Novel Molecular Targets for Genistein

in Prostate Cancer Cells

by

Kendra Merchant

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This dissertation was prepared under the direction of the candidate's dissertation advisor, Dr. James Kumi-Diaka, Department of Biological Sciences, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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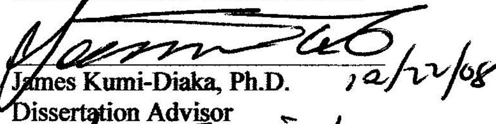

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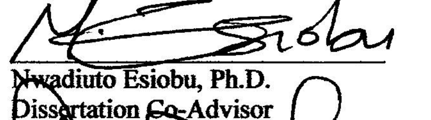

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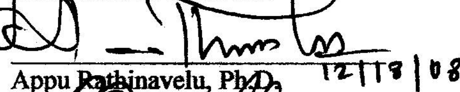

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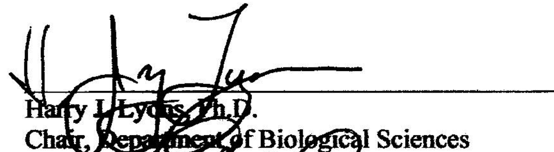

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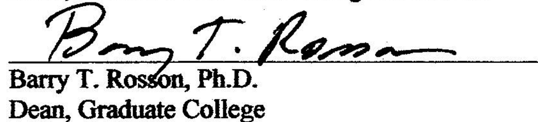

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To my husband, Kacey, thank you for the sacrifices you made, I am forever grateful. To my family, your love and support will never be forgotten. And most of all thanks to God for everything, without Him none of this would be possible.

Abstract

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Prostate cancer is the most common form of non-skin cancer and the second leading cause of cancer deaths within the United States. The five year survival rate has increased from 69% to 99% over the last 25 years for the local and regional disease, but has remained fairly low (approximately 34%) for the advanced disease. Therefore, current research is aimed at finding complementary or alternative treatments that will specifically target components of the signal transduction, cell-cycle and apoptosis pathways to induce cell death, with little or no toxic-side effects to the patient. In this study we investigated the effect of genistein on expression levels of genes involved in these pathways.

Genistein is a (4′, 5′, 7-trihydroxyisoflavone) is a major isoflavone constituent of soy that has been shown to inhibit growth proliferation and induce apoptosis in cancer

cells. The mechanism of genistein-induced cell death and potential molecular targets for genistein in LNCaP prostate cancer cells was investigated using several techniques. The chemosensitivity of genistein towards the prostate cancer cells was investigated using the ATP and MTS assays and apoptosis induction was determined using apoptosis and caspase assays. Several molecular targets were also identified using cDNA microarray and RT-PCR analysis.

Our results revealed that genistein induces cell death in a time and dose-dependent manner and regulates expression levels of several genes involved in carcinogenesis and immunogenicity. Several cell cycle genes were down-regulated, including the mitotic kinesins, cyclins and cyclin dependent kinases, indicating that genistein is able to halt cell cycle progression through the regulation of genes involved in this process. Several members of the Bcl-2 family which are involved in apoptosis were also affected and a number of genes involved in immunogenicity were up-regulated including the DefB1 and HLA membrane receptors.

The results of this study provide evidence of genistein's ability to inhibit growth proliferation and induce apoptosis and indicates its potential as an adjuvant in chemotherapy and immunotherapy.

Dedication

To Kacey, the love of my life and to my sunshine, Kyle; thank you for being my inspiration.

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1. Introduction

1.1. Background and Significance

Prostate cancer is the most common form of non-skin cancer and the second leading cause of cancer deaths in the United States (Jemal et al, 2008). The American Cancer Society estimates that there will be 186,320 new cases of prostate cancer, which will account for about 25% of all cancer cases in 2008. It is also estimated that there will be 28,660 deaths from this disease, accounting for 10% of all cancer deaths. Since 1992, cancer death rates have been steadily decreasing and have remained fairly constant since 1995 (Jemal et al, 2008). The five year survival rate has increased from 69% to 99% over the last 25 years for the local and regional disease, but has remained fairly low (approximately 34%) for the advanced disease (Stanford et al, 1999).

Cancer is a complex disease that occurs as a result of mutations in the DNA, resulting from damage that can be caused by both internal and external factors. The disease arises after repeated exposure to one or more of these factors and progresses due to malignant transformation of normal cells (Doucas et al, 2006; Hanahan, 2000). Documented risk factors for prostate cancer include age, race, and family history. Men with a family history of the disease are at a higher risk and those who have an affected father are more likely to be diagnosed at an earlier age (Cotter et al, 2002). About 5-10% of all cases are attributed to genetic predisposition and 64% of all cases are diagnosed in men 65 years or older (Jemal et al, 2008; Stanford et al, 1999).

There has been an increase in the number of cases among younger men (Lowe et al, 2003), which many speculate to be the result of new diagnostic and screening procedures. Jamaican men of African descent and African-Americans have the highest incidence rates in the world (Jemal et al, 2008) and epidemiological studies have shown that the incidence in African-Americans is twice as much as in Caucasian men (Ward et al, 2004). Studies also show that African-Americans with the metastatic disease have a worse prognosis than Caucasians and other ethnic groups (Thompson et al, 2001).

Other factors such as diet, environment and geographic location may also play a role in the progression of this disease (Colli et al, 2005; Hsing et al, 2000). Epidemiological studies have indicated that there is a correlation between cancer incidence and geographic location (Klassen et al, 2006; Mettlin, 1997; Sung et al, 1999; Yu et al, 1991). Western countries have higher incidence and mortality rates than Asian countries, which indicate that diet and lifestyle are factors that contribute to this disease. Several studies have shown that there is a link between diet and prostate cancer progression. These studies suggest that a diet high in animal fat and meat could promote tumor progression (Colli et al, 2005; Sung et al, 1999; Yu et al, 1991), while a diet rich in cereals and vegetables (which contain phytoestrogens) might reduce the risk of the disease (Colli et al, 2005; Hocman, 1989; Hodge et al, 2004; Sung et al, 1999).

Further evidence of environmental and dietary contributions is demonstrated by studies that show the difference in incidence rates between Asian men and those who have migrated to the United States. These studies reveal that there is a higher incidence rate for first and second generation Asian men who are living in the US compared to

those who are still living in their home countries (Cook et al, 1998; Marks et al, 2004; Mettlin, 1997).

Increases in relative survival rates in the US may be due partly to new diagnostic tools and/or improvement in diagnostic methods and an increase in testing for biologic markers such as prostate specific antigen (PSA). This leads to early diagnosis and better treatment of the early-stage disease (Baade et al, 2004; Gao et al, 1997).

1.2. Prostate cancer therapy

Current research is aimed at finding new prognostic markers and targets for therapy. The apoptotic and cell cycle pathways provide many avenues for researchers and specific targets for cancer therapy. Current treatments for the localized disease include surgery, hormone therapy or radiation therapy, but once the cancer has metastasized, these treatments become ineffective (Garnick et al, 1998). Therefore, research is ongoing to find successful methods for treatment of the advanced disease, with fewer side effects and less damage to normal cells. In the early stages of the disease, the cancer cells depend on androgens for survival and proliferation. In the advanced stages, the cells often lose this dependence (Craft et al, 1999; Pilat et al, 1999) and this is seen as a survival mechanism. Therefore, effective treatments will be those that are successful in inhibiting growth of both androgen dependent and androgen-independent cells. Researchers are also interested in combination therapies where alternative treatments are used in conjunction with conventional therapies. This includes the use of dietary and chemotherapeutic agents used in conjunction with conventional therapies such as radiation and hormonal therapy.

Other approaches include gene therapy and immunotherapy, either alone or in combination with other conventional therapies. Gene therapy includes the introduction of a gene of interest within the tumor cells whose expression will significantly alter the growth of the cells. Delivery of the gene requires a vector that can be viral or non-viral. Limitations of this current method include lack of efficient delivery of the target gene and the use of safe and non-toxic vectors (Figueiredo et al, 2007; Mazhar et al, 2004).

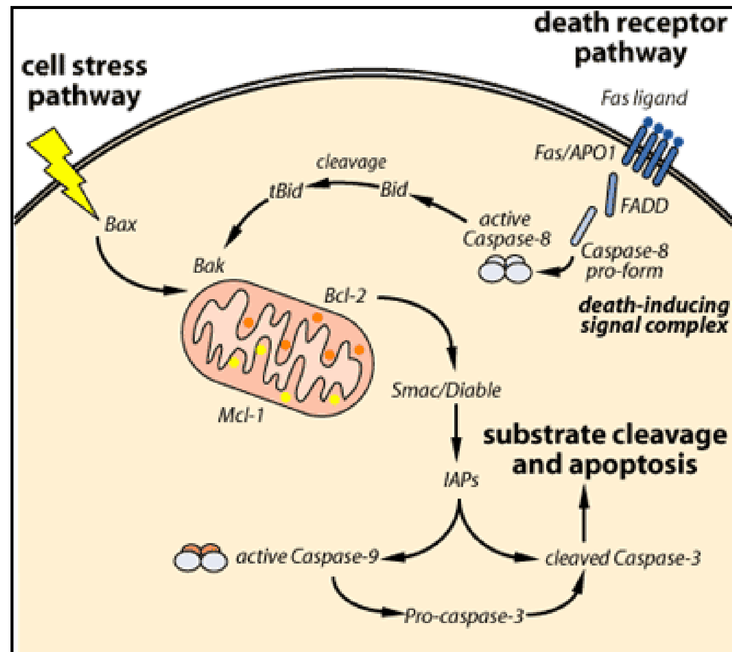
Immunotherapy involves the use of agents to activate an immune response within the body that will specifically target the tumor cells. The prostate has specific tumor associated antigens, such as PSA that are a unique target for these agents (Kiessling et al, 2008). There are ongoing studies investigating the use of vaccines that will stimulate the immune system and target the tumor cells (Kaufman et al, 2004; Sanda et al, 1999). Other studies include the use of agents that activate immune cells that are able to recognize and kill cancer cells. One such study involves the use of a vitamin-D3 binding protein, GcMAF (Gc Macrophage Activating Factor) to activate macrophages (Yamamoto et al, 2008). Cancer cells secrete α-N-acetylgalactosamine (Nagalase) that inactivates Gc proteins preventing them from activating macrophages. In this study they showed that the activated macrophages were able to recognize and kill cancer cells with no serious side effects to the patients, indicating that immunotherapy is a viable option for successful treatment of this disease.

1.3. Apoptotic Mechanisms

Both chemotherapeutic agents and ionizing radiation utilize apoptotic mechanisms to induce death in cancer cells (Bold et al, 1997; Fulda et al, 2003; Hu et al,

2003; Ventimiglia et al, 2001). There are two main types of cell death: apoptosis and necrosis. Apoptosis (programmed cell death) is a process that is characterized by morphological and biochemical changes such as cell shrinkage, nuclear DNA fragmentation and membrane blebbing. It plays a crucial role in tissue homeostasis (Bold et al, 1997; Hu et al, 2003). This process occurs by way of two major pathways: the mitochondria-apoptosome-mediated (intrinsic) pathway and the death-receptor-induced (extrinsic) pathway. Both pathways involve caspase activation and cleavage of specific cellular substrates that ultimately lead to the biochemical and morphological changes characteristic of apoptosis. Another pathway that triggers apoptosis and does not involve caspases involves reactive oxygen species.

Figure 1. Representation of the intrinsic (cell stress pathway) and extrinsic (death receptor pathway) apoptotic pathways within the cell. Both pathways involve activation of the caspases and cleavage of specific cellular substrates that ultimately lead to cell death.



www.scq.ubc.ca/.../2006/07/apoptosispathways.gif

Necrosis, which is cell death due to injury or disease, involves cellular swelling, organelle dysfunction, mitochondrial collapse and cellular disintegration (Bold et al, 1997). Unlike necrosis, apoptosis does not elicit an inflammatory response because the integrity of the cell membrane is maintained, preventing release of the cellular components (Ventimiglia et al, 2001).

Apoptosis can be regulated by manipulation of the cell cycle (Gastman, 2001) and chemotherapeutic agents that target these checkpoints and induce apoptosis are under investigation. Effective agents would be those that control or inhibit growth in tumor

cells while having little or no effect on normal cells. Therefore, a greater understanding of the mechanisms of this pathway could provide more effective therapy options.

1.4. Phytochemicals as therapeutic agents

Phytochemicals are low molecular weight, biologically active secondary plant metabolites, which have the potential to reduce the risk of certain chronic diseases such as cancer (Hasler et al, 1999). The goal of phytochemical research is to identify natural substances that will affect components of cancer growth and eventually lead to a decrease in cancer incidence. These components include signal transduction pathways, cell cycle regulatory molecules, growth factors and cell death pathways.

A major phytochemical that has been shown to have chemopreventive properties is genistein isoflavone. Genistein (4′, 5′, 7-trihydroxyisoflavone) is a major isoflavone constituent of soy that is found naturally as the glycoside genistin, but intestinal microflora are able to hydrolyze it to genistein. Genistein can be further metabolized within the body to produce several different metabolites (most of which are biologically inactive) depending on the bacterial environment within the intestines (Heinonen et al, 2002; Steer et al, 2003). Genistein is structurally related to estrogen (therefore it is called a phytoestrogen) and can exhibit both estrogenic and anti-estrogenic activity (Dixon et al, 2002; Murphy et al, 2002; Ososki et al, 2003). Due to its structural similarity to estrogen, genistein has the ability to bind estrogenic receptors and can therefore regulate cell proliferation and prevent hormone dependent growth of cancer cells (Dixon et al, 2002; Sarkar et al, 2002).

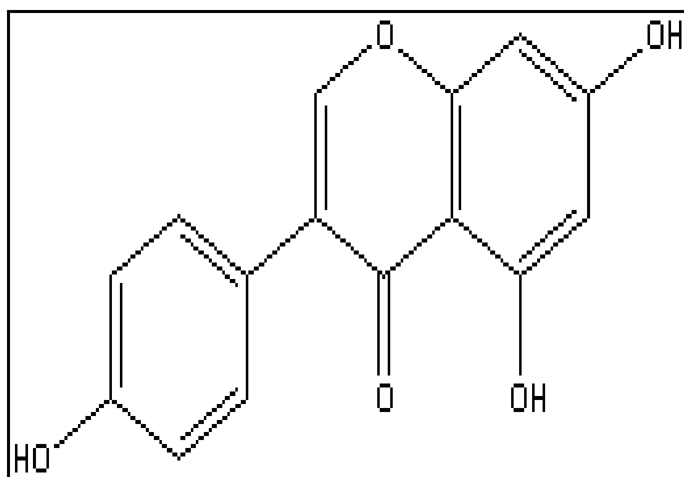


Figure 2. The Molecular Structure of Genistein

Studies have shown that genistein also exhibits several pharmacological effects such as inhibition of tyrosine kinases, DNA topoisomerases, microsomal lipid peroxidation, and angiogenesis (Dalu et al, 1998; Dixon et al, 2002; Sarkar et al, 2002) and has been shown to act as an antioxidant and a cell cycle inhibitor by arresting the cell cycle at the G2/M phase (Chen et al, 2004; Dixon et al, 2002).

Soymilk supplements can result in decreased oxidative DNA damage (Mitchell et al, 1999) and lowered prostate cancer risk (Jacobsen et al, 1998). Research in animal models has revealed that dietary phytoestrogens can inhibit growth and decrease androgen receptor expression in rat prostates (Lund et al, 2004) as well as reduce tumor cell proliferation, increase apoptosis and inhibit angiogenesis in mice (Zhou et al, 1999). Genistein has also been shown to alter growth factor signaling in rat prostates (Dalu et al, 1998; Wang et al, 2004). Unlike other isoflavonoids, the concentration at which genistein exerts its toxicity is far above that at which it exerts biological and pharmacological

effects. This property makes genistein highly favored as a potential chemopreventive agent (Dixon et al, 2002).

Combination studies involving genistein have shown that phytochemicals can act synergistically with other agents to inhibit the growth of cancer cells (Khoshyomn et al, 2002; Kumi-Diaka, 2002; Sakamoto, 2000). Genistein enhances radiosensitivity of prostate cancer cells (Ejima, 2003), and acts as an adjuvant to cytotoxic therapies (Kakeji et al, 1997). Gene studies have revealed that genistein regulates expression of genes involved in cell growth and apoptosis and can also inhibit bone metastasis by regulation of genes involved in this process (Li et al, 2004).

Along with other isoflavones, the effect of genistein on signal transduction pathways, cell cycle regulatory molecules and cell proliferation was investigated. In this study, it was found that genistein altered cell signaling pathways and the cell cycle, inhibited cell growth and led to cell death in human prostate carcinoma cells (Agarwal, 2000). Genistein was also proven to induce apoptosis and activate caspase-3 activity in cancerous testicular cells (Kumi-Diaka, 2000). Another investigative study proved that a large number of prostate cancer patients that were supplemented with soy isoflavones in their diet, showed a decrease in PSA and testosterone levels in the early stage disease, which led to a decrease in proliferation of the prostate cancer cells (Kumar et al, 2004).

1.5. Molecular targets for genistein treatment

1.5.1. Cell cycle genes

1.5.1.1. Kinesins

Kinesins are a large family of motor proteins that are involved in the transport of molecules along microtubules within the cell. Mitotic kinesins form a subfamily that are involved in a wide range of cellular processes such as cargo transport, spindle and chromosome movement and microtubule polymerization (Bergnes et al, 2005). The family members can be divided into 14 groups based on sequence similarity. Three main types have been shown to play a role in cell division. These include the Kin-N which have an N-terminal motor domain and travel toward the plus end of a microtubule, the Kin-C which have a C-terminal motor domain and travel toward the minus end of a microtubule and the Kin-I which have an internally placed motor domain and are involved in destabilization of microtubules (Bergnes et al, 2005; Hirokawa et al, 2008).

During mitosis, sister chromatids separate and are moved to opposite sides of the cell along the mitotic spindle. The chromatids are attached to the mitotic spindle by the kinetochore complex which forms at the centromere. The kinetochore complex plays an important regulatory role in the mitotic checkpoint and if there is no interaction of the kinetochore and mitotic spindle, the cell cycle will not progress to the next phase (Saitoh et al, 2005). Kinesins are involved in the formation of the mitotic spindle and movement of the chromosomes along the microtubules (Goshima et al, 2005) so any absence or mutation in any of these genes would affect cell cycle progression.

1.5.1.2. Cyclins and cyclin dependent kinases

There are various checkpoints in the cell cycle that control replication and prevent mutations (Ford et al, 1999). The cell cycle ceases if damage is detected and the cell is destroyed if this damage is irreparable. Due to molecular alterations in a cancer cell, the cell cycle progresses even if there is damage and the cell does not undergo apoptosis. (Gurumurthy et al, 2001). Cyclins and cyclin dependent kinases (cdks) are regulators of these cell cycle checkpoints. Key proteins involved in these checkpoints include Cdk1, Cdk2, Cdk4 and cyclins A, B, D and E. Both the cyclins and their related kinases are potential targets for cancer therapy and regulation of these genes could halt progression in cancer cells and induce apoptosis.

1.5.2. Apoptosis-related genes

1.5.2.1. Caspases

Caspases are intracellular cysteine proteases, which when triggered, cleave specific protein substrates that result in morphological and biochemical changes in the cell and eventually lead to cell death (Fulda et al, 2003; Gastman, 2001). Fourteen caspases have been isolated so far and three specific types have been identified. These are cytokine activators, apoptosis initiators and apoptosis effectors, with the latter two playing a key role in apoptosis (Sattar et al, 2003; Ventimiglia et al, 2001). The initiators (caspase 2, 8, 9 and 10) activate signals, which lead to cleavage and activation of other caspases, while the effectors (caspase 3, 6 and 7) serve to activate or inactivate specific protein substrates (Gurumurthy et al, 2001; Shi, 2002). Chemotherapeutic agents may initiate apoptosis either by direct activation of these caspases, triggering TRAIL receptors

or regulating activity of the Bcl-2 family of proteins (Gastman, 2001; Hu et al, 2003), that would also activate the caspases and ultimately lead to cell death.

1.5.2.2. BcL-2 family

The BcL-2 family members are integral membrane associated proteins that include both pro- and anti-apoptotic members. Anti-apoptotic members Bcl-2, Bcl-x and Bcl-w are associated with the outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane and serve to maintain the integrity of these structures within the cell (Cory et al, 2002; Shore et al, 2005). Pro-apoptotic members include Bax, Bak, Bim and the BH-3 only proteins that are also associated with the outer mitochondrial membrane. BH3 works by inhibiting the activity of the anti-apoptotic members while Bax and Bak associate with the outer mitochondrial membrane increasing its permeability. This results in the release of apoptotic proteins such as cytochrome c that lead to caspase activation and eventually cell death (Cory et al, 2002; Shore et al, 2005). Therapeutic agents that activate pro-apoptotic members or inhibit the activity of anti-apoptotic members provide a good option for cancer therapy.

1.5.2.3. Maspin

Maspin is a member of the serpin (serine protease inhibitor) family that was first identified in breast carcinoma and normal breast epithelia (Zou et al, 1994). It is expressed in normal breast epithelia, prostate, epidermis, lung and stromal cells of the cornea (Bailey, 2006). Maspin possesses certain structural features that are different from other serpins, which may suggest a unique role within cells, possibly in tumor progression (Sheng, 2006). Studies have shown that it is involved in cancer metastasis,

apoptosis and angiogenesis and so far is the only pro-apoptotic member of the serpin family (Bailey, 2006; Khalkhali-Ellis, 2006).

Maspin is regulated by p53 and was found to inhibit the metastasis of prostate cancer cells. Loss of maspin expression from these cells correlated with a higher tumor stage and systemic tumor progression (Machtens et al, 2001). Recent studies have shown that maspin can sensitize prostate and breast carcinoma cells to drug-induced apoptosis (Jiang et al, 2002; Liu et al, 2004, Tahmatzopoulos et al, 2005), which could provide alternative therapeutic options for prostate cancer therapy. Other studies have also shown that maspin could inhibit tumor progression at the stage of cell invasion and metastasis, which could provide therapeutic options for the advanced disease. Maspin may play a significant role in regulating processes that are associated with the progression and metastasis of prostate cancer and could therefore be a unique target for genistein.

2. Hypotheses

2.1. The molecular mechanism of apoptosis induction by genistein in LNCaP prostate cancer cells is mediated through induction of the caspase protease death signal and/or regulation of expression levels of Bcl-2 family members;

2.2. Genistein will up-regulate maspin expression levels in LNCaP prostate cancer cells;

2.3. Genistein will regulate the expression levels of several cell cycle and apoptosis related genes in LNCaP cells.

3. Specific Aims/Objectives

3.1. To determine the influence of genistein concentration on growth proliferation and apoptosis induction in LNCaP cells;

3.2. To determine the molecular mechanism(s) of genistein-induced apoptosis in LNCaP cells;

3.3. To compare gene expression levels in genistein-treated and untreated cells using cDNA microarray technology.

3.4. To investigate genistein-induced expression of novel genes in LNCaP cells.

4. Materials and Methods

4.1. Cell Culture

LNCaP cells were cultured in RPMI-1640 media supplemented with 10% FBS, 1% penicillin/streptomycin and L-glutamine. Cells were incubated for 24-48 hrs at 37^0C and 5% CO_2 to achieve 60-80% confluence. After incubation, the media was removed and replaced with media alone as a negative control or media containing various concentrations of genistein (25, 50, 75, 100 μM) and incubated for 24-48 hrs.

4.2. ATP Assay

The cytotoxicity of genistein on LNCaP cells was determined using the ATP assay. ATP is a source of energy in viable cells and is required for many cellular processes, including apoptosis. Cells that are dying are unable to perform normal metabolic functions and can no longer manufacture ATP. Therefore a decrease in ATP levels can indicate cell death, whereas an increase will indicate cell proliferation. The ATP assay is based on bioluminescent detection of ATP levels within the cell and offers a sensitive determination of even small amounts of ATP. The assay utilizes the D-luciferin substrate which when oxidized in an ATP-dependent process catalyzed by firefly luciferase, generates chemiluminiscence at 560nm.

The assay is optimized for fast determination of low levels of ATP. Concentrations down to 0.1 pmol can be accurately determined using the luminescent signal of the luciferase reaction.

For ATP determination, cells were plated at a density of 5×10^4 cells per well in a 96-well plate and incubated for 24 hrs to achieve confluence. After incubation, the cells were treated with varying concentrations of genistein, or 1% Triton-X as a positive control. The cells were then incubated for 24 hrs under standard conditions. After incubation, 50 µl of lysis solution was immediately added to each well. After 5 mins, 50 µl of solution was transferred from each well of the 96-well plate to its corresponding well in a white plate. 50 µl of luciferin/luciferase solution was then added to each well. Luminescence was determined after 5 mins using a luminometer. ATP concentration was determined by comparison of luminescent values against a standard curve. % inhibition was calculated using the following formula and the EC_{50} was determined from the graph.

$$\% \text{ inhibition} = \frac{(lum_c - lum_s)}{lum_c} \times 100$$

where lum_c = luminescence of control sample

lum_s = luminescence of treated sample

4.3. MTS Assay

The MTS assay was also performed to determine the cytotoxicity of genistein towards LNCaP cells. This assay utilizes MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, to determine cell viability. In the presence of viable cells, the substrate is reduced to a colored formazan product. In active mitochondria, the tetrazolium ring is cleaved in the presence of dehydrogenase enzymes, producing a product that is soluble in tissue culture medium. The color intensity is directly proportional to the number of viable cells; therefore relative cell numbers can be determined based on optical absorbance from each well of the microtiter plate.

Briefly, cells were plated at a density of 5×10^4 cells per well in a 96-well plate and incubated for 24 hrs to achieve confluence. After incubation, the cells were treated with varying concentrations of genistein, or 1% Triton-X as a positive control. After 48hrs incubation, 10 µl of MTS reagent was added to each well of the 96-well plate. The plates were then incubated at 37^0C and 5% CO_2 for 4 hrs and the absorbance was read at 490 nm using a plate reader. % inhibition was calculated using the following formula:

$$\textbf{\% inhibition} = \frac{(abs_c - abs_s)}{abs_c} \times 100$$

where abs_c = absorbance of control sample

abs_s = absorbance of treated sample

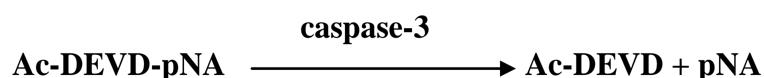
4.4. Determination of Apoptosis Induction using Flow Cytometry

Apoptosis is characterized by morphological changes within the cells such as fragmentation of nuclear chromatin and cell shrinkage. These changes can be detected by exposing the cells to certain dyes which when taken up by the cells will cause fluorescence, which indicates the level of cell death. Apoptosis induction was determined using the Vybrant ® apoptosis assay kit. This assay utilizes two dyes, Propidium Iodide (PI) and YO-PRO®-1. The green fluorescent dye YO-PRO®-1, can enter early apoptotic cells, whereas the red fluorescent dye PI will only enter dead cells. Therefore, use of both dyes provides a sensitive indication of apoptosis. After staining cells with both dyes, live cells show little or no fluorescence, early apoptotic cells will show green fluorescence and dead cells (membrane compromised cells) will show both red and green fluorescence. These can be detected by fluorescence microscopy or flow cytometry.

Cells were harvested and plated in 6-well plates and incubated for 24 hrs to achieve confluence. After incubation, cells were treated with varying concentrations of genistein, or topotecan (50 µM) as a positive inducer of apoptosis. The cells were then incubated for 48hrs. After incubation, cells were harvested and washed with cold PBS and cell density was adjusted to 1 x 10^6 cells/ml in PBS. 1 µl of YO-PRO®-1 stock solution (Component A) was added followed by 1µl of PI stock solution (Component B), to each 1ml of cell suspension. The cells were incubated on ice for 20-30 mins then analyzed on the Accuri C6 flow cytometer® using 488nm excitation with green fluorescence emission for YO-PRO®-1 and red fluorescence emission for PI. Data was then analyzed using the CFlow Plus® software.

4.5. Caspase Protease Analysis

The involvement of caspase-3 in the molecular pathway of genistein induced apoptosis was determined by using a caspase-3 colorimetric activity assay. The assay involves the caspase mediated cleavage of a labeled peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-*p*NA). The *p*-nitroaniline (*p*NA) product can be quantified using a spectrophotometer or plate reader at 405 nm. This gives a direct indication of caspase-3 activity.



For caspase-3 determination the cells were treated with varying concentrations of genistein or topotecan (50 µM) as a positive inducer of apoptosis. The cells were counted and collected by centrifugation at 1500 rpm for 5 mins. 2 – 4 x 10^6 cells were then resuspended in 200 µl of lysis buffer and incubated on ice for 20 mins. After incubation, the solution was centrifuged for 5 mins at 10,000 x g and the supernatant was collected. The BCA protein assay kit (Pierce) was used to determine the protein concentration of the samples. 30 µg of protein lysate for each sample was then assayed for caspase activity. Fold-increase in caspase-3 activity was then calculated by comparing the absorbance values of the treated samples to that of the control sample.

4.6. RNA Extraction

After treatment of the cells with genistein, the RNA was extracted using the RNeasy mini kit (Qiagen). Cells were collected by centrifugation and approximately

1×10^6 cells were collected and lysed using buffer RLT. The RNA was then extracted according to the manufacturer's protocol. RNA quality was determined by separation on a 1% agarose gel and confirmed by spectrophotometry. Good quality RNA was obtained by observing the 18S and 26S bands on the agarose gel and absorbance values giving an $A_{260/280}$ ratio of between 1.8 and 2. The RNA was quantified by spectrophotometry and used for microarray and RT-PCR analysis.

4.7. cDNA Microarray Analysis

RNA samples were prepared as previously described and were submitted to the Genomics Core of the Scripps Research Institute (Jupiter, Fl) for microarray analysis. Briefly, RNA samples were quantified using the NanoDrop ND-1000. Double-stranded cDNA was prepared from 1 µg of total RNA using the Affymetrix cDNA synthesis kit and then in vitro transcribed using an IVT labeling kit (Affymetrix) with the cRNA product purified using a GeneChip Sample Cleanup Module (Affymetrix). 20 µg biotin-labeled cRNA was fragmented and hybridized to Affymetrix Human Gene chip U133 plus 2.0 array overnight in the Affy 640 hybridization oven with a speed of 60 rpm for 16 hr. Microarrays were washed and stained using the Affymetrix Fluidics Station FS400. GeneChip arrays were scanned using a GeneChip Scanner 3000 (Affymetrix). The probe set intensities were quantified using the GeneChip Operating Software (GCOS) and analyzed with GCRMA normalization using Array Assist Software (Stratagene, La Jolla, CA). All hybridized chips met standard quality control criteria, and mean fluorescence values of each array were scaled to a mean intensity of 500. Gene expression of genistein treated cells was compared with control (untreated) cells to determine changes in

expression of target genes. Functional cluster analysis was done using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Glynn et al, 2003). Results of the microarray analysis were verified by RT-PCR.

4.8. RT PCR Analysis

The RNA extracted from the cell samples was subjected to RT-PCR analysis using the Access RT-PCR System (Promega). Selected genes were reverse transcribed to verify the results of the array gene expression changes. The Access RT-PCR System is designed for convenient and sensitive detection and analysis of RNA by one-step RT-PCR. cDNA synthesis and PCR are performed in a single tube using gene specific primers (4μM) and 1 μg of total RNA. The specific primers used were bax, bcl-x, vegf, mdm-2 and beta-actin as an internal loading control (Table 1). The amplified products were separated on a 1% agarose gel stained with ethidium bromide. The results of this experiment were compared to that of the microarray analysis to determine if similar changes in gene expression were observed in target genes.

Table 1. Primer sequences for RT-PCR analysis of selected genes

Gene name	Primer sequence
Bax	Forward 5' - ATCCAGGATCGAGCAGGGCG -3' Reverse 5' - GGTTCTGATCAGTTCCGGCA - 3'
Bcl-x	Forward 5' ATGTCTCAGAGCAACCGGGA - 3' Reverse 5' - TCATTTCCGACTGAAGAGTG - 3'
Mdm-2	Forward - 5' CTGGGGAGTCTTGAGGGACC - 3' Reverse - 5' CAGGTTGTCTAAATTCCTAG - 3'
VEGF	Forward primer: 5' - TCGGGCCTCCGAAACCATGA -3' Reverse primer: 5' – CCTGGTGAGAGATCTGGTTC - 3'
Maspin	Forward 5' - TGCTGCCTACTTTGTTGGCAAGT - 3' Reverse 5'- TCTGTATGGGAAACATTGACAGTATCA - 3'
β-actin	Forward 5' - GTGGGGCGCCCCAGGCACCA - 3' Reverse 5' - CTCCTTAATGTCACGCACGATTTC - 3'

5. Statistical Analysis

Experiments were performed in duplicates to confirm similar results. Significance of the differences in mean values was determined using the Student's t-test and considering $P < 0.05$ to be statistically significant.

6. Results

6.1. Growth Inhibition of LNCaP Cells

The MTS and ATP assays were done to determine the cytotoxicity of genistein on LNCaP cells. The data obtained shows that genistein is able to induce in cancer cells which is consistent with previous studies done. The ATP assay showed a steady increase in growth inhibition as genistein concentration increased (Figure 3). Being a more sensitive assay, we were able to see a significant difference after just 24 hrs of treatment. There was a significant increase in growth inhibition of the cells from 0 to 60 µM ($p < 0.05$). Above 60 µM, there was a further increase in inhibition but this was not significant ($p > 0.05$).

The MTS assay also showed a steady increase in inhibition (Figure 4). At 24 hrs there was no significant increase from 25 to 50 µM ($p > 0.05$). Above 50 µM, there was a significant increase in growth inhibition in the cells ($p < 0.05$). At 48 hrs, there was a significant increase in growth inhibition from 0 to 50 µM ($p < 0.05$), but no significant increase was observed beyond that concentration ($p > 0.05$). At 25 µM, there was no difference between the effect seen at 24 and 48 hrs, but at higher concentrations, the difference in growth inhibition observed was significantly higher at 48 hrs ($p < 0.05$). This is further evidence that the effects of genistein are both time and dose dependent. The EC_{50} of genistein in LNCaP cells was determined from the data obtained and was found to be about 50-60 µM.

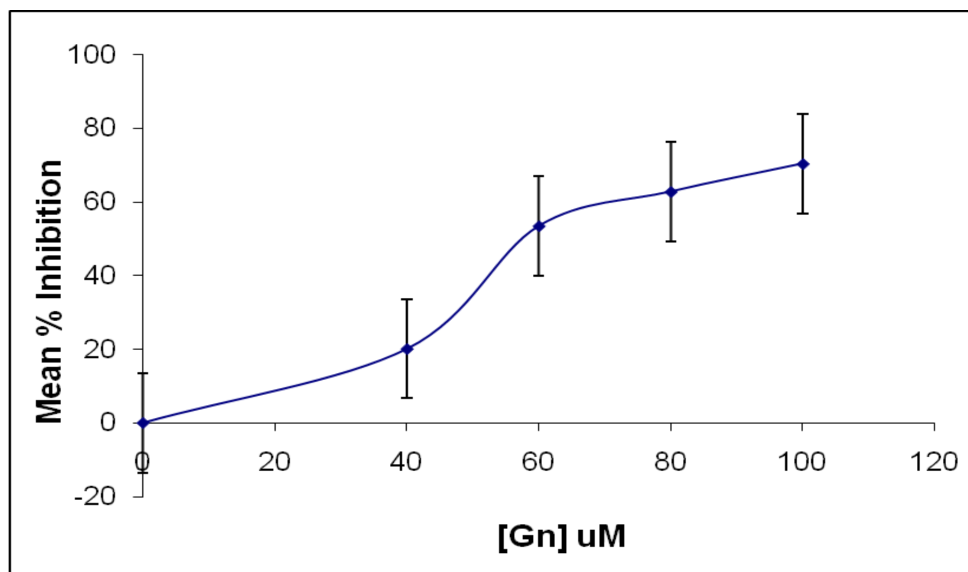


Figure 3. Determination of cytotoxic effect of genistein on growth inhibition of LNCaP cells using the ATP assay. Cells were treated with varying concentrations of genistein and % inhibition was determined using the ATP assay. Data are representative of two independent experiments and are expressed as the mean ± SEM. Bar =SEM

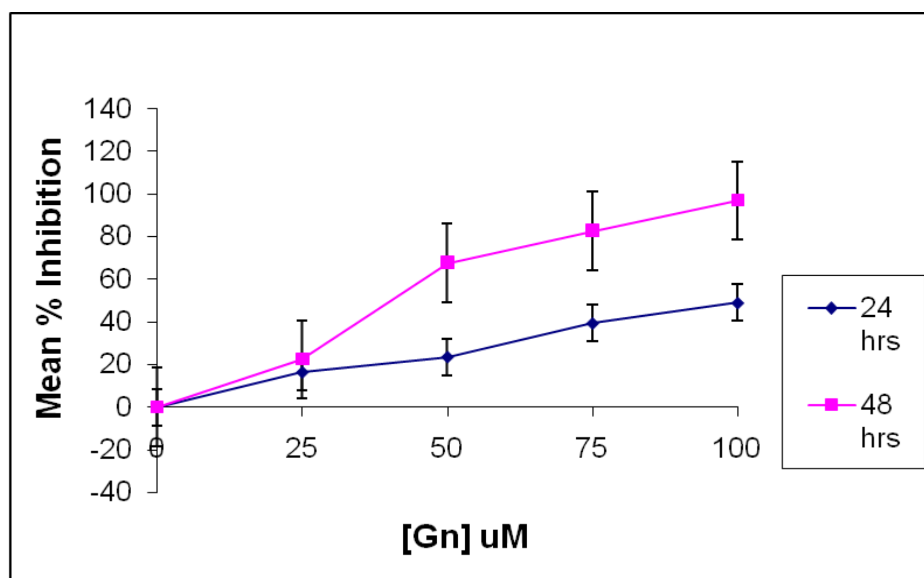
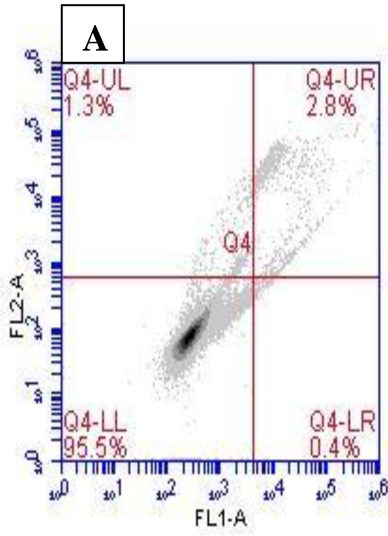


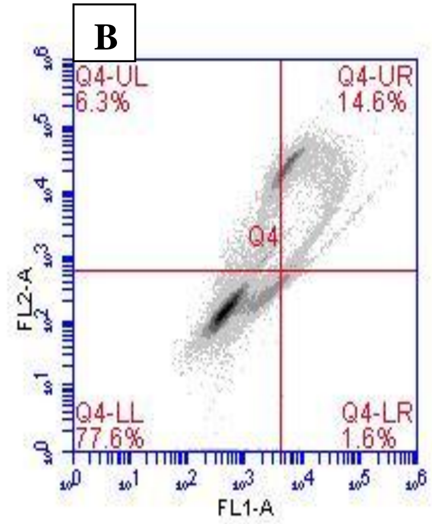
Figure 4. Determination of the cytotoxic effect of genistein on growth inhibition of LNCaP cells using the MTS assay. Cells were treated with varying concentrations of genistein and % inhibition was determined using the MTS assay. Data are representative of two independent experiments and are expressed as the mean ± SEM. Bar =SEM

6.2. Apoptosis Induction by Genistein

Apoptosis induction in genistein treated cells was investigated and the results show that genistein has a dose-dependent effect on LNCaP cells. Cells were stained with the fluorescent dyes YO-PRO®-1 and PI and showed an increased level of apoptosis with increasing genistein concentration. The dot plots in figure 5 show cell distribution with respect to their level of fluorescence. The FL1-A channel indicates fluorescence of the YO-PRO®-1 dye and the FL2-A channel indicates fluorescence of PI. Figure 5A shows the results for untreated LNCaP cells. One main population of cells is observed with a low level of fluorescence, indicating that these are viable cells that were able to exclude

most of the dye. As the concentration of genistein increases, there is a separation of the cells into subpopulations showing different fluorescence levels (Figure 5B-E). Cells in early apoptosis are able to exclude PI while taking up YO-PRO®-1, while late apoptotic cells will take up both dyes. An increase in the level of green fluorescence at 25 μM genistein exposure (Figure 5B) indicates cells undergoing apoptosis. There is a decrease in the percentage of viable cells, while the percentage of cells in early apoptosis increases from 0.5% in untreated cells, to 22.4% in cells treated with 100 μM of genistein (Figure 5E). The percentage of cells undergoing late apoptosis increases as well (1.4% in untreated cells to 38.8% in 100 μM genistein treated cells). The bar chart in figure 6 shows a comparison of the percentages of live and apoptotic cells seen in the dot plots and further indicates that apoptosis within cells increases with increasing genistein concentration.


A
Q4-UL
1.3%
Q4-UR
2.8%
Q4
Q4-LL
95.5%
Q4-LR
0.4%
FL2-A
FL1-A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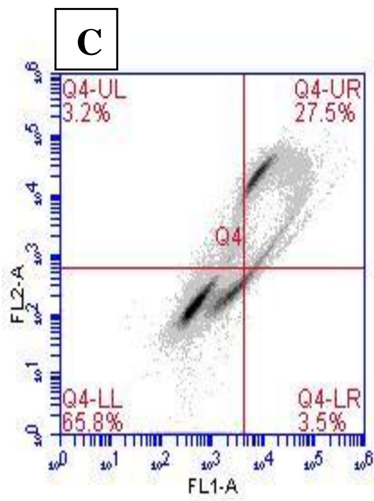
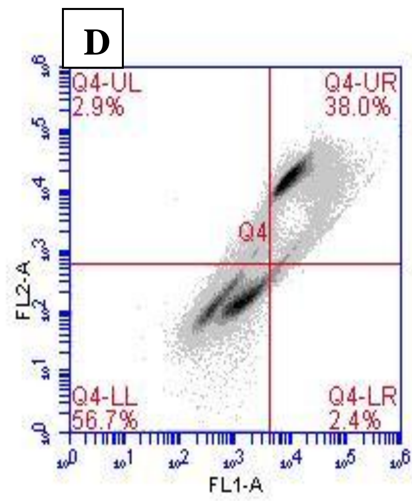
Q4-UR
14.6%
Q4
Q4-LL
77.6%
Q4-LR
1.6%
FL2-A
FL1-A


C
Q4-UL
3.2%
Q4-UR
27.5%
Q4
Q4-LL
65.8%
Q4-LR
3.5%
FL2-A
FL1-A


D
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Q4-UR
38.0%
Q4
Q4-LL
56.7%
Q4-LR
2.4%
FL2-A
FL1-A

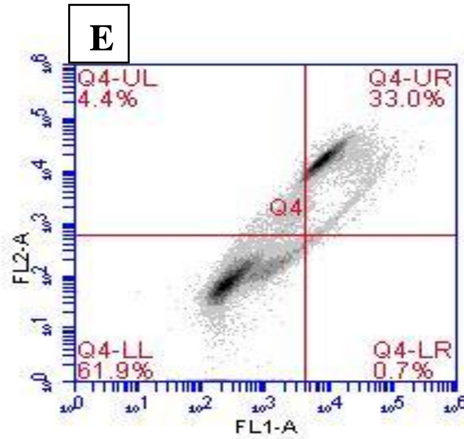


Figure 5. Flow cytometric determination of apoptosis induction in LNCaP cells by genistein. Cells were treated with genistein for 48 hrs, harvested and stained with YO-PRO®-1 and PI to detect apoptosis induction. FL1-A channel represesents YO-PRO®-1 fluorescence and the FL2-A channel represents PI fluorescence. A. Control cells stained with both dyes after 48 hrs treatment . B-E Genistein treated cells (25, 50, 75, 100 µM) stained with both dyes. Data is representative of two independent experiments.

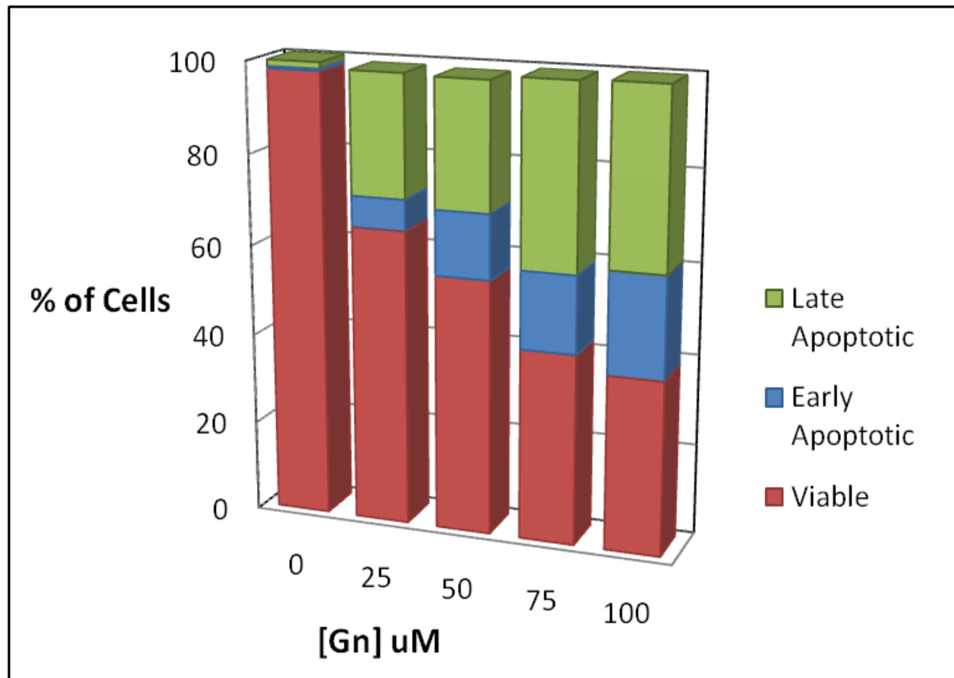
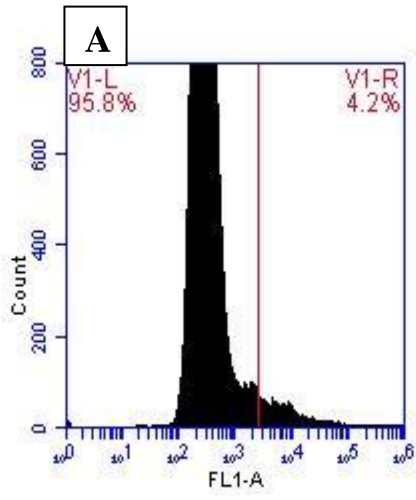
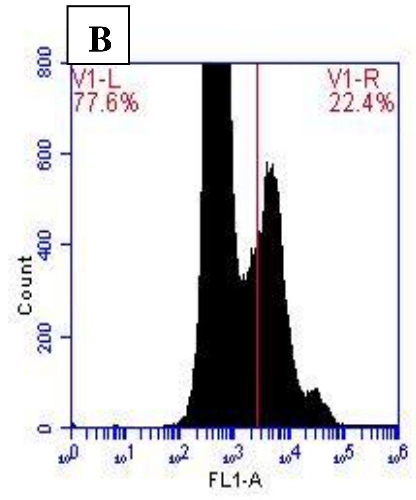


Figure 6. Bar chart showing distribution of viable and apoptotic cells with respect to concentration of genistein. Cells were treated with varying concentrations of genistein and analyzed by flow cytometry, using YO-PRO®-1 and PI stains to detect apoptosis induction. Data is representative of two independent experiments.

In figures 7 and 8 the histogram plots show a similar result. The figures show the relative fluorescence levels of the cells versus the number of events (counts). In the control sample, 95.8% of the cells show little or no YO-PRO®-1 fluorescence. As genistein concentration increases, the percentage of cells within this population decreases, indicating an decrease in the number of cells that are able to exclude the dye (Figure 7). The percentage of cells showing that become permeable to the PI dye show a similar trend (Figure 8), indicating a decrease in the number of viable cells or an increase in the number of dead cells (cells becoming permeable to the dyes).


A
V1-L
95.8%
V1-R
4.2%
Count
FL1-A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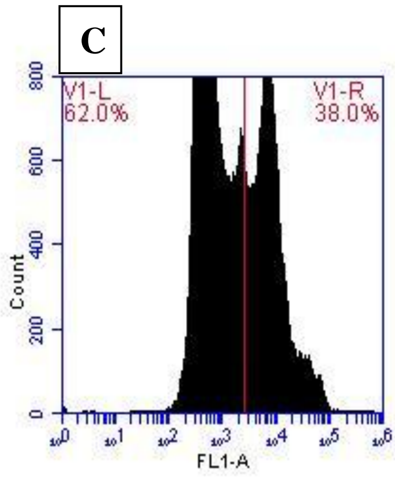
V1-L
77.6%
V1-R
22.4%
Count
FL1-A


C
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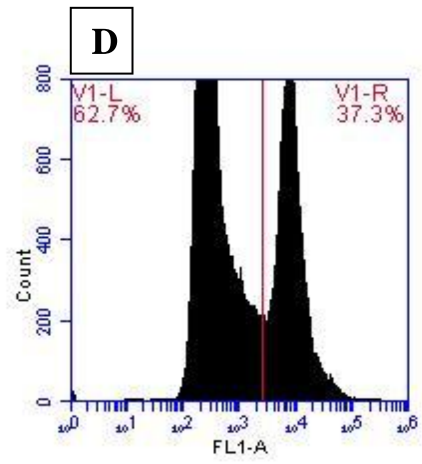
62.0%
V1-R
38.0%
Count
FL1-A


D
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62.7%
V1-R
37.3%
Count
FL1-A

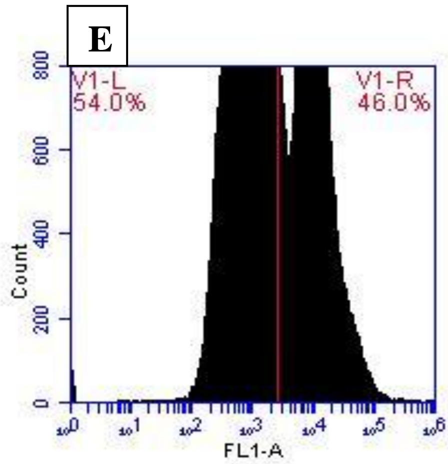
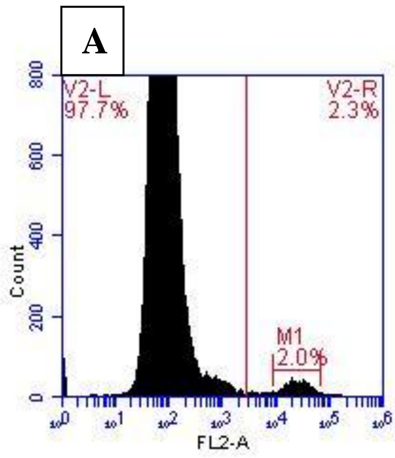
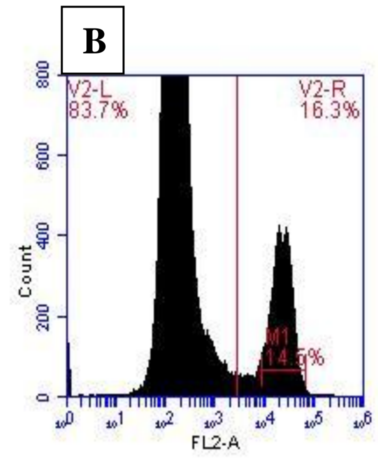


Figure 7. Flow cytometric determination of apoptosis induction in LNCaP cells by genistein. Cells were treated with genistein for 48 hrs, harvested and stained with YO-PRO®-1 and PI to detect apoptosis induction. Histogram plots show percentage of cells detected on FL1-A (YO-PRO®-1) channel. A. Control cells stained with both dyes after 48 hrs treatment . B-E Genistein treated cells (25, 50, 75, 100 uM) stained with both dyes. Data is representative of two independent experiments.


A
V2-L
97.7%
V2-R
2.3%
M1
2.0%
Count
800
600
400
200
0
10^0
10^1
10^2
10^3
10^4
10^5
10^6
FL2-A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83.7%
V2-R
16.3%
M1
Count
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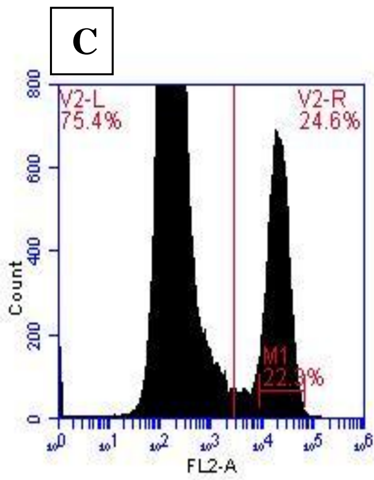
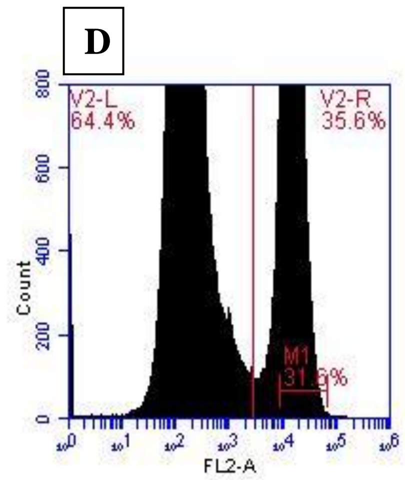
600
400
200
0
10^0
10^1
10^2
10^3
10^4
10^5
10^6
FL2-A


C
V2-L
75.4%
V2-R
24.6%
M1
Count
800
600
400
200
0
10^0
10^1
10^2
10^3
10^4
10^5
10^6
FL2-A


D
V2-L
64.4%
V2-R
35.6%
M1
Count
800
600
400
200
0
10^0
10^1
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10^5
10^6
FL2-A

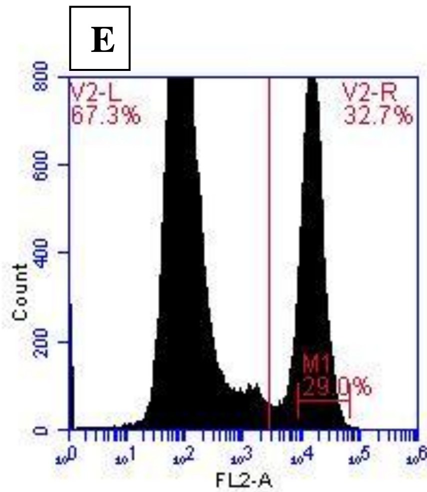

Figure 8. Flow cytometric determination of apoptosis induction in LNCaP cells by genistein. Cells were treated with genistein for 48 hrs, harvested and stained with YO-PRO®-1 and PI to detect apoptosis induction. Histogram plots show percentage of cells detected on FL2-A (PI) channel. A. Control cells stained with both dyes after 48 hrs treatment . B-E Genistein treated cells (25, 50, 75, 100 uM) stained with both dyes. Data is representative of two independent experiments.

6.3. Caspase-3 Induction by Genistein

The mechanism of apoptosis induction was investigated using the caspase-3 colorimetric assay. Data obtained indicate that caspase-3 induction increases in a time and dose-dependent manner. Experiments were performed in the presence and absence of a caspase-3 inhibitor and the data show that there was a significant difference in caspase-3 activity between the two treatment groups. In the presence of the caspase-3 inhibitor, expression was the same after 24 hrs even at high concentrations of genistein (Figure 9). However, in the absence of any inhibition there was a 1.5 fold increase at 50 µM after just 24 hrs and almost a 5-fold increase at the same concentration after 48 hrs (Figure 10). Even though there was some increase in caspase-3 activity after 48 hrs in the presence of

the inhibitor, there was significantly more activity without it (Figure 10). These results correlate with the data obtained in the apoptosis assay and indicate that genistein-induced cell death involves the activation of the caspase pathway.

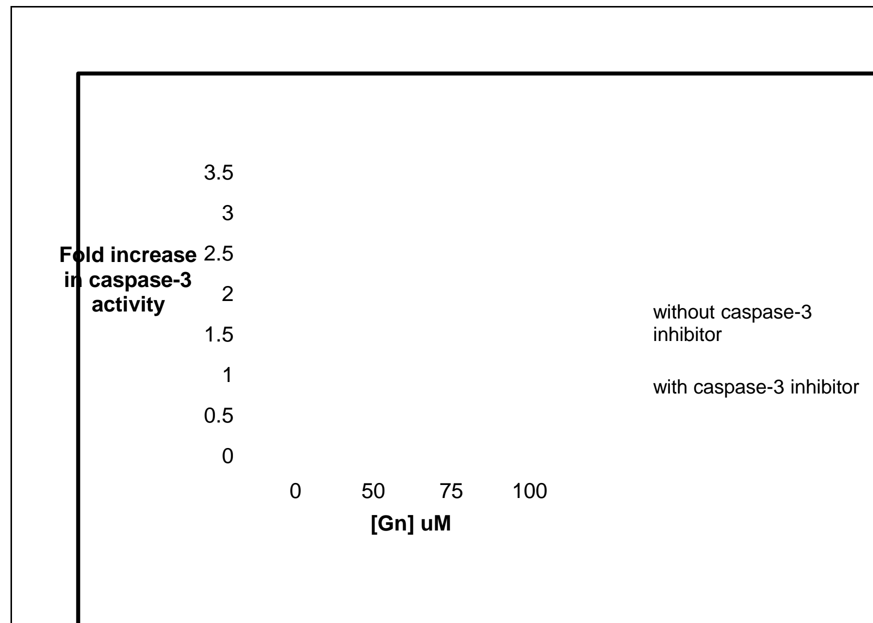

Figure 9. Effect of genistein treatment on caspase-3 activity in LNCaP cells after 24 hrs. Cells were treated changes in caspase-3 activity were determined. Data is representative of two independent experiments.

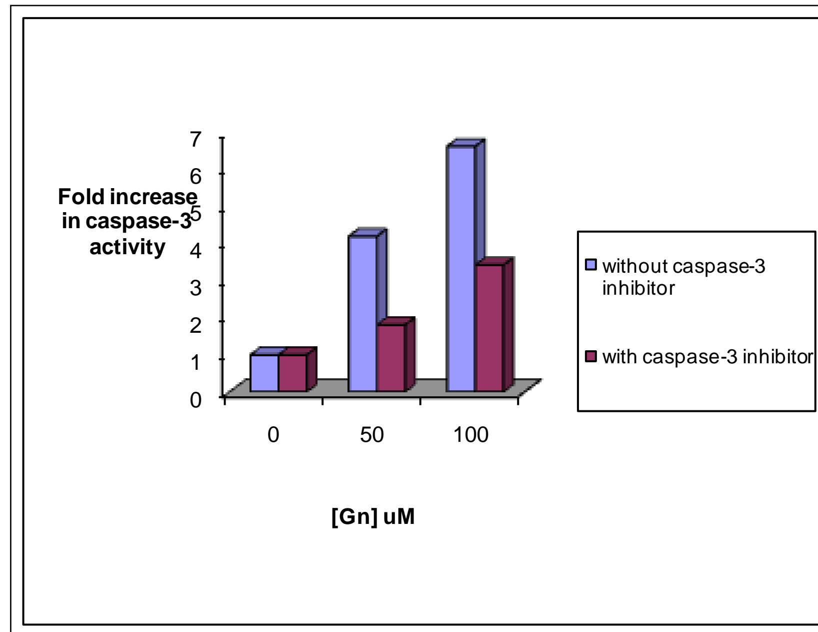

Figure 10. Effect of genistein treatment on caspase-3 activity in LNCaP cells after 48 hrs treatment. Cells were treated and changes in caspase-3 activity were determined. Data is representative of two independent experiments.

6.4. Microarray Analysis

The effect of genistein on gene expression in LNCaP cells was investigated using microarray analysis. The Affymetrix human gene chip U133 plus 2.0 array was used to compare expression of genes in genistein treated and untreated LNCaP cells. We found that there was differential expression of numerous genes following genistein treatment. A 2-fold difference in expression, compared to the control, was considered significant. A functional annotation cluster analysis of all these genes was done and changes in specific pathways were investigated based on KEGG annotations (Kanehisa et al, 2002). A number of pathways were found to be affected by genistein treatment. The cell cycle pathway was the most affected, with 51 genes being down-regulated (Appendix). The p53 signaling pathway was also affected with 14 genes being up-regulated and 14 being down-regulated.

The main cell cycle genes affected include the kinesins, the cyclins and cyclin dependent kinases that are involved in regulation of the cell cycle (Table 2). Other genes that were down-regulated include TGFB2, and TP53 Li Fraumeni (the mutated form of the p53 gene), which are known to be involved in tumor progression. Up-regulated genes include p53 related proteins, mdm-2 and cdk inhibitors 1a and 2b (Table 2).

Table 2. Cell cycle genes showing differential expression after treatment with genistein

Gene symbol	Gene name	Fold change
Down-regulated genes		
CCNB1	Cyclin B1	13.4
CCNB2	Cyclin B2	13.2
CCNH	Cyclin H	3.0
CCNE2	Cyclin E2	5.4
CNNE1	Cyclin E1	2.7
CCNF	Cyclin F	5.2
CDK2	Cyclin dependent kinase 2	4.5
CDK3	Cyclin dependent kinase 3	7.1
CDK4	Cyclin dependent kinase 4	2.2
CDK5	Cyclin dependent kinase 5	2.7
TP53	Tumor protein p53 (Li Fraumeni syndrome)	2.6
TGFB2	Transforming growth factor beta 2	3.7
tfdp1	Transcription factor dp-1	5.2
Up-regulated genes		
Gene symbol	**Gene name**	**Fold change**
Mdm2	Double minute 2 protein	3.7
TP53AP1	TP53 activated protein 1	2.0
TP53INP2	Tumor protein p53 inducible nuclear protein 2	6.6
CDKN1A	Cyclin dependent kinase inhibitor 1a	3.2
CDKN2B	Cyclin dependent kinase inhibitor 2b	5.3

A number of apoptosis related genes were affected as well, (Table 3) including members of the Bcl-2 family. Bcl-2 and Bcl-x which are anti-apoptotic members were found to be down-regulated following genistein treatment and Bam which is an apoptosis inducer was found to up-regulated. Other genes that were affected include members of the TNF superfamily which are involved in the apoptotic pathway. Genistein was found to have no effect on bax, maspin and caspase-3 expression levels.

Table 3. Apoptosis-related genes showing differential expression after treatment with genistein

Down-regulated genes		
Gene symbol	**Gene name**	**Fold change**
Bcl-2	B-cell CLL/lymphoma 2	4.8
Bcl-X	Bcl-2 like-1	3.0
BCLAF1	Bcl-2 associated transcription factor 1	2.4
BCL11A	B-cell CLL/lymphoma 11a (zinc finger protein)	2.1
NFKB1	Nuclear factor NF-kappa-B p105 subunit	2.8
FAIM2	Fas apoptotic inhibitory molecule 2	16.1
MAP2K5	Mitogen activated protein kinase 5	2.8
Down-regulated genes		
Gene symbol	**Gene name**	**Fold change**
Fas	TNFreceptor superfamily member 6	4.0
TNFSF9	Tumor necrosis factor (ligand) superfamily member 9	2.9
BIRC3	Bacuviral IAP repeat-containing 3 (Apoptosis inhibitor 2)	2.2
BAM/BIM/ Bcl2L11	Bcl-2 like-11	6.9
BCOR	Bcl6 corepressor	3.7

A few genes involved in antigen signaling and presentation were up-regulated such as the MHC cell surface receptors and a few cytokines that are involved in immune cell recognition (Table 4). Other genes affected include integrins (4, 6 and 8), lamins b1 and b2 and connexins which are involved in cell-cell adhesion or communication (Table 4). Genistein had no significant effect on VEGF expression within the cells.

Table 4. Cell adhesion and communication genes showing differential expression after treatment with genistein

Down-regulated genes		
Gene symbol	**Gene name**	**Fold change**
ITGB4	Integrin beta 4	2.8
ITGB8	Integrin beta 8	10.7
Itga6	Integrin alpha 6	2.6
col4a2	Collagen type IV alpha 2	24.6
LMNB2	Lamin b2	4.5
Lmnb1	Lamin b1	9.1
LAMB1	Laminin beta 1	3.0
ctnnb1	Catenin (cadherin-associated protein) beta 1/Beta catenin	2.9
RAC2	ras-related c3 botulinum toxin substrate 2	3.1
PAK6	p21 (cdkn1a) – activated kinase 6	2.4
GJB3	Gap junction protein beta 3 (connexin 31)	4.9
Gjb2	Gap junction protein beta 2 (connexin 26)	4.5
KRT19	Keratin 19	2.2
KRT7	Keratin 7	4.5
CTGF	Connective tissue growth factor	9.3
Up-regulated genes		
Gene symbol	**Gene name**	**Fold change**
HLA-E	Major histocompatibility complex class I,E	3.0
HLA-G	Major histocompatibility complex class I,G	2.2
HLA-B	Major histocompatibility complex class I,B	2.0
HLA-C	Major histocompatibility complex class I,C	2.0
HLA-DMA	Major histocompatibility complex class II, DM alpha	2.3
ICOSLG	Inducible T-cell co-stimulator ligand	2.5
PDGFA	Platelet-derived growth factor alpha polypeptide	2.0
ITGA3	Integrin alpha 3	3.3
LAMA 3	Laminin alpha 3	6.5
col6a1	Collagen type VI alpha 1	2.1
LAMC2	Laminin gamma 2	2.8

There were 67 genes down-regulated by genistein showing a 20-fold expression change or higher. These genes were further analyzed using the DAVID annotation tool and a functional annotation cluster was performed. The results revealed three gene clusters containing genes with similar functions (Table 5). Group 1 had 24 genes and groups 2 and 3 both had 3 genes; the other 38 genes were omitted from the cluster by the program.

In group 1, the kinesins were significantly affected showing fold-changes ranging from 22 to 87. Other genes in the group were found to be involved in regulation of the cell cycle. The second group (Table 5) included the MCM10 gene, which is involved in formation of the pre-replication complex. MCM10 interacts with the origin of recognition complex (ORC) and two other regulatory proteins CDC6 and CDT1. The genes that code for the ORC subunit were also down-regulated (ORC1 – 15.4, ORC2 – 2.9 and ORC6 – 5.8). CDC6 (12.4) and CDT1 (32.9) were not included in the cluster but the microarray analysis revealed that they were down-regulated as well. This indicates that genistein-induced cell death involves halting cell cycle progression and preventing further replication of cancer cells. The third group contained genes that code for transcription factors E2F2 and E2F8, that are involved in transcription of cell cycle related genes.

There were 52 genes that were found to have a 10-fold or higher increase in expression levels. These genes were also analyzed further and clustered according to function. Only one functionally similar group of genes was found. Genes within this group included CD68, GPNMB and NEU1, which seem to play a role in immune cell recognition of the cancer cells. A number of other genes that play a role in tumor suppression and apoptosis induction were also up-regulated (Table 6).

Table 5. Functional cluster analysis of down-regulated genes showing differential expression change of 20-fold or higher

Group 1 – Gene name and function	Fold change
KIF14, kinesin family member 14 – involved in cytokinesis	87.0
KIF2C , kinesin family member 2C - important for anaphase chromosome segregation and coordinates onset of sister chromosome separation	60.3
KIF15, kinesin family member 15 – involved in mitosis	49.0
KIF4A, kinesin family member 4A - may play a role in mitotic chromosomal positioning and bipolar spindle stabilization	39.5
KIF23, kinesin family member 23 – involved in organelle transport and movement of chromosomes during cell division	22.0
KIF20A, kinesin family member 20A – required for cytokinesis; targets PLK1 during anaphase and telophase; involved in transport of Golgi membranes and vesicles along microtubules	57.7
BUB1, budding uninhibited by benzimidazoles 1 homolog – involved in spindle checkpoint function	44.7
PLK1, polo-like kinase 1 – regulates dissociation of cohesion from chromosomes in mitosis	31.2
PLK4, polo-like kinase 4 – complexes with microtubule-based structures found in centrosomes and regulates centriole duplication during the cell cycle	22.9
CENPF, centromere protein F, 350/400ka (mitosin) – plays a role in chromosome segregation during mitosis	33.9
ESPL1, extra spindle poles like-1 – caspase-like protease;0 plays central role in chromosome segregation during anaphase	30.7
DLG7, discs large homolog 7 – cell cycle regulator; regulates adherens junction integrity and differentiation that may be involved in adhesion and signaling	28.3
TTK, TTK protein kinase – involved in cell proliferation	26.3
NEK2, NIMA (never in mitosis gene a)-related kinase 2 – may play a role	

in centrosome splitting during G2/M transition	39.9
Mki67, antigen identified by monoclonal antibody ki-67 – required for maintaining cell proliferation	33.9
CCNA2, cyclin A2 – expressed in G2 phase; plays a role in control of cell cycle at G1/S and G2/M transitions	25.9
NCAPH, chromosome-associated protein/barren homolog 1 – regulatory subunit of condensing complex that converts chromatin to mitotic-like condense chromosomes during interphase	25.5
CDC25C, cell division cycle 25C – inducer of mitotic control; plays a role in progression of cell cycle	28.9
BIRC5, Baculoviral iap repeat-containing 5 (survivin) – member of IAP (inhibitor of apoptosis) gene family that prevents apoptosis; component of chromosomal passenger complex (CPC) that is key regulator of mitosis; needed for proper chromosome alignment and segregation; plays role in chromatin-induced microtubule stabilization and spindle assembly	75.7
SPC25, spindle pole body component 25 homolog – involved in kinetochore-microtubule interaction and spindle checkpoint activity	64.0
CENPO (centromere protein O), hypothetical protein mgc11266 – component of complex that is involved in assembly of kinetochore proteins, mitotic progression and chromosome segregation	36.3

Group 2 – Gene name and function	**Fold change**
MCM10, minichromosome maintenance deficient 10 – key component of pre-replication complex; plays role in initiation of genome replication	37.0
NEIL3, - involved in base excision repair	24.9
ST18, binds to DNA sequence and represses basal transcription activity from target promoters	23.1

Group 3 – Gene name and function	**Fold change**
E2F2, e2f transcription factor 2 – transcriptional activator involved in cell cycle regulation and DNA replication; involved in cell-cycle progression from G1 to S phase	31.0
E2F8, e2f transcription factor 8 – involved in repressing expression of e2f target genes in S-phase of cell cycle	37.2

Other down-regulated genes not included in the functional cluster

Gene name and function	Fold change
PHF19, phd finger protein 19 – involved in regulation of transcription	24.9
TCF19, transcription factor 19 – potential trans-activating factor; could play a role in transcription of genes during cell cycle progression	23.1

Table 6. Functional cluster analysis of up-regulated genes showing differential expression change of 10-fold or higher

Group 1 – Gene name and function	**Fold change**
NEU1, sialidase 1 (lysosomal sialidase) – important for function of macrophages	12.1
CHPF, chondroitin polymerizing factor – involved in transfer of glucoronic acid to non-reducing end of elongating chondroitin polymer	10.4
GPNMB, glycoprotein (transmembrane) nmb – tumor associated antigen	26.3
CD68, CD68 molecule – plays a role in phagocytic activity of macrophages	12.1
Insig1, Insulin induced gene 1 – ER membrane protein that plays a role in regulating cholesterol levels in cell	10.1
Other up-regulated genes not included in cluster	
Gene name and function	**Fold change**
BrunoL6, bruno-like 6 RNA binding protein – involved in regulation of pre-mRNA alternative splicing and mRNA editing and translation	116.0
DEFB1, defensin beta 1 – antimicrobial peptide involved in resistance of epithelial surfaces to microbial colonization; plays role in innate immune response	28.8
TP53INP1, tumor protein 53 inducible protein 1 – promotes phosphorylation of p53 and apoptosis	14.3
GSN, gelsolin (amyloidosis) – actin binding protein that acts as regulator of cell growth and apoptosis	13.4

6.5. RT-PCR Analysis

A few genes were selected and subjected to RT-PCR analysis to confirm the results of the microarray. The selected genes include bax, bcl-x, mdm-2, vegf and b-actin, which was used as a loading control. The results indicate that mdm-2 showed an increase in expression when cells were treated with genistein (Fig 11), which correlates with the microarray results (Table 2). Vegf and bax expressions showed no significant change after genistein treatment (Fig 11), which also correlates with microarray results. Bcl-x showed a slight decrease in expression level as revealed by both PCR (Fig 11) and microarray analysis (Table 3). These results indicate that the PCR results are consistent with the microarray data obtained and confirms the effects of genistein on gene expression within the prostate cancer cells.

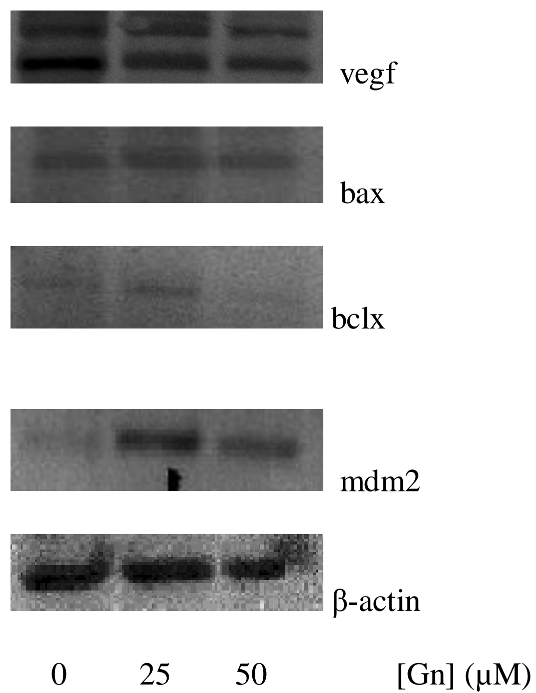

Figure 11. RT-PCR analysis of selected genes showing differential expression in LNCaP cells. LNCaP cells were treated with genistein at concentrations of 25 and 50 uM. After 48hrs treatment, RNA samples were extracted and subjected to PCR analysis using gene specific primers. PCR products were analyzed on a 1% agarose gel stained with ethidium bromide. β-actin was used as a loading control.

7. Discussion

7.1. Growth inhibition of LNCaP cells

Genistein has been shown to induce cell death in cancer cells through apoptosis and previous studies have indicated that this is a time and dose dependent effect (Kumi-Diaka et al, 2004; Merchant, 2005). The results of the ATP and MTS proliferation assays are consistent with these studies and indicate the chemosensitivity of LNCaP prostate cancer cells towards genistein. Based on our results the EC_{50} of genistein was found to be between 50 and 60 μM. This is also consistent with the results of previous studies. Previous studies reported an EC_{50} of about 40 μM (Onozawa et al, 1998; Shen et al, 2000) while another study found the EC_{50} to be about 50 μM (Zhou et al, 1999).

Genistein is also able to exert its effects *in vivo*, which also seem to be dose-dependent. In a recent study, plasma concentrations of genistein ranging from 1.4 to 6 μmol/L, were detected in healthy women after consumption of soymilk powder (2-8 mg/kg of body weight) (Xu et al, 1995). Another study showed that increasing concentrations of genistein reduced tumor volumes in mice by as much as 40% (Zhou et al, 1999). Genistein is able to exert its effects at low physiological concentrations, but there is a significantly greater effect on apoptosis within the cancer cells at higher concentrations, indicating that other effects such as regulation of gene expression may be dose-dependent as well. In Japanese men, soy consumption is very high and plasma levels detected ranged from 7.8 – 276 nmol/L (Adlercreutz et al, 1993). In individuals

who do not have a high level of soy in their diet plasma concentrations can range from 4.3 to 16.3 μM after consumption of about 150 mg in the form of a capsule (Takimoto et al, 2003). In this study, physiological levels were found to affect tyrosine phosphorylation *in vivo*, but since genistein's effect has been proven to be dose-dependent, our study used a concentration range close to the EC_{50} value that was found in our preliminary studies.

7.2. Apoptosis and caspase-3 induction

Activation of caspases results in DNA fragmentation and ultimately cell death. The results of the apoptosis and caspase-3 assays revealed that genistein can induce apoptosis and increase caspase-3 activity within the cells, as shown previously (Jagadeesh et al, 2006; Kumi-Diaka et al, 2000). Genistein's effect on apoptosis in the LNCaP cells was found to be dose-dependent, as observed from the results of the flow cytometry experiment.

Cells in early apoptosis were detected as low as 25 μM of genistein treatment and this percentage increased with the concentration of genistein. At higher doses, the percentage increase was not significant as that seen in lower doses, which correlates with our observations of growth inhibition of genistein, where the effects seen were significant at or around the EC_{50} value. Previous studies have shown that genistein not only induces apoptosis in prostate cancer but in other cancers as well (Buchler et al, 2003; Gercel-Taylor et al, 2004; Kumi-Diaka et al, 2000; Nakagawa et al, 2000), suggesting a potential therapeutic role for genistein in other cancers.

Caspase-3 activity was also increased after exposure to genistein. Caspase-3 is an effector caspase and plays a central role in apoptotic cell death, so detection of this caspase indicates activity of other up-stream caspases. Once activated it has been shown to initiate fragmentation of DNA which ultimately leads to cell death (Shi, 2002). The apoptotic pathway is regulated by a number of genes within the cell, so investigating the effect of genistein on the expression of these genes may help to identify potential targets for cancer therapy.

7.3. Gene expression profiles

The microarray results revealed that a number of genes were affected by genistein. Several major pathways were shown to be affected including the cell cycle pathway, p53 signaling pathway, and apoptosis pathways. From previous studies, we know that genistein affects gene expression at even low concentrations. Takahashi et al (2004) found that at physiologically achievable concentrations of genistein (< 10 µM), some pathways were affected, but other pathways, such as the cell cycle, DNA damage and stress response pathways, needed higher concentrations to elicit a significant response. Another study showed that at very high genistein concentrations (100 µM), not more than four genes were down-regulated and only seven genes were up-regulated by more than a 2-fold change in expression (Suzuki et al, 2002). This suggests a concentration dependent effect of genistein as observed in this study.

Previous studies done with the PC3 cell line show similar results indicating that genistein affects a number of major pathways within the cell (Li et al, 2002). In that study, the authors reported changes in gene expression after just 6 hrs of treatment.

Similar results have also been reported in other cancer types such as bladder, pancreatic and breast cancer (Bai et al, 2004; Chen et al, 2000; Chen et al, 2003). These studies also indicate that genistein affects genes that are involved in the cell cycle and signal transduction pathways. The results of our study are in conformity with these reported observations.

7.4. Down-regulation of cell-cycle related genes by genistein

Genes showing a > 20-fold inhibition were clustered according to function and we found that the majority of the genes within the cluster were involved in the cell cycle. This suggests that the major mechanism of treatment-induced cell death involves regulation of genes that control the cell cycle. If cell cycle genes are down-regulated this will halt progression of the cell cycle leading to apoptosis within the cell.

7.4.1. Kinesins

Gene expression for the kinesin family was significantly affected. Kinesins are a large super-family of motor proteins that are involved in a wide range of cellular processes. These ATPases are involved in the movement of molecules along microtubules within the cell. Kinesins also support several cellular processes such as mitosis, meiosis and cargo transport. Genistein treatment resulted in the down-regulation of a number of mitotic kinesins, indicating that these genes may be molecular targets for cancer treatment.

Kinesin 14 and 2C were the most down-regulated genes (fold-changes of 87 and 60.3 respectively). Kinesin 14, has been found to play a major role in cytokinesis and

previous studies indicate that silencing of this gene results in cell cycle disruption, cytokinesis failure and decrease in metastatic potential (Carleton et al, 2006) Over-expression was associated with a poor prognosis in several cancer types (Corson et al, 2006; Corson et al 2007). Kinesin 2C is involved in chromosome segregation in mitosis and over-expression has been demonstrated to be associated with progression of breast carcinogenesis (Shimo et al, 2008). All the other kinesins affected presently were found to be involved in cell cycle regulation including kinesin 4A, which is also involved in chromosome segregation and spindle formation. Deletion of this gene in mice has previously been shown to be associated with the DNA damage response (Wu et al, 2008).

Kinesins are involved in other cellular processes, but these data collectively suggest that genistein is targeting the mitotic kinesins in order to disrupt the cell cycle and induce cell death. This further indicates the mechanistic specificity of genistein treatment. Potentially this suggests that there may be less toxic side effects with the use of this phytochemical in cancer therapy.

7.4.2. Other cell cycle-related genes

A number of other cell cycle related genes were significantly down-regulated by genistein. These include PLK1, PLK4 and NEK2 which are involved in regulation of the centrosome cycle and formation of the mitotic spindle and BUB1 and TTK. Both are involved in the spindle assembly checkpoint that directs proper attachment of the chromatids to the mitotic spindle. Studies have found that alterations or deletions in any of these genes result in mitotic arrest and ultimately apoptosis within the cell (Malumbres et al, 2007).

BIRC5 (survivin) is a component of the chromosomal passenger complex (CPC), a key regulator of mitosis. This gene was also significantly down-regulated by genistein (75.7 fold-change). Decreased expression of this gene has been shown to be associated with inhibition of cellular proliferation and increase in apoptosis while over-expression was associated with increased cell proliferation and aggressive phenotypes in prostate cancer (Koike et al, 2007).

7.4.3. Cyclins and cyclin dependent kinases

The cyclins and cyclin-dependent kinases (Cdks) play a major role in cell cycle regulation. The cell cycle has two major checkpoints; G1/S checkpoint and G2/M checkpoint. These checkpoints are controlled by the cyclins and Cdks to ensure that the cell cycle does not progress if there is damage within the cell (Foster, 2008). Our data revealed that cyclin A2 was the most down-regulated gene from this family (25.9 fold-change). The G2/M checkpoint is controlled by interaction of cyclin A/cdk2 and silencing of this gene was found to delay entry into mitosis (De Boer et al, 2008). Over-expression of this gene was associated with poor prognosis in breast cancer patients (Husdal et al, 2006). Cyclin B1 and B2 were also down-regulated and in a study that was conducted to investigate cyclin B levels and cell cycle progression, co-depletion of both cyclins resulted in mitotic arrest within the cells (Soni et al, 2008). These key players in the cell cycle are often seen as potential therapeutic targets for cancer treatment; down-regulation of these genes by genistein is further indication of genistein's potential as an anticancer agent.

7.5. Up-regulation of cell-cycle related genes by genistein

Bruno-L6 was the most up-regulated gene, showing a fold-change of 116.0. This gene plays a role in mRNA processing and alternative splicing and belongs to a family of proteins that regulate mRNA stability and translation (Ladd et al, 2004). A study of the *Xenopus* homolog of this gene revealed that it mediates sequence specific deadenylation and repression of Eg5 (Kinesin 5) (Paillard et al, 1998), indicating that up-regulation of this gene by genistein may play a role in the down-regulation of the kinesins.

GPNMB, which was found to have a fold-change of 26.3 is involved in growth delay and metastatic potential and increased expression has been demonstrated to be correlated with increased survival in glioblastoma patients (Kuan et al, 2006).

7.5.1. p53-related genes

TP53INP1, TP53INP2 and TP53AP1 were also found to be up-regulated by genistein. TP53INP1 is expressed during cellular stress and over-expression induces apoptosis (Tomasini et al, 2003). It was also found to interact with HIPK2 to regulate p53 activity. TP53AP1 is a p53 target protein that also functions in response to cellular damage (Takei et al, 1998). Another p53 associated protein found to be up-regulated by genistein is gelsolin (GSN). GSN functions as a regulator and effector of apoptosis (Kwiatkowski, 1999) and high levels have been associated with longer survival in bladder cancer patients while low levels were found to be associated with p53 mutation (Sanchez-Carbayo et al, 2007).

In our study, mdm-2, which inhibits p53 activity, was also up-regulated by genistein (3.7 fold-change). This expression change was confirmed using PCR analysis.

Increased expression of mdm-2 was unexpected and may explain why we didn't see a greater increase in the expression levels of these p53 associated proteins. Without increased mdm-2 expression we may have seen a greater effect on apoptotic induction.

7.5.2. Apoptosis related genes

7.5.2.1. Caspases

There was no significant change in the expression of caspase-3 after genistein treatment, even though the caspase-3 assay showed increased activity after treatment. Caspases are synthesized as pro-enzymes that are inactive and require proteolytic activation (Shi, 2002). A possible explanation could therefore be that genistein is involved in post-translational activation of caspase-3 and not regulation of gene expression within the cells.

7.5.2.2. Bcl-2 family

The anti-apoptotic proteins Bcl-2 and Bcl-x were down-regulated and Bim was up-regulated by genistein. A decrease in Bcl-x expression was observed using both microarray and PCR analysis. Cell survival depends on a proper balance of these proteins so effective agents are those that shift the balance in favor of apoptosis (Shore et al, 2005). As seen from our results, genistein induced cell death is aided by an increase in expression of pro-apoptotic member Bim and a decrease in expression of anti-apoptotic members Bcl-x and Bcl-2.

Gene expression of bax showed no significant change following genistein treatment (which was confirmed by PCR analysis), although protein levels were

increased (results not shown). This suggests involvement of genistein in post-translational activation of bax, as was seen with caspase-3.

7.5.2.3. Other apoptosis-related genes

Other apoptosis-related genes that were affected include members of the TNF superfamily that are involved in the extrinsic apoptotic pathway. The TNF receptor Fas and TNF ligand 9 (TNFSF9) were up-regulated by genistein and the Fas apoptotic inhibitory molecule 2 (FAIM2) was down-regulated. Expression changes of these genes involved in the extrinsic pathway suggest that treatment-induced cell death potentially involves both apoptotic pathways.

Maspin expression levels showed no significant change. This was confirmed by western blot analysis (results not shown). Maspin expression is decreased in prostate cancer (Zou et al, 2002) and expression levels were not detectable by PCR analysis. Therefore, our hypothesis that genistein is able to induce maspin expression levels was rejected.

7.5.3. Genes involved in cell adhesion and communication

A number of cell adhesion genes were affected by genistein. Down-regulated genes included integrin beta 4, 6 and 8 which are cell surface receptors involved in cell attachment and signal transduction and laminin beta 1which is the major component of the basal lamina. Lamins are nuclear proteins that are the major component of the nuclear membrane and were also found to be down-regulated as well. Lamins have been reported to interact with DNA histones and play a major role in the breakdown of the nuclear

envelope and post-mitotic nuclear reassembly in mitosis (Gotzmann et al, 1999). These cell-adhesion proteins play a role in metastasis, so down-regulation of these genes suggests a mechanism by which genistein may be able to inhibit metastasis in cancer cells.

MMPs and VEGF play a major role cancer metastasis and previous studies done correlate genistein treatment with decreased protein activity (Guo et al, 2007; Kumi-Diaka et al 2006; Merchant, 2005). Presently, however, microarray and PCR analysis revealed no significant changes in gene expression levels following treatment, which may again indicate involvement of genistein in post-translational activation of these genes.

7.5.3.1. Genes involved in immune response

Genistein treatment resulted in up-regulation of a number of genes that play a role in the immune response within the body. Neu1 and CD 68 were both up-regulated and are both involved in immune recognition of cells. Neu1 is involved in differentiation of monocytes into macrophages and is important for the primary function of macrophages (Liang et al, 2006) while CD68 is an antigen marker that promotes phagocytosis and mediates recruitment and activation of macrophages.

Another group of genes affected by genistein include the MHC proteins (major histocompatibility complex) that are cell surface antigens involved in immune recognition of cells. Foreign particles or proteins are displayed on the cell surface by MHC receptors, which present them to immune cells. Cancer cells are able to evade the immune system by mutating these receptors or hiding them, so up-regulation of genes

that code for these receptors indicate that genistein my play a role in activation of the immune response within the body.

DefB1 expression is normally lost during prostate cancer (Donald et al, 2003) and was found to be up-regulated by genistein (28.8 fold-change). This gene has antimicrobial activity towards bacteria and fungi and is involved in chemo-attraction of immune cells (Yang et al, 1999; Zucht et al, 1998).

Up-regulation of these genes, suggests a role for genistein as an adjuvant in immunotherapy. Current research trials in immunotherapy have shown that activation of immune cells such as macrophages or dendritic cells enables recognition of the cancer cells and leads to eradication of the tumor and disease-free survival of patients (Simons et al, 2006; Yamamoto et al, 2008). In our studies genistein both up-regulated and down-regulated several genes that are involved in carcinogenesis and immunogenicity. This implies that genistein as a therapeutic adjuvant may enhance standard immunotherapy and chemotherapy, help to reduce toxic side-effects and increase the patient’s response to therapy.

8. Conclusion

Our study provides evidence that genistein induces apoptosis and regulates the expression of several genes including genes that are involved in carcinogenesis and immunogenicity. A number of other molecular targets were also identified and further research will enable us to elucidate the significance of these genes and the mechanisms of genistein-induced apoptosis in prostate cancer cells.

9. Appendix

Table 7. Pathways affected by genistein treatment

Down-regulated genes involved in various pathways in the cell

KEGG Pathway	Gene count	%	p-Value
Cell cycle	51	2.9	1.2E-22
Pyrimidine metabolism	29	1.6	3.6E-09
Purine metabolism	36	2	0.00000012
DNA polymerase	11	0.6	0.000032
Pentose and glucuronate interconversions	11	0.6	0.000049
One carbon pool by folate	8	0.5	0.00036
Androgen and estrogen metabolism	15	0.9	0.00046
Porphyrin and chlorophyll metabolism	12	0.7	0.0012
Starch and sucrose metabolism	17	1	0.0035
Small cell lung cancer	17	1	0.0072
p53 signaling pathway	14	0.8	0.011
Methionine metabolism	6	0.3	0.023
Ether lipid metabolism	7	0.4	0.078
Alanine and aspartate metabolism	7	0.4	0.088
Lysine degradation	9	0.5	0.09

Up-regulated genes involved in various pathways in the cell

KEGG Pathway	Gene count	%	p-Value
Glycan structures - degradation	11	0.6	0.00016
Sphingolipid metabolism	11	0.6	0.0012
p53 signaling pathway	14	0.8	0.0043
ABC transporters - General	10	0.6	0.012
Antigen processing and presentation	14	0.8	0.017
Neurodegenerative Diseases	8	0.5	0.044
Glycan structures - biosynthesis 1	17	1	0.047
SNARE interactions in vesicular transport	8	0.5	0.05
N-Glycan degradation	5	0.3	0.051
Glycosaminoglycan degradation	5	0.3	0.051
Cholera - Infection	8	0.5	0.056
Aminosugars metabolism	6	0.3	0.085
Nucleotide sugars metabolism	3	0.2	0.087
Heparan sulfate biosynthesis	5	0.3	0.098

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