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THE EFFECTS OF JASMONATES ON THE PROLIFERATION OF HUMAN PROSTATE CANCER CELL LINES IN CULTURE

by

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ABSTRACT

The Effects of Jasmonates on the Proliferation of Human Prostate Cancer Cell Lines in Culture

by

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Jasmonic acid (JA), cis-Jasmone (CJ), and Methyl jasmonate (MJ), belong to a family of plant stress hormones known as the jasmonates. In plants, these compounds function as activators of cellular responses to diverse situations including cell death. Proliferation and cytotoxicity assays showed that the jasmonates inhibited the proliferation of the prostate cancer cell lines (PC-3 and DU-145), cultured *in vitro*. In addition, the type of inhibition exhibited by these agents was analyzed using flow cytometry (BrdU assay and Annexin V-FITC/PI staining) and fluorescence microscopy. The mechanism of inhibition

was studied using 5-lipoxygenase enzyme. All agents (CJ, JA and MJ) inhibited proliferation of the cells in dose- and kinetic-dependent manners. All the assays confirmed that the inhibition was through the induction of apoptosis and that the mechanism of inhibition may involve the 5-lipoxygenase pathway. The results indicated a potential role for these compounds in the treatment of human prostate cancer.

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CHAPTER 1

INTRODUCTION

Cancer is a disease condition in which abnormal cells grow without control as either benign or malignant tumors. In most cases, this often results in death. At present, there is no clear understanding as to what constitute the causal factors for this disease; however, some causal factors have been categorized into sporadic and congenital factors. Sporadic factors are those causal factors that are associated with the individual's way of life such as smoking and exposure to chemicals or radiation. Congenital factors on the other hand involve those causal factors that are genetically controlled and can be transferred from the parents to the offsprings. Congenital factors include inherited mutations in the genes that can affect the immune condition as well as the hormone function of an individual.

The time it takes for cancer to fully manifest in an individual varies depending on the type of cancer involved therefore giving enough treatment opportunities using surgery, chemotherapy, radiation, cryotherapy, hormone

therapy or a combination of two or more of these options. However, due to side effects resulting from some of these treatment options, it becomes important to look into the role of natural plant compounds in the treatment of these cancers since these compounds are less toxic and are usually present in food consumed by humans.

1.1 Purpose of the study/Research question

Naturally occurring phytochemicals have been used in cancer treatment. For instance, in 1984, Elegbede *et al.* [1] first reported that the monoterpene, d-limonene, inhibited the development of chemically-induced mammary tumors in rats. Later, Elegbede *et al.* [2] reported that d-limonene caused the regression of frank mammary tumors in laboratory rats. In another study, by Vij and Kumar [3], Asian men were observed to have less prostate cancer incidence and mortality rate than American men; this was attributed to the type of food being consumed in those areas. In another cohort study by Kumar *et al.* [4], they found that when prostate cancer patients that are still at the early stages of the disease are supplemented with isoflavones (a phytochemical), there was a reduction in serum prostate-specific antigen (PSA) and free testosterone in a large number of these patients [4]. The mechanisms of

action the phytochemicals on cancer cells have not been fully understood. However, isoflavones and estrogens have been shown to inhibit the proliferation of prostate tumors by competitively interfering with the action of testosterone (a hormone that has been implicated in prostate cancer progression) [3, 4]. These phytochemicals influence the action of testosterone by increasing the level of sex hormone-binding globulin that binds testosterone, resulting in lower free testosterone levels and subsequent decrease in the rate of prostate cancer stimulation [5].

Prostate cancer has been shown to be hormone-dependent, that is, an increase in the level of serum testosterone will result in an increase in the rate of prostate cancer proliferation [6]. Based on this fact, hormone therapy is viewed by many researchers as what might bring an end to the ordeal experienced by prostate cancer patients. As explained later in this chapter, hormone therapy has been used to treat prostate cancer patients who are resistant to chemotherapy or other treatment options and those that are still at the locally advanced stage of the disease. However, a lot of side effects have been experienced with this treatment option [6]. Due to these side effects, there is a need for research into other

possible ways through which this disease can be eliminated or reduced.

Therefore, we proposed to study the chemotherapeutic values of a new class of phytochemicals that are found in plant foods consumed by humans. The purpose of this study was to observe the effects of the jasmonates on prostate cancer cells cultured *in vitro*. The jasmonates are plant stress hormones derived from an omega-3 fatty acid (alpha linolenic acid). Earlier studies by Fingrut and Flescher [7] showed that the jasmonates possessed anticarcinogenic effects against some cancer cell lines.

1.2 Significance of the study

This study is very significant since prostate cancer still remains the second most common cause of cancer deaths among men in the United States [8]. It has been predicted that 230,110 new cases and 29,900 deaths will occur in 2004 [8].

Prostate cancer incidence rates are higher in African American men compared to Caucasian men [8]. Table 1 compares prostate cancer death rate to that of other cancer types; due to the number of deaths resulting from prostate cancer, it is important that more studies be done in this area which directly justifies the importance of this study.

Table 1: Estimated new cancer cases and deaths in men, US, 2004. Adapted from ref. [8]

Cancer Type	Estimated new cases	Estimated deaths
Lung and bronchus	93,110	91,930
Prostate	230,110	29,900
Colon	50,400	28,320
Liver and intrahepatic bile duct	12,580	9,450
Melanoma-skin	29,900	5,050
Tongue	7,320	1,100
Brain and other nervous system	1,130	110

1.3. Epidemiology of Prostate Cancer

Prostate cancer is one of the leading causes of cancer deaths among men in most developed countries, yet only little knowledge about its causal factors are known [9]. Positive family histories of prostate cancer, old age as well as African ancestry have all been implicated as risk factors for prostate cancer [9]. However, the risk for development of prostate cancer may involve interactions of two or more of these factors.

There are a number of new studies ongoing to identify the risk factors involved with this disease such as the influence of polyunsaturated fatty acid on prostate cancer progression [10]; however, more studies are required to confirm what the role of these polyunsaturated fatty acids are, with respect to cancer treatment [10].

Studies of migrant Asian men done by Shimizu *et al.* [11] showed that environmental, dietary, or social factors may be involved with the etiology of prostate cancer [11]. They discovered that when migrant workers moved from a low-risk country such as Japan to the United States, there is an increase in the incidence and mortality rate resulting from prostate cancer and that this increase became several folds higher than observed in their native Japanese counterparts [11]. In addition to that, they found a positive correlation between the number of years since migration to the United States and incidence of prostate cancer [11]. They concluded that the increase in the incidence may be related to the type of food consumed in the United States such as a change in the diet from low fat to high fat [11]. The conclusion reached by Shimizu *et al.* was later confirmed by Kakehi in 1998, who studied the epidemiology and clinical features of prostate cancer in Japan and was able to show that high fat intake is positively associated with increased risk and may in part, explain the rising incidence of prostate cancer in Japan, as dietary habits become more Westernized [12].

Another risk factor that has been implicated with the development and/or etiology of prostate cancer is family history. It was reported by Schuurman *et al.* in

1999 that men with prostate cancer are two to three times more likely to have at least one first- or second-degree relative with prostate cancer [13]. Even before Schuurman *et al.* made their suggestions, Keetch *et al.* in 1995 showed that a patient with prostate cancer is between 3.1 to 4.3 times more likely than a control to have a history of prostate cancer in his father and brothers, respectively [14].

The role of vasectomy in prostate cancer progression remains controversial. Although retrospective and prospective cohort epidemiological studies have demonstrated a relative risk of approximately 1.6 in men who underwent vasectomy [15] other studies [16] did not confirm this.

Finally the molecular mechanisms of the disease as well as the role of specific gene activity however remain largely unsolved and continue to be an area of active research [10].

1.4. Classification of Prostate Cancer

Prostate cancer, like other types of cancers, develops via a multi-stage carcinogenesis process with three distinct phases: initiation, promotion, and progression [[reviewed in 17]]. At the initiation phase, the tumor is

difficult to detect clinically, due to its microscopic nature at this stage and it is confined to the prostate but undetectable by digital rectal examination (DRE), ultrasound or by any other screening method [[reviewed in 17]]. At the promotion phase, the tumor which is already in the prostate gland can be detected by DRE or ultrasound [[reviewed in 17]]. In the final stages of prostate cancer development, the cancer cells may have spread to organs close to the prostate, such as the bladder, the pelvic and other lymph nodes, organs, or bones distant from the prostate [reviewed in 17]. According to the American Cancer Facts and Figures, Prostate cancer can be classified into four stages:

Stage I or Stage A: The tumor cannot be felt during a rectal examination. It may be found by accident during surgery for other reasons such as benign prostatic hyperplasia (BPH).

Stage II or Stage B: The tumor involves more tissue within the prostate and therefore can be felt during a rectal exam, or detected with a biopsy.

Stage III or Stage C: The tumor has spread outside of the prostate to nearby tissues.

Stage IV or Stage D: At this stage of development, the tumor must have spread to the lymph nodes and/or to other distant parts of the body [8].

This proposed research will evaluate the effects of plant stress hormones on prostate cancer cell lines (PC-3 and DU-145) obtained from the American Type cell culture (ATCC; Mannasas, VA).

1.5 Influence of hormone on prostate cancer progression.

Androgens are steroid hormones whose production requires stimulation by leutinizing hormone and follicle stimulating hormone from the pituitary gland (Figure 1, pg 13). Once produced, androgen stimulates the prostate gland by binding to a steroid receptor protein known as androgen receptor (AR) [18]. This receptor is found in the nucleus of the epithelial cells [18].

Usually, when androgen binds to its receptor (AR), it stimulates proliferation of the cells and diverts prostate cancer cells from undergoing apoptosis [18]. This was documented in an earlier study by Kyprianou and Isaacs in 1998 [19] using rodent models. They showed that when there is a decrease in the amount of circulating androgens, prostate cancer cells start undergoing apoptosis [19]. This idea is the basis for hormone ablation therapy [18].

However, when prostate tumor cells are deprived of hormone, treatment only last for a while before the cancer cells develop resistance to hormone [19]. This often leads to androgen independence thus, making it more difficult to regulate tumor cell growth using hormone ablation. This transformation from hormone-dependent to hormone-independent tumor has been linked to many factors including change in the androgen receptor gene expression. [18]. The testes produce testosterone (T), and also dihydrotestosterone (DHT) which is the active metabolite of T. 5-alpha reductase converts T to DHT which is directly involved in prostate cancer progression (Figure 1; pg 13).

1.6 Apoptosis

The term "apoptosis" was devised by Kerr *et al.* [21] in 1972 to represent the type of cell death known as "programmed cell death". This distinguished apoptotic from other types of cell death such as necrosis and oncosis (cell death resulting from tumor formation and characterized by swelling) [21]. Change in the cell morphology has been one way used to differentiate apoptotic from non-apoptotic cells. Cells undergoing apoptosis are characterized by cell shrinkage, cellular budding and fragmentation, and chromatin condensation [21]. Apart from

programmed cell death, apoptotic death can also result from physical and toxic exposures to chemicals or radiation and cellular effects of cytokines such as tumor necrosis factor [reviewed in 22]. This indicates that multiple pathways of cell death may lead to similar morphological features found in typical apoptotic cells. Although it appears as if apoptosis may involve multiple pathways, it is still possible to make a strong case for a final common pathway for apoptosis since bcl-2 can still protect cells against apoptosis induced by some of these stimuli [reviewed in 22]. Bcl-2 may be protecting cells against apoptosis via the inhibition of a central step involved in apoptosis or protecting an important constituent of the cell that is a target of apoptotic program [reviewed in 22].

Other features of apoptosis such as the externalization of phosphatidylserine from the inner to the outer membrane of the cell plasma membrane have been employed to quantify the number of apoptotic cells in a cell population. Annexin V-FITC has the ability to bind to the externalized phosphatidylserine at the onset of apoptosis [23]. Apoptotic cells also show fragmented DNA, this property is again very useful in the study of apoptosis. Assay such as BrdU are used to stain the fragmented DNA, also DAPI dye can be used to stain the

fragmented DNA. BrdU stains the 3-OH end of the DNA and DAPI stains the double stranded DNA [24, 25]. Hoechst and propidium iodide are also dyes used in detecting apoptosis in cells. Propidium iodide stains necrotic cells thereby helping in differentiating apoptotic cells from necrotic cells. On the other hand, Hoechst stains live cells and so differentiates them from apoptotic cells [26]. The greatest benefit of the study of apoptosis is that it has led to the discovery of new genes and their effects in regulating apoptosis [reviewed in 22].

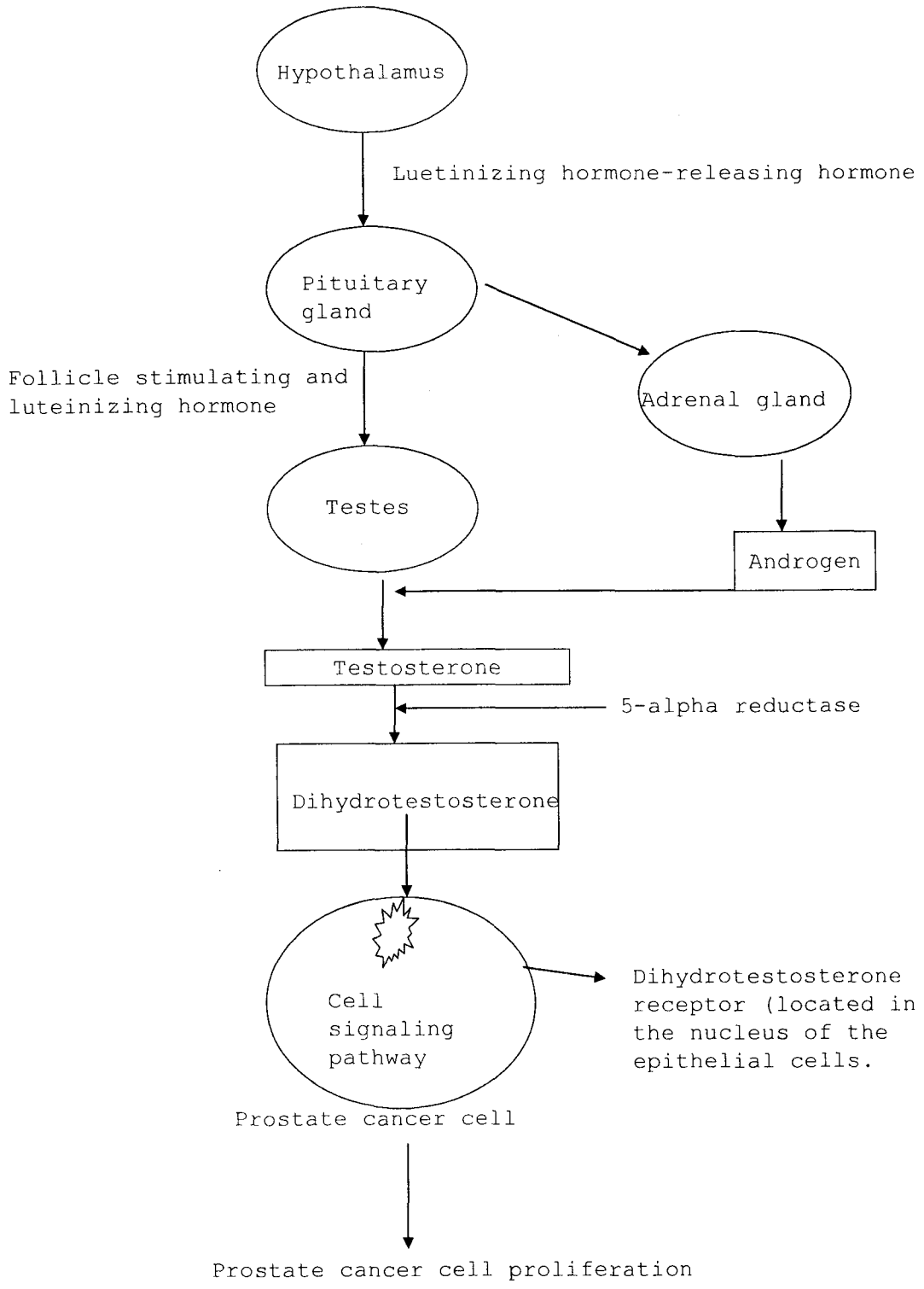


Figure 1: The influence of hormones on prostate cancer cell proliferation. [Adapted from ref.20]

1.7 Brief description of the cell lines

There are three prostate cancer cell lines that are commonly used in research; DU-145, PC-3 and LNCap. DU-145 and PC-3 are hormone-independent cell lines while LNCap cell line is hormone-dependent. Prostate cancer cells usually progress from hormone-dependent to hormone-independent stages, thus, hormone-independent prostate cancers are more difficult to treat because the growth of the cancer cells can no longer be regulated using hormone [6]. We studied the effects of the jasmonates on prostate cancer cell lines DU-145 ad PC-3 cultured *in vitro*. PC-3 is a human epithelial prostate cancer cell line of grade IV prostate cancer [27]. It is an adherent cell line and has a doubling time of about 18 hr. DU-145 is also a human prostate adenocarcinoma cell line; it is also an adherent cell line just like PC-3 [27]. The cell lines were procured from the American Type cell culture (ATCC; Mannasas, VA). Many studies have been done using these cell lines, some of the known facts about these two human prostate cancer cell lines are summarized in Table 2.

Table 2: Facts about prostate cancer cell lines (PC-3 and DU-145).

Cell line	p53	p21	Bcl-2	Bax	retinoblastoma (RB)	Cell cycle arrest		5-HETE	IL-6
PC-3	negative [28]	10-hydroxycamptothecin caused expression of p21 [29]	Wild-type Bcl-2 expression [30]	Null [31]	Expressed pRB [32]	As_2O_3 arrested cell growth of PC-3 in G_2/M [34]	terazosin-induced G_0/G_1 arrest [35]	500nM made cells grow rapidly [36]	Expressed [37]
DU-145	mutant [28]	inhibition of mouse doubling minute 2 (MDM2) expression resulted in expression of p21 [29]	wild-type Bcl-2 expression [30]	Null [31]	Negative [33]	As_2O_3 arrested cell growth of DU-145 G_2/M [34]	terazosin-induced G_0/G_1 arrest [35]	Not reported	Expressed [37]

PC-3 is also known to express heat shock transcription factor 1 (HSF-1) [38].

1.8 Jasmonates

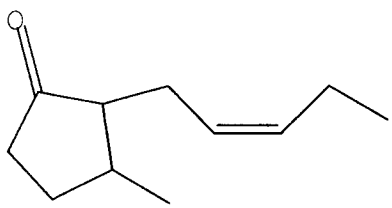
The jasmonates (Figure 2; pg 17), consisting of cis-jasmone (CJ), jasmonic acid (JA) and methyl jasmonate (MJ) are a novel class of plant compounds first isolated from the jasmine plant, are natural bioregulators [39] and are involved in intracellular signaling and defense in plants in response to injury [40, 41]. The jasmonates were reported to cause the formation of a zone of dead cells around an infection site and the synthesis and accumulation of antimicrobial agents [42]. The layers of dead cells that surround the site of pathogen entry or wounding are thought to function as a physical barrier that inhibits further proliferation and spread of the pathogen [42]. Other roles for the jasmonates in plants include: the inhibition of processes such as growth and germination,

promotion of senescence, abscission, tuber formation, fruit ripening, pigment formation, tendril coiling, and others [41].

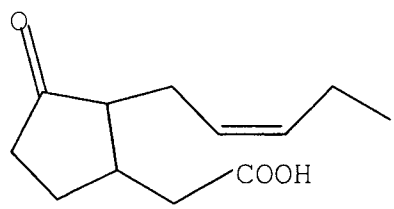
Recent studies by Fingrut (personal communication) showed that *cis*-jasmone induced cell death in some cancer cell lines. In addition, analogs of cis-jasmone (jasmonic acid and methyl jasmonate) inhibited the proliferation and induced cell death in lymphoblastic leukemia (MOLT-4) and breast cancer (MCF7) cells cultured *in vitro* but did not affect normal human blood lymphocytes or erythrocytes [7]. They also reported that JA and MJ caused apoptotic death, as determined by morphological changes that accompany apoptosis as well as caspase-3 activity. In addition, "the jasmonates induced mitochondrial membrane depolarization as determined by flow cytometry" [7].

The jasmonates are synthesized in plants from alpha linolenic acid through the lipoxygenase pathway (Figure 3, pg 18) [43]. As shown in figure 3, the pathway starts with the stimulation of a membrane receptor which results in the activation of phospholipase (PLD). This is followed by the release of alpha-linolenic acid which is further converted to 13-hydroperoxylinolenic acid by lipoxygenase enzyme. The formation of 12-oxo-phytodienoic acid marks the end of jasmonate biosynthesis in the chloroplast. The 12-oxo-

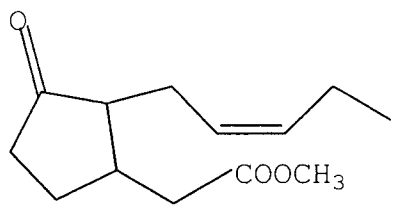
phytodienoic acid then leaves the chloroplast to act as signal in the peroxisome. In the peroxisome, 12-oxo-phytodienoic acid undergoes a reduction reaction catalyzed by 3-oxo-2(2pentenyl)-cyclopentane-1-octanoic acid as well as beta-oxidation reaction leading to the formation of jasmonic acid (Figure 3, pg 18). Jasmonic acid can then be methylated by S- adenosyl-L-methionine:jasmonic acid carboxylmethyltransferase to form methyl jasmonate or undergo decarboxylation reaction to form the cis-jasmone (Figure 3, pg 18).



cis-Jasmone (CJ)



Jasmonic acid (JA)



Methyl jasmonate (MJ)

Figure 2: Structures of the jasmonates

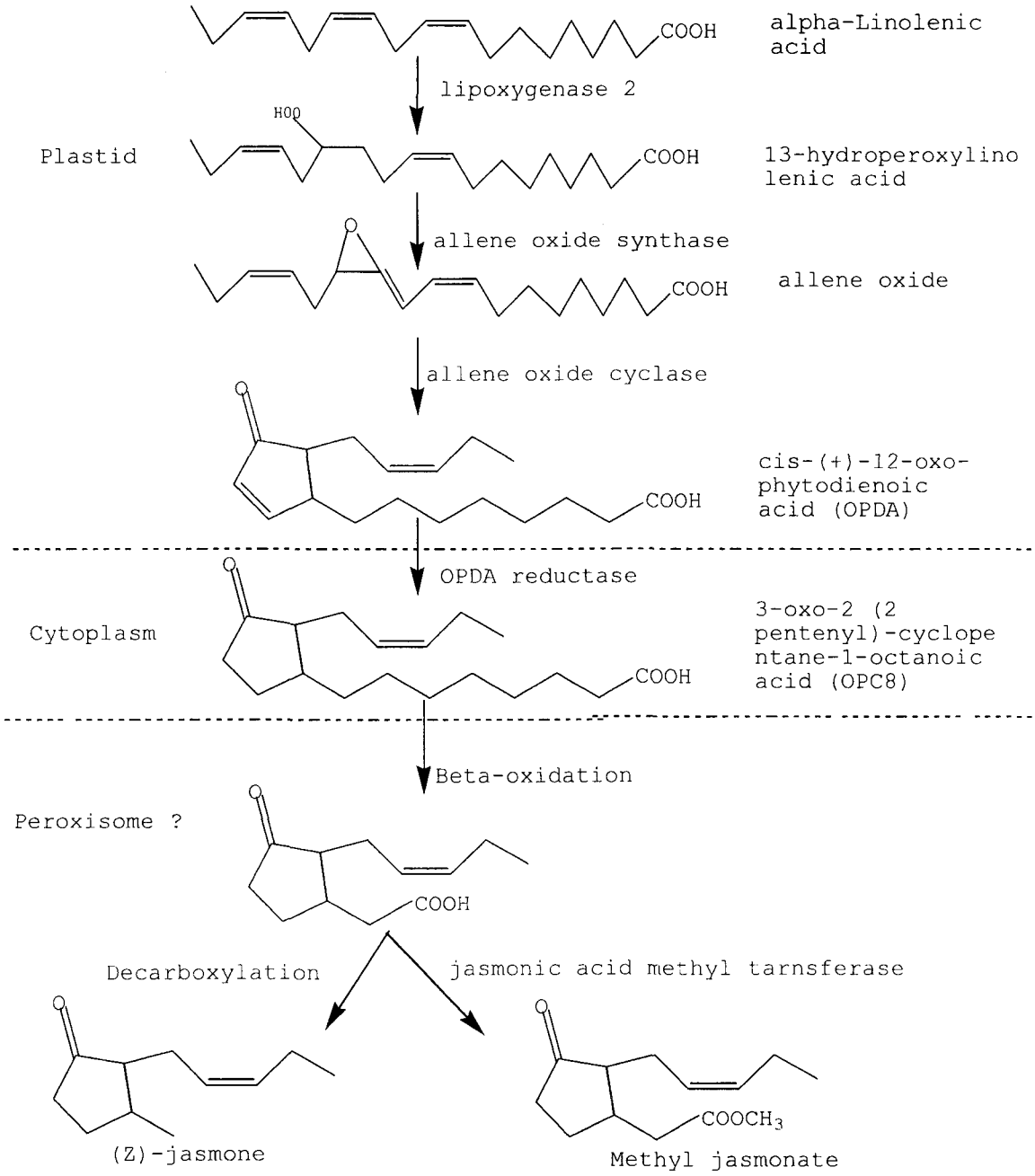


Figure 3: Model for the octadecanoid biosynthetic pathway following wounding or pest attack. Adapted from ref.[40].

1.9 5-Lipoxygenase pathway

As shown in Figure 4, pg 21, the 5-lipoxygenase pathway begins with the intracellular release of arachidonic acid, mediated by phospholipase A (PLA_2) [reviewed in 44]. In leukocytes, Clark *et al.* [45] showed that cytokines such interleukin-1 (IL-1) and tumor necrosis factor can activate PLA_2 by stimulating a phospholipase-activating protein [45].

Once released, arachidonic acid can either pass through the cyclooxygenase pathway and get converted to prostaglandin or it can pass through the action of 5-, 12-, or 15-lipoxygenases into the corresponding HETEs (Figure 5; pg 21). 5-lipoxygenase in the presence of FLAP (5-LO activating protein) catalyzes the oxygenation of arachidonic acid into 5-hydroperoxyeicosatetraenoic acid (5-HPETE), followed by a second reaction in which 5-HPETE is reduced in a glutathione-dependent reaction to 5-hydroxyeicosatetraenoic acid (5-HETE) [reviewed in 44].

Recent study on the localization of 5-lipoxygenase enzyme showed that it varies depending on the cell type [reviewed in 44]. For example, in B-cells, it is found in the nucleus [46], in HL-60 cells, 5-LO is found in the cytosol [47], and in RBL (rat basophilic leukemia), it is situated in both the nucleus and the cytosol [47]. 5-

lipoxygenase has also been shown to move from one area of the cell to the other. For instance, in human polymorphonuclear leukocytes (PMNs), 5-lipoxygenase translocates from the cytosol to the nuclear membrane when activated with Ca^{2+} [reviewed in 44].

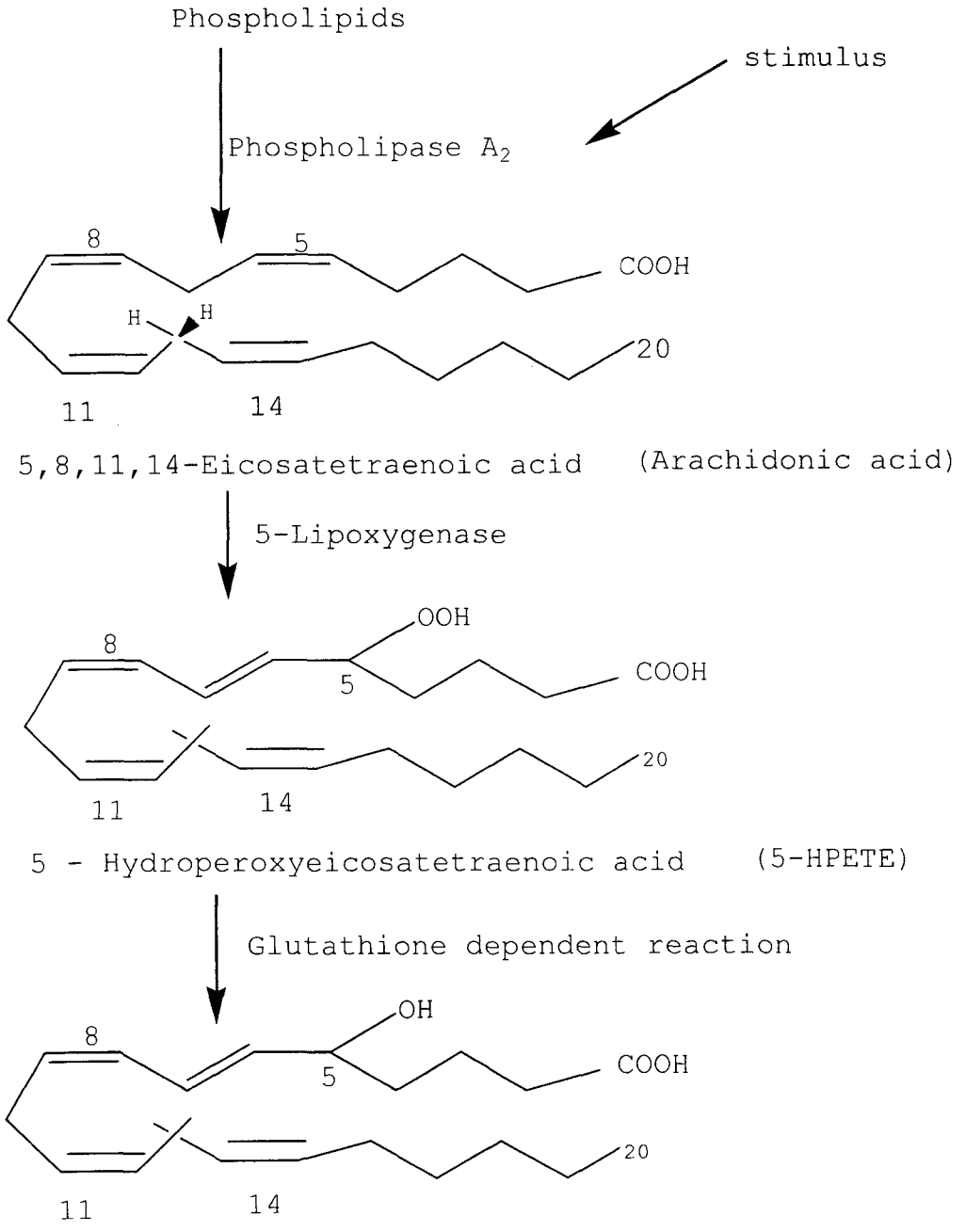


Figure 4: Lipoxygenase pathway showing the conversion of arachidonic acid (C20: 4) into 5 -HETE. Adapted from ref. [44]

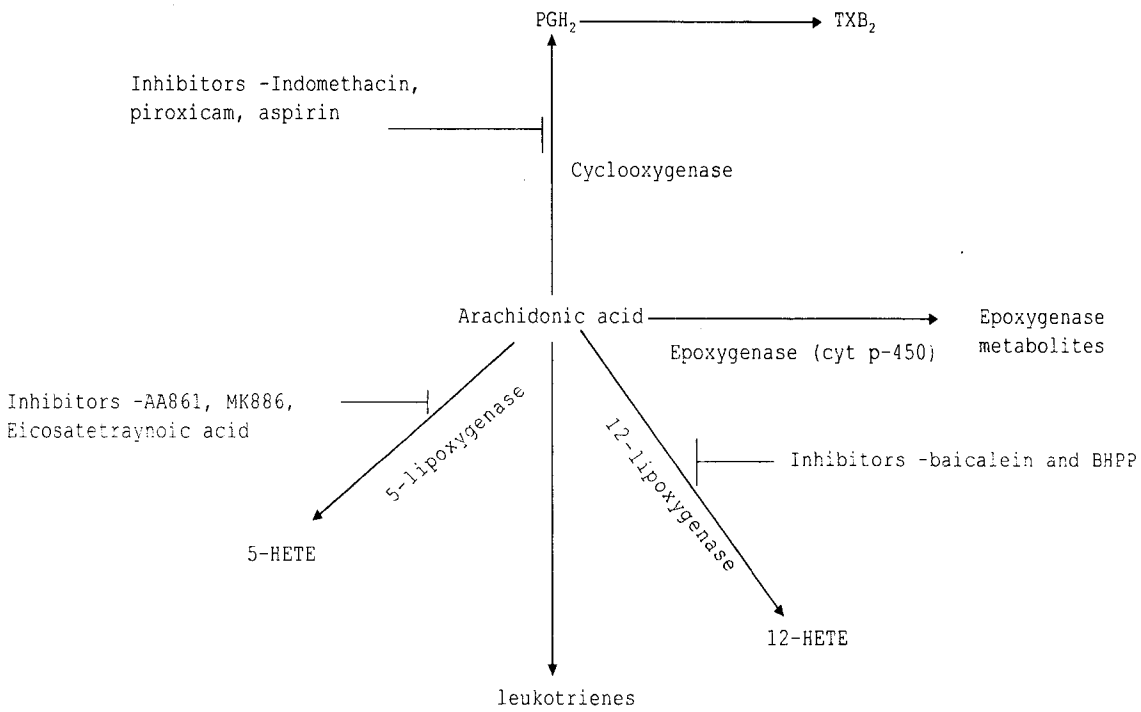


Figure 5: The eicosanoid pathway. Adapted from ref. [48, 49, 50, 51, 52].

Studies linking the eicosanoid pathway to prostate cancer progression have been documented. For example, linoleic acid (a precursor of arachidonic acid) stimulated cell growth in experiments conducted by Rose and Connolly [48] using human prostate cancer (PC-3 and DU-145)cells, whereas indomethacin, esculetin, and piroxicam (eicosanoid inhibitors) (Figure 5) inhibited it, indicating the involvement of eicosanoids in prostate cancer cell proliferation [48]. However, later studies by Anderson *et*

al. [49] showed that cyclooxygenase (COX) inhibitors indomethacin and aspirin, failed to reduce human prostate PC3 cell DNA synthesis, although the arachidonic acid antagonist, eicosatetraynoic acid, did reduce synthesis, suggesting that LO products are essential in modulating prostate DNA synthesis [49].

Other studies done by Anderson *et al.* [50] showed that with the specific 5-LO inhibitor (A63162), there was reduction in DNA synthesis and growth inhibition of prostate cancer (PC-3) cells, this also supports the involvement of the LO metabolic pathway in prostate cancer growth [50]. Recent study done by Ghosh and Myers [51] using PC-3 cells provides convincing evidence that the 5-LO metabolic pathway stimulates prostate cancer cell growth, they confirmed this by adding selective inhibitors of the different metabolic pathways of arachidonic acid such as SKF-525A for cytochrome P-450, ibuprofen for cyclooxygenase, baicalein and BHPP for 12-lipoxygenase, AA861 and MK886 for 5-lipoxygenase and found that only the 5-LO specific inhibitors were able to inhibit the proliferation of PC-3 cells [51]. In another study, both MK-886 and AA-861 which are 5-lipoxygenase inhibitors blocked prostate tumor proliferation induced by arachidonic

acid, whereas the COX inhibitor ibuprofen and 12-LO inhibitors baicalein and BHPP were ineffective [52].

On the basis of this information, agents that directly or indirectly interfere with the synthesis of lipoxygenase metabolites may be effective in preventing cancer. However, recent studies have shown that some phytochemicals especially those that are derived from omega-3 fatty acids inhibit the activities of 5-lipoxygenase and thereby function as both anticancer and anti-inflammatory agents [53]. Indeed, research into the possible role of phytochemicals especially the omega-3 derivates in the treatment of cancer has been promising.

Hypothesis

The hypothesis being tested in this thesis is whether jasmonates inhibit the proliferation and induce cell death in prostate cancer cells *in vitro* and if so what will be the possible pathway for the induction of cell death. These findings will identify a novel group of plant compounds for the prevention and/or therapy of prostate cancer in humans.

The model proposed to directly test these hypotheses is shown in Figure 6.

1.10 Proposed Model

The model proposed for this research was to study the effect of jasmonates on the proliferation of prostate cancer cells cultured *in vitro* and to propose a possible pathway through which these plant stress hormones elicit their effects.

Therefore, to test our hypothesis, we used both proliferation and cytotoxicity assays as well as apoptosis assays. We also, used the 5-lipoxygenase enzyme to trace the proposed pathway (Figure 6).

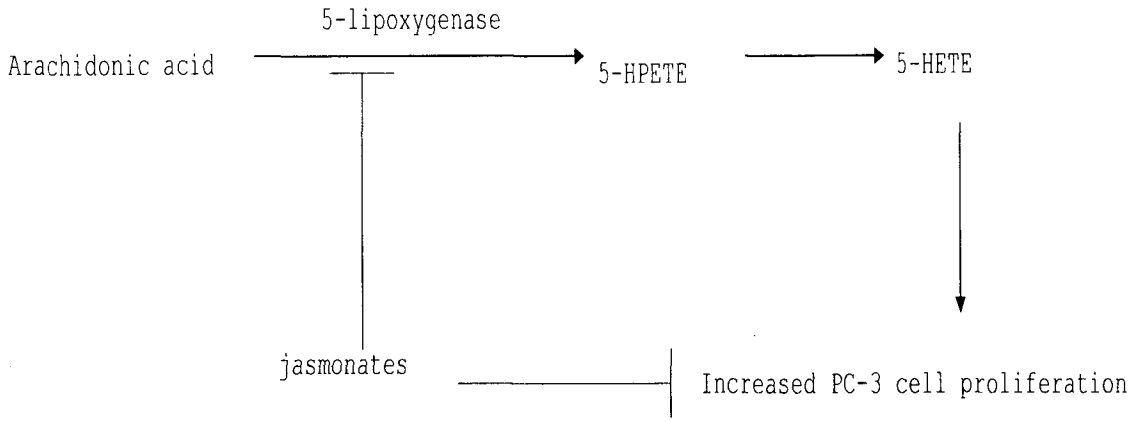


Figure 6: Proposed pathway model for jasmonate inhibition of prostate cancer cells.

CHAPTER 2

2.1 MATERIALS

2.1.1 Chemicals and Reagents

Jasmonic acid [3-oxo-2(2-pentenyl) cyclopentaneacetic acid], *cis*-Jasmone [3-oxo-2(2-pentenyl) cyclopentane], and methyl jasmonate [methyl 3-oxo- 2(2-pentenyl) cyclopentaneacetic acid], 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H- tetrazolium bromide (MTT) dye, Hoechst 33258 dye, propidium iodide (PI) and 4',6-diamidino-2-phenylindole hydrochloride (DAPI) were purchased from Sigma Chemical Co., St Louis, MO. Annexin V-FITC and BrdU assay kits were purchased from BD Biosciences PHARMINGEN, (San Diego, CA), 5-lipoxygenase was obtained from CalBiochem, CA, Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, (Logan, UT) and penicillin/streptomycin (P/S) was obtained from Invitrogen Incorporated(Grand Island, NY). Minimum essential medium (MEM Eagle) and Ham's F-12K medium were purchased from the American Type culture collection (ATCC; Manassas, VA). Stock solutions (1.0M) of each jasmonate were prepared by dissolving in

dimethylsulfoxide (DMSO), filter-sterilized and the aliquots stored at -20°C prior to use.

2.1.2 Cells and cell culture

Prostate adenocarcinoma (DU-145 and PC-3) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). DU-145 cells were cultured in Minimum Essential Medium (Eagle's modification) while PC-3 cells were cultured in HAMS F-12K medium. Each growth medium was supplemented with 10% FBS and 1% penicillin-streptomycin.

2.2 Methods

2.2.1 Cytotoxicity and Proliferation Assays

The cytotoxic effect of each agent was evaluated using the dye exclusion assay (DEA), mitochondrial dehydrogenase and clonogenic assays. The DEA measures the integrity of the cell membrane by measuring the permeability of the membrane to trypan blue, a charged molecule. Cells with an intact membrane exclude the charged dye while cells with compromised membrane take up the dye. Cells (1 million cells/plate) were cultured in 60-mm tissue culture plates and allowed to adhere overnight at 37°C in a humidified, 5% CO_2 atmosphere. The cells were treated with methyl jasmonate (MJ), cis-jasmone (CJ) or jasmonic acid (JA) at

concentrations ranging from 0-3mM. For the treatment, the medium in each plate was aspirated off and replaced with the appropriate treatment medium and incubated at 37°C in 5% CO_2 for 24 hr. At the desired time period, cells were harvested and counted using a hemacytometer. Live and dead cells were counted separately and the total number of live cells was determined. Cell viability was calculated as a percentage of live cells in the treatment groups relative to the control (100%).

2.2.2 Mitochondrial Dehydrogenase Assay (MTT)

The MTT assay measures the activity of mitochondrial dehydrogenase, an enzyme present in live but not in dead cells [54]. Briefly, cells (5,000/well) were cultured in 96-well tissue culture plates overnight to adhere and then treated with concentrations (0.5-3mM) of CJ, MJ or JA in 100µl of medium for up to 72 hr. The control cells received medium with DMSO (≤0.1%, v/v). After exposure to the agent for a specified period of time, the treatment medium was aspirated off and replaced with 100µl of fresh complete medium and 10µl of MTT (5mg/ml) in each well and the plate incubated at 37°C in a humidified, 5% CO_2 atmosphere for 3 hr. The formazan crystals formed were solubilized with 100µl of the stop solution (0.1N HCl in isopropanol). The optical densities (OD) of the resulting

solutions were determined at 570nm with a reference at 630nm using a microplate reader (Packard Instruments, Meriden, CT). Cell viability (%) was calculated by comparing the OD of the treatment group relative to that of the control cells (100%). Percent viability was taken as a measure of the cytotoxicity of each agent.

2.2.3 Colony Formation Assay (CFA)

The long-term effect of the jasmonates on the proliferation of prostate cancer cells was also assessed by using colony formation assay (CFA) as previously described [1]. Briefly, cells (500/well) were cultured in 6-well plates and allowed to adhere for 12-18 hr. The following day, the culture medium was removed and replaced by control or treatment medium (containing 0.5 or 1.0mM) and the cells incubated for 24 hr. At the end of the incubation period, cells were rinsed twice with phosphate bovine serum (PBS), once with complete culture medium and then incubated in complete culture medium for up to 14 days. The plates were viewed under the microscope every other day to monitor cell growth. At termination, the growth medium was removed from each well; the cells were washed with sterile PBS, and then stained with crystal violet (0.5g/100ml in 95% ethanol). Colonies, each containing at least 50 cells, were counted

and the cell proliferation was assessed by comparing the number of colonies formed in the treatment group to that of the control (100%).

2.2.4 Apoptosis Assays

2.2.4.1 Flow Cytometric Analyses

To determine the effects of the jasmonates on cell cycle progression and apoptosis, cells in exponential growth were treated with or without the jasmonates for specified period of time and analyzed by flow cytometry. Cells (100,000/plate) were cultured in 100mm tissue culture plates overnight to adhere. The cells were then exposed to control or varying concentrations (0.5-3mM) of MJ for up to 72 hr. At the end of the exposure period, the cells were harvested, rinsed with PBS, and fixed with 90% ethanol. For DNA content analysis, the cells were re-suspended in DNA staining solution (150µg/ml PI: 0.1% Triton-X 100: 1mg/ml RNase A (DNase-free) in PBS lacking Ca^{2+} or Mg^{2+}; 1:1:1 by volume) and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). PI fluorescence was linearly amplified, and both the area and width of the fluorescence pulse were measured using CellQuest acquisition software (Becton Dickinson, San Jose, CA). Twenty thousand events were acquired and the

percentages of hypodiploid (apoptotic; sub-G_0/G_1) populations and cells in G_0/G_1, S or G_2-M phases were determined using ModFit LT version 3.0 analysis software (Verity Software House, Inc., Topsham, ME).

2.2.4.2 Microscopic determination of apoptosis.

To confirm the mode of cell death, changes in the morphology of treated and control cells were analyzed using dual (Hoechst and PI) or DAPI staining. Following exposure to control or MJ, cells (50,000/well) plated in 12-well plates were washed three times with PBS and stained with Hoechst (1µg/ml) for 5 min and then with propidium iodide (5µg/ml) for 10 min at room temperature. Hoechst dye stained live or dying cells while PI stained dead cells. To further confirm apoptosis, cells treated as described above were fixed, on ice, with 3% paraformaldehyde and 0.1% Triton X-100 in PBS for 1 hr. Fixed cells were stained for 10 min with DAPI (1µg/ml) in distilled, de-ionized water, Changes or alterations in nuclear morphology were observed and photographed with a fluorescence microscope (Nikon Eclipse TE2000-U, equipped with a UV2A filter) to determine the presence or absence of essential morphological characteristics such as chromatin condensation and fragmentation, a hallmark of apoptosis.

2.2.4.3 Annexin V-FITC

The transversion of phosphatidylserine from the inner to outer plasma membrane leaflet, an initial event in the apoptotic pathway was assessed by dual dye staining using Annexin V-FITC/PI. Briefly, DU-145 and PC-3 cells (2 X 10^6) were cultured in 100mm dishes in the appropriate medium and allowed to adhere overnight, after which 2mM MJ was added and cells exposed for 72 hr. At the end of exposure time, both the untreated and treated cells were harvested and washed with cold PBS and then pelleted for 5 minutes at 400xg. The number of cells was adjusted to approximately 500,000 cells per ml in PBS before pelleting. Cells were washed in 2.0 mL of 1 x Annexin-V Binding Buffer (BD PHARMINGEN, San Jose, CA) for 5 minutes, centrifuged at 500Xg and then treated with Annexin-V-FITC (BD PHARMINGEN, San Jose, CA) and incubated in the dark for 15 minutes. The volume of cells-conjugated mixture was adjusted up with addition of 450µL of 1 x Annexin-V binding buffer, (each tube contains cells-conjugate mixture of approximately 500µL). Acquisition to discriminate between apoptotic and necrotic or dead cells was done by staining the cells-conjugate mixture with 10µL PI (500µg/mL) solution (BD PHARMINGEN, San Jose, CA). Acquisition was done on BD's FACSCalibur Cytometer on the FL1 (Annexin) and FL3 (PI)

channels with threshold on FSC and Duplet Discriminating Module (DDM) set at FL1. The level of shift in events distribution in the treated cells stained with Annexin-V only and Annexin-V-PI populations in comparison to untreated controls is indicative of level of apoptosis that can be induced by MJ. Numeric quantitations of these events were accomplished with population gating. In each case, 10,000 events were analyzed by flow cytometry.

2.2.4.4 Terminal deoxynucleotide transferase (TdT)-mediated dUTP nick-end labeling (TUNEL)

Apoptotic cells were visualized using DeadEnd colorimetric detection system (Phoenix Flow System Inc.,CA) according to the manufacturer's instructions. Briefly, cells ($2x10^6$) of Jurkat T-lymphocyte, DU-145 or PC-3 were fixed with formaldehyde to retain (crosslink) fragmented DNA within apoptotic cells and the 3'OH termini of DNA strand breaks were labeled with BrdUTP (this is a reaction catalyzed by the terminal deoxynucleotidyl transferase) while cellular DNA was counterstained with PI. Jurkat T-lymphocyte cells were used in this study as internal control for the two prostate cancer cell lines. The green and red fluorescence of cells probed for DNA strand breaks and DNA content was measured using 488 nm laser excitation in FACSCalibur flow cytometer (Becton Dickinson, San Jose,

CA). To calculate the proportion of cells in the respective phases of the cell cycle, the DNA content frequency histograms were deconvoluted using the MultiCycle software (Phoenix Flow Systems, San Diego, CA) (data not shown). Most experiments were run in triplicate and the experiments were repeated.

2.2.4.5 Mechanism of Inhbition

Since we proposed that the jasmonates may be inhibiting prostate cancer cell proliferation via the lipoxygenase pathway, we tested this model using the MTT assay, briefly; PC-3 cells (10,000/well) were plated in a 96-well plate. Cells were allowed to adhere overnight and the medium was then aspirated off. Cells were exposed to treatment medium containing 0.0001, 0.001, 0.01, 0.1, or 1 Units (U) of 5-lipoxygenase enzyme only; 0.0001, 0.001, 0.01, 0.1, or 1 Units (U) of 5-lipoxygenase enzyme supplemented with 2mM methyl jasmonate; 2mM methyl jasmonate only or untreated (control) for 72 hr. At the end of the exposure time, the treatment medium was aspirated off and replaced with 100µl fresh medium and 10µl MTT dye. Cell viability was assessed as described on pg 28-29.

2.3 Statistical Analysis

Results were expressed as mean ±S.D. of replicate analyses. Data analyses were performed (where appropriate) using ANOVA for a three factor, factorial treatment model with one way blocking to examine the effects of the three factors and differences at the many levels within each factor. The effectiveness of concentration levels compared to control was determined by Dunnet's two-sided test while Turkey's LSD was used to analyze the effects of MJ on the proliferation of human prostate adenocarcinoma cells at different concentration levels using multiple comparisons. Differences with p-value <0.01 were considered statistically significant.

Chapter 3

3.1 RESULTS AND DISCUSSION

We performed cytotoxicity and proliferation assays using DEA, MTT and clonogenic (CFA) techniques to determine the effects of the jasmonates on the proliferation of prostate cancer cells in culture. The effects of jasmonates on cell viability, as measured using trypan blue are as shown in Figure 7; pg 46. Exposure of cells up to 3mM of agent (CJ, JA or MJ) resulted in a dose-dependent decrease in the total number of live cells (as well as percentage of viable cells). In DU-145, MJ at 3mM showed about 50% inhibiton compared to JA treated group and about 50% inhibition of proliferation compared to CJ treated group. Both CJ and JA did not show much effect in DU-145 cell. However, with PC-3 cells at 3mM, the percent inhibitions for the three agents are MJ (35%), CJ (20%) and JA (15%) These results indicate that the jasmonates inhibit the proliferation of these prostate cancer cells at the conditions used during these experiments with MJ

significantly ($p<0.01$) causing the greatest effect at lowering the number of live cells.

The cytotoxicity of the jasmonates on the proliferation of DU-145 (Figure 8; pg 47) and PC-3 (Figure 9; pg 48) cells in culture were assessed using the MTT assay. The MTT assay measures the activity of mitochondrial dehydrogenase; a class of enzymes that are only active in live, but not in dead cells. Therefore a decrease in the proliferation of the prostate cancer cells is a measure of the cytotoxicity of the agent(s) being tested. MJ significantly ($p<0.01$) inhibited the proliferation of both cell lines compared to CJ or JA at 2mM except at 24hr (Figures 8 and 9; pg 47-48). The effective dose of MJ that inhibited cellular proliferation by 50% (ED_{50}) at 24, 48, or 72 hr were 1.6, 1.1, and 0.5mM for PC-3 cells (Table 3; pg 58) and 2.8, 2.3, and 2.0 for DU-145 cells (Table 3; pg 58), respectively. At 72 hr of exposure, a comparison of the ED_{50} showed that PC-3 cells (ED_{50}=0.5mM) were significantly ($p<0.01$) more sensitive to MJ than DU-145 cells (ED_{50}=2.0mM). Both CJ and JA minimally suppressed the proliferation of DU-145 cells (Figure 8; pg 47), however CJ showed about 50% inhibition at 72 hr with PC-3 cells (Figure 9; pg 48), JA did not show much effect after exposure for 48 or 72 hr. Also Figures 8 and 9, (pg

47 to 48) indicate that at 3mM concentration of MJ, almost all the cells were killed. Therefore, concentrations ranging from 0-1mM were used to study the long term effects of these agents on the proliferation of human prostate cancer cells.

The long-term effects of exposure of PC-3 and DU-145 cells to jasmonates on proliferation of the cells were studied using colony formation assay. MJ at 1mM concentration inhibited the proliferation of DU-145 cells by 80% ($p<0.01$) compared to the control cells (Figure 10; pg 49). Equivalent concentrations of CJ or JA caused 30 and 15% inhibition of proliferation in DU-145 cells respectively (Figure 10; pg 49). In PC-3 cells, both MJ and CJ inhibited proliferation by approximately 50-60% while JA caused about 30% inhibition of proliferation (Figure 10; pg 49). Compared to control cells, both MJ and CJ significantly ($p< 0.01$) and JA ($p< 0.05$) inhibited the long-term proliferation of PC-3 cells (Figure 10; pg 49). In earlier reports, the effects of JA and MJ (0.5-3mM) on human leukemia (Molt-4), prostate (LNCap), melanoma (SK28), and breast (MCF7) cells cultured *in vitro* was studied [7]. The highest concentration (3mM) was equivalent to the highest non-toxic pharmacological concentration of the non-steroidal anti-inflammatory agent, salicylic acid (SA),

used in humans [55]. Fingrut and Flescher reported that MJ was the most effective when compared to JA or SA at inducing cell death in every cell line studied. At 3mM concentration, MJ reduced the viability of Molt-4, LNCap, SK28 and MCF7 cells by approximately 90, 70, 40 and 35% respectively [7]. Further examination of their data showed that at 2mM, MJ reduced the viability of LNCap, SK28 and MCF7 cells by 35, 15 and 10% respectively after 24 hr exposure [7]. Our results, demonstrating the effectiveness of MJ on the prostate adenocarcinoma cell lines PC-3 (ED_{50} = 1.6mM) and DU-145 (ED_{50} = 2.8mM) at 24 hr (Figure 10; pg 49) compared very favorably with the results obtained earlier [7] for the exposure of the prostate adenocarcinoma cell line (LNCap) to MJ. In addition, our results showed that MJ was the most effective among the jasmonates in eliciting cytotoxic effect on prostate cancer cells, therefore, only MJ was employed to further study the mechanism of inhibition of proliferation of prostate cancer cells in culture. Since MJ was the most studied of all the jasmonates in this research, comparing the percent inhibition observed from dye exclusion, MTT and colony formation assays, in DU-145 cells, using dye exclusion, MJ at 3mM showed 50% inhibition at 24 hr compared to the untreated group; with MTT assay, MJ at 3mM, showed about

55% inhibition at 24 hr compared to the untreated group in DU-145 cells. In PC-3 cells, dye exclusion result showed about 30% inhibition of MJ at 3mM, where as MTT assay showed 90% inhibition after 24 hr exposure. This descripancy in percent inhibition can be explained by the fact that while dye exclusion assay measures the ability of the agent to inhibit proliferation, MTT assay measures the cytotoxicity of the agent being tested.

3.1.1 MJ induced cell cycle arrest and apoptosis.

Since MJ was more effective than CJ or JA as an inhibitor of proliferation, we investigated whether the observed inhibition of proliferation was via the induction of apoptosis. We used flow cytometric methods to study the effects of MJ on cell cycle progression and apoptosis in prostate cancer cells exposed for up to 72 hr. MJ caused cell cycle arrest in the G_2/M phase in DU-145 cells (Table 4; pg 59). In PC-3 cells, MJ caused cell cycle arrest in the S-phase arrest at 48 and 72 hr (Table 4; pg 59). The cell cycle arrest was accompanied by increase in hypodiploidy as the exposure time increased, although in PC-3 cells, there was a reduction in apoptosis observed at 72 hr compared to 48 hr, however, this calls for more studies so as to have a better understanding of what is going on. Plots of the apoptotic index (which was

calculated as a ratio of the mean values of sub-G_0/G_1 populations of the treatment group relative to that of the respective control) for the two cell lines are shown in Figure 11 (pg 50). This apoptotic index plot date were obtained from an earlier experiment which shows the same trend as that seen in Table 4.

3.1.2 Confirmation of apoptosis

To confirm apoptosis, we used dual (Hoechst and PI) staining of treated cells and fluorescence microscopy techniques. Live and dying cells fluoresced with Hoechst stain. When the same field was stained with PI and viewed using the fluorescence microscope, dead cells fluoresced (Figure 12 & 13; pg 51-52) suggesting that there are some necrotic cells. The Hoechst stain also indicated a decrease in the cell population in the MJ-treated group compared to the control (Figure 12 & 13; pg 51-52). When we stained the nuclei with DAPI dye, chromatin condensation and fragmentation were observed (Figure 14; pg 53) which confirmed that the observed cell death was via the induction of apoptosis rather than necrosis.

Since apoptotic cells lose membrane phospholipid asymmetry, resulting in the externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane, apoptosis is often quantified by measuring this

PS externalization using Annexin V-FITC staining. Annexin V is often used in conjunction with vital dyes such as propidium iodide (PI), which bind to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is breached, as occurs in the later stages of apoptosis or in necrosis. PI is a non-specific DNA intercalating agent, which is excluded by the plasma membrane of living cells, and thus can be used to distinguish necrotic cells from apoptotic and living cells. To quantify the mode of cell death (apoptosis or necrosis) induced by MJ, cells were treated as described in chapter two (pg 32-33). In the results (Figures 15 & 16; pg 54-55), Annexin V^- and PI^- (LL); Annexin V^+ and PI^- (LR); Annexin V^- and PI^+ (UL); Annexin V^+ and PI^+ (UR) represent live cells (LL), early apoptotic cells (LR), necrotic cells (UL) and late apoptotic cells (UR) respectively. As shown in Figures 15 & 16; pg 54-55, treatment of human prostate adenocarcinoma (DU-145 or PC-3) cells with 2mM MJ resulted in decrease in the live (LL)cell population as well as increase in apoptotic populations (LR + UR) when compared to their respective controls (untreated groups). For instance, in DU-145 cells, the result showed a decrease in the number of live cells from 82.69% in the control to 49.76% in the treated group with 11.1% apoptotic cells in the control and

33.49% apoptotic cells in the treated group. A similar trend was observed with PC-3 cells, the number of live cells decreased from 87.50% in the control group to 51.63% in the treated group with 12.02% apoptotic cell population in the control and 37.74% apoptotic cell population in the treated group.

To further confirm the induction of apoptosis by MJ on these prostate cancer cells, cells were treated and assayed using Terminal deoxynucleotide transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay as described in chapter two (pg 33-34). The result (Figure 17; pg 56) shows that MJ at 2mM caused a shift to the right of the histograms in treated groups compared to the untreated control. M1 and M2 represent live and apoptotic cells respectively. These values were obtained using the method described by Phoenix Flow System, San Diego, CA. in which the controls are used as templates to determine the level of apoptosis in the treatment groups. In DU-145 cells, (Figure 17; pg 56) M1 was 90.94% while M2 was 9.20% in the control, however, in the treated group, M1 showed about 50.83% with M2 showing 49.27%. In PC-3 cells, (Figure 17; pg 56) M1 was 91.16% and M2 was 8.95% in the untreated group, in the treated group, M1 decreased to 29.90% as M2 increased up to 70.23%. These sets of results (Figure 17;

pg 56) confirmed that MJ induced apoptosis in these cancer cells. Jurkat T-Lymphocyte cells which were used as internal control however, showed about 8.18% apoptosis in the control and 69.06% in the treatment group, this does not tell us much about the mechanism of inhibition caused by MJ and so more studies are necessary to help elucidate the possible mechanism of induction of apoptosis by MJ on the two prostate cancer cell lines (DU-145 and PC-3).

3.1.3 Mechanism of Inhibition

Figure 18; pg 57 shows that when PC-3 cells were treated with 2mM MJ , there was an inhibition of about 90%, however, when the same concentration of MJ was supplemented with different doses of 5-lipoxygenase, 5-LO was able to stimulate the cells and thus reduce the inhibitory effects of MJ on PC-3 cells. This suggests that 5-lipoxygenase affects the inhibitory effect of methyl jasmonate, thus 5-lipoxygenase pathway may have a role to play in the proliferation of prostate cancer cell line (PC-3). Also, when cells were treated with 5-LO only, there was a slight inhibition rather than stimulation, suggesting that 5-LO might actually be affecting the chemical structure of MJ thus reducing its (MJ) inhibitory effects on the cancer cells. The result (Figure 18; pg 57) validates our earlier proposal that MJ may be inhibiting the proliferation of

prostate cancer cells and that 5-lipoxygenase may be antagonist to MJ.

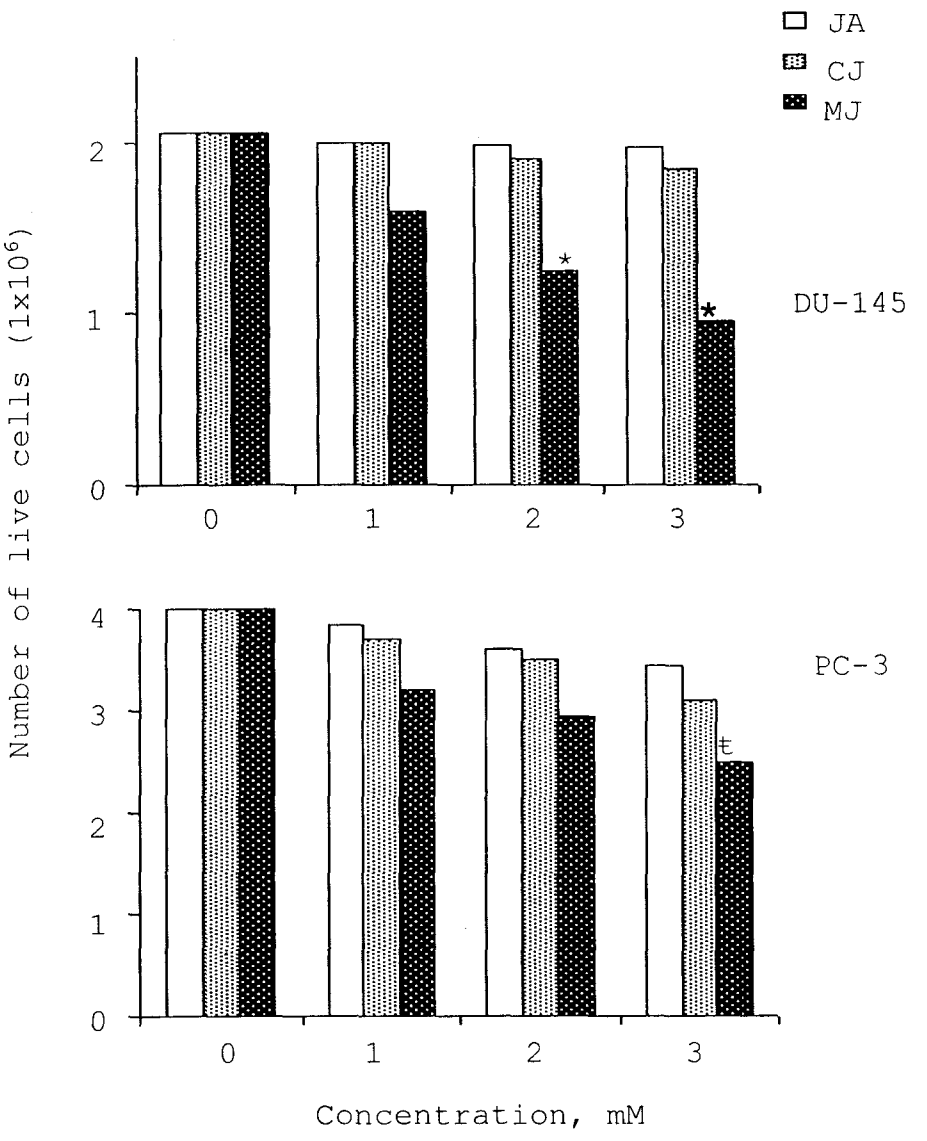


Figure 7: Effects of jasmonates on the proliferation of human prostate adenocarcinoma cells (DU-145 and PC-3) exposed for 24 hr. Jasmonic acid (JA), cis-jasmone (CJ) and methyl jasmonate (MJ) inhibited the proliferation of prostate cancer cells in a dose-dependent fashion with MJ showing the greatest effect. Error bars are so small as not to be visible. * $p<0.01$ for MJ versus either CJ or JA at 2mM or 3mM. ŧ $p<0.05$ for MJ at 3mM versus control for MJ in the two cell lines

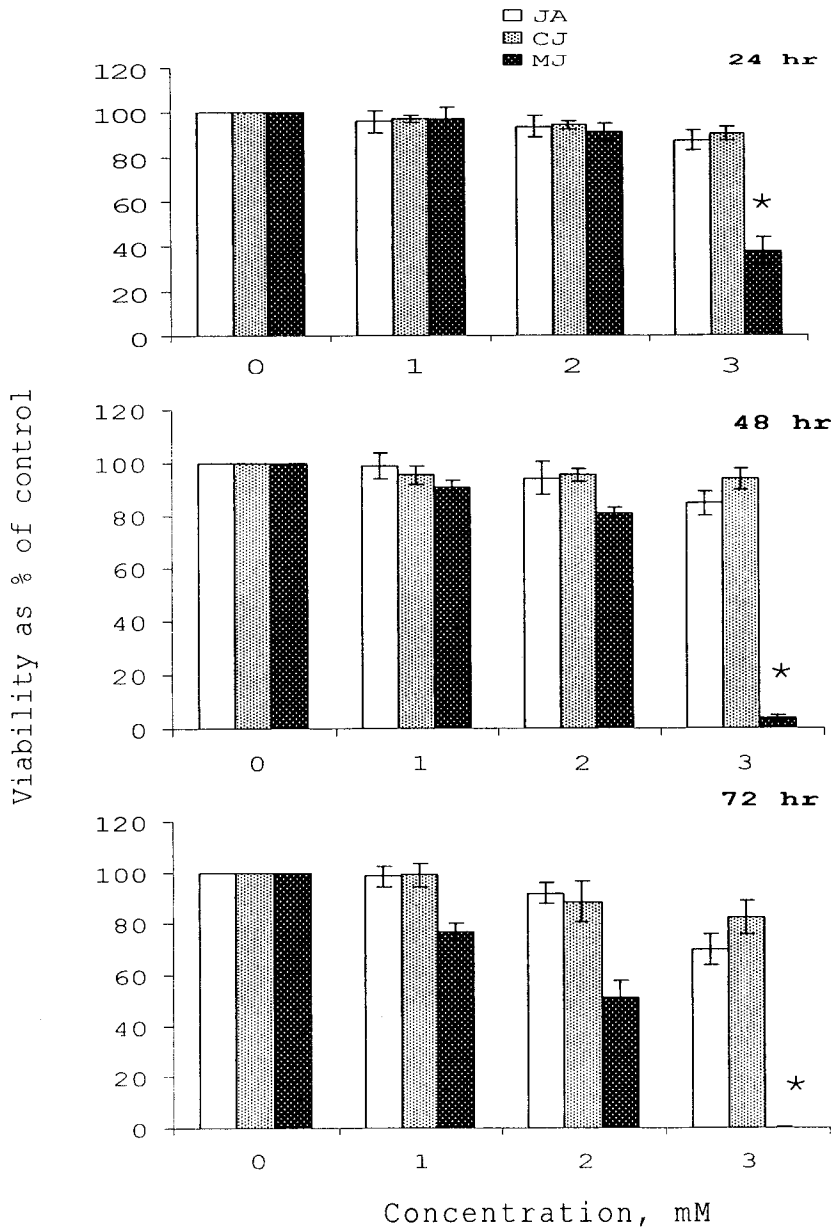


Figure 8: Jasmonates-induced inhibition of proliferation of DU-145 cells in culture using MTT assay. JA and CJ showed little or no inhibition of proliferation while MJ showed the greatest effect. The proportion of viable cells (Mean ± S.D.; n=12) was plotted against jasmonate concentrations (mM). The figure is representative of at least three separate experiments. * $P<0.01$ for MJ versus either CJ or JA at 3mM after 24, 48 or 72 hr of exposure. There were no significant ($p<0.01$) difference between CJ and JA at all concentrations and time points. There were no error bars in the control since they were used to normalize the treatment group.

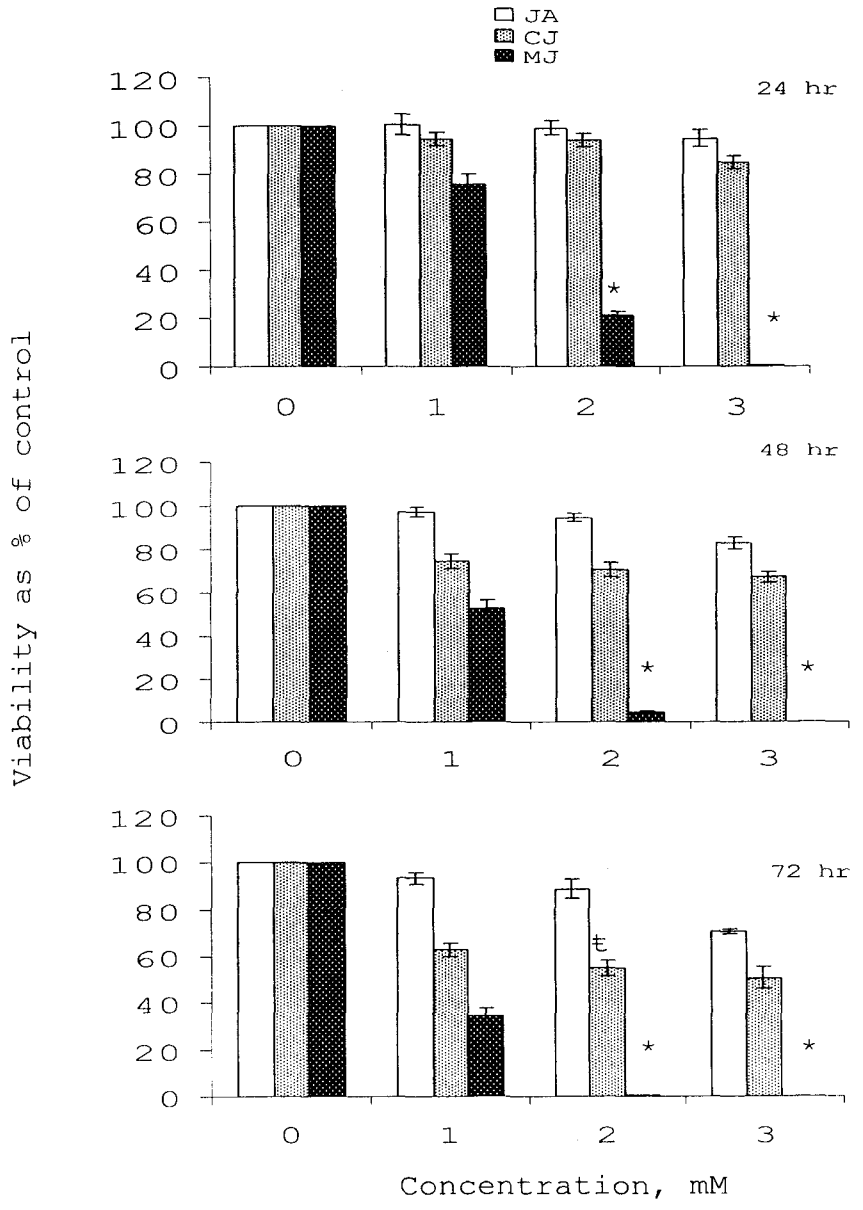


Figure 9: Jasmonate-induced inhibition of proliferation of PC-3 cells in culture using MTT assay. MJ elicited cytotoxic effects on the cells. CJ shows little effect whereas JA shows no inhibition of proliferation even at 3mM after 72 hr. The concentrations of jasmonates that caused 50% inhibitions of proliferation are shown in table 3, pg 57. * $P<0.01$ for MJ versus either CJ or JA at 2 or 3mM after 24, 48 or 72 hr of exposure. ŧ $p<0.05$ for CJ versus JA at 72 hr. There were no significant ($p<0.05$) difference between CJ and JA at 24hr. There were no error bars in the control since they were used to normalize the treatment group.

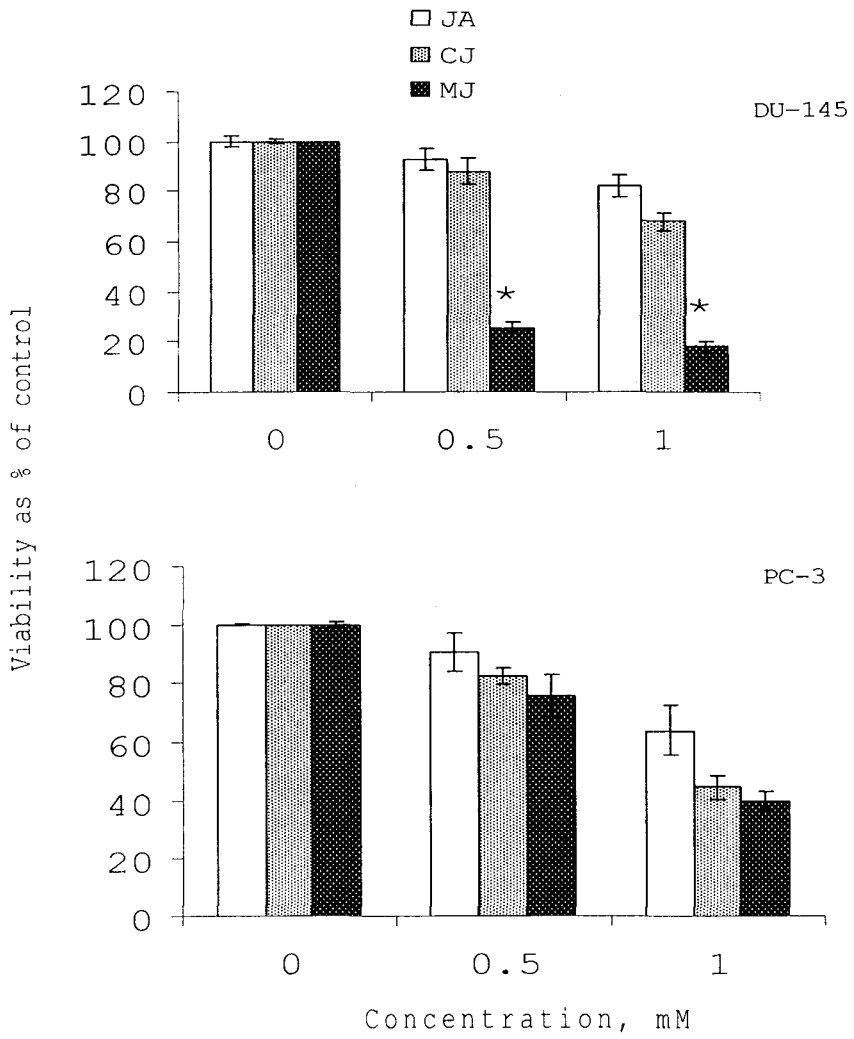


Figure 10: The jasmonates delayed the proliferation of prostate cancer cells cultured *in vitro*. Cells were exposed to 0, 0.5 or 1.0mM jasmonic acid (JA), *cis*-jasmone (CJ) or methyl jasmonate (MJ) for 24 hr. The treatment medium was aspirated off and the cells were washed twice with PBS and once with medium. The complete medium was added and the cells incubated for 14 days at 37°C in a humidified, 5% CO_2 atmosphere. The cells were then washed, and stained with crystal violet and the colonies counted. Cell viability was calculated as a percentage relative to the untreated (control) cells (100%). Values plotted were Mean ± S.D. (n=3) versus the concentration of jasmonates (mM). The figure is representative of two separate experiments. * $P<0.01$ for MJ at 0.5 and 1mM versus control in DU-145. Both MJ and CJ significantly ($p< 0.01$) and JA ($p< 0.05$) inhibited the long-term proliferation of PC-3 cells.

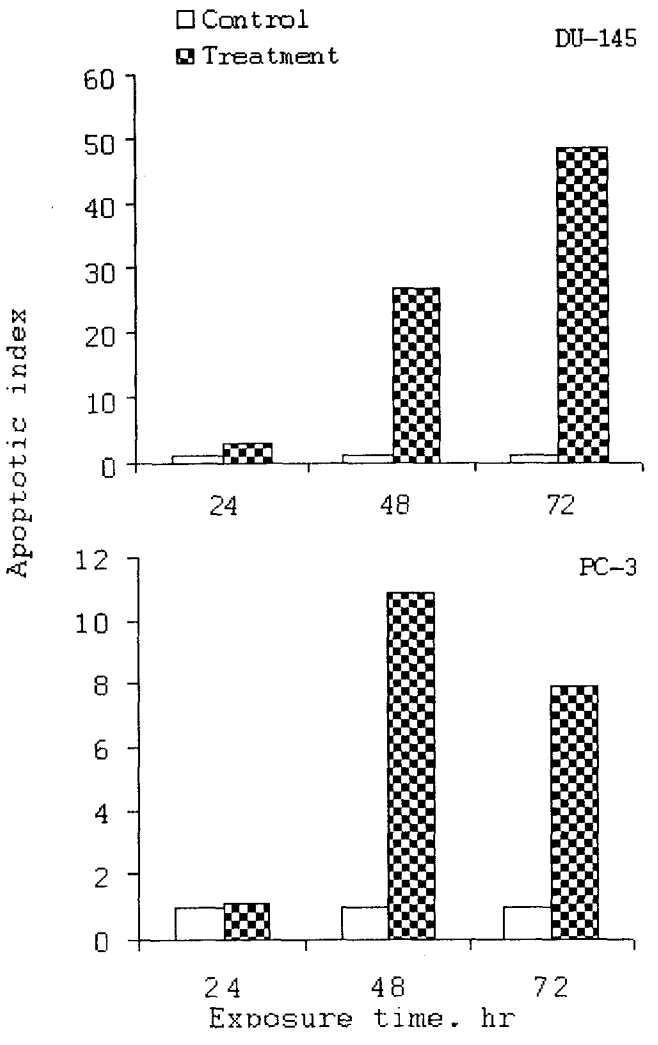


Figure 11: Methyl jasmonate (MJ) induced time-dependent increase in sub- G_0/G_1 populations in prostate cancer cells in culture. Cells were treated in triplicates with 0 or 2mM MJ for 24, 48 or 72 hr, harvested, fixed, stained with PI and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The data were acquired as described for Table 4 and the Apoptotic Index was calculated as a ratio of the Mean values of sub-G_0/G_1 populations of the treatment group relative to that of the respective Control. The figure represents a plot of the Apoptotic Index (arbitrary units) versus exposure time. The figure is representative of two separate experiments.

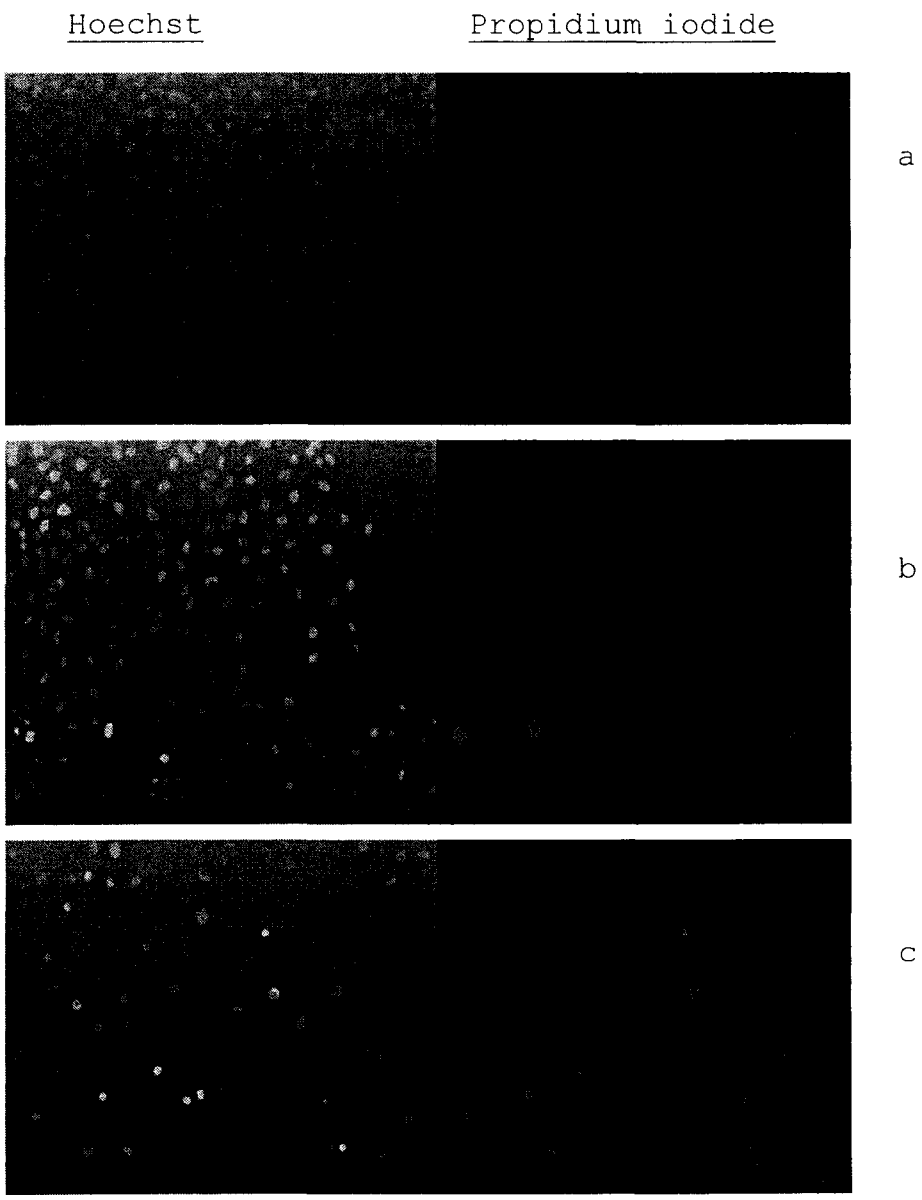


Figure 12: Methyl jasmonate (MJ) induced apoptosis in prostate adenocarcinoma (DU-145) cells in culture after 72 hr exposure. Cells 50,000/well) were double-stained with Hoechst (1µg/ml) and propidium iodide (5µg/ml). The fluorescence of live or dying cells (Hoechst fluorescence) and dead cells (PI fluorescence) were acquired using fluorescence microscopy (Nikon TE 2000U, Lake Forest, CA). a, b and c correspond to untreated (control), 1mM and 2mM MJ respectively.

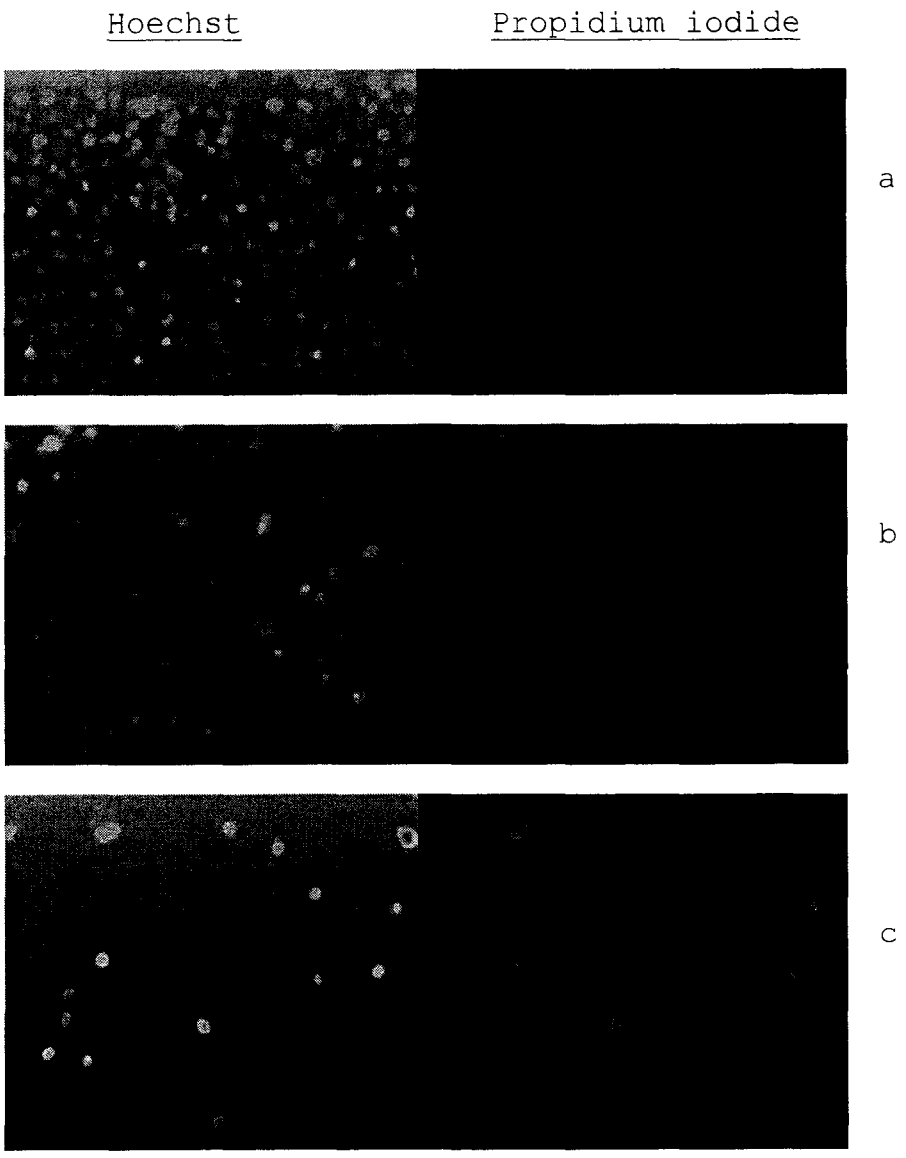
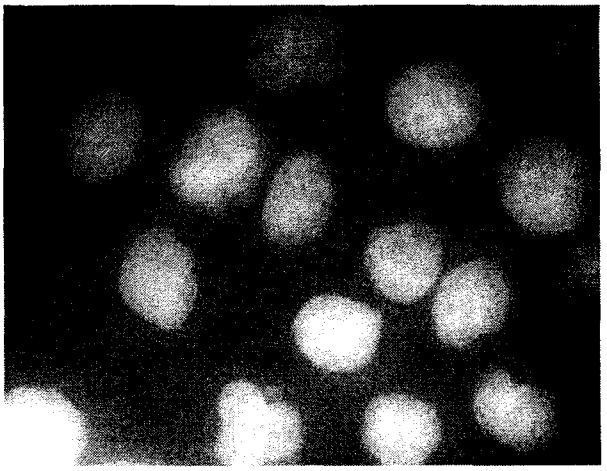


Figure 13: Methyl jasmonate (MJ) induced apoptosis in prostate adenocarcinoma (PC-3) cells in culture after 72 hr exposure. Cells 50,000/well) were double-stained with Hoechst (1µg/ml) and propidium iodide (5µg/ml). The fluorescence of live or dying cells (Hoechst fluorescence) and dead cells (PI;fluorescence) were acquired using fluorescence microscopy (Nikon TE 2000U, Lake Forest, CA). a, b and c correspond to untreated (control), 1mM and 2mM MJ respectively.

Control



MJ

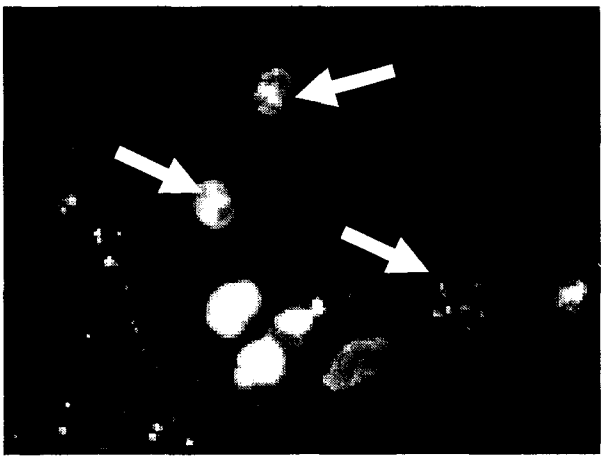
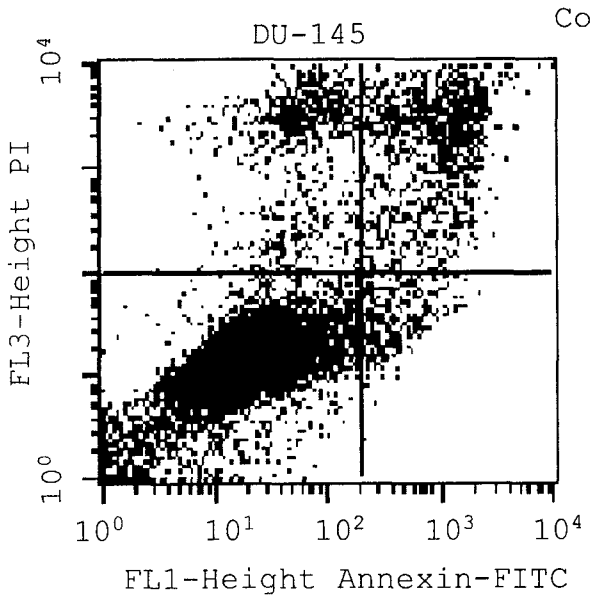
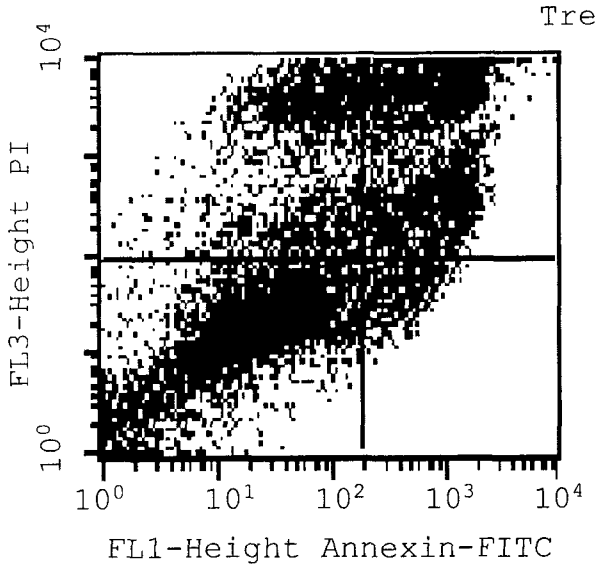


Figure 14: Methyl jasmonate induced nuclear fragmentation in DU-145 cells. Cells, both the control and treated group were fixed in 3% paraformaldehyde and stained with DAPI for 10 minutes. Changes in the nuclear morphology of the cells were observed at 40X magnification and then photographed using a fluorescence microscope. Arrows show nuclear chromatin condensation and fragmentation which are hallmarks of apoptosis.

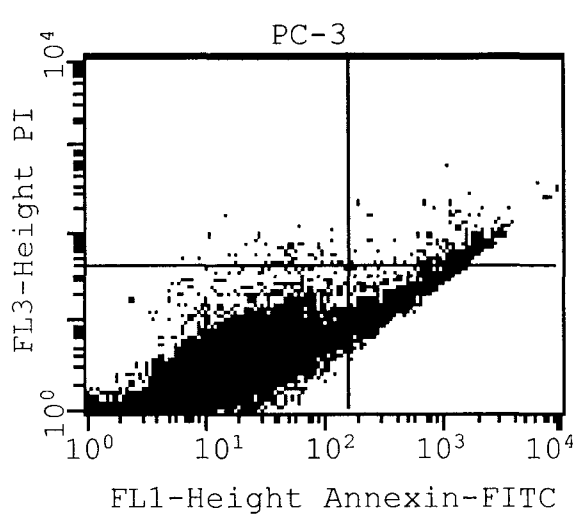


Quadrant	% Total
UL	6.21
UR	8.87
LL	82.69
LR	2.23



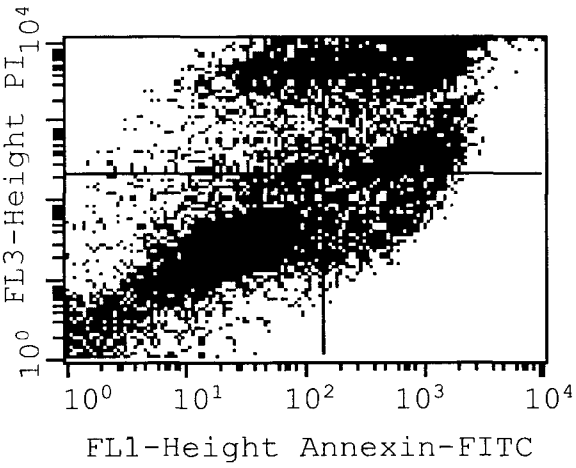
Quadrant	% Total
UL	16.74
UR	29.14
LL	49.76
LR	4.35

Figure 15: DU-145 cells contour diagram of FITC-Annexin V/PI flow cytometry of cells treated for 72 hr. Untreated and treated cells were harvested and washed with 5 ml wash buffer. Lower left quadrants (LL) of each point show the viable cells. The upper right quadrant (UR) represents late apoptotic cells. The lower right quadrants (LR) represent the early apoptotic cells and the upper left (UL) quadrant represents the necrotic cells. 2mM MJ was used because at concentrations above 2mM the cells were almost wiped out, and because 2mM MJ was used in most of the assays as optimum concentration, it is necessary to be consistent.



Control

Quadrant	% Total
UL	0.48
UR	4.47
LL	87.50
LR	7.55



Treatment

Quadrant	% Total
UL	10.63
UR	27.94
LL	51.63
LR	9.80

Figure 16: PC-3 cells contour diagram of FITC-Annexin V/PI flow cytometry of cells treated for 72 hr. Untreated and treated cells were harvested and washed with 5 ml wash buffer. Lower left quadrants (LL) of each point show the viable cells. The upper right quadrant (UR) represents late apoptotic cells. The lower right quadrants (LR) represent the early apoptotic cells and the upper left (UL) quadrant represents the necrotic cells. 2mM MJ was used because at concentrations above 2mM the cells were almost wiped out, and because 2mM MJ was used in most of the assays as optimum concentration, it is necessary to be consistent.

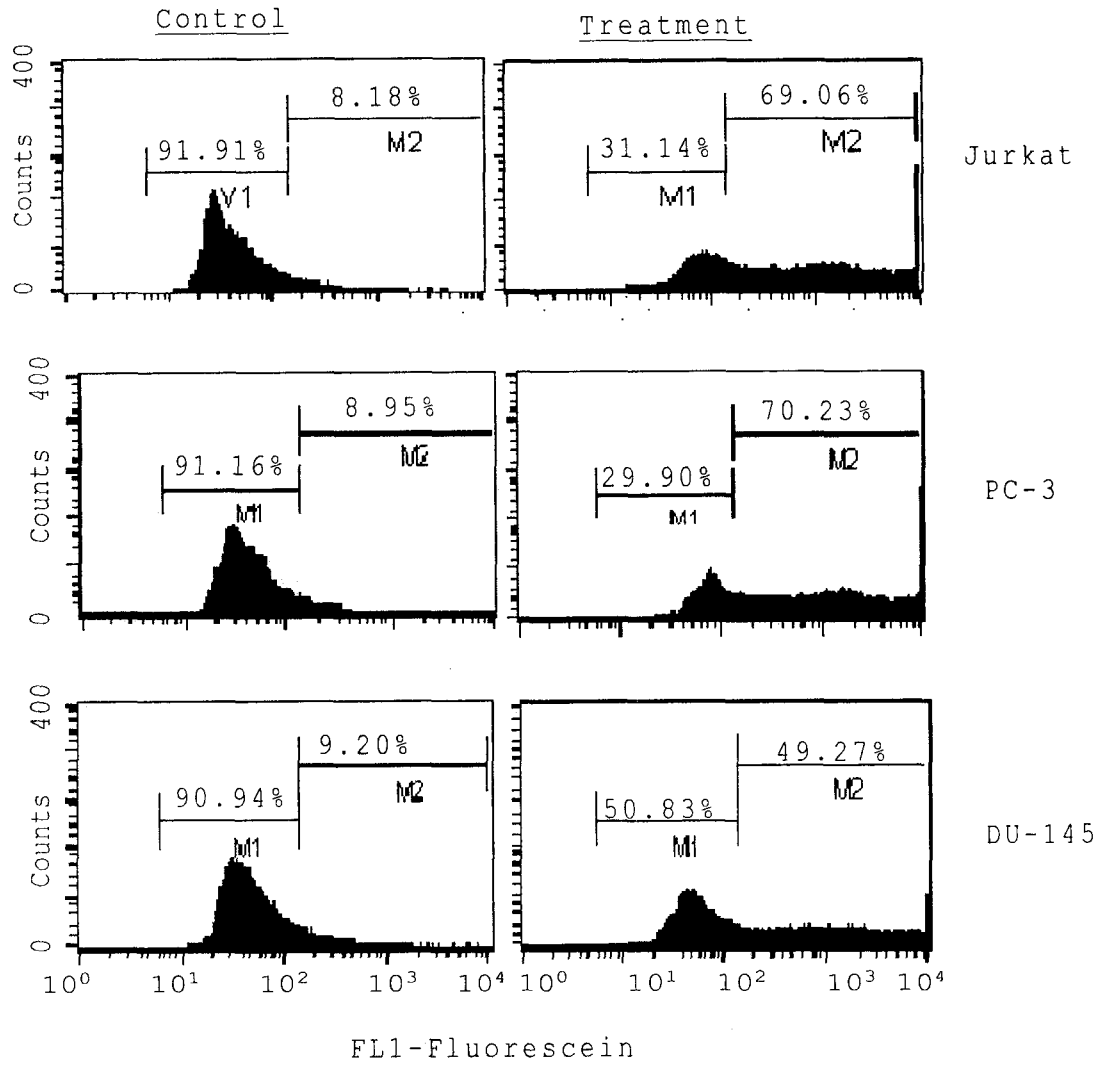


Figure 17: Jurkat T-lymphocyte, PC-3 and DU-145 cell death analysis using BrdUrd labelling. Untreated and treated cells for 72 hr were harvested and washed with 5 ml of wash buffer. Cells were incubated in DNA labelling for 1 hr at 37°C in water bath. After incubation, cells were rinsed twice with 1.0ml rinsing buffer and resuspended in 0.1ml of Fluorescein-PRB-1 Antibody solution for 30 minutes in the dark. 0.5ml of propidium iodide/RNase A solution was added and cells incubated for 30 minutes. Cell distribution was analyzed using flow cytometry. M1 and M2 represents live and apoptotic cells respectively. Jurkat cells were used as internal control. The experiment was repeated twice. 2mM MJ was used because at concentrations above 2mM the cells were almost wiped out, and because 2mM MJ was used in most of the assays as optimum concentration, it is necessary to be consistent.

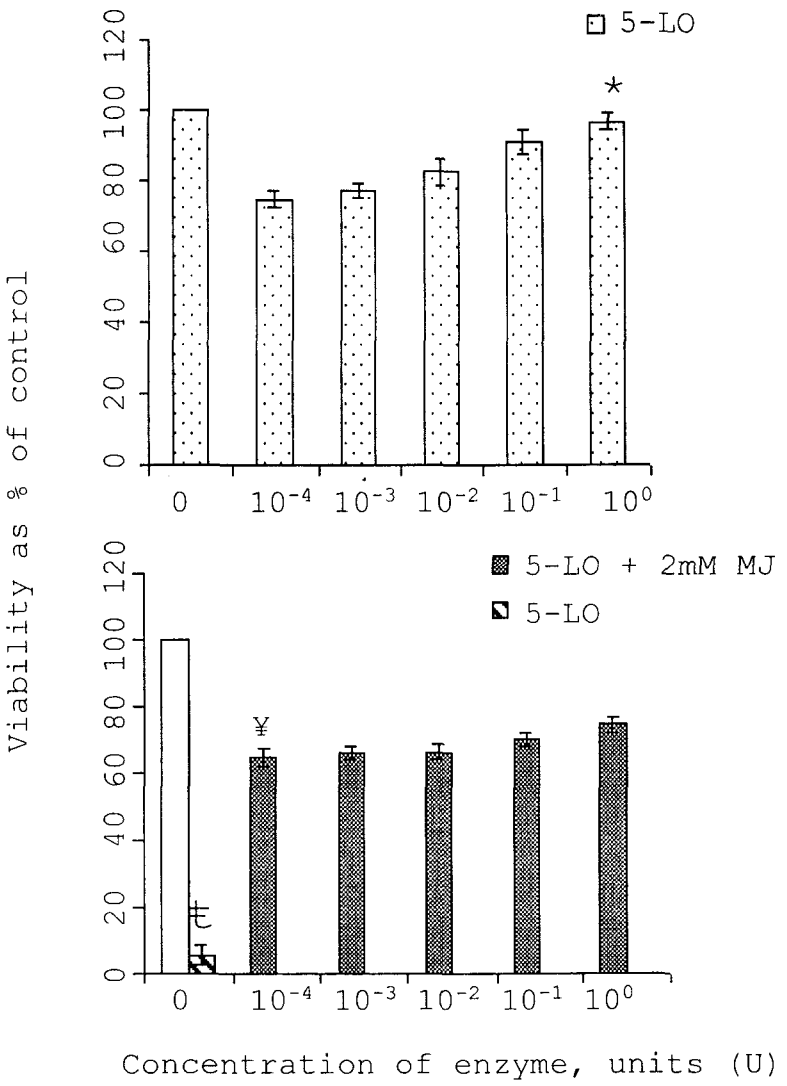


Figure18: Effect of 5-lipoxygenase on PC-3 cell growth. $1x10^4$ cells were plated per well of 96-well plate and allowed to adhere overnight. Cells were then stimulated with different concentrations of 5-lipoxygenase only or 5-lipoxygenase supplemented with 2mM MJ and exposed for 72 hr. Cells were then stained with alamar blue dye. Absorbance was taken at excitation of 590nm and emission wavelength of 530nm. Data are the mean ± SD (N=4). * No significant ($p<0.05$) difference between 5-LO at 1U versus control in the 5-LO-only treated group. ŧ $p<0.01$ for MJ-only treated group versus control and ¥ $P<0.05$ for MJ-only treated group versus MJ supplemented with 1U 5-LO.

Table 3: Concentration of MJ (mM) that inhibited proliferation of PC-3 or DU-145 cells by 50% (IC_{50}).

		24 hr	48 hr	72 hr
Cell line	Agent	IC50(mM)	IC50(mM)	IC50(mM)
PC-3	MJ	1.6	1.1	0.5
DU-145	MJ	2.8	2.3	2.0

Table 4: Effects of methyl jasmonate (MJ) on PC-3 and DU-145 cells in culture

		PC-3				DU-145			
	Treatment	G_0/G_1	S	G_2/M	Sub-G_0/G_1	G0/G1	S	G_2/M	Sub-G_0/G_1
24Hr	Control	51.7 ±1.4	33.8 ±1.0	14.5 ± 0.7	0.01 ± 0.02	51.6 ±1.4	14.5 ±0.6	16.2 ±1.1	0.01 ± 0.2
	2.0mM	59.7 ±5.4	26.1 ±9.0	14.2 ±3.6	0.15 ± 0.1	63.5 ±2.0	16.2 ±0.3	26.5 ±2.7	0.14 ± 0.1
48Hr	Control	56.1 ±1.0	26.3 ±0.6	17.6 ±0.4	0.01 ± 0.0	53.6 ±0.1	15.3 ±0.6	9.1 ±0.9	0.84 ±0.1
	2.0mM	54.2 ±2.5	34.7 ±2.5	10.9 ±0.6	5.45 ± 2.6	62.2 ±1.0	20.1 ±0.4	27.5 ±6.2	6.89 ±0.3
72Hr	Control	56.4 ±1.0	25.6 ±0.2	17.9 ±0.9	0.01 ±0.0	56.2 ±0.7	13.3 ±1.3	6.6 ±0.1	0.68 ±0.6
	2.0mM	51.8 ±0.6	37.4 ±0.5	10.8 ±0.12	0.03 ± 0.02	58.8 ±1.6	17.5 ±1.1	20.6 ±2.1	11.0 ±0.8

Prostate adenocarcinoma cells (PC-3 & DU-145) were treated with jasmonate (MJ) for 24-72 hr. The cells were harvested, fixed with ethanol, stained with propidium iodide and analyzed for cells in different phases of the cycle using a FACSCalibur flow cytometer. Values are Mean±SD of triplicate analyses. The experiment was performed twice.

Chapter 4

4.1 CONCLUSION

The jasmonates inhibited the proliferation of human prostate adenocarcinoma cells in culture. MJ was the most potent of the three members of this class significantly inhibiting proliferation and inducing cell death. Although CJ showed some cytotoxic effects against PC-3 cells (Figure 9; pg 48), it did not show much inhibition of proliferation or induce cell death in DU-145 cells at the concentrations tested (Figure 8; pg 47). JA was the least effective of the jasmonates (Figure 8 and 9; pg 47-48) when compared to CJ or MJ. These results are in agreement with Fingrut and Flescher who reported that MJ was a more potent inhibitor of proliferation than JA in human leukemia (MOLT-4), breast (MCF7) and prostate (LNCap) cancer cells in culture [7]. MJ was reported to be relatively non-toxic to whole animals. Fingrut and Flescher administered MJ (236 mg/kg body weight) intraperitoneally to mice daily for 30 days. They found that "this dose, and even a two times higher

dose, did not cause any apparent toxic effect". These researchers also reported that there were no changes in the activity level, feeding and appearance of the treated mice compared to the controls [7].

We confirmed using flow cytometry, Annexin V-FITC, tunnel (BrdU) and fluorescence microscopy assays that the mechanism for the observed inhibition of proliferation of prostate cancer cells by MJ was via cell-cycle arrest and the induction of apoptosis. Epidemiological [56, 57] and experimental [36] studies have indicated a link between arachidonic acid metabolism and the proliferation of human prostate adenocarcinoma cells. Arachidonic acid, through its conversion to 5-hydroxyeicosatetraenoic acid (5-HETE) in the lipoxygenase pathway strongly stimulated the growth of hormone-responsive (LNCaP) and hormone-refractory (PC-3) human prostate cancer cell lines in culture [36]. 5-Lipoxygenase, a key enzyme in the synthesis of 5-HETE, was over expressed in prostate adenocarcinoma cells (PC-3) [58]. Furthermore, the inhibition of arachidonate 5-lipoxygenase activity by the specific inhibitor MK886 blocked 5-HETE production and induced apoptosis in human prostate cancer cells (PC-3) [36]. Both the inhibition of lipoxygenase activity by MK886 and the induction of apoptosis in prostate cancer cells were reversed by the

addition of 5-HETE [36]. Since MJ is a downstream product of the lipoxygenase pathway in plants [43], we proposed that the mechanism of action of MJ may involve the inhibition of the lipoxygenase pathway, however, our results show slight inhibition of cells when treated with 5-lipoxygenase as shown in our results (Figure 18; pg 57) in human prostate cancer cells. Such inhibition would block the synthesis of 5-HETE, an important compound needed for the survival and proliferation of the prostate cancer cells. [36]

4.2 Recommendation for future study

Having confirmed that these agents, especially MJ, inhibited the proliferation of human prostate adenocarcinoma cells via the induction of apoptosis, it would be pertinent to investigate the cellular and molecular mechanisms that are involved in the response of prostate cancer to MJ. This can be achieved by studying the effects of jasmonates on the synthesis of 5-HETE, since we proposed a 5-HETE dependent pathway (figure 6, pg 25), if COX inhibitors are applied to these prostate cancer cell, we would think that there will be cell stimulation as already confirmed in an earlier study [36], since arachidonic acid will all be diverted to the lipoxygenase

pathway, therefore, since we proposed that MJ would inhibit the synthesis of 5-HETE, addition of MJ would reduce the stimulatory effects of 5-HETE on the cell. Also this 5-HETE can be quantified to give a better understanding of whether MJ is inhibiting its synthesis of not. It will also be important to study the role of p53 in MJ-induced apoptosis in prostate cancer cells, however, it has been reported earlier that PC-3 for instance lack p53 [26], it is still worth studying to know if MJ can cause p53 expression in these cancer cells. Finally, the roles of other pro and anti-apoptotic proteins such as Bcl-2, Bax, Rb, Bcl-x etc. will be necessary to look into since that will help to elucidate the proper mechanism/pathway involved in these reactions.

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