A NOVEL CHEMOTHERAPEUTIC DRUG COMBINATION FOR PROSTATE CANCER

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ABSTRACT

A NOVEL CHEMOTHERAPEUTIC DRUG COMBINATION FOR PROSTATE CANCER

Prostate cancer as a multistep and complicated cancer type is regulated by androgens at the cellular level and remains as the second most common cause of death among men. The discovery of new chemotherapeutics through the development of novel agents which enable cell death rapidly without exerting serious toxic effects to healthy tissues might alter the depressing aspects of chemotherapy.

In the current study, anti-cancer activity of a novel heterodinuclear copper(II)Mn(II) complex (Schiff base) and its P85 combination were evaluated by cell proliferation analysis, gene and protein expression assays, invasion experiments and antimicrobial analysis *in vitro*; reflecting the detailed molecular mechanisms, potential inhibitory role on metastasis and anti-inflammatory action. In order to assign maximum tolerated dose, toxicology analysis were performed on C57/B16 mice by determining blood counts, enzyme activities and histopathological examination of multiple organs. Tramp-C1 model was used to discover anti-tumor activity through the tumor volume measurements and Gleason score analysis *in vivo*.

Overall, a remarkable anti-cancer activity was observed for Schiff base-P85 combination *in vitro* and *in vivo* for prostate cancer; however, a set of experiments are ongoing to elucidate exact mechanism at the molecular and physiological level. This study is the first one in the literature which represents the anti-cancer activity of a novel Schiff base derivative synthysized by our group and its P85 combination on prostate cancer.

ÖZET

PROSTAT KANSERİ İÇİN YENİ BİR KEMOTERAPİK İLAÇ KOMBİNASYONU

Çok aşamalı ve karmaşık bir hastalık olan prostat kanseri, hücresel düzeyde androjenler tarafından kontrol edilmektedir ve erkek bireyler içinde ikinci en yaygın ölüm nedeni olma özelliğini korumaktadır. Sağlıklı dokulara ciddi zarar vermeden hızlı hücre ölümü sağlayabilen yeni kemoterapotiklerin keşfedilmesi kemoterapideki olumsuz tabloyu değiştirebilir.

Söz konusu çalışmada; detaylı moleküler mekanizmaları, metastas ve inflamasyonun durdurulmasını gösteren in vitro hücre proliferasyon analizleri, gen ve protein anlatım deneyleri, invazyon ve antimikrobiyal analizler yapılarak yeni bir heterodinüklear bakır (II)mangan(II) kompleksi ve P85 kombinasyonun antikanser etkisi değerlendirildi. Maksimum tolere edilebilecek dozu belirlemek için C57/B16 fareleri üzerinde kan hücre sayımları, enzim aktiviteleri ve organlarda histoloji değerlendirmeleri yapılarak toksikoloji analizleri tamamlandı. Antitümör aktivite, tümor hacim hesaplamaları ve Gleason skor analizleri ile Tramp-C1 prostat kanser modeli kullanılarak yapıldı.

Schiff bazı-P85 kombinasyonun moleküler ve fizyolojik düzeydeki kesin mekanizmasını tam olarak belirleyebilmek için çalışmalar devam etse de, söz konusu formülasyonun *in vitro* ve *in vivo* çalışmalar sonucunda son derece etkin bir antikanser aktivitesi olduğu gözlemlendi. Bu çalışma, grubumuz tarafından sentez edilen yeni bir Schiff bazı ve P85 kombinasyonun prostat kanseri üzerindeki antikanser aktivitesini gösteren literatürdeki ilk çalışmadır.

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5-FU	5-fluorouracil
AAH	Atypical adenomatous hyperplasia
ABC	Adenosine triphosphate-binding cassette
ACTH	Adrenocorticotropic hormone
ADT	Androgen deprivation therapy
Akt	Protein Kinase B
ALT	Alanine transaminase
AMACR	α-methylacyl-CoA racemase
AMPK	AMP-activated protein kinase
AR	Androgen receptors
AST	Aspartate transaminase
BAX	Bcl-2-associated X protein
BBMEC	Bovine brain microvascular endothelial cells
BCRP	Breast cancer resistance protein
BPH	Benign prostatic hyperplasia
CDK	Cyclin-dependent protein kinase
CMC	Critical micelle concentrations
CRPC	Castration resistant prostate cancer
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's Modified Eagle's Medium
E2F	Elongation factor 2
EBV	Epstein-Barr virus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Elk-1	ETS domain-containing protein
ErbB2	Erythroblastic leukemia viral oncogene homolog
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
GnRH	Gonadotropin-releasing hormone

GnRHA	Gonadotropin releasing hormone agonist
GSH	Glutathione
GSTP1	Glutathione S-transferase P1
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HER-2/neu	Human epidermal growth factor receptor–2
HGPIN	High grade prostatic intra-epithelial neoplasia
HIF1	Hypoxia inducible factor
HLB	Hydrophilic-lipophilic balance
HPV	Human papilloma viruse
HTLV	Human T-lymphotropic virus
IAD	Intermittent androgen deprivation therapy
KSHV	Kaposi's sarcoma-associated herpes-virus
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
MAP	Microtubule associated proteins
MBC	Minimum bactericidal concentration
MCV	Merkel cell polyomavirus
MDM2	Mouse double minute 2 homolog
MDR	Multidrug resistance
MFC	Minimum fungicidal concentration
MIC	Minimum inhibition concentration
MRP1	MDR-associated protein
MTD	Maximum tolerated dose
MTS	3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4 sulfo-phenyl)-2H-tetrazolium
NF-κB	Nuclear Factor kappa B
PEO	Polyethylene oxide
P-gp	P-glycoprotein
PI3K	Phosphoinositide 3-kinase
PIN	Prostatic intra-epithelial neoplasia
PPO	Polypropylene oxide
PSA	Penicillin/Streptomycin/Amphotericin

PSA	Prostate specific antigen
PSMA	Prostate–specific membrane antigen
PTCH	Patched receptor
PTEN	Phosphatase and Tensin Homolog
RB	Retinoblastoma
RNR	Ribonucleotide reductase
ROS	Reactive Oxygen Species
SEER	The Surveillance, Epidemiology, and End Results
SHBG	Sex hormone binding globulin
SHH	Sonic hedgehog
SNPs	Single nucleotide polymorphisms
TAE	Tris-Acetate-EDTA
TGF-β	Transforming growth factor –β
TSP-1	Thrombospondin-1
VEGF	Vascular Endothelial Growth Factor

1. INTRODUCTION

1.1. CANCER

Cancer as a multistep process refers to many diseases with uncontrolled cell division and invasion to the other tissues. Tumor cells are characterized by their abnormal proliferation potential and comprise a cell population diverse from normal cells [1]**.** These cells are able to spread to the other tissues of the body from primary site. Cancer is classified as a group of disease with different features. Cellular and molecular mechanism of each cancer has distinct features in detail. Although each cancer type has spesific properties, there are characterized hallmarks for most of cancers [2]. Six hallmarks of cancer were defined by Hanahan and Weinberg in 2000. In the year 2011 two recent ones were added to list named as'' reprogramming of energy metabolism and evading immune-destruction''. Other six well known characteristics of cancer can be classified as ''self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis'' [3-4]**.**

Cell division and homeostasis in normal tissue is strictly regulated by production and secretion of growth promoting signals which are able to activate cell growth and proliferation [3-4]. Altered growth signaling in cancer cells is required for uncontrolled cell division and can be obtained in many alternative ways. Cancer cells enable to generate growth signals in an autocrin manner or trigger normal cells at the surrounding tissue for production and release of several growth factors [5,3-4]. Overexpression of tyrosine kinase activity providing-growth factor receptors that are localized on the cell surface were found in tumor cells. Up-regulation of growth factor receptors provides the increased rates of response to the proliferation signals contributing the uncontrolled cell propagation. Instead of growth factor receptors, growth stimulating downstream pathways are also disregulated in cancer cells leading to unresponsiveness to apoptotic signals [3-4]. In addition to the altered growth stimuli regulation, cancer cells can block anti-growth signals. In order to maintain homeostasis in normal tissue, some types of anti-proliferative signals are produced. Cancer cells successfully evade growth suppressor with using different

mechanisms. Tumor suppressor genes as regulators of cell growth and proliferation are important for response to the antigrowth-signals. Cancer cells have deregulations at tumor suppressor gene pathways (Retinoblastoma and P53) and therefore able to evade death stimulators**.** Increased growth stimuli are not the only reason for high proliferation rate of cancer cells. Apoptosis as a key barrier mechanism for uncontrolled cell division is disrupted in cancer cells. Cancer cells activate or de-activate several mechanisms to evade apoptosis and promote tumorigenesis subsequently [3-4]. Downstream and upstream regulators of apoptotic pathway can be differentiated in cancer cells. Cancer initiation and progression depend on apoptotic pathways and several genes involved in the process [6].
Alterations in cell signaling pathways that regulate growth and cellular metabolism are necessary for carcinogenesis but they are not enough to create a tumor tissue including cells with unlimited life span and division capacity. Each cell has an intrinsic mechanism named as telomerase activity for regulation of cell life span [7]. Telomere shortening, which drives many of the events that are related with cell senescence leading to cell death, is overcome in cancer cells. Telomere maintenance is required for all cancer cells to conserve their replicative potential and neoplastic state [3-4]**.**

Oxygen and nutrient requirement is indispensible for normal tissue growth and homeostasis. Increased metabolic activity leading to hypoxia was observed during tumorigenesis [8]. Therefore angiogenesis is a clear need for tumor vasculature as normal tissues. Development of adult vasculature starts during embryogenesis and comes to a quiescent state excepting some pathological conditions such as inflammation, atherosclerosis, wound healing or cancer [9,4]. The angiogenic switch mechanism depends not only the inducers (growth factors mainly VEGF or FGF) [3]. but also on the inhibitor molecules (thrombospondin-1-TSP-1) [10-11]. Tumor development process is tightly related with new blood vessel formation and angiogenesis procedure. Normal cells preserve their stable location in the body and are not able to migrate to the other sites. Cancer is a disease characterized by metastasis from the original site to the other parts of the body. This trait is referred as the invasion and metastasis capability of cancer. Cancer cells should alter cell to cell and cell to extracellular matrix interactions to migrate other tissues. Loss of E-cadherin and increased levels of N-cadherin are observed during invasion procedure. Metastasis and invasion procedure requires several proteases and starts with the local invasion to the surrounding tissue. Cancer cells transfer to the blood stream

and lymph vessels (lymphoangiogenesis) followed by infiltration into the new tissue [3-4,12]. Hallmarks of cancer also refer to the properties related with events occurring during tumorigenesis process. Instead of these characteristics genomic instability of cancer cells inflammation are also important for cancer cells to survive. Cancer cells should reregulate their energy metabolism such as enhancing the glucose uptake or glycolysis rate to provide a replicative and proliferative advantage [8]. Another important issue for cancer promotion is the suppression of immune response. Immune system is the most sufficient factor for limiting the tumor formation and growth. Therefore as a new hallmark evading immune response is need to be further explained [4].

General traits of cancer are similar for almost all types of malignancies but classification depends on different parameters. Cancer stage or grade can be used for classification especially for diagnosis and treatment. The origin and histological area of tissue can be used for classification. Cancers originated from epithelial cells are termed as carcinomas. The term sarcoma is used to refer mesenchymal originated cancers. Glandular epithelium derived cancers are classified as adenocarcinoma [2,13,1].

As a big group of diseases and multistage process cancer may have several possible reasons. Causes of cancer can be examined at the cellular, metabolic or molecular levels. The most well known reason for cancer is the exposure to the chemical carcinogens. Carcinogenic chemicals are converted to ultimate carcinogens to react with target molecules in the cells with the exception of alkylating agents. After proper metabolic activation (such as Cytochrome P-450–Mediated activation) they bind to the macromolecules and start carcinogenic procedure. Carcinogens exert their effect commonly by inducing the DNA adduct formation leading to mutations at oncogenes and tumor suppressor genes [14-15]. Acquired mutations activating cellular oncogenes (Ras) and inactivating tumor suppressor genes (p53) is the reason for many type of cancer [13]. In addition to DNA adduct formation, epigenetic changes including methylation and acetylation occur during carcinogenesis induced by chemical agents [13]. Hypermethylation of the promoter regions associated with tumor suppressor genes is a common epigenetic change observed in carcinogenesis [16]. In the case of cancer initiation chemical induced carcinogenesis and UV radiation include same mechanisms. Damage to the DNA activates the repair mechanisms that generally end up with mistakes causing

inheritable mutations. The result is the malignant transformation leading to the tumorigenesis [17-18,13]. Reactive oxygen species (ROS) as the result of almost all metabolic reactions and aging are well characterized within the context of potent carcinogens. Damage to the DNA, proteins or membranes describes the role of ROS in carcinogenesis[19,13]. Apart from chemical carcinogens or radiation, viral infection is regarded to be a reason for human cancers. Viruses cause the carcinogenesis by directly affecting the genome or by indirectly activating cellular machinery (for example the proliferation state) leading to cancer [13]. There are seven viruses identified to have role in cancer process: Epstein-Barr virus (EBV), Human papilloma viruses (HPV), Kaposi's sarcoma-associated herpes-virus (KSHV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human T-lymphotropic virus (HTLV), Merkel cell polyomavirus (MCV) [20].

Cancer as a complicated and multi-step process is appeared by the accumulatin of mutations, delayed or failed DNA repair mechanisms and acquisition of a malignant phenotype. Cancer cells gain new features such as genetic instability and cellular changes associated with behavior or interactions that enable them to proliferate rapidly [21,13]. In order to acquire a malignant phenotype several biochemical events occur at the cellular level. Transformation process leads to obtain an infinite life span and unlimited dividing capacity. Malignant transformation is mediated by several growth factors in an autocrine or paracrine mode that are involved in intracellular or intercellular connections. These communications are necessary for cell proliferation and the aggressiveness of cancerous tissue. Presence of growth factors is required for an active cell division state (mitosis). The decision for entering the cell cycle or stay at the quiescent state is regulated by several factors including oncogenes and their protein products [22]. Transmission into the S phase of cell cycle is regulated by cyclin-dependent protein kinases (CDKs) and their inhibitors in mammalian system. Instead of oncogenes, tumor suppressor genes (retinoblastoma-Rb and p53) are also important for cancer cell cycle regulation. For example inactivation of Rb gene is observed in many malignancies. Similar to the Rb gene, the tumor suppressor p53 is also necessary for cell cyle check-point control. Mutations of p53 hinder the cell cycle arrest which allow DNA repair [23].

1.1.1. Oncogenes and Tumor Supressor Genes

When considered as a multistep and complex process carcinogenesis occurs at the end of the biochemical, physical or genetic alterations [24]. On the basis of molecular genetics of cancer there are two subset of gene regulating the tumor formation and carcinogenesis. In cancer cells activation of the oncogenes and suppression of the tumor suppressors are the key molecular mechanisms for cancer initiation and promotion [25]. According to the somatic mutation hypothesis, specific mutations at genetic information initiates the carcinogenesis [26]. If oncogenes are mutated by chromosomal translocations and gene amplifications at critical gene loci, they can be activated. In contrast to oncogenes, tumor suppressor genes are inactivated by genetic changes such as missense mutations, mutations affecting the protein product or epigenetic alterations [27]. In normal physiological situations cells give response to the growth stimulatory signals from surrounding cells and environment. Oncogenes and their protein products enable cells to proliferate even in the absence of growth stimulation [25]. Such an example to this idea is the ras oncogene which is found to be frequently mutated in human tumors. Growth factor binding to the receptor activates the Ras protein in normal situations. However the mutated gene product acts completely in an opposite way and stimulated without growth factor [28]. This gene pathway leads the uncontrolled cell proliferation which is crucial for carcinogenesis.

The other groups of genes affecting the tumorigenesis process are the tumor suppressor genes which are able to protect cells by growth suppression. Loss of function mutations are able to cause cancer when observed in tumor suppressor genes. According to the two-hit hypothesis both alleles of tumor suppressor genes generally should be disrupted [29]. In haploinsufficiency situations even though one allele was mutated and inactivated, the other one is functional [30]. Tumor suppressor genes can be classified as gatekeepers and caretakers [31]. Caretakers protect cells by preventing the generation mutations by different mechanisms such as inhibiting the DNA damage or providing the DNA repair properly. On the other hand gatekeepers act on the cells by triggering the apoptotic pathway or inducing the cellular senescence [32]. The most well-known two tumor suppressor genes are RB (Retinoblastoma) and TP53 which are classified as gatekeepers [33]. These are not only affected tumor suppressor gene pathways but, most of the cancers have altered Rb and p53 gene pathways [27].

p53 gene is one of the most important tumor suppressor gene and it's protein product as a transcription factor, programmes cells to the death in the presence of stimulators [34-36]. Instead of p53's transcription factor activity, the gene is involved in many cellular processes such as DNA synthesis, repair mechanisms and apoptosis [37]. Mutations of p53 in cancers can be observed during the early or late stages of carcinogenesis [38].
The other important tumor suppressor gene is the Rb found as the reason of childhood tumor retinoblastoma [39]. Retinoblastoma has both hereditary and sporadic forms. Both copies of the gene should be inactivated for cancer formation [25]. However the Rb gene is not only involved in retinoblastoma process, but also regulates many other cancer types. The protein is important for cell cycle regulation, cellular differentiation and cell survival. Moreover it has been considered that Rb regulation of carcinogenesis is highly associated with cell type and the pathway that is controlled by Rb. Rb tumor suppressor gene is very important for cancer because the pathway comprises a complicated network which regulates many cellular processes [40].

In addition to the oncogenes and tumor suppressor genes, another class of genes are ''stability genes''. These types of genes are associated with DNA repair mechanisms and help to fix harmful effects of DNA damage [41,27]. A cell bearing a mutated gene product of tumor suppressor gene lose the ability to give response to the growth inhibitory signals as in the example of transforming growth factor –β (TGF-β) [25]. TGF-β as a negative regulator of cell growth inhibits proliferation in low concentrations [42]. In the case of mutated retinoblastoma (Rb) gene, cells may not give the right response to the TGF-β and continue to grow [43].

1.1.2. Cancer Initiation, Promotion and Progression

As it is known that tumorigenesis is a multistep process, tumor initiation and progression are occurred by different mechanisms which are classified into three categories; initiation, promotion and progression [44,13]. The initiation and promotion phases of the carcinogenesis process require agents which have either initiating or promoting activity [13]. If cellular damage aroused at the initiation step, it becomes permanent, proliferated cells start to expand and enter to the progression step. Genetic alterations and instability that are supported by chromosomal translocations and mutations are the characteristic

features of progression phase [45]. Uncontrolled cell growth and division, invasiveness to the surrounding tissue and metastatic capability of cancer cells are the results of genetic changes [13]. The initiation phase is a short period of time caused by an agent, however promotion of carcinogenesis is no as slow as the initiation phase and exposure of the agent should be long [13]. Cell proliferation occurs at the promotion phase and clonal cells bearing the damage enter to the progression phase and tumor formation occurs. Although the carcinogenic process has three main steps, molecular mechanism of carcinogenesis and the molecular alterations are complicated [24]. Initiation phase starts with mutations leading to genetic change by directly affecting the DNA or indirectly causing epigenetic changes [46]. At this point the important thing is the period that molecular events occur. Initiation and progression phases require genetic changes but promotion phase does not include the genetic changes. In this phase, promoting agents such as environmental factors, wound formation or inflammation stimulate tumorigenesis [47,13]. Therefore the progression phase is the cell proliferation step for carcinogenesis. The last step of the process is a long time period and involves the differentiation, invasion and metastasis to the other tissues [13]. In this phase, proliferated cells should gain adaptations to the hard conditions like hypoxia or acidic environment and increase their invasiveness to the other tissues [48]. High rate of cell growth and proliferation are advantages for cancer cells but intact basement membrane restricts the blood supply which creates hypoxia [46]. Cells need to metastasize to other tissues to complete tumor formation. A fully completed tumor with all structural components and genetic alterations is able to invade to the other tissues with high mortality.

1.1.3. Metabolism of Cancer

Apart from characteristic alterations in cell cycle regulation, gene expression or mutational profile of cancer cells, metabolic regulation has been the aim of interest as a biochemical marker in recent years. Cancer cells prefer to use glucose and are prone to utilize glycolytic pathway under normal oxygen conditions. The metabolic situation is called as Warburg effect [49]. ATP is required for normal cellular functions and biochemical events but cancer cells distinct from normal cells with their demand for ATP to provide rapid cell proliferation. Moreover cancer cells are able to block checkpoints which are activated under the hard metabolic situations observed during tumor formation and progression [49].

Whether the metabolic changes are only observed in cancer cells or just required for rapid cell growth and proliferation is not fully elucidated [8]. Different genes including tumor suppressor genes and oncogenes have roles in the regulation of altered metabolic pathways in human malignancies. Akt (Protein Kinase B) which is activated by PI3K (Phosphoinositide 3-kinase) is responsible for increased glucose uptake and glycolysis [50,49]**.** Cell growth and homeostasis are complicated events conducted by several processes such as lipid biosynthesis [51]. Lipids are not only utilized as energy supplies but also required for maintanence of cell growth by serving as functional molecules for biosynthesis and signaling pathways. Although there is not a clear relationship between cancer and lipid synthesis, lipid production is necessary for cell growth and structural integrity. Akt is known to stimulate lipid production leading to cell growth instead of enhancing the glycolytic pathway [52]. Another PI3K downstream element HIF1 (Hypoxia inducible factor) is also involved in glucose metabolism by enhancing the glucose transport and conversion [53]. Although HIF1 is known to be increased under hypoxia, cancer cells are able to increase HIF 1 levels dramatically under normal oxygen conditions to enhance glucose metabolism [49]. As a cellular checkpoint AMP-activated protein kinase (AMPK) is activated in the case of energy stress and block cell proliferation by arousing the oxidative metabolism that is restricting the cell proliferation rate [54]. In addition to its role in apoptosis and DNA damage, p53 is determined to have impact on glucose metabolism in cancer cells. Some glycolytic pathway enzymes and intermediate molecules are synthesized by p53 activation and stimulate the energy metabolism. Therefore suppression of the p53 in cancers may lead to switch to the glycolytic phenotype [49].

1.1.4. Cancer Diagnosis

Cancer diagnosis is very important for surveillance of the patient. Diagnosis techniques used should identify the disease at the right stage and time for efficient treatment. Cancer tissue and normal tissue are different at biological, metabolic, genetic or phenotypic level. These differences comprise the basis of diagnosis.

Several diagnosis techniques are used for cancer identification. Genomic, proteomic, epigenetic analysis, biomarkers and electro-magnetic systems are used for diagnosis of different cancers [13]. DNA based markers are associated with mutations, DNA

hypermethylation or viral DNA detection. Antibody-based techniques, enzymatic analysis and microarray studies are also used for cancer diagnosis [55]. Microscopic evaluation of tissue samples is the oldest method for cancer diagnosis. Although the cancer cell phenotype is different from the normal cells and microscopic analyses give result about the staging of the cancer, the result is a predictable biological assessment. Serum markers specific to the certain cancer types are also used for cancer diagnosis. The problem with serum markers is the inadequacy of the number. It is necessary to use more than one marker for proper diagnosis [56].

In this case development of more specific markers for cancer diagnosis has been the aim of interest such as DNA microarrays. DNA microarray analysis can give idea about the gene expression patterns of individuals which is beneficial for improvement of treatment strategies for each individual [57]. The classical diagnosis methods are generally histological analysis and serum markers. The emerging demand for new molecular based technologies is because of the unsensitive classical methods [55]. For example prostate specific antigen (PSA) is used for prostate cancer screening as a molecular marker [58]. However high level of PSA is not always an indicator for prostate cancer. Therefore patient sometimes can take unnecessary treatment because of wrong diagnosis.

1.1.5. Cancer Epidemiology

The observation of the cancer as a worldwide disease, vigorous incidence of cancer is the consequence of several factors incluiding the nation, country, human populations, daily habits (diet, smoking, alcohol uptake) and the sex. There are several factors affecting the epidemiology of cancer and different type of cancers. Exposure to the cancer causing agents affects the incidence of different cancers as an environmental factor. Genetic susceptibility is another crucial factor for cancer epidemiology that determines the predisposition for some of the specific types of cancers. Aggregation of some specific types of cancers in the family is the result of genetic factors. The average age of the population is an effective factor for cancer incidence as in the example of prostate cancer [13]. Sex also determines the chance of cancer incidence. Lung cancer is the most common cancer observed in man population but breast cancer frequency is high in women population [59]. Another example is associated with the development state of the country.

Stomach and uterine cervix cancers are common in developing countries, colorectal and prostate cancers are common in developed countries. As a multistep and wide variety disease, the reason for some types of cancers is not known. This complicated situation makes the epidemiology analysis more difficult. For example although it is not the only reason, smoking is known to cause lung cancer [60]. Epidemiologic analysis is required to identify the risc factors affecfing the cancer initiation which may help to prevent cancer.

1.2. PROSTATE CANCER

Prostate cancer can be defined as the cancer that occurs in the prostate gland. The prostate is an exocrine gland localized in the pelvis and surrounded by rectum and bladder [61]. It has a secretion necessary for sperm motility. Prostate gland is a branching organ with ducts. It has epithelial and basal cells which are able to secrete PSA and prostatic acid phosphatase [62]. The stroma part of the gland contains fibroblast cells, muscles, nerves and lymphatic vessels. The stroma part of the prostate produces different growth factors that are important in prostate carcinogenesis [63].

In the prostate tissue there are three main cell types that are distinguishing each other with their phenotypic and functional characteristics. Androgen dependent luminal cells are the predominant type and secrete the prostatic proteins. Other cell type is the basal cells found on the basement membrane. These cells do not produce the prostatic proteins necessary for secretions [64]. The third type of cell is the androgen-independent neuroendocrine cells. These cells are found rather small amounts in the prostate gland. In the aggressive type of prostate cancers high amounts of neuroendocrine cell differentiation was observed [65].

In the conditions of benign enlargement of the prostate, epithelial tissue and fibrous tissue of the prostate gland start to grow which creates the prostatic cancer [66]. Almost all types of cancers have a similar development procedure including a series of genetic and biological events resulting in an invasive and metastatic disease. However prostate carcinogenesis is not as same as other malignancies [67]. Prostatic intra-epithelial neoplasia (PIN) is accepted as an adenocarsinoma lesion characterized by intact basement membrane and differentiated phenotype. This type of neoplasm includes malignant cells and prostatic carcinomas may have these foci [68]. Prostatic carcinomas include high grade

prostatic intra-epithelial neoplasia which generally expand in the peripheral zone of the gland proves that this neoplasia is the precursor for prostate cancer. Proliferative inflammatory atrophy (PIA) is another type of lesion thought to be an initiator for PIN or prostatic carcinoma because of inflammatory phenotype that is also observed in prostate cancer [69-71]. Prostate cancer can arouse as more than one PIN lesion or can arouse in a prostate gland as a sole lesion [67]. Formation of the high grade PIN (HGPIN) leads the formation of carcinoma in approximately ten years of time [72]. In this step PIN lesion can be characterized with differentiated invasive cells (this situation is not observed in all types of PIN), altered expression of cancer markers such as reduced cadherin and vimentin expression. Moreover PIN lesions are different from benign prostatic hyperplasia (BPH) and atypical adenomatous hyperplasia (AAH) at the cytological level. These are not precursor lesions for prostate cancer. On the other hand PIN lesions do not produce and secrete PSA. Thus the biopsy analysis is the only way to detect these lesions. At the end of this initiation step loss of chromosome 8p [73] and NKX3.1 occurs [74]. For the chromosomal region 8p there two or three potential regions for looses [75]. While 8p12-21 losses are observed at the early stages of prostate cancer, 8p22 losses are identified at later stages because of its specificity to advanced prostate cancer [64]. Since NKX3.1 is a regulatory homebox gene in the prostate tissue [76], inactivation of the gene locus leads to cancer initiation. Losses of 10q and 8p chromosomal regions show compliance in prostate canrcinogenesis. While the loss of 8p choromosomal region is observed in PIN lesions indicating its role in early prostate carcinogenesis, the loss of 10q chromosomal region is observed in carcinoma [77]. Phosphatase and Tensin Homolog (PTEN) is a lipid phosphotase which is using the PIP-3 as substrate and activates the PKB/AKT pathway. Deletion of the PTEN gene is found to be involved in the several type of carcinogenesis such as glioblastoma, breast, endometrial cancers and prostate cancer [78]. Loss of PTEN is a late stage marker for prostate carcinogenesis as 60% of advanced prostatic lesions exert the deletion of PTEN [79]. Therefore PTEN inactivation occurs during prostate cancer progression as a key event. Retinoblastoma (Rb) gene is located at the 13q chromosomal region and regulates the cell proliferation. As a tumor suppressor gene Rb suppress the aberrant cell growth and proliferation [80]. Loss of Rb gene function or mutations are frequently observed in advanced prostate cancer [81]. As the prostate cancer proceeds to the advanced tumor stage, lots of genetic alteration occurs which lead the cell proliferation and inhibition of the apoptosis. In this case prostate carcinogenesis is thought

to be related with cell cycle regulatory genes and their function. p27kip1 is a CDK inhibitor was detected to be deleted in advanced prostate carcinoma [82]. p16 is another cell cycle controlling gene that is found to be mutated at the advanced stage of the metastatic prostate cancer [83]. p16 has been reported to have a dual role for prostate cancer progression. In addition to mutations or deletion observed in prostate cancer, upregulation of the p16 protein levels was observed in prostate cancer [84]. p16 gene alterations occur frequently in consistent with Rb gene pathway. Phosphorylated Rb triggers the release of elongation factor 2 (E2F) and induce the expression of E2F associated proteins (for example cyclin A) leading to by-pass of cellular senescence [85]. p16 and Rb pathway impairments are required to skip tumor suppressor control mechanisms that induce the tumorigenesis [86]. p16 prevents the phosphorylation of Rb by inhibiting the relation of cyclin D with cdk4 and cdk6 which is the key event for cell cycle blockage [87]. Progression of the prostate cancer from PIN stage to the invasive carcinoma is tightly regulated by aging and telomerase activity. Prevention of the telomere shortening is required for prostate cancer, like all other types of cancers. Telomerase activation generally occurs to inhibit cell senescence [88]. After PIN lesions become an invasive carcinoma and switch to the metastatic prostate cancer, additional gene alterations come about to cause cancer progression. One of the important altered regulation pathway observed at the advanced stages of prostate cancer is the androgen signaling pathway. Although the androgen deprivation therapy is used in the treatment because of clinical efficacy, tumors are able to change androgen dependent phenotypes at the advanced stages of the carcinoma [89]. Several possible mechanisms can be involved in androgen independency such as androgen receptor activity or function. Although p53 mutations are not frequently observed in prostate cancer, loss of the region on the 17p chromosome containing p53 can usually occurs [90]. In addition to the p53 mutations, as an anti-apoptotic gene Bcl2 is overexpressed in advanced prostate cancer particularly for hormone independent disease [91]. Therefore it can be considered that prostate cancer is a multistep disorder that requires many physiological changes.

1.2.1. The Endocrinology of Prostate Cancer

Prostate cancer is a multistep and complicated cancer type which is also related with hormone regulation. As a hormone responsive cancer type, the initiation and progression of

the prostate cancer is mainly regulated by androgen action at the cellular and molecular level. The basic of the prostate cancer treatment today depends on the castration therapy that was first shown in 1940's [92]. In order to regulate normal and cancerous prostate tissue function, androgens are required. Testosterone is the most important androgen which comprises the maximum amount of the androgens in the body [93]. Steroidogenesis in the leydig cells provides the major contribution to the prostate tissue function and organized by the luteinizing hormone (LH). However androgen synthesis in adrenal glands is regulated by the adrenocorticotropic hormone (ACTH) and in normal situation is not required for prostate function [94]. In the circulation system testosterone is generally found to be bound with sex hormone binding globulin (SHBG). In the prostate tissue testosterone is converted to 5α-dihydrotestosterone (DHT). Prostate tissue is androgen dependent and the consequence of this dependence is the affect of androgens in prostate cancer [95]. Development of the androgen independent prostate cancer is one of the big problems that occurs during the treatment of prostate cancer. There are some different mechanisms suggested for the mechanism of androgen independency. Accumulation of the mutations may lead to development of more resistant prostate cancer cells that are able to grow without androgens [96]. Androgen receptor amplification, enhancement of the sensitivity of androgen receptors, increasing androgen levels, androgen receptor mutations, growth factor related pathway genes and apoptotic pathway genes are regulatory elements and events that are crucial for the development of the androgen independent prostate cancer [95].

1.2.2. Molecular Biology of Prostate Cancer

As a histologically heterogenic malignancy, prostate cancer is complicated at the molecular level too. Although there is not an exact mechanism defined for prostate carcinogenesis, different pathways associated with prostate cancer were identified with molecular studies [71]. Prostate cancer is generally diagnosed by using Gleason score system [67]. Gleason score system is an accurate and acceptable system for determination of prostate carcinogenesis but in the case of localized and metastatic tumors, it is difficult to score both. Therefore identification of the more sensitive molecular pathways regulating prostate cancer may allow diagnosing malignancies at an early stage.

There are different pathways and several molecules which are known to affect prostate cancer initiation and progression.

1.2.2.1. Androgen Receptor

Androgens and their receptors are one of the most well known ones for prostate cancer [97]. High levels of androgen (for example testosterone) is considered to be a risk factor for prostate cancer, even though there is not an exact relation between cancer and serum levels of androgens [98-99]. Normal prostate tissue development and cancer progression is highly associated with androgens that affects on cells carrying androgen receptors (AR). ARs are important regulators of prostate cancer at the molecular level. AR overexpression, mutations and activation of genes related with AR are some of the mechanisms involved in prostate cancer progression [100]. AR in the normal cell cytoplasm is found to be bound with the heat shock protein Hsp90. AR is activated by androgens, dissociated from the heat shock protein and translocates to the nucleus to activate several genes associated with cell growth [67,101,97].

Although most of the prostate cancer tumors are androgen dependent and give response to the androgen deprivation, they become androgen independent finally. Prostate cells require AR for cell survival and growth even they became hormone refractory [102]. AR is involved in the appearance of androgen-refractory prostate cancer. AR function can be maintained by prostate cancer cells in the absence of androgens by using different mechanisms. Hormone refractory prostate cancer cells may increase their sensitivity to low levels of androgens by the overexpression of AR [71]. AR gene amplification is observed in hormone independent prostate cancers [103]. Additionally receptor activating mutations of AR was found in androgen refractory tumors in previous studies [104]. Therefore receptors can response to the nonandrogenic signals [70]. AR signaling pathway is related with other signaling pathways to restore androgen function that is necessary for disease progression. There are studies suggesting the overexpression of ErbB2 (Erythroblastic leukemia viral oncogene homolog/ human epidermal growth factor receptor–2) in prostate cancer while some others have controversial results [105]. The higher expression levels of HER-2/neu (human epidermal growth factor receptor–2) in hormone-independent prostate cancer were proved with later studies [106].

1.2.2.2. Growth Factors and Receptors

The role of growth factors and their membrane receptors has been shown to have role on the prostate cancer progression [63]. Growth factors of EGF family such as epidermal growth factor (EGF) and transforming growth factor (TGF) have been determined in prostate cancer cells and act in an autocrine and paracrine manner [107-108]. Epidermal growth factor receptor (EGFR) is most well known member of ErbB famiy which includes four receptors: EGFR (or ErbB-1); Her 2/neu (ErbB-2); Her 3 (ErbB-3); and Her 4 (ErbB-4) [109]. EGFR receptors are localized on the membrane as inactive monomers. Receptor activation occurs by binding to specific ligand to the receptor following the homo/heterodimerization of the receptor. Autophosphorylation of the tyrosine kinase domain leads the initiation of intracellular signaling pathways that are regulating the cell growth [108]. EGFR is important in the autocrine pathway regulation in prostate cancer cells and direct cell proliferation, apoptosis and invasion to the other tissues [108,110,97]. Although EGFR is overexpressed in primary and metastatic cells, overexpression of EGFR and its critical role in androgen independent prostate cancer has been shown with many studies [111].

1.2.2.3. Apoptotic Genes

Apoptosis at the molecular level in prostate cancer has defects as other type of cancers. Upregulation of Bcl-2 gene level in prostate cancer cells is one of the features for late stage cancer and aggressive behavior of prostate cancer [112]. Overexpression of Bcl2 in advanced prostatic cancer and hormone refractory prostate cancer [113] may be a useful approach for development of therapeutic strategies against prostate cancer. p53 as an apoptotic gene was also found to be mutated in prostate cancer [114]. p53 metabolic pathway is regulated by many molecules such as MDM2 (Mouse double minute 2 homolog). MDM2 is a negative regulator in the p53 pathway and direct the p53 to proteosomal degredeation [115]. MDM2 overexpression in prostate cancer was observed in previous studies [116].

1.2.2.4. Glutathione S-Transferase P1

Defects in the gene coding glutathione S-transferase P1 (GSTP1) enzyme is frequently observed in prostate cancer. These enzymes enable to conjugation of glutathione and carcinogenic chemicals which prevents the body against cancer. Decreased expression or

silence of the GSTP1 is observed in prostate cancer [117]. Hypermethylation of the CpG island in the promoter region was observed in almost all prostate cancer cases [118].

1.2.2.5. Phosphate and Tensin Homolog (PTEN)

PTEN is a tumor supressor gene which encodes a lipid phosphatase is generally deleted in prostate cancers. The lipid phosphatase is important for signaling pathways in the cellular metabolism and antagonist of the Akt pathway. Dephosphorylation of the PIP-3 inhibits the Akt pathway. Deletion of the PTEN activates the PKB/AKT kinase which prevents cell death and cause cell growth [119,64].

1.2.2.6. NK3 Transcription Factor Related, Locus 1

The chromosomal region containing homeobox gene NKX3.1 is frequently lost in prostate cancer. NK3 transcription factor related, locus 1 is not a tumor suppressor gene but the regulation is androgen dependent and necessary for normal prostate tissue and prostate cancer development [120]. As a transcriptional regulator NKX3.1 also have role in PSA gene expression [121].

1.2.2.7. Hedgehog Pathway

Sonic hedgehog (SHH) pathway was shown to be important in prostate cancer progression and specifically in metastasis. When SHH molecules bind to their receptors (patched receptor-PTCH), they prevent the inhibition of the G protein coupled membrane receptor smoothened which has a role for activation of tumor promoting genes. Inhibition of the hedgehog signaling affects the growth of prostate cancer [122,67]. SHH pathway have role after androgen independence for epithelial cells. Moreover researchers suggested that prostate stem cells which have active SHH pathway are involved in prostate carcinogenesis [123-124].

Although above listed are the main pathways and molecular systems regulating the prostate cancer progression, there are also several genes and genetic events that are thought to be related with prostate cancer.

1.2.3. Diagnosis of Prostate Cancer

Diagnosis of the prostate cancer by histological evaluation is difficult. Although there are some specific criteria (perineural invasion, glomerulations, and collagenous micronodules)

for histological analysis, diagnosis is completed by the assessment of cellular and structural parameters [125]. The needle biopsy does not cause tissue morbidity but the procedure is difficult and the amount of sample is too small. Identification of the malignant disease is succeeded by the morphological analysis of the tissue sample but the misleading result is the problem of biopsy [126]. Therefore new challenging methods and markers have improved for diagnosis of prostate cancer such as PSA. Measurement of PSA levels is common among other markers because of the potential for detection of the cancer at early stages and observation of the response to therapies [127]. PSA is a serine protease from glandular kallikrein related peptidases family which is produced in normal prostate tissue and prostate cancers [128-129]. The physiological action of the PSA is to liquefaction of the seminal secretion. It degrades the proteins such as semenogelin I and II in the seminal fluids to prevent gelation [130]. Androgen regulation of the PSA expression is carried out by the androgen response elements that are located at the promoter region of PSA gene [127]. PSA may be free or bound with serum proteins in the blood. Free or bound PSA can be used for diagnosis of prostate cancer [131]. PSA levels may also be found at high levels in some special conditions such as age. PSA increase after the age of 50 may be the reason for missing cases in older man [132]. PSA measurements are not only used for diagnosis of prostate cancer but also used for the following of the treatment period. Although PSA is a reliable marker for prostate cancer detection, Gleason score should be used after the prostatectomy operations [131]. Gleason score is the combination of two dominant patterns observed in prostate cancer as it has distinct patterns. These two patterns were analyzed and graded between 1 (well differentiated) and 5 (less differentiated). The overall result is obtained by addition of the two score [133]. High scores indicate the metastatic and aggressive carcinoma. Gleason score is very important for prediction of the cancer and prognosis of the cancer after treatments. The Gleason score also affects the treatment procedure and determines whether the tumor should be removed surgically [71].

Serum levels of the PSA and histological analysis based on the biopsy and Gleason score are reliable methods for prostate cancer diagnosis. In addition to these markers different genetic, cellular and biomarkers are also used. 35 identified single nucleotide polymorphisms (SNPs) were characterized as genetic markers for prostate cancer [134]. Basal cell layer of the prostate epithelium can be labeled for high molecular weight

cytokeratin as a marker for prostate cancer. A similar sensitivity was observed for p63 in the basal cell layer [135]. α-methylacyl-CoA racemase (AMACR) is a marker that is only upregulated in prostate cancer compared to normal glands [136]. A correlation between Gleason score and the AMACR was reported in previous studies [137]. Prostate–specific membrane antigen (PSMA) is an upregulated marker in prostate cancer and expressed on cell membrane [138]. Overexpression of the protein can be observed at late stages of the prostate cancer and hormone refractory cancer [131]. These are the most well known markers for prostate cancer diagnosis. Additionally there are different biomarkers in urine and blood which may be used for diagnosis too [131]. Gleason score analysis and PSA detection are generally used for grading of the prostate cancer before the determination of the treatment method.

1.2.4. Epidemiology of Prostate Cancer

Prostate cancer is the second most common cancer in men with a high prevalence of 25,3/100,000 person. Prostate cancer is generally observed after the age of 50. The incidence differs depend on the countries and the population. The difference may be related with the genetic background, risk factors or environmentally conditions. Different incidence ratios between the Asia and U.S.A. regions are tightly related with ethnic populations and their differences [139]. Genetic susceptibility plays a crucial role in prostate cancer incidence. It was shown with previous studies that man whose brother has prostate cancer is more prone to have the disease [140]. Diet and nutrition intake also affect the prostate cancer incidence. High amounts of fat, meat or calcium intake increase the risk of prostate cancer [141]. AMACR which is an indicator marker for prostate cancer, is also important for oxidation of dietary fatty acids. People with prostate cancer have high levels of this enzyme indicating that fat intake may cause prostate cancer [142]. On the other hand lycopene, vitamin E or Selenium uptake can prevent prostate cancer by triggering the antioxidant pathways or by leading to apoptosis [139]. Moreover androgens, mainly the testosterone is important for prostate cancer incidence and differs between populations [139]. According to the current reports belongs to the years between 2005 and 2009, derived from SEER (The Surveillance, Epidemiology, and End Results); incidence of prostate cancer was 154.8 per 100,000 in men. Mortality rate was 23.6 per 100,000 men only in U.S.A. According to the statistical analysis of 2012 data obtained from USA,

prostate cancer is the most common cancer in man with a 29% incidence. It is found to be second lethal cancer with a 9% mortality ratio after lung cancer (29%) [143].

1.3. TREATMENT of CANCER

Cancer treatment depends on many factors including the age, genetic background and the other environmental conditions. Different methods according to the cancer type and stage of the disease may be preferred. In the current treatment methods; chemotherapy, radiation therapy, hormone therapy, surgical treatments, transplantation therapy and biological therapy are used for cancer treatments. Treatment methods are generally intended to kill cancer cells by inducing the apoptosis except surgical treatment.

Radiation therapy also known as the radiotherapy is widely used for cancer at particular times in the treatment period. Radiotherapy is generally used to control cancer progression locally. Response to the radiotherapy can be different according to the cancer type [144]. The main principle of radiation therapy is killing cancer cells by using ionizan radiation. Radiotherapy exerts its effect on DNA of cancer cells by causing the double strand breaks. In this method high energy radiation is used. Besides the direct effect on DNA, radiation therapy indirectly affects the cancer cells by causing the formation of free radicals [145]. Radiation therapy can be used to treat cancer as a single method but mostly it is used to prevent recurrence together with other treatment methods such as surgical removal of the tumor or chemotherapy. One of the important types of treatment method is the hormone therapy which is particularly associated with the stereoid hormones. This type of treatment is generally used for the tissues that are able to give response to the hormones such as breast, prostate or endometrium. Orchiectomy and hormone deprivation therapies, hormone agonists are widely used for cancer treatments [146]. Biological therapy which comprises the application of monoclonal antibodies or biologically active molecules such as interferon is also used for cancer treatment for long years [147]. For example bevacizumab is a monoclonal antibody against VEGF and used as an inhibitor of angiogenesis [148]. Although treatment principles change according to the cancer type, biological therapies are right choices for eliminating the cancer because of their specificity. Chemotherapy as a different area in cancer research has been the aim of interest for a long

time. Development of non toxic agents is one of the big research topics in the developing world.

1.3.1. Chemotherapy

Chemotherapy is the most important and developed treatment method for cancer and started to be improved at 1955's during World War II [149]. First attempts for chemotherapy was started at 1940's by using the mustard gas for lymphoma. Chemotherapeutic drug applications with cytotoxic effects were started to be used in 1970's for Hodgkin's disease first [150]. The principle of chemotherapeutic drugs is the treatment by using cytotoxic drugs [151]. The term is used for all drugs that are able to kill cancer cells. The general mechanism for chemotherapeutics is the disruption of DNA or inhibition of the chromosomal replication which cause cell death (generally apoptosis). The advantage of chemotherapy compared to surgery or radiotherapy is the systemic delivery of drugs to the each tissue. On the other hand widespread dispersion of the drug to all body parts causes the toxicity to other healthy cells. These agents are generally natural compounds derived from bacteria or plants. They are chemically developed for cancer treatment [152]. These drugs can be categorized according to their effects on the cell cycle. Cell-cycle-phase– nonspecific (alkylating agents) and cell-cycle-phase–specific (cell death ratio does not depend on drug dosage.) types of drugs are currently used for cancer treatment [153]. The cell death pathways induced by drugs are also important for mechanism of action. Although apoptosis is the basic pathway for cell death and many chemotherapeutics trigger the apoptosis, ‘’necrosis, autophagy, mitotic catastrophe, and senescence’’ are other pathways targeted for therapy [154].

In addition to cellular pathways that are leading to cell death, mechanism of action for the drugs is different depending on their activity. DNA, RNA and proteins are potential targets for the alkylation by alkylating agents which makes covalent bonds with active molecular sites (for example amino, carboxyl or phosphate groups) of the targets [155]. Resistance to these drugs is gained by DNA repair mechanisms or glutathione conjugation. Cisplatin is a well known alkylating agent that inhibits the DNA, RNA and protein synthesis [156]. In addition to the alkylation of DNA and RNA, synthetic analogs of the molecules that are involved in the synthesis processes can be used for cancer treatment. These types of agents

are effective at the S phase of cell cycle because they are active at the DNA replication step. Therefore antimetabolites are effective against proliferative tumor cells. 5-fluorouracil (5-FU) and methotrexate are well known examples for antimetabolites used in cancer treatment. As an example 5-FU binds to the active site of thymidylate synthase and hinders enzyme activity leading to the inhibition of the DNA synthesis [157]. Natural compounds from bacteria, fungi or plants are widely used for cancer treatment. Antibiotics with antitumor activity can be derived from bacteria and fungi are used for cancer therapies. These agents cause either double strand breaks or inhibit the DNA topoisomerase enzymes [153]. In the field of chemotherapy the most well known and widely used agents are derived from plants such as taxane derivatives: Paclitaxel and Docetaxel (Taxotere). Paclitaxel cause the assembly and stabilization of microtubules and block cell cycle during the mitosis phase [158].

Although new aspects in drug development have been improved to increase the effectiveness of the therapy and quality of life for patients, recurrence is still a big problem after first line treatment. At the beginning of the treatment cancer cells are sensitive to the chemotherapeutic agents. At the later stages they became insensitive to the same drugs by their ability to acquire resistance [159-160]. Development of the drug resistance in the host body depends on the several factors including the genetic factors, metabolism, tumor type or drug itself [159]. Multidrug resistance at the cellular level is related with the altered expression of cellular regulators which control the drug transport or accumulation [161]. Adenosine triphosphate-binding cassette (ABC) transporter superfamily and solute carrier transporters are two classes of membrane proteins that are associated with the development of Multidrug resistance (MDR) in cancer cells. ABC drug transporter family inhibits the accumulation of drugs in the cells. Solute carrier transporters also affect the uptake of drugs to the cells [162]. ABC drug transporter family members efflux chemotherapeutics out of cell by hydrolyzing the ATP. The ABC family has 7 subfamily and 49 total proteins. P-glycoprotein (P-gp; MDR1/ABCB1), MDR-associated protein (MRP1; ABCC1), and breast cancer resistance protein (BCRP; ABCG2) are most well known ones for their resistance mechanism [163]. P-gp expression may change according to the mutations in the cell. For example p53 gene mutations or p63 overexpression causes altered regulation of P-gp expression. MRP1 is basoletarally localized on the cell membrane and pumps chemotherapeutics to the body. Doxorubicin, epirubicin and vinblastin toxicity is related with the MRP1 overexpression. These receptors act by interacting with the Glutathione

(GSH) [164]. Breast cancer resistance protein is also important for development of the resistance against the several agents such as doxorubicin, epirubicin or methotrexate. High expression levels were observed in mammary gland and this transporter is affected from estrogen, progesterone and testosterone [165]. Development of the drug resistance is an important obstacle for successful chemotherapy. In addition to the development of effective agents without side effects, researchers are trying to improve methods to overcome drug resistance in cancer cells.

Chemotherapy can be classified according to the type of application. Palliative chemotherapy can not be used to treat the cancer but can relief the patient. This type of chemotherapy is generally used for the metastatic disease and is not able to remove the disease completely. Curative chemotherapy is used to treat cancers which are able to response to cytotoxic agents. This type of therapy can be highly toxic. Adjuvant chemotherapy is applied after the first treatment to eradicate the micrometastasis. Neoadjuvant therapy is applied before the first line therapy to collect the tumor in one side and reduce the malignant area which is required for surgery [152].

One of the important issues about chemotherapeutics is the side effects of the agents that are used to kill cancer cells. Nausea and vomiting are the most well known drawbacks of the chemotherapeutics such as cisplatin. Antagonists of the emetic receptors are generally used to overcome this side effect after chemotherapy [166]. As bone marrow and gastrointestinal epithelium are consisted of rapidly dividing cells, these parts of the body are mostly affected areas from chemotherapeutics. Following the myelosupression by chemotherapeutic agents, blood counts of the cells (neutrophils, platelets, etc.) reduced under normal values [167]. Gastrointestinal system is another target for cytotoxic agents because of the highly proliferative epithelium. Ulcers are generally occurs after chemotherapeutic applications [152]. Alopecia during the chemotherapy occurs because of the same reason with myelosupression and gastrointestinal problems. Hair follicle contains highly proliferating keratinocytes which are necessary for normal hair growth. Cytotoxic drugs affects on all dividing cells in the body and blocks the proliferation of hair follicles [168]. In addition to these well-defined side effects, neurological, genitourinary, hepatic and cardiac problems are often observed [152].

1.3.2. Treatment of Prostate Cancer

Prostate cancer is a common type of cancer in adult malignancies among men. There are combined treatment methods for prostate cancer which recover each other. Using only one treatment method is not successful for the prostate cancer. Additionally, stage of the cancer and conditions of the host are important factors for treatment options. Surgical removal of the tumor can be considered if the tumor is trapped into the prostate tissue. Prostatectomy can be applied by using different approaches [169]. After surgical removal; radiation therapy to control local disease is used. Hormonal therapy by using antiandrogens and chemotherapy are applied to control recurrence and spread. If radiation therapy is used for the first choice of treatment, cancer should be confined to the prostate and close surrounding tissue. Different types of radioation therapies are useful for palliative therapies [170].

As the prostate is a hormone dependent tissue, hormone therapy is necessary to overcome the progression of the disease. ‘’Bilateral orchiectomy, estrogen therapy, luteinizing hormone-releasing hormone (LHRH) agonists, antiandrogens’’ are different hormone therapy methods. Bilateral orchiectomy is advantegous because of the easy surgical access, easy operation and rapid decrease of the testosterone levels. However, it has psychologic and physiologic effects resulting from the lack of testosterone such as osteoporosis [171-172]. Application of estrogens and agonists can be used to reduce the levels of testosterone but have several side effects such as cardiovascular diseases [173]. The castration of hormones medically by using gonadotropin releasing hormone agonists (GnRHAs) were started in 1982’s [174]. In addition to the GnRHAs, androgen receptor antogonists (for example flutamide, bicalutamide) or agents that block the P450 enzyme can be used for androgen ablation therapy. Testosterone conversion to dihydrotestosterone is required for androgen receptor activation. Finasteride for example inhibits the key enzyme 5α-reductase and blocks the conversion [175]. Although the hormone therapy is essential for the management of prostate cancer there are several side effects. In the case of long term hormone therapy, development of insulin resistance, diabetes, cardiovascular disorders, obesity and bone problems can be observed [176].

1.3.2.1. Chemotherapy of Prostate Cancer

Chemotherapy of the prostate cancer is not as effective as combination therapies. Applying the cytotoxic agent to the tumor site does not provide a permanent solution to the cancer. Although there are promising candidate chemotherapeutic drug formulations that managed to enter clinical trials, cytotoxic treatments are not effective as hormone therapies. Systemic application of the drug can be applied after hormone ablation therapy [177]. Yagoda and Petrylak reviewed the chemotherapy in hormone refractory prostate cancer and concluded that cytotoxic agents are not effective in hormone refractory disease [178]. In 1997, Raghavan and colleagues proved that cytotoxic therapy is not well known for its clinical relevance [179]. Prostate cancer is a heterogenic disease consisted of different cell types each has a different response to the treatment. Moreover the hormone dependence of the disease requires using combination therapy strategies [180]. Although combination of the chemotherapeutic agents is considered to be effective against prostate cancer, there is not a reported data about the increased survival rates or improved patients life quality. Actually combination of the cytotoxic agents has been reported to increase the toxicity but enhancement of the survival rates are not at the same extent [181]. Improvement of new drugs or combinations, particularly for hormone refractory metastatic prostate cancer is of interest in the cancer research. Researches about the chemotherapeutic drugs in prostate cancer showed that mitotic inhibitors which belongs to the taxanes such as paclitaxel and docataxel are effective [182]. Docetaxel (Sanofi- Taxotere® docetaxel) and paclitaxel (Phyton Biotech- TAXOL®) are agents which are able to stabilize microtubules and block the cell division. Administration of these two cytotoxic agents alone is not an accepted therapy strategy for prostate cancer [180,177]. They are generally combined with estramustine which is an estrogenic and alkylating cytotoxic agent and able to inhibit microtubule stabilization by inactivating microtubule related proteins [183]. Taxanes and estramustine affects on the different molecules of the microtubule system and combination of these cytotoxic agents increases the benefit. Estramustine can be combined with different types of microtubule stabilizers such as vinblastine or vinorelbine [184]. Docetaxel is a recently developed cytotoxic agent for prostate cancer was found to be effective as single treatment agent or together with entramustine [185]. Docetaxel exerts cytotoxic effect by binding to the β subunit of the tubulin which leads to polymerization. Stable polymerization of microtubules prevents the disassembly and cause cell cycle arrest. The process ends up with the apoptosis. In addition to docetaxel's effect on mitotic

spindle, bcl-2 is another target for the cytotoxic effect of docetaxel. As an antiapoptotic protein bcl-2 protects prostate cancer cells. Phosphorylation of the bcl-2 is an important step for preventing the apoptosis [186]. Docetaxel leads the phosphorylation of bcl-2 after microtubule stabilization and forced cell to enter the apoptotic process [180]. Bcl-2 is not the only target of apoptosis for docetaxel. p53 induction, antiangiogenic propertis or inhibition of multidrug resistance are other mechanisms [185]. Docetaxel is a first line therapy agent for prostate cancer but there are some palliative agents used in prostate cancer therapy to provide relief for the patient. Mitoxantrone and prednisone combination was proven to be beneficial for pain relief in advanced prostate cancer patients [187].
However, single or combination therapies for prostate cancer may enhance the cytotoxic response and survival; they have a broad range of toxic effects in the body such as cardiac ischemia and thrombosis [188]. The dose of chemotherapy for prostate cancer should be decreased because of the risk group. Elderly people are more likely to develop prostate cancer and more sensitive to drug toxicity. Myelosupression, infection, cardiac problems, nausea and vomiting are symptoms due to the toxic effects of drugs [189].

Therefore, there is an urgent demand for chemotherapeutic agents which can either be used as adjuvant or neo-adjuvant agents for chemotherapy. Prostate cancer is difficult to treat with chemotherapy because of the complicated nature of the disease. Hormone dependence and heterogenic cell populations make the treatment period more difficult. There are recently developed and highly effective anticancer agents for prostate cancer that were demonstrated to be successful during treatment. Because of the adverse side affects, these cytotoxic agents also are not used efficiently.

1.4. SCHIFF BASES

Schiff bases were identified and characterized by Hugo Schiff in 1864. Schiff bases are derived from the condensation reaction of primary amine and carbonyl compound [190-194]. Schiff bases are formed by the replacement of carbonyl group of the aldehyde or ketone with an imine or azomethine group [194]. Schiff base as a term is used similar with azomethine with the chemical formula RHC=N-R1. R and R1 could be alkyl, aryl, cyclo alkyl or heterocyclic groups. They are also known as anils, imines or azomethines. They have carbon-nitrogen double bond (C=N group) as functional group which allows them to

be used in the chemistry for obtaining carbon-nitrogen bonds [191]. There are several methods for synthesis of the schiff bases [195]. These complexes are synthsesized by a two step chemical reaction. In the first step a carbinolamine intermediate is formed by the condensation reaction of primary amine and carbonile group. The second step is the dehydration of the carbinolamine leading to the formation of the Schiff base. Schiff bases were reported to have crystalline structure and are only soluble in organic solvents in previous studies [196]. Schiff bases are characterized at labile bonds and the synthesis occurs more rapidly at alkaline pHs. Formation of the Schiff bases is relatively more efficient at a pH interval of 9-10 [197]. Different types of carbonyl and amine groups can form a broad range of Schiff bases with different chemical and biological properties.

Schiff bases are involved in many enzymatic reactions by contributing the interaction of the enzyme with specific amino and carbonyl groups. Primary amine groups that belongs to the enzyme react with a carbonyl group found in the substrate and generates the Schiff bases dependent biochemical reactions [191]. In the biochemistry they were shown to act as catalytic active domains of metalloenzymes [198].

Schiff bases are able to generate highly stable complexes with metal ions which make them attractive sources for biological applications [191]. Their ability to form stable reactions with metal ions and regulating the metal ion activity makes them potential candidates in biological applications and industry [199-200]. They are able to form metal complexes by imine- nitrogen group which is associated with the aldehyde ketone. Schiff bases are accepted to have biological activity because of donor atoms that they have such as N, O and S. Metal binding to these donor sites increases the activity and biological advantages of the schiff bases [201]. These clorful metal complexes of Schiff bases have attractive chemical propeties. Copper complexes of Schiff bases are examples to this feature which mimics metalloproteins [202]. Several types of reactions can be catalyzed by the metal complexes of Schiff bases such as carbonylation, hydroformylation, reduction, oxidation and hydrolysis. Several methods can be used to obtain a metal Schiff base complex. Metal acetate salt of the related metal ion is used under reflux conditions by heating. Schiff base complexes with copper and nickel are synthesized by this reaction. In addition to the acetate salts, metal halides can also be used for a direct reaction [203]. As many other drugs schiff bases exert more activity when forms complexes with metal ions [204].

Therefore solving the biological properties of these metal ligands is essential for both to the chemistry and biology. Because of their pharmacological activities they are also used in the pharmaceutical research [205].

Different schiff base derivative compounds in the literature were reported to be antiinflamatuar [206], antifungal [207], antimicrobial [208] and antihypertensive [209]. Instead of their comprehensive biological activities, anticancer activity of different schiff base derivatives was reported in the previous studies [210]. Therefore these type of chemical agents can be used because of their biological activities in different fields. In the recent years they become popular because of their antimicrobial properties. Combining their antimicrobial properties with anti tumor effects may enhance their cytotoxic activity on cancer cells.

1.4.1. Antimicrobial Effects

Infectious disorders associated with antibiotic resistance of bacteria are long-standing problems observed all over the world [211]. Development of new agents to overcome resistance mechanisms is a promising effort. A similar situation is observed for fungal infections which were reported to be dangerous [212]. Therefore, improvements in antifungal research are required to provide efficient agents in this field. Schiff bases were reported to have antibacterial and anti-fungal properties. Although their antimicrobial mechanism is unknown, they were proven to be effective for several types of microorganism. Moreover highly selective Schiff base derivatives were tested in previous studies. For example N-(salicylidene)-2-hydroxyaniline was used against *Mycobacterium tuberculosis* without any cytotoxic effect on macrophages even at high doses [213]. A similar observation for *Mycobacterium tuberculosis* was evaluated for isoniazid-derived Schiff base which is selectively lethal for microorganism but is not toxic for VERO cells [214]. Morpholine derived Schiff bases were evaluated for their antimicrobial effects on *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus* and *Escherichia coli* [215]. N-(Salicylidene)-2-hydroxyaniline 4 is a type of Schiff base was found to be effective against phytopathogenic fungi *Alternaria brassicae* and *Alternaria brassicicola* [216]. Clinically important fungi such as *Aspergillus fumigatus*, *Aspergillus flavus*, *Trichophyton mentagrophytes* and *Penicillium marneffei* were also be inhibited by using 2,4-dichloro-5-

fluorophenyl bearing Schiff bases [217]. Development of the new agents like Schiff bases and their metal complexes to fight against microorganisms is a major field in the biological applications.

1.4.2. Anti-tumor Effects

Schiff bases and their metal complexes were found to be effective against cancer cells. Although the exact mechanism is not known and the effects can be alter according to the cancer type and the compound itself, there are different hypothesis for their anti-tumor activities. Hydrogen bond formation between N–H and the nitrogen atoms of DNA and copper binding to the DNA or protein could provide the anti –tumor effect [210]. Ribonucleotide reductase (RNR) is a critical enzyme for DNA synthesis in all dividing cells and can be a target key regulator in cancer treatment [218]. Different schiff base derivatives such as hydroxysemicarbazide have been tested as RNR inhibitors for cancer treatment [219]. Organotin (IV) complexes of Schiff bases were used in vitro for ‘’A498, EVSA-T, H226, IGROV, M19 MEL, MCF7 and WIDR’’ cell lines with their apoptotic effects. These compounds are able to interact with DNA by binding to sugar and nitrogen atoms of the nucleic acids [220]. Another mechanism for schiff bases is the inhibition of DNA synthesis by acting on topoisomerase II. Copper containing quinolinone Schiff base was tested against hepatocellular cancer and was found to be effective on topoisomerase II [221]. In another previous work, schiff base derivatives containing sulfonamide moiety were determined for their anticancer effect on MCF-7 cells. Sulfonamides were reported to have anticancer activity by differrent mechanisms such as microtubule stabilization or the disruption of angiogenesis [222]. Cytotoxic evaluation of copper containing oxindole-Schiff bases were carried out by using SH-SY5Y cell in vitro. In the study DNA binding activity and ROS producing ability for Schiff base complexes were detected. DNA cleavage that is leading to apoptosis makes these complexes potential anticancer drug candidates [223]. Mononuclear copper complexes bearing a Schiff base ligand were used against MCF-7 cells exerted ctyotoxicity. In the study it was hypothesized that Schiff base complexes increased caspase levels leading to activation of apoptotic pathway and inhibit Akt [224]. Copper containing another schiff base derivative taurine complex were investigated for antitumor effects. Proteosomal activity increased ubiquitinated proteins

and Bax that lead to apoptosis [225-226]. Other mechanisms which are predicted for Schiff bases are tyrosine kinase inhibition and cyclin-dependent kinases inhibition [227].
Different schiff base derivatives were used for prostate cancer *in vitro* to assess the anti-tumor affects of Sciff bases [228-231]. None of these studies used normal healthy cell lines in order to check the cytotoxic effects of the drug combinations which are required to assess the reliability of the drug. There is a big demand for both to evaluate the effects of Schiff bases on cancer treatment and toxicity on the normal adult tissues.

1.5. PLURONIC TRIBLOCK COPOLYMERS

Polymer based technology has widely started to be used in pharmaceutical research and applications. Pluronic triblock copolymers also known as poloxamers are widely used in biological applications as vehicles for drugs, growth factors and genes. Pluronics are named as P for paste, F for flake and L for liquid with two or three digits.the number of digits are used to calculate percentage of polyoxyethylene units and the molecular mass of the polyoxypropylene chain. Hydrophilic poly(ethylene oxide-PEO) and hydrophobic poly(propylene oxide-PPO) units are arrenged in an A-B-A triblock structure: PEO-PPO-PEO [232]. Different types of pluronics may be found with different numbers of PPO and PEO units. Altered numbers of these units can change the hydrophilic-lipophilic balance (HLB) of block copolymers. The triblock structure includes the amphiphilic character to the polymer which is necessary for surfactant properties [233].

In aqueous solutions higher than the critical micelle concentrations (CMC), these polymers are able to form micelles that have a diameter range between 10nm and 100nm [232]. These micelles can encapsulate drugs and facilitate the transport by interacting with the membrane. In addition to micelle forming ability they can also form unimers below the critical micelle concentration which enables to incorporate to the cellular membranes [234]. Unimers which are single copolymer molecules and formed under CMC can interact with the cellular membranes and translocate through the membranes. The hydrophobic PPO chains interact with the membranes to change membrane structure and decrease membrane viscosity. On the other hand, pluronics form micelles at high concentrations [235].

Accordingly they are used in many biological applications incluiding drug delivery. Inhibition of P-glycoprotein or MRP by pluronics may be one of the important mechanisms for overcoming the cancer cell drug resistance. Increasing the cyctochrome c release and ROS by pluronics leads the apoptosis [236]. They are specifically trigger the apoptotic pathway in MDR cells [237]. Moreover blocking the glutathione/glutathione S-transferase detoxification system by pluronics is important for drug metabolism [235]. Instead of the inhibiton of drug resistance proteins, pluronics decrease the ATP production that indirectly increases the sensitivity of the cancer cells to the chemotherapeutic agents [235]. As pluronics are able to interact with cellular membranes their translocation through the membranes is mediated by caveole-mediated endocytosis [238] directing them to different cellular compartments. They may interact with the mitochondrial membrane leading to disruption of the membrane structure and cause apoptosis [237].

One of the most important fields that pluronics can be used is gene therapy to increase transfection efficiency. Pluronic block copolymers increase plasmid DNA transfection in mice antigen presenting cells and increased the plasmid DNA expression in the skeletal muscle, spleen, and lymph nodes [239-240]. In this study, we focused on which has been reported to have different pharmaceutical properities.

1.5.1. P85 Pluronic Block Copolymers

P85 (PEO_{39}-PPO_{52}-PEO_{39}) pluronic block copolymer is a symmetric tri-block copolymer that is consisted of a central poly(propylene oxide) block with poly(ethylene oxide) blocks on the each end. It is in paste form and soluble in water at a degree of 10% [234]. The polymer can be referred as a nonionic and non toxic surfactant. P85 has been reported to increase transport of drugs from the cellular membrane *in vitro* [241]. P85 has been demonstrated to increase transport of digoxin from blood brain barrier *in vivo* [242]. Pharmacokinetic studies showed that P85 can be a versatie drug delivery system *in vivo* and tissue distribution is dependent on PPO segments [243]. P85 has a remarkable membrane permeabilization potential below the CMC which is observed when combined with drugs interacting with ATP-dependent efflux pumps [241]. This effect was observed by using rhodamine-123 efflux from the apical site of the cells. Although P85 concentrations below CMC can inhibit apical efflux transporters because of unimer

components found as trace amounts, significant inhibiton can be observed above CMC too. Unimers contribute to the drug transport by interacting with membrane and efflux pumps, endocytosis and followed by recycling from the apical site. These effects were shown with several drugs including doxorubicin and taxol. P85 does not act as a substrate of drug transporters. This pluronic causes conformational change of the drug pupms by membrane fluidization or inhibition of drug binding site [244]. One another possible explanation for pluronics effect on drug transport is P85's fusogenic activity in the vesicles which translocates through the cell mebrane by endocytosis. This activity provides the release of molecules (drugs) to the cytoplasm [243].

These polymers inhibit P-gp drug efflux system and increase the uptake of P-glycoprotein dependent drugs (Pgp) to the cells. P85 has the ability of permeabilization at concentrations below the CMC [245,234], especially when combined with a substrate of an ATP-dependent drug efflux mechanism [246,235,234]. P85 is widely used to enhance of drug transport to the brain because it can easily transport from the blood brain barrier. P85 is generally used in bovine brain microvascular endothelial cells (BBMECs) to inhibit drug transporters [247,234] or against cancer cells along with anticancer agents [248,234]. This pluronic has been used to increase cellular uptake of analgesic peptides such as biphaline and morphine below CMC [245]. Membrane fluidization, P-gp ATPase inhibition and ATP depletion contribute to the effects of P85 on drug transport efficiency.

1.6. AIM OF THE STUDY

The introduction of many novel targeted therapeutics into the clinical practice is a rapidly growing field in medical oncology. Chemotherapy of cancers as well as prostate cancer involves targeting cancer cells with cytotoxic agents. Chemotherapy has been considered as a solution for the treatment of all cancers, resulting in destruction of malignant cells, while having a non specific toxicity on all cell types. Current chemotherapeutic approach for prostate cancer is to use single or combined chemotherapeutic agents to increase survival rates. Therapeutic strategies are able to devastate tumor but can not effective on advanced prostate cancer. Even so, developments of new chemotherapeutic agents that enable the inhibiton of prostate cancer progression are the aim of interest. Moreover, the

impact of cytotoxic chemotherapy is not limited to the cancer tissue. Therefore, future treatments will include cytotoxic tools that are specifically kills tumor cells.

The challenge for the future prospects of this study is to discover a targeted and cost effective chemotherapeutic drug with specific toxicity for prostate cancer cells. Because the signaling pathways that are involved in cancer initiation and cell death are different, mechanism of the chemotherapeutic agents should be identified. The purpose of the current study is to determine the mechanism of newly developed drug combination on prostate cancer cells. The breakthrough of the study is the improvement of a new specific chemotherapeutic drug that is non-toxic for healthy tissues.

2. MATERIALS AND METHODS

2.1. *IN VITRO* STUDIES

2.1.1. Cell Lines

Tramp-C1 (CRL 2730, mouse prostate cancer cells), PC-3 (CRL 1435, human prostate cancer cells), DU 145 (HTB 81, human prostate cancer cells), LNCaP (CRL 1740, human prostate cancer cells) and L-929 (CCL 1, mouse fibroblast cells) cells were purchased from ATCC (Rockville, MD). Normal prostate epithelium cells (PNT1A) were purchased from Sigma-Aldrich (USA). Foreskin primary human fibroblast cells (HF) were isolated from neonatal foreskin after obtaining the informed consent of patient's parents and ethics committee approval of Kocaeli University according to the standard procedure described before [249]. All cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM, #41966-029, Invitrogen, Gibco, UK) supplemented with 10% fetal bovine serum (FBS, #10500-064, Invitrogen, Gibco, UK) and 1% Penicillin/Streptomycin/Amphotericin (PSA, Invitrogen, Gibco, UK) in a humidified chamber at 37 °C and 5% CO_2. Cells were trypsinized using 0.25% trypsin-EDTA (#25200-056, Invitrogen, Gibco, UK) as they reach enough confluence (≈80%).

2.1.2. Schiff base preparation

Heterodinuclear copper(II)Mn(II) complex (Schiff base) was synthesized and characterized by our group as described previously [250]. Schematic representation of the synthesis of the complex was given in Figure 2.1. The complex was kept at room temperature in a light-protected tube until use. Pluronic P85, (#30085877, BASF Corporation, Badische Anilin und Soda-Fabrik, USA) was prepared in Phosphate Buffered Saline (PBS, #P04-36500, Pan-Biotech, Germany) at a stock concentration of 10% (w/v) by incubating at 4 °C overnight to provide complete dissolving. Main stock solution (10%) was diluted to 1% in complete DMEM for cell culture experiments and 0.05% (w/v) P85 was combined with Schiff base for *in vitro* analysis.

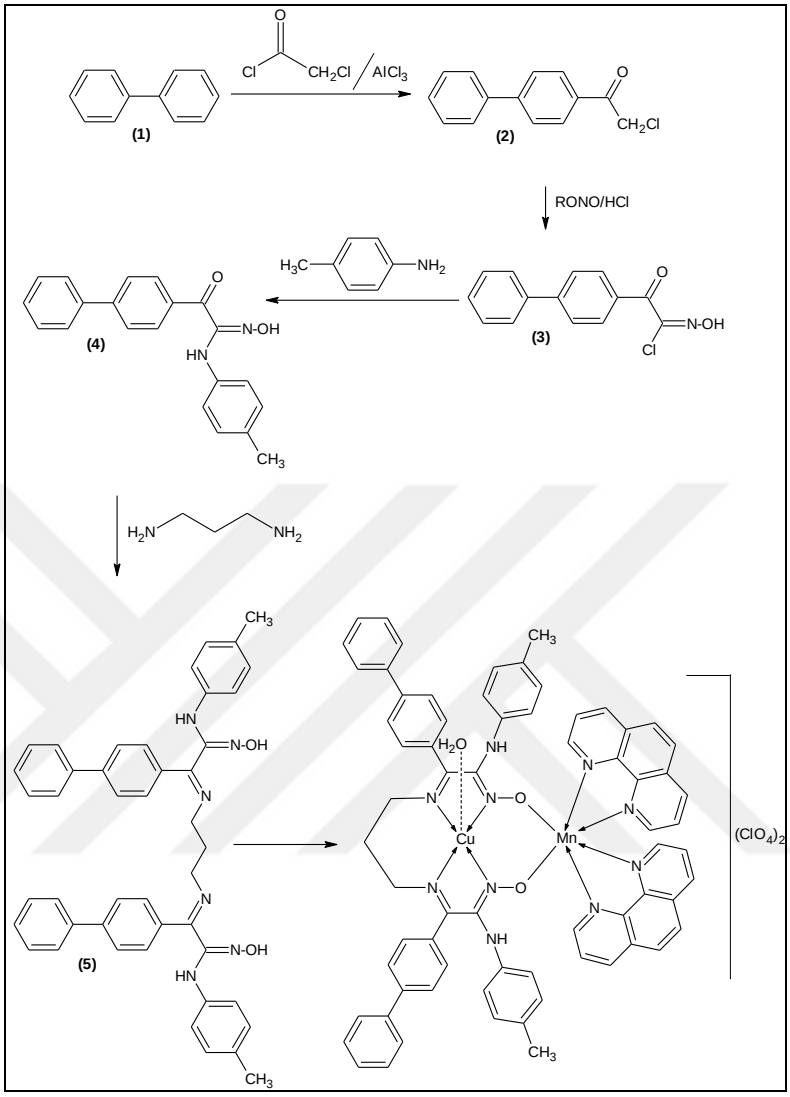

Figure 2.1. Synthesis of the Schiff base. The final product is the Heterodinuclear copper(II)Mn(II) complex. (1) Biphenyl, (2) 2-Chloro-1-(4-phenylphenyl)ethan-1-one, (3) N-Hydroxy-2-oxo-2-(4-phenylphenyl)ethenecarbonimidoyl chloride, (4) N'-Hydroxy-N-(4-methylphenyl)-2-oxo-2-(4-phenylphenyl)ethenimidamide, (5) N'-Hydroxy-2-{[3-({[N'-hydroxy-N-(4-methylphenyl)carbamimidoyl](4-phenylphenyl)methylidene}amino)propyl]imino}-N-(4-methylphenyl)-2-(4-phenylphenyl)ethenimidamide

2.1.3. Cell Viability Assay

Cell viability analyses were completed to determine potential cytotoxic effects of formulations on prostate cancer cell lines (Tramp-C1, PC-3, DU 145 and LNCaP), and healthy cell lines (PNT1A, L-929 and HF). Cell viability was measured using 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS)-assay (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) as described previously [234]. Docetaxel (#114977-28-5, Santa cruz, USA), an effective chemotherapeutic agent used in prostate cancer treatment was used as positive control throughout the study [251]. Schiff base was dissolved in dimethyl sulfoxide (DMSO, #D4540, Sigma-Aldrich, USA) at a stock concentration of 1mg/ml and diluted to 10µg/ml in complete DMEM supplemented with 10% FBS and 1% PSA. Cells were exposed to 0.5, 1, 2 and 5µg/ml of Schiff base and Docetaxel prepared in complete growth medium. Pluronic P85 was dissolved in PBS at 10% (w/v) concentration by incubating overnight at 4 °C and subsequently diluted to 1% (w/v) in complete medium. For all cell culture experiments, 0.05% (w/v) of P85 was used as working solution. Cells were seeded onto 96-well plates (#CLS6509, Corning Plasticware, Corning, NY) at a cell density of 5×10^3 cells/well and incubated in a humidified incubator at 37 °C and 5% CO_2 overnight. Indicated concentrations of compounds were added to the cell culture medium and cell viability was analyzed for 3-day incubation period (24, 48 and 72h). Briefly, 10µl MTS reagent was added to the wells of 96-well plates at the end of each time point and incubated at 37 °C for 1–3 h in a humidified and 5% CO_2 atmosphere. Absorbance was measured at 490nm by using an ELISA plate reader (Biotek, Winooski, VT).

2.1.4. Caspase Assay

Colorimetric Caspase 3 assay was used to detect Caspase 3 enzyme levels in the chemotherapeutic reagent administrered prostate cancer lines (Tramp-C1, PC-3, DU 145 and healthy cell line (PNT1A). Assay was performed by using commercial kit (#G7351, Promega, Madison, USA) according to the manufacturer's instructions. The 2µg/ml concentration of Schiff base, maximum tolerated toxic concentration for a 12h exposure time, 2µg/ml of Docetaxel and their pluronic P85 (0.05% w/v) combinations were used in Caspase 3 assay. Briefly, cells were seeded onto 6-well cell culture plates (#3516, Corning

Plasticware, Corning, NY) at a cell density of 2×10^5cells/well. Schiff base, Docetaxel and their combinations with P85 were administered to the cells for 24h. Briefly, total protein was isolated from the drug treated cells using RIPA Buffer (#sc-24948, Santa Cruz, USA) and protein concentrations were estimated using BCA assay (#23227, Pierce, Rockford, USA). 100µg protein samples, caspase asay buffer and substrate solutions were mixed and incubated at 37 °C for 4h. Absorbance was measured at 405nm by using an ELISA plate reader (Biotek, Winooski, VT). Caspase 3 activity was indirectly determined by measuring the absorbance value of released chromophore p-nitroaniline (pNA) from the substrate upon cleavage by Caspase 3.

2.1.5. Cell Migration Assay

In order to evaluate the effect of drug combinations on metastatic properties of prostate cancer cells, *in vitro* cell migration assays, scratch and trans-well assays, were conducted. The concentration of 0.5µg/ml for Schiff base and 12h incubation time were selected for cell migration assays as higher doses exerted cytotoxic effects in a short period of time and prevented migration detection. Cells were seeded onto a 12-well plate (#92012, TPP, Switzerland) at a cell density of 1×10^5 cells/well and incubated overnight in a humidified incubator at 37 °C and 5% CO_2. Next day, cells were scratched using a sterile 200µl pipet tip. Cell culture medium was immediately replaced with fresh medium to prevent re-attachment of scratched cells. Indicated concentrations of Schiff base, Docetaxel and their pluronic combinations were added to the cell culture medium. Pictures were taken after 12h using Zeiss PrimoVert light microscope with an AxioCam ERc5s camera and Zen 2011 software (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) and cell migration to the wounded area was measured by using image analysis software (ImageJ, NIH, Bethesda, MD).

Secondly, trans-well cell migration and invasion analyses were conducted using CytoSelectTM 24-well kit (#CBA-100-C, Cell Biolabs Inc., San Diego, CA, USA) according to the manufacturer's instructions. In short, cells were seeded onto trans-wells with 8µm pores (upper chamber) at a cell density of 1.5×10^5 cells/well in serum free DMEM containing specified concentration of the formulations (Schiff base, Docetaxel and their pluronic combinations). DMEM supplemented with 10% FBS was added to the lower

chambers of the trans-well migration system as chemoattractant media. 24h later cells were stained with Cell Stain Solution (supplied with the kit) by incubating for 10 min at room temperature. After rinsing three times with distilled water, pictures were taken and migration was quantitatively analyzed by dissolving the dye in extraction solution supplied by the kit. Briefly, dye was dissolved by adding 200 µl extraction solution to each well and incubating 10 minutes. Absorbance of the samples were taken at 560nm in a plate reader and analyzed.

2.1.6. Ethidium Bromide Displacement Assay

DNA binding properties of the Schiff base were evaluated by ethidium bromide displacement assay as described previously [252]. Fluorescence spectra for 2ml of reaction solution-(RS) (50 mM NaCl and 5 mM Tris–HCl) containing 6µg calf thymus DNA (#15633-019, Invitogen, UK) and 2µg ethidium bromide (EtBR, #E1510, Sigma, USA) was measured using excitation wavelength of 478nm and the emission range set between 520 and 685nm. RS with only EtBR was used as negative control. Different concentrations of the Schiff base ranging from 0.5 to 100µg/ml were added to the RS to observe DNA binding affinity of Schiff base in a dose dependent manner. It was confirmed that the Schiff base does not quench the EB fluorescence before conducting the experiment.

2.1.7. DNA Cleavage Assay

DNA cleavage assay was performed to detect DNA cleavage activity of the Schiff base according to the protocol described in the literature [253]. Briefly, 1µg of supercoiled plasmid (pMD2.G, #12259, Addgene, MA, USA) was treated with various concentrations of the Schiff base (10-100µg/ml) in a reaction volume of 20µl (50mM NaCl and 5mM Tris-HCl, pH 7.5). The solutions in test tubes were incubated for 6h at 37 ºC in a humidified chamber and subjected to gel electrophoresis for 1.5h at 60V on 0.8% agarose gel, prepared in Tris-Acetate-EDTA (TAE, Sigma-Aldrich, Stenheim, Germany) buffer, containing 0.5µg/ml of EtBR. DNA bands (supercoiled, open nicked and linear) were visualized under UV light, photographed using Molecular Imager Gel Doc XR System (BioRad, USA).

2.1.8. Angiogenesis Assays

2.1.8.1. Tube Formation Assay

Tube formation assay was performed to test potential anti-angiogenic properties of drug combinations as described previously [254]. Briefly, pre-chilled 48-well plates (#92096, TPP, Switzerland) were coated with growth factor reduced matrigel (#354230, Becton-Dickinson Biosciences, Bedford, MA) on ice and incubated for 30 min at 37 °C to provide matrigel polymerization. Human umbilical vein endothelial (HUVEC, ATCC-CRL 1730) cells were seeded onto matrigel-coated wells at a cell density of 2×10^4 cells/well. Aforementioned concentrations of the chemicals were added to the wells. The tube-like structures were observed after 7h using a light microscope (Nikon Eclipse TS100, Nikon, Tokyo, Japan). Number of branches was calculated from randomly selected five areas.

2.1.8.2. Aortic Ring Assay

Pre-chilled 48-well tissue culture plates were coated with 75µl of growth factor reduced Matrigel on ice and incubated for 30 min at 37 °C and 5% CO_2 to provide polymerization. Thoracic aortas were removed from 8- to 10-week-old male Sprague Dawley rats and fibroadipose tissues were carefully removed. The aortas were cut into 1-mm-long cross-sections, placed on Matrigel-coated wells, and covered with an additional 75µl of Matrigel, followed by additional 30 min polymerization period at 37 °C and 5% CO_2. EGM-2 (250µl, Endothelial growth factor medium-2) consisting of endothelial basal medium (EBM-2, #CC-3156, Lonza, USA) and endothelial growth factors provided with EGM-2 Bulletkit (#CC-3202, Lonza, USA) was added into each well and plates were incubated 24h at 37 °C and 5% CO_2 atmosphere. The culture medium was changed with EBM-2 supplemented with 2% FBS and 1% PSA containing specified drug compositions or 0.5% DMSO as vehicle. After a 7-day incubation period, area of angiogenic sprouting was monitored. Docetaxel was used at the concentration of 10^{-8}M as indicated in the literature [255].

2.1.9. Micro-well dilution assay

Minimum inhibition concentrations (MIC) for microbial strains tested (Table 1) were determined by micro-well dilution assay as described [256]. Microbial inoculums prepared from fresh cultures were adjusted to McFarland (0.5) standard turbidity. Schiff base

dissolved in DMSO and Schiff base-P85 were first diluted to 2.5mg/ml to be tested and serially diluted to obtain concentrations range from 0.6-2500µg/ml in sterile 15ml test tubes containing tryptic soy broth (#CM0129, Oxoid, UK) for bacteria, sabouraud dextrose broth (#CM0129, Oxoid, UK) for candidal and fungal species. 95µl of respective broth and 5µl of respective inoculums were put in the wells of 96-well plate. 100µl of serially diluted Schiff base solutions were added into each consecutive well. The last well of each column was prepared by dispensing 195µl of respective medium and 5µl of inoculum to be used as a positive control. The plate were covered with a sealer and mixed at 300 rpm for 20 seconds. The inoculated plates were incubated for 24h at 36±1 ºC for bacteria, 48h at 36±1 ºC for candida and 72h at 27±1 ºC for fungal species. At the ends of incubation periods, microbial growth was detected by reading the respective absorbance at 600 and 530nm for bacterial and fungal isolates, respectively.

2.1.10. Minimum bactericidal (MBC) and fungicidal (MFC) concentration

MBC/MFC values of the Schiff base and Schiff base-P85 combination for each microorganism tested were determined by pouring 5µl of each well prepared for micro-well dilution assay onto tryptic soy agar (#22091, Sigma-Aldrich, USA), sabouraud dextrose agar (#CM0041, Oxoid, UK), potato dextrose agar (#110130, Merck, Darmstadt, Germany) for bacteria, candida and fungi, respectively. The plates were incubated for 24h at 36±1 ºC for bacteria, 48h at 36±1 ºC for candida and 72h at 27±1 ºC for fungal species. MBC/MFC values were determined as the lowest concentration of the Schiff base at which no microbial growth were observed. Microbial species used in experiments were listed in Table 2.1.

Table 2.1. Microbial species used in experiments

Microbial Species
Escherichia coli ATCC 10536
Staphylococcus aureus ATCC 6538
Pseudomonas aeruginosa ATCC 15442
Proteus mirabilis ATCC 15146
Klebsiella pneumoniae ATCC 13883
Yersinia enterocolitica ATCC 27729
Aspergillus niger ATCC 16404
Candida albicans ATCC 10231

2.1.11. Quantitative Real time PCR (RT-PCR) Analysis

Primers for Protein kinase B (Akt), Androgen receptor (AR), Bcl-2-associated X protein (BAX), B-cell lymphoma 2 (Bcl-2), ATP-binding cassette sub-family G member 2 (BCRP), Caspase-3, Epidermal growth factor receptor (EGFR), ETS domain-containing protein Elk-1 (Elk-1), Multidrug resistance-associated protein (MRP) and Nuclear Factor kappa B (NF-κB) (Table 2.2) were designed using Primer-BLAST software from the National Center for Biotechnology (Bethesda, MD, USA) and synthesized by Macrogen (Seoul, Korea). β-actin sequence was used as reported elsewhere (Kafienah et al., 2006). RT-PCR reagents and conditions were given in Table 2.3 and 2.4, respectively. Total RNAs from drug treated cancer and healthy cells were isolated using RNAeasy plus mini kit (#74136, Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized using High Fidelity cDNA synthesis kit (#05081955001, Roche, USA). Reverse transcription polymerase chain reaction (RT-PCR) with SYBR Green method was used to detect mRNA levels of the target genes. cDNAs were mixed with primers, SYBR-mix (#K0221, Fermentas, USA) and PCR grade distilled water (#SH30538.02, Hyclone, Utah, USA) in a final volume of 20µl. β-actin was used for normalization of data. All RT-PCR experiments were conducted using CFX96 RT-PCR system (Bio-Rad, Hercules, CA).

Table 2.2. Primers used in RT-PCR assays

Gene	Species	Sequence	Product length
Akt	Mouse	F 5’ GGGACCTGAAGCTGGAGAA 3’ R 5’ CCTGGTTGTAGAAGGGCAGG 3’	240bp
Akt	Human	F 5’ GAAGCTGCTGGGCAAGGGGCA 3’ R 5’ GTGGGCCACCTCGTCCTTGG 3’	124bp
AR	Mouse	F 5’ TAGGGCTGGGAAGGGTCTAC 3’ R 5’ CTATGTTAGCGGCCTCAGGG 3’	129bp
AR	Human	F 5’ TGTAAGGCAGTGTCGGTGTC 3’ R 5’ GAAGCTGTTCCCCTGGACTC 3’	73bp
BAX	Mouse	F 5’ TTGGAGCAGCCGCCCCAGG 3’ R 5’ CGGCCCCAGTTGAAGTTGCC 3’	188bp
BAX	Human	F 5’ TGCAGAGGATGATTGCCGCCG 3’ R 5’ ACCCAACCACCCTGGTGTTGG 3’	250bp
Bcl-2	Mouse	F 5’ AGAGCAACCCAATGCCCGC 3’ R 5’ CAACGAGGGGCCTGAGAGG 3’	180bp
Bcl-2	Human	F 5’ AACGGAGGCTGGGATGCCTTTGTG 3’ R 5’ ACCAGGGCCAAACTGAGCAGAGT 3’	104bp
BCRP	Mouse	F 5’ CCTCACCTTACTGGCTTCCG 3’ R 5’ ATCCGCAGGGTTGTTGTAGG 3’	112bp
BCRP	Human	F 5’ CACAACCATTGCATCTTGGC 3’ R 5’ GAGAGATCGATGCCCTGCTT 3’	192bp
Caspase-3	Mouse	F 5’ GGGAGCAAGTCAGTGGACTC 3’ R 5’ CCGTACCAGAGCGAGATGAC 3’	136bp
Caspase-3	Human	F 5’ GAGGCGGTTGTAGAAGAGTTCGTG 3’ R 5’ TGGGGGAAGAGGCAGGTGCA 3’	177bp
EGFR	Mouse	F 5’ TCTCCAAAATGGCCCGAGAC 3’ R 5’ ACTCAGAGAGCTCAGGAGGG 3’	215bp

Akt: Protein kinase B, AR: Androgen receptor, Bax: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2, BCRP: ATP-binding cassette sub-family G member 2, EGFR: epidermal growth factor receptor.

EGFR	Human	F 5’ AATGCAACATCCTGGAGGGG 3’ R 5’ AGGTGATGTTCATGGCCTGG 3’	99bp
Elk-1	Mouse	F 5’TTGGAGCAGCCGCCCCAGG 3’ R 5’CGGCCCCAGTTGAAGTTGCC 3’	188bp
Elk-1	Human	F 5’ CGCATCCCTCTTTAACAGTACCCCT 3’ R 5’ GCCCGGCTGAGCTTGTCGTA 3’	239bp
MRP	Mouse	F 5’ CAGGAACCTGTGCTGTTTGC 3’ R 5’ CTCTCACCAACCAGGGTGTC 3’	158bp
MRP	Human	F 5’ GAGGACACGTCGGAACAAGT 3’ R 5’ TCGCATCCACCTTGGAACTC 3’	142bp
NF-κB	Mouse	F 5’ ACACGAGGCTACAATCTGC 3’ R 5’ GGTACCCCCAGAGACCTCAT 3’	164bp
NF-κB	Human	F 5’ GCCACCCGGCTTCAGAATGGC 3’ R 5’ TATGGGCCATCTGCTGTTGGCAGT 3’	147bp
PTEN	Mouse	F 5’ TGTGGTCTGCCAGCTAAAGG 3’ R 5’ AGGTTTCCTCTGGTCCTGGT 3’	215bp
PTEN	Human	F 5’ TGTGGTCTGCCAGCTAAAGG 3’ R 5’ ACACACAGGTAACGGCTGAG 3’	106bp
β-actin	Mouse/ Human	F 5’ GACAGGATGCAGAAGGAG 3’ R 5’ TGATCCACATCTGCTGGA 3’	141bp

EGFR: epidermal growth factor receptor, Elk-1: ETS domain-containing protein Elk-1, MRP: Multidrug resistance-associated protein, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells, PTEN: Phosphate and tension homologue deleted on chromosome 10, β-actin: Beta actin.

Table 2.3. RT-PCR reagents

Reagents	**Volume**
Maxima™ SYBR Green qPCR Master Mix	10 µl
Primer Forward (10pmol)	1 µl
Primer Reverse (10pmol)	1 µl
Distilled water	5 µl
Template (100ng/ml)	5 µl

Table 2.4. RT-PCR conditions

Cycle	Repeats	Step	Dwell time	Set point
Initial Denaturation	1	1	3 min	93 °C
Denaturation	36	1	30 sec	93 °C
Annealing		2	40 sec	61 °C
Extension		3	45 sec	72° C
Final extension	1	1	10 min	72 °C
Melt curve	110	1	12 sec	-0.5 °C/cycle
Hold	1	1	-	4°C

2.1.12. Western Blot Analysis

All chemicals used in immunoblotting assays were purchased from Biorad Laboratories (Richmond, CA). Solutions prepared for western blot analysis is given in Table 2.5. Primary antibodies against Akt (#9272), NF-κB (#8242) and GAPDH (#8884), purchased from Cell signaling technology (Beverly, MA, USA), were used to detect marker proteins for cancer and healthy cells treated with drug compositions. Briefly, total protein was isolated from the drug treated cells using RIPA Buffer (#sc-24948, Santa Cruz, USA) and protein concentrations were estimated using BCA assay (#23227, Pierce, Rockford, USA). Protein samples were loaded to Any kD™ Mini-PROTEAN® TGX™ precast gels (#456-9033, Biorad, USA) at 30µg/lane and electrophoresed by applying 90V for 100 min. Then, proteins were transferred to nitrocellulose membranes (#162-0115, Biorad, Germany) with a pore size of 0.45µm using semi-wet transfer technique at 175mA for 90 min. The membranes were incubated with blocking solution containing 5% non-fat dry milk powder (#170-6404, Biorad, USA) prepared in Tris-Buffered Saline and Tween-20 solution (TBS-T). Membranes were incubated with primary antibodies (dilution 1:5000) at 4 °C for 16h. After washing with TBS-T three times, membranes were incubated with anti-rabbit secondary antibody (sc-2004, dilution 1:5000, Santa Cruz Biotech Inc., USA) prepared in blocking buffer for 1h at room temperature. GAPDH was used as an internal control and images were taken by using the luminometer system (Biorad, USA). Band intensities were

calculated using Image J software and normalized to the respective GAPDH band intensities. Results were represented as fold change of control.

Table 2.5. Western blotting solutions

TBS-T	20mM Tris-HCl 150mM NaCl 0.1% Tween 20, pH 7.6
Running Buffer	25mM Tris base 190mM Glycine 0.1% SDS, pH 8.3
Transfer Buffer	25mM Tris base 190mM Glycine 20% Methanol
Blocking buffer	5% Non-fat dry milk prepared in TBS-T

2.2. *IN VIVO* STUDIES

2.2.1. Animals

Healthy male C57/Bl6 mice (n=16; n=8/group) weighing 20±2g were obtained from Yeditepe University (Istanbul, Turkey) for *in vivo* anticancer activity assay and healthy male C57/Bl6 mice (n=25; n=5/group) weighing 20±2g were obtained from Elazığ University (Elazığ, Turkey) for *in vivo* toxicology analysis.

The animals were housed individually in disinfected cages and subjected to a constant temperature of 23±1 °C, relative humidity of 60±10%. The mice were maintained at 12-h light/dark cycle and fed with food and water *ad libitum*. All respective procedures were approved by Yeditepe and Elazığ Universities Ethics Committee of Experimental Animal Use and the Research Scientific Committee at the same institutions.

2.2.2. Toxicology Analysis

Acute toxicity analysis of Schiff base and P85 combination in C57/Bl6 mice was completed according to the protocol described previously (Uckun ve ark., 2002). In short, mice (n=5) treated intraperitoneally with vehicle alone (2. 5% DMSO/PBS, 0.2ml) or four different doses of Schiff base (0.1, 0.25, 0.5 and 1mg/kg) combined with 500mg/kg pluronic P85. Daily examinations for mortality and morbidity were conducted for a course of 7 days. At the end of 7 days, animals were sacrificed by cervical dislocation and whole blood was collected in heparinized tubes to analyze blood parameters. Blood samples were centrifuged at 3000g for 10 min, serum was separated and stored at -20°C. Blood parameters were measured using a biochemical analyzer (Olympus AU-600, Tokyo, Japan). In addition, multiple organs and tissues were kept in 10% formaldehyde (#252549, Sigma-Aldrich, USA) for histopathological examinations. The number of animals subjected to drug application and histology for toxicology experiments are shown in Table 2.6.

2.2.3. Development of Tumor Model and Drug Application

Prostate cancer was created on dorsal side of C57/Bl6 mice near to tail side according to the protocol described by Young and his colleagues with slight modifications [257]. Under moderate ether anesthesia, 2×10^7 Tramp-C1 cells were injected subcutaneously and the tumor development was monitored daily. Tumors generally became visible at the end of 30 days. Tumors were resected and histopathological examinations were conducted.

For the examination of the Schiff base's anticancer activity, starting from one week after cell injection, specified concentrations of Schiff base (0.5mg/kg) and pluronic P85 (500mg/kg) combination were given intraperitoneally every four days for 52 days. Control groups received same volume (200µl) of sterile PBS containing 2.5%DMSO. Mice were examined for mortality and morbidity throughout the study. At the end of treatment period, mice were sacrificed by cervical dislocation and multiple organs and tissues were maintained in 10% formaldehyde solution for histopathological examinations.

Table 2.6. Tissues examined histopathologically and numbers of mice for each experimental groups for toxicology analysis

Treatment	**Vehicle**		**Schiff base-P85**		**Schiff base-P85**		**Schiff base-P85**		**Schiff base-P85**	
Treatment Dose	**Control**		**G1**		**G2**		**G3**		**G4**	
#Mice/Group	**5**		**5**		**5**		**5**		**5**	
Tissue	No Exam	%	No Exam	%	No Exam	%	No Exam.	%	No Exam.	%
Lung	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Kidney	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Spleen	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Heart	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Pancreas	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Brain	4/5	80	4/5	80	4/5	80	4/5	80	4/5	80
Intestine, Large	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Intestine, Small	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Stomach	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100

Testes	5/5	50	5/5	50	5/5	50	5/5	50	5/5	50
Skeletal Muscle	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Skin	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Bone Marrow	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Uterus	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Urinary bladder	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Spinal Cord	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Thymus	3/5	60	3/5	60	3/5	60	3/5	60	3/5	60
Tongue	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Epididiymis	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Ozephagus	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Salivary gland	4/5	80	4/5	80	4/5	80	4/5	80	4/5	80

Notes: G1: 0.1mg/kg Schiff base-500mg/kg P85, G2: 0.25mg/kg Schiff base-500mg/kg P85, G3: 0.5mg/kg Schiff base-500mg/kg P85 and G4: 1mg/kg Schiff base-500mg/kg P85.

2.2.4. Tumor Volume Measurements

During experiments and after resection of tumors, their sizes were measured by a caliper according to the formula 2.1 given below [258]:

$$Tumor\ volume = (\text{Lenght} \times \text{width} \times \text{height})/2 \quad (2.1)$$

2.2.5. Pathological analysis

Paraffin slides were taken and stained with hematoxylin and eosin for Gleason score analysis according to the previously described protocol [259-260]. Briefly, slides were placed in an incubator at 55 °C for 30 min to remove paraffin. Then, slides were deparaffinized immersing in xylene (#108685, Merck, Darmstadt, Germany) two times for 10 min each. Slides were rehydrated through a decreasing series of ethanol (100%, 95% and 70%). Sections were rinsed gently under running tap water for 10 min and stained with hematoxylin (#HHS16, Sigma, St Louis, USA) for 2 min. Then, slides were washed under tap water for 10 min and stained with eosin (#HT110216, Sigma, St Louis, USA) for 30 seconds. Slides were washed under tap water and dehydrated increasing concentration of alcohol (70%, 95% and 100%). Then, they were cleared with xylene and mounted by Canada balsam (#101691, Merck, Darmstadt, Germany).

2.3. STATISTICAL ANALYSIS

The data were statistically analyzed using one-way analysis of variance and Tukey post hoc test. The values of $P<0.05$ were considered statistically significant.

3. RESULTS

3.1. CELL VIABILITY ANALYSIS

Cell viability analyses were completed in order to investigate potential cytotoxic effects of drug combinations on prostate cancer cell lines (Tramp-C1, PC-3, DU 145 and LNCaP) and healthy cell lines (PNT1A, L-929 and HF). Docetaxel, an accepted and effective chemotherapeutic regimen for prostate cancer treatment [251], was used as positive control throughout the study. Various concentrations of Schiff base and Docetaxel (0.5, 1, 2 and 5µg/ml) were used to detect cell viability of prostate cancer cells and healthy cell lines. The results revealed that all concentrations of Schiff base and its pluronic combinations significantly decreased cell viability of Tramp-C1 cells for three days in a time dependent manner. At the end of 3-days incubation period, while Schiff base application reduced cell viability to an average ratio of 40%, its pluronic combinations decreased the cell viability ratio to 30% on average, indicating synergistic cytotoxic activity of pluronic P85 with Schiff base against Tramp-C1 cells (Figure 3.1A). The same response was detected for Docetaxel treatment. Viable cell ratio of Tramp-C1 cells reduced to 40 and 30% in Docetaxel and its pluronic combination groups, respectively, at the end of 3 days (Figure 3.1B). However, pluronic treatment did not significantly change cell viability of Tramp-C1 cancer cells (Figure 3.1).

PC-3 cells exhibited relatively more resistant phenotype compared to Tramp-C1 cells and viable cell ratio only reduced to 80% at the highest dose of Schiff base (5µg/ml) and its pluronic combination for day 1 and day 2. While there was not any significant difference between moderate concentrations of Schiff base (2, 1 and 0.5µg/ml) and growth medium treated control cells, significant reduction at viable cell ratio (42%) was observed in 5µg/ml of Schiff base treated groups at day 3 (Figure 3.2A). However, combining pluronic P85 with intermediate concentrations of Schiff base (2, 1 and 0.5µg/ml) increased anticancer activity against PC-3 cells. Docetaxel was found to be more effective against PC3 cells compared to Schiff base. Significant toxicity was noted for both Docetaxel and its pluronic combinations for day 1, 2 and 3. Moreover, Docetaxel application alone was found to be more efficient compared to its pluronic combination at day 1 and 2, whereas

combinations were more effective than stand-alone Docetaxel application at day 3 with an average 40% viable cell ratio (Figure 3.2B). As for Tramp-C1 cells, pluronic treatment did not significantly change cell viability of PC-3 cells (Figure 3.2).

All doses of Schiff base tested (except 5µg/ml) were not found to be cytotoxic against DU 145 cells after 24h treatment and the concentration of 5µg/ml provided an average proliferation inhibition of 50%. Anticancer activity of Schiff base against DU 145 cell significantly increased when specified concentrations were combined with pluronic P85 (Figure 3.3). After 24h, Schiff base-P85 treatment significantly decreased viable cell ratio of DU 145 cells in a dose dependent manner. At day 2, the highest concentration of Schiff base tested (5µg/ml) and all concentrations of Schiff base-P85 group decreased viable cell percentage to 20% and other stand-alone Schiff base concentrations (2, 1, and 0.5µg/ml) significantly decreased viable cells to a percentage of 50% on average. All experimental treatments except pluronic alone reduced viable cell ratio to an average of 50% at day 3. (Figure 3A). Docetaxel displayed quite similar effect as Schiff base. Stand-alone application of Docetaxel even the highest concentration was not efficient as in Schiff base treatment for day 1, whereas viable cell ratio reduced to almost 20% at day 2 (Figure 3B). Combination of P85 with Docetaxel gave the best growth inhibition rate for all time intervals with respect to Docetaxel alone administration. However, pluronic P85 application alone did not significantly change cell viability of DU 145 cells at the end of 3 days incubation period (Figure 3).

As in Tramp-C1 and PC-3 cells, growth of LNCaP cells treated with the Schiff base and Schiff base-P85 were repressed in a time and dose dependent manner. Significant reduction in viable cell ratio (up to 80% for highest concentration of Schiff base) was observed at day 3 (Figure 3.4A). Synergistic anticancer activity between the Schiff base and P85 was detectable only at day 3. While the Schiff base treatment provided an average inhibition ratio of 25% at the end of 3-days incubation period, P85 combination provided 52% inhibition ratio on average. Docetaxel also significantly reduced cell viability for three days. Interestingly, Docetaxel alone was noted to be more efficient than its combination with P85. Average cell viability ratios reduced to 30% and 40% in Docetaxel alone and its pluronic combination treated groups, respectively, after 72h **(**Figure 3.4B).

Pluronic P85 treatment alone did not significantly change cell viability of LNCaP cells at the end of 3 days incubation period (Figure 3.4).

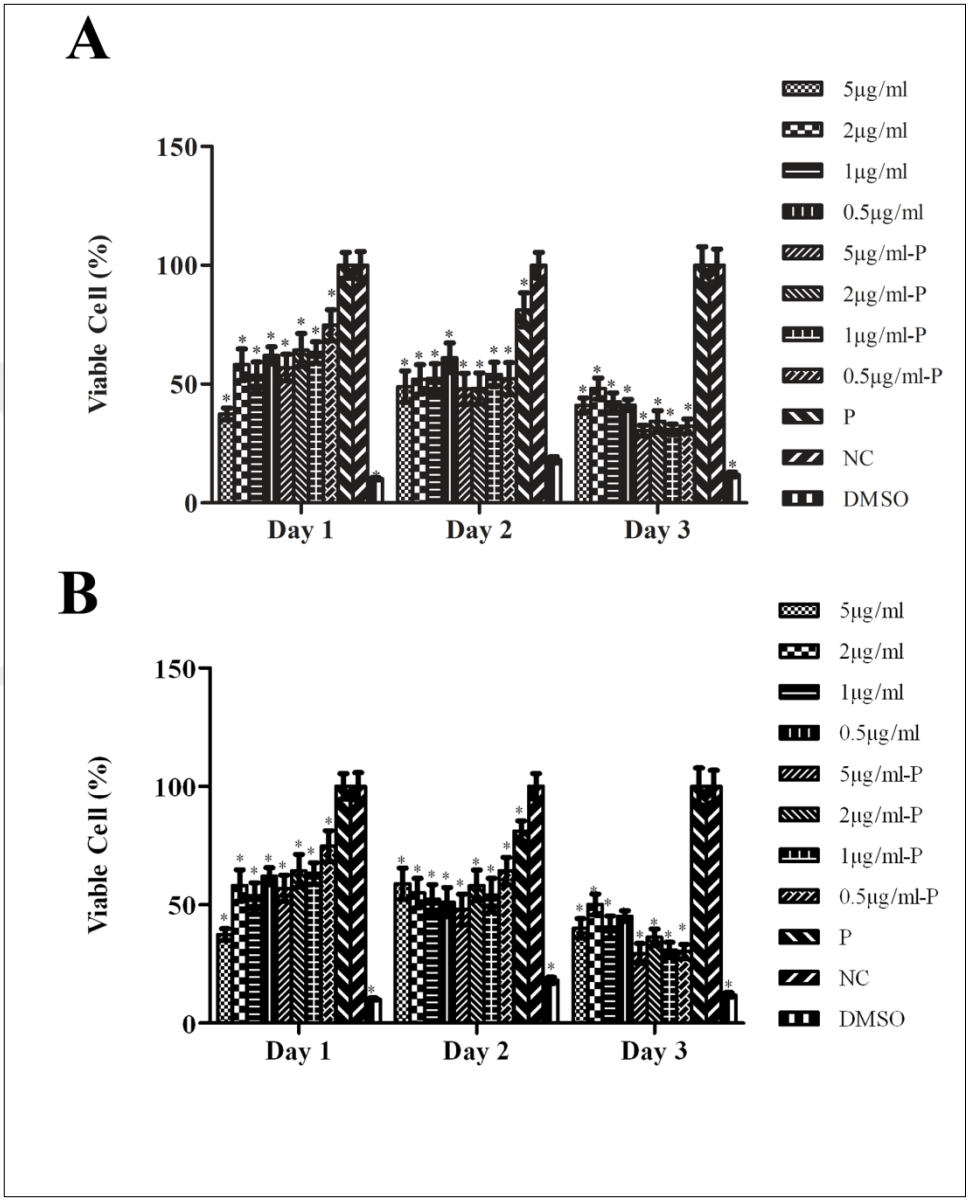

Figure 3.1. Effect of various concentrations of Schiff Base and its combination with Pluronic P85 (A) and Docetaxel and its combination with P85 (B) on the cell viability of Tramp-C1 cells. Abbreviations: P: Pluronic P85 (0.05% w/v) containing growth medium, NC: Negative Control (Growth medium), DMSO: Dimethyl sulfoxide (20% v/v) containing growth medium, *P<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

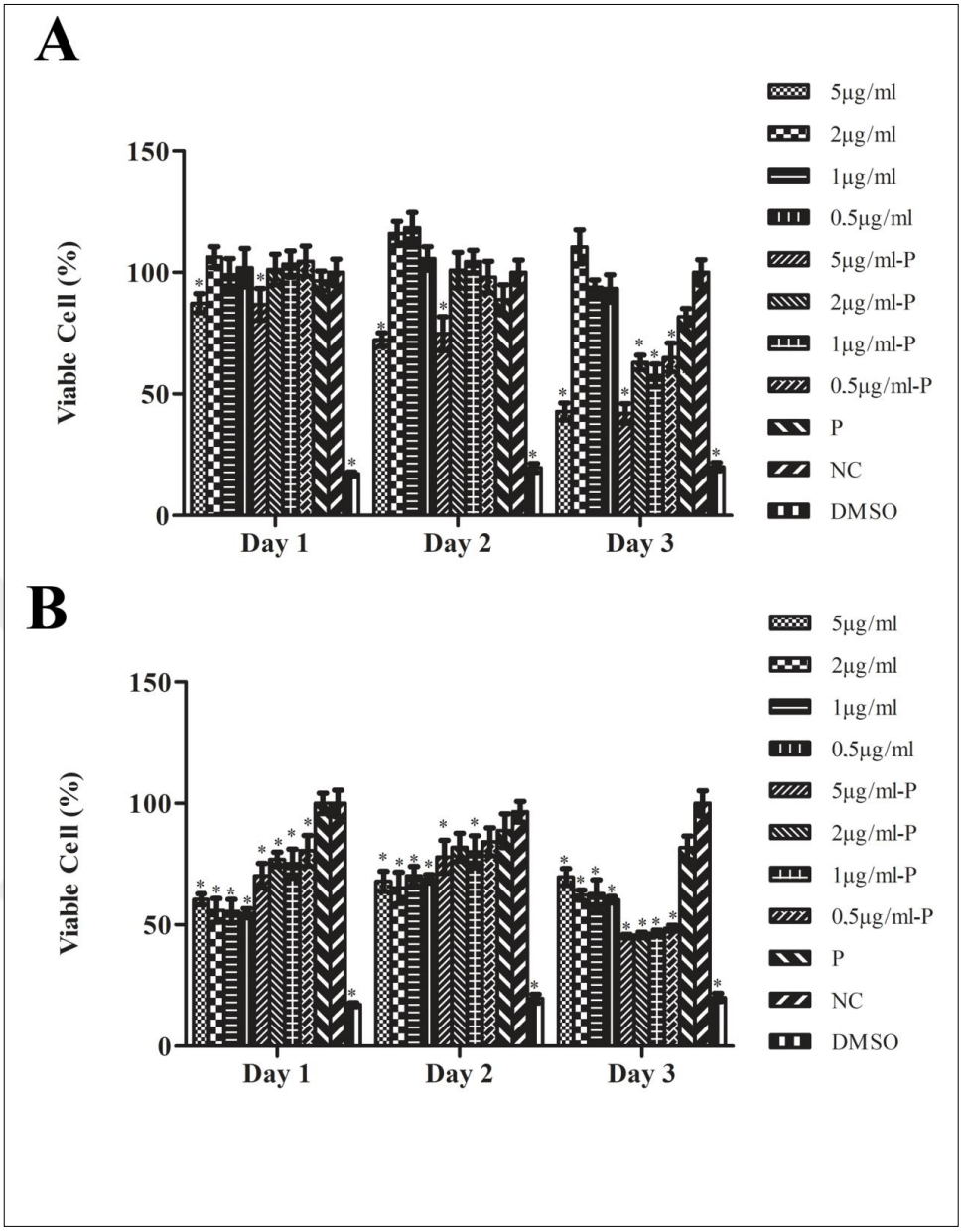

Figure 3.2. Effect of various concentrations of Schiff Base and its combination with Pluronic P85 (A) and Docetaxel and its combination with P85 (B) on the cell viability of PC-3 cells. Abbreviations: P: Pluronic P85 (0.05% w/v) containing growth medium, NC: Negative Control (Growth medium), DMSO: Dimethyl sulfoxide (20% v/v) containing growth medium, **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

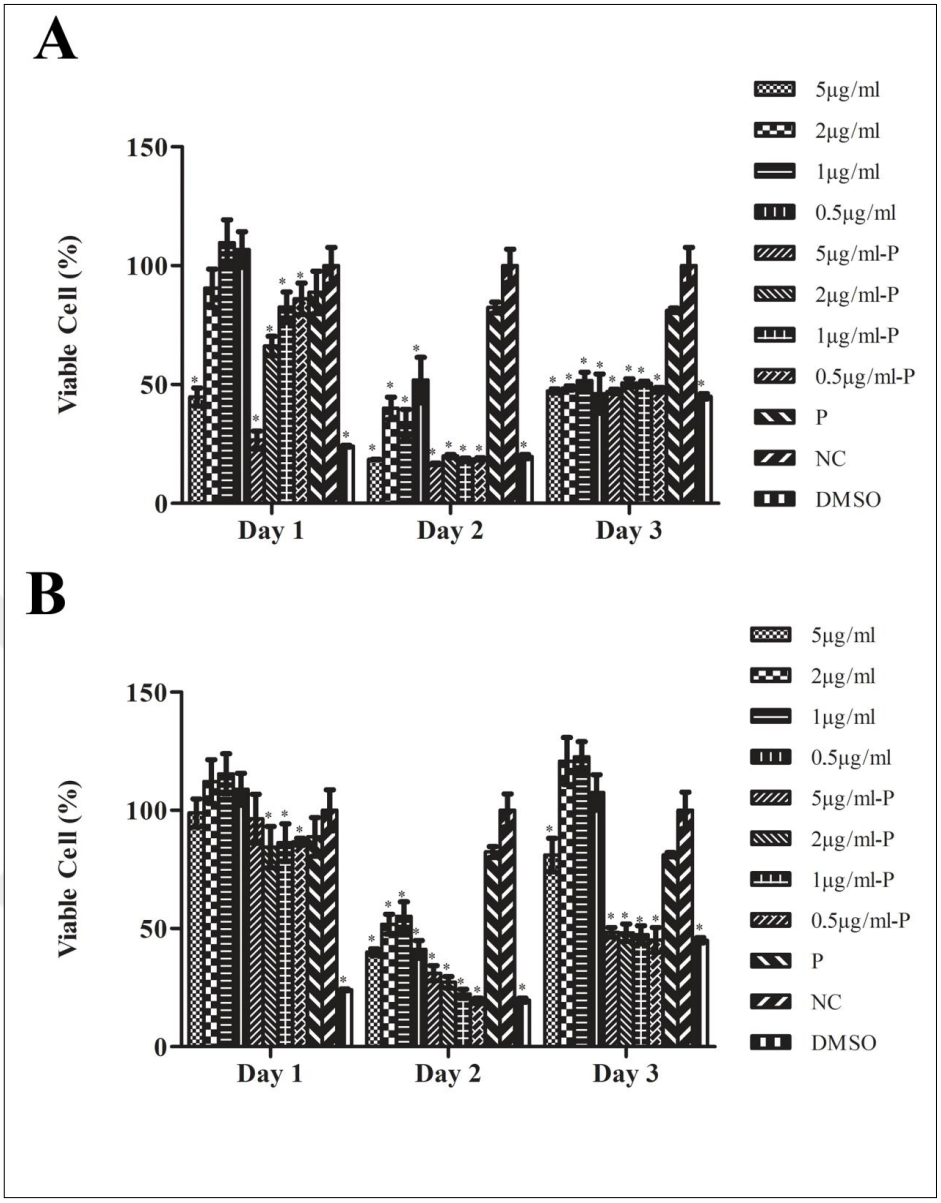

Figure 3.3. Effect of various concentrations of Schiff Base and its combination with Pluronic P85 (A) and Docetaxel and its combination with P85 (B) on the cell viability of DU 145 cells. Abbreviations: P: Pluronic P85 (0.05% w/v) containing growth medium, NC: Negative Control (Growth medium), DMSO: Dimethyl sulfoxide (20% v/v) containing growth medium, **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

Proliferation of PNT1A cells, healthy prostate epithelium cells, was also inhibited by both Schiff base, Docetaxel and their pluronic combinations. Schiff base exerted advanced toxicity at high doses, whereas its toxicity was acceptable for the concentrations of 1 and 0.5µg/ml at day 1 (Figure 3.5). However, all concentrations of Schiff base and Schiff base-P85 combinations (except 0.5µg/ml of Schiff base) displayed significant levels of toxicity at day 2 and 3 (Figure 5A). Docetaxel alone and its pluronic combinations decreased viable cells in time dependent manner. While the viable cell ratio was around 60% at the first two days, Docetaxel and Docetaxel-P85 combination decreased viable cell ratio to 40% on average at day 3 (Figure 5B). Pluronic P85 treatment did not result in a significant change in cell viability of PNT1A cell for all three days (Figure 5).

HF cells were used as another healthy cell line for *in vitro* toxicology analysis. Fibroblast cells were found to be more resistant to chemical's toxicity in comparison with PNT1A cells. There were no toxicity of Schiff base alone (except 5µg/ml) for all 3 days, whereas Schiff base-P85 combination displayed significant levels of toxicity at day 3 in a dose dependent manner (Figure 3.6A). On the other hand, Docetaxel and its pluronic combination reduced cell viability for all time intervals. Pluronic P85 administration, however, did not significantly change cell viability of HF cells (Figure 3.6).

L-929 cells were used as mouse fibroblast cell line to investigate whether Schiff base and its pluronic combination exerted species-specific response. Interestingly, Schiff base did not significantly reduce the viable cell ratio of L-929 cells for the first 48h as it decreased in PNT1A and HF experiments. On the other hand, stand-alone applications of 5, 2 and 1µg/ml of Schiff base reduced the viable cell numbers at day 3. None of the Schiff base concentrations combined with pluronic P85 displayed any cytotoxicity against L-929 cells for all 3 days (Figure 3.7A). Although Docetaxel administration to L-929 cell was found to be safe for the first 48h, it is alone or combination with P85 was found to be completely toxic to L-929 cells at day 3 (Figure 3.7B). Pluronic P85 at 0.05 (w/v) concentration did not significantly change cell viability of mouse fibroblast cells (Figure 3.7).

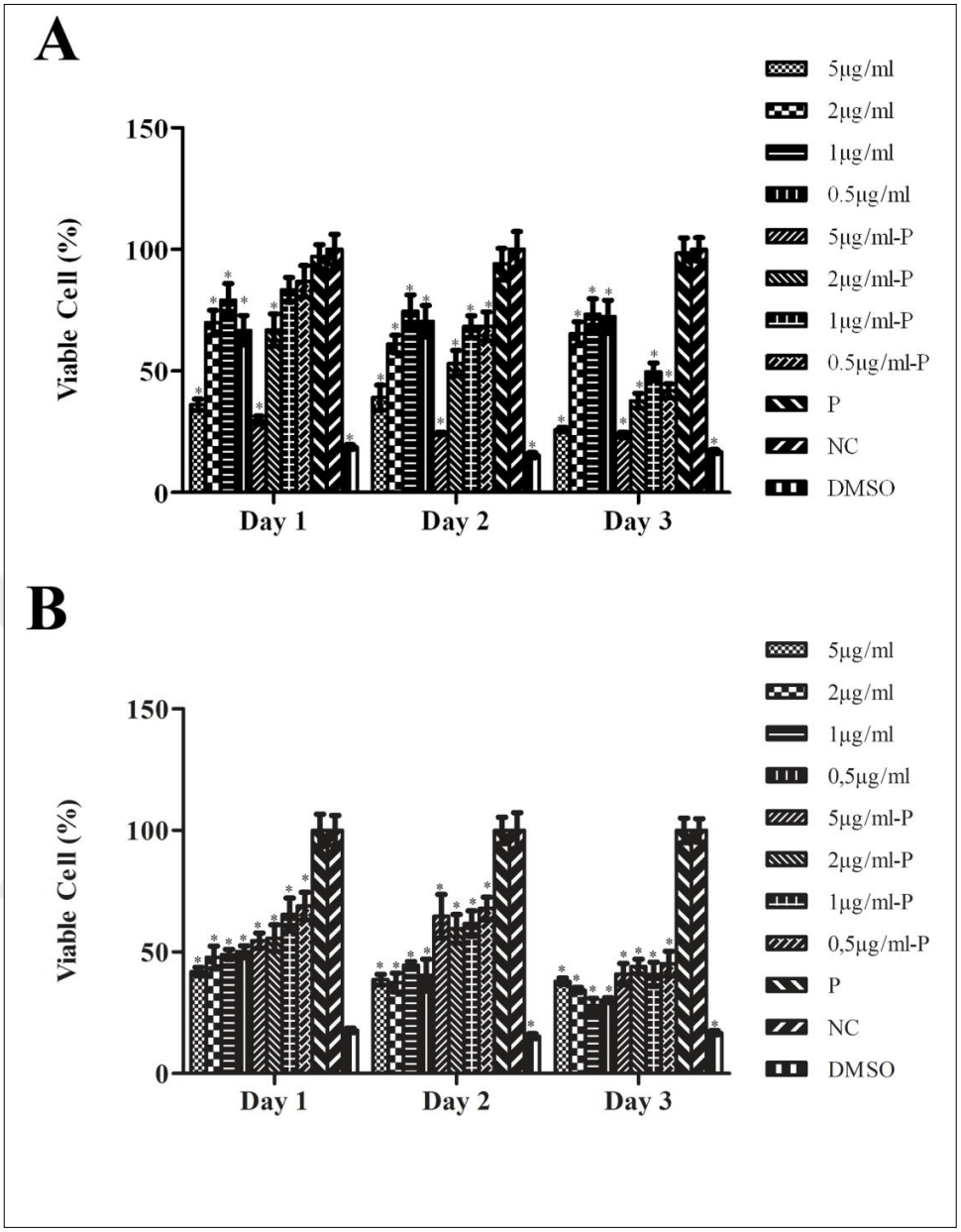

Figure 3.4. Effect of various concentrations of Schiff Base and its combination with Pluronic P85 (A) and Docetaxel and its combination with P85 (B) on the cell viability of LNCaP cells. Abbreviations: P: Pluronic P85 (0.05% w/v) containing growth medium, NC: Negative Control (Growth medium), DMSO: Dimethyl sulfoxide (20% v/v) containing growth medium, **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

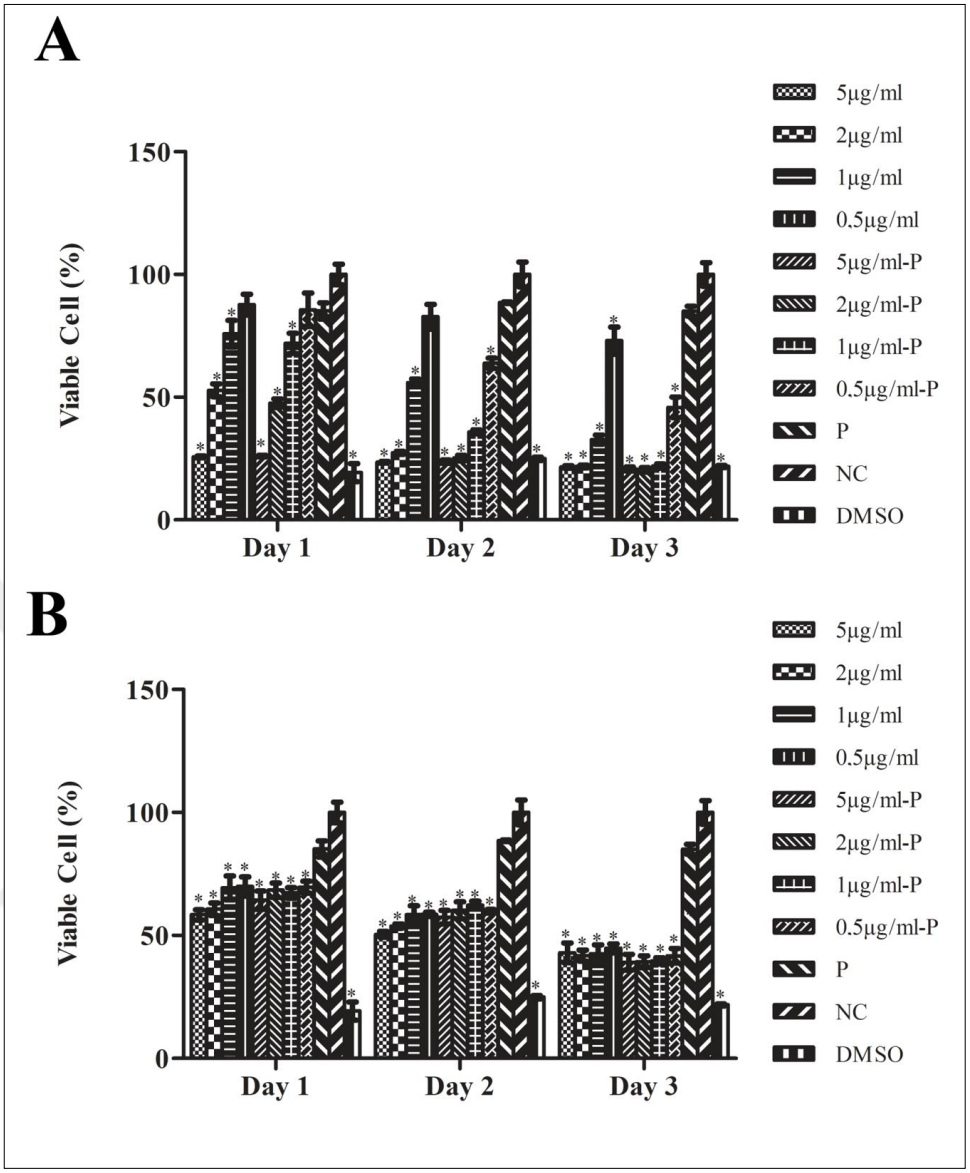

Figure 3.5. Effect of various concentrations of Schiff Base and its combination with Pluronic P85 (A) and Docetaxel and its combination with P85 (B) on the cell viability of PNT1A cells. Abbreviations: P: Pluronic P85 (0.05% w/v) containing growth medium, NC: Negative Control (Growth medium), DMSO: Dimethyl sulfoxide (20% v/v) containing growth medium, **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

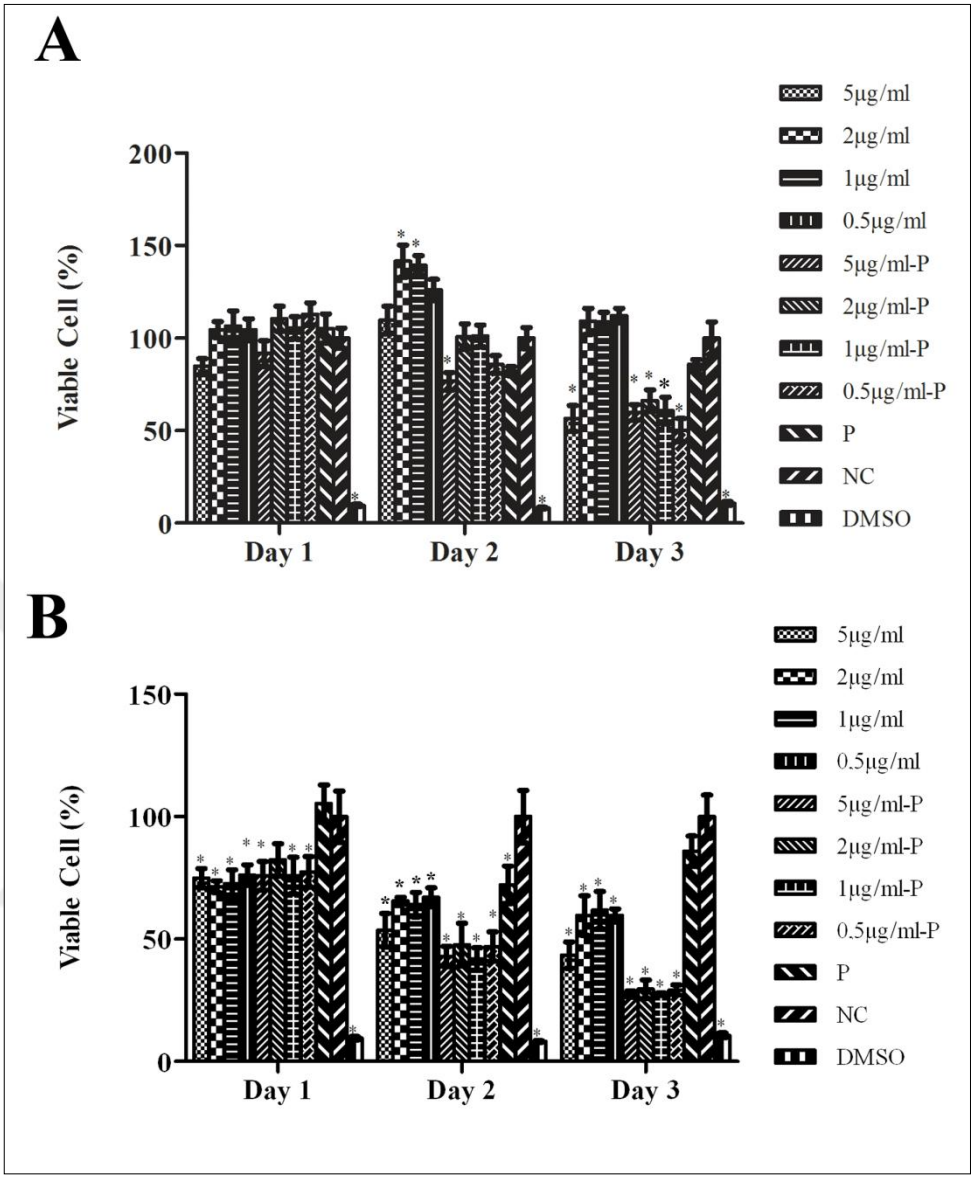

Figure 3.6. Effect of various concentrations of Schiff Base and its combination with Pluronic P85 (A) and Docetaxel and its combination with P85 (B) on the cell viability of human fibroblast cells. Abbreviations: P: Pluronic P85 (0.05% w/v) containing growth medium, NC: Negative Control (Growth medium), DMSO: Dimethyl sulfoxide (20% v/v) containing growth medium, **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

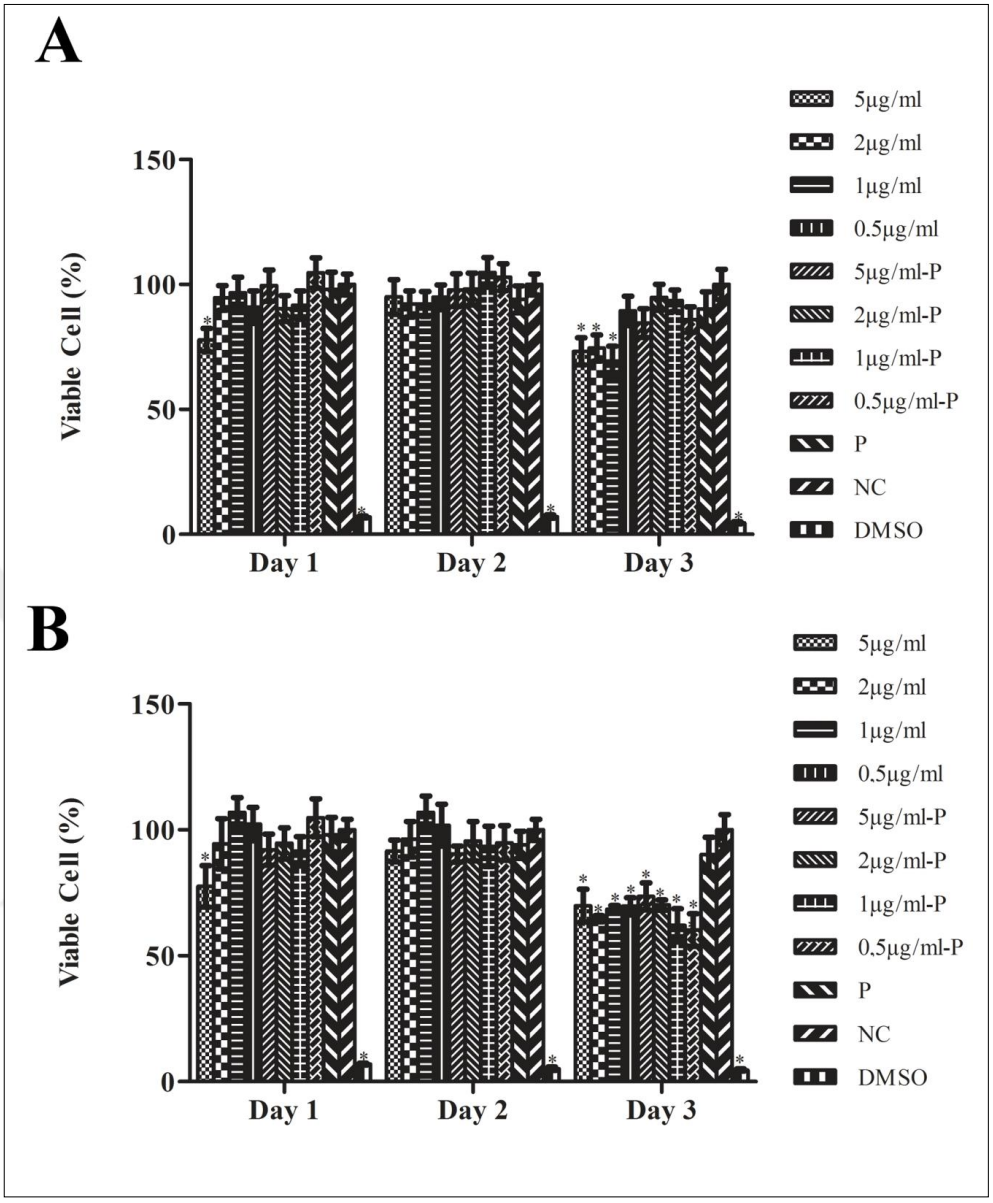

Figure 3.7. Effect of various concentrations of Schiff Base and its combination with Pluronic P85 (A) and Docetaxel and its combination with P85 (B) on the cell viability of L-929 cells. Abbreviations: P: Pluronic P85 (0.05% w/v) containing growth medium, NC: Negative Control (Growth medium), DMSO: Dimethyl sulfoxide (20% v/v) containing growth medium, **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

In vitro toxicology analysis results revealed that both agents (Schiff base-P85 and Docetaxel) were significantly reduced the cell viability of cancer cells. The significant reduction on the cell viability was detected at day 3 for almost all cell types. Schiff base was found to exert remarkable anticancer activity against prostate cancer cells as effective as Docetaxel. On the other hand, effect of Schiff base on healthy cell lines was detected to change depending on cell type.

3.2. CASPASE ASSAY

Cancer cells exposured to chemotherapeutic formulations were subjected to Caspase 3 assay analysis. Apoptotic status of cancer cells (LNCaP, DU 145, PC-3 and Tramp-C1) and healthy prostate cell (PNT1A) were determined. Results were represented as the amount of PNA release indicating the Caspase 3 activity. Schiff base, Docetaxel and their pluronic formulation significantly increased the enzyme activity in DU 45 cells. 1.4 fold increase was observed in Schiff base and Schiff base-P85 group. Docetaxel-P85 group caused a 2.2 fold increase in enzyme activity for DU 145 cells. Pluronic P85 decreased Caspase 3 enzyme activity in cultured DU 145 cells. LNCaP cells responded to chemotherapeutics more efficiently. Schiff base, Schiff base-P85 and Docetaxel-P85 increased the caspase3 enzyme activity about 4 fold compared to control LNCaP cells. Pluronic P85 also increased enzyme activity (2 fold) in LNCaP cells. PC-3 cells exhibited a similar outcome as LNCaP cells. PNA release increased to 2.8μM in Schiff base-P85 treated cells when control is 0.4 μM. Schiff base and Docetaxel-P85 caused an approximately 4-fold increase in enzyme activity. . Pluronic P85 also increased enzyme activity (2 fold) in PC-3 cells. Tramp-C1 cells showed a different phenotype for Caspase 3 assay. The significant enhance was observed in Schiff base-P85 group. PNA release increased to 10μM in Schiff base-P85 group when control is 4μM. Docetaxel and its pluronic P85 combination did not cause a significant increase in enzyme activity. On the other hand Schiff base application decreased the PNA release to 1.4 μM in Tramp-C1 cells. PNT1A cells were used as healthy prostate cancer cells and Schiff base-P85 and Docetaxel-P85 significantly increased the PNA release as an indicator of Caspase 3 activity. An approximately 1.3 fold increase was obtained in Schiff base-P85 and Docetaxel-P85 groups (Figure 3.8).

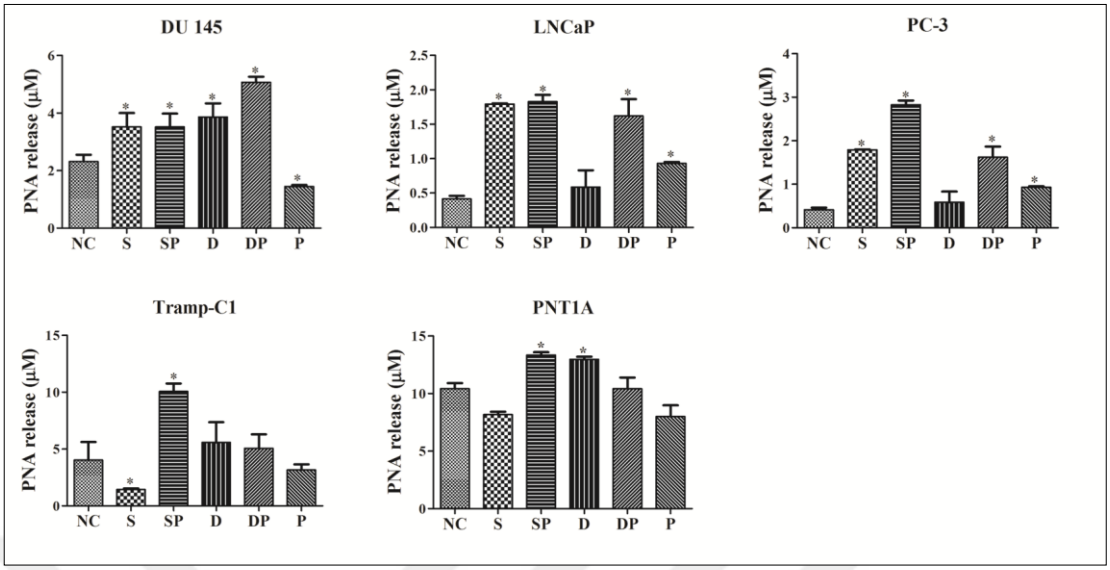

Figure 3.8. PNA release indicating the Caspase 3 enzyme activity in prostate cancer and healthy cells treated with Schiff Base, Docetaxel and their combination with P85. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml).

3.3. RT-PCR ANALYSIS

LNCaP, DU 145, PC-3, PNT1A and Tramp-C1 cells were treated with chemotherapeutics (2µg/ml of Schiff base or Docetaxel) and their combinations with pluronic P85 (0.05% w/v) in order to detect gene expression levels of specific cancer and apoptosis related markers.

3.3.1. DU-145 Cells

RT-PCR assay results showed that apoptotic BAX gene levels significantly increased (2.5-fold) in Docetaxel-P85 combination treated group compared to growth medium treated group. Although P85 application results in a slight increase in anti-apoptotic Bcl-2 gene expression, no significant change was detected between all experimental groups. Caspase-3 and AR gene levels enhanced in Schiff base-P85 combination group for an approximately 2- and 2.5-fold, respectively, while Docetaxel application reduced mRNA levels. Akt, NF-κB, ELK-1, BCRP, EGFR and MRP gene expression levels significantly decreased 2- to 5-fold in Schiff base and Schiff-P85 group. Although BCRP, EGFR, ELK-1 and MRP gene

levels also reduced in Docetaxel treated cells, Schiff base application was found to be more potent. Pluronic P85 did not significantly change most of the gene expression levels except ELK-1 (Figure 3.9).

3.3.2. LNCaP Cells

As in DU 145 cells, P85 treatment did not cause any significant change in any gene expression levels examined in LNCaP cells. Approximately 2-fold decrease in AR expression was noted in all chemotherapeutic groups. BAX and Caspase-3 mRNA levels increased 2.5-fold on average in Schiff base-P85 applied group. Although stand-alone Docetaxel and P85 treatments did not cause any significant change in Bcl-2 and EGFR mRNA levels, combination of those chemicals markedly augmented the gene expression levels 1.7- and 1.3-fold, respectively (Figure 3.10). On the other hand, combining P85 with Schiff base decreased Akt, NF-κB, EKL-1, EGFR, MRP and BCRP gene expression levels, whereas Schiff base alone enhanced levels of Akt, NF-κB, EKL-1 and did not significantly change EGFR and MRP mRNAs, indicating synergistic activity between the Schiff base and P85 (Figure 3.10).

3.3.3. PC-3 Cells

BCRP, EGFR and MRP gene expression levels were detected to augment and levels of Bcl-2 mRNA remained constant in all experimental groups. On the other hand, Akt, NF-κB and ELK-1 mRNA levels reduced in Schiff base and Schiff-P85 groups. While low level of Caspase-3 mRNA was noted in Schiff base and Docetaxel treated cells, high Caspase-3 expression was detected in P85 combination groups (≈1.5-fold). In addition, AR mRNA levels decreased in only P85 combination treated cells. Chemotherapeutic application decreased the ELK-1 expression 2-fold in all chemotherapeutic treated groups (Figure 3.11).

3.3.4. PNT1A Cells

Bcl-2 and NF-κB gene levels in all experimental groups were not different from the baseline. However, BAX, Akt, ELK-1, MRP, BCRP and EGFR levels were found to be 2-

fold lower in all application groups (except P85) compared to control group. In contrast to cancer cell lines, Schiff-P85 treatment did not result in a significant increase in Caspase-3 gene expression levels. Along with a slight decrease in Akt and BAX levels, pluronic P85 application did not significantly change gene expression levels (Figure 3.12).

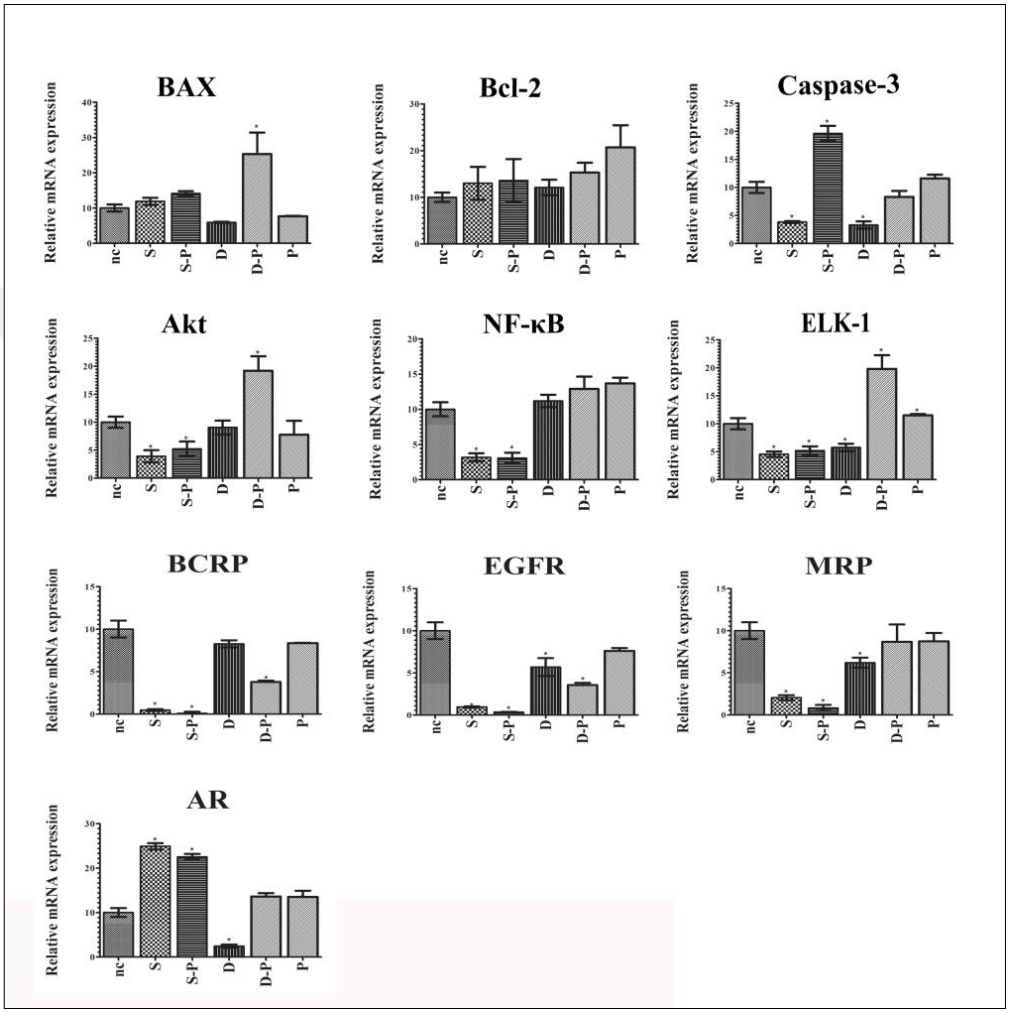

Figure 3.9. Effect of Schiff Base, Docetaxel and their combinations with P85 on gene expression profile of DU 145 cells. Abbreviations: P: Pluronic P85 (0.05% w/v), nc: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

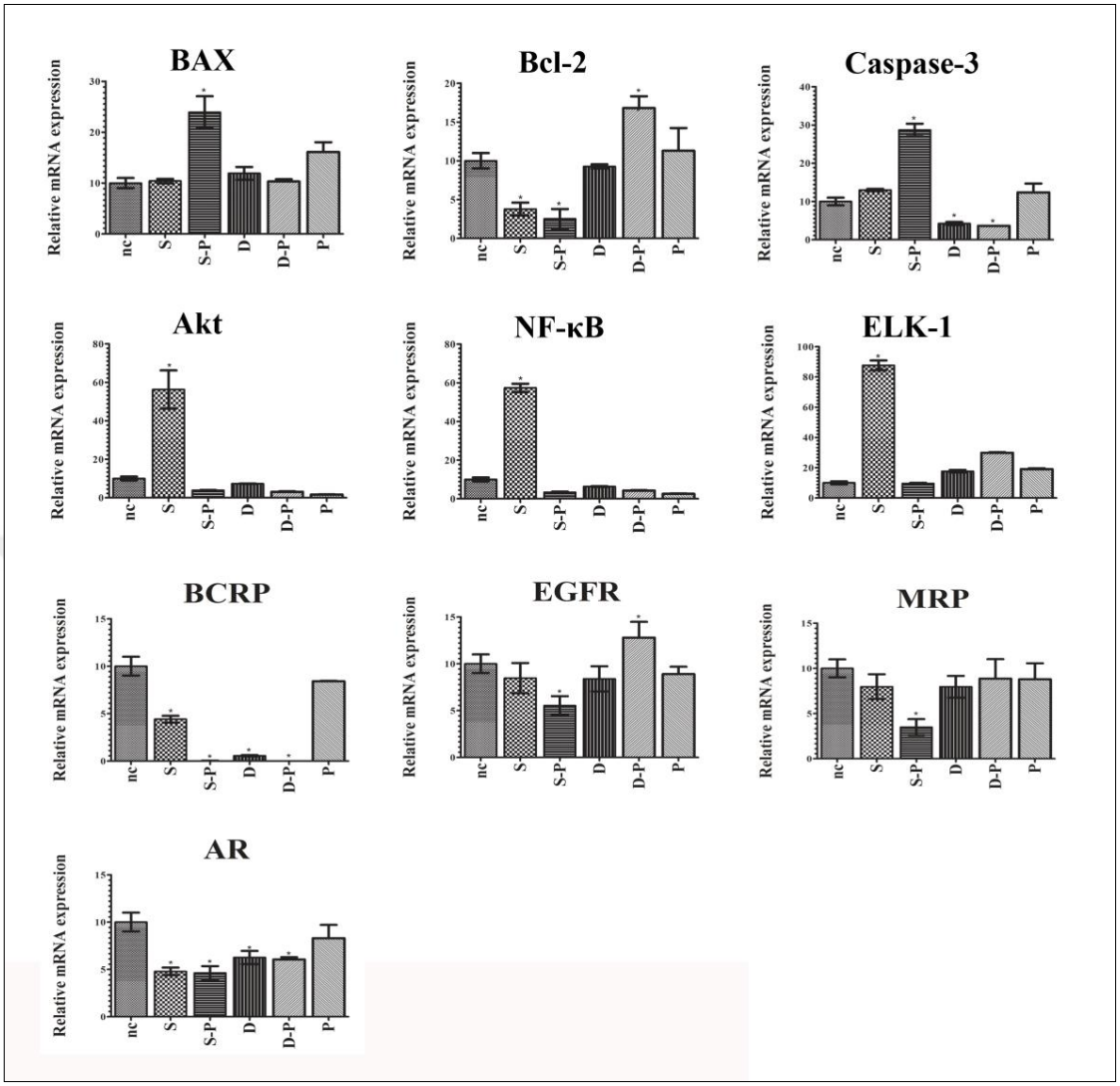

Figure 3.10. Effect of Schiff Base, Docetaxel and their combinations with P85 on gene expression profile of LNCaP cells. Abbreviations: P: Pluronic P85 (0.05% w/v), nc: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

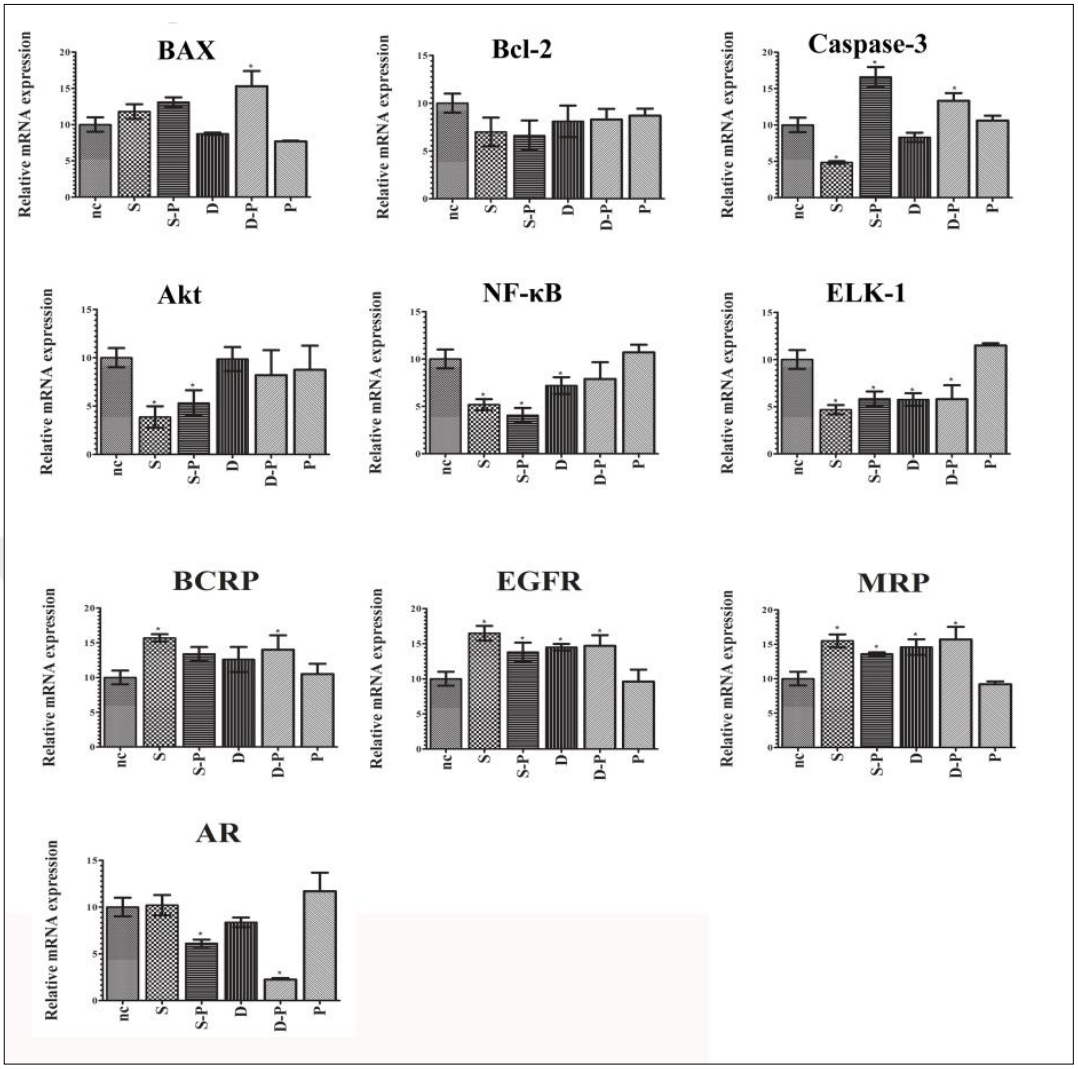

Figure 3.11. Effect of Schiff Base, Docetaxel and their combinations with P85 on gene expression profile of PC-3 cells. Abbreviations: P: Pluronic P85 (0.05% w/v), nc: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

3.3.5. Tramp-C1 Cells

Schiff-P85 treatment significantly augmented BAX and Caspase-3 levels in Tramp-C1 cells, whereas Schiff base and P85 alone decreased mRNA expression. Likewise, although Docetaxel treatment decreased BAX and Caspase-3 levels, P85 combination partially reversed the negative effect. ELK-1 mRNA levels were found to remain stable in all groups.

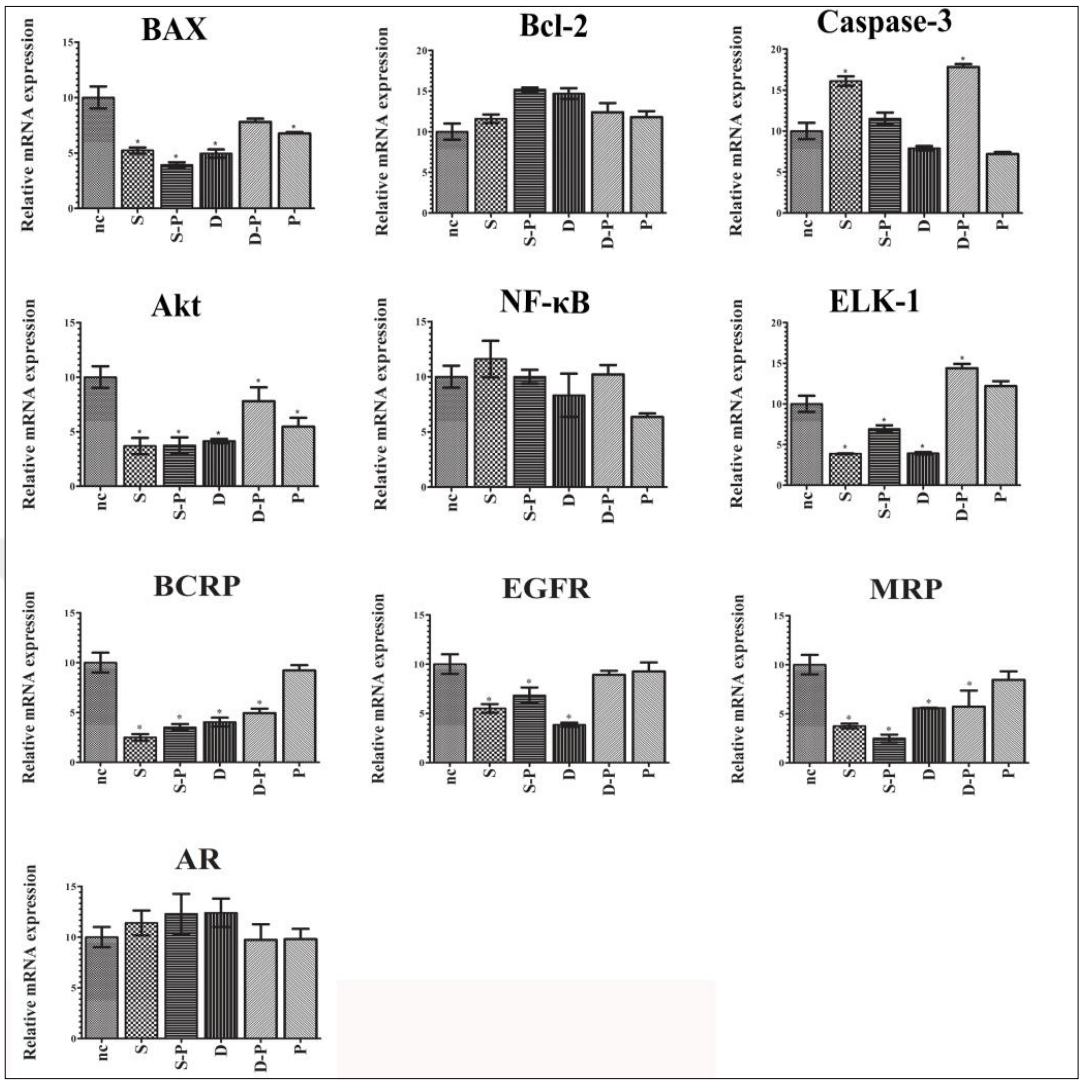

Figure 3.12. Effect of Schiff Base, Docetaxel and their combinations with P85 on gene expression profile of PNT1A cells. Abbreviations: P: Pluronic P85 (0.05% w/v), nc: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

Although BCRP and EGFR gene expression levels decreased up to 5-fold in all drug treated groups, MRP expression increased in Schiff base and Schiff-P85 administered groups. All test conditions also resulted in significant reduction in AR (except Pluronic P85) and Bcl-2 (except Docetaxel-P85) mRNA levels. Interestingly, Tramp-C1 cells treated with Schiff base and Schiff-P85 expressed high levels of Akt and NF-κB (Figure 3.13).

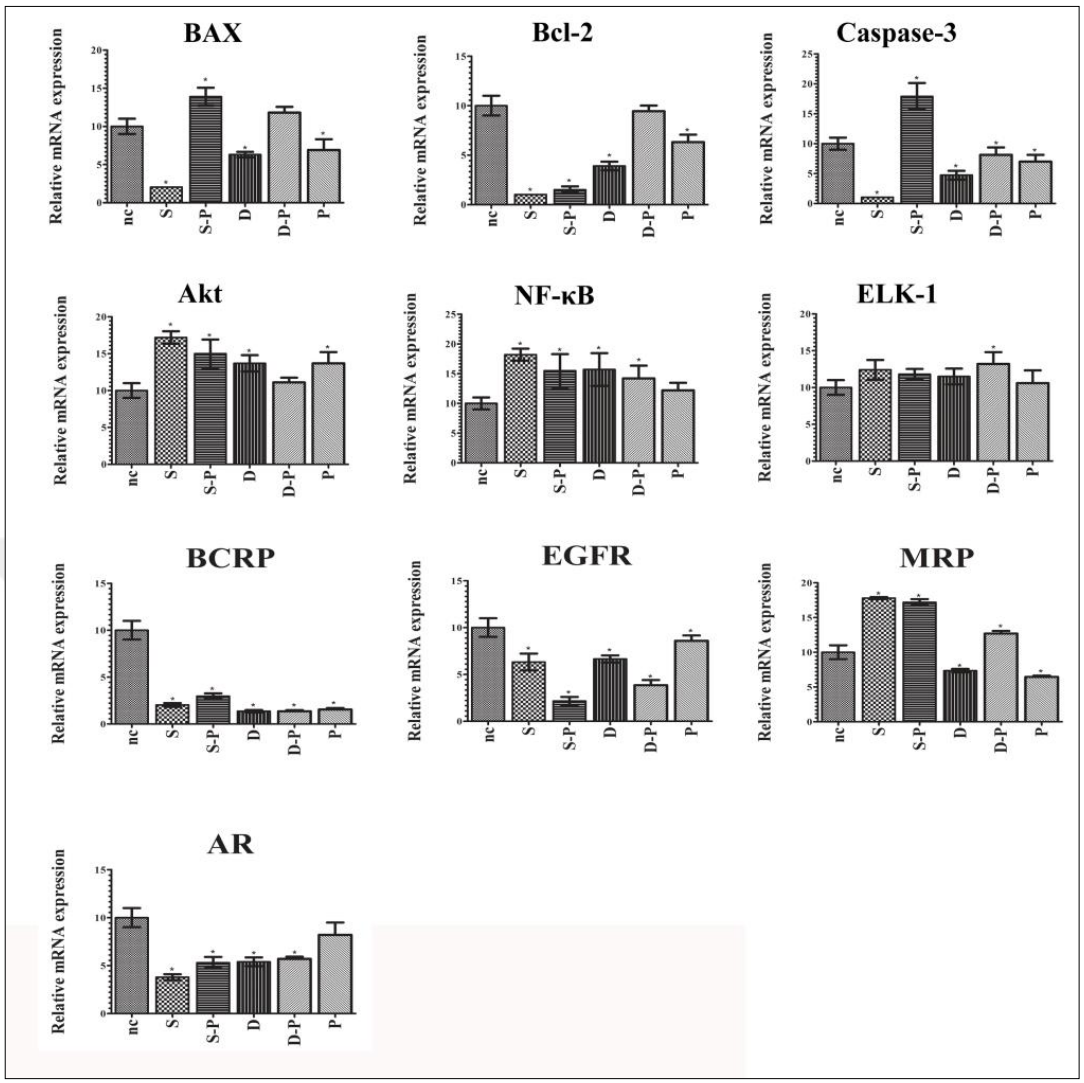

Figure 3.13. Effect of Schiff Base, Docetaxel and their combinations with P85 on gene expression profile of Tramp-C1 cells. Abbreviations: P: Pluronic P85 (0.05% w/v), nc: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

3.4. WESTERN BLOT ANALYSIS

Akt and NF-κB protein levels in Schiff base, Docetaxel and their combinations treated prostate cancer and health cells were detected by western blot analysis. The results revealed that while Akt protein levels did not significantly change in DU 145 cells, NF-κB expression was significantly decreased 0.3-fold on average by Schiff base, Docetaxel, Docetaxel-P85 and P85 treatments (Figure 3.14). However, LNCaP cells expressed

significantly low levels of Akt and NF-κB in Schiff base-P85 groups. In addition, although NF-κB expression in Schiff base alone treated LNCaP cells was not significantly different from the control group, Akt protein levels in this group were detected to be lower (Figure 3.15). Akt and NF-κB protein levels were found to be decreased in Schiff base, Schiff base -P85, Docetaxel-P85 and P85 treated PC-3 cells. Significant reduction was observed in Schiff base -P85 group for Akt protein expression. Docetaxel alone did not cause a significant reduction for both Akt and NF-κB (Figure 3.16). In mouse prostate cancer cells, Tramp-C1, treated with Schiff base alone or in combination with P85, were found to express low levels of NF-κB (≈0.7-fold) compared to growth medium treated cells, whereas Akt levels was similar to each other. On the other hand, NF-κB expression was about 1.3-fold higher in P85, Docetaxel alone or in combination with P85 groups compared to control group (Figure 3.17). Protein levels in PNT1A were quite similar to other cancer cells. Akt and NF-κB protein expressions in Schiff base and Schiff-P85 groups were significantly lower than control, P85 and Docetaxel groups (Figure 3.18).

3.5. CELL MIGRATION ASSAY

In vitro cell migration assay (Scratch assay) was performed to evaluate whether Schiff base affects cell migration capacity of cancer cells. According to the results obtained, both Docetaxel and Schiff base-P85 reduced cell migration and decreased the closure rate of cancer cells. Schiff base alone administration remained ineffective against cancer cell migration, whereas combination of pluronic P85 with Schiff base and Docetaxel increased respective migration inhibition activity and decreased cell motility. Stand-alone Docetaxel administration, on the other hand, was also found to inhibit cell migration. Pluronic P85 treated LNCaP and Tramp-C1 cells moved slower compared to control group (Figure 3.19).

3.6. TRANSWELL CELL MIGRATION ASSAY

Transwell cell migration assay was conducted to evaluate the effects of drug formulations on the metastatic properties of cancer cells. In consistent with cell migration assay's results, although Schiff base and pluronic treatments were found to be ineffective on cell migration (except Tramp-C1 migration), Schiff base-P85 combination drastically decrease

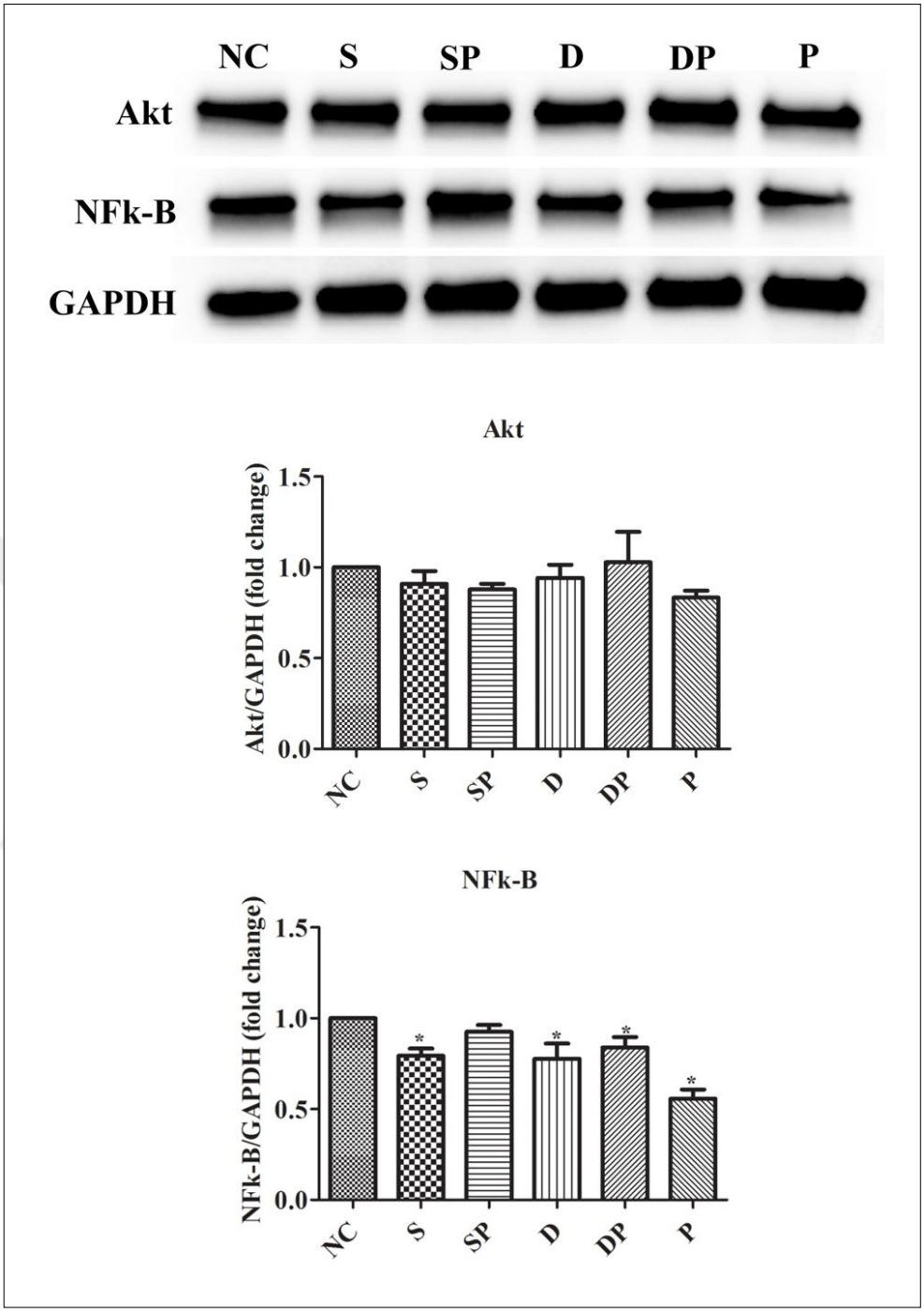

Figure 3.14. Western blot analysis of DU 145 cells. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

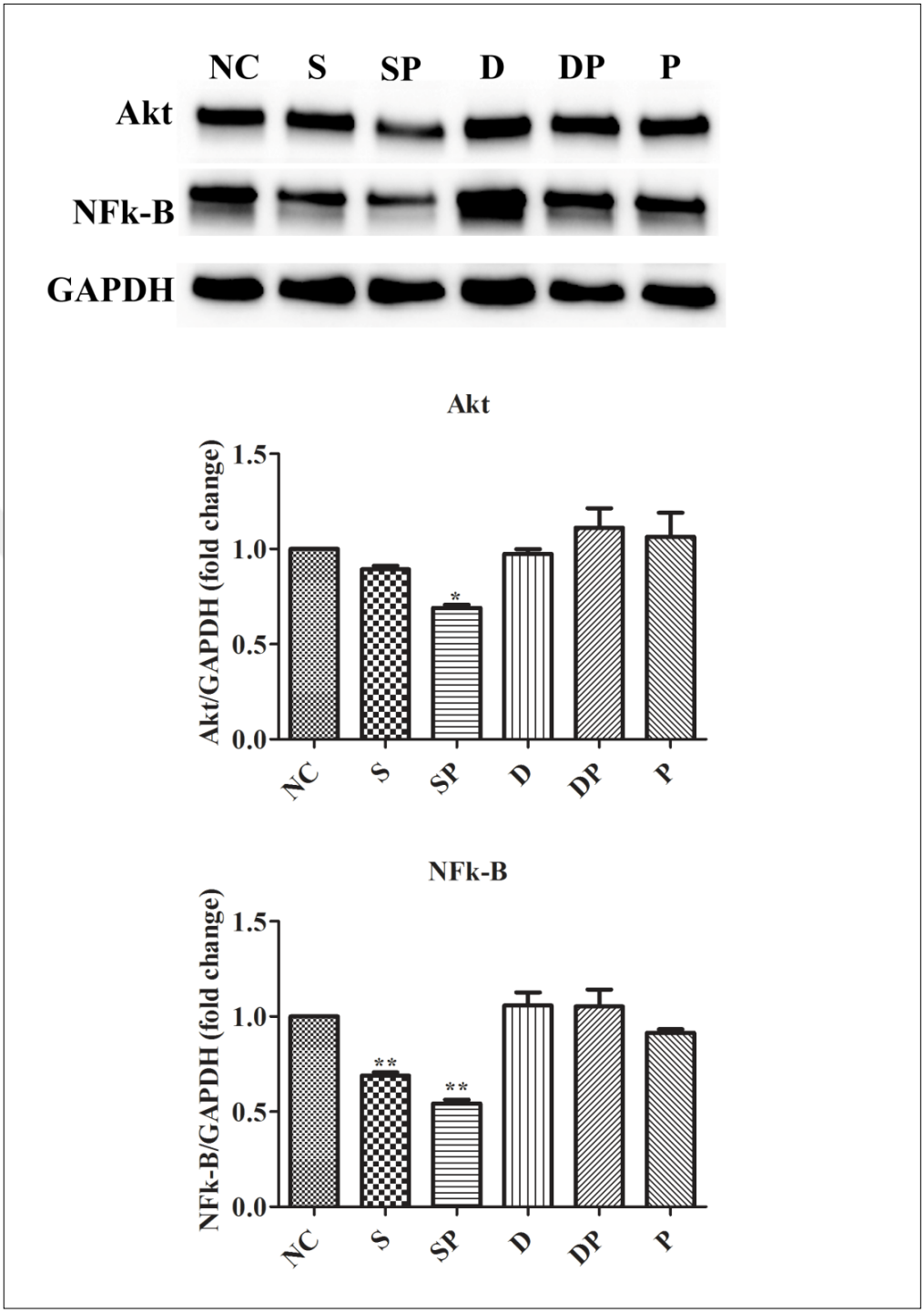

Figure 3.15. Western blot analysis of LNCaP cells. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

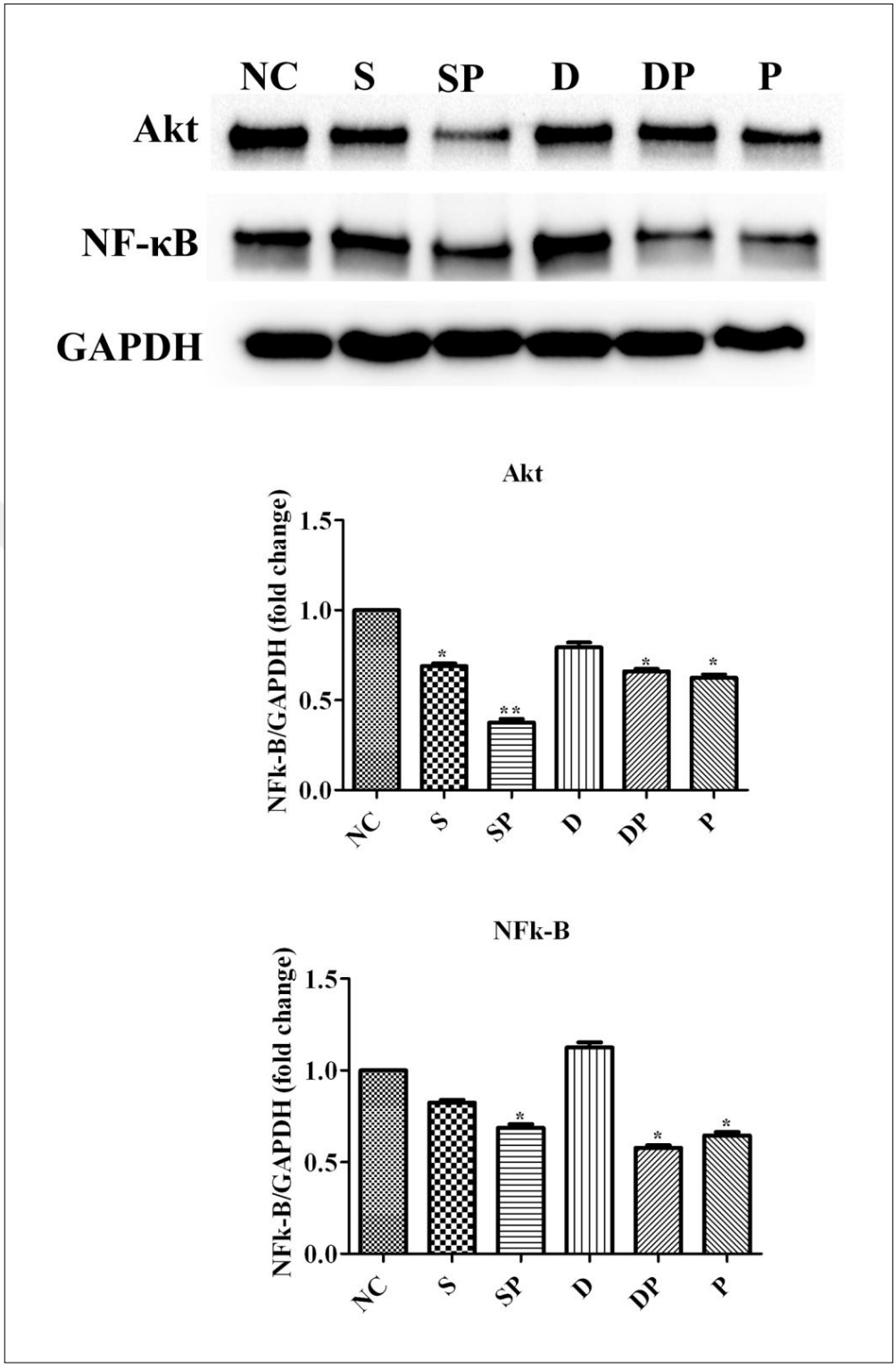

Figure 3.16. Western blot analysis of PC-3 cells. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

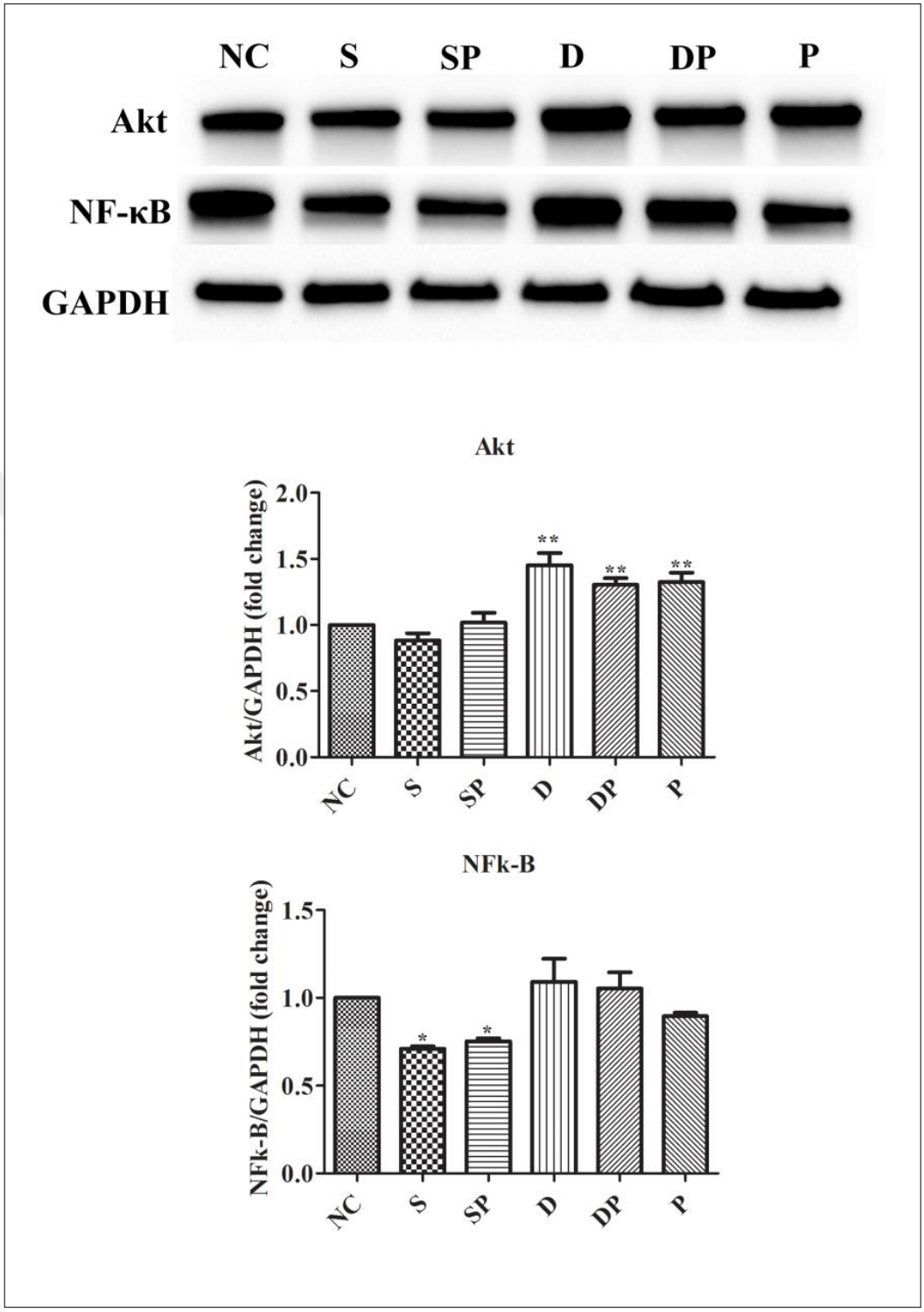

Figure 3.17. Western blot analysis of Tramp-C1 cells. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

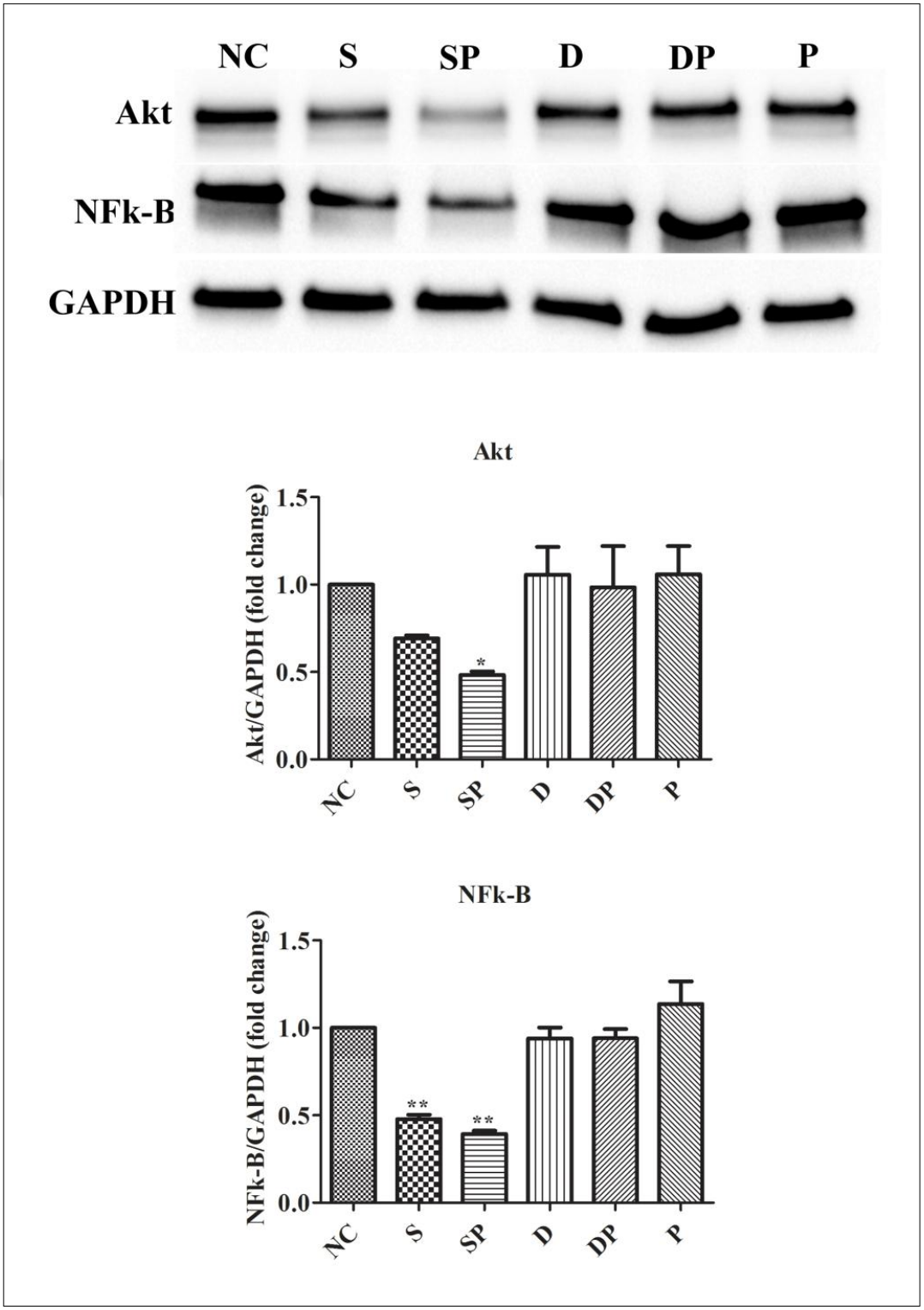

Figure 3.18. Western blot analysis of PNT1A cells. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control (Growth medium), S: Schiff base (2µg/ml), D: Docetaxel (2µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

cancer cell motility through the transwell membrane, indicating synergistic inhibitory activity of P85 and Schiff base. Docetaxel also significantly repressed the number of migrating cells for Tramp-C1, PC-3 and LNCaP cells but not DU 145 cells, whereas migrated PC-3 cells in Docetaxel-P85 combination group were not different from baseline (Figure 3.20).

3.7. TRANSWELL CELL INVASION ASSAY

Transwell cell invasion assay was used to evaluate the effects of chemotherapeutics on the invasive characteristics of prostate cancer cells. Similar to transwell cell migration assay, Schiff base and P85 combination considerably decreased cancer cell invasion. Although P85 combination with Schiff base provided superior inhibition activity compared to Schiff base alone treatment, combination of P85 with Docetaxel did not display significant difference compared to Docetaxel alone treatment. Docetaxel reduced the number of invasive cells for Tramp-C1, PC-3 and LNCaP cells, but not DU 145 cell, and Docetaxel and pluronic combination decreased cell invasiveness of Tramp-C1, DU 145 and LNCaP cells but not PC-3 cells (Figure 3.21).

3.8. ANGIOGENESIS ASSAY

To observe whether Schiff base and its pluronic combination is effective on angiogenesis, a very important process for cancer metastasis and progression, two different angiogenesis experiments were conducted: Tube formation assay and aortic ring assay.

3.8.1. Aortic Ring Assay

Aortic ring assay was conducted to analyze endothelial cell sprouting from rat aortas treated with Schiff base or Schiff base-P85 combination. The results revealed that Schiff base inhibited cell spreading from aortas in a dose dependent manner. While cell spreading from aortic rings were moderately repressed in 1 and 0.5µg/ml Schiff base application group, high doses of Schiff base treatments (2 and 5 µg/ml) and all concentrations of Schiff base-P85 combination group tested inhibited the endothelial cell spreading. Tested concentration of Docetaxel (10^{-8}M) also completely hampered cell sprouting from rat

aortas. On the other hand, pluronic P85 did not inhibit the microvessel growth (Figure 3.22).

3.8.2. Tube Formation Assay

HUVEC cells cultured on matrigel were treated with aforementioned drug combinations to test their effect on tube-like structure formation capacity of endothelial cells. Figure 3.23B illustrates that treatment of 5, 2, 1 and 0.5µg/ml of Schiff base resulted in 85%, 36%, 36%

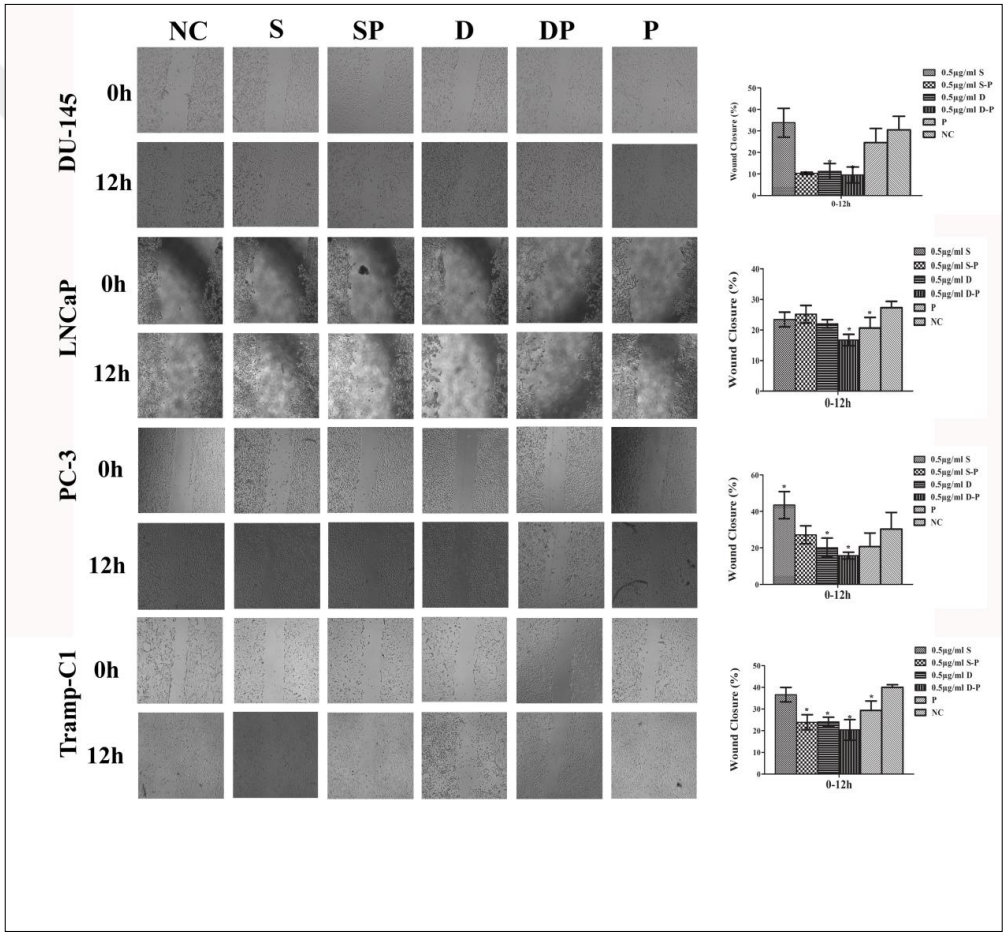

Figure 3.19. Effect of Schiff Base, Docetaxel and their combinations with P85 on prostate cancer cell migration evaluated by in vitro Scratch assay. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control (Growth medium), S: Schiff base (0.5µg/ml), D: Docetaxel (0.5µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

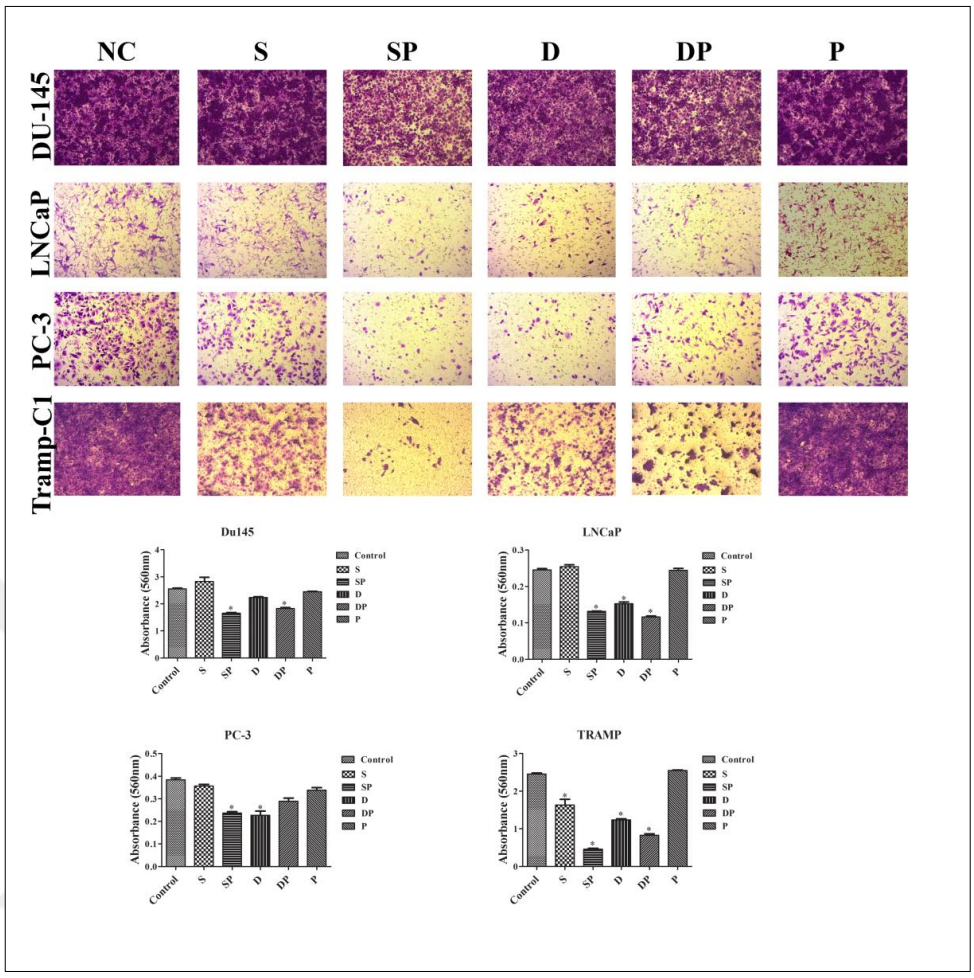

Figure 3.20. Effect of Schiff Base, Docetaxel and their combinations with P85 on prostate cancer cell migration evaluated by Transwell migration assay. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control (Growth medium), S: Schiff base (0.5µg/ml), D: Docetaxel (0.5µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

and 42% decrease in tube-like structure formation, respectively, in comparison with the control group. Pluronic P85 combination further increased the anti-angiogenic activity of the Schiff base and number of branches was 93%, 84%, 81.5% and 84% lower in 5, 2, 1 and 0.5µg/ml of Schiff base combined with 0.05% (w/v) P85, respectively, compared to the control group. As an interesting finding, pluronic P85 also caused a 32% decrease in branch formation of HUVEC cells (Figure 3.23C).

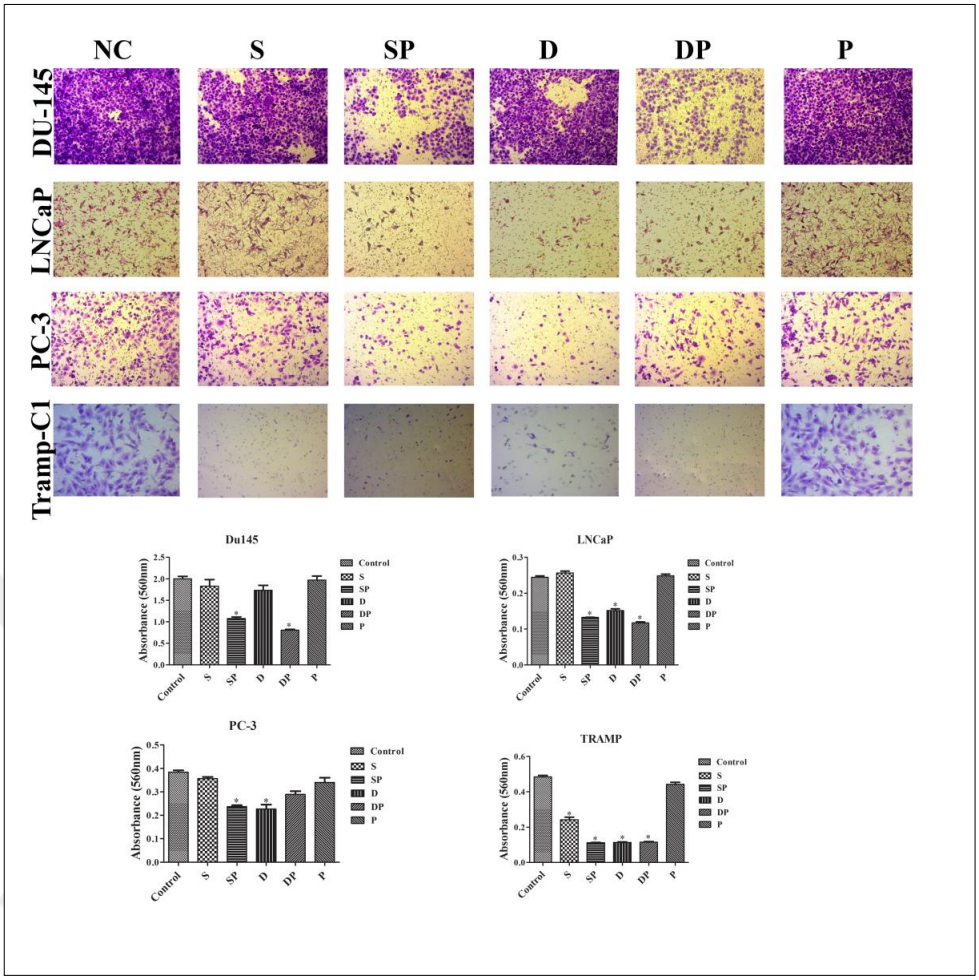

Figure 3.21. Effect of Schiff Base, Docetaxel and their combinations with P85 on prostate cancer cell invasion evaluated by Transwell invasion assay. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control (Growth medium), S: Schiff base (0.5µg/ml), D: Docetaxel (0.5µg/ml), **P*<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's posttest.

3.9. DNA BINDING ASSAY

Figure 3.24 summarizes data from EtBR displacement assay conducted to examine the ability of the Schiff base to interact with calf thymus DNA. Fluorescence measurements were collected at various concentrations of Schiff base mixed with a fixed amount of DNA and EtBR. It was found that as the Schiff base was introduced into calf thymus DNA-EtBR solution, EtBR was displaced and fluorescence intensity decreased, indicating the Schiff

base was able to be in a strong interaction with calf thymus DNA. Fluorescent intensity decrease was found to be strictly in correlation with the dose of Schiff base (Figure 3.24).

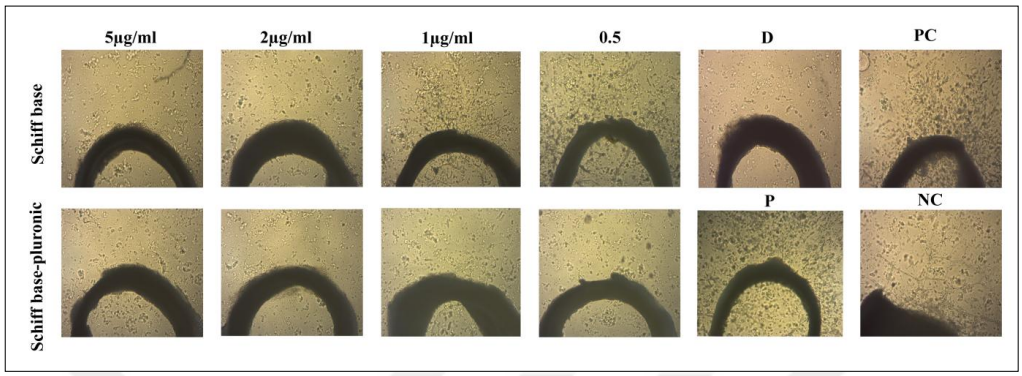

Figure 3.22. The effect of Schiff base, Schiff base-P85 combination and Docetaxel on microvessel growth in aortic ring assay. Abbreviations: P: Pluronic P85 (0.05% w/v), D: Docetaxel (10^{-8}M), NC: Negative Control (EBM-2 growth medium), PC: Positive Control (50ng/ml VEGF containing EBM-2 growth medium), **P*<0.05.

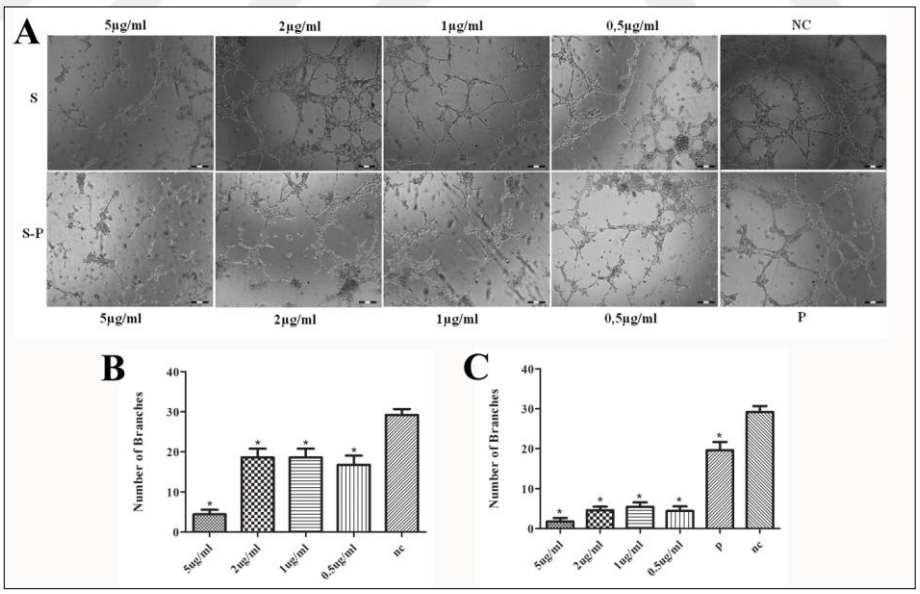

Figure 3.23. Effect of Schiff Base and Schiff base-P85 combination on tube-like structure formation ability of HUVEC cells. (A) Photographic representation of tube-like structure formation in the presence of Schiff base and its P85 combination. (B) Number of branches after Schiff base treatment, (C) Number of branches after Schiff base-P85 combination treatment. Abbreviations: P: Pluronic P85 (0.05% w/v), NC: Negative Control.

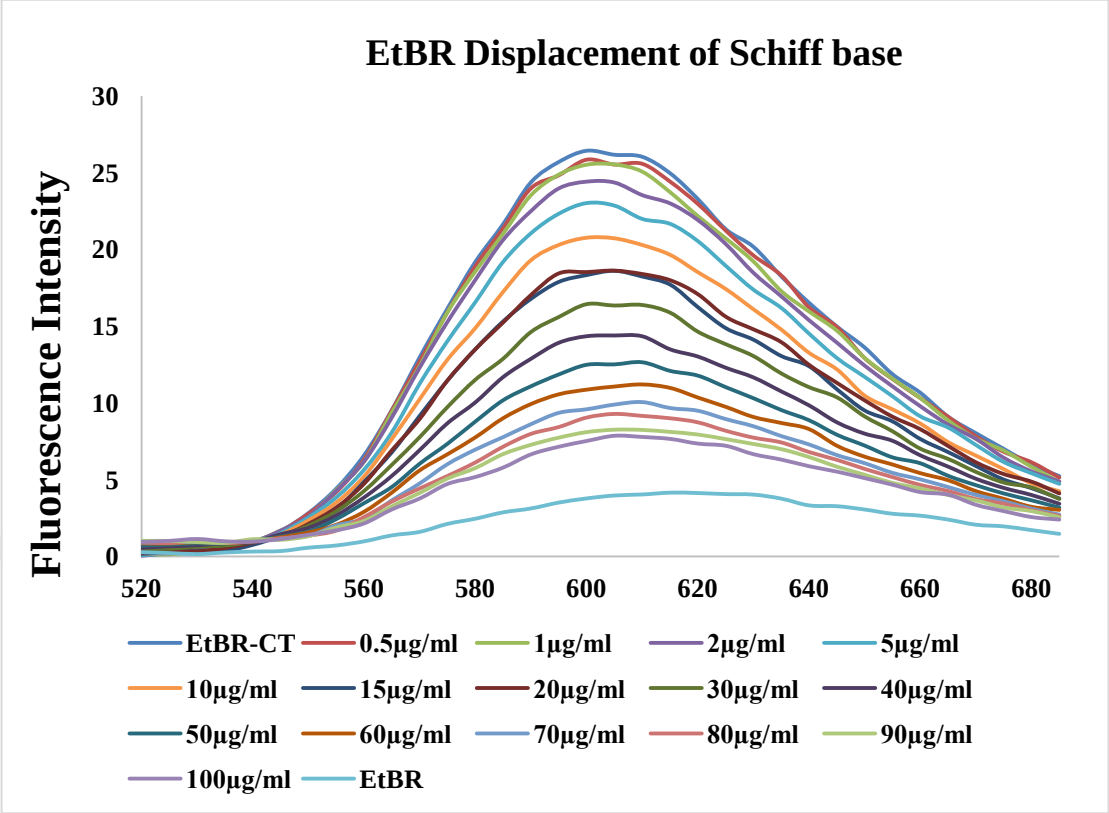

Figure 3.24. The emission spectra of EtBR which is bound to calf thymus DNA in the presence of various concentrations of the Schiff base. Abbreviations: EtBR: ethidium bromide (2µg), CT: calf thymus DNA (6µg).

3.10. DNA cleavage assay

As it was found that the Schiff base interacted with DNA, plasmid DNA was treated with different concentrations of the Schiff base to examine its potential DNA cleavage activity. The ligand converted supercoiled plasmid structure into open circular and linear form in a dose dependent manner, proving remarkable DNA cleavage activity of the compound.

Increasing concentrations of Schiff base exhibited a vigorous cleavage activity in which supercoiled plasmid DNA was cleaved and converted into open circular and linear form. DNA cleavage activity of Schiff base was shown with agarose gel electrophoresis experiments indicating the activity of Schiff base for binding and breaking the DNA strands (Figure 3.25).

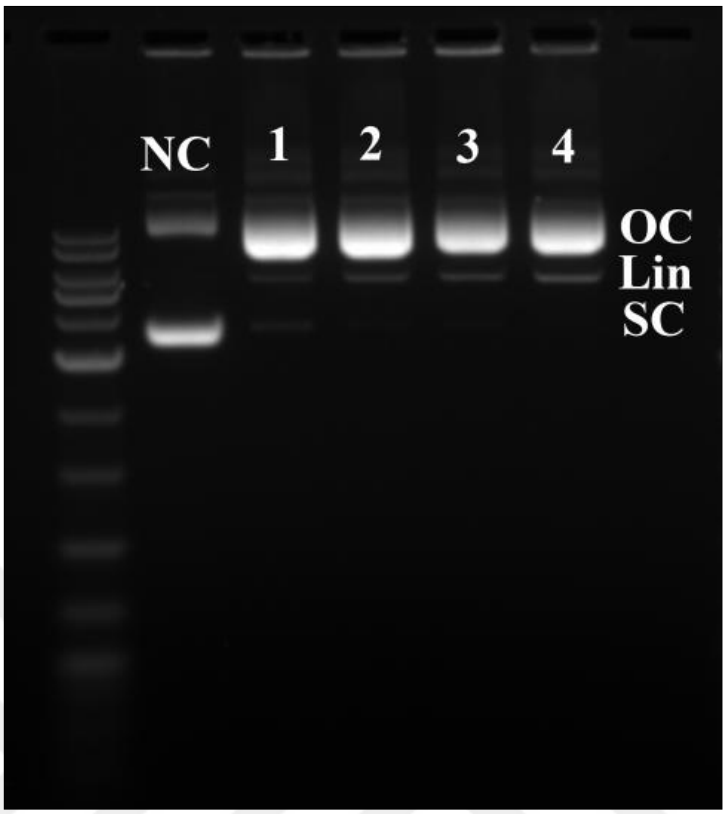

Figure 3.25. Ethidium bromide stained agarose gel electrophoresis photograph of pMD2.G plasmid DNA treat with different concentration of the Schiff base. NC: pMD2.G plasmid DNA, 1–4: DNA + Schiff base [10, 20, 50 and 100µg/ml, respectively], OC: open circular, Lin: linear and SC: supercoiled.

3.11. Antimicrobial assay

Antimicrobial activity of the Schiff base and Schiff base-P85 combination on gram positive and negative bacteria, yeast and fungi were qualitatively investigated. MBC/MFC values were determined as the lowest concentration of the Schiff base at which no microbial growth were observed. Results showed that Schiff base exerted a broad range of antimicrobial (antibacterial, anticandidal and antifungal) characteristics. MIC value of Schiff base for *Escherichia coli* and *Candida albicans* was 9.77µg/ml, while it was 4.88µg/ml for *Aspergillus niger* and *Staphylococcus aureus*. *Pseudomonas aeruginosa, Proteus mirabilis* and *Yersinia enterocolitica* were found to be most resistant bacteria tested against Schiff base with the MIC and MBC values of 156.25µg/ml. In addition, MBC value for *E. coli* and *S. aureus* was 9.77µg/ml, while MIC and MBC value for

Klebsiella pneumoniae was 78.13µg/ml. For fungal species tested, *A. niger* and *C. albicans*, the MFC value was noted to be 19.53µg/ml (Table 3.1). Addition of P85 did not chance the antimicrobial activity of Schiff base.

Table 3.1. MIC and MBC/MFC values of Schiff base complex determined by micro-well dilution assay

	Schiff base		**Schiffbase-P85**	
Microbial Species	**MIC**	**MBC/MFC**	**MIC**	**MBC/MFC**
Escherichia coli ATCC 10536	9.77µg/ml	9.77µg/ml	9.77µg/ml	9.77µg/ml
Staphylococcus aureus ATCC 6538	4.88µg/ml	9.77µg/ml	4.88µg/ml	9.77µg/ml
Pseudomonas aeruginosa ATCC 15442	156.25µg/ml	156.25µg/ml	156.25µg/ml	156.25µg/ml
Proteus mirabilis ATCC 15146	156.25µg/ml	156.25µg/ml	156.25µg/ml	156.25µg/ml
Klebsiella pneumoniae ATCC 13883	78.13µg/ml	78.13µg/ml	78.13µg/ml	78.13µg/ml
Yersinia enterocolitica ATCC 27729	156.25µg/ml	156.25µg/ml	156.25µg/ml	156.25µg/ml
Aspergillus niger ATCC 16404	4.88 µg/ml	19.53µg/ml	4.88 µg/ml	19.53µg/ml
Candida albicans ATCC 10231	9.77µg/ml	19.53µg/ml	9.77µg/ml	19.53µg/ml

MIC: Minimum inhibition concentration, MBC: Minimum bactericidal concentration, MFC: Minimum fungicidal concentration, Pluronic P85 (0.05% w/v)

3.12. *IN VIVO* TOXICOLOGY ANALYSIS

Prior to *in vivo* anticancer activity assay, acute toxicology analysis was conducted using blood samples of various concentrations of Schiff base treated mice in order to detect proper drug dose. Liver function was evaluated determining aspartate transaminase (AST) and alanine transaminase enzyme (ALT) levels. Enzyme levels increased in Schiff base-P85 combination treated animals in a dose dependent manner with respect to vehicle receiving mice, showing potential liver damage due to chemical treatment. Similarly,

creatine kinase, an important kidney damage marker, was found to augment in chemotherapeutic treated animals in a dose dependent manner.

Pancreas toxicity of the complex was analyzed in terms of amylase enzyme levels in the blood samples. Amylase enzyme levels increased in direct proportion of the ligand concentration. Severe liver damage was not detected as there was only a slight increase in bilirubin and albumin levels in all Schiff base treated mice. As indicators of kidney and heart toxicity, creatinine, ure and total protein levels were detected elevated in high concentration of Schiff base treated animals (Table 3.2).

Table 3.2. Enzyme and protein parameters evaluated in *in vivo* toxicology analysis

Parameter	**G1**	**G2**	**G3**	**G4**	**Control**
AST (U/L)	218.75±9.26	230.25±9.75	255.0±6.14	419.25±3.15	176.25±3.86
ALT (U/L)	104.5±4.09	94.00±6.70	100.50±3.89	186.75±2.14	99.75±6.33
CK (U/L)	522.25±7.56	839.75±3.50	803.50±8.18	871.00±3.16	775.25±6.21
AmylaseU/L)	395.25±3.81	409.75±3.16	414.75±6.52	419.25±6.22	382.50±5.40
Bilurubin (mg/dl)	0.05±0.00	0.06±0.01	0.06±0.01	0.07±0.01	0.05±0.01
Albumine (mg/dl)	2.65±0.06	2.68±0.08	2.70±0.08	2.73±0.11	2.43±0.10
Creatinine (mg/dl)	0.50±0.04	0.55±0.06	0.58±0.06	0.83±0.04	0.48±0.04
Ure (mg/dl)	43.25±3.55	47.25±3.33	45.25±4.52	102.50±4.36	40.50±2.49
Protein (g/dl)	6.45±0.14	6.43±0.01	6.48±0.18	6.60±0.24	6.33±0.28

AST**:** Aspartate transaminase, ALT: Alanine transaminase, CK: Creatine kinase
Notes: G1: 0.1mg/kg Schiff base-500mg/kg P85, G2: 0.25mg/kg Schiff base-500mg/kg P85, G3: 0.5mg/kg Schiff base-500mg/kg P85 and G4: 1mg/kg Schiff base-500mg/kg P85.

Secondly, blood parameters and cell counts were examined in serum samples to determine general toxicity of Schiff base-P85 combination for *in vivo* conditions. The results showed that blood parameters and cell numbers were not drastically changed by the complex application. White and red blood cells, eosinophils, basophils and platelet numbers in Schiff base-P85 group were found to be almost same with the vehicle received control group. However, Schiff base-P85 application increased eosinophilia in a dose dependent manner indicating possible allergic reactions after chemotherapeutic administration. In contrast, lymphocyte number slightly decreased but lymphocyte percentage was stable with respect to the control group. Numbers of monocytes and neutrophils were moderately higher in the ligand received mice compared to the control animals. Hematocrit levels were decreased by chemotherapeutic administration while hemoglobin level remained constant. All other parameters related to erythrocytes, hemoglobin and oxygen storage (MCV, MCH, MCHC and RDW-CV), and platelet related parameters (MPV, PDW and PCT) were not significantly different from the control group (Table 3.3). Macroscopic examination disclosed that no morbidity or gross lesions were noted in any experimental group. Multiple tissues including lung, liver, kidney, spleen, heart, pancreas, brain, intestine (large and small), stomach, testis, skeletal muscle, skin, bone/bone marrow, uterus, urinary bladder, spinal cord, thymus, tongue, epididymis, esophagus and salivary glands were subjected to histopathological analysis to observe microscopic toxicity signs. Significant toxicity symptoms were not observed in low dose treatments (Table 3.4). Kidney pyelonephritis indicating the mild inflammation was detected in group 1 (0.1mg/kg of Schiff base) (Figure 3.26A), whereas lung lymphocytic infiltration, focal lymphocytic infiltration in kidney, central vein congestion and focal parenchymal necrosis in liver were noted for group 2 (0.25mg/kg of Schiff base) (Figure 3.26B-C-D), and slight infiltration in small intestine was found to be the only toxic mark for group 3 (0.5mg/kg of Schiff base) (Figure 3.26E). On the other hand, significant toxicity in terms of hydrophobic degeneration of kidney tubules, lymphocytic infiltration in kidney, focal necrosis and hydrophobic degeneration of liver tissue was detected in group 4 (1mg/kg of Schiff base) (Figure 3.26F-G-H). These results indicated the tolerability rhtym of 0.5mg/kg of Schiff base-P85 combination for further animal experiments.

Table 3.3. Blood parameters of mice treated with different concentrations of Schiff base-P85

Parametre	G1	G2	G3	G4	Control
WBC (m/mm^3)	6.94±0.27	6,98±0.26	6.97±0.11	7.33±0.23	7.17±0.69
RBC (m/mm^3)	7.78±0.53	7.41±0.58	7.54±0.58	7.76±0.25	7.89±0.38
PLT (m/mm^3)	701.00±26.42	704.5±33.7	708.75±34.1	703.00±5.3	709.00±8.9
EOS#	0.07±0.01	0.07±0.01	0.06±0.01	0.06±0.01	0.07±0.01
EOS%	0.78±0.08	0.87±0.09	0.90±0.04	0.88±0.05	0.80±0.18
LYM#	4.78±0.16	4.63±0.60	4.46±0.47	4.79±0.16	4.93±0.26
LYM%	73.91±1.91	74.25±1.54	72.11±0.69	73.70±2.76	73.78±2.18
BAS#	0.02±0.00	0.03±0.01	0.02±0.00	0.02±0.00	0.02±0.00
BAS%	0.28±0.04	0.28±0.06	0.28±0.04	0.28±0.07	0.28±0.04
MON#	0.43±0.03	0.41±0.04	0.44±0.07	0.45±0.05	0.41±0.03
MON%	4.80±0.53	4.93±0.37	4.98±0.24	4.95±0.29	4.73±0.27
NEU#	1.32±0.04	1.31±0.03	1.34±0.01	1.27±0.05	1.13±0.06
NEU%	20.93±1.32	20.53±0.98	21.43±0.99	20.75±0.97	19.00±1.73
HGB (g/dl)	15.20±0.34	14.75±0.30	14.88±0.45	14.80±0.42	15.05±0.21
HCT%	42.10±3.49	48.00±6.52	44.03±4.17	48.83±5.72	51.75±4.42
MCV (fL)	51.25±0.80	50.05±0.65	51.15±3.70	50.68±2.48	51.80±0.55
MCH (pg)	19.40±0.22	19.50±0.90	19.48±1.07	19.55±0.68	19.58±0.37
MCHC(g/dL)	38.50±0.22	39.03±0.85	39.23±1.20	39.38±1.09	38.38±0.76
RDW-SD (fL)	27.48±1.93	28.98±1.04	29.43±2.51	29.63±1.62	27.93±0.98
RDW-CV%	14.10±0.41	14.05±0.56	14.28±0.75	14.25±0.71	14.23±0.39
MPV (fL)	6.03±0.15	6.00±0.21	5.95±0.21	6.05±0.12	5.98±0.08
PDW	14.55±0.19	14.80±0.47	14.93±0.47	14.98±0.17	14.75±0.06
PCT%	0.42±0.01	0.43±0.02	0.45±0.03	0.46±0.03	0.44±0.03

WBC: White blood cell, RBC: Red blood cell, PLT: Platelet, EOS: Eosinophil, LYM: Lymphocytes, BAS: Basophil, MON: Monocyte, NEU: Neutrophil, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RDW: Red blood cell distribution width, MPV: Mean platelet volume, PDW: Platelet distribution width, PCT: Platelet crit

Notes: G1: 0.1mg/kg Schiff base-500mg/kg P85, G2: 0.25mg/kg Schiff base-500mg/kg P85, G3: 0.5mg/kg Schiff base-500mg/kg P85 and G4: 1mg/kg Schiff base-500mg/kg P85.

Table 3.4. Histopathological examinations of mice treated with different concentrations of Schiff base-P85

Treatment	**Vehicle**		**Schiff base-P85**		**Schiff base-P85**		**Schiff base-P85**		**Schiff base-P85**	
Treatment Dose	**Control**		**G1**		**G2**		**G3**		**G4**	
#Mice/Group	**5**		**5**		**5**		**5**		**5**	
TISSUES	No Exam	%	No Exam	%	No Exam	%	No Exam	%	No Exam	%
LUNG										
Within normal limits	5/5	100	5/5	100	4/5	80	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	1/5	20	0/5	0	0/5	0
Pulmonary edema	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Lymphocytic infiltration	0/5	0	0/5	0	1/5	20	0/5	0	0/5	0
LIVER										
Within normal limits	5/5	100	5/5	100	4/5	80	5/5	100	4/5	80

Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	1/5	20	0/5	0	1/5	20
Focal necrosis and hydropic degeneration	0/5	0	0/5	0	1/5	20	0/5	0	1/5	20
Infiltration	0/5	0	0/5	0	1/5	20	0/5	0	1/5	20
Congestion	0/5	0	0/5	0	1/5	20				
KIDNEY										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	1/5	20	1/5	20	0/5	0	4/5	80
Edema	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Pyelonephritis	0/5	0	1/5	20	0/5	0	0/5	0	0/5	0
Lymphocytic infiltration	0/5	0	0/5	0	1/5	20	0/5	0	2/5	40
hydropic degeneration	0/5	0	0/5	0	0/5	0	0/5	0	2/5	40
SPLEEN										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100

Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
HEART										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
PANCREAS										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	1/5	20	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
BRAIN										
Within normal limits	4/4	100	4/4	100	4/4	100	4/4	100	4/4	100
Not examined	0/4	0	0/4	0	0/4	0	0/4	0	0/4	0
Notable or abnormal	0/4	0	0/4	0	0/4	0	0/4	0	0/4	0
INTESTINE, Large										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100

Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Infiltration	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
INTESTINE, Small										
Within normal limits	5/5	100	5/5	100	5/5	100	4/5	80	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	1/5	20	0/5	0
Infiltration	0/5	0	0/5	0	0/5	0	1/5	20	0/5	0
STOMACH										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
TESTES										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0

SKLETAL MUSCLE										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
SKIN										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Edema										
BONE/BONE MARROW										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
UTERUS										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100

Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
URINARY BLADDER										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
SPINAL CORD										
Within normal limits	5/5	100	5/5	100	4/5	80	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	1/5	20	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
THYMUS										
Within normal limits	3/3	100	3/3	100	3/3	100	3/3	100	3/3	100
Not examined	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0
Notable or abnormal	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0
TONGUE										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100

Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
EPIDIDIYMIS										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
OSEPHAGUS										
Within normal limits	5/5	100	5/5	100	5/5	100	5/5	100	4/5	80
Not examined	0/5	0	0/5	0	0/5	0	0/5	0	1/5	20
Notable or abnormal	0/5	0	0/5	0	0/5	0	0/5	0	0/5	0
SALIVARY GLAND										
Within normal limits	4/4	100	4/4	100	4/4	100	4/4	100	4/4	100
Not examined	0/4	0	0/4	0	0/4	0	0/4	0	0/4	0
Notable or abnormal	0/4	0	0/4	0	0/4	0	0/4	0	0/4	0

Notes: G1: 0.1mg/kg, G2: 0.25mg/kg, G3: 0.5mg/kg and G4: 1mg/kg Schiff bases and 500mg/kg pluronic P85 combination.

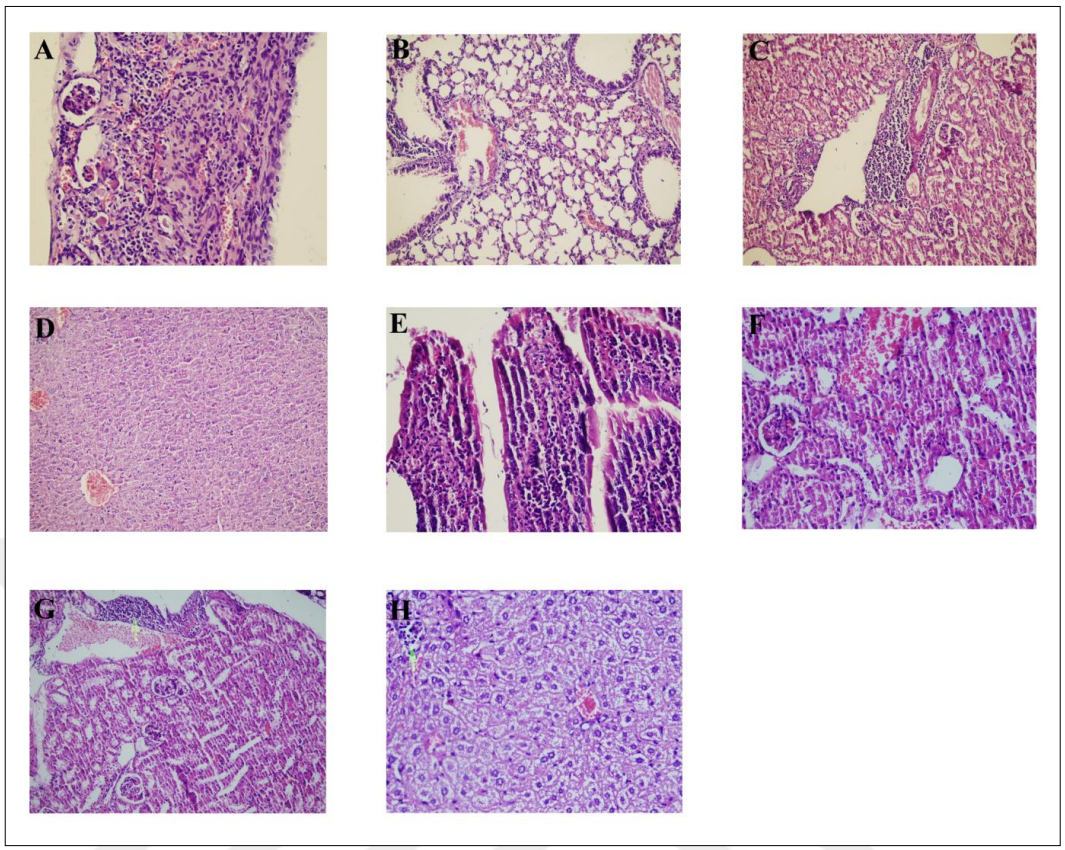

Figure 3.26. Representative H&E sections from different concentrations of Schiff base-P85 treated mice tissues. (A) Kidney pyelonephritis in group 1, (B) Lung lymphocytic infiltration in group 2, (C) Focal lymphocytic infiltration in kidney in group 2, (D) Central vein congestion and focal parenchymal necrosis in liver in group 2, (E) Slight infiltration in small intestine in group 3, (F) Hydrophobic degeneration of kidney tubules in group 4, (G) lymphocytic infiltration in kidney in group 4, (H) focal necrosis and hydrophobic degeneration of liver in group 4. Notes: G1: 0.1mg/kg Schiff base-500mg/kg P85, G2: 0.25mg/kg Schiff base-500mg/kg P85, G3: 0.5mg/kg Schiff base-500mg/kg P85 and G4: 1mg/kg Schiff base-500mg/kg P85.

3.13. ANIMAL EXPERIMENTS and DRUG APPLICATION

Tramp-C1 cells were injected subcutaneously to the dorsal side of C57/Bl6 mice near to tail side. Suitable concentration of Schiff base for *in vivo* anticancer activity assay was determined as 0.5mg/kg from toxicology analysis. Therefore, Schiff base (0.5mg/ml) and P85 (500mg/kg) combination or vehicle (2.5%DMSO/PBS 0.2ml) were applied

intraperitoneally every four days for 52 days, starting from one week after cell transplantation. Animals were weighed at each injection time, and monitored for mortality and morbidity throughout the study. Drug injection was ended when control group's animals started to die (at day 60 of cell transplantation). Mice were sacrificed by cervical dislocation and tumor volumes were measured.

3.13.1. Animal Weights

There was not a significant difference in weights of control animals between consecutive injections. Weight of the animals significantly increased at only time of 8. injection (day 40 of cell transplantation) for control group. Initial average weight for control animals was recorded as 21g and final average weight was noted as 24g at the end of 13. injection (day 60 of cell transplantation). On the other hand, animals of Schiff base-P85 group gained more weight compared to control group. Similar to control group, significant increase in weight was observed at 8. injection. Final average weight for Schiff base-P85 animals was 26g at the end of 13. injection (Figure 3.27).

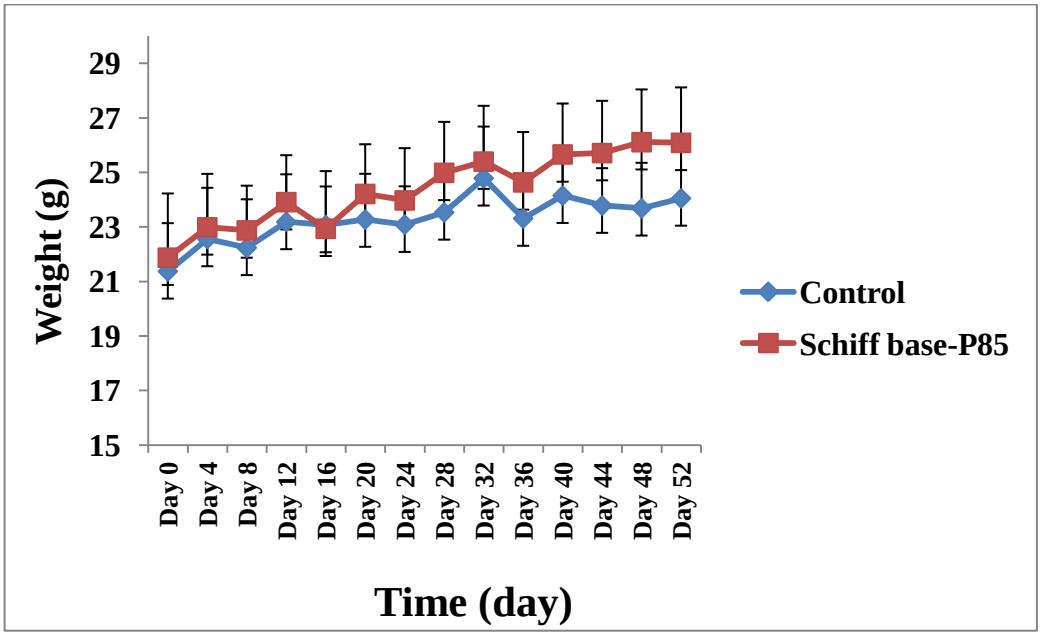

Figure 3.27. Average weights of animals during in vivo experiments. Control: Cancer cell injected animals receiving vehicle (2.5%DMSO/PBS). Schiff base-P85: Cancer cell injected animals receiving Schiff base-P85 combination.

3.13.2. Tumor Volume Measurements

Tumors started to appear approximately 30 to 40 days after cell injection. At the end of experiment (day 60 of cell transplantation), 6 out of 8 control animals had visible tumors (C1-C2-C3-C5-C6-C7). However, it was found that as the animals were necroscopied, solid tumors were detected in all control group animals. Tumors of C2 and C3 mice started to be visible at day 40 of cell transplantation (Figure 3.28A and C). Figure 3.28B illustrates that tumor volumes in the control group increased steadily over the course of experiment. Among experimental animals, C3 with 14 cm^3 tumor on its dorsal side dead first, which was able to live until day 60 of cell injection (Table 3.5). Tumors were not visible for C4 and C8 animals until the necropsy day. On the other hand, no visible tumor growth was detected in chemotherapeutic combination treated animals until necropsy day. Animals in the ligand treated group were healthy during the experimental procedure. The ligand injected animals were also sacrificed at day 60 of cell transplantation and immature solid tumor formations were observed in only D4, D5 and D8 mice with volumes of 0.001cm^3, 0.0045cm^3 and 0.03cm^3, respectively (Figure 3.29) (Table 3.6).

3.13.3. Histopathological Analysis

Gleason scores of tumors in vehicle or Schiff base-P85 treated animals were determined by histopathological analyses. Prostatic adenocarcinomas with high Gleason scores and aggressive phenotypes were diagnosed in all control group animals. Tumor of C6 mouse could not be detected for a Gleason score, and tumors of C3 and C4 were found to have necrotic foci (Figure 3.30, Table 3.7). In contrast to control mice, Schiff base-P85 treated animals showed relatively healthy phenotypes. Although prostatic adenocarcinoma was also observed in D4, D5 and D8 with high Gleason scores, no tumor formation was detected for other drug treated animals. D3 mouse had a solid tumor-like formation with edema, chronic inflammation and fibrosis on the dorsal area but the structure was not diagnosed as tumor. (Figure 3.31, Table 3.8). Metastatic foci were not observed in organs of both control and Schiff base-P85 group animals. While liver congestion and lymphocyte infiltration were detected in tumor carrying control and drug group animals, there were no significant toxicity in liver, kidney, testis and spleen tissues (Figure 3.32).

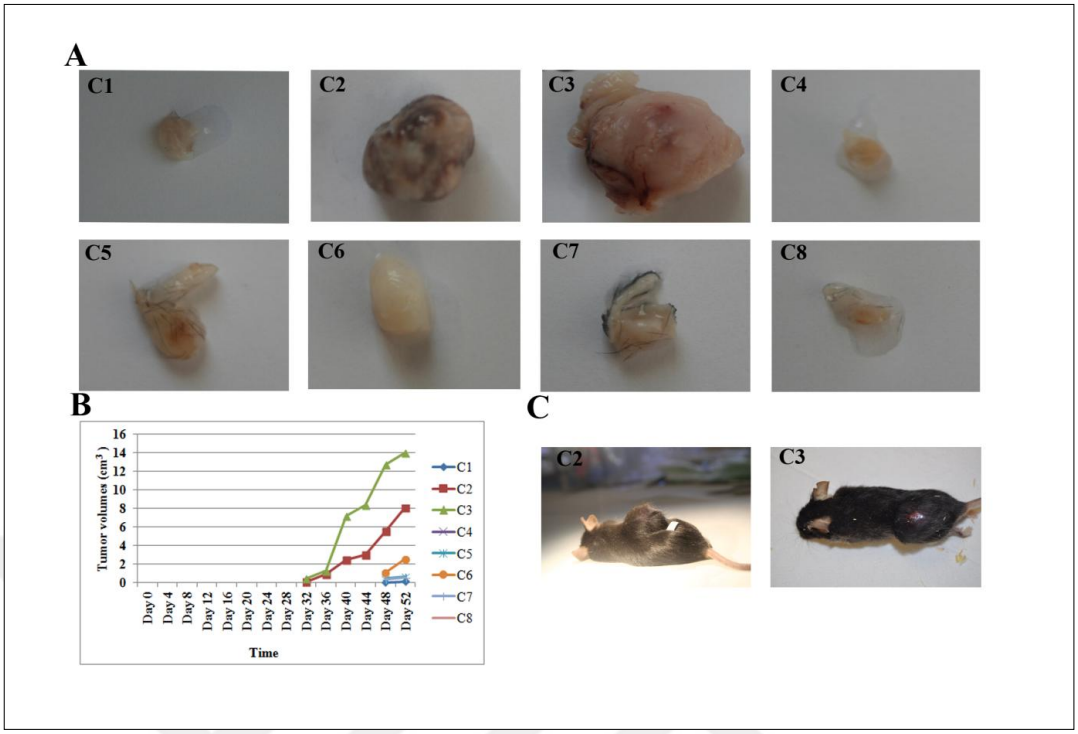

Figure 3.28. Tumor growth in control group animals. (A) Tumor appearance after resection. (B) Tumor growth rate in control group animals. (C) Exterior appearance of C2 and C3 mice carrying large tumors at day 60 of cell transplantation.

Table 3.5. Tumor volumes of control group animals during *in vivo* experiments

Groups/Tumor volume (cm^3)	**Day 40**	**Day 44**	**Day 48**	**Day 52**	**Day 56**	**Day 60**
C1	-	-	-	-	0.075	0.126
C2	0.125	0.9405	2.53	3.06	5.6	8.1
C3	0.5	1.275	7.1875	8.4375	12.75	14
C4	-	-	-	-	-	0.012
C5	-	-	-	-	0.45	0.65
C6	-	-	-	-	1.125	2.55
C7	-	-	-	-	0.405	0.6
C8	-	-	-	-	-	0.003

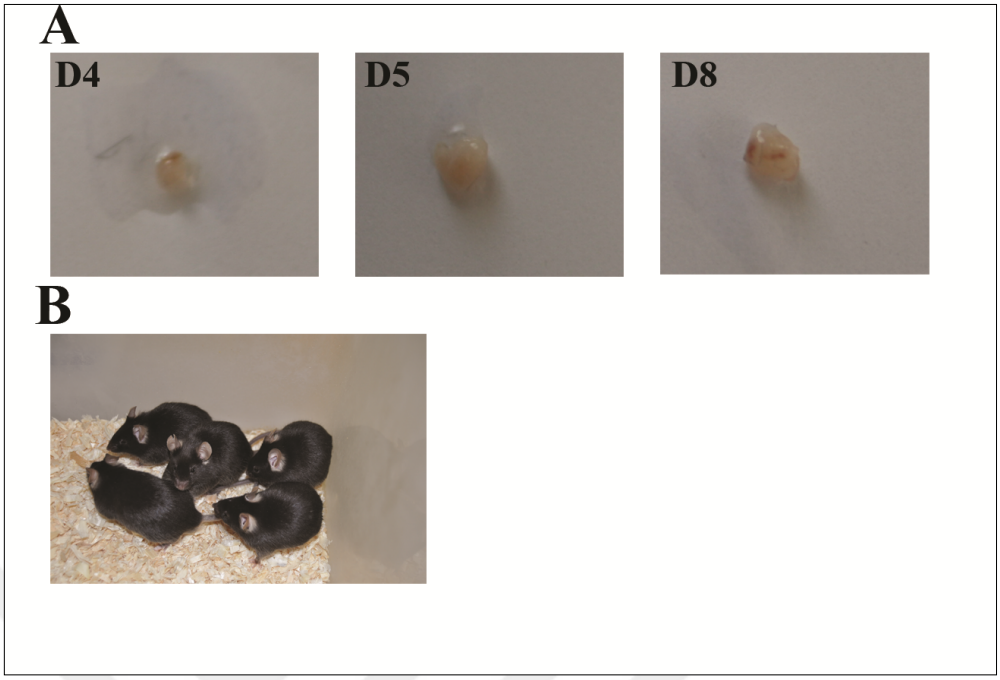

Figure 3.29. Tumor growth in Schiff base-P85 treated animals. (A) Tumor appearance after resection. (B) Exterior appearance of Schiff base-P85 treated animals with no naked-eye visible tumor formation.

Table 3.6. Tumor volumes of Schiff base-P85 group animals during *in vivo* experiments

Groups/Tumor volume (cm^3)	**Day 40**	**Day 44**	**Day 48**	**Day 52**	**Day 56**	**Day 60**
D1	-	-	-	-	-	-
D2	-	-	-	-	-	-
D3	-	-	-	-	-	-
D4	-	-	-	-	-	0.001
D5	-	-	-	-	-	0.0045
D6	-	-	-	-	-	-
D7	-	-	-	-	-	-
D8	-	-	-	-	-	0.003

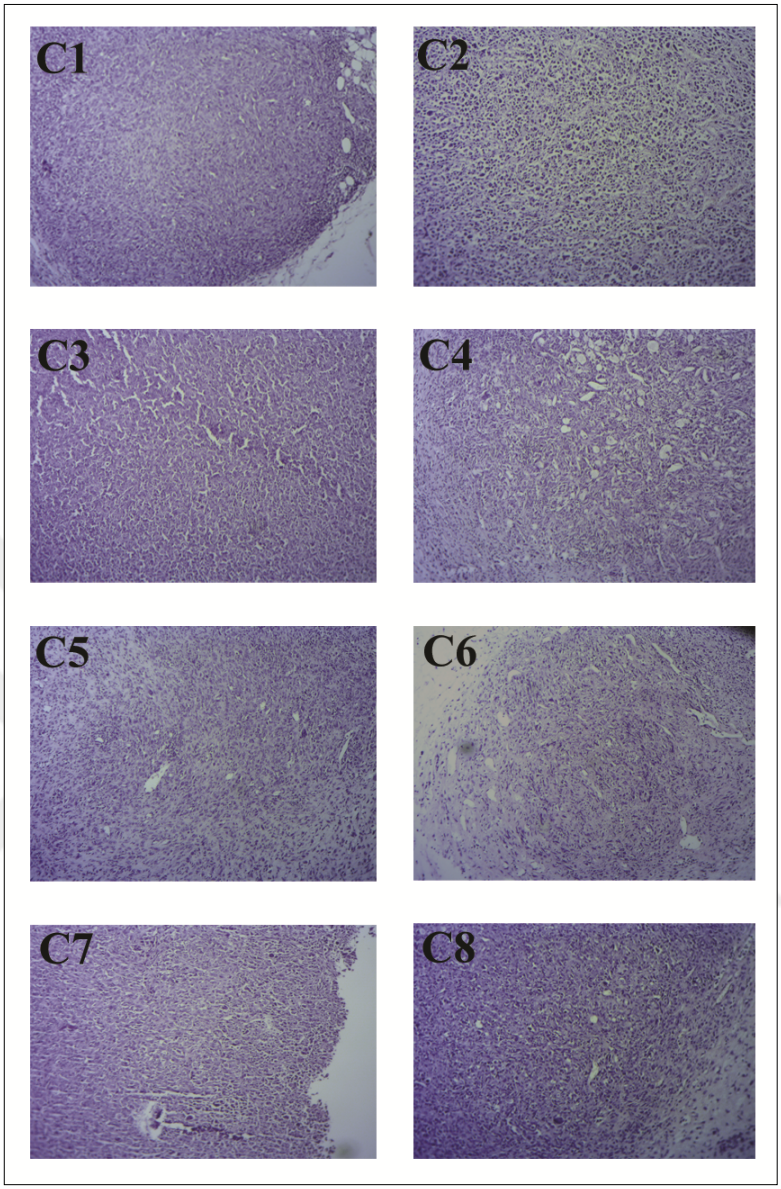

Figure 3.30. Histopathological examinations of control group tumors. Magnification**:** 40x

Table 3.7. Gleason score analysis and tumor patterns of control group animals.

Group (Control)	Tumor	Gleason Score
C1	Prostatic adenocarcinoma	9 (4+5)
C2	Prostatic adenocarcinoma	9 (4+5) solid tumor-necrosis
C3	Prostatic adenocarcinoma	9 (5+4) solid tumor-necrosis-apoptotisis
C4	Prostatic adenocarcinoma	9 (4+5) solid tumor-necrosis-apoptotisis
C5	Prostatic adenocarcinoma	10 (5+5)
C6	Prostatic adenocarcinoma	Not detected
C7	Prostatic adenocarcinoma	10 (5+5) solid tumor
C8	Prostatic adenocarcinoma	9 (5+4) solid tumor

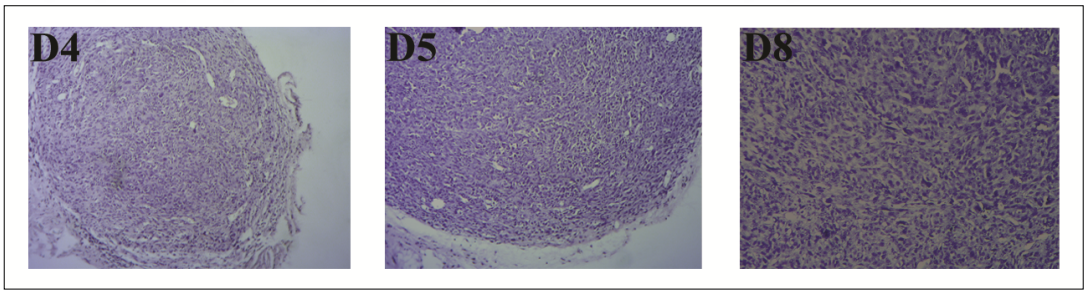

Figure 3.31. Histopathological examinations of Schiff base-P85 group tumors. Magnification**:** 40x

Table 3. 8. Gleason score analysis and tumor patterns of Schiff base-P85 treated animals.

Group (Drug)	Tumor	Gleason Score
D1	-	-
D2	-	-
D3	Edema, chronic inflammation, fibrosis	-
D4	Prostatic adenocarcinoma	9 (5+4) solid tumor-necrosis-apoptotisis
D5	Prostatic adenocarcinoma	10 (5+5) solid tumor-necrosis-apoptotisis
D6	-	-
D7	-	-
D8	Prostatic adenocarcinoma	10 (5+5) solid tumor

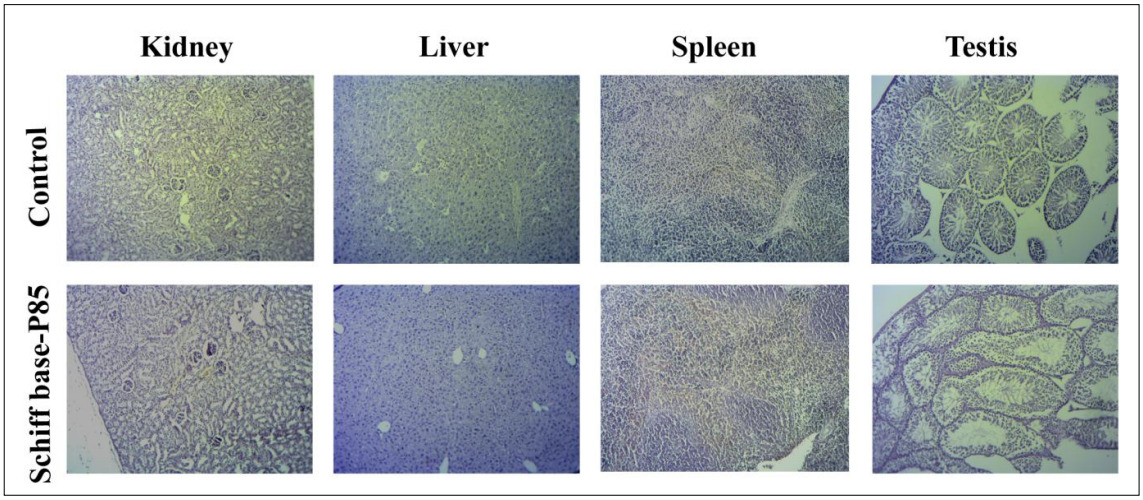

Figure 3.32. Histopathological examinations of kidney, liver, spleen and testis of control and Schiff base-P85 group animals. Magnification**:** 40x, Control: PBS Drug: Schiff base (0.5mg/kg)-P85 (500mg/kg)

4. DISCUSSION

Cancer as a worldwide problem has gained lots of interest over the past century. The observations on tumor metabolism, progression, molecular regulations including oncogene and tumor suppressor functions, tumor microenvironment, cancer prevention and treatment have made the cancer research one of the most popular area of biology. Detailed statistical analyses have suggested that cancer is the leading reason for death in developing countries [143]. Prostate and breast cancers have been reported as the major cancer types and second most common cause of death among cancer types for male and female, respectively, in the US [261]. An approximately 258.000 death per year has been reported for prostate cancer and death occurs one to two years after diagnosis [262]. Prostate cancer emerges in the prostate gland as an androgen dependent cancer and gains a resistant phenotype to conventional therapies referred to as androgen independent metastatic prostate cancer [263]. Due to the lack of screening methods, prostate cancer diagnosis at both slow growing and more advanced stage is difficult [264]. Treatment methods are generally chosen depending on the cancer localization and stage. Prostatectomy, radiation and chemotherapy are applied in combination with androgen deprivation therapy (ADT) which are effective at the primary stage but become ineffective at the later stages because of acquired resistance [265]. At the metastatic stage, tumors become refractory to established treatments and cause serious problems at the clinics [266].

Data obtained from clinical trials provide better understanding and knowledge for the diversity among treatment options which are variable between individuals regardless of tumor's Gleason score. Prostate cancer recognized as a hormone dependent disease and therefore, ADT is used as a first line treatment option for men at any stage. Although ADT initially inhibits the tumor progression, prolongs the life span and provide a favorable response, androgen independent prostate cancer (castration resistant prostate cancer-CRPC) is eventually developed either in a few weeks or years after hormone castration therapy [267-268]. ADT contributes to overall survival about approximately two years starting from diagnosis to death just in case of diagnosis at early stage of cancer [269]. However, additional treatment modalities and combinational therapies are required to obtain an effective response in patient populations with metastatic disease (late stage).

Moreover, despite encouriging results for ADT were obtained for prostate cancer patients, side effects such as high body temperature, erectile function and osteoporosis restricts the long term usage. Intermittent ADT (IAD) as a temporary solution has been introduced to clinical applications [270] which was defined by Bruchovsky and co-workers, claiming exposure of ADT in different time intervals may reduce the risk of resistance [271]. Currently, several treatment options (radiotherapy, prostatectomy, chemotherapy) are being used in addition to ADT in the clinics. Surgical castration or medical castration are effective methods generally applied at the first stage of the disease. Concurrent use of gonadotropin-releasing hormone (GnRH) agonists and antiandrogens are also used as treatment options either itself or in addition to castration [272]. As prostate cancer has bone metastasis at the later stages, radiotherapy is required after surgical operation together with chemotherapy or alone. Radiotherapy induced cell death releases tumor antigens and enhances the activity of chemotherapeutics in patients with CRPC. Radiotherapy alone [273] or in combination with chemotherapy [274] have been shown for its positive effects in clinical trials of prostate cancer with exerting severe toxicity. Apart from other treatment options, chemotherapy has also been shown to increase survival rate in prostate cancer as a palliative option [177]. Neoadjuvant therapy before surgical operation has shown impressive results when starts with doxorubicin and ketaconazole combination followed by alteration to vinblastin and estramustine. Neoadjuvant therapy applied before radiotherapy is not a widely used application; however, there are a few studies which has been conducted with estramustine and vinblastin [275]. There are several clinical trials using a variety of drugs including Docetaxel, leuprorelin, paclitaxel and estramustine as adjuvant chemotherapy after surgery or radiotherapy to provide a survival advantage in men with metastatic prostate cancer. [276]. Despite benefits of either neoadjuvat or adjuvant chemotherapy in clinics and their palliative role, prolonged survival rates could not be observed in metastatic disease along with CRPC [277]. Although Docetaxel is used in prostate cancer treatment efficiently since 1990’s, acquired resistance and side effcets are major obstacles for clinicians. Drug resistance is gained by tubulin mutations and activation of drug efflux pumps and becomes fatal for the patient [278]. Myelosupression, generally febrile neutropenia is the most dangerous hematological side effects observed during Docetaxel treatment and may lead to life- threatening infection. Hypersensitivity reactions including pain and rash, edema, nail problems, asthenia and neuropathy are other major problems which restrict drug dosage and application period [279]. Therefore, there is

a remarkable interest in novel chemotherapeutic agents that could be applied at either localized or metastatic prostate cancer alone or combined with other agents and treatment options. Current chemotherapeutics are relatively efficient as used in neoadjuvant therapies; however, adjuvant chemotherapy trials are mostly disappointing. Development and optimization of such new drugs for late stage cancer patients has been the aim of interest in recent years for prostate cancer.

In the current study, we aimed to develop a new chemotherapeutic agent fromulation for the treatment of prostate cancer and show its *in vitro* and *in vivo* anticancer effects. Cytotoxic properties of a novel Schiff base derivative were tried to be increased by combining with pluronic P85. In this thesis, activity of the newly developed Schiff base derivative and P85 combination were tested on prostate cancer cell lines (PC-3, DU 145, LNCaP, Tramp-C1) which have different androgen response phenotypes and metastatic grades. Anticancer activity of Schiff base derivative and P85 combination were also investigated *in vivo* using a Tramp-C1 prostate cancer model.

To assess whether cell proliferation, a crucial event during cancer progression, could be efficiently inhibited by newly developed chemotherapeutic formulation (Schiff base derivative and P85 combination), two androgen sensitive (LNCaP and Tramp-C1) and two androgen insensitive (PC-3 and DU 145) prostate cancer cell lines, having different metastatic and agressive properties, were used. Due to the cytotoxicity of a chemotherapeutic drug is strongly associated with the aggressive phenotype of cancer cells, androgen sensitive LNCaP and Tramp-C1 responded to applied agents more effectively. Tramp-C1 viable cell ratio decreased in a time-dependendent manner regardless of the concentrations for both Schiff base, Docetaxel and their P85 combinations. P85 combination reduced the viable cell ratio for Schiff base and Docetaxel especially at day three. The applied concentrations for Docetaxel were adequate to provide remarkable cell death in cultured TRAMP-C1 cells as it was reported in the literature [280]. Although Schiff base alone exerted relatively poor efficiency compared to Docetaxel in cell viability analysis of LNCaP cells, P85 combination increased the cytotoxicity of Schiff base in a concentration-dependent manner. Overall, there was a remarkable inhibitory effect in Docetaxel applied cells in concordant with the previous studies [281-282]. However, high concentration of Schiff base in combination with P85

was the most efficient treatment for cultured LNCaP cells. Drug applied LNCaP and Tramp-C1 cells increased Caspase-3 enzyme activity levels comparable to control group, and P85 combination activates the apoptotic pathway in such short incubation period (24h). As Docetaxel was reported to induce apoptosis after 72h in LNCaP cells [283], the results obtained for Docetaxel treated cells for Caspase 3 enzyme activity is in consistent with the literature.

PC-3 is a more aggressive cell type and referred to as highly metastatic compared to other prostate cancer cell types. Although Docetaxel seemed to supress cell proliferation more efficiently with respect to Schiff base, the exact inhibition was observed at day three for both agents. Using the compounds in combination with P85 to increase inhibitory activity worked well and significantly decreased cell viability as evidenced by literature [284]. As PC-3 is an androgen independent cell type, Docetaxel has been combined with other chemotherapeutics, receptor antagonists or apoptotic agents in order to increase inhibitory activity for PC-3 cells in previous studies. Sodium selenite, gossypol and estramustine exerted synergistic effect against PC-3 cells when used in combination with Docetaxel [285-287]. Docetaxel and Schiff base displayed a dose- and time-dependent activity against DU145 cells at the end of two days incubation when either individual agents were used alone or combined with P85. Due to DU 145 cells have been obtained from metastatic prostate cancer, significant drug response could only be observed at day 2. Schiff base and Schiff base-P85 at high dosage (5µg/ml) merely reduced viable cell ratio at day one. In general, Schiff base and its P85 combination was more efficient compared to Docetaxel after a three days incubation period. Interestingly, Docetaxel did not decrease viable cell ratio when used alone at day three. This situation might be explained by lack of Docetaxel amount in cell culture medium at the end of three days. A similar correlation was observed for Caspase 3 activity in PC-3 and DU 145 cells, indicating both agents induced apoptosis.

Unfortunately, Docetaxel resulted in a crucial reduction at viable cell ratio of PNT1A cell. This situation has not been addressed in the literature by using Docetaxel yet, however, palladium compounds and clusterin isoforms have been shown to exert remarkable cytotoxicity on PNT1A cells [288-289]. Similar inhibitory effect was observed upon Schiff base and Schiff base-P85 application in a dose- and time-dependent manner. Individual Schiff base treatment at 0.5µg/ml concentration was not toxic for the PNT1A cells for the

first two days. Caspase 3 enzyme activity was increased in PNT1A cells for Schiff base-P85 and Docetaxel confirming the apoptotic status of cells. As fibroblast cells (HF and L929) are mainly being used in toxicity analysis to mimic a healthy metabolism, they were used to evaluate the toxicity of Schiff base and Docetaxel on healthy cells. Mouse fibroblasts (L929) were found to be more resistant to the chemotherapeutics compared to human fibroblasts. Docetaxel, Schiff base and their pluronic combinations exerted cytotoxicity only at day three for cultured L929 cells. In contrast to L929 cells, HF cells were more sensitive and Docetaxel treatments for three days caused significant cell death starting from the first day. On the other hand, Schiff base-P85 reduced cell viability only at day three. Throughout the literature, many studies lack the use of healthy cell lines for chemotherapeutic drug screening. Although Docetaxel has been accepted as a single-agent against prostate cancer [185] and has been used in many preclinical and clinical studies, none has done *in vitro* cell culture analysis with healthy cell lines. Our results, therefore, lead an explanation to chemotherapy induced toxicity observed by commercially available drugs. Current cell viability analysis not only provided a reference to a commercially available drug (Docetaxel), proven to be effective by clinical studies and used for cancer treatment worldwide, but also introduced a new promising option (Schiff base) for chemotherapy. Although combining Docetaxel with P85 did not contribute too much to cytotoxicity *in vitro*, Schiff base-P85 combination specifically inhibited cell proliferation in cultured PC-3 and DU 145 cells. Having limited data from published research, literature is silent about Docetaxel and P85 combination *in vitro*. There is just one study in which intravenous delivery of Docetaxel loaded P85 based nanoparticles to target tumor has shown notable anticancer effect against breast cancer *in vivo* [290-291]. When P85 is used at concentrations under CMC to carry the drugs through the cell membrane, they can typically forms unimers, interact with the membrane and drug efflux pumps resulting in membrane fluidization, conformational changes in drug transporters and ATPase inhibition [244,243]. The concentration of P85 (0.05%) used in this thesis for *in vitro* analysis played a pivotal role for drug transition to the cells and probably inhibited the drug transporters found on cell membrane of cancer cells. The membrane fluidization capacity of P85 by changing fatty acid content of the lipid bilayer which was first reported by our group [234] might be one possible explanation for the easy transport of Schiff base to the cytosol. Whereas cell viability analysis, an initial and prequisite step for anticancer research, provided encouraging results for Schiff base-P85 combination therapy, more refined

studies addressing the molecular and physiological mechanisms are highly warranted to elucidate potential use and limitations of the formulation.

Docetaxel acts as a potent inducer for microtubule polymerization and inhibits cell division followed by binding to β-tubulin as many other types of taxanes [185]. In spite of Docetaxel's ability to slow down microtubule depolymerization, identified mutations on β-tubulin for binding sites of Docetaxel affect the tubulin polymerization capacity [292]. In addition to mutations, occur on β-tubulin or actin cytoskeleton, microtubule associated proteins (MAPs) such as tau or MAP2 stabilize the microtubules and result in drug resistance [293]. DNA binding agents are potential alternatives to slow down acquired resistancy. Chemotherapeutic agents with the ability of binding to DNA on major or minor groves, lead to DNA breakage followed by the inhibiton of replication and transcription, and subsequently cause cell death [294-295]. Addition of transition metal complexes such as Cu^{2+}, Fe^{3+} or Mn^{2+} increases the interaction with DNA chains due to their high nucleolytic capacity which leads to DNA break. Because Schiff base used in our study is a Cu(II) Mn(II) complex, it could easily bind to DNA and cause DNA breakage which was confirmed by *in vitro* ethidium bromide displacement and DNA cleavage assays. The only acquired mechanism for resistance to DNA binding agents is DNA repair mechanisms [296]. However, combining Schiff base with P85 rapidly kills cancer cells which might prevent the initiation of DNA repair mechanisms.

Understanding the possible molecular events occuring after Schiff base-P85 administration, gene and protein expression analysis were performed. Activation of Akt and the downsteam gene NFκ-B are major regulators for cancer cell survival, growth and proliferation. Signals activating the PI3K pathway and upregulating the Akt and NFκ-B are required to trigger cancer cell response such as proliferation or motility [297]. Schiff base and P85 combination decreased the Akt and NFκ-B gene and protein levels in prostate cancer cells *in vitro*. Some of the aggressive cell types such as DU-145 and Tramp-C1 did not show a considerable decrease for the expression of these genes. In consistent with our study, the results from the previous studies using prostate cancer cell lines have indicated that one day incubation period could not be sufficent to induce these proteins expression [298]. One other possible explanation for this situation might be the initiation of apoptosis after down-regulation of the PI3K/Akt signaling at the timing (24h) of our experiments.

Bcl-2 as an anti-apoptotic mediator decreased in Schiff base-P85 applied Tramp- C1 cells similar to Docetaxel treated cells. Schiff base-P85 treated Tramp-C1 and LNCaP cells, androgen sensitive and less agressive cell types, upregulated a network of apoptotic genes such as BAX and Caspase 3, in levels comparable to other groups. Active Caspase 3 as the key final element of apoptosis causes cleavage of several cellular components and proteins consequently, which have been reported to play fundamental role in prostate cancer cell death [299]. The current study laid notable results for Caspase 3 mediated apoptosis of prostate cancer cell lines, showed increased Caspase-3 enzyme levels in Schiff base-P85 treated prostate cancer cells (PC-3, DU 145, LNCaP and Tramp-C1) and stable levels in healthy cells (PNT1A), providing a proof for the chemotherapeutic potential of Schiff base-P85 combination. The finding that Docetaxel did not increase the Caspase 3 levels markedly was not surprising for the study, because Docetaxel has been reported to induce caspase 3 levels after a four days period in a previous study [300]. AR dependent growth of prostate cancer cells is mediated by activating Elk-1 binding sites and promoter activation through phosphorylation and thus enabling cell growth [301]. Phosphorylation of Elk-1 activates the EGFR gene which is the binding site for epidermal growth factor (EGF) [302], leading to cell proliferation and growth in the metastatic prostate cancer in an androgen independent way. In the current study, expression levels of Elk-1, AR and EGFR were decreased in Schiff base-P85 group as Docetaxel treated prostate cancer cells or healthy cells, illustrating how Schiff base-P85 or Docetaxel might have decreased viable cell ratio.

Drug efflux pumps (P-gp, BCRP, MRP1 and MRP2) which are potent target sites for P85 on the membrane are expressed on both healthy and tumor tissues. The expression levels of these multidrug resistance pumps on cancer cell membranes are tremendously high compared to normal cells [303]. Moreover, localization of these drug transportes on blood-testis barrier which protects germ cells against many potential harmful substances of the circulation hinders chemotherapeutic transport to the testis tissue [304]. A similar formation has been discovered for the prostate tissue in 2000 by Fulmer and Turner [305] and named as blood-prostate barrier. This barrier like blood-brain barrier and blood-testis barrier acts as an obstacle for drug transport and should be overcome for chemotherapy [306]. The inhibitory role of P85 on these drug transporters could be an advantage for *in vitro* and *in vivo* drug delivery compared to convential therapies. The results from the gene

expression analysis for MRP and BCRP genes showed a remarkable decrease in prostate cancer and healthy cells for Schiff base-P85 treatment indicating a strong correlation between Schiff base and pharmokinetic properties of P85 to hinder drug resistance. The disparate results of PC-3 cells could be explained by their aggressive phenotype and previous studies describing the high expression levels of MRP even in PC-3 cells after Docetaxel treatment for 48h [307]. While the inhibition of drug transporters and cancer cell proliferation are the initial steps for cancer chemotherapy, the current research should be enriched by metastasis and angiogenesis analysis to determine whether Schiff base-P85 combination can abolish tumor growth by supressing metastasis and angiogenesis. The capacity of cancer cells to migrate and invade within the surrounding tissue, allowing to entrance to the circulating system are major challenges for cancer therapy and established by drug resistant and aggressive cells residing in the malignant tumor [308-309]. In the current study both agents (Schiff base and Docetaxel) exhibited migration inhibitory activity and diminished cell invasion, indicating the anti-metastatic potential of Schiff base and its P85 combination as well as Docetaxel. As growing tumor tissue require more oxygen and nutrient supply, formation of new blood vessels and vascular network are indispensable for proliferation and metastasis of cancer cells [310]. In order to assess anti-angiogenic properties of Schiff base-P85 combination, vessel sprouting from rat aortas and tube formation analysis were completed. Docetaxel is a well known anti-angiogenic agent even used at low doses and causes centrosome reorientation, changing microtubule plasticity and inhibiting the cell migration ability of endothelial cells [255]. Although Schiff base as a single agent suppressed endothelial cell dispersal in a concentration dependent manner, Schiff base-P85 combination was more effective similar to Docetaxel, independent from concentration used. Tube formation assay confirmed the anti-angiogenic acitivty. Schiff base-P85 combination reduced branch like structure formation more effectivly compared to Schiff base alone treatment. This situation might be explained by our previous study showing inhibitory activity of Schiff base on HUVEC cell migration *in vitro* (data not shown). Considering these findings, it became possible to show anti-cancer activity of Schiff base-P85 on prostate cancer *in vitro*. In addition to remarkable activity on cancer cell proliferation, migration and invasion, Schiff base-P85 combination was evaluated for its antimicrobial activity to observe whether inflammation in the prostate tissue caused by microbial infection, one of the reasons for cancer progression, could be removed by Schiff base-P85 combination. Inflammation and the resulting prostatitis are

very common in prostate tissue, leading to epithelial cell proliferation and hyperplasia which directly contribute to the initiation of prostate cancer [311]. Common microorganisms found in the prostate tissue of cancer patients were selected for the current study and used in microbial analysis. Notably, significantly low values of MIC, MBC and MFC for Schiff base alone or Schiff base-P85 combination were obtained against all bacteria, yeast and fungi tested, reflecting the potential use of Schiff base-P85 in clinics to avoid inflammation in prostate tissue either for prostatitis or cancer patients.

To realize entire chemotherapeutic capacity of Schiff base-P85 combination, toxicology analysis and tumor model experiments were conducted in C57/B16 mice to confirm anticancer activity *in vivo*. Alternatively, dog and rat models have been suggested for the prostate cancer researches to develop a more analogous model for human prostate cancer. However, the limitations of these models including cost, long gestation periods, lack of metastasis and difficulties in tumor formation for these animal models remains as major impediments to the laboratory cancer research [312]. Therefore, development of mouse models for prostate cancer is necessary to cope with difficulties in drug research and screening analysis. The lifespan and anatomically different prostate tissue of mice compared to human body and different metastatic pathways in cancer progression restrict the use of this model in pharmacokinetic analysis of drug studies. Although mouse have several disadvantages for studying human cancers, similar genetic background with human genome, short gestation period and same cancer initiation pattern have made mouse an preferable model animal for human prostate cancer analysis [313]. Since chemotherapy induced toxicty affects the treatment regimen by changing drug resistance phenotype due to misuse, determining the limits of maximum tolerated dose (MTD) is crucial for cytotoxic chemotherapy [314]. Acute toxicology analysis was performed as decribed previously to determine appropriate dose of Schiff base-P85 combination [315-316].

MTD for Schiff base-P85 combination (0.5mg/kg) was selected based on enzyme activities reflecting the multiple organ toxicity, blood counts indicating the myelosupression and histoplathological analysis showing the inflammation in the organs. Tolerability rhytm of 0.5mg/kg of Schiff base-P85 combination was better compared to equivalants used in previous preclinical studies. 20mg/kg/wk of Docetaxel administration has been found effective for pancreatic cancer treatment in mice and resulted in remarkable toxicity [316].

Because the transport of drug molecules in the blood and diffusion from barriers (blood-brain barrier, testis- and prostate-blood barriers) and membranes could be enhanced by combination of drugs with P85, Schiff base was combined with P85 to increase efficiency as reported in the previous mice studies proving the non-toxicty of selected concentration for this block copolymer [317,242]. On the other hand, similar to the current work, 2.5, 10 and 33mg/kg of Docetaxel combined with a micellar agent has been shown for rapid distribution in the plasma indicating severe toxicity [318].

Subcutaneously injected Tramp-C1 prostate cancer model was chosen for the study as it is practical for rapid screening of chemotherapeutics *in vivo*. This model has been shown for its effectiveness, which starts with the PIN followed by HGPIN and prostatic carcinoma [319]. Tramp-C1 cells were obtained from the epithelial cells of prostate and display quite similar histological and biochemical properties of human prostate cancer. The dorsal area of mouse is more sutibale for tumor cell injection compared to prostate tissue because mouse prostate has a lobular structure which does not resemble the human prostate anotomy [313]. In addition, dorsal area is visible to monitor tumor progression with eye. Although some of the control group animals had visible tumors approximately 40 days after cell injection and some others developed visible tumors steadily over the course of experiment, tumors did not appear in Schiff base-P85 administered group. The difference for latency period of tumors arise from injected cells in contol group might be explained by the metabolic variations of animals. Despite the silence of literature about this situation, tumor initiation and progression were dependent on each animal metabolism and can be slow for some animals. The weights of mice exposed to either vehicle or Schiff base-P85 were identical at the first 32 days until the tumor mass was appearent. Control group animals, carrying tumors on the back region lost comparable weights with respect to Schiff base-P85 treated animals. No metastatic foci or significant toxicity was observed at the multiple organs, confirmed by histopathological evaluations. While big tumors, of those the largest one was $14cm^3$, were observed in control group, the biggest tumor with a $0.003cm^3$ volume was detected on the back of Schiff base-P85 group. All tumors resected from vehicle or Schiff base-P85 received groups had high Gleason scores indicating the aggressive phenotype of tumors. Schiff base-P85 combination, applied at a truly low dose inhibited the tumor progression with a 63% success reflecting the encouraging anticancer activity of the current combination. Since the ultimate aim of the current study is to find

out the effect of a drug combination prepeared with P85 and a novel Schiff base derivative which have been shown for its anti-cancer activity against colon and liver cancer *in vitro* and *in vivo* in our previous studies [320-321], the obtained results were compared with the available chemotherapeutics. Despite the remarkable activity and widespread use of Docetaxel, proven for effectiveness in preclinical and clinical studies, it has been shown to be ineffective for inhibition of tumor growth completely at 10mg/kg dose in a clinical trial [322]. In a mouse xenograft model, 12.5 mg/kg of Docetaxel injection (i.p.) has reduced tumor volume to $0.1cm^3$ while the control is $1cm^3$ when combined with Sabutoclax and stand alone Docetaxel has not exerted tumor supression role [323]. In another study, 40mg/kg Docetaxel and 50mg/kg Paclitaxel have exerted 60% and 40% tumor regression respectively in human fibrosarcoma xenograft model which could be referred as a slight effect compared to Schiff base-P85[324].

As Schiff base is a DNA binding agent, comparison of results with other DNA binding agents is necessary. Apigenin, a well-known DNA binding agent, has inhibited 48.5% of cancerous lesion formation in mice [325]. These preclinical data demonstrate that Schiff base-P85 combination functionally inhibits tumor formation and therefore, appears as a promising candidate for prostate cancer chemotherapy. As expected from the published studies, combination of P85 provided the micellar encapsulation of Schiff base which enabled the easy transport of Schiff base through the membrane by bocking drug transporters. Although unimers below the CMC was used for cell culture experiments in order to avoid dose dependent toxicty of P85 in micellar concentration *in vitro*, micelle forming concentration was selected for *in vivo* experiment. Micelles carrying Schiff base in the core might be highly stable, preventing the distribution of drug in circulation and elimination from kidney as reported in the literature [326]. Easy diffusion of micelles from the membrane and inhibiton of drug transporters might probably lead the rapid drug release to the cytosol and cause inhibition of cell proliferation.

Overall, a remarkable anti-cancer activity was observed for Schiff base-P85 combination *in vitro* and *in vivo* for prostate cancer; however, a set of experiments are ongoing to elucidate exact mechanism at the molecular and physiological level. Due to the limitations of the current study, several further experiments should be conducted to explore anticancer activity. Labeled drug molecules should be traced in the cell and animal body to observe

translocation way and method. As Tramp-C1 model has some limitations and could only be useful for drug screening in mouse cancer studies, nude mice and xenograft tumor models should be used to determine the effects of the drug combination on human tumors before clinical analysis. Pharmacokinetics, half life and stability analysis should be completed to design the formulation and determine proper dose, application volume and frequency for further clinical and phase studies.

5. CONCLUSION

Apart from many other types of cancer, prostate cancer is the most widespread cancer in men all around the world. Chemotherapy is applied either to control remaining cancer cells that are confined in the prostate tissue followed by initial treatment (prostatectomy) or prevent cell spreading from prostate to surrounding tissue, blood and lymph vessels. Despite the presence of available chemotherapy alternatives, the goal of this work is to develop a new alternative which is more efficient, less toxic and able to replace other chemotherapeutics.

Overall data suggest that Schiff base-P85 combination could be used as a non-toxic and effective agent against prostate cancer compared to many other chemotherapeutics in the market including Docetaxel. Although Docetaxel has been used in prostate cancer treatment for several years, high doses required for an efficient therapy, side effects observed during treatment period, demand of combination therapies to increase anticancer activity and the development of resistance increased the necessity of new options. Schiff base-P85 combination showed remarkable results at the low doses and should be improved for further preclinical and clinical analysis. This is the first study in the literature respresenting the anti-cancer activity of a novel Schiff base derivative synthysized by our group and its P85 combination on prostate cancer. The current formulation was developed by our group and has a pending patent application (TR-2014/01073).

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