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**ELLAGIC ACID-MEDIATED CK2 INHIBITION, A NATURAL,
MULTIFUNCTIONAL STRATEGY TO TRIGGER CERVICAL
CANCER CELL DEATH IN VITRO AND IN VIVO**

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To My Dear Parents Fathi & Ezziah

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ABSTRACT

Targeting CK2 for cancer therapy has proven effective in inhibiting tumor growth in several histology's. The main goal of this study was to acquire pre-clinical data on a naturally occurring CK2-inhibitor (ellagic acid) in support of a human clinical trial. In vitro analysis included testing the spectrum of ellagic acid anti-cancer activity, defining the mode of growth inhibition in cancer cells and its impact on biochemical and molecular pathways. In vivo studies included determination of ellagic acid inhibitory effect in animal tumor models and toxicity analysis. We found that CK2 inhibition mediated by ellagic acid, was effective in inhibiting the growth of a wide spectrum of human cancer cells. These include breast cancer (MCF-7), kidney cancer (ACHN), colon adenocarcinoma (SW480), glioblastoma (U87), prostate cancer (DU-145), melanoma (WM164) and HPV -positive (HeLa) and -negative (C33A) cervical carcinomas. We used HeLa and C33A cell lines to determine EA mechanisms of action and impact on oncogenic, proliferative, and apoptotic pathways. Since CK2 controls over 300 target proteins involved in major biochemical and molecular pathways, we found that ellagic acid treatment was able to inhibit HPV oncogene expression, interrupt oncogenic pathways and induce anti-proliferative and pro-apoptotic pathways in HeLa cells. In

C33A cells, CK2 inhibition bypassed tumor suppressor p53 and pRb mutation and induced apoptosis by inhibition of anti-apoptotic proteins IAPs. Comparing HeLa and C33A cells response to ellagic acid treatment, C33A cells were more sensitive to ellagic acid treatment compared to HeLa. The expression of HPV oncogenes in HeLa cells could delay HeLa response to death stimuli exerted by CK2 inhibition. CK2 inhibition and subsequent IAPs repression is the common event occurring in HeLa and C33A cells.

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CHAPTER I

INTRODUCTION

Cancer has now become the leading cause of death worldwide. It's a disease that's characterized by irregular proliferation, inappropriate cell survival, decreased apoptosis (programmed cell death), immortalization, invasion of surrounding tissue and metastasis to other organs. Cancer is responsible for high morbidity and mortality rates worldwide, specifically in the United States. According to National Cancer Institute (NCI) Surveillance Epidemiology and End Results (SEER), the estimated number of men and women diagnosed with cancer of all sites is 1,529,560 of which 569,490 die in 2010 statistics. Relating cancer immortality to perturbed proteins signal transduction and to subsequent uncontrolled cellular growth and tumor development have originated the concept of Signal Transduction Therapy (STT) in molecular medicine (1). Protein kinases are the regulators of cell signaling pathways controlling cellular growth, proliferation and apoptosis. The fact that some kinases abnormal activity can cause major diseases, in

addition to their ability to be readily inhibited by catalytic site-directed molecules, has resulted in kinases emerging as the drugable kinome that can be targeted for therapy of most human diseases. As a general rule in cancer therapy, an effective target for cancer treatment must have a biological function that is indispensable in the cell, its abnormal activity and dysregulation must be related to the malfunction of the cell, and be specific to cancer cells compared to the normal one (2). Based on growing evidence in cancer research, one can argue that protein kinase CK2, previously known as Casein Kinase II demonstrates these traits. Therefore, the emergence of CK2 as suitable target for cancer therapy is put forth in this work.

1.1 CK2 Characteristics.

Protein Kinase CK2 is a serine/threonine kinase (3). It recognizes acidic sites generally specified by clusters of carboxylic acid and/or pre-phosphorylated side chains downstream from the Ser/Thr residues with the one at position $n+3$ determining the minimum consensus sequence S/T-X-X-D/E (3) (4). CK2 exists as a tetramer composed of two catalytic subunits α (42-44 kDa), and α' (38 kDa), which exists in heterogeneous or homogenous nature, and two regulatory subunits β (25 kDa) (3, 5). Even though, the catalytic subunits exhibit more than 90% sequence similarity, they are the product of different genes and each has very distinctive C-termini, which suggests functional specialization (4, 6). A natural property of CK2- α catalytic subunit is the ability to utilize both ATP and GTP as phosphate donors while β subunit confers stability and substrate specificity of the enzyme (4, 7). CK2 phosphorylates more than 300 proteins which accounts for 18-27% of the cellular phosphoproteome localized in various cellular

compartments (3, 8). Protein kinase CK2 targets are involved in essential signaling pathways controlling vital cellular mechanism such as cell cycle, apoptosis, DNA replication and gene expression (4).

1.2 CK2 Is Indispensible.

CK2 is essential to cellular growth, proliferation and survival. Previous knockout study of CK2 α in *Saccharomyces Cerevisiae* resulted in a drastic decrease in yeast viability (9). In mice models, disruption of CK2 α lead to embryonic death with multiple defects found in heart and neural tube (10). In different studies, CK2 β knockout mice proved to cause embryonic death as well, while CK2 α' knockouts produced sterile mice offspring (11, 12). Therefore, CK2 function in the cell has been established to be indispensable.

1.3 CK2 in Cancer versus Normal Tissue.

Protein kinase CK2 is ubiquitously expressed in all cell types. However, CK2 distribution and expression level differ from one cell type to another and discriminates between normal and malignant cells. This difference in expression and distribution correlates with cellular state of proliferation and growth. CK2 is over-expressed in tissues involved in organogenesis or normal highly proliferative cells as well as all types of cancer tissue (7, 13). Comparing CK2 activity within normal tissue, it was found to be higher in the testis, brain and liver whereas in kidney, spleen, skeletal muscle, cardiac muscle and adrenal gland is relatively lower (7). However, over-expression of CK2 in normal highly proliferative tissue is only transient and much lower compared to the

persistent over-expression in cancer cells (13). CK2 localization pattern is distributed among almost all cellular compartments (as a holoenzyme or separate subunits) including cytoplasm, nucleus, plasma membrane, cytoskeleton components, and endoplasmic reticulum (7). CK2 subunits are expressed in different proportions and may shuffle between cellular compartments as a holoenzyme or individual subunits in accordance with the functional requirements of the cell. For example, CK2 α becomes rich in the nucleus at the late G₁ early S phase of the cell cycle and remains there for the rest of the cycle, which indicates a correlation between CK2 subunits localization and cellular function and activity. CK2 holoenzyme translocation was evident in normal cells while studies done on cancer cells models indicate the possibility of individual subunits migration between cellular compartments (14-17). The discrimination between normal and malignant cells was further illustrated in the subcellular localization of CK2 subunits. CK2 is diffused in all cellular compartments in normal cells however, in cancer cells, CK2 is mainly localized in the nucleus (13).

1.4 CK2 Dysregulation Is Tightly Related To Tumorigenesis.

Several convincing evidences have implicated CK2 in the tumorigenesis and transformation of almost all cancer types. CK2 was found to be overexpressed and its catalytic activity elevated in several malignancies (1, 18). CK2 can desensitize cells to vital cellular checkpoints and elimination mechanism such as programmed cell death (apoptosis). CK2 promotes cellular growth and proliferation through the regulation of vital pro-survival signal transduction pathways such as NF- κ B, Wnt, and P13K signaling (18, 19). Identifying the different signaling pathways that CK2 can perturb is

fundamental to understanding the mechanism of action of CK2 in promoting transformation and tumorigenesis.

1.4.1 NF- κ B Signaling Pathway.

Dysregulated activity of the transcription factor NF- κ B was implicated in multiple malignancies. NF- κ B promotes the activation of anti-apoptotic proteins including Bcl-xl and cIAPs, and cell cycle regulator cyclin D1. The inhibitor of κ B (I κ B) directly interacts with NF- κ B to inactivate it and to hold it in the cytoplasm of most cells. Activation of NF- κ B requires the proteasome dependent degradation of I κ B. This degradation was found to be enhanced by CK2-mediated increase of IKK kinases expression or direct phosphorylation of I κ B. Either pathway will activate proteasome-mediated destruction of I κ B (20). As a result NF- κ B is activated to translocate to the nucleus and induce transcriptional activation of anti-apoptotic proteins and cell cycle regulators, ultimately inducing uncontrolled cellular growth and inhibiting apoptosis.

1.4.2 Wnt Signaling Pathway.

Wnt pathway is known as a key player in cancer development specifically in mammary gland and colorectal cancer (21, 22). It regulates cell proliferation, polarity and adhesion by keeping a sufficient level of β -catenin, a cofactor of transcription factor (TCF/LEF) family. β -catenin is necessary for transcription activation of *c-myc*, *cyclin D* and a member of the inhibitor of apoptosis protein family (IAP) *Survivin* (19). Wnt is an extracellular protein which targets β -catenin for degradation. This destruction is inhibited by stabilizing protein dishevelled (Dvl). In this pathway, CK2 come into play by

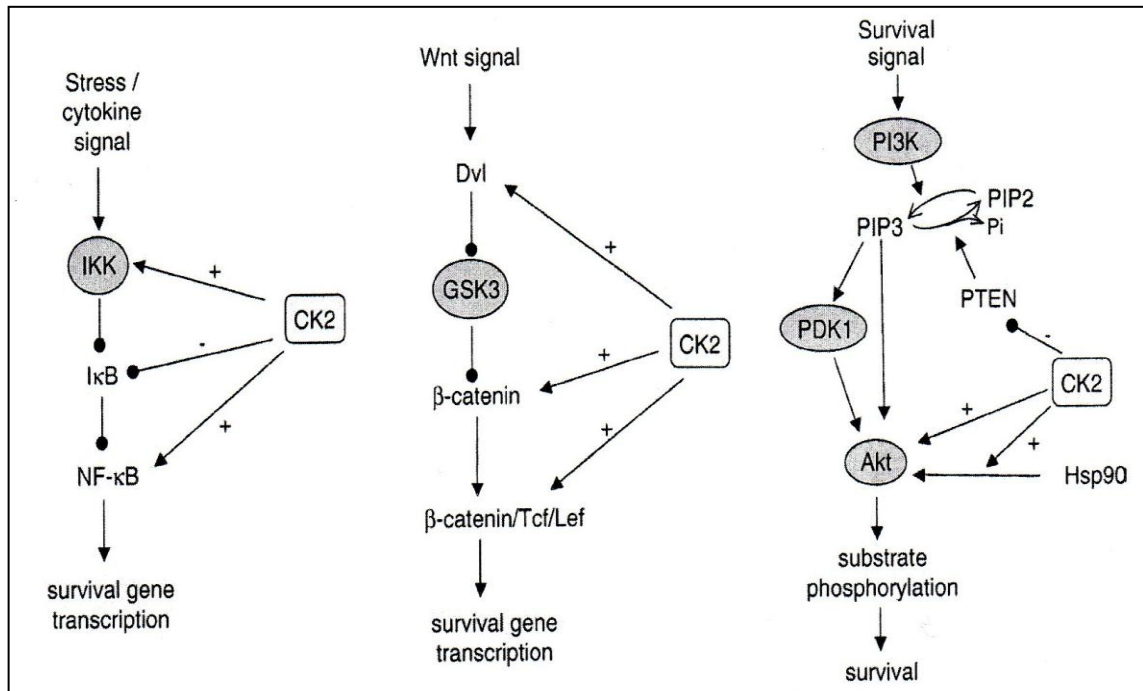


Figure 1.1 Schematic presentation of CK2 implication in carcinogenesis through regulation of NF-κB, Wnt and P13K oncogenic pathways. (Figure from: *Pinna et al. 2010.*)

phosphorylating Dvl, β -catenin and the complex β -catenin/Tcf/Lef. This phosphorylation activity promotes stabilization of targeted proteins and leads to increased β -catenin-mediated transcription activation of multiple pro-survival and anti-apoptotic genes including Survivin (19, 23, 24).

1.4.3 P13K/Akt Signaling Pathway:

The pro-survival/anti-apoptotic signaling pathway induced by P13 kinase (P13K) targets protein kinase B (PKB) or Akt that induces survival through direct inhibition of caspase 9, and pro-apoptotic protein BAD. This pathway is regulated by tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10). Removal of PTEN renders P13K-mediated Akt induction constitutively active (18). CK2 intervenes at two sites in this transduction pathway. First CK2 targets PTEN for degradation by the proteasome (25). Second, CK2 directly phosphorylates Akt and promotes its activation to further induce cell survival (26, 27). Overall, CK2 over-expression and dysregulated activity perturbs significant pro-survival and anti-apoptotic signaling pathways leading to cell transformation and tumorigenesis.

1.5 CK2 A Key Suppressor of Apoptosis.

In addition, recent studies have shown that CK2 plays a key role in apoptosis suppression. Apoptosis is initiated via two pathways: the intrinsic and extrinsic pathways. Intrinsic apoptosis is initiated when pro-apoptotic Bcl2 family of proteins stimulates the mitochondrial release of a potent pro-apoptotic cytochrome c protein into the cytosol. This event initiates the activation of pro-caspase 9 and pro-caspase 3 which in turn

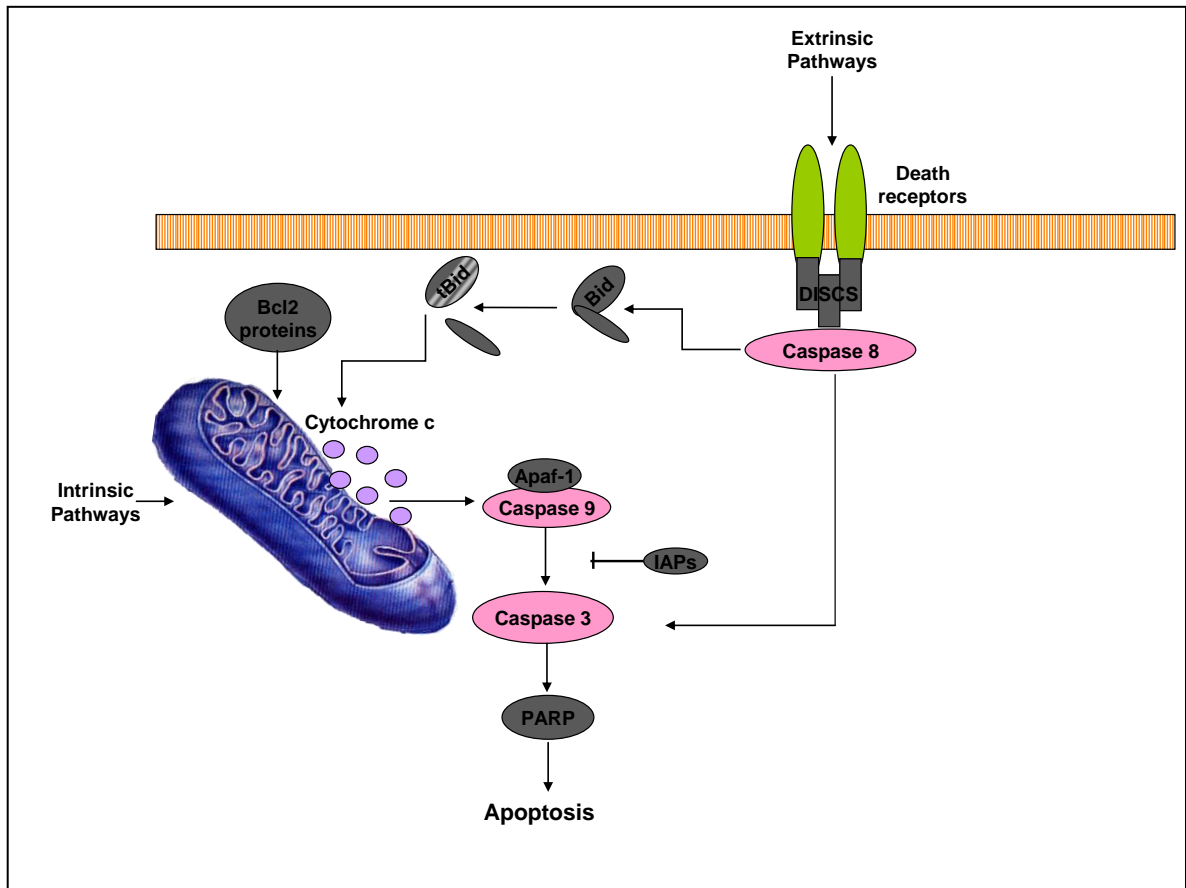


Figure 1.2 Apoptosis Pathways: Apoptosis cascade is initiated by extrinsic or intrinsic pathways, which can combine and lead to common execution of caspase cascade that result in DNA degradation and cell death.

cleaves poly (ADP) ribose polymerase (PARP) and cause DNA damage and cell death (28-30). The extrinsic pathway is triggered by the interaction of tumor necrosis factor (TNF) receptors superfamily which includes Fas, TNFR1, DR4 and DR5 to their corresponding ligand FasL, TNF, and TNF-related apoptosis-inducing ligand (TRAIL). Death receptors are transmembrane proteins that, upon activation, oligomerize forming death inducing signaling complex (DISC) which subsequently activate pro-caspase8. Active caspase 8 can directly cleave pro-caspase 3 (28, 30). Caspase 8 can cross talk with intrinsic pathway through the cleavage of Bid (pro death member of Bcl2 family) and formation of truncated-Bid (tBid). tBid stimulates mitochondrial release of pro-apoptotic protein cytochrome c and induces subsequent caspase activation (28, 30).

Protein kinase CK2 was found to block cell response to death stimuli at multiple levels in the apoptotic cascade. CK2 phosphorylates Bid at serine and threonine residues located in caspase-8 recognition site which inhibits caspase-8 induced tBid formation (31-34). In addition, CK2 activates ARC (apoptosis repressor with caspase recruitment domain) to inhibit caspase-8 and block apoptosis cascade (35). Also, the implication of CK2 in NF-kB, Wnt and P13K pathways increases IAPs expression which inhibits caspase activity and block apoptosis.

1.6. CK2 Enhances The Activity of Oncogenes.

CK2 was found to be upregulated and over-expressed in almost all malignancies. Dysregulation of CK2 kinase activity was found to enhance the oncogenic ability of some genes and lead to malignant transformation of tissue. For example, transfected animals with the catalytic subunit of CK2 in addition to *c-myc* or *Tal-1* oncogenes enhanced

tumor phenotype specifically in leukemia and lymphoma models (3, 21, 36). Similar results observed in mammary gland models where over-expression of CK2 α and MMTV enhanced breast cancer phenotype (10, 21). In addition, CK2 enhances oncogenic activity of Human Papilloma virus (HPV) oncogene E7. CK2 phosphorylates E7 at CR2 domain that contains the binding site of Rb proteins. This phosphorylation was found to enhance transformed phenotype by removing constraints on S-phase progression (37). Investigators have reported CK2 α to behave as an oncogene. CK2 α nuclear localization reflects the state of aggressiveness of prostate cancer as well as its role as a prognostic marker in squamous cell carcinoma of the lung (38, 39). In conclusion, CK2 over-expression and dysregulation is tightly related to the transformation and tumorigenesis of almost all cancer types.

1.7 Targeting CK2 Activity to Treat Cancer.

Taking together the fact that CK2 is involved in several essential cellular pathways, and implicated in tumorigenesis and transformation of tissue have set mounting evidence to propose CK2 as a promising target for cancer therapy. Fortunately, protein kinases can be shut down using small molecules targeting their ATP binding site. There is a growing list of small ATP-site directed molecules that can be used to inhibit CK2 kinase activity. Each compound displays a different selectivity and specificity towards CK2 (Table 1). With growing interest in naturally occurring compounds therapy, Ellagic Acid (EA) with its high selectivity and specificity towards CK2 displays the most efficient molecule for CK2 inhibition.

1.8 CK2 Inhibitor: Ellagic Acid

Ellagic acid (4,4',5,5',6,6' hexahydroxydiphenic acid 2,6,2',6'-dilactone) is a naturally occurring, relatively stable, polyphenolic compound that is found in raspberries (1500 µg/g dry weight), strawberries (630 µg/g), cranberries (120 µg/g), and walnuts (590 µg/g) (40, 41). Ellagic acid has high selectivity and specificity towards CK2 with $IC_{50} = 0.04 \mu M$ and $K_i = 20 \text{ nM}$ indicating the highest affinity to CK2 compared to other inhibitors (3, 18). Ellagic acid displays a unique binding mode in which it binds simultaneously to the phosphate binding region in the ATP binding pocket, and the hinge region through hydrogen bonding (42). EA also forms hydrophobic interactions with several CK2 residues that further stabilizes the EA-CK2 complex (43). It is soluble in dimethyl sulfoxide (DMSO), ethanol, and polyethylene glycol (PEG400). Ellagic acid demonstrates higher solubility in PEG400 (8.3mg/ml) compared to ethanol (0.21 mg/ml) and DMSO (2.5mg/ml), thus PEG400 provides the highest drug concentration with minimal toxicity (41).

1.9 Ellagic Acid Anti-Cancer Activity

Ellagic acid was reported to have in vitro anti-cancer activity and in vivo anti-tumor activity in several types of cancer (44, 45). In vitro studies reported that ellagic acid induces cell cycle arrest and apoptosis in human cervical cancer CaSki cells, colon carcinoma, and bladder cancer (46-48). It had also been reported that ellagic acid induces apoptosis through inhibition of NF- κ B in pancreatic cancer MIA PaCa-2 and PANC-1 cells (49). Moreover, animal studies demonstrated that ellagic acid has chemopreventive activity against chemically induced cancers of the skin, esophagus, lung and liver (50-

54). However, the impact of ellagic acid on cellular biochemical and molecular pathways, and its therapeutic potential in animal models remain unclear. Herein we report a pre-clinical study that investigates ellagic acid spectrum of anti-cancer activity, its impact on oncogenic and apoptotic pathways, and its therapeutic potential and tissue toxicity in animal models. Identifying ellagic acid mechanism of action could promote it for cancer therapeutics clinical trials.

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CHAPTER II

EFFECT OF POLYETHYLENE GLYCOL 400 (PEG400) ON ELLAGIC ACID ANTI-CANCER ACTIVITY

2.1 Abstract:

In vitro ellagic acid cytotoxicity studies were mostly performed using dimethyl sulfoxide (DMSO) as a solvent. Even though these studies have showed no cytotoxicity effect from DMSO by itself on cells screened, DMSO is known to be harsh solvent that can induce cell toxicity if used at concentrations > 1%. In this study, polyethylene glycol 400 (PEG400) was used as ellagic acid vehicle. The efficacy in tumor inhibition was compared between PEG400- and DMSO-dissolved ellagic acid treatments. This was accomplished using in vitro anti-proliferative assay to screen a panel of 7 cancer cell lines of different histology including: brain carcinoma cell line U87, HPV positive and HPV negative cervical cancers Hela and C33 cell lines respectively, Breast adenocarcinoma MCF7, Melanoma WM167, colon cancer SW480 cell line, and ACHN kidney cancer.

They all showed sensitivity to increasing micromolar concentration of EA/PEG400 and EA/DMSO at different exposure times. However, C33 and MCF7 cell lines showed chemosensitivity to DMSO treatment. PEG400 showed less toxicity and no interference with EA anti-cancer activity. When cervical cancer cell lines response to EA treatment was compared at the same exposure time, C33 showed two fold increase in sensitivity compared to HeLa.

2.2 Introduction:

Polyethylene Glycol (PEG) is a long chain polymer made of ethylene glycol subunits. PEG400 is a member in the PEGs family with a molecular weight of 400. PEGs have multiple applications in pharmaceutical industry as drug delivery vehicle such as ointment, capsules, and suppositories makeup (1). At higher molecular weight polyethylene glycol by itself can be used for constipation treatment, bowel cleansing and as an ingredient in artificial tear eye drops (1, 2). Also, PEG400 was determined to be widely used in the bases of cosmetics and skin creams (1). The wide use of PEG400 implies the safety and minimal cytotoxicity that can be induced if used as solvent for drug delivery. In addition, ellagic acid solubility studies have determined PEG400 to have higher efficiency in dissolving ellagic acid (solubility = 8.3mg/ml) compared to ethanol (0.21 mg/ml) and DMSO (2.5mg/ml) (3), therefore, providing higher concentration of drug and less solvent.

This study was designed to evaluate PEG400 as a safe and efficient vehicle that would not interfere with EA cancer inhibition effect, and to determine the spectrum of EA anti-cancer activity. During phase I of the study, growth-based cytotoxicity assay was used to survey a panel of 7 aggressive human cancer-cell lines of different histology against PEG400-EA and DMSO-EA treatments at different exposure times ranging from 96-144 hr. The panel screened includes colon adenocarcinoma (SW480), kidney carcinoma (ACHN), melanoma (WM164), brain cancer (U87), breast carcinoma (MCF7), HPV positive and HPV negative cervical carcinomas HeLa and C33. The results from this survey led to phase II of the study in which, a metabolism-based cytotoxicity assay was

used to compare the effect of PEG400-EA treatment on two cell lines of the same histology HeLa and C33 at the same exposure time of 96hr.

2.3 Experimental Procedures:

Drugs and chemicals.

The present study used Ellagic Acid Dihydrate (lot#D00076152), molecular weight = 338.2 provided by Calbiochem. Polyethylene Glycol (PEG) molecular weight 400 was purchased from Fisher Scientific lot# 062734, and Dimethyl Sulfoxide (DMSO) molecular weight = 78.13 was obtained from aMRESCO lot# 2890C058.

Cell Culture.

All cancer cell lines surveyed were maintained in RPMI (Cellgro), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Cellgro). Cells were incubated in a humidified atmosphere with 5% CO₂ at 37° C.

Preparation of EA/PEG400 and EA/DMSO formulations.

In accordance with solubility studies of Ellagic acid, stock solution of EA/PEG400 was made by dissolving 30 mg EA in 4 ml PEG400, Incubated in the dark at 37° C water bath for 5 minutes or until solution became clear and homogenous. EA/DMSO stock was made with 10 mg EA in 3 ml DMSO mixed and kept in the dark. Both solutions were filtered in 0.2 µm syringe filter. Solutions were kept at -20° C after usage.

SRB Antiproliferative Assay.

Growth inhibition effect of ellagic acid was assessed by Sulforhodamine-B (SRB) colorimetric assay (4). HeLa, C33, U87, MCF-7, WM164, SW480 and ACHN were plated in 2- 96 well-plates at concentration of 2000 cells/well. After 24 hours of

incubation, cells were fixed with cold 10% trichloroacetic acid (TCA) in one plate (to determine growth baseline) and the other plate was treated with increasing concentration of ellagic acid dissolved in PEG400 in one trial and DMSO in another. Two controls were set for each cell line, one untreated and one treated with the vehicle only at a concentration equal to that in highest ellagic acid dose. Cells were allowed to grow for 96-144 hours before test was stopped by aspirating the medium and fixing cells with 10% cold TCA for >1 hour. Fixed cells were then washed 3 times with water and dried. Fixed cells were then stained with 0.4% (w/v) SRB dye (Sigma), dissolved in 1% acetic acid for 30 minutes at room temperature. Dye was then extracted in mild basic conditions using 10 mM unbuffered Tris base solution PH 10.5 for 1 minute. The absorbance of dye released from cells was measured by microplate reader at 570 nm. Optical density measurements (OD) of the SRB dye is linear with the number of cells fixed at the bottom of the 96-well plate.

MTS viability assay.

Cytotoxic effect of ellagic acid was assessed using the MTS colorimetric assay. HeLa and C33 cervical cancer cell lines were plated in 96-well plates at concentration of 5×10^3 cells/well. After 24 h of incubation, cells were treated with increasing concentration of ellagic acid dissolved in PEG400 and control was treated with the vehicle (PEG400). Cells were allowed to grow for 96 h before a 20 μ l MTS solution was added to the wells and incubated in the dark at 37° C for 3 h. Optical density (OD) was read at 492 nm using a 1420 WALLAC counter. Optical density readings of the formazan product are linear with the number of viable/metabolically active cells.

Statistical analysis.

Data are expressed as mean \pm SEM from at least 3 independent experiments. Statistical analysis was done using student t-test. $P < 0.05$ was considered statistically significant.

2.4 Results

During phase I of the study, cells were screened with PEG400, DMSO, and ellagic acid in doses dissolved in PEG400 parallel to DMSO dissolved treatments. Cytotoxicity of solvents-treated cells was compared to untreated control. The statistical difference between untreated and solvent treated cells was calculated using t-test. Comparing PEG400-treated and untreated cells, t-test showed a P-value > 0.05 . This indicates no significant difference; therefore, we concluded that PEG400 has no cytotoxic effect on all cancer cell lines surveyed. On the other hand, MCF7 and C33A cells treated with DMSO showed P-value < 0.05 compared to untreated control. Therefore, these cell lines were screened using ellagic acid dissolved in PEG400 only. Overall evaluation of PEG400 versus DMSO revealed that there was no chemosensitivity induced by PEG400 on all carcinoma cell lines tested, while some cell lines showed sensitivity to DMSO. Therefore, PEG400 was determined a safer vehicle for ellagic acid administration compared to DMSO.

To investigate ellagic acid spectrum of anti-cancer activity, we screened different cancer cell lines using ellagic acid doses (6.125-50 $\mu\text{mol/l}$). Using SRB assay, we determined IC_{50} values of cancer cell lines surveyed. MCF-7 cell line demonstrated growth inhibition at 96 hours with $\text{IC}_{50} = 20 \mu\text{M}$, while WM164 had $\text{IC}_{50} > 50 \mu\text{M}$, SW480 $\text{IC}_{50} = 20 \mu\text{M}$, U87 and ACHN IC_{50} were $< 6.25 \mu\text{M}$, HeLa $\text{IC}_{50} = 40 \mu\text{M}$ and C33A cells showed IC_{50} of $10 \mu\text{M}$ at 144 hr of exposure time. This data revealed potent anti-cancer activity by ellagic acid in several cancer types.

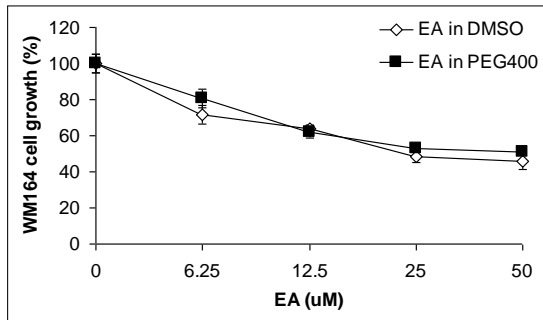
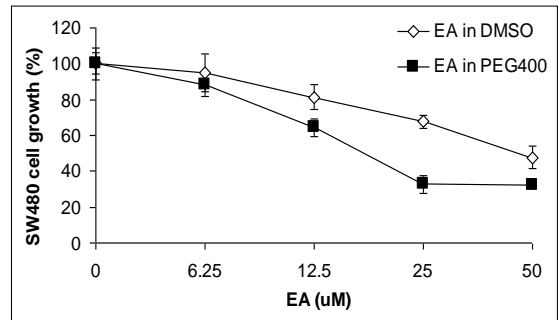
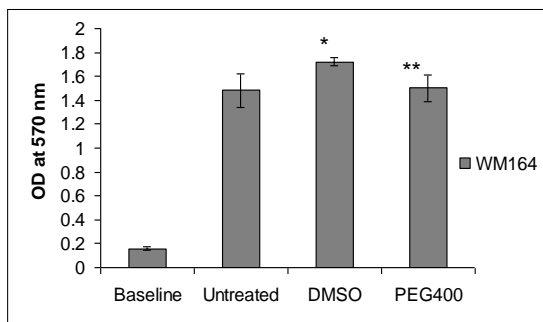
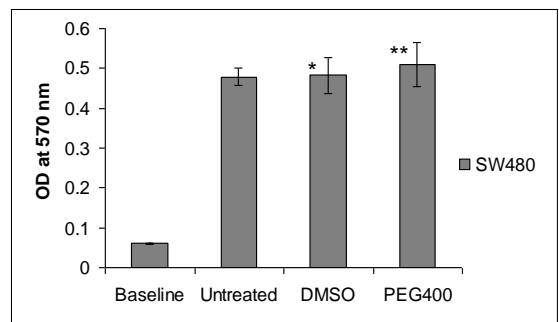
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Figure 2.1. Effect of EA/ DMSO vs. EA/PEG400 formulations on WM164 and SW480 cell growth. A,C. WM164 and SW480 cells were treated with 6.25-50 μ M EA/DMSO and EA/PEG400 for 120 h. Data represent the mean \pm SEM of 8 different cell density readings. B, D. * $p > 0.05$ for DMSO treated vs. untreated control group and ** $p > 0.05$ for PEG400 treated vs. untreated control.

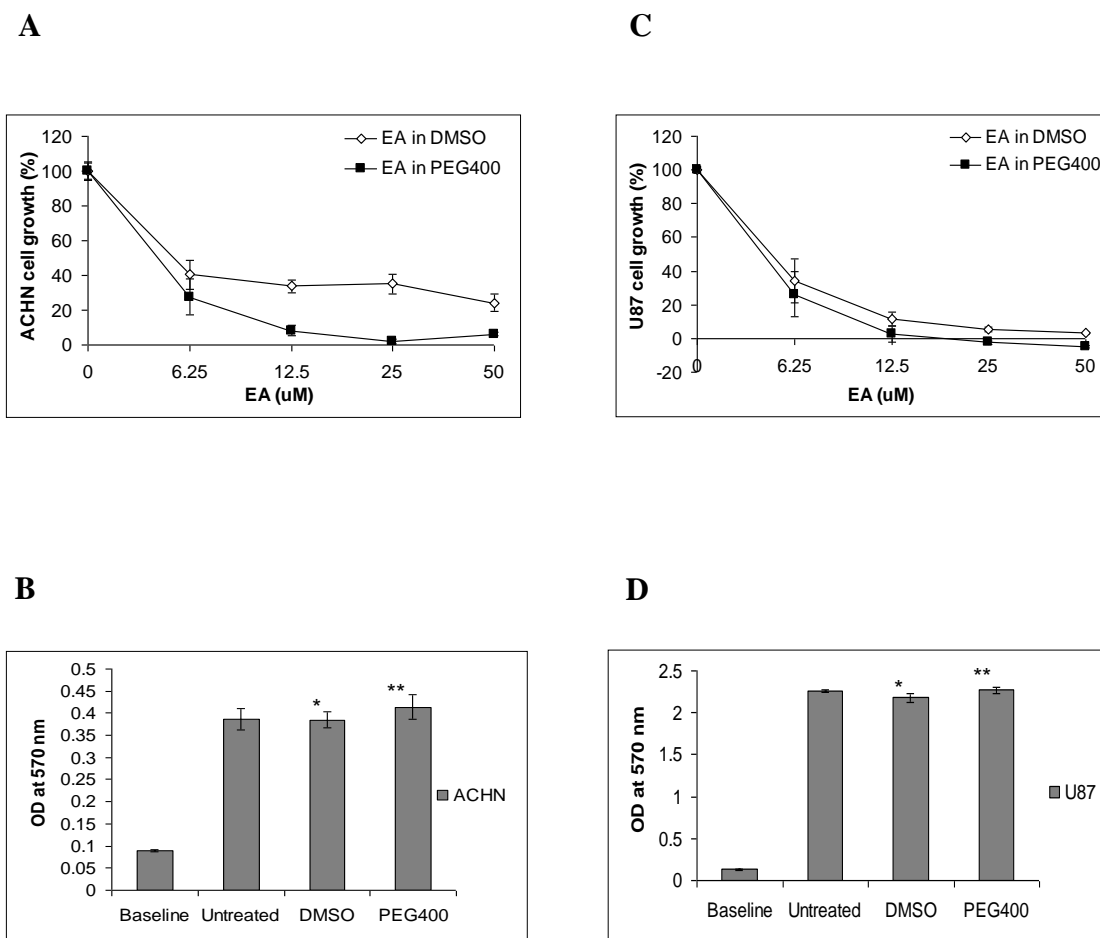


Figure 2.2. Effect of EA/DMSO vs. EA/PEG400 formulation on ACHN and U87 cell growth. **A, C.** ACHN and U87 cells were treated with 6.25-50 μ M EA/DMSO and EA/PEG400 for 120 and 96 h respectively. Data represent the mean \pm SEM of 8 different cell density readings. **B, D.** * $p > 0.05$ for DMSO vs. untreated control and ** $p > 0.05$ for PEG400 vs. untreated group for both cell lines.

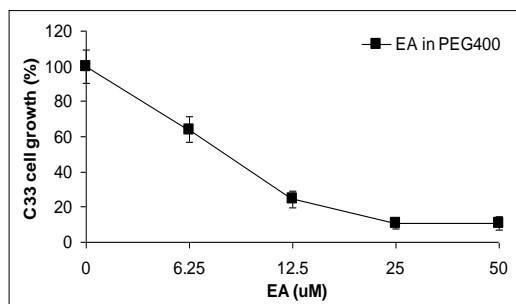
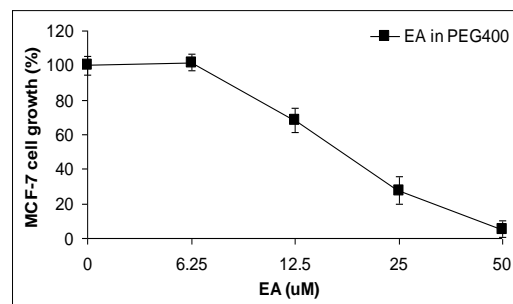
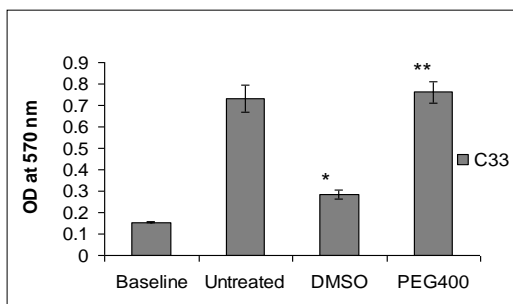
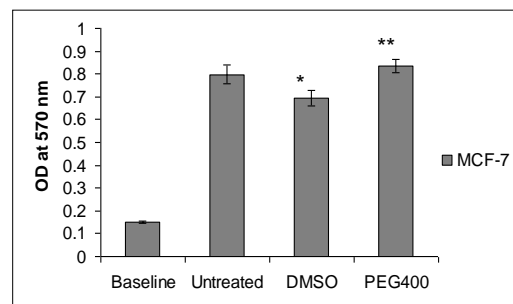
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Figure 2.3. Effect of EA/DMSO vs. EA/PEG400 formulation on C33 and MCF-7 cell growth. **A, C.** C33 and MCF-7 cells were treated with 6.25-50 μ M EA/DMSO and EA/PEG400 for 144 and 96 h respectively. Data represent the mean \pm SEM of 8 different cell density readings. **B, D.** * $p < 0.05$ for DMSO vs. untreated control and ** $p > 0.05$ for PEG400 vs. untreated group for both cell lines.

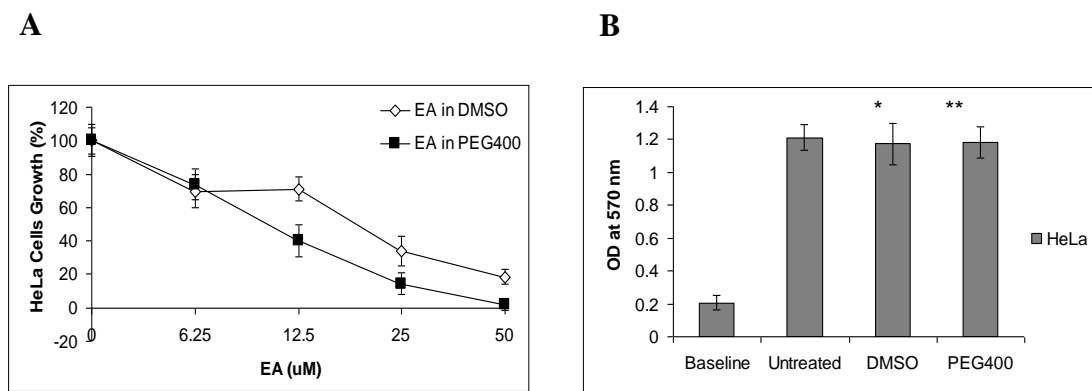


Figure 2.4. Effect of EA/DMSO vs. EA/PEG400 formulations on HeLa cell growth.
A. HeLa cells were treated with 6.25-50 μ M EA/DMSO and EA/PEG400 for 96 h. Data represents mean \pm SEM of 8 different cell density readings. **B.** * $p > 0.05$ for DMSO vs. untreated control, and ** $p > 0.05$ for PEG400 vs. the untreated group.

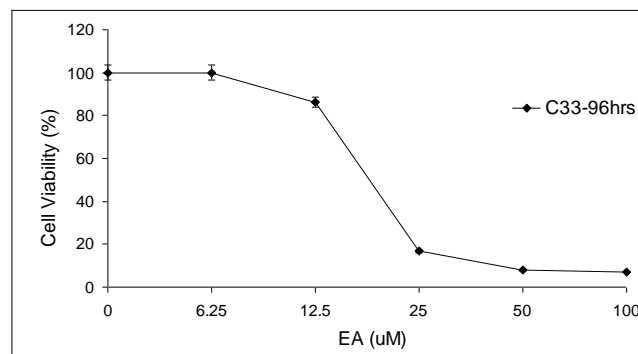
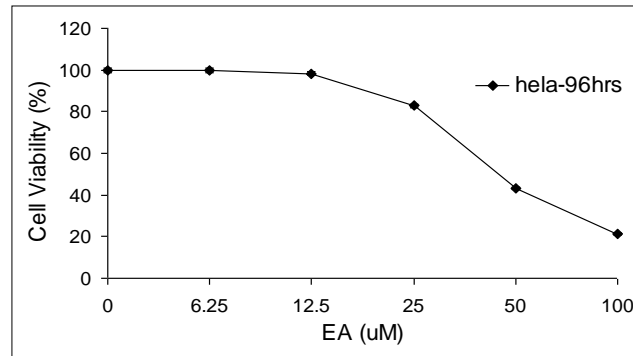


Figure 2.5. Comparison analysis of EA/PEG400 effect on HeLa vs. C33 cell lines. Cells were treated with 6.25-50 μ M EA/PEG400 doses for 96 h. Data represents mean \pm SEM of 2 independent MTS assays.

Cell Line	IC₅₀ (μM)
MCF7 (Breast Cancer)	18
WM164 (Melanoma)	50
U87 (Brain Cancer)	4
SW480 (Colon Adenocarcinoma)	18
ACHN (Kidney Cancer)	4
HeLa (HPV+Cervical Cancer)	45
C33 (HPV-Cervical Cancer)	18.7

Table 2.1. Determination of the EA IC₅₀ values for each cell line screened.

2.5 Discussion

The potential use of ellagic acid for in vivo pharmaceutical treatment requires safe vehicle to deliver the drug. This initial study points out the potential of PEG400 as a particularly safe vehicle for ellagic acid delivery for in vivo studies or clinical trials.

Testing the toxicity of the solvent PEG400 revealed no growth inhibition or viability reduction on all cell lines screened. Moreover, its use as a solvent for ellagic acid therapy did not compromise ellagic acid anti-cancer activity. However, for drug development and discovery, other factors like cellular uptake and metabolism, and its effect over long-term drug regimentation should be assessed in future studies.

As already mentioned in the results section, PEG400-ellagic acid was able to induce cancer cell growth inhibition at micromolar concentrations when screened against a panel of seven aggressive human malignancies. This anti-proliferative activity presented in this study reinforces the previously documented anti-cancer ability of ellagic acid in a wide range of cancer types. However, the novelty of this study is represented in the use of PEG400 for EA administration for maximal safety and efficiency.

It is noteworthy that the SRB assay used to assess ellagic acid inhibitory effect on different cancer cell lines from different tissues is used for large scale anti-cancer drug discovery program at the National Cancer Institute (NCI) (5, 6). However, this assay depends on the growth rate of cells. Therefore, exposure time differs from one cell line to another depending on how fast these cells grow and reach 70-80% confluence before the test can be terminated. Therefore, to compare two cell line's response to ellagic acid

treatment, exposure time has to be fixed which is not possible with SRB assay. The MTS reagent is metabolically reduced by mitochondria to formazan colored products (7, 8). It is a time sensitive method but it is not affected by growth rate thus exposure time can be controlled for comparison purposes. Upon comparison, cervical cancer cell lines HeLa and C33 responded differently to ellagic acid treatment at 96 hr. This difference in sensitivity raises the question of why HeLa responds differently to ellagic acid treatment compared to C33. We initially hypothesized that HPV oncogenic activity may delay HeLa response to EA-induced death stimuli. This suggests differences in the underlying mechanism of action of ellagic acid in HeLa and C33 cells. Therefore, HeLa and C33 are suitable models to study the role of EA-mediated CK2 inhibition in cancer cells. The upcoming studies are focused on identifying EA mechanism of action through the evaluation of the differences in CK2 inhibition, and its impact on HPV oncogenic activity, cell cycle progression, and apoptotic pathways. In vivo studies are also performed to validate EA suitable for a clinical trial.

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CHAPTER III

HPV-POSITIVE CERVICAL ADENOCARCINOMA:

ELLAGIC ACID INHIBITS MULTIPLE ONCOGENIC PATHWAYS IN HELA CELL LINE IN VITRO AND EXERTS ANTI-TUMOR ACTIVITY IN VIVO.

3.1 Abstract

Cervical cancer is the second most common malignancy among women worldwide. Herein, we report that ellagic acid (EA), a natural polyphenolic compound, exerts anti-proliferative and pro-apoptotic activity in HPV18 (+) human cervical adenocarcinoma cells (HeLa) in a dose- and time-dependent manner. Inhibition of casein kinase 2 (CK2) activity by ellagic acid resulted in abrogation of HPV oncogene E6 and E7 expression. The decrease in E6 expression correlated with stabilization of p53 and apoptosis induction. EA induced mitochondrial-mediated caspase activation via the release of cytochrome c into the cytosol and subsequent poly (ADP) ribose polymerase (PARP)

fragmentation and cell death. The decrease in E7 correlated with inhibition of cyclin A expression, and inhibition of cell cycle progression. Finally, treatment of athymic-nude mice treated EA resulted in inhibition of cervical cancer xenograft growth and longer survival compared to non-treated mice, confirming the potent anti-tumor activity of EA. Our data strongly suggest that EA has anti-tumor activity which could make it suitable for the prevention of HPV induced cervical carcinoma.

3.2 Introduction:

Cervical cancer is the second most common malignancy among women worldwide with >80% of the cases occurring in developing countries (1). The geographic distribution of cervical cancer is highest in Latin America, sub-Saharan Africa, south Asia and Southeast Asia (2). Indonesia, with a population of 200 million, has 79.14 million women age 15 years and older who are considered to be at high risk of developing cervical cancer. According to World Health Organization (WHO) 2010 data, cervical cancer cases in Indonesia were estimated to be 13,762 with a mortality rate of 7,493 women per year. Cervical cancer ranks as the second most prevalent cancer among women between 15 and 44 years of age in Indonesia (3). Human papilloma virus HPV16 and 18 DNA was discovered in cervical malignancies by Harald zur Hausen (winner of the Nobel Prize in Medicine, 2008). High risk types HPV 16 and 18 account for almost 50% of all female genital HPV infections, and they are present in 90% of human cervical carcinomas (4, 5). Therefore, HPV16 & 18 has been identified as the most prominent etiologic factor in cervical malignancy. According to WHO Information Center on HPV and Cervical Cancer, 80.1% of invasive cervical cancers in Indonesia are related to HPVs 16 and 18.

Viral survival in host cells depends on disrupting normal cellular machinery, inhibiting cellular defense mechanisms and exploiting cell signaling pathways to stimulate viral DNA replication and the production of viral particles. Persistent HPV infection leads to viral DNA integration into the host genome. Oncogenesis results when cells escape normal constraints on cell survival and proliferation. Loss of cellular control

over HPV oncogene transcription activity leads to induction of viral oncogene E6 and E7 expression (6). E6 removes normal constraints on cell survival while, E7 removes normal constraints on cell proliferation. Thus, elevated viral oncogene expression is essential to the transformation and immortalization of human cervical epithelial cells (7, 8).

High risk (HR) HPV E6 targets wild-type (wt) tumor suppressor protein p53 for ubiquitin-mediated degradation (7, 9). p53 plays an essential role in the host's defensive mechanism against DNA damage. Tumor suppressor protein p53 can induce apoptosis in a transcription-dependent pathway by increasing expression of DR5 and FAS/APO1 (membrane death receptors) and the pro-apoptotic Bcl2 family members (10-12). In addition, p53 exerts its tumor suppressive activity via a transcription-independent pathway involving p53 translocation to the mitochondria. At the mitochondria, p53 causes oligomerization and activation of the pro-apoptotic Bcl2 proteins that increases mitochondrial outer membrane permeability for the pro-apoptotic protein cytochrome c to be released into the cytosol and activate the caspase cascade (4, 13). Oncoprotein E7 causes dysregulation of the cell cycle by binding pRb, p107 and p130 (14, 15). HPV-E7 specifically targets pRb at the E2F binding site. pRb-free-E2F are active transcriptional factors that induce gene expression of proteins controlling S-phase progression of the cell cycle such as cyclin E and cyclin A which leads to uncontrolled cell cycle progression and cell proliferation (16). Hence, HPV oncogenic activity mediates malignant transformation of epithelial tissue.

This study demonstrates the in vitro and in vivo effect of EA on HPV18-positive cervical cancer cells. The HeLa cell line expresses elevated levels of CK2 as well as

HPV18 oncoproteins E6 and E7; thus it represents a good model to study the role of EA-mediated inhibition of CK2 activity on cell proliferation, survival, and HPV oncogene expression.

3.3 Experimental Procedures:

Cell culture.

HeLa cells were maintained in RPMI (Cellgro), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Cellgro). Cells were incubated in 5% CO₂ at 37° C.

CK2 activity assay

CK2 activity in HeLa cells was measured using whole cell lysates. Cells were extracted using ice-cold hypotonic buffer containing 20 mM Tris buffer, pH 8.0, 10% glycerol, 0.05% nonidet-P40, 2.0 mM EDTA, 2.0 mM EGTA and a cocktail of protease and phosphatase inhibitors. Cell suspension was kept on ice for 30 minutes, and then centrifuged at 10,000 xg for 10 minutes at 4° C. Endogenous kinase activity of CK2 was measured by CKII assay Kit (Upstate Biotechnology/ Millipore). 1 µg protein of cell lysates was added to a total volume of 40 µl containing assay dilution buffer (20mM MOPS, pH7.2, 25mM β-glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, and 1mM dithiothreitol), 0.4 mM of CK2-specific substrate peptide (R₃D₃SD₃), inhibitor cocktail to block activity of other serine/threonine kinases in lysates, and diluted [γ -³²P] ATP in MgCl₂. The mixture was incubated at 30°C for 10 minutes, and then reaction was stopped by adding 20 µl of 40% trichloroacetic acid (TCA). Twenty-five microliter of reaction mixture was transferred to P81 paper discs and allowed to bind for 30 seconds. Paper discs were washed 3 times with 0.75% phosphoric acid for five minutes each and one time with acetone, allowed to dry before they were transferred to scintillation vials with 2 ml of scintillation cocktail. Radioactivity of CK2 was counted using scintillation

counter, expressed as c.p.m percentage of control radioactivity. Parallel to CK2 activity assay, CK2 expression level was analyzed by standard method for western blotting.

Cell cycle analysis

HeLa cells were plated at density of 1×10^5 cell/well and incubated for 24h with 50 μ M EA. The cells were then harvested and fixed in 90% ethanol at 4° C. Cells were then washed with PBS and resuspended in a solution containing propidium iodide, sodium citrate, triton X and RNase, and incubated at 37° C in the dark. After 30 min of incubation, samples were analyzed by Cytomics FC500 flow cytometer (Beckman Coulter), Quantitative cell cycle analysis was performed using CXP software.

Trypan Blue Assay.

Trypan blue dye exclusion assay was done to test cytotoxicity of cervical cancer cell lines to ellagic acid in time dependent manner. HeLa cells were treated with 50 μ M ellagic acid for 24, 48, 72 and 96 h. At specified time points samples were trypsinized and harvested and an aliquot of 500 μ l was counted using Beckman Coulter Vi-Cell XR cell viability counter. Results were then compared to control at (0 h) which was treated with Peg400 only and collected after 96 h of incubation time.

SYBR Green-quantitative polymerase chain reaction (syber green-qPCR) detection.

Total RNA from treated cells was extracted using RNeasy mini kit (QIAGEN). cDNA was synthesized according to manufacturer's protocol for reverse transcriptase (RT). Primers for HPV18-E6/E7 and β -actin were synthesized by Invitrogen. Sequence for

HPV18-E6 primer:

5'-GGTGCCAGAAACCGTTGAATC-3',

3'-CGAATGGCACTGGCCTCTATAG-5'

HPV18-E7 primer:

5'-CGAACCACAACGTCACACACAAT-3'

3'-TGCTGGAATGCTCGAAGGT-5'

β - Actin primer:

5'-CCGAGGACTTTGATTGCACA-3'

3'-AGTGGGGTGGCTTTTAGGAT-5'

HPV Levels of m-RNA for HPV18 oncoproteins in treated HeLa cells were determined using SYBR green master mix (Clontech) and Fast 9400 real time PCR system (Applied Biosystems). The qPCR reaction total final volume was 20 μ l consisting of 10 μ l SYBR green 2x master mix, 0.2 μ l of forward and reverse primers (final concentration 0.2 pmol/ μ l) as well as 0.2 μ l of ROX reference dye and 9.4 μ l of H₂O. Instrument was set for regular 2 hours qPCR running conditions. Instrument run started with holding stage at 50°C for 20 seconds, and 96°C for 10 minutes. This was followed by 40 amplification cycles of 95°C for 15 sec, 60°C for 1 min and the melting curve to assess specificity of PCR product. The data were analyzed using comparative C_T ($\Delta\Delta C_T$) and target genes were quantified using the formula ($2^{\Delta\Delta C_T}$) and normalized to an endogenous reference (β -actin) that served as loading control. Final analysis of target genes expression was compared to

control group (Time 0). Average gene expression values were then blotted in bar graph from n=6 for HPVE7 and n=3 for HPVE6.

Oncoproteins E6 and E7, as well as their downstream targets (p53 and cyclin A) expression levels were evaluated by resolving 35 µg of total cell lysate using western blots and probing with HPV18-E6 and HPV18-E7 antibody (Abcam).

Annexin-V assay/ flow cytometry.

HeLa cells were treated with 50 µM ellagic acid and Peg400 (control) and incubated at 37°C. Cells were trypsinized and harvested at 24, 48, 72 and 96 h, washed once with cold PBS and resuspended in 100 µl Annexin-V binding buffer at concentration of 1×10^3 cells /µl. According to the manufacturer's protocol, cells were then incubated with FITC-Annexin V and propidium iodide (PI) solution (FITC Annexin V Kit II, BD pharminogen) for 15 min at room temperature in the dark. Apoptosis induction was then analyzed on Cytomics FC500 flow cytometer (Beckman Coulter), using CXP software.

Apoptosis array analysis.

HeLa cells were plated in 100 mm dishes at a density of 1×10^5 cells/ml. Cells were treated with 0 or 50 µM ellagic acid and incubated at 37° C for 96 h. After trypsinizing and harvesting of cells, they were rinsed with PBS and extracted at 1×10^7 cells/ml in kit lysis buffer (Human Apoptosis Array Kit; R&D Systems). The lysates were resuspended and rocked gently at 4° C for 30 min. After centrifuging at 14,000 x g for 5 min, the supernatant was transferred into a clean tube. 400 µg protein was incubated with apoptosis array membrane according to manufacturer's protocol. Arrays were read by the

typhoon and quantitative analysis of protein levels was done using Image Quant TL v2005.

Immunoblot.

Total cell lysate was prepared by lysing the cells in a buffer containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), and 50 mM Tris at pH 8.0 with a cocktail of protease inhibitors. Protein concentrations were determined using BCA protein assay (Biorad). An aliquot of 35 µg protein was taken from all samples and resolved on 12% polyacrylamide-SDS gel and transferred to PVDF membrane using semi dry transfer method. The membrane was blocked with 5% milk and incubated with anti-PARP antibody, anti-Caspase 3 (Cell Signaling).

Cytochrome c release

HeLa cells treated with 50 µM ellagic acid for 0, 24, 48, and 72 hours were swelled using hypotonic solution containing (20 mM HEPES, pH 7.5, 10 mM KCL, 1.9 mM MgCl₂, 1.0 mM EGTA and 1.0 mM EDTA, and a cocktail of protease inhibitors on ice for 30 minutes, then homogenized using dounce homogenizer. Cell lysate was centrifuged at 800 xg for 15 minutes at 4° C to pellet nuclei and cellular debris. Supernatant was transferred to new microfuge tubes and spun down at 6000 xg for 15 minutes at 4° C to pellet mitochondria (17). Supernatant from second centrifugation were transferred to new microfuge tubes, protein was measured using BCA assay (Biorad), and

resolved on 12% polyacrylamide gel. Gel was then transferred on PVDF membrane, blocked with 5% milk and probed with anti-cytochrome c antibodies.

Ellagic acid effect on HeLa xenograft.

Animal experiments were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic. Female athymic nu/nu mice were purchased from Case Comprehensive Cancer Center (CWRU). Cell inoculation was achieved by injecting mice subcutaneously with 1×10^6 HeLa cells suspended at 1×10^7 cells/ml. Mice were randomized into two groups and were monitored daily until tumors reached approximately 100 mm^3 in volume. Control groups received vehicle (Peg400) given intratumorally (IT) in a volume of 25 μl . Second group received ellagic acid treatment intratumoral at concentration 8 mg/kg. Treatments were given three times a week, tumor dimensions were measured using calipers and volume of tumor was calculated using the formula ($\text{Volume} = \frac{4}{3} \pi a^2 b$, where a=minor radius and b=major radius of the tumor).

Histology and immunohistochemistry.

Animals were euthanized and organs (liver, kidney, lungs, and spleen) along with tumors were harvested and fixed in 10% neutral buffered formalin. Sections of organs and tumors were stained with hematoxylin and eosin (H&E). Slides were viewed microscopically.

3.4 Results

Ellagic acid inhibits CK2 kinase activity.

To confirm the inhibitory effect of ellagic acid on CK2 activity in HeLa cells, kinase activity was measured in the presence of a specific peptide substrate. A decrease in CK2 kinase activity following EA treatment was at maximum at 72 h followed by a drug rebound effect evident at 96 h (Fig. 3.1A). CK2 expression was not decreased, at the contrary; there is an increase in CK2 expression after 72 h of ellagic acid treatment (Fig. 3.1B)

In vitro anti-proliferative and pro-apoptotic effect of EA.

To assess the effect of EA on cell cycle progression, flow cytometry was performed. Ellagic acid induced marked increase of S- phase from 15% to 32.3%, with a concomitant decrease in G₀/G₁ phase from 51.3% to 22.1% (Fig. 3.2). To test EA effect on viability of HeLa cells, cell count of trypan blue stained cells was performed. Treatment with fixed concentration of 50 μ M EA resulted in inhibition of HeLa cells viability in time dependent manner. HeLa IC₅₀ value was determined to be 45 μ M EA (Fig. 3.3)

Inhibition of E6 and E7 expression level, and its effect on downstream target proteins.

Quantitative PCR analysis showed that EA caused a 40% reduction in the level of E6 and E7 mRNA at 24 hours. The inhibition gradually increased to > 80%, reducing E6 and E7 mRNA to 20% of control (Fig. 3.4A). Similar reduction in protein expression was also seen with immunoblotting (Fig. 3.4B). To correlate the inhibition of E6 and E7

expression on their downstream protein targets, we tested the expression of p53 and E2F transcriptional target, cyclin A (Fig. 3.4C). Inhibition of E6 resulted in increased expression of p53 in HeLa cells, and cyclin A was reduced in expression following decreased E7. Overall, our data demonstrates that EA inhibits E6 and E7 expression, thus neutralizing their pathological effect on these critical downstream target proteins.

Ellagic acid induces apoptosis.

In figure 3.5A, we measured apoptosis by Annexin V-FITC staining which detects phosphatidyle serine (PS) exposure in the outer leaflet of the plasma membrane. A fixed 50 μ M EA treatment caused a shift to FITC-annexinV-positive/PI-negative cells (early apoptosis) and FITC-annexinV-positive/PI-positive cells (late apoptosis). Figure 3.5B, a quantitative time- dependent analysis indicates a significant increase in the total number of apoptotic cells (early + late) with increased time of EA treatment.

Ellagic acid targets activates apoptotic proteins in HeLa cells.

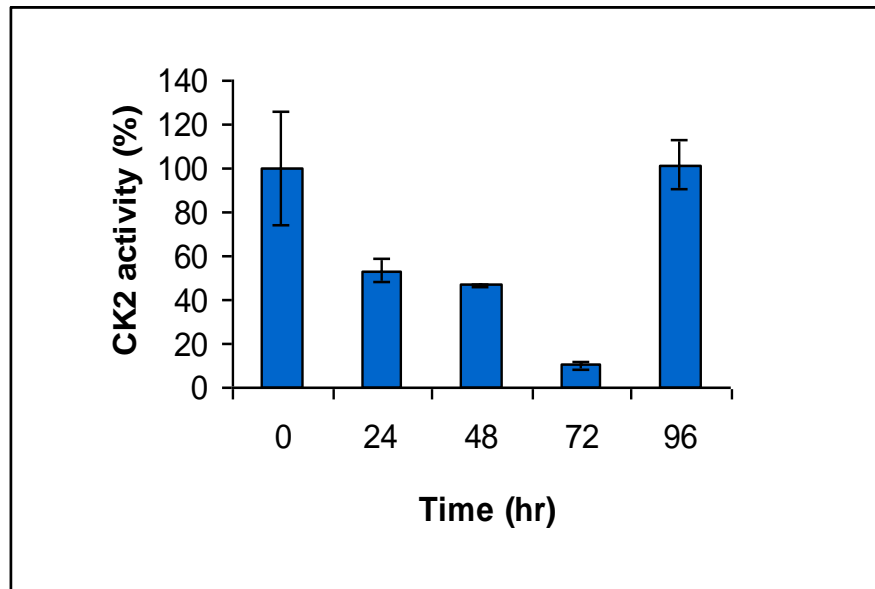
We next examined the effect of EA on various pro-apoptotic and anti-apoptotic components by fractionation, western blotting, and array analysis. Consistent with the observed apoptosis induction, we found that EA treatment induced caspase 8 cleavage, cytochrome c release into the cytosol and subsequent caspase-3 and PARP cleavage (Fig. 3.6). Array analysis showed that incubation of HeLa cells with EA results in a dramatic decrease in expression in the IAP's family of apoptosis inhibitors specifically cIAP-1, XIAP and survivin, and an increase in TRAIL R2/ DR5 death receptor (Fig. 3.7A, B).

This data implicates upregulation of pro-apoptotic proteins and downregulation of anti-apoptotic proteins in the EA mechanism of action.

In vivo anti tumor effect of ellagic acid.

Both intra-tumoral and intra-peritoneal administration of ellagic acid resulted in inhibited tumor growth in HeLa xenografts. All vehicle-treated mice exhibited progressive increase in HeLa tumor volume. HeLa tumors showed a 55% reduction in IT-treated tumors and 87% in IP (Fig. 3.8, 9). To further prove the effect of ellagic acid on cervical cancer tumors, we treated HeLa control animals with 8mg/kg ellagic acid intratumorally starting day 57. Treated tumors became black and necrotic. With H&E tissue analysis of control and treated tumor sections, treated tumor area appeared to be vacuolated containing cellular debris which is indicative of necrosis (Fig. 3.10). H&E staining of major organs revealed no tissue toxicity following EA treatment (Fig. 3.11).

A



B

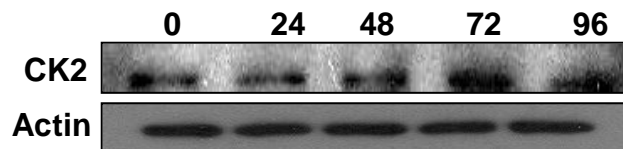


Figure 3.1. Ellagic acid inhibits endogenous CK2 phosphorylation activity.

A. CK2 phosphorylation activity was drastically inhibited in hela cells treated with 50 μ M ellagic acid for 24, 48 and 72 hours. CK2 activity was calculated compared to control sample at time 0 and the mean of a triplicate run \pm SE are shown. At 96h a drug rebound effect was evident. **B.** Immunoblot representing CK2 expression level with ellagic acid treatment and β -actin as an internal control. Immunoblots showing that ellagic acid inhibits CK2 kinase activity but does not decrease CK2 expression.

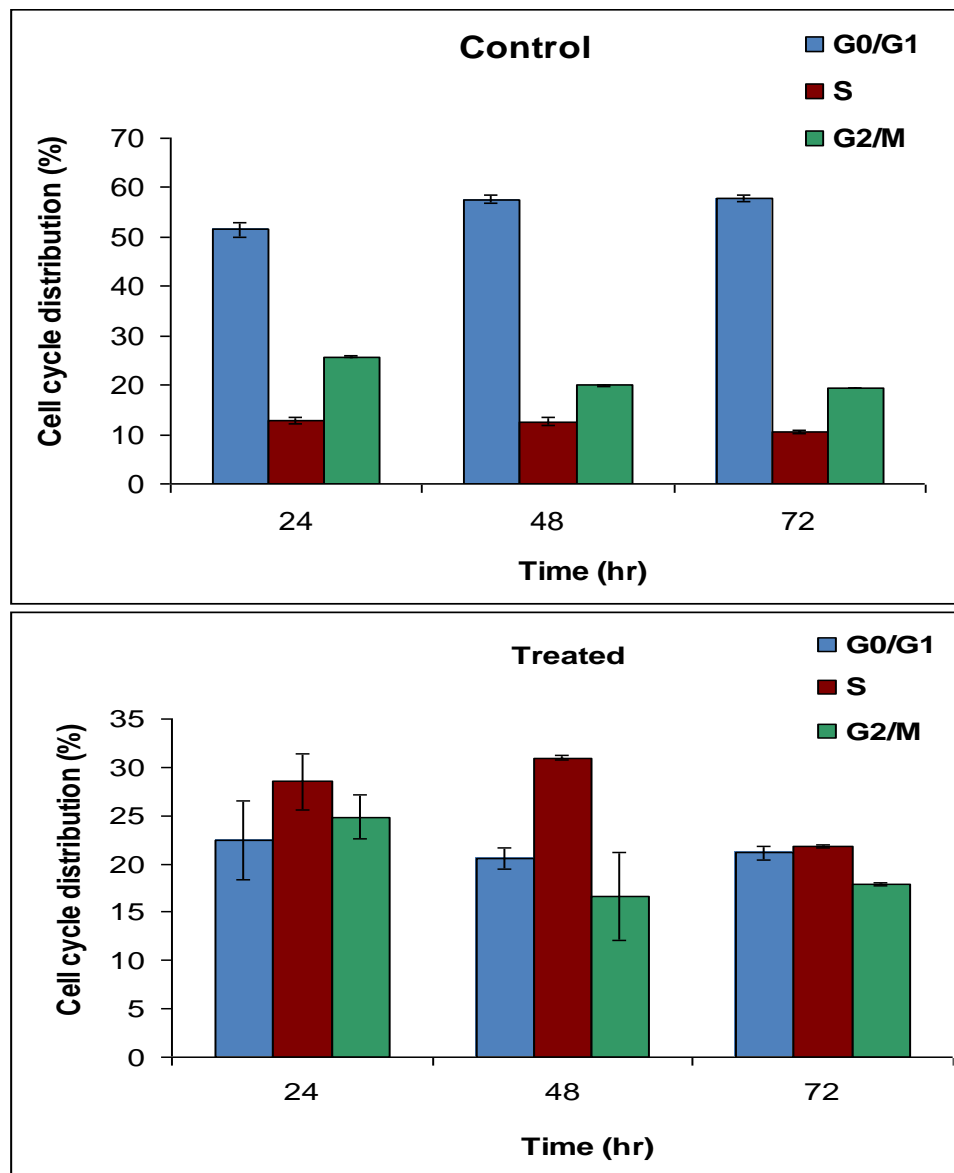


Figure 3.2. Ellagic acid causes S-phase arrest in HeLa cells.

Flow cytometry analysis of HeLa cells exposed to 50 μ M ellagic acid at indicated time points shows significant increase in cells accumulated in S-phase relative to control cells. Data shown are based on the mean of $n=3 \pm \text{SE}$.

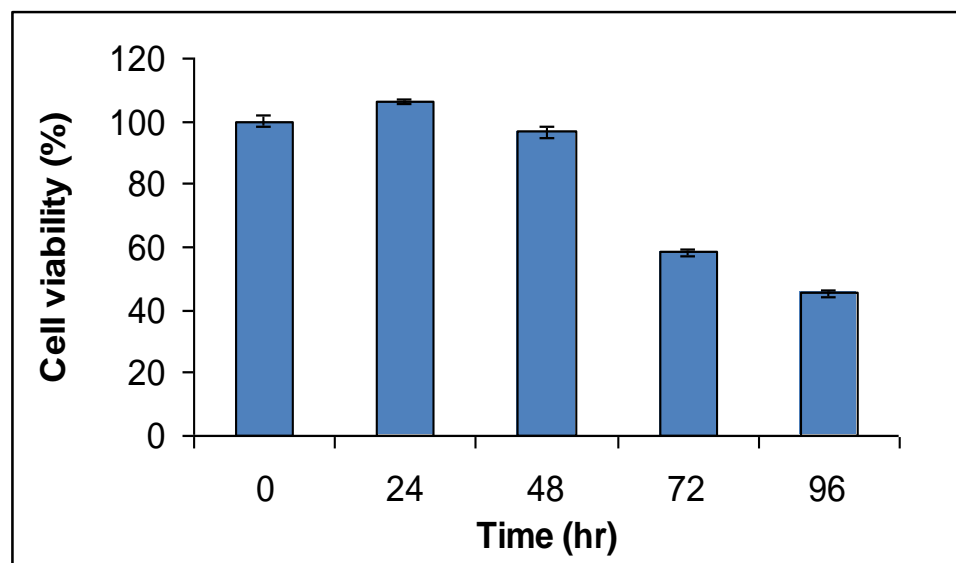


Figure 3.3. The effect of Ellagic acid treatment on HPV (+) cervical carcinoma viability.

A 50 μ M ellagic acid dose induced marked reduction in HeLa cells viability in time dependent manner. A single dose administration was effective up to 96 h of incubation time.

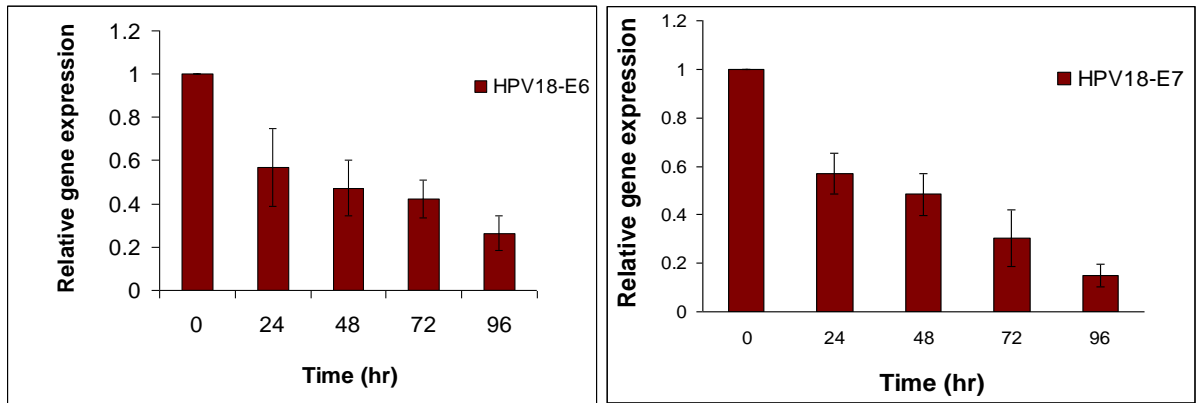
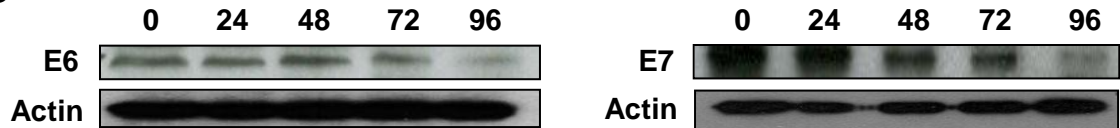
A**B****C**

Figure 3.4. Ellagic acid inhibits HPV18 oncogenes E6 and E7 expression.

A. Utilizing quantitative PCR analysis and based on 6 separate experiments, E6- and E7-mRNA levels were markedly decreased following a single dose of 50 μ M ellagic acid. E6- and E7- mRNA expression was compared to internal control (β -actin) and data presented in mean \pm SE. **B.** Representative immunoblots of E6 and E7 protein expression showing a decrease in protein levels as well. **C.** Data present stabilization of p53 and decrease in cyclin-A protein level as a consequence of E6 and E7 inhibition.

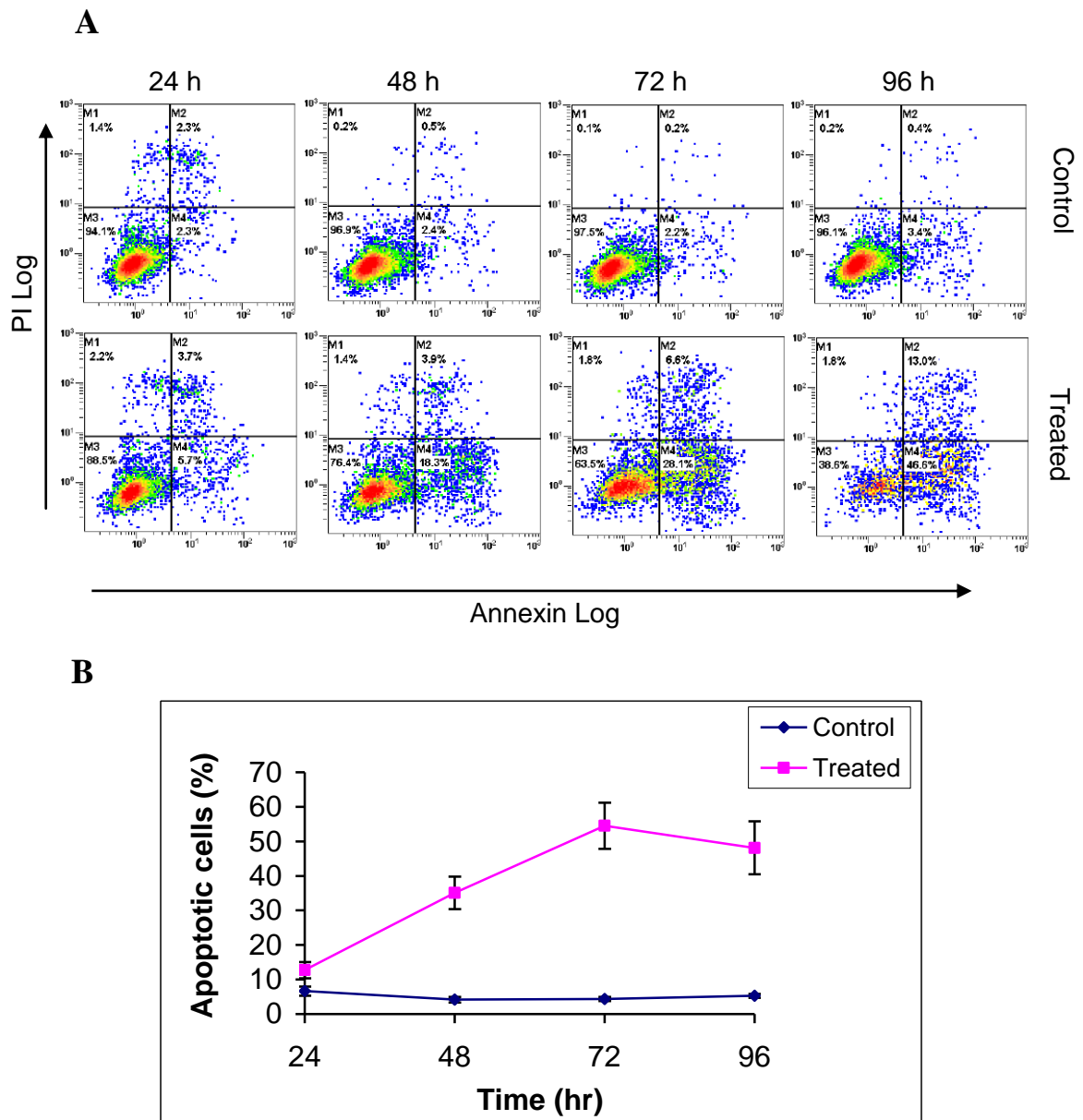


Figure 3.5. FITC Annexin V staining illustrates apoptosis induction.

A. A density scattergram showing viable untreated (control) HeLa cells mostly concentrated in M3 (PI negative, FITC Annexin V negative). With increasing time of EA treatment, HeLa cells are tracked in M4 (PI negative, FITC Annexin V positive) and M2 (PI positive, FITC Annexin V positive) which indicate early apoptotic and dead cells respectively. **B.** Representative quantitative analysis showing gradual increase of early apoptotic and dead cells with increasing exposure time to EA treatment. Results are presented as mean \pm SE of $n=3$.

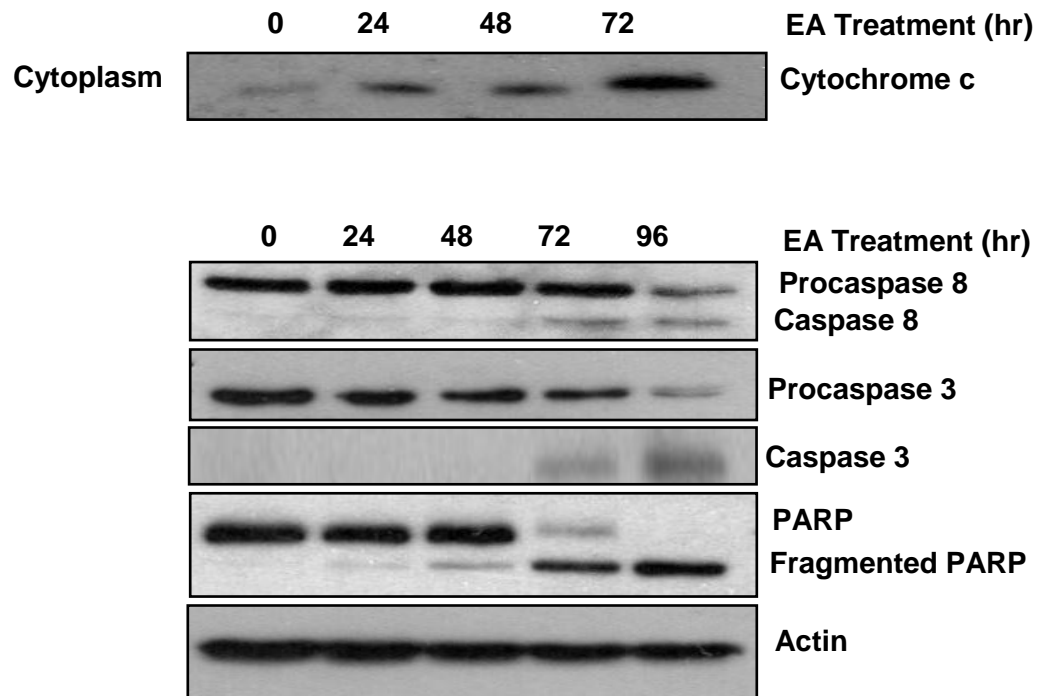


Figure 3.6. Ellagic acid treatment induces caspase-mediated apoptosis.

Immunoblot analysis of apoptosis executioner proteins, indicate the activation of caspase3 and caspase8 and the involvement of mitochondrial apoptotic pathway by the realease of cytochrome c into the cytosol.

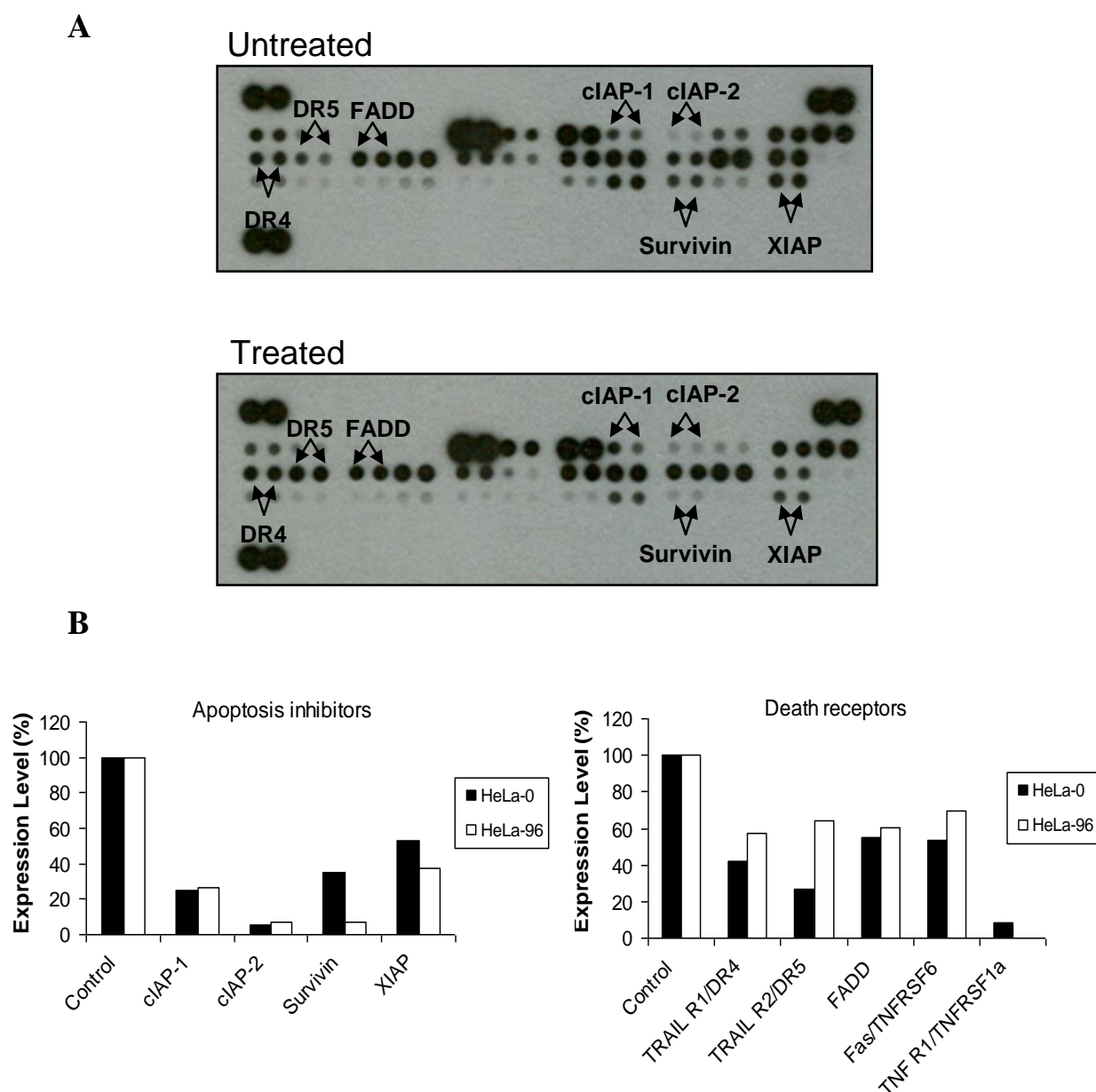


Figure 3.7. Apoptosis array analysis EA-treated HeLa cells.

A. Apoptosis array profile of control and treated HeLa cells. Most significant differential expression was noticed in apoptosis inhibitors and receptors in control vs. treated proteome. **B.** Quantitative analysis was done using the average pixel density of duplicate protein spots compared to positive control. P-value calculated for surviving, XIAP DR4 and DR5 pixel density < 0.05.

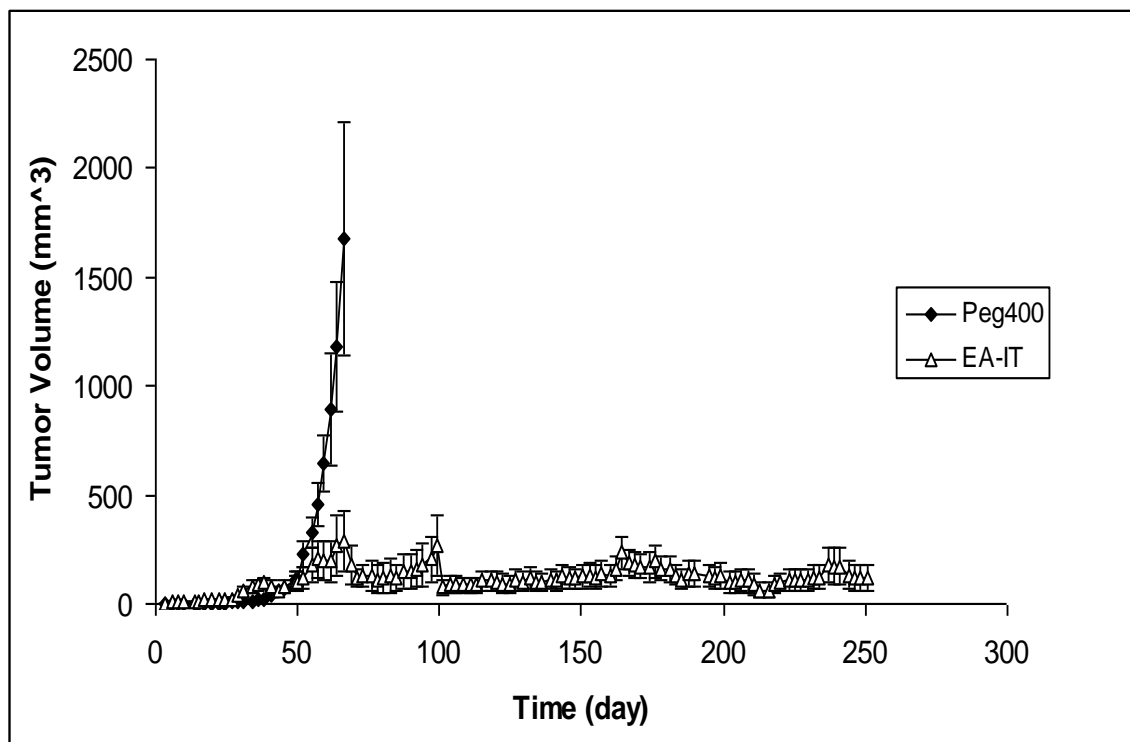


Figure 3.8. Tumor growth response to IT-EA treatment

Ellagic acid inhibited the growth of HeLa xenografts via intratumoral administration. Data represent the mean of 6 tumors for control and 8 for treated group. Treatment was terminated on day 94. Tumor volume did not increase even after termination of treatment.

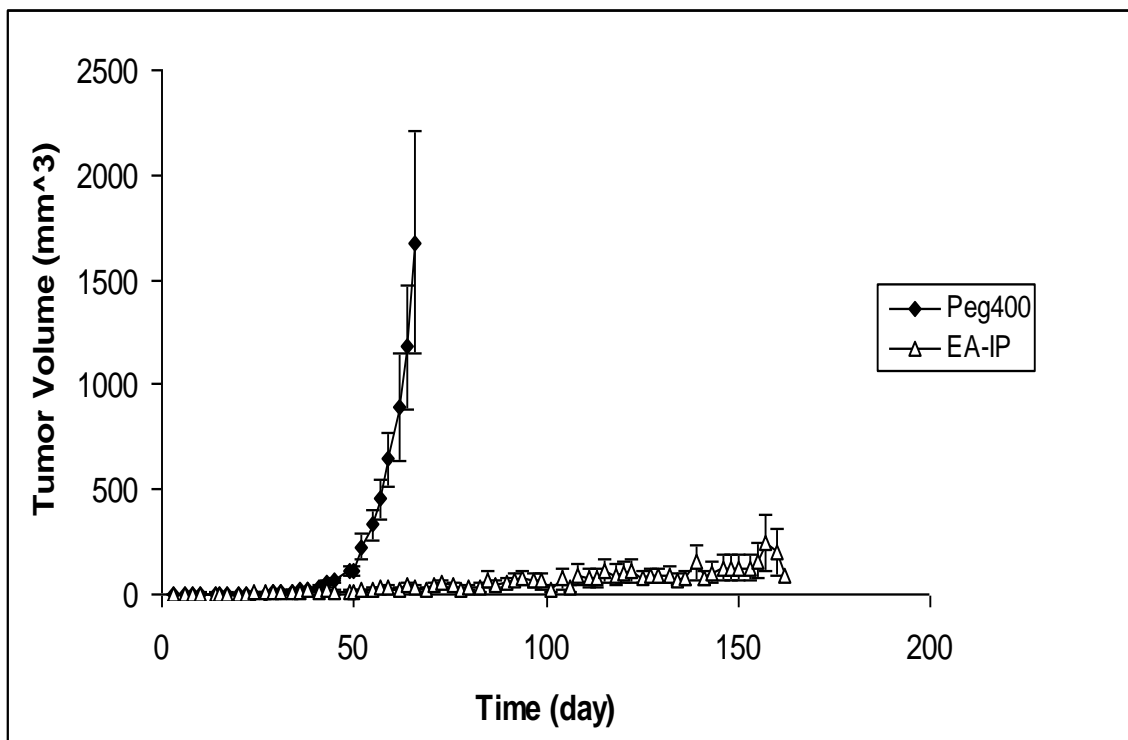


Figure 3.9. Tumor growth response to IP-EA treatment.

Mice were treated with ellagic acid via IP injections. Control group received vehicle (Peg400). Control tumor volumes continued to grow while treated- tumor growth was repressed. Data points represent the mean of $n=6$ tumor measurements \pm SEM. Treatment was terminated at day 94 and tumor growth was monitored until animal model ceased.

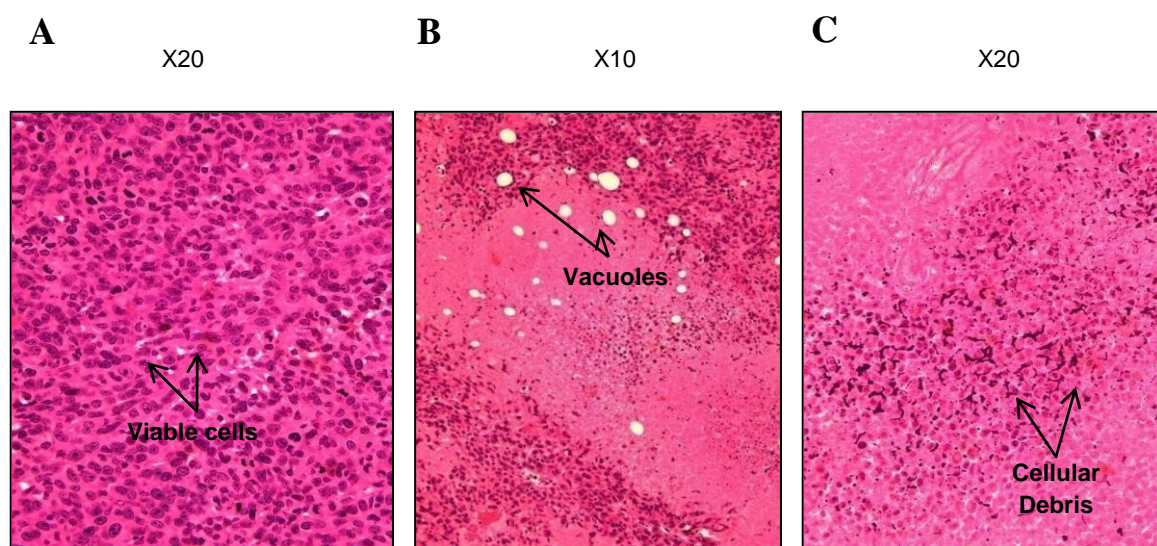


Figure 3.10. Tumor sections H&E staining.

A. control tumor section showing viable tumor cells with no sign of necrosis (vacuolization or cellular debris). **B & C.** intratumorally treated sections showing vacuolization and dead cells debris.

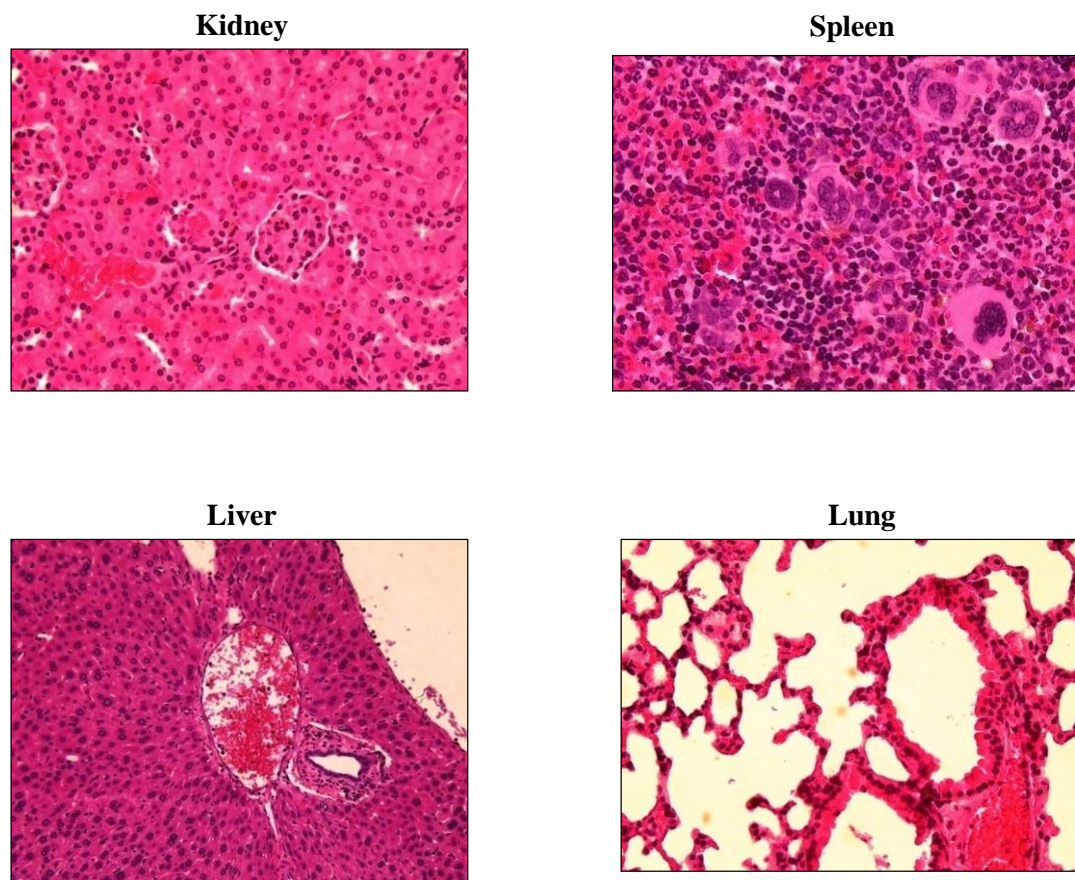


Figure 3.11. H&E staining of organ sections from EA-treated xenografts
HeLa xenografts organ sections showing no tissue toxicity or necrosis.

3.5 Discussion

Our data demonstrates that ellagic acid inhibits CK2 kinase activity in HeLa cells. Maximum inhibition occurred at 72h after ellagic acid treatment, and was followed by kinase activity rebound at 96 h. This could be a result of a decline in Ellagic acid inhibitory effect due to instability after 72 h. The increase in CK2 expression at 72 and 96 h may be due to cells compensating for the loss of CK2 activity, which further explains the increase of CK2 acitivity at 96 h. Results in this study suggest that EA interruption of CK2 signaling pathways is, at least in part, responsible for the inhibition of E6 and E7 expression at the mRNA and protein levels. E6 and E7 abrogation of host defense mechanisms plays a key role in the development of cervical cancer. Inhibition of HPV oncogene expression restores tumor-suppressor protein levels and sensitizes cells to apoptosis. This series of events will likely cause a phenotype of tumor growth inhibition and apoptosis. However, the exact mechanism of CK2-mediated down regulation of E6 and E7 remains to be elucidated.

E7 repression was associated with decreased expression of cyclin A. Given that S-phase progression is controlled by CDK2/ cyclin A complex (18) and cyclin A transcription is controlled by E2F, cell accumulation in S-phase can be associated with E7 down regulation. The inhibitory effect of ellagic acid on E7 led to increased stability of pRb, and inhibited transcriptional activity of E2F therefore decreasing cyclin A expression. The latest event would induce S-phase arrest, which was observed in EA-treated-HeLa cells. In addition, E6 inhibition stabilizes tumor suppressor protein p53

levels. HeLa cells express wt-p53 at low levels. Raising p53 to normal levels may restore its normal activity as a pro-apoptotic protein.

Inhibition of CK2 kinase activity can induce multiple apoptotic pathways. Array analysis of HeLa apoptotic proteome showed an increase in the expression of death receptors most significantly was the increase in TRAIL R2/ DR5 receptor. DR5 expression is regulated by p53 thus; it can be associated with stabilized p53 level. DR5 bind death stimuli and cause activation of pro-caspase 8. Cross talk between the extrinsic (via death receptors) and the intrinsic (via the mitochondria) apoptotic pathways can occur through BID cleavage by caspase-8 and p53 upregulation of Bcl2 pro-apoptotic proteins expression (19). Inhibition of CK2 kinase activity renders BID susceptible to caspase-8 cleavage and restores p53 level. Therefore, either signal is capable of inducing the intrinsic pathway. This was indicated by the detection of mitochondrial release of pro-apoptotic cytochrome c into the cytosol. The final outcome of extrinsic, intrinsic pathways is the activation of caspase 3, a terminal effector caspase that targets PARP for fragmentation (20, 21).

Most malignancies over-express IAP proteins to protect against death stimuli hence several anti-cancer therapeutics target IAP's to induce cancer regression (22-24). IAPs including cIAP-1, cIAP-2, XIAP and survivin interfere with apoptosis pathways. Most potent apoptosis suppressor is XIAP that binds and inhibit activated caspase-3, caspase-7 and caspase-9 (25). Controlling IAPs expression level is fundamental to sensitize cells to apoptosis (26). In this study, EA showed potent inhibition effect of IAP's specifically XIAP and survivin which render cells susceptible to apoptosis signaling.

Our in vivo evaluation of EA tumor-suppressive potential in mouse HeLa xenografts, via intratumoral and intra-peritoneal administration, showed high efficiency of EA in reducing HPV (+) cervical cancer tumor growth. EA treatment had no organ toxicity and resulted in relatively longer survival of tumor xenografts.

Overall evaluation of EA mechanism of action in HeLa cells indicates that EA, by targeting a multifunctional enzyme (CK2), inhibited HPV oncogene expression, and stabilizes tumor suppressor proteins p53 and pRb. EA inhibitory effect involves the activation of multiple pro-apoptotic proteins and the repression of anti apoptotic ones. Mechanism of action was mainly mediated by extrinsic apoptosis pathway and incorporated the intrinsic pathway as well. Multifunctional targets and activation of multiple apoptotic pathways is essential in cancer therapeutics to prevent drug resistance development in cancer.

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CHAPTER IV

HPV-NEGATIVE CERVICAL CARCINOMA:

ELLAGIC ACID INHIBITS C33A CELLS GROWTH IN VITRO AND HAVE ANTI-TUMOR ACTIVITY IN VIVO.

4.1 Abstract.

In this study, human cervical carcinoma cell line negative for human papillomavirus (HPV) DNA, was analyzed for its response to ellagic acid (EA) treatment. Cervical carcinoma cell line C33A, expressing mutated p53 and RB genes was highly sensitive to EA treatment. There was a marked inhibition of CK2 kinase activity with EA treatment. Apoptosis appeared to be the major mode of inhibition exerted on C33A cells by EA. Analysis of apoptosis inhibitors revealed a proportional relationship with cell toxicity. In vivo results showed that ellagic acid has potent anti-tumor activity in C33A xenografts.

These results indicate that EA can bypass tumor suppressor proteins p53 and pRb function and induce cytotoxicity through inhibition of CK2 kinase activity.

4.2 Introduction:

Despite recent improvements in prevention, diagnosis and treatment, invasive cervical cancer remains a disease with high mortality rate in females in the United States and in developing countries (1, 2). High risk human papilloma virus (HR-HPV) was found to be the major etiologic factor of 90% of all cervical dysplasia diagnosed (3-7). The remaining 10% can develop due to other unknown causes. However, there are factors that have been identified by the World Health Organization/ Information Center of Oncology (WHO/ICO) to be contributing to cervical cancer development. These include smoking, oral contraceptive use, fertility rate, sexual behavior and HIV prevalence.

Cellular homeostasis is defined as the process of balancing cell proliferation and cell death. Homeostasis is crucial to normal survival and development of normal tissue. It is regulated by multiple cellular proteins that form biological checkpoints to control cell growth and eliminate abnormally proliferating cells (8, 9). Cancer cells are usually defective in their ability to maintain normal cellular growth and to execute apoptosis when necessary. This could be a result of a genetic mutation, or an acquired oncogenic activity that interferes with normal cellular homeostasis machinery. In cervical cancer, the loss of p53 and pRb tumor suppressor activity is a common feature among HPV (+) and (-) cells, and fundamental to the development of malignant transformation of cervical epithelial tissue (10). As we mentioned in previous report the mechanism of malignant transformation of HPV-infected primary epithelial cells, have been related to HPV oncogenes E6- and E7- mediated inhibition of tumor suppressor proteins p53 and pRb respectively (3, 11-13). However, in HPV (-) C33A cell line abrogation of p53 and pRb

tumor suppressor activity is achieved by gene mutation. P53 gene mutation is mapped to core domain. A substitution of CGT→TGT at codon 273 results in amino acid change of Arg → Cys. Amino acids at core domain are required for direct contact with DNA (14). Thus this mutation causes accumulation of inactive p53 aggregates in C33A cells. On the other hand, a single G→A mutation in the intron/exon 20 splice junction of pRb-encoding gene was found in C33A cells. This point mutation results in inactive protein that is defective in its ability to be phosphorylated (10). An effective anti-cancer drug is the one that can inhibit acquired oncogenic agents or bypass the genetic mutation effect, and render cancer cells responsive to death stimuli.

To complement our evaluation of the ellagic acid inhibition of HPV (-) cervical cancer, we decided to assess this beneficial effect through animal studies. Several in vivo assays have assessed the importance of ellagic acid in tumorigenesis inhibition. Ellagic acid chemopreventive activity against chemically induced cancers of skin, esophagus, lung and liver were demonstrated by animal studies. In 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced skin cancer mice models, ellagic acid topical application inhibited biochemical markers of skin cancer development including DNA synthesis, ornithine decarboxylase, and hydroperoxide production (15). Ellagic acid dietary supplement was proven to effectively inhibit N-nitrosomethyl- benzylamine (NMBA) - and methybenzyl nitrosamine (MBN) - induced esophageal cancer (16-18). EA oral administration resulted in reversed fibrosis and significant reduction in elevated liver enzymes, lipid peroxidase and liver dihydroxy proline (19). In rat model of Crohn's disease, (10-20 mg/kg) EA oral administration effectively diminished intestinal injuries, alleviated oxidative stress and restored pro-inflammatory proteins expression to normal

levels (20). It is well known that the gold standard assay in assessing a therapeutic potential of a compound is done through in vivo trials, thus, in vivo testing is fundamental to understanding tumor suppression activity of ellagic acid in cervical cancer xenografts.

We have demonstrated previously that ellagic acid (EA) induced anti-proliferative effect on broad spectrum of malignancies including HPV (+) HeLa and HPV (-) C33A cells. Our investigation of EA mechanism of action in HeLa cells revealed that CK2 inhibition leads to abrogation of HPV oncogene expression. As a consequence p53 and pRb normal levels and function as tumor suppressors were rescued. pRb inhibited the expression of cyclin A, and p53 induced death receptor activation. Apoptotic pathway involved extrinsic and intrinsic routes and a concomitant inhibition of IAP's that could be induced by CK2 inhibition. In this report, we are investigating CK2 inhibition downstream mechanism of action in the absence of HPV oncogenic activity and the irreversible loss of p53 and pRb normal activity.

4.3 Experimental Procedures:

Cell culture.

C33A cells were cultured in RPMI (Cellgro), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Cellgro). Cells were maintained in 5% CO₂ at 37° C.

CK2 activity assay

CK2 activity in C33A cells was measured using whole cell lysates. Cells were extracted using ice-cold hypotonic buffer containing 20 mM Tris buffer, pH 8.0, 10% glycerol, 0.05% nonidet-P40, 2.0 mM EDTA, 2.0 mM EGTA and a cocktail of protease and phosphatase inhibitors. Cell suspension was kept on ice for 30 minutes, and then spun down at 10,000 xg for 10 minutes at 4° C. Endogenous kinase activity of CK2 was measured by CKII assay Kit (Upstate Biotechnology/ Millipore). 1 µg protein of cell lysates was added to a total volume of 40 µl containing assay dilution buffer (20mM MOPS, pH7.2, 25mM β-glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, and 1mM dithiothreitol), 0.4 mM of CK2-specific substrate peptide (R₃D₃SD₃), inhibitor cocktail to block activity of other serine/threonine kinases in lysates, and diluted [γ -³²P] ATP in MgCl₂. The mixture was incubated at 30°C for 10 minutes, and then reaction was stopped by adding 20 µl of 40% trichloroacetic acid (TCA). Twenty-five microliter of reaction mixture was transferred to P81 paper discs and allowed to bind for 30 seconds. Paper discs were washed 3 times with 0.75% phosphoric acid for five minutes each and one time with acetone, allowed to dry before they were transferred to scintillation vials

with 2 ml of scintillation cocktail. Radioactivity of CK2 was counted using scintillation counter, expressed as c.p.m percentage of control radioactivity. CK2 expression level was assessed by resolving 35 µg protein on 12% polyacrylamide-SDS gel, transferred to PVDF membrane and blotted with anti-CK2 α antibody.

Cell cycle analysis

C33A cells were plated at density of 1×10^5 cell/well and incubated for 24h with 50 µM EA. The cells were then harvested and fixed in 90% ethanol at 4° C. Cells were then washed with PBS and resuspended in a solution containing propidium iodide, sodium citrate, triton X and RNase, and incubated at 37° C in the dark. After 30 min of incubation, samples were analyzed by Cytomics FC500 flow cytometer (Beckman Coulter), Quantitative cell cycle analysis was performed using CXP software.

Trypan Blue Assay.

Trypan blue dye exclusion assay was done to test cytotoxicity of C33A to ellagic acid in time dependent manner. Cells were treated with 50 µM ellagic acid for 24, 48, 72 and 96 h. At specified time points. Cells were collected and an aliquot of 500 µl was counted using Beckman Coulter Vi-Cell XR cell viability counter. Results were compared to control which was treated with the vehicle (Peg400) and collected after 96 h of incubation time.

Annexin-V assay/ flow cytometry.

50 µM ellagic acid-treated cells were trypsinized and harvested at 24, 48, 72 and 96 h, washed once with cold PBS and resuspended in 100 µl Annexin-V binding buffer at

concentration of 1×10^3 cells / μ l. According to the manufacturer's protocol, cells were then incubated with FITC-Annexin V and propidium iodide (PI) solution (FITC Annexin V Kit II, BD pharmingen) for 15 min at room temperature in the dark. Apoptosis induction was then analyzed on Cytomics FC500 flow cytometer (Beckman Coulter), using CXP software.

Apoptosis array analysis.

C33 cells were plated in 100 mm dishes at a density of 1×10^5 cells/ml. Cells were treated with 0 or 50 μ M ellagic acid and incubated at 37° C for 96 h. After trypsinizing and harvesting of cells, they were rinsed with PBS and extracted at 1×10^7 cells/ml in lysis buffer (Human Apoptosis Array Kit; R&D Systems). The lysates were resuspended and rocked gently at 4° C for 30 min. After centrifuging at 14,000 x g for 5 min, the supernatant was transferred into a clean tube. 400 μ g protein was incubated with apoptosis array membrane according to manufacturer's protocol. Apoptosis array data developed on X-ray film were quantified and analyzed using Image Quant TL v2005. Pixel density of protein spots were analyzed and exported to excel. Average pixel density of 2 spots per protein was calculated. Negative control was used as background and subtracted from the mean. Results were then normalized to the positive control.

Immunoblot.

C33A cells were treated with 50 μ M ellagic acid, and incubated for 24, 48, 72 and 96 hours. Time point 0 was treated with the vehicle PEG400 only. Cells were harvested at indicated time points, washed with PBS and lysed in a buffer containing 150 mM sodium

chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), and 50 mM Tris at pH 8.0 with a cocktail of protease inhibitors. Protein concentrations were determined using BCA protein assay (Biorad). An amount of 35 µg protein was taken from all samples and resolved on 12% polyacrylamide-SDS gel and transferred to PVDF membrane using semi dry transfer method. The membrane is then blocked with 5% milk and incubated with anti-PARP antibody (Cell Signaling).

Ellagic acid effect on C33A xenograft.

Animal experiments were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic. Female athymic nu/nu mice were purchased from Case Comprehensive Cancer Center (CWRU). Cell inoculation was achieved by injecting mice subcutaneously with 1×10^6 C33A cells suspended at 1×10^7 cells/ml. Mice were randomized into two groups and were monitored daily until tumors reached approximately 100 mm³ in volume. Control groups received vehicle (Peg400) given intratumorally (IT) in a volume of 25 µl. Second group received ellagic acid treatment intratumoral at concentration 8 mg/kg and third group received 30 mg/kg via intraperitoneal route. Treatments were given three times a week, tumor dimensions were measured using calipers and volume of tumor was calculated using the formula (Volume = $\frac{4}{3} \pi a^2 b$, where a=minor radius and b=major radius of the tumor).

Histology and immunohistochemistry.

Animal models were euthanized and tumors were harvested and fixed in 10% neutral buffered formalin. Sections of tumors were stained with hematoxylin and eosin (H&E). Slides were viewed microscopically.

Statistical analysis

Data are expressed as mean \pm SEM from at least 3 independent experiments.

4.4 Results

The inhibitory effect of ellagic acid on CK2 activity in C33A cells.

A single administration of 50 μ M ellagic acid resulted in inhibition of endogenous CK2 phosphorylation of the CK2-specific peptide substrate (R₃D₃SD₃). Maximum inhibition of CK2 activity occurred after 48 h of EA treatment (Fig. 4.1A). CK2 activity rebound effect was evident after 72 h. As we have mentioned in previous report, 72 h might be the maximal effective time for 50 μ M EA after which a second dose is required. Figure 4.1B shows an evaluation of CK2 expression and found that EA exerts its effect on catalytic activity of CK2 only and not on its expression level.

Ellagic acid decreases viability and has no cell cycle effect in C33A.

Next, we assessed the effect of ellagic acid on cell cycle progression (Fig. 4.2). We previously showed that EA induced S-phase arrest in HeLa cells and we indicated the correlation between HPV oncogenic activity and cell cycle modulation in HeLa cells. As expected, HPV (-) C33A cells did not show any significant cell cycle modulation following EA treatment. However, a single dose of 50 μ M ellagic acid reduced viability of C33A cells to < 60% after 48 h. After 96 h of ellagic acid treatment, viability was reduced to < 20% (Fig. 4.3).

Ellagic acid Induces apoptosis in C33A cells.

Ellagic acid increased percent apoptotic cells (early+late) in a time-dependent manner. Apoptosis was detected by phosphatidylserine (PS) externalization. Exposed PS on the outer cellular membrane is an early sign of apoptosis induction and was tagged by FITC

annexin V staining. Positively stained cells shift rightward with increased time of EA treatment (Fig. 4.4A). Figure 4.4B illustrates quantitative analysis of the increased cell population in early and late stage apoptosis

Apoptosis effector proteins are modulated by ellagic acid treatment.

To identify effector proteins involved in ellagic acid-induced apoptosis in C33A cells, we evaluated the difference in pro-apoptotic and anti-apoptotic proteins expression in untreated-C33A and treated-C33A cells. Figure 4.5 A, B, C show that ellagic acid does not exert a modulation on the expression of p53 and cyclin A. Fragmentation of PARP was evident at 48 h after ellagic acid treatment. Array analysis indicates that death receptor-mediated apoptosis pathway is not involved in the mechanism of C33A cell death. However, Ellagic acid stimulated the repression of apoptosis inhibitors family of proteins IAP's including cIAP-1, cIAP-2, XIAP and Survivin (Fig. 4.6A, B).

Impact of ellagic acid treatment on C33A xenografts.

Next, we wanted to investigate whether ellagic acid in vitro inhibitory effect can have therapeutic benefits in C33A animal models. Inhibition of CK2 kinase activity blocked tumor growth in C33A xenografts treated via intra-peritoneal and intra-tumoral routes with a more profound effect in IT-treated animal models (Fig. 4.7, 8). When HPV-negative cervical cancer tumors reached a volume of 100 mm³, the mice were randomly divided into 3 groups and treated as explained in the experimental methods section. Control tumors continued to grow progressively. Mice treated with intratumoral injections of EA exhibited

dramatic decrease in tumor growth. Mice treated with intraperitoneal injections showed decreased tumor volume compared to control mice. To assess the impact of CK2 inhibition by EA in C33A tumors, control groups large tumors were injected with EA intratumorally. These tumors developed black, ulcerated lesions which upon examination by H&E staining was found to be necrotic tissue (Fig. 9).

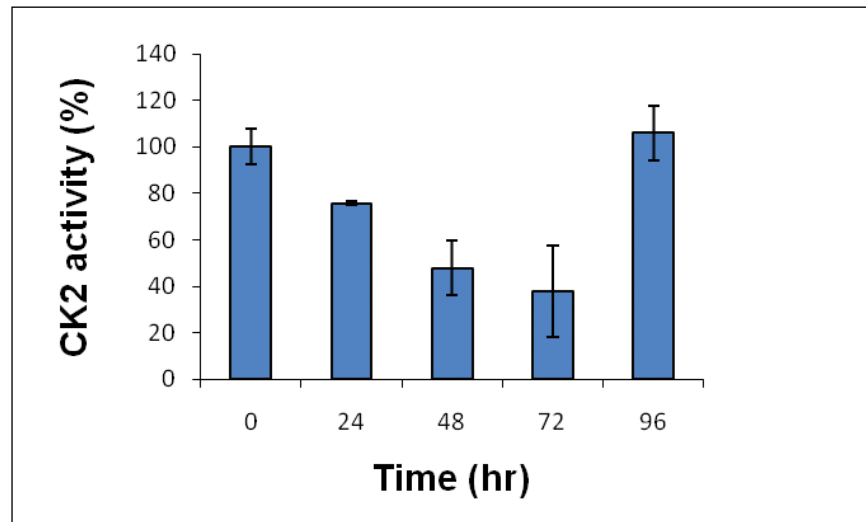
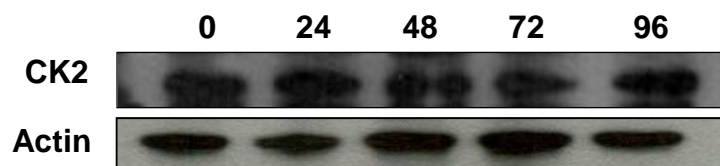
A**B**

Figure 4.1. Ellagic acid inhibits CK2 kinase activity.

A. CK2 phosphorylation assay of specific peptide was evaluated in the presence of 50 μ M ellagic acid at different time intervals. Data based on 3 independent studies, normalized to control (sample at time 0) and expressed as mean \pm SEM. **B.** CK2 expression by immunoblot showing stable expression of CK2 protein.

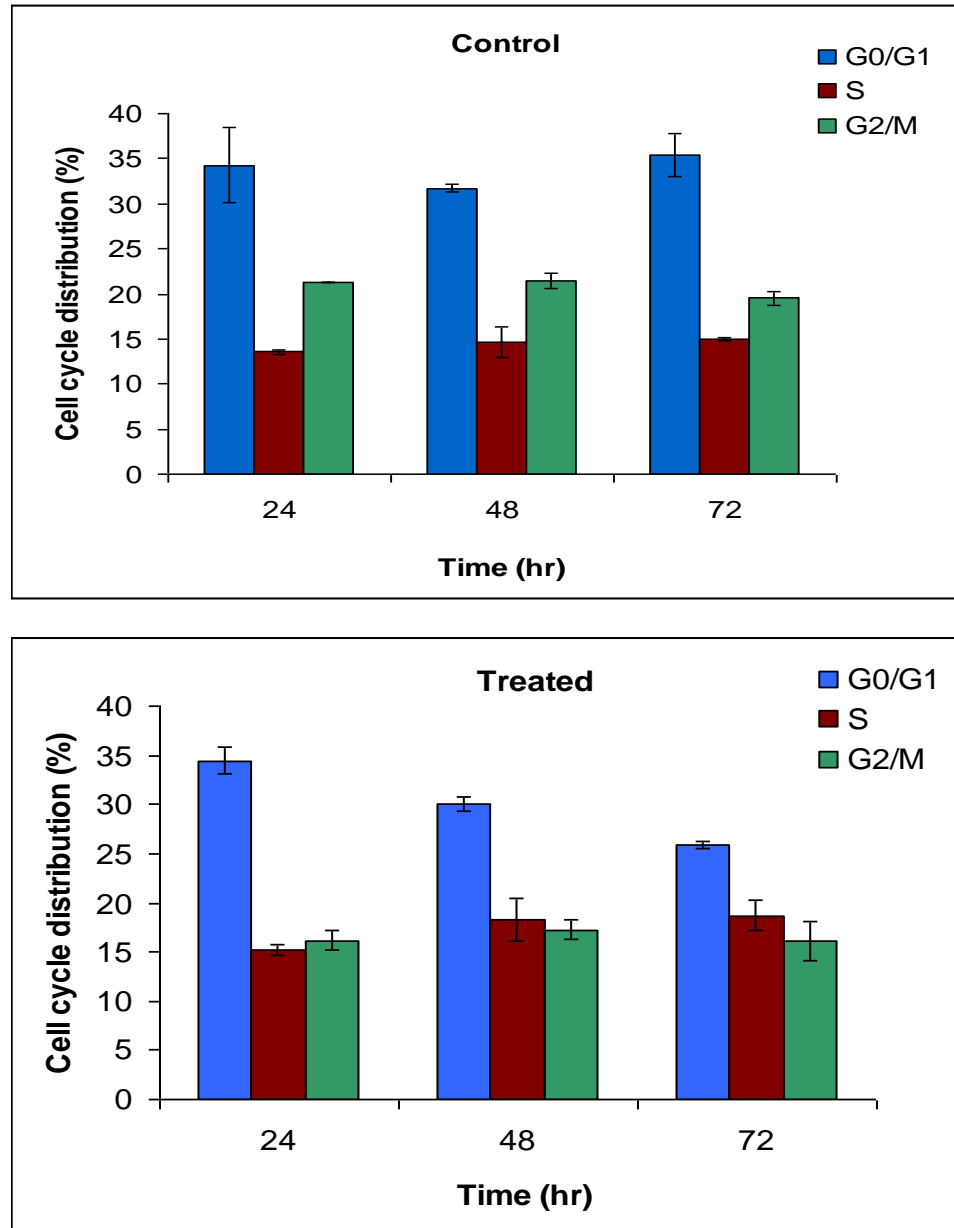


Figure 4.2. Minimal change in cell cycle distribution in C33A cells.

Ellagic acid-treated cells sorted by flow cytometry analysis of DNA content into G0/G1, S and G2/M phases, revealed no effect on cell cycle progression. Quantitative data represents mean of 3 independent assays \pm SEM.

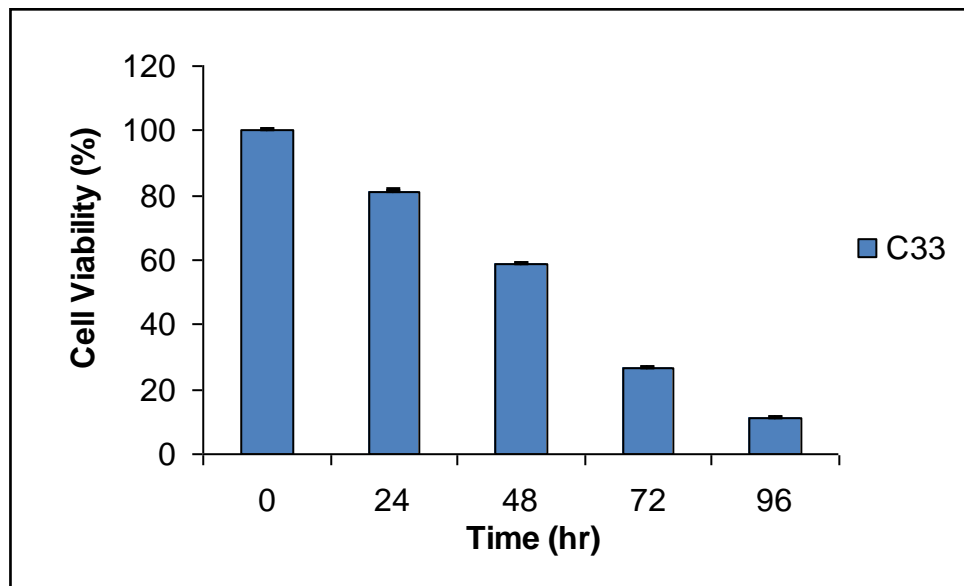


Figure 4.3. Ellagic acid-induced CK2 inhibition decreases cell viability in vitro. Trypan blue cell viability assay of C33A cells treated with 50µM ellagic acid at different treatment exposure times. Data is based on 3 independent studies and expressed as mean \pm SEM.

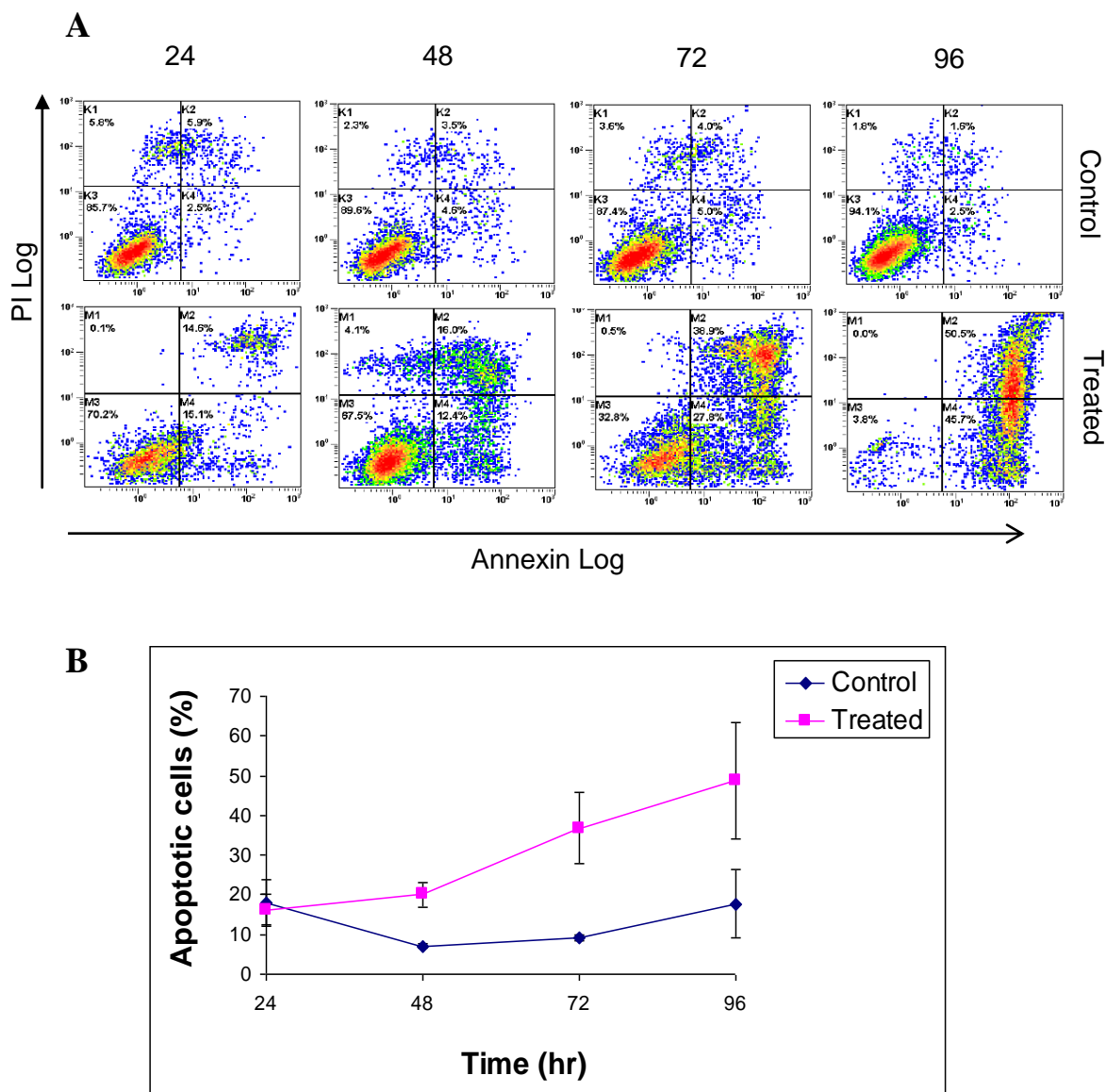


Figure 4.4. Ellagic acid induces apoptosis in C33A cells in a time-dependent manner. **A.** Density blot showing increase of cells shift rightward with increased time of ellagic acid treatment, compared to control. Cells shifting to the right lower quadrant are positive for annexin V (early apoptosis) and cells shifting to right-upper quadrant are positive for annexin V + PI staining (late apoptosis). This shift is indicative of PS externalization as a measure of apoptosis induction. **B.** A quantitative histogram of 3 independent annexin V assays. Data represent mean of (early + late apoptotic cells) \pm SEM.

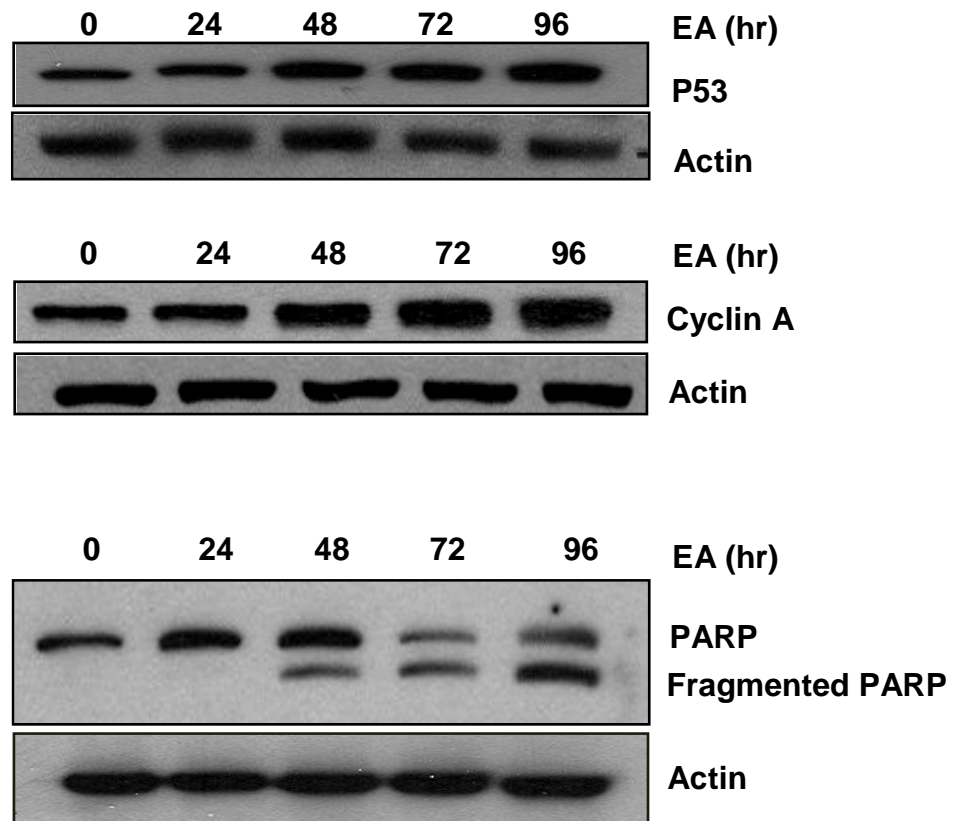
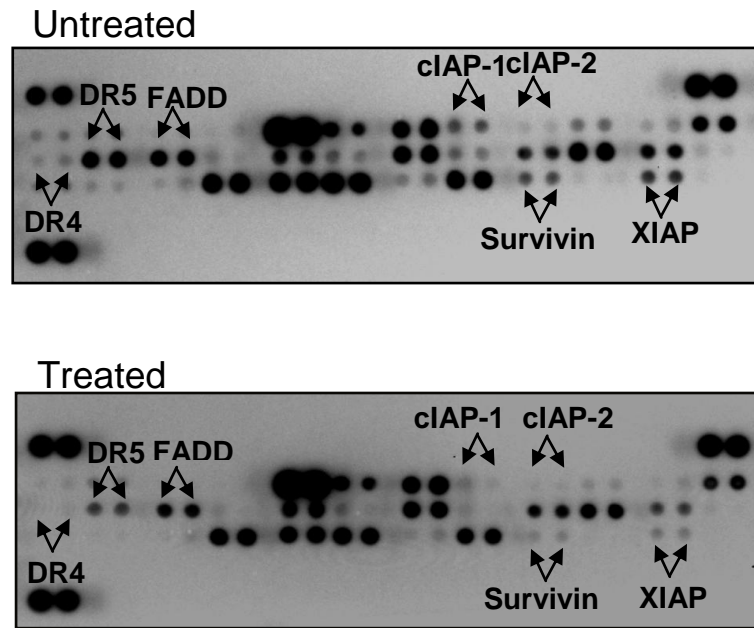


Figure 4.5. Ellagic acid effect on p53, pRb and apoptosis terminal effector protein PARP. Immunoblot analysis of C33A cells showed elevated levels of p53 and pRb with no change in expression with ellagic acid treatment. PARP fragmentation illustrated at 48 h of ellagic acid treatment is indicative of caspase-mediated apoptosis induction.

A



B

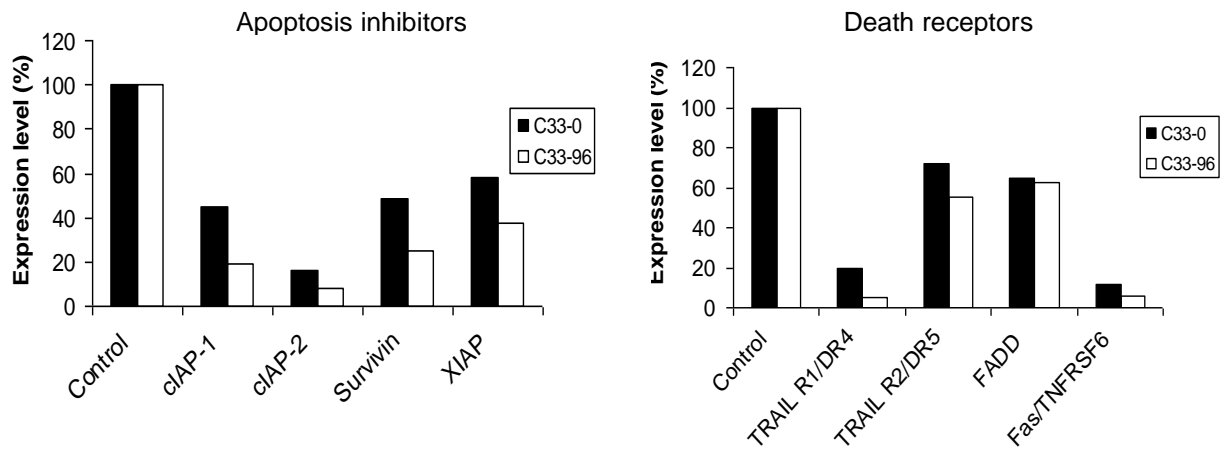


Figure 4.6. Apoptosis array analysis identifies apoptosis effector proteins involved in growth inhibition of C33A cells. A. TNF family of death receptors and IAP's expression was evaluated in ellagic acid treated and untreated C33A samples. Each protein is represented by two duplicate expression dots on the array. **B.** Quantitative analysis was done by pixel density measurement normalized to positive control.

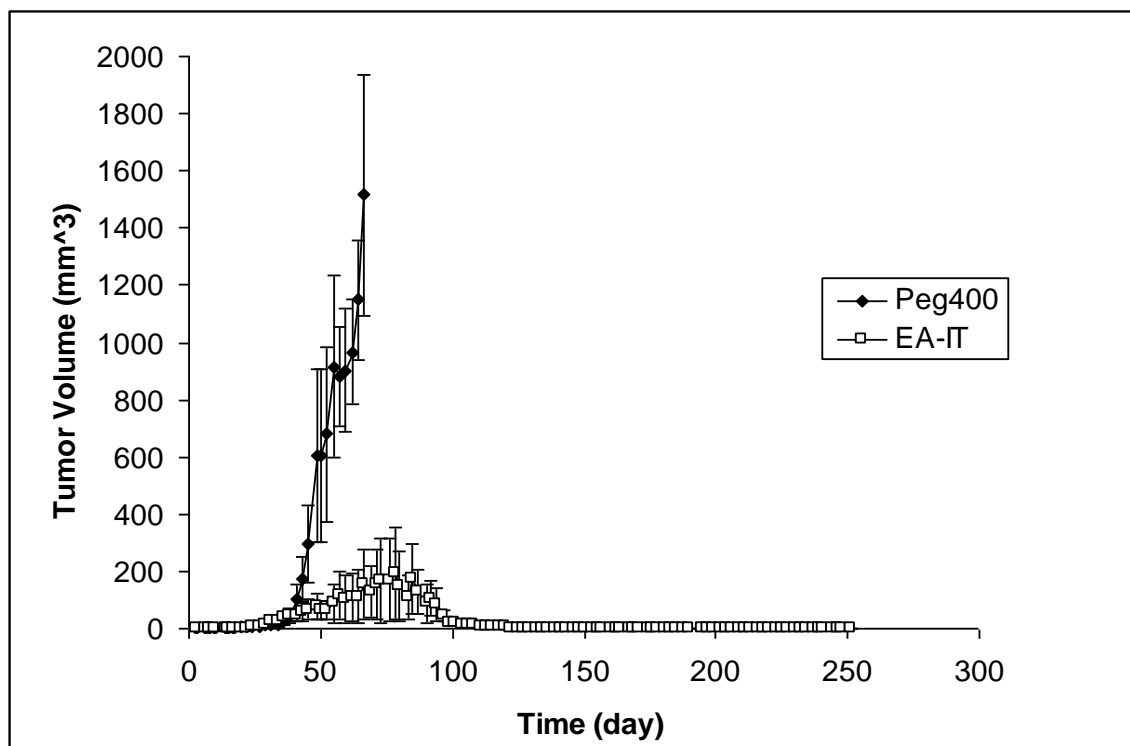


Figure 4.7. In vivo anti-tumor activity of intratumoral ellagic acid treatment.

Data represent the average volume of 6 tumors treated via intratumoral injections three times a week \pm SEM. Tumor volume decreased dramatically compared to Peg400 control group. Treated animals survived for 250 days.

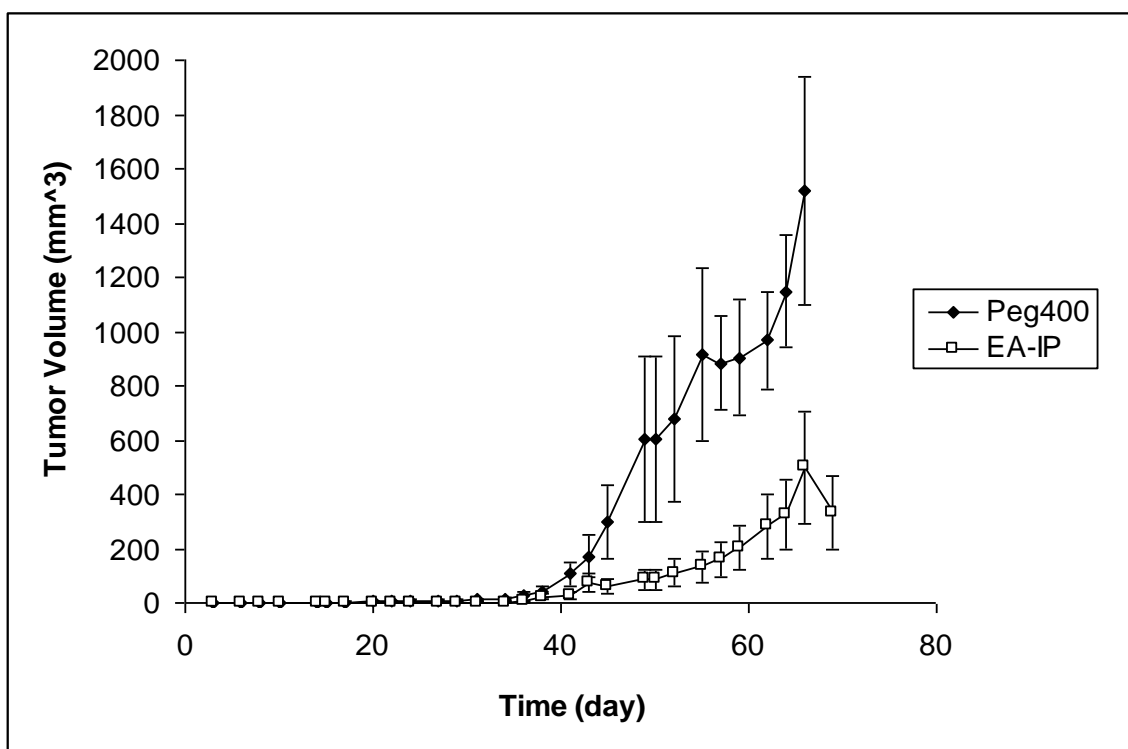
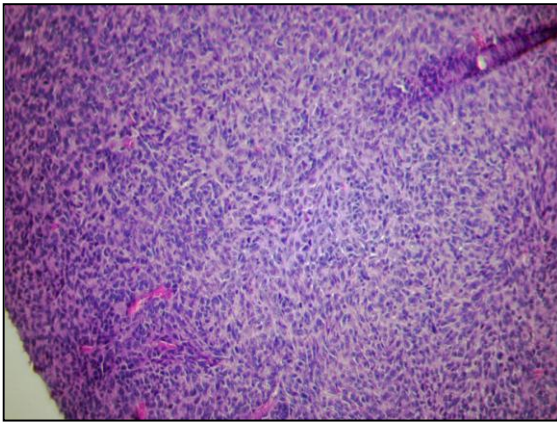


Figure 4.8. In vivo anti-tumor activity of intraperitoneal ellagic acid treatment. Quantification of C33A tumor growth from intra-peritoneally injected ellagic acid compared to Peg400 treated control. Data presented as mean of 6 tumors in control and treated groups.

Viable tumor



Necrotic tumor

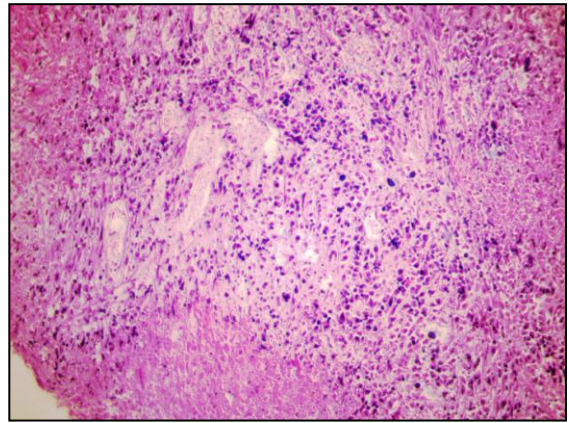


Figure 4.9. Development of necrosis in tumors following ellagic acid treatment.

Control tumors were injected intratumorally with 8 mg/kg ellagic acid. Black necrotic tissue developed after 3 EA-treatments, which upon evaluating by H&E staining was found to be necrotic tissue.

4.5 Discussion

We investigated the anti-proliferative and anti-tumoral effects of EA in HPV (-) C33A cells and the mechanism of apoptosis induction in the absence of p53 and pRb tumor suppressor activity. Our in vitro results show that C33A cells were sensitive to micromolar doses of EA in time and dose dependent manner. EA induces CK2 kinase activity inhibition with no effect on CK2 expression in C33A cells. Tumor suppressor's p53 and pRb are mutated in C33A cells thus; p53 and pRb expression was not affected by EA treatment. Ellagic acid mode of inhibition in C33A cells did not involve cell cycle arrest. However, flow cytometry analysis of PS early exposure as well as PARP fragmentation indicates caspase-mediated apoptosis induction. Decreased expression of XIAP, survivin, cIAP1 and cIAP2 is essential in the execution of apoptosis. IAPs are overexpressed in a variety of malignancies. They inhibit caspase activity and play a critical role in chemotherapy resistance. Repression of IAPs removes the block on caspase activity and renders cancer cells susceptible to apoptosis execution. In vivo study indicate EA is efficient in inhibiting HPV (-) cervical tumor growth by intratumoral and intraperitoneal treatment.

These results suggest that, ellagic acid-mediated CK2 inhibition is effective in inducing apoptosis in the absence of p53 and pRb tumor suppressor activity via IAP's inhibition. Inhibiting a multifunctional target such as CK2, can bypass tumor suppressor gene mutation and induce cancer cell death.

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CHAPTER V

OVERALL CONCLUSION

It is now well known that HPV infection is a sexually transmitted disease that when persistent, can cause vaginal, penile, vulva, anal canal and head and neck cancers (1-4). The newly developed vaccine containing noninfectious peptide representing high risk (HR) HPV strains is effective at preventing HPV infections and subsequent dysplasia (4, 5). Drug and Food Administration (FDA) has approved HPV vaccination for females aged 9-26. Recently females of any age are recommended to receive the vaccine as long as they test negative for HPV-DNA. This vaccine is a valuable tool in preventing HR-HPV infection and cervical dysplasia (4, 6). However, implementation of HPV vaccination is very limited in developing countries where cervical cancer results in high mortality rate. The population who receive the vaccine is limited to females who are negative for HPV-DNA, while males are also at risk of developing HPV-mediated cancers (4). HPV vaccine protects against 70% of HPV strains but the duration of this

immunity is still uncertain. Women who receive the vaccine have to follow up with a yearly Papanicolaou (Pap-smear) to screen for the remaining 30% of HPV strains against which the vaccine does not provide immunity (4). The limitations of HPV vaccine leave a large population that remains at risk of developing HPV-related cancers.

Early detection of cervical cancer is necessary for optimal treatment outcome. Women have to undergo a yearly Papanicolaou for early detection of cervical dysplasia. Currently the standard care for abnormal cytology, which is determined by pap-smear screening, is immediate colposcopy and biopsy of all suspected lesions. Biopsy specimen analysis determines the histologic diagnosis. Both cytology and histology analysis can then determine the degree of cervical dysplasia according to the Bethesda System (7). This categorization system divides abnormal cytology into two main groups: squamous and glandular origins. Squamous cell origins include atypical squamous cells, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma. Glandular cell origins include: atypical cells, atypical favoring neoplasia, endocervical adenocarcinoma in situ, adenocarcinoma (7). Cytology diagnosis is further confirmed by histology diagnosis, which is categorized into Low-grade cervical intraepithelial neoplasia (CIN1) which falls under LSIL; and Moderate and severe CIN2 and CIN3 (CIN3 indicates carcinoma in situ) which fall under HSIL (7, 8). Once cervical carcinoma is confirmed by cytology and histology examination, a clinical staging has to be determined according to International Federation of Gynecology and Obstetrics (FIGO). Depending on clinical staging of cervical neoplasia, treatment can be determined (8).

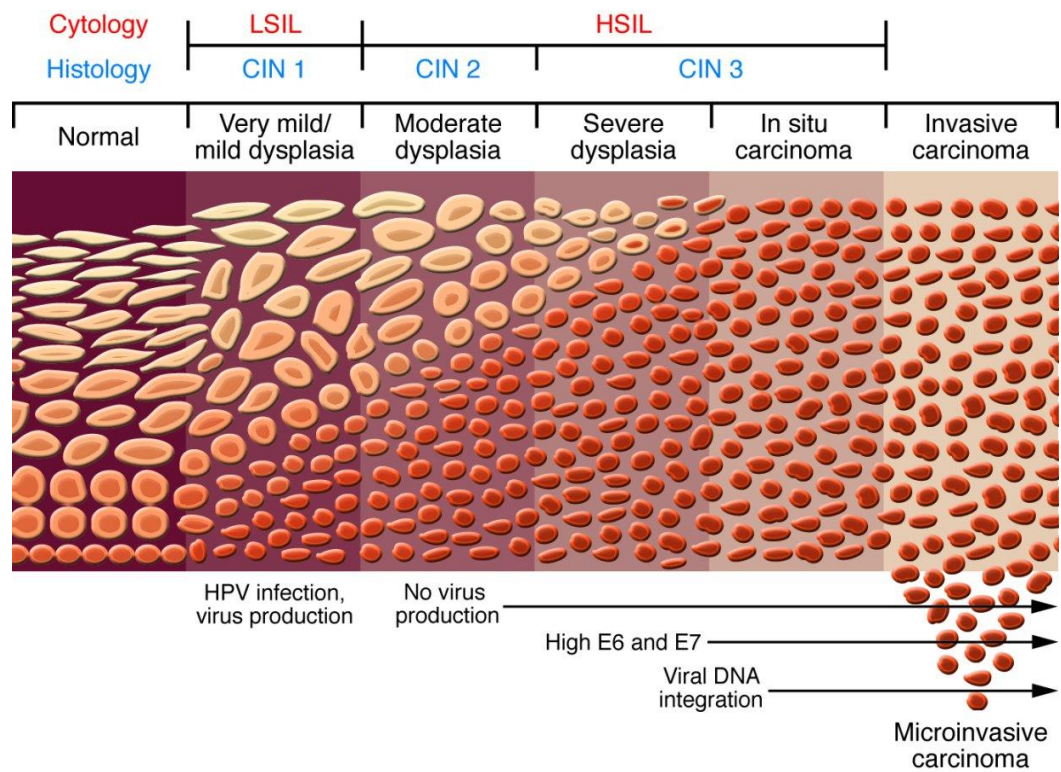


Figure 5.2. Cervical dysplasia classification according to cytology and histology analysis. From Lowy & Schiller, J Clin Invest, 116:1167-73, 2006

Cervical cancer treatment is aggressive and can include; radiation (external beam radiation or intra-vaginal), chemotherapy (cisplatin (CDDP) and 5-fluorouracil (5-FU)), surgery which may involve radical hysterectomy and pelvic lymphadenectomy (8). In most cervical cancer cases combined therapy is necessary. Combined radical surgery and radiation is used to treat certain stages of cervical cancer, however, this therapy is associated with toxic side effects (8). Combined chemo-radiotherapy is considered the gold standard treatment for cervical carcinoma. Although, the later therapy improves survival rate in cervical cancer patients, it can cause severe acute and late toxic effects (9, 10). Acute toxicity of concurrent chemotherapy and radiotherapy treatment manifest in hematologic and gastrointestinal complications that may require hospitalization (10). Late toxicity can include small bowel obstruction, ureteric fibrosis, pulmonary embolus, sepsis and toxic death (9).

The aggressiveness of these therapies and the development of chemo- and radio-therapy resistant tumor cells increased the need for an improved anti-cervical cancer drug (11). We found that ellagic acid-mediated CK2 inhibition has antiproliferative and apoptotic activity against cervical carcinoma in vitro and in vivo. Ellagic acid appears to present an anti-viral effective strategy that is safe, multifunctional (to avoid cancer cells resistance development), and has the ability to bypass tumor suppressors' mutation to induce cancer cell death.

Comparing HPV (-) C33A and HPV (+) HeLa cervical cancer cells in their response to ellagic acid- mediated CK2 inhibition, we found that C33A showed greater sensitivity in time- and dose- dependent manner compared to HeLa cells. We hypothesized that this

difference was due to differential gene expression. Therefore, we analysed major oncogene expression differences via immunoblotting (Fig. 2). Immunoblot analysis in HeLa cells confirm the expression of HPV18-E6 and -E7, show normal level of cyclin A as a product of pRb-E2F transcription activity, and undetectable p53. C33A on the other hand, is negative for HPV-E6 and E7 expression, expresses elevated level of mutated p53 and normal expression level of cyclin A. When we compared CK2 expression, we found that it is expressed at elevated level in C33A cells compared to HeLa. This suggests that HeLa might be more sensitive to ellagic acid inhibitory effect compared to C33A. However, as indicated in our previous reports, C33A is more sensitive than HeLa. This suggests that HPV-oncogenic activity delays HeLa response to CK2 inhibition and hinders it from being as sensitive to ellagic acid treatment as C33A.

Comparing ellagic acid mechanism of action in HeLa and C33A cells will provide insight into the molecular mechanism of action of ellagic acid in different proteome profiles. This comparison study revealed apoptosis as the common inhibitory mechanism in both C33A and HeLa cells in time- and dose- dependent manner. Cell cycle arrest was partly involved in ellagic acid inhibitory effect in HeLa cells. This specificity to HeLa cells is associated with the oncogenic activity of HPV-E7 as the later plays an important role in regulating cell cycle machinery. We predicted major differences in the underlying mechanism of apoptosis induction in HeLa and C33A cells. This was confirmed by different expression of effector proteins involved in apoptosis induction in HeLa and C33A cells. HeLa showed a combination of TRAIL death receptor activation, mitochondrial involvement, and IAP's inhibition. In contrast, C33A cells showed IAP's

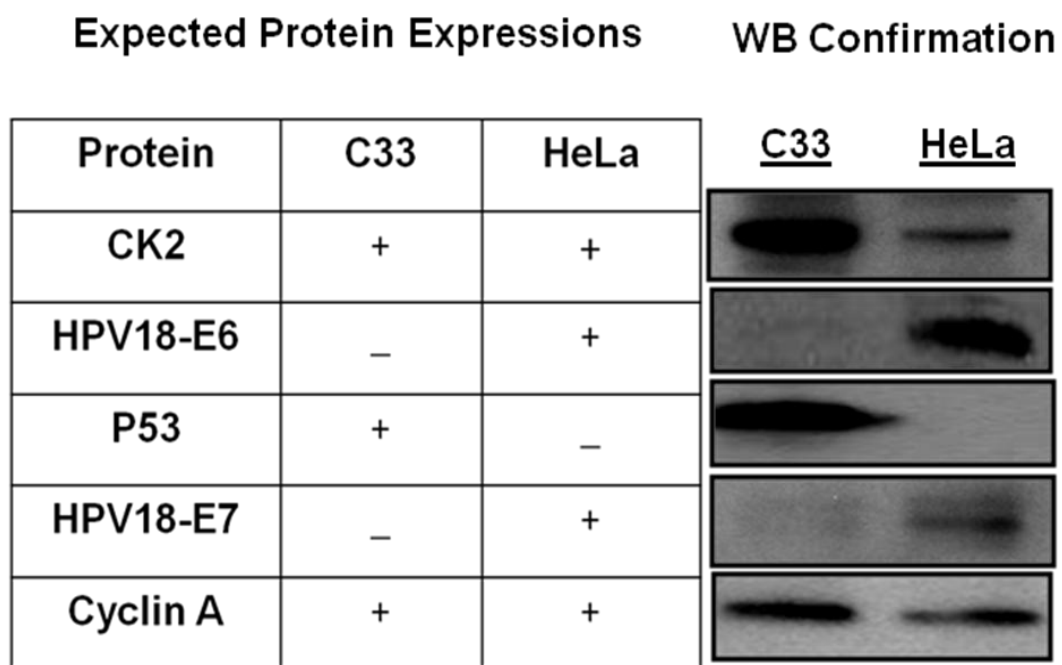


Figure 5.3. Immunoblot comparison analysis of gene differential expression in HeLa and C33A cells.

as the major effector proteins involved in apoptosis induction.

In vivo study of ellagic acid effect on HeLa and C33 tumors was consistent with previous in vitro results. At low dose application of ellagic acid, HeLa exhibited less sensitivity to intratumoral treatments than C33. However, at higher dose, HeLa and C33 tumors demonstrated similar response to intra-peritoneal administration of ellagic acid. It is noteworthy that upon treating HeLa and C33 tumors in control groups with ellagic acid, both tumors developed areas of necrosis. This further confirms ellagic acid effectiveness in tumor growth inhibition.

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