Schiff Base-Poloxamer P85 Combination Prevents Prostate Cancer Progression in C57/Bl6 Mice

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BACKGROUND. Prostate cancer which is the second most common cause of death among men has a high incidence in recent years. Current therapeutic regimens should be improved to overcome drug resistance. At the metastatic stage, tumors become refractory to established chemotherapeutic treatments and cause serious problems at the clinics. Development of new drug molecules that are able to transport through the membrane easily and kill tumor cells rapidly is of great interest.
METHOD. In the current study, a novel Heterodinuclear copper(II)Mn(II) Schiff base complex combined with P85 was used for prostate cancer treatment in vivo. Tramp-C1 cells injected animals were subjected to chemotherapeutic formulation treatment and results were analyzed by toxicology analysis, tumor volume measurements, and histopathological analysis. 0.5 mg/kg Schiff base was selected and combined with 0.05% P85 according to the toxicology analysis showing the enzyme levels, blood parameters, and multiple organ toxicity.
RESULTS. Results demonstrated that Heterodinuclear copper(II)Mn(II) complex-P85 combination decreased tumor formation and tumor volume steadily over the course of experiments.
CONCLUSIONS. Overall, Heterodinuclear copper(II)Mn(II) complex-P85 exerted remarkable anti-cancer activity in vivo in C57/B16 mice. *Prostate 76:1454–1463, 2016.*

KEY WORDS: **Schiff base; pluronic; P85; prostate cancer; tramp-C1**

INTRODUCTION

Prostate cancer is the second most common cancer in men with a high incidence of 26% among other cancers [1] which is generally observed after the age of 50. It is found to be second lethal cancer with a 9% mortality ratio after lung cancer [1]. Different incidence ratios between Asia and the United States of America regions are tightly related with ethnic populations, genetic background, risk factors, or environmentally conditions [2].

Conflicts of interest: The authors declare that they have no conflict of interest.

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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 at the initial steps where the cancer is still responding hormone deprivation [3]. 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 [4]. Treatment of prostate cancer at this metastatic stage is not possible by using just one option. Surgical removal of tumor tissue, radiation therapy, or chemotherapy should be applied to fight against aggressive prostate cancer.

Researches about the chemotherapeutic drugs in prostate cancer showed that mitotic inhibitors which belongs to the taxanes such as paclitaxel and docataxel are effective [5].

Docetaxel (Sanofi- Taxotere® docetaxel) and paclitaxel (Phyton Biotech- TAXOL®) are agents which are able to stabilize microtubules and block the cell division [6,7] are generally combined with estramustine which is an estrogenic and alkylating cytotoxic agent and able to inhibit microtubule stabilization by inactivating microtubule related proteins [8]. Estramustine can be combined with different types of microtubule stabilizers such as vinblastine or vinorelbine [9]. Instead of first line therapy agents, some palliative drug formulations used in prostate cancer to provide relief for the patient. Mitoxantrone and prednisone combination was proven to be beneficial for pain relief in advanced prostate cancer patients [10]. Although single or combination therapies for prostate cancer may enhance the cytotoxic response and survival, acquired resistance mechanisms and broad range of toxic effects observed in the body restricts the therapeutic potential. Therefore, there is an urgent demand for alternative chemotherapeutic agents which can either be used as adjuvant or neo-adjuvant agents without exerting cytotoxicity to the host.

Schiff bases derived from the condensation reaction of primary amine and carbonyl compound were first identified and characterized by Hugo Schiff in 1864 [11]. Schiff bases are able to generate highly stable complexes with metal ions which make them attractive sources for biological applications [12].

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, Schiff bases and their metal complexes were found to be effective against cancer cells. 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 [13]. 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 [14]. Instead of highly active chemotherapeutic formulations, there is a remarkable demand for easy transport of these molecules from biological membranes.

Polymer-based technology has widely started to be used in pharmaceutical research and applications. Pluronic triblock copolymers also known as poloxamers, which consist of hydrophilic poly(ethylene oxide-PEO) and hydrophobic poly(propylene oxide-PPO) units are widely used in biological applications as vehicles for drugs, growth factors, and genes [15]. These polymers are able to form micelles in aqueous solutions and can encapsulate drugs and facilitate the transport by interacting with the membrane. P85 which is one of the pluronic type has a remarkable membrane permeabilization potential when combined with drugs interacting with ATP-dependent efflux pumps [16]. Inhibition of P-glycoprotein or MRP by P85 may be one of the important mechanisms for overcoming the cancer cell drug resistance [17]. P85 is widely used to enhance of drug transport to the brain because it can easily transport from the blood brain barrier which could enable the usage of this polymer for cancer chemotherapy [18]. P85 has been used in bovine brain microvascular endothelial cells (BBMECs) to inhibit drug transporters in previous studies [16]. Therefore, in the current study, we aimed to enhance anticancer activity of a novel Heterodinuclear copper(II)Mn(II) complex (Schiff base) by combining with P85 against prostate cancer.

MATERIALS AND METHODS

Preperation of Drug Combination

Heterodinuclear copper(II)Mn(II) complex (Schiff base) was synthesized and characterized by our group as described previously [19]. Schematic representation of the complex was given in Figure 1. Synthesized complex was kept at room temperature in a dark place until use. Pluronic P85, (BASF Corporation,

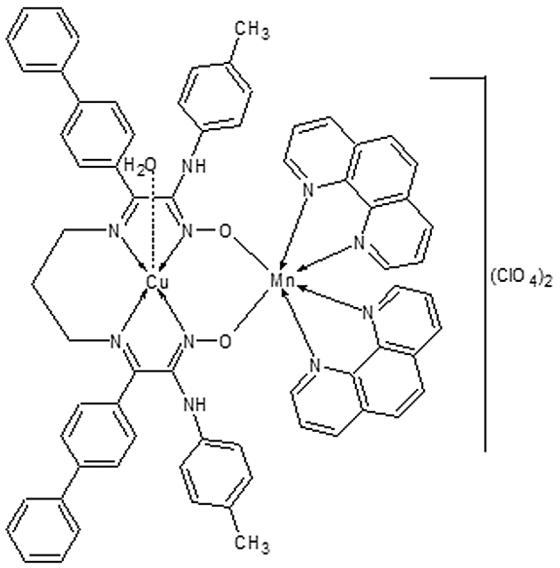

Fig. 1. Schematic representation of Heterodinuclear copper(II) Mn(II) complex.

Badische Anilin und Soda-Fabrik) was dissolved in Phosphate Buffered Saline (PBS, Pan-Biotech, Germany) at a stock concentration of 10% (w/v) by incubating at 4°C overnight and vortexing subsequently. Main stock solution (10%) was diluted to 1% in PBS and 0.05% (w/v) P85 was combined with Schiff base for in vivo analysis.

Animals

Healthy male C57/Bl6 mice (n = 16; n = 8/group) weighing 20 ± 2 g 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 ± 2 g 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-hr 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.

Toxicology Analysis

Acute toxicity analysis of Schiff base and P85 combination in C57/Bl6 mice was completed according to the protocol described previously [20]. In short, mice (n = 5) treated intraperitoneally with vehicle alone (PBS, 0.2 ml) or four different doses of Schiff base (0.1, 0.25, 0.5, and 1 mg/kg) combined with 0.05% 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 3,000*g* 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 (Sigma–Aldrich) for histopathological examinations.

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 [21]. 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 1 week after cell injection, specified concentrations of Schiff base (0.5 mg/kg) and pluronic P85 (0.05% w/v) combination were given intraperitoneally every 4 days for 52 days. Control groups received same volume (200 μl) of sterile PBS. 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.

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 [22].

$$\text{Tumor volume} = (\text{Length} \times \text{width} \times \text{height})/2$$

Pathological Analysis

Paraffin slides were taken and stained with hematoxylin and eosin for Gleason score analysis according to the previously described protocol [23,24]. Briefly, slides were placed in an incubator at 55°C for 30 min to remove paraffin. Then, slides were deparaffinized immersing in xylene (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 (Sigma, St Louis) for 2 min. Then, slides were washed under tap water for 10 min and stained with eosin (Sigma, St Louis) for 30 sec. 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 (Merck, Darmstadt, Germany).

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.

RESULTS

Blood Parameters

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 (Table I).

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 I).

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 (Table II). 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 II).

In Vivo Organ Toxicity

Macroscopic examination disclosed that no morbidity or gross lesions were noted in any experimental group. Multiple tissues including lung, liver, kidney,

TABLE I. 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
Amylase (U/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.
G1: 0.1 mg/kg Schiff base-500 mg/kg P85, G2: 0.25 mg/kg Schiff base-500 mg/kg P85, G3: 0.5 mg/kg Schiff base-500 mg/kg P85, and G4: 1 mg/kg Schiff base-500 mg/kg P85.

TABLE II. Blood Parameters of Mice Treated With Different Concentrations of Schiff Base-P85

Parameter	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 ± 33.76	708.75 ± 34.16	703.00 ± 5.33	709.00 ± 8.91
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.
G1: 0.1 mg/kg Schiff base-500 mg/kg P85, G2: 0.25 mg/kg Schiff base-500 mg/kg P85, G3: 0.5 mg/kg Schiff base-500 mg/kg P85, and G4: 1 mg/kg Schiff base-500 mg/kg P85.

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 (data not shown). Kidney pyelonephritis indicating the mild inflammation was detected in group 1 (0.1 mg/kg of Schiff base) (Fig. 2A), whereas lung lymphocytic infiltration, focal lymphocytic infiltration in kidney, central vein congestion, and focal parenchymal necrosis in liver were noted for group 2 (0.25 mg/kg of Schiff base) (Fig. 2B–D), and slight infiltration in small intestine was found to be the only toxic mark for group 3 (0.5 mg/kg of Schiff base) (Fig. 2E). 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 (1 mg/kg of Schiff base) (Fig. 2F–H). These results indicated the tolerability rhtym of 0.5 mg/kg of Schiff base-P85 combination for further animal experiments.

Animal Weights and Tumor Volume Measurements

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 0.5 mg/kg from toxicology analysis. Therefore, Schiff base (0.5 mg/ml) and P85 (0.05% P85) combination or vehicle (0.2 ml PBS) were applied intraperitoneally every 4 days for 52 days, starting from 1 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.

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 21 g and final average weight

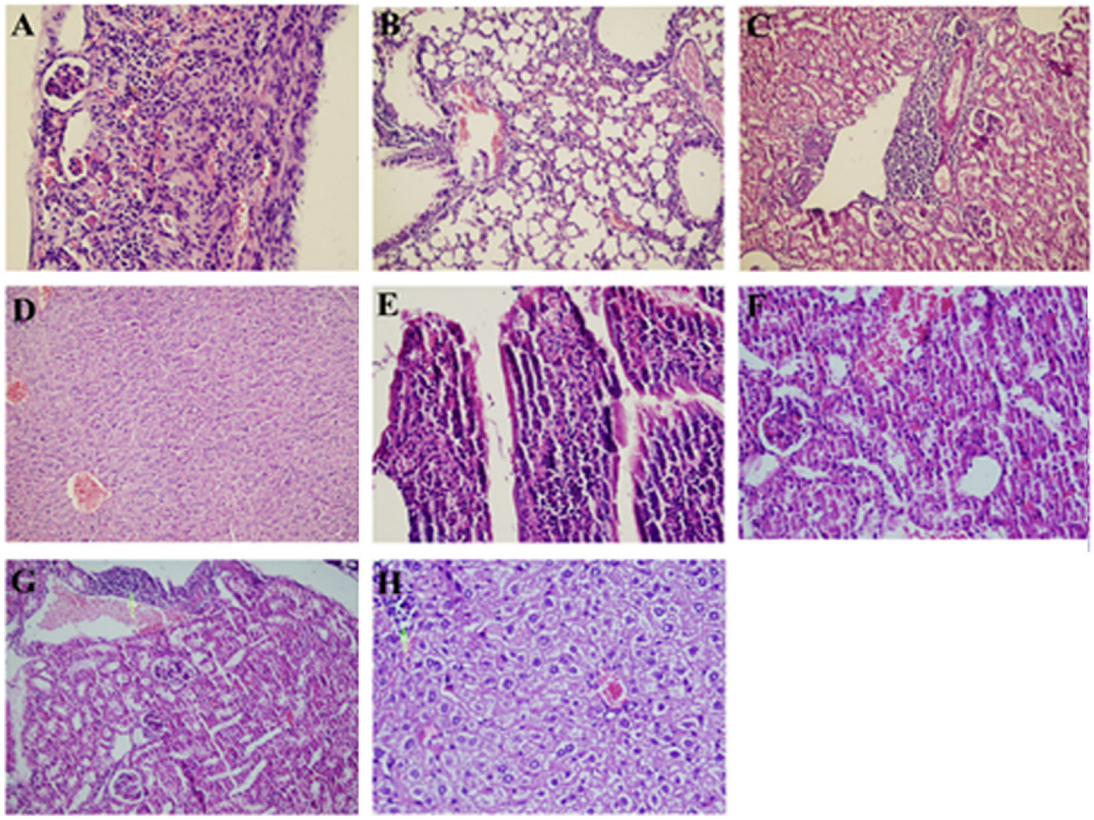

Fig. 2. Representative H&E sections from different concentrations of Schiff base-P85 treated mice tissues. (**A**) Kidney pyelonephritis in group 1, (**B**) Lung lymphocytic infiltration in group 2, (**C**) Focal lymphocytic infiltration in kidney in group 2, (**D**) Central vein congestion and focal 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: 0.1 mg/kg Schiff base-0.05%w/v P85, G2: 0.25 mg/kg Schiff base-0.05%w/v P85, G3: 0.5 mg/kg Schiff base-0.05%w/v P85, and G4: 1 mg/kg Schiff base-0.05%w/v P85.

was noted as 24 g 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 26 g at the end of 13. injection (Fig. 3).

Tumors started to appear approximately 30–40 days after cell injection. At the end of experiment (day 60 of cell transplantation), six out of eight control animals had visible tumors (C1-C2-C3-C5-C6-C7). However, it was found that as the animals were necropsied, 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 (Fig. 4A and C). Figure 4B 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 III). 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

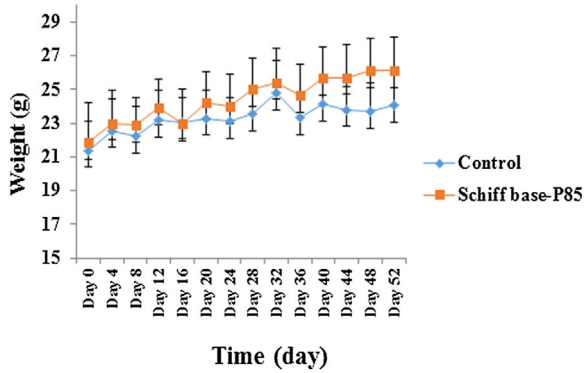

Fig. 3. Average weights of animals during in vivo experiments. Control: Cancer cell injected animals receiving vehicle (PBS). Schiff base-P85: Cancer cell injected animals receiving Schiff base-P85 combination.

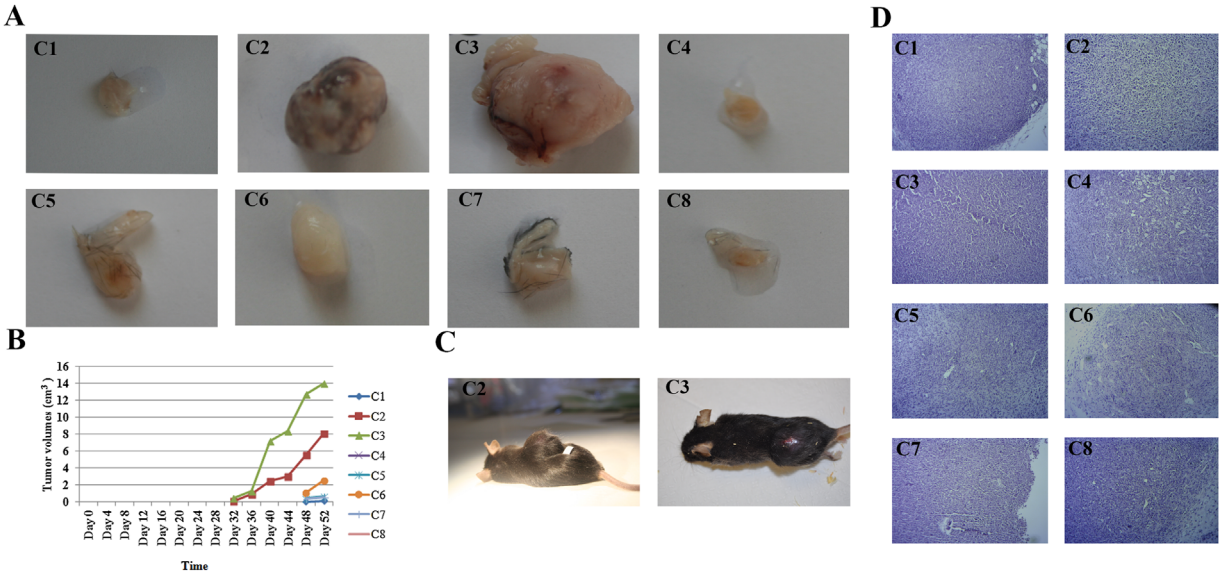

Fig. 4. Tumor growth in control group animals. (**A**) Tumor appearance after resection. (**B**) Tumor growth rate in control group animals. (**C**) Exterior appearance of C2 and C3 mice carrying large tumors at day 60 of cell transplantation. (**D**) Histopathological examinations of control group tumors. Magnification: 40×.

of cell transplantation and immature solid tumor formations were observed in only D4, D5, and D8 mice with volumes of 0.001 cm^3, 0.0045 cm^3, and 0.03 cm^3, respectively (Fig. 5) (Table IV).

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. Tumors of C3 and C4 were found to have necrotic foci (Fig. 4). 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 (Fig. 5). 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. 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 (Fig. 6).

TABLE III. 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

DISCUSSION

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. Prostate cancer is the second most common cause of death for men among other cancers. 220,800 new cases and 27,540 deaths were reported for the United States in 2015 [1]. Due to the lack of screening methods, prostate cancer diagnosis at both slow growing and more advanced stage is difficult [25]. 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 [26]. Chemotherapy as a palliative option has

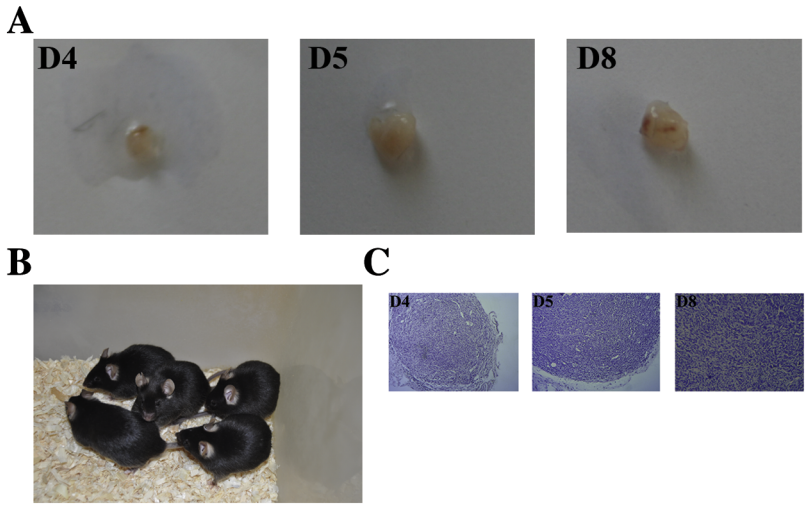

Fig. 5. Tumor growth in Schiff base-P85 treated animals. (**A**) Tumor appearance after resection. (**B**) Exterior appearance of Schiff base-P85 treated animals with no naked-eye visible tumor formation. (**C**) Histopathological examinations of Schiff base-P85 group tumors. Magnification: 40×.

emerged among other treatment methods by either increasing survival rates or extending patients life. Despite benefits of either neoadjuvant or adjuvant chemotherapy in clinics and their palliative role, prolonged survival rates could not be observed in metastatic disease along with CRPC [27]. Therefore, development of new chemotherapeutics which exert less cytotoxicity to healthy tissues and easily transport to the cancer cells is required for prostate cancer chemotherapy in clinics. In the current study, anticancer activity of a novel Schiff base derivative and P85 combination was investigated in vivo using a Tramp-C1 prostate cancer model.

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. Since chemotherapy-induced toxicity 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 [28]. MTD for Schiff base-P85 combination (0.5 mg/kg) was selected based on enzyme activities reflecting the multiple organ toxicity, blood counts indicating the myelosuppression, and histopathological analysis showing the inflammation in the organs. Tolerability rhythm of 0.5 mg/kg of Schiff base-P85 combination was better compared to equivalents used in previous preclinical studies.

TABLE IV. 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

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-toxicity of selected concentration for this block copolymer.

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 control group might be explained by the metabolic variations of 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 apparent. 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 14 cm^3,

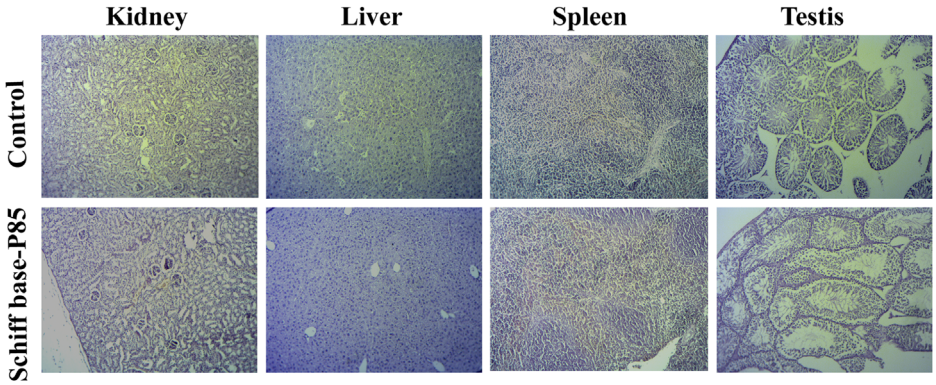

Fig. 6. Histopathological examinations of kidney, liver, spleen, and testis of control and Schiff base-P85 group animals. Magnification: 40×, Control: PBS Drug: Schiff base (0.5 mg/kg)-P85 (0.05% w/v).

were observed in control group, the biggest tumor with a 0.003 cm^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 prepared with P85 and a novel Schiff base derivative which have been shown for its anti-cancer activity against colon and liver cancer in vivo in our previous studies [29,30], 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 10 mg/kg dose in a clinical trial [31]. In a mouse xenograft model, 12.5 mg/kg of Docetaxel injection (i.p.) has reduced tumor volume to 0.1 cm^3 while the control is 1 cm^3 when combined with Sabutoclax and stand alone Docetaxel has not exerted tumor suppression role [32]. 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 [33]. Easy diffusion of micelles from the membrane and inhibition of drug transporters might probably lead the rapid drug release to the cytosol and cause inhibition of cell proliferation.

CONCLUSIONS

Overall, a remarkable anti-cancer activity was observed for Schiff base-P85 combination 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. 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.

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