Methyl jasmonate and cis jasmone induce apoptosis in Pc-3 and LncaP prostate cancer lines

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METHYL JASMONATE AND CIS JASMONE INDUCE APOPTOSIS IN PC-3 AND LNCaP PROSTATE CANCER LINES

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Methyl Jasmonate and CIS Jasnone Induce Apoptosis in PC-3 and LNCaP Prostate Cancer Lines

is approved in partial fulfillment of the requirements for the degree of
Master of Science in Biochemistry

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ABSTRACT

Methyl Jasmonate and Cis Jasmone Induce Apoptosis in PC-3 and LNCaP Prostate Cancer Lines

by

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Methyl jasmonate (MJ) and cis jasmone (CJ), lipid-derived plant stress hormones, were studied for their effects against both hormone dependent (LNCaP) and hormone independent (PC3) human prostate cancer cell lines. Both 2 mM MJ or CJ inhibited the growth of the cell lines. An alamar blue assay was used to determine IC\textsubscript{50} values for MJ and CJ in both cell lines at 24 hrs, 48 hrs and 72 hrs. At 24 hrs the MJ IC\textsubscript{50} values were 2.25 and 2.05 while the CJ values were 3.00 and 1.25 in the PC3 and LNCaP lines respectively. Cell cycle analysis revealed that MJ and CJ induced apoptosis in both cell lines as well as activated caspase-3. An \textit{in vitro} assay showed that MJ and CJ did not significantly inhibit either 5-lipoxygenase or 15-lipoxygenase. 5-HETE was able to stimulate cell growth in the presence or absence of MJ in both the cell lines.
# TABLE OF CONTENTS

ABSTRACT............................................................................................................ iii

LIST OF FIGURES ................................................................................................ v

LIST OF TABLES.................................................................................................. vii

CHAPTER 1 INTRODUCTION .............................................................................. 1

Purpose of the study........................................................................................... 10

Hypothesis .......................................................................................................... 11

CHAPTER 2 LITERATURE REVIEW .................................................................. 12

CHAPTER 3 MATERIALS AND METHODS ....................................................... 17

Materials ............................................................................................................. 17

Methods .............................................................................................................. 18

CHAPTER 4 RESULTS AND CONCLUSIONS .................................................. 25

CHAPTER 5 CONCLUSIONS .............................................................................. 50

APPENDIX........................................................................................................... 53

REFERENCES .................................................................................................... 57

VITA..................................................................................................................... 63
LIST OF FIGURES

Figure 1  The caspase cascade ..........................................................................5
Figure 2  Biosynthetic pathway of jasmonic acid .................................................7
Figure 3  Chemical structures of MJ and CJ .......................................................8
Figure 4  Arachidonic acid metabolism .............................................................9
Figure 5  Structure of 5-HETE ......................................................................10
Figure 6  Hypothesis .......................................................................................11
Figure 7  Proliferation assay on PC3 .................................................................35
Figure 8  Proliferation assay on LNCaP ............................................................36
Figure 9  Alamar blue assay on PC3 .................................................................37
Figure 10 Alamar blue assay on LNCaP ............................................................38
Figure 11 Colony formation assay ....................................................................40
Figure 12 Flow cytometry with MJ on PC3 ......................................................41
Figure 13 Flow cytometry with CJ on PC3 .......................................................42
Figure 14 Flow cytometry with MJ on LNCaP ..................................................43
Figure 15 Flow cytometry with CJ on LNCaP ..................................................44
Figure 16  In vitro lipoxygenase assay with MJ and CJ ....................................45
Figure 17  In vitro lipoxygenase assay with MJ ..................................................46
Figure 18 Alamar blue assay with 5-HETE .......................................................47
Figure 19 Caspase-3 activities with MJ on PC3 ..............................................48

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Figure 20  Caspase-3 activity with CJ on PC3 ...................................................49
Figure 21  Conclusion .........................................................................................52
LIST OF TABLES

Table 1  IC$_{50}$ of MJ and CJ in PC3 and LNCaP ................................ 39
Table 2  Cell cycle analysis with MJ on PC3......................................................53
Table 3  Cell cycle analysis with CJ on PC3 ................................................54
Table 4  Cell cycle analysis with MJ on LNCaP ...........................................55
Table 5  Cell cycle analysis with CJ on LNCaP ............................................56
CHAPTER 1

INTRODUCTION

The male sex hormones called androgens are involved in the growth and development of the prostate gland, an endocrine gland supporting male reproductive system. Prostate cancer is usually an adenocarcinoma, a cancer of the epithelial cells which compose the inner lining of endocrine glands. Prostate cancer is typically a slow growing cancer. Prostate cancer cells can metastasize to the lymph nodes, bones and other organs [1]. Cancer occurrence and the death rates vary among racial and ethnic groups. African American men have a 24% higher prostate cancer incidence rate than caucasians. Prostate cancer death rates in African American men are more than twice the rates in Caucasian men [2]. The estimated prostate cancer cases in 2006 will be 234,460 and the estimated prostate cancer deaths will be 27,350 [3]. Prostate cancer is the second most common cause of cancer death among men aged 80 years and older [3].

Prostate cancer can be treated in several different ways. Since testosterone is the most important male sex hormone that influences the behavior of the prostate gland and prostate cancer, its removal or inhibition can effectively slow down the growth of prostate cancer [4]. The major disadvantage
of hormonal therapy is that the prostate cancer cells eventually become resistant to hormonal therapy. Both radiation therapy and chemotherapy are used to treat prostate cancer. These treatment methods are not totally effective because they yield too many side effects, and sometimes the cancer cells grow back. The treatment options available for prostate cancer patients are considered to be ineffective, because, such treatment methods designed to kill cancer cells are dangerous to healthy cells [5]. Therefore, research is on going to discover new treatment methods and new agents that directly and selectively kill human cancer cells while leaving the healthy cells untouched.

Several plant compounds have been discovered in recent years that show cytotoxic effects against various human cancer cells. A number of plant stress hormones have been shown to suppress the proliferation and induce apoptosis in various human cancer cells [6]. Further research is required to understand the properties and their mode of action on cancer cells. Jasmonates are among such compounds.

Cell death can occur in two different ways, necrosis or apoptosis. Cell death due to injury such as exposure to toxic chemicals or mechanical damage in which the cell ruptures open is known as necrosis [7, 8]. Another form of cell death is programmed cell death or apoptosis. In apoptosis, the cells shrink; the chromatin in the nucleus condenses / degrades and mitochondria release cytochrome c [9]. Apoptosis is an evolutionarily conserved process. Apoptosis is required for normal development. During development of an organism the surplus cells are eliminated by apoptosis. For example, the establishment of
synapses between neurons in the brain requires the surplus cells be eliminated by apoptosis. Cells of the immune system such as cytotoxic T lymphocytes kill virus-infected cells by inducing apoptosis [19].

The central component of apoptotic machinery is a proteolytic system involving a family of proteases known as caspases. To date, 14 caspases have been identified in mammals [20]. Caspases are intracellular cysteine proteases that cleave certain proteins after specific aspartic acid residues [21]. The precursor forms of caspases are expressed as proenzymes, consisting of three domains: an NH$_2$-terminal domain, a large subunit and a small subunit. The active forms of caspases are generated by cleavage between the domains to liberate one large and one small subunit, which associate to form an active enzyme. The NH$_2$ terminal domain is involved in the regulation of activation of caspases [19]. Caspases can be grouped in to three different categories: initiator caspases (caspases-2, -8, -9, -10), apoptotic executioners (caspases-3, -6, -7) and cytokine processors (caspases-1, -4, -5, -13). Initiator caspases activate or cleave the effector caspases, which degrade or activate other cellular proteins.

Apoptosis can occur by several different pathways (Figure 1, page 5). The death ligands such as Fas-L, when bound to the cell surface receptors induce the caspase cascade [10]. Apoptosis can also be induced through a mitochondrial pathway [10]. During the caspase cascade inactive forms of caspases are converted to active forms [10]. The initiator caspases that are first activated in the cascade are caspase-8 and caspase-9 [11]. Fas-L leads to the activation of caspase-8, which activates the apoptotic executioners like caspase-3 and
caspase-7 [12]. Cytochrome c is released from mitochondria when the mitochondrial pathway is activated during apoptosis. Cytochrome c associates with Apaf-1 (apoptotic protease activating factor-1) and caspase-9 to form the "apoptosome" [22]. Apaf-1 processes pro-caspase-9 to an active form. Active caspase-9 initiates the caspase cascade involving other downstream executioners like caspase-3 and -7 [23]. Mammalian caspase-3 plays important and specific roles in apoptosis, and is important for cell shrinkage, chromatin condensation and DNA fragmentation [24, 13]. The proteolytic activity of these caspases leads to digestion of structural proteins and cleavage of a broad range of cellular targets that are essential for cell survival, chromosomal degradation and phagocytosis of the cell [25]. Caspase-3, -6 and -7 are involved in the cleavage of DNA repair enzyme poly (ADP-ribose) polymerase, whose cleavage is required for apoptosis induction [25].
Fig 1: The caspase cascade. C3=caspase 3, C7= caspase 7, C8=caspase 8 and C9=caspase 9. Cyt= cytochrome c and Apaf = apoptosis protease activating factor. Caspases in circles are in the procaspase form while the caspases in the crescents are in the active form. Adapted from ref. [12].

Jasmonates are plant stress hormones, found in a wide variety of plants. Jasmonates are lipid derived compounds formed from linolenic acid, a 18-C fatty acid. Jasmonates play an important role in gene regulation in response to wounding, defense against pathogens, metabolic activities, reproduction, etc. [14]. Jasmonic acid biosynthesis begins with linolenic acid (see figure 2). The most important enzyme involved in Jasmonic acid biosynthesis is lipoxygenase. Oxygenation of linolenic acid by lipoxygenase gives 13-hydroperoxylinolenic acid, which is then acted upon by the enzyme hydroperoxide dehydrase to give
12,13-epoxy-linolenic acid, which undergoes cyclization by alleneoxide cyclase to yield the cyclopentanone derivative, 12-oxo-phytodienoic acid. 12-oxo-phytodienoic acid then undergoes reduction and three steps of β-oxidation to yield jasmonic acid [15].
Fig. 2 Biosynthetic pathway of jasmonic acid. Adapted from ref [15].
Metabolic transformation of jasmonic acid yields methyl jasmonate (MJ) and cis jasmon (CJ) (figure 3). MJ and CJ are involved in different biological activities in plants. Jasmonates are involved in regulation of gene expression in response to certain pathogens and wounding [14].

![Chemical structure of Methyl Jasmonate](image1)

a) Methyl Jasmonate

![Chemical structure of Cis-Jasmon](image2)

b) Cis-Jasmon

Fig:3 Chemical structures of Methyl Jasmonate and Cis-Jasmon

Recent research and growing evidence suggests that arachidonic acid (a 20-carbon eicosanoid precursor) increases the progression of human prostate cancer [16]. Arachidonic acid metabolism yields prostaglandins, leukotrienes, and other eicosanoids [16]. As shown in figure 4 arachidonic acid metabolism through 5-lipoxygenase leads to the formation of 5-hydroxyeicosatetraenoic acid
(5-HETE) (Figure 5, page 9). 5-HETE is important for the survival and progression of human prostate cancer cells [17].

Fig: 4 Arachidonic acid metabolism. Adapted from ref. [16].

Fig: 5 Structure of 5-HETE.
The aim of this study is to examine the effect of plant stress hormones, MJ and CJ on the growth and proliferation of human prostate cancer cells. MJ and CJ are downstream products of linolenic acid metabolism in plants, produced by lipoxygenase enzyme [15]. The growth and proliferation of human prostate cancer cells is dependent on the presence of 5-HETE, a metabolite, of the lipoxygenase pathway [18]. Since MJ and CJ are derived from a similar lipoxygenase pathway in plants, we hypothesize that, both MJ and CJ will be involved in feed back inhibition of 5-lipoxygenase in human prostate cancer cell lines (Figure 6, page11). The two human prostate cancer cell lines used in this study are, LNCaP (hormone dependent) and PC3 (hormone independent).
Figure 6: Hypothesis, Inhibition of 5-lipoxygenase by methyl jasmonate (MJ) and cis jasmine (CJ).
CHAPTER 2

LITERATURE REVIEW

The present options for the treatment of prostate radiation therapy, chemotherapy, are all largely ineffective because of too many side effects and the cancer cell recurrence of growth. There is a need for the discovery of effective treatment methods which efficiently and selectively kill cancer cells without affecting normal healthy cells. In recent years use of plant compounds in treating cancer diseases like has increased because of the natural availability of these compounds, and their tendency to reduce the side effects and be less toxic than chemically synthesized compounds. Among such plant products, jasmonates have been tested for their ability to kill cancer cells [6]. Jasmonic acid formed from the precursor linolenic acid through the lipoxygenase pathway is involved in several physiological processes in plant growth and development [15].

In recent years plant compounds such as perillyl alcohol (POH) and perillic acid (PA) have been tested for their effects on several types of human cancer cell lines including, human lung carcinoma, and human head and neck carcinoma [6]. Jasmonates have been shown to induce apoptosis in human leukemia, breast and prostate cancer cells [6]. Several other plant compounds
such as silibinin and silymarin (extracts from the seeds of *Silybum marianum*) possess antiproliferative effects on human prostate cancer cells [26]. These plant compounds being tested were antiproliferative and induced apoptosis in cancer cell lines.

Another plant stress hormone, sodium salicylate (SA) showed antiproliferative effects on prostate, breast, lymphoblastic leukemia and melanoma human cancer cells [27]. Jasmonic acid (JA) and methyl jasmonate (MJ) were shown to induce apoptosis through caspase-3 activity in Molt-4 cells. JA and MJ decreased the proliferation and induced cell death in a dose dependent manner in hormone dependent human prostate cancer cell line, LNCaP [27].

Antiapoptotic protein family members such as Bcl-2 and Bcl-X<sub>L</sub> are involved in maintaining the integrity and stability of the mitochondrial membrane. Antiapoptotic proteins interact with the permeability transition pore complex within the mitochondrial membrane to inhibit membrane permeabilization (in turn inhibiting the release of cytochrome c) whereas the pro-apoptotic proteins such as Bax and BaK enhance the membrane permeabilization. Mitochondria are shown to be the best targets for the action of anticancer drugs [28].

The plant stress hormone MJ induced the release of cytochrome c from the mitochondria isolated from Molt-4 cells [29]. MJ has also been shown to activate MAPK (mitogen activated protein kinase) and induce differentiation in leukemia cells [30].
Arachidonic acid has been shown to stimulate the proliferation of prostate cancer cells [31]. Arachidonic acid is metabolized by three different pathways: cyclooxygenase pathway, where it is metabolized to prostaglandins, epoxygenase pathway, where it is metabolized to epozym metabolites and lipoxygenase pathway, where it is metabolized to hydroxyderivatives of fatty acids and leukotrienes. Metabolites of the lipoxygenase pathway were found to be elevated in prostate cancer tissue [32]. In the lipoxygenase pathway 5-lipoxygenase (5-LO) is a key enzyme and has been shown to be elevated in human prostate cancer patients [32]. Inhibition of 5-LO by specific inhibitors like MK886 induced apoptosis in human prostate cancer cell lines and resulted in the reduced production of 5-HETE [33]. The survival of both hormone-responsive and hormone-non responsive human prostate cancer cells can be dependent on 5-HETE [33]. Inhibition of the 5-HETE family of eicosanoids from arachidonic acid, triggered apoptosis in prostate cancer cell lines [17].

Arachidonic acid metabolism through the action of lipoxygenase enzymes lead to the formation of eicosanoid products such as 5-hydroxyeicosatetraenoic acid (5-HETE). This 5-HETE eicosanoid has been reported to be important for the growth of prostate cancer cells [33]. Specific inhibitors of 5-LO such as AA861 and MK886 resulted in the inhibition of growth stimulated by arachidonic acid [33]. But, the addition of a 5-LO product, 5-HETE, stimulated the growth of prostate cancer cells [33, 34]. It has been reported that metabolic conversion of arachidonic acid by the lipoxygenases is necessary to show its mitogenic activity [33]. Recent studies have shown that the hydroxyderivatives of fatty acids and its
metabolites (such as 5-HETE) but not the leukotrienes are involved in the proliferation of PC3 (hormone independent human prostate cancer cell line) [33]. Diets rich in fatty acids such as arachidonic acid stimulate the proliferation of human prostate cancer cells [33].

5-LO, a key enzyme in the lipoxygenase pathway and, has been the focus of intense research during the last few years. The eicosanoid 5-HETE, which is the down stream product of 5-LO was shown to be an essential survival factor for human prostate cancer cells [34]. But the molecular mechanisms involved in the 5-HETE dependent survival of prostate cancer cells have not been elucidated. Six conserved histidine residues in the lipoxygenase enzyme that are important for the enzyme activity have been reported [36].

Stress activated protein kinases have been shown to play an important role in apoptosis. Inhibition of the 5-LO enzyme has been shown to activate JNK (c-jun N-terminal kinase), a stress activated protein kinase, in prostate cancer cells [35]. 5-HETE was also been shown to prevent the activation of JNK, which is important for apoptosis [35].

Jasmonates, especially methyl jasmonate was shown to induce cell death via induction of Bax/Bcl-Xs and activation of caspase-3 and -9 in A549 human lung adenocarcinoma cells [37]. This apoptosis was shown to be mediated by the production of reactive oxygen species [37].

Caspases play an important role in the cell death via apoptosis [12]. In various human cancer cells caspases are inactive and are inhibited [38]. In PC3, LNCaP and DU145 human prostate cancer cell lines, the caspases-1 and -3
have been reported to be expressed at low levels, compared to normal cells [39].

Staurosporine, a protein kinase C inhibitor, induces apoptosis in LNCaP cells [40]. Recent research shows that, staurosporine induces the release of cytochrome c from the mitochondria to the cytosol, and activates the caspase-3 and -7. The DNA repair enzyme poly (ADP) ribose polymerase (PARP) has been shown to be cleaved by caspase-3 and -7 in staurosporine treated LNCaP cells [40]. These studies indicate that, activation of caspase-3 and -7 are important events during apoptosis in LNCaP cells.
CHAPTER 3

3.1 MATERIALS

3.1.1 Chemicals and Reagents

Methyl jasmonate [methyl 3-oxo-2 (2-pentenyl) cyclopentanecacetic acid], cis-Jasmone [3-oxo-2 (2-pentenyl) cyclopentane], and propidium iodide (PI) and 5-HETE (5-Hydroxy-eicosatetraenoic acid) were purchased from Sigma Chemical Co., (St Louis, MO). AlamarBlue dye was purchased from BioSource International, (Camarillo, CA). BCA protein assay kit was purchase from Pierce, (Rockford, IL). Lipoxygenase inhibitor screening assay kit was purchased from Cayman chemicals, (Ann Arbor, MI). Caspase-3 Activity Assay Fluorometric Kit and Ham's F-12K medium was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, (Logan, UT), penicillin/streptomycin (P/S) was purchased from Invitrogen Incorporated (Grand Island, NY). Stock solutions of 1.0 M methyl jasmonate and cis jasmone were prepared in dimethylsulfoxide (DMSO) and the aliquots were stored at -20°C prior to use.

3.1.2 Cells and cell cultures

Hormone dependent (LNCaP) and hormone independent (PC3) human prostate adenocarcinoma cell lines were purchased from the American Type
Culture Collection (ATCC, Manassas, VA). Both cell lines were cultured in Ham's F-12K medium, which was supplemented with 10% FBS and 1% penicillin-streptomycin.

3.2 METHODS

3.2.1 Cytotoxicity Assay

The cytotoxic effects of methyl jasmonate and cis-jasmone were evaluated using alamarBlue assay. The alamarBlue reagent has a redox indicator which exhibits both fluorescence and colorimetric change based on metabolic activity of cells. Metabolic activity or growth of cells results in chemical reduction of alamarBlue, i.e., the redox indicator in the alamarBlue changes from oxidized (non-fluorescent, blue) to reduced (fluorescent, red). The fluorescence can be measured with excitation wavelength at 530-560nm and emission wavelength at 590 nm. Briefly, 5,000 cells/well were plated in 96-well plates and the plates were incubated overnight to allow cells to adhere and then treated with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM) of MJ and CJ in 100 µl of medium for up to 72 hrs. The control cells received medium with DMSO (≤0.1%, v/v). After exposure at the desired time periods, the treatment medium was aspirated off and replaced with 100 µl of fresh medium and 10 µl of alamarBlue in each well and the plates were incubated at 37 °C in a humidified, 5% CO₂ atmosphere for 3-4 hrs. Cell viability was assessed by Genios plate reader at 530-560 nm excitation and 590nm emission wavelengths. Cell viability in the treatment groups was compared to the control.
3.2.2 Colony Formation Assay (CFA)

The long-term effect of the methyl jasmonate and cis-jasmone on the proliferation of hormone dependent – LNCaP cell line and hormone independent – PC3 cell line was assessed by colony formation assay. Approximately 500 cells/well were plated in 6-well plates and incubated overnight to allow the cells to adhere. The following day, the control wells received medium containing 0.1% DMSO and the treatment wells received media with 2 mM MJ and 2 mM CJ, respectively. The 6-well plates were incubated for 24 hrs. After the treatment period, the medium from all the wells was aspirated off and the cells were rinsed twice with sterile PBS (phosphate buffer saline) and once with fresh medium. Fresh medium was added to all the wells of the 6-well plates and the cells were grown for 10-14 days. The plates were observed under the microscope every other day to check cell growth. At the end of the desired time period, the medium was aspirated off and the cells washed with sterile PBS and then stained with crystal violet (0.5 g/100ml in 95% ethanol). The colonies (each colony containing ≥ 50 cells) were counted in control wells and treatment wells. The number of colonies formed in the treatment group was compared to controls.

3.2.3 Proliferation Assay

The effect of MJ and CJ on the proliferation of human prostate adenocarcinoma cell lines was assessed by proliferation assay. Approximately 100,000 cells were plated in each p100 plate. The cells were allowed to adhere by incubating overnight at 37°C in the incubator. The experiment was divided into three groups: control group, “24-hrs drug exposure and wash-off” group and
treatment group (10 plates in each group). Following adhesion the control group received 0.1% DMSO. The wash-off group and the treatment group received 2 mM MJ or CJ. The plates were incubated for 10 days (10 plates in each group). After one-day, one p100 plate from each of the three groups (control, wash-off and treatment) was harvested with trypsin-EDTA and the number of live and dead cells was counted separately using a hemocytometer. The remaining nine plates from the wash-off group were rinsed with PBS twice and with fresh medium once. Fresh medium was added to all the plates of the wash-off group and incubated. After two days of exposure, one plate from each group: control, wash-off and treatment, were harvested using trypsin-EDTA and live and dead cells were counted using a hemocytometer. This procedure was followed everyday for up to 5 days and then the cells were harvested and counted every other day (7th, 9th, 11th, 13th, and 15th day). The effect of MJ and CJ on the growth and proliferation of LNCaP and PC3 cell lines was assessed by comparing the number of live and dead cells in the wash-off and treatment groups.

3.2.4 Lipoxygenase Assay

The effect of MJ and CJ on 5-lipoxygenase and 15-lipoxygenase was assessed by an in vitro assay, using a lipoxygenase inhibitor screening assay kit, purchased from Cayman Chemicals. This assay detects and measures the hydroperoxides produced in the lipoxygenation reaction using a purified lipoxygenase. This in vitro assay was carried out in a 96-well plate. The blank wells received 100 µl of the assay buffer, control wells received 90 µl of 5-lipoxygenase or 15-lipoxygenase enzyme with 10 µl of assay buffer and the treatment wells received
90 µl of 5-lipoxygenase or 15-lipoxygenase enzyme with 10 µl of the inhibitor (MJ or CJ). The concentrations of MJ and CJ used in this assay were 10 mM, 50 mM, 100 mM and 500 mM. The reaction was initiated by adding 10 µl of arachidonic acid (substrate) to all the wells. The 96-well plate was kept on shaker for 5 min. 100µl of chromogen was added to all the wells to stop the enzyme catalysis and to develop the reaction. The plate was again kept on a shaker for 5 min. The absorbance was then read at 490-500 nm using a plate reader. The percent inhibition of 5-lipoxygenase and 15-lipoxygenase enzymes by MJ and CJ were plotted.

3.2.5 Assay With 5-HETE

The effect of MJ in presence of 5-HETE was evaluated in cytotoxicity assay using alamarBlue. Both PC3 and LNCaP cell lines were tested. Briefly, 5,000 cells/well were plated in 96-well plate and the plate was incubated overnight to allow the cells to adhere. First treatment group received 2 mM MJ in 100 µl of medium; the other treatment group received 2 mM MJ with 1000 nM of 5-HETE and the third treatment group received 1000 nm 5-HETE alone, for 48 hrs. The control cells received medium with DMSO (≤0.1% v/v). After exposure to desired time period, the treatment medium was aspirated off and replaced with 100 µl of fresh medium and 10 µl of alamarBlue in each well and the plate was incubated at 37°C in a humidified, 5% CO₂ atmosphere for 3-4 hrs. Cell viability was assessed by Genios plate reader at 530-560 nm of excitation and 590 nm of emission wave lengths. Cell viability in the treatment groups was compared to the control.
3.2.6 Apoptosis Assays

3.2.6.1 Flow Cytometry DNA Analysis using Propidium Iodide Stain

To assess the effect of MJ and CJ on cell cycle progression and apoptosis, cell cycle analysis was carried out using flow cytometry. One million PC3 cells and LNCaP cells were cultured in 100 mm tissue culture plates. The cells were allowed to adhere to the bottom by incubating overnight. The following day, the control plates received 0.1% DMSO and the treatment plates received 1 mM, 2 mM and 3 mM MJ and CJ. The plates were incubated for 24 hrs, 48 hrs and 72 hrs. At the end of exposure time the cells were harvested from the control plate and the treatment plate using trypsin-EDTA and centrifuged at 850 rpm for 5 minutes. The supernatant was aspirated off and the pellet was resuspended in appropriate volume of fresh media. Total number of cells was counted in the control and treatment groups using a coulter counter. From the control and the treatment group, a cell suspension containing 500,000 cells was centrifuged. The pellet was resuspended in an appropriate volume of PBS, and centrifuged at 850 rpm for 5 min. The supernatant was removed and to the cell pellet 100 ul of the PBS was added and 900 ul of ethanol (95%) was added while vortexing. The samples were transferred to +4 °C refrigerator.

For DNA content analysis, the ethanol fixed cells were centrifuged at 850 rpm for 5 min to remove ethanol supernatant. To the pellet, DNA staining solution (0.1% Triton-X: 1 mg/ml RNase: 150 µg/ml PI in 1:1:1 ratio by volume) was added and the samples were incubated in the dark at room temperature for 30 minutes. The stained cells were used for the flow assay. Both the area and
width of the fluorescence pulse were measured using Cell Quest acquisition software (Becton Dickinson, San Jose, CA). Ten thousand events were acquired and the percentage of cells in G1, S and G2/M phases and apoptosis were determined by ModFit analysis, using ModFit LT version 3.0 analysis software (Verity Software House, Inc., Topsham, ME).

3.2.6.2. Caspase-3 Fluorometric Assay

To determine whether or not the caspase cascade is activated by MJ in PC3 cells, caspase-3 fluorometric assay was performed. The caspase-3 activity assay detects the caspase-3 activity in the cell lysates. Cell lysates were prepared from 2 mM MJ treated PC3 cells. Briefly, 5 million cells were cultured in 100mm tissue culture plates. Cells were allowed to adhere to the bottom of the plates by incubating overnight. Following day, the control plate received 0.1% DMSO and the treatment plates received 2 mM MJ. The plates were incubated for 3 hrs, 6 hrs, 12 hrs, and 24 hrs. At the end of the exposure time, the cells were harvested using trypsin-EDTA and centrifuged at 850rpm for 5 minutes. The cell pellet was resuspended in an appropriate volume of fresh media and the numbers of cells were counted in control and treatment group using a hemocytometer. The cell suspension was again centrifuged at 850rpm for 5min. The pellet is washed with PBS, centrifuged at 850rpm and resuspended in 1 ml of PBS. The cell suspension was transferred to microfuge tubes and centrifuged at 1000rpm at 4°C for 10min. The supernatant was removed and the pellets were stored at -80°C. Cell lysates were prepared from the pellets using the
sample buffer/extraction buffer provided in the kit. Both positive and negative controls were included according to the instructions provided in the kit.

Caspase-3 assay was performed in a 96-well plate. The blank wells received 50 µl of the sample/extraction buffer. The control cell lysate (50 ul) was added to the control well and 50 µl of each of the treatment cell lysates (prepared from PC3 cells treated with 2 mM MJ for 3 hrs, 6 hrs, 12 hrs, 24 hrs and the lysates prepared from PC3 cells treated with 2 mM CJ for 3 hrs, 6 hr, 12 hrs and 24 hrs) were added to the test wells. The assay buffer (50 ul) was added to all the wells including blank and control. Caspase-3-substrate (10 ul) conjugate was added to all the wells. The plate was read immediately after the addition of substrate, using a fluorescent plate reader at 360nm excitation and 465 nm emission (0 hr reading) and then the plate was incubated at 37°C and read every 30 minutes. A total of 8 readings were taken (0 hr, 30 min, 1 hr, 1.5 hr, 2.0 hr, 2.5 hr, 3.0 hr and 3.5 hrs reading). The data was expressed as relative fluorescence units with respect to the control.
CHAPTER 4

RESULTS AND DISCUSSIONS

The effects of methyl jasmonate (MJ) and cis jasmone (CJ) were tested on both PC3 and LNCaP prostate cancer cells in culture. Results with a dye exclusion (proliferation) assay showed both MJ and CJ can inhibit the growth of human prostate cancer cell lines. When exposed to 2 mM MJ, PC3 cell proliferation was decreased compared to the control as the time of exposure to the agent increased (Fig 7 panel A, page 35). When 2 mM MJ was washed-off after 24 hrs exposure in PC3 cells and supplemented with fresh media, no recovery of proliferation was observed. As expected the control (without MJ), showed increasing proliferation with time.

The effect of CJ was also tested on PC3 cells. These results were similar to that of MJ treatment on the PC3 cell line. As the number of days of exposure to CJ increased, the number of cells decreased (Fig 7 panel B, page 35). When CJ was exposed only for 24 hrs and the drug was washed away, and supplemented with fresh media, no recovery of proliferation was observed. Control cells proliferated with time. These results indicate that both the plant stress hormones MJ and CJ induce cell death at 2 mM.
MJ and CJ were also tested at 2 mM on the proliferation of hormone-dependent human prostate cancer cell line, LNCaP. As shown in the Fig 8, page 36, both MJ and CJ decreased the proliferation of LNCaP cells at 2 mM. As the number of days of exposure to MJ and CJ increased, proliferation decreased correspondingly. When either MJ (panel A) or CJ (panel B) were washed-off following a 24 hr exposure and LNCaP cells supplemented with fresh medium, the proliferation of the cells was not restored. In the control, the proliferation increased with time.

Alamar Blue assay was conducted to measure the cytotoxicity of MJ and CJ on both PC3 and LNCaP cells. Fig 9 (page 37) shows the cytotoxicity of these agents on the PC3 cell line. MJ inhibited the proliferation of PC3 (panel A) cells at all the concentrations tested (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM and 3.0 mM). The inhibition of proliferation was dose and time dependent. The concentration of the MJ that inhibits 50% proliferation of PC3 cells (Inhibitory concentration-IC\textsubscript{50\%}) at three different time points 24 hrs, 48 hrs, and 72 hrs were 2.75 mM, 2.37 mM, 1.50 mM respectively (Table 1, Page 39). These results indicate that MJ is toxic to the PC3 cells and the cytotoxicity increases as the time of exposure to the cells increases. The IC\textsubscript{50\%} concentrations decreased as the time of exposure to the agent increased.

The cytotoxicity of CJ was also tested on PC3 cells (Figure 9 panel B, page 37) at the concentrations: 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM and 3.0 mM. Viability in the treatment group was calculated as % viability compared to the control. The % viability in the treatment groups decreased as the
concentration of the drug increased and as the time of exposure to the drug increased. The IC$_{50}$% concentrations for the CJ at three different time points 24 hrs, 48 hrs and 72 hrs were observed to be 3.0 mM, 3.0 mM and 2.0 mM respectively (Table 1, page 39).

Both the jasmonates were tested for their cytotoxicity on the hormone dependent prostate cancer cell line LNCaP. The alamar Blue assay with MJ and CJ on the LNCaP cell line, indicated that LNCaP cell line is more sensitive to MJ and CJ compared to PC3 cell line, as indicated by the IC$_{50}$% concentrations. The concentrations of MJ and CJ used were 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM and 3.0 mM (Figure 10 page 38). MJ decreased the viability of the LNCaP cells compared to control (Figure 10 panel A), in all the treatment groups with IC$_{50}$% of 2.05 mM, 1.25 mM and 1.18 mM for 24 hrs, 48 hrs and 72 hrs respectively (Table 1, page 39). CJ was found to be more effective than MJ on LNCaP cell line (Figure 10 panel B). The effect of CJ on LNCaP cells was more pronounced at 72 hrs with IC$_{50}$% of 0.62 mM. For 24 hrs and 48 hrs of exposure CJ induced cytotoxicity with IC$_{50}$% of 1.25 mM and 0.75 mM respectively.

The alamar Blue assay using MJ and CJ on PC3 and LNCaP cell lines indicate that, both MJ and CJ are cytotoxic and cause a decrease in the proliferation of these cell lines. This inhibition of proliferation was dose and time dependent except for LNCaP cells treated with CJ which failed to show time dependence. At the highest concentration tested (3 mM) MJ or CJ, killed almost all the cells. The concentration of MJ and CJ which is near or slightly greater than
the IC_{50%}, for both PC3 and LNCaP, was used in the further experiments. (i.e., 2 mM MJ and 2 mM CJ were used).

The long term effects of the methyl jasmonate and cis jasmone were tested on the proliferation of PC3 and LNCaP cells, using a colony formation assay. Figure 11 (page 40) shows the long term effect of MJ and CJ on PC3 cells (panel A). MJ (2 mM) inhibited the proliferation of PC3 cells by 79% and 2 mM CJ inhibited the proliferation by 79%. The cells were exposed to MJ or CJ for 24 hrs, and the drug was then washed off and the cells were provided with fresh media. The cells were allowed to grow and form colonies for about 10-14 days. The number of colonies (> = 50 cells) formed in the treatment group are compared to the control. These results indicate that both MJ and CJ effectively inhibit the long term proliferation of PC3 cell line.

Colony formation assay with LNCaP cell line (Fig 11, panel B, page 40) showed that, MJ inhibited 83% of colony formation and CJ inhibited 87%. These results are consistent with the alamar blue assay, which showed that the LNCaP cell line is more sensitive to MJ and CJ than the PC3 cell line and CJ is more lethal to LNCaP cells compared to MJ.

Cell cycle analysis using flow cytometry was conducted using both MJ and CJ at 1 mM, 2 mM and 3 mM, to study the effects of these agents on cell cycle progression and apoptosis. The PC3 and LNCaP cells were exposed for 24 hrs, 48 hrs and 72 hrs to MJ or CJ, PI stained and analyzed using flow cytometry. The values of the percentage of cells in each phase of cell cycle and apoptosis in both PC3 and LNCaP cells with MJ or CJ are shown in the appendix (Table 2-5,
Figure 12, page 45 shows the effect of 1 mM MJ, 2 mM MJ and 3 mM MJ on cell cycle progression and apoptosis of PC3 cell line. When PC3 cells were exposed to MJ for 24 hrs, (panel A) 1 mM MJ caused an increase in G1 population. S-phase arrest was observed in 48 hrs and 72 hrs of exposure to 2 mM and 3 mM MJ (panel B and C).

Following 24 hrs exposure as the concentration of MJ increased, the percentage of apoptotic cells also increased. At 24 hrs 1 mM MJ induced 1.21% of apoptosis, 2 mM MJ and 3 mM MJ induced 7.32% and 11.23% apoptosis respectively (Figure 12, panel A). The control had only 0.87% of apoptosis. At 48 hrs, 1 mM MJ, 2 mM MJ and 3 mM MJ induced 5.14%, 5.56% and 12.84% of apoptosis respectively, but the control also had 5% apoptosis (panel B). The percentage of apoptosis increased at 72 hrs with 3 mM MJ (13.37%). At 72 hrs, 1 mM and 2 mM MJ induced 11.57% and 15.18% apoptosis while the control had 7.37% apoptosis (panel C). These results indicate that MJ causes cell death in PC3 cells by inducing apoptosis and cell cycle arrest at S-phase (as observed with 2 mM and 3 mM MJ treatment at 48 hrs and 72 hrs).

Figure 13, page 42 shows the effect of 1 mM, 2 mM and 3 mM CJ on cell cycle progression and apoptosis in PC3 cells. All the three different concentrations of CJ tested, induced cell cycle arrest at S-phase and also induced apoptosis in a dose dependent manner. At 24hrs of exposure to CJ, 1 mM, 2 mM and 3 mM treatment groups showed 65.05%, 70.91% and 62.45% of cells in the S-phase, compared to the control which showed only 49.42% of cells in the S-phase (panel A). The percentage of cells that progressed to G2/M
phase, in the treatment groups decreased correspondingly. The control showed 18.35% of cells in G2/M phase whereas, 1 mM, 2 mM and 3 mM CJ treatment groups showed only 8.50%, 0.35% and 0.88% of cells in G2/M phase.

At 48hrs of exposure to CJ, similar results were obtained. All the three different treatment groups showed 68.95%, 66.25% and 63.63% of cells in S-phase (Figure 13, panel B). The control showed only 27.38% of cells in the S-phase. The percentage of cells that progressed to G2/M phase decreased. 1 mM, 2 mM and 3 mM CJ had, 0.36%, 0.33% and 0.57% of cells in G2/M phase, whereas control group had 30.81% of cells in G2/M phase.

Similar results were obtained at 72 hrs exposure to CJ (Figure 13, panel C). All three concentrations of CJ, 1 mM, 2 mM and 3 mM, induced cell cycle arrest at the S-phase. The control showed 30.62% of cells in the S-phase, whereas the three treatment groups showed 64.26%, 57.62% and 61.34% of cells in the S-phase of cell cycle, which indicates cell cycle arrest at S-phase. The control group showed 24.36% of cells in the G2/M phase. 1 mM CJ, 2 mM CJ and 3 mM CJ treatment groups showed only 4.34%, 12.73% and 0.45% of cells in G2/M phase. This indicates poor progression of cells from S-phase to G2/M phase, in the CJ treatment groups.

There was an increase in the percentage of apoptotic cells in the CJ treatment groups compared to the control. At 24 hrs of exposure, an increase in the concentration of CJ increased the percentage of apoptotic cells compared to the control. 1 mM CJ, 2 mM CJ and 3 mM CJ induced 1.93%, 1.93% and 11.94% of apoptosis compared to the control which had only 0.07% of apoptotic cells. At
48 hrs of exposure CJ induced 4.17%, 4.68% and 10.92% of apoptosis in the three treatment groups respectively. Control at 48hrs showed 1.57% of apoptosis. As the time of exposure to CJ increased, the number of apoptotic cells increased. This can be clearly viewed at 72 hrs of exposure to CJ. At 72 hrs 3mM CJ induced 23.66% of apoptosis. 1 mM and 2 mM CJ induced 4.80% and 5.57% apoptosis respectively compared to control, which had 2.37% of apoptotic cells.

Figure 14, page 43 shows the effect of MJ at 1 mM, 2 mM and 3 mM, on LNCaP cell line. At all the three concentrations tested no cell cycle arrest at any particular phase was observed. The percentage of apoptotic cells in the 1 mM, 2 mM and 3 mM MJ treatment groups, at 24 hrs were 3.69%, 2.11% and 10.41% (panel A). The control showed 0.45% apoptotic cells. At 48 hrs, the percentage of apoptotic cells were 1.43%, 5.95% and 6.93% in 1 mM, 2 mM and 3 mM treatment groups respectively (panel B). While the control showed 1.46% apoptosis. At 72 hrs of exposure to MJ (panel C), the percentage of apoptotic cells were 3.2%, 1.62% and 4.46% at 1 mM, 2 mM and 3 mM MJ treatment groups respectively, whereas the control has only 0.39% of apoptotic cells.

Figure 15, page 44 shows the effect of CJ, at 1 mM, 2 mM and 3 mM on the LNCaP cell line, at three different time periods, 24 hrs, 48 hrs and 72 hrs. At all the three concentrations tested, no cell cycle arrest at any particular phase was observed. But a dose response was observed in the percentage of apoptotic cells in all the three treatment groups. At 24 hrs, with 0 mM CJ the percentage of apoptosis was only 0.23%, which increased to 0.75%, 5.73% and 32.77% with 1 mM, 2 mM and 3 mM CJ treatments respectively (panel A). At 48 hrs, with 0
mM CJ the percentage of apoptosis was 0.26%, which increased to 1.67%, 12.22% and 50.74% with 1 mM, 2 mM and 3 mM CJ treatments (panel B). At 72 hrs with 0 mM CJ the percentage of apoptosis was only 0.14%, which increased to 6.40%, 9.00% and 52.61% with 1 mM, 2 mM and 3 mM CJ treatments (panel C).

We hypothesized that methyl jasmonate and cis jasmone inhibit the 5-lipoxygenase in arachidonic acid metabolism and cause a decrease in the production of 5-HETE eicosanoid, thereby increasing the cell death. Several studies on arachidonic acid metabolism and 5-HETE showed that, 5-HETE is essential for the survival of human prostate cancer cell lines [35]. To test this hypothesis, we performed an in vitro assay, to study the effects of MJ and CJ on 5-Lipoxygenase (5-LO) and 15-Lipoxygenase enzymes (15-LO).

Fig 16, page 45 shows the in vitro lipoxygenase enzyme assay with MJ and CJ. The concentrations of MJ and CJ used in this study were 10 mM, 50 mM, 100 mM and 500 mM. The percentage activity of 5-lipoxygenase with both MJ and CJ was around 80% at 10 mM concentration i.e., MJ and CJ at 10 mM concentration could inhibit only <20% of 5-lipoxygenase activity in vitro (panel A). The percentage activity of 15-lipoxygenase with 10 mM MJ was ~65% (panel B). While 10 mM CJ did not inhibit the 15-lipoxygenase (panel B). These results indicate that MJ and CJ did not inhibit the 5-lipoxygenase enzyme activity in vitro. We then tested the activity of 5-lipoxygenase enzyme with different concentrations of MJ. Lower concentrations of MJ were tested – 5 mM, 10 mM, 30 mM and 50 mM as shown in the figure 17, page 46. Inhibition of 5-
lipoxygenase with 5 mM MJ was only ~20%. At the concentrations 1 mM, 2 mM and 3 mM (which we used for other assays in culture), only ~5% inhibition of 5-lipoxygenase by MJ was observed.

In order to test whether MJ acts downstream of 5-HETE in the arachidonic acid metabolic pathway, we performed alamar Blue assay with 0.001 mM 5-HETE. As shown in the figure 18, page 47, panel A, treatment with 0.001 mM 5-HETE increased the viability of PC3 cells by ~30%. Treatment with 2 mM MJ alone decreased the percent viability by >75%. In the other treatment group where 0.001 mM 5-HETE was added together with 2 mM MJ, 5-HETE still increased the viability of PC3 cells by ~30%.

Similar results were obtained with the LNCaP cell line. The treatment group with 0.001 mM 5-HETE alone increased the proliferation of LNCaP cells by ~30% (panel B). 2 mM MJ alone decreased the viability by >60%. In the other treatment group, with 0.001 mM 5-HETE and 2 mM MJ, 5-HETE increased the viability of LNCaP cells by ~30% even in the presence of 2 mM MJ.

We performed fluorometric assay for caspase activity, in PC3 cell line, to study whether apoptosis in the human prostate cancer cells occur though caspase activity, induced by Methyl Jasmonate.

We have analyzed the mechanism by which MJ and CJ induce apoptosis in PC3 lines. We tested whether MJ or CJ induced apoptosis involves caspase-3. Our data shows that both MJ and CJ induce apoptosis through activation of caspase-3, in PC3 cell line (Figure 19 and 20, page 48 and 49). Caspase-3 is one of the apoptotic executioners and plays an important role in cell shrinkage,
chromatin condensation and DNA fragmentation [24]. Caspase-3 is involved in the cleavage of DNA repair enzyme poly (ADP-ribose) polymerase, which is required for apoptosis induction [25].

Cell lysates prepared from 2 mM MJ or CJ treatments for 3 hrs, 6 hrs, 12 hrs and 24 hrs were used in this assay. This fluorometric assay detects the caspase-3 activity in the cell lysates, when incubated with caspase-3 substrate conjugate. The cell lysates were incubated with the caspase-3 substrate conjugate for 3.5 hrs, and the enzyme activity was read every half-hour, and the data was represented as relative fluorescence units of the enzyme corrected for any activity in the control.

Figure 19, page 48 shows that caspase-3 activity was induced in all the treatment groups (3 hrs, 6 hrs, 12 hrs and 24 hrs exposure to 2 mM MJ). The amount of product fluorescence increased with time. Among the different treatment groups 3 hr showed lowest activity while the 6 hr and 12 hr groups had the highest.

Figure 20, page 49 shows similar results of caspase-3 fluorometric assay, with 2 mM CJ on PC3 cell line. Again 3 hr treatment group had the least activity while the 12 hr group had the highest activity.
Fig 7: Proliferation assay on PC3. 2 MJ and CJ induced cell death in PC3 cell line using dye exclusion assay. Cells were exposed to MJ or CJ for the time indicated. In another treatment group, cells were exposed for 24hrs, drug was then washed off and let grow for 15 days. The error bars represent the standard deviation of the means (n=4).
Fig 8: Proliferation assay on LNCaP. 2 MJ and CJ induced cell death in LNCaP cell line using dye exclusion assay. Cells were exposed to MJ or CJ for the time indicated. In another treatment group, cells were exposed for 24hrs, drug was then washed off and let grow for 15 days. The error bars represent the standard deviation of the means (n=4).

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Fig: 9 Alamar blue assay on PC3. MJ and CJ inhibited the proliferation of PC3 cells at the concentrations tested, using alamar Blue assay. PC3 cells were exposed to MJ and CJ for 24hrs, 48hrs and 72hrs. Error bars represent the standard deviation of the means (n=6).
Fig10: Alamar blue assay on LNCaP. MJ and CJ inhibited the proliferation of LNCaP cells at the concentrations tested, using alamar Blue assay. LNCaP cells were exposed to MJ and CJ for 24hrs, 48hrs and 72hrs. Error bars represent the standard deviation of the means (n=6).
Table 1: IC$_{50}$ values for MJ and CJ (mM) in PC3 and LNCaP cells

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<th>Cell line</th>
<th>Agent</th>
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<th>48hr</th>
<th>72hr</th>
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<td>CJ</td>
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<td>CJ</td>
<td>1.25</td>
<td>0.75</td>
<td>0.62</td>
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Fig 11: Colony Formation Assay, 2mM MJ and CJ inhibited the long term proliferation of PC3 cell and LNCaP cells. Cells were exposed to MJ and CJ for 24hrs, then the drug was washed off, cells were supplemented with fresh media and let grow for 10-14 days. The error bars represent the standard deviation of the means (n=4).
Fig 12: Flow cytometry with MJ on PC3. MJ induced S phase block and apoptosis in PC3 cells, exposed to MJ for 24hr, 48hr and 72hr, PI stained and analyzed through flow cytometry.
Fig 13: Flow cytometry with CJ on PC3. CJ induced S phase block and apoptosis in PC3 cells, to CJ for 24hr, 48hr and 72hr, PI stained and analyzed through flow cytometry.
Fig 14: Flow cytometry with MJ on LNCaP. MJ induced apoptosis in dose and time dependent manner in LNCaP cells, exposed to MJ for 24hr, 48hr and 72hr, PI stained and analyzed through flow cytometry.
Fig 15: Flow cytometry with CJ on LNCaP. CJ induced apoptosis in dose and time dependent manner in LNCaP cells, exposed to CJ for 24hr, 48hr and 72hr, PI stained and analyzed through flow cytometry.
Fig 16: *In vitro* lipoxygenase assay, MJ and CJ tested at the concentrations indicated for their effects on 5-LO and 15-LO enzymes. The error bars represent the standard deviation of the means (n=2).
Fig 17: *In vitro* lipoxygenase assay, MJ tested at concentrations indicated for its effect on 5-LO and 15-LO enzymes. The error bars represent the standard deviation of the means (n=2).
Fig 18: Alamar blue assay with 5-HETE. 2 mM MJ tested in presence and absence of 0.001 mM 5-HETE for 24hrs on the proliferation of both PC3 and LNCaP cells, using alamar Blue assay. Each graph represents the average of two different experiments. The error bars represent the standard deviation of the means of two experiments.
Fig 19: Caspase-3 assay with MJ on PC3. 2 mM MJ induced caspase-3 activity in the PC3 cell lysates, using a fluorometric assay. PC3 cells were exposed to 2 mM MJ for the time indicated and the cell lysates were tested for caspase-3 activity by adding the caspase-3 substrate conjugate.
Fig 20: Caspase-3 assay with CJ on PC3. 2 mM CJ induced caspase-3 activity in the PC3 cell lysates, using a fluorometric assay. PC3 cells were exposed to 2 mM CJ for the time indicated and the cell lysates were tested for caspase-3 activity by adding the caspase-3 substrate conjugate.
CHAPTER 5

CONCLUSIONS

The two plant stress hormones methyl jasmonate (MJ) and cis jasmone (CJ) tested in this study, induced cell death in both hormone dependent (LNCaP) and hormone independent (PC3) human prostate cancer cell lines. MJ and CJ also inhibited the proliferation of PC3 and LNCaP cells. Inhibition of proliferation and cell death were confirmed by cytotoxicity assays using alamarBlue. The IC\textsubscript{50} concentrations calculated from the alamar Blue assay showed that MJ and CJ are more lethal in LNCaP than PC3. CJ was shown to be more lethal than MJ in LNCaP but not in PC3 cells.

Cell cycle analysis using flow cytometry showed that both MJ and CJ at 1 mM, 2 mM and 3 mM concentrations induced an S-phase block and apoptosis in PC3 cells. In LNCaP cells both MJ and CJ at 1 mM, 2 mM and 3 mM concentrations induced apoptosis in a dose dependent manner. Flow cytometry showed no cell cycle arrest in LNCaP cells with either MJ or CJ.

Recent research indicated that the ecosanoid products of arachidonic acid metabolism especially the 5-HETE is essential for prostate cancer progression [28]. 5-LO is an important and key enzyme in the arachidonic acid pathway involved in the production of 5-HETE [25]. We tested whether MJ or CJ were
involved in inhibiting the 5-LO enzyme, thereby decreasing the production of 5-HETE and increasing the cell death. The in vitro Lipoxygenase assay with MJ and CJ confirms that both MJ and CJ do not inhibit the 5-LO enzyme and at the concentrations which we used for other assays in culture (1 mM, 2 mM and 3 mM), only ~5% inhibition of 5-lipoxygenase by MJ was observed. These results indicate that MJ and CJ do not inhibit the 5-lipoxygenase enzyme. Experiments with 5-HETE and MJ on both PC3 and LNCaP cell lines showed that 5-HETE increased the viability of PC3 and LNCaP cells even in the presence of 2 mM MJ. This indicates that MJ is not involved in inhibiting the 5-LO enzyme and it does not act downstream of 5-HETE.

Finally, these results demonstrate that the hypothesis, MJ and CJ act via arachidonic acid metabolism and inhibit the 5-lipoxygenase, to be false (Figure 21, page 52). These results do clearly indicate that both MJ and CJ induce apoptosis via the activation of caspase-3. Further studies will be required to determine the mechanism by which MJ and CJ induce apoptosis in prostate cancer lines.
Fig 21: Conclusion, MJ and CJ activate caspase-3 and induce cell death in PC3 and LNCaP prostate cancer cells.
APPENDIX

Table 2: Cell cycle analysis with MJ on PC3 cell line

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<tr>
<th>Time Period</th>
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<th>G0/ G1</th>
<th>S</th>
<th>G2/M</th>
<th>Apoptosis</th>
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Table 3: Cell cycle analysis with CJ on PC3 cell line

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<th>S</th>
<th>G2/M</th>
<th>Apoptosis</th>
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Table 4: Cell cycle analysis with MJ on LNCaP cell line
Table 5: Cell cycle analysis with CJ on LNCaP cell line

<table>
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<th>Time Period</th>
<th>Treatment</th>
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<th>S</th>
<th>G2/M</th>
<th>Apoptosis</th>
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</table>
REFERENCES


Bax/Bcl-Xs and activation of caspase-3 via ROS production in A549 cells.


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