MOLECULAR MECHANISM(S) OF ACTION OF PHYTOCHEMICALS IN CANCER CHEMOPREVENTION

Thesis

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DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that the thesis entitled "***Molecular Mechanism(s) of Action of Phytochemicals in Cancer Chemoprevention"*** and submitted by **Rajesh Loganathan Thangapazham** ID No **2001PH29102** for award of Ph. D. Degree of the Institute, embodies original work done by him under my supervision.

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DEDICATION

I dedicate this thesis to my parents, Mr R.L.Thangapazham and Mrs Leela Thangapazham. They more than anyone else, have been the best role models I could have hoped for. Any accomplishment of mine is due in no small part to their constant support and encouragement. Thank you Mom and Dad.

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To Cuttima, My Love.

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Words fail me to express my appreciation to my better half; Well …. I would rather say my best half, my wife, Sangeetha whose dedication, love and persistent confidence in me, has taken loads off my shoulder. She helped me a great deal in proof reading my thesis, paper and taking excellent care of me always and especially at times when I was sick.

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LIST OF ABBREVIATIONS

AP-1 – activator protein 1

AR - androgen receptor

ARE - antioxidant-responsive element

bFGF - basic fibroblast growth factor

CAPE - caffeic acid phenethyl ester

CASE - cellular activation of signaling ELISA

CDK - cyclin-dependent kinases

COX-2 – cycloxygenase-2

DDIT3 - DNA-damage-inducible transcript 3

EGCG - epigallocatechin gallate

ER - estrogen receptor

GADD45 – growth arrest and DNA damage inducible 45

GAPDH – glyceraldehyde – 3- phosphate dehydrogenase

GTP – green tea polyphenols

HO-1 – heme oxygenase-1

HUVEC - human umbilical vein endothelial cells

Keap1 - *K*elch-like *E*CH-*a*ssociated *p*rotein 1

LEF - lymphoid enhancer factor

MAPK - mitogen activated protein kinase

MMP - matrix metallo proteinases

MVD – microvessel density

NF-κB – Nuclear Factor kappa B

NRF2 - (NF-E2)-related factor 2

PCNA – proliferating cell nuclear antigen

PI3K - phosphoinositdol kinase

PKB – protein kinase B

PSA – prostate specific antigen

TCF - T-cell factor

TUNEL – Terminal deoxynucleotidyl transferase(TdT) mediated D-Uridine TriPhosphate nick end labeling

VEGF - vascular endothelial growth factor

ABSTRACT

Carcinogenesis is generally recognized as a multistep process in which distinct molecular and cellular alterations occur. The tumor development is considered to encompass three closely linked stages viz. tumor initiation, promotion and progression. Initiation is a result of rather rapid and irreparable assault to the cell which may be due to the initial uptake of a carcinogen and the subsequent stable genotoxic damage caused by its metabolic activation. In contrast to initiation which is rapid and irreversible, tumor promotion is a considerably lengthy and reversible process in which actively proliferating pre-neoplastic cells accumulate. The final step of transformation is the progression stage which involves the growth of tumor with invasive and metastatic potential. As the neoplastic transformation takes a considerably long time to develop to a fully blown metastatic disease, intervention of the stages of carcinogenesis may lead to delayed incidence or better prognosis of the disease.

Chemoprevention broadly implies the use of a chemical substance of either natural or synthetic origin, to prevent, hamper, arrest or reverse a disease. Phytochemicals are bioactive non-nutrient components of various plant parts like seed, leaves, rhizome etc. Recent epidemiological and preclinical testings have revealed the significant potential of phytochemicals in combating chronic diseases like cancer resulting due to oxidative stress induced by free radicals. Recent studies have found numerous phytochemicals which may have the potential to develop into a cancer chemopreventive agent. Therefore, in the present investigation attempts were made to explore the cancer chemopreventive effects of novel phytochemicals like green tea polyphenol, Brahma

rasyana and curcumin in hormone-dependent and independent cancers of the prostate gland or breast.

Tea [Camellia sinesis (Theacacea)] is considered second only to water as the most popular beverage consumed worldwide. Consumption of tea has been associated with many health benefits and their role and mechanism in cancer chemoprevention has been extensively reviewed. The biological activity of green tea is due to different catechins like epigallocatechin-3-gallate (62%), epicatechin-3-gallate (24%), epigallocatechin (5%), epicatechin (6%). Epigallocatechin gallate (EGCG) is identified as the principal antioxidant contributing approximately 30% of the total antioxidant capacity of green tea. In this study, the anti-proliferative activity of GTP and its key constituent EGCG was evaluated using MDA-MB- 231 breast carcinoma cell line *in vitro* and in nude mice xenograft model *in vivo*. Both GTP and EGCG decreased the proliferation of the tumor cell line by arresting the progression of the cell through G1 phase of the cell cycle. Furthermore, GTP and EGCG were capable of delaying the tumor incidence as well as reducing the tumor burden in the athymic nude mice. The results showed that EGCG and GTP induced apoptosis and suppressed invasiveness of MDA-MB-231 cells in a dose dependent manner. A fragmented DNA ladder was detected by electrophoresis in cells treated with polyphenols indicating apoptosis. AKT, which is a serine/threonine kinase, regulates cell survival and invasion. Treatment of cells with varying concentrations of EGCG and GTP inhibited AKT at both the RNA and protein level. Moreover, EGCG and GTP treatment resulted in lesser phosphorylation of AKT compared to untreated controls. Polyphenol treatment in this cell line decreased the level of beta-catenin in the cytoplasm and also reduced its accumulation in the nucleus. These studies have greater clinical

significance since the ability of polyphenol to activate the apoptotic program and decrease the invasiveness of tumor cells might determine the success of chemotherapy.

Brahma rasayana is a popular rasayana which contains several plant extracts and has been shown to have maximum immunomodulatory activity against tumor cells and have been indicated in immunostimulation in normal and disease state. Our results suggest that Brahma rasayana reduces tumor incidence, tumor growth and metastatic spread caused by MAT-LyLu cells in Copenhagen rats. The effect of Brahma rasayana treatment on Factor VIII expression was analyzed, which was found to be significantly lower than the control untreated animals. Similar results were observed when tissues were stained for VEGF, MMP-9 and MMP-2. Western blotting and zymogram analysis of the tumor tissue also showed down regulation in the pro-angiogenic factors like MMP-2, MMP-9 and VEGF which might help in explaining the reduced tumor nodules in the lungs. Methanolic extract of Brahma rasayana was also found to inhibit the proliferation, reduce cell migration, attachment and tube formation on matrigel in an *in-vitro* model of angiogenesis using HUVEC. These results suggest that Brahma rasayana may inhibit tumor promotion and progression by inhibiting angiogenesis.

Turmeric (*Curcuma longa* Linn), is a crystalline compound which has been traditionally used in medicine and cuisine in Asian countries. Curcumin (diferuloylmethane) is the major active component of turmeric and has been shown to be cancer chemopreventive agent in several different animal tumor bioassay systems including colon, duodenal, stomach, prostate and breast carcinogenesis both *in-vitro* and *in-vivo.* Although, previous reports suggest that curcumin down regulates AR transactivation, the effect of curcumin on AR target genes like NKX3.1 and other alternate pathways that might activate the

androgen receptor signaling in prostate cancer cells have not been identified. In the present study, we have evaluated the effects of curcumin on cell growth as well as evaluated the molecular target(s) related directly and indirectly to androgen signaling in both androgen responsive and independent cell lines. To better understand the biological mechanisms of the chemopreventive potential of curcumin in cancer, we performed a temporal gene expression analysis of the curcumin-gene expression response (Cu-GER) using hormone-responsive and non responsive human prostate cancer cell line, LNCaP and C4-2B respectively. Total RNAs from these cells were prepared at 3, 6, 12, 24 and 48 h after the treatments using Qiagen kit (Valencia, CA). High-density oligonucleotide Affymetrix human genome array GeneChip® HG U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) that contains 54,675 probe sets and about 38,500 annotated human genes were used for hybridization with biotin labeled and fragmented aRNA(amplified RNA). Hierarchical clustering methods and functional classification of the Cu-GER showed temporal co-regulation of genes involved in specific biochemical pathways involved in the oxidative stress response. Androgen up regulated genes like NKX3.1 has also been found to be down regulated by curcumin treatment at both RNA and protein level in our study. PSA expression was significantly down regulated by curcumin which confirms further the down regulation of AR activity by curcumin. In our study, both EGFR and ERBB2 receptor expression were found to be down regulated several fold in curcumin treated LNCaP and C4-2B cells as analyzed by microarray. EGFR signaling pathway plays a role in stimulating the AR pathway and in restoring AR function to prostate cancer cells in the absence of testosterone. In spite of its promising therapeutic index, the biological activity of curcumin is severely limited due to its poor bio-

availability. Hence several liposome formulations of curcumin were evaluated and conditions for encapsulating curcumin were optimized. The efficacy of liposome formulation with free curcumin on prostate cancer cells was studied by comparing the anti-proliferative effects of free curcumin and liposomal curcumin using a tetrazolium dye-based (MTT) assay. The results from the cell proliferation assays provide strong evidence for liposomes as effective nano-delivery vehicles that increase the bio-availability of curcumin.

INTRODUCTION

Cancer is the result of a multistage carcinogenesis process which involves distinguishable but closely connected stages like initiation (normal cell→transformed or initiated cell), promotion (initiated cells→ pre-neoplastic cells) and progression (pre-neoplastic cells →neoplastic cells) (Brennan 1975). Initiation is a result of rather rapid and irreparable assault to the cell and may be due to the initial uptake of a carcinogen and the subsequent stable genotoxic damage caused by its metabolic activation (Lee and Surh 2005). Other causes of cancer initiation may involve oxidative stress (Surh et al 2005), chronic inflammation (Surh et al 2005), hormonal imbalance (Russo and Russo 1998), etc. The transformed cells undergo lot of changes to form pre-neoplastic cells and this process is not as rapid as initiation. Lot of pathways undergoes dysfunction to cause deregulation in cell cycle and apoptotic function of cells, which causes the transformation from normal to malignant phenotype. Dys-regulated signal transduction pathways like serine threonine kinase, AKT, activator protein 1(AP-1), nuclear factor kappa B (NF-κB), mitogen activated protein kinase (MAPK), androgen receptor (AR), estrogen receptor (ER), and Raf/Ras pathway also contribute to carcinogenesis.

Chemoprevention broadly implies the use of a chemical substance of either natural or synthetic origin, to prevent, hamper, arrest or reverse a disease. The term 'chemoprevention' was coined by Michael Sporn in the mid-1970s. The rationale behind his work on retinoids against chemical carcinogenesis (Sporn et al 1976) showed the length that cancer takes to develop in humans through initiation, promotion and progression stages. Phytochemicals are bioactive non-nutrient components of various plant parts like seeds, leaves, rhizomes etc. Recent epidemiological and preclinical

testings have revealed the great potential of phytochemicals in combating chronic diseases like cancer resulting due to oxidative stress induced by free radicals (Liu 2004). The neoplastic transformation takes a considerably long time to develop to a fully blown metastatic disease. Hence intervention with cancer chemopreventive phytochemicals, which help in delaying or arresting either any of the three stages of carcinogenesis or all of them, may lead to delayed incidence or better prognosis of the disease. Recently, phytochemicals such as curcumin, epigallocatechin gallate (EGCG), resveratrol, sulphoraphane and many others have been identified to possess cancer chemopreventive activities and several pre-clinical studies have been initiated. As per the conventional classification originally proposed by Lee Wattenberg, chemopreventive agents are subdivided into two main categories, blocking agents and suppressing agents (Wattenberg 1985). Blocking agents prevent carcinogens from reaching the target sites, from undergoing metabolic activation or from subsequently interacting with crucial cellular macromolecules (for example, DNA, RNA and proteins). Some compounds that belong to chemical classes like reductive acids, tocopherols and phenols prevent formation of carcinogen from precursor compounds. One of the most impressive findings in the field of chemoprevention is the very large number of compounds that have been demonstrated to prevent the occurrence of cancer. Compounds belonging to over 20 different classes of chemicals like phenols, indoles, aromatic isothiocyanates, cooumarins, flavones, dithiothiones, diterpenes, dithiocarbamates, phenothiazines, barbiturates and trimethyl quinolones have been shown to have chemopreventive capacities by preventing the initiation of cancer at different levels. Suppressing agents, on the other hand, inhibit malignant transformation of initiated cells, in either the promotion

or the progression stage (Figure 1). These include chemical classes like retinoids, caroteniods, selenium salts, protease inhibitors, inhibitors of arachidonic acid metabolism, cyanates, isothiocyanates, phenols, plant sterols, methylated xanthines etc (Wattenberg 1985).

Cancer results and progresses due to deregulation of various processes. Few of them are introduced here. Cell division in a normal cell is an ordered, tightly regulated process involving multiple checkpoints that assess extracellular growth signals, cell size, and DNA integrity. The replication of DNA occurs in S phase and segregation of the chromosomes into daughter progeny occurs during mitosis (M phase). The two ‘‘gap’’ phases include G1, during which the cell prepares for DNA synthesis, and G2 during which the cell prepares for mitosis. Cyclins and their associated cyclin-dependent kinases (CDK) are the central machinery that control cell cycle progression. Once activated, the cyclin/CDKs form complexes that initiate phosphorylation of other proteins and downstream cyclin/CDK complexes. Alterations in these proteins, which lead to failure of cell cycle arrest, may thus serve as markers of a more malignant phenotype.

Apart from the dys-regulated cell cycle in cancer one other important hallmark characteristic of cancer cells is their ability to evade apoptosis. Apoptosis is a process of deliberate life relinquishment by a cell in a multicellular organism. It is one of the main types of programmed cell death (PCD), and involves an orchestrated series of biochemical events leading to a characteristic cell morphology and death. The apoptotic process is executed in such a way as to safely dispose of the cell corpses and fragments.There are two fundamental pathways in apoptosis, the death receptor pathway and the mitochondrial pathway. In cancer the normal functioning of the pathway has been

disrupted in such a way as to impair the ability of the cell to undergo normal apoptosis. This results in a cell to replicate and pass on any faulty machinery to its progeny, increasing the likelihood of the cell becoming cancerous or diseased.

Another fundamental step in the transition of tumors from a dormant state to a malignant state is angiogenesis. Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels. Once malignant transformation has occurred, tumor cells, like all cells, require oxygen and nutrients for expansion. When tumors are small (<1 mm in diameter), they rely on diffusion to meet these needs. In order to grow larger, tumors must create a new blood supply to deliver nutrients and oxygen as well as to remove waste products. Angiogenesis is the process by which new capillary beds are formed from pre-existing vessels. The angiogenic process is regulated by a balance between stimulatory and inhibitory factors which may be released by the tumor itself or by the surrounding tissues. Tumors induce blood vessel growth (angiogenesis) by secreting various growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and others. These growth factors can induce capillary growth into the tumor, which supply required nutrients allowing for tumor expansion.

Inflammation acts as a key regulator in promotion of these initiated cells possibly by providing them with proliferating signals and by preventing apoptosis (Philip et al 2005). Non-inflammatory parameters like hypoxia also regulate carcinogenesis. Hypoxia induces VEGF expression in tumor cells and also induces matrix metalloproteinases (MMP's) expression in endothelial cells leading to angiogenesis and tumor cell invasion (Cross and Welsh 2001). In contrast to initiation which is rapid and irreversible, tumor

promotion is a considerably lengthy and reversible process in which actively proliferating pre-neoplastic cells accumulate. The final stage of transformation is the progression stage which involves the growth of a tumor with invasive and metastatic potential. The role of inflammation in tumor induction and the subsequent malignant progression has been well reviewed (Balkwill et al 2005). Inflammatory response also produces cytokines which may be growth and/or angiogenic factors stimulating transformed cells to proliferate and undergo promotion. Leukocytes produce cytokines, angiogenic factors as well as matrix-degrading proteases which allows the tumor cells to proliferate, invade and metastasize. Tumor infiltrating lymphocytes secrete matrix degrading proteinases like MMP 9, thereby promoting neoplastic proliferation, angiogenesis and invasion (Owen et al 2004). These details suggest the role of inflammation in all the three stages of carcinogenesis. Substantial evidence for the role of inflammation in cancer can be understood by the frequent up-regulation of inflammation mediators like NF-κB. The pathways activated by NF-κB up-regulation are implicated not only in tumor growth and progression but also in cancer cells developing resistance to anticancer drugs, radiation, and death cytokines which reveals NF-κB as an excellent target for anti-cancer therapy (Luo et al 2005).

Signaling through MAPKs, Phosphoinositdol kinase (PI3K), AKT, AP-1 and NF-κB favors cell proliferation and survival. Several key molecules in these pathways (e.g. growth-factor receptors, RAS, PI3K, PKB and NF-κB) are over expressed or constitutively up-regulated in many types of cancer, suggesting that their inhibition or down-regulation might induce tumor cells to undergo cell-cycle arrest or apoptosis. Many dietary compounds, when tested *in vitro*, have proved effective in suppressing tumorigenic signaling (Manson 2003). AP-1 is another transcription factor that regulates

expression of genes that are involved in cellular adaptation, differentiation and proliferation. Curcumin, Gingerol, Capsaicin, EGCG, Genistein, resveratrol, caffeic acid phenethyl ester (CAPE), sulphoraphane, silymarin, apigenin, emodin, quercetin and anethole are some of the phytochemicals that have been reported to suppress the activation of NF-kB and AP1 and might contribute to their Chemopreventive and/or cytostatic effects (Surh 2003) (Figure 2).

Cells are protected against oxidative stress by an interacting network of antioxidant enzymes known as phase II enzyme system. Their induction is an important component of the cellular stress response in which a diverse array of electrophilic and oxidative toxicants can be removed from the cell before they are able to damage the DNA. Antioxidants exert their protective effects by scavenging reactive oxygen species. They also protect the cells by inducing *de novo* expression of genes that encode detoxifying/defensive proteins, including phase II enzymes. Many xenobiotics activate stress-response genes in a manner similar to that achieved by antioxidants. Some of the enzymes that are part of this protective system are glutathione peroxidase, gamma-glutamylcysteine synthetase, glutathione-s- transferase and heme oxygenase-1. The genes that code for these enzymes contain a common *cis*-element, known as the antioxidant-responsive element (ARE). ARE is regulated and activated by the inactivation of Nrf2-Keap1 complex (Balogun et al 2003). (NF-E2)-related factor 2 (Nrf2) is a transcription factor which regulates the expression of conjugating enzymes like GST, HO-1 via ARE (antioxidant response element) (Itoh et al 1997). Nrf2 activity is regulated by its sequestration in the cytoplasm by Kelch-domain containing protein, Keap1 (*K*elch-like *E*CH-*a*ssociated *p*rotein 1). Keap1 releases Nrf2 in the presence of oxidants and

chemoprotective agents thereby leading to the activation of ARE and expression of phase II enzymes (Pool-Zobel et al 2005). Curcumin, sulphoraphane and caffeic acid phenethyl ester can disrupt the Nrf2-Keap1 complex and stimulate nuclear translocation of Nrf2, which subsequently activates ARE leading to phase II enzyme gene induction (Surh 2003) (Figure 3).

Beta -catenin is another important target of chemopreventive phytochemicals. Beta-catenin has many functions. It was initially identified as a component of cell adhesion machinery. The catenins link the cytoplasmic domain of classic cadherins to the cytoskeleton. The catenin family comprises the α-, β- and γ-catenins, with β- and γ-catenin sharing the greatest homology. They bind directly to the cytoplasmic tail of E-cadherin in a mutually exclusive manner and α-catenin then links the bound β- and γ-catenin to the actin microfilament network (Mohan et al 2007). Given the essential role in mediating cell adhesion by E-cadherin and association with the Wnt-mediated transcriptional pathway, the catenins are potentially important regulators of pathways involved in tumor progression. When beta-catenin is not assembled in complex with cadherins, it can form a complex with axin. While bound to axin, beta-catenin can be phosphorylated by GSK-3, which creates a signal for the rapid ubiquitin dependent degradation of beta-catenin by proteosome signals such as the Wnt signaling pathway. Wnt pathway can inhibit GSK-3-mediated phosphorylation of beta-catenin (Liu et al 2005) allowing beta-catenin to go to the cell nucleus, interact with transcription factors, and regulate gene transcription. Once beta-catenin is stabilized and translocates into the nucleus, it interacts with lymphoid enhancer factor (LEF)/T-cell factor (TCF) transcription factors, resulting in transcriptional activation of various genes which are

involved in processes such as cell-cycle regulation, cell adhesion and cellular development. Curcumin, caffeic acid phenethyl ester, EGCG, resveratrol are some of the phytochemicals which target the beta-catenin mediated signaling pathway (Surh 2003) (Figure 4).

In short, chemopreventive phytochemicals can block or reverse the premalignant stage like initiation and promotion of multistep carcinogenesis. They can also halt or at least retard the development and progression of precancerous cells into malignant ones. Recent advances in our understanding of the carcinogenic process at the cellular and molecular level have shown this blocking and suppressing categorization to be an oversimplification, and numerous cellular molecules and events that could be potential targets of chemopreventive agents have been more specifically identified (Surh 2003). In many different cell types, a range of dietary constituents can induce cell-cycle arrest and/or apoptosis. Studies have identified key molecules, the expression of which might be altered to contribute to these effects. Furthermore, tumor cells seem to be more sensitive to these influences than normal cells (Manson 2003). Therefore, the ability of any single chemopreventive phytochemical to prevent tumor development should be recognized as the outcome of the combination of several distinct sets of intracellular effects, rather than a single biological response. Hence, in the present investigation attempts were made to explore the cancer chemopreventive effects of phytochemicals in hormone-dependent and independent cancers of the prostate gland or breast. One major aim has been to relate the cancer biology to potential targets for therapeutic chemoprevention using both *in vitro* and *in vivo* models. In an attempt to define the pathways regulated by the phytochemicals, we have also analyzed the gene expression

profiles in cancer cells using focused microarrays as well as Affymetrix oligonucleotide arrays. Furthermore, delivery of potential chemotherapeutics or preventive agents employing liposomal phospholipids nanovehicles have been employed to enhance the bioavailability.

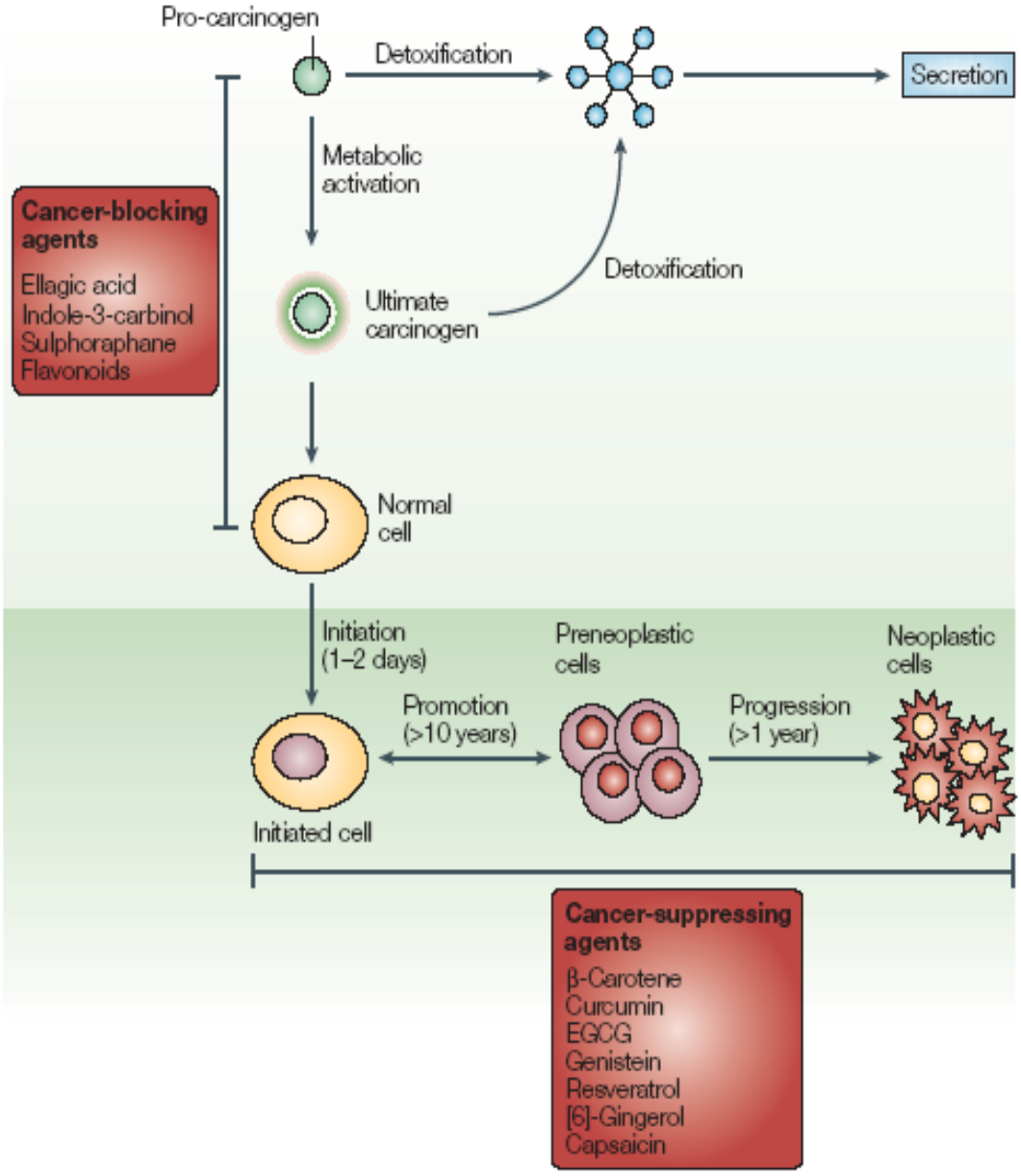

Figure 1. Dietary phytochemicals that block or suppress multistage carcinogenesis. Courtesy Surh 2003

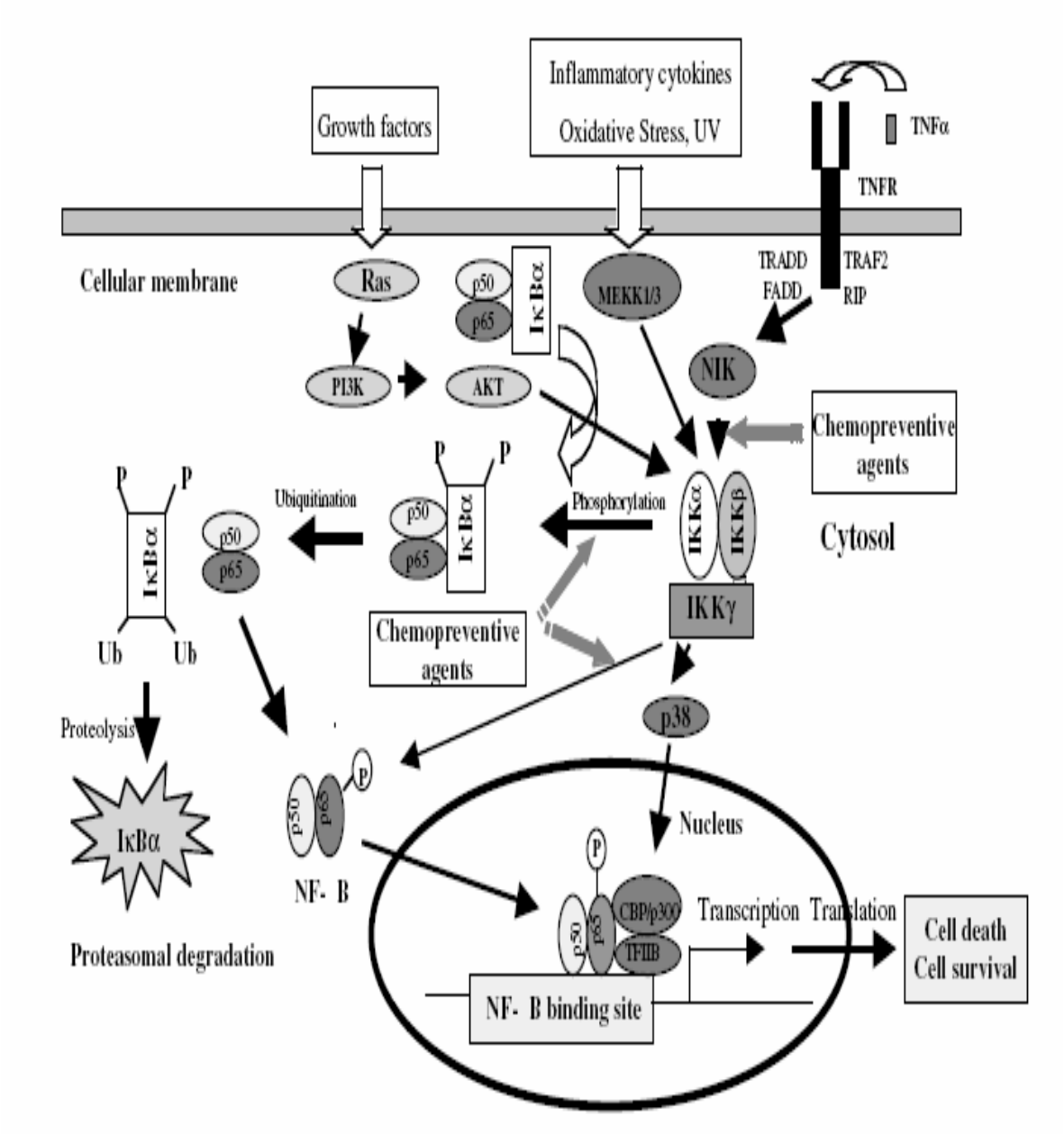

Figure 2. Effect of phytochemicals on activation of NF-κB. Courtesy Shen 2005

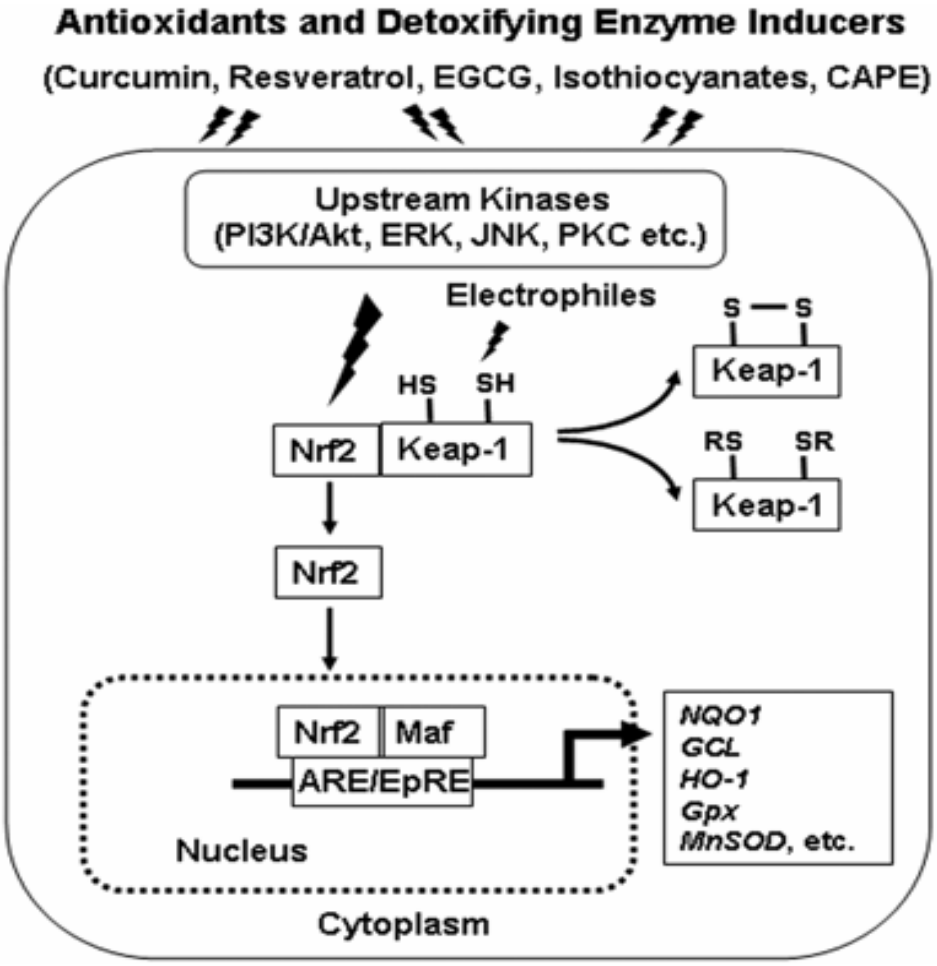

Figure 3. Activation of Nrf2 signaling and induction of phase II detoxifying and antioxidant genes. Chemopreventive phytochemicals activate diverse upstream kinases, which in turn stimulate dissociation of Nrf2 from Keap-1. Courtesy Surh 2005

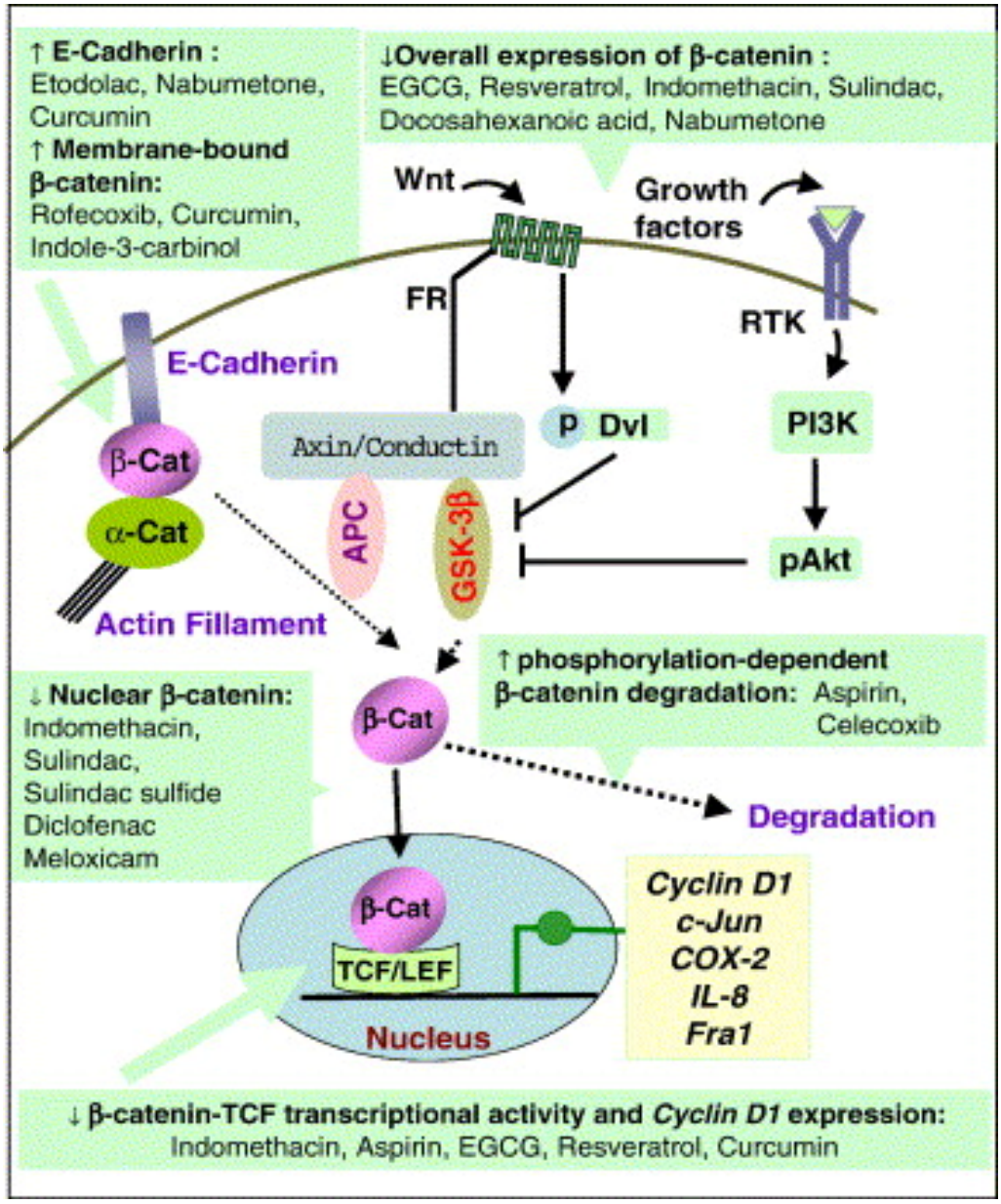

Figure 4. Modulation of aberrant β-catenin mediated signaling by phytochemicals and anti-inflammatory substances. Courtesy Kundu 2006

Chapter 1

Part 1: Green tea polyphenols and its constituent epigallocatechin gallate inhibit proliferation of human breast cancer cells *in vitro* and *in vivo*

INTRODUCTION

A report from National Cancer Institute on breast cancer statistics for the years 2001 through 2003 estimates that 12.7 percent meaning 1 in 8 of women born in United States today will develop breast cancer at some time in their lives. Premalignant progression and chronic administration of frequently used hormone responsive breast cancer treatments like aromatase inhibitors and specific estrogen receptor modulators may result in a hormone non-responsive form of the disease (Nandi et al 1995, Leary et al 2006). MDA-MB-231 is an estrogen receptor negative, highly invasive human breast cancer cell line and has been used as a relevant model system to evaluate chemopreventive agents against highly invasive and hormone non-responsive breast cancer phenotypes.

Tea [Camellia sinesis (Theacacea)] is considered second only to water as the most popular beverage consumed worldwide. Consumption of tea has been associated with many health benefits and its role and mechanism of action in cancer chemoprevention has been extensively reviewed (Adhamni and Mukhtar 2006, Beltz et al 2006, Hou et al 2004, Moyers and Kumar 2004, Surh 2003). Green, black and oolong tea are the three major commercial types of tea (Graham 1992). They differ in the way they are produced

and also in their chemical composition. Only 20% of the tea manufactured is green tea and it is processed to prevent the oxidation of green leaf polyphenols. Majority of the polyphenols are oxidized during black tea production and oolong tea consists of a partially oxidized product (Graham 1992). The biological activity of green tea is due to different catechins like epigallocatechin-3-gallate (62%), epicatechin-3-gallate (24%), epigallocatechin (5%), epicatechin (6%). Epigallocatechin gallate (EGCG) is identified as the principal antioxidant contributing approximately 30% of the total antioxidant capacity of green tea (Stewart et al 2005). Green tea's beneficial effect in prostate cancer has been extensively studied using pre-clinical transgenic models (Adhami et al 2004, Caporali et al 2004, Gupta et al 2001, Saleem et al 2005, and Sartor et al 2005) and nude mice xenograft models (Liao et al 1995). Breast cancer is the most common cancer in women and makes upto one tenth of all new cancer diagnoses worldwide (Bray et al 2004). Epidemiological studies suggest that increased consumption of green tea is also related to improved prognosis of human breast cancer (Nakachi et al 1998). An inverse association between the risk of breast cancer and the intake of green tea has also been reported in Asian- Americans (Wu et al 2003, Wu et al 2003,). The medicinal properties of these phytochemicals are often attributed to their antioxidative and/or anti-inflammatory activities (Yoneda et al 1995). Recent studies have shown that multiple mechanisms are involved in their beneficial effect with respect to tumor initiation, promotion and progression (Fassina et al 2004, Yamakawa et al 2004, Mittal et al 2004, and Yang et al 2003). However, it is still not clear whether these actions occur in animals or humans because of the limited bioavailability of EGCG following oral administration (Zhu et al 2000). Studying the cancer chemopreventive effect of polyphenols and the pathways

affected by them has been useful in understanding the molecular mechanism(s) involved in cancer promotion as well as to understand the properties of cancer cells.

Although green tea and its constituents have been shown to inhibit breast cancer (Kavanagh et al 2001, Sartippour et al 2001, Zhou et al 2004), the mechanism(s) of inhibition is not completely known. In this study, the anti-proliferative activity of GTP and its key constituent EGCG was evaluated using both *in vitro* with MDA-MB- 231 breast carcinoma cell line and *in vivo* with nude mice xenograft model. Both GTP and its constituent EGCG decreased the proliferation of the tumor cell line by arresting the progression of the cell through the G1 phase of cell cycle. Furthermore, GTP and EGCG were capable of delaying the tumor incidence as well as reducing the tumor burden in-vivo using MDA-MB-231 human breast carcinoma xenograft in athymic nude mice. These results suggest that green tea constituents may affect breast cancer cell on a cellular level and further studies are important to elucidate the precise mechanism(s) of inhibition of breast cancer by green tea.

MATERIALS AND METHODS

Materials

EGCG and GTP were obtained from LKT laboratories (St Paul, Minnesota, MN). The estrogen receptor-negative MDA-MB-231 breast cancer cell line was obtained from ATCC (Manassas, VA). The cells were maintained in monolayer in Eagle's minimum essential medium supplemented with 5% fetal bovine serum, nonessential amino acids, 2X vitamin solution, penicillin and streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air and were maintained at 70–90%

confluence in T-150 flasks. Medium was changed daily. Cells were dislodged for both passaging and harvesting by a brief incubation in 0.25% trypsin and 0.02% EDTA. Cells were stained with trypan blue and counted using a hematocytometer. For injection, the cells were re-suspended in the medium at a concentration of $5X10^6$ cells/ml.

Cell proliferation assay

MDA-MB-231 cell proliferation in the presence of various concentrations of EGCG and GTP was determined using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) cell proliferation kit (Boehringer Manheim, Indianapolis, IN) as per manufacturer's protocol. Briefly, cells were plated in 96-well tissue culture plates at a range of 3000 cells/well to a final volume of 100 µl of medium and were allowed to attach overnight. The cells were then treated once with varying doses of EGCG and GTP and observed after 24, 48, 72 and 96 hr. After completion of the treatment, the cells were incubated with MTT for 3-4 hr at 37°C. Cells were lysed, and the reduced intracellular formazan product was dissolved in the solubilization buffer provided in the kit. MTT is reduced to a colored, water insoluble formazan salt only by metabolically active cells which is quantitated in a conventional ELISA plate reader at 570 nm.

Cell cycle analysis

The effect of EGCG and GTP on cell cycle distribution was determined by flow cytometry after staining the cells with propidium iodide. Briefly, $5X10^5$ cells were seeded and allowed to attach overnight. The medium was replaced with fresh complete medium containing the desired concentration of EGCG and GTP. Both the floating and adherent

cells were collected, washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol at -80°C. The fixed cells were then centrifuged and washed with PBS. The cells were then treated with 1 unit of DNase-free RNase to the cell suspension (10^6 cells in 1 ml), and incubated for 30 min at 37°C. 50 µl of 1 mg/ml propidium iodide was added directly to the cell suspension and were passed in a Coulter Epics XL Flow Cytometer. The data was analyzed using modfit software.

Western blotting

Cells were harvested, pelleted and homogenized with ice cold lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% TritonX100, 1% sodium deoxycholate, 0.1% SDS to which 1mM DTT, 1 mM PMSF, 1 mg/ml pepstatin A, 10 mg/ml Leupeptin, 10 mg/ml aprotinin, 25 mM NaF, and 100 mM Na_3VO_4. The homogenate was passed 10 times through a 25-guage needle and centrifuged at 14,000Xg for 20 min at 4°C. The supernatant protein extract was transferred to fresh tubes, aliquoted and stored at -80°C. 50 µg of protein was suspended in 13 µl of lysis buffer thawed on ice, mixed with 2 µl of 10X sample reducing agent (Invitrogen, Carlsbad, CA) and 5 µl of 4X sample buffer (Invitrogen, Carlsbad, CA) and were heated at 100°C for 5 min. They were centrifuged and loaded on 4-12% Bis-Tris gels for fractionation at 120 V. Proteins on the gel were blotted onto nitrocellulose membrane at 225 mA for 120 min at 4°C. After transfer, the membranes were incubated with blocking buffer (5% skim milk in wash buffer (1 x TBS and 0.1% Tween 20)) for 1 hr at room temperature. Cell cycle protein expression was detected by incubating with primary antibodies (BD biosciences) in dilutions as specified by manufacturer in blocking buffer. The membranes were washed three times with wash

buffer and were then incubated with the appropriate secondary antibody (Santa Cruz Biotechnology) at dilutions of 1:2000 in blocking buffer. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) according to the specifications of the manufacturer (Santa Cruz Biotechnology). Blots were scanned and optical densities of the bands were quantitated. The expression cell cycle proteins were normalized with beta actin.

Animals

5 weeks old female athymic nude mice (NCr-nu/nu) were purchased from NCI (Frederick, MD) and were maintained in cages with housing in a specifically designed pathogen-free isolation facility. All the experiments were done in accordance with the United States Public Health Service policy and the University laboratory animal review board.

Tumor cell inoculation

The mice (n=45) were weighed and were anesthetized followed by an inoculation of $5X10^6$ cells in 100 µl of culture medium in the mammary fat pad. Mice were allowed to recover and were randomized into three groups of 10 animals each. 15 animals, which were not manipulated, were divided into 3 groups of 5 each and were used as control. The mice were weighed and the inoculation site was palpated at weekly intervals. The growth rates were determined by weekly measurement of two diameters of the tumor with a vernier caliper. Experiment was terminated 10 weeks after tumor cell inoculation. At the time of animal sacrifice, macroscopic examination of metastases was noted in lung, liver

and kidney. Part of the lung and tumor tissue was immediately frozen and the rest were fixed in 10% neutral-buffered formalin and were embedded in paraffin.

Feeding regimen

One group of animals received 1% GTP (made in autoclaved distilled water) as a sole source of drinking water and the other group received a dose of 1 mg/animal of EGCG in 100 µl of autoclaved distilled water. The control group was similarly handled and received 100 µl of autoclaved distilled water. The treatment was started the day after cell inoculation. Throughout the experiment, the animals had access to laboratory chow ad libitum.

Proliferating Cell Nuclear Antigen (PCNA) Staining

PCNA expression in tissue sections was analyzed by immunostaining using a monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Deparaffinized sections were rehydrated and treated with 10 mM citrate buffer (pH 6.0) at 95°C for 20 min. Endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide in phosphate buffered saline (PBS) followed by two washes with PBS. The sections were then treated with normal horse serum (Vector Laboratories, Burlingame, CA) followed by a wash with PBS. The sections were incubated with mouse monoclonal anti-PCNA antibody overnight at 4°C in a humidified chamber. Slides were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 10 min at room temperature with pre and post washes with PBS. Sections were incubated with conjugated horseradish peroxidase streptavidin complex for 5 min followed by incubation

with DAB substrate for peroxidase (Vector Laboratories, Burlingame, CA) for 7 min and a wash with PBS. The sections were counterstained with Hematoxylin.

In situ Apoptosis Detection by TUNEL Staining

Apoptotic cell death in deparaffinized tissue sections was determined by Terminal deoxynucleotidyl transferase (TdT) mediated D-Uridine Tri Phosphate nick end labeling (TUNEL) technique using apoptag peroxidase *in situ* apoptosis detection kit (Chemicon International Inc., Temecula, CA) as per manufacturer's information. This method is based on the specific binding of TdT to 3'-OH end of DNA and ensuring synthesis of a polydeoxynucleotide polymer. Briefly sections were digested using proteinase K and the endogenous peroxidase activity was blocked using 3% hydrogen peroxide in PBS. The sections were then placed in equilibration buffer and incubated with working strength of TdT enzyme in a humidifying chamber at 37°C for 1 hr. The reaction was terminated with stop/wash buffer, provided with the kit. The apoptotic nuclei were stained by direct immuno peroxidase detection of digioxigenin-labeled DNA in test sections.

Quantitative analysis of proliferation and apoptosis

We examined 10 fields (PCNA immunostaining or TUNEL) that were blinded and randomly selected for each slide. Each field was then photographed at a magnification of 20X. For each photomicrograph, we manually counted the number of total cells and the number of positive for anti-PCNA or TUNEL. The percentage of positive cells of the total number of cells was calculated for each image and a mean value was obtained for treated and untreated groups.

Statistical analysis

Unless otherwise indicated, data are presented as means±SEM of 3–5 different observations. Data were analyzed by using one-way analysis of variance (ANOVA). Significance was defined as a P-value of less than 0.05.

RESULTS

EGCG and GTP treatment inhibits growth of MDA-MB-231 in-vitro

The effect of increasing concentration of EGCG and GTP on the growth of the estrogen receptor-negative MDA-MB-231 human breast cancer cells for 24, 48, 72 and 96 hr was examined by MTT assay. Treatment with EGCG (1–200 μg/ml) and GTP (10– 150 μg/ml) inhibited the growth of MDA-MB-231 breast cancer cells in a concentration dependent manner with an IC50 of 50 μg/ml and 40 μg/ml for EGCG and GTP, respectively, at 48 h (Figure 5). Extensive inhibition of cell growth was observed in polyphenol treatment groups receiving higher concentrations (50 μg/ml) of either EGCG or GTP.

EGCG and GTP treated cells are arrested in G1 phase of the cell cycle

Cell cycle distribution analysis of MDA-MB-231 cells treated with varying concentration of EGCG (50 and 80 μg/ml) and GTP (40 and 60 μg/ml) and harvested at 12 and 24 h showed a G1 arrest (Table 1). Both EGCG and GTP treatments increased the percentage of cells in G1 at 12 hr (56% control vs. 76% treatment) and at 24 hr (43% control vs. 71% treatment). The percentage of cells in S-phase was significantly reduced by both EGCG and GTP at 12 hr followed by a reduction of cell in G2 in 24 hr. In order to examine the molecular mechanism(s), underlying changes in cell cycle pattern, the effect

of EGCG and GTP on various cyclins and CDKs involved in cell cycle control was investigated. MDA-MB-231 was treated with two different concentrations of EGCG (50 and 80 µg/ml) and GTP (40 and 60 µg/ ml) on MDA-MB-231 by treating them for a period of 12 and 24 hr (Figure 6A). More than 50% reduction in the expression of Cyclin E, Cyclin D and CDK 4 in treatment involving both concentrations EGCG and GTP for 12 hr (Figure 6B) was observed. A similar pattern was observed in 24 hr treatment (Figure 6C). Reduction in the expressions of PCNA and CDK 1 were also observed for both EGCG and GTP treatments. A moderate reduction in other Cyclins and CDKs were also seen.

Effect of oral administration of EGCG and GTP on the growth of human tumor xenograft in nude mice

EGCG solution (1 mg/0.1 ml/mouse) was administered by oral gavage and 1% GTP was administered as the sole source of water to nude mice bearing tumors established from MDA-MB-231. All of the animals appeared healthy with no loss of body weight (Figure 7A). The average GTP consumption was constant throughout the cages and was estimated at around 3 mg/mouse/day. The tumor incidence was decreased (25 and 55%) in EGCG and GTP treated animals as compared to the untreated control after 2 weeks of treatment. At the end of 10 weeks, 10 and 20% of mice in EGCG and GTP treatment, respectively, did not develop tumors whereas all the animals in the untreated control group developed tumors. Although the difference in the tumor development was not significant after 10 weeks, there was a delay in the average time of onset (Figure 7B). At the end of 10 weeks, the tumor volume was also reduced by 45 and 61% in the EGCG

treated and GTP treated, respectively, as compared to the untreated controls and was found to be statistically significant ($P<0.05$) (Figure 7C).

EGCG and GTP decrease proliferation and increase apoptosis of human tumor xenograft in nude mice

As there was a significant reduction in the tumor volume by the polyphenol treatment, the effect of EGCG and GTP on the tumor growth in xenograft was examined by performing TUNEL assay. The tissues were also stained with PCNA, a proliferation biomarker. EGCG and GTP fed animals showed increased apoptosis (Figure 8 A–C) and decreased proliferation (Figure 8 D–F) when compared with the untreated controls. GTP treatment showed a higher tumor cell death *in vivo* as around 80±10 SEM cells/field were apoptotic, whereas, it was around 60±8 SEM cells/ field in EGCG as compared to 23±5 SEM cells/field in control (Figure 9). The PCNA-positive cells in control mice xenograft was 87±12 SEM cells/field. A decrease in the PCNA positive cells in EGCG (24±5.0 SEM cells/field) and GTP (33±4.3 SEM cells/field) indicated the anti-proliferative effect of these polyphenols (Figure 9).

DISCUSSION

Despite significant advances in the treatment of breast cancer, this disease not only remains the second most frequent cause of cancer death but also one of the most commonly diagnosed cancers among women in 2005 (Jemal et al 2005). Epidemiological data from more than 250 case control and cohort studies show an inverse relationship between the risk of certain types of cancer and consumption of dietary phytochemicals

and fibers (Borek 2004). Previous studies in mice supported the anti-metastatic potential of green tea (Awad et al 2001, Baliga et al 2005), which is rich in polyphenols. Green tea has also been shown to significantly reduce the risk of breast and ovarian cancer in Asian women (Wu et al 2003, Zhang et al 2002). One of the key issues in chemoprevention with phytochemicals is to find out whether the activity and molecular mechanism(s) of the single active compound isolated and the extract are similar. Once its found that they are similar, the extract may preferentially replace single active compound, as the cost incurred to identify and to develop these isolated compounds are very expensive. The present research documents a parallel study showing the effect of GTP and EGCG treatment *in vivo* in a human tumor xenograft in nude mice as well as in *in vitro* cell culture models involving estrogen receptor negative MDA-MB-231. The data presented herein suggests that GTP and its principal constituent EGCG are effective in suppressing the proliferation of MDA-MB-231, a highly invasive estrogen receptor- negative breast cancer cell line as shown by growth inhibition and apoptosis induction both in-vivo and in-vitro. The study also explains the antiproliferative effect of these compounds as a function of their G1 blocking capacity. Many of the molecular alterations that accompany carcinogenesis lead to uncontrolled proliferation and growth and the ability of the transformed cells to evade apoptosis. Both EGCG and GTP had a significant antiproliferative effect. It has been shown that the anticancer effect of some potential phytochemicals like resveratrol and sulphoraphane take place through differential regulation of the cell cycle and subsequent events leading to cell death (Pozo-Guisando et al 2002, Jackson and Singletary 2004). Flow cytometry analysis showed that both EGCG and GTP treatments had a profound effect on the cell cycle control as cells accumulated

in the G1 phase of the cell cycle. Cyclin E, one of the key regulators of cell cycle has been found to be over-expressed in primary breast carcinoma tissue. Cyclin E has the potential to be used independently to predict the risk of visceral breast cancer relapse after surgery (Kim et al 2001). Cyclin E expression was generally high in estrogen receptor-negative tumors, suggesting a potential role for cyclin E in mechanisms responsible for estrogen-independent tumor growth. (Nielsen et al 1996). Over expression of cyclin D1 in the mammary gland of transgenic mice induces mammary carcinoma (Wang et al 1994). Cyclin D expression is deregulated frequently in human neoplasias and agents that can down regulate cyclin D1 expression could be helpful in the prevention as well as treatment of human neoplasias (Sausville et al 2000). Subsequent analysis of the cell cycle protein expression after EGCG and GTP treatment showed down-regulation of cyclin D and cyclin E, key cell cycle proteins, involved in G1/S progression. This indicates GTP and EGCG may be potential chemopreventive agents against breast tumors. EGCG was found to induce G1 phase cyclin-dependent kinase inhibitors and thereby causing an arrest in G1 phase of the cell cycle in prostate carcinoma cells (Gupta et al 2003). Furthermore, we also observed a tumor inhibitory activity of both EGCG and GTP in nude mice xenograft model. Both tumor incidence and mean tumor volume were significantly reduced by GTP and EGCG treatment. Immunohistochemical staining showed a decrease in the proliferating cell nuclear antigen and an increase in apoptosis in tumors from animals treated with EGCG and GTP. Earlier reports have indicated that induction of apoptosis by green tea catechin treatment may be due to a decrease in Bcl-2 and an increase in Bax level (Vergote et al 2002). Failure of normal apoptotic machinery in neoplastic cells underpin both tumorigenesis and drug

resistance. Neoplastic cells undergo changes that diminish their susceptibility to apoptosis (Hersey and Zhang 2003, Johnstone et al 2002, Kaufmann and Vaux 2003). Thus for, the phytochemical agent to be accepted and developed as a potential anti-cancer drug, it has to demonstrate a direct toxic activity on these resilient neoplastic cells. Our data indicate that both EGCG and GTP effectively suppress the proliferation as well as induce apoptosis in highly invasive estrogen negative MDA-MB-231 both *in vitro* and *in vivo*. Moreover, GTP as a mixture was needed in less concentration when compared to EGCG to induce similar cytotoxic effects in this cancer cell line. Though epidemiological studies and preclinical experimental studies, including ours, suggest the cancer chemopreventive and chemotherapeutic nature of these phytochemicals, we acknowledge that human intervention trials will be the ultimate proof of the aforementioned beneficial nature of these phytochemicals.

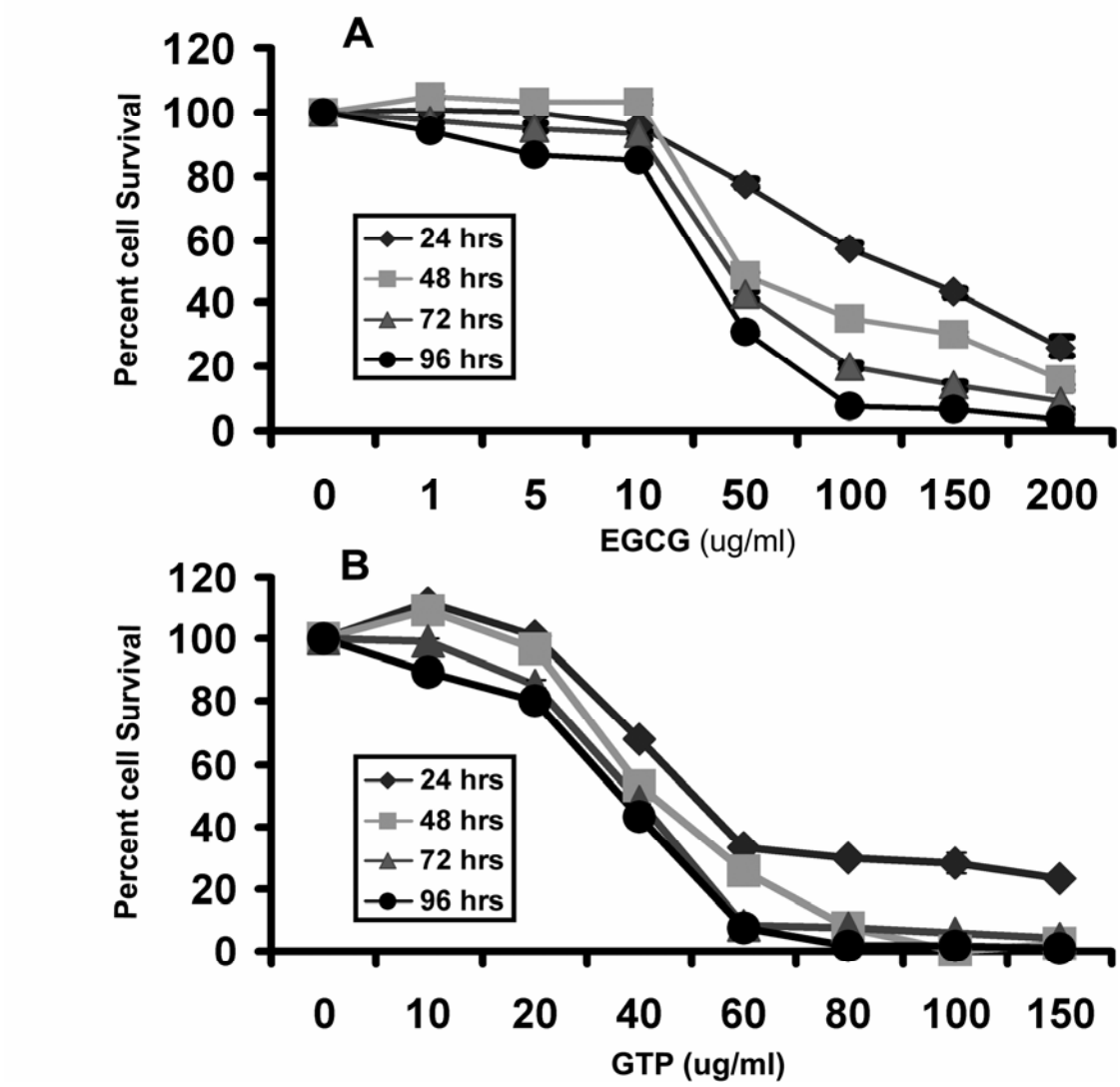

Figure 5. EGCG and GTP inhibit the proliferation of MDA-MB-231 cells. (A) Effect of different concentrations of EGCG on the proliferation of MDA-MB-231 cells incubated for 24,48,72,96 h. (B) Effect of different concentrations of GTP on the proliferation of MDA-MB-231 cells incubated for 24,48,72,96 h. The data are presented as the mean plus/minus SE(n=10).

Table 1. Flow cytometric analysis of the effect of EGCG and GTP on MDA-MB-231 cells

	12 hrs			24 hrs		
	G1	S	G2	G1	S	G2
CONTROL	56.21	42.54	1.25	43.23	31.15	25.62
EGCG50	76.04	19.32	4.64	71.12	20.69	8.19
EGCG80	74.87	19.86	5.27	71.75	19.51	8.74
GTP40	74.8	20.12	5.08	70.05	23.45	6.5
GTP60	77.19	18.7	4.11	72.46	20.36	7.18

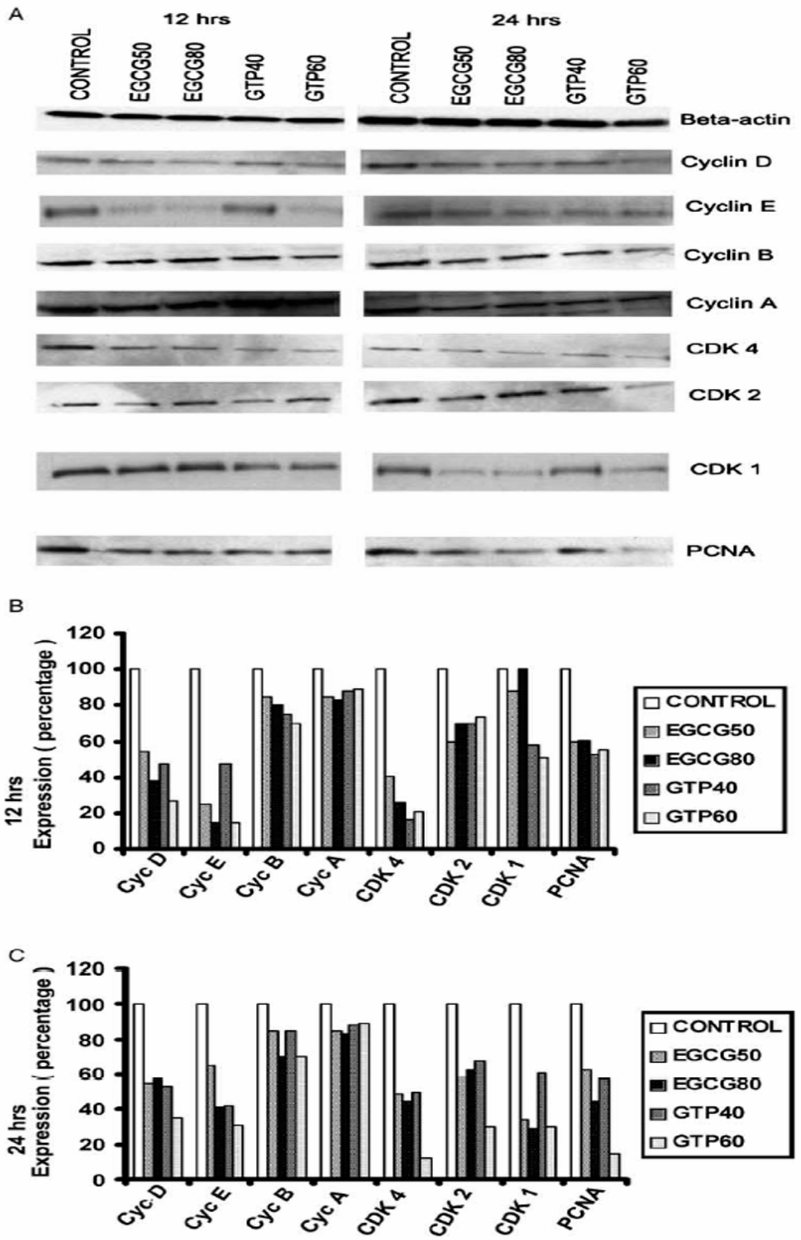

Figure 6. Effect of EGCG and GTP on Cyclins and CDK. (A) Representative picture of Western blot of cell cycle proteins of MDA-MB-231 cells treated with 50 and 80 ug/ml of EGCG and 40 and 60 ug/ml GTP for 12 and 24 h. (B) Quantitation of 12 h expression of cell cycle proteins. (C) Quantitation of 24 h expression of cell cycle proteins. Data are representative of two individual experiments.

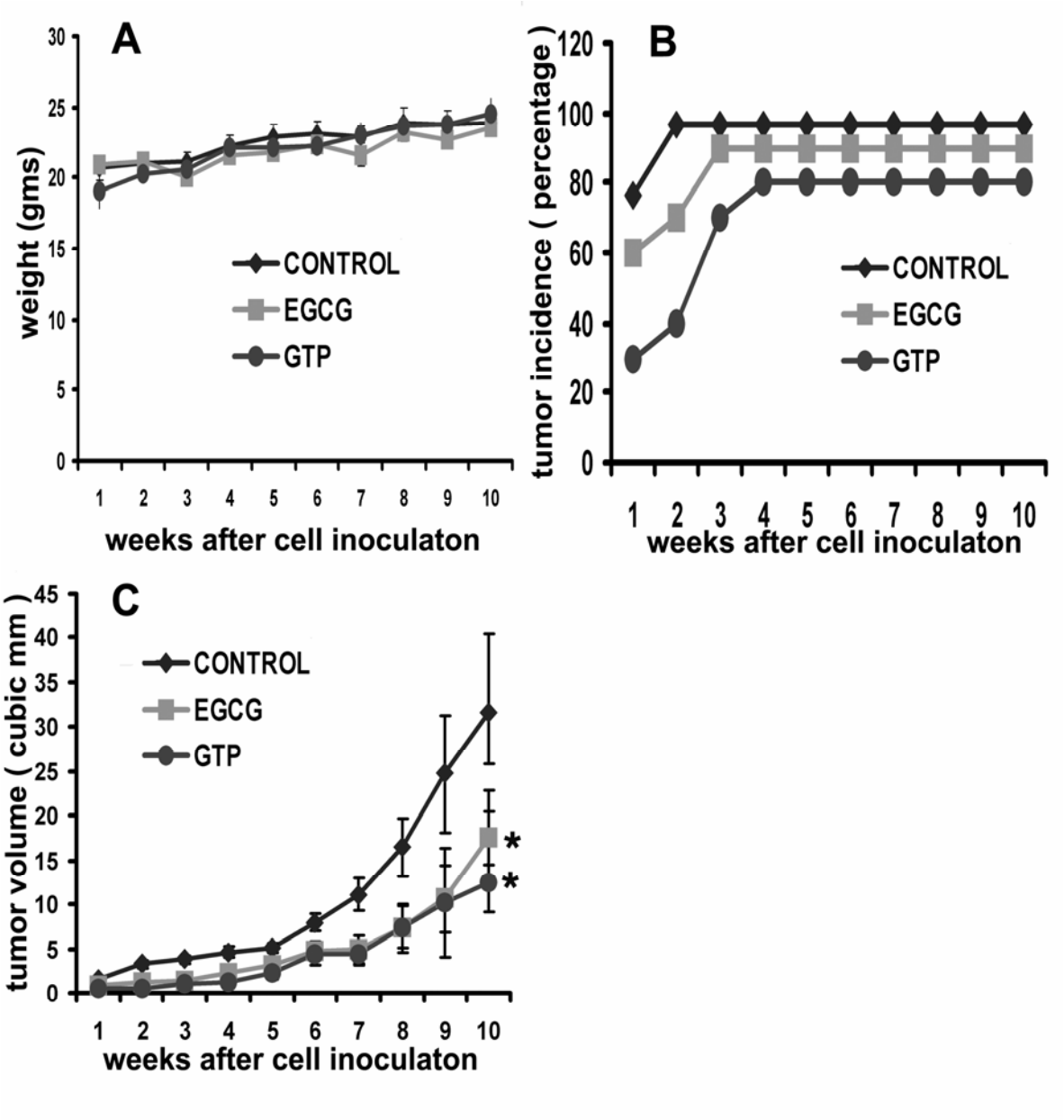

Figure 7. Effect of EGCG and GTP *in vivo*. (A) Effect of EGCG and GTP on body weight of nude mice. (B) Effect of EGCG and GTP on tumor incidence of nude mice. (C) Effect of EGCG and GTP on tumor volume. Data for weight and tumor volume are mean plus/minus SE (n=10). *Significantly different compared with controls, $P<0.05$.

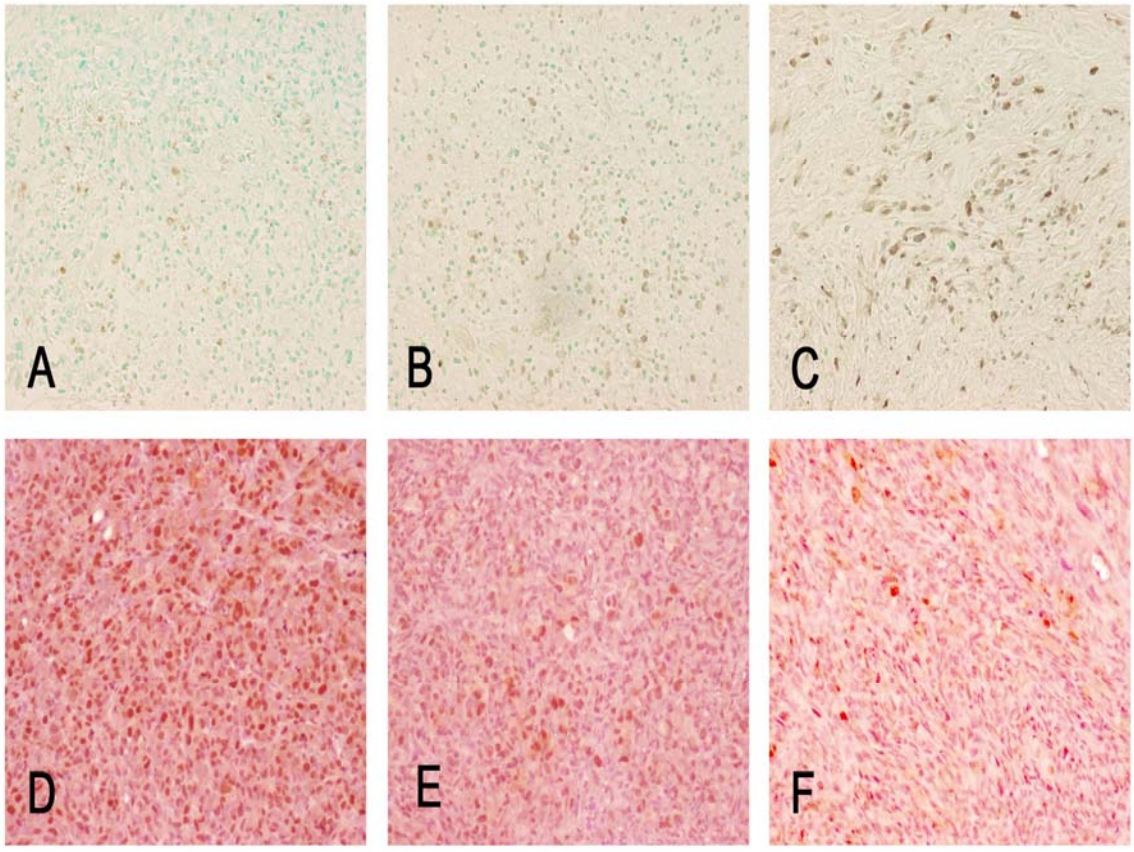

Figure 8. Immunohistochemical staining for TUNEL and PCNA in MDA-MB-231 xenograft tumor masses. Top panels: TUNEL staining in tumor masses from (A) control, (B) EGCG treated, and (C) GTP treated animals. The TUNEL staining was pronounced in the center of the tumor mass. A greater number of TUNEL positive cells were observed in tumors from polyphenol treated animals. Nuclei were counterstained with methyl green. Bottom panels: PCNA staining from tumor masses in (D) control, (E) EGCG treated, and (F) GTP treated animals. Polyphenol treatment decreases the number of proliferating cells in tumor masses. Counterstained with hematoxylin. All images are 200X.

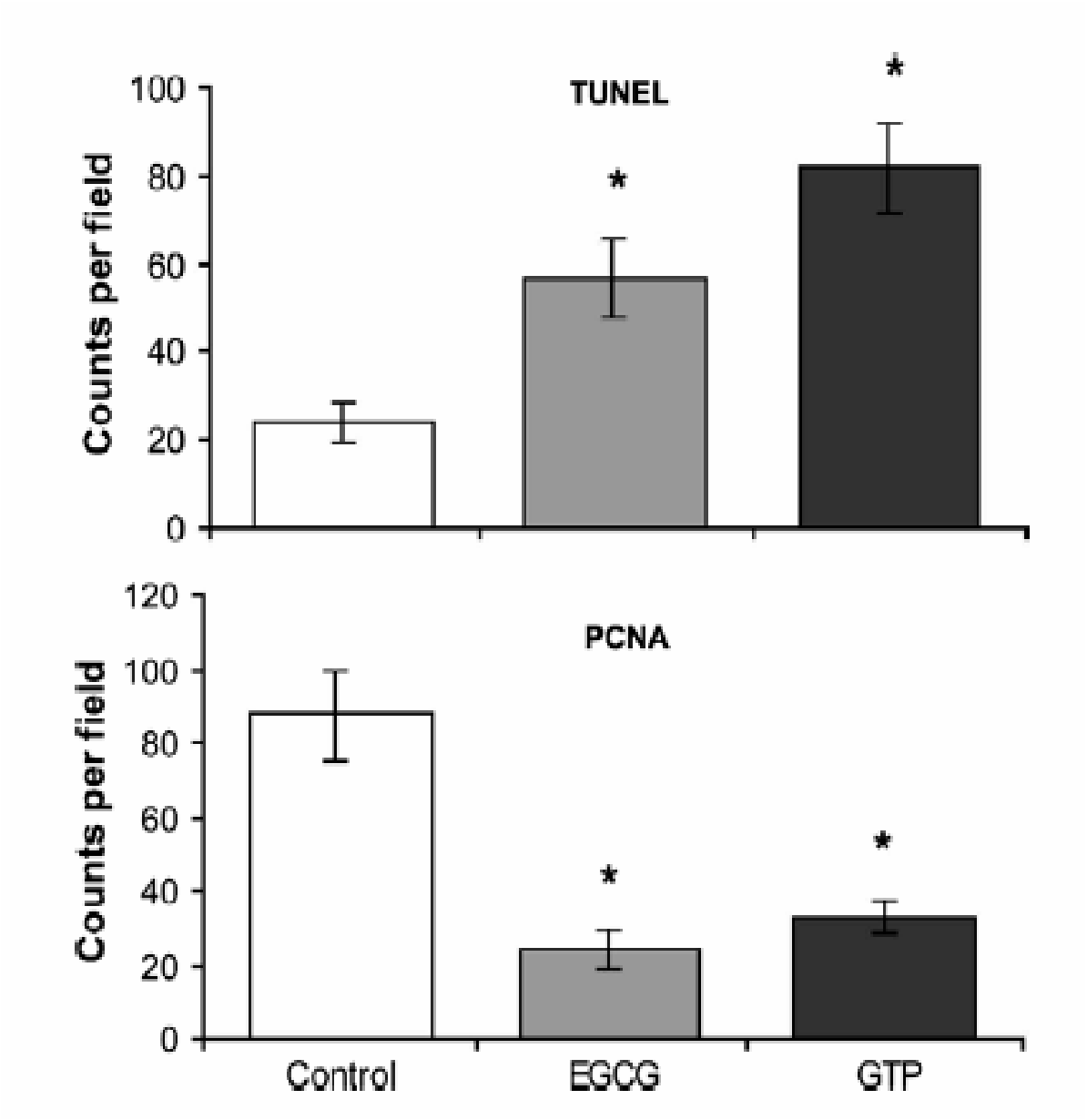

Figure 9. The number of TUNEL and PCNA positive cells in microscopic (20X) fields from 6 samples were averaged. Error bars indicate SEM. *Significantly different compared with controls, $P<0.05$.

Chapter 1

Part 2: Green Tea Polyphenol and Epigallocatechin Gallate Induce Apoptosis and Inhibit Invasion in Human Breast Cancer Cells

INTRODUCTION

In recent studies, GTP and EGCG have been shown to have therapeutic potential in treatment of cancers using MDA-MB-231 as a model system. GTP and EGCG have been shown to suppress MDA-MB-231 tumors *in vivo* (Thangapazham et al 2006) and exhibit proapoptotic, anti-invasive and antiproliferative properties *in vitro* (Thangapazham et al 2006, Bigelow and Cardelli 2006).

In the present study, we have investigated the possible mechanism(s) involved in pro-apoptotic and anti-invasive effects of EGCG and GTP in MDA-MB-231 cells. The results showed that EGCG and GTP induced apoptosis and suppressed invasiveness of MDA-MB-231 cells in a dose dependent manner. A fragmented DNA ladder was detected by electrophoresis in cells treated with polyphenols indicating apoptosis. AKT, which is a serine/threonine kinase, regulates cell survival and invasion. Treatment of cells with varying concentrations of EGCG and GTP inhibited AKT at both the RNA and protein level. Moreover EGCG and GTP treatment resulted in lesser phosphorylated AKT when compared to untreated controls. Polyphenol treatment in this cell line decreased the level of beta-catenin in the cytoplasm and also reduced its accumulation in the nucleus. Our results suggest that down-regulation of AKT plays an important role in proapoptotic and anti-invasive potential of green tea. These studies may have greater clinical significance

since the ability of polyphenol to activate the apoptotic program and decrease invasiveness of tumor cells might determine the success of chemotherapy.

MATERIALS AND METHODS

Materials

Antibodies for proteins involved in apoptosis were purchased from BD Biosciences (San Jose, CA). Beta-catenin antibodies and AKT antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Phospho-AKT (Ser-473) antibody was purchased from cell signaling technology, Danvers, MA.

Methods

DNA Fragmentation

To measure DNA fragmentation, 1 x 10^6 untreated or treated MDA-MB-231 cells were washed with PBS and then resuspended in 10mM of Tris (pH 8), 10 mM of EDTA (pH 8). Proteinase K was added to a final volume of 200 µg/ml followed by 10 % SDS to obtain a final concentration of 0.5 %. Samples were incubated at 55˚C for 4 h. 5 M NaCl was added to a make a final concentration of 1M and incubated on ice for 45 min. Protein precipitates were sedimented by centrifugation at 5000 g for 15 min and the supernatant was transferred to a fresh tube. DNase free RNase was added to a final concentration of 25 µg/ml and was incubated at 37˚C for 60 min. DNA was precipitated overnight at -20˚C after adding 2 volumes of 80 % ethanol. DNA was pelleted by centrifuging at 10,000g for 30 min at 4ºC. The pellet was resuspended in 10 mM of Tris (pH 8), 10 mM of EDTA(pH 8) and were fractionated on 1.5% agarose gel.

Western blotting was performed as described in page 18

Invasion Assay

MDA-MB-231 cells were was grown to 70-80% confluence. Cell suspension were prepared by trypsinizing the monolayer and resuspending in medium without FBS at $5x10^4$ cells/ml. BD Falcon HTS FluoroBlok 24-Multiwell Insert plate system (catalog No. 354165) was prepared by rehydrating BD Matrigel Matrix coating with PBS for 2 hr at 37˚C. The rehydrating solution was carefully removed, 0.75 ml of medium containing 10% FBS (chemoattractant) was added to the well and 0.5 ml of cell suspension was added to each insert well. EGCG and GTP were added to the medium in both upper and lower chambers along with the cells and chemoattractant solution. Uncoated insert plate BD Falcon HTS FluoroBlok 24-Multiwell Insert system (catalog No. 351157) included as migration control, was used without rehydration. Following incubation for about 24 hr, the medium was removed from upper chamber and the entire insert plate was transferred to a BD Falcon 24-well plate for post cell invasion labeling (catalog no. 351147) containing 0.5ml/ well of 4 ug/ml Calcein AM in Hanks buffered saline. The plate was incubated for one hr at 37˚C and read in a fluorescence plate reader with bottom reading capabilities without further manipulation.

Isolation of RNA

Cells grown with or without GTP and EGCG were trypsinised, pelleted and washed with PBS and transferred to 1.5 ml microfuge tubes. Total RNA was extracted using TriZol kit (Invitrogen life technologies, Carlsbad, CA) and quantitated spectrophotometrically using Beckman DU640 Spectrophotometer (Beckman instruments Inc., Columbia, MD, USA). RNA quality was determined by electrophoresis on 1% agarose formaldehyde gel.

Oligo GEArray Microarray

Human Breast Cancer Biomarker Oligo GEArray Microarray kit (Superarray Bioscience Corporation, Frederick, MD) was used and experiments were carried out as per the manufacturer's protocol. cRNA probe was synthesized from 1µg of RNA using TrueLabeling- AMP Linear RNA Amplification Kit, SuperArray Bioscience Corporation, Frederick, MD. The amplified cRNA was purified using spin column (ArrayGrade™ cRNA Cleanup Kit, SuperArray Bioscience Corporation, Frederick, MD) and quantitated spectrophotometrically. Array membranes were prehybidized for 2hr/60°C/ 10-20 rpm hybridization solution followed by overnight hybridization with 4µg of cRNA mixed in 750µl of hybridization buffer at 60°C/ 10-20 rpm in hybridization chamber. Array membranes were then washed for 15 min each at 60°C/10-20 rpm with pre-warmed wash solution 1 (2XSSC, 1% SDS) and wash solution 2 (0.1XSSC, 0.5%SDS) respectively. Blocking was done for 2hr/10-20 rpm at room temperature (RT) with 2ml of blocking solution Q followed by incubation with 2ml of binding solution (1:15000 Ap-strep in 1xBuffer F). Array membranes were then washed with 1X buffer F and rinsed with buffer G. Detection was done by incubating the membranes with 1ml of CDP star substrate

supplied with the kit for 3min, 10-20 rpm/ RT. X-ray films (Kodak Scientific Imaging Film, Eastern Kodak Company, Rochester, NY) were exposed to the membrane for different time periods and films were developed using Kodak Image developer. X-ray images were scanned and converted to digital image for further analysis.

Analysis: Image analysis was done using GEArray Expression Analysis Suite (Superarray Bioscience, Frederick, MD). Gene densities were expressed as average density (total density divided by the number of pixels). Background detection was done locally i.e. each expression value was individually subtracted with value from the area outside the capture grid but within the spot cell area. Data normalization was done with non-modulating house keeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A gene was considered "absent" where the average density of the spot was less than the mean value of the local backgrounds of the lower 75 percentile of all non-bleeding spots. All other spots were considered “present”.

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

cDNA was synthesized using the Superscript first strand synthesis system for RT-PCR kit (Invitrogen Inc. Carlsbad, CA). Briefly, primer mix (1ugRNA, dNTP, oligo dts) was incubated at 65°C for 5 min, then mixed with reaction mixture (10X PCR buffer, 25mM MgCl2, 0.1M DTT, RNase inhibitor) and incubated at 42°C for 2 min. cDNA synthesis was done using RT enzyme (SSII) at 42°C for 50 min. Reaction was stopped by incubating at 70°C for 15 min. Residual RNA was digested by E. coli RNase H at 37°C for 20 min and samples were stored at -20°C. PCR products were visualized by electrophoresis over 1.2% agarose gel and staining with ethidium bromide. Specific

amplification was determined by comparing the product size on the gel relative to known DNA molecular weight marker. The primers for AKT1 and c-myc were previously described (Jordan et al 2004, Park et al 2005).

Zymogram

Cells were seeded onto six-well plates in the propogation media mentioned above and cultured up to 80% confluence. Cells were subsequently washed in Ultra culture serum free media twice and the cells were then treated with TPA (80 nM) and/or varying doses of EGCG and GTP in the serum free medium for an additional 24 hr. The conditioned media from different treatments was collected and standardized to cell number and was concentrated 5 times, mixed with non reducing sample buffer and were loaded on 10% Zymogram (Gelatin) gels (Invitrogen life technologies, Carlsbad, CA) and electrophoresis was carried out in novex protein gel running tank(X Cell II, Novex Experimental Technologies) at 110° volts for 3 hr at 4°C. Gels were then taken out by breaking the cast, making sure that gels do not dry, then washed with 2.5% triton X 100 for 30 min over shaker (32 cycles/min) with one change of solution after 15 min followed by the washing with double distilled water for 20 min over shaker (32 cycles/min) with one change of water after 10 min. Gels are then treated overnight with substrate buffer (TrisCl 50 mM, CaCl2 5 mM, Tween 20 0.02%) at 37°C followed by the staining with 0.5% Comassie blue R-250 solution (Comassie R-250: 0.5%, Acetic Acid 10%, Methanol 40%) for 1 hr over shaker (32 cycles/min). Subsequent destaining was done with destaining buffer (Acetic Acid: Methanol: water:: 1:4:6) for 20 min with one change of buffer after 10 min. Gels were washed with double distilled water and kept in distilled

water overnight. The molecular weights of the proteolytic bands were determined in relation to the reference marker proteins, which were simultaneously loaded in the gel.

Cellular Activation of Signaling ELISA for AKT S473

Cellular Activation of Signaling ELISA (CASE™) Kit for AKT S473 (Superarray Bioscience, Frederick, MD) was used to analyze AKT phosphorylation levels in polyphenol treated cells. The CASE™ kit for AKT is designed to analyze AKT phosphorylation in cultured human and mouse cell lines. This cell-based ELISA kit quantifies the amount of activated (phosphorylated) AKT protein relative to total AKT protein. The AKT phosphorylation site is serine 473. This cell-based ELISA kit quantifies the amount of activated (phosphorylated) AKT protein relative to total AKT protein and was performed according to manufacturer's protocol. Briefly, 5000 cells are seeded into 96-well plates. After the experimental treatment, the cells are fixed to preserve any activation-specific protein modification, such as phosphorylation. Two primary antibodies are included in the kit. One antibody recognizes only the activated (phosphorylated) form of the specific target protein, while another recognizes the specific target protein regardless of its activation state. Following incubation with primary and secondary antibodies, the amount of bound antibody in each well is determined using a developing solution and an ELISA Plate Reader. The absorbance readings are then normalized to relative cell number as determined by a cell staining solution. The amount of phosphorylated protein, once normalized to the amount of total protein, is then directly related to the extent of downstream pathway activation.

Nuclear extract

Nuclear extracts were prepared with a NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce) according to the manufacturer's protocol. NE-PER® Nuclear and Cytoplasmic Extraction Reagents enable stepwise separation and preparation of cytoplasmic and nuclear extracts from mammalian cultured cells or tissue. Cells treated with different treatment of polyphenol were pelleted out by centrifugation. 200 µl of ice-cold CER I reagent from the kit was added to the cell pellet and vortexed vigorously on the highest setting for 15 sec to fully resuspend the cell pellet. 11 µ l of ice-cold CER II was added to the tube and was vortexed for 5 sec on the highest setting. The tube was then incubated on ice for 1 min followed by 5 sec vortexing. The samples were centrifuged for 5 min at maximum speed (~16,000 x *g*). The supernatant (cytoplasmic extract) fraction was immediately transferred to a clean pre-chilled tube. The insoluble (pellet) fraction which contains nuclei was resuspended in 100 µl of ice-cold NER and vortexed on highest setting for 15 sec. The vortexing was continued for 15 seconds every 10 min, for a total of 40 min. The samples were then centrifugd for 10 min and the supernatant (nuclear extract) fraction were immediately transferred to a clean pre-chilled tube. All extracts were stored at -80°C until use. Addition of the first two reagents to a cell pellet causes disruption of cell membranes and release of cytoplasmic contents. After recovering the intact nuclei from the cytoplasmic extract by centrifugation, the nuclei are lysed with a third reagent to yield the nuclear extract. Extracts obtained with this product generally have less than 10% contamination between nuclear and cytoplasmic fractions which is sufficiently pure for most experiments involving nuclear extracts. The levels of

beta-catenin in the cytoplasmic and nuclear extracts were assessed by immunoblotting with anti- β-catenin.

RESULTS

Effect of EGCG and GTP on DNA fragmentation

Typical DNA ladder pattern of internucleosomal fragmentation is known as a biological hallmark of apoptosis. To determine whether EGCG and GTP induce apoptosis, DNA was isolated from treated and untreated cells and was then fractionated by agarose gel electrophoresis. A typical DNA ladder pattern was observed in cells treated with both EGCG and GTP which was dose dependent, thus showing that both EGCG and GTP treatment induce apoptosis in MDA-MB-231 cells (Figure 10).

Effect of EGCG and GTP on DNA proteins involved in apoptosis

Western blot analysis showed that EGCG and GTP increased Bax and decreased Bcl2 in a dose dependent manner (Figure 11). After treatment with EGCG and GTP for 24 h, the full-length form of the PARP protein (116 kDa), a substrate for caspase-3, degraded into the cleaved form (85 kDa) (Figure 11). Thus, it appears that EGCG and GTP may induce apoptosis by altering the Bax/Bcl2 ratio favoring apoptosis and up regulating proteases and inducing PARP cleavage.

EGCG and GTP treatment decreases MDA-MB-231 cell invasion through Matrigel in-vitro

The effects of EGCG and GTP on tumor cell invasion were investigated *in vitro* by utilizing a synthetic basement membrane system (a modified Boyden chamber). Tumor cells were plated on a "partition barrier," consisting of a porous filter (8-micron pores) which was coated with a reconstituted basement membrane matrix (Matrigel), and induced to migrate across the barrier with medium enriched with 5% FBS (chemoattractant). The cells that invaded matrigel cells were fluorescence labeled with calcein AM, which is a cell-permeant dye that can be used to determine cell viability in most eukaryotic cells. In live cells, the non-fluorescent calcein AM is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. Quantitative Fluroscence labeling showed a 24-28 % reduction in the invasion by EGCG and 15-23 % reduction by GTP in a dose dependent manner (Figure 12).

Effect of EGCG and GTP on MMP-9 Expression

To compare the differential gene regulation in polyphenol treated and untreated cells, we did a focused microarray with the breast cancer biomarker gene array panel. The analysis indicated more than fivefold down-regulation of MMP-9 in both EGCG and GTP treated cells compared with the untreated controls (Figure 12). To confirm the results of the microarray experiment, we did a reverse transcriptase PCR reaction for the RNA isolated from cells treated and untreated with the polyphenol. The results showed that MMP-9 was down-regulated in the treated cells when compared with the untreated cells thus confirming the results of the microarray experiment (Figure 12). Moreover, the inhibition

of MMP-9 transcript was dose dependent. To compare MMP-9 activity in cell supernatants, conditioned media from EGCG and GTP treated and untreated cells were subjected to gelatin zymography. The result indicated that increasing doses of EGCG and GTP caused gradual decrease in MMP-9 expression (Figure 12).

Effect of EGCG and GTP on AKT and AKT phosphorylation

It has been shown that constitutively active AKT protects cells from apoptosis and also aids in invasion. Western blot analysis using anti-phosphospecific-AKT antibody showed that both EGCG and GTP suppressed AKT phosphorylation in a dose dependent manner (Figure 13). Cellular Activation of signaling ELISA for AKT S473 also confirmed that EGCG and GTP inhibited AKT phosphorylation in a dose dependent manner (Figure 13). Furthermore, EGCG and GTP inhibited AKT1 protein levels as revealed by western blots (Figure 14). AKT1 has recently been found to be a target gene for beta-catenin. Reverse transcriptase PCR for AKT1 revealed a dose dependent down-regulation of AKT1 gene in both EGCG and GTP treatments (Figure 14). C-myc a well known target gene of beta-catenin induced transcription and was also found to follow the same pattern as AKT1 in EGCG and GTP treated samples (Figure14).

Effect of EGCG and GTP on beta-catenin

The activation of TCF regulated genes results from the accumulation of beta-catenin in the nucleus. We performed western blot analysis for the cytoplasmic and nuclear fraction of cell lysates which were previously treated with EGCG and GTP for beta-catenin. The amount of beta-catenin protein was reduced both in the cytoplasmic as well as in the

nuclear fraction in the treated group when compared to the untreated control (Figure 14). Therefore, the data suggests that decreased amount of beta-catenin would have resulted in reduced formation of beta-catenin-TCF-4 complex culminating in down-regulation of beta-catenin/Tcf regulated genes like c-myc and AKT1.

DISCUSSION

SERM and Aromatase inhibitors are currently the popular treatment options for hormone dependent breast cancer. Resistance eventually develops to all forms of treatment and hormone non-responsive form of breast cancer is extremely difficult to manage as it progresses to a metastatic stage disease. Hence it becomes pivotal to investigate other strategies to control tumor proliferation. In this study, we investigated apoptotic and anti-invasive properties of GTP and EGCG in estrogen receptor negative MDA-MB-231 cells. Our results suggest that EGCG induces apoptosis and reduces invasive property of the aggressive MDA-MB-231 cells. These data also suggest that the beneficial properties of GTP and EGCG may be due to AKT inhibition.

Many of the molecular alterations that accompany carcinogenesis lead to uncontrolled proliferation and the ability of transformed cells to evade apoptosis. Both GTP and EGCG were pro-apoptotic as shown by intra-nucleosomal cleavage of DNA, which was visualized by a typical DNA ladder pattern. Induction of apoptosis was primarily due to Bax/Bcl2 ratio favoring apoptosis. PARP cleavage was also observed in polyphenol treated groups. Metastasis plays a major role in increasing the mortality of breast cancer patients and phytoestrogens are found to reduce the metastatic potential of breast cancer cells in-vitro(Menon et al 1998). Green tea polyphenol has recently been shown to have anti-metastatic effect on metastasis-specific mouse mammary carcinoma 4T1 cells(Baliga

et al 2005). We have shown the anti-invasive property of EGCG and GTP in modified Boyden chamber in highly invasive MDA-MB-231 breast cancer cell line. Invasion being a prominent initial step in metastasis, anti-invasive capacity of these phytoestrogens might have implications in increasing the survival time and decreasing morbidity of breast cancer patients.

For tumor angiogenesis as well as tumor invasion, degradation of ECM by MMPs is a critical process. Main function of MMPs is degradation of extracellular matrix contributing to cancer whereby tumor cells utilize the matrix degrading capability of these enzymes to spread to distant sites. Additionally MMPs are also thought to promote growth of these tumor cells once they have metastasized (John and Tuszynski 2001). Although many MMPs have been identified, MMP-2 (Gelatinase-A) and MMP-9 (Gelatinase-B) are thought to be the key enzymes as they degrade type IV collagen, a main component of ECM(Stamenkovic 2003, Hojilla et al 2003). In our study, MMP-9 was found to be down-regulated at the transcriptional level as revealed by focused microarray analysis, which was further confirmed by RT-PCR. Zymographs of cell culture supernatants revealed that GTP and EGCG modulate gelatinolytic activities of MMP-9 indicating that they may be exerting their invasion inhibitory effect by inhibiting proteinases.

Not only has PI3K/AKT signaling been found to be involved in survival and proliferation of a variety of tumor cells, their hyperactivation has resulted in altering the response of tumor cells to chemotherapy and irradiation (Kuo et al 2006). Recent study shows AKT to be one of the major pathways for MMP-9 expression and revealed that isoginkgetin, a biflavanoid for Dawn redwood to potently inhibit MMP-9 expression and tumor cell

invasion through the inhibition of AKT pathway (Dihlmann et al 2005). In our study, AKT was found to be inhibited both at the RNA and the protein level by polyphenol treatment. Moreover, EGCG and GTP decreased AKT phosphorylation as shown by western blotting analysis.

Beta-catenin signaling pathway contributes to transcriptional regulation of AKT1(Dihlmann et al 2005) and hence AKT1 can be up-regulated by a deregulated beta-catenin signaling. Also, beta-catenin signaling is activated in breast cancer and is up-regulated by numerous pathways in more than 50% of breast tumors (Cowin et al 2005). Thus, we investigated whether beta-catenin signaling is affected by EGCG and GTP. Beta-catenin protein level was decreased in cytoplasm and nucleus with polyphenol treatment. C-myc, one of the prominent among beta-catenin regulated genes was also found to be down-regulated by EGCG and GTP treatment and followed same pattern as AKT1 suggesting that beta-catenin can be involved in polyphenol mediated down-regulation of AKT1.

These data provide a basic mechanism for chemotherapeutic properties of EGCG and GTP in human breast cancer cells. Since deregulation of AKT pathway has been implicated in cancer, investigation of the molecular mechanism(s) that govern the expression of AKT should provide insight into its biological functions and can be used to design treatment strategies to target this pathway for prevention and treatment of cancer. Further *in vivo* studies are pivotal to confirm the translation of these *in vitro* mechanisms and are required to determine future therapeutic applications and chemopreventive effects of EGCG and GTP against breast cancer.

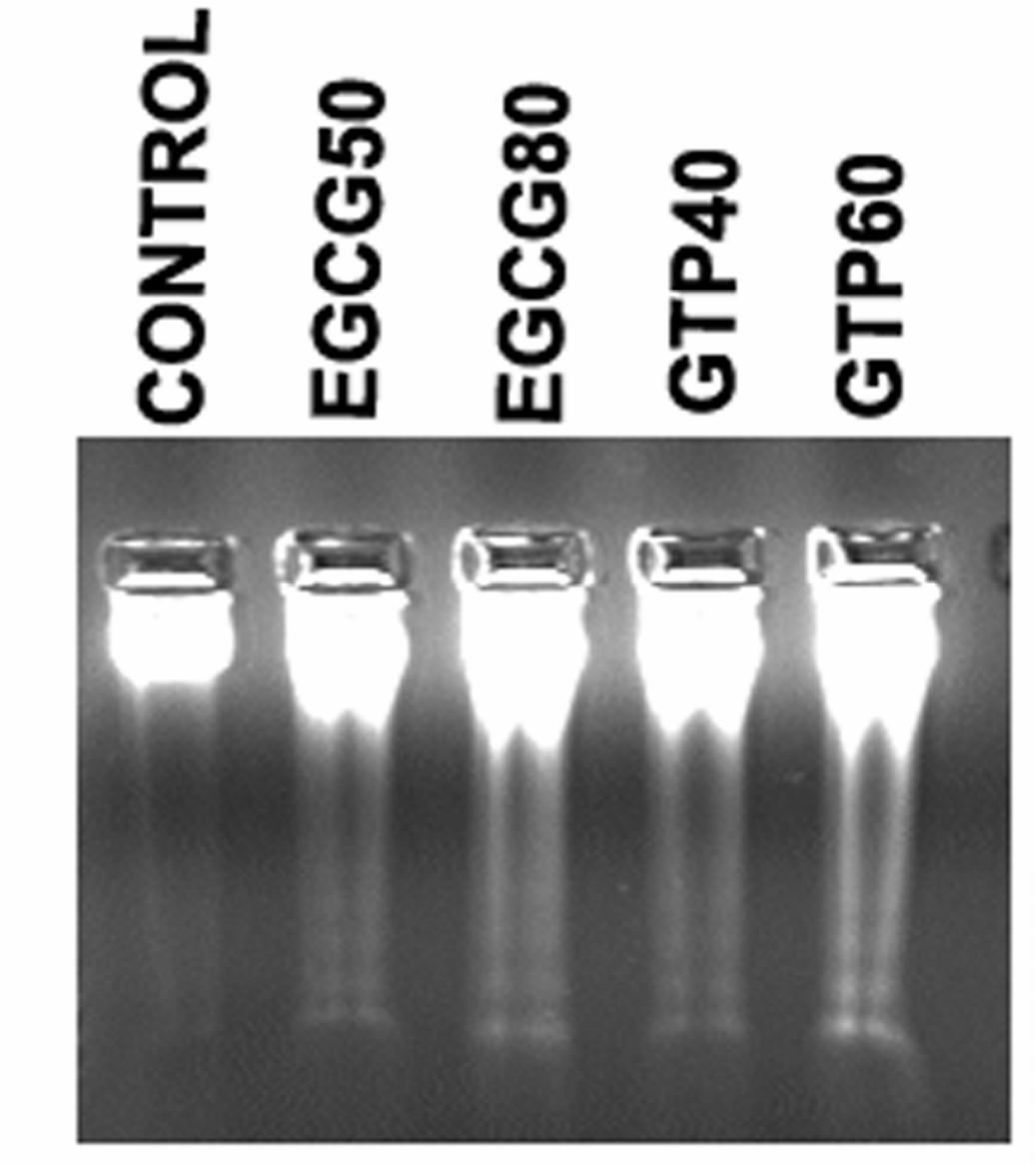

Figure 10. Effect of EGCG and GTP on DNA fragmentation. Representative picture from three independent experiments.

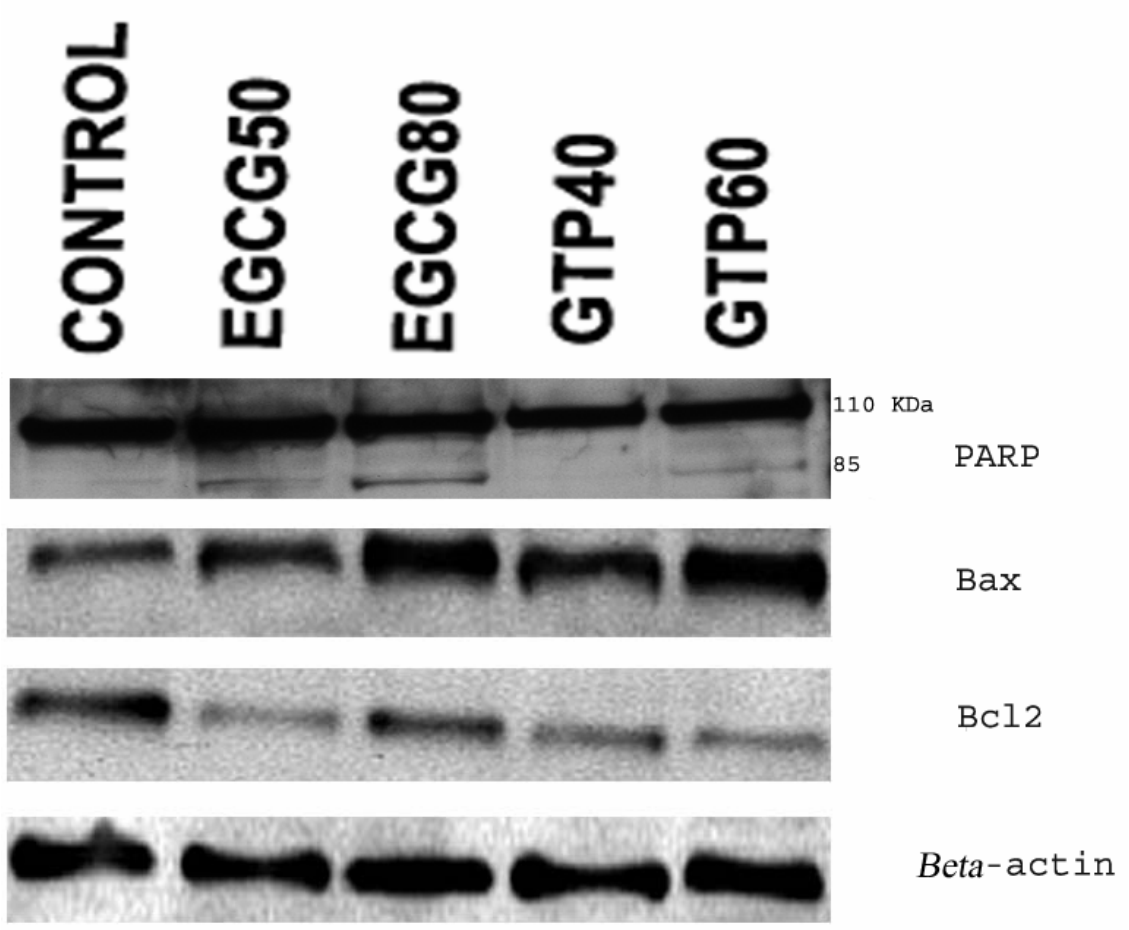

Figure 11. Effect of EGCG and GTP on proteins involved in apoptosis. Representative picture of western blot of cell cycle proteins of MDA-MB-231 cells treated with 50 and 80 ug/ml of EGCG and 40 and 60 ug/ml GTP 24 hrs from two independent experiments.

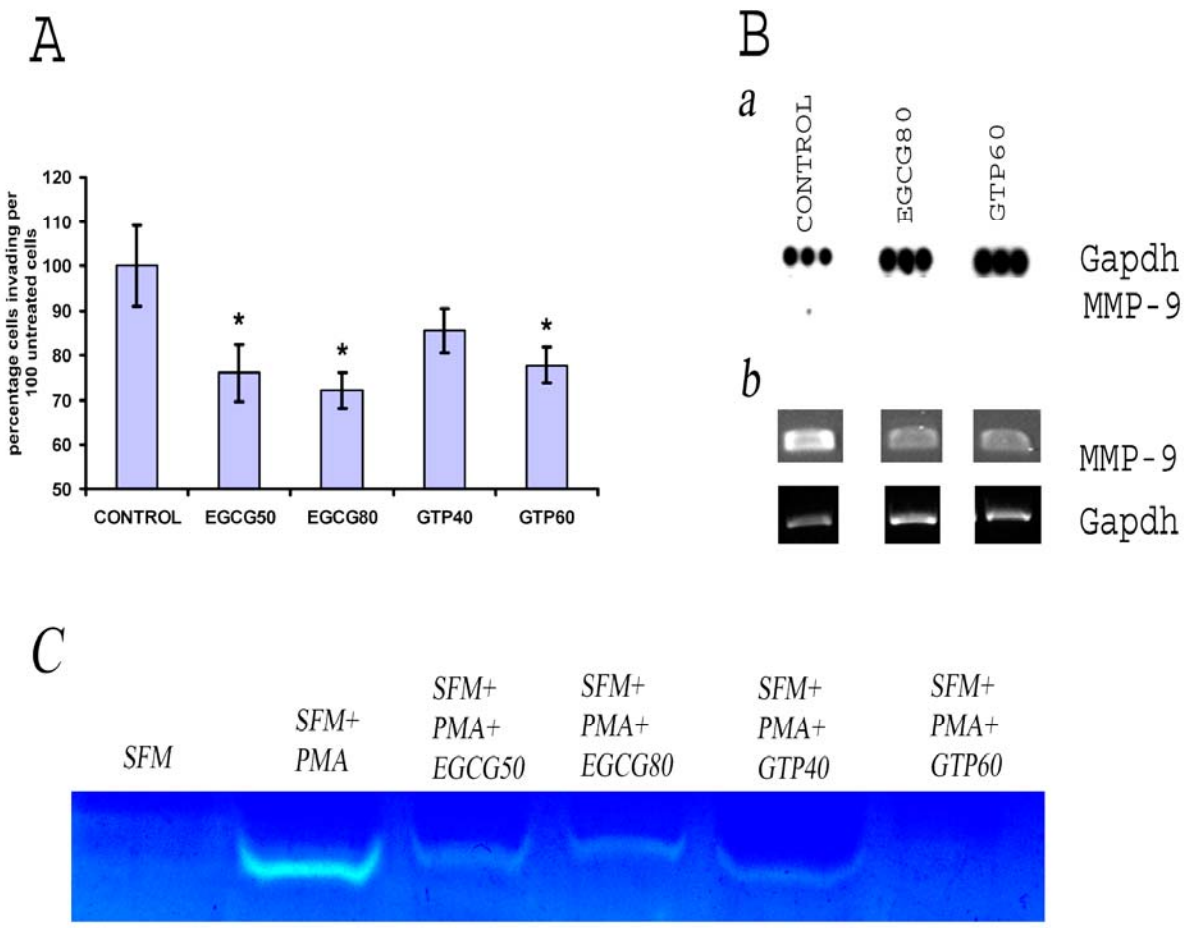

Figure 12. Effect of EGCG and GTP on invasion. 3A) EGCG and GTP inhibit the invasive capacity of MDA-MB-231 cells *in vitro* in a synthetic basement membrane system. Error bars indicate SEM (n=4). * Significantly different compared with controls, $P < 0.05$. 3B) EGCG and GTP down regulate MMP-9 transcript. a) Microarray analysis b) Reverse Transcriptase Polymerase chain reaction. 3C) MMP gelatinolytic activity was performed with the conditioned media from MDA-MB-231 cell culture. Data represent three individual experiments.

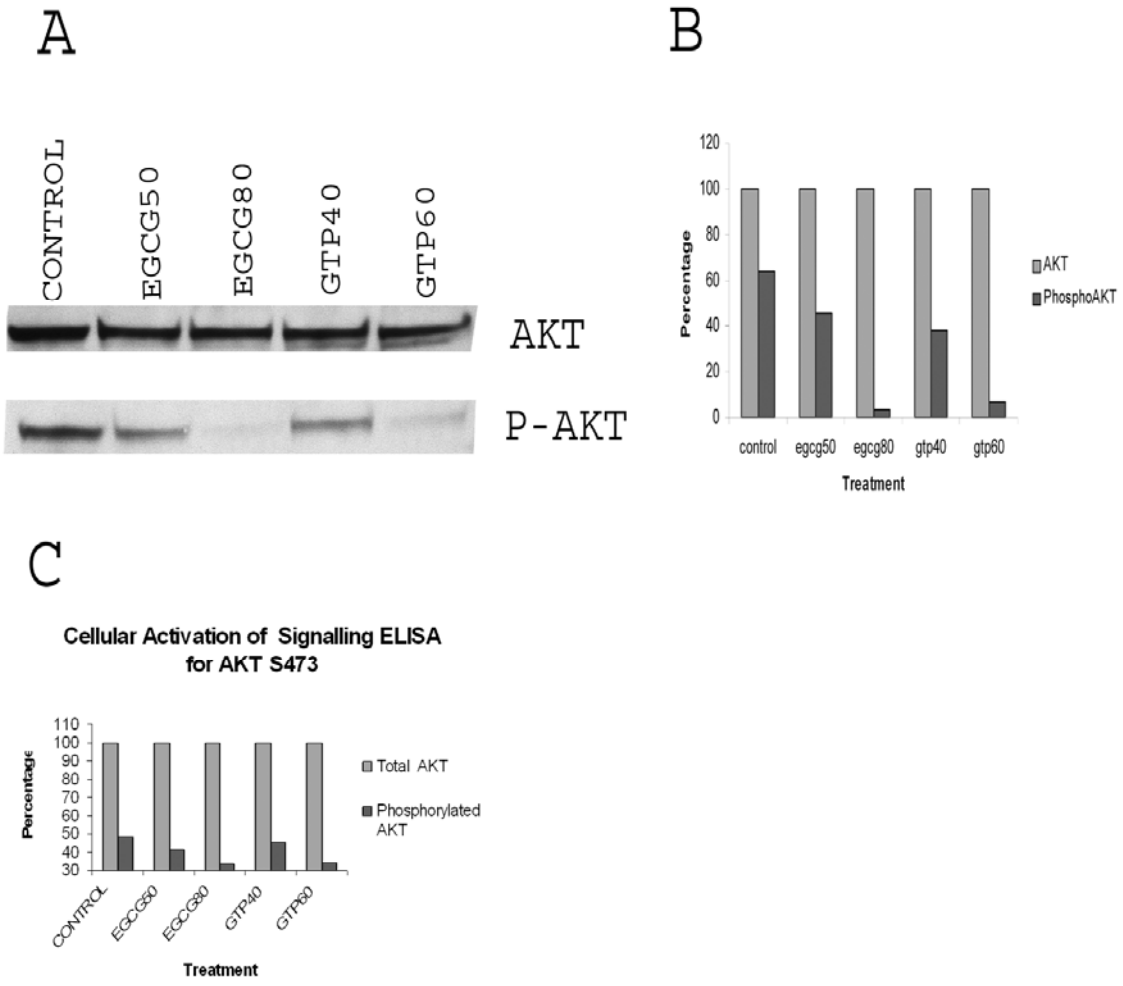

Figure 13. Effect of EGCG and GTP on AKT Phosphorylation. A) Representative picture of Western blot of AKT and Phospho-AKT of MDA-MB-231 cells treated with 50 and 80 ug/ml of EGCG and 40 and 60 ug/ml GTP 24 hrs. B) Quantitation of western blot of AKT and Phospho-AKT of MDA-MB-231 cells treated with 50 and 80 ug/ml of EGCG and 40 and 60 ug/ml GTP for 24 hrs. C) Cellular Activation of signaling ELISA for S473. Representative figure from two independent experiments.

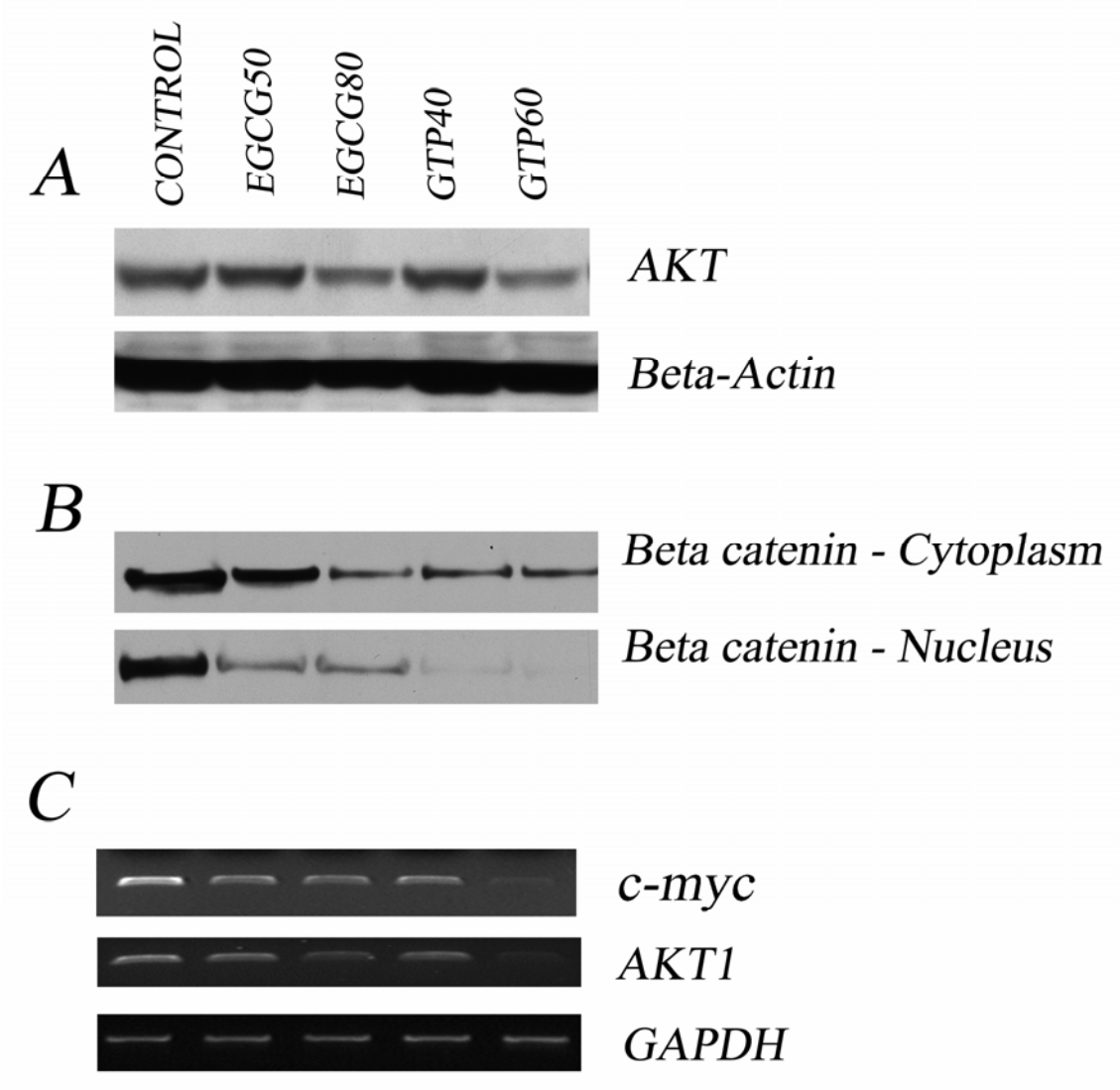

Figure 14. Effect of EGCG and GTP on AKT. A) Representative picture of Western blot of AKT of MDA-MB-231 treated with 50 and 80 ug/ml of EGCG and 40 and 60 ug/ml GTP 24 hrs. B) Western blot of cytoplasmic and nuclear beta-catenin of MDA-MB-231 treated with 50 and 80 ug/ml of EGCG and 40 and 60 ug/ml GTP 24 hrs. C) Reverse Transcriptase Polymerase chain reaction for c-myc and AKT1 of MDA-MB-231 cells treated with 50 and 80 ug/ml of EGCG and 40 and 60 ug/ml GTP 24 hrs. GAPDH is used as normalization control. Data is representative of two independent observations.

CHAPTER 2

Part 1: Protective effect of a polyherbal preparation, Brahma rasayana against tumor growth and lung metastasis in rat prostate model system

INTRODUCTION

The most common cancer in men is the malignancy of prostate and is the leading site for cancer incidence, accounting for 31% of new cancer cases in men (Greenlee et al 2001). Prostate cancer (PCa) is a leading cause of cancer related deaths among males in the U.S (Greenlee et al 2001). Adenocarcinoma of prostate has become the most common cancer in American men and was responsible for an estimated 30,200 deaths in 2002. The age adjusted prostate cancer mortality rates in the United States have increased by 28% over the last 35 years (Mettlin 1997). The current therapy for prostate cancer includes radical prostatectomy, radiation therapy and hormonal ablation. Chemotherapy has also provided good efficacy against prostate cancer. Patients succumb to the disease when it progresses to androgen-independent prostate cancer and ultimately metastasizes. Hence, the evaluation of complementary and alternate therapies, which can help in reducing the disease progression with minimal side effects, becomes pivotal.Although several advances have been made in the diagnosis and treatment modalities, an increase in the use of alternative medicine was observed during the last decade; therapies included herbal medicine, massage, megavitamins, homeopathy and others (Eisenberg et al 1998). Patients are increasingly seeking these unconventional therapies to improve their

prospects for good health, relieve the disease related pain and/or symptoms and reduce the side effects associated with conventional therapies. Adaptation of these methods varies and depends on several factors such as region, culture, philosophy, religion, socio-economic status and others. Of all the alternative therapies, herbal remedies have long histories and are practiced in all parts of the world. Ayurveda is a traditional system of medicine popular in India and was in use for more than 3000 years. A large percentage of population uses this system exclusively or in combination with conventional modern medicine. Ayurveda (knowledge of life) is a science that promotes health through an appropriate diet and life style. Traditional Ayurveda has eight branches: internal medicine; diseases of the head and neck; surgery; toxicology; pediatrics; aphrodisiacs and rejuvenation therapy (Glazier 2000). According to Ayurvedic literature rejuvenation therapy promotes the practice of rasayana (rejuvenation) in daily life, which prevents diseases and offsets the aging process. Rasayanas are based on nature's principle that for every part of the human physiology there exist herbal synergies with a matching vibration. The matching rasayana resets the vibration in the cells and reminds them of their correct function and order is restored (Bloomfield 2000). Ayurvedic rasayanas are mostly derived from plants and each compound used in the treatment of the disease contains several herbal products. Some of them have been shown to possess antioxidant (Sharma et al 1992, Sharma et al 1995,) and immunomodulatory properties (Kumar et al 1999). Brahma rasayana, Narasimha rasayana, Ashwagandha rasayana and Amruthaprasham are popular rasayanas. Of these, Brahma rasayana has been shown to have maximum immunomodulatory activity against tumor cells (Kumar et al 1999) and

indicated usefulness of these rasayanas for immunostimulation in normal and disease state.

According to Ayurvedic literature, Brahma rasayana is a formulation that retards the aging process and prolongs life expectancy. It is thought to create a disease free, youthful state and protect from age related diseases. In this study, the protective effect of Brahma rasayana for its efficacy in controlling tumor incidence and tumor metastasis in Copenhagen rat model using MAT-LyLu cells was assessed. The results suggest that Brahma rasayana reduces tumor incidence, tumor growth and metastatic spread caused by MAT-LyLu cells in Copenhagen rats.

MATERIALS AND METHODS

Brahma Rasayana

Brahma rasayana was obtained from Vaidyaratnam Oushadha Sala, Thrissur, India. It was prepared in a single large batch with established good manufacturing practice and good laboratory practice (GMP/GLP) guidelines. The manufacturer provided a certificate that it was free from pesticides and metals. Brahma rasayana is prepared from the fruits of *Emblicaofficinalis* and several other herbs as described in *Charaka Samhita* (Sharma 1983) (Table 2). Preparation of Brahma rasayana is a complex process and it is in paste form.

Cells

MAT-LyLu cells obtained from ATCC (Manassas,VA) were maintained in RPMI 1640 medium supplemented with 250 nM dexamethasone, 90%; fetal bovine serum, 10% in a humidified incubator containing 5% CO2 at 37oC.

Animals and Treatment

The study was performed on four to five weeks old male Copenhagen rats (Charles River Laboratories) following the model described by Tiwari et al., (Tiwari et al 1999) with few modifications. All the experiments were performed in compliance with the Public Health Service policy on humane care and use of animals and with the approval of the Institutional Animal Care and Use Committee, Uniformed Services University of the Health Sciences. Rats were randomly divided into eight groups (n = 8 each) viz. group 1-normal animals, Groups 2, 4, 6 and 8 were inoculated with 10,000 MAT-LyLu cells in 100 µl phosphate buffered saline by intradermal injection. From the second day of cell inoculation, animals were treated with various doses of rasayana [groups 3 and 4 - 250 mg (BR 250); groups 5 and 6 - 1,000 mg (BR 1000) and groups 7 and 8 - 1,500 mg (BR 1500) per kg body weight of animal per day]. Treatment was by feeding the rasayana suspension in 1 ml of water using an oral gavage and was continued daily until the end of the experiment. Other groups served as controls and were similarly handled and fed an equivalent volume of water. All animals were housed in individual cages and had *ad libitum* access to food and water. Body weight of the animals was measured weekly. Onset of tumor was assessed by daily palpation of cell inoculation site. Tumors were measured weekly with calipers and tumor volume was calculated. The formula for

volume is (V) = 0.5236*a*b*c where a, b and c were the three radii (Gleave et al 1992). At the end of five weeks, animals were euthanized. Blood, tumor mass and lungs were collected. Lungs were inspected for visible morphological changes. Tumor mass and lungs were weighed and utilized for histopathological studies.

Histopathologic Studies

All tissues collected at euthanasia were fixed in 10% neutral buffered formalin. The lung tissue was embedded in paraffin and 5 μm thick sections were obtained. The sections were stained with hematoxylin and eosin. A pathologist blinded to the treatment details carried out all histological examinations. Tissue sections were visualized and captured using a Nikon Eclipse E400 microscope with Nikon digital camera DXM1200.

Immunohistochemical detection of PCNA and *In situ* apoptosis detection by TUNEL staining was performed as described in page 20, 21.

Quantitative Analysis of Proliferation and Apoptosis

We examined either 10 fields (with anti-PCNA immunostaining or TUNEL) that were randomly selected for each slide. Each field then was photographed at a magnification of 600 X. Rest of the procedure is described in page 21.

Determination of Serum Testosterone Levels

Blood samples were collected at euthanasia by intracardiac puncture. Samples were allowed to clot at room temperature and the serum fraction was collected after

centrifugation. Serum specimens were stored at -20°C until further use. Testosterone levels were determined by using a commercially available testosterone ELISA kit (Alpha Diagnostic International, Inc., San Antonio, TX).

Statistical Analysis

Mean weekly body weight and geometric mean tumor volume were compared using repeated measures analysis of variance, followed by Bonferroni-adjusted post-hoc comparisons where appropriate. Average tumor weight at time of euthanasia was compared using 2-sided Student's t test for independent samples and the number of animals with visible lung nodules was compared using Fisher's exact test. Serum levels were analyzed by using one-way analysis of variance (ANOVA) and the Tukey's post-hoc pair wise comparisons. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Effect of Brahma Rasayana Treatment on Body Weight

The effect of Brahma rasayana treatment on the body weight was analyzed by measuring the body weight once weekly for all treated and untreated animals. Mean body weight per group is presented in Figure 15. Animal groups treated with three different concentrations of Brahma rasayana showed no change in mean body weight compared to controls. No significant weight loss or gain was observed reflecting the effect of Brahma rasayana treatments on the well being of the animals. Even at the highest concentration of 1,500 mg per kg body weight the animals showed no visible toxicity as measured by loss of appetite, hair loss or lack of movement.

Effect of Brahma Rasayana Treatment on Tumor Incidence

Inoculation of MAT-LyLu cells resulted in induction of tumors in 100% of untreated Copenhagen rats. However, Brahma rasayana treatment resulted in decreased tumor incidence with tumors occurring in only 25-37% of the animals. Tumor free survival functions were constructed using Kaplan-Meier method for each treatment group and compared using the log rank test (Figure 16). Comparisons using the Wilcoxon (Gehan) statistics indicates that tumors appear significantly earlier in control group than Brahma rasayana treated groups ($p=0.0232$, 0.0028 and 0.0054 for BR 250, BR 1000 and BR 1500 groups, respectively). The differences between the various doses of Brahma rasayana treated groups were not significant.

Effect of Brahma Rasayana on Tumor Growth

Tumor size was measured every seventh day after the onset of tumors once they attained measurable size. Tumor volumes were calculated and tumor burden was compared in animals treated with Brahma rasayana and untreated controls (Figure 17). Some animals were euthanized before the end point of the experiment as the tumors grew very large resulting in loss of weight and mobility of the animals. Tumors in some animals had ulcerated; severely affected rats were euthanized before the end point. For such animals with early end points, the last observed value at euthanasia was used at subsequent time points. By the fifth week after tumor initiation, a significant increase in tumor growth was observed in controls as tested by post-hoc comparisons using Tukey's adjustment method. A three fold difference in the geometric mean tumor volume after 5 weeks of

inoculation, clearly demonstrates the anti-tumor effect of Brahma rasayana treatment. Animals receiving Brahma rasayana showed a plateau in the tumor growth rate. Tumor weight was measured at the time of euthanasia and animals with no tumor were given 0 value. Average tumor weight comparison (Figure 18) indicates lower average tumor weight for rasayana treated groups compared to the untreated group. The differences were significant among groups with p=0.016. However, post-hoc pair wise comparisons using Tukey's method indicates significant difference in average tumor weights between control and BR 250 and BR 1500 groups. BR 1000 group showed tumors only in two animals and differences in individual animals might have contributed towards higher average volume. The decrease in average tumor weight was 15-20 g (approximately 60-80%) over the control group.

Effect of Brahma Rasayana on Lung Metastasis

The visible lung metastases in tumor bearing animals showed a considerable variation between the Brahma rasayana treated and untreated groups. The median number of nodules appeared to decrease with an increase in the dose of Brahma rasayana. However, the differences were not significant when compared to the untreated group. Histological evaluation of lungs showed numerous unencapsulated metastases in untreated groups whereas fewer and smaller metastases were noted in the Brahma rasayana treatment groups (Figure 19). The lungs of rats in the untreated MAT-LyLu group had metastatic nodules that measured 0.5 mm^2 to 1 mm^2 diameter and were composed of individual or small clusters of round to irregularly shaped neoplastic cells with moderate amounts of granular, eosinophilic cytoplasm and round to oval nuclei usually with a single prominent

nucleolus. Mitosis was generally 1 per 40-60X field. Neoplastic cells were supported by a fine fibrovascular stroma and tumor nodules often contained mixed inflammatory cell infiltrates composed mainly of lymphocytes. The histologic features of tumor nodules in the rats of the treatment group that had lung metastases were generally similar to those in the untreated group.

Effect of Brahma Rasayana on Cell Proliferation in Tumors

We evaluated the percentage of proliferating cells in the tumors from Brahma rasayana treated and untreated animals by immunohistochemical localization of PCNA in tumor sections. Compared to untreated, Brahma rasayana treated tumors showed significant decrease in the percentage of PCNA positive cells (Figure 20). This decrease in nuclear staining in tumors from Brahma rasayana treated animals was by 20% and was found to be statistically significant ($p < 0.05$) (Figure 21).

Apoptosis in Brahma Rasayana Treated Tumors

Apoptotic effect of Brahma rasayana treatment on tumors was assessed by immunohistological staining using *in situ* end labeling technique of apoptotic nuclei. Figure 22 shows the representative results for TUNEL staining of tumor sections from Brahma rasayana treated and untreated animals. Brahma rasayana treated tumors possessed around 45% of TUNEL-positive cells where as untreated tumors showed only 8% of the cells to be TUNEL positive (Figure 23). These results suggest that significant number of cells were undergoing apoptosis in the tumors treated with Brahma rasayana.

Effect of Brahma Rasayana Treatment on Serum Hormonal Levels

Serum testosterone levels were measured by ELISA using the serum obtained at the time of euthanasia (Figure 24). Testosterone levels in normal animals (5.4 ng/ml) were significantly higher compared to MAT-LyLu cell inoculated tumor bearing animals (2 ng/ml). In animals with Brahma rasayana treatment and without MAT-LyLu cell inoculation, no significant difference in serum testosterone levels was observed compared to normal animals. However, Brahma rasayana treated and MAT-LyLu inoculated groups showed an increase in serum testosterone levels ranging 2.6-4 ng/ml. Statistical analysis shows significant increase in testosterone levels only in the BR 250 group. BR 1000 and BR 1500 showed some increase but not significant compared to the levels in normal animals. This could be due to the presence of very few animals with tumors (two out of four animals) in these groups.

DISCUSSION

In recent years, use of unconventional therapies or complementary and alternative medicines (CAM) is on the rise and 7-64% of the population with diseases like cancer had tried these therapies (Zimmerman, Thompson 2002, Shraub 2000, Cassileth 1999). A survey conducted by a Canadian hospital indicated the use of complementary therapies among 27.4-38.9% of prostate cancer patients (Nam 1999). The most used CAM therapies include nutritional and herbal medicine, homeopathy and mind/body therapies (Monfort 2000). Herbal formulations are popular in Asian countries and are being adopted globally. This attention in natural remedies has reawakened the interest in Ayurveda (Science of life), an ancient Indian system of health care and longevity. Ayurvedic medicinal system is based mostly on plant products. Although little is known

about Ayurveda in Western cultures, it offers many health promotive interventions that can help and fulfill the needs of patients who seek a level of wellness not offered by conventional medicine. Ayurveda promotes the practice of rasayana (rejuvenation) in daily life. The word Rasayana (rasa = essence, water, plasma; ayana = path) refers to the acquisition of nutrition and its movement in the body tissues (Singh 2002). These unconventional therapies often do not undergo the rigorous scientific evaluation for their safety and effectiveness, which is an integral part of conventional therapies. To fill the void in our knowledge with the rasayanas, we have initiated a study for evaluating the effectiveness of one Ayurvedic preparation, Brahma rasayana against tumor growth and development in a prostate cancer cell model system in the Copenhagen rat. The findings as summarized in Table 3 indicate that treatment with Brahma rasayana reduces MAT-LyLu induced tumor incidence in Copenhagen rats. The results also demonstrate that treatment with Brahma rasayana reduces MAT-LyLu cell induced lung metastasis. Although, there is some reduction in tumor volume on Brahma rasayana treatment, the reduction does not appear to be so profound. However, comparison of size of metastatic nodules between Brahma rasayana treated and untreated controls confirms its role in reduction of tumor size. Apoptosis was suggested to play a pivotal role in the control of tumor growth by counterbalancing proliferation (Wu 1996). Furthermore, it has been suggested that tumor cell proliferation and apoptosis are inversely associated and interrelated with the efficiency of regulation in tumor growth and progression (Hanahan, Weinberg 2000). The findings concerning the decreased cell proliferation and enhanced apoptosis in Brahma rasayana treated tumors are consistent with the reduced tumor growth. According to the Ayurvedic texts Charaka Samhita and Sushrita Samhita,

rasayanas arrest aging and increase intelligence, vigor and resistance to diseases (Chopra, Doiphode 2002). They are used clinically either alone or as adjuvants with other modalities of treatment to improve immunity. They are available in different forms and same herb can be prescribed for different diseases in different forms. Apart from the therapeutic and medicinal value, the safety of a particular medicine is an important factor in the development of therapeutics. A rigorous analysis of herbal remedies for their efficacy has been recommended before prescribing these remedies to patients (De Smet 2002). Analysis of several rasayanic plants used in Ayurvedic medicines including *Emblica officinalis*, a major ingredient of Brahma rasayana was found to be safe in both acute and sub acute cytotoxicity studies (Rege et al 1999). Rasayanas can be used by anyone, young or old as they are believed to be free from any adverse side effect. However when the patient is suffering with some acute disease they should not take the place of a qualified doctor. These herbal preparations are administered orally as powders, decoctions and tablets. Some of them are reported to have immunomodulatory and antioxidant activities. The formulations contain extracts from tree barks, vegetables, spices, pulses and cereals. Some of the rasayanas were reported to have antioxidant activity higher than ascorbic acid and α-tocopherol. Among the different rasayanic plants *Emblica officinalis*, *Curcuma longa*, *Mangifera indica*, *Momordica charantia*, *Santalum album*, *Seertiachirata* and *Withania somnifera* contain antioxidant principles (Scartezzini, Speroni 2000). The major component of Brahma rasayana *Emblica officinalis, has* been shown to be a potent antioxidant *in vivo* and antimutagen *in vitro*. Treatment with extracts of *Emblica officinalis*, significantly enhanced the levels of superoxide dismutase, catalase, glutathione and glutathione peroxidase reduced by chemical carcinogens and

inhibited the tumor incidence in mice indicating their anti-carcinogenic activity (Vayalil et al 2002, Jose et al 2001, Jeena et al 1999). It is also used as an anti-inflammatory (Ilhantola-Vormisto et al 1997) and anti-stress agent (De Smet 2002) and is recommended not only in disease conditions but also for the general maintenance of health. Two tannins Emblicanin A and B were identified as active principles from *Emblica officinalis* (Bhattacharya et al 1999). Pyrogallol, a component of *Emblica officinalis* extract inhibits growth in human cell lines (Khan et al 2002). *Terminalia chebula,* the second major component of Brahma rasayana contains tannins, gallic acid and chebulinic acid and has antioxidant activity (Naik et al 2003). Methanol extracts of this plant have been shown to inhibit the growth of several malignant cell lines, including human prostate cancer cell line PC-3 (Saleem et al 2002). *Centella asaitica,* another plant commonly used in Ayurvedic system of medicine that is a part of Brahma rasayana has been shown to possess selective toxicity towards tumor cells (Lin et al 2002). *In vivo* studies involving mice also indicated *Centella asaitica*'s tumor reducing property and its stimulation of the immune system (Babu et al 1995). The antitumor activity of phytochemicals has been attributed to polyphenols and specifically bioflavonoids (Barnes 2001). Recently different cellular signaling mechanisms of some phytochemicals in reference to cancer chemoprevention were presented (Surh 2003). Rasayanas contain a combination of several medicinal plants that might exert an additive/synergistic antiproliferative activity by acting on multiple targets simultaneously.

Detailed investigations of the major individual components and the whole preparation may elucidate the molecular nature of these compounds as well as their mechanisms of action. The development and progression of prostate cancer are dependent on testosterone

and dihydrotestosterone; the androgen receptor is the vehicle through which these androgens exert their regulation on prostate cellular proliferation and differentiation. Analysis of serum testosterone in three groups of prostate cancer patients showed altered levels suggesting inhibition of serum testosterone with the development of prostate cancer (Zhang et al 2002). In our study, there was an up-regulation of serum testosterone following Brahma rasayana treatment and this phenomenon could be one of the many protective mechanisms of action. Few available studies suggest immunostimulation and antioxidant activities as the possible mechanisms of protection. In summary, the results demonstrate that Brahma rasayana can significantly reduce tumor incidence and tumor growth in the Copenhagen rat and MAT-LyLu cell model. Also the proliferation and apoptotic markers analyzed support these observations. Additional investigation is needed to explore individual components as well as the specific mechanisms.

Table 2. Constituents of Brahma Rasayana. Reference Ashtangahridayam

	INGREDIENTS FOR DECOCTION	QTY (g)	PART USED	SOURCE
1	*Terminalia Chebula*	0.9	Seed Coat	Market
2	*Embilica officinalis*	0.375	Fruit	Market
3	*Gmelina arborea*	0.075	Root	Market
4	*Aegle marmelos*	0.075	Root	Market
5	*Strereospermum Chelenoides*	0.075	Root	Market
6	*Oxoxylum indicum*	0.075	Root	Market
7	*Premna latifolia Roxb*	0.075	Root	Market
8	*Desmodium gangeticum*	0.075	Root	Herbal Garden
9	*Pseudarthria viscida*	0.075	Root	Herbal Garden
10	*Aerva lanata*	0.075	Root	Herbal Garden
11	*Solanum indicum*	0.075	Root	Herbal Garden
12	*Tribulus terrestris*	0.075	Seed	Market
13	*Desmostachya bipinnata*	0.075	Root	Herbal Garden
14	*Saccharum spontaneum*	0.075	Root	Herbal Garden
15	*Saccharum officinarum*	0.075	Root	Market
16	*Saccharum arundinaceum*	0.075	Root	Market
17	*Hygroryza aristata*	0.075	Root	Market
18	*Sida retusa*	0.075	Root	Market
19	*Boerhaavia diffusa*	0.075	Root	Herbal Garden
20	*Ricinus communis*	0.075	Root	Herbal Garden
21	*Teramnus labialis*	0.075	Root	Market
22	*Vigna pilosa*	0.075	Root	Market
23	*Asparagus racemosusl*	0.075	Tuber	Herbal Garden
24	*Coccinia grandis*	0.075	Fruit	Herbal Garden
25	*Holostemma adakodein*	0.075	Rhizome	Herbal Garden
26	*Ipomea palmate*	0.075	Tuber	Herbal Garden
	INGREDIENTS FOR POWDER	**QTY (g)**	**PART USED**	**SOURCE**
1	*Cinnamomum zeylanicum*	0.03	Bark	Market
2	*Elettatia cardamomum*	0.03	Seed	Market
3	*Cyperus rotundus*	0.03	Tuber	Herbal Garden
4	*Curcuma longa*	0.03	Rhizome	Herbal Garden
5	*Piper longum*	0.03	Berry	Herbal Garden
6	*Santalum album*	0.03	Wood	Market
7	*Aquilaria agallocha*	0.03	Wood	Market
8	*Centella asiatica*	0.03	Whole plant	Herbal Garden
9	*Datura stramonium*	0.03	Root	Herbal Garden
10	*Clitoria ternatia*	0.03	Root	Herbal Garden
11	*Acorus calamus*	0.03	Rhizome	Herbal Garden
12	*Pentapetes phoenicea*	0.03	Flower	Market
13	*Glycyrrhiza glabra*	0.03	Root	Market
14	*Embelia ribes*	0.03	Seed	Market
	GENERAL	**QTY (g)**		
1	*Sugar Candy*	8.25		
2	*Honey*	2.4		
3	*Seasmum Oil*	1000		
4	*Ghee*	1.5		

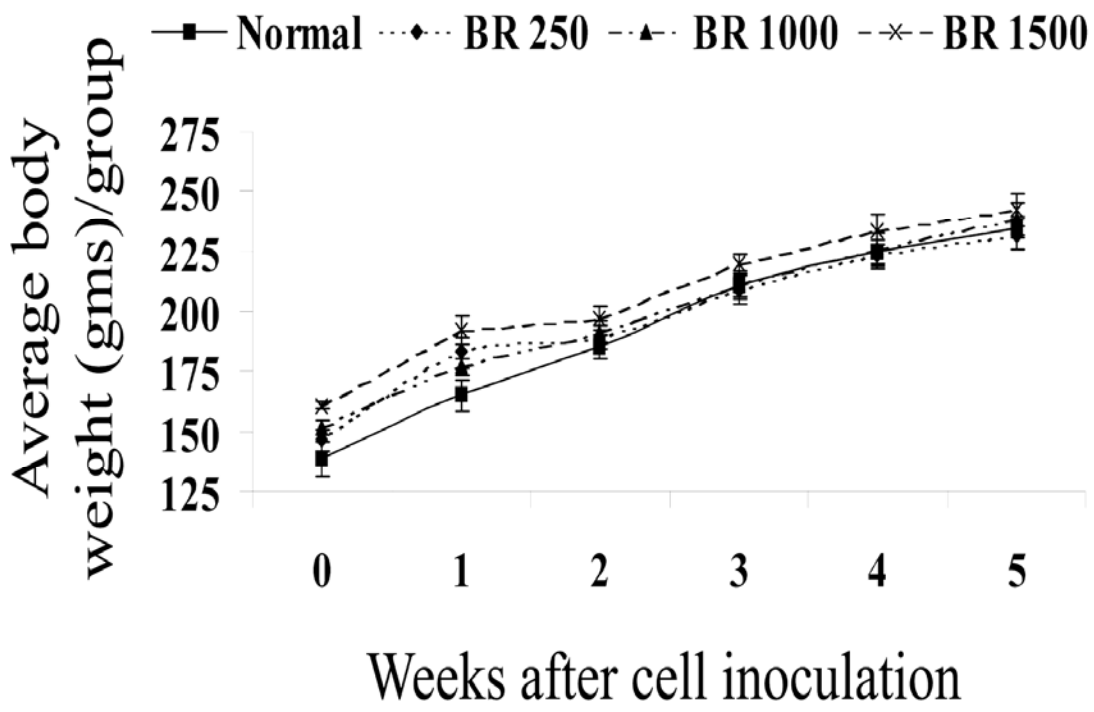

Figure 15. Average body weight in grams per group in Copenhagen rats. Comparison of average body weight between the groups using repeated measures analysis of variance showed no significant difference among the groups ($p = 0.35$) and also no differential effect of time in the different treatment groups ($p = 0.54$). Values are mean ± SEM of 8 animals.

Table 3. Tumor incidence, average tumor volume and average tumor weight in Brahma rasayana treated and untreated Copenhagen rats at the time of sacrifice. 'Group' represents the number of animals in each treatment (n=8) and 'Tumor' represents number of animals with tumors for that treatment.

	MAT-LyLU		BR 250 + MAT-LyLu		BR 1000 + MAT-LyLu		BR 1500 + MAT-LyLu	
	Group	Tumor	Group	Tumor	Group	Tumor	Group	Tumor
Tumor incidence	8	8	8	3	8	2	8	2
Tumor volume (cm^3)	2.45	2.45	0.52	1.39	1.41	5.64	1.25	1.83
Tumor weight (gms)	25.135	25.13	8.13	21.69	10.27	41.08	5.24	20.96

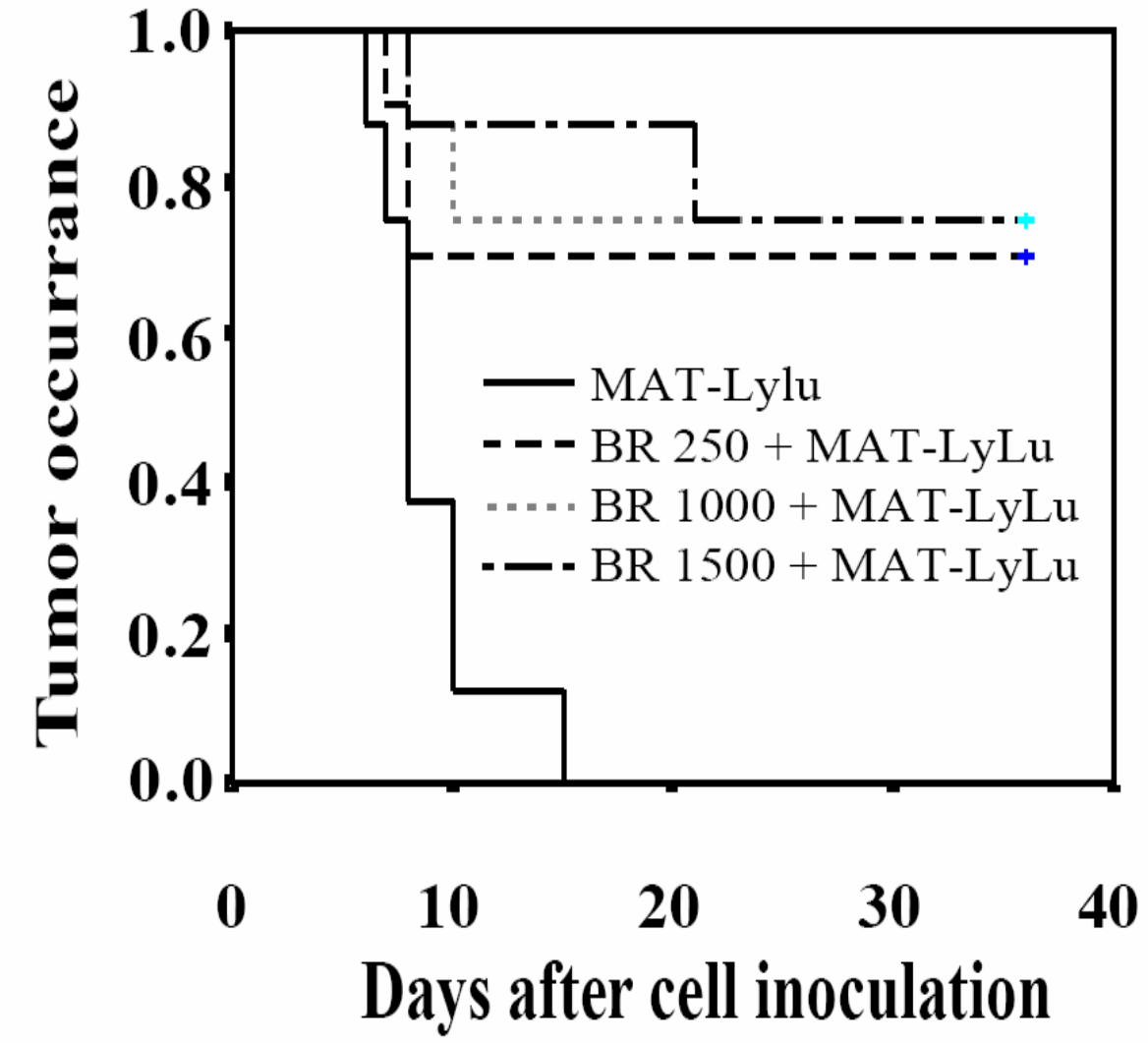

Figure 16. Kaplan-Meier analysis of Brahma rasayana treatment and tumor incidence in Copenhagen rats inoculated with MAT-LyLu cells.

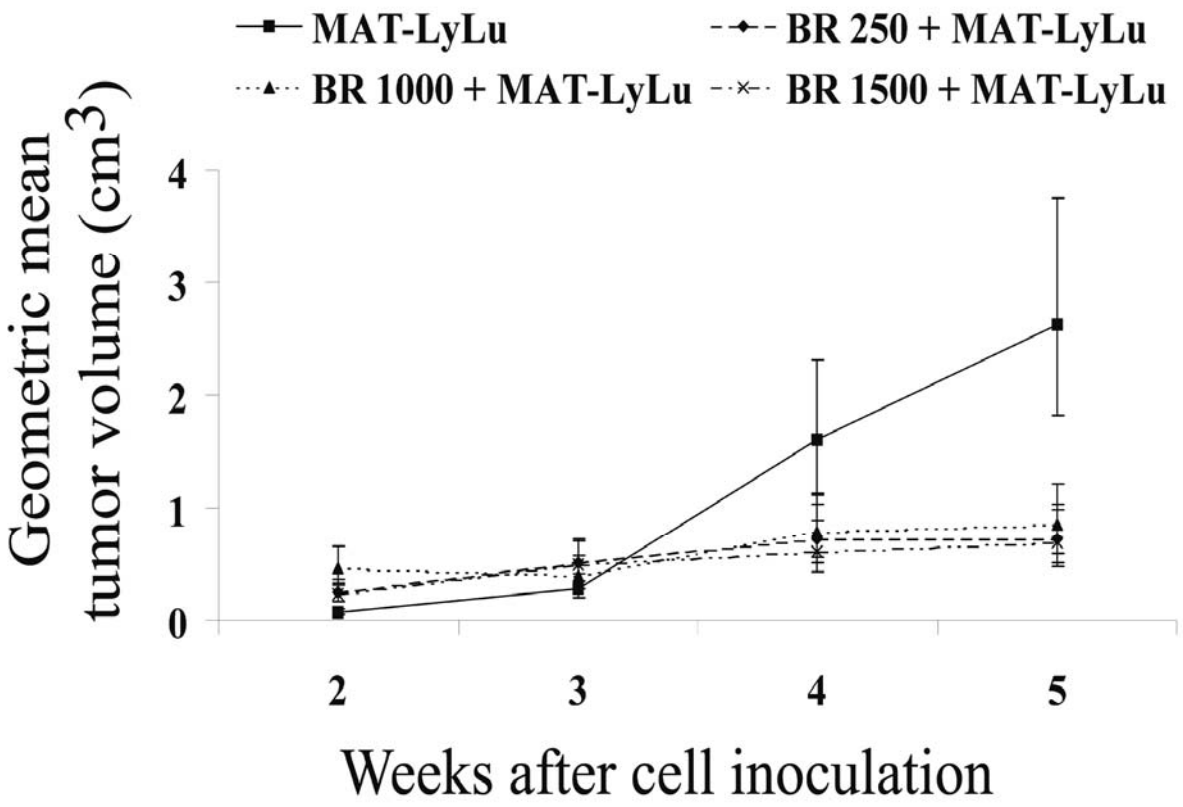

Figure 17. Effect of Brahma rasayana treatment on tumor growth in Copenhagen rats inoculated with MATLyLu cells. The geometric mean volume of MAT-LyLu group was significantly higher than Brahma rasayana treated groups by fifth week ($p < 0.05$). Values are mean ± SEM of 8 animals.

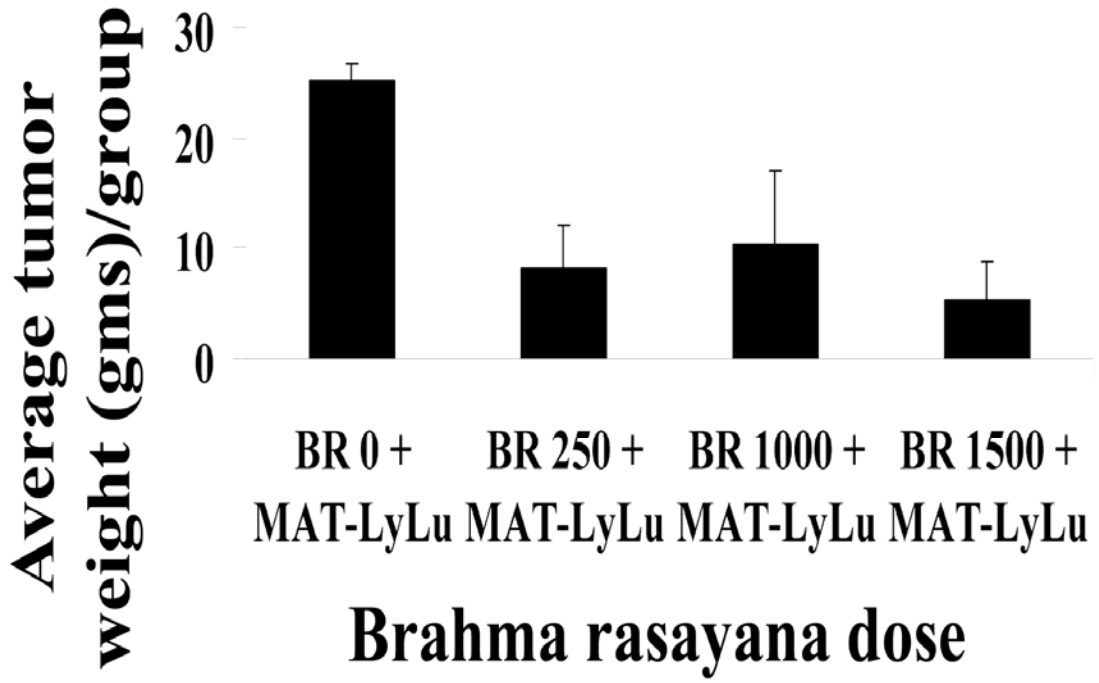

Figure 18. Effect of Brahma rasayana treatment on tumor weight at euthanasia. Comparison of average tumor weight at the time of euthanasia indicates 60- 80% reduction in tumor weight in Brahma rasayana treated animals. Values are mean ± SEM of 8 animals.

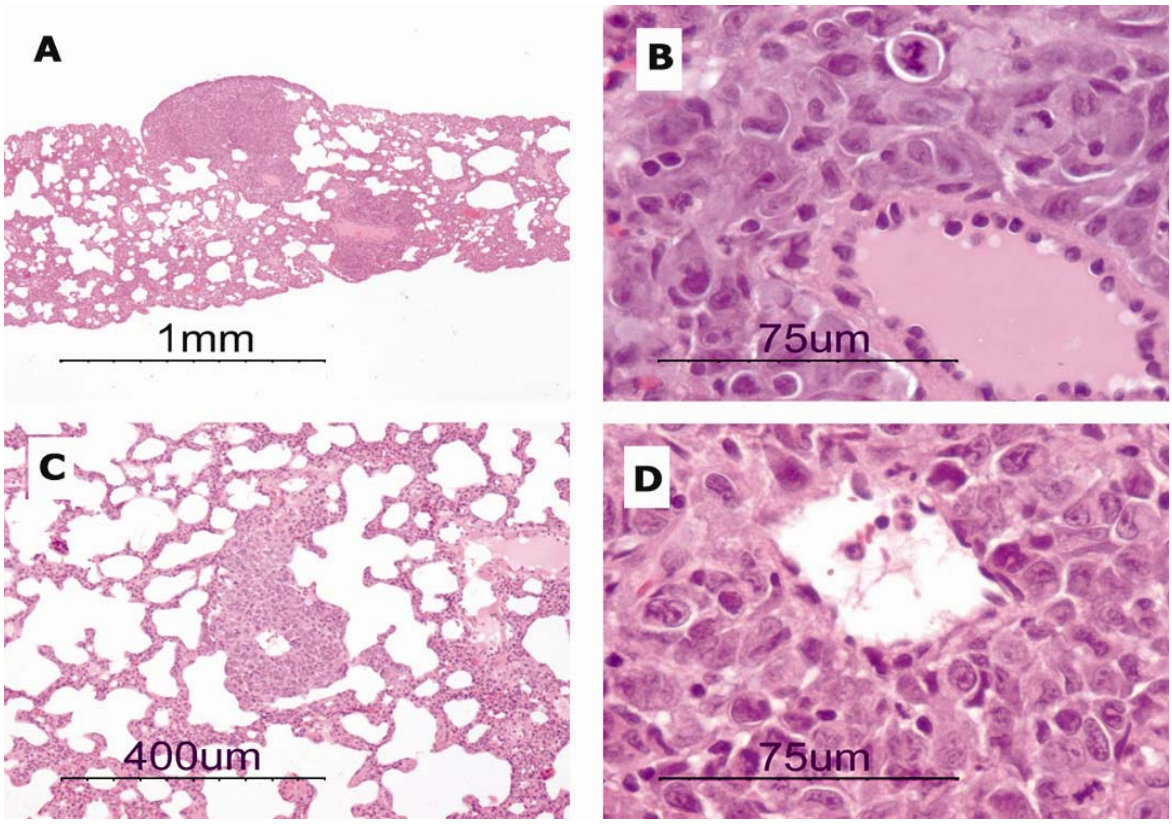

Figure 19. Hematoxylin and eosin stained lung sections showing pulmonary tumor metastases A) MAT-LyLu cells inoculated control at low magnification with pulmonary metastases oriented around blood vessels and focally elevating the pleura. B) Higher magnification (60X Objective magnification) of A showing cellular pleomorphism and mitoses. C) MAT-LyLu cell inoculated and Brahma rasayana treated rat with pulmonary metastasis that was evident histologically only. D) Higher magnification (60X) of C with similar cellular morphology as in B.

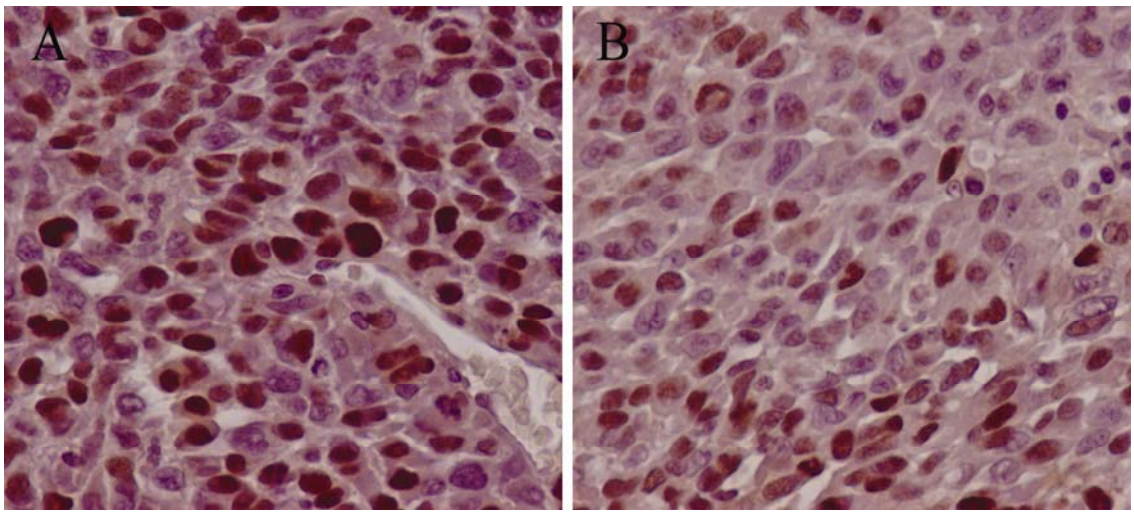

Figure 20. Representative picture of histological detection of cellular proliferation in tumors. Tumor tissue sections were stained by antibody against PCNA. A) MAT-LyLu cells inoculated untreated control and B) MAT-LyLu cell inoculated and Brahma rasayana treated; (600 X).

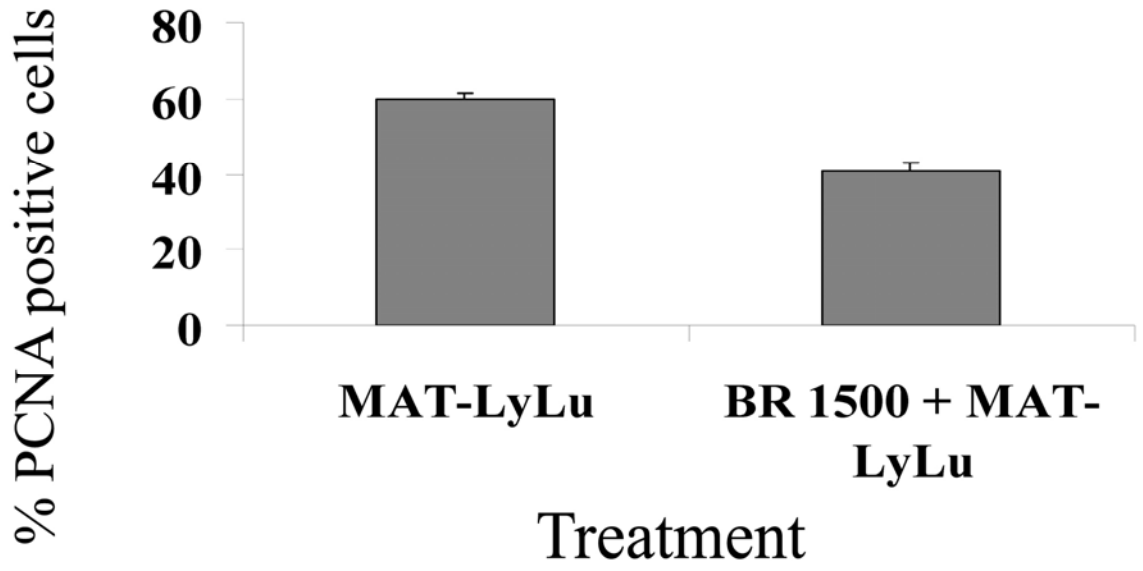

Figure 21. Quantitation of PCNA positive cells expressed as percentage of total cells in Brahma rasayana treated and untreated tumors. Brahma rasayana treatment showed significant reduction (20%) in PCNA positive cells compared to untreated tumors (* $p < 0.05$). Values are mean ± SEM of 4 animals.

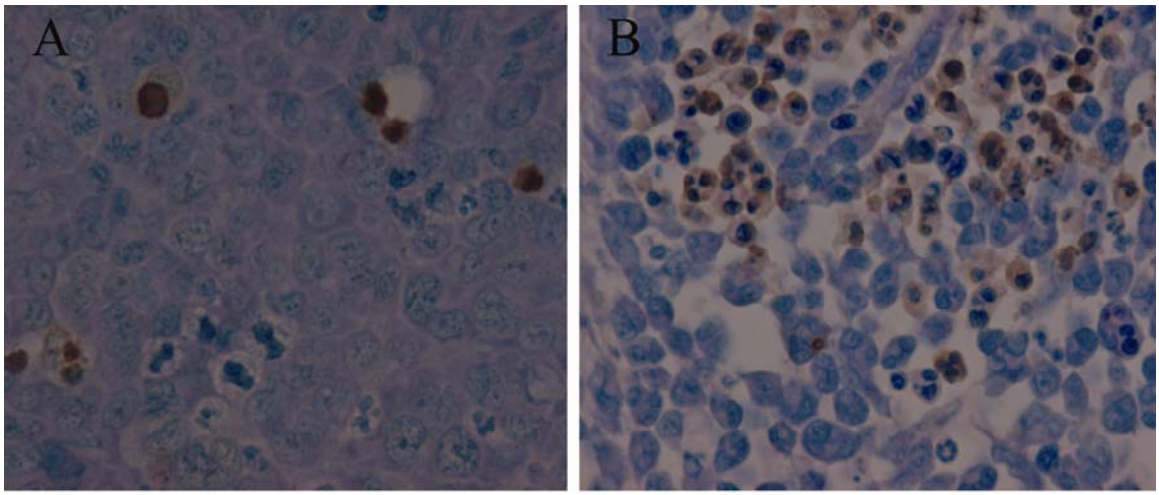

Figure 22. Representative picture of histological detection of apoptosis in tumors. Tumor tissue sections were stained by TUNEL. A) MAT-LyLu cells inoculated untreated control and B) MAT-LyLu cell inoculated and Brahma rasayana treated; (600 X).

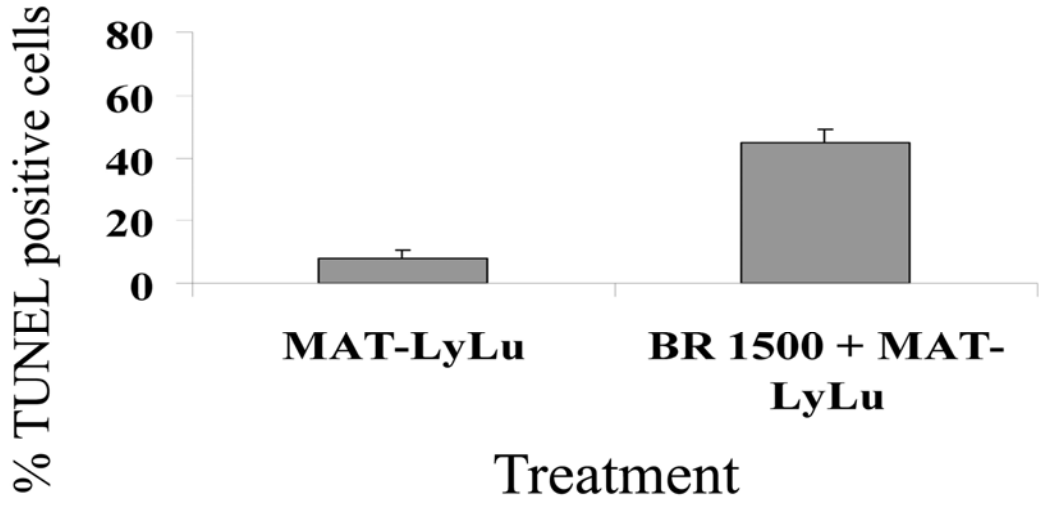

Figure 23. Quantitation of TUNEL positive cells expressed as percentage of total cells. Brahma rasayana treatment showed 35% increase in TUNEL positive cells (* $p < 0.05$). Values are mean ± SEM of 4 animals.

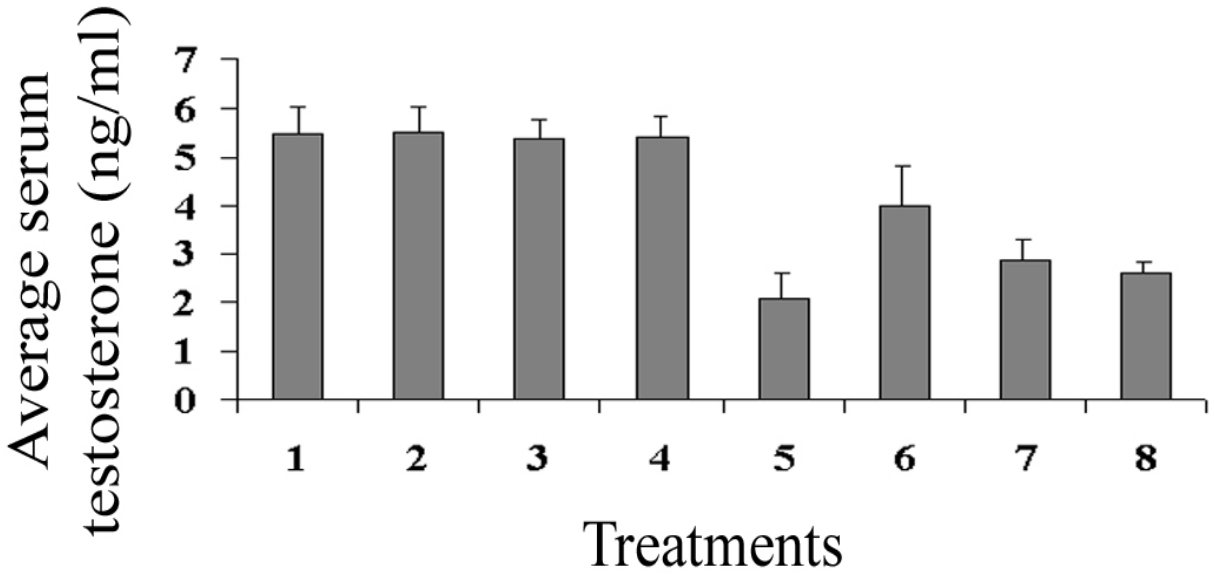

Figure 24. Effect of Brahma rasayana treatment on serum testosterone levels. Lanes: 1. Normal, 2. BR 250, 3. BR 1000, 4. BR 1500, 5. MAT-LyLu, 6. BR 250 + MAT-LyLu, 7. BR 1000 + MAT-LyLu and 8. BR 1500 + MAT-LyLu. Serum levels differed among the groups. MAT-LyLu cell inoculation caused a significant reduction in serum testosterone ($p < 0.05$). Normal, Brahma rasayana treated and BR 250 + MAT-LyLu treated groups showed no significant difference. Values are mean ± SEM of 4 animals.

Chapter 2

Part 2: Mechanism(s) of Inhibition of tumor angiogenesis by Brahma Rasayana

INTRODUCTION

It has been observed that well-vascularized tumors expand both locally and by metastasis, whereas nonvascular tumors do not grow beyond a diameter of 1 to 2 mm (Folkman 1976). Angiogenesis is one of the most important factors, which provides an important path for metastasis. Vascular Endothelial Growth Factor (VEGF) has been implicated as important and clinically relevant to the induction of angiogenesis in prostate cancer (Strohmeyer et al 2004). Expression of pro-angiogenic factors like Matrix Metalloproteinase-2 (MMP-2), Matrix Metalloproteinase-9 (MMP-9) and VEGF have also been shown to correlate with the metastatic potential of prostate cancer cells (Aalinkeel et al 2004). We have shown in part 1, that Brahma rasayana reduces the incidence, promotion and progression of tumor (Gaddipati et al 2004). The animal model has been used to evaluate the efficacy of various chemopreventive agents like Indole-3-carbinol and calcitrol to name a few (Garikapaty et al 2005, Lokeshwar et al 1999). This study was focused to investigate the regulation of molecular markers of angiogenesis by Brahma rasayana using both *in-vitro* and *in-vivo* models. The effect of Brahma rasayana treatment on Factor VIII expression was analyzed, which was found to be significantly lower than the control untreated animals. Similar results were observed when tissues were stained for VEGF, MMP-9 and MMP-2. Western blotting and zymogram analysis of the tumor tissue also showed down-regulation in the pro-angiogenic factors like MMP-

2, MMP-9 and VEGF which might help in explaining the reduced tumor nodules in the lungs. Methanolic extract of Brahma rasayana was also found to inhibit proliferation, reduce cell migration, attachment and tube formation on matrigel in an *in-vitro* model of angiogenesis using HUVEC. These results suggest that Brahma rasayana may inhibit tumor promotion and progression by inhibiting angiogenesis.

MATERIALS AND METHODS

Research strategy and study sample

The approach was to investigate the effect of Brahma rasayana on the factors associated with the regulation of angiogenesis. As mentioned earlier, we have observed a considerable decrease in visible lung metastases of rats inoculated with MAT-LyLu cells treated with Brahma rasayana compared to the MAT-LyLu inoculated untreated ones. Our analysis consisted of tumor tissue collected from MAT-LyLu injected Copenhagen rats that were treated with Brahma rasayana and water fed control from our previous experiment (6). MAT-LyLu cell culture and animal experiment has been performed as described (6). Briefly randomized Copenhagen rats were inoculated with 10,000 MAT-LyLu cells in 100 µl phosphate buffered saline by intradermal injection. From the second day of cell inoculation, animals were treated with Brahma rasayana. Treatment was by feeding the rasayana suspension in 1ml of water using an oral gavage and was continued daily until the end of the experiment. Other groups served as controls and were similarly handled and fed an equivalent volume of water. All experiments were done in accordance with the United States Public Health Service policy and the University laboratory animal review board.

Immunohistochemical Detection of VEGF, MMP-2 and MMP-9 Expression, Factor VIII staining and Micro Vessel Density (MVD) Quantitation

Deparaffinized sections were rehydrated and treated with 10 mM citrate buffer (pH 6.0) at 95°C for 20 min. Endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide in methanol followed by two washes with PBS. The sections were then treated with normal horse serum (Vector Laboratories, Burlingame, CA) followed by a wash in PBS. The sections were incubated overnight with anti-VEGF (1:200), anti-MMP-9 (1:100), anti-MMP-2 (1:100) and anti-factor VIII primary antibodies Santa Cruz Biotechnology Inc., Santa Cruz, CA. Slides were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 10 min at room temperature with pre and post washes with PBS. Sections were incubated with conjugated horseradish peroxidase streptavidin complex and counterstained with Hematoxylin. We examined 10 fields with immunostaining, which were randomly selected for each slide. Each field then was photographed at a magnification of 200 X. For MVD, two individuals counted the number of microvessels in each pictograph independently.

Western Blot

Frozen tissues extracts (50 µg) in 13 µl of lysis buffer and western blotting was done as described previously. VEGF protein expression was detected by incubating with primary antibodies (Santa Cruz Biotechnology) at 1:1000 dilutions in blocking buffer. The membranes were washed three times with wash buffer and were then incubated with the appropriate secondary antibody (Santa Cruz Biotechnology) at dilutions of 1:2000 in

blocking buffer. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) according to the specifications of the manufacturer (Santa Cruz Biotechnology). Blots were scanned and optical densities of the bands were quantitated. The expression of VEGF was normalized with beta actin.

Zymogram

Frozen tumor tissues were homogenized in homogenization buffer (50mM TRIS-Cl, 50mM Tris base, 20mM CaCl2, 150mM NaCl, 0.25% Triton X, 0.02M Sodium azide) and the protein amount was quantitated using BCA assay. Zymography was performed on 10 µg of the samples as described in page 39. Similar procedure was carried out from the protein isolated from the HUVEC lysates and supernatants that were treated with different concentration of methanolic extract of Brahma rasayana.

***In-vitro* studies**

Cells

HUVEC were purchased from Clonetics, San Diego, CA. Media-199, fetal bovine serum (FBS), streptomycin, penicillin, gentamicin, fungizone, phorbol-12-myristate 13-acetate (PMA), and 0.05% trypsin-EDTA were obtained from Gibco BRL, Gaithersburg, MD. Endothelial cell growth supplement (ECGS) and matrigel were purchased from Collaborative Research, Bedford, MA. Heparin, glutamine, phenylmethylsulfonyl fluoride (PMSF), N-ethylmalemide (NEM), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA) and 10% neutral buffered formalin were purchased from Sigma Chemical, St. Louis, MO.

Culture of human umbilical vein endothelial cells (HUVEC)

Cultures of HUVEC were maintained in media-199, supplemented with 2 or 10% FBS, 50 µg/ml of ECGS, 100 U penicillin, streptomycin, and fungizone, 50 µg/ml gentamicin and 50 U/ml heparin in 75% N_2, 5% CO_2, and 20% O_2 at 37°C. To maintain uniform conditions all experiments were carried out between cell passages 2 and 7.

Methanolic extract of Brahma rasayana

The solubility of BR was evaluated in water, absolute alcohol, methanol and various other organic solvents. Most of the Brahma rasayana was soluble in methanol. Hence we choose to evaluate the methanolic extract of Brahma rasayana. Brahma rasayana was weighed and dissolved in Methanol. Suspension was centrifuged at 10000g and clear supernatant was collected and re-centrifuged. Clear suspension was vacuum dried to get a thick semi solid extract and stored at -20°C. Pellet was re-suspended in the culture media and double filtered before use.

Cell Proliferation Assay

HUVEC proliferation in the presence of various concentrations of Brahma rasayana was determined using the Cell Proliferation Kit I {MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)} obtained from Boehringer, Manheim, Indianapolis, IN as described previously in page 17.

Capillary tube formation and endothelial cell migration

150 µl matrigel was plated in each well in a 24 well culture plate and allowed to solidify at 37°C for 2 hr. HUVEC (15000 cell each well) were then plated over the matrigel and treated with the respective doses of Brahma rasayana. Plates were then incubated at 37°C for 20 hr and fixed with 10% buffered formalin. Cells were stained with crystal violet for 10 min and capillary formation was studied under light microscope.

Statistical analysis

Unless otherwise indicated, data are presented as means±SEM of 3–5 different observations. Data were analyzed by using student *t* test. Significance was defined as a P value of less than 0.05.

RESULTS

Brahma rasayana reduces Microvessel density (MVD) in prostate tumor tissue

MAT-LyLu cells inoculated rats were treated with Brahma rasayana and the tumor tissue was analyzed for micro vessels density and was compared with the untreated controls. The analysis was done by immunohistochemistry for Factor VIII and the intratumoral microvessel density was quantified by counting the stained vessels in the tissue. Quantitation showed 14.33% ± 2 microvessels per field in control tumor as compared with 7.33 ± 1 microvessels. The decrease in microvessels density in Brahma rasayana fed group was around 50% ($P<.05$) over that of control (Figure 25).

Brahma rasayana reduces VEGF expression in tumor tissue

Immunohistochemical detection for VEGF resulted in more expression of the proteins in untreated controls and the vessels were observed to be more dilated in the same when compared with Brahma rasayana treated group (Figure 26). Western blot analysis showed a reduction in VEGF expression by around 40% compared with the controls and their P value was less than 0.05 (fig 2B and fig 2C). Protein concentration was estimated and normalized by BCA assay and the blots were normalized with actin expression.

MMP's expression is decreased by Brahma rasayana

Degradation of ECM can be a major way through which the tumor cell as well as endothelial cell migrate from their origin to other locations due to loss of cell–matrix contacts and cell–cell contacts. Immunohistochemical detection of MMP-9 and MMP-2 resulted in sparse expression around the blood vessels in Brahma rasayana treated when compared with untreated controls (Figure 27A and Figure 27B). Zymographic analysis of the Brahma rasayana treated tumor tissue resulted in around 25% and around 30% expression in MMP-9 and MMP-2 respectively compared with the tissues from untreated MAT-LyLu inoculated untreated rats (Figure 27C and Figure 27D).

Brahma rasayana inhibits proliferation of HUVEC

We examined the ability of Brahma rasayana to inhibit proliferation of the HUVEC. Dose-dependent decrease in the rate of proliferation was seen with the methanolic extract of Brahma rasayana. A significant (50%) reduction in cell population was observed when treated with 2000 µg/ml of Brahma rasayana at 24 hr and 1000 µg/ml at 48 hr (Figure 28). The calculation of doses and treatment duration that were used to treat the

endothelial cells for MMP evaluation and endothelial cell migration were done cautiously such that the toxic doses were not considered for treatment.

Brahma rasayana reduces capillary tube formation and endothelial cell migration

The effects of Brahma rasayana on the inhibition of migration and capillary formation were tested using an *in-vitro* model using HUVEC on matrigel. Brahma rasayana showed a dose dependent inhibition whereas the control untreated cells showed an extensive network of tubes. The inhibition of tube formation was evident from the lower concentration and it was significant at 1500 µg/ml (Figure 29).

Brahma rasayana down regulates MMP 9 expression in HUVEC

Reduction of MMP expression was observed in the zymograms of cell lysates of HUVEC treated with different concentrations of Brahma rasayana for 12 hr (Figure 30). A significant and dose dependent reduction in the expression of MMP-9 was observed with Brahma rasayana. A similar pattern was also observed in the zymograms of the HUVEC supernatant with the MMP-9 expression in the 24 hr treatment (Figure 30). Protein concentration was estimated and normalized by the BCA assay.

DISCUSSION

The efficacy of herbal mixtures in the treatment of prostate cancer has been examined recently (Ikezoe et al 2003, Sliva et al 2003, Darzynkiewicz et al 200, Kubota et al 200, Tiwari et al 1999, DiPaola et al 1998). Rasayanas deal with components, which accelerate or trigger an early onset of aging and reverse them (Newton 2001). Brahma rasayana is one such polyherbal preparation, which is used widely in India for

rejuvenation so as to prevent disease, and offsets the aging process. The health benefits of these Rasayanas are attributed to the herbal combination which acts as a synergistic blend, but the purported synergy has never been tested. Rasayanas are found rich in antioxidant properties (Hanna et al 1994, Sharma et al 1995, Rekha et al 200, Rekha et al 2001) and have been found to inhibit lung metastasis induced by B16F-10 melanoma cells (Menon et al 1997). Quantitation of microvessel density (MVD) has been shown to be an independent prognostic marker in transitional cell carcinoma (Chaudhary et al 1999). In prostate cancer, the MVD is shown to significantly correlate with clinical stage and progressiveness of the disease indicating that MVD can be used to forecast the disease specific survival (Borne et al 1998). The Brahma rasayana treated tumor tissue showed a statistically significant reduction in the MVD when compared to the untreated tumor. This indicates that Brahma rasayana treatment may result in decreased progression of the tumor. The anti-angiogenic effect of Brahma rasayana could have been contributed by its inhibitory effect on VEGF expression, as elucidated both by immunohistochemical staining and western blotting of the tumor tissue. The inhibitory effect on both MVD and VEGF could be responsible for the significant decrease in tumor cell proliferation. High MVD has been shown to be associated with the advancement of non-small cell lung cancer and it was observed to be particularly apparent in conjunction with high VEGF (Ushijima et al 2001)**.** Brahma rasayana's significant reduction of disease progression and reduction in the lung nodules and lesser MVD may be attributed to the suppression VEGF expression. Among many MMPs that have been identified, MMP-2 (Gelatinase-A) and MMP-9 (Gelatinase-B) are thought to be the key enzymes as they degrade type IV collagen, the main component of ECM (Hojilla et al 2003). MMP-9 expression in

Dunning tumor rats have been found to be a very useful marker to monitor the progression of prostate cancer as well as to evaluate the efficacy of various drugs (Jung et al 2003). Serums levels of MMP-2 have been shown to play a major role in development and extension of prostate cancer (Gohiji et al 1998). MMP mediated proteolysis involvement in tumor angiogenesis as well as tumor invasion, degradation of the extracellular matrix (ECM) has been reviewed (Sternlicht, Werb 2001). Experimental models have also successfully demonstrated that MMP-2 and MMP-9 cooperate in promoting the *in vivo* invasive and angiogenic phenotype (Masson et al 2005). In this study, we noticed that Brahma rasayana caused the reduction in MMP activities. Further more *in vitro* analysis showed that Brahma rasayana inhibited the proliferation of the HUVEC cells in a dose dependent manner. Not only does Brahma rasayana inhibit the proliferation of HUVEC, it was also found to down regulate MMP-2 and MMP-9. Brahma rasayana was also found to reduce capillary tube formation on matrigel in an i*n-vitro* model of angiogenesis using HUVEC, which also suggests the anti-angiogenic, and anti-invasive potential of Brahma rasayana. The results have encouraged us to pursue future study with transgenic adenocarcinoma of mouse prostate model to establish the efficacy and anti tumor potential of Brahma rasayana. Efforts are being undertaken to characterize and match the major constituents of the methanolic extract and in the serum of Brahma rasayana treated animals. Elucidating the precise molecular mechanism for the beneficial effects of Brahma rasayana requires fractionation of Brahma rasayana components as well as *in-vitro* validation and screening of these various fractions.

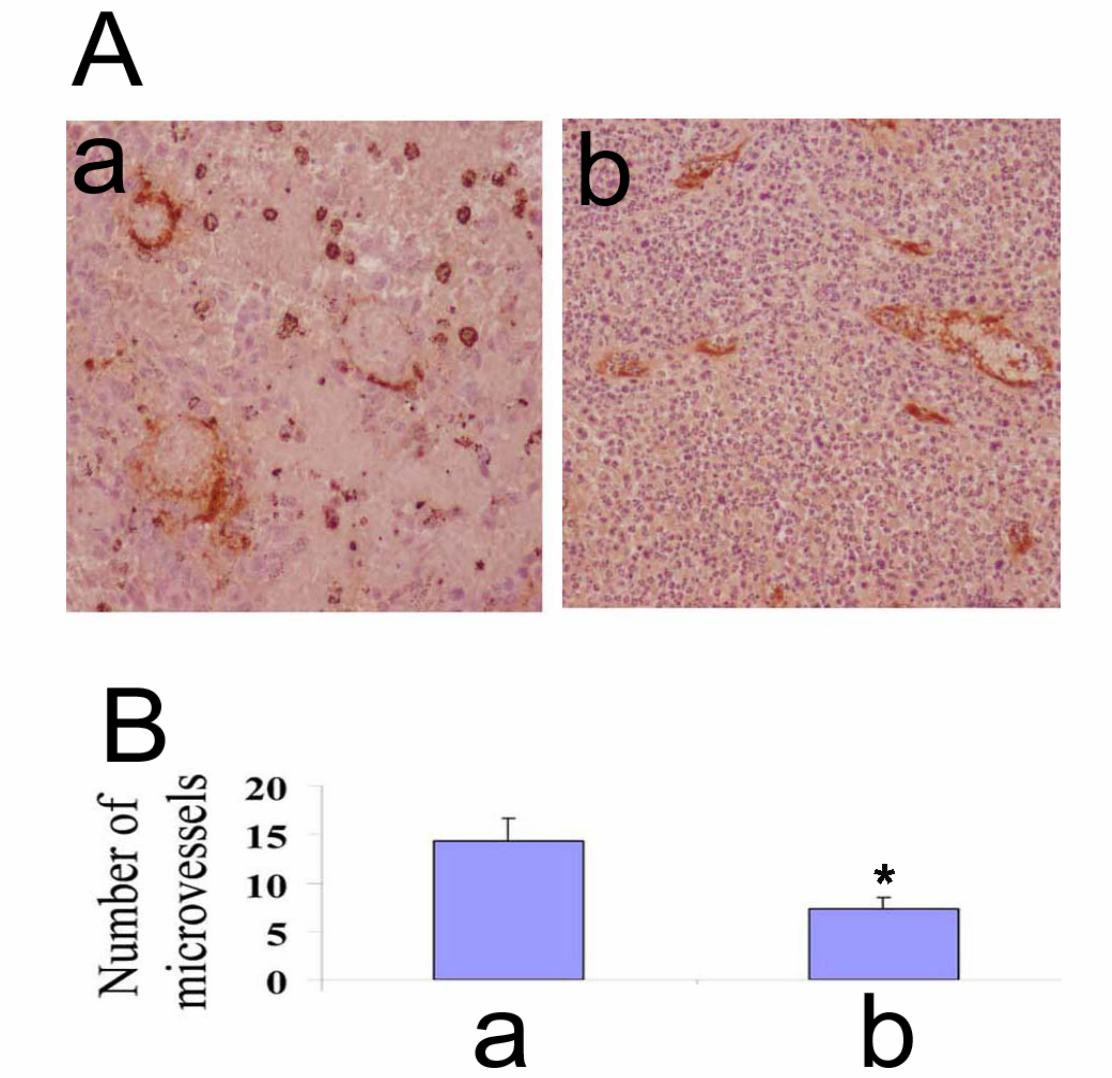

Figure 25. Effect of Brahma rasayana on MVD in rat prostate tumor tissue. (A) Representative picture of Factor VII staining (200X) a) MAT-LyLu b) MAT-LyLu + Brahma rasayana. (B) MVD Quantitation expressing percentage of microvessels in Brahma rasayana treated and untreated tumors. a) MAT-LyLu. b) MAT-LyLu + Brahma rasayana. *Significantly different compared with controls, $P < 0.05$.

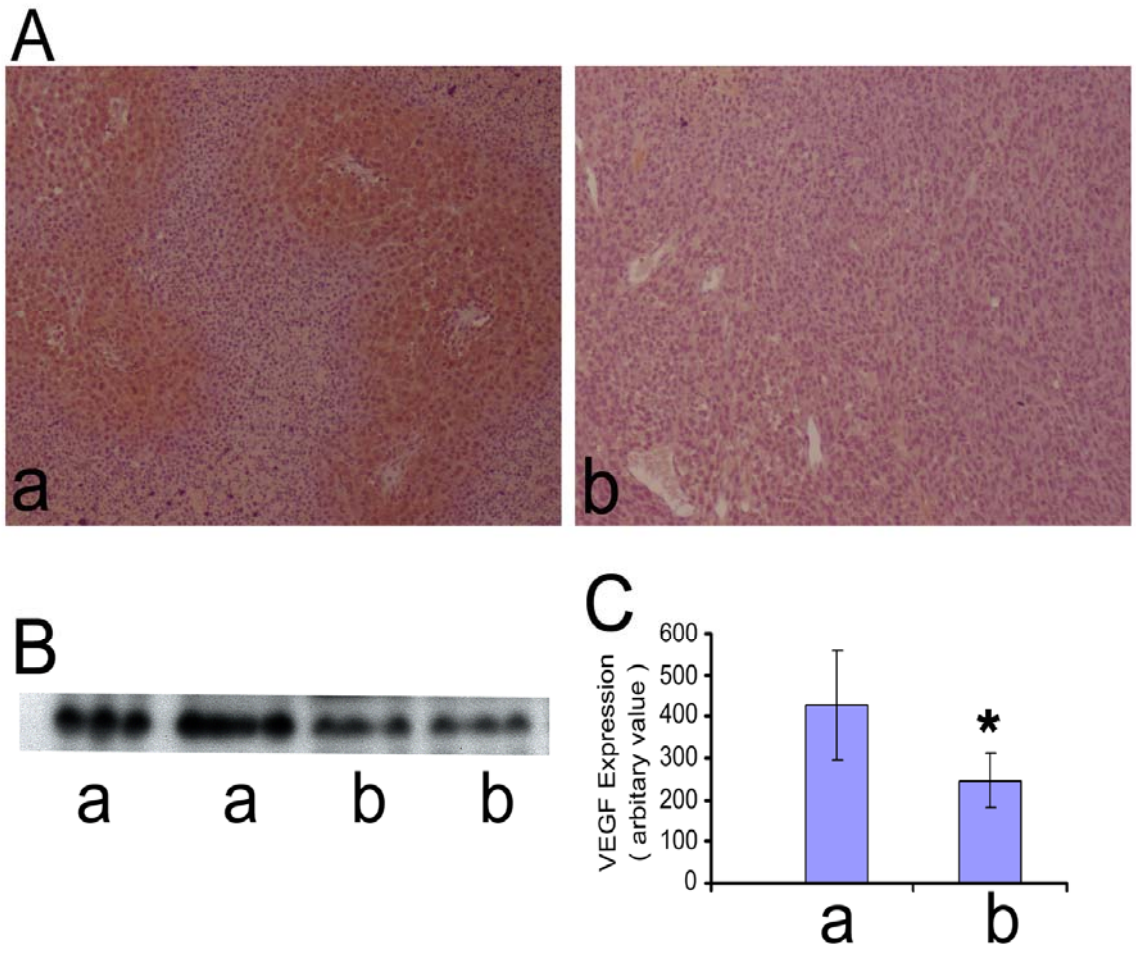

Figure 26. Effect of Brahma rasayana on VEGF expression in the rat prostate tumor tissue. Representative picture of immunohistochemical detection of VEGF (200 X). a.) MAT-LyLu. b.) MAT-LyLu + Brahma rasayana . (B) Western blotting for VEGF. a.) MAT-LyLu. b.)MAT-LyLu + Brahma rasayana. (C) Quantitation of VEGF expression in Brahma rasayana treated and control tumors. a.) MAT-LyLu b.) MAT-LyLu + Brahma rasayana. *Significantly different compared with controls, $P < 0.05$.

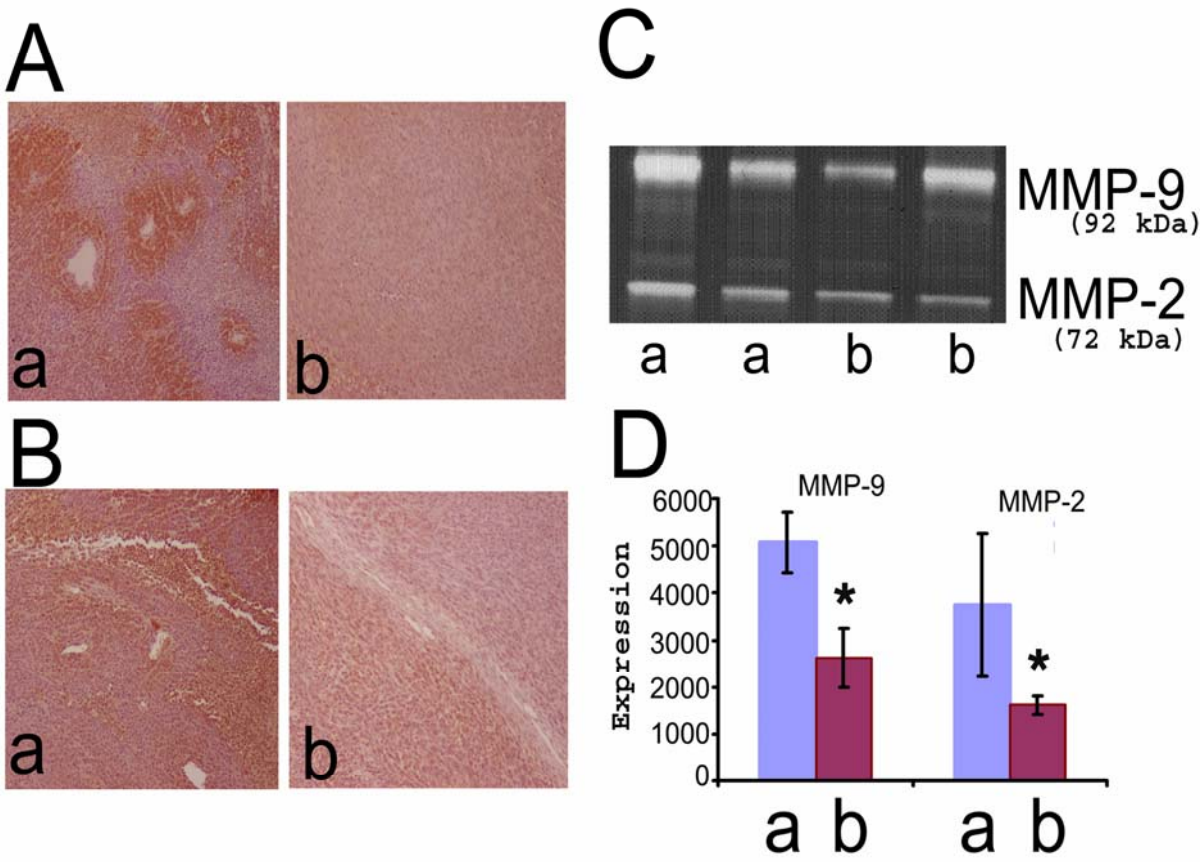

Figure 27. Effect of Brahma rasayana on MMP-9 and MMP-2 Expression in the rat prostate tumor tissue. (A) Representative picture for immunohistochemical detection of MMP-9. a) MAT-LyLu b)MAT-LyLu+ Brahma rasayana (B) Representative picture for Immunohistochemical detection of MMP-9 a) MAT-LyLu b) MAT-LyLu + Brahma rasayana (C) Zymographic Analysis of MMP-2 and MMP-9. a) MAT-LyLu b) MAT-LyLu + Brahma rasayana D) Quantitation of MMP expression. a) MAT-LyLu b) MAT-LyLu + Brahma rasayana. *Significantly different compared with controls, $P < 0.05$.

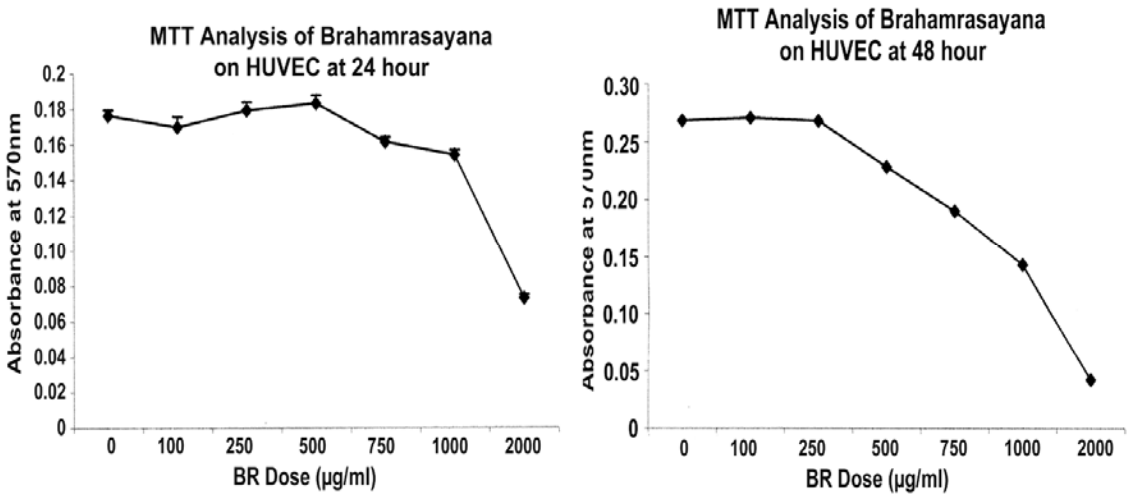

Figure 28. Effect of methanol extract of Brahma rasayana on proliferation of HUVEC. Brahma rasayana inhibited the HUVEC proliferation on a dose and time dependent manner. The data are presented as the mean plus/minus SE (n=10).

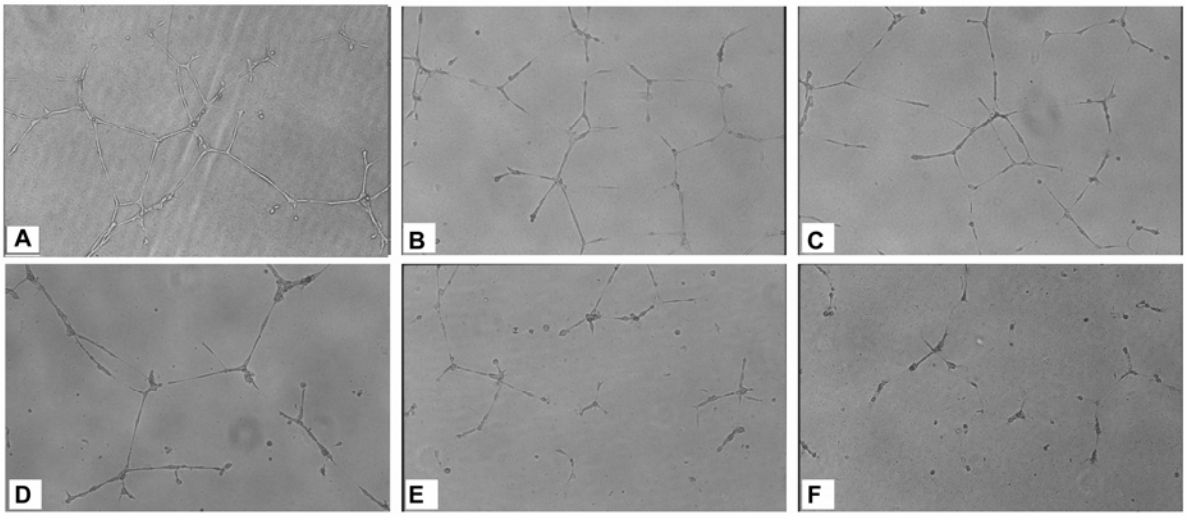

Figure 29. Effect of methanol extract of Brahma rasayana on capillary tube formation. Brahma rasayana inhibited tube formation by HUVEC on matrigel in a dose dependent manner. Doses of Brahma rasayana are A) 0 ug/ml. B) 250 ug/ml. C) 500 ug/ml . D) 1000 ug/ml. E) 1500 ug/ml. F) 2000 ug/ml.

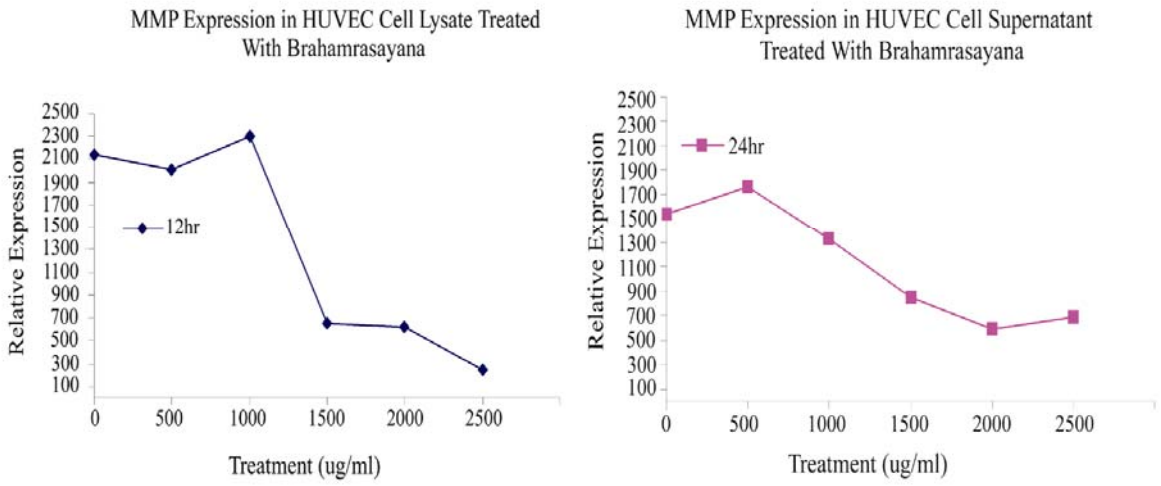

Figure 30. Effect of methanol extract of Brahma rasayana on MMP-9 expression in HUVEC. Brahma rasayana inhibited the MMP-9 expression in HUVEC in a dose and time dependent manner.

Chapter 3

Part 1: Curcumin induced gene expression response in human prostate cancer cells

INTRODUCTION

Prostate cancer is a common epithelial malignancy in elderly men of the western countries. Androgens are hormones which are not only responsible for prostate cell growth, proliferation and differentiation but also play a significant role in prostate tumorigenesis (Dehm and Tyndal 2006). The hormone dependent disease when treated by androgen deprivation, which clinically is known as androgen ablation therapy responds initially but reverts to a hormone independent phenotype which is hard to manage (Scher et al 1995). In addition to androgens there may be alternate pathways stimulating the androgen receptor (AR) signaling and restoring AR function to prostate cancer cells. Modulating AR function and alternate pathways that trigger AR activation could be novel and effective for prostate cancer chemoprevention.

Turmeric (*Curcuma longa* Linn), is a crystalline compound which has been traditionally used in medicine and cuisine in India. Curcumin (diferuloylmethane) is the major active component of turmeric (Ammon and Wahl 1991). Studies have shown that curcumin has a dose-dependent chemopreventive effect in several different animal tumor bioassay systems including colon, prostate, duodenal, stomach, esophageal, skin oral and breast carcinogenesis (Aggarwal et al 2005, Choudhuri 2005, Dorai 2001, Singh et al 1998, Huang et al 1999, Huang et al 1994, Azuine et al 1992). Low incidence of bowel cancer

in Indians and increase in the incidence of large bowel cancers in immigrants and urban Indians compared to rural populations supports a role for environmental risk factors including diet and the use of curcumin in Indian cookery (Mohandas and Desai, 1999). The molecular basis of anticarcinogenic and chemopreventive effects of curcumin is attributed to its effect on several targets including transcription factors, growth regulators, adhesion molecules, apoptotic genes, angiogenesis regulators and cellular signaling molecules. Curcumin has been shown to be a potent blocker of NF-κB activation through the inhibition of IκB kinase (IKK), a kinase that is needed for NF-κB activation (Aggarwal and Shishodia 2004). The biological effect of curcumin and its recently identified targets like NF-κB, beta-catenin have been reviewed in detail (Maheshwari et al 2006, Thangapazham et al 2006). Laboratory studies have shown the effectiveness of curcumin in prostate cancer chemoprevention both using *in vitro* and *in vivo* models (Dorai 2001, Hong 2007 and Nonn et al 2006). Recently, synergism in the inhibition of growth of PC3 prostate tumor xenografts in nude mice was demonstrated with curcumin in combination with phenethyl isothiocyanate was demonstrated showing that the effect of these two compounds together was stronger than each agent alone (Khor et al 2006).

Absence of dose limiting toxicity, when curcumin is administered up to 8 g/day in human clinical trials, reveals the possibility of curcumin in the prevention and treatment of cancer (Aggarwal et al 2003, Cheng et al 2001).

AR antagonism and down-regulation of AR and its related cofactors has been shown to be a potential mechanism for curcumin's role in prostate cancer chemoprevention (Nakamura et al 2002, Ohtsu et al 2002). Although, previous reports suggest that curcumin down regulates AR transactivation, the effect of curcumin on AR target genes

like NKX3.1 and other alternate pathways that might activate the androgen receptor signaling in prostate cancer cells have not been identified. In the present study, using high-density oligonucleotide Affymetrix human genome array, we analyzed the temporal expression profiles of curcumin regulated genes in widely used hormone responsive LNCaP cells and non-responsive C4-2B cells. The gene chip data was analyzed by hierarchical clustering methods and functional classification. Further more, we have evaluated the effects of curcumin on cell growth and identified its key molecular targets related directly and indirectly to androgen signaling in both androgen responsive and independent cell lines.

MATERIALS AND METHODS.

Materials

Curcumin was obtained from LKT laboratories (St Paul, Minnesota, MN). Prostate cancer cell lines LNCaP and C4-2B cells were kind gifts from Dr. Shiv Srivastava, CPDR, Rockville, MD, USA. Synthetic Androgen R1881 and non steroidal anti androgen bicluatamide (casodex) were also obtained from Dr. Shiv Srivastava. EGFR, ERBB2 antibodies were obtained from cell signaling technologies. PSA, NKX3.1 and AR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell proliferation inhibition studies by MTT

Human LNCaP and C4-2B prostate cancer cells were seeded in 96-well plates. After 24 hr, the cells were treated with varying doses of curcumin for 24, 48 and 72 hr. Control

cells were treated with 0.1% DMSO (vehicle control). Proliferation was determined using the MTT assay as desribed previously in page 17.

Total RNA was extracted as described in page 37.

Gene Chip Analysis - Hybridization, Staining and Scanning of the GeneChip

RNAs were prepared at 3, 6, 12, 24 and 48 hr after the treatments with Qiagen kit (Valencia, CA) according to the manufacturer's instructions. High-density oligonucleotide Affymetrix human genome array GeneChip® HG U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) that contains 54675 probe sets and about 38,500 well characterized human genes was used for hybridization with biotin labeled and fragmented aRNA(amplified RNA). Briefly, biotylation of aRNA was carried out by *In vitro* transcription using MEGA script T7 *in vitro* Transcription Kit (Ambion, Austin, TX, USA) and biotinylated UTP and biotinylated CTP (ENZO, Farmingdale, NY, USA). The biotin labeled cRNA was purified using the QIAGEN RNeasy spin columns (QIAGEN, Valencia, CA) following the manufacturer's protocol. The biotin labeled cRNA was fragmented in a 40 µl reaction mixture containing 40 mM tris-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate incubated at 94°C for 35 min and then put on ice. Hybridization was done at 42°C in a rotisserie hybridization oven (Model 320, Affymetrix) at 60 rpm for 16 hr. Following hybridization, the GeneChip arrays were washed 10 times at 25°C with 6X SSPE-T buffer using the automated fluidics station protocol. GeneChip arrays were incubated at 50°C in 0.5X SSPE-T, 0.005% Triton X-100 for 20 min at 60 rpm in the rotisserie oven. The arrays were

stained for 15 min at room temperature and at 60 rpm, with streptavidin phycoerythrin stain solution. GeneChip arrays were then be washed twice at room temperature with 6X SSPE-T buffer and scanned with the HP GeneArray Scanner (Hewlett-Packard, Santa Clara, CA) controlled by GeneChip 3.1 Software (Affymetrix).

Image Analysis and Data Collection and Data Analysis

Intensity values for probes were captured from the GeneChip images using the GCOS (GeneChip® Operating System). Affymetrix GeneChip® Microarray Analysis Software, version 3.1 and Affymetrix Micro DB and Data Mining Tool version 2.0 (Affymetrix), Microsoft Excel 2000 (Microsoft, Seattle, WA) and Statistica version 4.1 (Stat Soft, Inc., Tulsa, OK) were used. Normalized data in Microsoft XL format From GeneChip® CEL. files were obtained by using Partek Pro software. Gene expression data analysis was done using Microsoft XL 2003. Net work analysis for the selected genes was done using Genomatix pathway edition of Bibliosphere as described by the vendor (Genomatix GmbH, Munich, Germany, www.genomatix.de).

cDNA Microarray

cDNA microarray was performed for the human androgen signaling panel and human angiogenesis panel using cDNA microarray kit (Superarray Bioscience Corporation, Fredrick, MD) as per the manufacturer's protocol. Briefly, the procedure can be divided into 3 parts I.) Probe synthesis II.) Hybridization and III.) Detection. Probe was synthesized using True Labeling-RT kit (Superarray Biosc Coop., Frederick, MD).

Annealing mix was incubated at 70°C for 3min, cooled and maintained at 42°C for 2 min. RT cocktail was added to the annealing mix and reverse transcription was carried out at 42°C for 90 min. Reaction was stopped using buffer C. Probe was denatured by adding buffer D to the reaction mixture and incubated at 68°C for 20 min followed by neutralization with buffer E and incubation for another 10 min. Prehybridization was done for 2 hr at 60°C with GEAhyb Hybridization solution supplemented with 100µg/ml of denatured salmon sperm DNA followed by overnight hybridization with hybridization solution (probe resuspended in 750ul of prehybridization solution). Membranes were then washed and chemiluminescence was developed using Av-streptavidin followed by incubation with CDP-Star substrate.Image development and analysis was done as described previously in page 38.

RT-PCR

Expression of AR mRNA was analyzed by RT-PCR. Reverse transcription was done using the first strand synthesis kit (Invitrogen life technologies Carlsbad, CA) as per the manufacturer's instruction. PCR was done using PCR supermix (Invitrogen life technologies Carlsbad, CA) as per the manufacturer's protocol. Primers for PCR were: AR sense primer (5'-TCTCAAGAGTTTGGATGGCTCC-3'), AR antisense primer (5'-TCACTGGGTGTGGAAATAGATG-3'). GAPDH forward primer (5′-CGGAGTCAACGGATTTGGTCGTAT-3′), GAPDH reverse primer (5′-GCTCCTGGAAGATGGTGATGG-3').Initial denaturation was done at 94°C for 3 min; then denaturation at 94°C for 40 min, annealing at 60^0C for 40 sec, extension at 72°C for 40 min and 30 sec for 28 cycles; a final extension was done at 72°C for 8 min. PCR

product was visualized by electrophoresis on 1% agarose gel stained with ethidium bromide. Specific amplification was determined by comparing the size of the product on the gel relative to known DNA molecular weight marker. NKX3.1, EGFR, ERBB2 primers were purchased from superarray.

PSA Elisa

Conditioned medium from LNCaP and C4-2B cells treated with and without curcumin were collected and the protein concentration in the conditioned medium was quantified. The conditioned medium with equal amount of protein for each sample was subjected to PSA detection using the PSA enzyme immunoassay kit from United Biotech (Mountain View, CA) according to the manufacturer's protocol.

Western blotting was performed as described in page 18

RESULTS

Inhibition of cell proliferation by curcumin

We examined the effect of increasing concentrations of curcumin on the proliferation of human prostate cancer cells for 24, 48 and 72 h using MTT assay. We found that the treatment of LNCaP and C4-2B prostate cancer cells with curcumin resulted in a dose- and time dependent inhibition of cell proliferation (Figure 31). Extensive inhibition of cell growth was being observed in treatment groups receiving high concentrations (>40um) of curcumin.

Curcumin-Gene Expression Response (Cu-GER): GeneChip

We analyzed the Curcumin-Gene Expression Response (Cu-GER) in LNCaP and C4-2B cells between the 1–48 hr time points. Consistent androgen responsiveness of LNCaP and androgen insensitive property of C4-2B cells made them ideal for this study because we could compare the difference in effect of curcumin between less aggressive LNCaP tumor cell line and its isogenic derivative, the highly aggressive C4-2B cells. When we applied arbitrary cutoffs of >four fold induction or <four fold repression over the control level, a maximum of 181 genes were up-regulated at 12 hr and 245 genes were down-regulated at 24 hr in LNCaP cells treated with curcumin. In C4-2B cells a maximum of 27 genes were up-regulated at 12 hr and 453 genes were down-regulated at 48 hr with curcumin treatment (Table 4). For other time points please refer Table 4. As noted in Table 5 oxidative stress response and other cellular protective pathways are turned on in response to curcumin. There was a maximum of 25 fold increase in the expression of heme oxygenase-1 (HO-1), a stress response protein by curcumin. The DNA-damage-inducible transcript 3 (DDIT3) was also increased by curcumin treatment. ATF3 was identified to be one of the basic- leucine zipper transcription factors that activate HO-1 and GADD45A, a DNA damage inducible gene was also upregulated by curcumin. Stress-regulated protein tribbles homolog TRB3, a negative modulator of AKT was upregulated by curcumin. Table 5 and Table 6 show the list of genes that have been temporally regulated by curcumin treatment and a difference of at least 4 fold in their expression. NKX3.1, TMEPA1, KLK2, KLK3 (PSA), and TMPRSS2 were the AR regulated genes in curcumin treated LNCaP cells. Among those TMPRSS2 was consistently down-regulated in 4 out of the 5 time points. KCNN2 was down-regulated in

6, 12 and 24hr. In the cell cycle related genes, p21 was also upregualted and cyclin B1 was downregualted in both the cell lines by curcumin treatment. Figure 32 represents the coordinated gene expression pattern which defines biochemical pathways in response to curcumin treatment in prostate cancer cells. The pathways analyzed by the software suggest oxidative stress to be the target for chemopreventive potential of curcumin. Moreover p53, NF-kB and Jun nodes are highlighted by Genomatix-Bibliosphere analyses of transcriptions factors which also converge to these key stress response proteins (Figure 33). Figure 34 represents the difference in the number of regulated genes between LNCaP and C4-2B in response to curcumin treatment. It is remarkable to note that the magnitude of genes modulated by curcumin is significantly different between aggressive C42B tumor cell line and its isogenic derivative of less aggressive LNCaP cells. This suggests that the protective responses are more intact in LNCaP cells than C4-2B cells. This also suggests that C4-2B cells may be more resistant to the effects of curcumin than LNCaP cells. The cell proliferation assay shows that C4-2B is more resistant in gene response than LNCaP cells to curcumin up to 10 um concentration. C4-2B may take a little longer to respond to curcumin but the outcome is as robust as in LNCaP cells as observed in the 48 h time point down-regulation.

Effect of curcumin on gene expression in prostate cancer cells: Focused microarray and semiquantitative PCR

Microarray analysis indicated down-regulation of NKX3.1, EGFR and ERBB2 by more than 5 fold when LNCaP and C4-2B cells were treated with curcumin (Figure 35A). The

results of the microarray experiments were confirmed by semi quantitative RT PCR (Figure 35B). This result suggested that curcumin might have the ability to down regulate the AR gene as well. RT PCR indicated that in curcumin treated prostate cancer cells, the expression of AR gene was down-regulated in a dose dependent manner (Figure 35B).

Effect of curcumin on proteins involved in AR signaling in prostate cancer cells

To examine the proteins levels of AR and NKX3.1, western blot analysis of the total protein of cells treated and untreated with different concentrations of curcumin was performed. NKX3.1 and AR protein expression was found to be down-regulated in a dose dependent manner by curcumin in both LNCaP and C4-2B cells (Figure 36). Moreover, PSA and EGFR protein levels were also found to be down-regulated in a dose dependent manner. Our findings emphasize that the level of AR regulated molecules like NKX3.1 and PSA expression in the curcumin treated prostate cancer cells are down-regulated as a consequence of AR down-regulation (Figure 37).

AR Rescue

In androgen dependent cells curcumin acts similar to the well know androgen antagonist casodex. Addition of androgen to the curcumin treated androgen sensitive LNCaP cells rescues the androgen receptor levels to the normal levels. However in androgen independent C4-2B cells, androgen has no effect and the AR levels remain down-regulated in curcumin treated cells whereas casodex had no effect in AR levels (Figure 38). This shows that curcumin has the ability to down regulate AR in androgen independent phenotypes as well.

DISCUSSION

The LNCaP and C4-2B cell lines are well established androgen responsive and non-responsive cell lines respectively. To better understand the mechanism of the chemopreventive potential of curcumin in cancer, we performed a temporal gene expression analysis of the Curcumin-Gene Expression Response (Cu-GER) using Affymetrix oligonucleotide gene chip microarray in hormone-responsive and non responsive human prostate cancer cell line, LNCaP and C4-2B respectively. Hierarchical clustering methods and functional classification of the Cu-GER showed temporal co-regulation of genes involved in specific biochemical pathways involved in the oxidative stress response. Some of the genes like HO-1, ATF3, NKX 3.1(McNally et al 2007, Yan et al 2005, Zhang et al 2007) have been shown to be modulated in very recent studies which give confidence to the data presented here. Some of the novel targets modulated by curcumin presented in this study include TRIB3, AKAP12, TncRna, TMPRSS2, KLK2, TMEPA1 etc. There was a maximum of 25 fold increase in the expression of heme oxygenase-1 (HO-1), a stress response protein by curcumin. The DNA-damage-inducible transcript 3 (DDIT3) was also increased by curcumin treatment. Selenium was found to increase the expression of a DNA damage-inducible gene 153 (CHOP/GADD153) whose mRNA sequence represents the DDIT-3 gene (Wu et al 2005). ATF3 was identified to be one of the basic- leucine zipper transcription factors that activate HO-1. It was also up-regulated by curcumin. Another DNA damage inducible gene GADD45A was also found to be up-regulated by curcumin. Recent study identified GADD45A as a critical mediator of apoptosis and growth arrest in response to NSAID in cancer cells (zerbini et al 2006). Stress-regulated protein tribbles homolog TRB3 is a negative modulator of

AKT. TRB-3 inhibits AKT activation by preventing its phosphorylation. The proposed mechanism for this effect is a direct TRB binding to the activation domain of AKT and therefore masking the Thr308 phosphorylation site. A major role for the oxidative stress response in the pro-apoptotic action of cannabinoids on tumor cells was found to be mediated by TRIB3 over expression which was up-regulated by curcumin in our study (Carracedo et al 2006).

Androgen signaling pathway alone and by its interaction with other pathways like Wnt has been implicated in the development, growth, and progression of prostate cancer (Terry et al 2006). Recent review has indicated the importance of AR signaling in the development of prostate cancer, as well as in the development of hormone resistance and relapse of the disease (Singh and Agarwal 2006). Hence androgen receptor becomes an attractive target for chemoprevention of prostate cancer and there are several cancer chemopreventive phytochemicals like resveratrol (Mitchell et al 1999) and quercetin (Xing et al 2001) targeting the AR signaling and associated molecular events. It is conceivable that using phytochemicals that target AR could help contain prostate cancer growth and progression. In the present study, curcumin has been found to suppress AR not only at the protein level but also at the transcriptional level. The androgen up-regulated gene NKX3.1 has also been found to be down-regulated by curcumin treatment at both RNA and protein level in this study. PSA, a well known AR downstream target gene is a serine protease which can activate biological proteins and indirectly regulate prostate cancer promotion and progression (Charlesworth et al 1999, Diamandis et al 2000). PSA expression was significantly down-regulated with curcumin treatment which further confirms down-regulation of AR activity by curcumin. Recently it was shown that

in a majority of prostate cancer TMPRSS2 fuses with ETS transcription factor members like ERG or ETV1 and androgen responsive elements in the TMPRSS2 guides the overexpression of the ETS family members resulting in progression of the disease(Tomlins et al 2005). In our study the TMPRSS2 was down-regulated significantly and may extend to the reduction in the transcription of the fusion gene.

EGFR signaling pathways play a role in stimulating the AR pathway and in restoring AR function to prostate cancer cells in the absence of testosterone. Il-6 and Her2 neu have been shown to stimulate AR through the MAPK pathway and may result in prostate cancer progression and androgen independence (Yeh et al 1999, Hobisch et al 1998). EGFR pathway is suggested to be one of the pathways responsible for androgen ablation resistant survival of prostate cancer cells. This may occur due to stimulation and activation of Her-2/neu which has been shown to occur in an androgen-depleted environment or due to AR inactivation (Berger et al 2006). Moreover, ligand independent growth was observed in androgen-dependent prostate cancer cells when Her-2/neu was over expressed (Craft et al 1999). Her-2/neu was not only responsible for stabilizing the AR protein levels and prevents its degradation; it also optimized the binding of AR to the promoter/enhancer regions of androgen-regulated genes which makes it a target for intervening hormone refractory prostate cancer (Mellinghoff et al 2004). Therefore, a chemopreventive agent which can down regulate both AR and EGFR receptors will be indispensable for prevention and treatment of prostate cancer. In our study, both EGFR and ERBB2 receptor expression were found to be down-regulated several fold in curcumin treated LNCaP and C4-2B cells as analyzed by microarray. The results were confirmed by reverse transcriptase PCR reactions. A recent study has shown that

curcumin inhibits EGFR transcription through reducing the trans-activation activity of Egr-1 in human colon cancer cells (Chen et al 2006). Studies have shown that using an epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib (Iressa) and nonsteroidal anti-androgen, bicalutamide (Casodex) in androgen dependent cell line has additive effect in controlling prostate cancer progression as well as cause a significant delay in the onset of androgen independence caused by EGFR signaling (Festuccia et al 2005). Curcumin was shown to inhibit AR not only in AR dependent LNCaP cell line but also in C4-2B cells whereas the well known androgen receptor antagonist bicalutamide did not down regulate AR expression in C4-2B cells. Therefore, these data suggest that the dual action of curcumin can be handy in increasing the therapeutic index of anti-androgen therapy and other treatment modalities or can be used currently in the treatment of hormone independent aggressive phenotype of the disease.

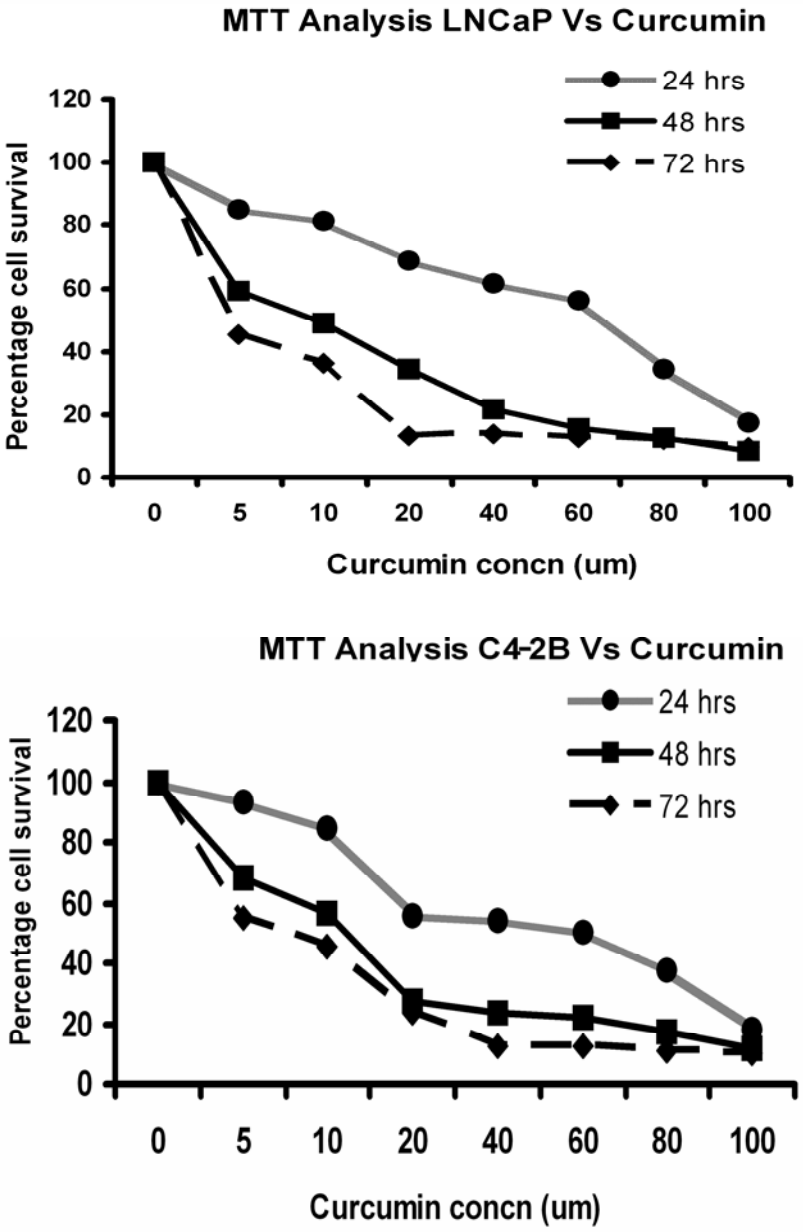

Figure 31. Curcumin inhibits the proliferation of LNCaP and C4-2B cells. (A) Effect of different concentrations of curcumin on the proliferation of LNCaP incubated for 24,48,72 h. (B) Effect of different concentrations of curcumin on the proliferation of C4-2B cells incubated for 24,48,72 h. The data are presented as the mean plus/minus SE (n=10).

Table 4. Summary of the number of genes regulated by curcumin in prostate cancer cells with respect to treatment time.

LNCaP	**Treatment Period**				
4-fold threshold	**3h**	**6h**	**12h**	**24h**	**48h**
Up-regulation	8	73	181	3	0
Down-regulation	48	15	13	245	0
5-fold threshold					
Up-regulation	5	40	83	2	0
Down-regulation	26	5	6	82	0

C4-2B	**Treatment Period**				
4-fold threshold	**3h**	**6h**	**12h**	**24h**	**48h**
Up-regulation	2	12	27	19	0
Down-regulation	26	2	13	2	453
5-fold threshold					
Up-regulation	2	8	15	13	0
Down-regulation	9	1	4	1	138

Table 5. Temporal expression profiles of curcumin treated LNCaP cells with treatment period at > 4 threshold. Fold change on each gene was calculated as intensity of treated/control cells. No transformation was applied to fold value. "---" represents unknown or expressed sequence tag.

Genbank	Gene	3h	6h	12h	24h	48h	GO Molecular Function Description
Up-Regulation							
NM_002133	HMOX1	6.38	18.30	25.39	5.83	1.15	heme oxygenase (decyclizing) activity
AA553477	---	6.94	15.72	21.91	4.51	1.25	---
NM_016415	---	5.69	15.45	19.99	1.77	1.09	---
AV702101	---	6.67	11.74	13.22	2.98	1.28	---
AI369956	---	2.31	5.70	12.77	3.14	1.08	---
NM_021158	TRIB3	2.21	6.38	11.63	2.25	0.88	transcription corepressor activity
NM_001674	ATF3	3.38	6.71	11.37	3.01	1.19	DNA binding /// transcription factor activity
BC003637	DDIT3	2.57	7.67	11.37	3.08	1.15	transcription factor activity
AI885066	---	3.32	7.99	10.29	2.13	1.15	---
AI640434	ZNF219	2.85	8.02	9.80	1.22	1.12	---
AY010114	---	2.90	8.43	9.74	1.09	0.97	---
AF070569	MGC14376	1.10	3.73	9.40	5.30	1.39	---
BF215487	PSMC2	2.34	6.13	9.00	1.49	1.38	---
AU134977	TncRNA	3.26	6.09	8.98	1.89	1.13	---
BF114967	AKAP12	2.83	5.96	8.61	1.04	1.01	protein binding /// protein kinase A binding
H06497	---	4.08	7.60	8.33	1.39	0.98	---
AL565238	LOC153222	3.99	6.75	8.07	1.25	1.14	transcription factor activity
NM_022157	RRAGC	2.35	5.21	7.70	1.39	1.13	nucleotide binding /// magnesium ion binding
R76550	ZNF219	3.11	7.61	7.69	1.34	1.10	---
BC043158	---	1.38	4.13	7.56	1.01	0.98	---
AW297143	HBS1L	2.20	4.82	7.46	1.33	1.23	nucleotide binding
AI807498	---	3.05	6.57	7.42	1.88	1.20	DNA binding /// zinc ion binding
BF131886	SESN2	3.31	6.06	7.42	1.85	1.17	---
AA001150	---	1.91	4.13	7.24	1.78	1.10	protein binding
AI912194	DERA	2.35	5.31	7.20	1.33	1.33	deoxyribose-phosphate aldolase activity
AW296039	VAPA	2.80	5.25	7.03	1.08	1.26	signal transducer activity
AA553959	C10orf99	1.35	3.76	6.84	1.35	1.09	---
AA889628	HNRPC	2.47	5.64	6.82	0.95	1.17	nucleotide binding /// RNA binding
AI494500	---	2.89	5.80	6.77	1.39	1.13	---
AW471220	ZA20D2	1.19	2.62	6.76	2.08	1.30	DNA binding /// zinc ion binding
W73819	DDR2	2.74	6.43	6.69	1.60	0.91	---
AL567411	CDK5R1	2.54	4.07	6.54	1.15	1.06	protein kinase activity /// calcium ion binding
AK024921	---	2.81	6.49	6.52	1.54	1.22	---
NM_005904	SMAD7	1.36	3.29	6.42	0.99	1.14	receptor signaling protein serine/threonine kinase
NM_002229	JUNB	1.65	3.69	6.41	1.88	1.31	transcription factor activity /
AI190292	---	1.64	3.73	6.39	1.19	1.06	---
AA678047	MMAA	3.18	5.84	6.37	1.17	1.11	nucleotide binding
AL136551	SESN2	1.59	3.43	6.30	1.77	1.01	---
NM_007146	ZNF161	2.13	4.53	6.30	1.28	1.29	DNA binding
NM_001806	CEBPG	1.41	3.06	6.26	1.52	1.09	transcription factor activity
D30658	GARS	2.60	4.25	6.26	1.24	0.95	nucleotide binding

D13889	ID1	1.49	2.39	6.26	2.22	2.20	protein binding
BE645231	SEC24A	1.31	3.41	6.13	1.26	1.20	protein binding
AI201880	HSPC128	1.37	3.47	6.03	1.18	1.18	---
AW979261	---	9.01	11.49	6.03	1.61	1.42	---
AI610347	---	3.29	5.10	5.89	0.96	0.92	actin binding /// calcium ion binding
AI420959	---	2.44	5.04	5.87	1.27	1.18	---
AI307750	LOC153222	3.26	5.94	5.80	1.07	1.17	transcription factor activity
BE964053	SUI1	2.25	3.63	5.78	1.43	1.06	translation initiation factor activity
AF250311	TRIB3	1.01	2.57	5.78	0.86	0.97	transcription corepressor activity
BF514098	---	1.24	3.54	5.71	2.27	1.22	---
BE764796	C10orf42	1.04	5.96	5.70	0.96	1.06	---
BC000527	EWSR1	2.03	3.56	5.69	1.51	1.16	nucleotide binding
AL833114	---	1.30	4.01	5.69	1.06	1.10	protein-methionine-R-oxide reductase activity
AW271626	---	2.18	4.66	5.68	1.09	1.09	DNA binding /// zinc ion binding
AL137331	---	2.67	4.98	5.62	1.23	1.03	---
H28915	---	1.35	3.62	5.61	1.36	1.28	---
AU143964	COPB	2.38	4.63	5.60	1.13	1.09	protein binding
NM_002359	MAFG	1.60	3.35	5.59	1.38	1.10	transcription factor activity
AW665538	---	1.41	3.34	5.43	1.40	1.25	---
AL834319	LOC221091	2.01	3.45	5.40	1.01	0.95	---
Y08613	NUP88	2.01	3.77	5.36	1.16	1.01	transporter activity
BC008846	---	2.20	4.41	5.36	1.13	1.06	---
BC002446	DNAJB6	1.53	3.14	5.35	1.45	1.34	heat shock protein binding
NM_006948	STCH	0.72	2.05	5.32	1.72	0.99	nucleotide binding
BE671060	---	1.77	3.35	5.31	1.23	1.09	---
NM_005335	HCLS1	1.32	2.82	5.30	1.34	1.12	transcription factor activity
AU146275	ZNF161	1.16	2.60	5.25	1.08	1.08	DNA binding
AB037732	RBM27	1.55	3.45	5.23	1.16	1.31	nucleotide binding
BC035096	---	1.92	4.35	5.22	1.05	1.05	---
BE672659	CACNA1D	1.15	3.31	5.22	0.72	0.94	calcium ion binding
AI493853	RBM7	3.57	4.30	5.19	1.39	1.08	nucleotide binding
AL037805	---	1.98	4.39	5.18	0.88	0.87	transcription factor activity
AK026760	C20orf119	2.83	5.78	5.18	0.98	0.98	nucleotide binding
BC021582	SCML4	0.93	3.32	5.15	0.88	1.08	---
AK024556	SPRY4	1.10	3.28	5.14	0.97	1.02	protein binding
T77543	---	1.38	4.44	5.14	0.91	0.83	cation transporter activity
BE378479	HDLBP	1.27	2.56	5.13	1.58	0.97	RNA binding /// lipid transporter activity
D29832	SERPINC1	1.34	2.68	5.10	1.05	1.03	serine-type endopeptidase inhibitor activity
NM_000078	CETP	0.99	2.49	5.09	1.28	1.17	catalytic activity /// lipid binding
AI829920	UBE2H	1.32	2.74	5.08	1.24	1.15	ubiquitin-protein ligase activity
BF723605	NRCAM	0.94	2.82	5.01	0.98	1.14	---
AI003508	---	1.21	3.06	5.00	1.25	1.25	---
BG537255	DNAJB1	1.24	2.61	4.95	1.09	1.31	heat shock protein binding
BF110588	FRMD3	0.90	2.79	4.93	1.16	1.12	binding /// cytoskeletal protein binding
AW006290	RIOK3	1.32	2.45	4.93	1.50	1.17	nucleotide binding
AI769477	---	1.55	2.82	4.92	1.04	1.16	---
AL049251	PLCL3	1.46	3.93	4.87	0.97	1.16	phosphoinositide phospholipase C activity
BF029081	MGC39715	3.40	4.69	4.86	0.93	1.08	protein binding /// phosphoinositide binding
BE893893	MAP1LC3B	1.00	2.37	4.86	1.70	1.39	protein binding
U87836	SFRS10	1.42	3.02	4.84	1.12	1.11	nucleotide binding
NM_002822	PTK9	2.39	3.86	4.82	0.84	1.16	protein-tyrosine kinase activity
W60953	VCP	2.36	4.39	4.81	1.52	1.22	nucleotide binding
AA005023	NOD27	1.05	3.70	4.80	0.93	1.09	nucleotide binding
AI950023	---	2.55	4.96	4.77	1.54	1.18	---
NM_014423	AFF4	1.36	2.63	4.76	1.21	1.19	transcription factor activity
AA972711	ZNF292	2.44	4.23	4.70	2.10	1.31	DNA binding /// zinc ion binding

BC013942	---	0.81	2.85	4.70	1.16	1.05	---
AA203136	ZNF585A	1.82	3.88	4.69	1.44	1.12	nucleic acid binding
BC006488	ASB3	1.16	3.01	4.69	1.04	1.23	---
NM_006182	DDR2	1.52	3.54	4.69	0.98	1.10	nucleotide binding
N66072	---	1.39	3.18	4.67	0.98	1.10	protein binding /// zinc ion binding
NM_024978	---	3.36	5.74	4.65	0.95	1.14	---
BC002490	CXXC5	2.18	4.05	4.61	0.78	1.20	DNA binding /// zinc ion binding
AI459194	EGR1	1.49	4.28	4.59	0.82	1.17	transcription factor activity
AA926831	---	1.79	3.38	4.57	0.93	1.09	---
AF087974	NKIRAS1	2.10	3.69	4.57	0.91	1.04	---
NM_022771	TBC1D15	1.10	2.44	4.57	1.40	1.04	GTPase activator activity
Y11162	RNU68	2.65	3.11	4.57	1.02	1.06	---
NM_000036	AMPD1	1.43	2.86	4.54	1.03	1.14	AMP deaminase activity
NM_006477	RRP22	1.20	2.37	4.51	0.86	0.97	nucleotide binding
NM_005083	U2AF1L1	1.72	3.36	4.51	1.17	1.05	nucleotide binding
AI005163	FLJ40427	0.86	2.44	4.51	0.87	1.24	microtubule motor activity
NM_002167	ID3	1.21	1.88	4.48	1.23	1.87	transcription corepressor activity
NM_016265	ZNF12	1.74	3.45	4.47	1.27	1.37	nucleic acid binding
AA714835	---	1.69	3.01	4.46	1.08	1.13	---
AW298070	BRD4	1.44	2.66	4.45	1.04	1.00	---
NM_005930	CTAGE5	1.89	3.66	4.43	1.14	1.14	3'-5'-exoribonuclease activity
BC041051	---	1.15	3.51	4.43	0.94	1.10	---
AF505656	ZNF12	1.61	3.31	4.43	1.24	1.36	nucleic acid binding
AW241864	CELSR1	1.57	3.05	4.43	1.02	1.01	---
U58658	FLJ10385	0.63	1.64	4.41	1.21	1.03	DNA strand annealing activity
AA351360	PTDSR	1.42	2.62	4.37	1.06	1.09	---
NM_003186	TAGLN	1.82	3.11	4.36	0.70	0.85	actin binding
AV716798	LEREPO4	1.25	2.36	4.35	0.95	1.27	nucleic acid binding /// zinc ion binding
BE622659	CEBPG	0.98	2.08	4.35	1.19	1.03	transcription factor activity
BQ024890	---	1.71	3.40	4.32	0.99	1.08	---
BC041468	LOC339988	3.50	5.02	4.31	0.99	1.09	---
AK026404	MUC17	1.30	2.43	4.30	1.10	1.37	extracellular matrix constituent, lubricant activity
AA083483	FTH1	1.88	3.29	4.30	1.82	2.46	ferroxidase activity
AW173076	SART3	1.28	2.79	4.29	0.98	1.14	nucleotide binding
BC001120	LGALS3	0.95	2.57	4.29	0.82	1.08	sugar binding
AW967619	CRSP7	1.45	2.90	4.28	1.13	1.23	transcription coactivator activity
BC002559	YTHDF2	1.53	2.56	4.27	0.80	1.11	---
AK001782	CXXC5	2.10	4.09	4.26	0.74	1.15	DNA binding /// zinc ion binding
W86831	SYT1	2.38	3.55	4.26	1.21	1.10	transporter activity
BG403486	PRO0149	1.43	2.59	4.25	1.16	1.28	---
BC041468	LOC339988	3.72	4.91	4.24	1.03	1.06	---
AI523450	C9orf126	0.91	3.29	4.23	0.83	1.21	---
NM_019058	DDIT4	4.35	6.05	4.22	0.87	0.90	---
NM_014167	HSPC128	1.04	2.41	4.22	1.00	1.18	---
AI800518	ANTXR1	1.65	3.48	4.21	0.96	1.14	receptor activity
AA705933	---	1.13	2.64	4.21	1.02	1.02	---
BG481972	EIF5	1.22	2.38	4.19	1.35	1.24	nucleotide binding
AA729232	---	1.42	2.80	4.19	1.24	1.08	---
NM_000389	CDKN1A	0.78	1.64	4.19	2.21	1.58	protein kinase activity
U89358	L3MBTL	2.14	4.07	4.18	1.17	1.09	transcription factor activity
BC039395	---	1.92	3.14	4.17	1.25	1.28	---
NM_173631	ZNF547	1.10	2.70	4.17	1.22	1.06	DNA binding
AI356405	IARS	1.64	4.05	4.14	1.00	1.08	nucleotide binding
AI459157	---	1.11	2.38	4.14	1.45	1.08	protein phosphatase type 2A regulator activity
BF674842	TDG	1.49	2.69	4.13	0.91	1.22	damaged DNA binding
AI435828	STC2	2.49	3.96	4.13	0.74	0.66	hormone activity

NM_152608	C1orf55	1.12	2.13	4.12	1.51	1.16	---
D14826	CREM	1.44	2.61	4.11	2.22	1.27	transcription factor activity
AA890703	LOC283680	1.26	2.72	4.10	1.29	1.23	---
NM_018293	ZNF654	0.96	2.11	4.10	1.05	1.17	nucleic acid binding
NM_004417	DUSP1	1.69	3.33	4.09	1.46	0.94	protein tyrosine phosphatase activity
BE219444	GTF2A2	1.63	3.24	4.09	0.97	1.35	---
AW270170	---	1.29	2.87	4.09	0.90	1.09	protein binding
NM_001695	ATP6V1C1	1.09	2.21	4.09	0.90	1.09	transporter activity
AI627532	KIAA1276	1.60	3.28	4.08	0.95	1.17	---
AI888057	---	1.43	2.83	4.08	0.92	1.08	---
NM_173829	FLJ36754	1.27	2.34	4.07	0.90	1.12	nucleic acid binding
R37780	NRCAM	0.78	2.32	4.07	0.85	1.17	protein binding
N22849	---	1.51	2.44	4.05	1.12	1.38	---
NM_001924	GADD45A	0.79	2.11	4.05	1.93	1.05	---
NM_002392	MDM2	1.12	2.21	4.05	1.26	1.07	ubiquitin-protein ligase activity
NM_004134	HSPA9B	1.22	2.20	4.04	1.18	1.19	nucleotide binding
AW970888	NSUN6	2.15	4.75	4.04	1.34	0.98	RNA binding
AI357655	---	1.51	2.89	4.04	1.12	1.13	---
AK024582	---	1.55	3.03	4.03	0.88	1.07	---
BG386322	PAFAH1B2	1.79	3.31	4.03	0.67	1.28	1-alkyl-2-acetylglycerophosphocholine
AW731710	---	1.37	3.24	4.03	1.06	1.31	---
AI538172	RBBP6	1.38	2.16	4.02	1.17	1.30	nucleic acid binding
NM_012234	RYBP	1.18	2.58	4.01	0.94	1.11	DNA binding
NM_017415	KLHL3	0.89	2.13	4.01	1.00	1.16	actin binding
AC003007	LOC23117	1.90	3.00	4.01	1.17	1.09	binding
AA553959	C10orf99	1.02	2.38	4.01	0.92	1.13	---
AF017307	ELF3	1.58	3.00	4.01	1.33	1.14	transcription factor activity
L13386	PAFAH1B1	1.17	2.24	4.01	1.27	1.24	dynein binding

Down-regulation

AF318340	---	0.16	0.24	0.25	0.27	1.02	ion transporter activity
AK023795	ADAMTS1	0.19	0.24	0.24	0.26	1.53	metalloendopeptidase activity
NM_024923	NUP210	0.53	0.38	0.24	0.23	1.13	protein binding
AA927670	NRP1	0.08	0.18	0.23	0.32	0.98	receptor activity
AW974642	LRRN3	0.53	0.46	0.23	0.27	1.17	serine-type peptidase activity
NM_138818	C9orf65	0.18	0.21	0.23	0.36	0.84	---
BF433975	ANK3	0.88	0.75	0.22	0.33	1.60	structural constituent of cytoskeleton
AI873273	SLC16A6	0.13	0.18	0.17	0.13	0.85	transporter activity
NM_001731	BTG1	0.44	0.28	0.17	0.26	0.89	transcription cofactor activity
AI660243	TMPRSS2	0.40	0.33	0.17	0.16	0.67	serine-type endopeptidase activity
NM_021614	KCNN2	0.54	0.39	0.17	0.15	0.79	ion channel activity
AI056992	RNU47	0.08	0.11	0.16	0.31	1.07	---
BC042961	---	0.15	0.15	0.11	0.26	1.06	---

Table 6. Temporal expression profiles of curcumin treated C4-2B cells with with treatment period at > 4 threshold. Fold change on each gene was calculated as intensity of treated/control cells. No transformation was applied to fold value. "---" represents unknown or expressed sequence tag.

Genbank	Gene	3h	6h	12h	24h	48h	GO Molecular Function Description
Up-regulation							
NM_002133	HMOX1	6.00	19.99	25.68	16.87	0.43	heme oxygenase (decyclizing) activity
AF070569	MGC14376	1.19	4.12	12.73	9.41	0.55	---
AF003934	GDF15	1.03	5.03	8.87	7.19	0.46	cytokine activity /// growth factor activity
BE465032	C6orf62	3.51	2.10	8.30	2.22	0.75	---
AW471220	ZA20D2	1.45	3.80	7.22	2.69	0.43	DNA binding /// zinc ion binding
AL136551	SESN2	0.89	3.73	6.83	2.15	0.58	---
AF115512	DNAJB9	1.47	1.99	6.39	2.34	0.51	chaperone regulator activity
BC003637	DDIT3	1.85	7.23	6.31	2.82	0.52	transcription factor activity
NM_021158	TRIB3	1.08	6.52	6.26	1.99	0.41	transcription corepressor activity
NM_001674	ATF3	1.48	8.07	6.21	2.45	0.48	DNA binding /// transcription factor activity
NM_001924	GADD45A	0.61	3.19	5.94	2.03	0.48	---
BE964053	SUI1	1.82	4.18	5.85	1.46	0.41	translation initiation factor activity
AU155361	TncRNA	2.46	3.45	5.73	2.30	1.02	---
NM_000389	CDKN1A	0.44	1.90	5.45	4.53	1.16	protein kinase activity
AI422414	---	0.80	1.37	5.15	1.49	0.61	carboxypeptidase A activity
AB040875	SLC7A11	0.83	2.16	4.76	6.46	0.39	cystine:glutamate antiporter activity
NM_014331	SLC7A11	0.86	1.96	4.61	7.31	0.64	cystine:glutamate antiporter activity
BE566894	LTB4DH	0.92	0.98	4.59	6.73	1.52	alcohol dehydrogenase activity
AF288391	C1orf24	0.70	1.73	4.46	2.77	0.27	heat shock protein binding
BF131886	SESN2	1.07	3.02	4.37	1.56	0.56	---
AU134977	TncRNA	2.29	2.92	4.27	3.61	0.59	---
NM_006815	RNP24	1.41	2.01	4.24	1.30	0.43	---
AA488687	SLC7A11	2.73	1.57	4.17	5.26	0.36	cystine:glutamate antiporter activity
AI927692	---	0.87	1.92	4.12	2.44	0.43	---
AF084462	RIT1	1.01	2.26	4.11	2.84	0.45	nucleotide binding /// calmodulin binding
NM_019058	DDIT4	2.57	8.57	4.11	2.29	0.38	---
NM_173617	FLJ36701	0.96	1.76	4.04	1.64	0.54	---
Down-regulation							
AA885753	PPFIA2	0.48	0.49	0.25	0.73	0.31	protein binding
AI961235	FLJ12505	0.48	0.68	0.24	0.40	0.17	---
NM_021643	TRIB2	0.28	0.40	0.23	0.46	0.28	nucleotide binding /// protein kinase activity
AI346835	TM4SF1	0.51	0.88	0.22	0.38	0.10	---
AF252283	KLHL1	0.53	0.91	0.22	0.29	0.20	actin binding /// protein binding
NM_003759	SLC4A4	0.53	0.88	0.21	0.55	0.34	inorganic anion exchanger activity
BF681305	---	0.50	0.37	0.21	0.57	0.21	---
NM_021614	KCNN2	0.35	0.59	0.21	0.50	0.34	ion channel activity /// calmodulin binding
AF247704	NKX3-1	0.36	0.43	0.20	0.33	0.29	transcription factor activity
AA622392	C1orf116	0.19	0.33	0.20	0.25	0.18	---
AI989530	SGEF	0.42	0.44	0.20	0.60	0.37	Rho guanyl-nucleotide exchange factor activity
AL157453	PPFIA2	0.47	0.56	0.19	0.72	0.29	protein binding
AL035541	TMEPAI	0.32	0.49	0.19	0.34	0.28	---

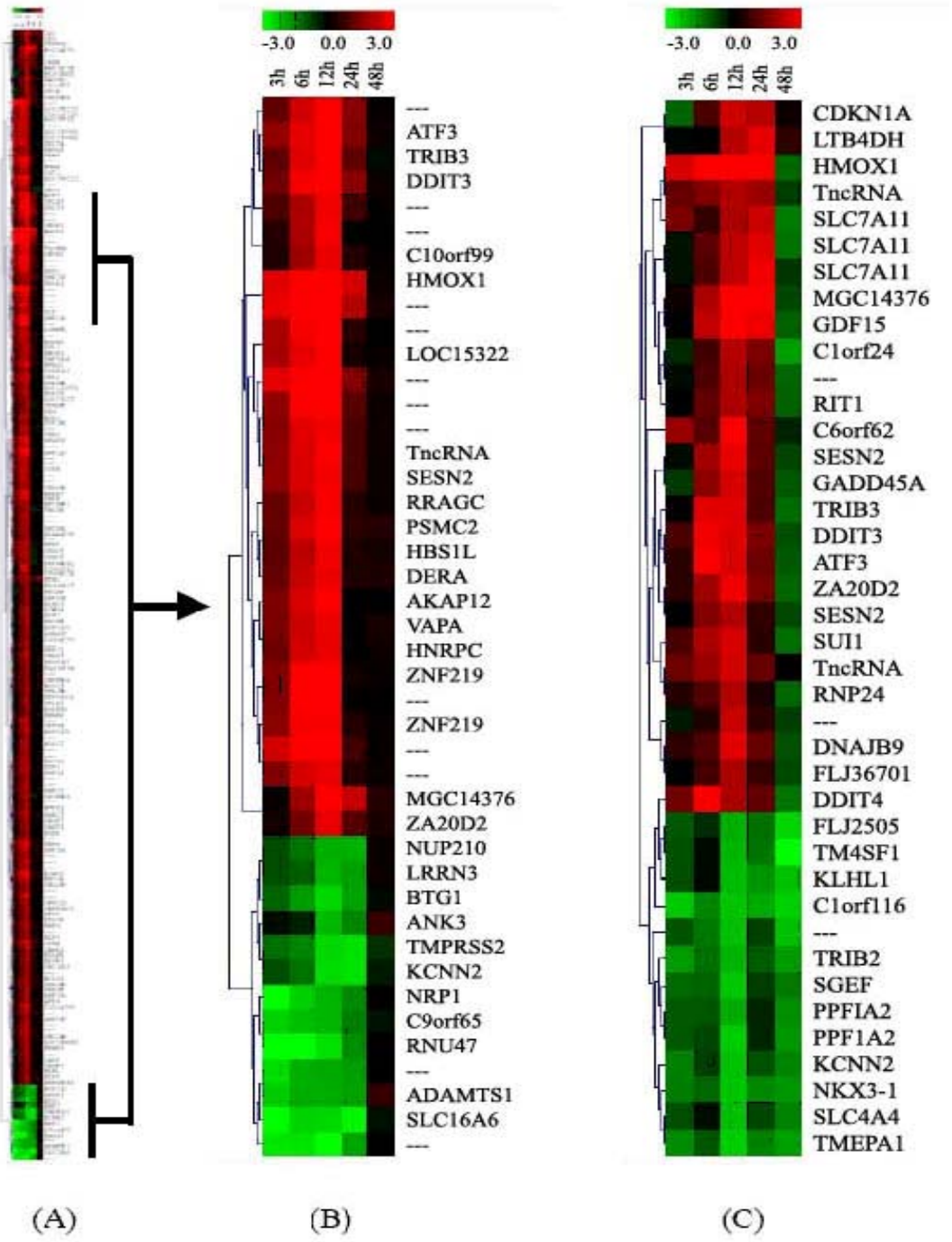

Figure 32. Hierarchical clustering using $\log_2$ transformed relative expression fold-changes with > 4 threshold over treatment periods in **LNCaP (A) and C4-2B (C)** cells. (B) The top 30 up-regulated genes (> 6.7 threshold) and all down-regulated genes were separately clustered and presented. Each row and column represents genes and treatment period with the representative colors of up-regulation (red) or down-regulation (green). "---" represents unknown or expressed sequence tag.

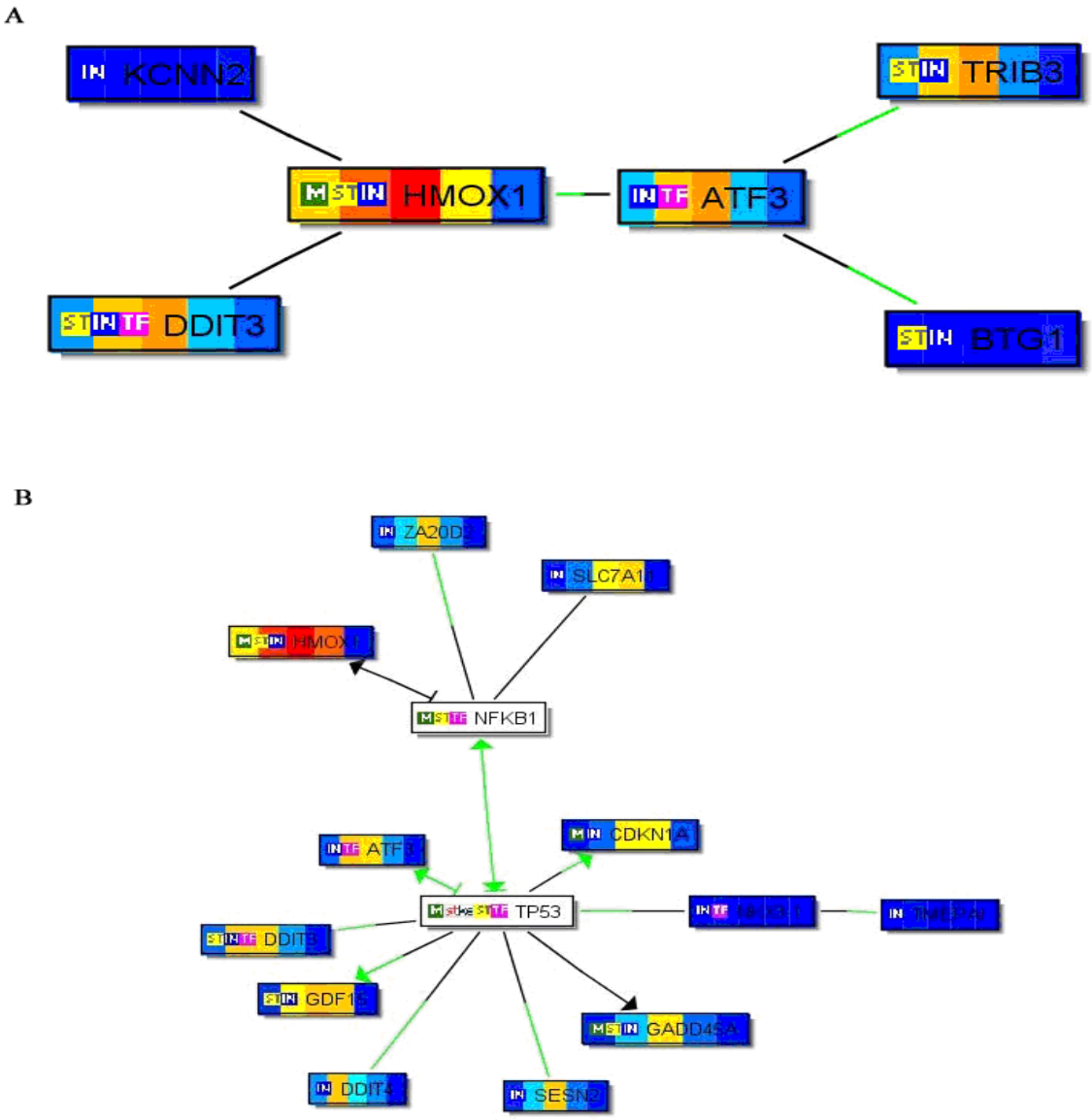

Figure 33. A. Pathway with the top 30 up-regulated and all down-regulated genes (13 genes) in LNCaP cells. B. Pathway with the up-regulated genes (40 genes, Table 4) in C4-2B cells. Each block contains five segments, representing the treatment period in order of 3, 6, 12, 24, and 48 h from left. Blue is the lowest expression and red represents the highest expression level.

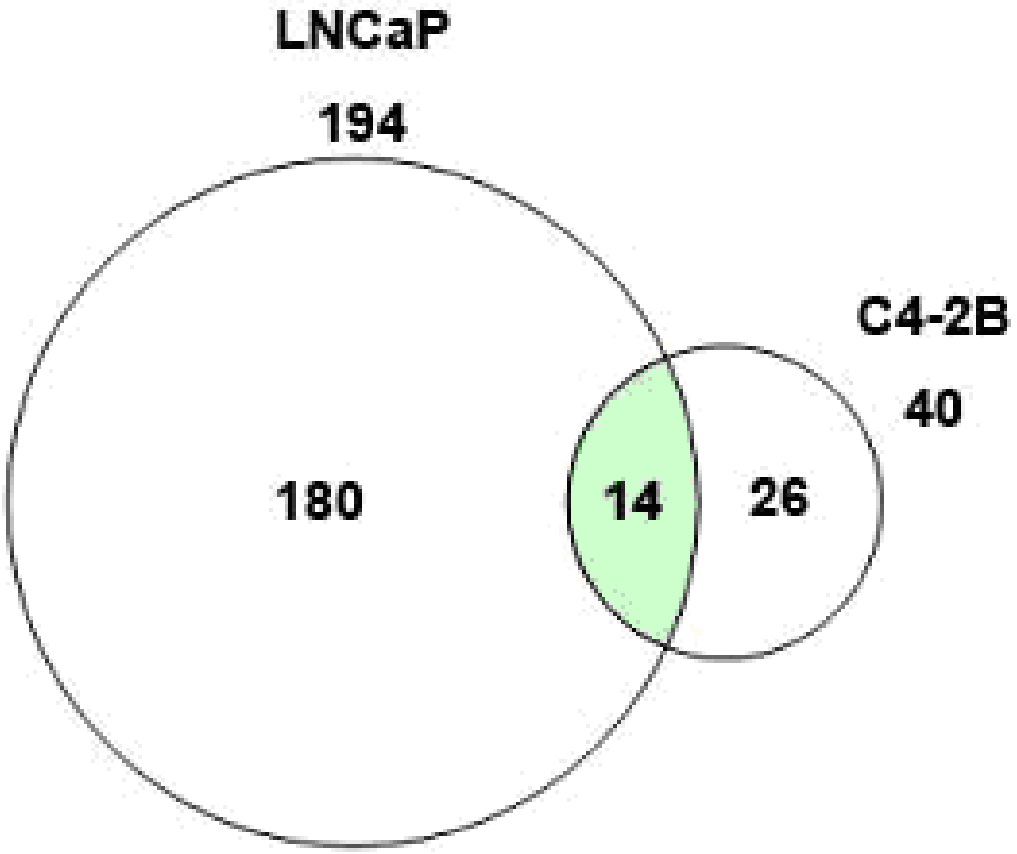

Figure 34. Difference in the number of regulated genes between LNCaP and C4-2B cells in response to curcumin treatment.

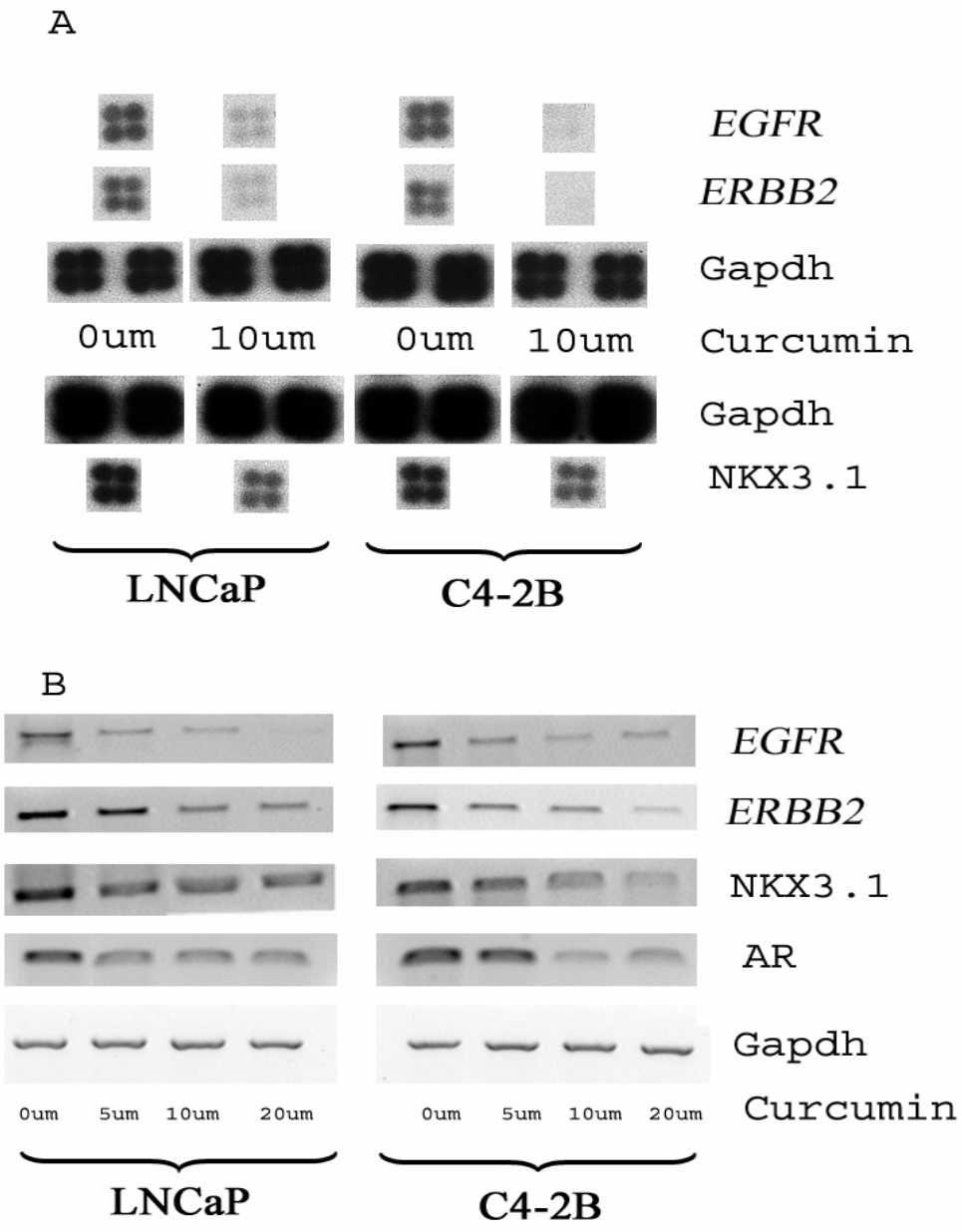

Figure 35. Effect of curcumin on gene expression in LNCaP and C4-2B cells. (A) Representative picture of cDNA Microarray of NKX3.1, EGFR, ERBB2 in LNCaP and C4-2B cells treated with 10μm of curcumin for 12 h. (B) Representative picture of RT-PCR of NKX3.1, EGFR, ERBB2 and AR in LNCaP and C42B cells treated with 5, 10, 20 μm of curcumin for 12 h.

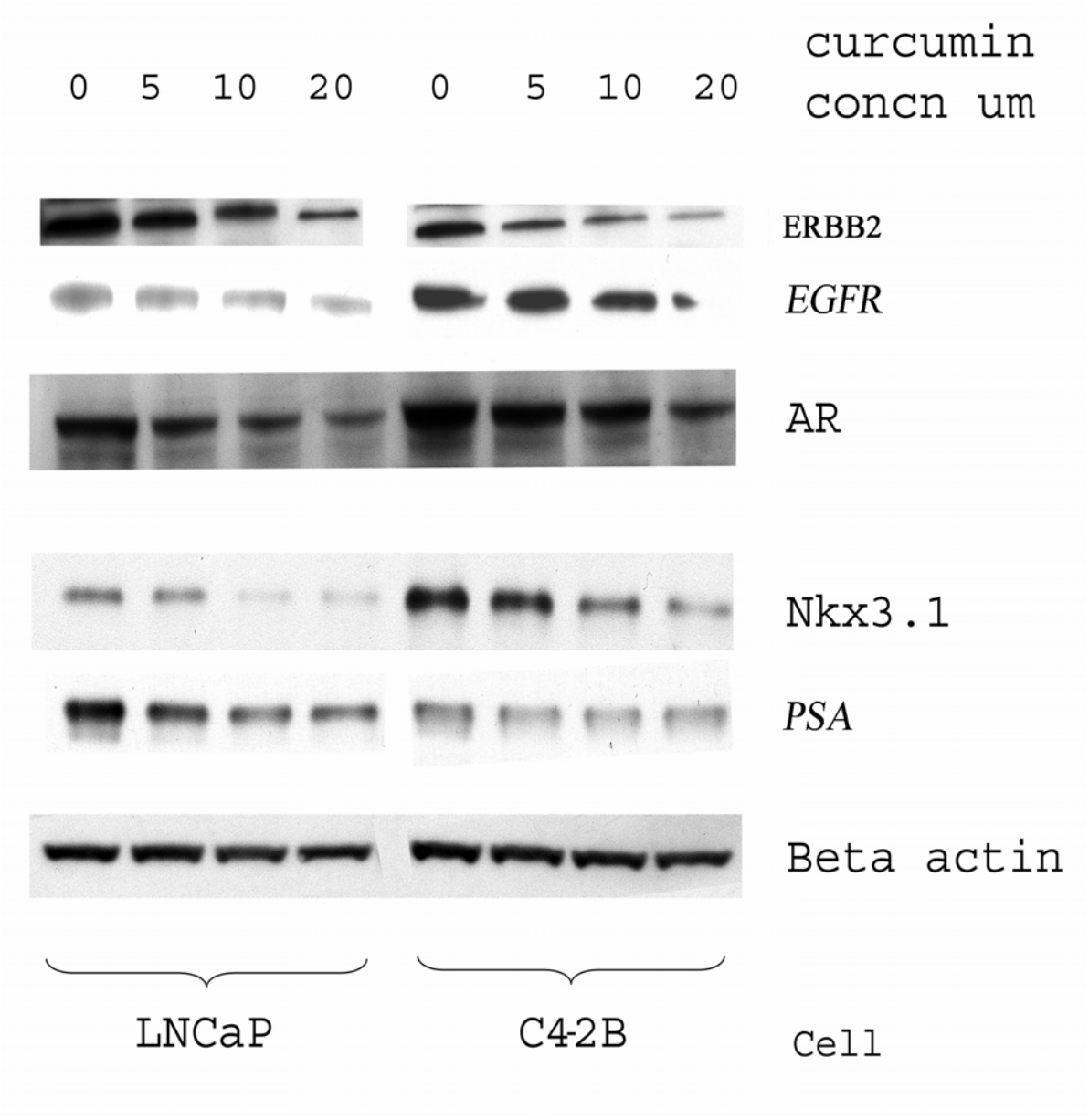

Figure 36. Effect of curcumin on protein expression in LNCaP and C4-2B cells. Representative picture of western blot of NKX3.1, EGFR, AR and PSA in LNCaP and C4-2B cells treated with 5, 10, 20 μm of curcumin for 12 h.

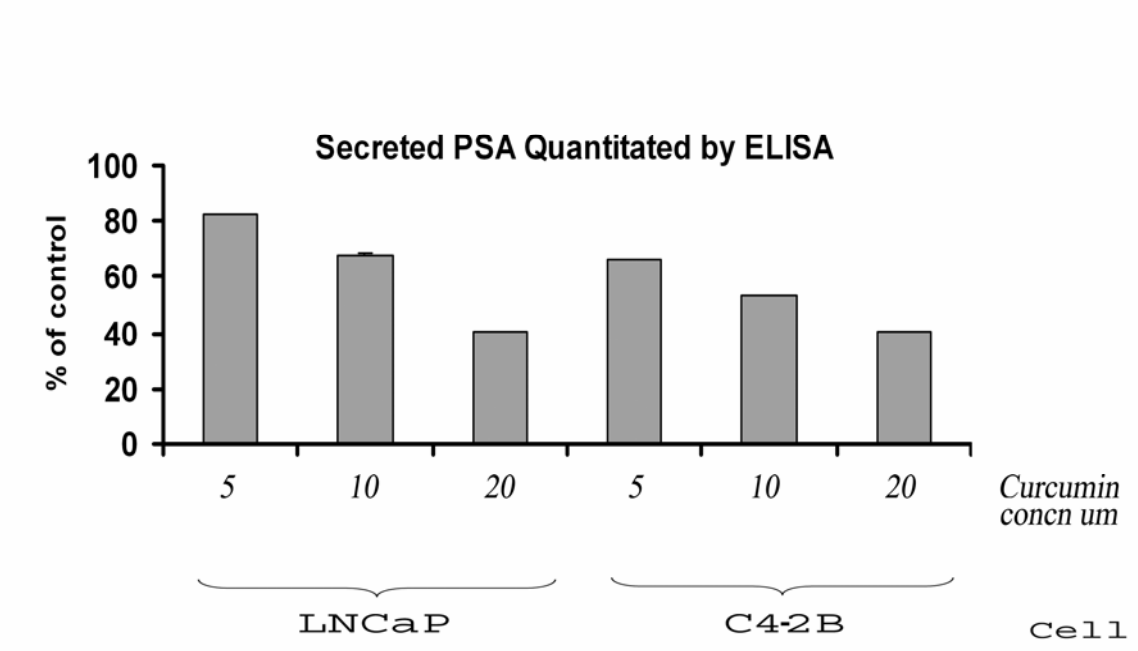

Figure 37. Effect of curcumin on secreted PSA levels in LNCaP and C4-2B cells. PSA Elisa showed that curcumin inhibits the secretion of PSA in LNCaP and C4-2B cells.

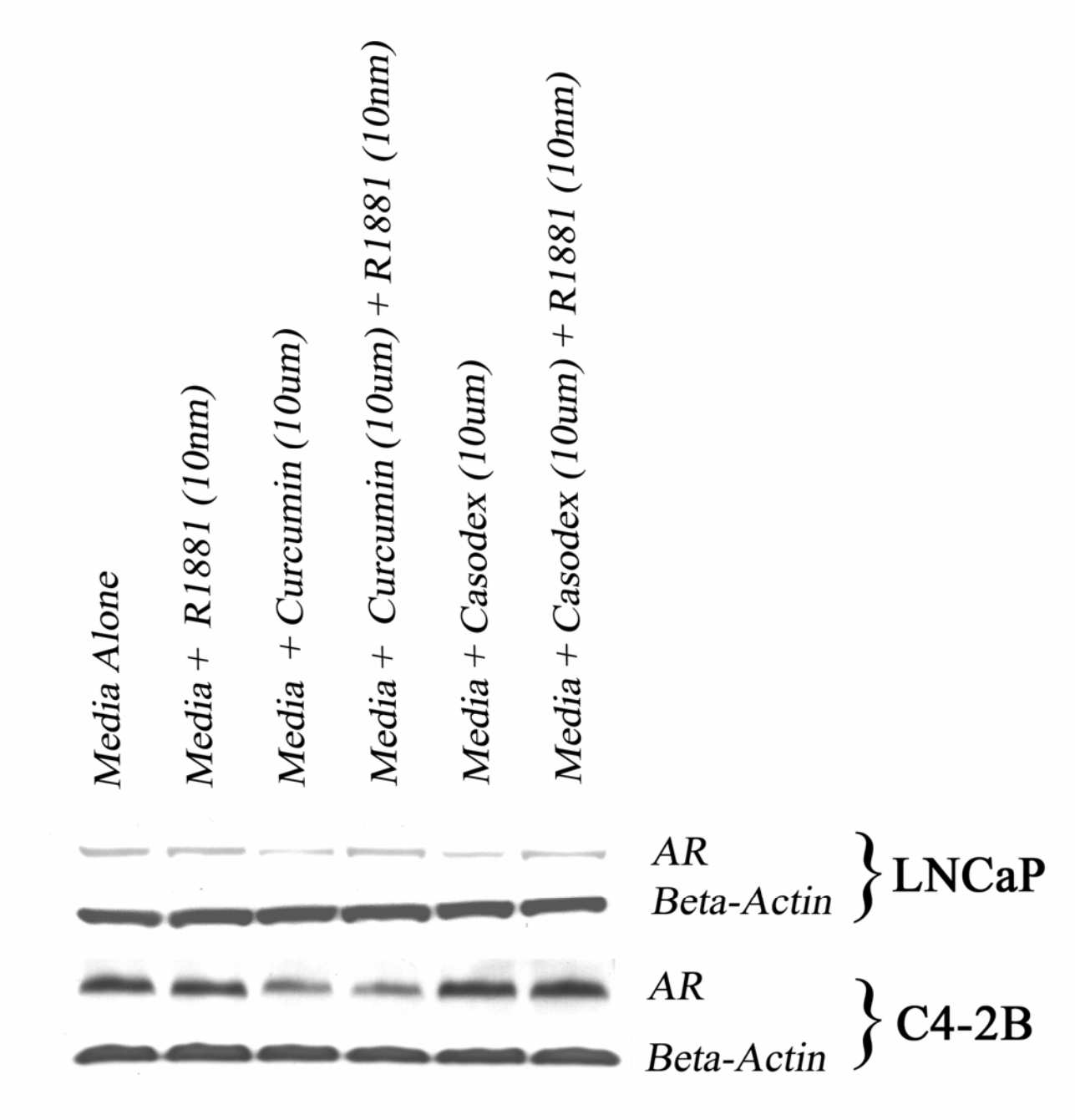

Figure 38. Effect of curcumin and bicalutamide on AR levels in the presence of R1881 in LNCaP and C4-2B cells.

Chapter 3

Part 2: Evaluation of a Nanotechnology based carrier for delivery of curcumin.

INTRODUCTION

In spite of its promising therapeutic index, the biological activity of curcumin is severely limited due to its poor bio-availability (Kostarelos et al 2005). Effective methods to deliver targeted therapy to solid tumors and/or to increase their bioavailability have been a major challenge in current biomedical research. Nanotechnology-based tools and techniques including liposomes are rapidly emerging in the fields of medical imaging and targeted drug delivery (Caruthers et al 2007). Among various drug delivery vehicles, liposomes have been explored for decades due to their biodegradability and the potential to load large concentrations of therapeutic agents. Liposomes have the capacity to alter the biodistribution of drugs they encapsulate through delayed clearance and longer intravascular circulation time (Allen 1997). Liposomes are spherical vesicles with a membrane composed of phospholipids and cholesterol bilayer. They are composed of naturally-derived phospholipids with mixed lipid chains like egg phophatidylethanolamine or surfactant. Liposomes are being widely used for the delivery of various pharmaceutical agents. The currently undergoing trials for various liposome formulations and the large number of commercially available therapeutics show its success rate as a widely used mode of delivery (Goyal et al 2005). Since curcumin is hydrophobic, it is considered to be a good candidate for liposome incorporation as it can

be encapsulated in the lipid layer of the liposome (Kunwar et al 2006). Recent studies have shown that the incorporation of curcumin into liposomes has increased the bio-availability of curcumin significantly (Li et al 2005, Kunwar et al 2006). Research has been pursued in the direction of encapsulating curcumin in liposomes. In a study conducted on human pancreatic carcinoma cells, the activity of liposomal curcumin was equal to or better than that of free curcumin at equimolar concentrations. Liposomal curcumin down-regulated NF-kB machinery, suppressed growth, and induced apoptosis of human pancreatic cells *in vitro*. Antitumor and anti-angiogenic effects were also observed *in vivo* (Li et al 2005). In another study, both liposomal and human serum albumin (HSA) vehicles were examined for the transfer of curcumin to spleen lymphocyte cells of the EL4 cell line. From these studies it was found that the liposomal vehicle was capable of loading more curcumin into cells than the HSA or the aqueous-DMSO vehicles (Kunwar et al 2006).

The aim of this study was to evaluate curcumin partitioning potential into the liposomes composed of phospholipids with a wide range of melting transition temperatures (Tm) and optimize conditions for encapsulating curcumin and examine the efficacy of curcumin-loaded liposome formulations on prostate cancer cells. We have chosen LNCaP and its isogenic more resistant derivative C4-2B as the *in vitro* model system The anti-proliferative effects of free curcumin and liposomal curcumin were studied using a tetrazolium dye-based (MTT) assay. Our studies show that curcumin selectively partitions with high efficiency into DMPC liposomes as compared to DPPC or egg PC. The high therapeutic index of liposomal curcumin when compared with free curcumin shows promise for future experiments on targeted delivery. The long term goal is to

couple new chemoprevention agents and therapeutics with advanced delivery strategies and design liposome nanovehicles with cell specific targeting moieties.

MATERIALS AND METHODS

Materials

Curcumin was obtained from LKT laboratories (St Paul, Minnesota, MN). Dimyristoyl Phosphatidylcholine (DMPC), Dipalmitoyl Phosphatidylcholine (DPPC), Egg Phosphatidylcholine (EGG PC) cholesterol (Figure 39) and cholesterol were obtained from Avanti polar lipids inc., **(**Alabaster, AL). Other reagents were for Sigma-Aldrich Co. (St. Louis, MO).

Liposome Preparation and Curcumin Encapsulation

Liposome (small unilamellar vesicles (SUV)) was prepared by probe sonication. The lipids and curcumin were mixed at a phospholipids:cholesterol:curcumin at a ratio of 90:10:10, wt:wt) in chloroform in a round bottom flask and the lipid/curcumin film was then formed by removing the solvent using a rotary evaporator. Any residual chloroform was removed by placing the films overnight in a vacuum desiccator. Multilamellar vesicles were formed by reconstituting the lipid film with HBSE buffer (10mM HEPES, 150mM NaCl, 9.1mM EDTA, pH 7.5) with vigorous vortexing. Unilamellar vesicles were then formed using a probe sonicator W-375 (Heat Systems-Ultrasonics, New York, USA) for 15 to 20 min on ice (2 min pulse with 30 sec interval between each pulse). After sonication, the liposomes were centrifuged at 2000 x g to pellet any curcumin not intercalated into the liposomes. The liposomes were then passed through a size exclusion

gel chromatography column (Bio-Rad Biogel-A5M, BioRad, Hercules, CA), equilibrated with HBSE buffer, pH 7.4, in order to separate any residual curcumin loosely associated with the liposome s. Fractions containing liposomes were pooled and filtered through .22μm filter, stored at 4^0C and used with in 48 hr for quantitation, sizing and further analysis.

Quantification of curcumin

Liposomes containing curcumin were prepared as described above. Liposomal curcumin was quantified using a simple colorimetric assay measured at 450 nm. A standard curve was formulated from known concentrations of curcumin HBSE-TX100 (10mM HEPES, 140mM NaCl, 4mM EDTA, 1% TX-100) and was used to determine curcumin concentration in liposomes subsequent to lysis with 1% TX-100. Measurement of liposome size and charge was conducted by dynamic light scattering techniques by the National Characterization Laboratory (Frederick, MD, USA) using a Zeta nanosizer (Malvern, UK).

Cell Proliferation Assay

LNCaP and C4-2B cell proliferation in the presence of various concentrations of liposomal and free curcumin was determined using the Cell Proliferation Kit as described previously in page 17.

RESULTS

Curcumin encaspulation efficiency

We prepared curcumin-loaded liposomes of various lipid compositions by sonication to an average size of 100-150 nm (Figure 40). Our initial efforts to partition curucmin into EGG PC liposomes were not successful. It was found that curcumin partitioned favorably into DMPC based liposomes. Encapsulation efficiency refers to the relative amounts of curcumin that intercalated into the liposomes. It was found that DMPC based liposomes allowed the greatest amount of curcumin to be intercalated into the lipid membrane, whereas Egg PC based liposomes had the lowest amount of curcumin intercalation (Table 7). All the formulations were made at a 1:10 curcumin:lipid ratio (w/w basis). We conclude that curucmin partition into liposome is dependent on the type of lipid used

Curcumin Quantification and Characterization

Although fluorescne based quantitation methods are available for determining curcumin concentration, we have quantitated curcumin in the presence of a detergent based on absorbance at 450 nm. A standard curve for curcumin concentration was formed via a colorimetric assay. Known concentrations of free curcumin in HBSE-TX100 (10mM HEPES, 140mM NaCl, 4mM EDTA, 1% TX-100) were read at 450nm. Varying amounts of curcumin liposomes were read in HBSE-TX100 buffer, and the resulting fluorescence values were used to determine curcumin concentration. For example, a 20ul curcumin liposome sample may elicit a fluorescence value of 850 (Figure 41). Using the standard curve, we see that this correlates to 2.5ug of curcumin in the 20ul sample, giving

us an estimation of 0.125mg/ml curcumin in the liposomes. The resulting liposomes were found to have an average particle diameter of 100nm as measured by dynamic light scattering.

Cytotoxicity Induced by Curucmin on LNCaP and C4-2B cells

DMPC liposomal curcumin (5-10 μM) for 24-48 hr at 37^{o}C resulted in at least 70-80% inhibition of cellular proliferation. On the other hand, free curcumin exhibited similar inhibition only at 10-fold higher doses (> 50 μM). It was also observed that LNCaP cells were relatively more sensitive to liposomal curcumin mediated block of cellular proliferation than C4-2B cells. 31 % and 70 % of LNCaP cells survived 10μM of liposomal and free curcumin treatment, whereas C4-2B cells were more resistant to liposomal treatment, surviving around 36% and 75% with 10μM of liposomal and free curcumin treatment, respectively (Figure 42).

DPPC and DMPC liposomal curcumin had improves efficacy than free curcumin in inhibiting the proliferation of prostate cancer cells (Figure 43). However among the liposome tested DMPC liposomal curcumin were found to be the most effective. Control liposomes did show some toxic effects (10-15%) at higher doses in our experiments which was consistent with earlier studies.

DISCUSSION

Chemoprevention is a promising preventive measure because of its overall availability and affordability. It is conceivable that in the future patients might only need to take specifically formulated pills that prevent cancer or delay its onset. Although

prostate cancer mortality has recently begun to decline in the US (2003), the cumulative costs of various treatments for early stage disease, treatment-related morbidity and treatment of biochemical failures remains substantial (Klein and Thompson 2004). Chemoprevention by natural products such as edible phytochemicals is a suitable alternative as an inexpensive, readily applicable, acceptable and accessible approach to cancer control and management (Klein and Thompson 2004). Hence, developing phytochemical which has shown tremendous promise *in vitro* and preclinical animal testing in a pharmaceutically acceptable dosage form becomes pivotal.

Although cancer chemoprevention studies and preclinical trials have shown curcumin as a favorable cancer chemopreventive agent in colon cancer and other gastro intestinal disorder, its efficacy in other organs is debatable due to its poor bioavailability. No curcumin was detected in the serum of human subjects administered up to 8 gms/day of curcumin (Lao et al 2006). Recent finding implicates rapid intestinal sulfation, glucuronidation, and reduction, especially in humans to be a plausible explanation for the poor systemic availability of curcumin (Ireson et al 2002). This situation is probably analogous to the low bioavailability of drugs, such as the oral contraceptive ethinylestradiol, which is thought to be caused by extensive sulfate conjugation. Another study from the same group clearly indicates GIT as a more approvable target for curcumin as it is significantly exposed to unmetabolized curcumin and the metabolized byproducts of curcumin have reduced ability to inhibit COX-2 expression (Ireson et al 2001). Level of curcumin and it metabolites in portal and peripheral blood, bile and liver tissue as measured by HPLC was found to be so low that it was unlikely to exert pharmacological activity in those human tissues (Garcea et al 2004). It becomes critical

for the drug to reach the infection site and attain a pharmacologically desired concentration to be considered a potential drug candidate.

Hence it becomes pivotal to develop a suitable delivery agent and ways to increase the bioavailability of curcumin to take the advantage of curcumin's chemopreventive potential to various other affected organs. Research is being pursued to increase the bioavailability of curcumin by various other means like formulating curcumin with phosphatidylcholine and soy-phospholipids (Marzylo et al 2006, Liu et al 2005).

Previous studies have reported successful use of various chemotherapeutic agents delivered in a liposome formulation (Vaage et al 1994, Hwang et al 2007, Webb et al 2006). In this study, we have considered the use of liposomes as a delivery model for curcumin.The results from the cell proliferation assays provide strong evidence for liposomes as effective nano-delivery vehicles that increase the bio-availability of curcumin. Currently, strategies to conjugate liposome formulations with prostate membrane specific antigen (PMSA) are being developed such that they can specifically target the prostate cancer cells to further improve therapeutic index efficacy of curcumin *in vivo.*

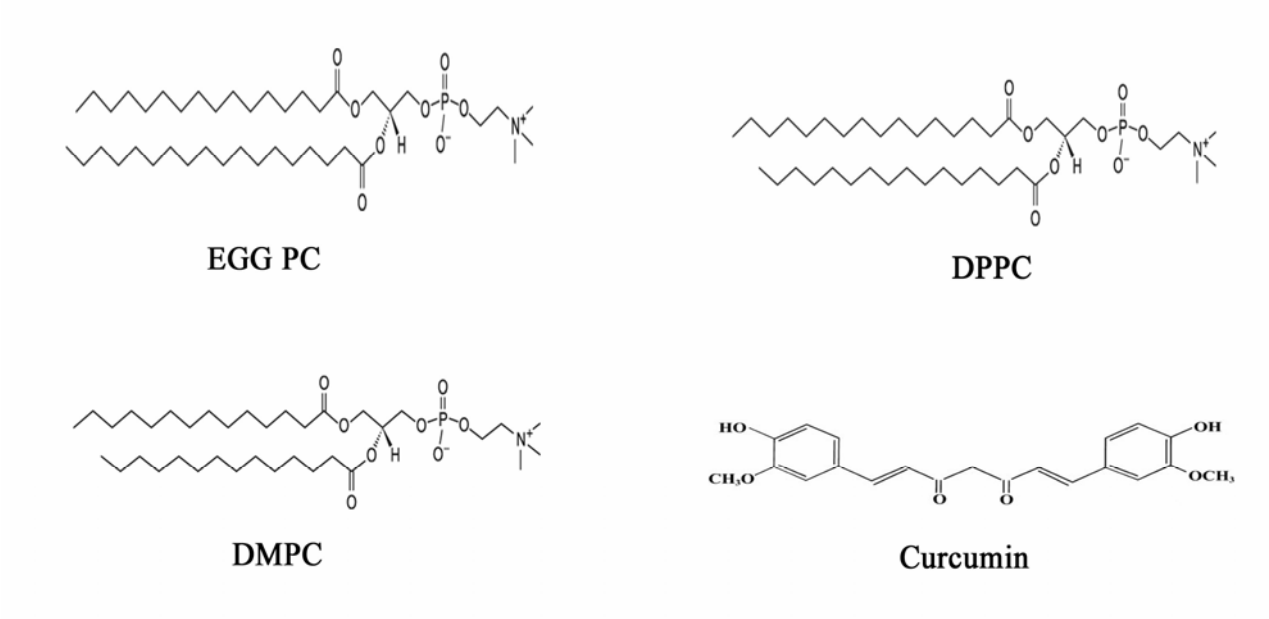

Figure 39. Lipid and curcumin structures. Egg Phosphatidylcholine (EGG PC) cholesterol, Dipalmitoyl Phosphatidylcholine (DPPC) and Dimyristoyl Phosphatidylcholine (DMPC).

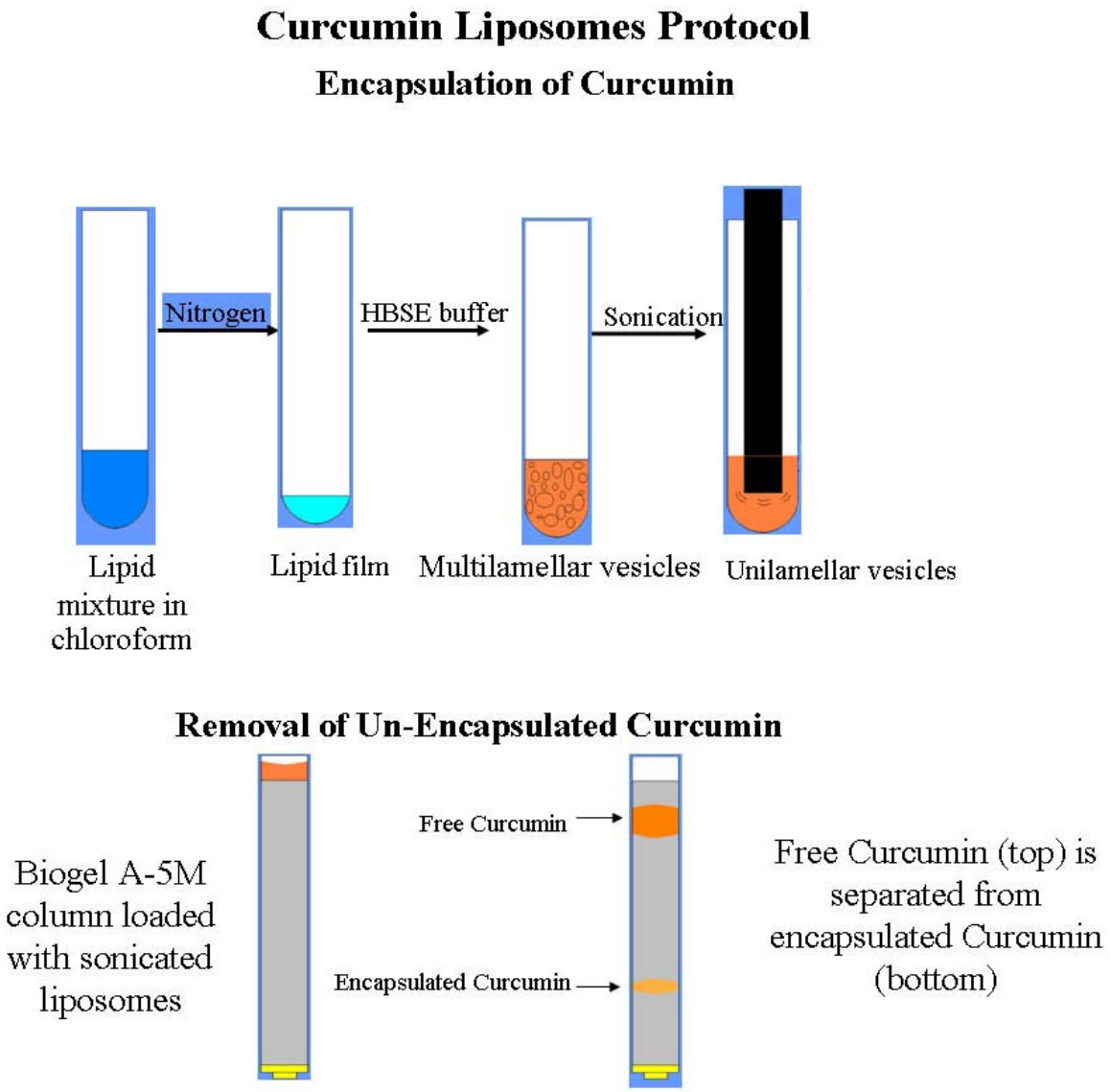

Figure 40. Cartoon depiciting the encapsulation of curcumin into liposomes. Liposomes are nanodelivery vehicles primarily composed of phospholipids. Free curcumin was separated from intercalated curcumin via a Biogel A-5m size exclusion column.

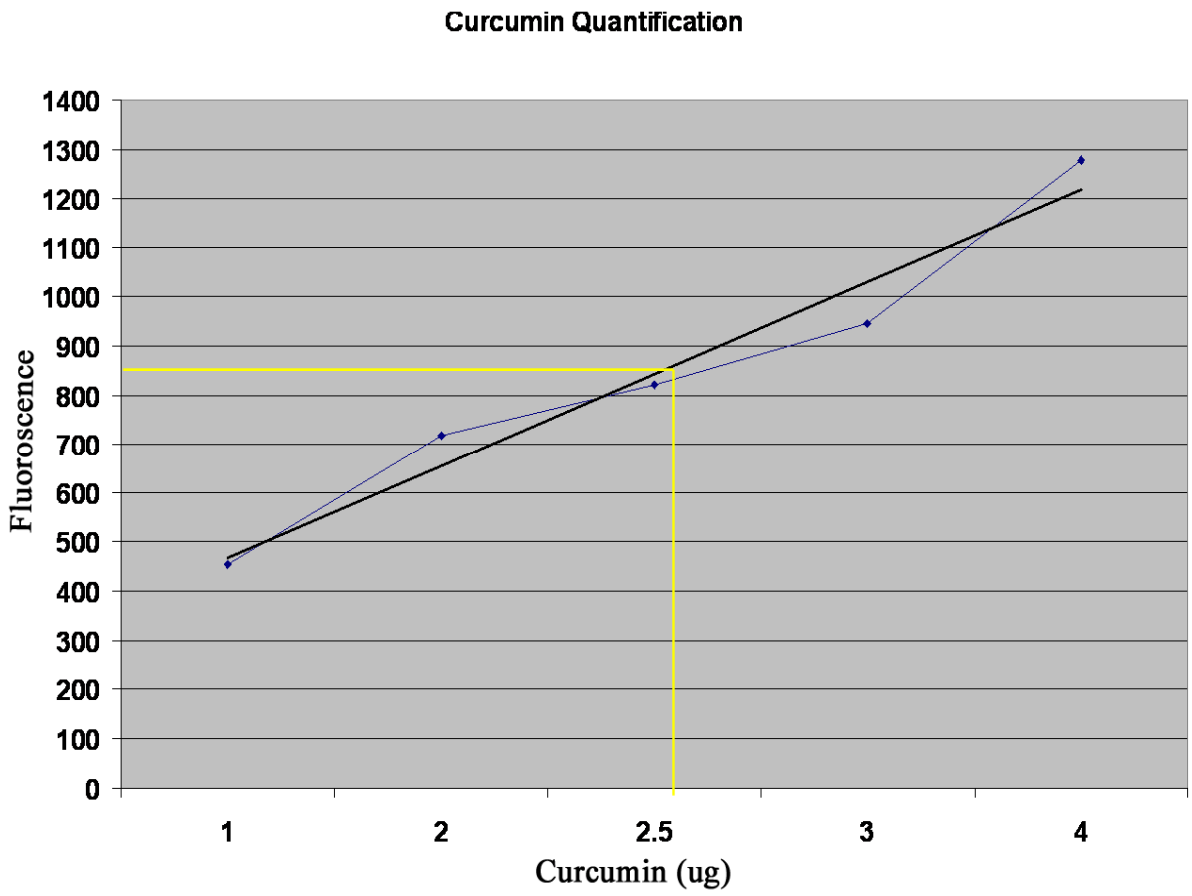

Figure 41. standard curve for curcumin concentration was formed via a colorimetric assay. Known concentrations of free curcumin in HBSE-TX100 (10mM HEPES, 140mM NaCl, 4mM EDTA, 1% TX-100) were read at 450nm.

Table 7. Encapsulation efficiency of different liposome formulations. Encapsulation efficiency refers to the relative amounts of curcumin that intercalated into the liposomes. It was found that DMPC based liposomes allowed the greatest amount of curcumin to be intercalated into the lipid membrane, whereas Egg PC based liposomes had the lowest amount of curcumin intercalation. All formulations were made at a 1:10 curcumin:lipid ratio.

Formulations	**Encapsulation Efficiency**
EGG PC based	Poor
DPPC based	Average
DMPC based	Good

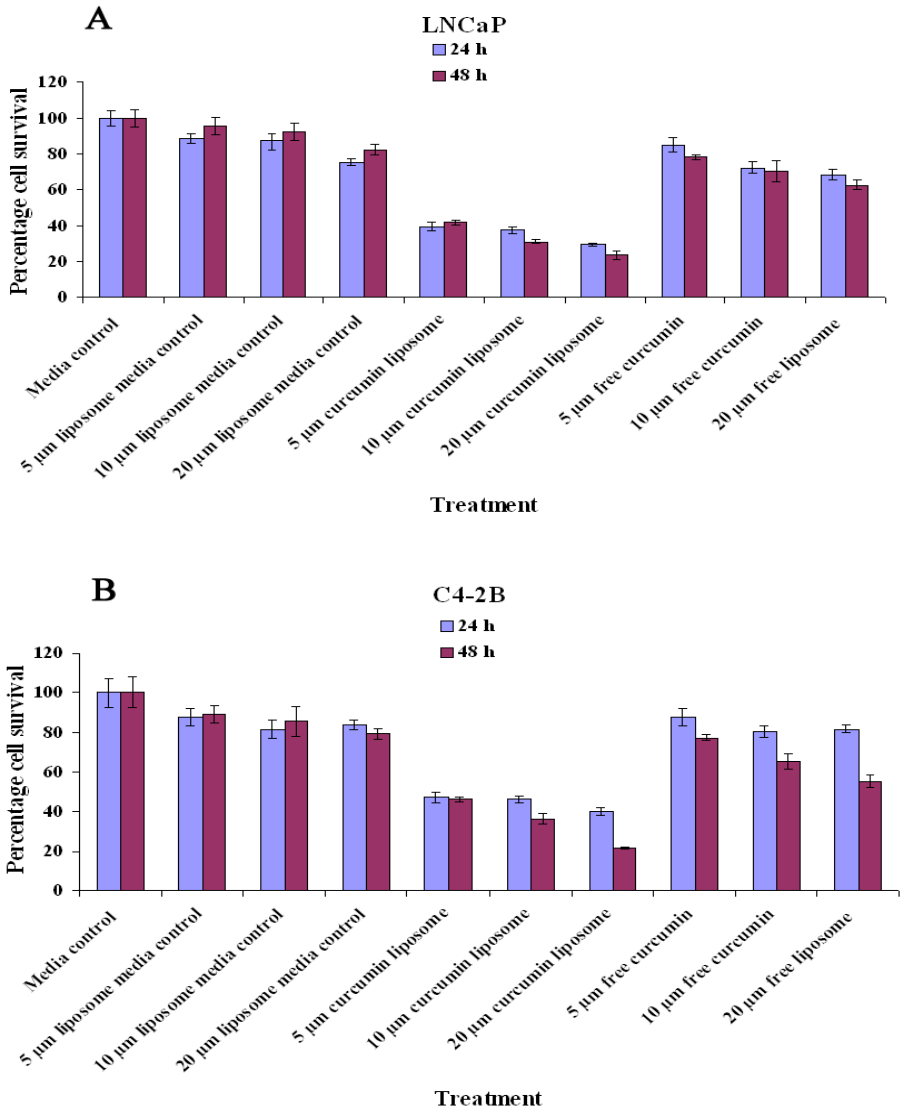

Figure 42. DMPC Liposomal curcumin inhibits the proliferation of LNCaP and C4-2B incubated for 24 and 48 hrs at 37°C. **(A)** Effect of different concentrations of liposomal curcumin on the proliferation of LNCaP. **(B)** Effect of different concentrations of liposomal curcumin on the proliferation of C4-2B. The results were compared with equivalent amount of free curcumin

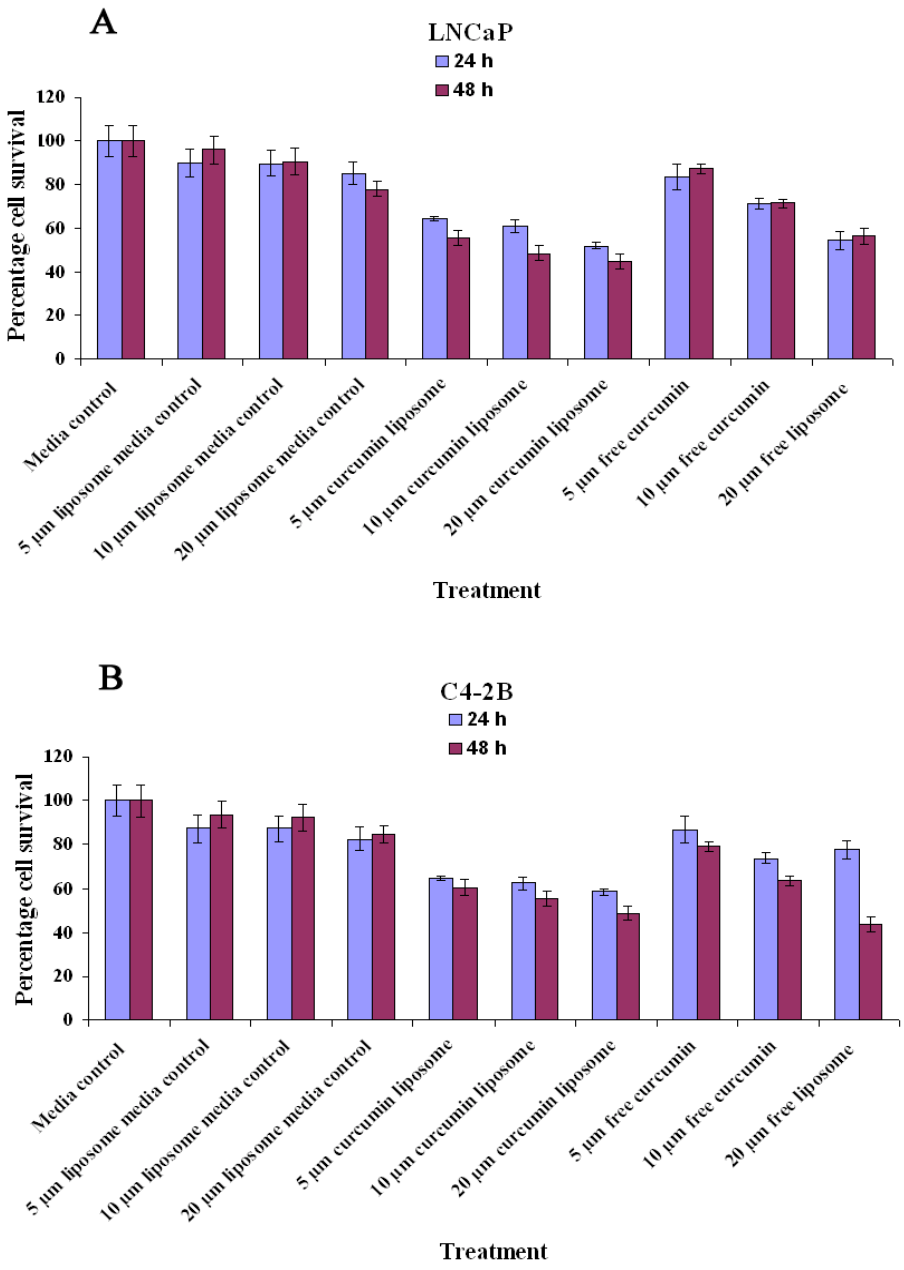

Figure 43. DPPC Liposomal curcumin inhibits the proliferation of LNCaP and C4-2B incubated for 24 and 48 hrs at 37°C. **(A)** Effect of different concentrations of liposomal curcumin on the proliferation of LNCaP. **(B)** Effect of different concentrations of liposomal curcumin on the proliferation of C4-2B. The results were compared with equivalent amount of free curcumin.

SUMMARY AND CONCLUSIONS

Plant products are considered the cheapest and safest medicine as they have been used for many centuries. The studies discussed in the chapters have shown the protective efficacy of plant products in a cancer chemoprevention set up. In summary, we have demonstrated the anti-proliferative effect of GTP and EGCG on the growth of human breast cancer MDA-MB-231. Both GTP and EGCG treatments had the ability to arrest the cell cycle at G1 phase as assessed by flow cytometry. The expression of Cyclin D, Cyclin E, CDK 4, CDK 1 and PCNA were down-regulated over time in GTP and EGCG treated experimental groups, compared to the untreated control group. Nude mice inoculated with human breast cancer MDA-MB-231 cells and treated with GTP and EGCG were effective in delaying the tumor incidence as well as reducing the tumor burden when compared to the water fed and similarly handled control. Moreover, in the in *vitro* human breast cancer model, EGCG and GTP induced apoptosis and significantly decreased invasion of breast cancer cells. Results revealed inhibition of MMP-9 expression at the transcriptional level. Moreover EGCG and GTP decreased AKT phosphorylation and AKT expression through the beta-catenin pathway. All these data suggest that GTP and EGCG treatment can induce apoptosis and inhibit the proliferation and invasion of MDA-MB-231 cells in-vitro and in-vivo. All together, these data sustain our contention that GTP and EGCG have anti-tumor properties (Figure 44).

Brahma rasayana can significantly reduce tumor incidence and tumor growth in the Copenhagen rat and MAT-LyLu cell model. Also the proliferation and apoptotic markers analyzed support these observations. The poly herbal preparation, Brahma rasayana may play a beneficial role in preventing tumor incidence, tumor growth and metastatic spread.

These are inexpensive preparations that have little or no adverse side effects with a potential as lead chemopreventive compounds and which might prove useful for the treatment of disorders such as human prostate cancer. These findings also suggest the possible mechanism(s) of action of BR in the reduction of tumor growth and metastatic spread (Figure 45). Additional investigation is needed to explore individual components as well as the specific mechanisms.

Turmeric (*Curcuma longa* Linn), is a crystalline compound which has been traditionally used in medicine and cuisine in India. Hierarchical clustering methods and functional classification of the Cu-GER showed temporal co-regulation of genes involved in specific biochemical pathways implicated in the oxidative stress response. There was a maximum of 25 fold increase in the expression of heme oxygenase-1 (HO-1), a stress response protein by curcumin. Interestingly, the magnitude of genes modulated by curcumin was significantly different between aggressive C42B tumor cell line and its isogenic derivative of less aggressive LNCaP cells, suggesting that C4-2B may be more resistant to the effects of curcumin than LNCaP cells. Alternate pathway which was also identified as a target for curumin is the AR pathway (Figure 46). Moreover to increase the bioavailability we evaluated several liposome formulations and optimized conditions for encapsulating curcumin and compare the efficacy of liposome formulation with free curcumin on prostate cancer cells.

One of the recent concerns is the mixed composition of plant products and the unidentified compounds present in the mixture. Recent NCI guidelines state that a single active compound must be isolated and the active compound must be co-developed with

the extract. If the effect and molecular targets of both the extract and the active compound are similar, the extract may be preferentially developed to be cost effective. Therefore evaluating and documenting chemical composition along with the pharmacological action of the extract becomes critical to avoid misinterpretation of the data. Hence putting forth biomarkers to determine the safety and efficacy of these natural products becomes pivotal. Even though these natural products are considered relatively safe and bypass the normal stringent FDA regulation that a drug would encounter, these dietary supplements should be evaluated both for chemical composition and potential side effects. Although preclinical studies in animal and cell cultures have been successful in establishing the chemo preventive potential of phytochemicals like curcumin and green tea, solid evidences compiled from epidemiological studies and results of clinical trials becomes pivotal to propagate the development of phytochemicals as cancer chemopreventive agents. Even though the evaluation of natural products is becoming popular and the results are viewed with great enthusiasm, there is a skepticism that is expressed towards their development as pharmacological agents. One should not forget that some of the popular agents like digoxin from foxglove, taxol from pacific yew, to name a few have all been huge success from folk medicine. Proper research and stringent conditions in the evaluation of plant products would help in developing a cost effective way of treating various diseases and increase the seriousness in plant products.

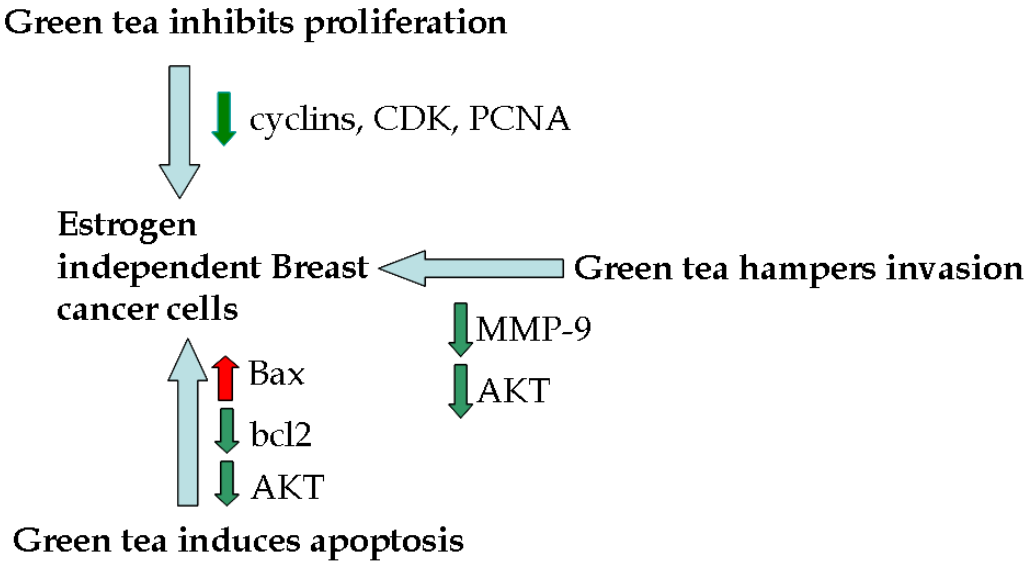

Figure 44. Molecular targets for chemoprevention in breast cancer by green tea.

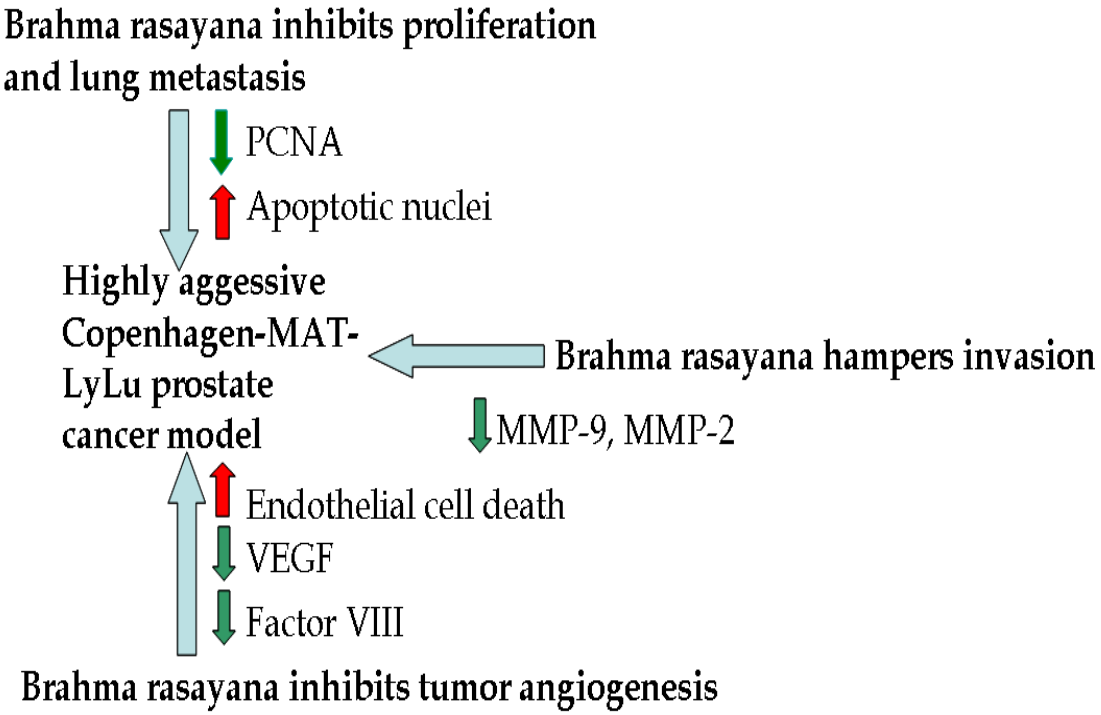

Figure 45. Molecular targets for chemoprevention in prostate cancer by Brahma rasayana.

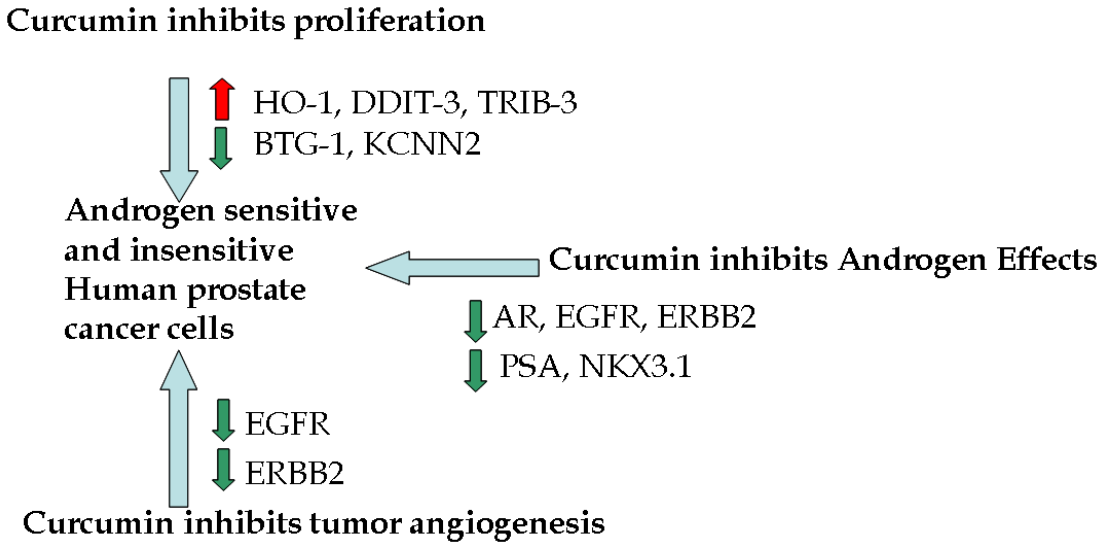

Figure 46. Molecular targets for chemoprevention in prostate cancer by curcumin.

REFERENCES

Aalinkeel, R., Nair, M. P., Sufrin, G., Mahajan, S. D., Chadha, K. C., Chawda, R. P., and Schwartz, S. A. (2004). Gene expression of angiogenic factors correlates with metastatic potential of prostate cancer cells. Cancer Res *64*, 5311-5321.

Adhami, V. M., Siddiqui, I. A., Ahmad, N., Gupta, S., and Mukhtar, H. (2004). Oral consumption of green tea polyphenols inhibits insulin-like growth factor-I-induced signaling in an autochthonous mouse model of prostate cancer. Cancer Res *64*, 8715-8722.

Adhami, V. M., and Mukhtar, H. (2006). Polyphenols from green tea and pomegranate for prevention of prostate cancer. Free Radic Res *40*, 1095-1104.

Aggarwal, B. B., Kumar, A., and Bharti, A. C. (2003). Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res *23*, 363-398.

Aggarwal, B. B., and Shishodia, S. (2004). Suppression of the nuclear factor-kappaB activation pathway by spice-derived phytochemicals: reasoning for seasoning. Ann N Y Acad Sci *1030*, 434-441.

Aggarwal, B. B., Shishodia, S., Takada, Y., Banerjee, S., Newman, R. A., Bueso-Ramos, C. E., and Price, J. E. (2005). Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB pathway in breast cancer cells and inhibits lung metastasis of human breast cancer in nude mice. Clin Cancer Res *11*, 7490-7498.

Allen, T. M. (1997). Liposomes. Opportunities in drug delivery. Drugs *54 Suppl 4*, 8-14.

Ammon, H. P., and Wahl, M. A. (1991). Pharmacology of Curcuma longa. Planta Med *57*, 1-7.

Awad, A. B., Williams, H., and Fink, C. S. (2001). Phytosterols reduce *in vitro* metastatic ability of MDA-MB-231 human breast cancer cells. Nutr Cancer *40*, 157-164.

Azuine, M. A., and Bhide, S. V. (1992). Chemopreventive effect of turmeric against stomach and skin tumors induced by chemical carcinogens in Swiss mice. Nutr Cancer *17*, 77-83.

Babu, T. D., Kuttan, G., and Padikkala, J. (1995). Cytotoxic and anti-tumour properties of certain taxa of Umbelliferae with special reference to Centella asiatica (L.) Urban. J Ethnopharmacol *48*, 53-57.

Baliga, M. S., Meleth, S., and Katiyar, S. K. (2005). Growth inhibitory and antimetastatic effect of green tea polyphenols on metastasis-specific mouse mammary carcinoma 4T1 cells *in vitro* and *in vivo* systems. Clin Cancer Res *11*, 1918-1927.

Balk, S. P. (2002). Androgen receptor as a target in androgen-independent prostate cancer. Urology *60*, 132-138; discussion 138-139.

Balkwill, F., Charles, K. A., and Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell *7*, 211-217.

Balogun, E., Hoque, M., Gong, P., Killeen, E., Green, C. J., Foresti, R., Alam, J., and Motterlini, R. (2003). Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. Biochem J *371*, 887-895.

Barnes, S. (2001). Role of phytochemicals in prevention and treatment of prostate cancer. Epidemiol Rev *23*, 102-105.

Beltz, L. A., Bayer, D. K., Moss, A. L., and Simet, I. M. (2006). Mechanisms of cancer prevention by green and black tea polyphenols. Anticancer Agents Med Chem *6*, 389-406.

Berger, R., Lin, D. I., Nieto, M., Sicinska, E., Garraway, L. A., Adams, H., Signoretti, S., Hahn, W. C., and Loda, M. (2006). Androgen-dependent regulation of Her-2/neu in prostate cancer cells. Cancer Res *66*, 5723-5728.

Bhattacharya, A., Chatterjee, A., Ghosal, S., and Bhattacharya, S. K. (1999). Antioxidant activity of active tannoid principles of Emblica officinalis (amla). Indian J Exp Biol *37*, 676-680.

Bigelow, R. L., and Cardelli, J. A. (2006). The green tea catechins, (-)-Epigallocatechin-3-gallate (EGCG) and (-)-Epicatechin-3-gallate (ECG), inhibit HGF/Met signaling in immortalized and tumorigenic breast epithelial cells. Oncogene *25*, 1922-1930.

Borek, C. (2004). Dietary antioxidants and human cancer. Integr Cancer Ther *3*, 333-341.

Borre, M., Offersen, B. V., Nerstrom, B., and Overgaard, J. (1998). Microvessel density predicts survival in prostate cancer patients subjected to watchful waiting. Br J Cancer *78*, 940-944.

Bray, F., McCarron, P., and Parkin, D. M. (2004). The changing global patterns of female breast cancer incidence and mortality. Breast Cancer Res *6*, 229-239.

Brennan, M. J. (1975). Endocrinology in cancer of the breast. Status and prospects. Am J Clin Pathol *64*, 797-809.

Caporali, A., Davalli, P., Astancolle, S., D'Arca, D., Brausi, M., Bettuzzi, S., and Corti, A. (2004). The chemopreventive action of catechins in the TRAMP mouse model of prostate carcinogenesis is accompanied by clusterin over-expression. Carcinogenesis *25*, 2217-2224.

Carracedo, A., Lorente, M., Egia, A., Blazquez, C., Garcia, S., Giroux, V., Malicet, C., Villuendas, R., Gironella, M., Gonzalez-Feria, L., *et al.* (2006). The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells. Cancer Cell *9*, 301-312.

Caruthers, S. D., Wickline, S. A., and Lanza, G. M. (2007). Nanotechnological applications in medicine. Curr Opin Biotechnol *18*, 26-30.

Cassileth, B. R. (1999). Complementary therapies: overview and state of the art. Cancer Nurs *22*, 85-90.

Charlesworth, M. C., Young, C. Y., Miller, V. M., and Tindall, D. J. (1999). Kininogenase activity of prostate-derived human glandular kallikrein (hK2) purified from seminal fluid. J Androl *20*, 220-229.

Chaudhary, R., Bromley, M., Clarke, N. W., Betts, C. D., Barnard, R. J., Ryder, W. D., and Kumar, S. (1999). Prognostic relevance of micro-vessel density in cancer of the urinary bladder. Anticancer Res *19*, 3479-3484.

Chen, A., Xu, J., and Johnson, A. C. (2006). Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1. Oncogene *25*, 278-287.

Cheng, A. L., Hsu, C. H., Lin, J. K., Hsu, M. M., Ho, Y. F., Shen, T. S., Ko, J. Y., Lin, J. T., Lin, B. R., Ming-Shiang, W., *et al.* (2001). Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res *21*, 2895-2900.

Chopra, A., and Doiphode, V. V. (2002). Ayurvedic medicine. Core concept, therapeutic principles, and current relevance. Med Clin North Am *86*, 75-89, vii.

Choudhuri, T., Pal, S., Das, T., and Sa, G. (2005). Curcumin selectively induces apoptosis in deregulated cyclin D1-expressed cells at G2 phase of cell cycle in a p53-dependent manner. J Biol Chem *280*, 20059-20068.

Cowin, P., Rowlands, T. M., and Hatsell, S. J. (2005). Cadherins and catenins in breast cancer. Curr Opin Cell Biol *17*, 499-508.

Craft, N., Shostak, Y., Carey, M., and Sawyers, C. L. (1999). A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med *5*, 280-285.

Cross, M. J., and Claesson-Welsh, L. (2001). FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends Pharmacol Sci *22*, 201-207.

Darzynkiewicz, Z., Traganos, F., Wu, J. M., and Chen, S. (2000). Chinese herbal mixture PC SPES in treatment of prostate cancer (review). Int J Oncol *17*, 729-736.

De Smet, P. A. (2002). Herbal remedies. N Engl J Med *347*, 2046-2056.

Dehm, S. M., and Tindall, D. J. (2006). Molecular regulation of androgen action in prostate cancer. J Cell Biochem *99*, 333-344.

Diamandis, E. P., Yousef, G. M., Luo, L. Y., Magklara, A., and Obiezu, C. V. (2000). The new human kallikrein gene family: implications in carcinogenesis. Trends Endocrinol Metab *11*, 54-60.

Dihlmann, S., Kloor, M., Fallsehr, C., and von Knebel Doeberitz, M. (2005). Regulation of AKT1 expression by beta-catenin/Tcf/Lef signaling in colorectal cancer cells. Carcinogenesis *26*, 1503-1512.

DiPaola, R. S., Zhang, H., Lambert, G. H., Meeker, R., Licitra, E., Rafi, M. M., Zhu, B. T., Spaulding, H., Goodin, S., Toledano, M. B., *et al.* (1998). Clinical and biologic activity of an estrogenic herbal combination (PC-SPES) in prostate cancer. N Engl J Med *339*, 785-791.

Dorai, T., Cao, Y. C., Dorai, B., Buttyan, R., and Katz, A. E. (2001). Therapeutic potential of curcumin in human prostate cancer. III. Curcumin inhibits proliferation, induces apoptosis, and inhibits angiogenesis of LNCaP prostate cancer cells *in vivo*. Prostate *47*, 293-303.

Eisenberg, D. M., Davis, R. B., Ettner, S. L., Appel, S., Wilkey, S., Van Rompay, M., and Kessler, R. C. (1998). Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. Jama *280*, 1569-1575.

Fassina, G., Vene, R., Morini, M., Minghelli, S., Benelli, R., Noonan, D. M., and Albini, A. (2004). Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate. Clin Cancer Res *10*, 4865-4873.

Festuccia, C., Gravina, G. L., Angelucci, A., Millimaggi, D., Muzi, P., Vicentini, C., and Bologna, M. (2005). Additive antitumor effects of the epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib (Iressa), and the nonsteroidal antiandrogen, bicalutamide (Casodex), in prostate cancer cells *in vitro*. Int J Cancer *115*, 630-640.

Folkman, J. (1976). Influence of geometry on growth of normal and malignant cells. Adv Pathobiol *4*, 12-28.

Gaddipati, J. P., Rajeshkumar, N. V., Thangapazham, R. L., Sharma, A., Warren, J., Mog, S. R., Singh, A. K., and Maheshwari, R. K. (2004). Protective effect of a polyherbal preparation, Brahma rasayana against tumor growth and lung metastasis in rat prostate model system. J Exp Ther Oncol *4*, 203-212.

Garcea, G., Jones, D. J., Singh, R., Dennison, A. R., Farmer, P. B., Sharma, R. A., Steward, W. P., Gescher, A. J., and Berry, D. P. (2004). Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration. Br J Cancer *90*, 1011-1015.

Garikapaty, V. P., Ashok, B. T., Chen, Y. G., Mittelman, A., Iatropoulos, M., and Tiwari, R. K. (2005). Anti-carcinogenic and anti-metastatic properties of indole-3-carbinol in prostate cancer. Oncol Rep *13*, 89-93.

Gelmann, E. P. (2002). Molecular biology of the androgen receptor. J Clin Oncol *20*, 3001-3015.

Gleave, M. E., Hsieh, J. T., Wu, H. C., von Eschenbach, A. C., and Chung, L. W. (1992). Serum prostate specific antigen levels in mice bearing human prostate LNCaP tumors are determined by tumor volume and endocrine and growth factors. Cancer Res *52*, 1598-1605.

Gohji, K., Fujimoto, N., Hara, I., Fujii, A., Gotoh, A., Okada, H., Arakawa, S., Kitazawa, S., Miyake, H., Kamidono, S., and Nakajima, M. (1998). Serum matrix metalloproteinase-2 and its density in men with prostate cancer as a new predictor of disease extension. Int J Cancer *79*, 96-101.

Goyal, P., Goyal, K., Vijaya Kumar, S. G., Singh, A., Katare, O. P., and Mishra, D. N. (2005). Liposomal drug delivery systems--clinical applications. Acta Pharm *55*, 1-25.

Graham, H. N. (1992). Green tea composition, consumption, and polyphenol chemistry. Prev Med *21*, 334-350.

Greenlee, R. T., Hill-Harmon, M. B., Murray, T., and Thun, M. (2001). Cancer statistics, 2001. CA Cancer J Clin *51*, 15-36.

Gupta, S., Hastak, K., Ahmad, N., Lewin, J. S., and Mukhtar, H. (2001). Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols. Proc Natl Acad Sci U S A *98*, 10350-10355.

Gupta, S., Hussain, T., and Mukhtar, H. (2003). Molecular pathway for (-)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells. Arch Biochem Biophys *410*, 177-185.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanna, A. N., Kauffman, E. M., Newman, H. A., and Sharma, H. M. (1994). Prevention of oxidant stress by Student Rasayana (SR). Adv Exp Med Biol *366*, 444-445.

Hersey, P., and Zhang, X. D. (2003). Overcoming resistance of cancer cells to apoptosis. J Cell Physiol *196*, 9-18.

Hobisch, A., Eder, I. E., Putz, T., Horninger, W., Bartsch, G., Klocker, H., and Culig, Z. (1998). Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. Cancer Res *58*, 4640-4645.

Hojilla, C. V., Mohammed, F. F., and Khokha, R. (2003). Matrix metalloproteinases and their tissue inhibitors direct cell fate during cancer development. Br J Cancer *89*, 1817-1821.

Hong, J. H., Ahn, K. S., Bae, E., Jeon, S. S., and Choi, H. Y. (2006). The effects of curcumin on the invasiveness of prostate cancer *in vitro* and *in vivo*. Prostate Cancer Prostatic Dis *9*, 147-152.

Hou, Z., Lambert, J. D., Chin, K. V., and Yang, C. S. (2004). Effects of tea polyphenols on signal transduction pathways related to cancer chemoprevention. Mutat Res *555*, 3-19.

Huang, M. T., Lou, Y. R., Ma, W., Newmark, H. L., Reuhl, K. R., and Conney, A. H. (1994). Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. Cancer Res *54*, 5841-5847.

Hwang, T. L., Lee, W. R., Hua, S. C., and Fang, J. Y. (2007). Cisplatin encapsulated in phosphatidylethanolamine liposomes enhances the *in vitro* cytotoxicity and *in vivo* intratumor drug accumulation against melanomas. J Dermatol Sci *29*, 29.

Ihantola-Vormisto, A., Summanen, J., Kankaanranta, H., Vuorela, H., Asmawi, Z. M., and Moilanen, E. (1997). Anti-inflammatory activity of extracts from leaves of Phyllanthus emblica. Planta Med *63*, 518-524.

Ikezoe, T., Chen, S. S., Tong, X. J., Heber, D., Taguchi, H., and Koeffler, H. P. (2003). Oridonin induces growth inhibition and apoptosis of a variety of human cancer cells. Int J Oncol *23*, 1187-1193.

Ireson, C., Orr, S., Jones, D. J., Verschoyle, R., Lim, C. K., Luo, J. L., Howells, L., Plummer, S., Jukes, R., Williams, M.*, et al.* (2001). Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat *in vivo*, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. Cancer Res *61*, 1058-1064.

Ireson, C. R., Jones, D. J., Orr, S., Coughtrie, M. W., Boocock, D. J., Williams, M. L., Farmer, P. B., Steward, W. P., and Gescher, A. J. (2002). Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. Cancer Epidemiol Biomarkers Prev *11*, 105-111.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I.*, et al.* (1997). An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun *236*, 313-322.

Jackson, S. J., and Singletary, K. W. (2004). Sulforaphane: a naturally occurring mammary carcinoma mitotic inhibitor, which disrupts tubulin polymerization. Carcinogenesis *25*, 219-227.

Jeena, K. J., Joy, K. L., and Kuttan, R. (1999). Effect of Emblica officinalis, Phyllanthus amarus and Picrorrhiza kurroa on N-nitrosodiethylamine induced hepatocarcinogenesis. Cancer Lett *136*, 11-16.

Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R. C., Ghafoor, A., Feuer, E. J., and Thun, M. J. (2005). Cancer statistics, 2005. CA Cancer J Clin *55*, 10-30.

John, A., and Tuszynski, G. (2001). The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. Pathol Oncol Res *7*, 14-23.

Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. (2002). Apoptosis: a link between cancer genetics and chemotherapy. Cell *108*, 153-164.

Jordan, N. J., Gee, J. M., Barrow, D., Wakeling, A. E., and Nicholson, R. I. (2004). Increased constitutive activity of PKB/AKT in tamoxifen resistant breast cancer MCF-7 cells. Breast Cancer Res Treat *87*, 167-180.

Jose, J. K., Kuttan, G., and Kuttan, R. (2001). Antitumour activity of Emblica officinalis. J Ethnopharmacol *75*, 65-69.

Jung, K., Krell, H. W., Ortel, B., Hasan, T., Romer, A., Schnorr, D., Loening, S. A., and Lein, M. (2003). Plasma matrix metalloproteinase 9 as biomarker of prostate cancer progression in Dunning (Copenhagen) rats. Prostate *54*, 206-211.

Kaufmann, S. H., and Vaux, D. L. (2003). Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. Oncogene *22*, 7414-7430.

Kavanagh, K. T., Hafer, L. J., Kim, D. W., Mann, K. K., Sherr, D. H., Rogers, A. E., and Sonenshein, G. E. (2001). Green tea extracts decrease carcinogen-induced mammary tumor burden in rats and rate of breast cancer cell proliferation in culture. J Cell Biochem *82*, 387-398.

Khan, M. T., Lampronti, I., Martello, D., Bianchi, N., Jabbar, S., Choudhuri, M. S., Datta, B. K., and Gambari, R. (2002). Identification of pyrogallol as an antiproliferative compound present in extracts from the medicinal plant Emblica officinalis: effects on *in vitro* cell growth of human tumor cell lines. Int J Oncol *21*, 187-192.

Khan, N., Afaq, F., Saleem, M., Ahmad, N., and Mukhtar, H. (2006). Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. Cancer Res *66*, 2500-2505.

Khor, T. O., Keum, Y. S., Lin, W., Kim, J. H., Hu, R., Shen, G., Xu, C., Gopalakrishnan, A., Reddy, B., Zheng, X., *et al.* (2006). Combined inhibitory effects of curcumin and phenethyl isothiocyanate on the growth of human PC-3 prostate xenografts in immunodeficient mice. Cancer Res *66*, 613-621.

Kim, H. K., Park, I. A., Heo, D. S., Noh, D. Y., Choe, K. J., Bang, Y. J., and Kim, N. K. (2001). Cyclin E overexpression as an independent risk factor of visceral relapse in breast cancer. Eur J Surg Oncol *27*, 464-471.

Klein, E. A., and Thompson, I. M. (2004). Update on chemoprevention of prostate cancer. Curr Opin Urol *14*, 143-149.

Kostarelos, K., Emfietzoglou, D., Papakostas, A., Yang, W. H., Ballangrud, A. M., and Sgouros, G. (2005). Engineering lipid vesicles of enhanced intratumoral transport capabilities: correlating liposome characteristics with penetration into human prostate tumor spheroids. J Liposome Res *15*, 15-27.

Kubota, T., Hisatake, J., Hisatake, Y., Said, J. W., Chen, S. S., Holden, S., Taguchi, H., and Koeffler, H. P. (2000). PC-SPES: a unique inhibitor of proliferation of prostate cancer cells *in vitro* and *in vivo*. Prostate *42*, 163-171.

Kumar, V. P., Kuttan, R., and Kuttan, G. (1999). Effect of "rasayanas" a herbal drug preparation on cell-mediated immune responses in tumour bearing mice. Indian J Exp Biol *37*, 23-26.

Kumar, V. P., Kuttan, R., and Kuttan, G. (1999). Effect of "rasayanas", a herbal drug preparation on immune responses and its significance in cancer treatment. Indian J Exp Biol *37*, 27-31.

Kundu, J. K., Choi, K. Y., and Surh, Y. J. (2006). beta-Catenin-mediated signaling: a novel molecular target for chemoprevention with anti-inflammatory substances. Biochim Biophys Acta *1765*, 14-24.

Kunwar, A., Barik, A., Pandey, R., and Priyadarsini, K. I. (2006). Transport of liposomal and albumin loaded curcumin to living cells: an absorption and fluorescence spectroscopic study. Biochim Biophys Acta *1760*, 1513-1520.

Kuo, P. L., Hsu, Y. L., and Cho, C. Y. (2006). Plumbagin induces G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells. Mol Cancer Ther *5*, 3209-3221.

Lanzilli, G., Fuggetta, M. P., Tricarico, M., Cottarelli, A., Serafino, A., Falchetti, R., Ravagnan, G., Turriziani, M., Adamo, R., Franzese, O., and Bonmassar, E. (2006). Resveratrol down-regulates the growth and telomerase activity of breast cancer cells in vitro. Int J Oncol *28*, 641-648.

Lao, C. D., Ruffin, M. T. t., Normolle, D., Heath, D. D., Murray, S. I., Bailey, J. M., Boggs, M. E., Crowell, J., Rock, C. L., and Brenner, D. E. (2006). Dose escalation of a curcuminoid formulation. BMC Complement Altern Med *6*, 10.

Leary, A., and Dowsett, M. (2006). Combination therapy with aromatase inhibitors: the next era of breast cancer treatment? Br J Cancer *95*, 661-666.

Lee, J. S., and Surh, Y. J. (2005). Nrf2 as a novel molecular target for chemoprevention. Cancer Lett *224*, 171-184.

Li, L., Braiteh, F. S., and Kurzrock, R. (2005). Liposome-encapsulated curcumin: in vitro and in vivo effects on proliferation, apoptosis, signaling, and angiogenesis. Cancer *104*, 1322-1331.

Liao, S., Umekita, Y., Guo, J., Kokontis, J. M., and Hiipakka, R. A. (1995). Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. Cancer Lett *96*, 239-243.

Lin, L. T., Liu, L. T., Chiang, L. C., and Lin, C. C. (2002). In vitro anti-hepatoma activity of fifteen natural medicines from Canada. Phytother Res *16*, 440-444.

Liu, A., Lou, H., Zhao, L., and Fan, P. (2006). Validated LC/MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin. J Pharm Biomed Anal *40*, 720-727.

Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr *134*, 3479S-3485S.

Liu, X., Rubin, J. S., and Kimmel, A. R. (2005). Rapid, Wnt-induced changes in GSK3beta associations that regulate beta-catenin stabilization are mediated by Galpha proteins. Curr Biol *15*, 1989-1997.

Lokeshwar, B. L., Schwartz, G. G., Selzer, M. G., Burnstein, K. L., Zhuang, S. H., Block, N. L., and Binderup, L. (1999). Inhibition of prostate cancer metastasis in vivo: a comparison of 1,23-dihydroxyvitamin D (calcitriol) and EB1089. Cancer Epidemiol Biomarkers Prev *8*, 241-248.

Luo, J. L., Kamata, H., and Karin, M. (2005). IKK/NF-kappaB signaling: balancing life and death--a new approach to cancer therapy. J Clin Invest *115*, 2625-2632.

Maheshwari, R. K., Singh, A. K., Gaddipati, J., and Srimal, R. C. (2006). Multiple biological activities of curcumin: a short review. Life Sci *78*, 2081-2087.

Manson, M. M. (2003). Cancer prevention -- the potential for diet to modulate molecular signalling. Trends Mol Med *9*, 11-18.

Marczylo, T. H., Verschoyle, R. D., Cooke, D. N., Morazzoni, P., Steward, W. P., and Gescher, A. J. (2006). Comparison of systemic availability of curcumin with that of curcumin formulated with phosphatidylcholine. Cancer Chemother Pharmacol *19*, 19.

Masson, V., de la Ballina, L. R., Munaut, C., Wielockx, B., Jost, M., Maillard, C., Blacher, S., Bajou, K., Itoh, T., Itohara, S., *et al.* (2005). Contribution of host MMP-2 and MMP-9 to promote tumor vascularization and invasion of malignant keratinocytes. Faseb J *19*, 234-236.

McNally, S. J., Harrison, E. M., Ross, J. A., Garden, O. J., and Wigmore, S. J. (2007). Curcumin induces heme oxygenase 1 through generation of reactive oxygen species, p38 activation and phosphatase inhibition. Int J Mol Med, *19,* 165-172.

Mellinghoff, I. K., Vivanco, I., Kwon, A., Tran, C., Wongvipat, J., and Sawyers, C. L. (2004). HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. Cancer Cell *6*, 517-527.

Menon, L. G., Kuttan, R., and Kuttan, G. (1997). Effect of rasayanas in the inhibition of lung metastasis induced by B16F-10 melanoma cells. J Exp Clin Cancer Res *16*, 365-368.

Menon, L. G., Kuttan, R., Nair, M. G., Chang, Y. C., and Kuttan, G. (1998). Effect of isoflavones genistein and daidzein in the inhibition of lung metastasis in mice induced by B16F-10 melanoma cells. Nutr Cancer *30*, 74-77.

Mettlin, C. (1997). Recent developments in the epidemiology of prostate cancer. Eur J Cancer *33*, 340-347.

Mitchell, S. H., Zhu, W., and Young, C. Y. (1999). Resveratrol inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. Cancer Res *59*, 5892-5895.

Mittal, A., Pate, M. S., Wylie, R. C., Tollefsbol, T. O., and Katiyar, S. K. (2004). EGCG down-regulates telomerase in human breast carcinoma MCF-7 cells, leading to suppression of cell viability and induction of apoptosis. Int J Oncol *24*, 703-710.

Mohan, A., Nalini, V., Mallikarjuna, K., Jyotirmay, B., and Krishnakumar, S. (2007). Expression of motility-related protein MRP1/CD9, N-cadherin, E-cadherin, alpha-catenin and beta-catenin in retinoblastoma. Exp Eye Res *9*, 9.

Mohandas, K. M., and Desai, D. C. (1999). Epidemiology of digestive tract cancers in India. V. Large and small bowel. Indian J Gastroenterol *18*, 118-121.

Montfort, H. (2000). A new homeopathic approach to neoplastic diseases: from cell destruction to carcinogen-induced apoptosis. Br Homeopath J *89*, 78-83.

Moyers, S. B., and Kumar, N. B. (2004). Green tea polyphenols and cancer chemoprevention: multiple mechanisms and endpoints for phase II trials. Nutr Rev *62*, 204-211.

Naik, G. H., Priyadarsini, K. I., Satav, J. G., Banavalikar, M. M., Sohoni, D. P., Biyani, M. K., and Mohan, H. (2003). Comparative antioxidant activity of individual herbal components used in Ayurvedic medicine. Phytochemistry *63*, 97-104.

Nakachi, K., Suemasu, K., Suga, K., Takeo, T., Imai, K., and Higashi, Y. (1998). Influence of drinking green tea on breast cancer malignancy among Japanese patients. Jpn J Cancer Res *89*, 254-261.

Nakamura, K., Yasunaga, Y., Segawa, T., Ko, D., Moul, J. W., Srivastava, S., and Rhim, J. S. (2002). Curcumin down-regulates AR gene expression and activation in prostate cancer cell lines. Int J Oncol *21*, 825-830.

Nam, R. K., Fleshner, N., Rakovitch, E., Klotz, L., Trachtenberg, J., Choo, R., Morton, G., and Danjoux, C. (1999). Prevalence and patterns of the use of complementary therapies among prostate cancer patients: an epidemiological analysis. J Urol *161*, 1521-1524.

Nandi, S., Guzman, R. C., and Yang, J. (1995). Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. Proc Natl Acad Sci U S A *92*, 3650-3657.

Narayanan, B. A. (2006). Chemopreventive agents alters global gene expression pattern: predicting their mode of action and targets. Curr Cancer Drug Targets *6*, 711-727.

Newton, K. G. (2001). The biology of aging (JARA): an ayurvedic approach. Bull Indian Inst Hist Med Hyderabad *31*, 161-179.

Nielsen, N. H., Arnerlov, C., Emdin, S. O., and Landberg, G. (1996). Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status. Br J Cancer *74*, 874-880.

Nonn, L., Duong, D., and Peehl, D. M. (2006). Chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells. Carcinogenesis *6*, 6.

Ohtsu, H., Xiao, Z., Ishida, J., Nagai, M., Wang, H. K., Itokawa, H., Su, C. Y., Shih, C., Chiang, T., Chang, E.*, et al.* (2002). Antitumor agents. 217. Curcumin analogues as novel androgen receptor antagonists with potential as anti-prostate cancer agents. J Med Chem *45*, 5037-5042.

Owen, J. L., Iragavarapu-Charyulu, V., and Lopez, D. M. (2004). T cell-derived matrix metalloproteinase-9 in breast cancer: friend or foe? Breast Dis *20*, 145-153.

Park, C. H., Hahm, E. R., Park, S., Kim, H. K., and Yang, C. H. (2005). The inhibitory mechanism of curcumin and its derivative against beta-catenin/Tcf signaling. FEBS Lett *579*, 2965-2971.

Perkins, S., Verschoyle, R. D., Hill, K., Parveen, I., Threadgill, M. D., Sharma, R. A., Williams, M. L., Steward, W. P., and Gescher, A. J. (2002). Chemopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of familial adenomatous polyposis. Cancer Epidemiol Biomarkers Prev *11*, 535-540.

Philip, M., Rowley, D. A., and Schreiber, H. (2004). Inflammation as a tumor promoter in cancer induction. Semin Cancer Biol *14*, 433-439.

Pool-Zobel, B., Veeriah, S., and Bohmer, F. D. (2005). Modulation of xenobiotic metabolising enzymes by anticarcinogens -- focus on glutathione S-transferases and their

role as targets of dietary chemoprevention in colorectal carcinogenesis. Mutat Res *591*, 74-92.

Rege, N. N., Thatte, U. M., and Dahanukar, S. A. (1999). Adaptogenic properties of six rasayana herbs used in Ayurvedic medicine. Phytother Res *13*, 275-291.

Rekha, P. S., Kuttan, G., and Kuttan, R. (2001). Antioxidant activity of brahma rasayana. Indian J Exp Biol *39*, 447-452.

Rekha, P. S., Kuttan, G., and Kuttan, R. (2001). Effect of Brahma Rasayana on antioxidant systems and cytokine levels in mice during cyclophosphamide administration. J Exp Clin Cancer Res *20*, 219-223.

Russo, I. H., and Russo, J. (1998). Role of hormones in mammary cancer initiation and progression. J Mammary Gland Biol Neoplasia *3*, 49-61.

Saleem, A., Husheem, M., Harkonen, P., and Pihlaja, K. (2002). Inhibition of cancer cell growth by crude extract and the phenolics of Terminalia chebula retz. fruit. J Ethnopharmacol *81*, 327-336.

Saleem, M., Adhami, V. M., Ahmad, N., Gupta, S., and Mukhtar, H. (2005). Prognostic significance of metastasis-associated protein S100A4 (Mts1) in prostate cancer progression and chemoprevention regimens in an autochthonous mouse model. Clin Cancer Res *11*, 147-153.

Sartippour, M. R., Heber, D., Ma, J., Lu, Q., Go, V. L., and Nguyen, M. (2001). Green tea and its catechins inhibit breast cancer xenografts. Nutr Cancer *40*, 149-156.

Sartor, L., Pezzato, E., Dona, M., Dell'Aica, I., Calabrese, F., Morini, M., Albini, A., and Garbisa, S. (2004). Prostate carcinoma and green tea: (-)epigallocatechin-3-gallate

inhibits inflammation-triggered MMP-2 activation and invasion in murine TRAMP model. Int J Cancer *112*, 823-829.

Sausville, E. A., Johnson, J., Alley, M., Zaharevitz, D., and Senderowicz, A. M. (2000). Inhibition of CDKs as a therapeutic modality. Ann N Y Acad Sci *910*, 207-221; discussion 221-202.

Scartezzini, P., and Speroni, E. (2000). Review on some plants of Indian traditional medicine with antioxidant activity. J Ethnopharmacol *71*, 23-43.

Scher, H. I., Steineck, G., and Kelly, W. K. (1995). Hormone-refractory (D3) prostate cancer: refining the concept. Urology *46*, 142-148.

Schraub, S. (2000). Unproven methods in cancer: a worldwide problem. Support Care Cancer *8*, 10-15.

Sharma, H. M., Hanna, A. N., Kauffman, E. M., and Newman, H. A. (1992). Inhibition of human low-density lipoprotein oxidation in vitro by Maharishi Ayur-Veda herbal mixtures. Pharmacol Biochem Behav *43*, 1175-1182.

Sharma, H. M., Hanna, A. N., Kauffman, E. M., and Newman, H. A. (1995). Effect of herbal mixture student Rasayana on lipoxygenase activity and lipid peroxidation. Free Radic Biol Med *18*, 687-697.

Shen, G., Jeong, W. S., Hu, R., and Kong, A. N. (2005). Regulation of Nrf2, NF-kappaB, and AP-1 signaling pathways by chemopreventive agents. Antioxid Redox Signal *7*, 1648-1663.

Singh, R. H. (2002). An assessment of the ayurvedic concept of cancer and a new paradigm of anticancer treatment in Ayurveda. J Altern Complement Med *8*, 609-614.

Singh, R. P., and Agarwal, R. (2006). Mechanisms of action of novel agents for prostate cancer chemoprevention. Endocr Relat Cancer *13*, 751-778.

Singh, S. V., Hu, X., Srivastava, S. K., Singh, M., Xia, H., Orchard, J. L., and Zaren, H. A. (1998). Mechanism of inhibition of benzo[a]pyrene-induced forestomach cancer in mice by dietary curcumin. Carcinogenesis *19*, 1357-1360.

Sliva, D., Sedlak, M., Slivova, V., Valachovicova, T., Lloyd, F. P., Jr., and Ho, N. W. (2003). Biologic activity of spores and dried powder from Ganoderma lucidum for the inhibition of highly invasive human breast and prostate cancer cells. J Altern Complement Med *9*, 491-497.

Sporn, M. B., Dunlop, N. M., Newton, D. L., and Smith, J. M. (1976). Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed Proc *35*, 1332-1338.

Stamenkovic, I. (2003). Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol *200*, 448-464.

Sternlicht, M. D., and Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol *17*, 463-516.

Stewart, A. J., Mullen, W., and Crozier, A. (2005). On-line high-performance liquid chromatography analysis of the antioxidant activity of phenolic compounds in green and black tea. Mol Nutr Food Res *49*, 52-60.

Strohmeyer, D., Strauss, F., Rossing, C., Roberts, C., Kaufmann, O., Bartsch, G., and Effert, P. (2004). Expression of bFGF, VEGF and c-met and their correlation with microvessel density and progression in prostate carcinoma. Anticancer Res *24*, 1797-1804.

Surh, Y. J. (2003). Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer *3*, 768-780.

Surh, Y. J., Kundu, J. K., Na, H. K., and Lee, J. S. (2005). Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. J Nutr *135*, 2993S-3001S.

Tang, F. Y., Nguyen, N., and Meydani, M. (2003). Green tea catechins inhibit VEGF-induced angiogenesis in vitro through suppression of VE-cadherin phosphorylation and inactivation of AKT molecule. Int J Cancer *106*, 871-878.

Terry, S., Yang, X., Chen, M. W., Vacherot, F., and Buttyan, R. (2006). Multifaceted interaction between the androgen and Wnt signaling pathways and the implication for prostate cancer. J Cell Biochem *99*, 402-410.

Thangapazham, R. L., Sharma, A., and Maheshwari, R. K. (2006). Multiple molecular targets in cancer chemoprevention by curcumin. Aaps J *8*, E443-449.

Thangapazham, R. L., Singh, A. K., Sharma, A., Warren, J., Gaddipati, J. P., and Maheshwari, R. K. (2007). Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in vitro and in vivo. Cancer Lett *245*, 232-241.

Tiwari, R. K., Geliebter, J., Garikapaty, V. P., Yedavelli, S. P., Chen, S., and Mittelman, A. (1999). Anti-tumor effects of PC-SPES, an herbal formulation in prostate cancer. Int J Oncol *14*, 713-719.

Tomlins, S. A., Rhodes, D. R., Perner, S., Dhanasekaran, S. M., Mehra, R., Sun, X. W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., Lee, C., Montie, J. E., Shah, R. B., Pienta, K. J., Rubin, M. A., and Chinnaiyan, A. M. (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science, *310,* 644-648.

Ushijima, C., Tsukamoto, S., Yamazaki, K., Yoshino, I., Sugio, K., and Sugimachi, K. (2001). High vascularity in the peripheral region of non-small cell lung cancer tissue is associated with tumor progression. Lung Cancer *34*, 233-241.

Vaage, J., Donovan, D., Loftus, T., Abra, R., Working, P., and Huang, A. (1994). Chemoprevention and therapy of mouse mammary carcinomas with doxorubicin encapsulated in sterically stabilized liposomes. Cancer *73*, 2366-2371.

Vayalil, P. K., Kuttan, G., and Kuttan, R. (2002). Rasayanas: evidence for the concept of prevention of diseases. Am J Chin Med *30*, 155-171.

Wang, T. C., Cardiff, R. D., Zukerberg, L., Lees, E., Arnold, A., and Schmidt, E. V. (1994). Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature *369*, 669-671.

Wattenberg, L. W. (1985). Chemoprevention of cancer. Cancer Res *45*, 1-8.

Webb, M. S., Johnstone, S., Morris, T. J., Kennedy, A., Gallagher, R., Harasym, N., Harasym, T., Shew, C. R., Tardi, P., Dragowska, W. H., *et al.* (2006). In vitro and in vivo characterization of a combination chemotherapy formulation consisting of vinorelbine and phosphatidylserine. Eur J Pharm Biopharm *17*, 17.

Wu, A. H., Tseng, C. C., Van Den Berg, D., and Yu, M. C. (2003). Tea intake, COMT genotype, and breast cancer in Asian-American women. Cancer Res *63*, 7526-7529.

Wu, A. H., Yu, M. C., Tseng, C. C., Hankin, J., and Pike, M. C. (2003). Green tea and risk of breast cancer in Asian Americans. Int J Cancer *106*, 574-579.

Wu, J. (1996). Apoptosis and angiogenesis: two promising tumor markers in breast cancer (review). Anticancer Res *16*, 2233-2239.

Wu, Y., Zhang, H., Dong, Y., Park, Y. M., and Ip, C. (2005). Endoplasmic reticulum stress signal mediators are targets of selenium action. Cancer Res *65*, 9073-9079.

Xing, N., Chen, Y., Mitchell, S. H., and Young, C. Y. (2001). Quercetin inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. Carcinogenesis *22*, 409-414.

Yamakawa, S., Asai, T., Uchida, T., Matsukawa, M., Akizawa, T., and Oku, N. (2004). (-)-Epigallocatechin gallate inhibits membrane-type 1 matrix metalloproteinase, MT1-MMP, and tumor angiogenesis. Cancer Lett *210*, 47-55.

Yan, C., Jamaluddin, M. S., Aggarwal, B., Myers, J., and Boyd, D. D. (2005). Gene expression profiling identifies activating transcription factor 3 as a novel contributor to the proapoptotic effect of curcumin. Mol Cancer Ther, *4*, 233-241.

Yang, C. S., Lambert, J. D., Hou, Z., Ju, J., Lu, G., and Hao, X. (2006). Molecular targets for the cancer preventive activity of tea polyphenols. Mol Carcinog *45*, 431-435.

Yeh, S., Lin, H. K., Kang, H. Y., Thin, T. H., Lin, M. F., and Chang, C. (1999). From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc Natl Acad Sci U S A *96*, 5458-5463.

Yoneda, T., Hiramatsu, M., Sakamoto, M., Togasaki, K., Komatsu, M., and Yamaguchi, K. (1995). Antioxidant effects of "beta catechin". Biochem Mol Biol Int *35*, 995-1008.

Zerbini, L. F., Czibere, A., Wang, Y., Correa, R. G., Otu, H., Joseph, M., Takayasu, Y., Silver, M., Gu, X., Ruchusatsawat, K., *et al.* (2006). A novel pathway involving melanoma differentiation associated gene-7/interleukin-24 mediates nonsteroidal anti-

inflammatory drug-induced apoptosis and growth arrest of cancer cells. Cancer Res *66*, 11922-11931.

Zhang, M., Binns, C. W., and Lee, A. H. (2002). Tea consumption and ovarian cancer risk: a case-control study in China. Cancer Epidemiol Biomarkers Prev *11*, 713-718.

Zhang, P. L., Rosen, S., Veeramachaneni, R., Kao, J., DeWolf, W. C., and Bubley, G. (2002). Association between prostate cancer and serum testosterone levels. Prostate *53*, 179-182.

Zhang, H. N., Yu, C. X., Zhang, P. J., Chen, W. W., Jiang, A. L., Kong, F., Deng, J. T., Zhang, J. Y., and Young, C. Y. (2007). Curcumin downregulates homeobox gene NKX3.1 in prostate cancer cell LNCaP. Acta Pharmacol Sin, *28,* 423-430.

Zhao, X., Tian, H., Ma, X., and Li, L. (2006). Epigallocatechin gallate, the main ingredient of green tea induces apoptosis in breast cancer cells. Front Biosci *11*, 2428-2433.

Zhou, J. R., Yu, L., Mai, Z., and Blackburn, G. L. (2004). Combined inhibition of estrogen-dependent human breast carcinoma by soy and tea bioactive components in mice. Int J Cancer *108*, 8-14.

Zhu, M., Chen, Y., and Li, R. C. (2000). Oral absorption and bioavailability of tea catechins. Planta Med *66*, 444-447.

Zimmerman, R. A., and Thompson, I. M., Jr. (2002). Prevalence of complementary medicine in urologic practice. A review of recent studies with emphasis on use among prostate cancer patients. Urol Clin North Am *29*, 1-9, vii.

PUBLICATION PROFILE

Papers Published/Accepted

1. **Thangapazham R.L.,** Shaheduzzaman S, Kee-Hong Kim, Neena Passi, Albert Dobi, Srivastava S and Maheshwari R.K. Molecular targets and signaling pathways of curcumin in prostate cancer chemoprevention (Communicated to Cancer Research)
2. **Thangapazham R.L.,** Passi N and Maheshwari R.K. Green tea polyphenol (GTP) and epigalloctechin gallate(EGCG) induces apoptosis and inhibits invasion of human breast cancer cells. (Communicated to Cancer Biology and Therapy)
3. **Thangapazham R.L**., Puri A, Tele S.C., Blumenthal R and Maheshwari R.K. Liposomal curcumin inhibits the proliferation of human prostate cancer cells in-vitro – A nano delivery approach (Under Revision in Frontiers in Bioscience)
4. **Thangapazham R.L.,** Singh A.K., Warren J., Seth P., Namita Misra, Shafali, Vijayavitthal T.Mathad, Kanwal Raj and Radha K. Maheshwari. Shikonin analogue (SA) 93/637 induces apoptosis by activation of caspase-3 in U937 Cells (Accepted in Frontiers in Bioscience).
5. **Thangapazham R.L.,** Sharma A and Maheshwari R.K. (2007) Protective effects of phytochemicals in cancer chemoprevention, wound healing and ischemia-reperfusion injury. Proceedings of National Society of Ethanopharmacology (In Press)
6. Anoop K. Singh, Anuj Sharma, James Warren, Subhashree Madhavan, Keith Steele, N.V. Rajesh Kumar N.V., **Thangapazham R. L.,** Shekhar C. Sharma,

Dinesh K Kulshreshtha, Jaya Gaddipati, and Radha K. Maheshwari (2007).Picroliv accelerates epithelialization and angiogenesis in rat wounds. Planta Med. 2007 Mar;73(3):251-6. Epub 2007 Feb 22.

7. **Thangapazham, R.L**, Singh, A.K., Sharma, A., Warren, J., Gaddipati, J., and Maheshwari, R.K. Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in-vitro and in-vivo. Cancer Letters. 2007 Jan 8;245(1-2):232-41. Epub 2006 Mar 6
8. **Thangapazham R.L.,** Sharma, A., Gaddipati, J.P., Singh, A.K., and Maheshwari, R.K. (2007). Inhibition of tumor angiogenesis by Brahama Rasayana (BR). Journal of Experimental Therapeutics and Oncology. 2006 Dec;6(1) :13-21
9. Jonas W.B., Gaddipati, J.P., Rajesh Kumar N.V., Sharma A., **Thangapazham R.L.**, Warren J., Singh A.K., Ives J.A., Olsen C., Mog S.R. and Maheshwari, R.K. Can homeopathic treatment slow prostate cancer growth? Integrative Cancer Therapies. 2006 Dec;5(4):343-9
10. **Thangapazham R.L.**, Rajesh Kumar N.V., Sharma A., Warren J., Singh A.K., Ives J.A., Gaddipati, J.P., Maheshwari, R.K and Jonas W.B. Effect of homeopathic treatment on gene expression in copenhagen rat tumor tissues. Integrative Cancer Therapies. 2006 Dec;5(4):350-5
11. **Thangapazham R.L.**, Gaddipati, J.P., Rajesh Kumar N.V., Sharma A., Singh A.K., Ives J.A., Maheshwari, R.K and Jonas W.B. Homeopathic medicines do not alter growth and gene expression in prostate and breast cancer cells in vitro. Integrative Cancer Therapies. 2006 Dec;5(4):356-61

12. Sharma A., Singh, A.K., Warren J., **Thangapazham, R.L,** and Maheshwari, R.K. Differential expression of angiogeneic genes in diabetic wound healing. Journal of Investigative Dermatology, 2006 Oct;126(10):2323-31
13. **Thangapazham R. L,** Sharma A, Maheshwari R. K. Multiple molecular targets in cancer chemoprevention by curcumin. Journal of the American Association of Pharmaceutical Sciences. AAPS J. 2006 Jul 7;8(3):E443-9. Review
14. **Thangapazham R. L,** Sharma A, Maheshwari R. K. Biomarkers of angiogenesis in wound Healing and cancer: role of botanicals and phytochemicals. Proceedings of the fourth international conference on quality and safety issues related to botanicals. Acta Horticulturae. 2006; Number 720, 129 -136
15. Gaddipati, J.P., Rajesh Kumar N.V., **Thangapazham R.L.**, Sharma A., Warren J., Mog S.R., Singh A.K., and Maheshwari, R.K. Protective effect of a polyherbal preparation, Brahma rasayana against tumor growth and lung metastasis in rat prostate model system. Journal of Experimental Therapeutics and Oncology .2004 Oct;4(3):203-12

Book Chapters

16. **Thangapazham R. L,** Sharma A, Maheshwari R. K. Beneficial Role of Curcumin in Skin Diseases. Molecular targets and therapeutic uses of curcumin in health and disease. Edited by Bharat B.Aggarwal, Young Jun-Surh and Shishir Shishodia.343-358. Adv Exp Med Biol. 2007;595:343-57.

Poster Presentations/Abstracts

1. **Thangapazham R.L**, Singh A.K, Seth P, Raj K, Maheshwari RK. Shikonin Analogue (SA) 93/637 induces apoptosis by activation of caspase-3 in U937 cells. Research Week. Uniformed Services University of the Health Sciences Research Day, Bethesda, MD, May 14-16, 2007
2. **Thangapazham R.L**, Tele SC, Puri A, Blumenthal R, Passi N, Maheshwari RK. Design of Nanodelivery system to enhance the bioavailability of curcumin. Research Week. Uniformed Services University of the Health Sciences Research Day, Bethesda, MD, May 14-16, 2007
3. **Thangapazham R.L**, Passi N, Maheshwari RK. Green tea induces apoptosis and inhibit invasion in human breast cancer cell line. Research Week. Uniformed Services University of the Health Sciences Research Day, Bethesda, MD, May 14-16, 2007
4. **Thangapazham R.L**, Passi N, Maheshwari RK. Curcumin induced gene expression response in human prostate cancer cells. Research Week. Uniformed Services University of the Health Sciences Research Day, Bethesda, MD, May 14-16, 2007
5. **Thangapazham R.L**, Tele SC, Puri A, Blumenthal R, Passi N, Maheshwari RK. Evaluation of a nanotechnology based carrier for delivery of curucmin. 7th Annual CCR fellows and young investigators retreat. Ocean city, MD, February 27-March1, 2007
6. **Thangapazham R.L**, Tele SC, Puri A, Blumenthal R, Passi N, Maheshwari RK. Liposomal curcumin inhibits the proliferation of human prostate cancer cells in-

vitro. Frontiers in cancer prevention research. Boston, MA, November 12-15, 2006

7. **Thangapazham R.L**, Singh AK, Gaddipati JP, Sharma A, Warren J, Maheshwari RK. Green tea induces cell cycle arrest estrogen independent human breast cancer cell line. Research Week. Uniformed Services University of the Health Sciences Research Day, Bethesda, MD, May 16-17, 2006
8. Maheshwari RK, Sharma A, **Thangapazham RL,** Singh AK, Kulreshtha D, Sharma S, Enhancement of woundhealing by novel phytochemicals in normal and diabetic impaired animals: A successful Indo-US collaboration. International conference on ethnopharmacology and alternate medicine, Amala Cancer Institute, Kerala, India, January 20-22, 2006
9. **Thangapazham R.L**, Gaddipati JP, Sharma A, Singh AK, Maheshwari RK. Evaluation of anti-angiogenic activity of Brahma Rasayana in a rat prostate tumor model system in-vivo and in HUVEC in-vitro. Research Week. Uniformed Services University of the Health Sciences Research Day, Bethesda, MD, May 17-19, 2005
10. **Thangapazham R.L**, Singh AK, Gaddipati JP, Sharma A, Warren J, Maheshwari RK. Green tea polyphenols and its constituent epigallocatechin gallate influences the proliferation of highly invasive estrogen independent breast cancer cell line. Uniformed Services University of the Health Sciences Research Day, Bethesda, MD, May 17-19, 2005
11. **Thangapazham R.L**, Singh AK, Gaddipati JP, Sharma A, Warren J, Maheshwari RK. Anti-proliferative, apoptotic and anti-invasive effect of green tea in estrogen

independent human breast cancer cell line. Combined Faculty Retreat, CCR, National Cancer Institute, NIH, Rocky Gap, MD, July 20-22, 2005.

12. **Thangapazham RL,** Singh AK, Sharma A, Gaddipati JP, Warren J, Maheshwari RK. Green tea induces cell cycle arrest and apoptosis in estrogen independent human breast cancer cell line. Frontiers in cancer prevention research. Baltimore, MD, October 30 – November 2, 2005
13. **Thangapazham R.L**, Gaddipati JP, Sharma A, Singh AK, Maheshwari RK. Molecular targets involved in the anti-angiogenic activity of a polyherbal chemopreventive agent Brahma Rasayana. 96th American Association for Cancer Research Annual Meeting, Anaheim, CA, April 16-20, 2005
14. **Thangapazham RL,** Singh AK, Gaddipati JP, Sharma A, Warren J, Maheshwari RK. Anti-proliferative effect of green tea polyphenols and its constituent epigallocatechin gallate against human breast cell line. 96th American Association for Cancer Research Annual Meeting, Anaheim, CA, April 16-20, 2005
15. Gaddipati JP, **Thangapazham R.L**, Sharma A, Singh AK, Maheshwari RK. Regulation of angiogenesis by Brahma Rasayana a polyherbal preparation in vitro and in vivo models. The American Society for Cell Biology 44th Annual Meeting Washington, DC, December 4-8, 2004
16. Jonas WB, Gaddipati JP, Rajeshkumar NV, Sharma A, **Thangapazham R.L,** Warren J, Singh AK, Ives J, Olsen C, Mog SR, Maheshwari RK. In-vitro and in-vivo assessment of homeopathic treatment for prostate cancer. Society of Integrative Oncology 1st International Conference, New York, November 18, 2004

17. **Thangapazham R.L,** Gaddipati JP, Rajeshkumar NV, Sharma A, Warren J, Mog SR, Singh AK, Maheshwari RK. Brahma Rasayana: A polyherbal preparation as a chemopreventive agent against prostate cancer. American Institute for Cancer Research-International Research Conference on Food, Nutrition and Cancer, Washington, DC, July 15-16, 2004
18. James Warren, Anoop Singh, **Thangapazham R.L**, Radha K Maheshwari. EGCG decreases MDA-MB-231 breast cancer cell tumor incidence and tumor size in Nude Mice. USUHS Research Day, May 2004