INVESTIGATING THE ROLE OF ZOLEDRONIC ACID ON INTERLEUKIN-6 CYTOKINE EXPRESSION IN PROSTATE CANCER CELL LINES

**A Thesis Submitted to
the Graduate School of Engineering and Science of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of**

MASTER OF SCIENCE

in Biotechnology

**by
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**June 2006
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ACKNOWLEDGEMENTS

First I would like to thank to everyone who had contributed to my education troughout my life.

Many thanks go to my advisor, Assist. Prof. Dr. Fuat DOYMAZ. Thank you for your knowledge, concern, humor, friendship and patience during my studies in İYTE.

I would like to thank Assoc. Prof. Dr. Çağ ÇAL for his inavulable ideas, supervision, throughout this study.I am grateful to Assist.Prof.Dr.Selim UZUNOĞLU for his valuble suggestions and supports in my thesis.

I would also like to thank Assist. Prof. Dr. Oğuz BAYRAKTAR for his ever-lasting encouragement and support in my life. I am particularly grateful to my friends Yonca BAŞSOY and Çığır BİRAY for their understanding, and endless patience.

Finally, I want to express my gratitude to my dear family for their excellent support, understanding and encouragement in all my life.

ABSTRACT

Investigating The Role Of Zoledronic Acid On Interleukin-6 Cytokine Expression In Prostate Cancer Cell Lines

Prostate cancer (CaP) is a common disease in aged men. Androgen deprivation therapy is the first line treatment for advanced disease. Following this therapy, within few months CaP cells become androgen independent in a few mounths. The mechanism responsible for development of androgen-independent cancer is not known. Evidences suggest that growth factors and cytokines including Interleukin–6 (IL-6) cast a main role in this mechanism. IL–6 is an important cytokine on CaP progression. Bisphosphonates inhibit the proliferation and induce the apoptosis of certain prostate cancer cell lines, *in vitro*. However, their mechanism of action is not clearly understood. One possible mechanism might be the IL-6 mediated pathway. This relation between zoledronic acid (ZA) and IL-6 hasn't been studied.

The objective of this study is to evaluate the cytotoxic and apoptotic effect of ZA on hormone independent CaP cell line (PC-3) and to understand the relation of underlying mechanism of ZA-mediated cytotoxicity and IL-6 expression.

PC-3 cells were treated with different concentrations of ZA. Drug-mediated cytotoxicity and apoptosis were determined. IL-6 levels were measured from the supernatants of PC-3 cells at 24, 48, 72 hrs.

Maximum cytotoxicity was achieved at 90 and 100 µM of ZA. ZA produced the classical DNA ladders on gel synonymous of apoptosis. The decrease in IL-6 secretion was found to be statistically significant compared to controls. The decreasing effect of ZA on IL-6 expression might suggest a new mechanism of action of ZA's antitumor activity. This outcome may offer a novel therapy that utilizes IL-6 antagonists along with ZA in CaP patients.

ÖZET

PROSTAT KANSERİ HÜCRE HATLARINDA ZOLEDRONİK ASİDİN İNTERLÖKİN-6 SİTOKİNİNİN EKSPRESYONU ÜZERİNDEKİ ROLÜNÜN ARAŞTIRILMASI

Prostat kanseri günümüzde ileri yaş erkekler arasında en sık görülen kanser tipidir. Hastalığın ileri evresinde kullanılan tedavi androjen baskılanmasıdır. Androjen baskılanmasından kısa bir süre sonra prostat kanseri hücrelerinin birçoğu hormondan bağımsız çoğalmaya devam ederler. Bu çoğalmanın mekanizması henüz bilinmemekle birlikte çeşitli büyüme faktörleri ve sitokinlerin bu mekanizmada önemli rol aldığı düşünülmektedir. İnterlökin–6 (IL–6) prostat kanserinin ilerlemesinde önemli bir sitokindir. Bifosfonatlar, çeşitli prostat kanser hücre hatlarında çoğalmayı önleyici ve apoptozisi tetikleyici bir etki göstermektedir, fakat etki mekanizmaları henüz bilinmemektedir. Olası mekanizmalardan biri IL–6 yolağı üzerinden olabilir. Şu ana kadar Zoledronik asit (ZA) ile IL–6 ilişkisini ortaya koyan bir çalışma yapılmamıştır.

Bu çalışmanın amacı, hormon-dirençli prostat kanseri hücre hattı (PC–3) üzerinde ZA'nın sitotoksik ve apoptotik etkisini göstererek ilaca bağlı meydana gelen bu sitotoksisitenin mekanizmasının IL–6 ile ilişkisini saptamaktır.

PC–3 hücreleri farklı konsantrasyonlarda ZA ile inkübe ediltikten sonra ZA'nın PC–3 hücreleri üzerindeki sitotoksisitesi ve apoptotik etkisi ölçüldü. Hücrelerin süpernatantlarındaki IL–6 düzeyleri 24, 48 ve 72 saatlerde ölçüldü.

Maksimum sitotoksisiteye ZA'nın 90 ve 100 µM konsantrasyonunda ulaşıldı. ZA ile inkübasyon sonrasında apoptozisin göstergesi olan DNA merdiven oluşumu jel üzerinde gözlendi. Kontrol grubuna oranla IL–6 salınımındaki azalmalar istatistiksel açıdan anlamlı bulundu. ZA'nın IL–6 ekspresyonu üzerindeki azaltıcı etkisi, ZA'nın antitümör aktivitesinde yeni bir mekanizmanın varlığına işaret etmektedir. Bu sonuç, prostat kanserli hastalarda zoledronik asitle birlikte IL–6 antagonistlerinin kullanılması ile yeni bir tedavi seçeneğine kapı açmaktadır.

To Selim Uzunoğlu, with all my respect and special thanks.

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CHAPTER 1

INTRODUCTION

Our growing understanding of cancer cell biology should lead to better ways of diagnosis and treating this disease. Anticancer therapies can be designed to destroy cancer cells preferentially by exploiting the properties that distinguish them from normal cells, including the defects they harbor in their DNA repair mechanisms, cell cycle check points, and apoptosis pathways. Tumors can also be attacked through their dependence on their blood supply. By understanding the normal control mechanisms and how they are subverted in specific cancers, it becomes possible to devise drugs to target cancers more precisely. Cancer cells are defined by two heritable properties: they and their progeny reproduce in defiance of the normal restraints on cell division and invade and colonize territories normally reserved for other cells. It is the combination of these actions that makes cancer dangerous. An isolated abnormal cell that does not proliferate more than its neighbors does no significant damage, no matter what other disagreeable properties it may have; but if its proliferation is out of control, it will give rise to a tumor, or *neoplasm*- a growing mass of abnormal cells. As long as the neoplastic cells remain clustered together in a single mass, the tumor is said to be benign. At this stage, a complete cure can usually be achieved by removing the mass surgically. A tumor is considered a cancer only if it is malignant, that is, only if its cells have acquired the ability to invade surrounding tissue. Invasiveness usually implies an ability to break loose, enter the bloodstream or lymphatic vessels, and form secondary tumors, called metastases, at other sites in the body. The more widely a cancer spreads, the harder it becomes to eradicate.

Prostate cancer is a common disease in aged men. Normal and early stage prostate cancer cells require androgen for growth and survival, which led to the formulation and successful application of androgen ablation and anti-androgen therapies as principal treatment modalities for the disease. However, beneficial effects of the hormonal therapies are often temporary, and the cancer regularly progresses to the androgen-refractory stage characterized by recurrent growth and metastasis, predominantly to bone (Feldman et al, 2001, Nelson et al, 2003). Despite decades of

intense laboratory and clinical research, to date there is no cure for androgen-refractory prostate cancer. The existence of androgen-independent prostate tumors suggests that release of locally produced and/or circulating growth factors such as Interleukin-6, which work through cellular receptors, can switch the prostate cells from an inactive to an activated phenotype leading to cellular proliferation. The majority of prostate cancers arise from epithelial cells, and in tissue cultures, epithelial prostate cancer cells produce factors that act in an autocrine fashion to regulate their growth and survival (Xie et al, 2002, Berger et al, 2003).

Interleukin-6 (IL-6) is a multifunctional cytokine which is involved in regulation of growth of various malignant tumors. IL-6 also regulates gene expression in a number of different organs, modulates immune function, stimulates the hypothalamic-pituitary axis, promotes osteoclasting resorption in bone, and stimulates bone marrow (Ershler et al, 2000). The biological activities of IL-6 are mediated by the IL-6 receptor, which binds IL-6 specifically and with low affinity, and gp130, which associates with the IL-6-IL-6 receptor complex, resulting in high-affinity binding and activation of intracellular signaling. The evidences were shown that IL-6 may be an important autocrine and/or paracrine growth factor for prostate cancer and may contribute to cancer progression (Siegall et al, 1990, Okamoto et al, 1997, Siegsmund MJ et al, 1994, Hobisch A et al, 2000). For example, inhibition of IL-6, with anti-IL-6 antibody, sensitizes androgen-independent prostate cancer cells to chemotherapeutic agents in vitro. Additionally, IL-6 induces androgen receptor (AR) activity through both increasing AR gene expression and activating the AR in the absence of androgen in prostate cancer cells (Lin et al, 2001). These data suggest that targeting IL-6 may have multiple benefits in prostate cancer patients.

Bisphosphonates inhibits the proliferation and induces the apoptosis of certain prostate cancer cell lines, *in vitro* (Lee et al, 2001). Zoledronic acid, nitrogen-containing bisphosphonate, is used in prostate cancer patients according to laboratory evidence documenting that prostate cancer bone metastases are associated with an increased osteoclast-mediated bone resorption (Ikeda et al, 1996, Maeda et al, 1997). Furthermore, preclinical studies suggest that bisphosphonates possess antitumor activity and can inhibit proliferation and induce apoptosis of tumor cell lines (Lipton et al, 2004).

In this study, we discuss prostate cancer as a model and try to find out drug-mediated cytotoxicity of zoledronic acid on hormone independent prostate cancer cell line and its mechanism of action. Thus, we first considered more closely what prostate

cancer is and its current treatments. Then we reviewed zoledronic acid and its role in prostate cancer treatment. Finally, we examined the function of interleukin-6 in prostate cancer cells. The objective of the study is to confirm the known apoptotic and cytotoxic effect of zoledronic acid in PC–3 prostate cell line and investigate the role of zoledronic acid on the expression of IL–6 in this cell line.

1.1 Prostate Cancer and Treatment

Prostate cancer is the fourth most common cancer in men, worldwide, with incidence and mortality rates that vary markedly among and within different countries. Since the early 1990s, new screening tests and improved treatments have been associated with dramatic shifts in the incidence, stage at diagnosis, and mortality of this disease. Major advances in molecular biology and epidemiology have provided new insights into the etiology and biology of prostate cancer. These developments promise to transform our understanding of this disease and will likely lead to new and better ways to prevent and treat prostate cancer in the future.Incidence and mortality rates vary tremendously among countries. Incidence and mortality rates are generally higher in Western countries than in developing countries. There are multiple complex causes for the worldwide and ethnic variations in prostate cancer incidence. The two major factors are genetics and environment. Prostate cancer is predominantly a disease of the elderly man, with more than 75% of new prostate cancers being diagnosed in men older than 65 years. The incidence of prostate cancer in men age 50 to 59 years, however, has increased substantially since the 1970s.

Although the specific causes of prostate cancer initiation and progression are not yet known, considerable evidence suggests that both genetics and environment play a role in the evolution of this disease. Classic and molecular epidemiology studies have identified a number of potential risk factors associated with the development of prostate cancer. A number of case-control studies have demonstrated familial clustering of prostate cancer, suggesting that some prostate cancers may be inherited. The clinical characteristics of familial prostate cancers have also been characterized to determine if hereditary prostate cancer may be more aggressive.

Prostate cancer initiation and progression are influenced by androgens. Prostate tumors are exquisitely sensitive to androgens and regress after medical or surgical

castration. Ross and colleagues hypothesized that the higher incidence of prostate cancer observed in African Americans may be related to elevated levels of circulating androgen (Ross et al, 1998).

Although the exact etiology of prostate cancer remains unknown, there has been tremendous progress in defining the molecular events responsible for the initiation and progression of this disease. This progress has resulted from a number of factors. Increased funding for prostate cancer research has focused and accelerated efforts in the field. Prostate cancers develop from the accumulation of genetic alterations that result in an increase in cell proliferation relative to cell death, arrest differentiation, and confer the ability to invade, metastasize, and proliferate in a distant site. Histologic changes are present in the prostates of men in their 20s, yet the diagnosis is typically made 3 to 4 decades later, which suggests that the development of the disease is a multistep process (Sakr et al, 1993). The alterations include somatic point mutations, gene deletions, amplifications, chromosomal rearrangements, and changes in DNA methylation (Nelson et al, 2003). It is believed that the accumulation of changes acting synergistically is more critical than the order in which the alterations occur. Identifying and understanding the events has implications for control of the disease at the earliest stages of transformation, for progression as an invasive tumor, for prognostication, and for points of therapeutic attack. Men who are castrated before the age of 40 years rarely develop prostate cancer (Montie et al, 1994). The evolution of the tumor is influenced by hormonal factors; it is also influenced by environmental, infectious/inflammatory factors, and, given the long history once the diagnosis is established, the specific therapy (ies) used to treat the disease. The alterations include a reduction in defense against carcinogen-induced damage, inflammation, and changes in androgen signaling and changes in growth-regulatory genes that contribute to cell proliferation, survival, and spread.

Neoplastic growth is the result of genetic, hormonal, environmental, and possibly infectious factors that modulate the expression of specific genes. The normal mutation frequency of DNA (1×10^{-10}) is too low to produce significant changes in overall gene expression. It is now believed that tumor cells themselves have an inherent genetic instability that results in the coexistence of multiple genetically related, yet distinct, clones within a tumor mass (Sarasin et al, 2003). Clones with a survival advantage continue to proliferate, whereas those that acquire changes that reduce viability undergo cell death.

Overexpression of certain growth factors, such as epidermal growth factor (EGF), basic fibroblast growth factor, and platelet-derived growth factor (PDGF), has been reported to be involved in prostate cancer as autocrine and paracrine signaling loops together with their corresponding receptors (EGFR, fibroblast growth factor receptor, and IL–6) (Scher et al, 1995, Uehara et al, 2003, Culig et al, 1996). The insulin-like growth factor–1 and -2 (IGF–1, IGF–2), and transforming growth factor-α and -ß (TGF-α, TGF-ß) and their receptors (IGFR and TGFR, respectively) have also been implicated (Cardillo et al, 2003, Tu et al, 2003). IGFs are potent mitogens for human prostate cancer cells and osteoblasts via interaction with IGF receptors. Elevated levels of serum IGF-binding proteins have been reported in metastatic human prostate cancer. HER–2/neu protein is a transmembrane tyrosine kinase receptor with strong homology to EGFR. Amplification of the *HER–2/neu* gene and overexpression of the protein have prognostic significance in breast cancer and are used to select therapy. *HER–2/neu* amplification in prostate cancer is uncommon. Nevertheless, increased HER2/neu protein was found in 20% of untreated hormone-naive primary tumors, whereas overexpression was observed in 80% of metastatic cases and more than 60% of primary tumors surviving after androgen ablation. Investigators have also reported HER2 can activate AR independent of ligand (Craft et al, 1999).

The major cause of death from prostate cancer is progressive castration-resistant disease, a tumor that continues to grow despite castrate levels of testosterone. As prostate cancers evolve to castration resistance, PSA synthesis resumes. The current view is that prostatic cancers at the time of diagnosis are composed of cells with three distinct cellular phenotypes: androgen-dependent, androgen-sensitive, and androgen-independent cells. Androgen-dependent cancer cells continuously require a critical level of androgenic stimulation for maintenance and growth (i.e. without adequate androgenic stimulation, these cells die) and, in this regard, are very similar to the androgen-dependent non-neoplastic cells of the normal prostate. The growth of androgen-sensitive cancer cells slows when androgens are withdrawn and they become dormant. They do not die. In contrast, the growth of androgen-independent cells does not change after androgen deprivation, no matter how complete; these cells are completely free of androgenic effects on growth (Arnold et al, 2002, Isaacs et al, 1992). In contrast to what can be accomplished *in vitro* using charcoal-stripped serum to eliminate androgens completely, *in vivo* it is virtually impossible to eliminate all androgens completely.

How do resistant tumor cells emerge during androgen ablation therapy? Some researchers theorize that selection of resistant cells occurs, whereas others suggest that, under the pressure of androgen-ablative therapies, cells that were sensitive only to the point at which growth was slowed without cell death adapt to the low androgen environment and, over time, acquire additional somatic changes that result in tumor regrowth (Isaacs et al, 1981, Craft et al, 1999, Bruchovsky et al, 1990). Clinically, selection of resistant or insensitive cells may be more relevant immediately after androgen withdrawal and adaptation more important later. The observation that basal epithelial cells preferentially survive androgen ablation (in contrast to secretory epithelial cells) demonstrates that intrinsically resistant cells do exist even in the normal prostate gland, in line with the selection hypothesis. This is consistent with the theorized role of basal cells as the stem cells for the prostatic epithelium (De Marzo et al, 1998, Bui et al, 1998, Isaacs et al, 1999). Unknown is whether the first transformed cell that ultimately develops into the self-renewing stem cell of a prostate cancer is dependent, sensitive, or insensitive to androgens. It is also difficult to determine whether the resistant/surviving cell population has a more basal or stem cell genotype, or a basal or a more differentiated cell that has been transformed. That hormonal ablation alone cannot eradicate the disease completely in either the primary site or in a metastatic focus suggests an intrinsic resistance and, at best, partial androgen sensitivity. Many pathways associated with resistance involve inhibition of proapoptotic molecules or the up-regulation of cell-survival molecules.

The androgen receptor (AR) is a member of a super-family of ligand-dependent transcription factors. Alterations in AR signaling that have been identified in human prostate cancer include alterations in steroid metabolism, an increase in the level of the protein, changes in co-regulator profiles, and ligand-independent activation. Changes in AR occur as the disease progresses from a clinically localized lesion in a non-castrate environment to a castrate metastatic lesion (Buchanan et al, 2001, Grossmann et al, 2001, Scher et al, 2001). All of these mechanisms are consistent with continued signaling through the receptor in castration-resistant lesions. The AR protein is expressed in prostate cancers of all clinical states. In upwards of 30% of cases, levels are higher in castration-resistant as opposed to non-castrate tumors (Culig et al, 1998, Tilley et al, 1994). Amplification of the *AR* gene itself has also been reported in approximately 22% of castration-resistant metastases (Bubendorf et al, 1999) and in

23% to 28% of recurrent primary tumors and is associated with increased levels of the AR and the proteins it regulates (Koivisto et al, 1996, Linja et al, 2001).

The most common mutations in the AR that have been identified in clinical specimens are in the ligand-binding domain. Virtually all are associated with a gain of function, as opposed to the loss of function mutations, which are most common among patients with androgen-insensitivity syndromes. Most disrupt a protein–protein interaction surface and result in an increase in the transactivation activity of the receptor in response to a range of classical and nonclassical ligands (Buchanan et al, 2000)

In addition to steroid hormones, growth factors, such as keratinocyte growth factor, IGF–1, and EGF; HER2; and cytokines, such as interleukin–6 (IL–6), can activate the AR independent of ligand (Culig et al, 1994, Ueda et al, 2002). Exactly how these factors activate signaling is an area of active study. This can also contribute to progression in castration-resistant disease.

Inhibitors of apoptosis are also implicated in the acquisition of the castration-resistant phenotype. Blocking cell death pathways that are normally induced by androgen ablation allows cells to survive. *bcl–2*, which inhibits the death of cancer cells without affecting their rate of proliferation, is essentially undetectable in most non-castrate lesions but is highly expressed in castration-resistant disease (McDonnell et al, 1992). Similarly, survivin, a member of the class of proteins called *inhibitors of apoptosis*, is highly expressed in benign and malignant prostate neuroendocrine cells (Xing et al, 2001).

Bone Metastasis

Bone metastases represent a major health and financial burden because of their frequency and the considerable morbidity they generate in cancer patients. They occur commonly in patients with advanced prostate carcinoma, affecting at least 70% of patients with metastatic disease (Adami et al, 1997).

Prostate cancer cells that have gained access to the circulation have a unique predilection for bone. The establishment of a metastatic focus in bone involves multiple steps, including adhesion of the tumor cells to endothelial cells in the marrow and migration through fenestrations in the endothelial cell layer. This migration is driven, in part, by a chemo attractant gradient of marrow- and stromal-derived growth factors. Once established, tumor cells and marrow-derived cells develop a bidirectional interaction that protects the epithelial cells and promotes tumor cell survival and proliferation (Cher et al, 2001).

Factors that contribute to cancer growth in bone are broadly divided into osteoblastic and osteolytic factors. Sources include the tumor cells themselves, normal bone cells, and reserves in the bone matrix that are released as part of the remodeling process. Many of the factors contributing to the osteoblastic phenotype, such as endothelin–1 (ET–1) (Nelson et al, 2003) and IL–6 can be targeted directly. ET–1, produced by prostate cells, stimulates the differentiation of osteoblast precursors, decreases osteoclastic bone resorption and motility, and augments the mitogenic effects of IGF–1, IGF–2. In normal bone, osteoblasts regulate osteoclastogenesis by interacting with mononuclear hematopoietic precursors. Osteoblasts express receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin. Osteoprotegerin level increases in patients with bone metastases (Jung et al, 2003). IL–6 is another cytokine released by prostate cancer cells that contributes to increased bone resorption (Keller et al, 2004). Binding of RANKL to RANK on osteoclastic precursors initiates intracellular signals that activate an osteoclastic phenotype.

Management of Prostate Cancer

The management of all stages of prostate cancer is sometimes highly contraversial. This disease often has a long natural history; therefore, substantial numbers of patients survive 15 years or longer after the diagnosis (even without treatment). Furthermore, because the disease occurs in older men, a large number of patients die from these conditions before they suffer symptoms or die from prostate cancer. Investigators and clinicians vary widely in their use of surgery, radiotherapy, hormonal manipulation, and other measures for treating each stage of disease. Most clinicians agree, however, that treatment of early stage disease with either surgery or radiotherapy results in comparable survival. It is unclear at time whether similar survival rates could be achieved with systemic therapies.

Endocrine therapy is mainstay of treatment for symptomatic advanced prostate cancer. Patients with asymptomatic advanced disease do not appear to have improved survival with treatment when compared with untreated cells. Thus, treatment of patients with asymptomatic, advanced disease is not essential. Orchiectomy (removal of testicles by surgery), luteinizing hormone-releasing hormone (LHRH) agonists, and antiandrogens are the available treatments. After androgen ablation treatment, usually prostate cancer cells undergo an active process of programmed cell death (apoptosis). Unfortunately, within 18–24 months after starting this treatment %80 relapse occurs and cells will be adept to survive without androgenic stimulation and become unresponsive

to androgen withdrawal. The mechanism responsible for development of androgen-independent cancer is not clear yet. Accumulating evidence suggests that growth factors and cytokines play an important role in this mechanism.

Other agents that may be helpful in management of prostate cancer include the following; progestins, corticosteroids, zoledronic acid for reduction in bone pain, in time to first skeletal related events and other drugs that inhibit androgen synthesis such as aminoglutethimide or ketoconazole. Chemotherapy also provides relief in 20 % to 30% of symptomatic patients with prostate cancer. Estramustine, cisplatine, 5-fluorouracil, vinorelbine, gemcitabine, paclitaxel are the most popular agents.

1.2 Zoledronic Acid

Bisphosphonates are pyrophosphate analogues in which a carbon atom has replaced the oxygen bridge. They bind strongly to hydroxyapatite in bone. The addition of different carbon side chains has generated a diverse group of compounds that can inhibit bone resorption via actions on the osteoclast. The more recently developed nitrogen containing bisphosphonates; such as pamidronate and zoledronic acid, have greatly enhanced potency. Zoledronic acid is a nitrogen-containing bisphosphonate that inhibits osteoclast activity and bone resorption. It is indicated for the treatment of hypercalcemia of malignancy and for the treatment of patients with multiple myeloma and patients with documented metastasis from solid tumors, in conjunction with standard antineoplastic therapy. Zoledronic acid is structurally similar to other bisphosphonates, having the required phosphorus-carbon-phosphorus core and a hydroxyl group at the R1 position (Green et al, 2001). However, it is the heterocyclic imidazole group attached to the R2 position that distinguishes zoledronic acid from other bisphosphonates (Widler et al, 2002). The chemical structure of zoledronic acid is shown in the Figure 1.1.

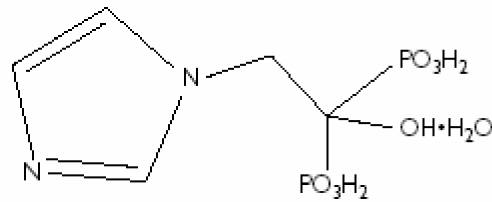


Figure 1.1. Chemical structure of zoledronic acid
(Source: Zometa®, insert package)

Bone metastases represent a major health and financial burden because of their frequency and the considerable morbidity they generate in cancer patients. They occur commonly in patients with advanced prostate carcinoma, affecting at least 70% of patients with metastatic disease (Adami et al, 1997). After development of hormone-refractory metastatic disease, prostate cancer is incurable. The metastasis of prostate cancer cells to the bone marrow constitutes the major source of morbidity and mortality in prostate cancer (Singh et al, 2006). Excessive osteoclast activity plays a central role in the pathophysiology of bone disease at each stage of prostate cancer disease progression (Saad et al, 2006). The bone marrow microenvironment is strictly involved in the evolution of the prostate disease progression by supporting cell growth and survival (Corso et al, 2005). Tumor cells then release a variety of growth factors that promote bone resorption and increase the risk of skeletal complications (Lipton, 2004). The activation of some signaling pathways within the bone stromal cells increases the productions of several cytokines which in turn favors the cell proliferation and survival and enhance the drug resistance by anti-apoptotic mechanisms (Corso et al, 2005).

Bisphosphonates have become a new form of medical therapy for tumor bone disease (Body et al, 1998, Powles et al, 2002). They are successfully used to treat the symptoms of metastatic bone disease, especially bone pain, and they reduce by up to 40% the long-term skeletal morbidity rate. Bisphosphonates interfere as well with bone microenvironment inhibiting the survival of stromal cells and hampering the contact between plasma and stromal cells (Corso et al, 2005). Zoledronic acid, a new-generation bisphosphonate is mainly osteolysis-inhibitory agent (Brubaker et al, 2006). These agents act primarily by initiating biochemical processes that ultimately result in apoptosis of osteoclasts, but they also have a number of other antitumor functions (eg, inhibition of angiogenesis) (Conte et al, 2004). Osteoclast inhibition was obtained with

bone-targeted zoledronic acid (Gao et al, 2005). Bisphosphonates are potent inhibitors of osteoclast activity that have demonstrated efficacy in the treatment of bone metastases (Lipton, 2004). Bisphosphonates bind eagerly to the bone matrix, are released during bone resorption, and are subsequently internalized by osteoclasts, where they interfere with biochemical pathways and induce osteoclast apoptosis (Lipton, 2004). Bisphosphonates also antagonize osteoclastogenesis and promote the differentiation of osteoblasts. As a result, bisphosphonates inhibit tumor-induced osteolysis and reduce skeletal morbidity (Lipton, 2004).

Zoledronic acid, a highly potent inhibitor of osteoclast-mediated bone resorption, has increased bone mineral density in men receiving androgen deprivation therapy and is the only bisphosphonate that has shown statistically significant reductions in skeletal morbidity in patients with bone metastases from prostate cancer (Saad et al, 2006). Due to its unique properties, zoledronic acid is a breakthrough in the management of metastatic bone disease in patients with advanced prostate cancer (Hoesl et al, 2006). Zoledronic acid should be considered for the prevention of skeletal morbidity in patients with prostate cancer throughout their treatment continuum (Saad et al, 2006).

Nitrogen containing bisphosphonates (e.g., pamidronate, ibandronate, zoledronic acid) inhibit protein prenylation, thus affecting cell function and survival (Green, 2003). In vitro, submicromolar concentrations of bisphosphonates inhibited tumor cell adhesion and reduced invasion through extracellular matrix. At higher concentrations, antiproliferative and proapoptotic effects have been reported (Green, 2003). Bisphosphonates exert their effects on osteoclasts and tumor cells by inhibiting a key enzyme in the mevalonate pathway, farnesyl diphosphate synthase, thus preventing protein prenylation and activation of intracellular signaling proteins such as Ras (Green, 2004). Recent evidence suggests that bisphosphonates also induce production of a unique adenosine triphosphate analogue (Apppi) that can directly induce apoptosis (Green, 2004). Their biochemical effects on protein prenylation, they induce caspase-dependent apoptosis, inhibit matrix metalloproteinase activity, and down regulate alpha (v) beta (3) and alpha (v) beta (5) integrins (Green, 2004).

Zoledronic acid also inhibits tumor cell adhesion to the extracellular matrix and has anti-angiogenic activity (Green, 2004).

Preclinical evidence suggests that zoledronic acid has antitumor activity in prostate cancer models probably due to their ability to interfere with several intracellular

signaling molecules (Melis et al, 2005, Saad et al, 2006, Green, 2005). Furthermore, preclinical studies suggest that bisphosphonates possess antitumor activity and can inhibit proliferation and induce apoptosis of tumor cell lines (Lipton, 2004). In addition, zoledronic acid, a new-generation bisphosphonate, appears to inhibit tumor cell invasion of the extracellular matrix (Lipton, 2004).

Bisphosphonates work by inhibiting osteoclast-mediated bone resorption and have also demonstrated antitumor activity in preclinical models (Saad, 2005). Specifically, zoledronic acid inhibits proliferation and induces apoptosis of human prostate cancer cell lines in vitro (Green, 2005). In a model of prostate cancer, zoledronic acid significantly inhibited growth of both osteolytic and osteoblastic tumors and reduced circulating levels of prostate-specific antigen. Bisphosphonates block the development of monocytes into osteoclasts and are thought to promote apoptosis of osteoclasts (Berenson, 2001). These agents prevent osteoclasts from moving to the bone surface and seem to inhibit the production of bone-resorbing cytokines such as interleukin-6 (IL-6) by bone marrow stromal cells (Berenson, 2001).

The therapeutic benefits of biphosphonates in patients with advanced prostate were proved in several studies. Zoledronic acid (Zometa; Novartis Pharmaceuticals Corp.; East Hanover, NJ and Basel, Switzerland) exerts synergistic antitumor activity when combined with other anticancer agents (Green, 2004). It was shown that the combination of zoledronic acid with selective EGFR-tyrosine kinase inhibitor (gefitinib) causes a cooperative antitumor effect accompanied by induction of apoptosis and regulation of the expression of mitogenic factors, proangiogenic factors and cell cycle controllers both in vitro and in xenografted nude mice (Melisi et al, 2005). These results provide new insights into the mechanism of action of zoledronic acid and a novel rationale to translate this feasible combination treatment strategy into a clinical setting (Melisi et al, 2005). The experimental data based on combinations of zoledronic acid with either gemcitabine or fluvastatin indicates its therapeutic role in treatment of bone metastasis of selected malignancies (Budman et al, 2006). The recent history of chemotherapy has shown that combinatorial therapy by using specific inhibitors of dominantly active signaling pathways opens new treatment perspectives in metastastic tumors. These studies suggest that zoledronic acid has the potential to inhibit bone metastasis and bone lesion progression in patients with prostate cancer (Green, 2005).

Recent studies have suggested that bisphosphonates may also exert direct antitumor effects on myeloma cells (Avcu et al, 2005). In vitro studies have

demonstrated the antitumor potential of zoledronic acid on myeloma cell lines (Corso et al, 2005). Zoledronic acid is able to affect the isoprenylation of intracellular small G proteins (Caraglia et al, 2004). The antitumor activity of Zoledronic acid combined with R115777 farnesyl transferase inhibitor (FTI) against epidermoid cancer cells was increased (Caraglia et al, 2004). In human epidermoid head and neck KB and lung H1355 cancer cells, 48 h exposure to pamidronate and zoledronic acid induced growth inhibition (25 and 10 µM, respectively) and apoptosis and abolished the proliferative and antiapoptotic stimuli induced by epidermal growth factor (EGF) (Caraglia M et al, 2004).

Zoledronic acid induced apoptosis through the activation of caspase 3. A strong decrease of basal ras activity was observed. These effects were paralleled by impaired activation of the survival enzymes extracellular signal regulated kinase 1 and 2 (Erk-1/2) and Akt that were not restored by EGF (Caraglia et al, 2004).

The combined treatment with Zoledronic acid and R115777 resulted in a strong synergism both in growth inhibition and apoptosis in KB and H1355 cells. The combination was highly effective in the inhibition of ras, Erk and Akt activity, while farnesol again antagonized these effects (Caraglia et al, 2004).

Zoledronic acid has been shown to inhibit the oncogenicity of Ras through the inhibition of prenylation of Ras and Ras-related proteins by the induction of S-phase cell cycle arrest and apoptosis (Chuah et al, 2005). Nitrogen containing-bisphosphonates have direct antitumor effects via the inactivation of Ras proteins (Sato et al, 2005). Zoledronic acid also can exert an antiangiogenic activity and inhibition of tumor cell bone invasiveness by a transient reduction of VEGF, bFGF circulating levels after infusion (Ferretti et al, 2005).

Although zoledronic acid is widely used in metastatic prostate cancer management, few data are available about its molecular effects in prostatic cells. The antitumor activity of biphosphonates that found in vitro and in vivo is interesting and has to be further assessed in laboratory and clinical studies. Further studies are needed to fully elucidate these biochemical mechanisms and to determine if the antitumor potential of bisphosphonates translates to the clinical setting.

Bone metastasis microenvironment-related growth factors such as insulin-like growth factor 1 (IGF-1), transforming growth factor beta 1 (TGF-beta1), basic fibroblast growth factor (bFGF) and interleukin 6 (IL-6) show survival factor activity by inhibiting chemotherapy-induced apoptosis of PC-3 prostate cancer cells in vitro (Tenta

et al, 2005). Recently, zoledronic acid has been shown to induce apoptosis in PC-3 prostate cancer cells (Tenta et al, 2005). These bone microenvironment-related growth factors, also can modify the actions of various pharmaceutical agents, including cytotoxic drugs in malignant cell lines (Tenta et al, 2006). Since bisphosphonates inhibit the proliferation and induce the apoptosis of certain prostate cancer cell lines, its mechanism of action is not clearly understood**.** IL-6 is an important cytokine on prostate cancer progression. One possible mechanism might be the IL-6 mediated pathway. The relation between zoledronic acid and IL-6 has not been known since there is no experimental data.

1.3. Interleukin-6 in Prostate Cancer

IL-6 is a 21–28 Kd cytokine containing 184 amino acids following cleavage of a 28 aa signal peptide (May et al, 1988). IL-6 belongs to the ‘‘IL-6 type cytokine’’ family that also includes leukemia inhibitory factor, IL-11, ciliary neurotrophic factor, cardiotrophin-1 and oncostatin M (Sehgal et al, 1995). In the normal homeostatic state, IL-6 levels are typically very low. However, in response to the appropriate stimulus (e.g. inflammation); a wide variety of cells produce IL-6. Many physiologic functions are attributed to IL-6 including promotion of antibody production from B lymphocytes, modulation of hepatic acute phase reactant synthesis, promotion of osteoclastic-mediated bone resorption, and induction of thrombopoiesis (Hirano et al, 1992). In addition, it is implicated in development and progression of tumors of various organs, in particular myeloma, renal and prostate cancer, and melanoma.

Multiple studies have demonstrated that IL-6 is elevated in the sera of patients with metastatic prostate cancer (Adler et al, 1999; Hoosein N et al, 1995; Twillie et al, 1995). Adler et al demonstrated that serum levels of IL-6 and transforming growth factor-b1 are elevated in patients with metastatic prostate cancer, and that these levels correlate with tumor burden as assessed by serum PSA or clinically evident metastases. In a similar fashion, Drachenberg et al reported elevated serum IL-6 levels in men with hormone-refractory prostate cancer compared to normal controls, benign prostatic hyperplasia, prostatitis, and localized or recurrent disease (Drachenberg et al, 1999). These observations suggest that IL-6 may be a surrogate marker of the androgen independent phenotype. IL-6 has been shown to be a candidate mediator of prostate

cancer morbidity and a candidate marker of disease activity for prospective clinical testing (Twillie et al, 1995). In addition to its role as a mediator of morbidity, IL-6 may also act as a growth factor, and protect prostate cancer cells from cell death induced by certain chemotherapeutic agents. IL-6 has been implicated in the modulation of growth and differentiation in many malignant tumors and is associated with poor prognosis in several solid and hematopoietic neoplasms such as renal cell carcinoma, ovarian cancer, lymphoma, and melanoma (Siegall et al, 1990). Thus, taken together, these data provide a large body of evidence that IL-6 is associated with prostate cancer in the clinical arena.

The androgen receptor (AR), which is expressed in normal prostate tissue and heterogeneously in prostate cancers, is a key transcription factor in the prostate (Hobisch et al, 1996; Hobisch et al, 1991; Van der Kwast et al, 1991). Activation of the AR in prostate cancer is being intensively investigated, and there is evidence that the AR could be stimulated by a number of nonsteroidal compounds, such as polypeptide growth factors, protein kinase A activators, vitamin D and neuropeptides (Culig et al, 1994, 1997; Nakhla et al, 1997; Nazareth et al, 1996; Zhao et al, 1997). IL-6 activates the AR in a ligand-independent manner and induces a synergistic AR response with very low concentrations of androgen (Chen et al, 2000; Hobisch et al, 1998). The interaction between IL-6 and AR might be particularly important in patients with advanced prostate cancer who have elevated serum levels of IL-6 (Adler et al, 1999; Hoosein et al, 1995; Twillie et al, 1995).

IL-6 and Signaling Pathways

IL-6 signals through the membrane receptor that is composed of the ligand-binding subunit gp80 which forms a low affinity complex with the cytokine and the signal transduction subunit gp130. The gp130 subunit's action is redundant since it is activated in response to IL-6-related cytokines, such as leukemia inhibitory factor (LIF) and IL-11. IL-6 receptors are expressed in a variety of benign and malignant cells. Following homodimerization of gp130, there is a formation of a hexameric complex consisting of two molecules each of IL-6, gp80, and gp130. This complex forms a high-affinity binding site for IL-6. IL-6 signaling is enhanced by the soluble IL-6 receptor, which can form a fully hexameric complex. One reason for induction of different responses is the ability of IL-6 to activate signaling through distinct pathways.

IL–6 is capable of activating three major proliferative pathways as shown in the Figure 1.2. The MAPK pathways and STAT transcription factor activation serves to

drive proliferation, as does the PI3K pathways. Activation of AKT inactivates many pro-apoptotic mediators. While this leads to cell survival in an immune related crisis, it can also lead to survival of DNA damaged cells, and potentially to neoplastic growth.

In various cell types, IL-6 binding to its receptor leads to phosphorylation of Janus kinases (JAK) that in turn phosphorylate the gp130 receptor subunits. The next step in IL-6 signaling is translocation and phosphorylation of signal transducers and transcription (STAT) factors by JAK. Among these transcription factors, STAT3 has a predominant role in IL-6 signal transduction. The role of STAT3 in malignant transformation of several cell lines has been well documented (Horiguchi et al, 2002).

Upon tyrosine phosphorylation, STATs (including STAT1, STAT3, and STAT5) translocate to the nucleolus as homodimers or heterodimers and bind to specific consensus DNA sequences of target-gene promoters and activate transcription (Fukada et al, 1996; Fourcin et al, 1996).

Another signaling pathway of IL-6 involves the GTP binding protein Ras, which may also be involved in other cytokine systems. GTP-binding motifs are present in the gp130 intracytoplasmic region; however, their precise role is unclear (Hibi et al, 1990). This Ras-dependent pathway includes intermediate steps involving Raf, MEK and MAPK (Chen-Kiang et al, 1995, Ernst et al, 1996, Kishimoto et al, 1994, Kishimoto et al, 1995, Akira et al, 1990). Following translocation into the nucleus, it is believed that MAP kinase activates the nuclear factor for IL-6 (NF-IL6) transcription factor to act on its target genes (Akira et al, 1990). The binding activity of NF-IL6 is most likely induced by IL-6 through the increased expression of the NF-IL6 gene, rather than through post-translational modification (Matsumoto et al, 1998). Other serine: threonine protein kinases can also be activated by IL-6 (Yin et al, 1994).

Moreover, IL-6 also activates phosphatidylinositol (PI3)-kinase through the activation of the p-85 subunit of PI3-kinase and contributes to the complexity of the cellular response to this cytokine (Boulton et al, 1994, Chen et al, 1999, Takahashi-Tezuka et al, 1997). Regulation of cell death by IL-6 is a subject of great importance. A typical feature of prostate cancer is a decreased rate of cell death. The PI3-K pathway is activated in response to IL-6 in PC-3 cells. This was demonstrated in experiments in which the tyrosine phosphorylation of p85, the regulatory subunit of the PI3-K was studied (Chung et al, 2000).

P85 coprecipitated with the signal-transducing subunit of the IL-6 receptor. IL-6 thus contributes to an increase in phosphorylation of Akt, which is a typical feature of

aggressive prostate cancer. Akt phosphorylation in prostate cancer Gleason scores 6 or 7 have a prognostic significance (Ayala et al, 2004).

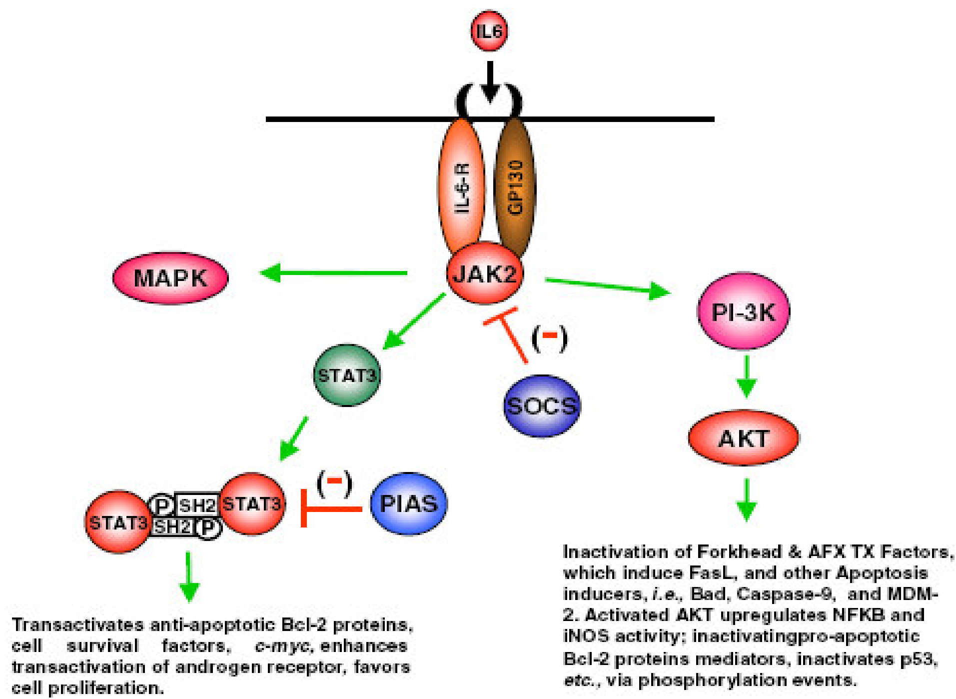


Figure 1.2. IL–6 and signaling pathways

(Source: David et al, 2005.)

Effects of IL- 6 on human prostate cancer cell lines

Defining mechanisms to control IL-6 or IL-6R expression may prove useful for therapy of the many clinical disorders in which IL-6 plays a role (Keller et al, 1996). Addition of anti-IL-6 antibody to the growth medium of the hormone independent cell lines DU145, PC-3 (Chung et al, 1999, Borsellino et al, 1995) and TSU (Chung et al, 1999) inhibits cell growth. In vitro studies have shown that the addition of anti-IL-6 enhances the cytotoxicity of certain chemotherapeutic agents in PC-3 cells, which are resistant to the drugs alone (Borsellino et al, 1995). Borsellino et al. later showed that the activity of IL-6 was more efficiently blocked with an IL-6R antagonist, Sant7, and that this too potentiated the sensitivity of PC-3 cells to etoposide-mediated cytotoxicity (Borsellino et al, 1999). In this study, the investigators also blocked signaling through gp130 in PC-3 using a gp130 antisense oligodeoxynucleotide. This inhibited cell growth and viability by about 20% and increased sensitivity to etoposide, confirming the

positive role of endogenous IL-6 in cell survival (Borsellino et al, 1999). These data suggest that endogenous IL-6 acts to protect tumor cells from drug induced cell death, and its neutralization may be a useful adjuvant to chemotherapy.

Studies of IL-6 expression in prostate cancer were initially carried out in a variety of prostate cancer cell lines. The androgen-refractory cell lines PC-3, DU145, and TSU secrete a number of cytokines including high levels of IL-6 (Chung et al, 1999). IL-6 was also detected in the supernatants of cultured prostatic stromal and epithelial cells (Hobisch et al, 2000, Chung et al, 1999, Degeorges et al, 1996). There have been conflicting reports as to whether or not the androgen-responsive cell line LNCaP secretes IL-6. Some investigators have observed IL-6 production in this cell line (Keller et al, 1996, Siegall et al, 1990) while others have reported minimal or no IL-6 production by LNCaP cells (Chung et al, 1999, Okamoto et al, 1997). In contrast to the conflicting data regarding IL-6, the presence of IL-6 receptor is more consistent. Specifically, the hormone refractory cell lines DU-145, PC-3 and TSU, and the hormone-dependent cell lines LNCaP, LNCaP-ATCC, and LNCaP-GW have been shown to express both components of the IL-6 receptor complex.

Proliferation studies carried out with prostate cancer cell lines revealed different effects of IL-6 on androgen-sensitive and insensitive cells (Chung et al, 1999). Just as there have been contrasting results regarding the production of IL-6 by LNCaP cells, a number of studies from various laboratories have yielded contrasting results regarding the effects of IL-6 on the growth of these cells. Chung et al showed that inhibition of IL-6 resulted in decreased cell growth of hormone-refractory cells, but had no effect on the growth of hormone-dependent cell lines (Chung et al, 1999). Addition of exogenous IL-6 to the culture media of LNCaP cells by several groups has resulted in a dose-dependent inhibition of cell growth (Chung et al, 1999, Degeorges et al, 1996, Ritchie et al, 1997, Hobisch et al, 2000). On the other hand, some researchers observed a stimulatory response after treatment with IL-6 (Qiu et al, 1998, Okamoto et al, 1997). The reasons for these differences have not been clarified to date but it seems that IL-6 in human prostate cancers exerts divergent effects and therefore it will be interesting to learn more about its co-localization with molecules that regulate cellular proliferation. It thus appears that IL-6 acts as an autocrine and paracrine growth factor in PC-3, TSU, and DU145 cells (Chung et al, 1999) and as a paracrine growth inhibitor in LNCaP cells (Chung et al, 1999, Degeorges et al, 1996). However, in the presence of androgen, IL-6 acts as an autocrine growth factor in LNCaP cells (Okamoto et al, 1997). Applying

these findings to behavior of prostate cancer in vivo, Chung et al. have suggested that IL-6 may undergo a functional transition from paracrine growth inhibitor to autocrine growth stimulator during the progression of CaP to the hormone refractory phenotype (Chung et al, 1999).

1.4. Characterization of PC-3 Prostate Cancer Cell Line

The human hormone-independent prostate cancer cell line PC-3 was used (Figure 1.3.) which is derived from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian (Kaighn et al, 1979). The cultured cells show anchorage-independent growth in both monolayers and in soft agar suspension and produce subcutaneous tumors in nude mice. Culture of the transplanted tumor yielded a human cell line with characteristics identical to those used initially to produce the tumor. Drug-resistant prostatic carcinoma cell-line PC-3 has a greatly reduced dependence upon serum for growth when compared to normal prostatic epithelial cells and does not respond to androgens, glucocorticoids, or epidermal or fibroblast growth factors. Karyotypic analysis by quinacrine banding revealed the cells to be completely aneuploid with a modal chromosome number in the hypotriploid range. At least 10 distinctive marker chromosomes were identified (Chen et al, 1993). The overall karyotypes as well as the marker chromosomes are distinct from those of the HeLa cell. Electron microscopic studies revealed many features common to neoplastic cells of epithelial origin including numerous microvilli, junctional complexes, abnormal nuclei and nucleoli, abnormal mitochondria, annulate lamellae, and lipoidal bodies. They have non-functional androgen receptor (AR), express the IL–6 receptor (Tilley et al, 1990) also secrete IL–6 (Chung et al, 1999).

Overall, the functional and morphologic characteristics of PC-3 are those of a poorly-differentiated adenocarcinoma. These cells should be useful in investigating the biochemical changes in advanced prostatic cancer cells and in assessing their response to chemotherapeutic agents.

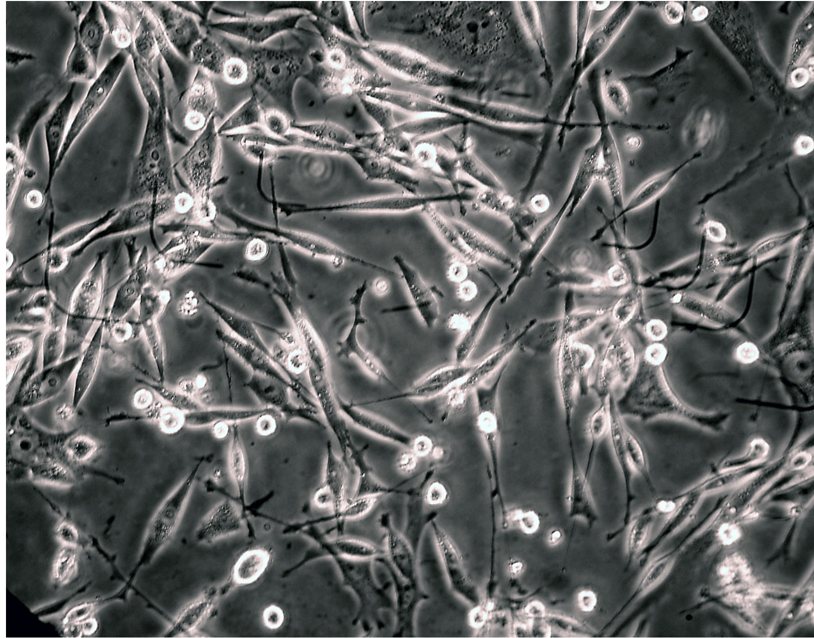


Figure 1.3. Morphology of PC-3 cells under a microscope (10x)

1.5. Methods for Studying Cytotoxicity

Cytotoxicity is the cell-killing property of a chemical compound (such as a food, cosmetic, or pharmaceutical) or a mediator cell (cytotoxic T cell). In contrast to necrosis and apoptosis, the term cytotoxicity does not indicate a specific cellular death mechanism. For example, cell-mediated cytotoxicity (that is, cell death mediated by either cytotoxic T lymphocytes [CTL] or natural killer [NK] cells) combines some aspects of both necrosis and apoptosis (Berke et al, 1991; Krahenbuhl et al, 1991).

Most current assays for measuring cytotoxicity are based on alterations of plasma membrane permeability and the consequent release (leakage) of components into the supernatant or the uptake of dyes, normally excluded by viable cells ("Dye exclusion method"). This permeability assays involve staining damaged cells with a dye and counting viable cells that exclude the dye. Counts can be performed manually using a hemocytometer. Counting cells by the use of a hemocytometer is a convenient and practical method of determining cell numbers and it gives us the opportunity to see what we are counting. If the cells were previously mixed with an equal volume of viability stain (e.g trypan blue) a viability determination may be performed at the same time. Most of the errors in this procedure occur by incorrect sampling and transfer of cells to

the chamber. Although the trypan stain distinction has been questioned, it is simple and gives a good approximation.

This method is also inexpensive, and requires only a small fraction of total cells from a cell population. Therefore, it is generally used to determine the cell concentration (cell number/ml) in batch cell cultures. This is helpful in ensuring that cell cultures have reached the optimal level of growth and cell density before routine sub-culture, freezing, or any experiment (Berridge et al, 1993).

A serious disadvantage of permeability assays is that the initial sites of damage of many, if not most cytotoxic agents are intracellular. Therefore, cells may be irreversibly damaged and committed to die and the plasma membrane is still intact. Thus, these assays tend to underestimate cellular damage when compared to other methods. Despite this fact, permeability assays have been widely accepted for the measurement of cytotoxicity.

Alternatively, metabolic activity can be measured by adding tetrazolium salts to cells. These salts are converted by viable cells (metabolically active cells) to colored formazan dyes that are measured spectrophotometrically. In contrast dead cells are unable to metabolize tetrazolium salts. Thus, tetrazolium salt-based colorimetric assays detect viable cells exclusively. This allows the use of the colorimetric assays MTT, XTT, or WST-1 to measure cell survival. Because they are sensitive, these assays can readily be performed in a microplate with relatively few cells. Since a cytotoxic factor will reduce the rate of tetrazolium salt cleavage by a population of cells, these metabolic activity assays are frequently used to measure factor-induced cytotoxicity or cell necrosis (Mosmann et al, 1983, Mosmann et al 1989). Applications include: Assessment of growth-inhibitory or cytotoxic effects of physiological mediators, analysis of the cytotoxic and cytostatic effects of potential anti-cancer and other drugs, analysis of cytopathic effects of viruses and screening of compounds with potential anti-viral activity and screening of antibodies for growth-inhibiting potential.

The most common used microplate-based metabolic activity assays are;

- **MTT Cell Proliferation Kit**, in which metabolically active cells cleave the tetrazolium salt MTT to a water-insoluble formazan that can be solubilized and quantitated with an ELISA plate reader

- **XTT Cell Proliferation Kit**, in which metabolically active cells cleave the modified tetrazolium salt XTT to a water-soluble formazan, which may be directly quantitated with an ELISA plate reader (Figure 1.4.)

• **Cell Proliferation Reagent WST-1**, a modified tetrazolium salt that can be cleaved by metabolically active cells to a water-soluble formazan, which may be directly quantitated with an ELISA plate reader

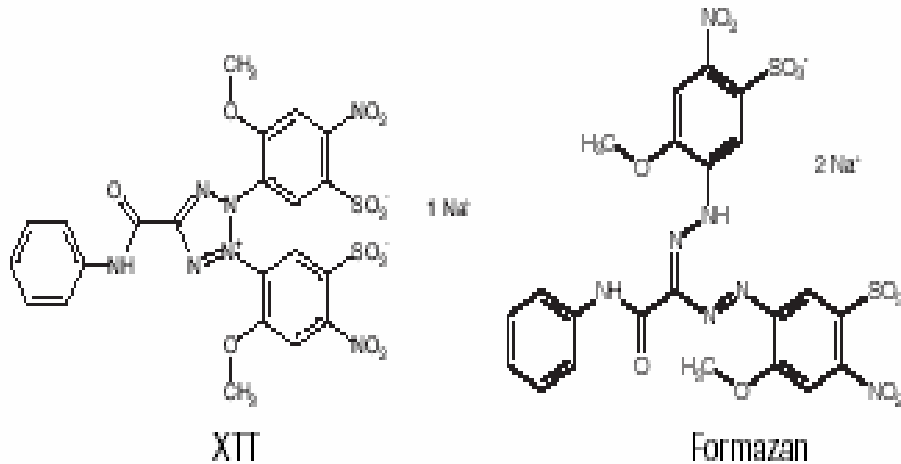


Figure 1.4. Metabolization of XTT to a water soluble formazan salt by viable cells. (Source: Roche Applied Science®)

1.6. Methods for Studying Apoptosis in Cell Populations

Cell death can occur by either of two distinct mechanisms (Schwartzman et al, 1993, Vermes et al, 1994) necrosis or apoptosis. The two mechanisms of cell death may briefly be defined:

Necrosis (“accidental” cell death) is the pathological process which occurs when cells are exposed to a serious physical or chemical insult. *Apoptosis* (“normal” or “programmed” cell death) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes.

There are many observable morphological and biochemical differences (Table 1.1.) between necrosis and apoptosis (Vermes et al, 1994). Necrosis occurs when cells are exposed to extreme variance from physiological conditions (*e.g.,* hypothermia, hypoxia) which may result in damage to the plasma membrane. Under physiological conditions direct damage to the plasma membrane is evoked by agents like complement and lytic viruses.

Table 1.1. Differential features and significance of necrosis and apoptosis.

Necrosis	**Apoptosis**
Loss of membrane integrity	Membrane blebbing, but no loss of integrity
Begins with swelling of cytoplasm and mitochondria	Begins with shrinking of cytoplasm and condensation of nucleus
Ends with total cell lysis	Ends with fragmentation of cell into smaller bodies
No vesicle formation, complete lysis	Formation of membrane bound vesicles
Random digestion of DNA (smear of DNA after agarose gel electrophoresis)	Non-random mono- and oligonucleosomal length fragmentation of DNA (Ladder pattern after agarose gel electrophoresis)
Postlytic DNA fragmentation	Prelytic DNA fragmentation
Affects groups of contiguous cells	Affects individual cells
Phagocytosis by macrophages	Phagocytosis by adjacent cells or macrophages
Significant inflammatory response	No inflammatory response
Loss of regulation of ion homeostasis	Activation of caspase cascade

Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (Van Furth et al, 1988).

Apoptosis, in contrast, is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy.

Cells undergoing apoptosis show characteristic morphological and biochemical features (Cohen et al, 1993). These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. *In vivo*, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells (Savill et al, 1989). Due to this efficient mechanism for the removal of apoptotic cells *in vivo*

no inflammatory response is elicited. *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse.

Scientists now recognize that most, if not all, physiological cell death occurs by apoptosis, and that alteration of apoptosis may result in a variety of malignant disorders. Consequently, in the last few years, interest in apoptosis has increased greatly. Great progress has been made in the understanding of the basic mechanisms of apoptosis and the gene products involved. Key elements of the apoptotic pathway include:

Death receptors:

Apoptosis has been found to be induced via the stimulation of several different cell surface receptors in association with caspase activation. For example, the CD95 (APO-1, Fas) receptor ligand system is a critical mediator of several physiological and pathophysiological processes, including homeostasis of the peripheral lymphoid compartment and CTL mediated target cell killing. Upon cross-linking by ligand or agonist antibody, the Fas receptor initiates a signal transduction cascade which leads to caspase-dependent programmed cell death.

Membrane alterations:

In the early stages of apoptosis, changes occur at the cell surface and plasma membrane. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell.

Protease cascade:

Signals leading to the activation of a family of intracellular cysteine proteases, the caspases, (Cysteinyl-aspartate-specific proteinases) play a pivotal role in the initiation and execution of apoptosis induced by various stimuli. Different members of caspases in mammalian cells have been identified. Among the best-characterized caspases is caspase-1 or ICE (Interleukin-1-Converting Enzyme), which was originally identified as a cysteine protease responsible for the processing of interleukin-1 (IL-1).

Mitochondrial changes:

Mitochondrial physiology is disrupted in cells undergoing either apoptosis or necrosis. During apoptosis mitochondrial permeability is altered and apoptosis specific protease activators are released from mitochondria. Specifically, the discontinuity of the outer mitochondrial membrane results in the redistribution of cytochrome C to the cytosol followed by subsequent depolarization of the inner mitochondrial membrane. Cytochrome C (Apaf-2) release further promotes caspase activation by binding to Apaf-

1 and therefore activating Apaf-3 (caspase 9). AIF (apoptosis inducing factor), released in the cytoplasm, has proteolytic activity and is by itself sufficient to induce apoptosis.

DNA fragmentation:

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability (prelytic DNA fragmentation). In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+} - dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments (Figure 1.4). In contrast, the DNA of the nucleosomes is tightly complexed with the core histones H2A, H2B, H3 and H4 and therefore is protected from cleavage by endonuclease (Burgoyne et al, 1974, Stach et al, 1979). The DNA fragments yielded are discrete multiples of a 180 bp subunit which is detected as a "DNA-ladder" on agarose gels after extraction and separation of the fragmented DNA. The enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cells is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown (Duke et al, 1986, Bonfoco et al, 1995).

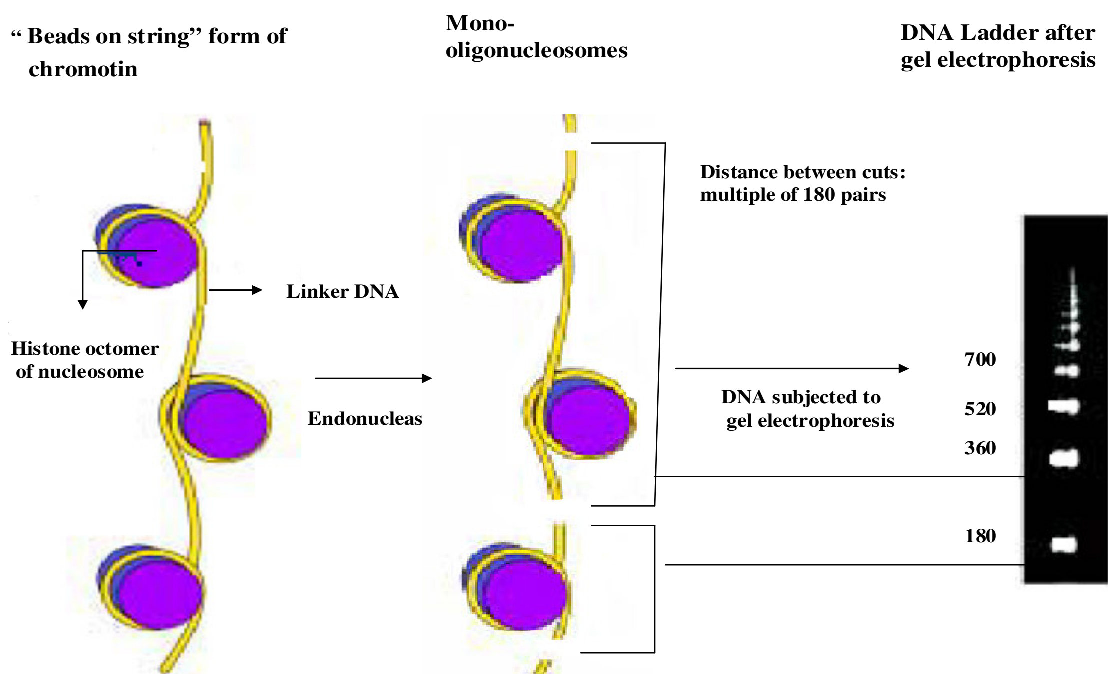


Figure 1.5. The biochemistry of DNA fragmentation and the appearance of the "DNA ladder". (Source: Roche Applied Science®)

A number of methods have now been developed to study apoptosis in cell populations. Because DNA cleavage is a hallmark for apoptosis, assays which measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death. The DNA fragments may be assayed in either of two ways:

-As “ladders” (with the 180 bp multiples as “rungs” of the ladder) derived from populations of cells. In this assay, DNA has to be isolated and analyzed by simple agarose gel in order to detect fragmented DNA in cells.

-By quantification of histone-complexed DNA fragments (mono- and oligonucleosomes) with an ELISA. It is an alternative method which circumvents the isolation and electrophoretic analysis of DNA, and is the immunological detection of DNA fragments by an immunoassay.

1.7. Methods for Measuring Interleukin-6 in the Supernatant of Cell Culture

Enzyme Linked Immunosorbent Assay (ELISA) combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily assayed enzyme that possesses a high turnover number. ELISA can provide a useful measurement of antigen or antibody concentration.

One of the most useful of the immunoassays is the two antibody “sandwich” ELISA. This assay is used to determine the antigen concentration in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amount of antigen in an unknown sample. The sandwich ELISA requires two antibodies that bind to epitopes that do not overlap on the antigen. This can be accomplished with either two monoclonal antibodies that recognize discrete sites or one batch of affinity-purified polyclonal antibodies.

To utilize this assay, one antibody is purified and bound to a solid phase typically attached to the bottom of a plate well. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a labeled second antibody is allowed to bind to the antigen, thus completing the “sandwich”. The assay is then quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of a colorimetric substrate by ELISA microplate reader (Figure 1.6). Major advantages of this technique are that the antigen

does not need to be purified prior to use, and that these assays are very specific. However, one disadvantage is that not all antibodies can be used. Monoclonal antibody combinations must be qualified as “matched pairs”, meaning that they can recognize separate epitopes on the antigen so they do not hinder each other’s binding.

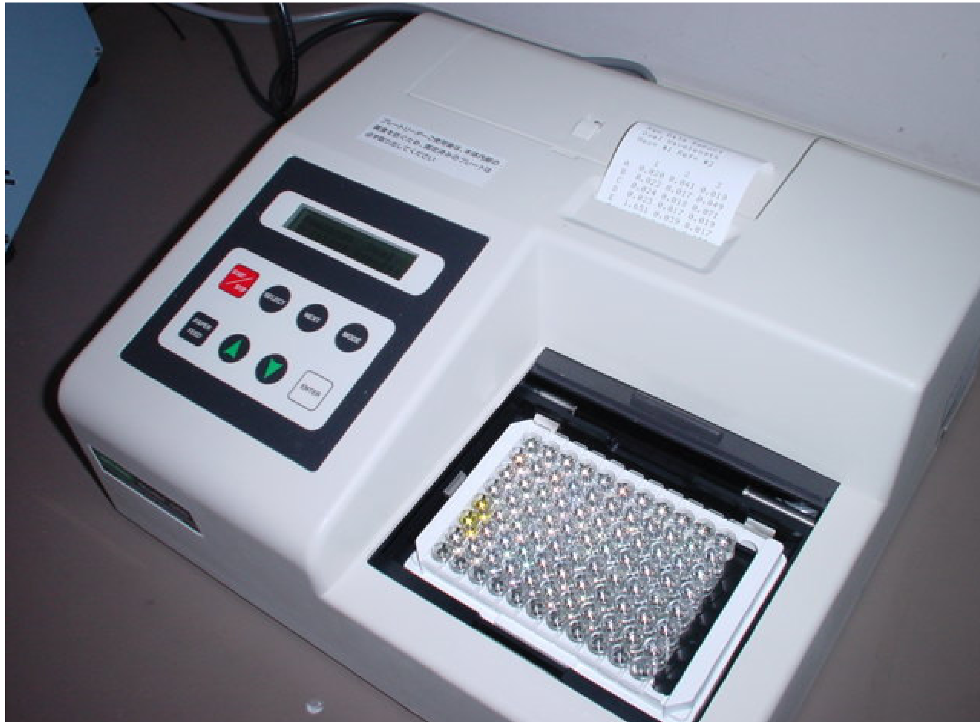


Figure 1.6. ELISA microplate reader

CHAPTER 2

MATERIALS AND METHODS

In this study, cell culture technique was used in order to grow prostate cancer cells, *in vitro.* The term *cell culture* refers to a culture derived from dispersed cells taken from original tissues, from a primary culture, or from a cell line or cell strain by enzymatic, mechanical, or chemical disaggregation. The major advantage of cell culture is control of the physiochemical environment (pH, temperature, osmotic pressure) also large quantities of well-defined cells can be obtained by using this technique.

2.1 Materials

The chemicals, reagents and kits used in our experiments are shown in Table 2.1. In The human hormone-independent prostate cancer cell line PC-3 was generously supplied by Dr Levent Türkeli Marmara University, İstanbul, Turkey. Zoledronic acid was obtained from Novartis Pharmaceuticals (Basel, Switzerland). 1mM stock solution of zoledronic acid was prepared by dissolving 4,016 mg dry powder zoledronic acid in 10 mL distilled water and aliquots were stored in -20º C. Because zoledronic acid acts as a chelating agent on Ca^{++} in cell culture media, also we assessed the effects of the knowing chelating agent, EDTA (1-100µM) on the growth of PC-3 cells, under identical experimental conditions. Laboratory equipments required in this study are shown in Table 2.2.

Table 2.1. List of chemicals used in experiments.

Chemicals	Trademark
RPMI 1640 with L-glutamine	Biological Industries
DMSO	Sigma Chemical Co.
Heat inactivated fetal bovine serum	Biological Industries
EDTA	Sigma Chemical Co.
Tyripsin-EDTA	Sigma Chemical Co.
Penicillin	Biological Industries
Streptomycin	Biological Industries
Trypan-Blue	Sigma Chemical Co.
Zoledronic acid	Novartis Pharma AG
Cell Proliferation Kit II (XTT)	Roche Diagnostic GmBH
Apoptosis Cell Death Detection ELISA ^{PLUS} Kit	Roche Diagnostic GmBH
Apoptotic DNA Ladder Kit	Roche Diagnostic GmBH
Human IL-6 ELISA Kit	Biosource Inc.

Table 2.2. List of equipments used in experiments.

Equipments	Trademark
Laminar-Flow Hood	NuAire,USA
CO_2 Incubator	NuAire,USA
Inverted Microscope	Olympus,Japan
Light Microscope	Olympus,Japan
Hemocytometer	Neubauer
Pipettors (10,20,100,1000µl)	Eppendorf
Plate Reader	Medispec ESR 200
Culture vessels	Corning
Pipetting Aid	Eppendorf

2.2 Methods

2.2.1 Cell Culture of PC-3 Cell Line

The human hormone-independent and drug-resistant prostatic carcinoma cell-line PC-3 was maintained in culture as adherent cells and cultured in Roswell Park Memorial Institute 1640 (RPMI 1640, Biological Industries, Israel) plus 5% heat inactivated fetal bovine serum (Biological Industries, Israel) added to 1% L-glutamine (Biological Industries, Israel) 1% nonessential amino acids (Biological Industries, Israel), 10.000 U/mL penicillin (Biological Industries, Israel) and 10 mg/mL streptomycin (Biological Industries, Israel). All cells were grown in humidified atmosphere at 37C° in 5% CO2. When the tumor cells were used as target cells, they were treated with trypsin–EDTA (Sigma Chemical Co.), washed, and resuspended in complete medium.

2.2.2 Assessment of Cytotoxicity Produced by Zoledronic Acid.

Trypan blue dye exclusion assay and XTT proliferation assay was used to confirm the drug-mediated cytotoxicity of zoledronic acid on PC-3 cells.

2.2.2.1 Trypan Blue Dye Exclusion Assay

Trypan Blue Dye Exclusion assay was used to assess the cytotoxic effect of zoledronic acid on PC-3 cells. This assay was also used to measure the proportion of viable cells following a potentially traumatic procedure, such as disaggregation, cell separation, or freezing and thawing during the cell culture. Briefly, assay involves staining damaged cells with a dye and counting viable cells that exclude the dye. Counts were performed manually using a hemocytometer and trypan blue (Figure 2.1).

The hemocytometer consists of two chambers, each of which is divided into nine 1.0 mm squares. A cover glass is supported 0.1 mm over these squares so that the total volume over each square is 1.0 mm x 0.1 mm or 0.1 mm^3, or 10^{-4} cm^3. Since 1 cm^3 is approximately equivalent to 1 ml, the cell concentration per ml will be the average count per square x 10^4. In order to determine the ability of zoledronic acid to inhibit the

growth of PC-3 cells, cells were plated at a cell density of 6x 10^3 cells/well in 24-well plates and grown with RPMI containing 5% FBS. Cells then treated with zoledronic acid in a dose- (10, 60, and100 µM) and time-dependent manner (24, 48, 72 hr). After incubation period, 50 µL cells were taken and mixed with an equal volume of trypan blue [0.4% (w/v) trypan blue in PBS]. Then this suspension was loaded to the counting chamber of hemocytometer. Using the microscope with a 10X ocular, the numbers of unstained cells (viable cells) in the 4 outer squares were counted. Then the numbers of viable cells per mL were calculated. Counting was repeated in order to check reproducibility. The cell concentration was calculated as follows:

Cell concentration per milliliter = average count per square x 10^4

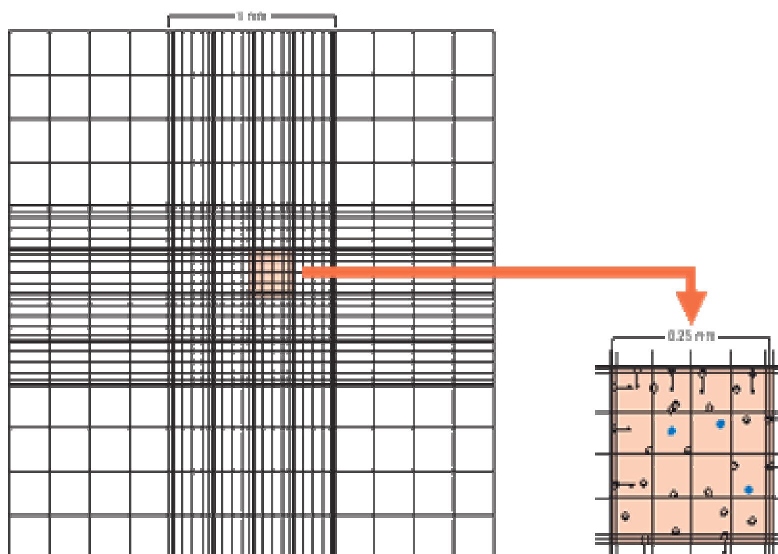


Figure 2.1. Measurement of cytotoxicity by counting the cells with a hemocytometer. (Source: Roche Applied Science®)

2.2.2.2 XTT Proliferation Assay

The effect of zoledronic acid (1-100µM) on PC-3 cell proliferation was investigated using a commercially available XTT proliferation kit (Roche Applied Science, Penzberg, Germany). The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange dye by metabolic active cells (Gerlier et al, 1986). While living (metabolically active) cells reduce tetrazolium salt to colored

formazan compound; dead cells do not. The formazan dye formed is soluble in aqueous solutions and is directly quantified using an ELISA microplate reader.

Briefly, the cells were plated in 96-well culture plates (Corning Glass Works, Corning, N.Y, USA) at a density of 1000 cells/well in 200-µl RPMI culture medium after verifying cell viability by trypan blue (Sigma Chemical Co.) dye exclusion test. After incubation with zoledronic acid for 48 and 72 hr, 50 µl of XTT labeling mixture (sodium3'-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate; mixed in proportion 50:1) was added to the each well. Then cells were incubated with this mixture for 4 hr in a humidified atmosphere. The optical density was measured at 450 nm with a reference wavelength at 650 nm in a Medispec ESR 200 microplate reader. The percentage of cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = 1- (A \text{ of experimental well}/ A \text{ of positive control well}) \times 100$$

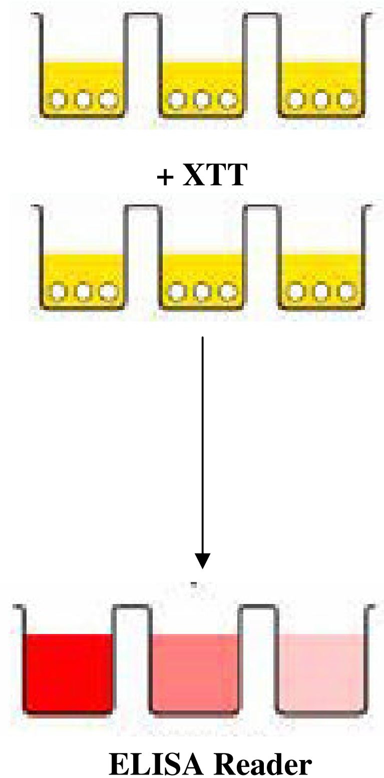


Figure 2.2. Measurement of metabolic activity using the tetrazolium salt XTT (Source: Roche Applied Science®)

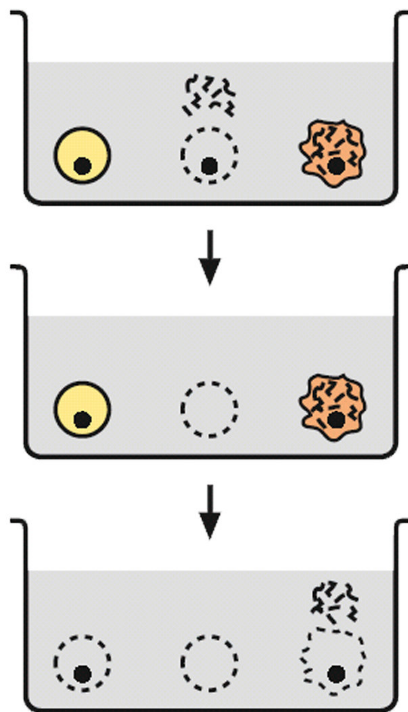
2.2.3 Evaluation of Apoptosis Induced by Zoledronic Acid

Mono-oligo nucleosome detection-based ELISA and DNA fragmentation on simple agarose gel electrophoresis assays were used to evaluate the apoptosis.

2.2.3.1 Mono-Oligo Nucleosome Detection-based ELISA

The commercially available Cell Death Detection ELISA kit (Roche Applied Science, Penzberg, Germany) was used to detect DNA fragmentation according to manufacturer's instructions (Aragene et al, 1998). This nonradioactive immunoassay has been designed for relative quantification of DNA fragmentation in cells and it measures the enrichment of histone-complexed DNA fragments (*mono- and oligonucleosomes*) in the cytoplasm of apoptotic cells.

Briefly, cytoplasmic lysates from control and zoledronic acid treated cells were transferred to a streptavidin-coated plate that was supplied with the kit. They were incubated for 2 hr in the presence of the immune reagent containing the antibodies against the histone proteins and DNA fragments (Anti-histone-biotin and Anti-DNA-POD). During the incubation period, the Anti-histone antibody bound to the histone-component of the nucleosomes and simultaneously captured the immunocomplex to the streptavidin-coated plate via its biotinylation. Additionally, the Anti-DNA-POD antibody reacted with the DNA component of the nucleosomes. After the removal of unbound components by a washing step, quantitative determination of the amount of nucleosomes was measured by the POD retained in the immunocomplex. POD is determined photometrically with ABTS as substrate, which then was read for optical density at 405 nm with a reference wavelength at 490 nm (Figure 2.3.). The enrichment of histones and DNA fragments in cell lysates were evaluated as indicator of apoptosis. The degree of apoptosis was displayed as percentage according to positive control cells.



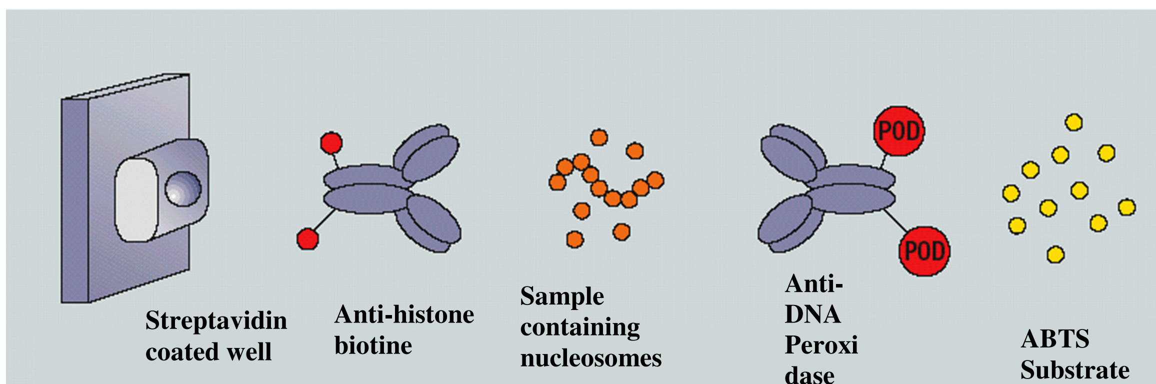


Figure 2.3. How the Cell Death Detection ELISA^{PLUS} works. (Sample preparation and ELISA) (Source: Roche Applied Science®)

2.2.3.2 DNA Fragmentation on Simple Agarose Gel

The Apoptotic DNA Ladder Kit (Roche Diagnostic, Mannheim, Germany) was used to detect the typical DNA ladder formation in zoledronic acid treated cells, which is the hallmark of apoptotic cells. DNA fragments reveal, upon agarose gel

electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit.

Firstly PC-3 cells were plated at a cell density of 2x 10^6 cells/well in 24-well plates and grown with RPMI containing 5% FBS and then treated with different concentration of zoledronic acid (1,10,60 and 90 μM) for 48 and 72 hr. After lysis of cultured cells in binding buffer, the cell lysates is applied to a filter tube with glass fiber fleece and passaged through the glass fiber by centrifugation. During this procedure nucleic acids bind specifically to the surface of glass fibers in the presence of chaotropic salts (Vogelstein et al, 1979). Residual impurities were removed by a wash step and subsequently DNA was eluted in elution buffer. The DNA is stable and can be used directly or stored at -20º C for later analysis.

For gel electrophoresis, DNA samples and positive control (apoptotic U937 cells) were run on 1% agarose gel stained with ethidium bromide. The voltage was set to 75V for 2 hours. DNA ladders indicative of nucleosomal DNA fragmentation became apparent after 2 hours. DNA ladders were visualized by placing gel onto an UV light source and photographed.

2.2.4 Detection of IL–6 Levels by ELISA

The Human IL-6 ELISA Kit (Biosource Inc., California, USA) was used for the quantititative determination of IL-6 in the supernatant from zoledronic acid treated PC-3 cells. Hu IL-6 kit is a solid phase sandwich ELISA. A monoclonal antibody specific for Hu IL-6 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu IL-6 content, control specimens, and unknowns, are pipetted into these wells, followed by the addition of a biotinylated monoclonal second antibody. During the first incubation, the Hu IL-6 antigen binds simultaneously to the immobilized antibody on one site, and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, Streptavidin- Peroxidase enzyme is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove the entire unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Hu IL-6 present in our sample.

Briefly, PC-3 cells were cultured for 24,48,72 hr at a concentration of $1x10^5$ cells/ml of RPMI medium supplemented with 5% FBS. Then cells were incubated in the same medium in the absence or presence of zoledronic acid (1, 10, 60, 90,100µM). Supernatants were collected for all culture conditions and stored at -20°C until the day of the assay. Before thawing the supernatants, Hu IL-6 standards was prepared by making serial dilutions of 500, 250, 125, 62.5, 31.2, 15.6 and 7.8 pg/mL Hu IL-6. 100 µL of supernatants and standards were added to the microplate wells. Then 50 µL of biotinylated anti-IL-6 solution was added into each well and plate was incubated for 2 hours at RT. After the washing, 100 µL Streptavidin-HRP working solution was added to each well and plate was incubated with this solution 30 minutes at RT. After washing thoroughly, 100 µL of Stabilized Chromogen was added to each well. The liquids in the wells began to turn blue. Plate was incubated for 30 minutes at room temperature and in the dark. By adding 100 µL of Stop Solution to each well process is completed and the solutions in the wells changed from blue to yellow (Figure 2.4.). The absorbance was measured at 450nm with ELISA microplate reader. The absorbance of the standards against the standard concentration was plotted on a graph paper and the best smooth curve trough these points was drawn to construct the standard curve. The Hu IL-6 concentrations for unknown samples were read from the standard curve.

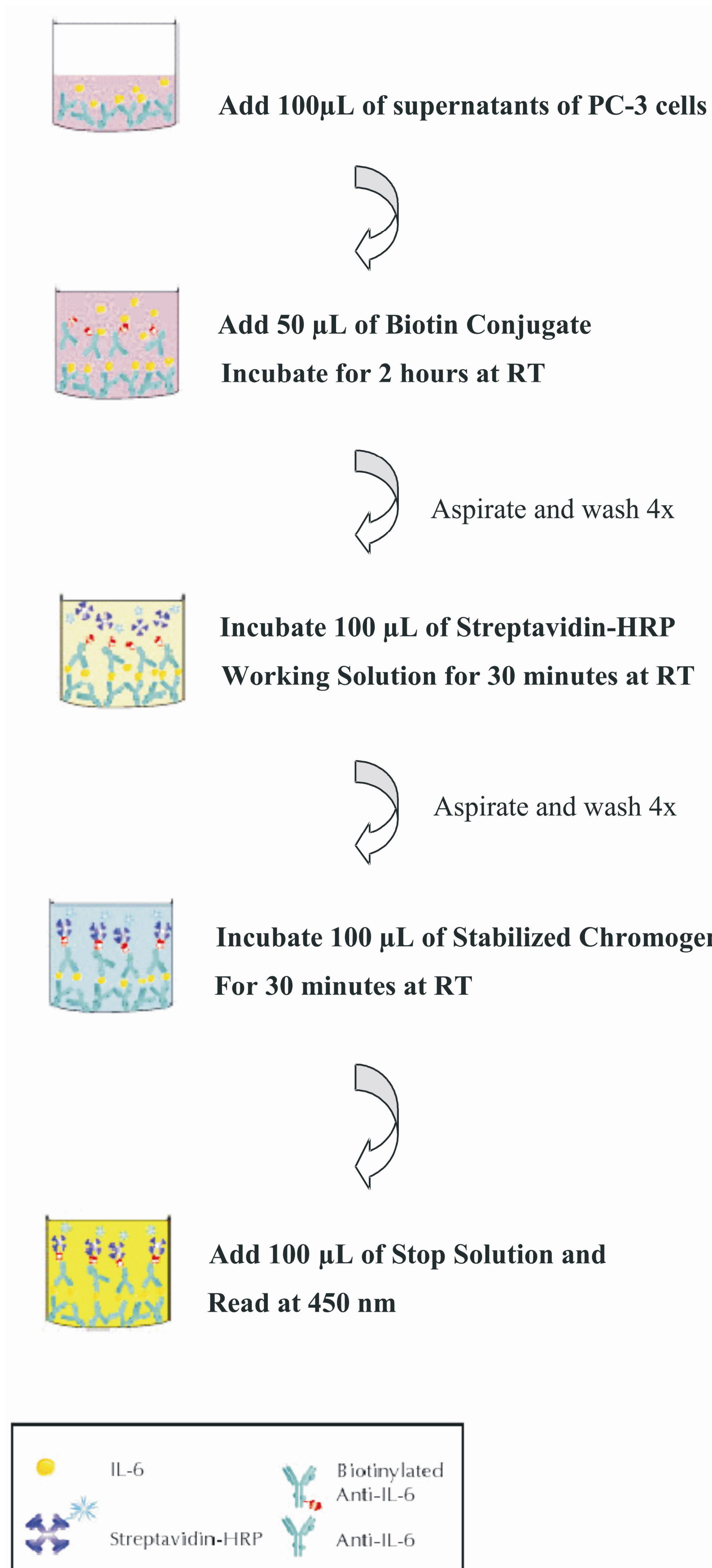


Figure 2.4. Human IL-6 assay summary

2.3. Statistical Analysis

All assays were set up in triplicate and the results were expressed as the mean±SD. Analysis of variance (one-way ANOVA) statistical methodology was performed to elucidate the mean effect of ZA treatment on IL-6 levels.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Results

Two different methods were used to confirme the cytotoxic effect of zoledronic acid on PC-3 cells. First, trypan blue dye exclusion assay was used to determine the inhibition of proliferation of PC-3 cells by zoledronic acid. After treatment of various concentration of zoledronic acid, cells were stained with trypan blue and then counted with hemocytometer. The addition of trypan blue helps to distinguish viable, unstained cells from non-viable, blue-stained cells (Figure 3.1.). The number of viable cells was calculated as previously described. Our data proved that the cytotoxic effect of zoledronic acid was dose and time dependent in PC-3 cells (Figure 3.2.).

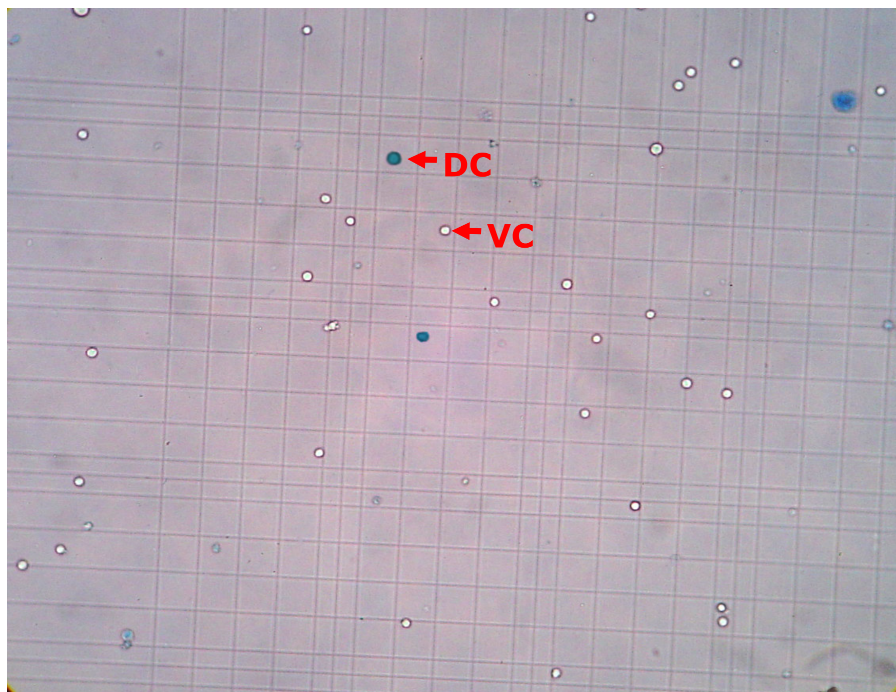


Figure 3.1. Trypan blue dye exculision assay (VC: Viable cell, DC: Dead cell)

In concert with trypan blue exclusion assay, XTT proliferation assay have confirmed the ability of zoledronic acid to inhibit the growth of PC-3 cells in a dose and time dependent manner (48 and 72 hr). Identical experiments with increasing doses of zoledronic acid produced an inhibition of the growth of PC-3 cells, at drug concentrations as low as 10 µM (48 and 72 hr) (Figure 3.3.).

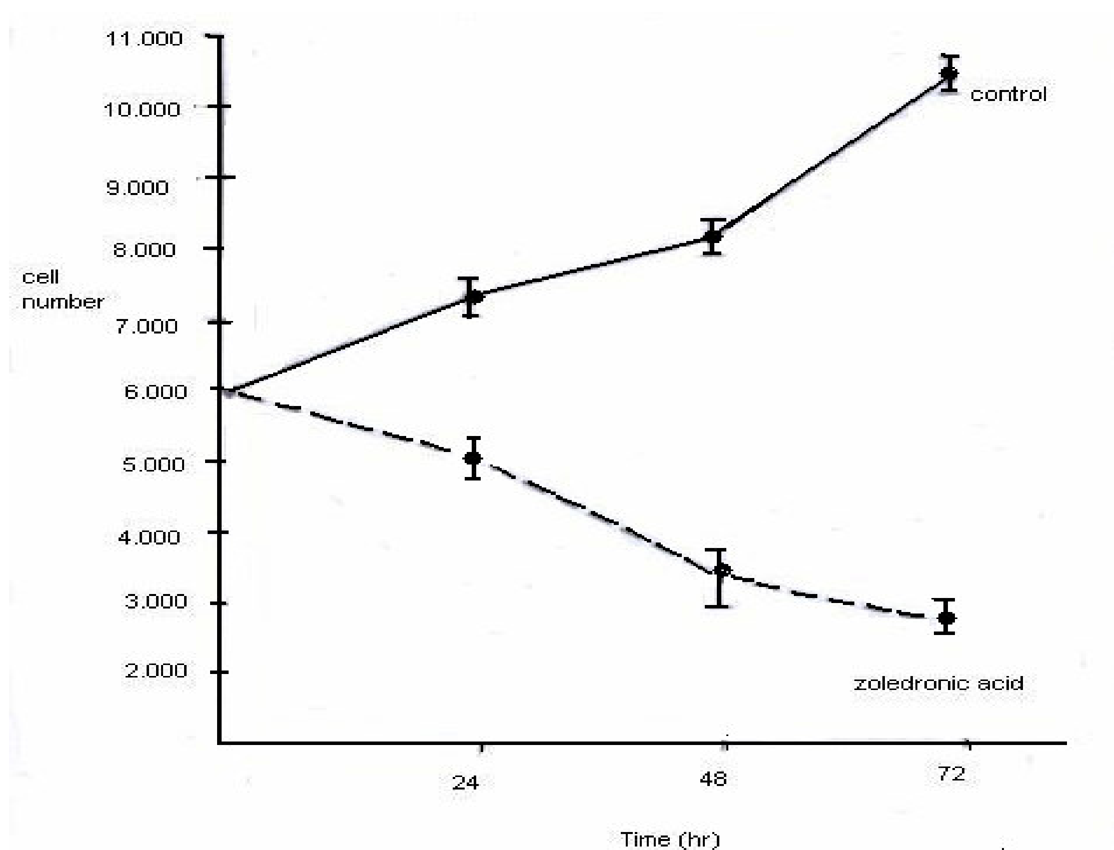


Figure 3.2. Cell counts of viable PC-3 cells as measured by trypan blue exclusion assay with zoledronic acid (100 µM for 24, 48 and 72 hr)

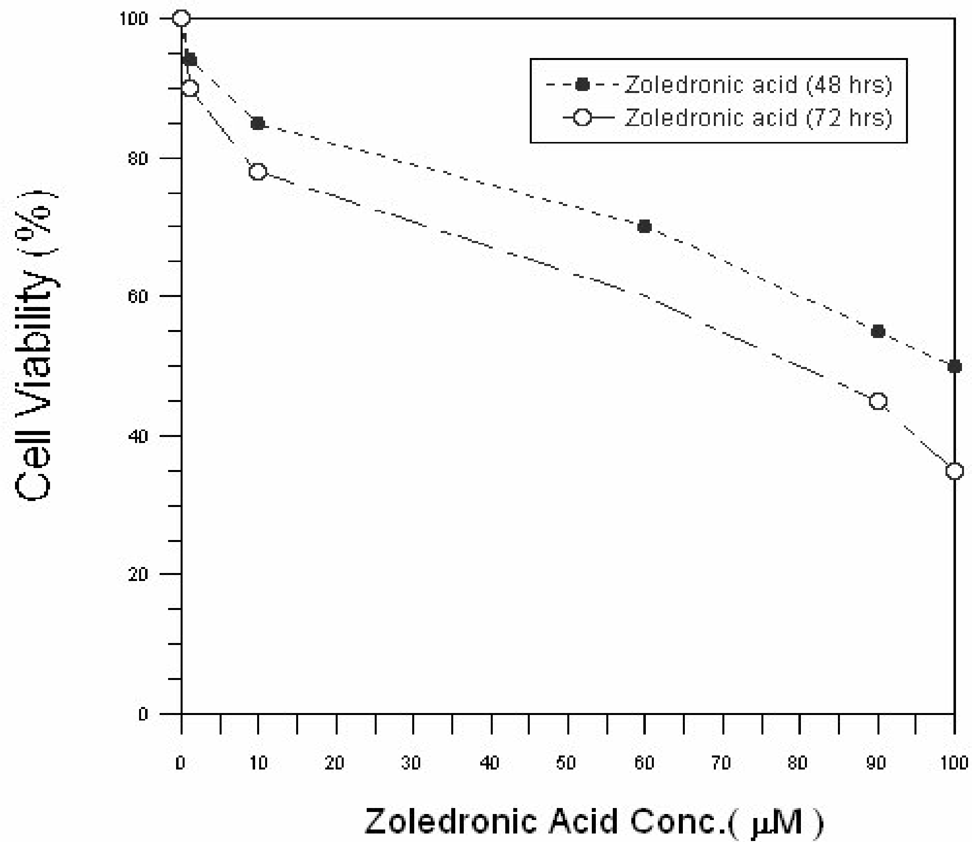


Figure 3.3. Dose–dependent cytotoxic activities of zoledronic acid against PC-3 cells (48 and 72 hrs)

We tested the apoptotic effect of zoledronic acid on PC3- cells by using two different apoptosis measurement assay. It was shown that zoledronic acid induces apoptosis and this effect was time and dose dependent manner as displayed in Figure 3.3. and 3.4.

First, the apoptotic effect of zoledronic acid was measured by Cell Death Detection ELISA kit (Roche Applied Science, Penzberg, Germany), and displayed as the percentage values based on positive control data. Apoptotic effect was dependent on time and dose exposure (1, 10, 60, 90, 100 µM) in PC-3 cells (Figure 3.4.).

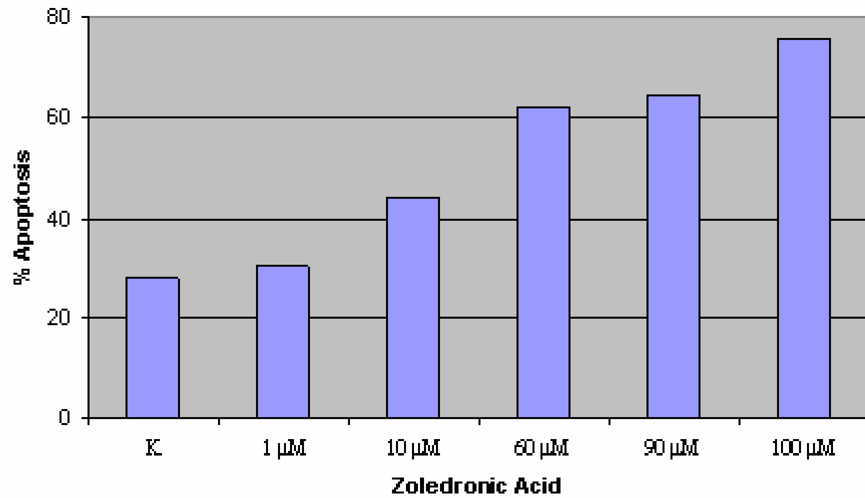


Figure 3.4. Evidence of the apoptotic effect of zoledronic acid (48 hrs).

Then apoptotic effect of zoledronic acid was assessed by Apoptotic DNA Ladder Kit (Roche Applied Science, Penzberg, Germany). The cells were cultured for 48 and 72 hr with various concentrations of zoledronic acid (1, 10, 60, 90, 100 µM). Fragmented DNA extracted from the cells was analyzed by agarose gel electrophoresis. Zoledronic acid (90 and100 µM for 72 hr) produced the classical DNA ladders on agarose gel characteristic of PC-3 cells undergoing apoptosis (Figure 3.4.).

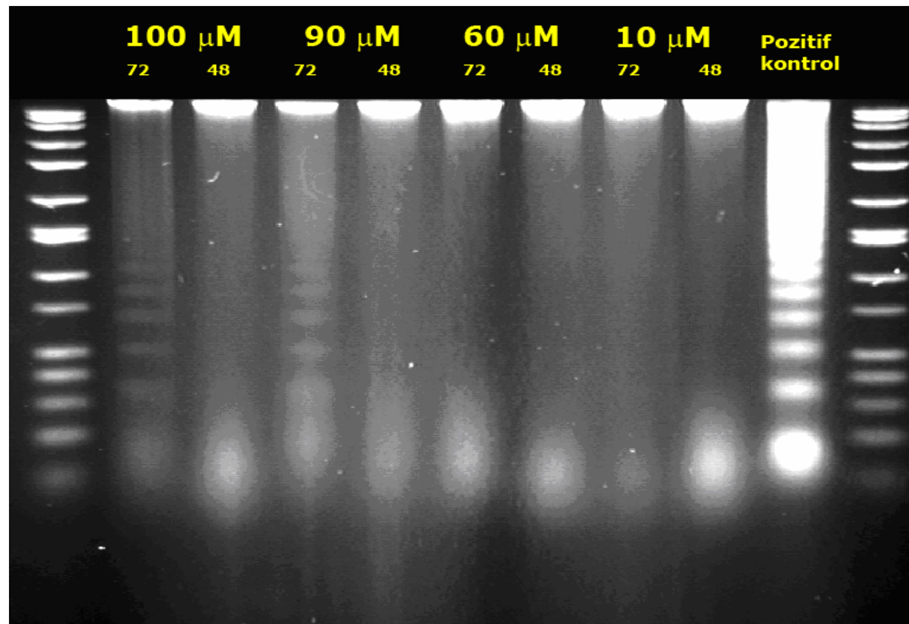


Figure 3.5. DNA fragmentation analysis of zoledronic acid treated PC-3 cells using simple agarose gel electrophoresis.

IL-6 levels produced *in vitro* by PC-3 cells were determined using The Human IL-6 ELISA Kit (Biosource Inc., California, USA). PC-3 cells produced 24.04 ± 2.6 pg/ml IL-6 (72 hr), under normal culture conditions in the absence of zoledronic acid. PC-3 cells were incubated with various concentrations of zoledronic acid (1, 10,30 , 60, 90, 100 µM) for 24,48 and 72 hrs. Then IL-6 levels were measured in supernatants of cell culture. IL-6 concentrations secreted by PC3 cells in the treatment of various zoledronic acid concentrations were lowered from 24 ± 0.4 pg/ml to 7 ± 0.8 pg/ml (Table 3.1.).

Table 3.1. IL-6 Levels in supernatants of PC-3 cells.

Time Point	Control (pg/ 10^5 viable cell)	Zoldronic Acid 1µM (pg/ 10^5 viable cell)	ZA 10µM (pg/10^5)	ZA 60 µM (pg/10^5)	Z A 90µM (pg/ 10^5)	ZA 100µM (pg/ 10^5)
24 hrs	10.10±0.84	3.57±1.04	2.31±0.40	2.33±1.17	1.93 ±1.08	3.45 ± 1.09
48 hrs	20.01± 0.33	11.12 ±0.31	9.16±0.41	10.44±0.61	10.72±1.53	8.9 ±0.94
72 hrs	24.04±2.60	10.99±0.62	8.36±1.10	8.16±0.80	7.76±0.41	7.24±0.85

There was significant decrease in IL-6 secretion in the 1-100 µM concentrations in PC-3 cells (Table 3.1.). The decrease in IL-6 levels in the presence of zoledronic acid concentrations comparing to control group was statistically significant ($p< 0.05$). However there was no significant difference between 1 µM and 100 µM zoledronic acid concentration except in 72 hours exposure. There was slide difference between 1 µM and 100 µM in 72 hours.

IL-6 levels were decreased by various zoledronic acid concentrations (1, 10, 60, 90, 100 µM) in time and dose dependent manner in PC-3 cell lines. The displayed data was average values from 24, 48 and 72 hr exposure time points (Figure 3.6.).

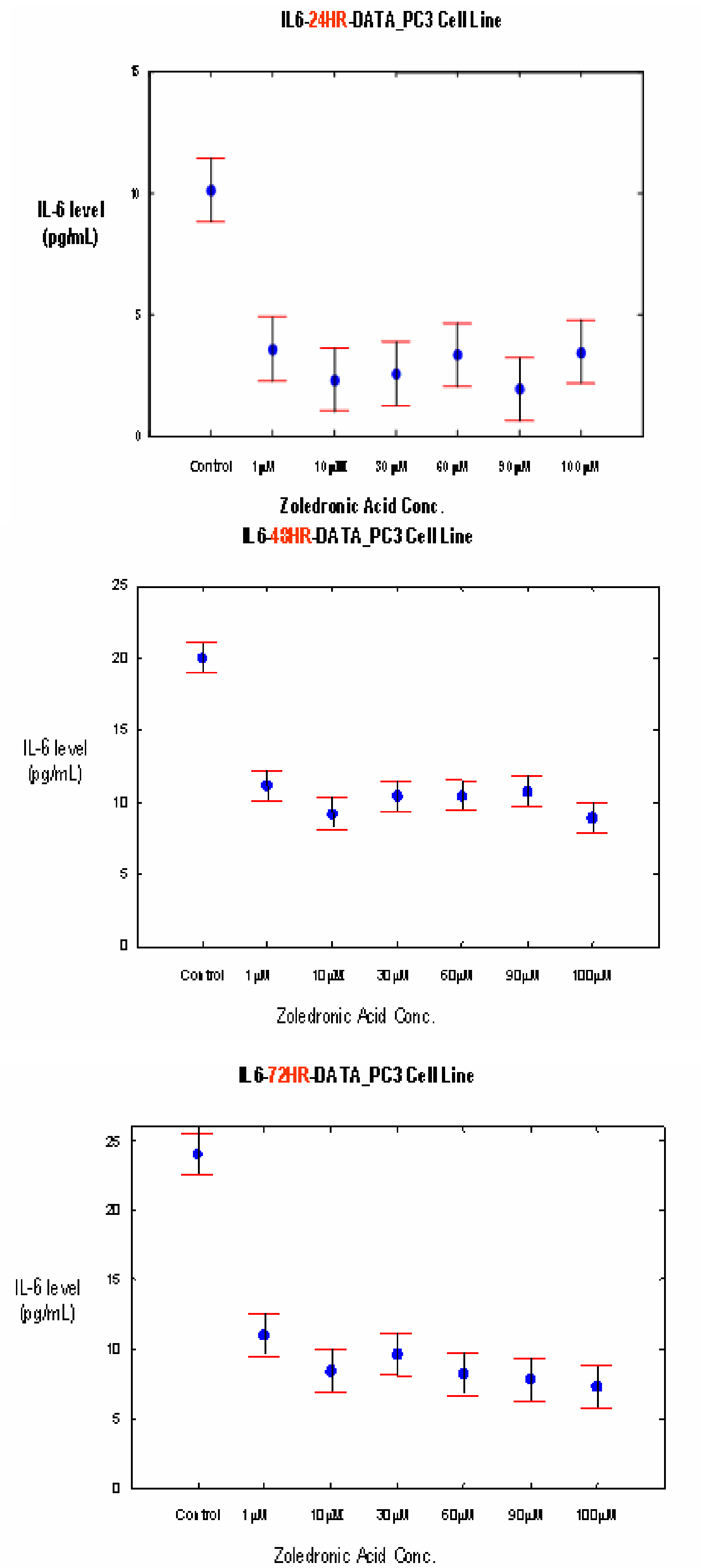


Figure 3.6. Time and dose dependent decrease of IL-6 levels in the supernatants of PC-3 cells. (24, 48 and 72 hrs)

These data suggest that zoledronic acid inhibits the expression and secretion of IL-6 normally produced in high levels in PC-3 cell lines. These data imply that anti tumor effects of zoledronic acid could be also mediated by interfering IL-6 signaling pathways in prostate cells.

3.2 Discussions

In the literature the first data on cytotoxic effect of zoledronic acid was produced by Aparicio A et al in myeloma cell lines (Aparici et al, 1998). Then several studies (Lee et al, 2001, Witters et al, 2002; Fromigue et al, 2000; Mundy et al, 2001, Jagdev et al, 2000, Jagdev et al, 2001, Derenne et al, 1999, Tassone et al, 2000, Corey et al, 2003, Lee et al, 2002) have suggested direct antiproliferative and pro-apoptotic effects of the bisphosphonates on breast, prostate, and myeloma cancer cells.

Lee et al studied the effects of pamidronate and zoledronic acid on prostate cancer cell growth in vitro. Three separate prostate cancer cell lines were seeded in 96-well plates and incubated in serum-free media with or without the bisphosphonates at various concentrations for up to 72 hours. The numbers of cells remaining were determined indirectly by measuring the absorbance of crystal violet dye stain in viable cells. In all cell lines, treatment with 100-µmol/L pamidronate induced significant cell death compared with untreated cells, whereas lower concentrations (25 and 50 µmol/L) caused cell stasis. In contrast, zoledronic acid 100 µmol/L did not cause cell death but caused cell cytostasis, even at concentrations as low as 10 µmol/L. In the presence of serum, both bisphosphonates inhibited prostate cancer cell growth, but only zoledronic acid was effective at lower concentrations (25–50 µmol/L). In a separate in vitro study of zoledronic acid with a cyclooxygenase inhibitor in the DU-145 human prostate carcinoma cell line (Witters et al, 2002) the effects on cell growth were determined using the 3,[4,5-dimethylthiazol-2-41]-2,5-diphenyltetrazolium bromide (MTT) dye assay. Cell exposure to zoledronic acid (1–10 µmol/L) resulted in a dose-dependent growth inhibition (8%–70%). The combination gave an additive inhibitory effect on cell number compared with zoledronic acid treatment (Witters et al, 2002). Studies have also shown that the combination of zoledronic acid plus docetaxel resulted in additive apoptotic effects on PC–3 and DU–145 prostate cancer cells (Wei et al, 1989).

Two in vitro studies (Corey et al, 2003, Lee et al, 2002) have further examined the exposed 2 prostate cancer cell lines—LnCaP and PC-3—to zoledronic acid to

determine its effects on apoptosis and cell cycle progression. Compared with unexposed cells, the proportion of apoptotic cells among the PC-3 cells was increased after 1 day of treatment, but there was no significant difference among the LnCaP cells.The proliferation of PC-3 cells was inhibited by either continuous or pulsatile exposures of zoledronic acid in a dose-dependent manner (Tenta et al, 2004). Tenta et al also showed that zoledronic acid induces apoptosis in PC-3 prostate cancer cells (Tenta et al, 2005).

In a recent study of the effects of several bisphosphonates on PC–3 prostate cancer cells, zoledronic acid markedly inhibited cell growth through a combination of cytostatic and apoptotic effects (Dumon et al, 2004). Notably, zoledronic acid was the most potent bisphosphonate, exerting profound antiproliferative effects more rapidly than either pamidronate or ibandronate.

The known effects of zoledronic acid were confirmed in our experimental conditions. Our data also proved that the cytotoxic effect of zoledronic acid was dose- and time- dependent in PC-3 cell line as displayed in several studies (Figure 3.3.). We also tested the apoptotic effect of zoledronic acid on PC-3 cell lines by using two different apoptosis measurement assay. It was shown that zoledronic acid induces apoptosis and this effect was also time and dose dependent manner (Figure 3.4. and 3.5.).

In summary we found that zoledronic acid have two main effects on PC-3 cell lines. The cytotoxic and apoptotic effect of zoledronic acid was dependent on time and concentration. The second finding was that the expression of IL-6 was downregulated in time and dose dependent manner by zoledronic acid.

IL-6 is produced by lymphoid, non-lymphoid cells, and cancer cells (Kishimoto et al, 1989). IL-6 is a multifunctional cytokine that plays an important role in the regulation of hematopoiesis, immune response, inflammation, bone metabolism, and neural development (Kishimoto et al, 1992). It has been demonstrated that normal prostate epithelial cells, cells derived from benign prostatic hyperplasia and primary prostate tumors secrete IL-6 in cell culture (Twillie et al, 1995).

IL-6 can increase the proliferation of prostate cancer cells (McCarty et al, 2004) and treatment of cultures of androgen insensitive prostatic cancer cell lines PC-3 and DU145 with anti-IL-6 antibodies decreases growth rates of both cell lines (Borsellino et al, 1995). In contrast, androgen sensitive cell line LNCaP showed some divergence in reported effects of IL-6. Both stimulation and inhibition of proliferation being reported in this cell line (Okamoto et al, 1997; Levesque et al, 1998).

It was shown that bisphosphonates induce both myeloma cell and bone marrow stromal cells (BMSCs) apoptosis. Furthermore, at lower concentrations, they induce a significant inhibition (40% and 60%, respectively) of the constitutive production of interleukin-6 (IL-6) by BMSCs. In conclusion, the apoptosis of myeloma cells and BMSCs and the inhibition of IL-6 production induced by bisphosphonates, mainly zoledronate, could have antitumoral effects in patients with MM (Derenne et al, 1999).

Interleukin-6 (IL-6) is the major growth and survival factor for multiple myeloma (MM), and has been shown to protect MM cells from apoptosis induced by a variety of agents (Tassone et al, 2002). IL-6 receptor antagonists, which prevent the assembly of functional IL-6 receptor complexes, inhibit cell proliferation and induce apoptosis in MM cells (Tassone et al, 2002).

It was shown that the IL-6 receptor super-antagonist Sant7 might enhance the antiproliferative and apoptotic effects induced by the combination of dexamethasone and zoledronic acid on human multiple myeloma (MM) cell lines. Sant7 significantly enhanced growth inhibition and apoptosis induced by dexamethasone and zoledronic acid on both MM cell lines. These results indicate that overcoming IL-6 mediated cell resistance by Sant7 potentiates the effect of bisphosphonates on MM cell growth and survival, providing a rationale for therapies including IL-6 antagonists in MM. (Tassone et al, 2002). Furthermore, exogenous administration of interleukin 6 (IL-6) inhibited the doxorubicin-induced apoptosis of PC-3 cells (Tenta et al, 2004).

In vitro studies have demonstrated the antitumor potential of zoledronic acid on myeloma cell lines, but few data are available on its effects on bone marrow stromal cells (BMSCs). In vitro exposure to the lowest concentrations of zoledronic acid decreased IL-6 production by on bone marrow stromal cells (BMSCs) (Corso et al, 2005).

In our study, we confirmed that zoledronic acid also inhibited the expression of IL-6 significantly in PC-3 prostate cell lines. There is no data on the decreasing of IL-6 levels induced by zoledronic acid in PC-3 cell lines in current literature. In addition we also found that decreasing of IL-6 expression by various zoledronic acid concentrations was not dose dependent. The data on the 1 μM and 100 μM zoledronic acid concentrations caused the more or less the same amount of decrease in IL-6 level. This was so significant that the 1 μM which is also achieved serum level in prostate patients switch off IL-6 secretion in significant level. This might be directly inactivation of IL-6

nuclear transcription factor by zoledronic acid. This new data implies new hypothesis that zoledronic acid may exert its antitumor effects via interfering IL-6 expression.

We speculate the effects of zoledronic acid on PC-3 cell lines might be mediated by interfering IL-6 signaling pathways. IL-6 has different effects on prostate cancer cell lines. The bases of these differences can be originated from multistep and alternative signal flow pathways used by IL-6 in different cells. For instance, there are two forms (membrane bound and soluble) of IL-6 receptor regulated tightly. Signal transducer gp-130 in membrane is common substrate for several growth factors. Therefore its activation is not only dependent on IL-6.

Moreover activated gp130 either simultaneously or preferentially triggers three intracellular pathways by altering its intracellular domain. IL-6 signaling was mediated by JAK-STAT, ras-raf-MAPK and PI3K-Akt signaling. It is suggested that, one or two alternative pathways are preferentially more active in different cell lines. IL-6 is also capable of up and down regulation by autocrine or paracrine effect and feed-back (Klein et al, 1989, Miki et al, 1989, Yee et al, 1989, Okamoto et al, 1997, Chung et al, 1999). The expression of IL-6 is regulated by several transcription factors such as AP-1, NFKapaB, CREB and c/EBP. It is considered that intracellular signaling pathways of IL-6 also regulate these transcription factors. Therefore mentioned IL-6 signaling pathways might be affected by zoledronic acid. The possible interacting proteins with zoledronic acid might be either the structural or regulatory component of the following signaling pathways. These are Ras-Raf-MAPK cascade involving cell proliferation, tumor suppressor and apoptotic pathways, cell cycle proteins (cyclins), posttranslational mechanisms of intracellular signaling proteins. Moreover, there may be not only one substrate for zoledronic acid; it may have multiple interacting substrates within the cell. At present, it is not certain which pathways in the upstream or downstream signal flow are disrupted by zoledronic acid. However, it was shown that zoledronic acid interferes with isoprenylation of intracellular small G proteins such as *ras* (Caraglia et al, 2004) by inhibiting the farnesyl pyrophosphate (FPP) synthase enzyme (Rogers et al, 2000), which is required for producing FPP and subsequently geranylgeranyl pyrophosphate (GGPP).

It is also shown that zoledronic acid induced apoptosis by either caspase 3 or caspase 9 (Oades et al, 2003). Although the apoptotic effect of zoledronic acid was diminished by co-treating with a broad spectrum caspase inhibitor, Z-VAD-FMK (Coxon et al, 2004), it is not clear whether zoledronic acid binds to one of caspases.

On the other hand the apoptotic effect of zoledronic acid may not be the premature action of zoledronic acid, since it is possible that zoledronic acid first inhibits signal transduction in the upstream regulation of apoptotic switch mechanism. This possibility may explain the apoptotic effect and decrease of IL-6 in PC-3 cells which was shown in our study. The observed apoptosis in PC-3 cells and decreases in IL-6 expression seem to be paralel events. But these two effects of zoledronic acid might be either independent or associated. However it is uncertain whether the apoptotic pathway was triggered by zoledronic acid mediated-decrease of IL-6 level. It is also unknown whether the decrease in IL-6 expression is caused by zoledronic acid induced apoptosis. There is also another hypothesis that if these two events are independently controlled in prostate cancer cells, then it might be interpreted that zoledronic acid can exert its effect by inhibiting IL-6 autocrine effect mechanism. Therefore, there is a need to carry out further studies in order to define the new zoledronic acid substrates mediating apoptotic effects.

Moreover it is also not certain that apoptotic pathway in these cell lines and IL-6 down regulation pathway are independently active or related each other. As displayed in several studies including our's, zoledronic acid switch on apoptotic pathway, therefore one of the outcome of apoptosis might lead to decreasing in expression of IL-6. But it has to be investigated further.

This study indicates that the molecular target of zoledronic acid is likely to be one of the molecules involving in IL-6 expression or signaling pathway. If it is true, it could be possible to develop new treatment strategies for prostate cancer. It suggests that the inhibition of IL-6 may be a useful adjunct to prostate cancer therapy regarding to *in vitro* studies. It could be speculated that a therapeutic intervention that interferes with IL-6 signaling may be justified both at early and late stages of prostate carcinogenesis and could decrease morbidity in patients with advanced disease. On the other hand, the importance of IL-6 for androgen independent growth of tumor cells makes it a good candidate for targeted therapy in human carcinoma of the prostate.

CHAPTER 5

CONCLUSIONS

Zoledronic acid is a potent, nitrogen-containing bisphosphonate that inhibits bone resorption by reducing osteoclast proliferation and inducing osteoclast apoptosis. Zoledronic acid exerts direct antitumor effects by inducing apoptosis, inhibiting cell adhesion molecules, preventing prostate and breast cancer cell adherence to bone, reducing IL-6 secretion from myeloma stromal cells, and affecting angiogenesis. However, zoledronic acid’s mechanism of action is not clearly understood. Also, its molecular substrates in PC-3 cell lines are not known.

In this study we have showed that zoledronic acid reduces IL-6 expression in significant level in prostate cancer cell line PC-3. The ability of IL-6 to activate signaling through several distinct pathways may exert differential effects on various cell lines. IL-6 is using three intracellular pathways to transducing IL-6 signaling by binding gp130 then activating signal transducer and activator of transcription (STAT)-3, mitogen activated protein kinases (MAPK), and phosphatidylinositol 3-kinase (PI3K) signaling pathways, each depends upon different regions of gp130.

Additional research is required to elucidate further the action of IL-6 and its role in the pathogenesis of advanced prostate cancer at the cellular and molecular levels. At present, it is not certain which pathways in the upstream or downstream signal flow are disrupted by zoledronic acid. New therapeutic strategies using anti-IL-6 monoclonal antibodies seem warranted in such patients for which conventional treatment holds little and may afford improved quality of life and survival benefit.

These original data on the decreasing of IL-6 secretion by zoledronic acid in PC-3 cells suggest that targeting IL-6 in future may have multiple benefits in prostate cancer patients in terms of developing alternative combined threapies in hormone refractory prostate cancer patients.

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