THE INVOLVEMENT OF CERAMIDE METABOLIZING GENES AND THEIR PRODUCTS IN DOCETAXEL INDUCED APOPTOSIS IN HUMAN PROSTATE CANCER CELLS

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by
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ABSTRACT

THE INVOLVEMENT OF CERAMIDE METABOLIZING GENES AND THEIR PRODUCTS IN DOCETAXEL INDUCED APOPTOSIS IN HUMAN PROSTATE CANCER CELLS

Patients diagnosed with prostate cancer initially respond to androgen ablation therapy with tumor cells undergoing apoptosis, but then the patients relapse in time and develop metastatic, androgen independent prostate cancer.

Docetaxel has been widely used for treatment of patients with advanced metastatic prostate cancer. The sphingolipid, ceramide, is a lipid second messenger that mediates a lot of functions as regulation of cell growth, proliferation, differentiation, senescence and apoptotic responses in various cancer cells. The enzyme, glucosylceramide synthase (GCS) is responsible for bioactivation of the proapoptotic mediator ceramide to antiapoptotic glucosylceramide. Likewise, sphingosine kinase-1 (SK-1) transforms apoptotic ceramide to antiapoptotic sphingosine 1-phosphate. Emerging results indicate that GCS and SK-1 are overexpressed in resistant cancer cell lines and cancerous tissue samples of patients. Moreover apoptosis and inhibition of cell proliferation and survival are induced by intracellular ceramide levels including enhancement in *de novo* ceramide production, exogenous delivery of cell permeable ceramide and inhibition of ceramide metabolism by affecting GCS and SK-1.

In this study, we applied exogenous ceramide and inhibitors of GCS and SK-1 in combination with docetaxel for sensitizing androgen independent prostate cancer cells to chemotherapy and provide their effectively utilization with minimizing side effects of the drugs. The *de novo* generation of ceramide is regulated by the genes (LASS1-6) in mammalian cells. Therefore in this study, we examined the possible roles of the ceramide/S1P and ceramide/GS by examining expression levels of GCS, SK-1 and LASS1,2,4,5,6 which can play important roles to overcome androgen independent prostate cancer both concurrently used chemotherapeutic agents and alone.

ÖZET

PROSTAT KANSERİ HÜCRELERİNDE SERAMİD METABOLİZMASI GENLERİNİN VE ÜRÜNLERİNİN DOSETAKSELİN TETİKLEDİĞİ HÜCRE ÖLÜMLERİ ÜZERİNE ETKİSİ

Prostat kanseri günümüzde erkeklerde görülen en sık malinite ve ikinci en sık ölüm nedenidir. Prostat kanseri hücreleri, standart androjen ablasyonu tedavisiyle ilk olarak apoptozise gitmelerine karşın, bazı hastalarda zamanla tedaviye direnç gelişmektedir.

Prostat kanseri hastalarında dosetaksel kullanımının hastanın yaşam kalitesini ve süresini uzattığı tespit edilmiştir. Bir sfingolipid olan seramid, hücrede ikinci haberci olup, apoptozisi yönetir. Glukozilseramid sentaz (GSS) enzimi, proapoptotik bir molekül olan seramidin antiapoptotik glukozilseramide dönüşmesinden sorumludur. Benzer şekilde sfingozinkinaz-1 (SK-1), hücrede seramidi hücresel farklılaşma ve çoğalma sağladığı gösterilen antiapoptotik sfingozin1-fosfat'a dönüştürür. Daha önce yapılan çalışmalar, GSS ve SK-1'in bir çok tümor tipinde fazla miktarda bulunduğu ve kanser hücrelerinde direnç gelişimine katkısı olduğu gösterilmiştir. Çeşitli araştırmalarda, vücutta *de novo* seramid üretimi arttırmakla, dışarıdan hücre zarı geçirgenliği özelliği olan seramid analogları eklenerek ya da GSS ve/veya SK-1 yolu inhibe edilerek hücre içi seramid miktarının arttırıldığı gösterilmiştir.

Araştırmamızda, androjenden bağımsız prostat kanseri hücrelerine dosetaksel ile beraber, seramid analoğu, GSS ve SK-1 inhibitörü uygulanararak hücre içi seramid seviyesinin artması sağlanmıştır, aynı zamanda dosetaksel ile kombinasyonu kullanılarak sinerjistik etkilerine bakılmıştır. Bu şekilde dosetakselin apoptotik etkinliğinin arttırılarak kullanım dozajının azaltılması ve yan etkilerinin minimize edilmesi hedeflenmiştir. Ceramidin *de novo* sentezi, memeli hücrelerinde LASS 1-6 genleri tarafından regüle edilir. Çalışmamızda GSS, SK-1, ceramid/S1P, ceramid/GS, LASS1, 2, 4, 5, 6 genlerinin ekspresyon düzeyleri araştırılarak dosetaksel ile ve tek başlarına etkilerine bakılmış, androjenden bağımsız prostate kanser hücreleri üzerindeki rolleri araştırılmıştır.

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ABBREVIATIONS

Akt	Protein kinase B
AR	Androgen receptor
CDK4	Cyclin dependent kinase 4
C1P	Ceramide-1-phosphate
Cer	Ceramide
CerS	Ceramide synthase
CERK	Ceramide kinase
CERT	Ceramide transport protein
DHT	Dihydrotestosterone
Doc	Docetaxel
EGF	Epidermal growth factor
ERK	Extracellular regulated kinases
FSH	Follicle-stimulating hormone
GCS	Glucosylceramide synthase
GlcCer	Glucosylceramide
AIPC	Androgen independent prostate cancer
IGF-1	Insulin-like growth factor-1
KGF	Keratinocyte growth factor
LASS	Longevity-assurance homologue
LH	Luteinizing hormone
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PSA	Prostate specific antigen
PTEN	The phosphatase and tensin
S1P	Sphingosine 1-phosphate
SM	Sphingomyelin
SMase	Sphingomyelinase
SK-1	Sphingosine kinase-1
Sph	Sphingosine
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 PROSTATE CANCER

Prostate cancer is nowadays the most frequent non-skin cancer in the western industrialized countries and the second leading cause of death in men (Visakorpi 2003, Schulz, et al. 2003). Charles Huggins first found androgen ablation therapy that metastatic prostate cancer responds to androgen ablation therapy. However in 2 to 3 year transition, androgen independent state occurs and results in more metastatic cancer and death. In addition, new invention on diagnosis with prostate specific antigen (PSA) testing increased prostate cancer incidence dramatically with early stage (Pienta and Smith 2005). Prostate cancer incidence increases with higher age and has a hereditary etiology. Accumulated data showed that incidence of prostate cancer occurrence is very high in man who have first degree relative with suffer from prostate cancer. Numerous studies have consistently reported that prostate cancer has a low incidence in Asians and highest in Scandinavians and African-Americans (Sakr, et al. 1998). Healthy consumptions of food and choices are also important in prostate cancer. Consciously consumption of diets by using specific fatty acids, antioxidant vitamins, carotenoids, and phytoestrogens may alter prostate cancer risk. Previous studies showed that changes in plasma levels of key hormones and associated molecules and naturally occurring variants in genes of the androgen also have a great importance. For instance, vitamin D, insulin-like growth factor 1 (IGF-1), and prostate cell growth regulatory pathways may affect prostate cancer risk.

It is well known that the normal prostate is a composite tubulo-alveolar gland, as a chestnut size and encompasses the urethra, under the bladder, below the bladder neck localization (Lang, et al. 2009). In prostate, some regions are well defined as a first

origin of prostate cancer and benign prostatic hyperplasia. These distinct regions are composed of transition, central and peripheral zone (Abate-Shen and Shen 2000). Benign prostatic hyperplasia can be derived from transition and central zone. It is different from prostate cancer and can cause enlargement of the prostate gland which leads to urinary obstruction. The peripheral zone is the site for origin of 60-70% of the carcinomas (Schulz, et al. 2003).

The normal growth and development of the prostate is stimulated by androgens such as testosterone and 5α-dihydrotestosterone (DHT). These hormones exert their growth stimulatory effect through binding to the intracellular androgen receptor (AR) (Chen, et al. 2008). The AR is a member of the steroid receptor hormone superfamily, and its gene is located on chromosome Xq11–12 (Gelmann 2002, Visakorpi 2003). The AR plays an important role on the initiation and growth of prostate cancers and in their reaction to therapy (Devlin and Mudryj 2009). Pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) influence releases testosterone from Leydig cells (Debes and Tindall 2002). Normally 90% of the testosterone is transformed to DHT intracellularly by the enzyme 5α-reductase.

As a potent AR ligand, DHT cause a conformational change that releases heat shock proteins and allows receptor dimerization. The AR then bind to specific DNA binding sequences, known as AR response elements, in the promoter and enhancer regions of target genes. There is an assembly of multi-protein complexes under the influence of coactivator proteins and transcriptional activation and AR acts as a transcription factor (Dehm and Tindall 2006).

Transition to androgen independent state after hormon therapy explained from different theories. The function of AR which is thought to be bypassed could be related with the AR or the development of alternative signaling pathways. Several target genes including those encoding PSA, insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and keratinocyte growth factor (KGF) are of great significance. These have been proved to play an important role in prostate cancer angiogenesis, metastases, and differentiation. In addition, a lot of hormones and cytokines have been shown to interact with the AR and influence transcription. Peptide growth factors such as KGF, IGF-1, and epidermal growth factor (EGF), IL-6, forskolin, cyclin E activate the AR in an androgen-deprived environment (Debes and Tindall 2002, Visakorpi 2003). In addition, after androgen ablation therapy, surviving

clonogenic tumor cells (stem cells) alter from an androgenic-dependent into an androgen-independent stage (Suzuki, et al. 2008).

New treatment options are emerged to date for early stage prostate cancer. Surgery, radiotherapy can cure organ restricted carcinomas. Sometimes as primary therapy and complete ablation of the prostate, cryotherapy, for slow growing carcinomas watchful waiting and hormone therapy also has been utilized (Marberger, et al. 2008, Schulz, et al. 2003). Treatment decisions are of great significance in terms of patients and clinicians. It depends on bases of the stage of tumor, age and life expectancy. If the patient diagnosed Gleason score of <7 and <10 year life expectancy, watchful-waiting is followed. For these type patients, treatment is not given but patient closely monitored (Heidenreich, et al. 2008).

On the other hand the advantages of radiotherapy and radical prostatectomy are clear; the intention of treatment is usually curative. Although nearly 60% - 80% metastatic prostate cancer patients first respond well to hormone treatment in 2 years, more than 50% become androgen independent (Feldman and Feldman 2001, Yu, et al. 2006). Metastasized prostate cancer can be treated with hormones, because most prostate cancers depend on androgens for their growth. Surgical or medical castration could contribute to improvement of bone pain, regression of soft-tissue metastases and diminish in serum PSA levels. However, tumor ultimately turns into androgen independent state, because most tumors will eventually gain mechanisms for independent growth (Korfage, et al. 2003). Currently, docetaxel (Doc) is advised from food and drug administration (FDA) in androgen independent prostate cancer (Madalinska, et al. 2001, Penson, et al. 2003).

1.2. Molecular Biology of Prostate Cancer

The phosphatase and tensin (PTEN) tumor suppressor gene is located on 10q23 and is mutated more frequently in hormone refractory prostate cancer (Karayi and Markham 2004). PTEN mutation provides activation of the cascade, which provides cellular survival and known as a negative regulator of the phosphatidylinositol 3-kinase (PI3K) signaling cascade, protein kinase B (Akt) (Abate-Shen and Shen 2000,

DeMarzo, et al. 2003, Marandola, et al. 2004). It has also been implicated that lessened sensitivity to apoptosis and abnormal cellular proliferation is shown by the result of PTEN loss (Abate-Shen and Shen 2000). Moreover it is exhibited that PTEN function correlated with cell resistance to some drugs (Sherbakova, et al. 2008). Several studies have shown that prostate cancer progression raised Akt activity and loss of function of the cyclin dependent kinase 4 (CDK4) inhibitor, $p27^{kip1}$ in advanced prostate cancer (Bott, et al. 2005). Therefore, tumor invasiveness related to Akt is enhanced parallel with enhanced grade of cancer (Shukla, et al. 2007). Besides, Akt results in activation of the kinase mammalian target of rapamycin (mTOR) (Cao, et al. 2006, McCarty 2004) and several key proteins that are overexpressed as a consequence of this event include c-myc, cyclin D1, and VEGF. Although overexpression of cyclin D1 is uncommon in primary prostate tumors, it is also found in metastatic prostate cells (Abate-Shen and Shen 2000). Additional, NKX3-1 which is expressed in normal prostate epithelium is found decreased in prostate tumor cells (Lee, et al. 2008, DeMarzo, et al. 2003). One of the most important tumor suppressor gene glutathione S-transferase (GSTP1) is also demonstrated very frequent that looses of the activation by hypermethylation in prostate cancer (Hughes, et al. 2005, Wagenlehner, et al. 2007).

Ras is thought to be the initiator of signaling in the Raf/MEK/ERK pathway regulated by binding to Ras. In a smaller group of advanced prostate cancer, extracellular regulated kinases (ERK) activation can be presented (Chatterjee 2003). Mutations in Ras gene are not common in prostate cancer cells (McCarty 2004, Karayi and Markham 2004). It is proved that a number of complex mixture of mutations that interrupt both cell cycle control and cell death pathways by activation of oncogenes and loss of tumor suppressor proteins. Frequently mutated cell cycle proteins not only at early but also in late stages of prostate cancer progression include Rb, p14, p16, p53 and p27 (Lee, et al. 2008). In metastatic or treatment-resistant disease, mutations of the p53 gene are more commonly observed (Reynolds 2008). Abnormal or reduced expression of E-cadherin has been also associated with advanced stage and poor prognosis in human prostate cancer (Abate-Shen and Shen 2000, DeMarzo, et al. 2003). Therefore, HER-2/neu overexpression promotes androgen independent prostate cancer cells proliferation via the Bcl-2 up-regulation, activating AR in the absence of androgen cycle control deficiencies (Shi, et al. 2006).

After all accumulation of multiple genetic deficiencies which result in tumor progression and somatic changes in suppressor genes occur in metastatic late phase

prostate cancer. Rb inactivation is common in metastatic and hormone refractory lesions and generally comes before the somatic modifications affecting the p53 and PTEN. Mutations of the Rb gene and loss of Rb protein expression have been described in both localized and more advanced prostate carcinomas (Abate-Shen and Shen. 2000). Interactions of these tumor suppressor genes thought to be very significant in terms of development of prostate cancer. Transition to a metastatic and hormone-insensitive state characterized by inadequate answer to chemotherapy may be result of increased levels of Bcl-2 protein (Gimba and Barcinski 2003). Overexpression of Bcl-2 in prostate cancer cells is a hallmark of advanced, hormone-refractory disease, and can be responsible for the resistance to apoptosis in late stages. Moreover, it has been shown that Bcl-2 can prevent activation of apoptosis of drugs related with the mechanisms which have microtubule assembly and disassembly functions. Thus it is also mentioned that Bcl-2 strongly connected with recurrence of this disease (Kaur, et al. 2004).

1.3. Treatment Strategies for Prostate Cancer

1.3.1. Chemotherapy for Androgen Independent Prostate Cancer

Chemotherapy is known as a major choice for effective treatment for androgen independent prostate cancer (AIPC). Various clinical trials have exhibited the role of both single agent and combination chemotherapy in the treatment of AIPC. Encouraging results have gained from some of these trials related with PSA response, overall survival, and improvement in quality of life (Basler and Groettrup 2007). Since two decades, studies demonstrated that prostate cancer cells have susceptibility to mitotic spindle inhibitors including vinblastine, paclitaxel, and Doc.

1.3.2. Docetaxel

Doc is a very effective and frequently used anticancer agent for the treatment of prostate cancer (Kish, et al. 2001, Schulz, et al. 2003). It is known as an inactive precursor and extracted from the needles of Taxus baccata. Doc as a semisynthetic taxane, likely has multiple antineoplastic activity functions (Basler and Groettrup 2007, Kraus, et al. 2003). Therefore, this most widely accepted mechanism of action involves binding of Doc to β-tubulin and helps polymerization impairs mitosis and thus retarding cell cycle progression in the G2/M phase and lead to the apoptosis (Ting, et al. 2007). Microtubules normally have a function as a cytoskeleton for the mitotic spindle in cell division. Thus they undergo polymerization in the attendance of microtubule related proteins. Doc inhibits microtubule depolymerization and microtubules can not be disassembled (Kolesnick, et al. 2007, Pienta and Smith 2005).

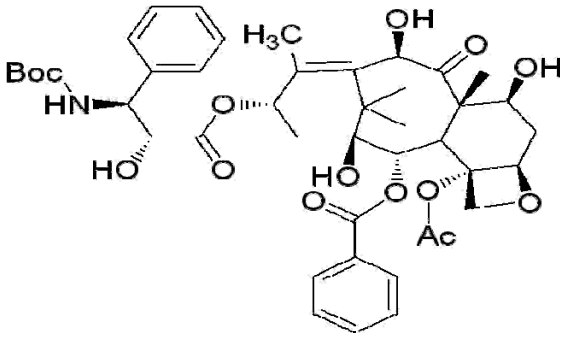

Figure 1.1. Chemical structure of docetaxel.
(Source: Docetaxel®, insert package)

The cytotoxicity of Doc is also demonstrated by Bcl-2 phosphorylation and involving the activation of the c-Raf-1/Ras or the p53/p21WAF1/CIP1 signalling pathway (Fabbri, et al. 2006). In prostate cancer cells, Bcl-2 overexpression increases survival of prostate cancer cells and prevents apoptosis after androgen withdrawal and plays roles on both chemo- and androgen-resistance. Inactivation of Bcl-2 by phosphorylation results in decreased binding to the proapoptotic protein, Bax, which starts the process for caspase cascade and leading to apoptosis in the G2/M phase (Lee, et al. 2008). Besides, Bcl-2-negative tumors development can be inhibited by Doc inducing overexpression of the cell cycle inhibitor, p27. In advanced hormone

refractory prostate cancer cells, p27 is generally found less (Schrijvers 2007). Accumulating data have shown that there are a number of proapoptotic effects of Doc. For instance, induction of p53, Bcl-xL effects and their role on subdue multidrug resistance and antiangiogenic properties in Doc-induced apoptosis in cancer cells (Pienta and Smith 2005). At the moment, FDA approved Doc for the treatment of patients with advanced metastatic prostate cancer, and it does show a survival benefit.

Consequently, from the clinical trials, it could be clearly concluded that Doc reduced PSA levels and relieved symptoms in patients diagnosed with AIPC (Petrylak 2006, Fitzpatrick, et al. 2008). Therefore, promising researches could be new avenue by contribution underlying molecular mechanisms to max out its effectiveness of combination therapy with other agents. However, besides its known mechanisms related to microtubules and apoptosis other mechanisms of Doc-induced apoptosis including ceramide metabolizing genes and their end products, are unknown.

1.4. Ceramide

1.4.1. Sphingolipid Metabolism and the Sphingolipid Rheostat

Ceramide can be generated via two ways. The first is *de novo* sythesis by serine and palmitate condensation to form 3-keto-dihydrosphingosine and acylation by a dihydro ceramide synthase (LASS or CerS) converted to dihydro-sphingosine, through the desaturation reaction (Hannun and Obeid 2008). In the second way, ceramide is generated by hydrolysation of the sphingomyelin via pH specific sphingomyelinases (acid SMase, neutral SMase, alkaline SMase).

It was shown that both of these pathways could act as producing cellular signals and the major difference between these signaling routes are duration. Activation of SMase to form ceramide was illustrated within few minutes whereas *de novo* synthesis to produce ceramide in several hours (Morales, et al. 2007). Ceramide could be utilized in different sphingolipid pathways by phosphorilation by ceramide kinase, and glucosylation by glucosyl ceramide synthases or for biosynthesis of sphingomyelin by

receiving phosphocholine (Hannun and Obeid 2008). Ceramide is transported to the golgi apparatus to form sphingomyelin by ceramide transport protein (CERT) and also through vesicular transport to cytosolic side of golgi. After synthesis of glucosylceramide, it is transferred by transfer protein FAPP2, as a precursor for complex glycosphingolipids which are then transferred in small vesicles to the plasma membrane (Hannun and Obeid 2008, Venkatamaran and Futerman 2000).

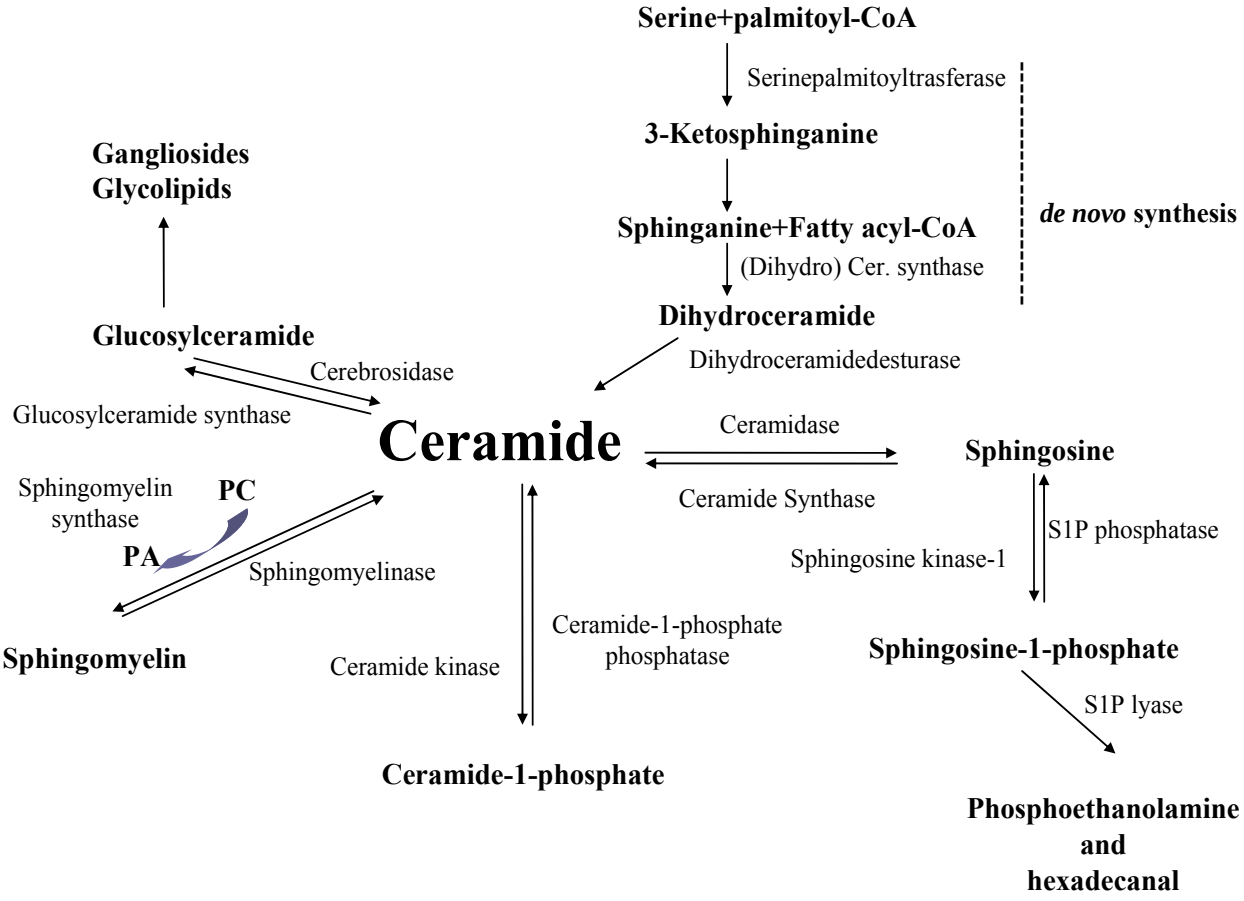

Figure 1.2. The synthesis and metabolism of sphingolipids
(Source: Pettus, et al. 2002)

Degradation of simple sphingolipids, such as sphingomyelin and ceramide, mainly takes places within the acidic late endosomes and lysosomes as well as in the non-lysosomal compartments such as the caveolae in the plasma membrane (Zeidan and Hannun 2007). In lysosomes, acidic SMase hydrolyses sphingomyelin to yield phosphocholine and ceramide. Ceramide is subsequently degraded by the acidic ceramidase to the sphingosine backbone and a fatty acid. In non-lysosomal compartments sphingomyelin is degraded into ceramide by secreted acidic SMase.

Thereafter, ceramide is deacylated to sphingosine and reutilized for glycolipid formation (Huwiler, et al. 2000). Alternatively, sphingosine can be first phosphorylated at the 1-hydroxyl group and subsequently cleaved by a lyase into a long chain aldehyde and ethanolamine phosphate (Huwiler, et al. 2000).

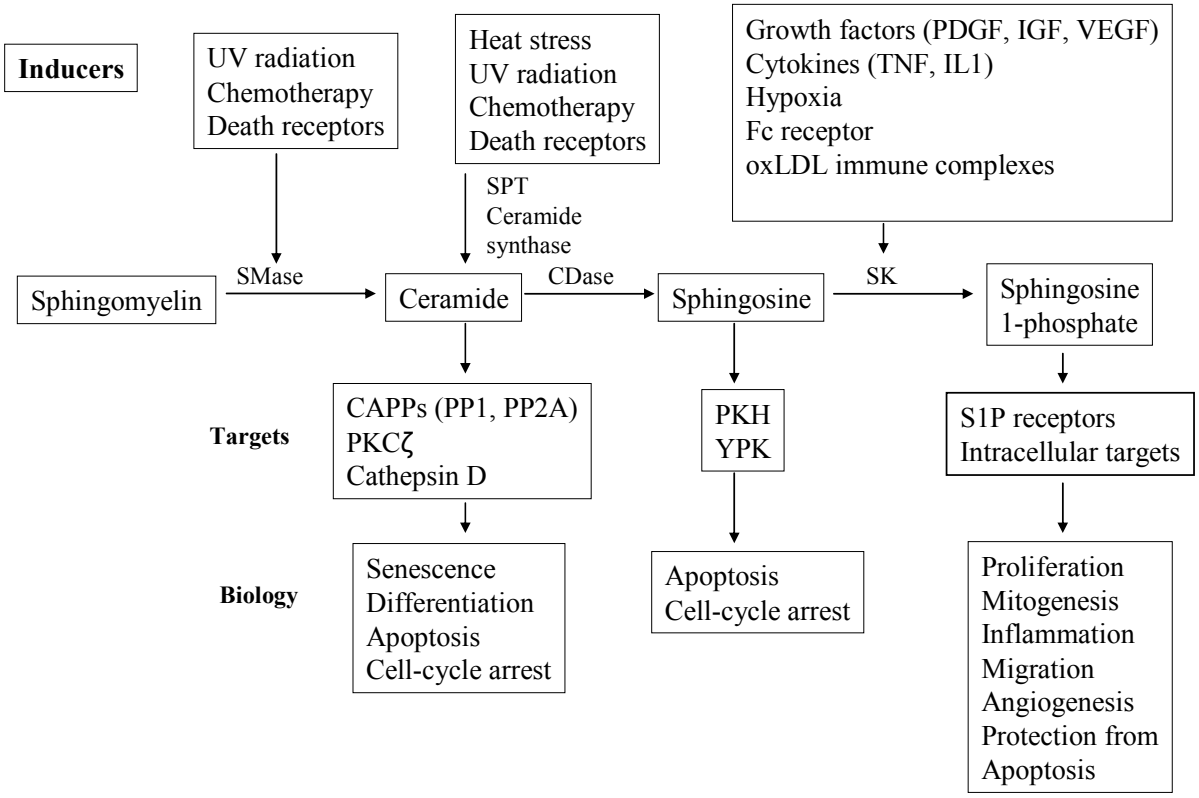

Figure 1.3. Stress factors influencing ceramide metabolism
(Source: Hannun, et al. 2008)

Ceramides are influenced from a number of stress factors such as cytokines (tumor necrosis factor-α and Fas), environmental stress (heat and hypoxia), radiation (UV and ionization), and chemotherapeutics (Fillet, et al. 2003). Exogenous sphingomyelinase or ceramide induced NF-kB activation has been known (Fillet, et al.2003) as well as selective induction of the stress activated protein kinases (SAPK/JNK) pathway such as JNK, PKC, kinase suppressors of Ras (KSR), and protein phosphatases, such as protein phosphatase 2A (PP2A) and PP1 and ceramide activated protein phosphotases (Chen, et al. 2008). After activation of PP2A and PP1 via ceramide, dephosphorylation happens to Bcl-2, Akt, retinoblastoma (RB), c-Jun, and the nuclear serine/arginine-rich domain proteins (Modrak, et al 2006). Researches showed that ceramide has been demonstrated to activate PKCζ, the kinase KSR and cathepsin D (Ogretmen and Hannun 2004).

Therefore ceramide directly activates PKCζ (Lu and Wong 2004). It has been shown to be involved in inhibition of AKT.

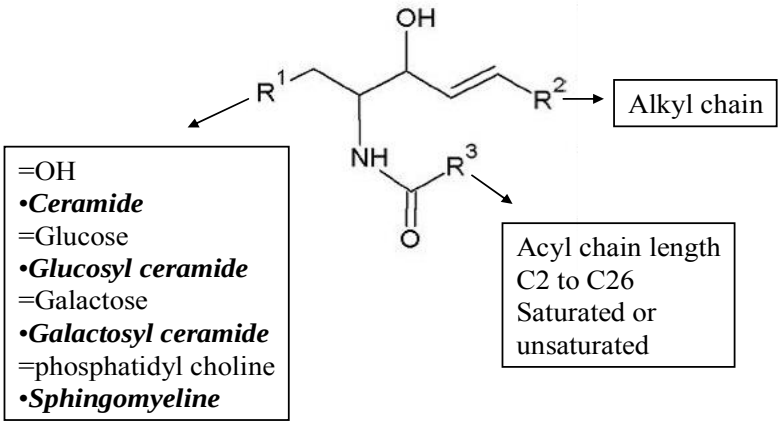

Figure 1.4. General structure of sphingolipids

Accordingly, as a possible therapeutic agent, ceramide could be used in tumor cells to trigger apoptosis. Cell permeable ceramide analogs have been developed for this purpose (Macchia, et al. 2003). It was shown that short-chain cell-permeable ceramide have antiproliferative and proapoptotic effects in numerous tumor cell types *in vitro* (Devalapally, et al. 2007). Consistent with the general idea that ceramide is a mediator of apoptosis and lack of ceramide generation has been linked to tumor cell resistance to death (Senchenkov, et al. 2001). Moreover, chemotherapy and radiotherapy bring out an increase in endogenous ceramide levels before the first biochemical signs of apoptosis and radio-resistant cell lines have been shown not to generate ceramide after irradiation of chemotherapy.

1.4.2. LASS Genes and Ceramide Sythhase

The *de novo* synthesis of ceramide is regulated in mammals by LASS (longevity assurance homologues) genes. First of all, two genes, Lag l and its homologue Lacl genes identified from *Saccharomyces cerevisiae* and this is proved to be necessary for ceramide synthesis. It is found that actually all members of the LASS family of proteins

have resembling in their transmembrane profiles (Spassieva, et al. 2006). Membrane localization of some of the members, including yeast Lac1p (longevity assurance gene cognate 1) motif is required for (dihydro)ceramide synthase activity of LASS proteins and they share the same amino acids in the motif. Mammalian LASS1, LASS4, LASS5, and LASS6 genes are established to be in the endoplasmic reticulum cytoplasmic leaflet.

Ceramides containing different fatty acids may play distinct roles in specific cell functions. It has been well documented previously that LASS1 regulates C18 ceramide and weakly C-20 ceramide, LASS2 and LASS4 enhances both C-22 and C-24 ceramides with longer chain lengths and LASS5 and LASS6 synthesize C-14 and C-16 and weakly C-12 and C-18 ceramides. LASS5 also shows C-18:1 ceramide synthesis different from LASS6 (Baran, et al. 2007, Mizutani, et al.2005). LASS genes also have different activities in different regions of tissues. As an illustrated in mouse, LASS1 is found mostly in brain, LASS2 in liver and kidney, LASS3 is found to be testis specific, LASS4 in skin, LASS5 in testis and kidney, LASS6 is found to be expressed in kidney and brain (Mizutani, et al. 2005, Mizutani, et al. 2006). By different fatty acids, it could be regulated distinct functions in specific cell regions. It also remains to be evolved.

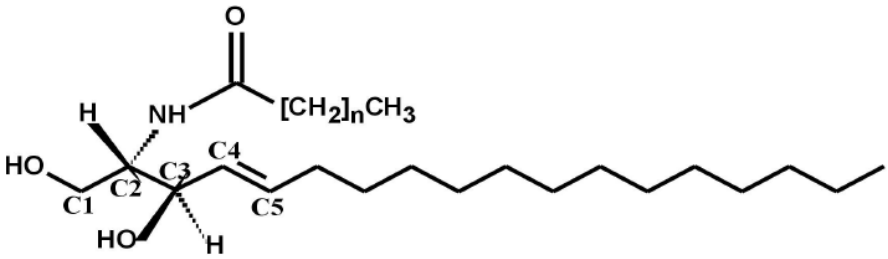

Figure 1.5. General structure of a ceramide
(Source: Siskind, et al. 2003)

In *de novo* synthesis pathway condensation of serine and palmtoyl-CoA results in 3-ketosphingosine and then sphinganine occurs by 3- ketosphinganine reductase. It is continue with the dihydroceramide synthase enzyme and dihydroceramide can be converted to ceramide by the introduction of a double bond between C4 and C5 on the sphingoid base catalyzed by dihydro ceramide desaturase (Matmati and Hannun 2008). In mammals, biosynthesis of ceramide is happened in the endoplasmic reticulum but to form glucosylceramide (GlcCer) or phosphocholine to generate sphingomyelin (SM)

occurs in the Golgi. Further glycosylation steps of GlcCer also operate in the Golgi (Sabourdy, et al. 2008).

1.4.3 Sphingosine-1-Phosphate

Ceramide hydrolyses to form sphingosine (Sph) by the enzyme ceramidase and after the generation of Sph, it is phosphorylated to sphingosine-1-phosphate (S1P) through the action of SK-1. Since this is the only pathway to form Sph, ceramidases also play an important role in this regulation (Mao and Obeid 2008).

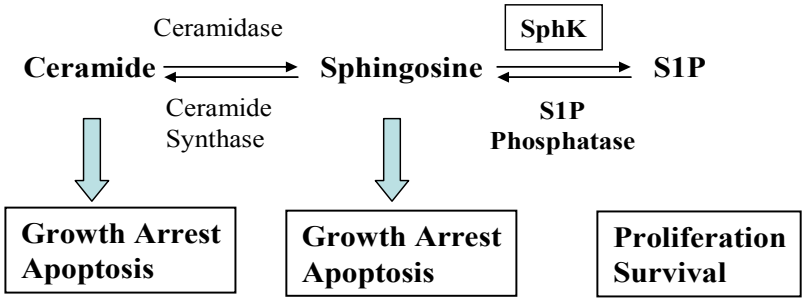

Figure 1.6. The sphingolipid rheostat

The balance between the levels of ceramide and S1P is very crucial in terms of SK-1 activity. S1P is known as a powerful anti-apoptotic molecule. SKs and S1P play very important roles on regulating many cellular responses including mitogenic signaling, chemotaxis, cytokine/chemokine generation, intracellular calcium signals, tumor metastasis, angiogenesis, and autoimmune diseases. It is thought that S1P is yielded at the inner leaflet of the plasma membrane in response to tumor necrosis factor-α (TNFα), and SK-1 is activated in a mechanism that is dependent on TRAF (TNFα receptor-associated factor) and ERK (Hannun and Obeid 2008). S1P acts by binding to cell surface receptors to activate them which are known as G protein coupled receptors with five subtypes S1P1-S1P5. Sphingosine kinases are encoded by two genes, SK-1 and SK-2. SK-1 and its product S1P are related with cancer cell survival.

Up to now, to reduce S1P activities, SK-1 and SK-2 enzyme inhibitors or unspecific compounds were used in researches and promising results encourage developing novel drugs as a potential therapeutic in the future (Huwiler and Pfeilschifter 2008, Melendez 2008). SK inhibitors rapidly promote the intrinsic apoptotic pathway via mitochondrial distribution (Leroux, et al.2007).

SK-1 and S1P play a role on inflamation and tumor immunology. Recent studies have shown that S1P1R presents at high levels on endothelial cells and is known to have important roles in angiogenesis. Thus, it is shown that by inhibiting COX-2, tumor size and metastasize distribution can be decreased (Melendez 2008). It has been reported that S1P can transactivate VEGF receptors in endothelial cells and could be responsible for release of pro-angiogenic cytokines, VEGF and IL-8 (Visentin, et al. 2006).

Moreover, recent evidence suggested that p53 upregulation can increase the synthesis of ceramide but decrease the synthesis of S1P (Panjarian, et al. 2008). Many growth factors, such as epidermal growth factor and platelet-derived growth factor, as well as the cytokines TNFα and IL-1, activate SK-1, resulting in transient elevations in the levels of S1P. This stimulation requires PKC, phospholipase D (PLD) and/or the extracellular signal-regulated kinase (ERK) MAPKs, which act upstream of SK-1 (Pitson, et al. 2003). PKC may act directly or indirectly through PLD and ERK, whereas it has been proposed that ERK directly phosphorylates SK-1 on residue Ser225 (Pitson, et al. 2003), which is required for its translocation to the plasma membrane. PLD generates phosphatidic acid, which may directly bind SK-1 and lead to its membrane association. Actually, transporting S1P from the inner leaflet of the plasma membrane to the cell exterior is performed through the ABC transporter ABCC1 (Hannun and Obeid 2008).

1.4.4 Glucosyl Ceramide Synthase

Specialized glucosyltransferases transfer a glucose residue in a α-glycosidic linkage to the Cl-hydroxyl of ceramide to produce glucosylceramide (GCS). Golgi is the main side of the synthesis of the glucosylceramidesynthase. This process is happened on the cytosolic surface of the Golgi (Morales, et al. 2007). The enzyme glucosylceramide

synthase, responsible for conversion of the apoptotic ceramide to an antiapoptotic glucosylceramide is characteristically overexpressed in many drug resistant tumors (Kiguchi, et al. 2006). Moreover, it is found that in breast cancer and other cancer cells, the expression of the drug resistance ABC transporter P-glycoprotein is related with GCS expression and drug resistance (Zeidan and Hannun 2007). Thus, it is demonstrated that multidrug resistant cancer cells contain high level of glucosylceramide (GlcCer) than drug-sensitive cells. The drug resistance pump, MDR1, is one of the transporters for GlcCer, interestingly involved in occurrence of neutral glycosphingolipids (Merrill, et al. 2007). Involved with proliferation, oncogenic transformation, differentiation and tumor metastasis, GlcCer and its derivatives are of great importance. Therefore, it is found that glucosylceramide synthase regulates cytotoxic response to chemotheraphy (Gouaze-Andersson, et al. 2007).

1.5. Aim of the Study

For a long time, androgen dependence and insensitivity were the main issues in prostate cancer research and prostate cancer. It is expected to reveal insights into the molecular mechanisms of carcinogenesis to aid prevention. As ceramide mediates antiproliferative responses, whereas S1P and GlcCer enhance several tumor-promoting responses, strategies that either mimic/antagonize these sphingolipids or modulate their levels could provide a novel avenue for cancer therapy. Similarly, modulating these pathways might enhance drug action, overcome drug resistance, protect normal cells from cytotoxic actions, and even create new strategies for chemoprevention. Taken together, mounting evidence points to close connections between cytotoxic agents and ceramide metabolism. This investigation may also show a promising clinical potential for this therapeutic strategy to increase chemotherapeutic efficacy.

The main objectives of the project are to examine the apoptotic pathways induced by Doc alone and/or in combination with specific chemicals which manipulate ceramide metabolism such as GCS inhibitor (PDMP), SK-1 inhibitor, exogenous C:8 ceramide in hormone resistant prostate cancer cells. Then, evaluating the involvement of ceramide metabolizing genes and their products in Doc-induced apoptosis in DU-145

and PC-3 cells, we aimed to increase the sensitivity of prostate cancer cells to Doc by targeting ceramide metabolizing genes and enzymes.

CHAPTER 2

MATERIALS & METHODS

2.1. Chemicals

The androgen independent prostate cancer cell lines, PC-3 and DU145, were obtained from German Collection of Microorganisms and Cell Cultures, Germany. RPMI1640, heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin EDTA were obtained from Biological Industries, Israel. Doc was obtained from Gulhane Medical School, Department of Hematology, Ankara, Turkey. SK-1 inhibitor was obtained from Cayman Chemical, USA. C:8 ceramide was obtained from Cayman Chemical, USA. GCS inhibitor, ((±)-*threo*-1-Phenyl-2-decanoylamino-3-morpholino-1-propanolhydrochloride C23H38N2O3·HCl) (PDMP) and all other chemicals were obtained Cayman Chemical, USA. RNeasy RNA isolation kit, QIAquick gel extraction kit, Taq DNA Polymerase was obtained from Finnzymes, FI. Bradford dye, 4-5 % SDS polyacrylamide gel, coommasie blue, tween-20, 10X tris-glycine-EDTA were obtained from Sigma, USA. dNTP set, DNA ladder was obtained from AMRESCO, USA. Caspase-3 colorometric assay kit was obtained from BioVision, USA. JC-1 mitiochondrial membrane potential detection kit was obtained from APO LOGIC^{TM} JC-1 from BACHEM, USA. Reverse-Transcription system and bovine serum albumine (BSA), trypan blue solution, β-mercaptoethanol, dimethyl sulfoxide (DMSO), agarose were obtained from Sigma, USA. 25 cm^2 and 75 cm^2 tissue culture flasks were obtained from Corning, USA.

2.2. Cell lines, Culture Conditions and Maintenance

PC-3 and DU145 cells were cultured in RPMI1640 supplemented with 15% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin. The cells were maintained in CO_2 incubator in the presence 5% CO_2 at 37 ˚C. Medium was refreshed in every 3 days. For maintenance of DU-145 and PC-3 cells suspension on adherent cells was removed from the tissue culture flask. After 5 min incubation with 4mL trypsin EDTA, cell suspension was taken from tissue culture flask into a sterile falcon tube and equal amount of RPMI1640 was added. The cells were centrifuged at 1,000 rpm for 10 min. After centrifugation, supernatant was removed and the pellet was resuspended in 15mL of RPMI1640 and transferred into a sterile 75 cm^2 tissue culture flask.

2.3. Thawing the Frozen Cells

Cells were removed from frozen storage and quickly thawed in a 37 ℃ water bath to obtain the highest percentage of viable cells. When the ice crystals melted, the content was quickly taken into a sterile falcon tube and washed with PBS. Then the cells were cultured in 25 cm^2 tissue culture flask in RPMI1640 medium.

2.4. Cell Viability Assay

Viable cells normally impermeable to trypan blue dye. Breakdown in membrane integrity cause the uptake of a dye in to cell. Cells will be observed and counted under microscope by using hemocytometer and calculated the percentage of unstained cells. Before each experiment, cell viability assay was conducted.

2.5. Cell Proliferation Assay

The effect of different concentrations of Doc and other chemicals on PC-3 and DU145 cell lines were evaluated by using XTT Cell Proliferation Kit II (Biological Industries, Israel). Cells at a concentration of 10^4 cells/well were plated in a 96-well plate in 200 µl culture medium after verifying cell viability by trypan blue exclusion test. After 72 hours incubation, 40 µl of XTT labeling mixture were added to each well. The optical density was measured at 492 nm with a reference wavelength at 650 nm in a microplate reader (Multimode Reader).

2.6. Evaluation of Apoptosis

2.6.1. Determination of Caspase-3 Enzyme Activity

Apoptotic effects of applied C:8 ceramide, GCS and SK-1 inhibitors alone or in combination with Doc were measured by caspase-3 colorimetric assay. Caspase-3 enzyme activity was determined using the caspase-3 colorimetric assay (R&D Systems, USA). After lysis, cell extracts were used for caspase-3 enzyme activity by addition of a caspase-3 specific peptide that is conjugated to the color reporter molecule p-nitroanaline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction. Caspase-3 activity levels were normalized to total protein amounts for each sample determined by Bradford Assay. Firstly, the cells $5x10^5$ in per well that had been induced to apoptosis were collected by centrifugation at 1000 rpm for 10 min. Then, the collected cells were lysed by adding 100 ml of cold lysis buffer. After incubation of the cells on ice for 10 min, they were centrifuged at 14000 rpm for 1 min. Supernatants were removed to new eppendorf tubes and the reaction

mixture was prepared in 96-well plates adding 20 ml assay buffer , 25 ml of sample, 50 ml of sterilized water and 5 ml of caspase-3 colorimetric substrate. After 2 h incubation at 37 ˚C, the plate was read under 405 nm wavelength light (Thermo Electron Corporation Multiskan Spectrum, Finland).

2.6.2. Detection of Mitochondrial Membrane Potential

APO LOGIX JC-1 Assay Kit (Cell Technology, USA) was used to measure the mitochondrial membrane potential in DU-145 and PC-3 cells as described previously. JC-1, a unique cationic dye, was used to determine the loss of the MMP. In the beginning, the cells, the $5x10^5$ for per well that had been induced to apoptosis were collected by centrifugation at 1000 rpm for 10 min. Supernatants were removed and 500 µl of JC-1 dye was added onto the pellets. After incubation at 37 ˚C in 5% CO_2 for 15 min, they were centrifuged at 1000 rpm for 5 min. Then, 2 ml of assay buffer was added onto the pellets which were centrifuged for 5 min at 1000 rpm. All pellets were resuspended with 500 ml assay buffer and 150 ml from each of them was added into the 96-well plate. The aggregate red form has absorption/emission maxima of 585/590 nm and the green monomeric form has absorption/emission maxima of 510/527 nm. The plate was read in these wavelengths by fluorescence Elisa reader (Thermo Varioskan Spectrum, Finland).

2.6.3. Total RNA Isolation from Cells and PCR

The role of ceramide metabolizing genes in Doc induced apoptosis were investigated by examining mRNA levels of SK-1, GCS, LASS 1, 2, 3, 4, 5, 6 in human prostate cancer cells exposed to different concentrations of Doc for 72 h. Total RNA's were extracted using RNA Isolation Kit (Macherey-Nagel) as described by manufacturer and eluted in DEPC-treated materials. Recovered RNA concentration was measured by Nanodrop ND-1000 (260/280 and 260/230 ratios). 1 μg of total RNA was

reverse transcribed using reverse transcriptase enzyme (Moroney Murine Leukemia Virus Reverse Transcriptase, Fermentas, USA). After 1 h incubation at 50 ˚C, the reactions were stopped at 95 C˚ for 5 min (Table 2.2). The resulting total cDNA was used in PCR to measure the mRNA levels of LASS 1, 2, 3, 4, 5, 6, SK-1, GCS and β actin, as internal positive control. PCR mixture was prepared in the sterile 0.5 mL eppendorf tubes (Table 2.3). Amplification conditions were maintained in 35 cycles (Table 2.4.). The mRNA levels were quantified using the imaging system after running PCR products in agarose gels, followed by ethidium bromide staining. Primer sequences were given in Table 2.1.

Table 2.1. Primers used in this study.

GCS	5'ATGACAGAAAAAGTAGGCT3'
GCS	5'GGACACCCCTGAGTGGAA3'
LASS1	5'CTATACATGGACACCTGGCGCAA3'
LASS1	5'TCAGAAGCGCTTGTCCTTCACCA3'
LASS2	5'CCCTCGAGGGATGGATTACAAGGATGACGACGATAAGATGCTCCAGACCTTGTATGATT3'
LASS2	5'CGGAATTCCGTCAGTCATTCTTACGATGGTT3'
LASS5	5'CCCTCGAGGGATGGATTACAAGGATGACGACGATAAGATGGCGACAGCAGCGCAGGGA3'
LASS5	5'CGGAATTCCGTTACTCTTCAGCCCAGTAGCT3'
LASS6	5'CCCTCGAGGGATGGATTACAAGGATGACGACGATAAGATGGCAGGGATCTTAGCCTGG3'
LASS6	5'CGGAATTCCGTTAATCATCCATGGAGCAGGA3'
SK-1	5'CCGACGAGGACTTTGTGCTAAT3'
SK-1	5'GCCTGTCCCCCCAAAGCATAAC3'
β actin	5'CAGAGCAAGAGAGGCATCCT3'
β actin	5'TTGAAGGTCTCAAACATGAT3'

Table 2.2. Ingredients of reverse transcription reaction.

Ingredients	**Amount**
RNAse Free Water	5 µL
Total RNA (5 µg)	5 µL
10X Buffer	2 µL
Random Primers (0.5 µg/L)	0.7 µL
RNAse Inhibitor (50U/µL)	0,7 µL
$MgCl_2$ (25 mM)	4 µL
dNTP (10 mM)	2 µL
Moloney Murine Reverse Transcriptase enzyme (200 U/µL)	0.7 µL
Total	20 µL

Table 2.3. Ingredients of PCR solutions for LASS1, LASS2, LASS4, LASS5, LASS6, SK-1, GCS genes.

Reaction Mixture	**LASS1, LASS2, LASS4, LASS5, LASS6, SK-1, GCS, β Actin**
PCR grade water	37.7
Reaction buffer (10x)	5
$MgCl_2$ (25 mM)	5
dNTP (10 mM)	4
Primer forward (25 pmol/µL)	1
Primer reverse (25 pmol/µL)	1
cDNA	1
Taq DNA Polymerase	0.3
Total Mixture	50

Table 2.4.Amplification conditions of LASS1, LASS2, LASS5, LASS6, SK-1, GCS, β Actin.

Steps	Temperature	Time
Initial Denaturatin	94 ˚C	5 min
Denaturation	94˚C	30 s
Annealing	55˚C	45 s
Extention	72˚C	1 min
Final Extention	72˚C	5 min

2.6.7. Statistical Analysis

All experiments were set up in triplicate and the results were expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using Student's *t*-test or ANOVA and $p<0.05$ was considered significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cell Proliferation Analysis on DU-145 and PC-3 cells

3.1.1. Cytotoxic Effects of Doc

Cytotoxic effects of Doc on DU-145 and PC-3 cells were examined by XTT cell proliferation assay. IC50 values of Doc were calculated from cell proliferation plots and were 30 nM and 38,4 nM in DU-145 (Figure 3.1) and PC-3 (Figure 3.2) cells exposed to increasing concentrations of Doc for 72 hours, respectively.

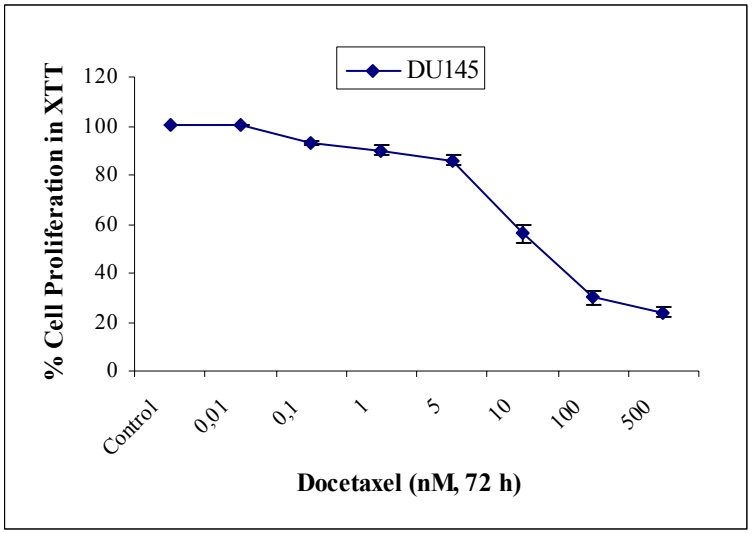

Figure 3.1. The cytotoxic effect of Doc on proliferation of DU-145 cells.

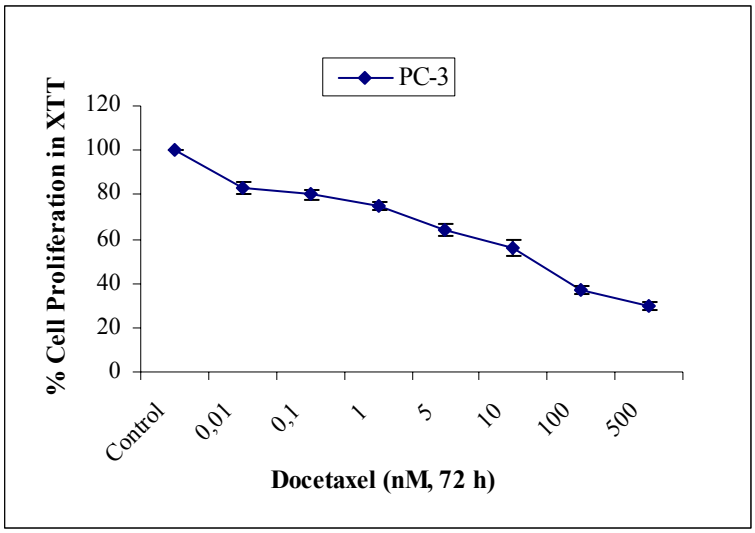

Figure 3.2. The cytotoxic effect of Doc on proliferation of PC-3 cells.

3.1.2. Cytotoxic Effects of PDMP

IC90 measures of both DU-145 (Figure 3.3) and PC-3 (Figure 3.4) cells were determined and found to be 100 µM and 85 µM respectively.

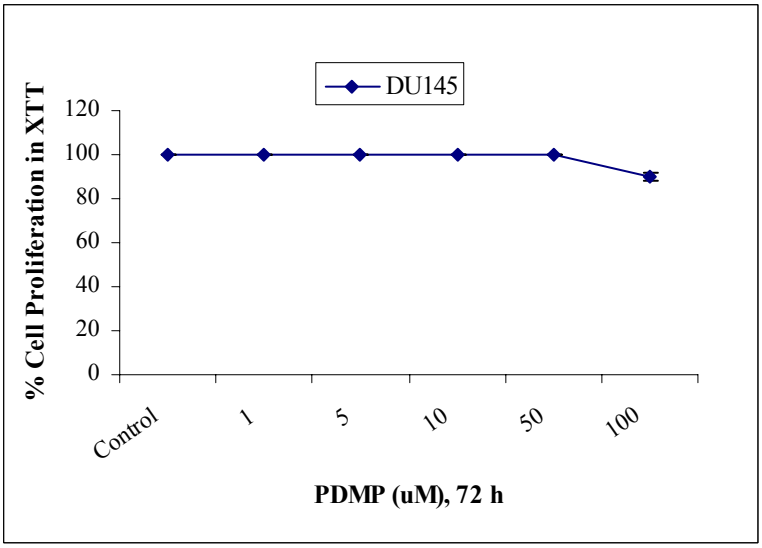

Figure 3.3. The cytotoxic effect of PDMP on proliferation of DU-145 cells.

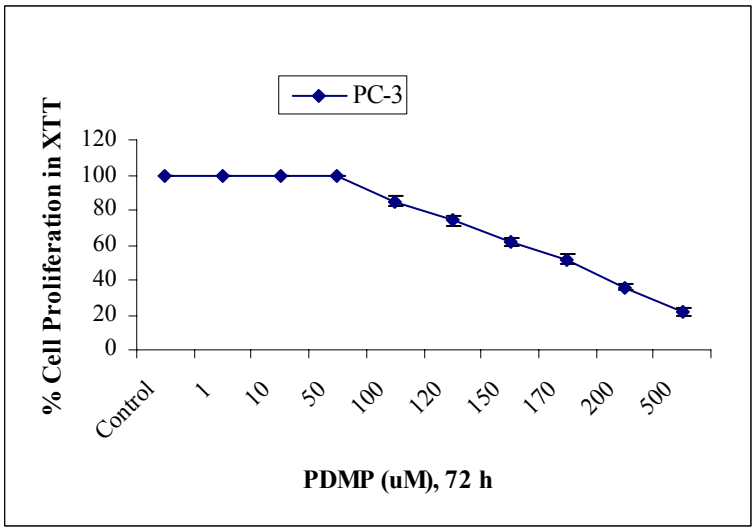

Figure 3.4. The cytotoxic effect of PDMP on proliferation of PC-3 cells.

3.1.3. Cytotoxic Effects of C:8 Ceramide

We have observed a dose dependent cytotoxic effects of C:8 ceramide on both DU-145 and PC-3 cells. IC50 values of C:8 ceramide were found as 33 µM and 35 µM in DU-145 (Figure 3.5) and PC-3 (Figure 3.6) cells exposed to increasing concentration of C:8 ceramide for 72 hours, respectively.

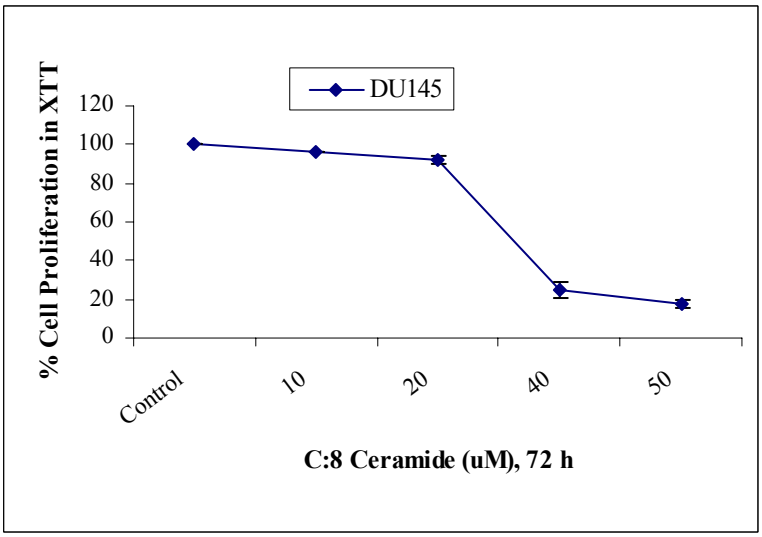

Figure 3.5. The cytotoxic effect of C:8 ceramide on proliferation of DU-145 cells.

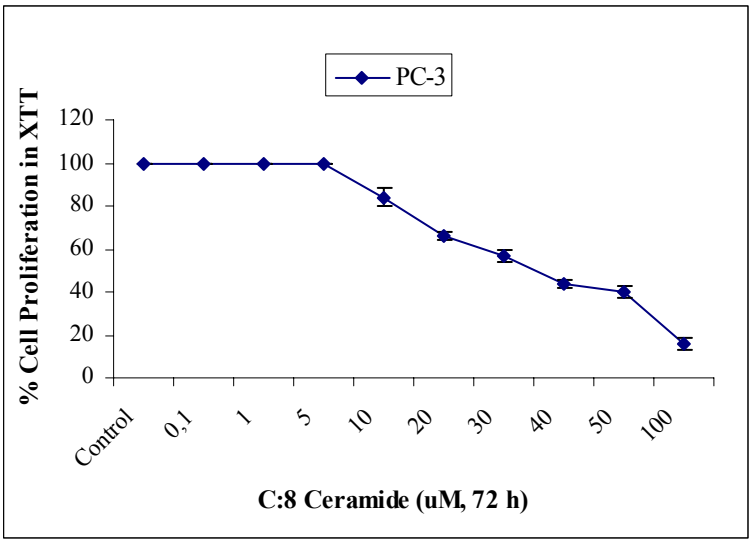

Figure 3.6. The cytotoxic effect of C:8 ceramide on proliferation of PC-3 cells.

3.1.4. Cytotoxic Effects of SK-1 inhibitor

The cytotoxic effects of SK-1 inhibitor also were found by using XTT assay. IC50 measurements of SK-1 inhibitor on DU-145 (Figure 3.7) and PC-3 (Figure 3.8) cells subsequent to 72 hours were found to be 32 and 92 µM respectively.

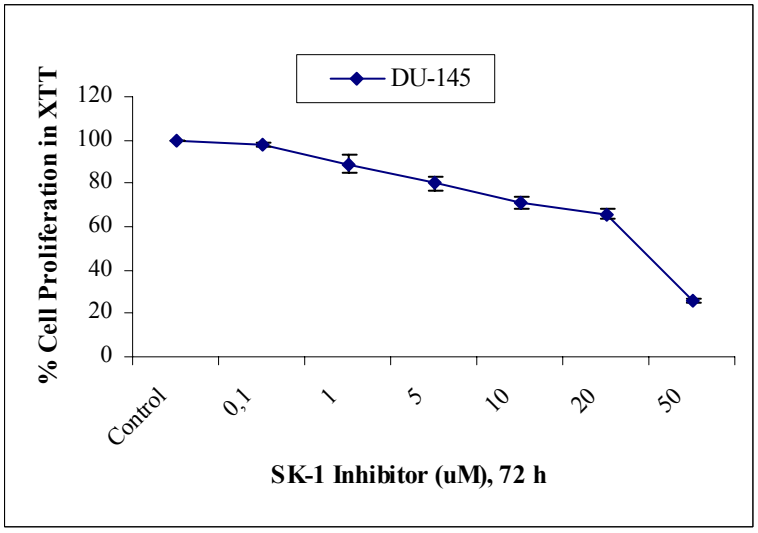

Figure 3.7. The cytotoxic effect of SK-1 inhibitor on proliferation of DU-145 cells.

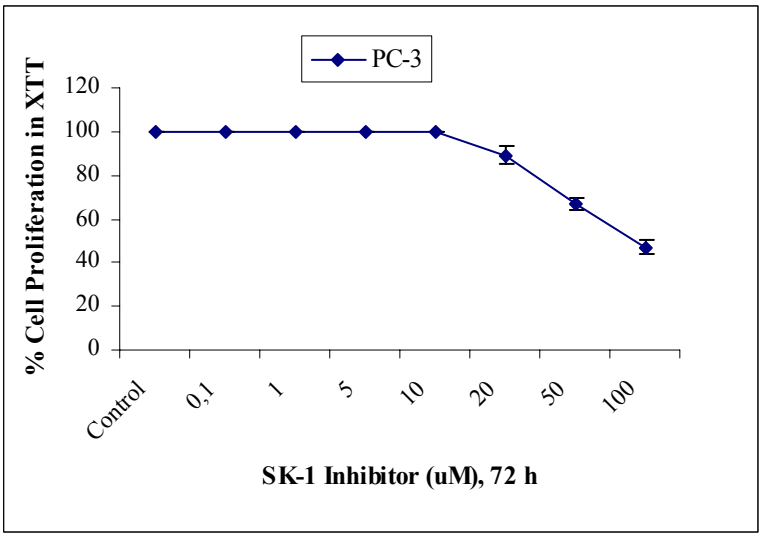

Figure 3.8. The cytotoxic effect of SK-1 inhibitor on proliferation of PC-3 cells.

3.1.5. Cytotoxic Effects of Combination of Doc/PDMP

To show possible synergistic cytotoxic effects of Doc/PDMP combination on prostate cancer cells, we applied increasing concentrations of Doc with 100 μM PDMP for DU-145 and 85 μM PDMP for PC-3 cells. There were 10-, 44-, 70% and 25-, 44-, 63% decreases in cell proliferation in 1-, 10-, 100 nM Doc exposed DU-145 and PC-3 cells for 72 hours, respectively. On the other hand, 1-, 10-, 50- nM Doc with a combination of 100 μM PDMP decreased cellular proliferation 13-, 73- and 89% in DU-145 cells (Figure 3.9). Likewise on PC-3, the same concentration of Doc with combination of 85 μM PDMP reduced cellular proliferation 73-, 74-, 74% (Figure 3.10).

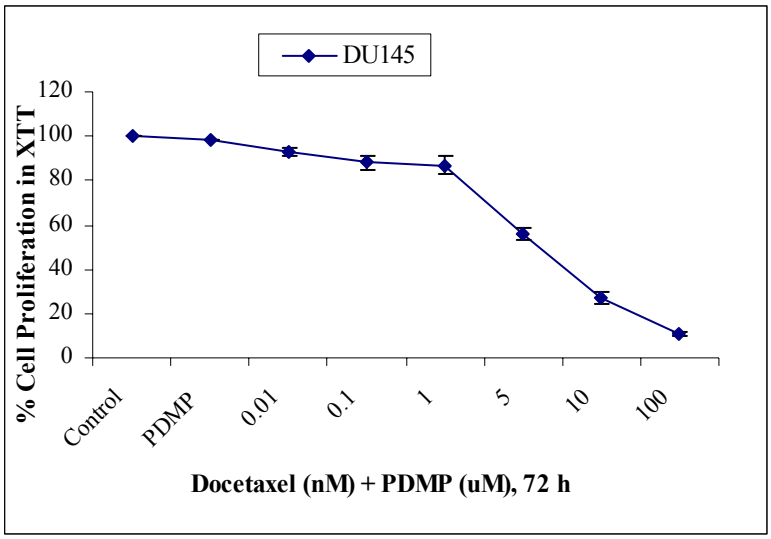

Figure 3.9. Antiproliferative effects of Doc and PDMP combination on DU-145 cells.

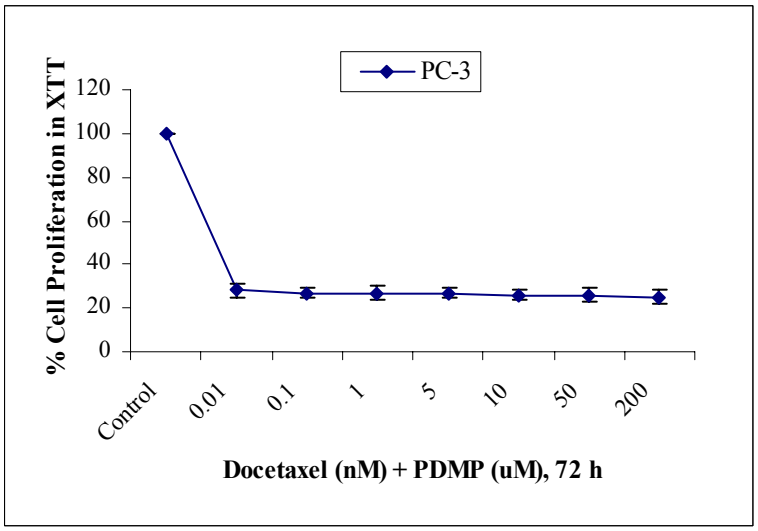

Figure 3.10. Antiproliferative effects of Doc and PDMP combination on PC-3 cells.

3.1.6. Cytotoxic Effects of Combination of Doc/C:8 Ceramide

While there were 10-, 44-, and 70% or 25-, 44-, and 63% decreases in cell proliferation in 1-, 10-, and 100 nM Doc treated DU-145 or PC-3 cells the same concentration of Doc with a combination of 33 and 35 μM C:8 ceramide decreased

cellular proliferation 78-, 80-, and 81% or 61-, 66-, and 69% in DU-145 (Figure 3.11) and PC-3 cells (Figure 3.12) in the same order.

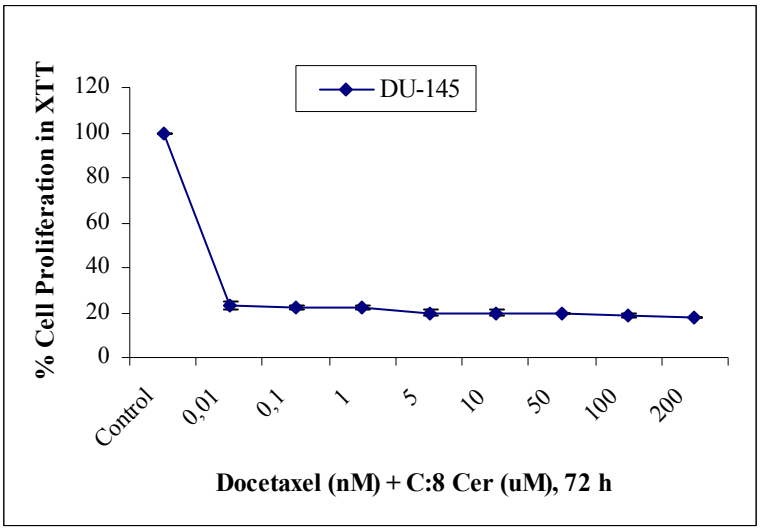

Figure 3.11. Antiproliferative effects of Doc and C:8 ceramide combination on DU-145 cells.

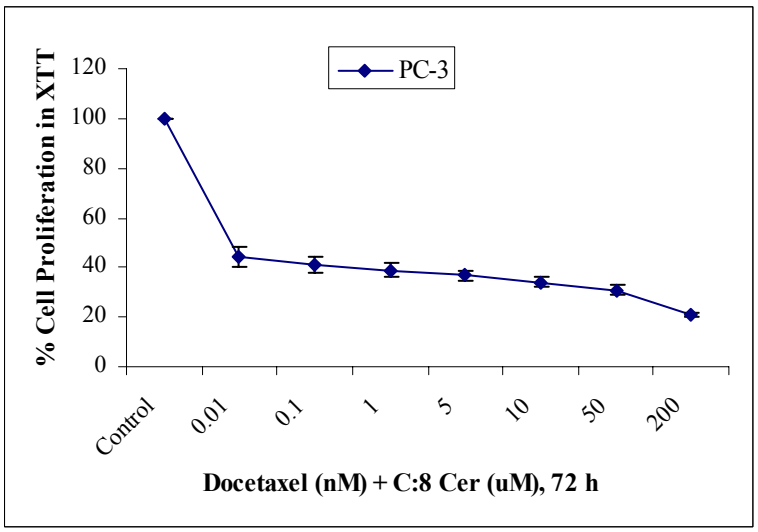

Figure 3.12. Antiproliferative effects of Doc and C:8 ceramide combination on PC-3 cells.

3.1.7. Cytotoxic Effects of Combination of Doc/SK-1 inhibitor

When same concentration of Doc and 32 µM SK-1 inhibitor were incubated simultaneously in culture for 72 h, there were 77-, 77- and 79% decreases in proliferation of DU-145 cells comparing to untreated controls (Figure 3.13). Likewise on PC-3 cells, 0,1-, 1-, 10 nM Doc with combination of 92 µM SK-1 inhibitor reduced cellular proliferation 74-, 75-, 76% (Figure 3.14) respectively.

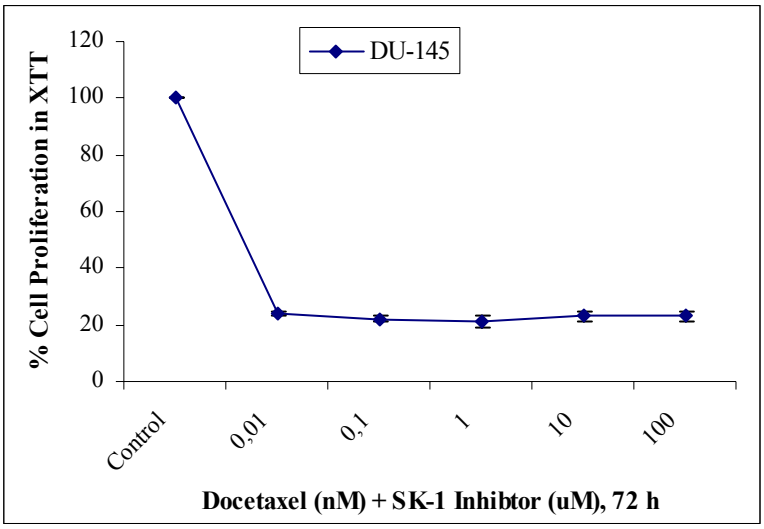

Figure 3.13. Antiproliferative effects of Doc and SK-1 inhibitor combination on DU145 cells.

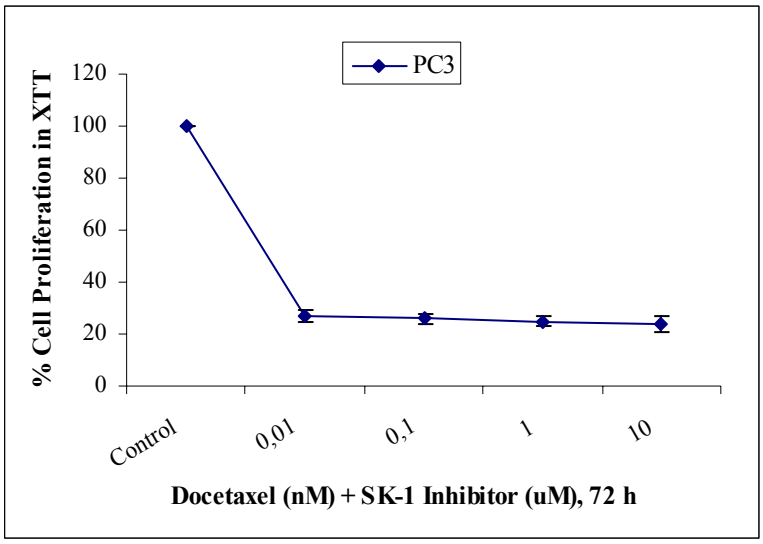

Figure 3.14. Antiproliferative effects of Doc and SK-1 inhibitor combination on PC-3 cells.

3.2. Evaluation of Apoptosis in DU-145 and PC-3 Cells

3.2.1. Synergistic Apoptotic Effects of Doc and PDMP

Treatment with Doc/PDMP combination caused a significant loss of mitochondrial membrane potential (MMP), as measured by increased accumulation of cytoplasmic/monomeric form of JC-1, in DU-145 and PC-3 cells as compared any agent alone.

There were 1.5- and 1.8-fold increase in cytoplasmic/monomeric JC-1 in 1- and 10 nM Doc applied DU-145 cells, respectively, while 100 μM PDMP decreased MMP 1.3 fold by itself. On the other hand, there were 1.8- and 2.6-fold increases in cytoplasmic/monomeric JC-1 in response to concentrations of Doc with 100 μM PDMP, respectively (Figure 3.15).

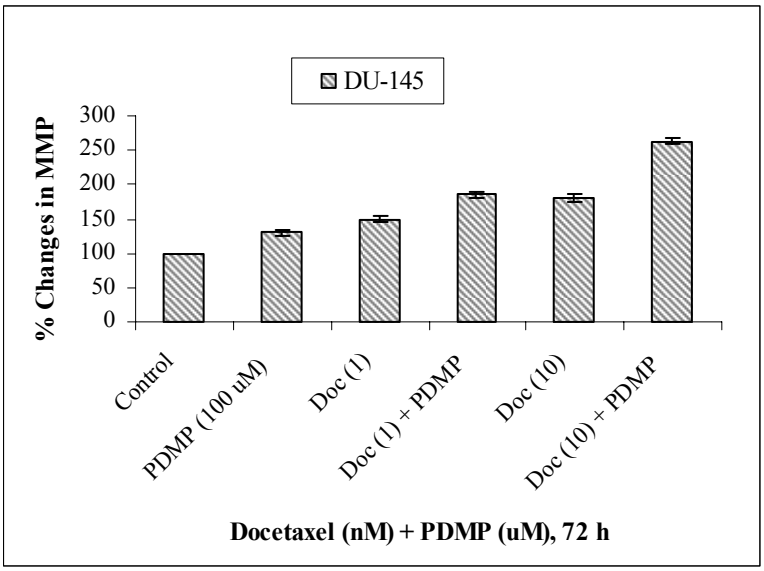

Figure 3.15. The changes in the MMP in DU-145 cells exposed to combination of Doc/PDMP.

In parallel with these results, there were 1.0- and 1.1-fold increase in cytoplasmic/monomeric JC-1 in 1- and 10 nM Doc applied PC-3 cells, respectively. As shown in Figure 3.16, the same concentration of Doc in combination with 85 μM PDMP increased the cytoplasmic/monomeric JC-1 1.6- and 1.7- fold, respectively, as compared to untreated controls.

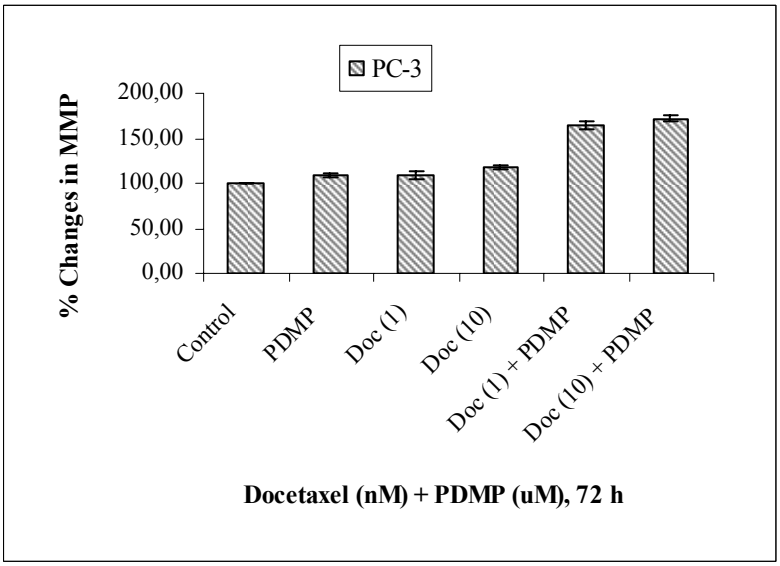

Figure 3.16. The changes in the MMP in PC-3 cells exposed to combination of Doc/PDMP.

In order to confirm these results, we determined the changes in caspase-3 enzyme activity in the same experimental setups. There were 1.1- and 1.3-fold increases in 1- and 10 nM Doc alone applied DU-145 cells, respectively, while 100 µM PDMP did not change the caspase-3 enzyme activity as compared to untreated controls. Doc in combination with PDMP with same concentrations increased the enzyme activity 1.4- and 2.0-fold on DU-145cells, respectively (Figure 3.17).

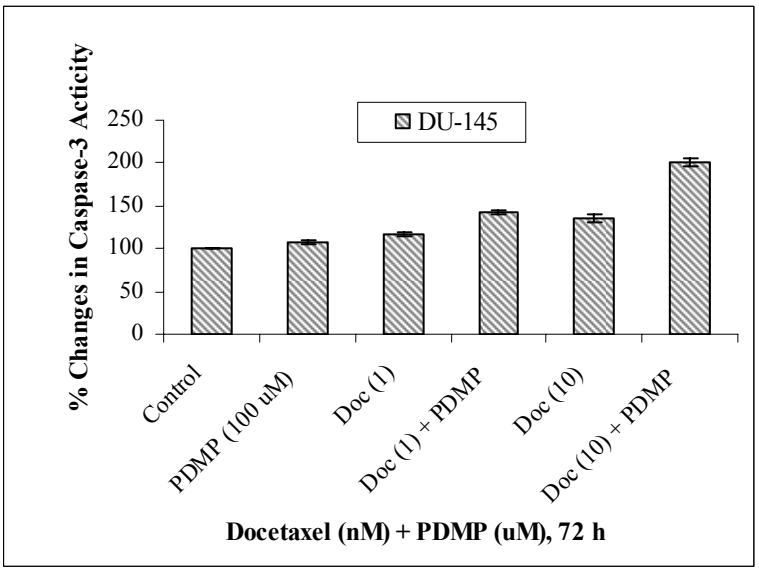

Figure 3.17. The changes in caspase-3 enzyme activity in the Doc/PDMP applied DU-145 cells.

In addition, the same concentration, of Doc were applied to PC-3 cells and 1.6- and 2.4-fold increase observed in caspase-3 enzyme activity. Caspase-3 enzyme activity was 1.2-fold increased in response to only 85 µM PDMP application. Finally, significant synergistic results were observed on 1- and 10 nM Doc in combination with 85 µM PDMP applied cells and the enzyme activity was raised 2.0- and 6.5-fold, respectively (Figure 3.18).

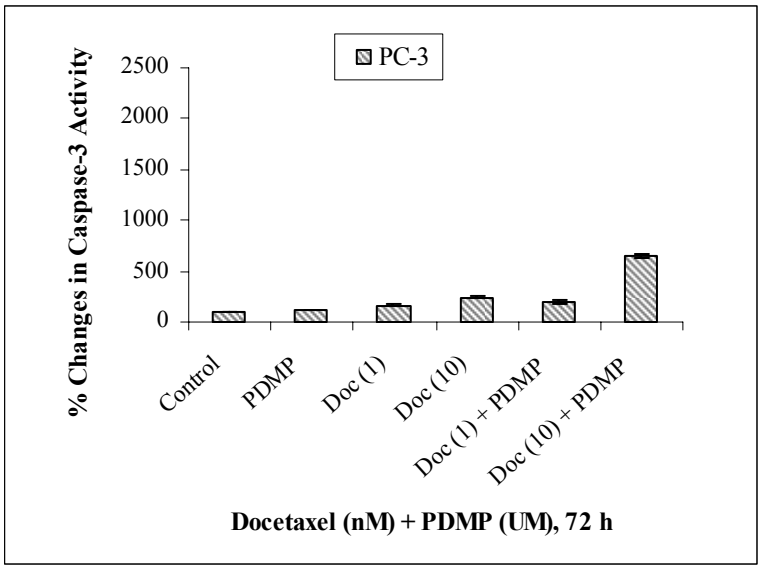

Figure 3.18. The changes in caspase-3 enzyme activity in Doc/PDMP applied PC-3 cells.

3.2.2. Synergistic Apoptotic Effects of Doc and C:8 Ceramide

The combination of Doc and C:8 ceramide showed important increases in cytoplasmic/monomeric JC-1 in prostate cancer cells. There were 1.1- and 1.2-fold increase in cytoplasmic/monomeric JC-1 in 1- and 10 nM Doc applied DU-145 cells, respectively. While the application of 33 μM C:8 ceramide increased cytoplasmic/monomeric JC-1, 1.4 fold by itself, the same concentrations of Doc combination with 33 μM ceramide increased cytoplasmic/monomeric JC-1, 2.5- and 2.7-fold, respectively (Figure 3.19).

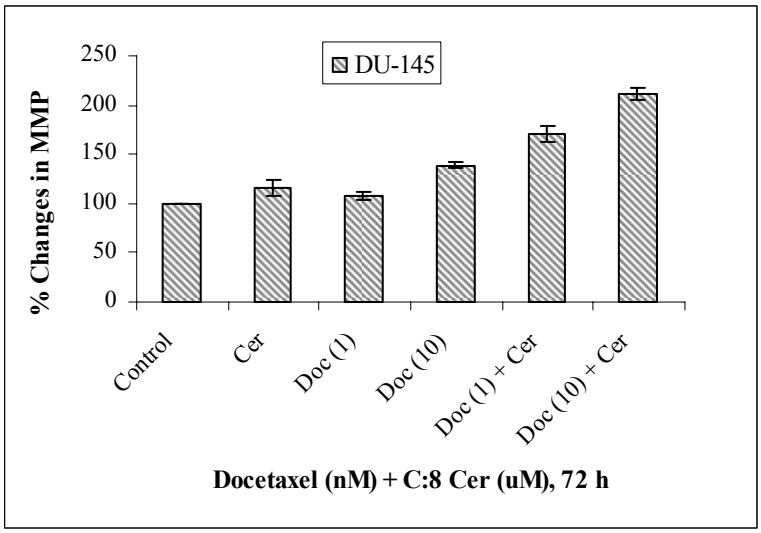

Figure 3.19. The changes in the MMP in DU-145 cells exposed to combination of Doc/C:8 ceramide.

Likewise, 1.0- and 1.1-fold increases in cytoplasmic/monomeric JC-1 were found in response to 1- and 10 nM Doc on PC-3 cells. While 35 μM C:8 ceramide increased cytoplasmic/monomeric JC-1, 1.2 fold by itself, there were 2.0- and 2.3-fold increases in cytoplasmic/monomeric JC-1 in PC-3 cells treated with combination of Doc and 35 μM C:8 ceramide (Figure 3.20) respectively.

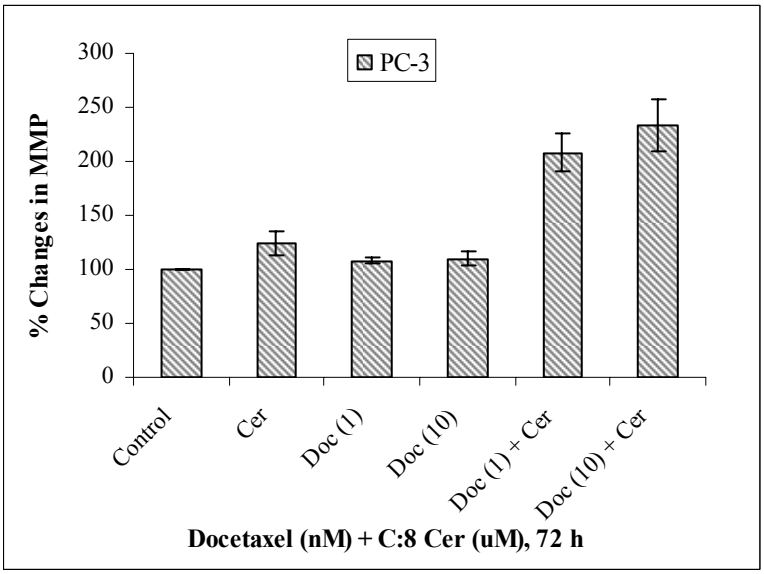

Figure 3.20. The changes in the MMP in PC-3 cells exposed to combination of Doc/C:8 ceramide combination.

In parallel with MMP results, caspase-3 enzyme activity analyses showed that there were 1.0- and 1.3-fold increases in DU-145 cells as they treated with 1- and 10 nM Doc, respectively. 33 μM C:8 ceramide were only increased the caspase-3 enzyme activity 1.1-fold. But Doc in combination with 33 μM C:8 ceramide increased the enzyme activity 1.7- and 2.1-fold (Figure 3.21) respectively.

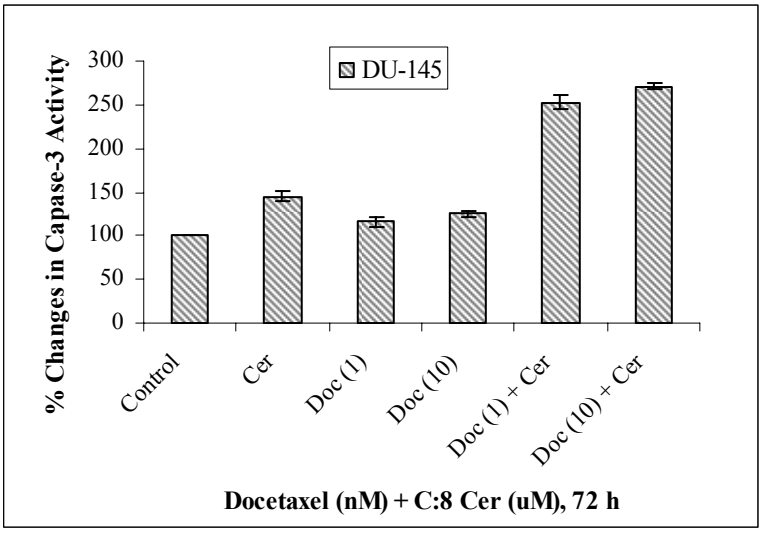

Figure 3.21. The changes in caspase-3 enzyme activity in the Doc/C:8 ceramide applied DU-145 cells.

Furthermore, caspase-3 enzyme activity analyses presented that there were 1.1- and 1.3-fold increment in 1- and 10 nM Doc alone applied PC-3 cells, by the same order. Treatmet of PC-3 cells with Doc and 35 μM C:8 ceramide (35 μM) resulted in significant activation of caspase-3 (about 3.4- and 3.8-fold, respectively), whereas treatment with only C:8 ceramide at same concentration enhanced the caspase-3 enzyme activity 1.9-fold (Figure 3.22).

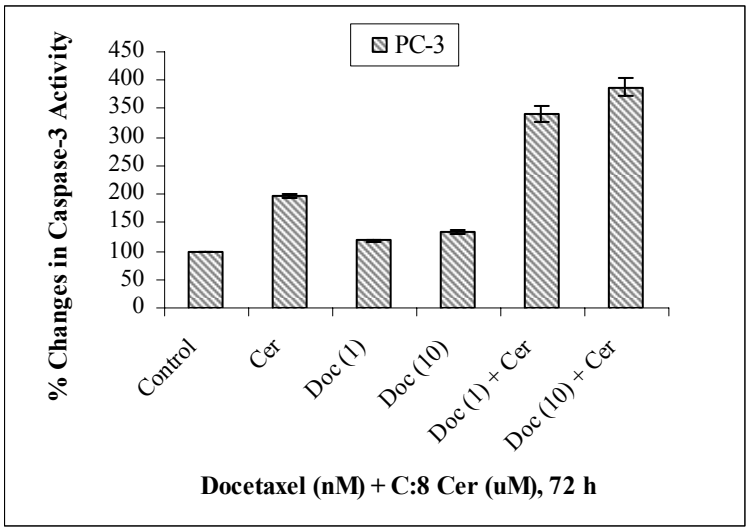

Figure 3.22. The changes in caspase-3 enzyme activity in the Doc/C:8 ceramide applied PC-3 cells.

3.2.3. Synergistic Apoptotic Effects of Doc and SK-1 Inhibitor

Increments of cytoplasmic/monomeric JC-1 in 1- and 10 nM Doc applied DU-145 cells were found as 1.0- and 1.2-fold from previous data. Treatment of DU-145 cells with 32 μM SK-1 inhibitor in the presence of 1- and 10 nM Doc also showed significant increases in cytoplasmic/monomeric JC-1 as 21.3- and 22.3-fold, respectively, compared to SK-1 inhibitor itself which was 15.3 fold (Figure 3.23).

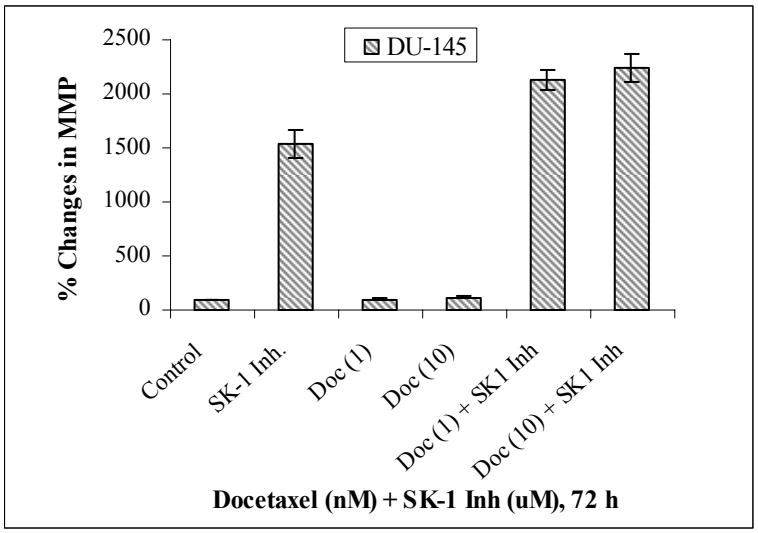

Figure 3.23. The changes in the MMP in DU-145 cells exposed to combination of Doc/SK-1 inhibitor.

To show effects of Doc and SK-1 inhibitor on MMP, we also detected changes in cytoplasmic/monomeric form of JC-1, on PC-3 cells. There were 1.0- and 1.3-fold increase in cytoplasmic/monomeric JC-1 in 1- and 10 nM Doc applied PC-3 cells, respectively. While 92 μM SK-1 inhibitor inceased cytoplasmic/monomeric form of JC-1, 20.0 fold by itself. On the other hand, the same concentrations of Doc with 92 μM SK-1 inhibitor increased cytoplasmic/monomeric JC-1, 29.0- and 30.0-fold, respectively (Figure 3.24).

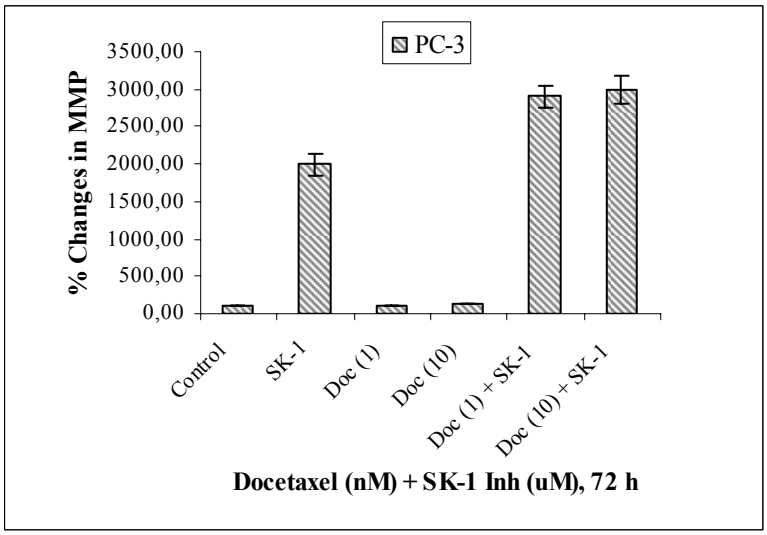

Figure 3.24. The changes in the MMP in PC-3 cells exposed to combination of Doc/SK-1 inhibitor.

Caspase-3 enzyme activity was also determined in DU-145 cells exposed to 1- and 10 nM Doc or 32 μM SK-1 inhibitor. Results showed that there was around 1.0- and 1.1-fold increase in 1- and 10 nM Doc or 1.5-fold increase in 32 μM SK-1 applied DU-145 cells. On the other hand, the same concentrations of Doc in combination SK-1 inhibitor increased the enzyme activity 2.4- and 3.0-fold (Figure 3.25), respectively.

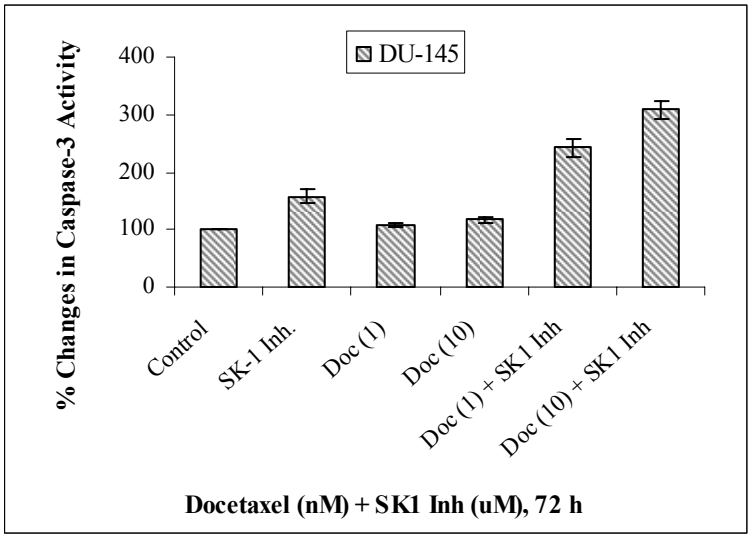

Figure 3.25. The changes in caspase-3 enzyme activity in the Doc/SK-1 inhibitor applied DU-145 cells.

Similarly, raise on caspase-3 enzyme activity was also demonstrated on PC-3 cells. Caspase-3 enzyme activity analyses showed that there were 1.3- and 1.4-fold increase in 1- and 10 nM Doc alone applied PC-3 cells, respectively. The same concentrations of Doc in combination with 92 μM SK-1 inhibitor increased the enzyme activity 2.3- and 2.4-fold, respectively by itself to untreated controls. SK-1 inhibitor increased the caspase-3 enzyme activity only 1.3-fold (Figure 3.26).

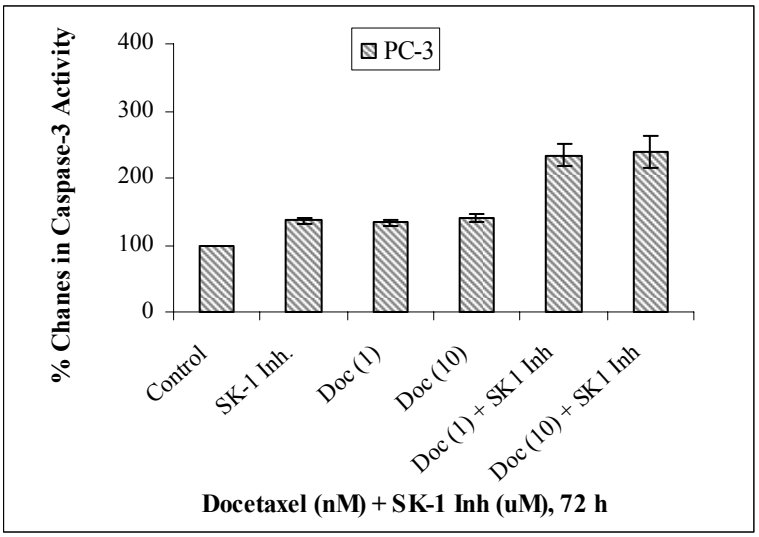

Figure 3.26. The changes in caspase-3 enzyme activity in the Doc/SK-1 inhibitor applied PC-3 cells.

3.3. Evaluation of PCR results

We treated androgen independent DU-145 and PC-3 cells with increasing concentrations of Doc as 0,1-, 1-, 10- nM for 72 h to determine if Doc somehow effects expression levels of GCS, SK-1 and LASS genes (1, 2, 4, 5, 6). There were significant decreases in expression levels of GCS in response to elevation of Doc as compared to untreated controls and of course internal positive control, β actin (Figure 3.27.).

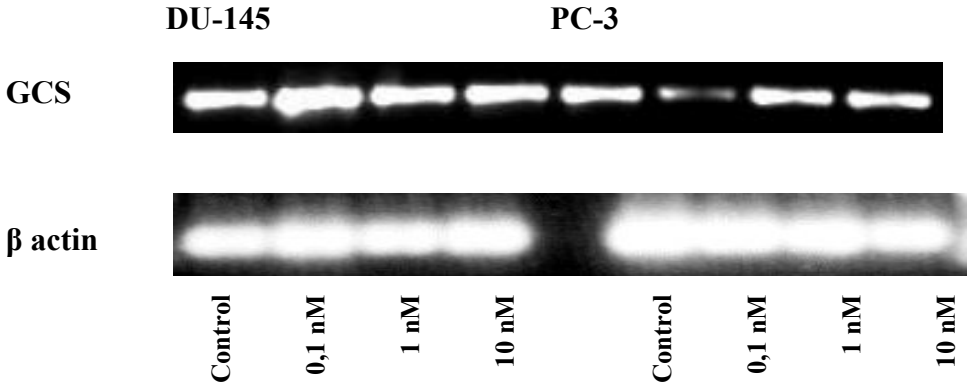

Figure 3.27. The expression levels of GCS in Doc treated DU145 and PC-3 cells.

The mRNA levels of SK-1 gene in Doc applied DU-145 and PC-3 cells by PCR. We compared expression levels of Doc applied cells with untreated controls and

normalized their levels to β actin levels. There were significant decreases in expression levels of SK-1 gene in both DU-145 and PC-3 cells in response to increasing concentration of Doc in a dose dependent manner (Figure 3.28.).

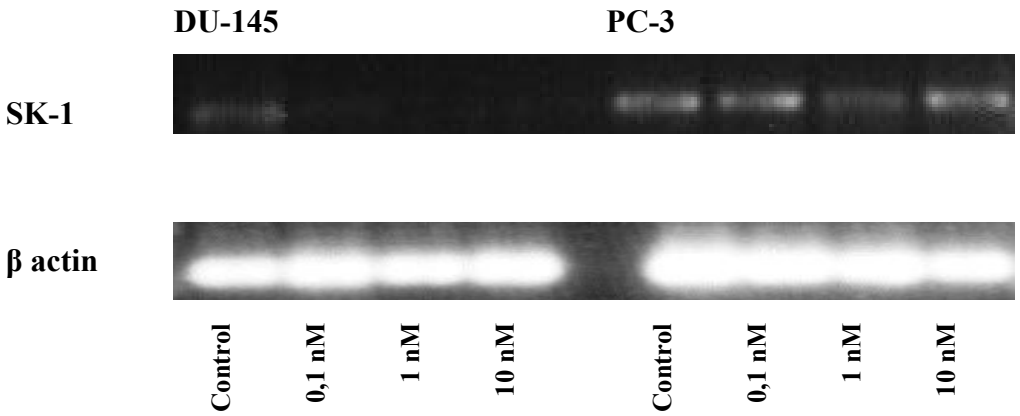

Figure 3.28. The expression levels of SK-1 in Doc treated DU145 and PC-3 cells

The expression levels of LASS1, -2, -4, -5, and -6 were determined by PCR in DU-145 and PC-3 cells exposed to 0,1-, 1-, 10- nM Doc for 72 h. PCR results revealed that there were significant increases in LASS1 gene expressions in dose-dependent manner as compared to untreated controls and normalized to β actin levels. On the other hand, LASS4 and LASS5 mRNA levels were declined when increased level of Doc concentration were applied in PC-3 cells. However there were increases in mRNA levels of LASS4 besides LASS5 expressions remain same in PC-3 cells. Thus, LASS2 and LASS6 mRNA expressions have similar preferences and did not show any such increase in DU-145 but there were only weak increase in PC-3 cells in mRNA expressions of LASS2. However, there were not significant differences in response to increased doc concentration. All LASS mRNA expression results were compared with untreated controls and normalized to β actin. In summary, LASS family mRNA expressions each have distinct preferences for DU-145 and PC-3 cells, although with some overlap (Figure 3.29).

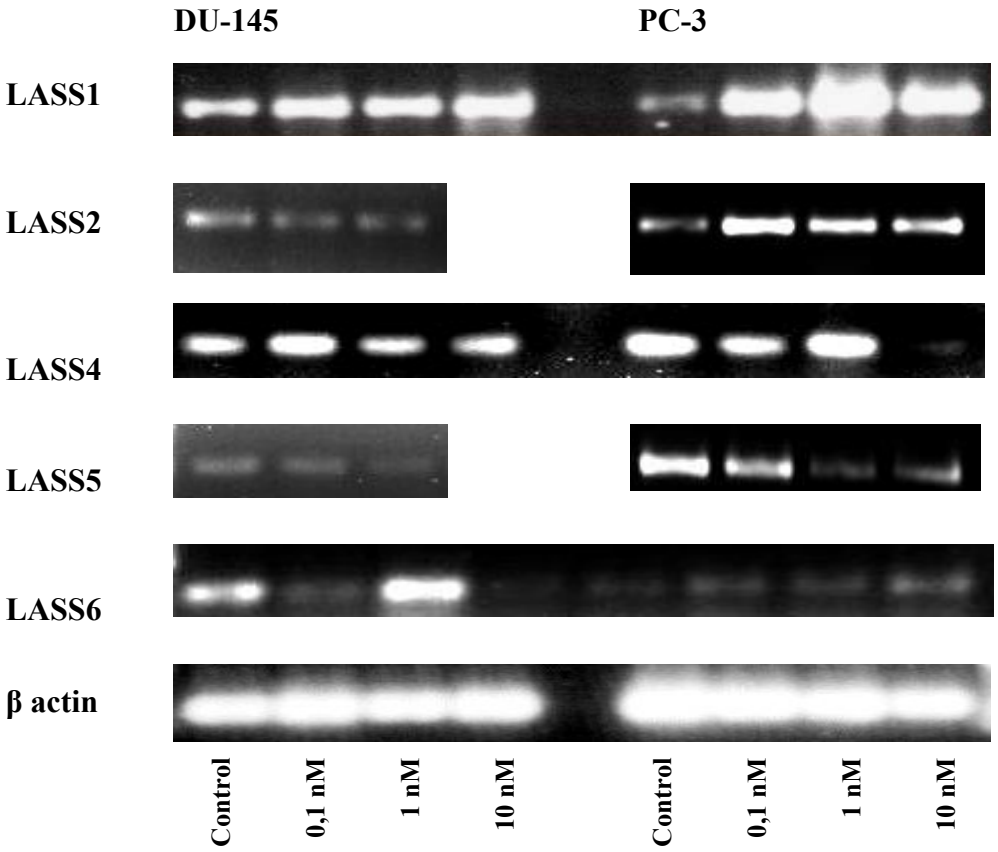

Figure 3.29. The expression levels of LASS 1, 2, 4, 5, 6 in Doc treated DU145 and PC-3 cells.

CHAPTER 4

CONCLUSION

For a long time, androgen dependence and insensitivity were the main issues in prostate cancer research. It is expected to reveal insights into the molecular mechanisms of carcinogenesis to aid prevention. As ceramide mediates antiproliferative responses, S1P and GlcCer enhance several tumor-promoting responces, strategies that either mimic/antagonize these sphingolipids or modulate their levels could provide a novel avenue for cancer therapy. Similarly, modulating these pathways might enhance drug action, overcome drug resistance, protect normal cells from cytotoxic actions, and even create new strategies for chemoprevention. Taken together, mounting evidence points to close connections between cytotoxic agents and ceramide metabolism.

Bioactive sphingolipid ceramide involves in mediating anti-proliferative responses via various distinct mechanisms in human cancer cells. It has been well documented that treatment with some anticancer drug, results in increased generation and/or accumulation of endogenous ceramide either via the activation of the *de novo* pathway, or by increased activity of sphingomyelinases (Baran, et al. 2007).

The way for treatment of cancer more than one agent is known as combination therapy. The main aim of the combination therapy is to get more effective result with at least two agents than any agent alone. For this purpose, several groups demonstrated that targeting the combination therapy increases the effect of one agent and it appears a very good alternative way for the treatment of cancer to protect some tissues from side effects of cancer therapy (Gore and Hermes-DeSantis 2008, Hind, et al. 2008). As known from the studies, chemotherapeutic agents induce apoptosis and they cause accumulation of ceramide during apoptosis in various cell types. In different cancer cells, arrangement of ceramide levels may enhance the effectiveness of some cancer therapies. From previous studies, it is known that ceramide usage with other anti-cancer agents importantly increased the apoptotic activity and therapeutic effectiveness in multidrug resistant tumor models. Therefore, Desai and friends exhibited that short chain ceramide coadministration with paclitaxel can important degree grow therapeutic efficacy in human ovarian adenocarcinoma cells and tumor models (Desai, et al. 2007).

To show enhancement of intracellular ceramide levels, many methods are used as exogenous delivery of cell permeable ceramide, and inhibition of ceramide metabolism by affecting GCS and SK-1 inhibitor.

It has been well documented that low ceramide levels or with a defect in ceramide generation leads cancer cells more resistant to apoptosis (Macchia, et al. 2003). Numbers of studies by using exogenous cell-permeable analogs of ceramide indicate evidence of their role in apoptosis (Akao, et al. 2002). Studies previously demonstrated that short-chain analog of ceramide is preferable since it has more water soluble characteristics than long-chain ceramides in experiments with cells in culture (Gomez-Munoz, et al. 2006). Short chain ceramide analogs are shown to be caused G0-G1 cell-cycle arrest in most cancer cell lines. Develapally and friends recently showed that coadministration of ceramide with paclitaxel significantly enhanced apoptosis in both sensitive and multidrug resistant ovarian cancer cells (Develapally, et al. 2007).

In order to potentiate the cytotoxicity of drugs, GCS inhibitor could be used for elevation of ceramide levels in various cancer cells (Reynolds 2004). Moreover in several cancer cells including in multidrug resistant melanoma, and resistant epidermoid and breast carcinoma cells, GlcCer was found and it is also believed that decreased the ceramide-mediated apoptotic signals, involving drug resistance (Modrak, et al. 2006, Ogretmen 2006). Thus, it was also shown that anti-androgen therapy in prostate cancer may accelerate ceramide levels by reducing the GCS levels (Radin, et al. 2003).

Sphingosine kinase plays a significant role for the balance of sphingolipid rheostat, by transforming sphingosine to pro-survival S1P (Segui, et al. 2006). Other previous studies have showed that SK-1 expression is up-regulated in colon carcinogenesis and in the tumors of the patients with lung cancer. More importantly, it has been demonstrated previously that modulation of S1P levels increases apoptosis and perturbations in the metabolism of ceramide via overexpression of SK-1 may play important roles in the development of resistance to anticancer agents (Baran, et al. 2007). Cyclooxygenase-2 expression function of SK-1 was also examined in colon carcinogenesis by Kawamori and co-workers and was found potential role of SK-1 to induction of carcinogenesis (Kawamori, et al. 2006). Recently Le Scolan et al. reported that up-regulation of the expression of the sphingosine kinase gene was found associated with a leukemic process *in vivo* (Le Scolan, et al. 2005). Therefore Leroux and friends recently reported that in the study with unspecific SK inhibitor, DMPS, treated PC-3 cells which are enforced BCL-2 expression was not observed apoptotic

resistance (Leroux, et al. 2007). Another studies showed that besides its antitumor activity, SK inhibitors have identified as in the absence of toxicity to the animal *in vivo* (French, et al. 2003). As for the sphingolipid rheostat which is known as balance of S1P, ceramide is able to act as a sensitizer of tumor cell apoptosis induced by taxol (Huwiler and Zangemeister-Wittke 2007). The similar effects were shown by Pchejetski and friends established that in prostate cancer cells SK-1 inhibition correlates with Doc and camptothecin sensitivity (Pchejetski, et al. 2008).

Several studies regarding ceramide-mediated apoptotic signaling have been reported. However, the mechanisms of ceramide-induced transcriptional regulation are less known. This study is the first to show that ceramide induces Doc with different agents which have an important role on ceramide biochemical pathways such as PDMP, ceramide analogue, C:8 ceramide, and SK-1 inhibitor. Therefore expression and the subsequent activation of LASS, GCS, and SK-1 genes were observed involved in Doc induced cell apoptosis.

Doc is commonly used anticancer agent in prostate cancer patients. Their combination therapy was tried with GCS and SK-1 inhibitors and ceramide analog C:8 ceramide. The signals inducing apoptosis could be blocked with S1P which mediate many important cellular processes including growth, survival, differentiation, angiogenesis and known proliferative effect on prostate cancer cells. In addition to its role as a mediator of cell death, and alterations in ceramide metabolism whereby pro-apoptotic ceramide is converted to the antiapoptotic GlcCer and S1P have been found in a number of cancer cells and also related with resistance mechanism. In this manner, we used SK-1 inhibitor which specifically inhibits SK-1 and decreases intracellular concentrations of S1P. It is generally assumed that GCS inhibitors exert their chemosensitizing effect by potentiation of drug-induced intracellular ceramide elevation. Chemosensitization effects of PDMP, GCS inhibitor, and exogenously applied C:8 ceramide which provide synthesis or accumulation of ceramides were used in combination of Doc in DU-145 and PC-3 cells.

In the first part of study, the roles and the mechanisms of action of ceramide metabolism in the regulation of Doc-induced cell death was investigated. PDMP/Doc, C:8 ceramide/Doc, and SK-1 inhibitor/Doc combinations enhanced apoptotic effect remarkably and found strongly synergistic. Cell viability and apoptotic assays also showed that PDMP, SK-1 inhibitor and C:8 ceramide in combination with Doc resulted in higher numbers of cells in apoptosis as compared to only Doc exposed cells. These

findings demonstrated that the ability to modulate ceramide metabolism might provide a new avenue by which drug sensitivity can be increased in androgen independent prostate cancer cells.

At the second part of the investigation, to evaluate the role of ceramide metabolizing genes and their products in the apoptotic process in human prostate cancer cell lines, DU-145 and PC-3, treated with Doc. The data presented here showed in Doc induced apoptosis in androgen independent prostate cancer cells that the perturbations of the balance between proapoptotic ceramide and anti-apoptotic S1P, with opposing functions respectively, by downregulation of SK-1, and increased level of LASS1, play an important role in the regulation of mechanisms underlying on apoptosis. To examine the potential role of LASS/SK-1 on sphingolipid rheostat, in Doc induced apoptosis, first, the mRNA levels of LASS1, LASS2, LASS4, LASS5, LASS6, SK-1 and GCS were measured in DU-145 and PC-3 cells exposed to increasing concentrations of Doc compared with untreated controls. The additional strong support related our results that the ceramide increase due to *de novo* synthesis through LASS1 gene in a dose-dependent manner as compared untreated cells. The data are also in agreement with the study showed that weak increase in LASS2 and LASS6 but inverse effect as a decrease in LASS4 and LASS5 genes on DU-145 and PC-3 cells. On the other hand, decreased expressions levels of SK-1 and GCS genes in response to Doc lead us to suggest that the sphingolipid rheostat changes on the behalf of ceramide accumulation via applied increased concentration Doc in androgen independent prostate cancer cells. It may offer a novel therapy that utilizes Doc combined with ceramide and/or agents affect ceramide metabolism in prostate cancer patients.

In summary, our studies suggest that it has been long recognized that these findings hold major biological implications to tumor progression and therapy. In this manner, we hypothesize that exogenous ceramide, PDMP, a strong GCS inhibitor and, SK-1 inhibitor administration in a combination with Doc may induce apoptosis more than any agent alone. Targeting ceramide metabolism by increasing its synthesis and/or modulating its metabolism might provide improved strategies for the treatment of prostate cancer which has a high rate of incidence and has been accepted as incurable when it is androgen independent. Mounting evidence indicates a role for ceramide not only in tumor survival but also in tumor migration and invasion. Revealed novel molecular mechanisms of Doc with ceramide will bring new understandings and approaches to androgen independent prostate cancer therapies.

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