Streptomycin.

HEPG2 and HEP3B hepatic cancer cells were culture in MEM Alpha media containing 10% fetal bovine serum and 1% Penicillin/Streptomycin supplemented with sodium pyruvate, sodium bicarbonate and non-essential amino acids.

**Detection of Cell Death by Trypan Blue Assay:** After treatment, medium was removed and cells were washed in 1X PBS. Cells were then harvested by trypsinization with Trypsin/EDTA for ~5 min at 37°C. Because some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1400 RPM for 5 min. The pooled cell pellets were resuspended and mixed with trypan blue dye. Trypan blue stain, in which blue dye-incorporating cells were scored as being dead, was performed by counting of cells using a light microscope and a hemacytometer. The number of dead cells was counted and expressed as a percentage of the total number of cells counted.

**Culture of Cells and Drug Treatments for Colony Formation Assays:** Cells were plated (250–1000 cells/well of a 6-well plate). 12 h after plating medium was removed and serum-free medium was added to the cells for 24 or 48 h as indicated. After this, the serum-free media was carefully removed and fresh media (with serum) was added. Colony formation assays were cultured for an additional 8-10 days, after which the media were removed, cells were fixed with methanol, stained with crystal violet, and counted manually.

**Immunoprecipitation and Western Blotting:** 12 hours after plating cells, they were treated with various drugs as indicated. 12, 24 or 48 h after drug treatment, cells were lysed in whole cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1%  $\beta$ -mercaptoethanol, 0.02% bromophenol blue).

12 hours after plating cells, they were treated with drugs as indicated and 24 h after drug treatment, cells were scraped in CHAPS buffer (10mM HEPES, 140mM NaCl, 1% CHAPS) and then active BAK, active BAX or Mcl-1 was immunoprecipitated. Samples were boiled for 10 min in whole cell lysis buffer. All samples were then loaded on 8%-16% Criterion pre-cast gels (BIORAD) after normalizing total protein and run for about 2 hours. Proteins were then electrophoretically transferred onto 0.22um nitrocellulose membranes and immunoblotted with various primary antibodies as indicated.

**Virus Infections:** Cells were infected 12h after plating with adenoviruses at an approximate multiplicity of infection of 30 for 4 h with gentle rocking, after which time the media was replaced. Cells were further incubated for 24 h to ensure adequate expression of transduced gene products before drug exposures.

**Transfection of Cells with Small Interfering RNA Molecules:** RNA interference for down-regulating the expression of various molecules was performed using validated target sequences designed by Qiagen. For transfection, 20-40 nM concentration of the annealed siRNA-targeting Mcl-1, Bcl-xl or Bcl-2, or the negative control (a "scrambled" sequence with no significant homology to any known gene sequences from mouse, rat, or human cell lines) were used. The siRNA molecules were transfected into cells according to the manufacturer's instructions. Cells were cultured for 24 h after transfection before any additional experimentation.

**Cell Fractionation:** 12h after plating cells, they were treated with drugs as indicated. 12 or 24 h later, medium from plates was aspirated and cells were scraped in buffer (75mM NaCl+8mMNa<sub>2</sub>HPO<sub>4</sub>+1mMNa<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>+0.5mMEDTA+ 0.5mMEGTA with freshly added 350ug/ml digitonin, 250mM sucrose, protease and phosphatase inhibitor cocktails (Roche)) and passed through a 25 gauge needle 10 times. After 15 to 30 minutes on ice, cells were spun down at 5000RPM for 1.5 minutes at 4°C to remove cell debris. Pellet was discarded and supernatant was transferred to a new tube and spun down at 13000 RPM for 25 minutes at 4°C. The supernatant obtained is the cytosolic fraction where as the pellet is the mitochondrial fraction. Whole cell lysis buffer was added to the supernatant and the pellet, boiled for 10 minutes and then western blot analysis was performed. This protocol was adapted from Leist M, *et. al.* (1998).

**CD95 cell surface localization:** Cells were washed once in DPBS and fixed with 4% paraformaldehyde in DPBS. Slides were washed again in DPBS and placed in a humidified chamber. Blocking solution (1% bovine serum albumin and 2% rat serum in DPBS) was

added to the cells for 1 hour at room temperature. 1:100 diluted CD95 antibody was then added to the cells that were then placed in a humidified chamber at 4 degrees Celsius overnight. The next day, cells were washed once in DPBS and secondary antibody (R-488) was added diluted 1:300 for an hour. Cells were then washed in DPBS and vectashield with Dapi stain was added to the cells which were then cover-slipped and read at 100X magnification.

**DAPI staining:** Cells were washed once with DPBS and fixed for 20 minutes with 4% paraformaldehyde (in PBS). Chamber covers were removed from the slides and cells were washed in DPBS once. Mounting media containing DAPI staining was added to cells and slides were coverslipped.

#### **D. Results:**

***Effect of sorafenib + vorinostat on cell survival and death:*** Colony formation assays show that sorafenib and vorinostat act synergistically in Panc1 cells to reduced cell survival (Table 1). Trypan blue analysis also showed that sorafenib and vorinostat combination can induce significantly higher levels of cell death compared to vehicle or individual drug treatments in Panc1 cells (Figure 30), HEP3B cells (Figure 31) and also in HEPG2 cells (Figure 32).

***CD95 pathway in sorafenib + vorinostat induced death:*** We wanted to confirm previous studies that showed that sorafenib and vorinostat mediated toxicity occurred via CD95 activation (Zhang G, *et. al.* 2008; Park MA, *et. al.* 2008). Knock-down of CD95 using siRNA protected Panc1 cells from sorafenib and vorinostat induced cell death as determined by trypan blue assay (Figure 33).

c-FLIP-s is a negative regulator of the CD95 pathway and hence, we wanted to investigate whether over-expression of c-FLIP-s would protect cells from sorafenib and vorinostat mediated toxicity (Krueger A, *et. al.* 2001). Results showed that over-expression of c-FLIP-s protected Panc1 cells from sorafenib and vorinostat induced cell death as determined by trypan blue assay (Figure 34). DAPI staining also showed that over-expression of c-FLIP-s protected cells from sorafenib and vorinostat induced DNA fragmentation (Figure 35).

***Effect of sorafenib + sodium valproate on cell survival and death:*** We wanted to determine whether sorafenib combined with sodium valproate (also a HDACI) would also act synergistically to kill cancer cells. For all short term cell viability experiments, we used



1mM dose of sodium valproate which is a clinically relevant dose (Ueshima S, *et. al.* 2008). Colony formation assays showed that sorafenib and sodium valproate act synergistically in Panc1 and HEP3B cells to reduced cell survival (Table 1, 2). Trypan blue analysis also showed that sorafenib and sodium valproate in combination can induce significantly higher levels of cell death compared to vehicle or individual drug treatments in Panc1 and HEPG2 cells (Figure 36, 37).

***Mitochondrial translocation of BAX upon sorafenib+sodium valproate treatment:*** We next investigated the proteins that may be involved in mediating sorafenib and sodium valproate toxicity. Cell fractionation studies showed that 12 h after treatment with sorafenib and sodium valproate combined in HEPG2 cells, BAX translocation from cytosol to mitochondrial was observed (Figure 38).

***BIM and BAK co-immunoprecipitation with MCL-1 in sorafenib + sodium valproate treated cells:*** In HEPG2 cells, immunoblotting studies failed to show any significant change in Mcl-1 expression level 6 h post sorafenib and sodium valproate treatment (Figure 39). However, at this time-point, immunoprecipitation studies showed that BAK association with Mcl-1 was decreased in cells treated with the drug combination compared to vehicle treated cells (Figure 39). 12 h after drug combination treatment, total Mcl-1 expression level as well as levels of BIM and BAK associated with Mcl-1 appeared to be significantly reduced compared to vehicle treated cells (Figure 39).

***CD95 cell surface localization in sorafenib + sodium valproate treated cells:*** 6 h after treating HEPG2 cells with vehicle, 3 $\mu$ M sorafenib alone, 1mM sodium valproate alone or the two drugs together, CD95 cell surface localization was determined (Figure 40). This

time point was chosen since previous studies have shown that sorafenib and vorinostat combined result in CD95 cell surface localization/activation as early as 6 h post drug treatment (Zhang G, *et. al.* 2008). We observed that 6h after drug treatment, CD95 localization at the cell surface was much greater in HEPG2 cells treated with sorafenib+sodium valproate compared to other conditions.

***Effect of obatoclox in combination with sorafenib alone:*** We wanted to investigate whether inhibition of Bcl-2 family members via obatoclox would enhance cancer cell killing when combined with sorafenib alone. Obatoclox treatment resulted in significantly higher cell death compared to vehicle treated HEPG2 cells (Figure 41). Obatoclox and sorafenib combined resulted in significantly higher cell death compared to sorafenib alone at 3 $\mu$ M and 6 $\mu$ M doses of sorafenib (Figure 41). At all other doses of sorafenib tested, the presence of obatoclox did not significantly enhance cell death compared to vehicle treated cells (Figure 41).

***Effect of obatoclox in combination with vorinostat alone:*** We wanted to investigate whether inhibition of Bcl-2 family members via obatoclox would enhance cancer cell killing when combined with vorinostat alone. Results showed that obatoclox alone induced significantly higher cell death in HEPG2 cells compared to vehicle treated cells (Figure 42). Increasing concentrations of vorinostat, alone or in combination with obatoclox, failed to have a significant effect on cell death compared to respective vehicle treated conditions (Figure 42).

***Effect of obatoclox in combination with sodium valproate alone:*** We wanted to investigate whether inhibition of Bcl-2 family members via obatoclox would enhance

cancer cell killing when combined with sodium valproate alone. In HEPG2 cells, obatoclax showed significantly higher levels of cell death compared to vehicle treated cells (Figure 43). Increasing concentrations of sodium valproate, alone or in combination with obatoclax, failed to have a significant effect on cell death compared to respective vehicle treated conditions (Figure 43).

***Effect of obatoclax on sorafenib + sodium valproate mediated cell death:*** We wanted to investigate whether inhibition of Bcl-2 family members via obatoclax would enhance cancer cell killing when administered to cells treated with sorafenib and sodium valproate simultaneously. Trypan blue analysis revealed that there were significant effects of sorafenib + sodium valproate treatment (at each dose combination tested) and also of obatoclax on HEPG2 cell death compared to vehicle treated cells (Figure 44). It was also found that the presence of obatoclax significantly enhanced sorafenib + sodium valproate induced cell death at each dose combination (Figure 44).

***Effect of CD95 knock-down in sorafenib+sodium valproate+obatoclax treated cells:*** Knock-down of CD95 in HEPG2 cells protected them from the effects of sorafenib and sodium valproate alone or in combination and also from obatoclax induced cell death (Figure 45). However, CD95 knock-down failed to protect HEPG2 cells from death induced by all three drugs (sorafenib+sodium valproate+obatoclax) in combination (Figure 45).

***Effect of c-FLIP-s over-expression in sorafenib+sodium valproate+obatoclax treated cells:*** c-FLIP-s over-expression in HEPG2 cells protected them from the effects of sorafenib and sodium valproate alone or in combination and also from obatoclax induced

cell death (Figure 46). However, c-FLIP-s over-expression failed to protect HEPG2 cells from death induced by all three drugs (sorafenib+sodium valproate+obatoclax) in combination (Figure 46 ).

***Effect of Bcl-xl, Mcl-1 or Bcl-2 knock-down on sorafenib and sodium valproate induced cell death:*** To investigate whether obatoclax mediated inhibition of any one Bcl-2 family member was sufficient to enhance sorafenib+sodium valproate toxicity, HEPG2 cells were transfected with siRNA to knock-down Bcl-xl, Mcl-1 or Bcl-2 expression and then treated with vehicle or sorafenib and sodium valproate combined. Trypan blue analysis was then performed to measure cell viability. Knock-down of Bcl-xl or Bcl-2, but not Mcl-1, enhanced sorafenib and sodium valproate induced cell death (Figure 47).

***Effect of Bcl-xl and Bcl-2 or Bcl-xl, Mcl-1 and Bcl-2 knock-down on sorafenib and sodium valproate induced cell death:*** HEPG2 cells were transfected with siRNA to knock-down Bcl-xl and Bcl-2 simultaneously or Bcl-xl, Mcl-1 and Bcl-2 simultaneously and then treated with vehicle or sorafenib and sodium valproate combined. Trypan blue analysis was performed to measure cell viability. Compared to siSCR treated cells, simultaneous knock-down of Bcl-xl and Bcl-2 significantly increased sorafenib and sodium valproate induced cell death (Figure 48). Simultaneous knock-down of Bcl-xl, Mcl-1 and Bcl-2 also significantly increased sorafenib and sodium valproate induced cell death compared to siSCR as well as compared to combined siBcl-xl and siBcl-2 transfected cells (Figure 48).

**Activation of BAK and BAX:** Treatment of HEPG2 cells with sorafenib and sodium valproate combined, obatoclastax alone or sorafenib + sodium valproate + obatoclastax combined resulted in increased BAK and BAX activation compared to vehicle treated cells suggesting that under these conditions, BAK and BAX are available to initiate cell death pathways (Figure 49).

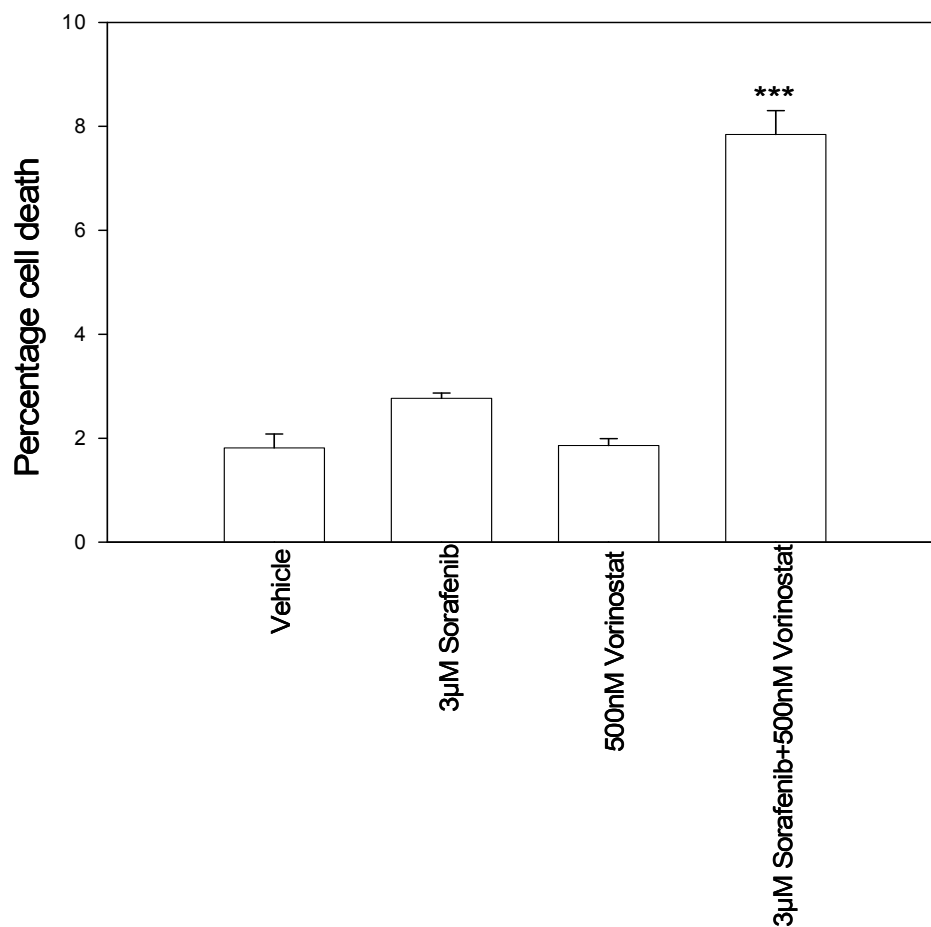
**Cytosolic release of cytochrome c in cells treated with sorafenib, sodium valproate and obatoclastax:** Cell fractionation assays revealed that HEPG2 cells treated with sorafenib + sodium valproate + obatoclastax showed significant release of cytochrome c into the cytosol and correspondingly, a decrease in the mitochondrial levels of cytochrome c was observed under the same treatment conditions (Figure 50). These results show that cytosolic cytochrome c is available to initiate cell death pathways under this condition. Expression of c-FLIP-s appeared to prevent the release of cytochrome c in these cells under basal and sorafenib + sodium valproate treatment conditions indicating that blocking the extrinsic cell death pathway prevents release of cytochrome c under these conditions (Figure 50). However, in spite of c-FLIP-s over-expression, cytosolic release of cytochrome c was observed in cells treated with obatoclastax alone or sorafenib + sodium valproate + obatoclastax combined (Figure 50). This confirms that in spite of blocking the CD95 pathway, treating cells with sorafenib + sodium valproate + obatoclastax combined can result in cytosolic cytochrome c release which is then available to activate cell death pathways.

Sorafenib (uM)	Vorinostat (nM)	Fa	CI
3.0	250	0.31	0.48
6.0	500	0.42	0.70
9.0	750	0.51	0.83

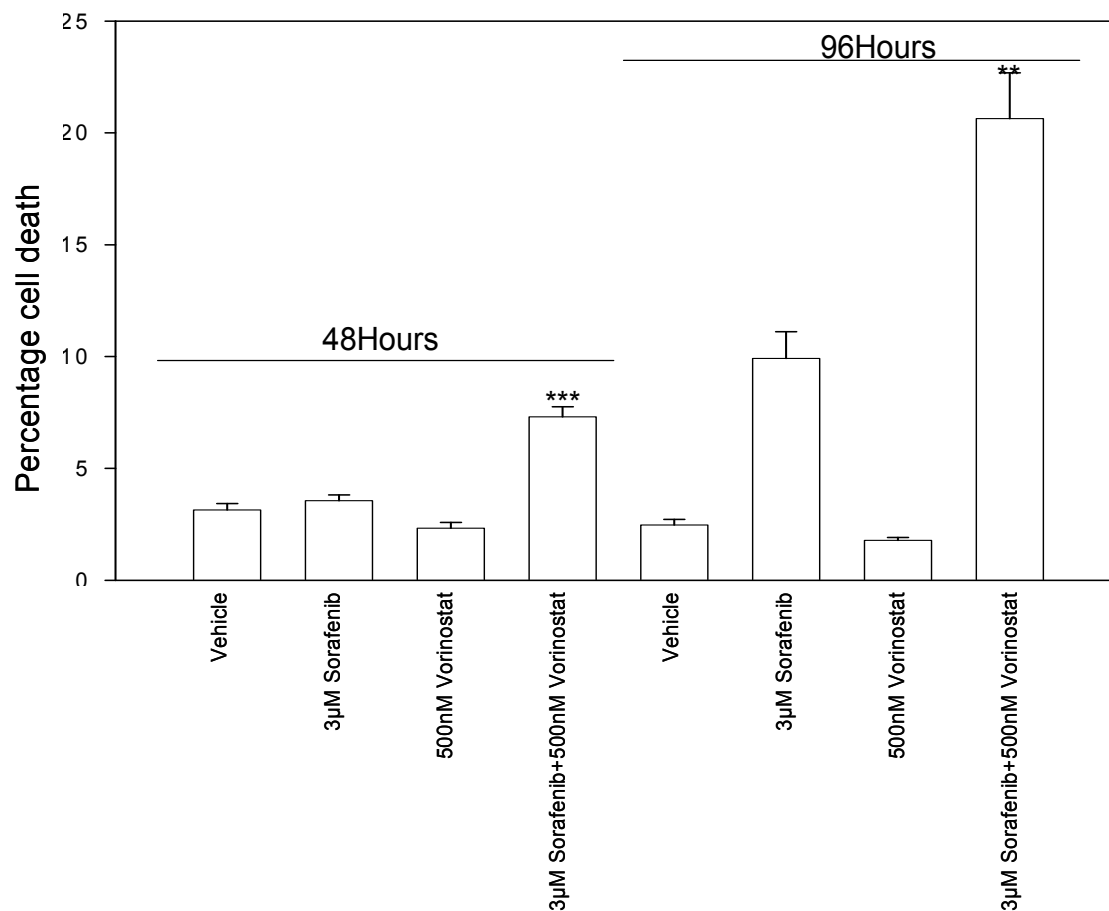
  

Sorafenib (uM)	Valproic Acid (mM)	Fa	CI
3.0	0.50	0.37	0.46
4.5	0.75	0.43	0.58
6.0	1.00	0.54	0.57
7.5	1.25	0.73	0.41
9.0	1.50	0.80	0.37

**Table 1: Sorafenib synergizes with vorinostat and with sodium valproate in Panc1 cells.** 12h after plating Panc1 pancreatic cancer cells, they were treated with vehicle (DMSO), sorafenib and sodium valproate or vorinostat, as indicated at a fixed concentration ratio to perform median dose effect analyses for the determination of synergy. After drug exposure (48h), the media was changed and cells cultured in drug free media for an additional 10-14 days. Cells were fixed, stained with crystal violet and colonies of > 50 cells / colony counted. Colony formation data were entered into the Calcsyn program and combination index (CI) and fraction affected (Fa) values were determined. A CI value of less than 1.00 indicates synergy and larger the Fa value, the lower the survival under that condition.



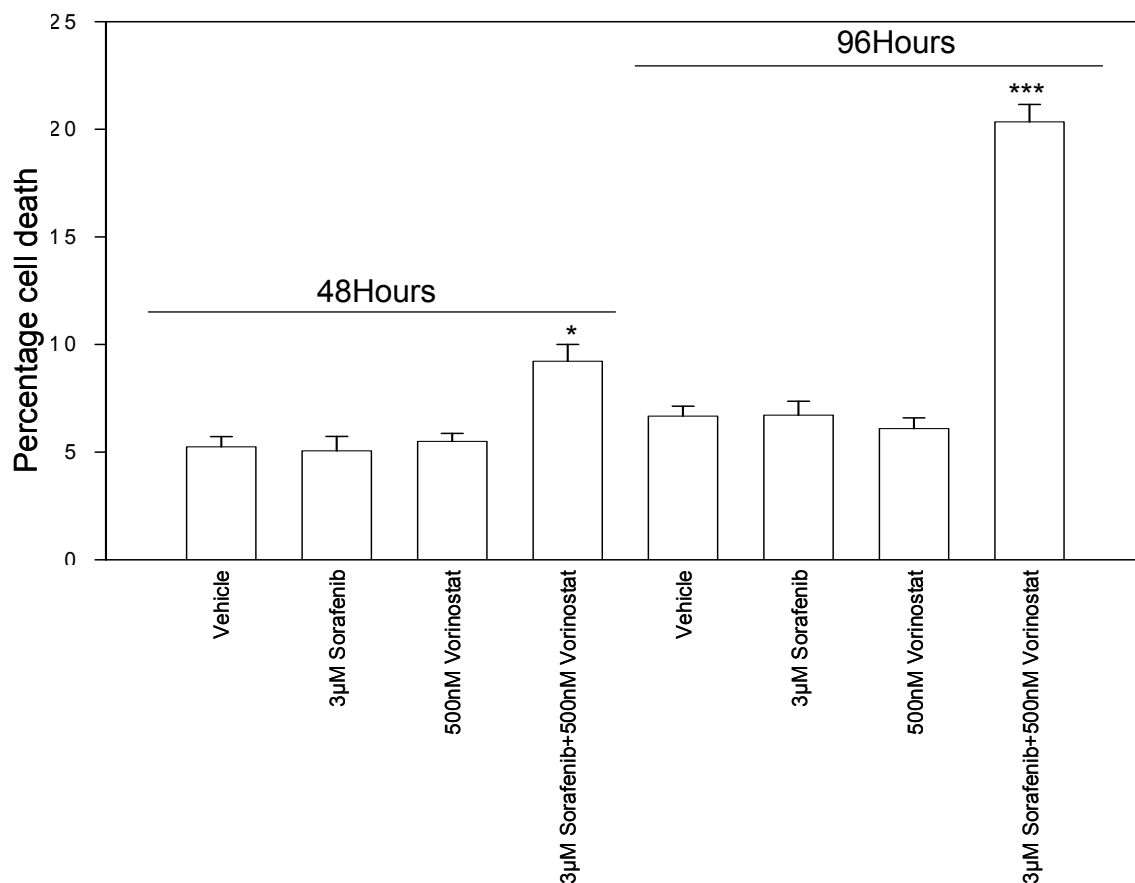
**Figure 30: Sorafenib and vorinostat combined enhance cell death in Panc1 cells.** 12 h after plating Panc1 cells, they were treated with Vehicle (DMSO), 3µM sorafenib, 500nM vorinostat or 3µM sorafenib and 500nM vorinostat combined. 48 h after drug treatment, cell viability was measured using the trypan blue exclusion assay  $\pm$ S.E.M.  $n=2$ . One way ANOVA with Bonferroni post-test revealed that sorafenib and vorinostat combined induced significantly higher levels of cell death (\*\*\*,  $p < 0.0001$ ,  $n=6$ ) as compared to all other treatment conditions. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



**Figure 31: Sorafenib and vorinostat combined enhance cell death in HEP3B cells.**

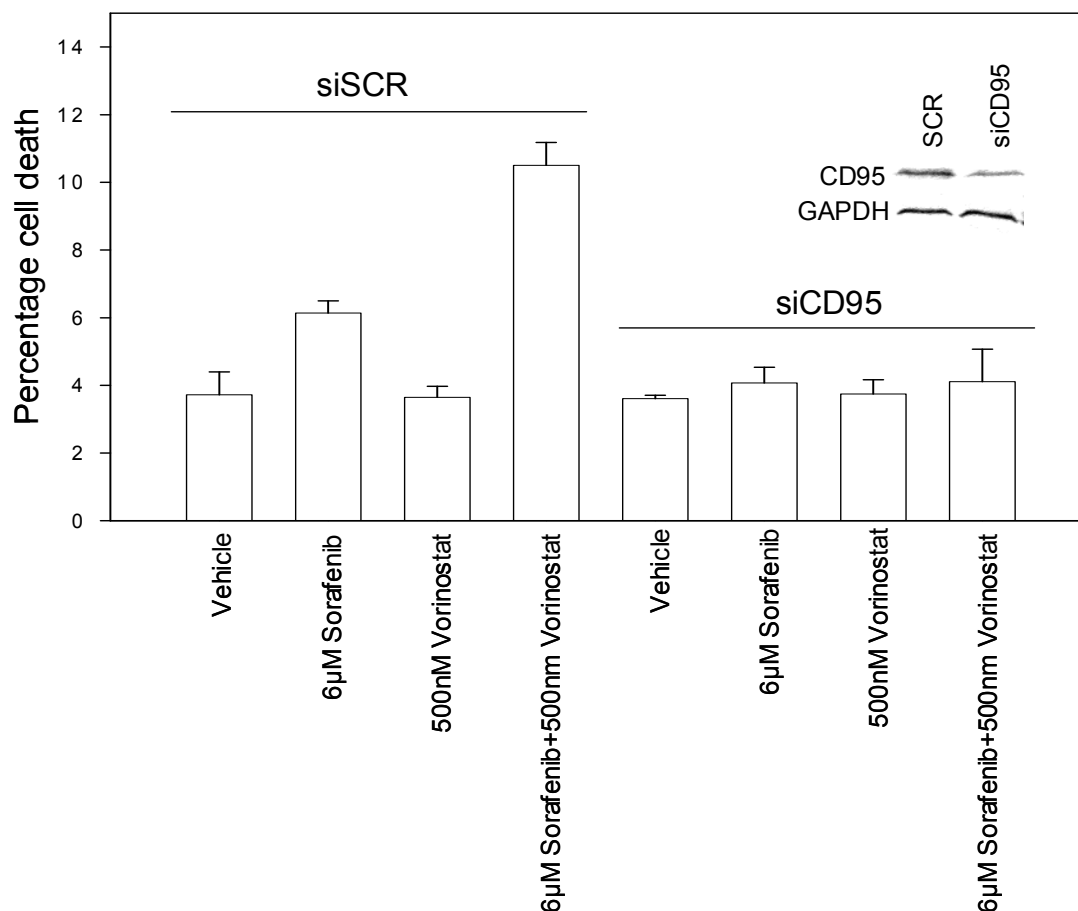
12 h after plating HEP3B hepatoma cells, they were treated with vehicle (DMSO), 3µM sorafenib, 500nM vorinostat or 3µM sorafenib and 500nM vorinostat combined. 48 or 96 h after drug treatment, cell viability was measured using the trypan blue exclusion assay. One way ANOVA with Bonferroni post-test revealed that sorafenib and vorinostat combined induced significantly higher levels of cell death (\*\*\*,  $p < 0.001$  at 48 h and \*\*,  $p < 0.001$  at 96 h,  $n=6$ ) as compared to all other treatment conditions. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



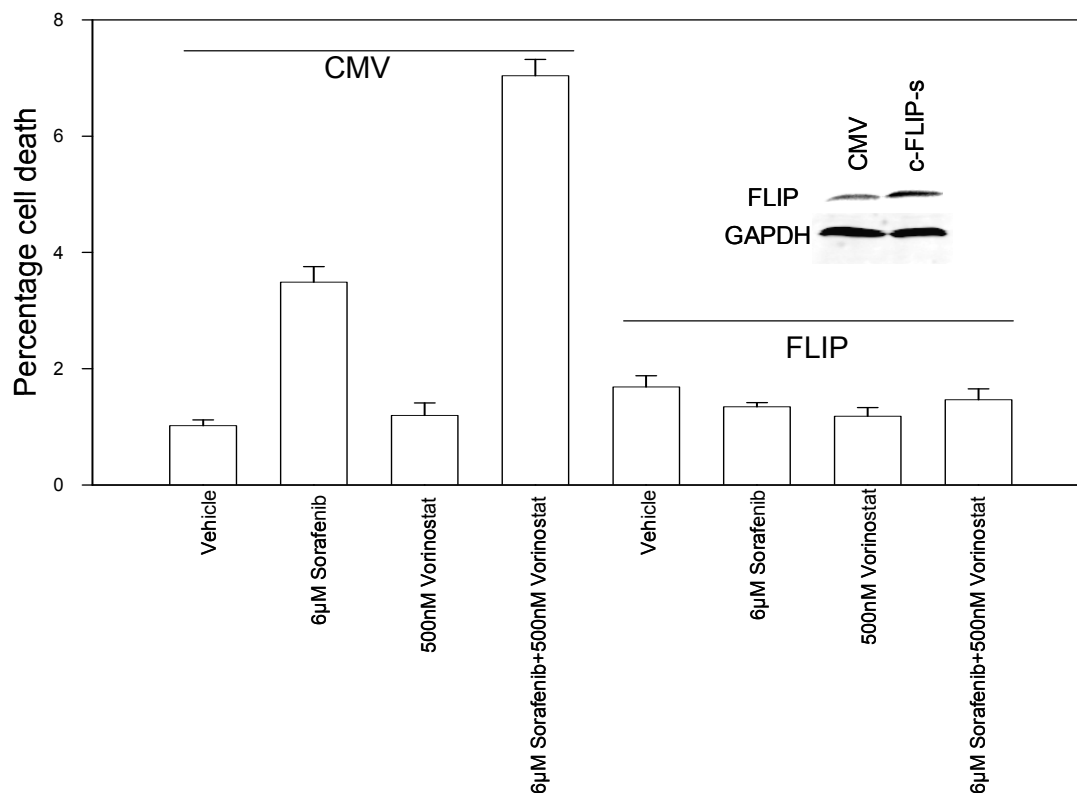


**Figure 32: Sorafenib and vorinostat combined enhance cell death in HEPG2 cells.**

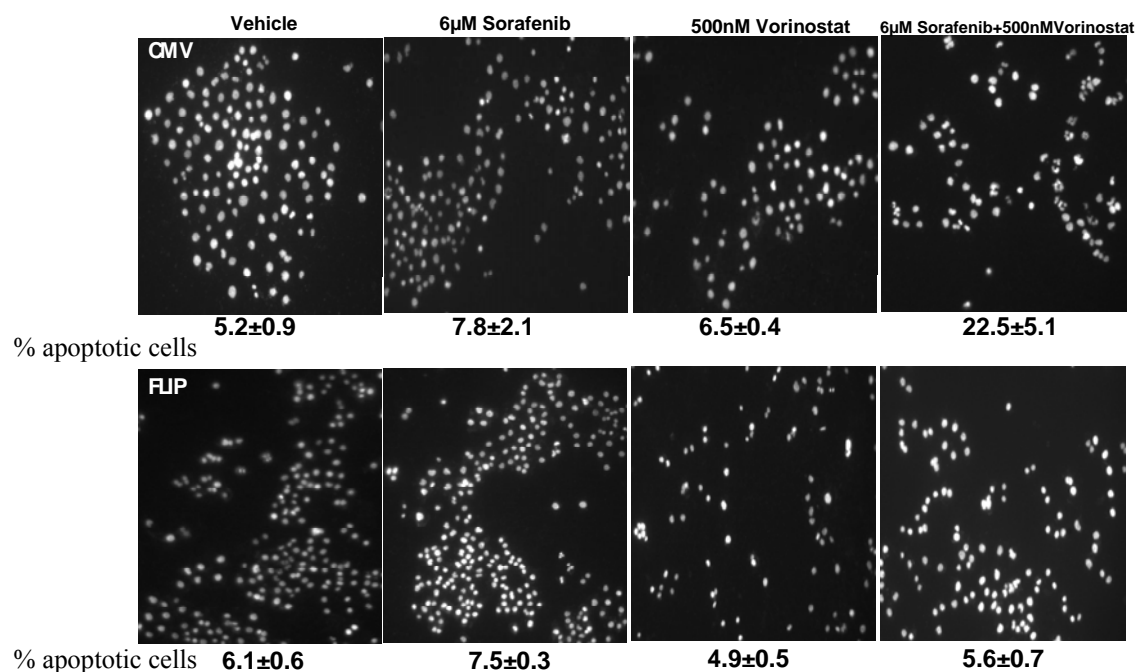
12 h after plating HEG2 hepatoma cells, they were treated with vehicle (DMSO), 3μM sorafenib, 500nM vorinostat or 3μM sorafenib and 500nM vorinostat combined. 48 or 96 h after drug treatment, cell viability was measured using the trypan blue exclusion assay  $\pm$ S.E.M.  $n=2$ . One way ANOVA with Bonferroni post-test revealed that sorafenib and vorinostat combined induced significantly higher levels of cell death (\*,  $p < 0.05$  at 48 h and \*\*\*,  $p < 0.0001$  at 96 h,  $n=6$ ) as compared to all other treatment conditions. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



**Figure 33: Knock-down of CD95 protects Panc1 cells from sorafenib+vorinostat combined lethality.** 12 h after plating Panc1 cells, they were transfected with scrambled siRNA (siSCR) or with siRNA to knock-down CD95 (siCD95). 24 h after transfection, cells were treated with vehicle (DMSO), 6μM sorafenib, 500nM vorinostat or 3μM sorafenib and 500nM vorinostat combined. 48 h after drug treatment, cell viability was measured using Trypan blue exclusion assay. There were significant effects of drug treatment and knock-down of CD95 on Panc1 cell death (two-way ANOVA,  $p < 0.0001$ ,  $n=6$ ). Bonferroni's post-tests revealed that CD95 knock-down significantly protected against cell death in the presence of sorafenib and vorinostat combined ( $p < 0.001$ ). Data (mean ± S.E.M.) are from two independent experiments combined.



**Figure 34: Over-expression of c-FLIP-s protects Panc1 cells from sorafenib+ vorinostat combined lethality.** 12 h after plating Panc1 cells, they were infected with an empty vector recombinant adenovirus (CMV) or a virus to express c-FLIP-s. 24 h after virus infection, cells were treated with vehicle (DMSO), 6μM sorafenib, 500nM vorinostat or 6μM sorafenib and 500nM vorinostat combined. 48 h after drug treatment, cell viability was measured using trypan blue exclusion assay  $\pm$ S.E.M.  $n=2$ . There were significant effects of drug treatment and FLIP expression on Panc1 cell death (two-way ANOVA,  $p<0.0001$ ,  $n=6$ ). Bonferroni's post-tests revealed that FLIP expression significantly protected against cell death in the presence of sorafenib or sorafenib and vorinostat combined ( $p < 0.001$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

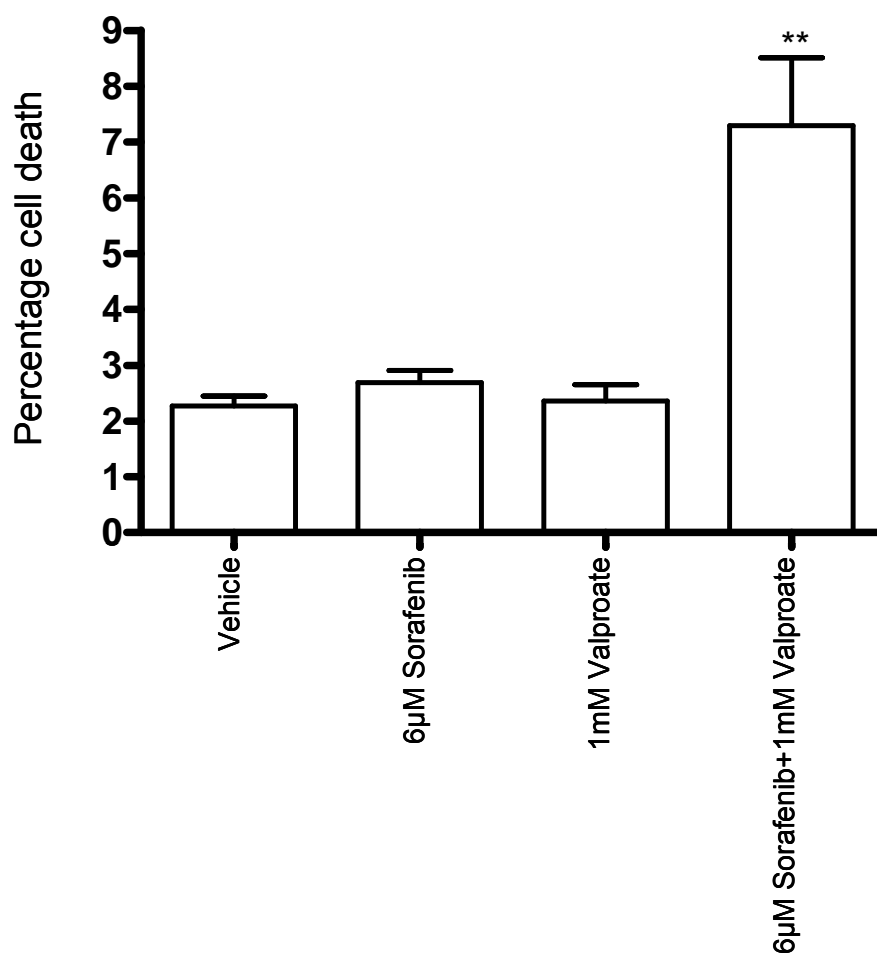


**Figure 35: Panc1 cells over-expressing c-FLIP-s fail to show nuclear fragmentation upon sorafenib+vorinostat combined treatment.** 12 h after plating Panc1 cells, they were treated with vehicle (DMSO), 6µM sorafenib, 500nM vorinostat or 6µM sorafenib and 500nM vorinostat combined. 48 h later, cells were fixed and DAPI staining was performed using mounting media containing DAPI stain. Apoptosis was measured (50 cells per condition) by expressing the nuclei with obvious DNA fragmentation as a percentage of DAPI positive nuclei (shown below each panel). Representative study images from two independent studies shown.

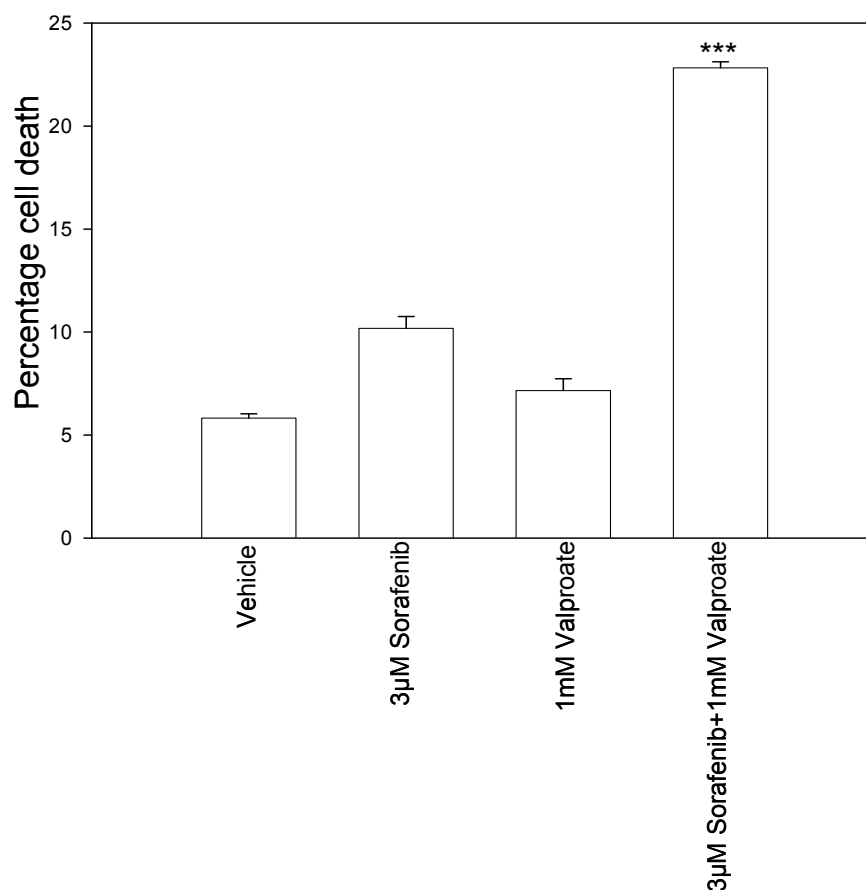
CI For experimental values Hep3B 48h			
Sor (uM)	Valproic (mM)	Fa	CI
2.25	0.75	0.12	0.691
3.00	1.00	0.19	0.687
3.75	1.25	0.36	0.540

**Table 2: Sorafenib synergizes with sodium valproate to reduce HEP3B cell survival.**

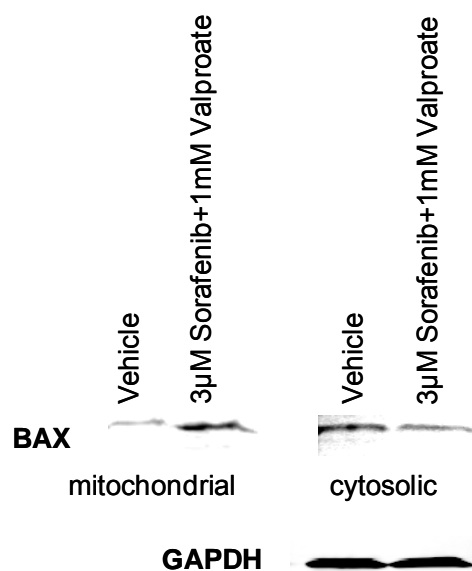
12h after plating HEP3B hepatoma cells, they were treated with vehicle (VEH, DMSO) or sorafenib and sodium valproate, as indicated at a fixed concentration ratio to perform median dose effect analyses for the determination of synergy. After drug exposure (48h), the media was changed and cells cultured in drug free media for an additional 10-14 days. Cells were fixed, stained with crystal violet and colonies of > 50 cells / colony counted. Colony formation data were entered into the Calcsyn program and combination index (CI) and fraction affected (Fa) values determined. A CI value of less than 1.00 indicates synergy and larger the Fa value, the lower the survival under that condition.



**Figure 36: Sorafenib and sodium valproate combined enhance cell death in Panc1 cells.** 12 h after plating Panc1 cells, they were treated with vehicle (DMSO), 6µM sorafenib, 1mM valproate or 6µM sorafenib and 1mM valproate combined. 48 h after drug treatment, cell viability was measured using the trypan blue exclusion assay. One way ANOVA with Bonferroni post-test revealed that sorafenib and valproate combined induced significantly higher levels of cell death (\*\*,  $p < 0.001$ ,  $n=6$ ) as compared to all other treatment conditions. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

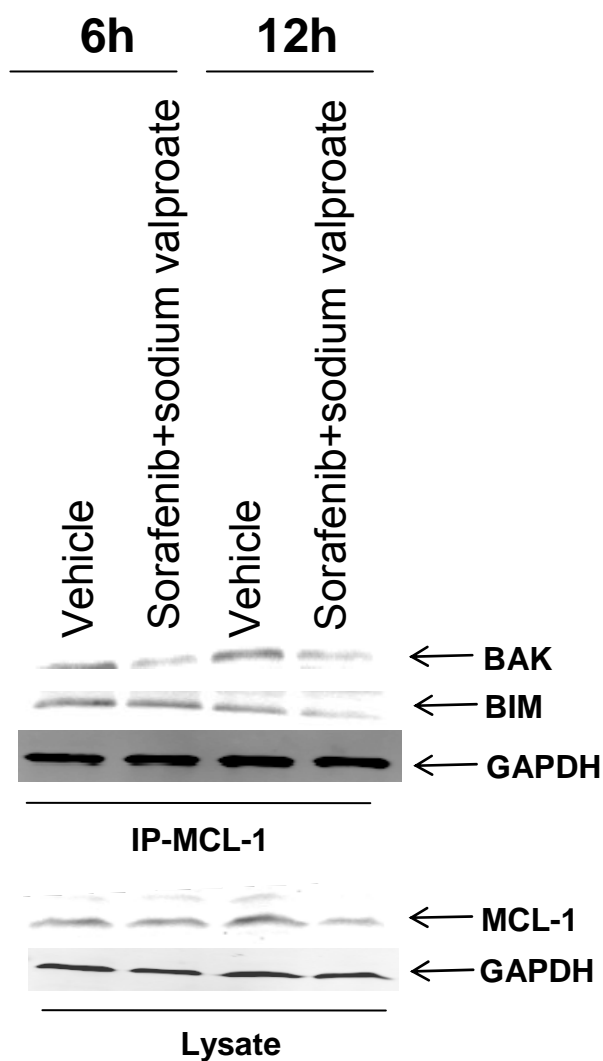


**Figure 37: Sorafenib and sodium valproate combined enhance cell death in HEPG2 cells.** 12 h after plating HEPG2 cells, they were treated with vehicle (DMSO), 3µM sorafenib, 1mM sodium valproate or 3µM sorafenib and 1mM sodium valproate combined. 48h after drug treatment, cell viability was measured using trypan blue exclusion assay. One way ANOVA with Bonferroni post-test revealed that sorafenib and valproate combined induced significantly higher levels of cell death (\*\*\*,  $p < 0.0001$ ,  $n=6$ ) as compared to all other treatment conditions. Data (mean ± S.E.M.) are from two independent experiments combined.

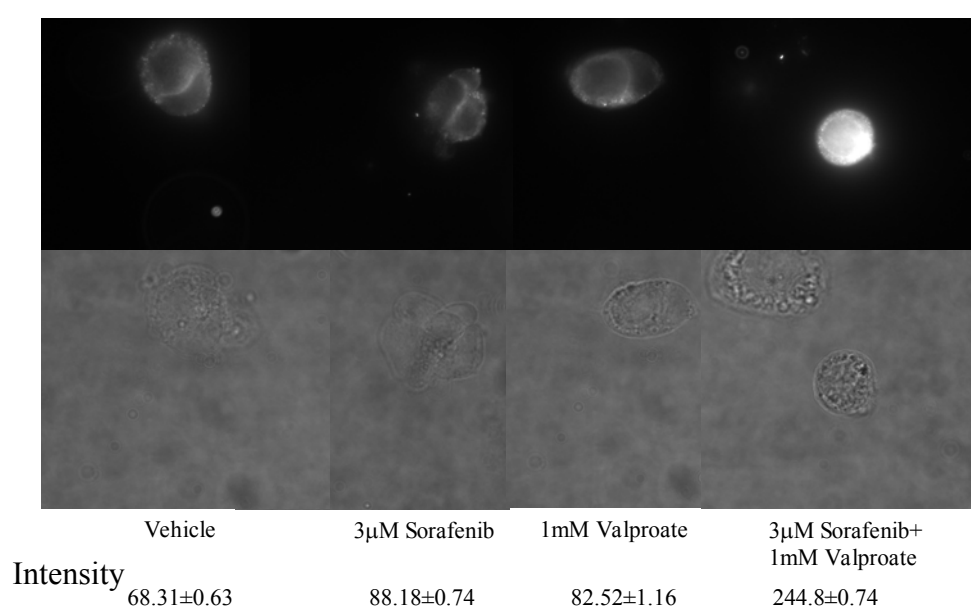


**Figure 38: Mitochondrial translocation of BAX in HEPG2 cells treated with sorafenib+ sodium valproate combined.** 12 h after plating HEPG2 cells, they were treated with vehicle (DMSO) or sorafenib and sodium valproate as indicated. 12 h after drug treatment, cell fractionation assay was performed to determine mitochondrial and cytosolic localization of BAX. Representative image from two independent studies shown.

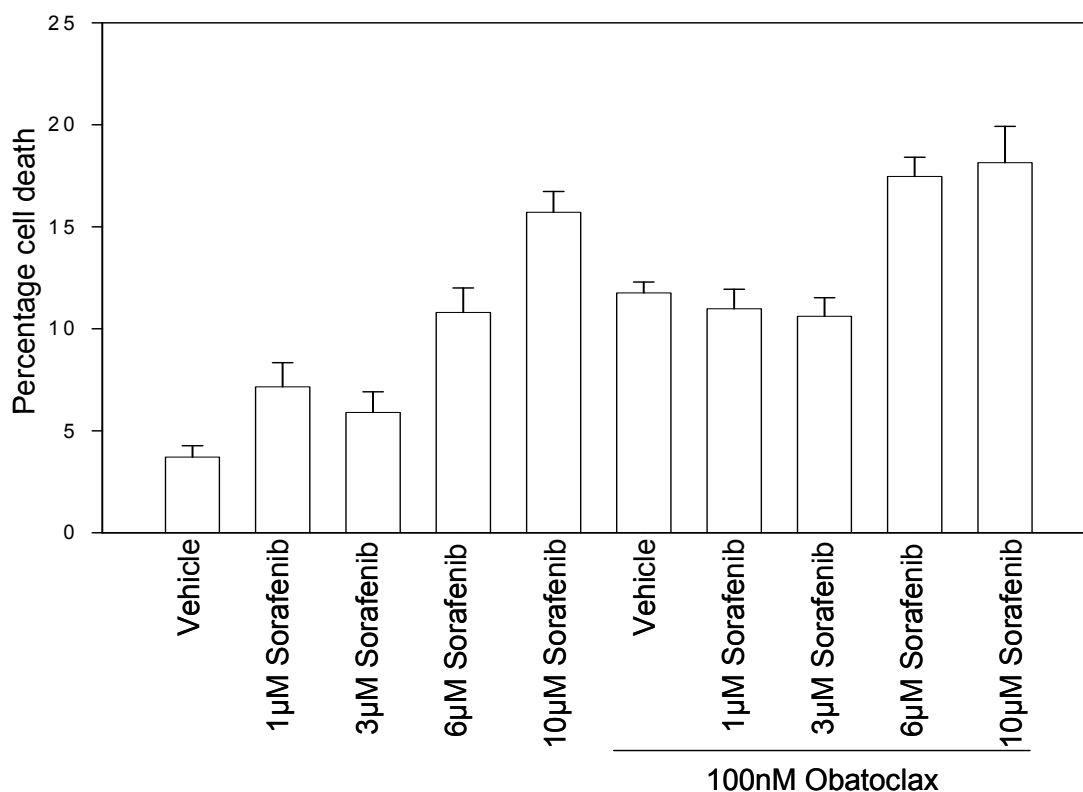




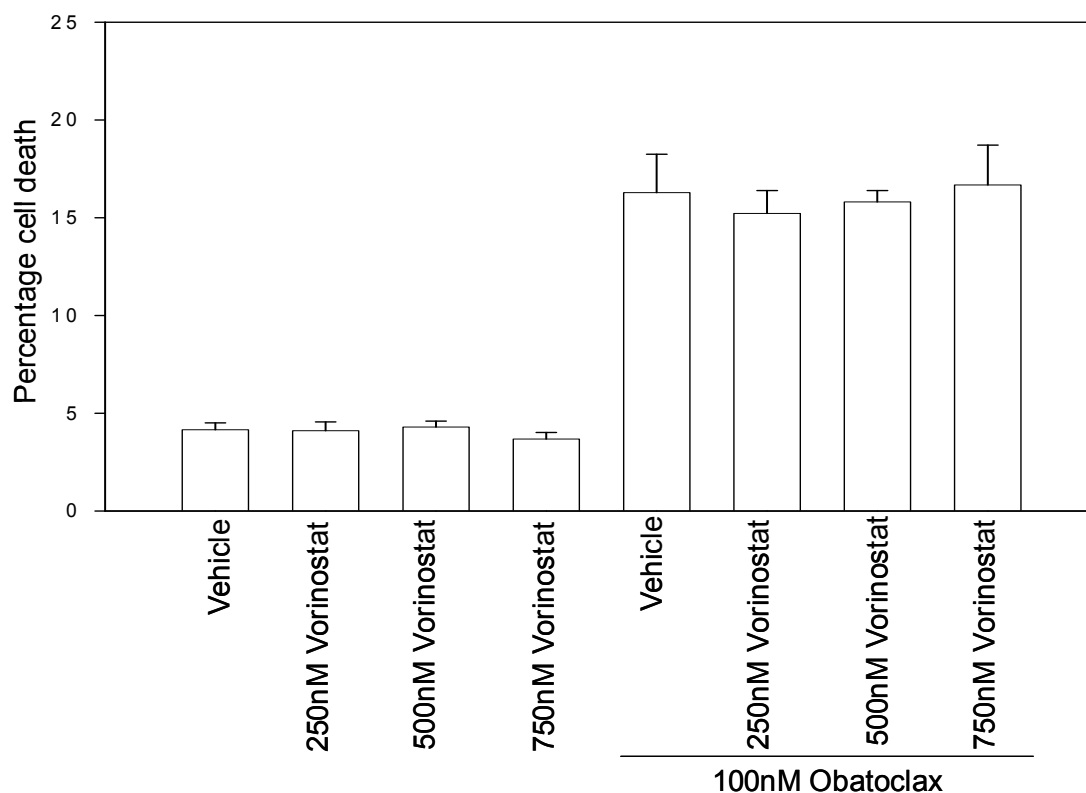
**Figure 39: BAK and BIM co-immunoprecipitation with Mcl-1 in sorafenib+sodium valproate combined treated HEPG2 cells.** 12 h after plating HEPG2 cells, they were treated with vehicle (DMSO) or 3 $\mu$ M sorafenib and 1mM sodium valproate as indicated. 6 h or 12 h after drug treatment, Mcl-1 was immunoprecipitated and immunoblotting was performed to determine levels of BIM or BAK co-immunoprecipitated with Mcl-1. Representative image from two independent studies shown.



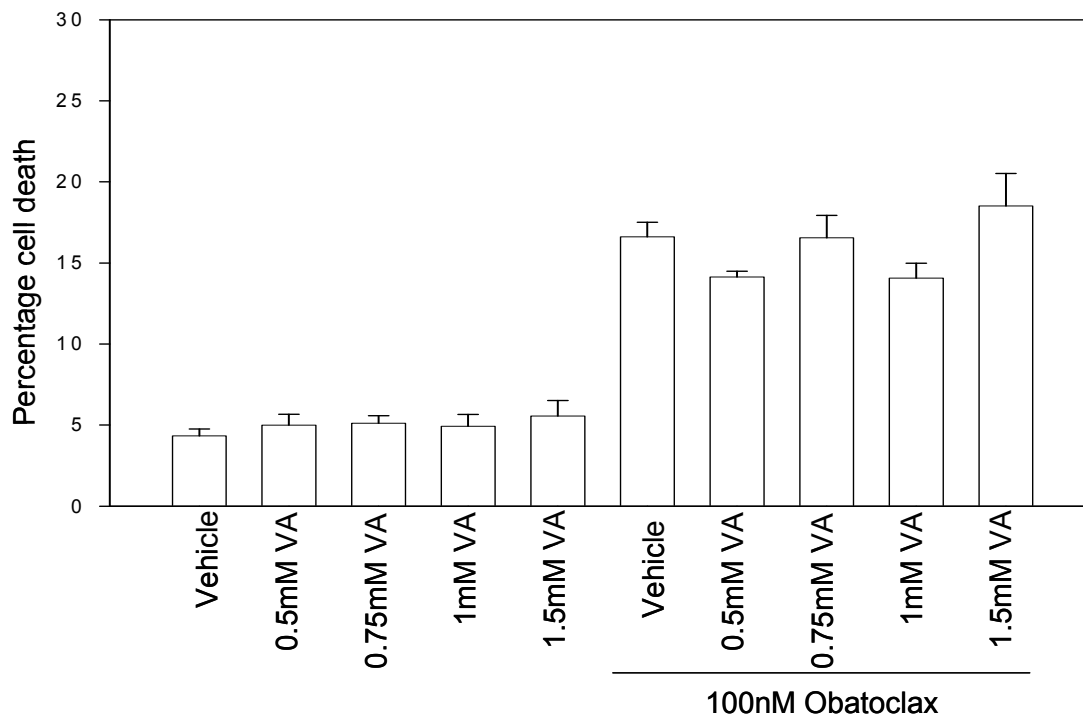
**Figure 40: CD95 cell surface localization in HEPG2 cells treated with sorafenib and sodium valproate.** 12 h after plating HEPG2 cells, they were treated with vehicle, sorafenib, sodium valproate or sorafenib and sodium valproate combined as indicated. 6 h post drug treatment, CD95 cell surface localization was performed. Representative images from one experiment are shown (n=2). Quantitative assessment of increase in fluorescence intensity was determined from fifty cells counted over two independent experiments.



**Figure 41: Sorafenib dose response in HEPG2 cells with or without obatoclox.** 12 h after plating HEPG2 cells, they were treated with increasing concentrations of Sorafenib as indicated along with Vehicle or 100nM Obatoclox. 48 h after drug treatment, cell viability was measured via trypan blue analysis. Two way anova (n=6) shows that there is an effect of Sorafenib dose ( $p < 0.0001$ ) and there is an effect of Obatoclox dose ( $p < 0.0001$ ). Bonferroni post-test shows that the presence of Obatoclox causes a significant increase in cell death only under Veh ( $p < 0.0001$ ) treated or 3 ( $p < 0.05$ ) and 6  $\mu$ M ( $p < 0.0001$ ) Sorafenib doses compared to identical conditions in the absence of Obatoclox. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

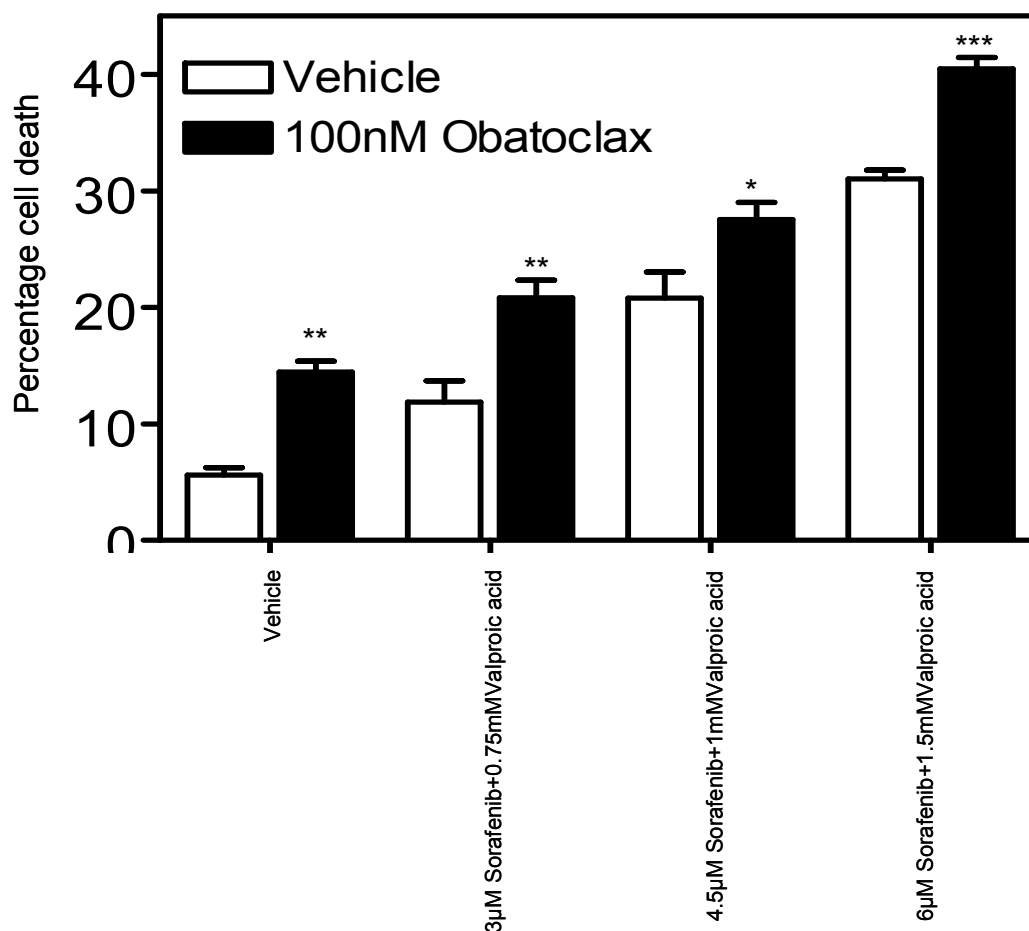


**Figure 42: Vorinostat dose response in HEPG2 cells with or without obatoclox.** 12 h after plating HEPG2 cells, they were treated with increasing concentrations of Vorinostat as indicated along with Vehicle or 100nM Obatoclox. 48 h after drug treatment, cell viability was measured via trypan blue analysis. Two way ANOVA ( $n=6$ ) shows that there is an effect of Obatoclox dose ( $p < 0.0001$ ) but no significant effect of increasing doses of Vorinostat on cell death. Bonferroni's post-test shows that the presence of Obatoclox causes a significant increase in cell death under Vehicle treated conditions and also with all doses of Valproate ( $p < 0.0001$ ) compared to identical conditions in the absence of Obatoclox. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

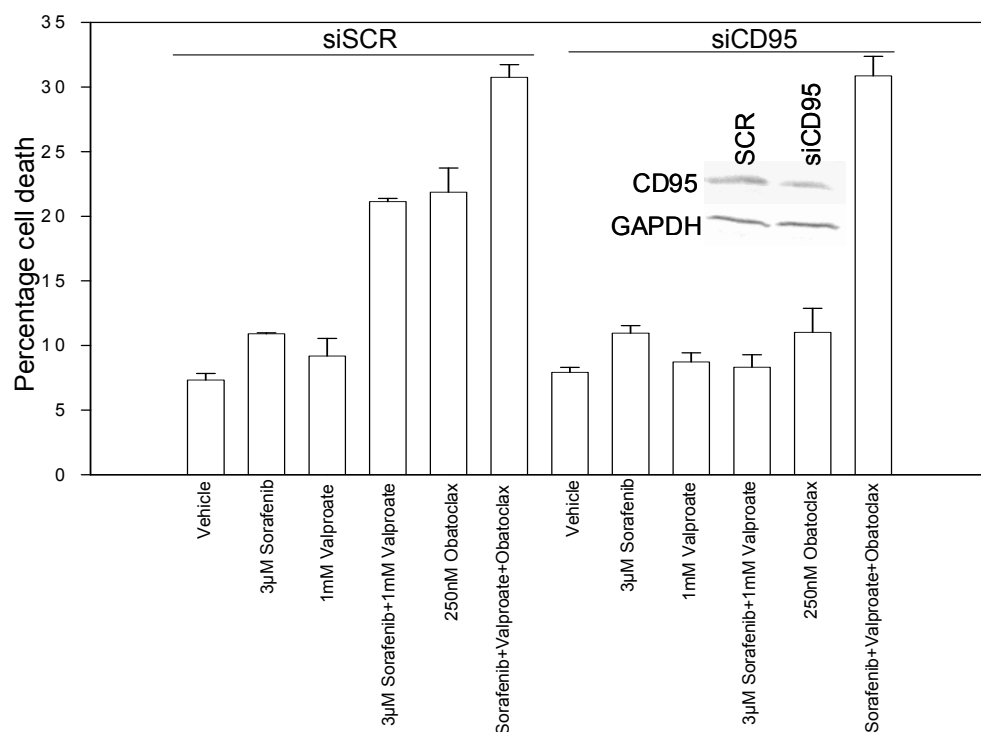


**Figure 43: Sodium valproate dose response in HEPG2 cells with or without obatoclax.**

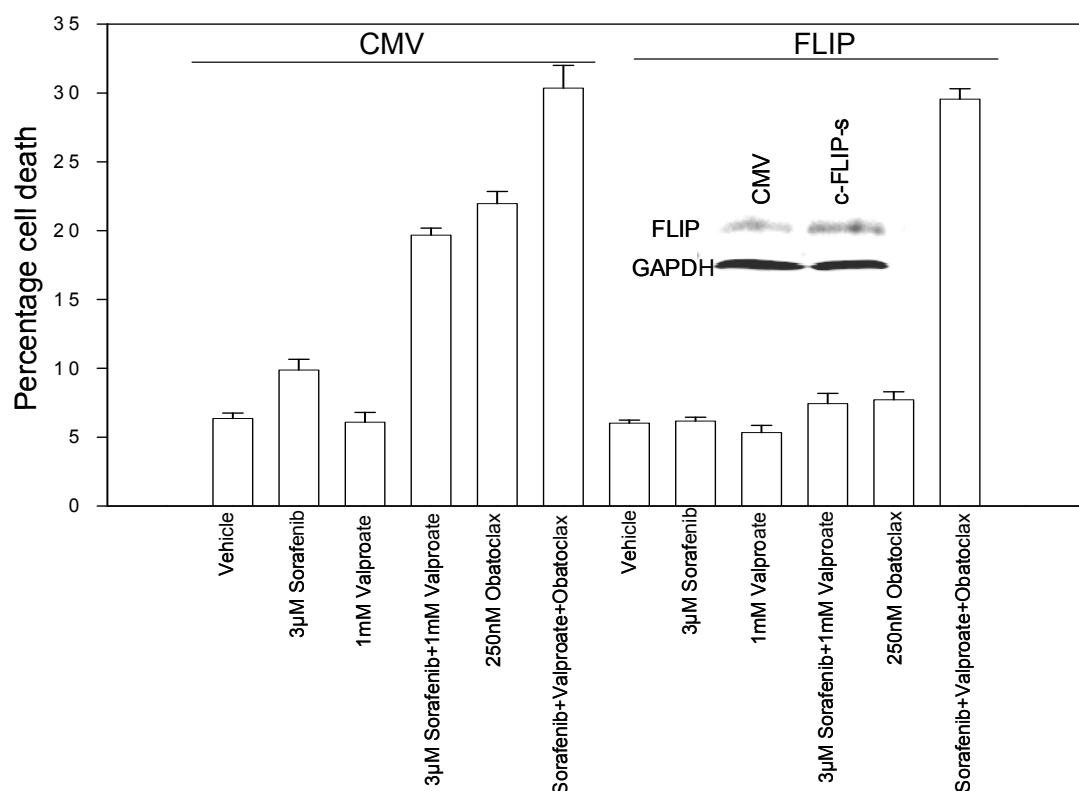
12 h after plating HEPG2 cells, they were treated with increasing concentrations of sodium valproate (VA) as indicated along with vehicle or 100nM obatoclax. 48 h after drug treatment, cell viability was measured via trypan blue analysis. Two way ANOVA (n=6) shows that there is an effect of obatoclax dose ( $p < 0.0001$ ) but no significant effect of increasing doses of valproate on cell death. Bonferroni's post-test shows that the presence of obatoclax causes a significant increase in cell death under vehicle treated conditions and also with all doses of valproate ( $p < 0.0001$ ) compared to identical conditions in the absence of obatoclax. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



**Figure 44: Cell death induced by sorafenib+sodium valproate combined in HEPG2 cells with or without obatoclax.** 12 h after plating HEPG2 cells, they were treated with Vehicle (DMSO) or various dose combinations of Sorafenib and Sodium valproate as indicated with or without 100nM Obatoclax. 48h after drug treatment, cell viability was measured using trypan blue exclusion assay. There were significant effects of sorafenib + sodium valproate treatment (at each dose combination tested) and also of obatoclax on HEPG2 cell death (two-way ANOVA,  $p < 0.0001$  in both cases,  $n=6$ ). Bonferroni's post-test revealed that the presence of obatoclax significantly enhanced sorafenib + sodium valproate induced cell death at each dose combination tested (\*  $p < 0.05$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.0001$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

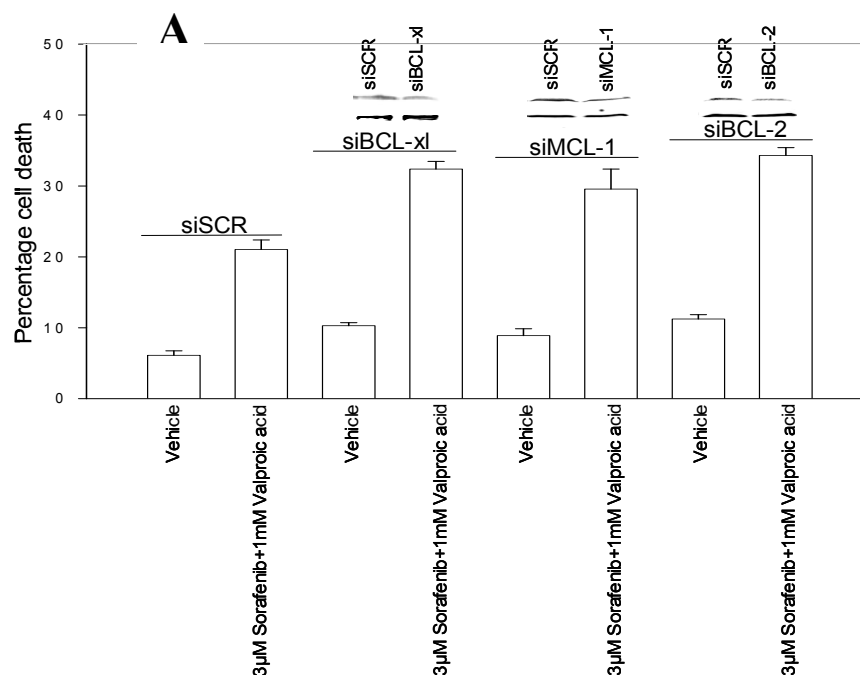


**Figure 45: Knock-down of CD95 failed to protect HEPG2 cells from obatoclax+ sorafenib+sodium valproate combined lethality.** 12 h after plating HEPG2 cells, they were transfected with scrambled siRNA (siSCR) or with siRNA to knock-down CD95 (siCD95). 24 h after being transfected with siRNA, cells were treated with vehicle (DMSO), 3μM sorafenib, 1mM valproate or 3μM sorafenib and 1mM valproate combined. 48 h after drug treatment, cell viability was measured in triplicate using trypan blue analysis. There were significant effects of drug treatment and knock-down of CD95 on HEPG2 cell death (two-way ANOVA,  $p < 0.0001$ ,  $n=6$ ). Bonferroni's post-tests revealed that CD95 knock-down significantly protected against cell death in the presence of sorafenib and sodium valproate combined or obatoclax ( $p < 0.001$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

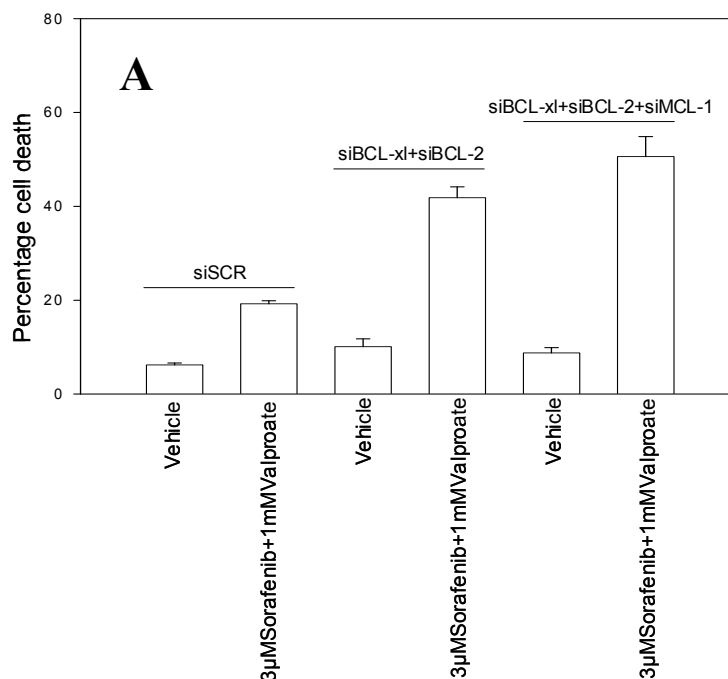


**Figure 46: Over-expression of c-FLIP-s failed to protect HEPG2 cells from obatoclox+ sorafenib+sodium valproate combined lethality.** 12 h after plating HEPG2 cells, they were infected with an empty vector recombinant adenovirus (CMV) or a virus to express c-FLIP-s. 24 h after virus infection, cells were treated with vehicle (DMSO), 3μM sorafenib, 1mM sodium valproate or 3μM sorafenib and 1mM sodium valproate combined. 48 h after drug treatment, cell viability was measured using trypan blue exclusion assay. There were significant effects of drug treatment and FLIP over-expression on HEPG2 cell death (two-way ANOVA,  $p < 0.0001$ ,  $n=6$ ). Bonferroni's post-tests revealed that FLIP over-expression significantly protected against cell death in the presence of sorafenib ( $p < 0.01$ ), sorafenib and sodium valproate combined or obatoclox ( $p < 0.001$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.

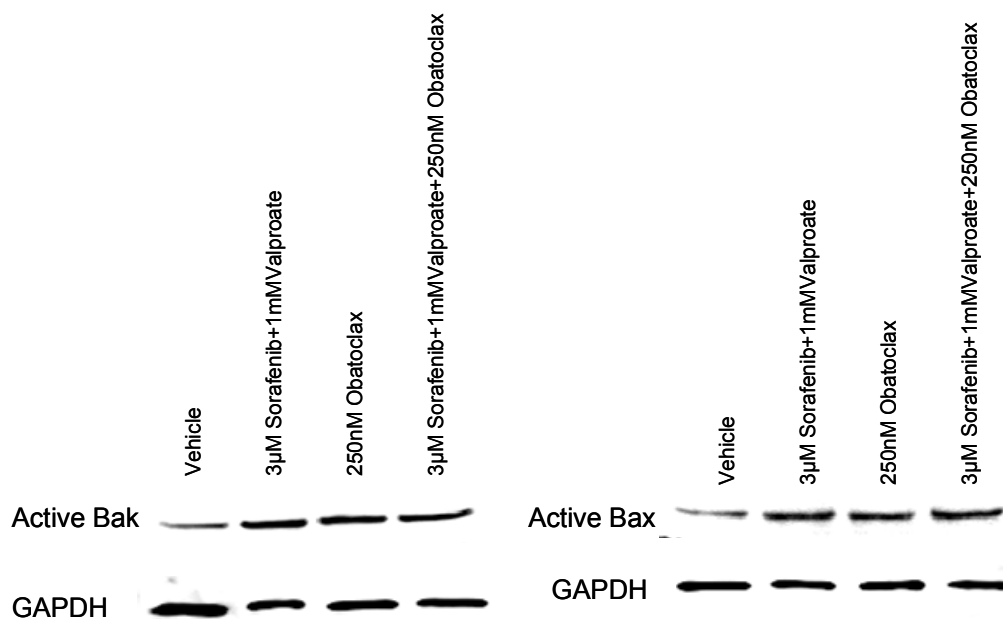




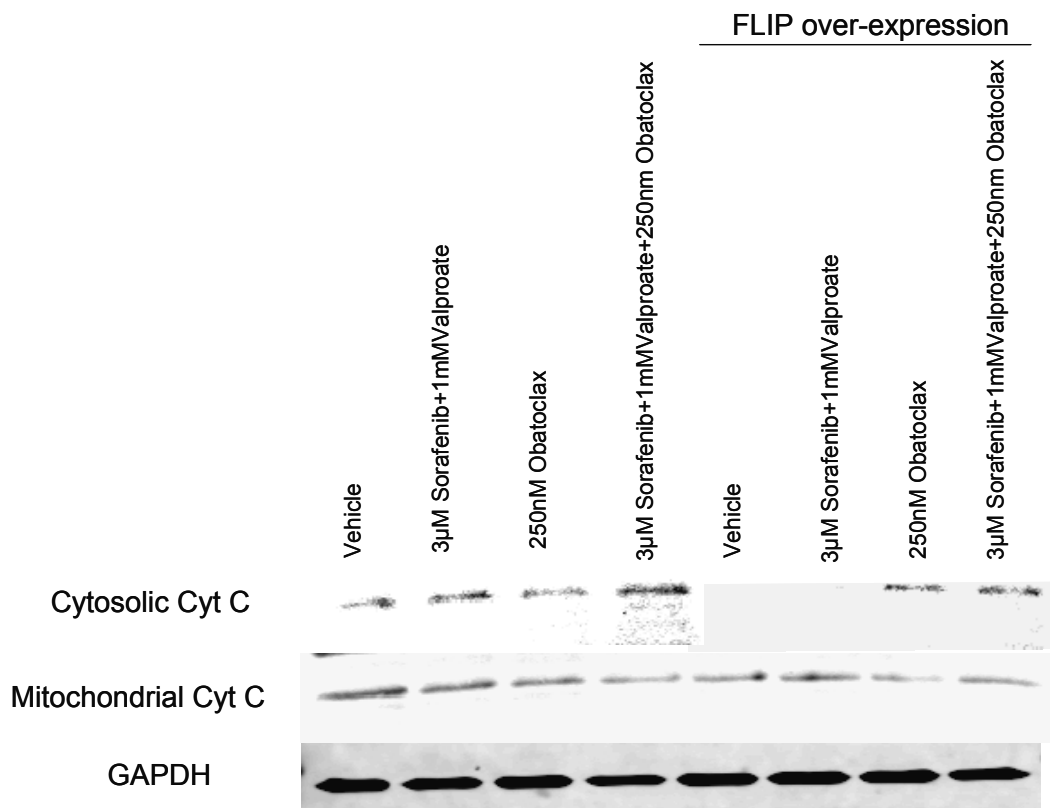
**Figure 47: Knock-down of Bcl-xl or Bcl-2, but not Mcl-1, enhanced sorafenib+ sodium valproate induced cell death.** 12 h after plating HEPG2 cells, they were transfected with scrambled siRNA (siSCR) or with siRNA to knock-down BclL-xl (siBcl-xl), Mcl-1 (siMcl-1) or Bcl-2 (siBcl-2). 24 h after transfection, cells were treated with vehicle (DMSO) or 3µM sorafenib and 1mM sodium valproate combined. 48 h after drug treatment, cell viability was measure using trypan blue assay. One way ANOVA with Bonferroni's post test showed that knock-down of Bcl-xl or Bcl-2, but not Mcl-1, enhanced sorafenib and sodium valproate induced cell death ( $p < 0.05$ ). Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



**Figure 48: Knock-down of Bcl-xl+Bcl-2 or Bcl-xl+Bcl-2+Mcl-1 enhanced sorafenib+ sodium valproate induced cell death.** 12 h after plating HEPG2 cells, they were transfected with control siRNA (siSCR), siRNA to knock-down Bcl-xl+Bcl-2 or Bcl-xl+Bcl-2+Mcl-1. 24 h later, cells were treated with vehicle (DMSO) or 3µM sorafenib and 1mM sodium valproate. One way ANOVA with Bon-ferroni's post test showed that knock-down of Bcl-xl+Bcl-2 and of Bcl-xl+Bcl-2+Mcl-1 significantly enhanced sorafenib and sodium valproate induced cell death ( $p < 0.001$ ) compared to siSCR treated cells. Data (mean  $\pm$  S.E.M.) are from two independent experiments combined.



**Figure 49: BAK and BAX activation in HEPG2 cells treated with sorafenib, sodium valproate and obatoclax.** 12 h after plating HEPG2 cells, they were treated with vehicle (DMSO), 3µM sorafenib and 1mM sodium valproate combined, 250nM obatoclax alone or all three drugs combined. 24 h after drug treatment, cells were isolated for immunoprecipitation to determine the amount of the activate BAX and BAK. Representative images shown (n=2).



**Figure 50: c-FLIP-s over-expression in HEPG2 cells prevents cytosolic cytochrome c release due to sorafenib+sodium valproate+obatoclax.** 12 h after plating HEPG2 cells, they were treated with vehicle (DMSO), 3µM sorafenib and 1mM sodium valproate combined, 250nM obatoclax alone or all three drugs combined. 24 h after drug treatment, cells were isolated for cell fractionation assay to determine cytochrome c release into the cytosol. A representative from two separate studies is shown.

#### **D. Discussion:**

We have attempted to determine whether sorafenib and sodium valproate interact to kill transformed cells, and whether obatclax mediated inhibition of mitochondrial Bcl-2 family protective proteins could enhance sorafenib + sodium valproate toxicity.

The results of the present study indicate that low concentrations of sorafenib and vorinostat as well as sorafenib and sodium valproate interact in a synergistic manner to kill pancreatic and liver cancer cells *in vitro*. Other studies have shown that sorafenib and vorinostat combined can induce cell death via ligand independent but ceramide dependent activation of the CD95 death receptor signaling pathway (Zhang G, *et. al.* 2008; Park MA, *et. al.* 2008). The CD95 pathway can be negatively regulated by FLIP proteins that have sequence homology to caspase 8 but lack the catalytic domain and hence when FLIP is recruited to activated death receptors rather than caspases, it can block activation of the caspase cascade and the cell death cascade (Krueger A, *et. al.* 2001). There are several splice variants of FLIP known to exist at the mRNA levels but three isoforms of FLIP protein namely, c-FLIP-s, c-FLIP-r and c-FLIP-l have been identified (Djerbi M, *et. al.* 2001; Rasper DM, *et. al.* 1998; Golks A, *et. al.* 2005). This study confirms that sorafenib + vorinostat induced cell death in cancer cells is mediated via CD95 signaling and shows that sorafenib + sodium valproate induced lethality in cancer cells also involves CD95 signaling. Knock-down of CD95 or over-expression of c-FLIP-s protected tumor cells from the effects of these drug combinations. Studies have suggested similar roles of c-FLIP-s and c-FLIP-r in death receptor mediated apoptosis (Golks A, *et. al.* 2005). We chose to over-express c-FLIP-s rather than c-FLIP-l because studies have confirmed the

anti-apoptotic role of c-FLIP-s as it can inhibit the activation of pro-caspase 8 and thereby inhibit CD95 induced cell death (Golks A, *et. al.* 2005). However, the role of c-FLIP-l in the activation of CD95 pathway remains controversial. Some studies suggest the c-FLIP-l is an anti-apoptotic molecule whereas other studies ascribe pro-apoptotic functions to c-FLIP-l referring to its assistance in pro-caspase 8 activation (Chang DW, *et. al.* 2003; Micheau O, *et. al.* 2002). Collectively, the present findings argue that pancreatic and hepatoma tumor cells are susceptible to being killed by sorafenib + HDACI exposure through a death receptor dependent mechanism.

Studies have shown that anti-apoptotic Bcl-2 family proteins namely, Bcl-xl and Mcl-1 play a role in maintaining mitochondrial integrity and prevent activation of cell death pathways (Certo M, *et. al.* 2006; Chipuk JE and Green DR. 2008). In unstressed cells, pro-apoptotic Bcl-2 family protein BAK has been shown to be sequestered primarily by Mcl-1 (Cuconati A, *et. al.* 2003). Upon death receptor and caspase 8 activation, BH-3 only protein BID is cleaved to form truncated BID (tBID) which is known to displace BAK from the Mcl-1-BAK complex allowing BAK to perform its pro-apoptotic functions (Clohessy JG, *et. al.* 2006). We observed that 6 h after sorafenib and sodium valproate treatment, no significant change in Mcl-1 level is observed compared to control treated cells. However, at this time point after sorafenib and sodium valproate combined treatment, a decrease in BAK association with Mcl-1 is observed suggesting that BAK is available to activate cell death pathways as early as 6 h post sorafenib and sodium valproate combined treatment. 12 h post sorafenib and sodium valproate treatment, a decrease in Mcl-1 level as well as a decrease in BIM co-immunoprecipitated with Mcl-1 in

observed. Studies have shown that in unstressed cells, BIM is sequestered by Mcl-1 and certain stressful stimuli can lead to caspase mediated degradation of Mcl-1 and this allows Mcl-1-free BIM to initiate cell death pathways (Han J, *et. al.* 2006).

It is known that the pro-apoptotic Bcl-2 family protein BAX primarily resides in the cytosol of unstressed cells and upon certain stressful stimuli, BAX is known to translocate to the mitochondria where it can damage mitochondrial integrity and initiate cell death pathways (Murphy KM, *et. al.* 2000). We found that sorafenib and sodium valproate combined induce mitochondrial localization of BAX within 12 h of treatment suggesting that upon exposure to the drug combination, BAX translocates to the mitochondria in order to initiate cell death pathways.

Studies have shown that in cancer cells HDACI alone can induce CD95 activation and sorafenib and vorinostat combined can cause an increase in CD95 cell surface localization/activation 6 h post drug treatment (Insinga A, *et. al.* 2005; Zhang G, *et. al.* 2008). Our results showed that 6 h after drug treatment, neither sorafenib nor sodium valproate alone induced CD95 cell surface localization but this was observed in cells treated with both drugs at the same time suggesting that the drug combination is able to activate CD95 cell death pathway as early as 6 h after treatment. This also indicates that CD95 activation is likely involved in mediating cell death induced by sorafenib and sodium valproate combination treatment.

An inability to express death receptors or the ability to over-express dominant negative forms of death receptors has been linked to apoptosis resistance (Walsh CM, *et. al.* 2003; Safa AR, *et. al.* 2008). An additional mechanism that could block toxic death receptor

signaling is constitutive high expression levels of c-FLIP-s (Safa AR, *et. al.* 2008).

Increased expression of mitochondrial protective proteins such as Mcl-1 and Bcl-xl have also been implicated in mediating drug resistance in cancer cells (Martin AP, *et. al.* 2008; Del Poeta G, *et. al.* 2008). As mentioned previously, our studies show that sorafenib + HDACI combined can trigger cell death via CD95 signaling. We wanted to enhance the toxicity of sorafenib and sodium valproate combined, as well as reduce the chance of resistance development in cancer cells treated with these agents. Therefore, it was logical to combine sorafenib and sodium valproate with obatoclax, which can facilitate cell death independent of CD95 signaling, target the mitochondrial protective proteins, and according to our results, obatoclax can enhance cell death in cancer cells when combined with sorafenib and sodium valproate at all dose combinations tested. Theoretically, using these three drugs in combination would reduce the likelihood of tumor cells being resistant as well as potentially circumventing the development of drug resistant cancers. We found that while knock-down of CD95 or over-expression of c-FLIP-s, protected tumor cells from sorafenib and sodium valproate combined lethality, additional treatment of cells with obatoclax under these conditions failed to protect them from cell death. This confirmed our hypothesis that treating cancer cells with obatoclax+sorafenib+sodium valproate induces cell death that is maintained even upon blockade of CD95 signaling.

No significant effect on cell death was found upon addition of obatoclax to cells co-treated with varying doses of vorinostat or sodium valproate. The presence of obatoclax, however, caused a significant increase in cell death when combined with 3 uM and 6 uM sorafenib compared to identical conditions in the absence of obatoclax. However, cell death was



enhanced most when obatoclax was combined with sorafenib and sodium valproate simultaneously.

As mentioned previously, obatoclax is a pan-inhibitor of the protective Bcl-2 family proteins which includes Bcl-xl, Mcl-1 and Bcl-2 (Trudel S, *et. al.* 2007). Since obatoclax enhanced sorafenib and sodium valproate mediated cell death, we determined whether knock-down of Bcl-xl, Mcl-1 or Bcl-2 alone would be sufficient to mimic the effects of obatoclax on sorafenib and sodium valproate treated cells. siRNA mediated individual knock-down of Bcl-xl or Bcl-2 , but not Mcl-1, significantly enhance sorafenib and sodium valproate induced cell death compared to control siRNA treated cells. Simultaneous knock down of Bcl-2 and Bcl-xl significantly increased sorafenib + sodium valproate toxicity compared to control siRNA treated cells. Furthermore, simultaneous loss of Bcl-2, Bcl-xl *and* Mcl-1 expression resulted in a significant increase in sorafenib + sodium valproate induced cell death compared to control siRNA and siBcl-xl + siBcl-2 treated cells. This suggests that inhibiting more than one pro-survival Bcl-2 family protein at the same time, rather than individual Bcl-2 family members, enhances sorafenib and sodium valproate mediated lethality to a greater extent.

Studies have shown that in the presence of cellular stress, BAK and BAX change conformation, oligomerize and form pores in the outer mitochondrial membrane that allows the release of proteins from the inter-mitochondrial space that can then activate cell death pathways (Antignani A and Youle RJ. 2006). Immunoblotting studies showed that compared to vehicle conditions, treatment of cells with sorafenib + sodium valproate combined, obatoclax alone or sorafenib + sodium valproate + obatoclax combined resulted

in increased “active” BAK and BAX proteins suggesting that in the presence of the above mentioned drug treatments, BAK and BAX are activated and hence available to initiate cell death pathways.

Upon activation, pro-apoptotic Bcl-2 family proteins BAK and BAX can lead to the release of cytochrome c from the mitochondria to cytosol (Wei MC, *et. al.* 2001; Desagher S, *et. al.* 1999). This is considered a key step in the initiation of cell death pathways as this can lead to formation of the apoptosome consisting of cytochrome c, its adaptor molecule called Apaf-1 (apoptotic protease activating factor 1) and pro-caspase 9 (Cain K, *et. al.* 2000). Binding of cytochrome c to Apaf-1 is thought to facilitate the binding of ATP to the apoptosome which then allows for caspase 9 activation (Adrain C, *et. al.* 1999). Active caspase 9 can then lead to activation of various other caspases including caspase 2, 3, 6, 7, 8 and 10 (Guerrero AD, *et. al.* 2008). Cells treated with sorafenib + sodium valproate + obatoclax simultaneously show a higher level of cytochrome c release into the cytosol as compared to other treatment conditions. Over-expression of c-FLIP-s abolishes basal and sorafenib + sodium valproate induced cytosolic release of cytochrome c. However, in spite of c-FLIP-s over-expression, cytosolic release of cytochrome c is observed in cells treated with either obatoclax alone or treated with sorafenib + sodium valproate + obatoclax combined. This indicates that cell death pathways can be activated via cytosolic release of cytochrome c in cells treated with sorafenib + sodium valproate + obatoclax in spite of loss of CD95 function mediated by c-FLIP-s over-expression.

As sorafenib+HDACI therapy is about to be explored in a phase I trial, our data suggest that the incorporation of obatoclax together with sorafenib+HDACI therapy may provide significant additional value in tumor control.

In conclusion, the results of the present study indicate that sorafenib and vorinostat or sodium valproate interact in a synergistic manner to kill pancreatic and liver tumor cells *in vitro* via activation of CD95. These effects are magnified when Bcl-2 family protein activity is inhibited and of novelty demonstrate that loss of extrinsic pathway activation does not diminish cell death levels induced by sorafenib and sodium valproate in combination with obatoclax.

## GENERAL DISCUSSION

We investigated the mechanism of lapatinib mediated cell death and lapatinib resistance in HCT 116 colon cancer cells. Lapatinib has been approved by the Food and Drug Administration to be used in the treatment for breast cancer as part of a combination therapy. Clinical trials have been planned and are underway to investigate the use of lapatinib in the treatment of other types of cancers including colorectal cancer (Clinicaltrials.gov). Hence, it is important to understand the mechanism of action of lapatinib in order to optimize its toxic effects on cancer cells and also identify other drugs that may be combined with lapatinib to enhance cancer cell killing and reduce occurrence of refractory cancers. Lapatinib has been known to cause its effects by inhibiting ERBB1 and ERBB2 tyrosine kinase receptors and their downstream signaling pathways (Wood ER, *et. al.* 2004; Rusnak DW, *et. al.* 2001). We expressed dominant negative ERBB1 and/or ERBB2 receptors in wild-type HCT116 colon cancer cells in order to mimic the effects of lapatinib and to investigate whether inhibition of these receptors was the sole mechanism of lapatinib mediated toxicity in these cells. Dominant negative ERBB1 and ERBB2 receptors were able to inhibit receptor activation similar to lapatinib but were unable to induce comparable levels of cell death as seen in the presence of lapatinib. It is possible that CD533 and CD572 inhibit the receptors but are unable to inhibit downstream ERK and Akt activation. Hence, we determined whether an inability to inhibit ERK1/2 and Akt pro-survival signaling pathways prevented CD533 and CD572 from inducing of cell death in WT cells. However, dominant negative ERBB1 and ERBB2 receptors inhibited

ERK1/2 and Akt phosphorylation in WT cells indicating that lack of inhibition of these pro-survival signaling pathways was not the reason for lack of cell death in cells treated with CD533 and CD572. This data suggests that lapatinib mediated effects in wild-type HCT 116 cells may not solely depend on inhibition of ERBB1 and ERBB2 pathways and that possibly other targets of lapatinib are involved. Several drugs have been developed to target specific, however, over the years, studies have shown that these agents can mediate their toxicity by inhibition of other target molecules as well. For instance, sorafenib was developed as a Raf-kinase inhibitor but later studies showed that it could mediate its effects via inhibition of other kinases such as VEGFR and PDGFR (Wilhelm SM, *et. al.* 2008). Hence, it is possible that further studies may reveal other targets of lapatinib.

The present study showed that lapatinib induced cell death in wild-type HCT 116 cells is caspase independent and is mediated by cytosolic release of apoptosis inducing factor (AIF) from the mitochondria which is thought to initiate cell death (Penninger JM and Kroemer G. 2003). Other chemotherapeutic agents have also been known to induce cell death via AIF activation. For instance, studies show that arsenic trioxide is effective in killing human cervical cancer cells via cytosolic release of AIF (Kang YH, *et. al.* 2004).

Chemotherapeutic drug resistance development has been shown to be a major problem in the process of treating cancer (Kobayashi S, *et. al.* 2005). Initial drug treatment may kill cancer cells but often times cells may become resistant to drugs used to treat the cancer initially and also to a variety of other chemotherapeutic agents making it refractory cancers very difficult to manage (Kobayashi S, *et. al.* 2005). Our studies show that it is possible for lapatinib resistance to occur in colon cancer cells. This suggests that lapatinib resistance

could occur in patients and understanding the mechanism of lapatinib resistance in colon cancer cells may enable us to diagnose and treat resistance development in the clinic. It may also assist us in designing combinatorial therapy regimes in order to reduce the chances of lapatinib resistance development in cancer patients.

Other studies have shown that in breast cancer cells, lapatinib resistance can occur due alterations in the PI3K pathway via loss-of-function mutations in the tumor suppressor PTEN, up-regulation of estrogen receptor or due to an inability of lapatinib to inhibit pro-survival ERK and Akt signaling downstream of ERBB receptors (Eichhorn PJ, *et. al.* 2008; Chen FL, *et. al.* 2008; Zhou H, *et. al.* 2004). However, in WT-AD cells, it was observed that inhibition estrogen receptor signaling did not restore lapatinib sensitivity in these cells suggesting that lapatinib resistance in WT-AD cells was unlikely to be mediated by changes in estrogen receptor signaling. Studies performed by others in our laboratory have also shown that inhibition of ERK1/2 and/or Akt in WT-AD cells did not enhance lapatinib mediated cell death suggesting that these proteins were not responsible for lapatinib resistance in WT-AD cells (Martin AP, *et. al.* 2008).

In WT-AD cells, increased expression Bcl-xl, Mcl-1 and p53 proteins, decreased expression of pro-death Bax protein and decreased phosphorylation of p53 was noted. It was also found that Bax and Bak proteins were activated in lapatinib treated wild-type HCT 116 cells but not in WT-AD cells. Hence, it appears that alterations in the expression and activation of Bcl-2 family proteins are involved in mediating lapatinib resistance in HCT 116 colon cancer cells. Other studies have shown that altered expression of Bcl-2 family proteins can confer multi-drug resistance phenotype in cancer cells by dramatically

reducing toxicity mediated by commonly used therapeutic agents such as bleomycin, cisplatin and etoposide (Raffo AJ, *et. al.* 1995; Minn AJ, *et. al.* 1995). Hence, several drugs have been developed to target Bcl-2 family proteins such as obatoclax that mimics the BH-3 domain of pro-death Bcl-2 family members and can inhibit the activity of pro-survival Bcl-2 members by interacting with them (Zhang L, *et. al.* 2007).

Further studies will be required to investigate the effects of Bcl-2 family inhibitors on WT and WT-AD cells but it can be expected that such inhibitors will prove to be toxic to lapatinib resistant WT-AD cells. If this is the case, it may prove beneficial to combine lapatinib with a Bcl-2 family inhibitor such as obatoclax in the clinic in order to reduce the likelihood of resistance development in patients.

Our studies confirmed previous finding that multi-kinase inhibitor sorafenib and vorinostat (a histone deacetylase inhibitor or HDACI) combined toxicity is mediated via CD95 death receptor pathway (Zhang G, *et. al.* 2008; Park MA, *et. al.* 2008). We have also shown that sorafenib interacts with another HDACI named sodium valproate in a synergistic manner to kill transformed cells via a mechanism involving the CD95 death receptor pathway.

Studies have shown that HDACIs themselves can cause an induction of the CD95/CD95L system and hence cells treated with HDACI alone may also show elevated CD95 surface localization/activation (Glick RD, *et. al.* 1999). However, at the time-points tested, we found that CD95 cell surface localization was elevated in cells treated with sorafenib and sodium valproate combined but not in cells treated with vehicle or individual drugs.

Previous studies have shown that in cancer cells, resistance to drugs that mediate their effects via the CD95 pathway can occur due to an inability of the cells to express death

receptors or by over-expressing dominant negative forms of death receptors (Park SM, *et. al.* 2005; Walsh CM, *et. al.* 2003). Since sorafenib+HDACI mediated effects involve CD95 activation, it is possible that alterations in the CD95 signaling pathway may render cancer cells resistant to this drug combination. Hence, we wanted to combine sorafenib and sodium valproate with a third agent i.e. obatoclax. Obatoclax mediated cell death is CD95 independent and therefore combining it with sorafenib+sodium valproate may reduce chances of resistance development occurring due to alterations in the CD95 signaling pathway. Obatoclax is a BH-3 mimetic and binds the BH-3 binding domain of the Bcl-2 family proteins and is known to mediate its effects in cancer cells via inhibition of Mcl-1-BAK interaction, BIM upregulation and cytochrome c release into the cytosol (Trudel S, *et. al.* 2007). Alterations in the Bcl-2 family proteins have also been implicated in mediating chemotherapeutic drug resistance in cancer (Martin AP, *et. al.* 2008) and based on previous, using obatoclax may likely reduce occurrence of resistance development in cancer cells via alterations in the Bcl-2 family proteins as well (Nguyen M, *et. al.* 2007). Results showed that obatoclax enhanced the toxicity of sorafenib and sodium valproate combined. Knock-down of CD95 or over-expression of c-FLIP-s (a negative regulator of the CD95 pathway) to mimic a non-functional CD95 pathway, protected cells from sorafenib+sodium valproate mediated cells death. However, no significant reduction in cell death levels was found in HEPG2 hepatoma cells treated with all three agents (i.e. sorafenib+sodium valproate+obatoclax) in spite of CD95 knock-down or over-expression of c-FLIP-s. This confirmed our hypothesis that combining sorafenib and sodium valproate



with obatoclax (which is known to mediate cell death independent of CD95 activation) maintains toxic effects in cancer cells even if the CD95 pathway is not functional.

In conclusion, the results of the present study indicate that sorafenib and vorinostat or sodium valproate interact in a highly synergistic manner to kill tumor cells *in vitro* via activation of CD95 pathway. These effects are magnified as well as maintained in spite of loss of CD95 death receptor pathway function when Bcl-2 family protein activity is inhibited in addition to sorafenib+sodium valproate treatment of HEPG2 liver cancer cells. This suggests that it may prove beneficial to combined sorafenib+HDACI with a Bcl-2 family inhibitor such as obatoclax in order to avoid occurrence of refractory cancers in patients.

### **List of references**

- Adachi M, *et. al.* 2000. Nuclear export of MAP kinase (ERK) involves a MAP kinase kinase (MEK)-dependent active transport mechanism. *J Cell Biol.* 148(5): 849-856.
- Adrain C, *et. al.* 1999. Regulation of apoptotic protease activating factor-1 oligomerization and apoptosis by the WD-40 repeat region. *J Biol Chem.* 274: 20855-20860.
- Alavi A, Hood JD, *et. al.* 2003. Role of Raf in vascular protection from distinct apoptotic stimuli. *Science.* 301(5629): 94-96.
- Allan LA, *et. al.* 2003. Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. *Nat Cell Biol.* 5(7):647-54.
- Alonso G, *et. al.* 2000. Differential activation of p38 mitogen-activated protein kinase isoforms depending on signal strength. *J Biol Chem.* 275(51): 40641-40648.
- Alsdorf R and Wyszynski DF. 2005. Teratogenicity of sodium valproate. *Expert Opin Drug Saf.* 4: 345-353.
- Amorino GP, *et. al.* 2002. Epidermal growth factor receptor dependence of radiation-induced transcription factor activation in human breast carcinoma cells. *Mol Cell Biol.* 13(7):2233-44.
- Antignani A and Youle RJ. 2006. How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane? *Curr Opin Cell Biol.* 18(6): 685-689.
- Ashkenazi A. 2002. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2: 420-430.
- Attardi LD, *et. al.* 2000. PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family. *Genes Dev.* 14(6): 704-18.
- Awwad RA, *et. al.* 2003. The role of transforming growth factor alpha in determining growth factor independence. *Cancer Res.* 63(15): 4731-4738.
- Baba I, *et. al.* 2000. Involvement of deregulated epiregulin expression in tumorigenesis in vivo through activated Ki-Ras signaling pathway in human colon cancer cells. *Cancer Res.* 60(24):6886-9.

Bae SS, *et. al.* 2003. Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. *J Biol Chem.* 278(49): 49530-6.

Bakin RE, *et. al.* 2003. Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. *Cancer Res.* 63(8): 1981-1989.

Baldwin AS. 2001. Control of oncogenesis and cancer therapy resistance by the transcription factor NF- $\kappa$ B. *J Clin Invest.* 107(3): 241-246.

Bali P, *et. al.* 2005. Activity of suberoylanilide hydroxamic Acid against human breast cancer cells with amplification of her-2. *Clin Cancer Res.* 11: 6382-6389.

Bange J, *et. al.* 2001. Molecular targets for breast cancer therapy and prevention. *Nat Med.* 7: 548-552.

Berthois Y, *et. al.* 1986. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci USA.* 83(8): 2496-2500.

Bewry NN, *et. al.* 2008. Stat3 contributes to resistance toward BCR-ABL inhibitors in a bone marrow microenvironment model of drug resistance. *Mol Cancer Ther.* 7(10): 3169-3175.

Bhana S and Llyod DR. 2008. p53-dependent global nucleotide excision repair of cisplatin-induced intrastrand cross links in human cells. *Mutagenesis.* 23(1): 43-50.

Bianco R, *et. al.* 2008. Vascular endothelial growth factor receptor-1 contributes to resistance to anti-epidermal growth factor receptor drugs in human cancer cells. *Clin Cancer Res.* 14(16): 5069-5080.

Birtwistle MR, *et. al.* 2007. Ligand-dependent responses of the ErbB signaling network: experimental and modeling analyses. *Mol Syst Biol.* 3:144.

Blagosklonny MV. 2000. p53 from complexity to simplicity: mutant p53 stabilization, gain-of-function, and dominant-negative effect. *FASEB J.* 14(13): 1901-7.

- Blaheta RA, *et. al.* 2005. Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. *Med Res Rev.* 25(4): 383-397.
- Bleeker FE, *et. al.* 2008. AKT1(E17K) in human solid tumours. *Oncogene.* May 26) (Kim MS, *et. al.* 2008. Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias. *Br J Cancer.* 98(9): 1533-5.
- Bolden JE, *et. al.* 2006. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov.* 5: 769-784.
- Broker LE, *et. al.* 2004. Cathepsin B mediates caspase-independent cell death induced by microtubule stabilizing agents in non-small cell lung cancer cells. *Cancer Res.* 64: 27-30.
- Bug G, *et. al.* 2005. Valproic acid stimulates proliferation and self-renewal of hematopoietic stem cells. *Cancer Res.* 65: 2537-2541.
- Bulavin DV, *et. al.* 1999. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J.* 18(23): 6845-6854.
- Bursch W. 2004. Multiple cell death programs: Charon's lifts to Hades. *FEMS Yeast Res.* 5(2): 101-110.
- Buschmann T, *et. al.* 2001. Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. *Mol Cell Biol.* 21(8): 2743-2754.
- Cabannes E, *et. al.* 1999. Mutations in the I $\kappa$ B $\alpha$  gene in Hodgkin's disease suggest a tumour suppressor role for I $\kappa$ B $\alpha$ . *Oncogene.* 18(20): 3063-3070.
- Cain K, *et. al.* 2000. Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4-MDa apoptosome complexes. *J Biol Chem.* 275: 6067-6070.
- Camirand A, *et. al.* 2002. Co-targeting HER2/ErbB2 and insulin-like growth factor-1 receptors causes synergistic inhibition of growth in HER2-overexpressing breast cancer cells. *Med Sci Monit.* 8(12): BR521-6.

cancer.org (1)

([http://www.cancer.org/downloads/STT/Global\\_Facts\\_and\\_Figures\\_2007\\_rev2.pdf](http://www.cancer.org/downloads/STT/Global_Facts_and_Figures_2007_rev2.pdf))

cancer.org (2) (<http://www.cancer.org/downloads/STT/2008CAFFfinalsecured.pdf>)

Cantley LC and Neel BG. 1999. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci.* 96: 4240-4245.

Cantley LC. 2002. The phosphoinositide 3-kinase pathway. *Science.* 296(5573): 1655-1657.

Catlett-Falcone R, *et. al.* 1999. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity.* 10(1): 105-115.

Certo M, *et. al.* 2006. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell.* 9: 351-365.

Chang DW, *et. al.* 2003. Interdimer processing mechanism of procaspase-8 activation. *EMBO J.* 22(16): 4132-4142.

Chen FL, *et. al.* 2008. Acquired resistance to small molecule ErbB2 tyrosine kinase inhibitors. *Clin Cancer Res.* 14(21): 6730-6734.

Chen N., *et. al.* 2001. Suppression of skin tumorigenesis in c-Jun NH(2)-terminal kinase-2-deficient mice. *Cancer Res.*, 61(10): 3908-3912.

Chen S, *et. al.* 2007. Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. *Cancer Res.* 67: 782-791.

Chen YR, *et. al.* 1996. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J Biol Chem.* 271(50): 31929-31936.

Chène P. 2001. The role of tetramerization in p53 function. *Oncogene.* 20(21): 2611-2617.

Chin KV, *et. al.* 1992. Modulation of activity of the promoter of the human MDR1 gene by Ras and p53. *Science.* 255(5043): 459-62.

Chipuk JE and Green DR. 2008. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol.* 18: 157- 164.

Chittenden T, *et. al.* 1995. A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *EMBO J.* 14: 5589-5596.

Cho NL, *et. al.* 2007. Estrogen receptors alpha and beta are inhibitory modifiers of Apc-dependent tumorigenesis in the proximal colon of Min/+ mice. *Cancer Res.* 67: 2366-2372.

Chong H, *et. al.* 2003. Mechanisms of regulating the Raf kinase family. *Cell Signal.* 15: 463-469.

Cinatl J. Jr., *et. al.* 1997. Sodium valproate inhibits in vivo growth of human neuroblastoma cells. *Anticancer Drugs.* 8: 958-963.

Clarke PG. 1990. Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol.* 181: 195-213.

Clinicaltrials.gov (<http://clinicaltrials.gov/ct2/show/NCT00574171>)

Clohessy JG, *et. al.* 2006. Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis. *J Biol Chem.* 281(9): 5750-5759.

Croce CM. 2008. Oncogenes and cancer. *N Engl J Med.* 358(5):502-11.

Cuconati A, *et. al.* 2003. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev.* 17(23): 2922-2932.

Cunningham MP, *et. al.* 2006. Responses of human colorectal tumor cells to treatment with the anti-epidermal growth factor receptor monoclonal antibody ICR62 used alone and in combination with the EGFR tyrosine kinase inhibitor gefitinib. *Cancer Res.* 66(15): 7708-7715.

Das B, *et. al.* 2005. A hypoxia-driven vascular endothelial growth factor/Flt1 autocrine loop interacts with hypoxia-inducible factor-1alpha through mitogen-activated protein

kinase/extracellular signal-regulated kinase 1/2 pathway in neuroblastoma. *Cancer Res.* 65(16): 7267-7275.

Dasmahapatra G, *et. al.* 2007. Synergistic interactions between vorinostat and sorafenib in chronic myelogenous leukemia cells involve Mcl-1 and p21CIP1 down-regulation. *Clin Cancer Res.* 13: 4280-4290.

Davis RJ. 2000. Signal transduction by the JNK group of MAP kinases. *Cell.* 103(2): 239-252.

Davies H, *et. al.* 2002. Mutations of the BRAF gene in human cancer. *Nature.* 217: 949-954.

de Bruin EC and Medema JP. 2008. Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treat Rev.* 34(8): 737-749.

Degterev A, *et. al.* 2003. A decade of caspases. *Oncogene.* 22: 8543-8567.

Del Poeta G, *et. al.* 2008. Deregulation of the mitochondrial apoptotic machinery and development of molecular targeted drugs in acute myeloid leukemia. *Curr Cancer Drug Targets.* 8(3): 207-222.

Dent P, *et. al.* 1992. A myofibrillar protein phosphatase from rabbit skeletal muscle contains the beta isoform of protein phosphatase-1 complexed to a regulatory subunit which greatly enhances the dephosphorylation of myosin. *Eur J Biochem.* 210(3):1037-44.

Dent P, *et. al.* 2003. MAPK pathways in radiation responses. *Oncogene.* 22(37): 5885-5896.

Der CJ and Cox AD. 1991. Isoprenoid modification and plasma membrane association: critical factors for ras oncogenicity. *Cancer Cell.* 3: 331-340.

Desagher S, *et. al.* 1999. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol.* 144(5): 891-901.

Dhanasekaran DN and Reddy EP. 2008. JNK signaling in apoptosis. *Oncogene.* 27: 6245-6251.



- Dittmer D, *et. al.* 1993. Gain of function mutations in p53. *Nat Genet.* 4(1): 42-46.
- Djerbi M, *et. al.* 2001. Characterization of the human FLICE-inhibitory protein locus and comparison of the anti-apoptotic activity of four different flip isoforms. *Scand J Immunol.* 54(1-2): 180-189.
- Dokmanovic M, *et. al.* 2007. Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res.* 5(10): 981-989.
- Donovan N, *et. al.* 2002. JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery. *J Biol Chem.* 277(43): 40944-40949.
- Dornan D, *et al.* 2004. The ubiquitin ligase COP1 is a critical negative regulator of p53. *Nature* 429: 86–92.
- Downward J. 2003. Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer.* 3(1): 11-22.
- Droga-Mazovec G, *et. al.* 2008. Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues. *J Biol Chem.* 283(27): 19140-19150.
- Duenaz-Gonzalez A, *et. al.* 2008. Valproic acid as epigenetic cancer drug: preclinical, clinical and transcriptional effects on solid tumors. *Cancer Treat Rev.* 34(3): 206-222.
- Eichhorn PJ, *et. al.* 2008. Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235. *Cancer Res.* 68(22): 9221-9230.
- EMD Biosciences  
(<http://www.emdbiosciences.com/Products/ProductDisplay.asp?catno=573095&>)
- Erster S, *et. al.* 2004. In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol Cell Biol.* 24(15): 6728-41.

Franke TF. 2008. PI3K/Akt: getting it right matters. *Oncogene*.27(50): 6473-88.

Friesen C, *et. al.* 1997. Deficient activation of the CD95 (APO-1/Fas) system in drug-resistant cells. *Leukemia*. 11: 1833-1841.

Fuchs SY, *et. al.* 1998. JNK targets p53 ubiquitination and degradation in nonstressed cells. *Genes & Dev*. 12: 2658-2663.

Fulda S and Debatin KM. 2006. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*. 25: 4798-4811.

Gannon JV, *et. al.* 1990. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J*. 9(5): 1595-1602.

Gardner AM, *et. al.* 1994. MEK-1 phosphorylation by MEK kinase, Raf, and mitogen-activated protein kinase: analysis of phosphopeptides and regulation of activity. *Mol Biol Cell*. 5(2): 193-201.

Geminx.com (<http://www.geminx.com/en/research/gx15070.php>)

GlaxoSmithKline ([http://us.gsk.com/products/assets/us\\_tykerb.pdf](http://us.gsk.com/products/assets/us_tykerb.pdf))

Glick RD, *et. al.* 1999. Hybrid polar histone deacetylase inhibitor induces apoptosis and CD95/CD95 ligand expression in human neuroblastoma. *Cancer Res*. 59(17): 4392-4399.

Glozak MA, *et. al.* 2005. Acetylation and deacetylation of non-histone proteins. *Gene*. 363: 15-23.

Golks A, *et. al.* 2005. c-FLIPR, a new regulator of death receptor-induced apoptosis. *J Biol Chem*. 280(15): 14507-14513.

Gollob JA. 2005. Sorafenib: scientific rationales for single-agent and combination therapy in clear-cell renal cell carcinoma. *Clin Genitourin Cancer*. 4:167-74.

Gozuacik D, Kimchi A. 2004. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene*. 23(16): 2891-2906.

Graeber TG, *et. al.* 1994. Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. *Mol Cell Biol.* 14(9): 6264-6277.

Grant S, *et. al.* 2002. Roles of ERBB family receptor tyrosine kinases, and downstream signaling pathways, in the control of cell growth and survival. *Front Biosci.* 7: d376-389.

Grant S and Dent P. 2004. Kinase inhibitors and cytotoxic drug resistance. *Clin Cancer Res*; 10:2205-7.

Green DR and Kroemer G. 2004. The pathophysiology of mitochondrial cell death. *Science.* 305(5684): 626-629.

Gregory PD, *et. al.* 2001. Histone acetylation and chromatin remodeling. *Exp Cell Res.* 265: 195-202.

Gross A, *et. al.* 1999. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem* 274 (2): 1156-1163.

Guerrero AD, *et. al.* 2008. Delineation of the caspase-9 signaling cascade. *Apoptosis.* 13(1): 177-86.

Gupta E, *et. al.* 1997. Modulation of glucuronidation of SN-38, the active metabolite of irinotecan, by valproic acid and phenobarbital. *Cancer Chemother Pharmacol.* 39: 440-444.

Gurvich N, *et. al.* 2004. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Res.* 64(3):1079-86.

Han J, *et. al.* 2006. Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis. *J Biol Chem.* 281(15): 10153-10163.

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

Herrmann JL, *et. al.* 1997. Bcl-2 suppresses apoptosis resulting from disruption of the NF-kappa B survival pathway. *Exp Cell Res.*237(1): 101-109.

Honegger AM, *et. al.* 1987. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. *Cell*. 51(2): 199-209.

Hoozemans JJ, *et. al.* 2004. Neuronal COX-2 expression and phosphorylation of pRb precede p38 MAPK activation and neurofibrillary changes in AD temporal cortex. *Neurobiol Dis*. 15(3): 492-499.

Houston A and O'Connell J. 2004. The Fas signalling pathway and its role in the pathogenesis of cancer. *Curr Opin Pharmacol*. 4(4): 321-326.

Huang Y, *et. al.* 2007. Distinct roles of VEGFR-1 and VEGFR-2 in the aberrant hematopoiesis associated with elevated levels of VEGF. *Blood*. 110(2): 624-631.

Hussain SP and Harris CC. 1998. Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. *Cancer Res*. 58(18): 4023-37.

Hynes NE and Lane HA. 2005. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer*. 5(5): 341-54.

Imai K and Takaoka A. 2006. Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer*. 9: 714-27.

Insinga A, *et. al.* 2005. Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. *Nat Med*. 11(1): 71-76.

Janne PA. 2008. Challenges of detecting EGFR T790M in gefitinib/erlotinib-resistant tumours. *Lung Cancer*. 60 Suppl2: S3-9.

Jaumot M and Hancock JF. 2001. Protein phosphatases 1 and 2A promote Raf-1 activation by regulating 14-3-3 interactions. *Oncogene*. 20(30): 3949-3958.

Jiang ZY, *et. al.* 2003. Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc Natl Acad Sci U S A*. 100(13): 7569-74.

Jimenez GS, *et. al.* 1999. p53 regulation by post-translational modification and nuclear retention in response to diverse stresses. *Oncogene*. 18: 7656-7665.

Johnson GL and Nakamura K. 2007. The c-jun kinase/stress-activated pathway: regulation, function and role in human disease. *Biochim Biophys Acta*. 1773(8):1341-8.

Kang YH, *et al.* 2004. Caspase-independent cell death by arsenic trioxide in human cervical cancer cells: reactive oxygen species-mediated poly(ADP-ribose) polymerase-1 activation signals apoptosis-inducing factor release from mitochondria. *64*(24):8960-7.

Kennedy NJ and Davis RJ. 2003. Role of JNK in tumor development. *Cell Cycle*. 2(3): 199-201.

Kischkel FC, *et al.* 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J*. 14: 5579-5588.

Kilic M, *et al.* 2002. Formation of noncanonical high molecular weight caspase-3 and -6 complexes and activation of caspase-12 during serum starvation induced apoptosis in AKR-2B mouse fibroblasts. *Cell Death Differ*. 9(2): 125-137.

Klas C, *et al.* 1993. Activation interferes with the APO-1 pathway in mature human T cells. *Int Immunol*. 5: 625-630.

Knudson A. 1971. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci* 68(4): 820-3.

Kobayashi S, *et al.* 2005. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 352(8): 786-792.

Kohler MF, *et al.* 1992. Overexpression and mutation of p53 in endometrial carcinoma. *Cancer Res*. 52(6): 1622-1627.

Kordes U, *et al.* 2000. Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells. *Leukemia*. 14(3): 399-402.

Korsmeyer SJ, *et al.* 2000. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ*. 7(12): 1166-1173.

- Kourtis N and Tavernarakis N. 2009. Autophagy and cell death in model organisms. *Cell Death Differ.* 16(1): 21-30.
- Krammer PH. 2000. CD95's deadly mission in the immune system. *Nature.* 407: 789-795.
- Krantic S, *et. al.* 2007. Apoptosis-inducing factor: a matter of neuron life and death. *Prog Neurobiol.* 81: 179-196.
- Krueger A, *et. al.* 2001. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol Cell Biol.* 21: 8247-8251.
- Kubbuttat MH, *et. al.* 1997. Regulation of p53 stability by Mdm2. *Nature.* 387(6630): 299-303.
- Kuendgen A, *et. al.* 2007. Valproic acid for the treatment of myeloid malignancies. *Cancer.* 110(5): 943-54.
- Kwon SH, *et. al.* 2002. Apicidin, a histone deacetylase inhibitor, induces apoptosis and Fas/Fas ligand expression in human acute promyelocytic leukemia cells. *J Biol Chem.* 277: 2073-2080.
- Kyriakis JM, *et. al.* 1992. Raf-1 activates MAP kinase-kinase. *Nature.* 358(6385):417-21.
- Lamb JA, *et. al.* 2003. JunD mediates survival signaling by the JNK signal transduction pathway. *Mol Cell.* 11(6): 1479-1489.
- Lawlor MA and Alessi DR. 2001. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci.* 114: 2903.
- Lei X, *et. al.* 2006. Gossypol induces Bax/Bak-independent activation of apoptosis and cytochrome c release via a conformational change in Bcl-2. *FASEB.* 20: 2147-2149.
- Leist M, *et. al.* 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med.* 185(8): 1481-1486.
- Leng RP, *et. al.* 2003. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. *Cell* 112: 779–791.

Letai A, *et. al.* 2002. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* 2(3): 183-192.

Levine B and Klionsky DJ. 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*. 6(4): 463-77.

Ley Y, *et. al.* 2003. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J Biol Chem*. 278:18811-6.

Li N, *et. al.* 2007. B-Raf kinase inhibitors for cancer treatment. *Curr Opin Investig Drugs* 2007; 8:452-6.

Liang XH, *et. al.* 1999. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*. 402(6762): 672-676.

Lin NU and Winer EP. 2004. Small molecule tyrosine kinase inhibitors. *Breast Cancer Res*. 6:204-10.

Ling YH, *et. al.* 2008. Erlotinib induces mitochondrial-mediated apoptosis in human H3255 non-small-cell lung cancer cells with epidermal growth factor receptor L858R mutation through mitochondrial oxidative phosphorylation-dependent activation of BAX and BAK. *Mol Pharmacol*. 74(3): 793-806.

Longley DB, *et. al.* 2006. c-FLIP inhibits chemotherapy-induced colorectal cancer cell death. *Oncogene*. 25: 838-848.

LoPiccolo J, *et. al.* 2008. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resist Updat*. 11(1-2): 32-50.

Los M, *et. al.* 1997. Cross-resistance of CD95- and drug-induced apoptosis as a consequence of deficient activation of caspases (ICE/Ced-3 proteases). *Blood*. 90(8): 3118-3129.

Lu Y, *et. al.* 2001. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst*. 93(24): 1852-1857.

- Luo J, *et.al.* 2003. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell.* 4(4): 257-262.
- Marchenko ND, *et.al.* 2000. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J Biol Chem.* 75(21): 16202-12.
- Maya R, *et. al.* 2001. ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev.* 15: 1067-1077.
- Maehama T and Dixon JE. 1999. PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends Cell Biol.* 9: 125-128.
- Marks P, *et. al.* 2001. Histone deacetylases and cancer: causes and therapies. *Nat Rev Cancer.* 1: 194-202.
- Marks PA, *et. al.* 2003. Histone deacetylases. *Curr Opin Pharmacol.* 3: 344-351.
- Marks PA, *et. al.* 2005. Histone deacetylase inhibitors: discovery and development as anticancer agents. *Expert Opin Investig Drugs.* 14(12): 1497-1511.
- Marks PA and Breslow R. 2007. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. *Nat Biotechnol.* 25: 84-90.
- Martin AP, *et. al.* 2008. Lapatinib resistance in HCT116 cells is mediated by elevated MCL-1 expression and decreased BAK activation and not by ERBB receptor kinase mutation. *Mol Pharmacol.* 74(3): 807-822.
- McDonnell TJ, *et. al.* 1992. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res.* 52: 6940–6944.
- Mcintyre A, *et. al.* 2005. Amplification and overexpression of the KIT gene is associated with progression in the seminoma subtype of testicular germ cell tumors of adolescents and adults. *Cancer Res.* 65(18): 8085-8089.
- Meijerink JP, *et. al.* 1998. Hematopoietic malignancies demonstrate loss-of-function mutations of BAX. *Blood.* 91: 2991- 2997.



Mendelsohn J. 2002. Targeting the epidermal growth factor receptor for cancer therapy. *J Clin Oncol.* 20(Suppl. 18):1S–13S.

Meng Y, *et. al.* 2008. Natural BH3 mimetic (-)-gossypol chemosensitizes human prostate cancer via Bcl-xL inhibition accompanied by increase of Puma and Noxa.. *Mol Cancer Ther.* 7(7): 2192-2202.

Micheau O, *et. al.* 2002. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem.* 277(47): 45162-45172.

Minn AJ, *et. al.* 1995. Expression of bcl-xL can confer a multidrug resistance phenotype. *Blood.* 86(5):1903-10.

Mitsiades CS, *et. al.* 2004. Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. *Proc Natl Acad Sci U S A.* 101(2): 540-545.

Miyashita T and Reed JC. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell.* 80: 293-299.

Modjtahedi N, *et. al.* 2006. Apoptosis-inducing factor: vital and lethal. *Trends Cell Biol.* 16(5): 264-272.

Moll UM and Petrenko O. 2003. The MDM2-p53 interaction. *Mol Cancer Res.* 1(14): 1001-1008.

Mori M, *et. al.* 2003. Activation of extracellular signal-regulated kinases ERK1 and ERK2 induces Bcl-xl up-regulation via inhibition of caspase activities in erythropoietin signaling. *J Cell Physiol.* 195:290–7.

Murphy KM, *et. al.* 2000. Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. *Cell Death Differ.* 7(1): 102-111.

Muschen M, *et. al.* 2000. CD95 ligand expression as a mechanism of immune escape in breast cancer. *Immunology.* 99(1): 69-77.

Nagata S. 2000. Apoptotic DNA fragmentation. *Exp Cell Res.* 256: 12-18.

Nahta R, *et. al.* 2007. Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling. *Mol Cancer Ther.* 6(2): 667-674.

Nair PN, *et. al.* 2001. Aberrant expression and activation of insulin-like growth factor-1 receptor (IGF-1R) are mediated by an induction of IGF-1R promoter activity and stabilization of IGF-1R mRNA and contributes to growth factor independence and increased survival of the pancreatic cancer cell line MIA PaCa-2. *Oncogene.* 20(57): 8203-8214.

National Cancer Institute. FDA Approval for Lapatinib Ditosylate Brand name: Tykerb® <http://www.cancer.gov/cancertopics/druginfo/fda-lapatinib>

Nelson MH and Dolder CR. 2006. Lapatinib: a novel dual tyrosine kinase inhibitor with activity in solid tumors. *Ann Pharmacother.* 40(2): 261-9.

Nemajerova A, *et.al.* 2005. Viral and cellular oncogenes induce rapid mitochondrial translocation of p53 in primary epithelial and endothelial cells early in apoptosis. *FEBS Lett.* 579(27): 6079-83.

Nguyen M, *et. al.* 2007. Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc Natl Acad Sci U S A.* 104: 19512–19517.

Offer H, *et. al.* 2002. The onset of p53-dependent DNA repair or apoptosis is determined by the level of accumulated damaged DNA. *Carcinogenesis.* 23(6): 1025-1032.

Olayioye, *et. al.* 2000. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.* 19: 3159–3167.

Oliner JD, *et. al.* 1992. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature.* 358: 80–83.

Otera H, *et. al.* 2005. Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. *EMBO J.* 24: 1375-1386.

Ozoren N and El-Deiry WS. 2003. Cell surface Death Receptor signaling in normal and cancer cells. *Semin Cancer Biol.* 13(2): 135-147.

Palmero I, *et. al.* 1998. p19ARF links the tumour suppressor p53 to Ras. *Nature*. 395: 125-126.

Pao W, *et. al.* 2005. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med*. 2(3):e73.

Papachristou DJ, *et. al.* 2003. Activation of the JNK-AP-1 signal transduction pathway is associated with pathogenesis and progression of human osteosarcomas. *Bone*. 32(4): 364-71.

Parreno M, *et. al.* 2008. Bobel-24 and derivatives induce caspase-independent death in pancreatic cancer regardless of apoptotic resistance. *Cancer Res*. 68(15): 6313-6323.

Parikh AA and Ellis LM. 2004. The vascular endothelial growth factor family and its receptors. *Hematol Oncol Clin N Am*. 18: 951-971.

Park MA, *et. al.* 2008. Vorinostat and sorafenib increase ER stress, autophagy and apoptosis via ceramide-dependent CD95 and PERK activation. *Cancer Biol Ther*. 7(10): 1648-1662.

Park SM, *et. al.* 2005. Non-apoptotic functions of FADD-binding death receptors and their signaling molecules. *Curr Opin Cell Biol*. 17: 610-616.

Peart MJ, *et. al.* 2005. Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. *Proc Natl Acad Sci USA*. 102(10): 3697-3702.

Penninger JM and Kroemer G. 2003. Mitochondria, AIF and caspases--rivaling for cell death execution. *Nat Cell Biol*. 5: 97-99.

Peschard, P. and Park, M. 2003. Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. *Cancer Cell* 3: 519–523.

Pfister S, *et. al.* 2008. BRAF gene duplication constitutes a mechanism of MAPK pathway activation in low-grade astrocytomas. *J Clin Invest*. 118(5): 1739-1749.

Prives C and Hall PA. 1999. The p53 pathway. *J Pathol*. 187(1): 112-26.

Qiao L, *et. al.* 2003. Bile acid regulation of C/EBPbeta, CREB, and c-Jun function, via the extracellular signal-regulated kinase and c-Jun NH2-terminal kinase pathways, modulates the apoptotic response of hepatocytes. *Mol Cell Biol.* 23:3052-66.

Qin B, *et. al.* 2006. Activated Src and Ras induce gefitinib resistance by activation of signaling pathways downstream of epidermal growth factor receptor in human gallbladder adenocarcinoma cells. *Cancer Chemother Pharmacol.* 58(5): 577-584.

Raffo AJ, *et. al.* 1995. Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. *Cancer Res.* 55(19): 4438-4445.

Rahmani M, *et. al.* 2005. Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. *J Biol Chem.* 280:35217-27.

Rahmani M, *et. al.* 2007a. The multikinase inhibitor sorafenib induces apoptosis in highly imatinib mesylate-resistant bcr/abl+ human leukemia cells in association with signal transducer and activator of transcription 5 inhibition and myeloid cell leukemia-1 down-regulation. *Mol Pharmacol* 2007; 72:788-95.

Rahmani M, *et. al.* 2007b. The kinase inhibitor sorafenib induces cell death through a process involving induction of endoplasmic reticulum stress. *Mol Cell Biol.* 27: 5499-5513.

Rasper DM, *et. al.* 1998. Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. *Cell Death Differ.* 5(4): 271-288.

Raspolini MR, *et. al.* 2004. c-KIT expression and correlation with chemotherapy resistance in ovarian carcinoma: an immunocytochemical study. *Ann Oncol.* 15(4): 594-597.

Read AP and Stachan T. 1999. Chapter 18: Cancer genetics. *Human molecular genetics* 2. New York: Wiley. ISBN 0-471-33061-2.

Reddel RR, *et. al.* 1983. Effects of biologically active metabolites of tamoxifen on the proliferation kinetics of MCF-7 human breast cancer cells in vitro. *Cancer Res.* 43: 4618-4624.

Reed JC, *et. al.* 1996. BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J Cell Biochem.* 60(1): 23-32.

Reed JC. 2002. Apoptosis-based therapies. *Nat Rev Drug Disc.* 1(2): 111- 121.

Reed JC. 2006. Proapoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, and therapeutic opportunities. *Cell Death Differ.* 13: 1378-1386.

Richon VM, *et. al.* 1998. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proc Natl Acad Sci U S A.* 95(6): 3003-7.

Richon VM. 2006. Cancer biology: mechanism of antitumour action of vorinostat (suberoylanilide hydroxamic acid), a novel histone deacetylase inhibitor. *Brit J of Cancer* 95:S2-S6.

Riggs BL and Hartmann LC. 2003. Selective estrogen-receptor modulators -- mechanisms of action and application to clinical practice. *N Engl J Med.* 348(7): 618-629.

Rini BI. 2006. Sorafenib. *Expert Opin Pharmacother.* 2006; 7:453-61.

Robert E. 1991. Teratogenic risks of epilepsy and anticonvulsants. *Pediatric.* 46: 579-583.

Rosato RR, *et. al.* 2007. The multikinase inhibitor sorafenib potentiates TRAIL lethality in human leukemia cells in association with Mcl-1 and cFLIPL down-regulation. *Cancer Res.* 67(19): 9490-9500.

Roth W, *et. al.* 2001. Soluble decoy receptor 3 is expressed by malignant gliomas and suppresses CD95 ligand-induced apoptosis and chemotaxis. *Cancer Res.* 61: 2759-2765.

Rowinsky EK. 2004. The erbB family: targets for therapeutic development against cancer and therapeutic strategies using monoclonal antibodies and tyrosine kinase inhibitors. *Annu Rev Med.* 55: 433-457.

Rusnak DW, *et. al.* 2001. The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. *Mol Cancer Ther.* 1(2): 85-94.

Safa AR, *et. al.* 2008. Cellular FLICE-like inhibitory protein (C-FLIP): a novel target for cancer therapy. *Curr Cancer Drug Targets*. 8: 37-46.

Salomon, DS, *et. al.* 1995. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit. Rev. Oncol. Hematol.* 19: 183–232.

Saporita AJ, *et. al.* 2007. Therapeutic targets in the ARF tumor suppressor pathway. *Curr Med Chem*. 14(17): 1815-27.

Savill J, *et. al.* 2003. Cell biology. Eat me or die. *Science*. 302(5650): 1516-1517.

Scaffidi C, *et. al.* 1998. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*. 17: 1675-1687.

Scheid MP, *et. al.* 1999. Regulation of bad phosphorylation and association with Bcl-x(L) by the MAPK/Erk kinase. , *J Biol Chem*. 274(43):31108-13.

Schmidt-Ullrich RK, *et. al.* 2003. ERBB receptor tyrosine kinases and cellular radiation responses. *Oncogene*. 22(37): 5855-5865.

Seabra MC. 1998. Membrane association and targeting of prenylated Ras-like GTPases. *Cell Signal*. 10: 167-172.

Sedlacek HH. 2000. Kinase inhibitors in cancer therapy: a look ahead. *Drugs* 59: 435-476.

Senzer N, *et. al.* 2007. p53 therapy in a patient with Li-Fraumeni syndrome. *Mol Cancer Ther*. 6: 1478-1482.

Shawver LK, *et. al.* 1997. Inhibition of platelet-derived growth factor-mediated signal transduction and tumor growth by N-[4-(trifluoromethyl)-phenyl]5-methylisoxazole-4-carboxamide. *Clin Cancer Res*. 3: 1167-1177.

Shinozaki T, *et. al.* 2003. Functional role of Mdm2 phosphorylation by ATR in attenuation of p53 nuclear export. *Oncogene*. 22: 8870-8880.

Shintani T and Klionsky DJ. 2004. Autophagy in health and disease: a double-edged sword. *Science*. 306(5698): 990-995.

Siliciano JD, *et. al.* 1997. DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev.* 11(24): 3471-81.

Sizeland AM and Burgess AW. 1992. Anti-sense transforming growth factor alpha oligonucleotides inhibit autocrine stimulated proliferation of a colon carcinoma cell line. *Mol Biol Cell.* 3: 1235–1243.

Sok JC, *et. al.* 2006. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. *Clin Cancer Res.* 12(17): 5064-5073.

Sridhar SS, *et. al.* 2005. Raf kinase as a target for anticancer therapeutics. *Mol Cancer Ther.* 4(4): 677-685.

Strano S, *et. al.* 2007. Mutant p53: an oncogenic transcription factor. *Oncogene.* 26(15):2212-9.

Strasser A. 2005. The role of BH3-only proteins in the immune system.. *Nat Rev Immunol.* 5: 189-200.

Strumberg D, *et. al.* 2005. Phase I clinical and pharmacokinetic study of the Novel Raf kinase and vascular endothelial growth factor receptor inhibitor BAY 43-9006 in patients with advanced refractory solid tumors. *J Clin Oncol.* 23: 965-972.

Sumitomo M, *et. al.* 1999. An essential role for nuclear factor kappa B in preventing TNF-alpha-induced cell death in prostate cancer cells. *J Urol.* 161(2): 674-679.

Sunpaweravong P, *et. al.* 2005. Epidermal growth factor receptor and cyclin D1 are independently amplified and overexpressed in esophageal squamous cell carcinoma. *J Cancer Res. Clin. Oncol.* 131: 111–119.

Szakacs G, *et. al.* 2006. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov.* 5(3): 219-234.

Taddei A, *et. al.* 2005. The effects of histone deacetylase inhibitors on heterochromatin: implications for anticancer therapy? *EMBO Rep.* 6(6): 520-524.

- Tang Y, *et. al.* 2008. Acetylation is indispensable for p53 activation. *Cell*. 133(4): 612-626.
- Taniguchi M, *et. al.* 1999. Effect of c-kit mutation on prognosis of gastrointestinal stromal tumors. *Cancer Res*. 59: 4297-4300.
- Thorton TM and Rincon M. 2009. Non-classical p38 map kinase functions: cell cycle checkpoints and survival. *Int J Biol Sci*. 5(1): 44-52.
- Toledo F and Wahl GM. 2006. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer*. 6(12): 909-23.
- Tournier C, *et. al.* 2000. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science*. 288(5467): 870-874.
- Trudel S, *et. al.* 2007. Preclinical studies of the pan-Bcl inhibitor obatoclax (GX015-070) in multiple myeloma. *Blood*. 109(12): 5430-5438.
- Tsujimoto Y, *et. al.* 1984. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science*. 226: 1097-1099.
- Ueshima S, *et. al.* 2008. *J Clin Pharm Ther*. 33(1): 31-38.
- Uren RT, *et. al.* 2005. Mitochondrial release of pro-apoptotic proteins: electrostatic interactions can hold cytochrome c but not Smac/DIABLO to mitochondrial membranes. *J Biol Chem*. 280: 2266-2274.
- Vakkila J and Lotze MT. 2004. Inflammation and necrosis promote tumour growth. *Nat Rev Immunol*. 4: 641-648.
- Valerie K, *et. al.* 2007. Radiation-induced cell signaling: inside-out and outside-in. *Mol Cancer Ther*. 6(3): 789-801.
- van Delft MF, *et. al.* 2006. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell*. 10(5): 389-399.



Vandenabeele P, *et. al.* 2005. Serine proteases and calpains fulfill important supporting roles in the apoptotic tragedy of the cellular opera. *Cell Death Differ.* 12: 1219-1224.

van Noesel MM, *et. al.* 2002. Tumor-specific down-regulation of the tumor necrosis factor-related apoptosis-inducing ligand decoy receptors DcR1 and DcR2 is associated with dense promoter hypermethylation. *Cancer Res.* 62: 2157-2161.

Vassilev LT, *et. al.* 2004. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science.* 303: 844-848.

Vassilev LT. 2005. p53 activation by small molecules: Application in oncology. *J Med Chem.* 48(14): 4491-4499.

Vousden KH and Lu X. 2002. Live or let die: the cell's response to p53. *Nat Rev Cancer.* 2(8): 594-604.

Waetzig V and Herdegen T. 2005. Context-specific inhibition of JNKs: overcoming the dilemma of protection and damage. *Trends Pharmacol Sci.* 26(9): 455-461.

Wagner J, *et. al.* 2005. p53-Mdm2 loop controlled by a balance of its feedback strength and effective dampening using ATM and delayed feedback. *Syst Biol.* 152(3): 109-118.

Walczak H and Krammer PH. 2000. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Exp Cell Res.* 256: 58-66.

Walsh CM, *et. al.* 2003. The "fuzzy logic" of the death-inducing signaling complex in lymphocytes. *J Clin Immunol.* 23: 333-353.

Wan P, *et. al.* 2004. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell.* 116: 855-867.

Wang CW and Klionsky DJ. 2003. The molecular mechanism of autophagy. *Mol Med.* 9(3-4): 65-76.

Wang W and El-Deiry WS. 2008. Restoration of p53 to limit tumor growth. *Curr Opin Oncol.* 20(1):90-96.

Wang WF, *et. al.* 2007. Apoptosis induction in human melanoma cells by inhibition of MEK is caspase-independent and mediated by the Bcl-2 family members PUMA, Bim, and Mcl-1. *Clin Cancer Res.* 13:4934-42.

Wei MC, *et. al.* 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science.* 292(5517): 727-730.

Whitmarsh AJ and Davis RJ. 1996. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med.* 74: 589-607.

Wilhelm S and Chien DS. 2002. BAY 43-9006: preclinical data. *Curr Pharm Des.* 8: 2255-2257.

Wilhelm SM, *et. al.* 2004. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res.* 64(19): 7099-109.

Wilhelm SM, *et. al.* 2008. Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol Cancer Ther.* 7(10): 3129-3140.

Willis SN, *et al.* 2005. Pro-apoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev.* 19: 1294-1305.

Wilson AJ, *et. al.* 2006. Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem.* 281: 13548-13558.

Witters LM, *et. al.* 2007. Synergistic inhibition of breast cancer cell lines with a dual inhibitor of EGFR-HER-2/neu and a Bcl-2 inhibitor. *Oncol Rep.* 17(2): 465-469.

Wise LD, *et. al.* 2007. Assessment of developmental toxicity of vorinostat, a histone deacetylase inhibitor, in Sprague-Dawley rats and Dutch Belted rabbits. *Birth Defects Res B Dev Reprod Toxicol.* 80: 57-68.

Wood ER, *et. al.* 2004. A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res.* 64(18): 6652-6659.

- Wu J, *et. al.* 1992. Renaturation and partial peptide sequencing of mitogen-activated protein kinase (MAP kinase) activator from rabbit skeletal muscle. *Biochem J.* 285: 701-705.
- Xia W, *et. al.* 2006. A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. *Proc Natl Acad Sci U S A.* 103(20): 7795-7800.
- Xu L, *et. al.* 2006. MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer. *Oncogene.* 25(21):2987-98.
- Xu W, *et. al.* 2007. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene.* 27: 5541-5552.
- Yacoub A, *et. al.* 2006. Radiotherapy-induced signal transduction. *Endocr Relat Cancer.* 13 Suppl 1:S99-114.
- Yang YM, *et. al.* 2003. C-Jun NH(2)-terminal kinase mediates proliferation and tumor growth of human prostate carcinoma. *Clin Cancer Res.* 9: 391–401.
- Yarden Y and Sliwkowski MX. 2001. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol.* 2: 127-137.
- Yip KW and Reed JC. 2008. Bcl-2 family proteins and cancer. *Oncogene.* 27: 6398-6406.
- van Oijen M and Slootweg PJ. 2000. Gain-of-function mutations in the tumor suppressor gene p53. *Clin Cancer Res.* 6: 2138-2145.
- Yorimitsu T and Klionsky DJ. 2005. Autophagy: molecular machinery for self-eating. *Cell Death Differ.* 12: 1542-1552.
- Zawacka-Pankau J, *et al.* 2007. Protoporphyrin IX interacts with wild-type p53 protein in vitro and induces cell death of human colon cancer cells in a p53-dependent and -independent manner. *J Biol Chem.* 282(4): 2466-2472.
- Zhang G, *et. al.* 2008. Vorinostat and sorafenib synergistically kill tumor cells via FLIP suppression and CD95 activation. *Clin Cancer Res.* 14(17): 5385-5399.

Zhang L, *et. al.* 2007. BH3 mimetics to improve cancer therapy; mechanisms and examples. *Drug Resist Updat.* 10(6): 207-217.

Zhou H, *et. al.* 2004. Effects of the EGFR/HER2 kinase inhibitor GW572016 on EGFR- and HER2-overexpressing breast cancer cell line proliferation, radiosensitization, and resistance. 58(2):344-52.

Zhou Y, *et. al.* 2006. Blockade of EGFR and ErbB2 by the novel dual EGFR and ErbB2 tyrosine kinase inhibitor GW572016 sensitizes human colon carcinoma GEO cells to apoptosis. *Cancer Res.* 66(1): 404-411.

## Curriculum Vitae

### Aditi Pandya Martin

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#### Education:

- 2005-2009      *Ph.D. Pharmacology & Toxicology*  
Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA.
- 2002-2005      *B.S. Microbiology*  
Michigan State University, East Lansing, MI.
- 2001-2002      B.S. Zoology (Freshman Year)  
Maharaja Sayajirao University of Baroda, Gujarat, India.

#### Research Experience:

- 2006-present      *Doctoral Research* (Advisor: Paul Dent, Ph.D.) Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA.
- *Dissertation:* 'Mechanisms of drug toxicity and resistance in cancer'.
  - Characterized the mechanism of resistance to the chemotherapeutic agent Lapatinib in HCT116 colon cancer cells.
  - Identified effects of the BCL-2 family inhibitor, Obatoclax, on HCT116 cells and the ability of Obatoclax to enhance Lapatinib toxicity in BT474 breast cancer cells.
  - Investigated the mechanism of combined Sorafenib and Valproate toxicity to HEPG2 cancer cells.
- 2002-2005      Undergraduate Research Assistant (Advisor: Frances Trail, Ph.D.), Department of Plant Biology, Michigan State University, East Lansing, MI.
- Characterized functions of the mycotoxin Zearalenone in *G. zeae*.

**Honors and Awards:**

2009	VCU School of Medicine Phi Kappa Phi scholarship award
2007	Phi Kappa Phi national honor society membership
2005-present	Pre-doctoral Fellowship, Department of Pharmacology and Toxicology, Virginia Commonwealth University
2004	Frank Howard Award for Undergraduate Research, American Phytopathological Society
2003	Global Spartan Scholarship for Outstanding International Students, Michigan State University

**Articles:**

**Martin AP**, Park MA, *et al.* BCL-2 family inhibitors enhance HDACI+sorafenib toxicity and overcome blockade of the extrinsic pathway to facilitate killing. (Submitted to Molecular Pharmacology).

Park MA, Walker T, **Martin AP**, *et al.* Ceramide/CD95- and PERK-dependent regulation of MDA-7/IL-24-induced cell killing in human renal carcinoma cells. (Submitted to Molecular Cancer Therapeutics).

**Martin AP**, Miller A, *et al.* Lapatinib resistance in HCT116 cells is mediated by elevated MCL-1 expression and decreased BAK activation and not by ERBB receptor kinase mutation. *Molecular Pharmacology*. 2008 Sep;74(3):807-22.

Park MA, Zhang G, **Martin AP**, *et al.* Vorinostat and sorafenib increase ER stress, autophagy and apoptosis via ceramide-dependent CD95 and PERK activation. *Cancer Biology and Therapy*. 2008 Oct 12;7(10).

Zhang G, Park MA, Mitchell C, Hamed H, Rahmani M, **Martin AP**, *et al.* Vorinostat and sorafenib synergistically kill tumor cells via FLIP suppression and CD95 activation. *Clinical Cancer Research*. 2008 Sep 1;14(17):5385-99.

Hamed H, Mitchell C, Park MA, Hanna D, **Martin AP**, *et al.* Human chorionic gonadotropin (hCG) interacts with lovastatin and ionizing radiation to modulate prostate cancer cell viability in vivo. *Cancer Biology and Therapy*. 2008 Jan 8;7(4).

Yacoub A, Park MA, Hanna D, Hong Y, Mitchell C, **Pandya AP**, *et al.* OSU-03012 promotes caspase-independent but PERK-, cathepsin B-, BID-, and AIF-dependent killing of transformed cells. *Molecular Pharmacology*, 2006 Aug;70(2):589-603.

**Presentations:**

- 2009 American Association for Cancer Research, Denver, CO (poster).
- 2008 36<sup>th</sup> Annual John C. Forbes Graduate Student Honors Colloquium, Virginia Commonwealth University (platform).
- 2005 22<sup>nd</sup> Annual Daniel T. Watts Research Symposium, Virginia Commonwealth University (poster).
- 2004 11<sup>th</sup> Annual Microbial Midwest Pathogenesis Conference (poster).

**Student Membership:**

- 2008-present President, Pharmacology and Toxicology Student Organization, Virginia Commonwealth University.
- 2008-present Member, American Association for Cancer Research.
- 2008-present Secretary, Virginia Academy of Science (Medical Sciences Section).
- 2007-2008 Vice President, Pharmacology and Toxicology Student Organization, Virginia Commonwealth University.
- 2006-2007 Secretary, Pharmacology and Toxicology Student Organization, Virginia Commonwealth University.
- 2006-2007 Cofounder and V.P. for Programming and Professional Development, Women In Science Organization, Virginia Commonwealth University.
- 2003-2004 Cofounder and Undergraduate Director of Public Relations, International Student Association, Michigan State University.

**Service:**

- 2007 Student Editor, Pharmacology and Toxicology Department newsletter.
- 2007 Student Representative, Pharmacology and Toxicology curriculum review committee.
- 2006-2007 Seminar Organizer, Women in Science Organization.
- 2006-2007 Annual “Questers Day” for teaching high school students basic laboratory science and procedures.