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Combinatorial Modulation of Multiple Signaling Pathways to Gain Therapeutic Response in Breast and Prostate Cell Carcinomas

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COMBINATORIAL MODULATION OF MULTIPLE SIGNALING PATHWAYS TO GAIN THERAPEUTIC RESPONSE IN BREAST AND PROSTATE CELL CARCINOMAS.

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

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Abbreviations

AG1478	tyrosine kinase inhibitor
AIF	apoptosis inducing factor
Ala	alanine
AP-1	aκpurinic binding site 1
ATM	Ataxia telangiectasia mutant
BCC	breast cell carcinoma
Bcl-2	B-cell lymphoma 2 protein
bFGF	basic fibroblast growth factor
BPH	benign prostatic hypertrophy
Cdk	cyclin dependent kinase
CENP-E	centromeric protein E
CENP-F	centromeric protein F
CMV	cytomegalovirus
CRD	cysteine-rich domain
CTP	C-terminal peptide
DAG	diacylglycerol
DcR	decoy receptors
DISC	death-induced signaling ligand
DMSO	dimethyl sulfoxide
DN-caspase-9	dominant negative caspase-9
DR	death receptor
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER-	estrogen receptor negative
ER+	estrogen receptor positive
ErbB	epidermal growth factor receptor gene family
ERK	extracellular related kinase
FADD	Fas-ligand associated death domain
FAK	focal adhesion kinase
FSH	follicle stimulating hormone
FTI	farnesyl transferase inhibitor
GAP	GTPase-activating proteins
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor

GGTI	geranylgeranyl transferase inhibitor
Gln	glutamine
Gly	glycine
GM6001	matrix metallo-protease inhibitor
GnRH	Gonadotropin releasing hormone
GPH	glycoprotein hormone
Grb2	growth factor receptor 2
GTP	guanine triphosphate
Gy	Gray (radiation dose equal to the amount absorbed)
H&E	hematoxylin and eosin
hCG	human Chorionic Gonadotropin
hCGα	human Chorionic Gonadotropin alpha subunit
hCGβ	human Chorionic Gonadotropin beta subunit
i.p.	intraperitoneal
IAP	inhibitors of apoptosis
IETD	caspase-8 inhibitor
IGF	insulin-like growth factor
IKK	inhibitor of κB-kinase
INK	family of inhibitors of cyclin dependent kinases
IP	inositol phospholipids
JNK	c-JUN N-terminal transcription factor kinase
Kg	kilogram
KSR	kinase suppressor of Ras
LEHD	caspase-9 inhibitor
LH	lutenizing hormone
LHRH	lutenizing hormone releasing hormone
Lova	lovastatin
LXL	lysine X lyseine sub-motif
LY294002	PI3 kinase inhibitor
MAP	mitogen-activated protein
MAPK	mitogen activated protein kinase
MEK	MAP kinase kinase
Mg	milligram
MKP-3	mitogen-activated protein kinase phosphatase-3
mm	millimeter
MNNG	N-methyl-N-nitrosoguanidine
MP1	mitogen adaptor protein 1
mU/ml	milliUnits per milliliter equivalent
NF-κB	nuclear factor kappa B transcription factor
nM	nanomolar
PARP-1	poly-(ADP-ribose) polymerase-1

PBS	phosphate buffered saline
PD032591	MEK1/2 inhibitor
PD183452	MEK1/2 inhibitor
PD-ECGF	platelet derived endothelial cell growth factor
PDGF	platelet-derived growth factor
PDK	protein dependent kinase
PgR	progesterone receptor
PH	plecstrin binding domain
PJ-34	PARP inhibitor
PKB	protein kinase B
PKC	protein kinase C
PLD	potentially lethal damage
PLGF	placenta growth factor
PP2A	protein phosphatase 2A
PTEN	lipid phosphatase and tensin homologue of chromosome 10
PTX	pertussis toxin
RBD	Ras binding domain
RIP	receptor-interacting protein
RKIP	Ras kinase inhibitor
RTK	receptor tyrosine kinas
SAPK	stress-activated protein kinase
SEK1	stress-activated extracellular regulated kinase
SEM	standard error of the mean
Ser	serine
SLD	sub-lethal damage
SOS	son of sevenless
SUR-8	Ras-8 suppressor
T/C	tumor to control ratio
TGF-α	transforming growth factor alpha
TGF-β	transforming growth factor beta
Thr	threonine
TNFR	tumor necrosis factor receptor
TNFα	tumor necrosis factor alpha
TRADD	tumor necrosis factor receptor-associated death domain
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
TSH	thyroid stimulating hormone
Tyr	tyrosine
µl	microliter
µm	micrometer
µM	micromolar
U/ml	enzyme Unit per milliliter

UCN-01	7-hydroxystaurosporine
VEGF	vascular endothelial growth factor
zVAD	pan-caspase inhibitor

Abstract

Combinatorial Modulation of Multiple Signaling Pathways to Gain Therapeutic Response in Breast and Prostate Cell Carcinomas

By William Tressel Hawkins II, M.S.

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

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Major Director: Paul Dent, Ph.D.
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Our laboratory is primarily interested in novel pharmacological intervention of cell proliferation and survival pathways expressed in various types of cancer. These cyto-protective pathways can be activated in response to growth factor stimulation, toxic insult and radiation. In our studies, we utilized novel drug combinations with and without radiation to enhance breast & prostate tumor cell death both in vitro and in vivo. Previous studies from our group have shown that UCN-01 and MEK1/2 inhibitors interact to cause tumor cell death in transformed cell lines in vitro. We extended this observation to an in vivo animal model system using the estrogen dependent breast cell carcinoma line MCF-

7 and the estrogen independent breast cell carcinoma line MDA-MB-231. This drug combination was shown to profoundly reduce tumor cell proliferation in vivo and also exhibited the ability to significantly reduce ex-vivo tumor cell colony formation 30 days after cessation of the combination drug treatment. In addition, tumor cell death coincided with decreased ERK1/2 phosphorylation, reduced immunoreactivity of Ki67 and CD31. Overall, these studies demonstrate that UCN-01 and MEK1/2 inhibitors have the potential to suppress mammary tumor growth in vivo which is independent of p53 status, estrogen dependency, caspase-3 levels or oncogenic K-RAS expression. In our LnCap prostate carcinoma cell studies we demonstrated the impact of hCG and lovastatin in combination with ionizing radiation to radiosensitize and enhance tumor cell lethality. This enhancement was attributed to the hCG-induced activation of ERBB1 via a GPCR, MEK1/2 and metalloprotease dependent paracrine mechanism which was further enhanced by radiation. This enhanced cell killing effect was shown to involve prolonged activation of PARP1 which could be suppressed by inhibition of ERBB1, MEK1, PI3 kinase or PARP1. Therefore, the combination of hCG, lovastatin and radiation may represent a novel approach to kill prostate cancer cells and potential new therapy.

Introduction

1.1 Cancer

Carcinomas are defined as malignant neoplasms of epithelial origin arising from any of the three germ layers; ectoderm, mesoderm or endoderm. Carcinomas are further classified upon the organ of origination and microscopic evaluation of cell morphology. Tumors displaying a glandular growth pattern are referred to as adenocarinomas, e.g. prostate adenocarcinoma and breast cell adenocarcinoma. And those tumors which produce distinctive squamous cells arising from any epithelium in the body are referred to as squamous cell carcinomas, e.g. bronchogenic squamous cell carcinoma. Neoplasia refers to "new growth" in which both benign and malignant tumors share two basic components: (1) the proliferation of new growth that makes up the parenchyma and (2) a supportive architecture made up of connective tissue and blood vessels. Malignant tumors share four characteristics which occur in phases: (1) a target cell undergoes a malignant change, referred to as transformation (2) proliferation of the transformed cell (3) marked by local invasion and (4) resulting in distant metastasis. Today, the common term for all malignant tumors is cancer. (Kumar, Abbas and Fausto (eds), 2005).

The beginning of the 21st century saw 10 million new cases of cancer and 6 million cancer related deaths worldwide (Parkin, 2000) . In the United States, figures released by the American Cancer Society estimate 556,000 cancer related deaths at a rate of 1500 per day and accounting for 23% of all deaths in the United States in 2003 (Jemel et al, 2003). However, advances in the treatment of cancers have resulted in a decline in cancer related mortality during the last decade of the 20th century (Simmonds, 2003). In addition, the dramatic improvements in the 5 year survival rates for many forms of cancer are continuing to this day.

The most common forms of cancer occurring in men are prostate, lung and colorectal, and the most common forms of cancer occurring in women are breast, lung, colon and rectal. Cancer of the lungs, female breast, prostate and colon/rectum comprise more than 50% of new cancer cases and deaths in the United States (Weir et al, 2003).

1.2 Breast Cell Carcinoma

Breast cell carcinoma is the most common form of non-skin cell cancer in woman. In 2006, breast cell carcinoma is estimated to account for approximately 62,000 newly diagnosed cases, 212,000 new cases of invasive cancer and 41,000 deaths in the United States. Better treatments and early detection and screening account for significant

increases year over year in the five year survival rate and breast cancer death rates have declined on average, 2.4% per year since 1990 (Smigal et al, 2006).

Breast cell carcinomas are manifested as invasive ductal carcinomas, medullary carcinomas, inflammatory carcinomas, papillary invasive ductal carcinomas, invasive lobular carcinomas and adenocarcinomas. They are further classified by their genetic expression profile or mutation, (e.g.) Steroid receptor status; i.e., estrogen receptor-alpha (ER), progesterone receptor (PgR), Growth factor status; i.e., epidermal growth factor receptor (EGFR), Her-2/neu (ERBB2), Transforming growth factor-alpha (TGF-α), germ-line mutation status; BRCA1/2, etc.

The three most commonly used (ER+) breast cell carcinomas (BCC) lines for in vivo studies in mice are MCF-7, T-47D and ZR-75-1 which require some form of estrogen supplementation for tumorogenesis to occur in nude mice. The two most commonly used (ER-) BCC lines are MDA-MB-231 and MDA-MB-435 which are more aggressive than their ER+ counterparts and MDA-MB-435 is the most likely to develop metastasis in nude mice. The number of established breast cancer cell lines for use in experimental research is remarkably small, only about 100 known and with three cell lines, MCF-7, T47D and MDA-MB-231 accounting for two-thirds of all abstracts reporting experimental study results found on Medline/PubMed (Lacroix and Leclercq, 2004).

Male breast cancer is rare, about 1.4% of all breast cancer cases occur in men. The average male has a lifetime risk of developing breast cancer of about 0.11%, while a woman's risk is 13%. Male breast cancer accounts for approximately 1500 new cases per year and about 400 deaths. Between 4 and 14% of male breast cancer is linked to a hereditary BRCA2 mutations. But male breast cancer is far less common in families with BRCA1 mutations. And 3-8% occurs in males with Klinefelter syndrome (male hypogonadism with an XXY karyotype). Pathology of male breast cancer is nearly identical to that of woman except that 81% of male breast cancers are estrogen receptor positive (ER+). Systemic treatment and prognostic factors are nearly similar for both men and women (Giorodano and Buzdar, 2002).

1.3 Prostate adenocarcinoma

In men, prostate cancer is the most common malignancy, which in the United States is estimated to account for an additional 230,000 cases diagnosed in 2006 and result in around 31,000 deaths (Sivaprasad et al, 2006).

Cancer of the prostate involves multimodality treatment including radiation, hormone therapy and surgery. Greater than 90% of all prostate cancer patients treated with any of the methods above live 15 years or longer after diagnosis (American Cancer Society,

2004). Surgical re-section (i.e. radical prostatectomy) and radiation therapy with either external focused-beam or brachytherapy (interstitial placement of radioactive seeds) are commonly used for the treatment of prostate cancer.

Hormonal manipulation or endocrine therapy is treatment of choice, for metastatic or advanced prostate cancer (Droller MJ, 1997). The dependence of prostate cells on androgens to sustain them allows for the use of endocrine manipulation to deprive the cells of testosterone. This strategy employs the administration of lutenizing hormone-releasing hormone (LHRH) agonists which suppresses testosterone production. However, during the course of prostate tumor cell progression, testosterone insensitive clones emerge during this type of therapy which results in a poor prognosis for the patient (Armas et al, 1994).

Initially, prostate cell growth is under the control of androgen regulation. As pathogenesis of the prostate progresses from benign prostatic hypertrophy (BPH) to prostate carcinoma, various growth determining changes occur. A survey of biopsy specimen's of BPH and prostate carcinoma cells found that as the disease progressed through its clinically defined stages, cellular events occurred that corresponded with their growth capabilities in vitro.

For example, both BPH cells and prostate carcinoma cells expressed epidermal growth factor (EGF) receptors and insulin-like growth factor (IGF) receptors. However, prostate carcinoma cell lines contained higher levels of EGFR than BPH. The increase in EGFR was also associated with a decline in androgen receptors and an increase in c-myc expression which coincided with poor differentiation of the tumor cells. The expression of H-ras was elevated to higher degree with the loss of glandular differentiation and K-ras expression was elevated in grade III prostate carcinomas. The loss of differentiation was also associated with the onset of bi-phasic IGF receptors in the higher grade tumors (Davies et al, 1988). While growth factor receptors are known to function as oncogenes in cancer, the transformation of androgen-dependent prostate carcinomas to androgen-independent carcinomas allows for the potential of pharmacological intervention by blocking these growth factor related, cell signaling pathways responsible for cell proliferation, cytoprotection and tumorogenesis.

1.4 Growth Characteristics of Cancerous Human Tumors.

Growth factors function as endogenous extra-cellular agents in normal cell physiology that moderate cell function or activate quiescent cell populations to proliferate by entering the cell or differentiate into its respective cell type. Regulation of this process occurs at several levels: (1) the bio-availability of the growth factor and its half life; (2) the capacity to respond to the growth factor and the regulation of the growth factor

receptor, which is influenced by the presence of its respective ligand; (3) the ability of the cell to relay the signal generated by the interaction of the growth factor and its receptor to downstream effector mechanisms within the cell (Davies et al, 1988).

The first quantitative study of growth kinetics in malignant human tumors was performed in 1956 by (Collins et al,) using serial chest x-rays to observe pulmonary metastases growth rates. The doubling time of human malignancies varies from patient to patient. (Tubiana et al, 1976) collected data from almost 400 patients with pulmonary metastasis which were classified into five histological categories. When arranged in rank order of magnitude with regard to doubling times, embryonic tumors had the fastest growth rate at 27 days, followed by malignant lymphomas at 29 days, mesenchymal sarcomas at 41 days, squamous cell carcinomas at 58 days and adenocarcinomas at 82 days.

Additionally, the degree of differentiation appears to be related to the doubling time of tumors, e.g. poorly differentiated cancerous tumors generally progress more rapidly (Hall EJ, 1987). Furthermore, when comparing the growth rates for primary and secondary metastasis in breast and bronchial tumors within the same patients, the primary tumors grew at a significantly slower rate than the secondary metastasis. In fact the metastatic tumors grew at almost twice the rate as the primary tumor, (i.e.) primary squamous cell carcinoma and adenocarcinomas doubled in volume in approximately 82 and 166 days

respectively, while the secondary metastatic tumors in the same patients doubled in size in 58 and 83 days respectively (Charbit et al, 1971.)

The overall pattern of malignant tumor growth depends on three factors: (1) the cell cycle of proliferative cells within the tumor population; (2) the fraction of cells within the population that are proliferating versus those that are quiescent; and (3) and the rate of tumor cell loss, either by cell death including apoptosis and necrosis, shedding or immunological attack (Hall EJ, 1987).

Proliferating tumor cells are unrestrained by any homeostatic control mechanisms and subsequently divide and proliferate as rapidly as they are able too. They are limited only by their own inherited characteristics and the availability of sufficient nutrients necessary to maintain growth.

Since tumors are not an organized tissue, they frequently outgrow their own blood supply. This results in areas of necrosis within the tumor which is often accompanied by hypoxic cells which can constitute as much as 15% of the total viable cell population within the tumor mass. This leads to another consequence of the outstretched blood, supply in that only a portion of the viable cells (the growth fraction) are able to proceed through the cell cycle and multiply. Since the limit of diffusion from blood capillaries is

approximately 150 μm for oxygen, the typical tumor has on average a growth fraction of between 30-50% which tends to be higher in areas close to blood capillaries and lower near necrotic areas. The potentially explosive growth of human tumors is rarely manifested in practice because of cell loss from metastasis, random cell death after mitosis or cell death in necrotic areas of the tumor. However, the hypoxic tumor cell fraction and the aerated quiescent cell population are recruited back into the cell cycle after the tumor has shrunk as a result of radiation or cytotoxic drug treatment. This results in tumor re-population and neo-angiogenesis all over again. This population presents the challenge to scientists and clinicians as well as representing the nemesis to patients (Hall EJ, 1987).

1.5 Tumor angiogenesis

In 1971 Judah Folkman proposed the idea that tumor growth is angiogenesis dependent. This hypothesis stated that a tumor would remain dormant at a size of less than a few millimeters unless neo-vascularization occurred to support tumor growth. But most importantly, Folkman introduced the term anti-angiogenesis to describe a novel but then unattainable potential therapy that would prevent neo-vascularization by the tumor. The idea was largely ignored because endothelial cells had never been successfully cultured in vitro and bioassay isolation methods for angiogenic factors were non-existent until the early 1980's (Folkman and Haudenschild, 1980).

Later studies on biopsies of breast tissue to evaluate the presence of vascular endothelial growth factor (VEGF), demonstrated that 60% of primary human breast cell carcinomas expressed mainly VEGF when they were first diagnosed (Relf et al, 1997). In fact breast cell tumors can express up to six angiogenic protein factors including vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factor (bFGF), tumor growth factor beta-1 (TGF-β), platelet-derived endothelial cell growth factor (PD-ECGF), placenta growth factor (PLGF) and pleitropin in human primary breast cancer as reported by Relf et al, 1997. In addition, the supporting stroma of the tumor can also express VEGF (Fukumura et al, 1998). The expression of angiogenic promoters, (i.e.) VEGF, has also been shown to increase the level of circulating endothelial precursor cells from the bone marrow and migrate to the source of the promoter (Monestrioli et al, 2001).

An article by St.Croix in Science, 2000, established that endothelial cells in the tumor bed contain 79 significant differences in gene expression than their counterparts in normal tissue.

In parallel with the studies mentioned above, was the search for angiogenesis inhibitors in which 11 were discovered in nearly 25 years since Folkman's published his hypothesis. These include endostatin, TNP-470, Thalidomide, 3-amino thalidomide, 2-

methoxyestradiol, Interferon α/β and angiostatin among others (Folkman, 2003 and Kerbel and Folkman, 2002). Of these compounds, several display mechanism characteristics that are of interest to our studies. TNP-470 for example has been shown to block cdk2, inhibit cdc and inhibit Rb-phosphorylation in endothelial cell cycle pathways at concentrations 3 fold lower than that which inhibits tumor cell proliferation (Ingber et al, 1990 and Sin et al, 1997). And endostatin has been shown to inhibit cyclin D1 in endothelial cells (Hanai et al, 2002).

1.6 Xenografts of human tumors in animal models.

A wide variety of human tumor cells are able to be successfully transplanted as xenografts in athymic nude mice for the development of experimental tumor models. It is estimated that over 300 different and individual human tumors have been evaluated in animal model systems (Steel et al, 1983). Typically, breast cell carcinomas and ovarian cancers are the most difficult to graft, while tumors of the colon, bronchus and melanoma are relatively successful.

Although, athymic nude mice are the easiest to successfully graft, some investigators have resorted to other manipulations in order to study various tumor cell types in vivo. These include immunosuppression by drugs, total body irradiation or a combination of the two. Approximately 6 Gy is sufficient to destroy the animal's immune system but this

type of treatment often results in the death of the animal or animals within the experimental group.

Human xenografts are able to retain human karotypes through serial passages of the cells in vitro and also maintain some of the response characteristics of the individual human tumor. To this extent they display great advantages for study over murine tumors alone. Of course, this is not without certain drawbacks, first as mentioned above, the tumor cells display tendencies to be rejected by some of the animals. Therefore the number of animals receiving the human xenografts must exceed the number of animals proposed for use in the planned study to insure an adequate number of animals per treatment group. Additionally, the failure of human xenografts of some cell lines to proliferate as tumors in animals can be misleading where tumor control is the observed end point. However, growth delay and cell survival studies are probably affected to a lesser extent in this type of cell line.

Secondly, human tumor cells can undergo changes in both cell kinetic and cell selection when they are transplanted in mice (Kallman RF, 1987). As an example of this observation, human xenografts typically have cell doubling times about one fifth the rate seen in humans, therefore an increase in responsiveness to proliferation-dependent chemotherapeutic agents should be expected in mice. Evidence for this comes from a

field review by Steel and his colleagues in 1983, who concluded that human tumor xenografts generally maintain the response characteristics of the class of tumor from which they are derived with regard to chemotherapeutic treatment. But the individuality of a tumor cell response also occurs between xenografts of the same class (Steel et al, 1983, Hermens and Barendsen, 1967 and Hermens and Barendsen, 1969).

Thirdly, while the histological characteristics and cell morphology features are usually well maintained in xenografts, the stromal tissue is of mouse origin. As a consequence, tumor cell xenografts of human origin are not any more valid than murine tumors for any studies evaluating the importance or effects on tumor vascular supply. As an example, the observed fraction of hypoxic cells in xenografts of human origin is much the same as in mice tumors (Tannock IF, 1968).

However, with regard to chemotherapeutic agents, there exists a strong correlation between a xenografts growth delay in vivo and clinical remission of the donor patients. For example, testicular cancer was the most responsive in the clinic and in xenografts, followed by breast cell carcinoma and small cell lung cancer. The least responsive were melanoma, colon and non-small cell lung which was last. This same pattern is also observed in studies using radiation therapy and tumor response evaluated as growth

delay, which in human xenografts exhibits a pattern similar in response to that of the clinical responsiveness in patients (Kallman RF, 1987).

1.7 Epidermal Growth Factor Receptors

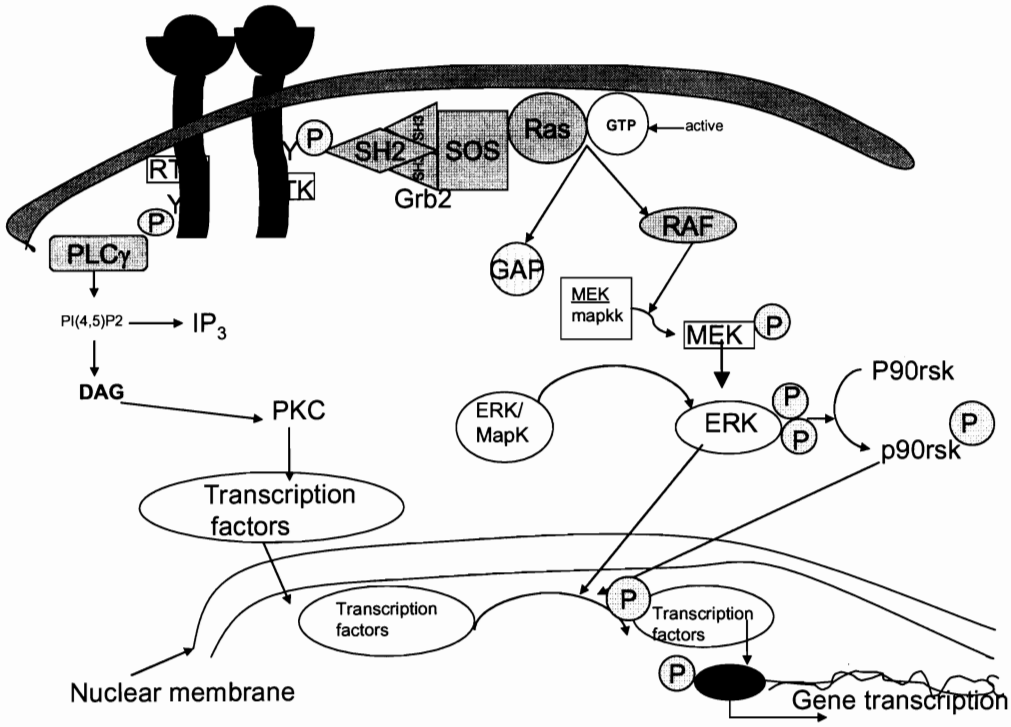
Epidermal growth factor receptor (EGFR) is one of the best examples of receptor tyrosine kinases in oncogenic cell signaling. The ErbB proto-oncogene family of receptor mediated tyrosine kinases are comprised of ErbB1, ErbB2, ErbB3 and ErbB4 and are often referred to as EGFR, HER2/Neu2, HER3 and HER4. (See Figure 2) The autocrine growth ligands epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) bind to EGFR which results in receptor homo- or hetero-dimerization with other ErbB receptors, which activates its intracellular tyrosine kinase domain and trans-phosphorylates the other. This action initiates the MAP-kinase cascade described below that includes protein kinase C and activates the transcription of pro-growth stimulatory proteins such as cyclin D. (See Figure 1 and Figure 2).

EGFR is over-expressed or amplified in some cancers e.g. mammary carcinomas and structurally altered in others. One example is EGFR VIII, a truncated form of EGFR found in glioblastoma. This form is auto-phosphorylated and independent of EGF stimulation because the kinase is constitutively active.

The ErbB2/HER2 receptor is activated by heregulin/neuregulin and its kinase cascade also includes MAPK and Akt. ErbB2 may facilitate the activation of the other ErbB receptors by heterodimerization. And along with ErbB1 may have a cytoprotective role

against cellular stress and insult. HER2 is over-expressed in 25-30% of breast cancers which eliminates its estrogen dependency (ER-) and is associated with a more aggressive form of breast cancer.

ErbB3 receptors appear to lack an active tyrosine kinase domain due to a substitution of asparagine for aspartic acid at its catalytic site and are reliant on heterodimerization with other ErbB family members to mediate signaling. ErbB3 receptors bind ligands of the heregulin/neuregulin family but not EGF or TNFα. ErbB4 receptors also bind heregulin/neuregulin, but unlike ErbB3, they possess a functional tyrosine kinase domain which implicates them in pathological processes such as cancer.

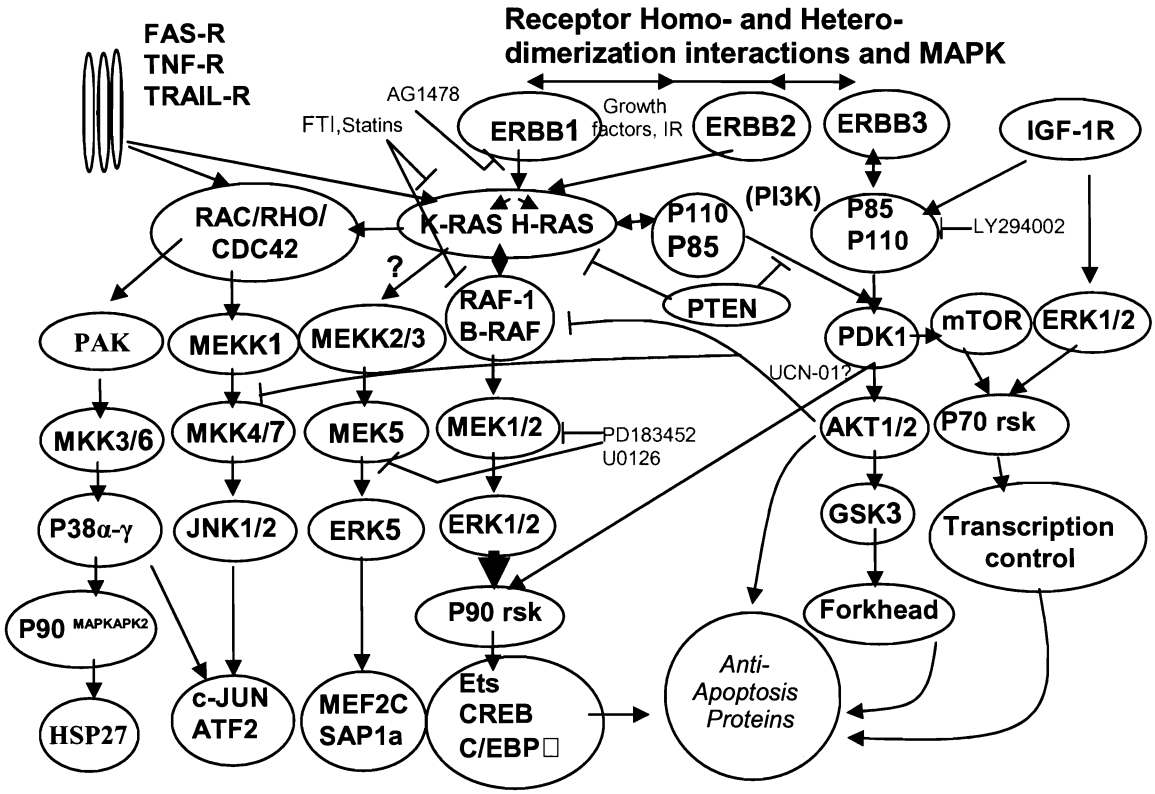


(Figure 1.) EGF Receptor tyrosine kinase activation (RTK) of downstream MAPK pathways. Growth factor (EGF) stimulation leads to RTK dimerization and autophosphorylation on multiple tyrosine residues. These residues serve as docking sites for adapter molecules such as Grb2-SOS complex. Adapter proteins tether multiple proteins to a single signaling pathway, e.g. MAPK. The RTK-Grb2-SOS complex triggers activation of Ras by the exchange of GDP for GTP. Ras then initiates an orderly phosphorylation cascade of Raf-MEK-ERK-Rsk which is required for cell proliferation. The end result is phosphorylation of multiple transcription factors which bind DNA and induce transcription.

1.8 Mitogen Activated Protein Kinase and Cell Signaling.

The most well known and studied signal transduction pathway is the Ras-Raf-MEK-ERK pathway. Ras is the primary activator of the Ras-Raf-MEK-ERK pathway and essentially functions as a GTP switching mechanism (Denhardt, 1996, Campbell et al, 1998 and Gille and Downward, 1999). (See Figure 1 and Figure 2).

Ras is activated by growth-factor mediated receptor tyrosine kinases (RTKs), as well as other stimuli. Ras is inactive in a GDP-bound state, but then activated in a GTP-bound state in which both states are facilitated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The RTKs are activated by growth factors e.g. epidermal growth factor (EGF) which results in the phosphorylation of tyrosine residues in their cytoplasmic domain. The phosphotyrosine residues then bind to different Src homology 2 (SH-2) sites on adapter proteins such as Shc, which is then, phosphorylated itself. Then growth factor receptor 2 (Grb2) can bind to certain phosphotyrosines on RTKs or Shc which are associated with the GEF, son of sevenless (SOS) which in turn is trans-located from the cytosol to the inner plasma membrane where it engages the membrane bound inactive form of Ras which causes Ras to release its bound GDP which is then replaced by GTP. In short, Shc, Grb2 and SOS proteins relay the mitogenic signal from the cell surface receptor (e.g. EGFR) to Ras.



(Figure 2.) EGFR activation of MAPK pathways and some known inhibitors (Modified from Dent et al, 2002).

Active Ras is then inactivated by a GAP e.g. p120 which facilitates the GTPase ability of Ras and reverts to the inactive Ras-GDP. The overall activity of the Ras-Raf-MEK-ERK pathway is determined by the activities of GEFs and GAPs. In human cancers, Ras mutations are frequently found on the Ras domain responsible for its GTPase activity. The most frequent mutations occur at Gly^{12}, Gly^{13}, Ala^{59} and Gln^{61}. Mutations at Gly^{12}, Gly^{13} or Ala^{59} interfere with the conformational changes induced by the binding of GAP which then interferes with the ability of Gln^{61} residue to re-position itself and catalyze the hydrolysis of GTP to form the inactive Ras (Scheffzek et al, 1998 and Macaluso et al, 2000). These mutations allow for what is known as "constitutively active or oncogenic Ras" found in human cancers.

Another Ras domain containing the amino acid residues 32-40 interacts with downstream Ras effector proteins (Campbell et al, 1998 and Webb et al, 1998) The domain is important for the activity of the Ras-Raf-MEK-ERK pathway in that mutations in this region (i.e.) amino acids at position 37 and 40 have been shown to cripple the ability of Ras to interact with and activate Raf (Web et al, 1998).

Raf, the second effector molecule in the Ras-Raf-MEK-ERK signal transduction pathway is a serine/threoinine family of kinases comprised of A-Raf, B-Raf and Raf-1 or C-Raf. Raf-1 or C-Raf has been the most extensively studied of the Raf family members.

Inactive Raf-1 is kept inactive by an adaptor protein, 14-3-3 when it is not interacting with Ras-GTP (Kolch, 2000 and Dhillon and Koch, 2002). The adaptor protein 14-3-3 contains a cysteine-rich domain (CRD) that interacts with the two phosphorylated inhibitory sites on the Raf-1 molecule, ser^{259} and ser^{261}. This interaction allows the CRD of 14-3-3 to associate with the kinase domain of Raf-1 and inhibit its kinase activity. Raf-1 is indirectly phosphorylated by binding to active Ras-GTP and phosphotidylserine by way of its RAS binding domain (RBD) which localizes Raf-1 to the cytoplasmic side of the plasma membrane. When activated Ras-GTP binds to Raf-1, the 14-3-3 adaptor protein is dissociated from the phosphoserine residue which results in the de-phosphorylation of serine ^{259} by protein phosphatase 2A (PP2A), which in turn results in the CRD of 14-3-3 to dissociate from the kinase domain of Raf-1. At this point, Raf-1 is primed for activation by other kinases, which phosphorylate Raf-1 at ser^{338} and tyr^{341} in its kinase domain which synergistically activate Raf-1 kinase activity and within the activation loop at thr^{491}, ser^{494} and ser^{499} (Kolch, 2000). If these sites are mutated, Raf-1 kinase activity is nearly abolished. The phosphorylated ser^{338} residue is important for both Raf-1 kinase activation and downstream activation of MEK and ERK kinases as well.

However, ser^{338} phosphorylation is not the only requirement for Raf-1 kinase activation, in that tyr^{341} phosphorylation has been shown to relieve the repression of the kinase

domain by the regulatory domain and correlates with the magnitude of Raf-1 activation as well. Other important phosphorylation sites occur within the activation loop as well. Thr^{491} and ser^{494} have been shown to become phosphorylated in a mitogen-dependent manner by Dhillon and Kolch, 2002. While phosphorylation of ser^{499} is involved in the activation of protein kinase C (PKC) but mutation of ser^{499} does not interfere with MEK activation but only Raf-1 auto-phosphorylation. These multiple phosphorylation sites within Raf-1 and the different levels of activity associated with these phosphorylation sites imply that different mitogens could activate Raf-1 to different extents specific for a particular mitogen activating factor. Or multiple mitogen activated pathways could converge on Raf-1 and synergize to produce maximal activity in these pathways (Denhardt DT, 1999).

Additionally, Raf-1 is also negatively regulated by phosphorylation. For instance, ser^{43} exists in an un-phosphorylated state in activated Raf-1; if it becomes phosphorylated by perhaps a MAPK then Raf-1 cannot maintain its affinity for Ras-GTP and is released from Ras. The release of Raf-1 allows Ras GAP to associate with Ras-GTP which results in the down regulation Ras signaling. Other proteins such as SUR-8 (a Ras-8 suppressor) can regulate Ras activity by forming a complex between Ras-GTP and the Raf-1 kinase domain (Kolch, 2000) and a scaffolding protein involved in the Ras-Raf-MEK-ERK pathway known as KSR (kinase suppressor of Ras) simultaneously binds Raf-1 and

MEK1/2 which facilitates the activation of MEK1/2 by Raf-1. KSR has also been shown to bind ERK1/2 which allows for the activation of ERK1/2 by MEK1/2 (Roy et al, 2002). And KSR is regulated by phosphorylation of ser^{392} which allows the binding of the 14-3-3 adaptor protein that confines KSR to the cytoplasm.

Other proteins that have been shown to have interactions with Raf-1 are the heat shock protein 90 (hsp90) and a Raf kinase inhibitor (RKIP). Hsp90 prevents the degradation of Raf-1 and when hsp90 activity is inhibited, Raf-1 is ubiquitinated and degraded. Hsp90 has been purported to be a chaperone for Raf-1 allowing it maintain its biological structure and activity (Schulte et al, 1997). While RKIP is a negative regulator of Raf-1 by preventing the interaction between Raf-1 and MEK1/2 which prevents the down-stream signaling of Raf-1 which itself can be abolished by KSR permitting Raf-1 to phosphorylate MEK1/2 (Yeung et al, 2000).

MEK1/2 is dual specificity kinase for threonine and tyrosine residues of ERK1/2. MEKs are regulated by regions of their C-terminal which may determine their cellular distribution and interaction with Raf-1 to activate ERK1/2 (Cha et al, 2001). A proline – rich region and multiple phosphorylation sites appear to regulate MEK1/2 activity. The investigation of mutated forms of MEK1/2 with C-terminal deletions demonstrated that MEK1/2 mutants were unable to phosphorylate ERK1/2 or become phosphorylated

themselves by constitutively active Raf-1. And they appeared to be associated with compartments in the plasma membrane instead of being distributed throughout the cytoplasm. Other proteins are also associated with MEK1/2 activity. The adaptor protein MP1 has been shown to interact with MEK-1 and ERK-1 by bringing the two into close proximity with each other and allowing MEK-1 to phosphorylate and activate ERK-1. MP1 acts in a preferential manner with MEK and ERK-1 which facilitates the activation of ERK-1 over ERK-2 (Kolch, 2000 and Dhillon and Kolch, 2002) but the physiological impact of this unknown.

ERK1/2 are serine/threonine kinases that phosphorylate and regulate various transcription factors and other proteins. As mentioned above, ERK1/2 phosphorylation is facilitated by scaffolding proteins like KSR which bring MEK1/2 into close proximity with ERK1/2. After which they are purported to be directed to their intracellular targets by way of docking-domains (Barsyte-Lovejoy et al, 2002). These so called "docking domains" are located on intracellular targets such as transcription factors. And ERKs also contain reciprocal docking domains which interact with sub-motifs of the target docking domain which consist of a series of basic amino acids and hydrophobic amino acids with LXL-sub-motifs (Barsyte-Lovejoy et al, 2002). These sub-motifs are then known to be slightly modified to ensure that they are activated by only one specific type of mitogen activated protein kinase (MAPK), (e.g. p38 or ERK1/2).

In addition to being regulated by docking domains, ERKs can be dephosphorylated by mitogen-activated protein kinase phosphatase-3 (MKP-3) which is a dual specificity kinase (Nichols et al, 2000). This group demonstrated that MKP-3 can both activate and dephosphorylate ERK. Studies done with p38/ERK chimeras indicated that MKP-3 binds to the C-terminal domain of ERKs and this binding site overlaps with the substrate specificity domain for the ERKs. This observation was consistent with the fact that some ERK1/2 substrates such as Elk-1 and $p90^{rsk}$ inhibit ERK-dependent activity of MKP-3.

Activation of the ERK1/2 pathway can result in a variety of cellular effects. Whether or not this activation results in regulation of cell proliferation versus differentiation and cell survival appears to be dependent on the amplitude and duration of ERK1/2 activation. A short activation of the ERK1/2 cascade has been shown to correlate with enhanced cell cycle progression through the G_1-S transition phase (Tombes et al, 1998). On the contrary, a prolonged ERK1/2 activation has been shown to inhibit DNA synthesis by the up-regulation of Cdk inhibitor protein $p21^{CIP1}$ by a process referred to as super-induction, which may result in cellular differentiation or death (Park et al, 2000). ERK1/2 activation may also play a larger role in cell cycle progression in addition to that of the G1-S phase transition. It may also be involved in the enabling of cells to progress through G2/M particularly following DNA damage-induced growth arrest (Vrana et al, 1999 and Hayne et al, 2000).

The Ras-Raf-MEK-ERK signal transduction pathway has also shown the ability to integrate growth factor stimulation with regulation of the G1/S restriction point and cell cycle progression by Aktas et al, 1997. Primarily this involves the regulation of Cdk4, Cdk6, cyclin D1 and $p27^{kip1}$ by Ras. The activation of Cdk4/6 by the binding of cyclin D1 along with the down-regulation of the Cdk inhibitor $p27^{kip1}$ is known to promote cell cycle progression. A dominant-negative Ras mutant was utilized to prove the induction of cyclin D gene expression and down-regulation of $p27^{kip1}$ gene expression resulted from Ras signaling. This study also demonstrated that the over-expression of cyclin D1 eliminated the necessity for active Ras to facilitate progression through the cell cycle. The up-regulation of Cdk4/6 activity is achieved by the ability of Ras to both increase cyclin D1 levels and decrease $p27^{kip1}$ activity which drives the cell out of G1 and into S phase.

Growth factors by themselves result in only a transient increase in ERK activity, especially in adherent cells, and in order for a cell to proceed through the G1/S checkpoint a sustained level of ERK activation must be maintained. Schwartz and Assoian, 2001 describe the engagement of integrins with the extracellular matrix as essential for sustained ERK activity.

1.9 Integrin Signaling

Integrins generally have a long extracellular domain and a short cytoplasmic domain devoid of kinase activity. This large group of heterodimers individually exists as a single α and single β chain. There are 16 different α and 8 different β chains that form the 22 known integrins (Hulleman and Boostra, 2001).

The cytoplasmic domains of the integrins typically interact with other intracellular proteins such as kinases or adapter proteins.

Of the known integrins, several have been shown to enhance cell proliferation by engaging with components of the extracellular matrix while the cells are exposed to growth factors. As an example, the integrin α5β1 has been shown in fibroblasts to up-regulate cyclin D1 expression by causing a sustained level of ERK activity in cells treated with growth factors (Roovers et al, 1999). While the integrin αvβ3, has been shown to promote cell cycle progression through the G1/S restriction point.

In one set of experiments, fibroblasts treated with platelet-derived growth factor (PDGF) were shown to enhance their proliferation when the integrin αvβ3was engaged with vitronectin (Schneller et al, 1997). In another set of experiments, vascular smooth muscle cells were exposed to epidermal growth factor (EGF) while the integrin αvβ3 was bound

to tenascin-C, these conditions resulted in enhanced epidermal growth factor receptor (EGFR) phosphorylation and cell proliferation only when the integrin-tenascin-C interaction was followed by stimulation with EGF in which integrin-tenascin-C mediated EGFR activation followed (Jones et al, 1997).

An integrin signal cascade with enhanced and sustainable ERK activation in cells treated with growth factors must be able to synergize with the Ras-Raf-ERK-MEK signaling pathway.

How this occurs may be due in part to focal adhesion kinases (FAK), which interact with the cytoplasmic domain of engaged integrin. This allows FAK to autophosphorylates itself, when it is associated with the activated integrin (Zhao et al, 1998). The autophosphorylation of FAK allows it to bind to the SH2 domain of Src which in turn results in an additional phosphorylation of FAK at tyr^{925}. The phosphorylation of tyr^{925} on FAK allows it to bind to Grb2/SOS complex which contributes to further activation of Ras signaling pathways which results in increased ERK activation as mentioned above. Additionally, integrin mediated cell-adhesion is known to promote the translocation of ERK1/2 to the cell nucleus and subsequent phosphorylation of Elk-1 (Aplin et al, 2001). As mentioned above, the increase in ERK activity results in increased expression of cyclin D1 and decreased expression of the Cdk inhibitor p21. Both of which are seen in fibroblasts transfected with constitutively activated FAK. Another study found that cell

adherence to an extracellular matrix and the engagement of growth factors is both required for maximal activation of cyclin E-cdk2 and Rb phosphorylation (Zhu et al, 1996).

1.10 Phosphatidyl Inositol 3-kinase (PI3K) pathway and Akt

Inositol phospholipids (IP) are intercellular signaling molecules known as "second messengers". Phospholipase C_γ cleaves IP into diacylglycerol (DAG) and IP_3 which is released into the cytoplasm to activate endoplasmic reticulum receptors and release intercellular stores of Ca^{2+} into the cytosol. Intercellular free Ca^{2+} and DAG cause the activation of Protein Kinase C (PKC) isoforms.

PI3 kinases are a class of enzymes that have a p110 catalytic subunit and a p85 regulatory subunit. The p85 subunit contains a phospho-tyrosine (SH2) domain while the p110 subunit phosphorylates inositol membrane bound phospholipids (e.g. Phosphatidyl inositol 4, 5 bis-phosphate) at the number 3 position of the inositol sugar ring. Activation of PI3K can occur via mitogenic stimulation by EGF and TGFα. Mitogenic activation results in the phosphorylation of tyrosine residues on ErbB receptors which provides biding sites for the SH2 domain of p85. The binding of p85 to activated ErbB receptors (i.e. ErB3) results in p110 activation of PI3K. If the other inositol ring positions are phosphorylated by PI4-kinase and PI5-kinase to yield 3, 4, 5-inositol triphosphate, this

becomes an acceptor site for plecstrin binding domains (PH domains) of the protein kinases PDK1 and Akt or protein kinase B (PKB). PDK1 is thought to phosphorylate and activate Akt and facilitate other protein kinases such as $p90^{rsk}$. PI3K-dependent phosphorylation of inositol can be reversed by the lipid phosphatase and tensin homologue on chromosome 10 (PTEN). Loss of PTEN expression is frequently found in glioblastoma multiforme which results in constitutive activation of PDK1 and Akt. The downstream signaling events of Akt and PDK1 to other protein kinases such as PKC isoforms, GSK3, mTOR, $p70^{S6K}$ and $p90^{S6K}$ have been reported to play key roles in mitogenic response of cells in addition to cytoprotective effects from noxious stress. In addition, other protein kinases such as p38 MAPK (which is suggested to be PDK2) can phosphorylate Akt independently of PDK1 resulting in modified Akt cytoprotective activity. Of note, oncogenic Ras can also be activated by the p110 subunit of PI3K. (Dent et al, 2002)

1.11 c-JUN NH_2-terminal kinase (JNK) pathway

JNK1/2 is a stress-induced protein kinase that phosphorylates the NH_2-terminus of the c-JUN transcription factor and is referred to as the stress activated protein kinase (SAPK) pathway. Cellular stress caused by both UV- and γ-irradiation, free radicals and cytotoxic drugs increase JNK1/2 activity. The activation of JNK1/2 results in the phosphorylation of NH_2-terminal sites Ser^{63} and Ser^{73} on c-JUN which enhance its ability to trans-activate

AP-1 enhancer elements in the promoters of many other genes. In a manner similar to those of MAPK, JNK1/2 activity is regulated by dual threonine and tyrosine kinases which are analogous to MEK1/2 and called stress-activated extracellular regulated kinase I (SEK1) or MEK4. An additional isoform of MEK4, called MEK7 also exists. And like MEK1/2, MEK4/7 is also regulated by dual serine phosphorylation. In contrast to MAPK pathway activation which utilizes three Raf family protein kinases to activate MEK1/2, MEK4/7 can be activated by at least ten kinases including Raf 1-4, TAK-1 and Tpl-2. In addition to Raf, several other upstream enzymes are also JNK pathway protein kinases such as Ste20-homologues and small GTP-binding proteins of the Rho family such as Cdc42 and Rac1. How these Rho family proteins are activated by growth factor receptors is unclear. Some suggested explanations are by the ras-proto-oncogene or by PI_3 kinase and/or PKC isoforms. In addition to those above, evidence exists for agonist activation of the tumor necrosis factor alpha (TNFα) receptor by way of the lipid second messenger ceramide (a sphingomyelinase enzyme product) that can activate the JNK pathway by mechanisms which may also act through Rho family GTPases. (Dent et al, 2002)

1.12 p38 MAP kinase pathway

The p38 MAPK pathway is also activated in to response to cellular stress much like JNK. It is known to promote cell growth and enhance survival as well as promote cell death.

P38 MAPK consists of four known isoforms p38α, p38β, p38γ and p38δ and several other downstream protein kinases including $p90^{MAPKAPK2}$, and MSK1/2 which activate HSP27 and the transcription factor CREB respectively. And Rho family GTPases appear to be involved in the upstream activation of the p38 MAPK pathway activity. This activity is known to be variable and inconsistent in response to radiation-induced cell stress which is in contrast to radiation-induced activation of MAPK and JNK pathways. In radiation induced activation of p38, it appears that p38γ may signal G2/M cell cycle arrest which is dependent upon the functional expression of ATM proteins.

1.12 Cell cycle regulation

In mammalian cells, the cell cycle is controlled and maintained by the cyclins and cyclin-dependent kinases (cdks) which are activated by cyclins, cdk inhibitors and cyclin activators (See Figure 3). The activation of cyclin-dependent kinase controls the transition from one cell cycle phase to the next. Each cdk is a holoenzyme complex which consists of a regulatory subunit, the cyclin, and a catalytic subunit, the cyclin-dependent kinase. Each phase of the cell cycle and its transition within the cell can be identified by the type of cyclin and their associated kinase activities. There are numerous cdks that function at various stages of the cell cycle. Progression through the G1 phase is modulated by the association/binding of the D family members (D1, D2, D3) to cdk4 or its homologue cdk6. D-family member cyclins are unstable, and their induction and expression are dependent on active mitogenic stimulation (Matsushime et al, 1994 and Sherr and Roberts, 1999). While cdk4 and cdk6 are stable and maintain constant levels throughout the cell cycle.

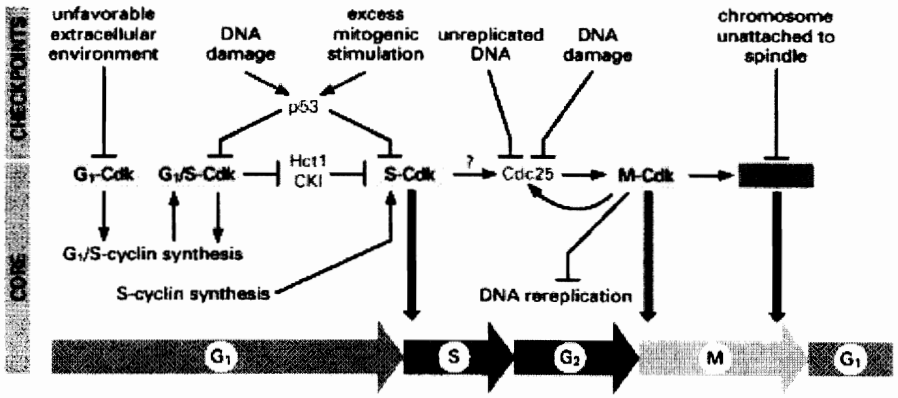
In mid to late G1 a second cyclin-dependent kinase complex appears which is cyclin E and cdk2. The cyclin E and cdk2 are expressed in all cell types and are not dependent on persistent mitogenic stimulation for their presence or assembly. Cyclin E binding to cdk2 is an additional requirement for G1 phase progression.

The binding of Cyclin A to cdk2 is then required for cell cycle progression through S phase. The activated forms of cdk4 or cdk6 and cdk2 are required for the phosphorylation of the retinoblastoma protein (Rb) which then activates the transcription of genes which are regulated by E2F family transcription factors. Both cyclin-D and cyclin-E associated kinase activities phosphorylate and inactivate the retinoblastoma gene product, pRB. Cyclin-D-cdk4/6 complexes initially phosphorylate and inactivate the Rb in mid G1. The sequence is completed later in G1 when cyclin-E-associated kinase phosphorylation further disrupts the pocket domain of pRb which dissociates the pRb-E2F complex and releases the E2F transcription factor (Harbour et al, 1999). One of the primary functions of E2F in cell cycle progression is to regulate the expression of cyclin A and therefore the entry into S phases (Weinberg, 1995).

The progression through the G2/M phases is activated by the binding of cyclin B to cdk1 which is equivalent to cdc2 (Assoian, 1997 and Heichman and Roberts, 1994).

Alternatively, the progression through G1 or G2/M can be inhibited by either of the cdk inhibitors families; (i.e.) INK4 family members (p15, p16, p18 and p19) or by the p21 family members p21cip1, p27kip1 and p53kip2. The INK4 family of cdk inhibitors binds to cdk4/6 which results in G1 arrest. The p21 family of cdk inhibitors binds to both cdk 4/6 and cdk2 which prevents progression into G1 or S phase.

DNA checkpoints in cell cycle



(Figure 3.) Simplified scheme for Cell cycle regulation and checkpoints.

1.13 Radiation-induced activation of cell signaling pathways,

EGFR is also activated in response to ionizing radiation in a number of carcinoma cell lines. Radiation-induced activation of EGFR results in the activation of MAPK pathways that is physiologically similar to EGF activated pro-growth stimulation. TGFα expression also increases in response to ionizing radiation which as described above can, also activates EGFR. TGFα has been described as a mediator of secondary activation of EGFR after radiation and its downstream MAPK and JNK pathways. Signaling activity by Ras, MAPK and p53 can also increase after radiation exposure which has the effect of up-regulating the expression of epiregulin and HB-EGF in epithelial cells. This in turn enhances the intrinsic cell proliferation and cytoprotective effects of ErbB receptor activation (Dent et al, 2002).

An increase in EGFR expression translates to an increase in cell proliferation and cyto-protection in the surviving cell fraction of the tumor population.

In an article by Dent et al, 2002, the authors astutely point out that this phenomenon may have a "self-limiting effect" on radiation therapy, and its induced cell lethality/toxicity, due to the increased activity of EGFR and its associated downstream MAPK activity. This observation necessitates the need for pharmacological intervention, and warrants the

use of MAP kinase inhibitors in combination with radiation therapy to achieve the desired clinical outcome.

Numerous investigations have demonstrated the effectiveness of growth receptor and MAP kinase cascade inhibition on enhancing cancer cell death. These interventions involve a multitude of approaches such as blockade of growth receptors like EGFR and their downstream effectors with antibodies (e.g. C225 and herceptin), genetic modification of growth receptors (e.g. dominant negative truncated EGFR-CD533 and antisense EGFR), receptor tyrosine kinase inhibitors (e.g. AG1478), MEK1/2 inhibitors (e.g. PD183452 and U0126), Ras inhibitors (e.g. FTI and statins) which enhance the effectiveness of radiation therapy and reduce tumor cell growth (Dent et al, 2002).

1.14 Ionizing Radiation and tumor cell killing

Ionizing radiation involves high speed γ-particles and slower speed α-particles that are employed as a component of multimodality therapy for the treatment of cancer. Radiation therapy is utilized in almost 80% of all cancer cases today. Dose determination is based on the type of cancer and its location in a patient. Studies conducted to determine the effectiveness of individual doses of radiation involve increasing the doses of radiation and measuring the surviving cell fraction in vitro. These types of experiments are ongoing and constitute a significant portion of radiation oncology research today.

Essentially, these studies create a survival-curve for individual cell lines by plotting increasing doses of radiation as the amount absorbed in Gray (Gy) or rads, versus the surviving cell population, yielding the reciprocals of the initial and final slopes of the curve, D_1 and D_0 respectively. The data is then fitted into a quadratic equation where they are related by the expression, ($\log_e\eta=Dq/D_0$) where η equals the extrapolation number, which is a measure of the shoulder of the curve and Dq is the quasi-threshold dose that produces no effect, which incidentally is non-existent. The slope of this curve yields the initial slope D_1 which is due to a "single-cell damaging event or killing" and the final slope D_0 "which is due to multiple-cell damaging event or killings". In either case these values represent the required amount of radiation to reduce the fraction of surviving cells to 37% of its previous value.

However, this introduces the concept of "dual radiation action", whereby there are two components to cell killing, one which is proportional to the dose and the other which is proportional to the square of the dose. This idea is the result of early radiation research studying chromosome breaks where observations were made noting that many chromosomal aberrations are the result of two separate breaks. By this model the cell-survival curve expression becomes ($S=_e-\alpha D-\beta D^2$) where S is the fraction of cells surviving a given dose D and α and β are constants. Then the components of a radiation damaging event are proportional to a single dose and the square of the dose and being

equal when $\alpha D=\beta D^2$ or $D=\alpha/\beta$. This is an important point in radiobiology in that the linear and quadratic contributions to cell killing are essentially equal at a dose that is equal to the ratio of α to β for that particular cell type (Hall EJ, 1988). For our purposes in the in vivo mammary carcinoma studies, the D_0 in mammary cells is 1.27 Gy or 127 rads (Hewitt and Wilson, 1961 and Gould and Clifton, 1977).

1.15 Ionizing radiation and cell cycle checkpoint inhibition

Ionizing irradiation is known to induce a tumor cell response that involves the temporary arrest of the cell cycle at the G2 checkpoint. As discussed below this checkpoint is activated by a signaling cascade which involves both ATM-dependent and ATM-independent pathways.

In a recent report, breast carcinoma cell lines (HCC1937 and MT-1), possessing either a defective or intact tumor suppressor protein, (BRCA1 genotype) respectively, were exposed to ionizing irradiation in the presence and absence of UCN-01. The response of the cells were then analyzed with regard to cell cycle distribution, G2 phase regulatory factors and clonogenic potential. The results of the study showed the radiation-induced G2 phase cell accumulation was preceded by a transient down-regulation of the G2 phase-specific plk-1 and cyclin B1 which require intact functioning of BRCA1 and

CHK1. The concomitant treatment of the BRCA1 positive cell line MT-1 with UCN-01 completely abolished the G2 phase accumulation of these cells and was confirmed with an exponential loss of colony formation efficiency (Ree et al, 2004). This report provides further support for the use of UCN-01 as a radiosensitizing agent.

Another report demonstrated that p53 transactivation and repression played distinct roles in preventing UCN-01 mediated abrogation of DNA-damage induced arrest at S and G2 cell cycle checkpoints (Levesque AA, 2005). Using the cell lines MCF10A, an immortalized breast cell carcinoma with wild-type p53 and cell lines MCF10A/EB and MCF10A/AL where transactivation of p53 was prevented by inhibition of tetramerization, the investigators were able to demonstrate that disruption of p53 tetramer formation rendered the cells susceptible to UCN-01 abrogation of S phase arrest, but they remained resistant to G2 checkpoint abrogation. The investigators attributed this response to the retention of p53 repressor function which suppresses other proteins including cyclin B. Further investigation using a MCF10A/Δp53 cell line in which p53 expression was ablated by short hairpin RNA (shRNA) revealed that transactivation of $p21^{waf1}$ was blocked and cyclin B was elevated in G2 in the cells responding to DNA damage. Interestingly, this particular cell line was sensitive to UCN-01 induced abrogation at both the S and G2 checkpoints. The researchers concluded that the transactivation and repression of p53 functions are important in the complete regulation of S and G2 cell

cycle arrest. As well as in cell protection from check point abrogators such as UCN-01 (Levesque et al, 2005).

1.16 Apoptosis or "programmed cell death"

"Programmed cell death" or apoptosis was originally defined as an internal series of events that culminate in the death of a cell by (Lockshin and Williams, 1965 and Kerr et al, 1972). Apoptosis is a highly regulated process by which cells shut down growth signaling, energy production and metabolism, DNA damage repair, transcription, translation and its cell cycle while maintaining its membrane encapsulation (Earnshaw et al, 1999). The classic features of apoptosis are chromatin condensation, with crescent shaped condensed chromatin on one side of the nuclear membrane, nuclear shrinkage and blebbing of ribonucleotide-filled membrane bound buds. This re-structuring of nucleus allows the cell to package itself as a membrane bound, non-inflammatory body which can be removed by phagocytosis without damaging neighboring cells.

The major mediators of apoptosis are a group of cysteine proteases referred to as caspases, Bcl-2 family member proteins, the p53 family and members of the tumor necrosis factor (TNF) receptor super family.

1.17 Caspases

There are at least 14 known caspases that have been identified in mammals. The caspases are divided into two groups, the initiator caspases which include caspase-2, caspase-8, caspase-9 and caspase-10, and the effector caspases which include caspase-3, caspase-6 and caspase-7. All of the caspases are produced as inactive zymogens and must undergo proteolytic cleavage to become active during apoptosis. The activation of effector caspases such as caspase-3 is performed by an initiator caspase (e.g. caspase-9) by cleavage of a specific Asp residue that separates the large (p20) and small (p10) subunits. Unlike the effector caspases, the initiator caspases are auto-activated. The activation of the effector caspases results in cleavage and inactivation of cellular targets such as DNA repair enzymes, lamins, MDM2 and a protein kinase C (PKC) that ultimately leads to cell death (Thornberry and Lazebnik, 1998). Active caspases are eliminated by the ubiquination-mediated proteosome degradation pathway (Suzuki et al, 2001). And the activity of caspases such as the initiator caspase-9 and the effector caspases-3 and 7 can be inhibited by the inhibitor of apoptosis (IAP) family of proteins such XIAP, cIAP and eIAP2 while the other caspases such as caspase 6 and 8 are not (Deveraux and Reed, 1999).

1.18 The Bcl-2 family

The pro-survival, B-cell lymphoma 2 (BCL-2) families of proteins are key regulators of caspase activation. This family includes the anti-apoptotic proteins BCL-2, BCL-$_{XL}$, BCL-w and two other groups of proteins that promote cell death (pro-apoptotic); the Bax group and BH3-only domain family which include BID, BAD, BIM and BIK. Both types of pro-apoptotic proteins are necessary for the initiation of apoptosis. BH3-only proteins serve as damage sensors, while Bax-like proteins act further downstream which may include mitochondrial disruption (Huang and Strasser, 2000).

The differing pro-apoptotic Bcl-2 family member activity depends on post-translational modifications. The activity of BAD has been shown to be regulated by Akt or protein kinase A-mediated phosphorylation (Zha et al, 1996). BID cleavage by caspase-8 mediates mitochondrial damage during the activation of Fas ligand/receptor complex Li et al, 1998). And the pro-apoptotic activity of BIM is regulated by interacting with the dynein motor complex (Puthalakath et al, 1999).

1.19 Tumor Necrosis Factor (TNF) Receptor Family

The TNFR super family can be divided into two groups; one class of receptors, termed the death receptor (DR), contains a cytoplasmic death domain while the other group does not. The death domain mediates the interaction of the DR group with adaptor proteins.

One of the primary adapter proteins is Fas-associated death domain (FADD) which activates specific caspases that initiate apoptosis (Sprick et al, 2000). Another adaptor called TNFR-associated death domain (TRADD) stimulates protein kinases to induce transcription of immune system modulating genes, which then can induce FADD (Hsu et al, 1996).

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL/APO-2L) is known to induce apoptosis in cancer cells (Wiley et al, 1995). The receptors for TRAIL include two death receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2 or KILLER) as well as two decoy receptors (DcR1 and DcR2) or TRAL-R3 and TRAIL-R4 (Wu et al, 1997). The complex interaction pattern and the complete physiological role of these receptors are not fully understood.

After the binding of Fas ligand or APO2L/TRAIL, the death receptor Fas, DR4 or DR5 assemble a complex for death-inducing signaling termed (DISC) through the FAS-associated death domain (FADD). Once completed, they recruit and activate the caspase initiators caspase-8 and caspase-10 which leads to apoptosis.

The binding of TNF to TNFR1 recruits TRADD as an adaptor and assembles signaling complexes through other adaptors such as DISC which involves FADD and caspase-8 and triggers apoptosis (Hsu et al, 1996). Another complex involves receptor-interacting

protein (RIP), which links receptor stimulation to the inhibitor of κB-kinase (IKK) cascade, which activates the nuclear factor (NF- κB) transcription factor. A third complex involves the TNFR-associated factor 2 (TRAF-2), which couples receptor activation to the JUN N-terminal kinase (JNK) cascade which stimulates the AP-1 transcription factor as does the DR6 death receptor which also signals through JNK/AP-1 pathway (Zhao et al, 2001).

1.20 p53 Family tumor suppressors

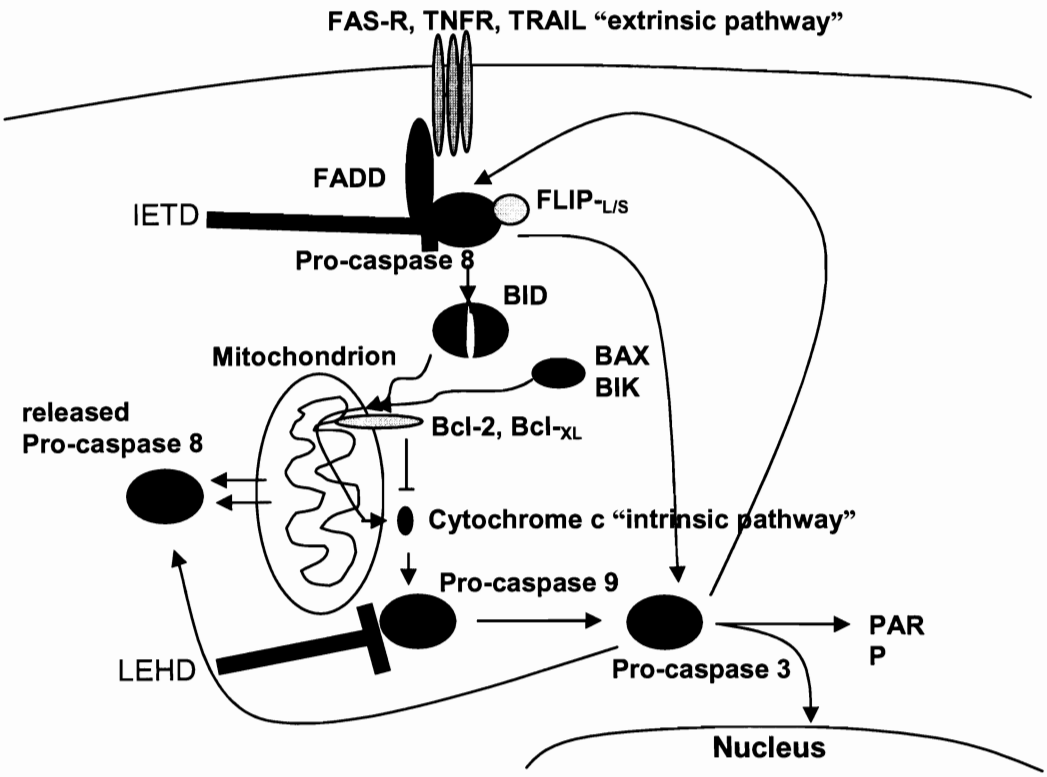
The p53 tumor suppressor belongs to small family of proteins which include p63 and p73. The p53 tumor-suppressor protein was first described as component of complex events that evolved to sense a variety of cellular stresses such as DNA damage and hyper-proliferative signaling. Once it is activated by genotoxic stress, p53 can either repress or activate differing gene targets for cell cycle regulation, DNA repair, angiogenesis and apoptosis (Ko and Prives, 1996). The primary role of p53 is to induce the apoptotic cascade associated with the release of cytochrome C and SMAC (Ryan et al, 2001) but has also been shown to activate gene expression which inhibits cell survival signaling (Hoffman et al, 2001). Additionally, p53 can repress gene expression as well as act independently of transcription regulation functions for the induction of apoptosis.

1.21 Apoptosis Signaling Pathways

The apoptotic cell suicide program utilizes two main signaling pathways the "intrinsic", and "extrinsic" pathways in mammalian cells (See Figure 4.) The intrinsic pathway is known as the mitochondrial pathway. This pathway triggers apoptosis in response to DNA damage, defective cell cycle, separation from the extracellular matrix, hypoxia, loss of cell survival factors and other severe stresses. The intrinsic pathway involves the activation of the pro-apoptotic Bcl-2 family members which in turn engage the mitochondria and causes the release of cytochrome c and SMAC/DIABLO into the cytosol (Hunt and Evan, 2001). Within the cytosol, cytochrome c binds the adaptor APAF-1 which forms a apopsome that activates caspase-9 which activates caspase-3, caspase-6 or caspase-7. SMAC/DIABLO promotes apoptosis by binding to inhibitors of apoptosis (IAP) proteins which prevents the attenuation of the caspase activity.

The "extrinsic pathway" triggers apoptosis in response to the coupling of death receptors by their ligands. This pathway stimulates the apoptotic caspases in a p53 independent manner. The extrinsic pathway is initiated by the TNF super family such as TRAIL/APO2L, FasL, and TNFα which bind to their respective receptors KILLER/DR5, DR4 (TRAIL-R1), and Fas/APO1 (Ashkenazi, 2002). The association of FasL and TRAIL with its receptor results in trimer formation and a clustering of the receptors death

domains (DD) leading to the formation of the death inducing signaling complex (DISC). This leads to the recruitment of the adaptor molecule FADD and activation of caspase-8 and caspase-10. Activated caspase 8 and 10 then cleave pro-caspase-3 which leads to the cleavage of death substrates such as the pro-apoptotic protein BID (Green, 2000)



Death receptor signaling (extrinsic pathway) through caspase 8 can lead to cell death. The intrinsic pathway, from the mitochondria via cytochrome c can also lead to cell death.

Figure 4. (Modified from Dent et al, 2002).

Experimental Drug mechanisms and targets

1.22 Staurosporine and 7-hydroxystaurosporine (UCN-01)

Staurosporine was initially discovered when Streptomyces extracts were being screened for compounds with protein kinase C (PKC) inhibitory activity (Omura et al, 1977). The development of staurosporine analogs was prompted by the discovery that PKC is activated in some types of human cancers (O'Brian et al, 1989 and Benzil et al, 1992). One of the most promising of these staurosporine analogs is 7-hydroxystaurosporine (UCN-01). However, certain cancers express abnormally low levels of PKC as well (Kopp et al, 1991). This prompted other investigators to questions the use of PKC inhibitors as target drugs. While others proposed that the growth inhibitory effects of staurosporine and its analogues are mediated by other mechanisms (Gesher, 2000). Numerous studies were undertaken to evaluate the mechanism of action of UCN-01 which revealed very complex insights. UCN-01 has been shown to inhibit Cdks 2, 4 and 6 at 50 nM, while blocking cell cycle progression in G1 (Kawaakami et al, 1996). However, (Nishi et al, 1998) demonstrated that there is also an accumulation of Cdk inhibitors such as p21 that are independent of G1 arrest which suggested that other mechanisms were also involved in UCN-01 activity. In certain hematological malignancies such as B-cell chronic lymphocytic leukemia, UCN-01 leads to the down

regulation of various anti-apoptotic proteins although the precise mechanism for this action is unknown (Kitada et al, 2000).

The most interesting activity of UCN-01 is the ability of the drug to abrogate the accumulation of cells with DNA damage in G2. Normally, in dividing cells those with DNA damage activate a cell cycle checkpoint and arrest in G2. This response occurs with the involvement of both p53 dependent and independent mechanisms. In fact, in cells treated with UCN-01 the arrest of cells in G2 was abrogated in an effect that was more pronounced in cells with defective p53 (Wang et al, 1996). The tumor-suppressor protein p53 is the primary regulatory protein of the G1 checkpoint in the cell cycle where it coordinates the cellular ability of DNA repair. It is also possesses a dual functional role in that it determines whether or not a cell should survive or undergo apoptosis (Fei and El-Diery, 2003 and Ronnison et al, 20001). Essentially, cells can re-enter the cell cycle if the damage has been adequately repaired, or they die by apoptosis if the damage is too great. The inherent ability of checkpoint abrogators to sensitize cells to DNA damaging agents and abrogate cell cycle arrest is related to the p53 status of the cell. Cells that possess intact wild-type p53 are resistant to sensitization of by checkpoint abrogators. The tumor suppressor gene p53 is the most commonly mutated gene in many forms of human malignancies. Loss of the p53 tumor suppressor gene has been associated with increased resistance to apoptosis in transformed cell lines and this observation has

resulted in efforts to re-introduce p53 into tumor cells through gene therapy. As mentioned above, p53 promotes G1 arrest which protects cells from the cytotoxic events that follow DNA damage. Even though it is not required for S or G2 cell cycle arrest, p53 reinforces these checkpoints. While cells that lack or have defective p53 are sensitive to checkpoint abrogators such as UCN-01 which can overcome S or G2 arrest and drive these cells through a lethal mitosis and enhance DNA damage cytotoxicity (Shao et al, 1997, Kohn et al, 2002 and Kohn et al, 2003). This regulation or lack thereof by p53 has been well documented in tumorogenesis studies (Fan et al, 1995, Powell et al, 1995, Russell et al, 1995 and Shao et al, 1997). This selectivity for p53 defective cells allows for a potentially tumor-specific therapeutic treatment in that, p53 defective tumor cells would be killed while normal cells with wild-type p53 would not.

In order to develop such a therapeutic strategy it is important to understand the mechanisms by which p53 regulates cell cycle arrest and check point activation. Cells with DNA damage are induced to arrest in the G1 phase of the cell cycle are dependent on p53 tumor suppressor protein activation. In cells without DNA damage, p53 is kept in an inactive state by MDM2, an E3 ligase that targets p53 for ubiquitin-mediated degradation. (Haupt et al, 1997 and Kubbutat et al, 1997). If DNA damage occurs, p53 is

phosphorylated on the N-terminus by several damage activated checkpoint proteins which include ATM (on serine 15) and Chk2 (on serine 20) (Banin et al, 1998, Canman et al, 1998, Hirao et al, 2000 and Shieh et al, 2000). These phosphorylation events result in the dissociation of p53 from MDM2 which promotes p53 stability and in increasing abundance within the cell. The p53 molecules then form a tetramer and bind to a p53-response elements and induce transcription of the Cdk inhibitor $p21^{waf1}$, which arrests cell cycle progression (Xiong et al, 1993 and Henstchlager et al, 1999).

In contrast to the G1 checkpoint regulation, the S and G2 checkpoints triggered by DNA damage are triggered in a p53-independent action. Damaged DNA causes a rapid activation of ATM and/or ATR kinases depending on the type of damage (reviewed in Sagata, 2002). ATR then phosphorylates and activates the checkpoint kinase Chk1 and ATM activates Chk2 by phosphorylation (Matsuoka et al, 2000 and Zhao and Piwnica-Worms, 2001). Chk1 and Chk2 then phosphorylate and thereby inhibit Cdc25C phosphotases that are essential for cell cycle progression. In the G2 checkpoint the most critical target of Chk1 and Chk2 is Cdc25C which would normally activate Cdk1 to promote the onset of mitosis (Matsuoka et al, 1998 and Zeng et al, 1998). With the S phase checkpoint, the cdc25A phosphatase is the critical target of Chk1 and/or Chk2. Subsequently, Cdc25A is degraded after phosphorylation by these kinases and prevents

the activation of Cdk2, which is a key mediator of S phase cell cycle progression (Mailand et al, 2000, Falck et al, 2001 and Zhao et al, 2002).

In both the S and G2 phases of the cell cycle, p53 activation in response to DNA damage can protect against cell cycle progression induced by either caffeine or UCN-01. The precise mechanism for protection of this nature has not been fully investigated.

Subsequent investigations found that this effect was eventually mediated by Cdk1, but also found to be a result of events further upstream that was identified as ATP-competitive inhibition of (chk1) checkpoint kinase 1, (Yu et al, 1998, Graves et al, 2000 and Zhao et al, 2002).

In the cell cycle and under physiological conditions, Chk1 phosphorylates cdc25 and excludes it from the nucleus which prevents it from dephosphorylating Cdk1. This in turn results in Cdk from becoming activated and the cells are arrested in G2. Conversely, the inhibition of Chk1 allows cdc25 to dephosphorylate Cdk1 and releases the cell cycle block. In cancer cells, this abrogation event would be associated with apoptosis. UCN-01 may then potentiate the cytotoxic effects of drugs that cause DNA damage, such cis-platinum or radiation therapy (Wang et al, 1996 and Xiao et al, 2002). This abrogation of Chk1 activity may also prevent p53-deficient cancer cells from repairing damaged DNA,

proceed into M phase and undergo apoptosis as a result of a "mitotic catastrophe" (Dixon and Norbury, 2002). One class of compounds the methylxanthines and specifically caffeine and pentoxifylline have been found to have similar effects on G2 arrest after DNA damage.

As with UCN-01 this effect is reported to be a possible consequence of the inhibition of Ataxia telangiectasia (ATM), a member of the phosphoinositide-3-kinase family and a protein kinase upstream of Chk1. The ATM –dependent pathway regulates the fast response of the S-phase checkpoint only, which is sensitive to inhibition by wortmannin. The slow-response element is controlled by an ATM-independent mechanism which is sensitive to caffeine and UCN-01 but resistant to wortmannin. Both the ATM-dependent and ATM-independent mechanisms are involved in the Chk1 pathway. And the fast and slow responses cooperate to maintain an intact S-phase checkpoint in mammalian cells after ionizing radiation induced DNA damage (Zhou et al, 2000).

As mentioned above, the ant-cancer effects of UCN-01 are primarily mediated by abrogation of the G2 checkpoint. In leukemia cells treated with cytostatic concentrations of the purine analog, gemcitabine, the cells were found to accumulate in S phase. After removal of gemcitabine and the addition of non-toxic concentrations of UCN-01 the cells

proceeded to undergo apoptosis without resuming DNA synthesis and without arresting in G2 (Shi et al, 2001).

(UCN-01) is presently being evaluated in clinical trials as an antineoplastic agent both alone, in combination with various chemotherapeutic agents and in conjunction with ionizing radiation (Mow et al, 2001). The first phase I trial results have been reported in 47 patients with refractory neoplasms (Sausville et al, 2001), results from the study indicate that the dose limiting toxicities were hyperglycemia with metabolic acidosis, pulmonary dysfunction, nausea, vomiting and hypotension. The plasma half-life of UCN-01 unexpectedly was reported to be 30 days which necessitated a longer interval between dosing cycles in patients (Senderowicz et al, 2000). The recommended phase 1 dose was 45 mg/m^2, occurring over a 3 day period, and using a constant infusion regimen with dosing every other week.

Initial investigations postulated that UCN-01 when administered at pharmacologically active concentrations promoted cell death by cyclin-dependent kinases (Cdk) dephosphorylation, but later studies indicate that inhibition of protein kinase C (PKC) enzymes and protein dependent kinase (PDK-1) are also involved in its mechanism of action (Wang et al, 1995). UCN-01 has demonstrated anti-proliferative activity both in

vitro and in vivo through a mechanism which purportedly involves inhibition of PKC isoforms (Mizuno et al, 1995).

UCN-01 has also been shown to enhance the cytotoxicity of chemotherapeutics by several different proposed mechanisms, one of which is the inhibition of Chk1 (Graves et al, 2000). Chk1 inhibition by UCN-01 may promote the activation of a protein phosphatase Cdc25C and also interferes with its elimination by blocking the binding of Cdc25C with 14-3-3 proteins required for its subsequent degradation. The down-regulation of Cdc25C results in the enhanced phosphorylation and the subsequent inactivation of Cdks such as $p34^{cdc2}$ which have been shown to be involved in G2/M cell cycle arrest following DNA damage (Peng et al, 1997).

UCN-01 has recently been shown to inhibit the downstream effector of PI3 kinase, PDK-1, in a similar concentration range used to inhibit PKC isoforms (Komander et al, 2003). Subsequently, UCN-01 functions as a checkpoint abrogator which is capable of enhancing cell killing effects of DNA-damaging agents such as cisplatin, Ara-C, camptothecin and ionizing radiation (Busby et al, 2000, Bunch et al, 1996, Tang et al, 2000 and Shao et al, 1997).

Multiple intracellular signal transduction pathways such as ERK1/2 and PI3K/AKT are of great interest to researchers as potential therapeutic targets for the treatment of many malignancies because they are often highly activated in tumor cells (Carter et al, 1998, Jarvis et al, 1998 and Wang et al, 1999). This strategy is warranted by the success of numerous investigators demonstrating that the inhibition of chemotherapeutic and radiation –induced growth factor receptor or signaling pathways by novel inhibitors of kinase domains enhances the toxicity of existing chemotherapy and radiation modalities (Park et al, 1999, Wang et al, 1999, Dent et al, 2003 and Dent et al, 2002). However an alternative approach to tumor cell killing without using the established cytotoxic therapies is to exploit the necessity of malignant tumor cells to rely on high levels of signaling pathway activity to maintain their growth and viability. Thus, the previous studies by our group cited above, have demonstrated that UCN-01 causes activation of the ERK1/2 pathway in transformed cell types at clinically relevant concentrations in vitro. Furthermore, prevention of ERK1/2 pathway activation by either MEK1/2 inhibition or inhibition of RAS function, enhanced UCN-01 induced tumor cell death in a synergistic manner (McKinstry et al, 2002, Dai et al, 2001, Dai et al, 2002, Yu et al, 2003 and Dai et al, 2005).

However, experiments utilizing non-transformed cell lines from multiple tissues were found to be insensitive to apoptotic events induced by this strategy in transformed cell

lines. The underlying strategy of blocking compensatory cell survival pathway activation results in tumor cell killing has been extended by our group and others using various small molecule inhibitors of kinases and other enzymes. These include PI3 kinase inhibitors and flavopiridol, histone deacetylase inhibitors and flavopiridol, MEK1/2 inhibitors and imatinib mesylate and histone deacetylase and perifosine (Yu et al, 2003, Gao et al, 2004, Rahman et al, 2005 and Yu et al, 2002).

The present studies were designed to determine if MEK1/2 inhibition combined with UCN-01 would suppress mammary tumor cell growth in vivo and with a similar synergy that was observed our in vitro studies.

1.23 Human Chorionic Gonadotropin

The first bioassay for human chorionic gonadotropin (hCG) was reported in 1927 for the diagnosis of pregnancy (Ascheim and Zondek, 1927). Over the next several decades, improvements and modifications of this assay were used clinically until immunoassays eventually replaced bioassays (Wide and Gemzell, 1960 and Vaitukaitis et al, 1972). The development of monoclonal antibodies enabled new assays to be developed for specifically degraded forms of hCG and its subunits. Today, these assays have found new clinical applications for the diagnosis of pregnancy, trophoblastic disease and non-

trophoblastic cancers and gonadal germ cell tumors (Stenman et al, 2004). Due to the high concentrations of hCG in the urine of pregnant women, this urine has been used to recover and purify hCG for both clinical and experimental use.

Human chorionic gonadotropin belongs to the glycoprotein hormone (GPH) family which also includes Lutenizing hormone (LH), Follicle stimulating hormone (FSH), and Thyroid stimulating hormone (TSH). The GPH family members are heterodimers which consist of an α-subunit (GPHα) and a β-subunit. The common α-subunit consists of 92 amino acids and the β-subunit which has various degrees of sequence homology confers the biological activity. Homology between the β-subunits of hcG and LH is about 80%. The LHβ-subunit consists of 121 amino acids while the hCGβ-subunit contains 145 amino acids. The difference between the two is due to a 24-amino acid extension called the C-terminal peptide (CTP) described in Pierce and Parsons, 1981. About one third of the total mass of hCG is made up of eight carbohydrate moieties, six are attached to the hCGβ and two are attached to the hCGα. The average molecular weight of a 'typical' hCG molecule is 38,931 g/mol (Birken et al, 2003). The hCG produced in trophoblastic cancer is higher than the hCG found in pregnancy (Mann and Karl, 1983). Interestingly though, the hCG recovered from the urine of some cancer patients is nicked on the beta subunit and lacks the CTP (Cole et al, 1982 and Cole et al, 1991).

The half-life of hCG is biphasic, in that the first half-life (rapid phase) occurs between 5-6 hours and the second (slower phase) occurs between 24-33 hours (Rizkallah et al, 1969 and Wehmann and Nisula, 1981). Peak concentrations of hCG or recombinant hCG occur at about 6 hours after injection. And most of the circulating hCG is metabolized by the liver with about 20% being excreted by the kidneys (Nisula et al, 1989). Although hCGβ remains in circulation longer than hCG itself (Korhonen et al, 1997).

The biological activity of hCG occurs through the LH/hCG receptor with its major function being the maintenance of progesterone production in the corpus luteum in early pregnancy. In early pregnancy hCG is produced by the cytotrophoblasts. The LH/hCG receptor is also present in the vasculature of the uterus as well as other tissues outside of the ovary but its functions are unknown (Filicori et al, 2005).

The secretion of pituitary hCG is regulated by GnRH which increase in post-menopausal women and in men over the age of 60. In men, gene expression of hCGβ occurs at very low levels without concomitant expression of hCGα in tissues such as the bladder, adrenal gland, thyroid gland and colon. Although the source of normal serum levels of hCGβ is unknown. While the expression of both hCG subunits are found in testis, prostate and skeletal muscle (Bellet et al, 1997). The serum levels of hCGβ in men and

non-pregnant woman are extremely low, even though they are detectable in some of the most sensitive assays, and do not appear to increase with age (Alfthan et al, 1992).

In the male prostate, it was recently demonstrated that both hCG subunits are expressed as well as the gonadotropin-receptor in benign prostatic hyperplasia (BPH) and prostate cancer. But whether or not this expression acts as an autocrine or paracrine modulator of prostate pathophysiology and which cell types are involved, is undetermined (Dirnhofer et al, 1998). However, it is known that the progression of prostate cancer from hormone dependent primary carcinoma to the increasingly androgen-independent metastatic lesions is associated with multiple molecular and genetic changes (Zhang et al, 1998).

In prostate cancer, hCG has been suggested to function as a both a growth factor and angiogenesis-stimulatory factor. In a review of tissue samples from patients diagnosed with localized prostate adenocarcinoma, those found to be positive for hCGβ determined by immunohistochemical staining, 75% of those developed metastasis and 92% died within 18 months (Sheaf et al, 1996).

HCGβ by itself does not appear to have hCG like activity but several investigations indicate that it may promote cell growth. It has been shown to enhance the growth of bladder cancer cells and anti- hCGβ antibodies have inhibited this action (Gillott et al,

1996 and Butler et al, 2003). But in rodent breast cell carcinoma, hCGβ was demonstrated to induce apoptosis (Srivastava et al, 1997). In another study involving choriocarcinoma cells, hCGβ expression was inhibited by antisense messenger RNA which suppressed cell proliferation and induced apoptosis (Hamada et al, 2005).

Although the underlying mechanisms for these activities of hCGβ are unknown, some researchers have proposed that hCGβ displays autocrine like growth effects and may interfere with the growth-inhibiting effects of transforming growth factor (TGF-β), platelet derived growth factor (PDGF-β) and nerve growth factor (Butler and Iles, 2004). On the other hand, hCGα does not possess any hCG like activity but has been shown to work synergistically with progesterone to stimulate endometrial stromal cells to differentiate into decidual cells and stimulate prolactin production in decidual cells (Moy et al, 1996).

In another in set of studies, investigators evaluated the effects of antisense c-myc, (AVI-126), and antisense β-hCG oligomers, (PMO-36-1), on two prostate cancer cell lines DU-145 which are (Rb-/-, p53-/- and androgen independent) and LnCap which are (Rb+/+, p53+/+ and androgen dependent) and both of which express the oncogene c-myc. The c-myc oncogene is a key regulator of cell proliferation and differentiation. Activation of c-myc occurs as a downstream effect of hCG signaling through G-protein coupled signal

transduction pathway. The results demonstrated that antisense β-hCG oligomer treatment of DU-145 cells caused selective growth inhibition but treatment with antisense c-myc oligomer had no anti-proliferative effects. In contrast, the treatment of LnCap cells with antisense β-hCG oligomer caused no growth inhibition but treatment with antisense c-myc oligomer significantly decreased LnCap cell viability. And in vivo, the inhibition of both c-myc and β-hCG by antisense oligomers potentiate the anti-proliferative effect in DU-145 cells over that of β-hCG inhibition alone (Devi et al, 2002).

1.24 Farnesyltransferase Inhibitors

Farnesyltransferase inhibitors (FTI) are analogs of imidazole that were developed to block the activity of Ras oncoproteins which are dependent on post-translational modification (prenylation) by the addition of a farnesyl isoprenoid membrane anchor. Prenylation is required for the translocation and association of Ras to the plasma membrane where Ras proteins function. Farnesylation of Ras by farnesyl transferase is an essential post-translational process for Ras activity. The Ras family of proto-oncogenes is an upstream mediator of essential signal transduction pathways involved in cell proliferation and survival. Ras mutations result in the abrogation of its inherent GTPase activity which subsequently causes permanent activation of Ras resulting in un-controlled cell growth and proliferation. Ras mutations occur in approximately 30% of all human

cancers including lung and colon and almost every form of pancreatic cancer (Sebti and Adjei, 2004).

The Ras genes were identified about 35 years ago as the primary oncogenes in two of the classic oncogenic viruses in mice. They are H-Ras in Harvey sarcoma virus and K-Ras in Kirsten murine sarcoma virus (Bos, 1989 and Shimizu et al, 1983). Ras proteins belong to the G-protein family which is able to bind and hydrolyze guanosine triphosphate reversibly. The Ras family members are small GTPases that occupy essential positions in the growth factor receptor-signal transduction pathways such as EGFR, FGF and VEGF (Cox and Der, 2002). These pathways are known to regulate cell differentiation, proliferation, migration and cell survival (Giehl, 2005). Inhibition of oncogenic Ras activity by FTI can reverse the Ras-mediated suppression of, and can lead to the up-regulation of Fas ligand and down regulation of vascular endothelial growth factor VEGF (Zhang et al, 2002).

It is now been shown that FTI have activity that is independent of Ras and has anti-tumor effects on tumors without Ras mutations (Giehl, 2005). The observation was also noted in studies employing R115777 which blocks prenylation of H-Ras but not K-Ras or N-Ras. In these studies, R115777 was able suppress tumor growth in cell lines with K-Ras or N-Ras mutations which suggested that FTI also targeted other proteins that are important for

tumor cell growth (Yao et al, 2006). While FTI is known to inhibit the farnesylation of H-Ras, K-Ras can serve as a substrate for geranylgeranyl transferase type I (GGTase I) and maintains its oncogenic function even in the presence of FTI. Although, FTI is still able to inhibit tumors with K-Ras mutations which demonstrates that FTI exhibits anti-tumor activity based on its effects on other cellular proteins beside Ras (Pan and Yeung, 2005).

Another, activity of FTIs that inhibit cancer cell growth is their ability to interfere with bipolar spindle formation during the prophase-metaphase transition during mitosis. The centromeric proteins, CENP-E and CENP-F are substrates for farnesyltransferase, even though FTI does not affect their localization at the kinetochore. However, cells treated with FTI-2153 displayed un-aligned chromosomes at the metaphase plate and the association of CENP-E with microtubules was altered (Crespo et al, 2001 and Ashar et al, 2001).

The inhibition of Akt/protein kinase B-mediated cell survival pathways may also be important in the anti-cancer effects of FTI. Although the mechanism is unclear, FTI has been shown to inhibit Akt activity in head and neck squamous cell carcinomas and decrease Akt expression in non-small cell lung cancer (Chun et al, 2003 and Lee et al, 2004).

Recent studies have demonstrated that FTI are effective against several malignancies including myeloid leukemia's (Zhu et al, 2005 and Selleri et al, 2003) And FTI in combination with other anti-neoplastic agents increase their cytotoxic effects, including that of radiation (Shi et al, 2000, Moasser et al, 1998 and Van Golen et al, 2002). Pre-clinical studies have demonstrated that FTI can inhibit breast cancer cell proliferation both in vitro and in vivo and phase II studies with the FTI, (R115777), in advanced breast cancer show promising results (Van Golen et al, 2002).

Increased expression of Ras and/or MAP kinase signaling, a downstream event of Ras activation, has been observed in 50% of human breast cell cancers and the upstream effectors of Ras proteins such as EGFR, HER2/Neu are often over-expressed in many human breast cell carcinomas (Hand et al, 1987).

In over-expressive N-Ras transgenic mice, which were susceptible to mammary and lymphoid tumors, FTI resulted in significant anti-tumor effects in this experimental model (Mangues et al, 1998). A similar growth inhibition effect was observed in K-Ras over-expressing transgenic mice which developed mammary tumors spontaneously but to a lesser extent when treated with FTI (Omer et al, 2000).

1.25 Statins (HMG-CoA reductase inhibitors)

The statins are 3-hydoxy-3-methylglutaryl-coenzyme A reductase inhibitors or (HMG-CoA-reductase inhibitors) which have been clinically approved for their cholesterol lowering ability and for the treatment of lipid disorders. The statin family includes lovastatin, simvastatin, atorvastatin, fluvastatin and pravastatin. Their primary mechanism of action involves the inhibition of the enzyme HMG-CoA-reductase which is upstream in the mevalonate biosynthesis pathway. HMG-CoA reductase is responsible for the conversion of HMG-CoA into mevalonate (Goldstein and Brown, 1990). Within this biosynthetic pathway, mevalonate can be converted into farnesylpyrophosphate (FPP). FPP is the precursor of many other mevalonate pathway products besides cholesterol; these include heme A, dolichols and ubiquinones and geranylgeranylpyrophosphate (GGPP). Both FPP and GGPP are essential for the activation of intracellular proteins such as Ras, Rho, rhodopsin kinase, transducins, nuclear lamins, and G-proteins (Casey, 1995). This activation is referred to as prenylation which is the coupling of a farnesyl or geranylgeranyl moiety to the target protein. This action is catalyzed by the enzymes farnesyl transferase or geranylgeranyl transferase.

Of these proteins, Ras has been the most studied because of its role in cell growth and differentiation. Since mutated forms of Ras have been identified in about 30% of human tumors, this aberrant Ras is thought to play a functional role carcinogenesis (Bos, 1989). The therapeutic potential of statins in the treatment of cancer is in its ability to inhibit HMG-CoA reductase activity and block the mevalonate biosynthetic pathway and subsequent activation of Ras. The initial focus on the therapeutic application of statins in cancer was based on the anti-tumor properties of specific farnesyl transferase (FTIs) and geranylgeranyl transferase (GGTIs) inhibitors. But extensive investigations into the statins have provided new evidence that other mechanisms are involved too (Gaaf et al, 2004).

In addition to HMG-CoA-reductase inhibition, the statins have demonstrated the ability to induce growth arrest and apoptosis as well as inhibit metastasis and possibly angiogenesis. In vitro studies have shown that a lovastatin induced inhibition of cell proliferation in C6 glioma cells can be reversed with the addition of geranylgeraniol (Crick et al, 1998). Lovastatin has also been shown to induce apoptosis in human medulloblastoma cells in vitro and which can be reversed by the addition of mevalonate to the culture (Macaulay et al, 1999, and Wang and Macaulay, 1999). Additionally, lovastatin in combination with phenyl acetate was shown to induce apoptosis in human malignant glioma cells (Schmidt et al, 2001).

In certain pediatric cancers and squamous cell carcinomas, lovastatin has demonstrated the ability to induce apoptosis and cell differentiation (Dimitroulakis et al, 2001). In addition several other investigators have focused on the ability of lovastatin to induce apoptosis and cell death in myeloma plasma cells and in acute myeloid leukemia as well multi-drug resistant myeloid leukemia (Van de Donk et al, 2002, Maksumova et al, 2000, Dimitroulakas et al, 1999 and Xia et al, 2001).

In breast cell carcinoma cell lines, Cerivastatin was shown to inhibit signaling pathways involved in metastasis and invasion in vitro, although it should be noted that Cerivastatin was withdrawn from the U.S. market in 2001 due to a higher than expected risk of hepatotoxicity (DeNoyelle et al, 2001). Also, Cerivastatin was shown to induce G1-arrest in breast cancer cells but without inducing apoptosis. And in prostate cell cancer, lovastatin was able to modulate E2F-1 transcription factor resulting in cell death and also induce apoptosis of LnCap cells by activating caspase-7 (Park et al, 2001 and Marcelli et al, 1998).

The ability of the statins to induce growth arrest and apoptosis appears to be dependent on statin concentration. Growth arrest and apoptosis are known to occur in vitro at lovastatin concentrations between 0.1 and 100 μM depending on which cell line was

being evaluated. In phase I trials of lovastatin using doses ranging from 2-25 mg/kg/day, the drug plasma levels in volunteers ranged from 0.1 to 3.9 μM. These values imply that lovastatin-induced apoptosis and anti-proliferative effect is clinically achievable for some forms of cancer. In those cell lines that require higher doses of statins to achieve these effects, statin therapy perhaps could be used in combination with other anti-tumor agents (Gaaf et al, 2004). And all statins are not equal in their inhibitory effects. Cerivastatin for example was shown to be 10 times as potent as the other statins for inducing apoptosis in acute myeloid leukemia cells (Wong et al, 2001). And the rank order of potency after cerivastatin is the equipotent group simvastatin, lovastatin, fluvastatin and atorvastatin, followed by pravastatin which is the least potent (Negre-Aminou et al, 1997).

In vivo, the statins have been demonstrated to have anti-tumor forming properties on colon cancer cell lines in rats and in H-ras transformed cell lines in nude mice (Gaaf et al, 2004).

1.26 Poly (ADP-ribose) polymerase-1

The nuclear enzyme poly-(ADP-ribose) polymerase-1 (PARP-1) is activated in response to DNA strand breaks. PARP-1 contributes to DNA repair by binding to DNA-repair enzymes and facilitating chromatin remodeling. This activation consumes NAD+ to form

poly-(ADP-ribose) on specific acceptor proteins. Excessive PARP-1 activation in response to extensive DNA damage can also promote cell death. The PARP-1 cell death pathway is caspase-independent of, and morphologically distinct from classical apoptosis. The major events in this PARP-1 induced cell death response are mitochondrial depolarization, mitochondrial permeability and the release of apoptosis-inducing factor (AIF) from the mitochondria. Mitochondrial AIF release is followed by its translocation to the cell nucleus which results in nuclear DNA fragmentation. This nuclear DNA fragmentation is considered to be an irreversible event in cell death. The down-regulation or ablation of PARP-1 is able to completely disrupt PARP-1 mediated cell death.

The ability of PARP-1 activation in the nucleus to trigger the release of AIF from mitochondria is not understood. Several explanations have been suggested by investigators as to how and why this occurs. One is that PARP-1 is present in the mitochondria as well as the nucleus which would eliminate speculation about communication events occurring between the mitochondria and the nucleus. Another is that PARP-1 activation results in the depletion of cytostolic NAD+ (which is independent of and apart from the mitochondrial NAD+ pool but not the nuclear NAD+ pool) which would lead to a block in glycolysis at the NAD+-dependent glyceraldehyde-3-phosphate dehydrogenase step and result in depriving the mitochondria of the glucose-derived

substrate pyruvate. If the NAD+ pool is replenished in experimental condition or if a non-glucose metabolic substrate is substituted, PARP-1 induced mitochondrial events and cell death can be prevented (Alano and Swanson, 2006).

In a recent study by Xu et al, 2006, the authors provided evidence for the involvement of c-Jun N-terminal kinase 1 (JNK1) in PARP-1 activation-induced cell death. JNK1 is associated with signal-transduction events and cell death by tumor necrosis factor-α (TNFα) and other noxious cell stimuli. The activation of PARP-1 in response to DNA damage generated by the alkylating agent, N-methyl-N'-nitrosoguanidine (MNNG) in mouse embryonic fibroblasts was followed by mitochondrial depolarization and AIF release. In addition, these actions coincided with the activation (phosphorylation) of JNK1 which could be blocked by PARP-1 inhibitors. Additionally, pharmacological intervention by JNK1 inhibitors and the deletion of genes encoding for JNK1 prevented mitochondrial depolarization but not the release of PARP-1. However, the mechanism for the upstream activation of JNK1 by PARP-1 is not understood (Alano and Swanson, 2006).

In our studies, we investigated the potential therapeutic use of pharmacological inhibitors on these important cell survival and proliferation pathways known to occur in cancer cells. Many traditional anti-cancer drugs are administered as sole agents in succession to limit systemic toxicity effects. However, our laboratory has focused on the use of non-

traditional chemotherapeutic drugs in combination at low doses. This combination of new MAP kinase inhibitors and check point abrogators among others described above appears to be better tolerated in most patients as well.

MATERIALS and METHODS

2.1 Materials for PD-183452 and UCN-01 Studies.

Phospho-ERK1/2, anti-BAX and total ERK1/2 antibodies were purchased from Cell Signaling Technologies (Worcester, MA). The anti-BAX monoclonal antibody 6A7 for immunoprecipitation of conformationally altered BAX was purchased from Sigma-Aldrich (St. Louis, MO). The anti-Ki67 antibody was purchased from Oncogene Research Products (San Diego CA). The anti-CD31/PECAM antibody was purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). And the cleaved caspase-3 antibody was purchased from Cell Signaling Technologies (Worcester, MA). All of the secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP, and anti-goat-HRP) were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA).

Enhanced chemiluminescence (ECL) and TUNEL reagent kits were purchased from NEN Life Science Products (Boston, MA) and Boehringer-Mannheim (Manheim, Germany), respectively. Trypsin-EDTA, RPMI culture medium, penicillin-streptomycin were purchased from GIBCOBRL (GIBCOBRL Life Technologies, Grand Island, NY). PD98059 and U0126 were obtained from Calbiochem/EMD Sciences (San Diego, CA). UCN-01 was generously provided by Dr. Janet Dancey at the National Cancer Institute, Developmental Therapeutics Program/CTEP.

PD184352 was synthesized in house based on the published structure of the compound and stored in powder form in a dry nitrogen atmosphere under light excluding conditions at -80°C. R115777 and UCN-01 was generously provided by Dr. Steve Grant, and stored under desiccant conditions at 4°C. Hydroxypropyl β-cyclodextrin was purchased from Sigma Chemical, St. Louis, MO.

Female athymic Ncr-nu/nu mice at 6 weeks old and weighing 20-25 grams were purchased from the National Cancer Institute Animal Resource Center, (National Cancer Institute at the National Institute of Health, Bethesda, MD) and Jackson Laboratories (Bar Harbor, ME).

2.2 Cell culture and in vitro drug treatment of mammary carcinoma cell lines.

MDA-MB231 (p53, pRB+, ER-) and MCF7 (ER+, pRB+, p53+) cells were cultured at 37°C in a 5% CO_2 atmosphere in vitro using RPMI supplemented with 5% (v/v) fetal calf serum. In vitro use of UCN-01, UO126, PD98059 and PD184352 as experimental treatment was made from 100 mM stock solutions of each drug and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v).

2.3 Cell culture and in vitro drug treatment of prostate carcinoma cell lines.

LnCap (p53+, pRB+, AR+, PTEN null, low beta hCG expressors), 22RW1 (Rb-/-, androgen (weakly)sensitive, EGFR responsive, low-expressors of beta hCG), and PC-3 (Rb+/+, p53+/+, androgen insensitive) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured at 37°C in a 5% CO_2 atmosphere in vitro using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum.

Phosphorylated and total (ERK1/2; JNK1/2; and p38 MAPK) antibodies, anti-PARP1 antibody, phosphorylated and total AKT (S473) and phosphorylated and total ERBB1 (Y1068, Y1173, Y845; Y992) antibodies were purchased from Cell Signaling Technologies (Worcester, MA). The anti-PARP1 ADP-ribosylation specific 10H monoclonal antibody was purchased from Axxora LLC. (San Diego, CA). All of the secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP and anti-goat-HRP) used for these studies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence (ECL) reagents and TUNEL kits were purchased from NEN Life Science Products (NEN Life Science Products, Boston, MA) and Boehringer Mannheim (Manheim, Germany) respectively. N-(6-oxo-5, 6-dihydrophenanthridin-2-yl)-2-(N, N-dimethylamino) acetamide hydrochloride (PJ-34, CAS number 344458-15-7) was purchased from Sigma (St. Louis, MO). Trypsin-EDTA, DMEM and RPMI media,

penicillin-streptomycin was purchased from GIBCOBRL (GIBCOBRL Life Technologies, Grand Island, NY). Pertussis toxin, caspase inhibitors (zVAD, LEHD, IETD), GGTI-286, AG1478, LY294002, farnesyl pyrophosphate, geranylgeranyl pyrophosphate and lovastatin were purchased from Calbiochem (San Diego, CA). Human chorionic gonadotropin was purchased from Pro-Spec-Tany TechnoGene Ltd., Weizmann Science Park, and P.O. Box 398, Rehovot 76103, Israel. PD184352 was chemically synthesized in house which was based on the published structure of the drug and stored in powder from in a nitrogen atmosphere and under light protected conditions at -80° C.

Methods.

2.4 Cell culture and in vitro drug exposure.

All cell lines used in these studies were cultured at 37°C, 5% (v/v) CO_2, in vitro using RPMI supplemented with 10% (v/v) fetal calf serum. In vitro AG1478, PD184352, Wortmannin and Lovastatin treatment was made from a 100 mM stock solution of each drug and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v).

2.5 In vitro cell treatments Clonogenic assays, Western blot and SDS-PAGE analysis.

For in vitro analyses of short-term apoptosis effects, cells were treated with vehicle (DMSO), AG1478, PD184352, LY294002, Lovastatin, hCG or their combination for the indicated time points. For the apoptosis assays, cells were pre-treated with zVAD, IETD or LEHD at 50 μM each. Following pre-treatment the cells were isolated by trypsin procedure and subjected to either trypan blue cell viability assay and counted under a light microscope or fixed to slides and stained using a commercially available TUNEL assay kit according to method instructed by the manufacture.

Cells for in vitro colony formation assays were plated at 250-4000 cells per well in sextuplicate and for in vitro assays 14 hours after plating were treated with either vehicle (DMSO), AG1478, PD184352, LY294002 or the indicated drug combinations for 48 hours followed by drug removal. Fourteen to twenty-eight days after drug exposure treatment or tumor isolation, the plates were washed in PBS, fixed with methanol and stained with a 5% crystal violet solution, rinsed with tap water and colonies were counted both manually (by eye) and digitally using a ColCount^{TM} plate reader (Oxford Optronics, Oxford England). Data presented is the arithmetic mean (+/- SEM) from both counting methods and from multiple studies. Colony formation was defined as a colony of 50 or more cells.

For SDS PAGE and immunoblotting, cells were plated at 5 x 10^5 cells/cm^2 and treated with drugs at the indicated concentrations and after the indicated time of treatment, and lysed in whole-cell lysis buffer (0.5M Tris-HCL, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue), and the samples were boiled for 30 minutes. The boiled samples were loaded onto 10-14% SDS-PAGE and electrophoresis was run overnight. Proteins were electrophoretically transferred onto a 0.22 μM nitrocellulose membrane and immunoblotted with various primary antibodies against different proteins of interest. All immunoblots were visualized by ECL or by using an Odyssey Li-COR system (Lincoln, Nebraska). For presentation, ECL immunoblots were digitally scanned at 600 dpi using Adobe PhotoShop 7.0, and their color removed for the generation of figures in Microsoft PowerPoint.

2.6 Recombinant adenoviral vectors; generation and infection in vitro.

Our laboratory generated and/or purchased previously described recombinant adenoviruses to express constitutively activated and dominant negative AKT and MEK1 proteins, dominant negative ERBB1 (COOH-terminal, 533 amino acid deletion; CD533), dominant negative JNK1, dominant negative caspase-9, dominant negative I Kappa B (S32A) and BCL-$_{xl}$ (18, 24-26) from (Vector Biolabs, Philadelphia, PA). Prostate cancer cells were infected with these adenoviruses at an approximate multiplicity of infection

(m.o.i) of 25-30. Cells were further incubated overnight for approximately 12 hours to ensure adequate expression of transduced gene products prior to any drug exposure or assays. After which, the culture media was changed to remove excess virus and replaced with fresh media.

2.7 Data Analysis.

Comparison of the effects of various treatments was performed using the Student's t-test. Differences with a p-value of <0.05 were considered to be statistically significant. Experiments shown are the means of multiple individual points (+/- SEM).

2.8 In vitro and ex vivo cell treatment.

In vitro analysis for determination of short-term apoptosis effects, cells were treated with vehicle, UCN-01, PD184352 or PD98059 or a combination for the indicated time points. In the apoptosis assays, cells were pre-treated with the caspase-8 inhibitor IETD and the caspase-9 inhibitor LEHD at 20 uM each. Cells were then isolated and fixed to slides and stained using a commercially available TUNEL assay kit according to manufacture's instructions. (McKinstry et al, 2002). For the cell cycle analysis, cells were treated with drug or drug combination for 48 hours then isolated and fixed in 70% (v/v) ethanol. After which they were RNAse and propidium iodide overnight at 4°C and subjected to flow cytometry analysis and the data processed by Verity Winlist software.

Cells for the in vitro or ex vivo colony formation assays were plated at 250-400 cells per well in 6-well plates. In the in vitro colony formation assay, cells were treated with vehicle (DMSO), UCN-01 (10-150 nM), PD 184352 (0.1-2.0 uM) or the drug combination 14 hours after plating. Then, 48 hours after drug exposure the culture medium was changed to remove the drug and replaced with fresh medium. Between 10-14 days post drug treatment or tumor cell isolation, the plates were washed with PBS and fixed with methanol before staining with a filtered solution of crystal violet 5% (w/v). The 6-well plates containing the fixed and stained cell colonies were then washed with tap water and counted both manually (by eye) and digitally using a ColCount^{TM} plate reader from (Oxford Optronics, Oxford, England). Colony formation is defined as an isolated cluster of cells containing 50 or more cells colony. The data was combined and is presented as the arithmetic mean (+/- SEM) from both counting methods from multiple studies for each experimental drug treatment.

2.9 In vivo exposure of mammary carcinoma cell tumors to experimental drug treatment.

Female athymic Ncr-nu/nu mice were obtained from the National Cancer Institute Animal Resource Center (NCI-National Institutes of Health, Bethesda, MD) and from Jackson Laboratories (Bar Harbor, ME). Experimental in vivo studies were performed by

three different operators independently. All mice used in these studies were maintained under pathogen-free conditions with a 12-hour light/ 12-hour dark cycle and were fed autoclaved standard murine chow and water ad libitum in the Massey Cancer Center Animal Vivarium at Medical College of Virginia Hospitals-Virginia Commonwealth University campus. All facilities are approved by the American Association for the Accreditation of Laboratory Animal Care and in accordance with the current standards and regulations set forth by the United States Department of Agriculture, Washington, DC and the National Institutes of Health and Human Services, Washington, DC as well as the National Institutes of Health, Bethesda, MD.

The generation of MCF7 tumors in female athymic Ncr-nu/nu mice was achieved by the implantation of 90 day slow release estrogen pellets (Innovative Research #NE-121; 0.72 mg of 17-β-estradiol) subcutaneously below the midline and between the scapulas. Cultured MCF7 and MDA-MB-231 cells for xenografts were isolated by trypsinization washed three times with cold phosphate buffered saline (PBS) and counted by using a hemacytometer. The cells were then re-suspended in PBS and $5x10^6$ cells/100µl of PBS were injected subcutaneously in the right rear flank of each mouse using a Tuberculin™ 28 gauge syringe. The xenografts were permitted to form over a period of 3-4 weeks until they achieved a volume of ~100 mm^3.

2.10 Experimental drug preparation, treatments and delivery.

PD184352 was aliquoted in ~50mg units/ vial and stored under vacuum at -20°C in a super cold room and protected from light. In the animal studies, PD184352 was dissolved first in DMSO in an amount equal to 4 times in micro-liters of the weight of PD184352 in milligrams, i.e., (50 mg of PD184352 dissolved in 200 µl of DMSO). Followed by an equal volume of Cremophor (Sigma, St. Louis, MO) and mixed by vortex. After thorough mixing, a 1:10 dilution was made with 0.9% sterile saline which yields a uniform suspension. The animals designated for treatment with PD184352 alone or in combination with UCN-01 were injected intraperitoneally (IP) with the PD184352 suspension at a dose equivalent to 25 mg/kg of body mass. Animals received an initial dose of PD184352 followed by two more doses 8 hours apart for 2 days. UCN-01 was administered IP at 45 minutes following the initial PD184352 injection for each of the 2 days of treatment.

UCN-01 was prepared by dissolving the solid powder form in a milliliter volume of sodium citrate equal to the amount of UCN-01 in mg. The UCN-01 vehicle consisted of a solution of 2% (w/v) of sodium citrate at a pH of 3.5. An aliquot of sodium citrate was filtered and sterilized using a 10 ml syringe fitted with a 0.2 µm filter.

The UCN-01 was diluted further with sodium citrate at a ratio of 1:20 for IP injections and had a final concentration of 0.2 mg/kg for MDA-MB-231 tumor bearing mice or 0.1

mg/kg for the MCF7 tumor bearing mice. Each of the designated control group animals received an injection of the vehicle equal to the volume administered to the animals receiving PD184352 or UCN-01 treatments.

The farnesyl transferase inhibitor, R115777, was prepared as follows. Four grams of hydroxypropyl β-cyclodextrin were dissolved in 10 ml of 0.1N HCL to yield a 40% stock solution. This was diluted 1:1 in 0.1N HCL to make a 20% solution for vehicle controls. R115777 was dissolved in 5ml of 40% hydroxypropyl β-cyclodextrin in 0.1N HCL and dissolved by sonication and further diluted 1:1 with 0.1N HCL to yield a 100mg/kg solution and delivered by oral gavage at 0.05 ml per 10g of body weight twice a day to achieve a 100mg/kg dose.

Tumor volumes were determined by caliper measurements in millimeters, every other day and tumor volume was determined by using the conventional formula [$V= (LxW^2)/0.5$] where L and W are the longest and shortest lengths of the tumor respectively and the volume (V) is reported in cm^3. Tumor growth was expressed as a relative fold increase in tumor volume starting with the initial tumor measurement prior to the onset of experimental drug treatment. By using the formula (T_x/T_0), where T is the mean tumor volume within each experimental group at a particular time in days X and T_0 is the initial tumor volume at Day 0 of the experiment.

2.11 Radiation therapy for the PD-183452 and UCN-01 combination treatment.

Female athymic nu/nu mice bearing MDA-MB-231 tumors were irradiated 24 hours after completion of the drug regimen. Anesthetized animals received a single dose of 2 Gy delivered from a Picker Zoneguard V4M60, ^{60}Co gamma irradiator (Picker X-ray Manufacturing, Cleveland, Ohio) at a rate of 1.9 Gy/minute. Immediately after the administration of radiation the animals were allowed to recover from the anesthesia on a heating pad set at medium (37-42° C) for 45 minutes.

2.12 Ex vivo mammary carcinoma tumor preparation for experimental assays.

At the predetermined time point, animals were euthanized by rapid CO_2 asphyxiation and placed in a BI.2 laminar flow cell culture hood on a sterile barrier field. The entire body of the mouse was soaked with 70% (v/v) ethanol and the skin around the tumor was removed using sterilized surgical scissors, forceps and a disposable scalpel with a #10 blade. These surgical implements were flame sterilized and dipped in 95% ethanol between removal of the skin layers and tumor resection. A piece of the tumor constituting ~50% of the total volume was placed in a 10 cm dish containing 5 ml of RPMI cell culture media and placed on ice. The remainder of the tumor was placed in 5-10 ml of 4% paraformaldehyde in a 50 ml conical tube and subjected to immersion fixation for a minimum of 2 weeks. The sample of the tumor placed in RPMI on ice was minced

manually with disposable sterile scalpels into the smallest pieces achievable and subsequently placed in a disposable digestion flask. The dish was then rinsed with 6.5 ml of media in 3.25 ml increments which was then added to the flask. A 10x solution of collagenase (Sigma, St. Louis, MO; 2.5 ml at a final concentration of 28U/ml) and 10x of enzyme cocktail containing DNAse (Sigma, St. Louis, MO; at a final concentration of 308 U/ml) and Pronase (EMD Sciences, San Diego, CA; at a final concentration of 22,500 U/ml) in a volume of 1 ml was added to the flask. The flask was then placed into an orbital shaker at 37°C for between 1.5 to 4 hours and rotated at 150 rpm. Once digestion of the tumor pieces appeared to be complete, the solution was passed through a 0.4 μm sterile filter into a 50 ml conical tube. After mixing by vortex, a 1 ml sample was removed of which 10μl aliquots in duplicate were taken for a determination of total and viable cell count using a hemacytometer.

Cells were then centrifuged at 500 x g for 4 minutes and the supernatant was removed and replaced with fresh RPMI media containing 5% FBS. This process was repeated twice to remove any residual enzyme cocktail before finally being re-suspended at a cell concentration of 1×10^6 cells/ml. These cells were then diluted and plated in 10 cm dishes in triplicate at a concentration of, 2×10^3 cells/dish for the control treatment group and 4,000 cells/dish for the PD184352, UCN-01 and drug combination groups for the colony formation assays.

2.13 Immunohistochemistry and H & E staining of fixed tumor sections.

Fixed tumors were washed 3 times in PBS for 15 minutes before being subjected to the dehydration/paraffin embedding process. Tumor sections were cut from paraffin blocks at a thickness of 10 μm using a microtome and floated in water bath at 40-50°C and dry mounted on Permafrost™ (Fisher Scientific) slides. Tumor sections were allowed to dry on a slide warmer at 40-45°C for 2 hours and then sit at room temperature overnight. The slides were placed in a 37°C oven overnight to melt the paraffin and then put through the standard de-paraffinization and re-hydration process. The tissue section containing slides were further hydrated by placing them in distilled water for 1 minute. After which, the tissue sections were treated to block endogenous peroxidase using 3% H_2O_2 in methanol for 20 minutes. The rinsed in tap water for 3 minutes and then placed in distilled water for 2 minutes. The slides were then subjected to the antigen retrieval process using 10 mM (w/v) Sodium Citrate/Citric Acid buffer, pH 6.5 and heated to 90°C using a constant temperature microwave for 12 minutes followed by cooling at room temperature for 20-30 minutes. Slides were the dipped in PBS 3 times followed by dipping in distilled water before being put back in PBS for 3 rinses at 20 minutes/rinse. The tissue sections were then treated with normal goat (NGS) or horse serum (NHS) e.g. (5% NHS; 0.3% Triton X) and incubated in a humidified chamber for 30 minutes. The slides were decanted of excess serum and the specific primary antibody of interest (i.e. CD31; phospho-ERK1/2;

Ki-67; cleaved caspase-3) was added to each section at 1:300 in (e.g. 2 ml of 3% NGS, 10 ul of APP). Slides were placed in a closed humidified chamber and incubated overnight at 4°C for 18 hours, then removed from the refrigerator and allowed to sit at room temperature for 10-15 minutes. Slides were then placed in PBS for 5 minutes to ensure hydration of all tissue slices. The slides were then treated with the secondary antibody i.e. biotin-conjugated anti-mouse secondary at a 1:500 dilution in NGS for 2 hours and then rinsed in PBS for 3 x 5 minutes changing the PBS every 5 minutes.

The slides were then developed using the avidin-biotin-peroxidase complex (ABC reagent kit; Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) kit was used at dilutions of 1:250 'A' and 1:250 'B' in 1% goat serum and placed back in the humidified chamber for 45-60 minutes. The processed slides were then washed in 0.1M phosphate buffer twice for 5 minutes and rinsed in PBS by dipping for 1 minute. Finally, the slides were placed in freshly prepared di-aminobenzidine (DAB) substrate solution according to the method described by (Vandesane, F, 1979) containing 0.1M phosphate buffer and 0.01 % H_2O_2 and incubated for 15 minutes. The slides were given a final rinse under tap water for 3 minutes.

The tissue sections were then dehydrated using a standard protocol, cleared and mounted with cover slips using Permount. The finished slides were then allowed to dry at room

temperature for 24-48 hours and photographed at the indicated magnifications. The area selected for all of the photographed images was the proliferative zone within 2 mm of, or juxtaposed to the leading edge of the tumor.

H & E staining of the fixed tumor sections were performed by the standard method describe in (Gridley MF, 1960).

2.14 Data Analysis and Statistics

Comparison of the effects on treatment groups was performed using a mixed effects model with an AR (1) error covariance structure. Tumor growth data shown here are the mean values for one representative experiment using 6-9 animals per treatment condition. Experiments performed on each of the two cell lines were done in triplicate with similar results. Differences between each treatment group with a p-value <0.05 were deemed statistically significant. The experimental data shown here are the mean values for multiple experiments and their individual points (+/- SEM).

RESULTS

3.1 Early In Vitro Studies

Our preliminary observations that demonstrated the MEK1/2 inhibitor PD184352 and UCN-01 interacted synergistically to kill mammary tumor cells in vitro was extended by the initial studies undertaken by (McKinstry et al, 2002). This earlier report demonstrated that 48 hours of exposure to PD184352 and UCN-01 resulted in a synergistic induction of cell killing in both MDA-MB-231 and MCF7 cells as measured by colony formation assays conducted 14 days after drug removal in vitro. Also, a note worthy observation was made when combined exposure of the paracrine TGFα /ERBB1-regulated MDA-MB-231 cells to the ERBB1 inhibitor AG1478 and UCN-01 unexpectedly resulted in an antagonistic interaction for cell killing.

While the treatment of MDA-MB-231 and MCF7 cells in vitro with UCN-01 and either of the MEK1/2 inhibitors, PD184352 or U0126 resulted in the promotion of apoptosis (Figure 5), UCN-01 and MEK1/2 Inhibition Promotes Apoptosis. Further evaluation demonstrated that treatment of MDA-MB-231 cells in vitro with the MEK1/2 inhibitor PD98059 and UCN-01 correlated with the cleavage of pro-caspase 9, pro-caspase 8, pro-caspase 3 as well as altered BAX confirmation and apoptosis when measured by flow cytometry and TUNEL assay (Figure 6), Pro-Caspase Cleavage and Flow Cytometry

Data. Additionally, these apoptotic effects were abolished by the pan-caspase inhibitor zVAD and partially attenuated by either the caspase 8 inhibitor (IETD) or the caspase 9 inhibitor (LEHD). In vitro however, non-transformed cells types such as primary mammary epithelial cells, CD34+ bone marrow progenitor cells and primary rat hepatocytes were all unaffected by the drug combination induced lethality seen in the mammary carcinoma cells (Figure 7), UCN-01 and MEK1/2 Inhibition Does Not Effect Non-transformed Cells.

3.2 In Vivo Studies for UCN-01 and PD184352

The in vivo studies consisted exclusively of athymic nude mice injected subcutaneously in the right rear flank with MDA-MB-231 or MCF7 cells and tumors were allowed to form over a 3-4 week period. Tumor bearing mice with tumors at 100-150mm^3 were injected with; vehicle, UCN-01, PD184352 or the drug combination for two days and tumor growth was monitored over the next 30 days. Treatment of mice with either PD184352 or UCN-01 alone resulted in transient suppression of tumor growth but this inhibition was relieved between 15-30 days after drug treatment. During this interlude no significant changes in tumor volume were observed in mice treated with vehicle, UCN-01 or PD184352 alone (Figures 8 and Figure 9), In Vivo Effect of UCN-01 and MEK1/2 Inhibition on MDA-MB-231 and MCF-7, respectively. In stark contrast to the tumor data generated from individual drug treatments, the combination of UCN-01 and PD184352

significantly reduced tumor growth for both types of mammary carcinoma cell tumors ($p<0.05$ for MDA-MB-231 and $p<0.01$ for MCF7). Thirty days after drug exposure the tumor control ratio (T/C) was 0.36 for MDA-MB-231 tumors and 0.36 for MCF7 tumors. These findings demonstrate that a transient 2 day combined drug exposure to MEK1/2 inhibition and UCN-01 results in tumor growth suppression for up to 30 days.

Since tumor growth had been both suppressed in the MCF7 cell line and reduced in the MDA-MB-231 cell line for 30 days after drug exposure, we decided to perform additional ex vivo studies aimed at examination of gross tumor histology including vascularization and cell viability at this late time point. In tumors exposed to the individual drug treatments compared to the vehicle controls, exhibited a reduced number of cells per field after H&E staining and a modest reduction in the levels of Ki-67 immunoreactivity (Figures 10 and 11), Colony formation assay, Immunohistochemistry and H & E staining of Tumors for MCF-7 and MDA-MB-231 respectively. In contrast to these findings in the individual drug treatment groups, the combined exposure to PD184352 and UCN-01 resulted in a profound reduction in tumor cellularity and almost abolished Ki-67 immunoreactivity, indicating that tumor regeneration had been suppressed. These tumors exposed to both PD184352 and UCN-01 also appeared to have less CD31 immunoreactivity and tissue morphology consistent with tumor vasculature

when compared to controls. This finding indicates that the combined drug treatment had also inhibited neo-angiogenesis and/or killed tumor endothelial cells.

In parallel, these isolated tumors were also macerated and digested to yield individual tumor cells which in turn were plated for ex vivo colony formation assays. In this set of experiments the single drug treated tumors exhibited an in vivo growth rate similar to that of controls for both the MDA-MB-231 and MCF7 cell lines, but the ex vivo growth rate for PD184352 and to a lesser extent UCN-01 treated tumor cells was significantly reduced compared to those of controls (Figures 10 and Figure 11), Colony formation assay, Immunohistochemistry and H & E staining of Tumors for MCF-7 and MDA-MB-231 respectively. The ex vivo colony formation data for in vivo treated tumor cells with both PD184352 and UCN-01 was significantly lower than would have been expected based on the additive combination of PD184352 and UCN-01 alone. Together, our findings indicate that the combined exposure to PD184352 and UCN-01 results in tumor cell killing in vivo and profoundly alters the ability of these tumor cells to proliferate in vitro the despite the absence of the drug in vivo or ex vivo.

Because of these findings illustrated in (Figures 8-11) for tumor growth and end-point analysis, we decided to perform additional studies to examine the cellular response of these tumors during and following cessation of drug treatment. The combination of

PD184352 and UCN-01 treatment compared with individual drug treatments resulted in a rapid induction of cell death revealed by H&E staining and examination of tumor cell morphology (Figures 12A & 12B), Tumor morphology, Days 1-5. The observations of tumor cell death in vivo made as a result of the H&E staining, corresponded to an increase in cleavage of pro-caspase 3 and a reduction in ERK1/2 phosphorylation (Figure 12C), Immunohistochemical staining for ERK1/2 phosphorylation. Corresponding with our in vitro observations that MDA-MB-231 cells treated with UCN-01 in vivo promoted ERK1/2 activation within 24 hours. The activation of ERK1/2 was still evident 24 hours following the last administration of UCN-01. However in this instance, activated ERK1/2 under these conditions appeared to be located in the nucleus (Figure 12D), Immunohistochemical staining shows activated ERK1/2 nuclear translocation. The transient exposure of MDA-MB-231 cells in vivo to either PD184352 or UCN-01 also suppressed CD31 immunoreactivity indicating that as individual agents the MEK1/2 inhibitor or UCN-01 caused endothelial cell death or suppressed tumor angiogenesis (Figure 12E and 14a), Immunohistochemical staining for CD31. The combined exposure to both PD184352 and UCN-01 suppressed CD31 immunoreactivity below that which was observed in tumors treated with either drug individually. Based on these observations and previous work done in our laboratory; the exposure of MDA-MB-231 tumor cells to the drug combination of PD184352 and UCN-01 results in a rapid induction of cell death

that corresponds to the activation of the intrinsic apoptotic pathway and reduced cellular proliferation.

Further studies were undertaken to examine the tumor histology approximately 5-20 days after drug treatment. When compared to either drug individually or vehicle controls, the combination of PD184352 and UCN-01 resulted in a prolonged disruption of cellularity as well as reduced Ki-67 immunostaining, ERK1/2 phosphorylation and CD31 immunostaining (Figures 10 and 14b). Another surprising finding was that in MDA-MB-231 tumors exposed to the UCN-01 and unexpectedly PD184352, the enhanced ERK1/2 phosphorylation persisted 5-10 days following the transient drug exposure and then dissipated to control levels by day 15 (Figure 13B): Immunohistochemical staining for ERK1/2 phosphorylation, Days 5-20. In the MCF7 tumor cell line, UCN-01 treatment promoted ERK1/2 phosphorylation up to 30 days after drug exposure and was abolished in tumors treated with UCN-01 and the MEK1/2 inhibitor. This same observation was also made in the MDA-MB-231 tumor cells (Figure 11). These findings together with those illustrated in (Figure 13a): H&E staining for tumor morphology, Days 5-20., demonstrate that the transient exposure of mammary carcinoma tumors in vivo to the MEK1/2 inhibitor PD184352 or UCN-01 results in the induction of a compensatory activation of ERK1/2 which leads to a transient (5-10 day) up-regulation of ERK1/2

activity above the basal levels of ERK1/2 signaling in control cells. This phenomenon is then subsequently abolished by the co-administration of these agents.

3.3 PD184532 and UCN-01 combined with 2Gy Ionizing Radiation

The multi-modality treatment using 2 Gy ionizing radiation, in combination with PD184352 and UCN-01, suppressed the growth of MDA-MB-231 mammary carcinoma tumors to a greater extent than the drug combination alone in vivo (Figure 15): 2 Gy IR and PD183452/UCN-01 combination. The suppression of MDA-MB-231 tumors by the radiation and UCN-01 and PD-183452 drug combination resulted in approximately a 50% delay in a 2 fold increase in tumor volume.

Compared to the previous effect of UNC-01 and PD183452 combination, this was surprising. However, as discussed in the introduction, the induction of MAPK by radiation and its inherent ability to produce cell killing by irreversible DNA damage, the outcome may be explained. The ability of UCN-01 to induce a hyper-stimulation of MAPK activity, which is blocked by PD183452 could be further enhanced by the radiation induced stimulation effect. Since radiation is known to activate EGFR cell signaling pathways involving MEK1/2. In addition, the ability of UCN-01 to abrogate the G2/M checkpoint would drive sub-lethal DNA damaged cells into mitosis without repair and cause further cell death via apoptosis.

3.4 Combined use of the Farnesyl Transferase Inhibitor R115777 and UCN-01 in vivo

The inhibition of farnesyl transferase by R115777, combined with UCN-01 was shown to suppress the growth of MDA-MB-231 tumors in vivo in a greater than additive fashion (Figure 16): Farnesyl transferase inhibition combined with UCN-01. Our results showed a significant growth delay of approximately 11 days or 33% for the drug combination group to reach the primary endpoint of a 4 fold increase in tumor volume. While the single treatment groups only experienced a marginal delay in tumor growth which reached control levels by day 21. The result of MDA-MB-231 cell treatment with R115777 and the inhibition of Ras prenylation are known to prevent downstream MAPK signaling essential for cell proliferation and survival. Additionally, the inhibition of geranylgeranyl prenylation of H-Ras by FTIs has been shown to prevent C6 glioma cells to progress into S-phase, and may involve the necessary prenylation of other proteins as well (Crick et al, 1998). And previous studies in our group have shown that UCN-01 activation of MAPK was opposed by PD183452 (McKinstry et al, 2002). This suggests that this UCN-01 induced activation of MAPK can be suppressed by inhibition of Ras function by the FTI R115777.

(Figure 5): UCN-01 and MEK1/2 Inhibition Promotes Apoptosis. MDA-MB-231 above left and MCF7 above right cells were treated with either Vehicle control (DMSO) or indicated concentrations of UCN-01 and PD184352 or UO126. Cells were isolated by trypsinization 48 hours after treatment, fixed to glass slides by cytospin and stained for double stranded DNA breaks using a TUNEL assay kit in triplicate from two separate experiments. From (McKinstry et al., 2002).

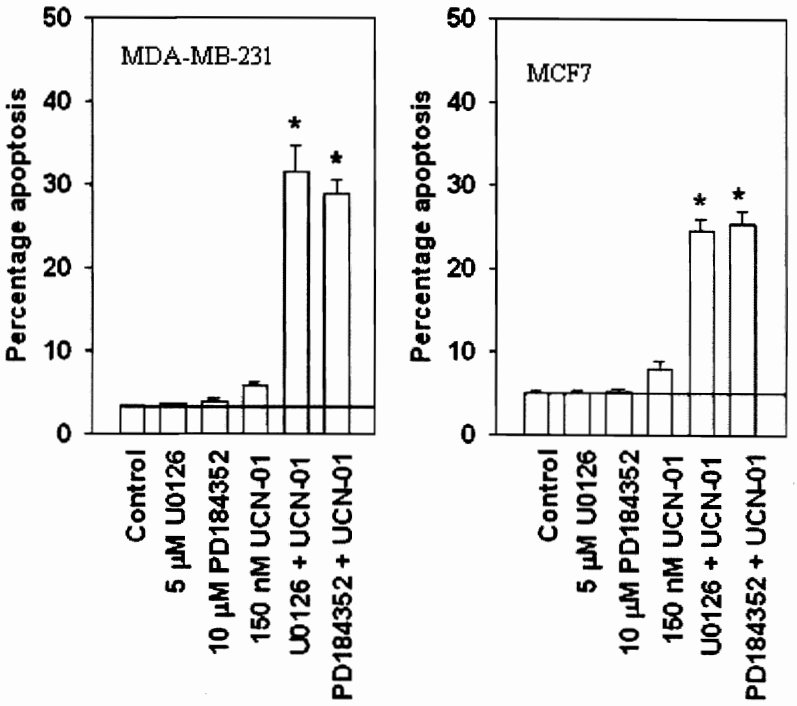

MDA-MB-231
MCF7
Percentage apoptosis
0
10
20
30
40
50
*
Control
5 μM U0126
10 μM PD184352
150 nM UCN-01
U0126 + UCN-01
PD184352 + UCN-01

Figure 5

(Figure 6): Pro-Caspase Cleavage and Flow Cytometry Data. Left panel: MEK1/2 (PD-98059, 25uM) inhibition interacts synergistically with UCN-01 (150 nM) to promote cell death in mammary carcinoma cells (MDA-MB231) that is blocked by inhibition of caspase 9 (LEHD, 20 μM) and caspase 8 (IETD, 20 μM) functions. Inset panel: Immunoblotting of the cleavage/integrity status of pro-caspase 3, pro-caspase 8, pro-caspase 9 and conformational change (increase expression) in BAX (after prior immunprecipitation), 24 hours after treatment. Right panel: MDA-MB231 cells were stained with propidium iodide and flow cytometric analysis was used to assess cell cycle progression and the percentage of sub-G1 DNA fragment content. Percentage of apoptotic cells was determined after 48 hours of treatment with either vehicle (DMSO), 10 μM PD 184352, 150nM UCN-01 or the drug combination. From (McKinstry et al., 2002)

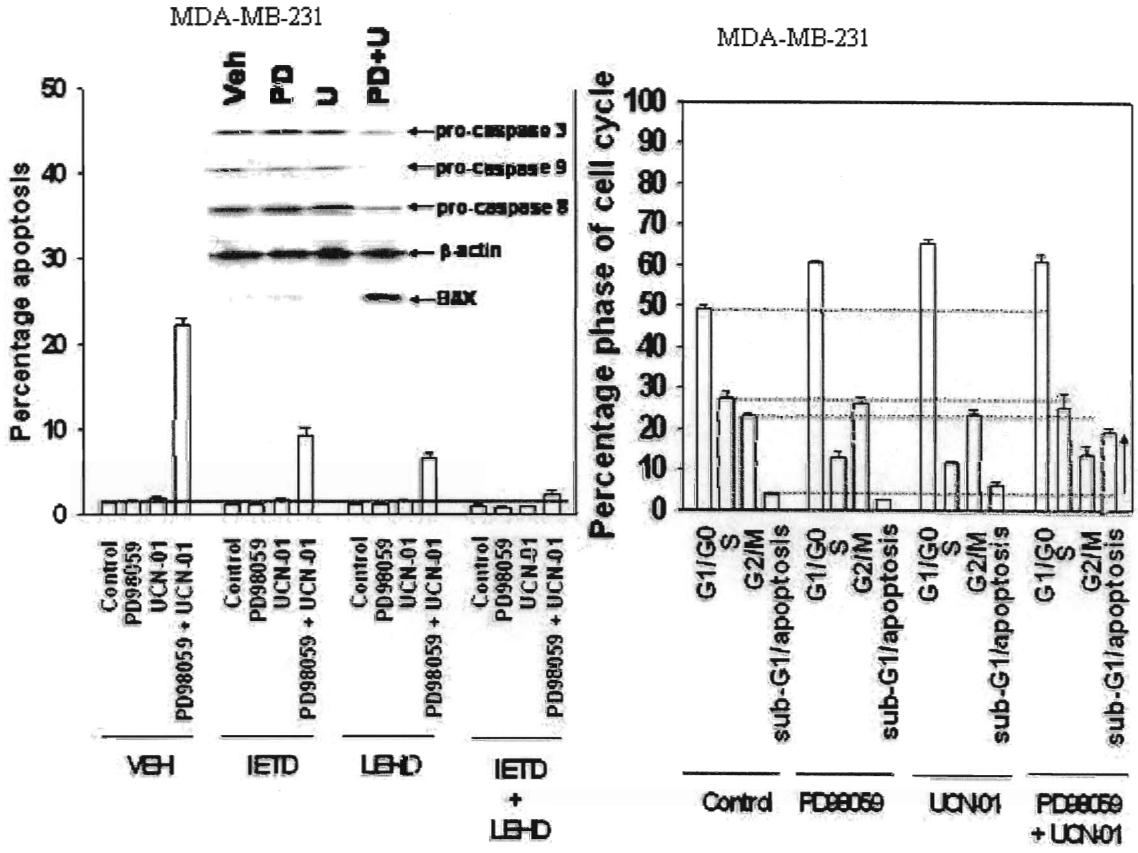

MDA-MB-231
Veh
PD
U
PD+U
pro-caspase 3
pro-caspase 9
pro-caspase 8
β-actin
Bax
Percentage apoptosis
Control
PD98059
UCN-01
PD98059 + UCN-01
VEH
IETD
LEHD
IETD + LEHD
MDA-MB-231
Percentage phase of cell cycle
G1/G0
S
G2/M
sub-G1/apoptosis
Control
PD98059
UCN01
PD98059 + UCN01

Figure 6.

(Figure 7): UCN-01 and MEK1/2 Inhibition Does Not Effect Non-transformed Cells. Primary cell types were cultured as described by McKinstry et al, 2002. Cells were isolated by trypsinization, fixed to glass slides by cytospin and stained for double stranded DNA breaks using a TUNEL assay kit in triplicate from two separate experiments. . Percentage of apoptotic cells was determined after 48 hours of treatment with either vehicle (DMSO), 10 μM PD 184352, 150nM UCN-01 or the drug combination. From (McKinstry et al., 2002).

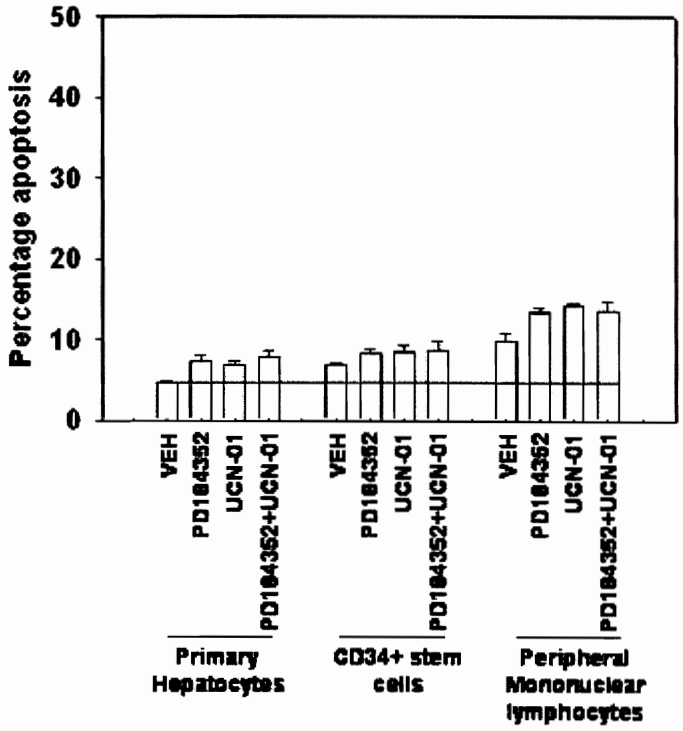

Percentage apoptosis
50
40
30
20
10
0
VEH
PD184352
UCN-01
PD184352+UCN-01
VEH
PD184352
UCN-01
PD184352+UCN-01
VEH
PD184352
UCN-01
PD184352+UCN-01
Primary Hepatocytes
CD34+ stem cells
Peripheral Mononuclear lymphocytes

Figure 7.

(Figure 8): In Vivo Effect of UCN-01 and MEK1/2 Inhibition on MDA-MB-231. PD184352 and UCN-01 combine to suppress the growth of established estrogen-independent MDA-MB-231 mammary carcinoma tumors in a greater than additive fashion. MDA-MB-231 xenografts containing 10 million cells in 50 µl of PBS were injected subcutaneously into the rear flank of female athymic nude mice; tumor take rate was over 90%.
Tumors were allowed to form for 20 days. Animals with palpable tumors (~100 mm^3) were divided into treatment groups with equal tumor distribution volume within each group (+/-) 10%. Animals were injected with vehicle, PD-184352, UCN-01 or the drug combination for two days as described in the Methods section. Tumor volume was determined by caliper measurement in millimeters and calculated as [(width^2 x length)/2] x 1000 to yield volume in cm^3. The mean tumor volume for all animals for each treatment condition was plotted (+/-) SEM, n=6 for experimental conditions and n=5 for vehicle control. Data are from a representative of three independent studies.

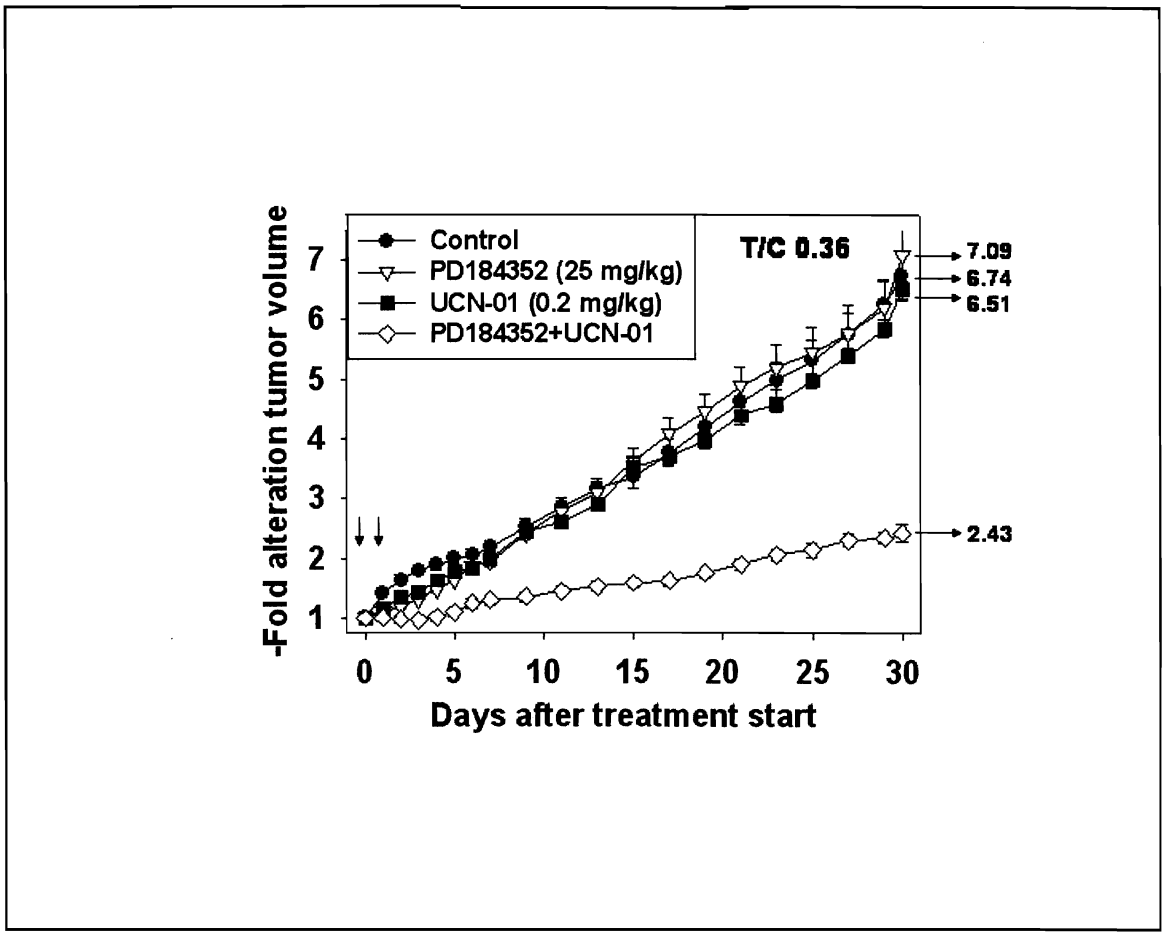

Control
PD184352 (25 mg/kg)
UCN-01 (0.2 mg/kg)
PD184352+UCN-01
T/C 0.36
7.09
6.74
6.51
2.43
-Fold alteration tumor volume
Days after treatment start
0
5
10
15
20
25
30
1
2
3
4
5
6
7

Figure 8.

(Figure 9): In Vivo Effect of UCN-01 and MEK1/2 Inhibition on MCF-7. PD184352 and UCN-01 combine to suppress the growth of established estrogen-dependent MCF7 mammary carcinoma tumors in a greater than additive fashion. Estrogen pellets (0.72mg/pellet/90-day release) were implanted in the sub scapular midline of female athymic mice. One week after implantation, MCF7 xenografts containing 5 million cells in 100 ul of reduced growth factor Matrigel were injected subcutaneously into the rear flank of the animal; tumor take rate was 60%.
Tumors were allowed to form for 30 days. Animals with palpable tumors (~150 mm^3) were divided into groups of eight with equal tumor distribution volume within each group (+/-) 10%. Animals were injected with vehicle, PD-184352, UCN-01 or the drug combination for two days as described in the Methods section. Tumor volume was determined by caliper measurement in millimeters and calculated as [(width^2 x length)/2] x 1000 to yield volume in cm^3. The mean tumor volume for all animals for each treatment condition was plotted (+/-) SEM, n=8. Data are from a representative of three independent studies.

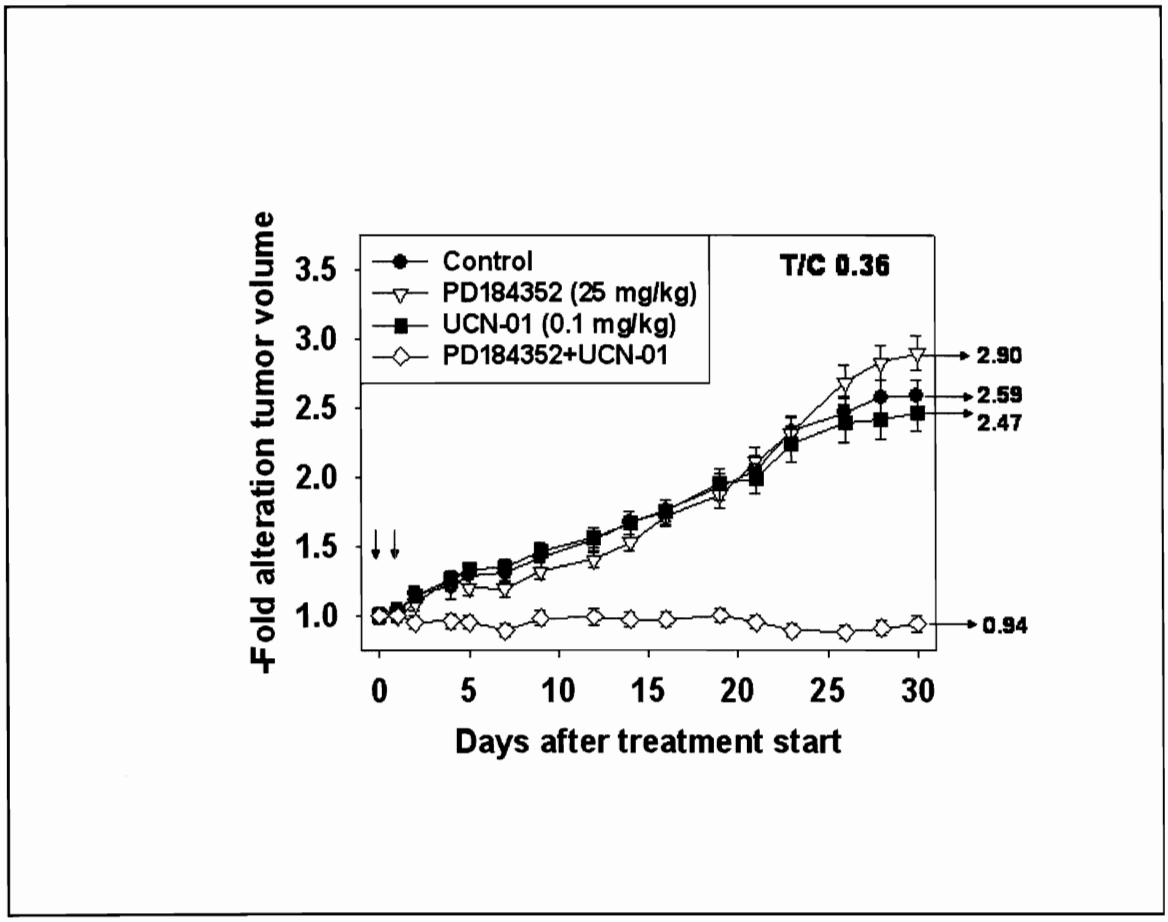

Fold alteration tumor volume
3.5
3.0
2.5
2.0
1.5
1.0
Control
PD184352 (25 mg/kg)
UCN-01 (0.1 mg/kg)
PD184352+UCN-01
T/C 0.36
2.90
2.59
2.47
0.94
0
5
10
15
20
25
30
Days after treatment start

Figure 9.

(Figure 10): Colony Formation Assay, Immunohistochemistry and H & E staining of MCF-7 tumors. PD-184352 and UCN-01 combine to suppress the cellularity of established estrogen-dependent MCF7 mammary carcinoma tumors 30 days post drug treatment which correlates with the suppression of ex vivo colony formation. One half was macerated and digested to obtain single cells that were plated in vitro to determine ex-vivo colony formation and the other half was fixed for paraffin embedding and subsequent immunohistochemistry. Immunohistochemistry results from 10 uM tumor sections for H & E determination of tumor morphology and cellularity, and Ki67 and CD31 immunoreactivity. Data are representative of images from three independent taken at 60X magnification except were noted using an Olympus microscope.

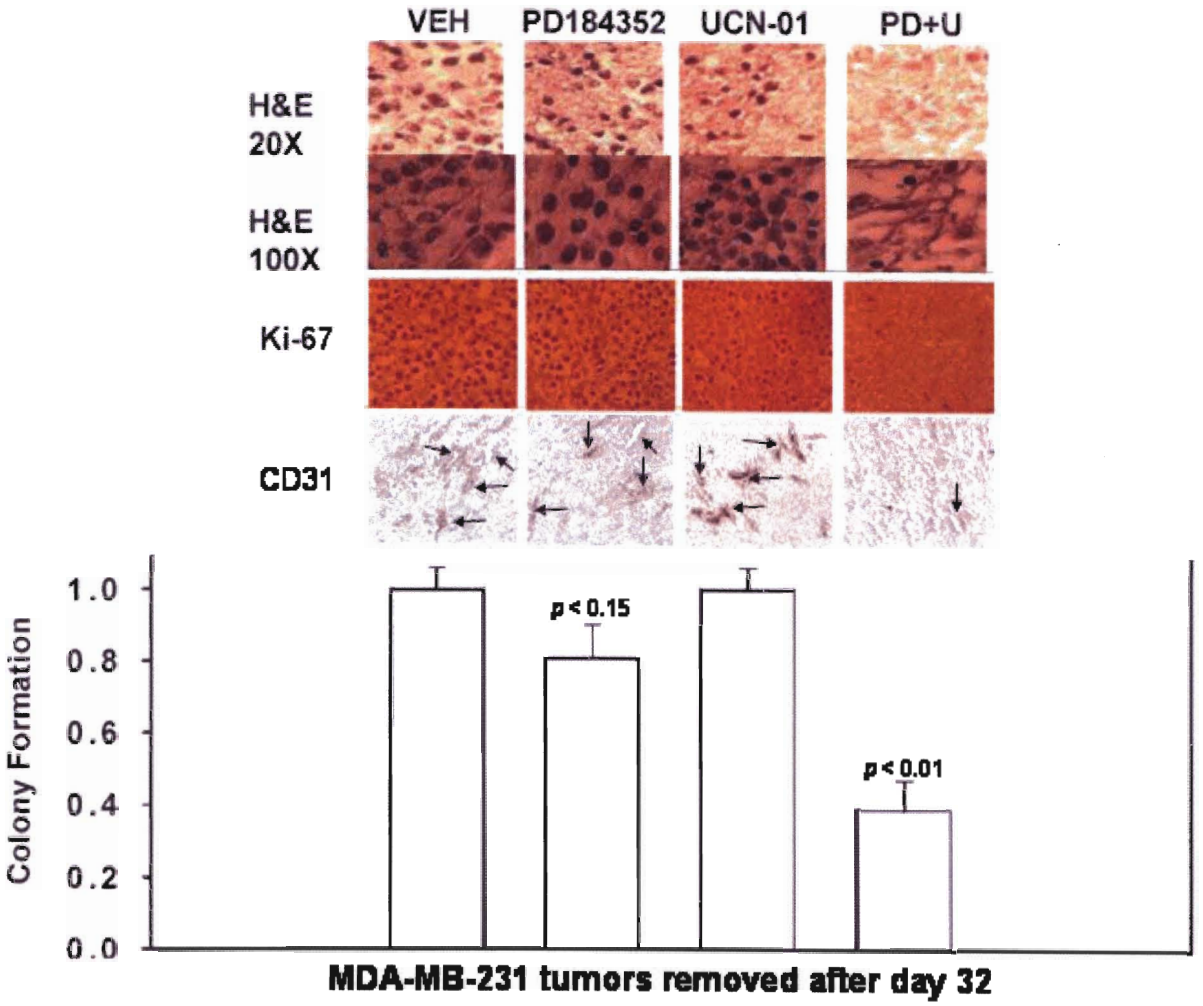

VEH
PD184352
UCN-01
PD+U
H&E 20X
H&E 100X
Ki-67
CD31
Colony Formation
1.0
0.8
0.6
0.4
0.2
0.0
p < 0.15
p < 0.01
MDA-MB-231 tumors removed after day 32

Figure 11.

(Figure 12a): Tumor morphology, Days 1-5. Combined PD 184352 and UCN-01 exposure causes a rapid induction of MDA-MB-231 cell death within two days of drug treatment in flank tumors. Tumors were recovered daily from animals 1-5 days after being treated with vehicle, PD-184352, UCN-01 or the drug combination. Isolated tumors were fixed and 10 μM sections taken to determine: (A) H&E staining for tumor morphology and cellularity for days 1-3.

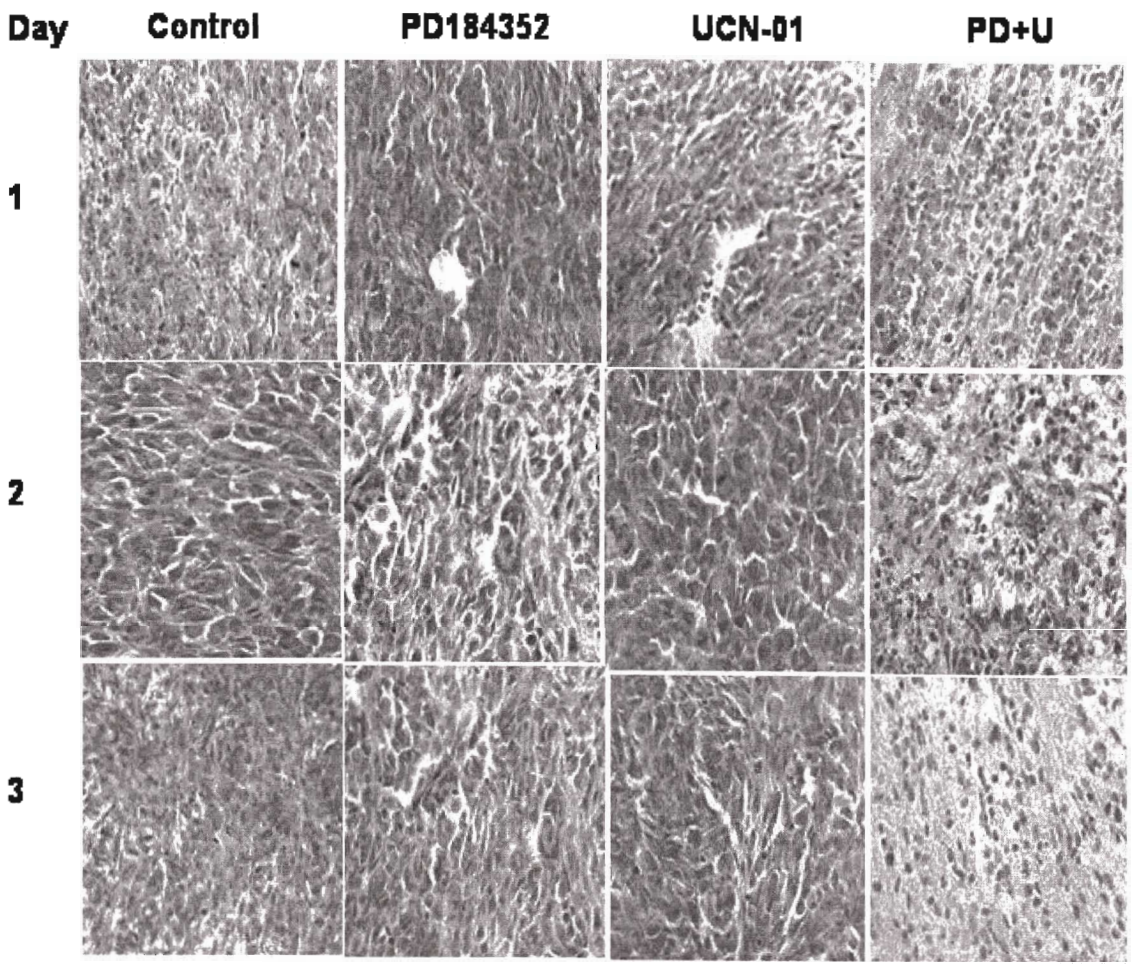


Figure 12a.

(Figure 12b): Tumor morphology, Days 1-5. Combined PD 184352 and UCN-01 exposure causes a rapid induction of MDA-MB-231 cell death within two days of drug treatment in flank tumors. Tumors were recovered daily from animals 1-5 days after being treated with vehicle, PD-184352, UCN-01 or the drug combination. Isolated tumors were fixed and 10 μM sections taken to determine: (B) H&E staining at 60X for tumor morphology and cellularity for days 4-5.

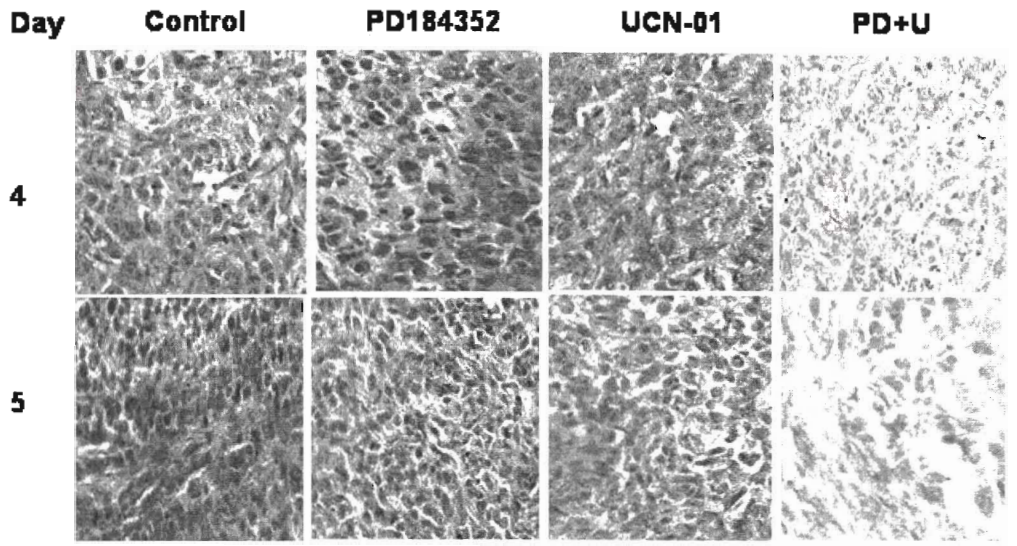


Figure12b.

(12c): Immunohistochemical staining for ERK1/2 phosphorylation, Days 1-5. Combined PD 184352 and UCN-01 exposure causes a rapid induction of MDA-MB-231 cell death within two days of drug treatment in flank tumors. Tumors were recovered daily from animals 1-5 days after being treated with vehicle, PD-184352, UCN-01 or the drug combination. Isolated tumors were fixed and 10 μM sections taken to determine: (C) Immunohistochemical staining for ERK1/2 phosphorylation at 10X.

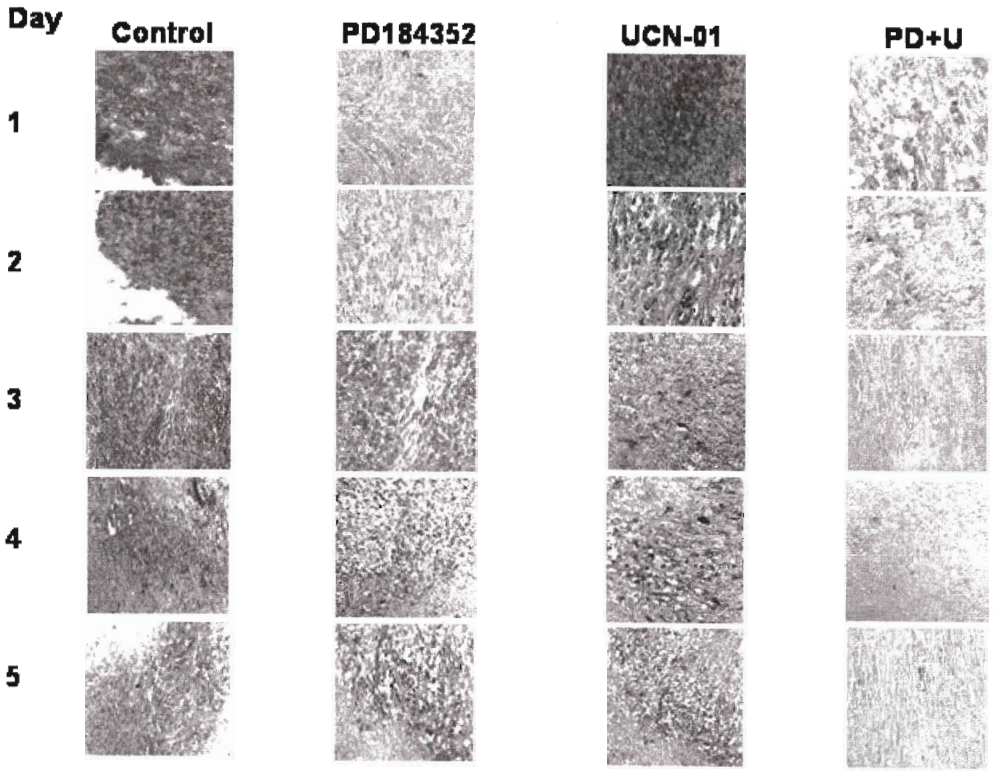


Figure 12c.

(12d-e), (D): Immunohistochemical staining shows activated ERK1/2 nuclear translocation Combined PD 184352 and UCN-01 exposure causes a rapid induction of MDA-MB-231 cell death within two days of drug treatment in flank tumors. Tumors were recovered daily from animals 1-5 days after being treated with vehicle, PD-184352, UCN-01 or the drug combination. Isolated tumors were fixed and 10 μM sections taken to determine: (D) Immunohistochemical staining for ERK1/2 phosphorylation after exposure to UCN-01 from a control image, a day 1 image at 40X and a day 3 image at 20X.
(E): Immunohistochemical staining for CD31 reactivity to indicate the presence or absence of endothelial cells. Images except where indicated taken at 20X using an Olympus microscope.

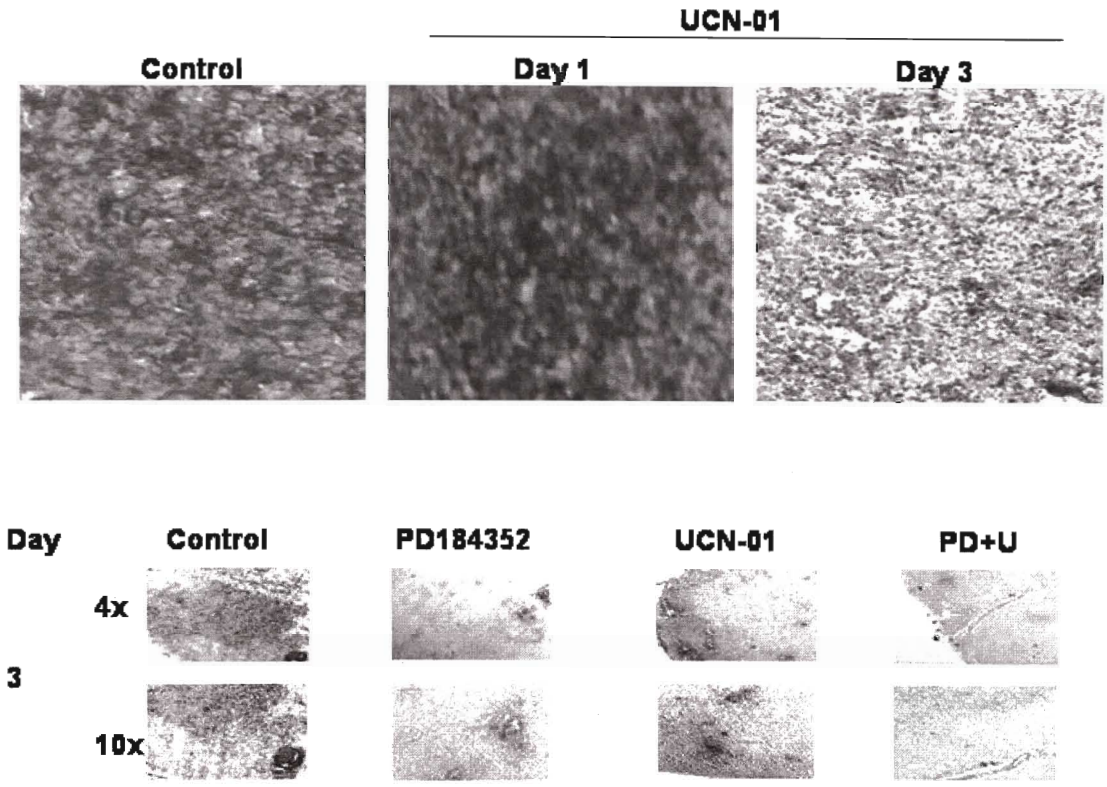

UCN-01
Control
Day 1
Day 3
Day
Control
PD184352
UCN-01
PD+U
4x
3
10x

Figure 12d-e.

(Figure 13a): H&E staining for tumor morphology, Days 5-20. Combined PD 184352 and UCN-01 exposure causes a prolonged reduction of MDA-MB-231 cellularity and ERK1/2 phosphorylation that is maintained for over 20 days post-drug treatment. Tumors were recovered from animals 5-20 days after being treated with vehicle, PD-184352, UCN-01 or the drug combination for 2 days. Isolated tumors were fixed and 10 μM sections taken to determine: (A) H&E staining for tumor morphology and cellularity for days 5-20. Images except where indicated taken at 60X using an Olympus microscope.

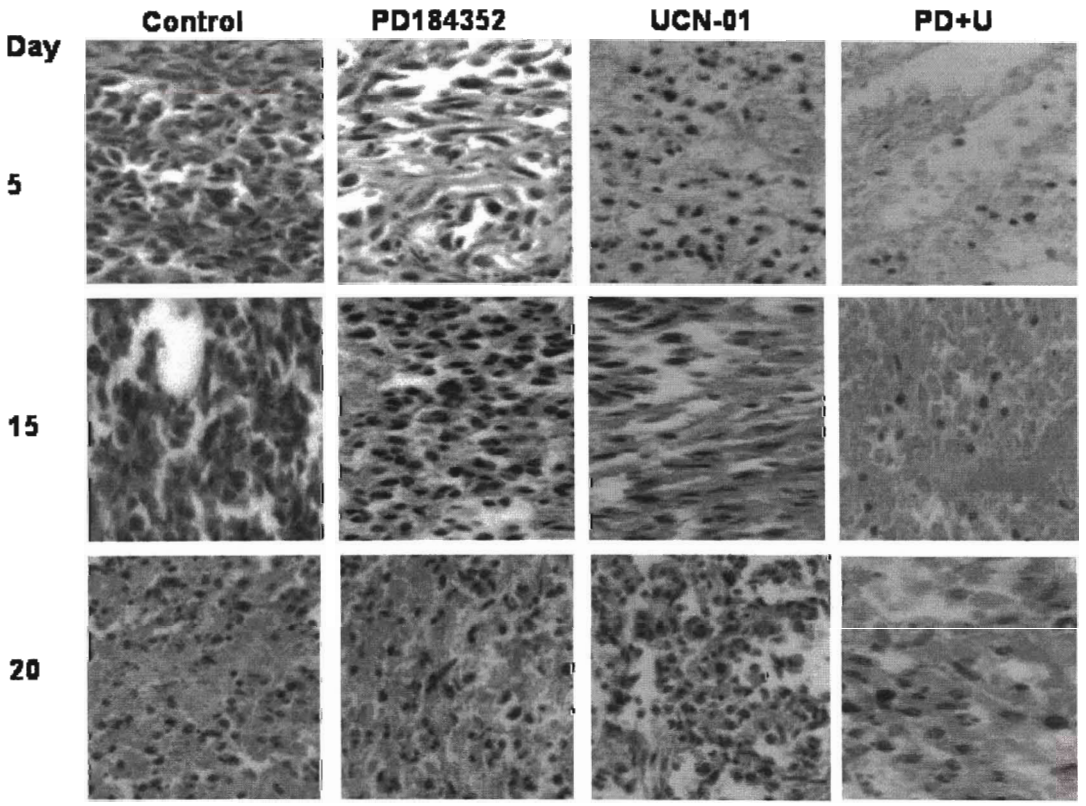


Figure 13a.

(Figure 13b): Immunohistochemical staining for ERK1/2 phosphorylation, Days 5-20. Combined PD 184352 and UCN-01 exposure causes a prolonged reduction of MDA-MB-231 cellularity and ERK1/2 phosphorylation that is maintained for over 20 days post-drug treatment. Tumors were recovered from animals 5-20 days after being treated with vehicle, PD-184352, UCN-01 or the drug combination for 2 days. Isolated tumors were fixed and 10 μM sections taken to determine: (B) Immunohistochemical staining for ERK1/2 phosphorylation for days 5-20. Images except where indicated taken at 60X using an Olympus microscope.

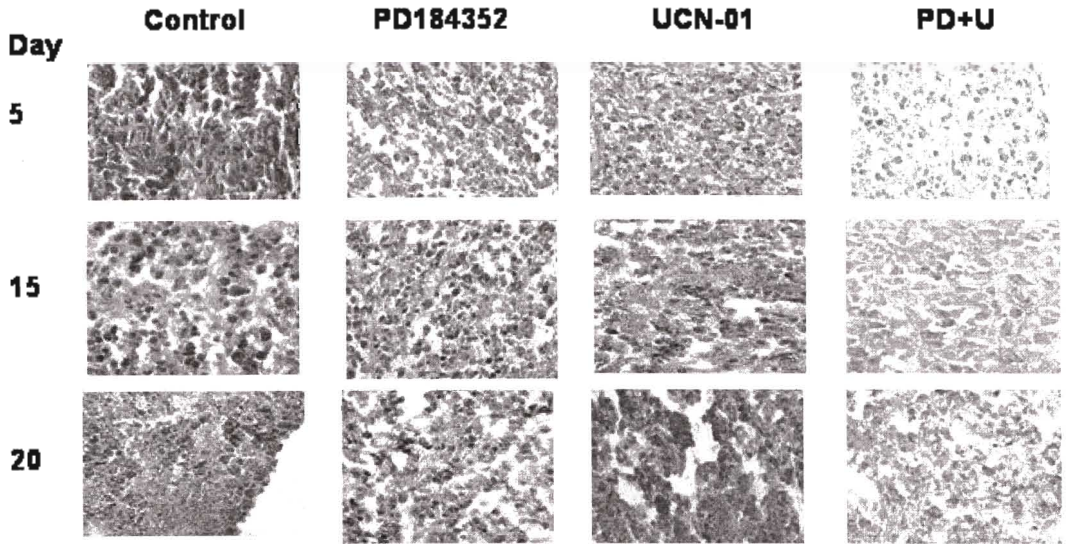


Figure 13b.

(Figure 14a-b): (A) Immunohistochemical staining for CD-31 reveals cross section of tumor vasculature i.e. arterioles and venules from animals treated with Vehicle control, PD-184352 or UCN-01 at 10X. But not in animals treated with the drug combination. (B) Both MCF7 cells and MDA-MB-231 tumor bearing animals treated with UCN-01 showed promotion of ERK1/2 phosphorylation up to 30 days following exposure at 10X but was abolished in tumors treated with the UCN-01 and MEK1/2 inhibitor drug combination.

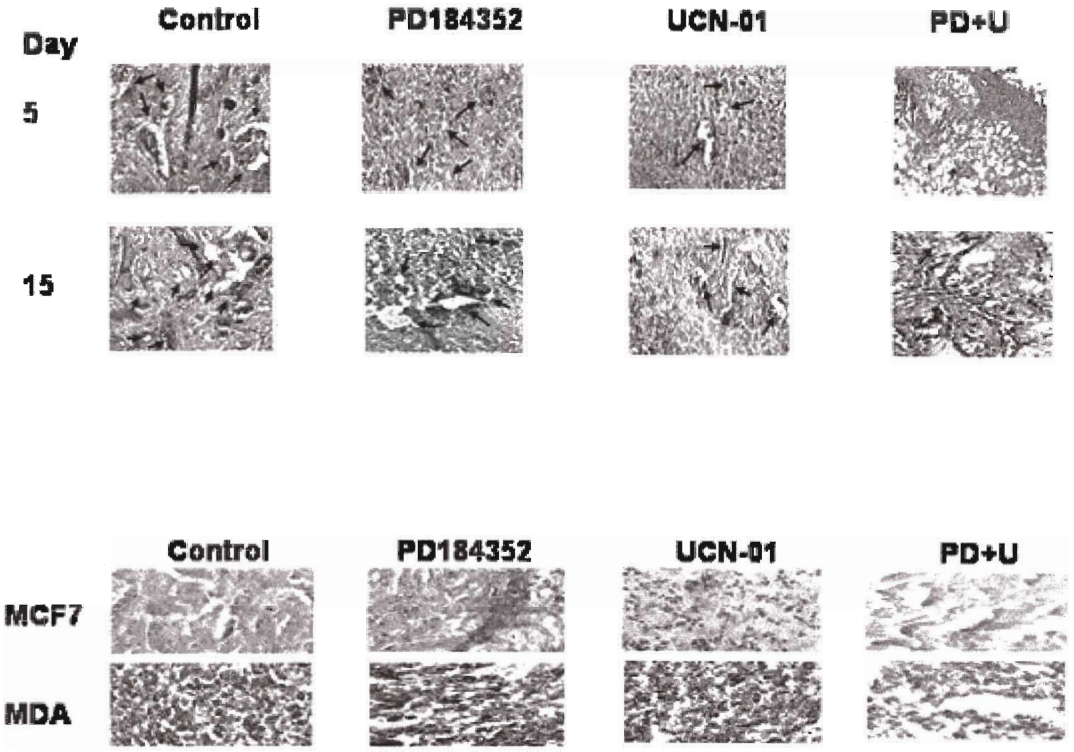

Day
Control
PD184352
UCN-01
PD+U
5
15
Control
PD184352
UCN-01
PD+U
MCF7
MDA

Figure 14a-b.

(Figure 15.): 2 Gy IR and PD183452/UCN-01 combination. The combination of 2Gy ionizing radiation with PD-184352 and UCN-01 suppress the growth of estrogen independent MDA-MB-231 mammary carcinoma tumors to greater extent than the combination of PD-184352 and UCN-01 alone.

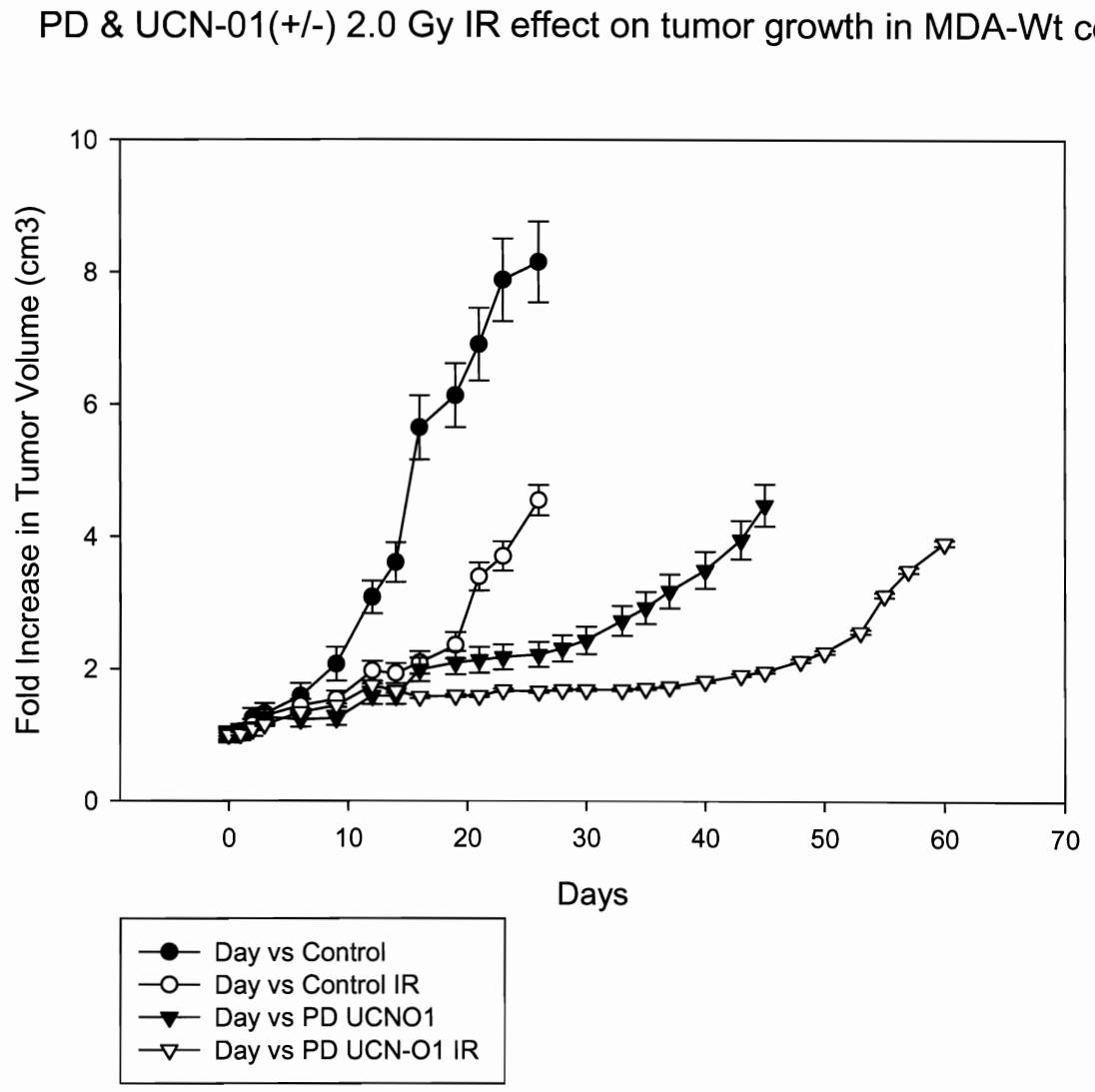

PD & UCN-01(+/-) 2.0 Gy IR effect on tumor growth in MDA-Wt cells
Fold Increase in Tumor Volume (cm3)
0
2
4
6
8
10
0
10
20
30
40
50
60
70
Days
Day vs Control
Day vs Control IR
Day vs PD UCNO1
Day vs PD UCN-O1 IR

Figure 15.

(Figure 16.): Farnesyl transferase inhibition combined with UCN-01. Farnesyl transferase inhibition by FTI-277, combined with UCN-01 suppresses the growth of estrogen independent MDA-MB-231 tumors in a greater than additive fashion.

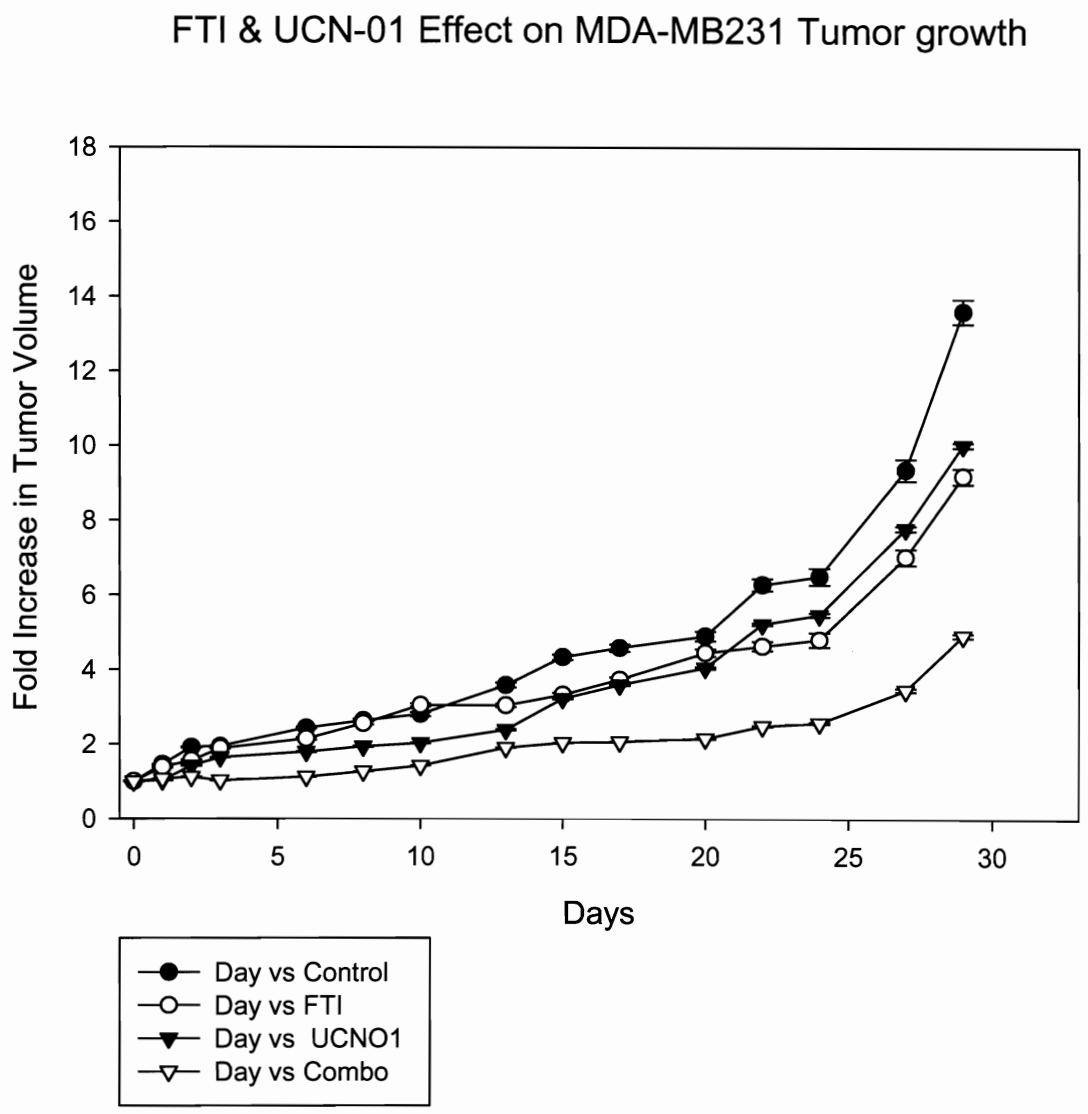

FTI & UCN-01 Effect on MDA-MB231 Tumor growth
Fold Increase in Tumor Volume
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
Days
Day vs Control
Day vs FTI
Day vs UCNO1
Day vs Combo

Figure 16.

3.5 In Vitro prostate cell line Studies with Radiation, HCG and lovastatin.

3.5.1 Studies with Radiation and HCG.

A previous report demonstrated evidence for the radiosensitization of the estrogen dependent MCF-7 breast cell line by hCG (Pond-Tor et al, 2002).The results in (Figure 17): IR Dose-response Effect of hCG Induced Radiosensitization of LnCap. , clearly demonstrate a significant dose-response effect of increasing doses of radiation on pre-treated LnCap cell viability with 2.0 mU/ml hCG as compared to vehicle control after 96 hour exposure. This result supports earlier reports of the potential for hCG induced radio-sensitization in cancer cells. Our investigation into enhanced cell death by the combined hCG and radiation treatment prompted us to explore which apoptotic mechanisms were involved. In (Figure 18): HCG and Radiation Induced Apoptosis, it is evident that pre-treatment of LnCap cells with hCG or vehicle alone resulted in approximately 17 % and 7 % cell death respectively. When used in combination with 4 Gy the percentage of cell death increased substantially to around 40% for hCG treated cells and 17 % for vehicle control.

To determine the mechanism of this hCG induced enhancement of cell death by radiation we used the caspase 8 inhibitor (IETD), caspase-9 inhibitor (LEHD) and the pan caspase inhibitor zVAD. Results form these experiments demonstrate that caspase-8 inhibition had an insignificant effect on cell death when compared to hCG and radiation combined.

But caspase-9 and pan caspase inhibition showed a decrease in cell death by approximately 25% for LEHD (50μM) pre-treated and 35% for zVAD (50μM) pre-treated cells when compared to the hCG and radiation combination. Additionally, lack of effect by IETD and the reduction in cell death by both LEHD and zVAD suggest that the "intrinsic" apoptotic pathway is at least partially responsible for hCG enhanced radiosensitization of LnCap cells.

Numerous reports as discussed above have shown that radiation can activate EGFR signaling pathways. Our investigation focused on the potential for hCG to induce EGFR activation in androgen receptor positive prostate carcinoma cell lines such as LnCap. To investigate this idea, we performed analysis for ERBB1 activity in LnCap cells treated in vitro with the MEK1/2 inhibitor PD184352 and subjected them to the hCG/combination radiation treatment. (Figure 19): ERBB1 Activity in LnCap Cells Treated with the MEK1/2 Inhibitor PD184352, demonstrated that in both vehicle and PD184352 treatment alone, little activation of ERBB1 occurred, indicated by p-ERBB1 immunoprecipitation. Treatment with hCG alone resulted in a significant level of ERBB1 activity; however when PD183452 was added to the hCG treated cells, the ERBB1 activity level dropped to that of vehicle control indicating that ERBB1 response is trans-activated by hCG. When 4 Gy radiation was combined with either vehicle or hCG, p-ERBB1 levels increased substantially. The addition of PD183452 to 4 Gy treated cells diminished p-ERBB1

levels when compared to hCG and 4 Gy treatment. In the hCG, PD184352 and 4 Gy radiation set, MEK1/2 inhibition was able to reduce the ERBB1 activity levels seen with treatment of both hCG and 4 Gy radiation, which accounted for the highest levels of p-ERBB1. With the hCG/4 Gy treatment condition showing the highest level of activity, which is attenuated by MEK1/2 inhibition, suggests that the hCG induced radiosensitization involves ERBB1 activation through a transactivation response. In (Figure 20): HCG-Induced Time-Dependent Transactivation, the hCG transactivation of ERBB1 is shown to occur in time-dependent manner. ERBB1 activity appears early after hCG stimulation at 5 minutes, and increases throughout the time course with a peak level of p-ERBB1 at 30 minutes. Basal level activity level is almost abolished by the tyrosine kinase inhibitor AG1478 when used alone, and the peak activity levels are significantly reduced when used in combination with hCG. The inhibition of hCG induced transactivation of ERBB1 by AG1478 demonstrates this process.

Further investigation into how this hCG induced radio-sensitization was occurring, explored the possibility that hCG stimulation resulted in the activation of PARP, (Figure 21): PARP Activation by hCG. Western blot analysis demonstrated that the combination of 4 Gy and hCG resulted in a high level of activated PARP well over that with either treatment condition alone. As indicated by the anti-body response (PARP 10H) against activated PARP, the levels of activated PARP seen with either 4 Gy alone or hCG

treatment were similar, with hCG providing a more enhanced effect. But when these levels were compared to the combination treatment, the combination effect was greater than additive or super-additive. This result strongly suggests that hCG induced radio-sensitization is in large part due to the observed hyper-activation of PARP. This hyper-activation ultimately results in the irreversible process of DNA fragmentation and cell death. To further test our findings, we used the PARP inhibitor PJ-34 (1 μM) to validate our results above (Figure 22): Inhibition of PARP Results in Decreased Cell Death. As was seen earlier, hCG induced-radiosensitization at 4 Gy resulted in a 2-fold increase in cell death when compared to hCG alone. In vehicle controls, radiation induced cell killing resulted in ~22% cell death while vehicle alone accounted for ~12% cell death. The addition of PJ-34 however produced a dramatic decrease in cell death with the hCG/4 Gy combination treatment. PJ-34 inhibited cell death to the extent that no significant differences were observed between the vehicle or hCG individual treatments or in combination with radiation. Therefore, the radio-sensitizing effect of hCG on LnCap cells was abolished by the PARP inhibitor PJ-34.

The decrease in PARP activation indicated by the results in (Figure 23): Inhibition of EGFR Results in PARP Accumulation, show that the activation/up-regulation of PARP by 4 Gy and hCG or the hyper-activation of PARP seen with the combination is largely

diminished in the presence of AG1478 at the same time-points corresponding to the peak levels of ERBB1 activation described earlier.

In (Figure 24): Metallo-protease Involvement in ERBB1 Trans-activation, the introduction of the metallo-protease inhibitor (GM6001) provided evidence that demonstrated G-protein induced metallo-proteases resulted in the transactivation of ERBB1 and pERK in response to hCG stimulation. Additionally in (Figure 25): Pertussis Toxin diminishes ERBB1 Induction; the hcG induced transactivation of ERBB1 was blocked by the G-protein inhibitor pertussis toxin. Providing more evidence that the hCG induced transactivation is occurring through a G-protein dependent activation. Furthermore in (Figure 26): Inhibition of G-protein Activation by Pertussis Toxin reduces activated ERK1/2 Levels, our laboratory demonstrated that pertussis toxin was able to diminish the hCG induced G-protein coupled transactivation by activated ERK in LnCap cells in vitro, linking hCG to ERBB receptor activation of downstream pathways.

3.5.2 In vitro studies of Human Chorionic Gonadotropin & Lovastatin

Investigations with lovastatin were undertaken to see if pharmacologically achievable lovastatin concentrations in combination with hCG could enhance the cell killing effect in prostate carcinoma cells as was demonstrated by our group in the hCG experiments on

LnCap. In (Figure 27): Dose-Reponse Effect of Lovastatin and hCG Combination, our initial dose-response studies in vitro, we observed a significant effect (2-fold difference) on cell survival when 0.6 μM Lovastatin was used in combination with hCG at both 1 and 2 mU/ml compared with lower concentrations of lovastatin. And this effect occurred in a dose-dependent fashion. In (Figure 28): Dose-Reponse Effect of Lovastatin and hCG Combination on 22RW1 cells. we evaluated this combined treatment on the 22RW1 cell line as well. In this study, we demonstrated a significant dose-response effect with the hCG and lovastatin combination over that of lovastatin alone. Treatment of 22RW1 with 0.6 μM lovastatin accounted for ~25% cell death, the combination of lovastatin and hCG at 2mU/ml resulted in a greater than 2-fold increase in cell death. Demonstrating that lovastatin enhances hCG-induced cell death in both LnCap and 22RW1 cell lines.

In (Figure 29): Cell Death Occurs via Activation of JNK and ERK1/2 illustrates a 6 hour time course after hCG stimulation in LnCap cells. Activated Erk1/2 occurs as an early response and remains relatively constant from 15 -60 minutes. At 3 hours, p-Erk1/2 levels have diminished considerably at which activated JNK1/2 levels are highest, indicating cell death. Activated Akt levels are also noticeably lower in hCG treated cells as compared with controls in the early (15-60 minutes) part of the time course. This result may indicate a decrease in Akt associated cytoprotection in LnCap cells. In the androgen independent cell line PC-3 (Figure 30): Reduced Akt Activation is Dose Dependent, p-

Erk1/2 levels are relatively constant at 96 hours but p-Akt levels decline in response to increasing doses of lovastatin in the presence of hCG. This decline in Akt associated cytoprotection suggests that lovastatin treatment can enhance cell death in PC-3 cells. This result suggests that lovastatin treatment can enhance cell death in PC-3 cells induced by hCG which involves the down regulation of Akt. In parallel (Figure 31): Enhanced Cell Death Occurs in a Dose-Dependent Fashion Involving Decreased Akt Expression, a similar dose-response experiment was performed in LnCap cells. These experiments show that increasing lovastatin and hCG concentration results in increased cell death 96 hours after exposure. Treatment with 2.0 mU/ml and 0.6 μM lovastatin resulted in ~70% cell death which amounts to a 40% increase in cell death over treatment using 1.0 mU/ml which resulted in ~50% cell killing. Additionally, both p-Erk levels declined with increasing doses of lovastatin used in combination with hCG as well as p-Akt levels.

Based on the previous experimental results, we wanted to determine if the mechanism by which lovastatin enhances hCG-induced cell lethality involving JNK, was linked to apoptosis. For the experiments shown in (Figure 32): Lovastatin and HCG induced Apoptosis Involves "Intrinsic Pathway". Pre-treatment with 50μM LEHD and zVAD followed by daily supplementation, significantly decreased LnCap cell death after exposure to lovastatin alone or in combination with hCG. A more pronounced effect was observed with the pan-caspase inhibitor, zVAD versus LEHD, which resulted in

diminished cell killing at the level of vehicle controls indicating the "intrinsic" apoptotic pathway is involved. The above result prompted us to further investigate the "intrinsic" apoptotic pathway. Results from our viral transduction studies in LnCap cells (Figure 33): Viral Mediated Evidence for "Intrinsic "Pathway Involvement., over-expressing either the anti-apoptotic protein BCL_{-XL} or dominant-negative caspase-9 (DN-caspase-9) or cytomegalovirus (CMV) vector control provided further evidence of "intrinsic" pathway involvement in hCG and lovastatin induced cell death. CMV expression alone did not suppress cell death in the drug combination treatment, and DN-caspase-9 expression resulted in significantly decreased cell killing compared with CMV. Over-expressing BCL_{-XL} resulted in profound inhibition of cell death when comparing the drug combination with vector control. Results shown in (Figure 34): PI3 Kinase Inhibition Enhances Cell Death Over that of AG1478, illustrates that ERBB1 inhibition by the tyrosine kinase inhibitor AG1478 is not enough to potentiate hCG induced cell death, but inhibition of PI3 kinase with LY294002 prevents the downstream activation of the cytoprotective Akt pathway, corresponding to down-regulation of Akt which further enhanced hCG induced cell death observed in Figures 29-31.

(Figure 17): IR Dose-Response Effect of hCG Induced Radiosensitization of LnCap. Radio-sensitization of LnCap prostate carcinoma cells due to treatment with 2.0 mU/ml hCG 30 minutes prior to exposure of increasing doses of radiation.

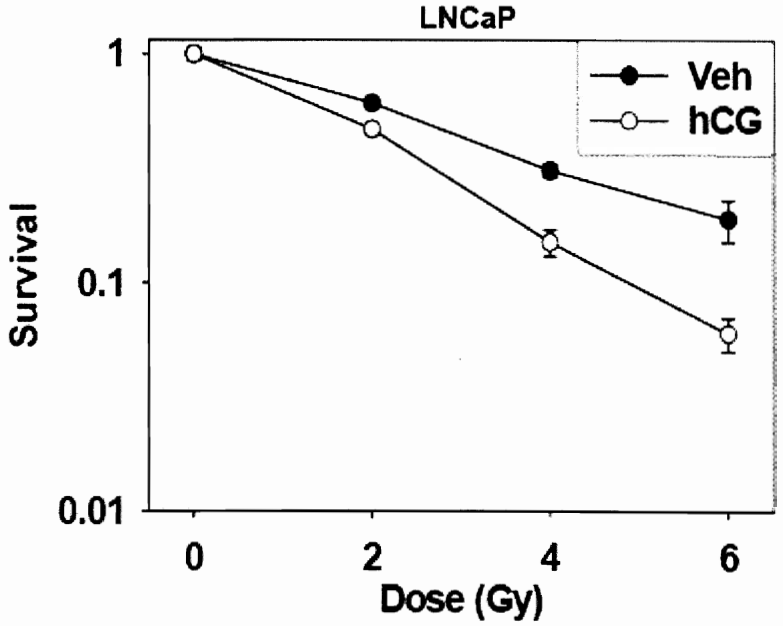


Figure 17

(Figure 18.): HCG and Radiation Induced Apoptosis. Cell death significantly decreased in response to pre-treatment with 50 μM LEHD, not IETD. Effect was further enhanced by pre-treatment with 50 μM zVAD, a known pan-caspase inhibitor. Cell death was determined by trypan blue exclusion method in which positive staining cells are identified as dead and clear cells are deemed viable.

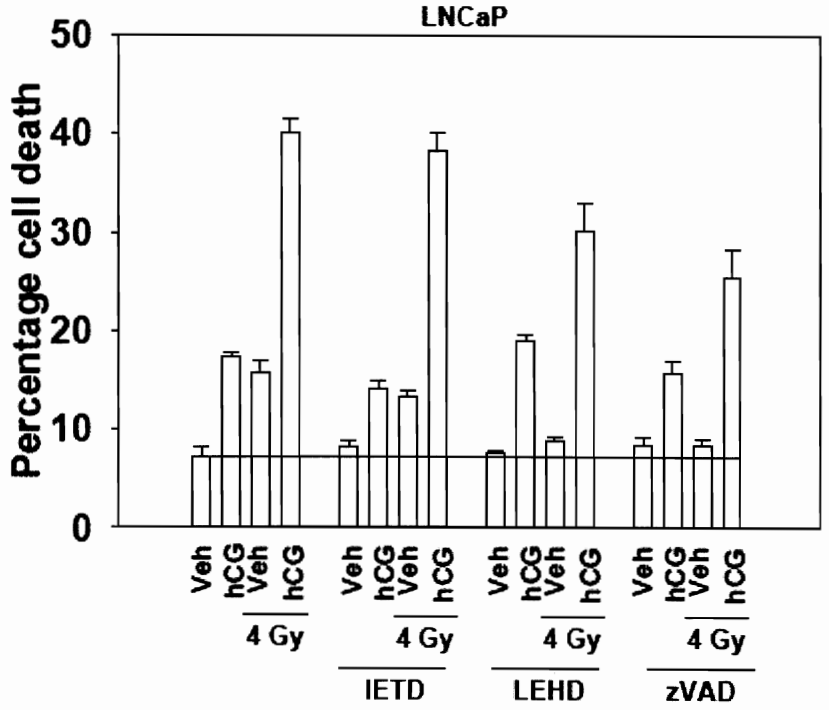

LNCaP
Percentage cell death
50
40
30
20
10
0
Veh
hCG
Veh
hCG
4 Gy
IETD
LEHD
zVAD

Figure 18.

(Figure 19.): ERBB1 Activity in LnCap Cells Treated with the MEK1/2 Inhibitor PD184352. Immunoprecipitation for ERBB1 activation enhanced by hCG and radiation. Reduction of ERBB1 activation by the MEK1/2 inhibitor PD184352 in hCG treated cells.

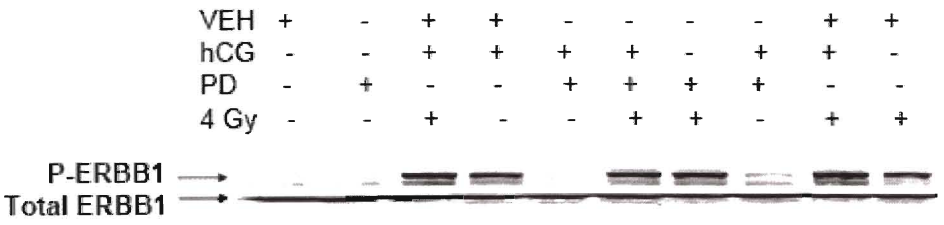


Figure 19.

(Figure 20.): HCG-Induced Time-Dependent Transactivation. The hCG induced time-dependent transactivation of ERBB1 peaks at 30 minutes and is blocked by the ERBB1 inhibitor AG1478.

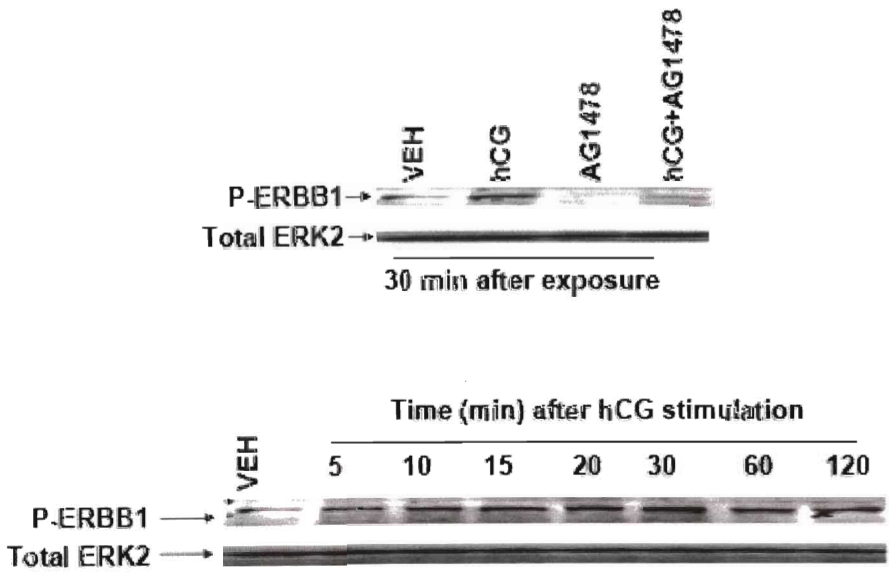

VEH
hCG
AG1478
hCG+AG1478
P-ERBB1
Total ERK2
30 min after exposure
Time (min) after hCG stimulation
VEH
5
10
15
20
30
60
120
P-ERBB1
Total ERK2

Figure 20.

(Figure 21): PARP Activation by hCG. 30 minute time point analysis of hCG and 4 Gy of LnCap prostate carcinoma cells shows evidence for transactivation occurring through the activation of PARP.

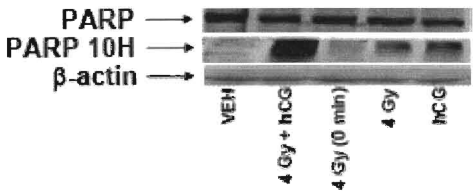


Figure 21.

(Figure 22): Inhibition of PARP Results in Decreased Cell Death. Radiosensitization of LnCap prostate carcinoma cells by hCG is blocked by the PARP inhibitor PJ-34. Cell death was determined by trypan blue exclusion assay.

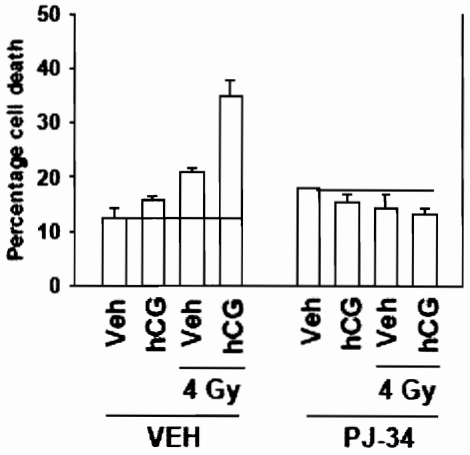

Percentage cell death
50
40
30
20
10
0
Veh
hCG
Veh
hCG
4 Gy
VEH
Veh
hCG
Veh
hCG
4 Gy
PJ-34

Figure 22.

(Figure 23.): Inhibition of EGFR Results in PARP Accumulation. AG1478 treatment results in accumulation of PARP in hCG and 4Gy combination treatment, which is otherwise activated by the hCG and 4 Gy radiation or their combination, suggests that ERBB1 is trans-activated by PARP.

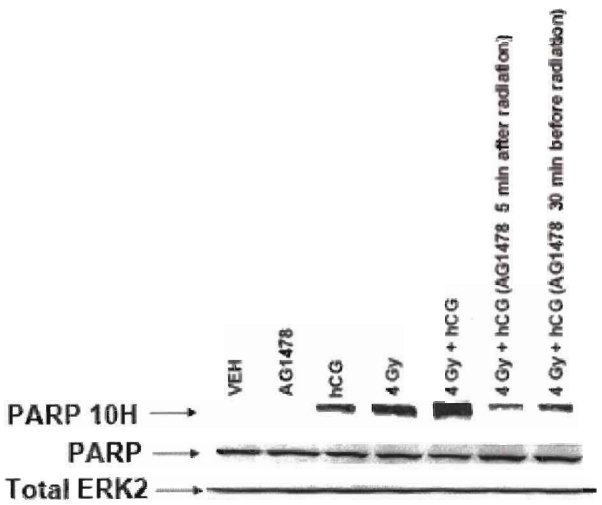

VEH
AG1478
hCG
4 Gy
4 Gy + hCG
4 Gy + hCG (AG1478 5 min after radiation)
4 Gy + hCG (AG1478 30 min before radiation)
PARP 10H
PARP
Total ERK2

Figure 23.

(Figure 24): Metallo-protease Involvement in ERBB1 Trans-activation. ERBB1 activation and corresponding ERK1/2 levels in LnCap prostate carcinoma cells are substantially diminished by the metallo-protease inhibitor GM6001.

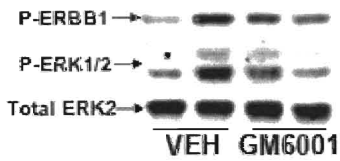


Figure 24.

(Figure 25): Pertussis Toxin diminishes ERBB1 Induction. The hCG induced transactivation of ERBB1 is blocked by the G-protein inhibitor pertussis toxin.

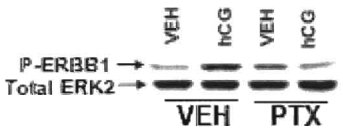


Figure 25.

(Figure 26): Inhibition of G-protein Activation by Pertussis Toxin reduces activated ERK1/2 Levels. Pertussis toxin (PTX) diminishes hCG- induced G-protein coupled transactivation indicated by decreased ERK activation in LnCap cells.

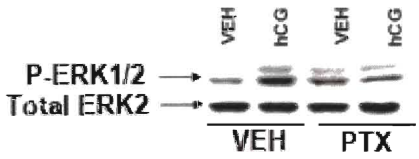


Figure 26

(Figure 27): Dose-Reponse Effect of Lovastatin and hCG Combination. Dose-response experiment showing decreased cell survival in LnCap prostate carcinoma cells comparing 1-2 mU/ml hCG and 0.0, 0.3 and 0.6 μM Lovastatin.

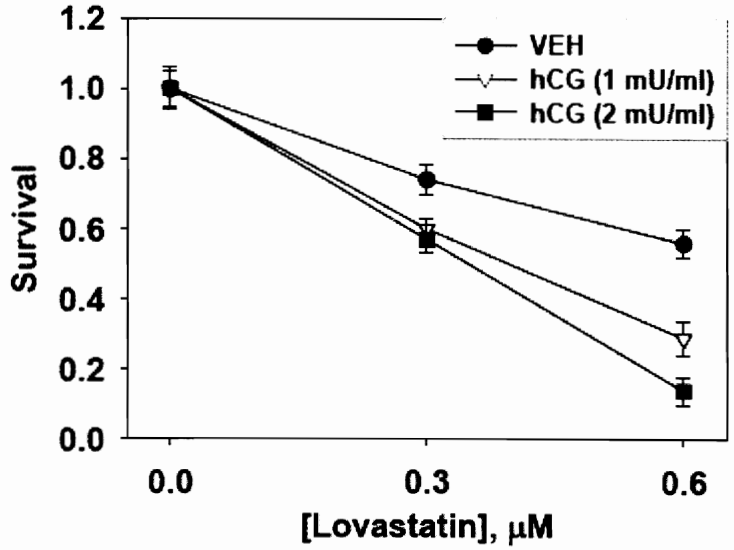

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Survival
VEH
hCG (1 mU/ml)
hCG (2 mU/ml)
0.0
0.3
0.6
[Lovastatin], μM

Figure 27.

(Figure 28): Dose-Reponse Effect of Lovastatin and hCG Combination on 22RW1 cells. Post-treatment effects for 96 hour time point of dose-response profile using 0.0, 0.3 and 0.6 μM Lovastatin and addition of 2.0 mU/ml hCG enhances 22RW1 prostate carcinoma cell death.

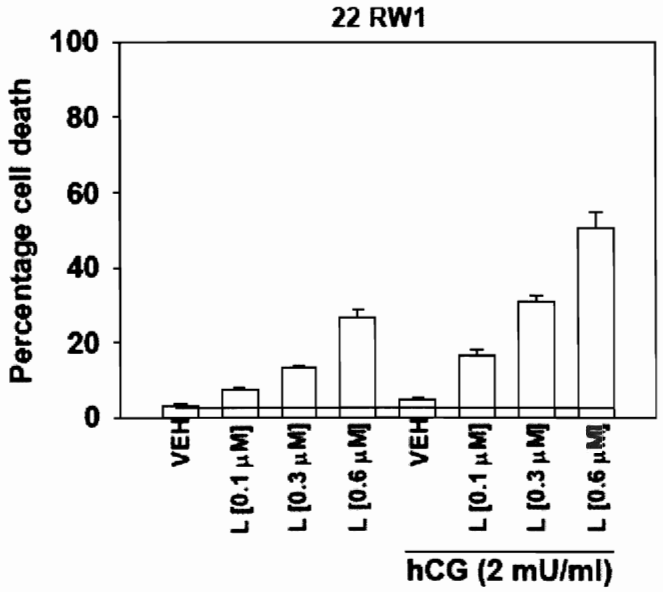

22 RW1
Percentage cell death
100
80
60
40
20
0
VEH
L [0.1 µM]
L [0.3 µM]
L [0.6 µM]
VEH
L [0.1 µM]
L [0.3 µM]
L [0.6 µM]
hCG (2 mU/ml)

Figure 28.

(Figure 29): Cell Death Occurs via Activation of JNK and ERK1/2. Western blot of six hour time course demonstrating hCG induced cell death in LnCap prostate carcinoma cells correlates with down-regulation of ERK1/2 activity and peak activation of JNK1/2 at 3 hours.

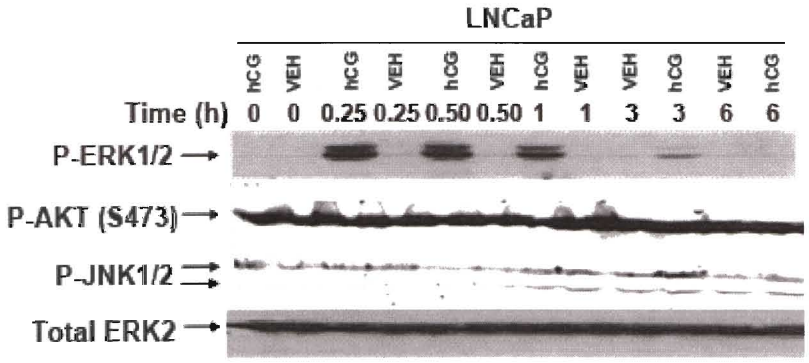

LNCaP
hCG VEH hCG VEH hCG VEH hCG VEH VEH hCG VEH hCG
Time (h) 0 0 0.25 0.25 0.50 0.50 1 1 3 3 6 6
P-ERK1/2
P-AKT (S473)
P-JNK1/2
Total ERK2

Figure 29.

(Figure 30): Reduced Akt Activation is Dose Dependent. Dose response experiment showing decreased cell survival in PC-3 prostate carcinoma cells comparing 1mU/ml hCG with 0.0, 0.3 and 1.0 μM Lovastatin. Inset: Immunoblotting for p-AKT and p-ERK shows ERK1/2 activity declining and AKT activation diminishing with increasing dose-response for lovastatin-hCG combination.

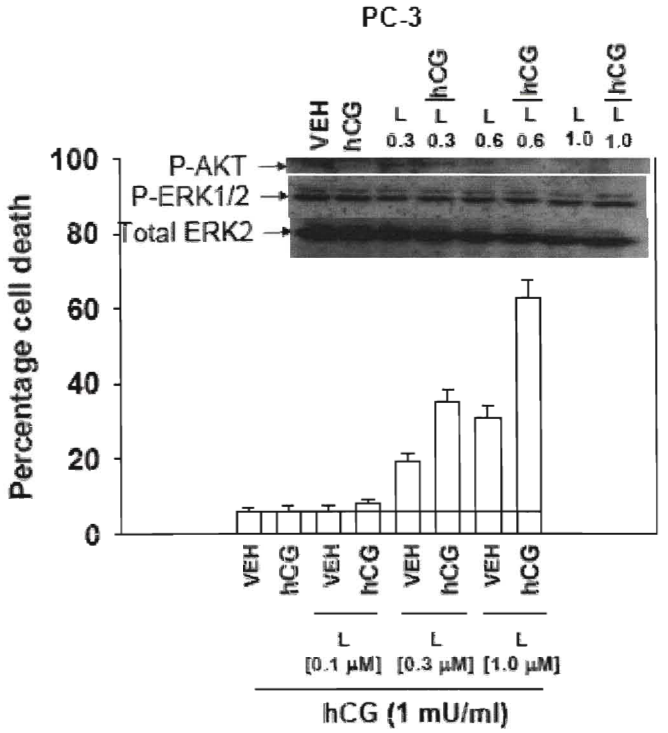

PC-3
VEH
hCG
L
L hCG
0.3
0.6
1.0
P-AKT
P-ERK1/2
Total ERK2
Percentage cell death
100
80
60
40
20
0
[0.1 μM]
[0.3 μM]
[1.0 μM]
hCG (1 mU/ml)

Figure 30.

(Figure 31): Enhanced Cell Death Occurs in a Dose-Dependent Fashion Involving Decreased Akt Expression. Dose response experiment showing decreased cell survival in LnCap prostate carcinoma cells comparing 1-2mU/ml hcG (+/-) 0.0, 0.3 and 0.6 μM Lovastatin. Inset: Immunoblotting for p-AKT and p-ERK show decrease ERK1/2 and AKT activation with increasing dose-response for lovastatin with hCG at 2.0 mU/ml. Cell death was determined by trypan blue exclusion assay.

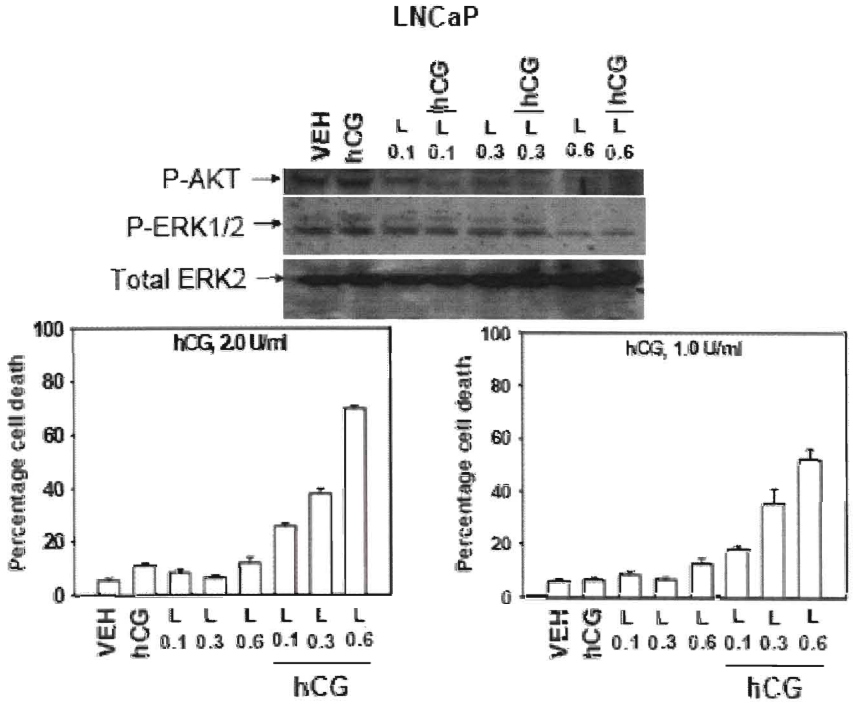

LNCaP
VEH
hCG
L
L/hCG
0.1
0.3
0.6
P-AKT
P-ERK1/2
Total ERK2
hCG, 2.0 U/ml
hCG, 1.0 U/ml
Percentage cell death
100
80
60
40
20
0
hCG

Figure 31.

(Figure 32): Lovastatin and hCG induced Apoptosis Involves "Intrinsic Pathway". Combination treatment with hcG and lovastatin induced cell death significantly decreased in response to pre-treatment with 50 μM LEHD, a caspase-9 inhibitor as compared to vehicle control. Effect was further enhanced by pre-treatment with 50 μM zVAD, a known pan-caspase inhibitor.

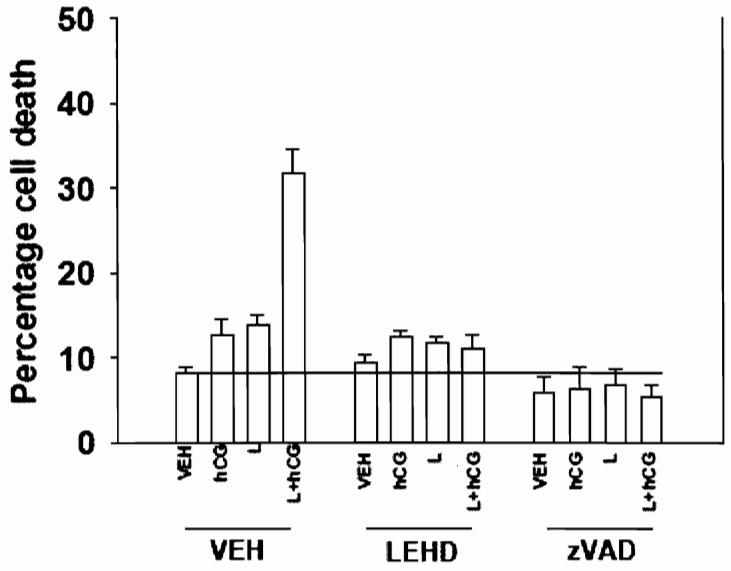

Percentage cell death
50
40
30
20
10
0
VEH
hCG
L
L+hCG
VEH
hCG
L
L+hCG
VEH
hCG
L
L+hCG
VEH
LEHD
zVAD

Figure 32.

Figure 33.) Viral Mediated Evidence for "Intrinsic "Pathway Involvement. Viral transduction of LnCap prostate carcinoma cells with either control CMV, over-expression of BCL-xl or DN-caspase-9 provides evidence for intrinsic pathway involvement.

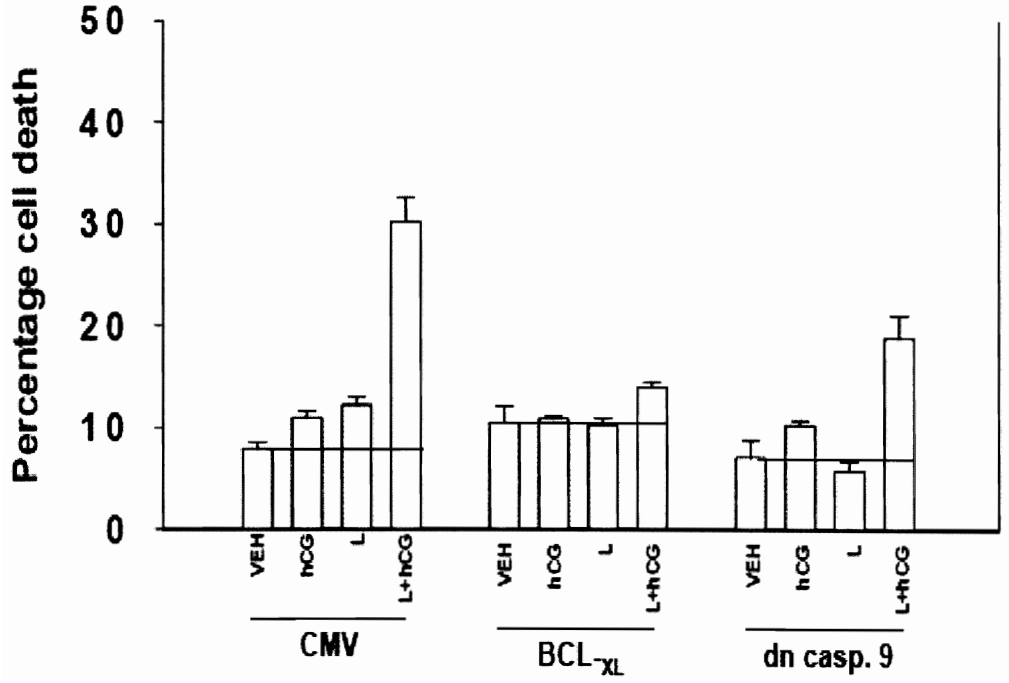

Percentage cell death
50
40
30
20
10
0
VEH
hCG
L
L+hCG
VEH
hCG
L
L+hCG
VEH
hCG
L
L+hCG
CMV
BCL-xL
dn casp. 9

Figure 33.

Figure 34. (Figure 34): PI3 Kinase Inhibition Enhances Cell Death Over that of AG1478. ERBB1 inhibition by AG1478 alone is not sufficient to potentiate hcG cell death in LnCap prostate carcinoma cells. However, inhibition of PI3 kinase with LY294002 prevents downstream activation of Akt which further potentiates hcG induced cell death in LnCap prostate and corresponds to down-regulation of Akt.

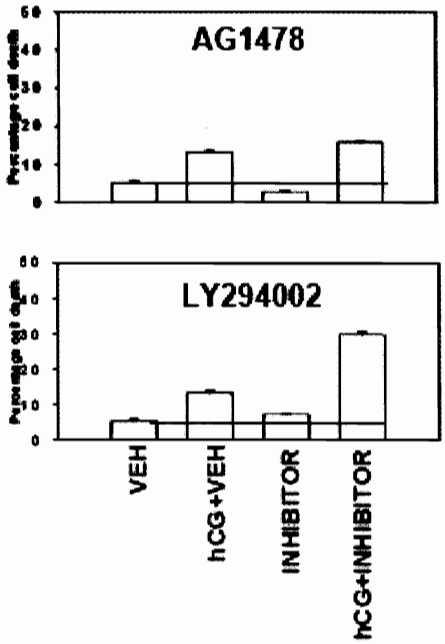


Figure 34.

Discussion

Combination PD183452 and UCN-01 In Vivo Studies

Previous experimental investigations by our group have found evidence that supports the idea that MEK1/2 inhibitors and UCN-01 interact in vitro to promote cell specific killing in a wide variety of malignancies including; breast cell carcinoma, prostate cell carcinoma and multiple hematological cell types and with further study can be successfully applied clinically. These studies were initiated to determine whether MEK1/2 inhibition combined with UCN-01 would interact to kill carcinoma cells in vivo.

As illustrated in the results section, the combined exposure of established mammary carcinoma tumors to MEK1/2 inhibitors and UCN-01 resulted in a substantially greater reduction in tumor growth than either drug administered separately. And that this reduction in tumor growth corresponded with cleavage of pro-caspase-3, a reduction in ERK1/2 phosphorylation and a prolonged suppression of Ki67 immunoreactivity. These observations also correspond with markedly reduced tumor cellularity and ex vivo colony formation, 30 days after cessation of drug treatment. Tumor angiogenesis was also disrupted by both the individual and combined MEK1/2 inhibitor and UCN-01 treatment as evidenced by diminished CD31 staining. Collectively, our findings demonstrate that the tumoricidal properties of the MEK1/2 and UCN-01 combination treatment observed

in our in vitro studies can be translated into several animal models using human xenografts of mammary breast cell carcinomas.

Our findings clearly demonstrate that UCN-01 and MEK1/2 inhibition potently inhibit mammary tumor cell growth in vivo and the effect is independent of p53 status, estrogen dependency, caspase-3 expression or oncogenic K-RAS expression.

The cytoprotective effect of MEK1/2-ERK1/2 pathway activation in tumor cells has been previously demonstrated to be a critical determinant of tumor cell survival (Dent et al, 2003 and Dent et al, 2002). Additionally these reports also reveal that the activation of this cell signaling cascade has been observed as a compensatory response of tumor cells to numerous environmental stresses including radiation and cytotoxic drugs. One of the more surprising and unanticipated findings in our present studies was the increase in phospho-ERK levels in MDA-MB-231 tumors treated with PD185342 and UCN-01 for 5-10 days following the drug treatment regimen. In the MCF7 tumors this UCN-01 mediated stimulation of ERK1/2 phosphorylation was observed for up to 30 days following drug treatment. Even though this increase in ERK1/2 phosphorylation had been observed by several other groups following exposure to UCN-01, a compensatory ERK1/2 response to MEK1/2 inhibition has not previously been reported (McKinstry et al, 2002, Dai et al, 2001 and Dai et al, 2002).

The absence of or diminished ERK1/2 phosphorylation in both MDA-MB-231 and MCF7 cell tumors treated with the combination of PD184352 and UCN-01 was not due to persistence of the MEK1/2 inhibitor in the animal tumors since PD184352 treated tumors showed both a suppression of phospho-ERK1/2 levels during drug exposure and then a sudden transient rebound in ERK1/2 phosphorylation above the staining intensity of control treated tumors 5-10 days following cessation of the drug treatment.

Additionally, the published pharmacokinetic data of UCN-01 generated in mice showing a murine plasma half-life of 4-7 hours, makes it unlikely that the UCN-01 administered in our MCF7 study protocol would have persisted as a pharmacologically active compound for 30 days to alter the phospho-ERK1/2 levels in this time-point (Patel et al, 2002). Therefore, our present findings are most consistent with the idea that tumor cells exposed to both PD184352 and UCN-01 were killed in an immediate fashion corresponding to the drug exposure and that following this combined treatment, these cells were incapable of a compensatory rebound of ERK1/2 pathway activation against either UCN-01 actions or MEK1/2 inhibition. This inability resulted in a prolonged suppression of tumor re-growth. As described earlier, a two day exposure of MCF7 tumor cells to UCN-01 elevated ERK1/2 phosphorylation for up to 30 days. In this study, our findings also

suggest that the transient exposure of mammary carcinoma cells to UCN-01 may modify the inherent tumor cell biology in vivo.

In addition to tumor cell killing the individual and combined treatment of MDA-MB-231 and MCF7 tumors with PD184352 and UCN-01 also reduced the CD31 immunoreactivity both during and shortly after drug exposure in the tumor itself. This suggests that either the endothelial cells within the tumor vasculature were directly killed or were being indirectly lost due to tumor cell death. It is also important to note, that the reduction in CD31 reactivity was greatest in tumors treated with PD184352 and UCN-01. It has also been reported that exposure to UCN-01 in vitro at clinically achievable drug concentrations suppresses angiogenesis although the mechanism for this observation has not yet been determined (Kruger et al, 1998-1999). And in vitro suppression of endothelial cell proliferation has also been observed using the MEK1/2 inhibitors (Giuliani et al, 2004). One potential explanation for this finding is that in a cell type dependent manner, inhibition of MEK1/2 can lead to the suppression of c-Jun and AP-1 dependent vascular endothelial growth factor (VEGF) promoter activity (Park et al, 2001). In our experimental system, the loss of CD31 immunoreactivity was maintained in tumors as long as 30 days which suggests that neo-angiogenesis following drug treatment had also been suppressed. Earlier findings in our laboratory have shown that non-transformed cell types are resistant to lethal effects of PD184352 and UCN-01 in vitro,

and additional studies are required to determine whether non-transformed endothelial cells within a tumor in vivo, are vulnerable to PD184352 and UCN-01 induced cell killing.

In other recent reports using UCN-01 or PD184352 as individual agents to treat animals, noticeably higher drug concentrations were used to demonstrate significant anti-tumor effects than the doses used in our studies. As an example, Patel et al., 2002, treated animals bearing head and neck tumors xenografts for five days with 7.5 mg/kg UCN-01 to achieve tumor regression. While Sebolt-Leopold et al., 1999 and McDaid et al., 2005, used PD184352 concentration in the 48-300 mg/kg range over a period of 14-20 days to achieve anti-tumor effects. A comparison between the doses used in the above referenced protocols and those used in our studies warrants consideration, in that they are important due to the plasma concentration levels of these drugs that are believed to be clinically achievable. The maximal concentration of bio-available or free drug concentration of UCN-01 in human plasma is reported to be at or below approximately 100 nM. While UCN-01 also exhibits a relatively long plasma half-life due it's binding to the human α-1-acidic glycoprotein. This is a considerably lower drug concentration level than that seen in rodent plasma (Sausville et al, 1998, Fuse et al, 1998 and Hagenauer et al, 2004). The maximal free drug concentration of PD184352 seen in most patients enrolled in Phase I/II

clinical trials was in the range of approximately 400 nM partially due to its apparent rapid metabolism profile.

For the treatment of mammary carcinoma tumors in our animal experiments, the theoretical peak UCN-01 concentration would be around 300 nM, based on instantaneous absorption of the entire agent. While using a dose of 25 mg/kg of PD184352 would result in a theoretical peak concentration of around 50 μM. Based on this assumption the UCN-01 concentration used in our experiments, could be achievable in human patients. Recently a newer second generation MEK1/2 inhibitor, PD0325901, has been developed which has been shown to have a significantly superior pharmacokinetic profile in humans than that of PD184352, it would be of great interest to determine if this inhibitor interacts with UCN-01 to suppress tumor growth as well in our experimental model (Thompson et al, 2005).

In our in vitro studies we observed that non-transformed cell types were seemingly less sensitive to the toxic effects of the drug combination of UCN-01 and PD184352 than the transformed cell types. In our in vivo studies using mice, we discovered that the combined exposure to doses of UCN-01 and PD184352 which were four times greater and twice as long as those used in vitro, did not cause weight loss, skin color changes, alterations in animal behavior or exhibit any other untoward effects, which suggests that

with this drug combination the toxicity is minimal in animals. These observations are complimentary to our in vitro studies. Additionally, more detailed analysis and study is required to definitively examine the toxicology profile of the combined use of PD184352 and UCN-01 in animals.

Breast cancer is often treated clinically using estrogen receptor inhibitors such as tamoxifen, growth receptor modulators such as Herceptin, cytotoxic chemotherapy such as Docetaxel and fractionated ionizing radiation therapy or taxanes (Dent et al, 2003, Dent et al, 2002, Kronblad et al, 2005, Kirsh et al, 2005 and Heys et al, 2004). In vitro studies have demonstrated that the combined MEK1/2 inhibition and UCN-01 drug treatment can radiosensitize human mammary carcinoma and prostate carcinoma cells (McKinstry et al, 2002). Furthermore, MEK1/2 inhibition is known to promote the lethality of taxanes both in vitro and in vivo (McDaid et al, 2005 and Yacoub et al, 2003). Further studies however are required to determine if the combined PD184352 and UCN-01 drug regimen can compliment the established cytotoxic drug therapies to enhance cell killing in vivo in combination with radiation or taxanes.

Other studies on hematological cell types have demonstrated that the lethal potential of UCN-01 is promoted by both MEK1/2 inhibitors and also by drugs that inhibit RAS processing; Farnesyl transferase inhibitors (FTI), (Dai et al, 2005 and Pei et al, 2005).

These studies were undertaken based on the rationale that RAS signaling is upstream of MEK1/2 and that upstream inhibition of MEK1/2 function should also promote UCN-01 induced lethality. In fact, UCN-01 and FTI also interacted synergistically to promote MDA-MB-231 and MCF7 cell death in vitro (Mitchell K, Dai W, Grant S and Dent P unpublished observations cited in Hawkins et al, 2005).

In ionizing radiation studies using MDA-MB-231 cells, our laboratory reported that paracrine signaling by transforming growth factor alpha/ERBB1 upstream of ERK1/2 plays an important role in cell survival and cell proliferation/re-population following radiation exposure (Park et al, 1999, Dent et al, 2003, Dent et al, 2002 and McKinstry et al, 2002). Since both MEK1/2 inhibitors and FTI were shown to promote UCN-01 lethality, further experiments determined whether ERBB1 inhibition could also promote UCN-01 lethality.

The findings unexpectedly showed that ERBB1 inhibition suppressed UCN-01 induced lethality. These findings suggested that the actions of UCN-01 could modify or promote the activity of ERBB1 and under such conditions ERBB1 is acting to promote mammary tumor cell death. These findings were not exceptionally novel since other groups using various cell lines have shown that ERBB1 can promote cell death via the extrinsic or intrinsic apoptotic pathways (Gulli et al, 1996 and Reinehr et al, 2004). However these in

vitro observations involving growth factor receptor signaling and subsequent MEK1/2 activation were the basis for our subsequent study involving upstream inhibition of MEK1/2 by the RAS inhibitor, FTI and combining it with UCN-01 in vivo using our animal model of mammary breast cell carcinoma.

Combination Radiation Therapy and UCN-01/PD184352 In Vivo Study

The suppression of MDA-MB-231 tumors by the radiation and UCN-01 and PD-183452 drug combination resulted in approximately a 50% delay in a 2 fold increase in tumor volume (Figure 15). As discussed above, when compared to the prior studies or the effect of UCN-01 and PD183452 combination in this study, the result was surprising. However, as discussed in the introduction, the induction of MAPK by radiation and its inherent ability to produce cell killing by causing irreversible DNA damage, the outcome may be explained as an additive effect of the combined treatments.

The ability of UCN-01 to induce a hyper-stimulation of MAPK activity, which is blocked by PD183452 (McKinsrty et al, 2002) could be further enhanced by the radiation induced stimulation effect (cited earlier as "self limiting") of radiation induced MAP kinase pathway signaling could contribute to our findings. In addition, the ability of UCN-01 to abrogate the G2/M checkpoint would drive sub-lethal DNA damaged cells into mitosis without repair and cause further cell death. Further more the concept of "dual radiation

action" discussed earlier and the fact that our dose of 2 Gy exceeds the D_0 of mammary carcinomas of 1.37 Gy may account for our growth delay results. This result may prove to be promising for the enhanced cell killing by PD183452 and UCN-01 in combination with radiation. Since, the effectiveness of pharmacological intervention with MAPK inhibitors and other cell signaling pathway inhibitors when combined with radiation has been widely demonstrated as discussed in (Dent et al, 2002).

R115777 (FTI) and UCN-01 In Vivo Studies

FTI and UCN-01 drug combination was evaluated as a potential therapeutic treatment for mammary breast cell carcinoma (Figure 16). As discussed above, other investigations on hematological cell types have demonstrated that the lethal potential of UCN-01 is promoted by both MEK1/2 inhibitors and also by drugs that inhibit RAS processing; Farnesyl transferase inhibitors (FTI), (Dai et al, 2005 and Pei et al, 2005). These studies were undertaken based on the rationale that RAS signaling is upstream of MEK1/2 and that upstream inhibition of MEK1/2 function should also promote UCN-01 induced lethality. In fact, UCN-01 and FTI also interacted synergistically to promote MDA-MB-231 and MCF7 cell death in vitro (Mitchell K, Dai W, Grant S and Dent P unpublished observations cited in Hawkins et al, 2005). And as described earlier, the inhibition of geranylgeranyl prenylation of H-Ras by FTIs has been shown to prevent C6 glioma cells

to progress into S-phase, and may involve the necessary prenylation of other proteins as well (Crick et al, 1998). Our results showed a significant growth delay of approximately 11 days or 33% for the drug combination group to reach the primary endpoint of a 4 fold increase in tumor volume. While the single treatment groups only experienced a marginal delay in tumor growth which reached control levels by day 21.

The result of MDA-MB-231 cell treatment with R115777 and the inhibition of Ras prenylation are known to prevent downstream MAPK signaling essential for cell proliferation and survival. And previous studies in our group have shown that UCN-01 activation of MAPK was opposed by PD183452 (McKinstry et al, 2002). This suggests that this UCN-01 induced activation of MAPK can be suppressed by inhibition of Ras function by the FTI R115777. However, UCN-01would still maintain its check point abrogating ability which drives the damaged cells into mitosis and ultimately resulting in apoptosis.

Human Chorionic Gonadotropin and Lovastatin in Vitro Studies

Human Chorionic Gonadotropin

It has been demonstrated that hCG may be useful for the potentiation of radiation therapy in prostate cancer. Our early investigations demonstrated this radiosensitization effect of hCG on LnCap cells. The results described above, clearly demonstrate a significant dose-response effect of radiation on pre-treated LnCap cell viability with 2.0 mU/ml hCG as compared to vehicle control.

We were also able to demonstrate in part, the mechanism of this hCG induced enhancement of cell death by radiation by evaluating the use the caspase-8 inhibitor (IETD), caspase-9 inhibitor (LEHD) and the pan caspase inhibitor zVAD. Results from these experiments demonstrate that caspase-8 inhibition had an insignificant effect on cell death when compared to hCG and radiation combined. But caspase-9 and pan caspase inhibition showed a decrease in cell death by approximately 25% for LEHD (50 μM) pre-treated and 35% for zVAD (50 μM) pre-treated cells when compared to the hCG and radiation combination. Additionally, lack of effect by IETD and the reduction in cell death by both LEHD and zVAD suggest that the "intrinsic" apoptotic pathway was at least partially responsible for hCG enhanced radiosensitization of LnCap cells. Other elements must be involved in the ability of hCG to radiosensitize LnCap cells, since we could only partially account mechanistically for our initial observations. It is known that growth factor mediated cell signaling pathways are activated along with the G-protein coupled androgen receptor as described in the introduction. This fact prompted us to

further explore the possibility that the EGFR pathway may also be involved in our observations thus far. Analysis for ERBB1 activity in LnCap cells treated in vitro with the MEK1/2 inhibitor PD184352 and subjected to the hCG/radiation treatment demonstrated that in both vehicle and PD184352 treatment alone, little activation of ERBB1 occurred, indicated by p-ERBB1 immunoprecipitation. Treatment with hCG alone resulted in a significant level of ERBB1 activity; however when PD183452 was added to the hCG treated cells, the ERBB1 activity level dropped to that of vehicle control indicating that ERBB1 response occurs through the MAP kinase pathway. When 4 Gy radiation was combined with either vehicle or hCG, p-ERBB1 levels increased substantially. The addition of PD183452 to 4 Gy treated cells diminished p-ERBB1 levels when compared to hCG and 4 Gy treatment. In the hCG, PD184352 and 4 Gy radiation set, MEK1/2 inhibition was able to reduce the ERBB1 activity seen in treatment with both hCG and 4 Gy radiation which accounted for the highest levels of p-ERBB1. With the hCG/4 Gy treatment set showing the highest level of activity, it was apparent that the hCG induced radiosensitization involves ERBB1 activation through a transactivation response.

In the hCG transactivation of ERBB1, it was shown to occur in time-dependent manner as discussed above. The initial ERBB1 activity appears early after hCG stimulation at 5 minutes, and increases throughout the time course with a peak level of p-ERBB1 at 30

minutes. The basal activity level can be abolished by the tyrosine kinase inhibitor AG1478 when used alone or the peak levels significantly reduced when used in combination with hCG. The inhibition of hCG induced transactivation of ERBB1 by AG1478 demonstrates this process.

Western blot analysis demonstrated that the combination of 4 Gy and hCG resulted in a high level of activated PARP, well over that with either treatment condition alone. As indicated by the anti-body response (PARP 10H) against activated PARP, the levels of activated PARP seen with either 4 Gy alone or with hCG treatment were similar, with hCG providing a more enhanced effect. But when these levels were compared to the combination treatment, the combination effect was greater than additive or super-additive. This result strongly suggests that hCG induced radio-sensitization is in large part due to the observed hyper-activation of PARP. This hyper-activation ultimately results in the irreversible process of DNA fragmentation and cell death. As described earlier, PARP activation results in the formation of polymeric PARP which requires the NAD+ moiety. This requirement can result in the depletion of cellular NAD+ stores and effectively deprive the mitochondria of energy substrate. As a result, AIF is released from the mitochondria and trans-locates to the nucleus where it initiates DNA fragmentation resulting in cell death.

As was described earlier, hCG induced-radiosensitization at 4 Gy resulted in a 2-fold increase in cell death when compared to hCG alone. In vehicle controls, radiation induced cell killing resulted in ~22% cell death while vehicle alone accounted for ~12% cell death. The addition of PJ-34 however produced a dramatic decrease in cell death with the hCG/4 Gy combination treatment. PJ-34 inhibited cell death to the extent that no significant differences were observed between the vehicle or hCG individual treatments or in combination with radiation. Therefore, the radio-sensitizing effect of hCG on LnCap cells could be abolished by the PARP inhibitor PJ-34. The results of the tyrosine kinase inhibitor AG1478 on PARP activation provided more evidence for the transactivation of ERBB1 by hyper-active PARP. The decrease in PARP activation described above in the results, show that the activation/up-regulation of PARP by 4 Gy and hCG and/or the hyper-activation of PARP seen with the combination is largely diminished in the presence of AG1478 at the same time-points corresponding to the peak levels of ERBB1 activation described earlier. This data provides additional evidence for the transactivation of ERBB1 occurring through hyper-active PARP.

The metallo-protease inhibitor (GM6001) results demonstrated that G-protein induced metallo-proteases resulted in the transactivation of ERBB1 by pERK in response to hCG stimulation. Additionally the hCG induced transactivation of ERBB1 was blocked by the G-protein inhibitor pertussis toxin. Providing more evidence that the hCG induced

transactivation is occurring through a G-protein dependent activation. In addition our group was able to demonstrate that pertussis toxin was able to abolish the hCG induced G-protein coupled transactivation by activated ERK in LnCap cells in vitro.

Human Chorionic Gonadotropin & Lovastatin

As was described above, the statins have been shown to exhibit anti-cancer properties in numerous reports and in various cell lines. This ability is mediated by the inhibition of the mevalonate biosynthetic pathway and subsequent inability of Ras to become prenylated which is required for its activity. The inhibition of geranylgeranyl prenylation of H-Ras by FTIs has been shown to prevent C6 glioma cells and others to progress into S-phase, which can be overcome by the addition of a farnesol or geranylgeraniol substrate in vitro, and may involve the requisite prenylation of other proteins as well (Crick et al, 1998).

As mentioned, Ras is an upstream moderator of the MAP kinase signaling cascade. Our early investigations with lovastatin were undertaken to see if pharmacologically achievable lovastatin concentrations in combination with hCG could enhance the cell killing effect in LnCap cells as were demonstrated by our group in the hCG experiments. In our initial dose-response studies in vitro, we observed a significant 2-fold effect on

decreased cell survival when 0.6 μM Lovastatin was used in combination with hCG at both 1 and 2 mU/ml compared with lower concentrations of lovastatin. In the 22WR1 cell line, we demonstrated a significant dose-response effect with the hCG and lovastatin combination over that of lovastatin alone. Treatment of 22WR1 with 0.6 μM lovastatin accounted for ~25% cell death, the combination of lovastatin and hCG at 2 mU/ml resulted in a greater than 2-fold increase in cell death. This was illustrated in a 6 hour time course study after hCG stimulation in LnCap cells. Activated Erk1/2 occurs as an early response and remains relatively constant from 15-60 minutes. At 3 hours, p-Erk1/2 levels have diminished considerably at which activated JNK1/2 levels are highest, indicating cell death. Activated Akt levels are also noticeably lower in hCG treated cells as compared with controls in the early (15-60 minutes) part of the time course. This result may indicate a decrease in Akt associated cytoprotection in LnCap cells. In the androgen independent cell line PC-3, p-Erk1/2 levels are relatively constant at 96 hours but p-Akt levels decline in response to increasing doses of lovastatin in the presence of hCG. This decline in Akt associated cytoprotection is reinforced, by the cell viability determination assay at 96 hours, in PC-3 treated cells with lovastatin alone or in combination with hCG.

This result suggests that lovastatin treatment can enhance cell death in PC-3 cells. In parallel, a similar dose-response experiment was performed in LnCap cells. These experiments show that increasing lovastatin and hCG concentration results in increased

cell death 96 hours after exposure. Treatment with 2.0 mU/ml and 0.6 μM lovastatin resulted in ~70% cell death which amounts to a 40% increase in cell death over treatment using 1.0 mU/ml which resulted in ~50% cell killing. Additionally, p-Erk levels declined with increasing doses of lovastatin used in combination with hCG as well as p-Akt. For the experiments shown in pre-treatment studies with 50 μM LEHD and zVAD followed by daily supplementation, significantly decreased LnCap cell death after exposure to lovastatin alone or in combination with hCG. A more pronounced effect was observed with the pan-caspase inhibitor, zVAD versus LEHD, which resulted in diminished cell killing at the level of vehicle controls. Results from our viral transduction studies in LnCap cells , over-expressing either the anti-apoptotic protein BCL_{-XL} or dominant-negative caspase-9 (DN-caspase-9) or cytomegalovirus (CMV)vector control provided further evidence of "intrinsic" pathway involvement in hCG and lovastatin induced cell death. CMV expression alone did not suppress cell death in the drug combination treatment, and DN-caspase-9 expression resulted in significantly decreased cell killing compared with CMV. Over-expressing BCL_{-XL} resulted in profound inhibition of cell death when comparing the drug combination with vector control. Results of the PI3 kinase inhibition study, illustrate that ERBB1 inhibition by the tyrosine kinase inhibitor AG1478, but inhibition of PI3 kinase with LY294002 prevents the downstream activation of the cytoprotective Akt pathway which further enhanced hCG induced cell death.

Data presented here, show that hCG-induced trans-activation of ERBB receptors occurs through G-protein coupled receptors. The resultant trans-activation leads to a hyper-activation of PARP which depletes NAD+ stores and results in cell death through the "intrinsic" pathway mechanism of apoptosis which corresponds to the activation of JNK and down-regulation of Akt.

Overall, the use of unconventional drugs, with novel mechanisms of action, in low-dose combination therapy with and without radiation has been successfully demonstrated by our group. This strategy has been shown to have significant benefit in the treatment of cancers both in vitro and in vivo. In vivo, the utilization of UCN-01 and MEK1/2 inhibitors in combination resulted in a greater than anticipated reduction in tumor proliferation compared to the individual drug alone in both the MDA-MB-231 and MCF-7 studies. This outcome also coincided with the cleavage of pro-caspase-3, substantially diminished ERK1/2 levels and a prolonged suppression of Ki-67 immunoreactivity. In addition, we observed reduced ability of the ex-vivo tumor cells to form colonies 30 days after drug cessation of the combination treatment. Neo-angiogenesis of the tumor was also disrupted by both individual and combined treatment of MEK1/2 inhibitor and UCN-01 determined by reduced CD31 immunoreactivity. Collectively, our findings demonstrate the potential therapeutic implications of the tumoricidal properties of the combined MEK1/2 and UCN-01 treatment to be successfully applied from a series of in

vitro studies to translation in a relevant animal model systems of breast cell carcinoma. In addition, our use of the MEK1/2 inhibitors and UCN-01 combination in both MCF-7 and MDA-MB-231 cell lines demonstrate the tumoricidal effect of this drug combination is applicable to breast cell carcinomas independent of their p53 status, estrogen dependency, caspase-3 levels or oncogenic K-RAS expression.

In our prostate cell carcinoma studies we demonstrated that hCG can radiosensitize human prostate carcinoma cells in vitro. The treatment of prostate carcinoma cells with hCG promoted the activation of ERBB1 which was enhanced by the use of hCG and radiation treatment. The hCG-induced activation of ERBB1 was indirect via a paracrine loop involving Gαi coupled GPCR followed by MEK1/2 activation and metalloprotease cleavage of a poorly defined ERBB1 receptor activating ligand that was not HB-EGF, TGFα or heregulin. The combination of hCG and radiation also profoundly increased the level of PARP1 activity which was dependent on ERBB1 and MEK1 signaling. Inhibition of PARP1 activity was shown to abolish the hCG and radiation exposure cell lethality. While inhibition of pan-caspase or AIF function reduced the lethality of hCG and radiation exposure by approximately 50%. Alternatively, the inhibition of MEK1/2 or JNK1/2 suppressed the lethality of hCG and radiation interactions, while inhibition of AKT promoted cell death in vitro. Furthermore, expression of constitutively active AKT circumvented the ability of dominant-negative IκB to enhance hCG-induced cell lethality

and restored near basal levels of BCL_{-XL} even when dominant-negative IκB was expressed. Over-expression of constitutively active BCL_{-XL} blocked lovastatin and hCG induced cell lethality. Our findings provide evidence for the cyto-protection of LnCap prostate cells by parallel signaling pathways of AKT and NFκB from the toxic effects of hCG and lovastatin treatment. With AKT as the primary upstream cell survival pathway and NFκB as the secondary signaling pathway for LnCap cell survival. In other prostate carcinoma cell lines with more active NFκB signaling, this cyto-protective pathway may play a more important role in maintaining cell viability after lovastatin and hCG exposure.

The use of MEK1/2 inhibitors, check point abrogators, farnesyl transferase inhibitors and the statins in combination with androgen stimulation have been demonstrated by our group as well as others as "non-traditional" pharmacological treatments with the potential benefit to off-set the limitations of traditional forms of therapeutic treatment against cancer. Hopefully, future studies which need to be undertaken will determine that these novel therapies can potentially be translated into clinical use.

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List of References

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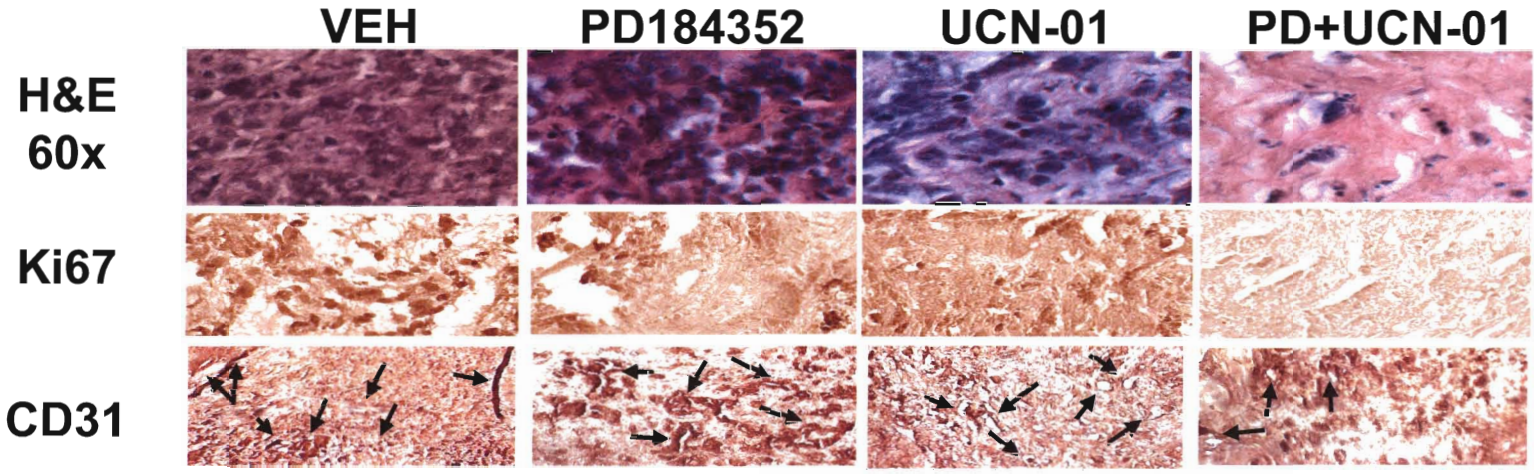
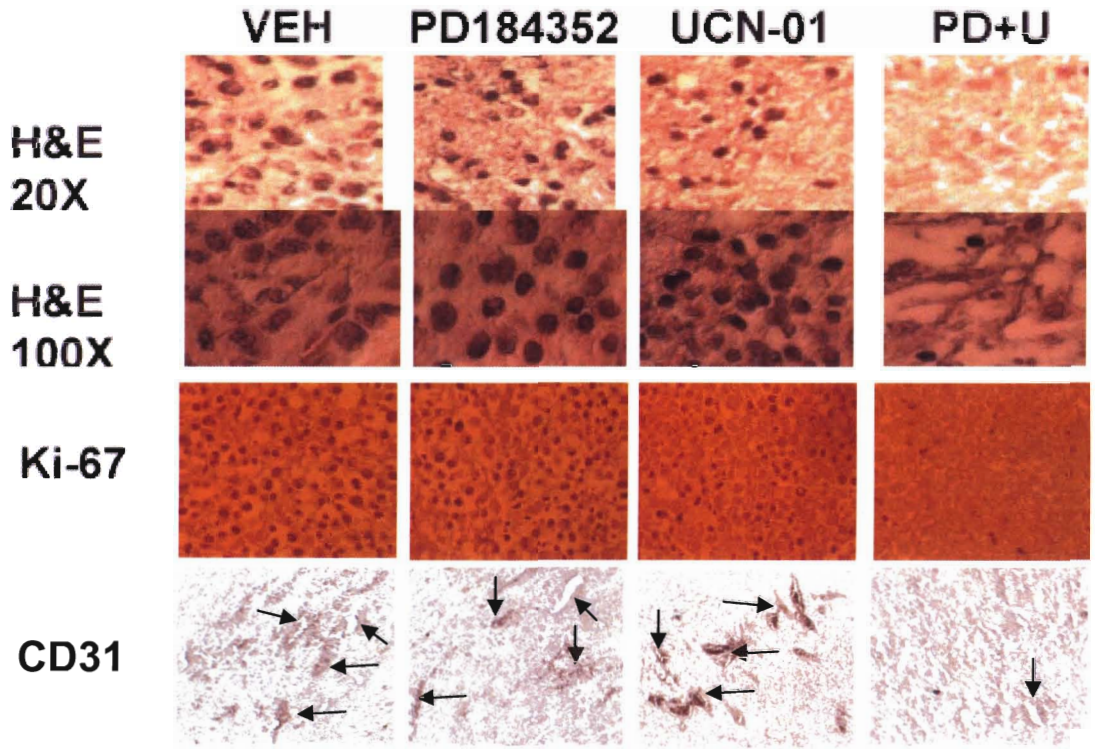
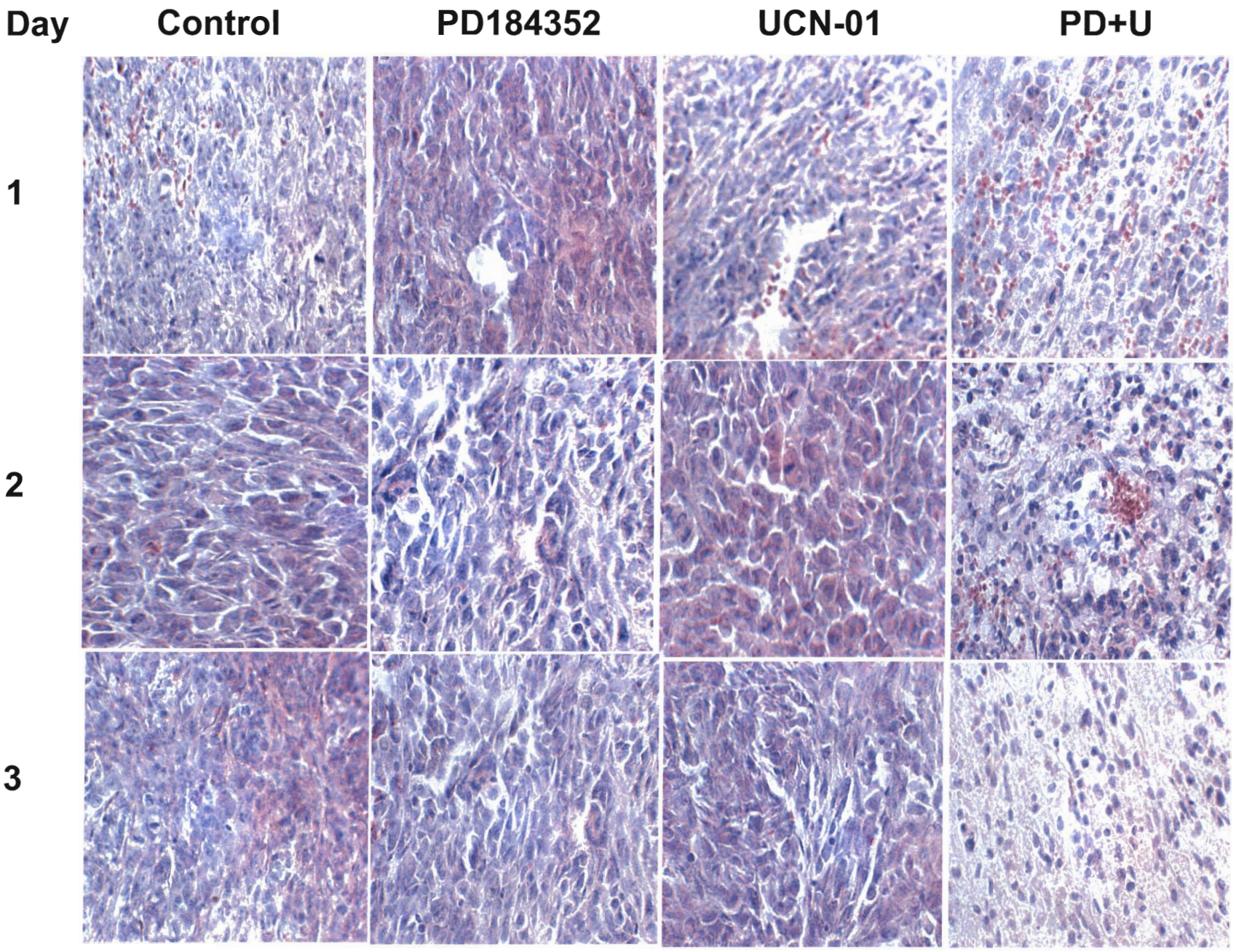
APPENDICES



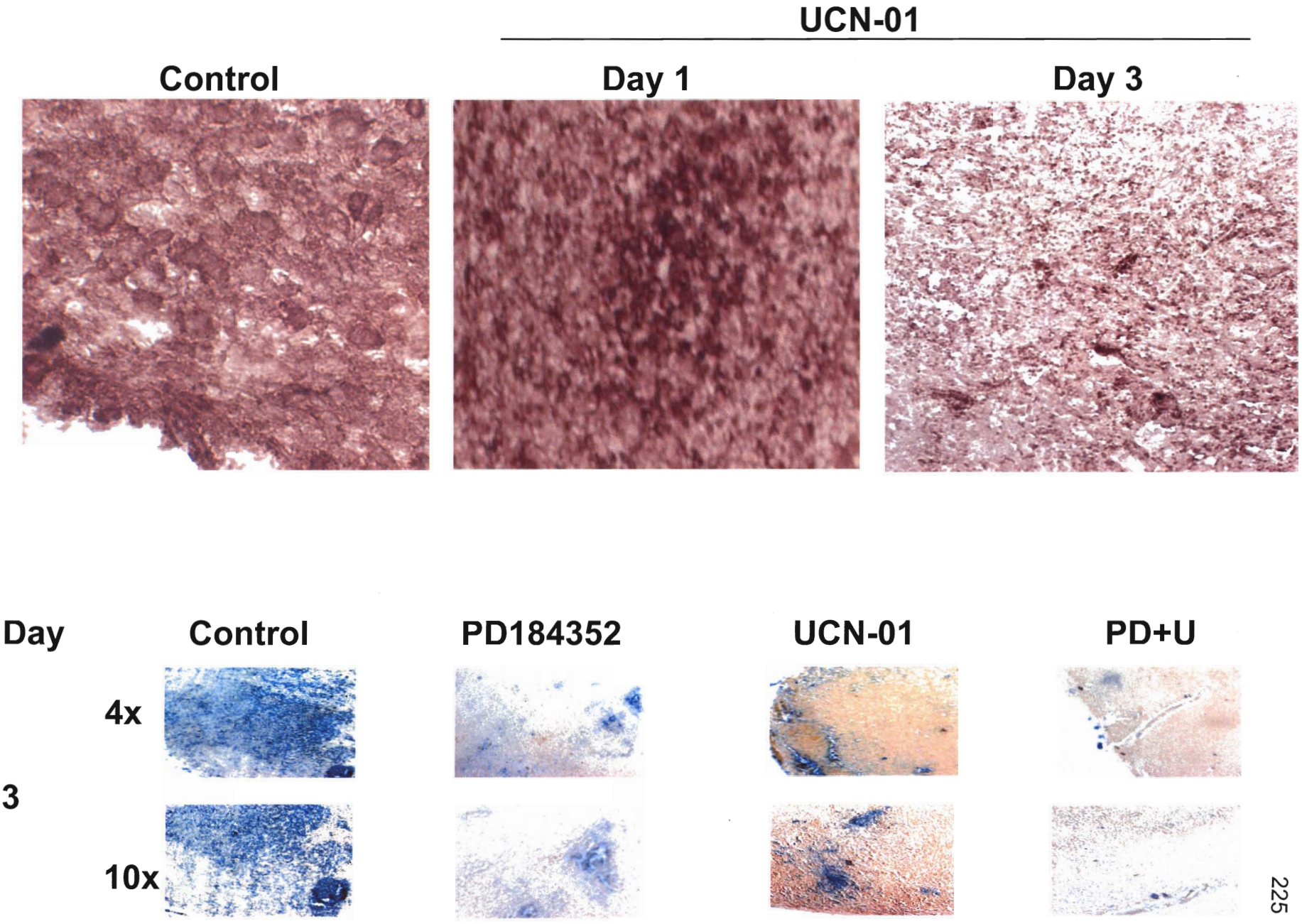
Appendix i: Figure 10. Expanded View



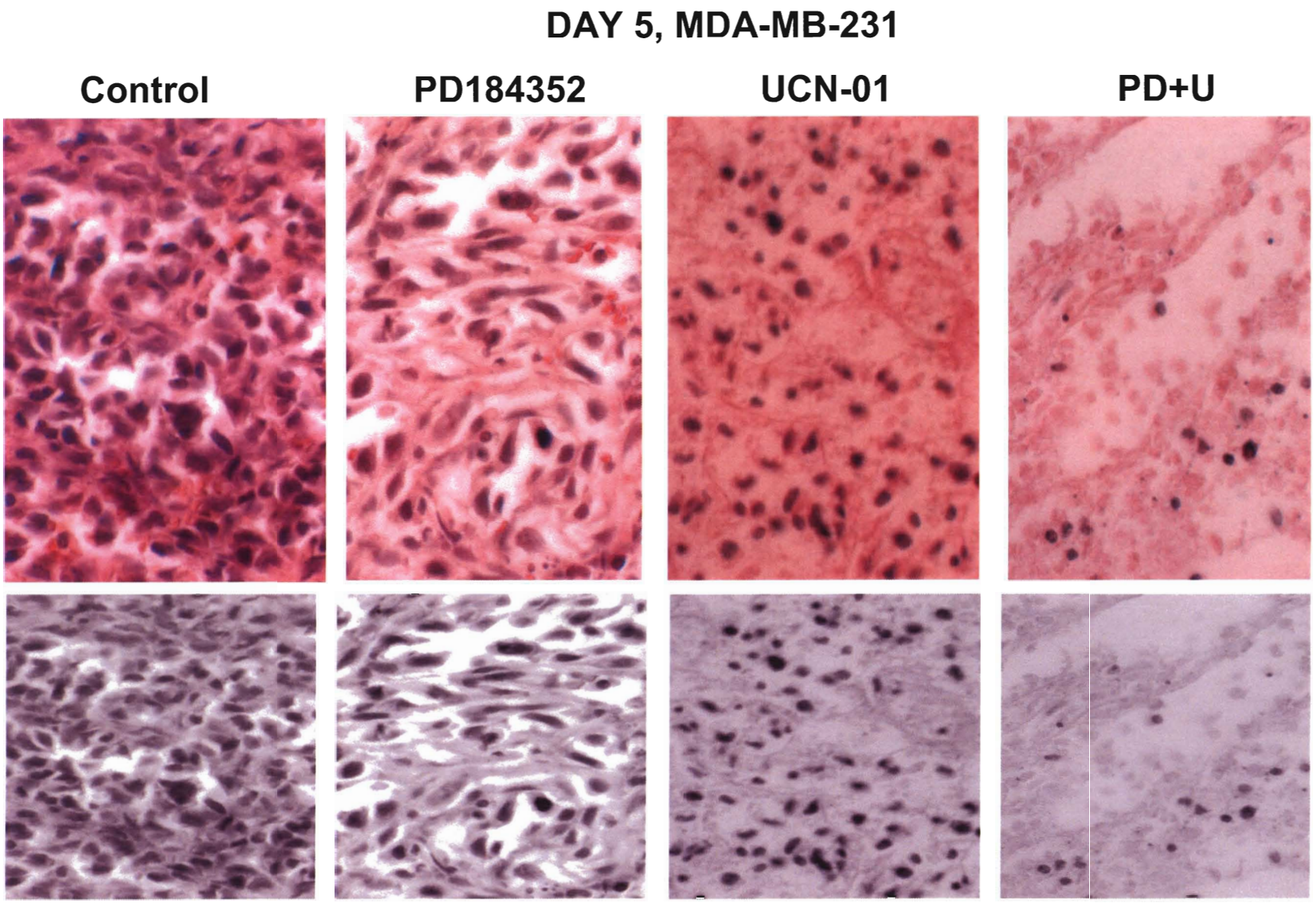
Appendix ii: Figure 11. Expanded View



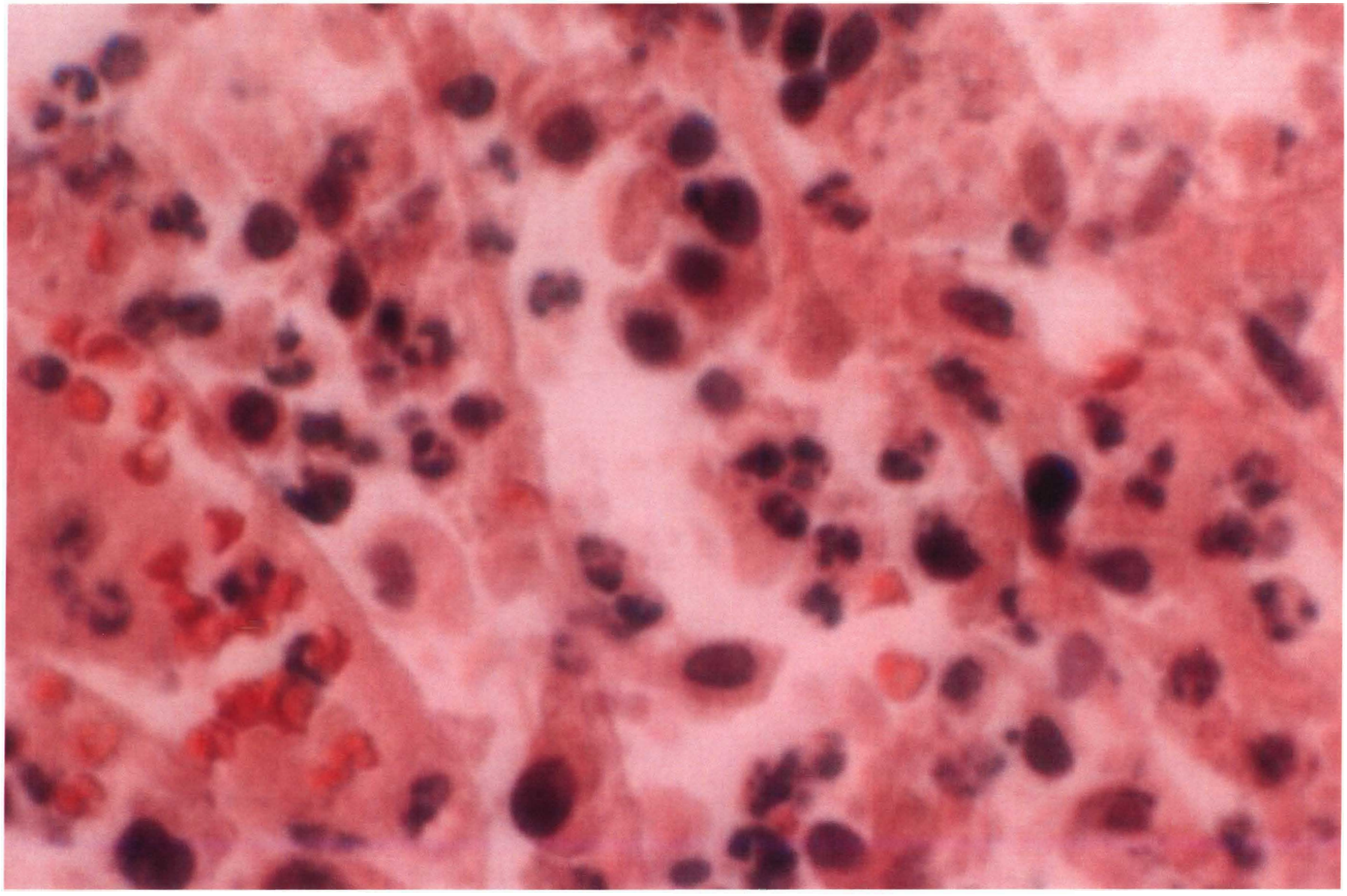
Appendix iii: Figure 12a. Expanded View



Appendix iv: Figure 12d-e. Expanded view



Appendix v: Figure 13a. Expanded View



Appendix vi: H&E stained MDA-MB-231 tumor treated with PD/UCN-01. Photo used for the cover of the November issue of Cancer Biology & Therapy which includes Hawkins et al., Vol. 4:11, pages 1275-84, 2005.

Vita

William T. Hawkins II was born a citizen of the United States of America in a United States Army hospital in LaRhoshelle, France and returned to Alexandria, Virginia at 9 months of age. He graduated from Midlothian High School, Midlothian Virginia and went on to receive a Bachelor of Science degree while majoring in Psychology & Chemistry in 1987 from Virginia Commonwealth University. He was employed as a laboratory specialist in the Department of Pharmacology & Toxicology and a laboratory specialist advanced in the Department of Pharmacy & Pharmaceutics at the Medical College of Virginia until 1991.

At that time he returned to the Department of Pharmacology & Toxicology as a laboratory specialist senior and pursued his Master's degree in Pharmacology & Toxicology which he received in 1997. He went on to work at the Massey Cancer Center in the Department of Radiation Oncology under the direction of the late chairman, Dr. Rupert Schmidt-Ullrich and Dr. Peck-Sun Lin until 2001 when he entered the Doctoral program in the Department of Anatomy & Neurobiology. He received his Doctor of Philosophy degree in Anatomy & Neurobiology in November, 2006. He is married and he and his wife, Wenjun, have one son, James. While working on his doctoral degree in the laboratory of Dr. Paul Dent, he received four publications for efforts in cancer research.