Poloxamer P85 increases anticancer activity of Schiff base against prostate cancer *in vitro* and *in vivo*

Selami Demirci[a], Ayşegül Doğan[a], Neşe Başak Türkmen[d], Dilek Telci[a], Ahmet B. Çağlayan[c], Mustafa Ç. Beker[c], Ertuğrul Kılıç[c], Ferda Özkan[b], Bülent Dede[e] and Fikrettin Şahin[a]

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Prostate cancer is the second most common cancer among men and the leading cause of death after lung cancer. Development of hormone-refractory disease is a crucial step for prostate cancer progression for which an effective treatment option is currently unavailable. Therefore, there is a need for new agents that can efficiently target cancer cells, decrease tumor growth, and thereby extend the survival of patients in late-stage castration-resistant prostate cancer. In the current study, a novel heterodinuclear copper(II)Mn(II) Schiff base complex combined with P85 was used to evaluate anticancer activity against prostate cancer *in vitro* and *in vivo*. Cell proliferation and cytotoxicity were evaluated by cell viability, gene, and protein expression assays *in vitro*. Results showed that the heterodinuclear copper(II)Mn(II) complex–P85 combination decreased cell proliferation by upregulating the apoptotic gene expressions and blocking the cell proliferation-related pathways. Tramp-C1-injected C57/B16 mice were used to mimic a prostate cancer model. Treatment combination of Schiff base complex and P85 significantly enhanced the cellular uptake of chemicals (by blocking the drug transporters and increased life time), suppressed tumor growth, and decreased tumor volume steadily over the course of the experiments. Overall, heterodinuclear copper(II)Mn(II) complex–P85 showed remarkable anticancer activity against prostate cancer in *in vitro* and *in vivo*. *Anti-Cancer Drugs* 28:869–879 Copyright © 2017 Wolters Kluwer Health, Inc. All rights reserved.

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[a]Department of Genetics and Bioengineering, Faculty of Engineering, [b]Department of Pathology, Faculty of Medicine, Yeditepe University, [c]Department of Physiology, Faculty of Medicine, Istanbul Medipol University, Istanbul, [d]Department of Pharmaceutical Toxicology, University of Inonu, Malatya and [e]Department of Chemistry, Faculty of Sciences and Arts, Süleyman Demirel University, Isparta, Turkey

Correspondence to Ayşegül Doğan, PhD, Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University Kayisdagi, 34755 Istanbul, Turkey
Tel: + 90 216 578 0619; fax: + 90 216 578 0829;
e-mails: aguldgn@gmail.com, aysegul.dogan@nih.gov

Present address: Selami Demirci: National Heart, Lung, and Blood Institute (NHLBI), NIH, Bethesda, Maryland, USA.

Present address: Ayşegül Doğan: National Cancer Institute, CDBL, NIH, Frederick, Maryland, USA.

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Introduction

Prostate cancer is the second most frequently diagnosed cancer among men and also the leading cause of death (9% mortality ratio) after lung cancer [1]. Regulation of prostate cancer is a multistep process controlled by androgens at the cellular and molecular levels through androgen and tyrosine kinase receptor mutations, amplifications, or enhancement the sensitivity of receptors to growth factors [2]. Development of androgen independency is one of the biggest problems during the treatment of prostate cancer. Androgen-deprivation therapy as a standard treatment can enhance the sensitivity of cells to chemotherapy and radiotherapy. Development of castration-resistant prostate cancer (CRPC) is a major challenge for prostate cancer therapy when hormone-deprivation therapy cannot prevent cancer growth [3]. Surgical removal, antiandrogens, chemotherapy, and radiotherapy are the treatment options generally applied for advanced-stage prostate cancer [4,5]. The heterogenic nature of prostate cancer is exemplified by the tumor response variability to chemotherapy underscoring the challenge [6]. Although a combination of chemotherapeutic agents is considered to be an effective treatment strategy for prostate cancer, there is a scarcity of data on the enhanced survival rates or improved quality of life of patients. Besides, patient survival rates are lower compared with the higher toxicity associated with combinatorial treatment [7]. There are several available Food and Drug Administration approved chemotherapeutics such as estramustine, mitoxantrone, docetaxel, and cabazitaxel for the prostate cancer treatment [4]. Docetaxel and cabazitaxel are taxane group chemotherapeutics, and act on the microtubule structure, P-glycoprotein, and multidrug-resistance protein-1 (MRP-1) [3]. The activity of these chemotherapeutics is impaired because of the amplification of these drug transporters [8]. Although docetaxel is the most promising available chemotherapeutic in the market, acquired resistance is a major problem and response in CRPC is not satisfactory. Therefore, development of new chemotherapeutics,

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particularly for late-stage metastatic prostate cancer, is an increasingly attractive field.

Schiff bases are derived as a result of the condensation reaction of primary amine and carbonyl compounds [9]. Different Schiff base derivatives or their metal complexes were identified for their anti-inflammatory [10], antifungal [11], antimicrobial [12], and antihypertensive [13] activities. In addition to their broad range of biological activities, the anticancer properties of various Schiff bases have been reported previously [14–16]. Their DNA-binding and cleavage activities favor the usage of these compounds as potential drugs in cancer treatment. The antitumor activity of Schiff bases has been attributed to DNA breakage through hydrogen bond formation with nitrogen atoms of DNA or copper binding to the DNA and/or to the proteins [17]. In the current study, we evaluated the anticancer activity of a novel Schiff base derivative, referred to as the heterodinuclear copper(II) Mn(II) complex, in combination with the poloxamer P85. In our previous studies, we have documented the antitumor activity of this Schiff base derivative against liver, colorectal cancer, and prostate cancer [18–20].

Polymer-dependent drug delivery to tumor tissues, on the basis of the encapsulation of drug molecules into stable micelles, is a new modality in chemotherapy research. Micellar encapsulation of drug molecules decreases the systemic degradation of drug molecules in the blood stream and increases the drug uptake by tumor cells [21]. Poloxamers, also known as Pluronics, arranged in an A–B–A triblock structure that consist of hydrophilic poly(ethylene oxide-PEO) and hydrophobic poly(propylene oxide-PPO) units have been used for the delivery of chemotherapeutics to cancer cells [22]. P85 among these copolymers have been combined with different molecules to inhibit ATP-dependent efflux pumps, increase membrane permeabilization [23], and overcome drug resistance in various cancer cells [24]. Their ability to transport drugs through blood barriers makes them potential candidates for chemotherapeutic delivery. P85 has increased anticancer activity of several chemotherapeutic drugs in the previous studies [25,26]. We have already evaluated the anticancer activity of the heterodinuclear copper(II)Mn(II) in complex with P85 for an early-stage prostate cancer model *in vivo* [18]. In the current study, we have conducted in-vivo experiments using a prostate cancer model in C57/Bl6 mice mimicking the late-stage disease, and in-vitro experiments, to mechanistically analyze the cytotoxic effect of the Schiff base–P85 complex by defining the molecular pathways involved.

Materials and methods

Preparation of drug combination

The heterodinuclear copper(II)Mn(II) complex (Schiff base) was synthesized and characterized by our group as described previously [27]. Preparation of the heterodinuclear Cu(II)-Mn(II) complex was performed in six steps. 4-(Chloroacetyl)biphenyl was prepared from chloroacetyl chloride and biphenyl in the presence of aluminum chloride according to Friedel–Crafts acylation. Chloroketooxime was obtained by reacting 4-(chloroacetyl)biphenyl with alkyl nitride in the presence of dry HCl gas. Ligand was prepared by the condensation reaction of chloroketooxime with *p*-toluidine and 1,3-propanediamine, respectively. The oily mononuclear Cu(II) complex was obtained by the addition of metal salts of Cu(II) to the solution of the ligand dissolved in acetone. Finally, the heterodinuclear Cu(II)–Mn(II) complex of the iminooxime ligand was precipitated by the reaction of the mononuclear Cu(II) complex with Mn(II) salt and 1,10-phenanthroline. The structure of the Schiff base used in this study is shown in Fig. 1a. The complex was stored at room temperature in a light-protected tube in a dry place. Pluronic P85 (BASF Corporation, Badische Anilin und Soda-Fabrik, Florham Park, New Jersey, USA) was prepared in PBS (Pan-Biotech, Aidenbach, Germany) at a stock concentration of 10% (w/v) by incubation at 4°C overnight for complete dissolution. The main stock solution (10%) was diluted to 1% in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Gibco, UK) containing 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin/amphotericin (PSA; Invitrogen) for cell culture experiments. P85 (0.05% w/v) was combined with the selected concentrations of the Schiff base in DMEM supplemented with 10% FBS and 1% PSA for in-vitro analysis. The main stock of P85 (10%) was diluted to 0.05% (w/v) in PBS for in-vivo experiments.

Cell lines

Tramp-C1 (CRL 2730, mouse prostate cancer cells), DU 145 (HTB 81, human prostate cancer cells), and LNCaP (CRL 1740, human prostate cancer cells) were purchased from ATCC (Rockville, Maryland, USA). All cell lines were incubated in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% PSA (Invitrogen) in a humidified chamber at 37°C and 5% CO_2.

Cell viability analysis

The cell viability of prostate cancer cell lines (Tramp-C1, DU 145, and LNCaP) was measured using the 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTT)-assay (#G3582; CellTiter96 Aqueous One Solution; Promega, Southampton, UK) as described previously [28]. The Schiff base was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri, USA) at a stock concentration of 1 mg/ml and diluted to 10 μg/ml in DMEM supplemented with 10% FBS and 1% PSA.

Cells were exposed 2 μg/ml of the Schiff base in complete growth medium and its P85 combination. Working solution for P85 was selected as 0.05% (w/v). Cell viability was analyzed at three different time intervals (24, 48, and 72 h). At the end of each time point,

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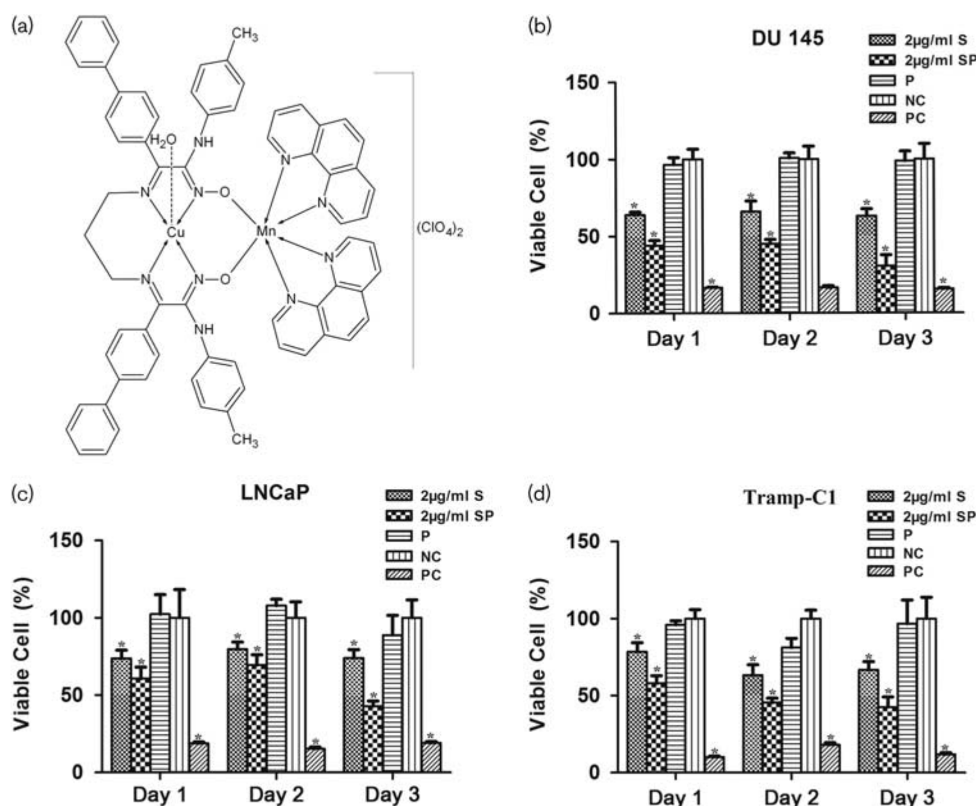
Fig. 1



Structure of Schiff base and toxicity analysis. (a) Chemical structure of the heterodinuclear copper(II)Mn(II) complex. Cytotoxicity of the Schiff base–P85 combination on (b) DU 145, (c) LNCaP, and (d) Tramp-C1 at day 1, 2, and 3. Cell viability was evaluated by comparison with NC. NC, negative control (growth medium); P, Pluronic P85 (0.05% P85); PC, 20% dimethyl sulfoxide; S, Schiff base (2 μg/ml); SP, Schiff base–P85 complex (2 μg/ml S–0.05% P85). $^{*}P < 0.05$.

absorbance at 490 nm was detected using an ELISA plate reader (Biotek, Winooski, Vermont, USA).

Real-time quantitative reverse transcription PCR analysis

RT-PCR experiments were conducted according to the previously described protocol [29]. Primers for Bcl-2-associated X protein (*BAX*), B-cell lymphoma 2 (*Bcl-2*), ATP-binding cassette subfamily G member 2 (*BCRP*), *caspase 3*, epidermal growth factor receptor (*EGFR*), MRP, and p53 were designed using Primer-BLAST software from the National Center for Biotechnology (Bethesda, Maryland, USA) and synthesized by Macrogen (Seoul, Korea). β-Actin was used as the housekeeping gene. Total RNA from drug-treated cancer and healthy cells were isolated using the RNeasy plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized using the High Fidelity cDNA synthesis kit (Roche, Indianapolis, Indiana, USA). RT-PCR with the SYBR Green (Foster City, California, USA), method was used to detect mRNA levels of the target genes. All RT-PCR experiments were conducted using the CFX96 RT-PCR system (Bio-Rad, Hercules, California, USA).

Western blot analysis

All chemicals used in immunoblotting assays were purchased from Bio-Rad Laboratories (Richmond, California, USA). Primary antibodies against Akt (#9272), nuclear factor-κB (NF-κB) (#8242) and GAPDH (#8884), all purchased from Cell Signaling Technology (Beverly, Massachusetts, USA), were used to detect marker proteins for cancer cells treated with drug compositions. Nitrocellulose membranes were incubated with primary antibodies (dilution 1 : 5000) at 4°C for 16 h. After washing with TBS-T three times, the membranes were incubated with the anti-rabbit secondary antibody (sc-2004, dilution 1 : 5000; Santa Cruz Biotech Inc., Dallas, Texas, USA) prepared in blocking buffer for 1 h at room temperature. GAPDH was used as an internal control and images were obtained using the luminometer system (Bio-Rad). Band intensities were calculated using NIH Image J software (NIH Image, Bethesda, Maryland, USA) and normalized to the respective GAPDH band intensities. Results were represented as fold change of control.

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Animals

Healthy male C57/Bl6 mice (total 32; $n=8$/group) weighing 20 ± 2 g were obtained from Yeditepe University (Istanbul, Turkey). The animals were housed individually in disinfected cages and acclimated to a constant temperature of $23\pm1°C$ and a relative humidity of $60\pm10\%$. The mice were maintained on a 12-h light/dark cycle and fed with food and water *ad libitum*. All respective procedures were approved by the Yeditepe University Ethics Committee of Experimental Animal Use and the Research Scientific Committee.

Development of the tumor model and drug application

Prostate cancer was developed on the dorsal side of C57/Bl6 mice by a Tramp-C1 cell injection according to the previously described protocol [18,30]. Briefly, 2×10^7 Tramp-C1 cells were injected subcutaneously under moderate anesthesia and the tumor development was monitored daily. For the examination of the Schiff base's anticancer activity, specified concentrations of Schiff base (0.5 mg/kg) and Pluronic P85 (0.05%) combination were administered intraperitoneally every 4 days after tumor volumes became $\sim0.34\ cm^3$. Control groups received the same volume (200 µl) of sterile PBS. Mice were examined for mortality and morbidity throughout the study and weight changes were recorded. Experiments were terminated for each group individually when the animals started to die. Mice were killed by cervical dislocation at the end of the experiment and tumor tissues were maintained in a 10% formaldehyde solution for histopathological examinations. The experimental design is shown in Table 1.

Tumor volume measurements

Throughout the experiments and after resection of tumors, their sizes were measured by a caliper according to the formula given below [31]:

$$\text{Tumor volume} = \frac{\text{Length}\times\text{width}\times\text{height}}{2}.$$

Pathological analysis

Paraffin slides were obtained and stained with hematoxylin and eosin for Gleason score analysis according to the previously described protocol [32,33]. Multiple organs were analyzed for metastasis.

Statistical analysis

The data were statistically analyzed using one-way analysis of variance and the Tukey post-hoc test, with a P-value less than 0.05 considered statistically significant.

Results

Cytotoxicity of Schiff base–Pluronic P85 in prostate cancer cell lines

The impact of various Schiff base concentrations and its Pluronic combinations on prostate cancer cells was evaluated to identify the minimum effective dose for further experiments [34]. A combination of 2 µg/ml Schiff base–0.05% P85 (SP) was used for cytotoxic analysis for 24, 48, and 72 h. Although stand-alone Schiff base application (S) decreased the viable cell ratio approximately to 65% compared with the baseline, Schiff base–0.05% P85 decreased cell viability to 30, 42, and 42% for DU 145 (Fig. 1b), LNCaP (Fig. 1c), and Tramp-C1 (Fig. 1d) cells, respectively, at the end of the 72 h incubation period. P85-only treatment remained ineffective on the viability of cancer cells, unless combined with the Schiff base chemical (Fig. 1).

Gene expression analysis

Apoptotic gene expression levels were examined in the drug-treated group to evaluate the mechanism of cytotoxicity. The results showed that Bax, caspase 3, and p53 expressions were significantly upregulated in the Schiff base–P85 (SP) group, indicating the onset of apoptosis in the Schiff base and P85 combination group. Although there was no significant change in the expression levels of antiapoptotic *Bcl-2* gene in DU 145 cells for the experimental groups, LNCaP and Tramp-C1 cells showed a significant reduction of Bcl-2 expression (Fig. 2). Although stand-alone Schiff base increased the expression of some of the apoptotic genes, the monotreatment with the Schiff base or P85 did not generally affect the apoptotic gene expression levels. *EGFR* expression, a vital gene in the prostate cancer progression during hormone-refractory disease emergence, reduced significantly in the S and SP groups (Fig. 3). *BCRP* and *MRP* expression levels were also found to be low in the S and SP groups with respect to the vehicle-treated control. The most significant reduction was observed in the SP group (Fig. 3).

Table 1 In-vivo experimental design

Groups	Treatment	Period (tumor formation) (days)
Control	20×10^6 Tramp-C1 injection + PBS solution injection	48
S (0.5 mg/kg)	20×10^6 Tramp-C1 injection + S solution injection	48
SP (0.5 mg/kg S–0.5% P85)	20×10^6 Tramp-C1 injection + SP solution injection	48
P (0.5% P85)	20×10^6 Tramp-C1 injection + SP solution injection	48

S, Schiff base, administration of S at 0.5 mg/kg of body weight; SP, Schiff base–P85 complex, administration of SP complex at 0.5 mg/kg S–0.05% P85 of body weight; P, Pluronic P85 (0.05% P85).

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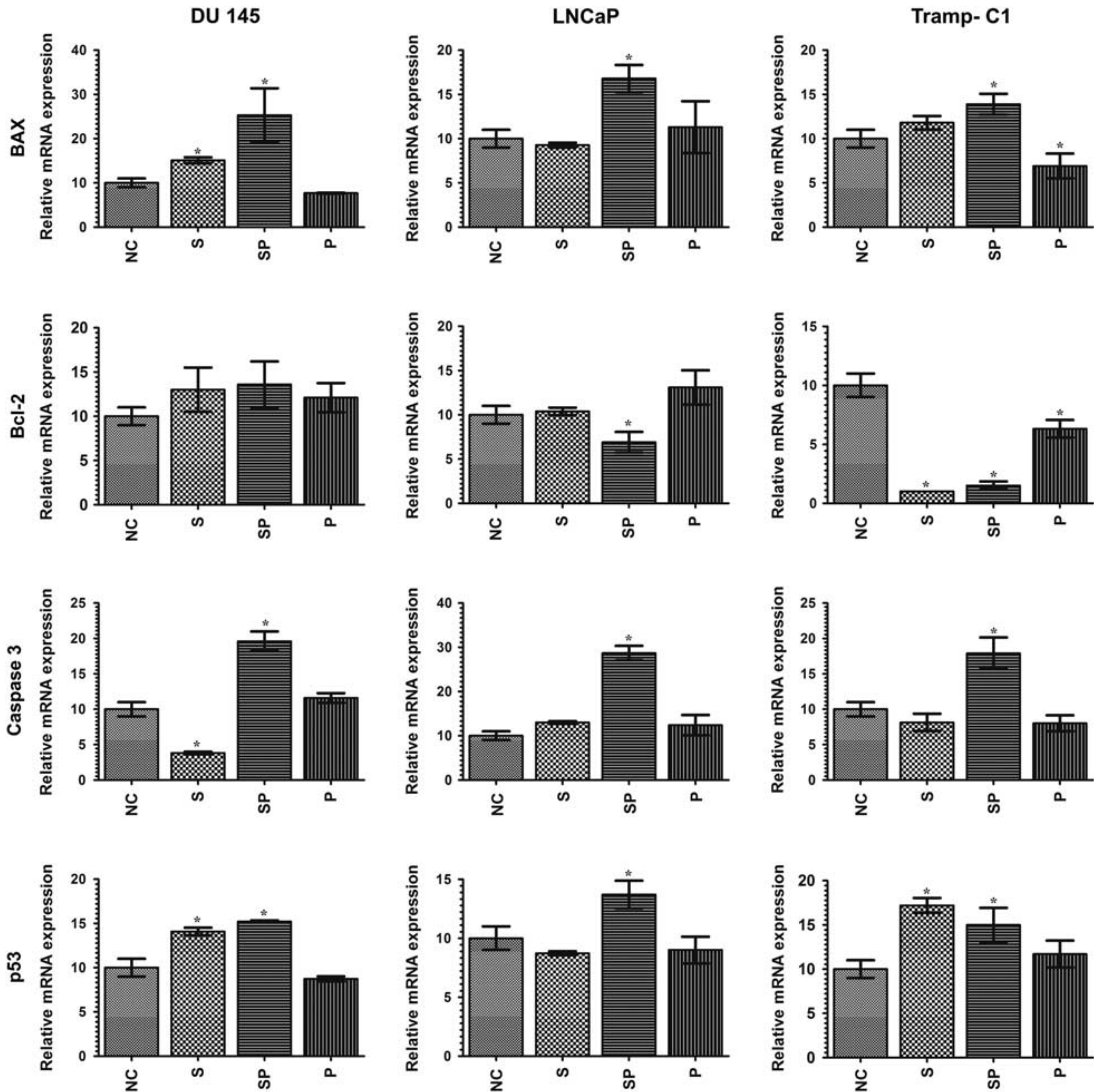
Fig. 2



Comparison of mRNA expression levels of Bax, Bcl-2, caspase 3, and p53 in the experimental groups. Gene expression was evaluated by comparison with NC. NC, negative control (growth medium); P, Pluronic P85 (0.05% P85), S, Schiff base (2 μg/ml); SP, Schiff base–P85 complex (2 μg/ml S–0.05% P85). $^{*}P < 0.05$.

Protein expression analysis

Akt and NF-κB protein expression levels were detected to observe the proliferative state of cancer lines treated with the Schiff base and its P85 combination. SP-exposed cells showed reduced levels of Akt and NF-κB proteins, which might be the primary explanation for decreased cell viability and enhanced cytotoxicity. S application also decreased the protein expressions in DU 145 cells for both proteins, and NF-κB protein expression in LNCaP and Tramp-C1 cells. Interestingly, Akt expression did not change in Tramp-C1 cells for either S or SP groups (Fig. 4).

Animal weights, life time, and tumor volumes

Forty-eight days after a subcutaneous Tramp-C1 cell injection was administered, tumors appeared on the dorsal side and animals were divided randomly into four groups as shown in Table 1. The maximum tolerated

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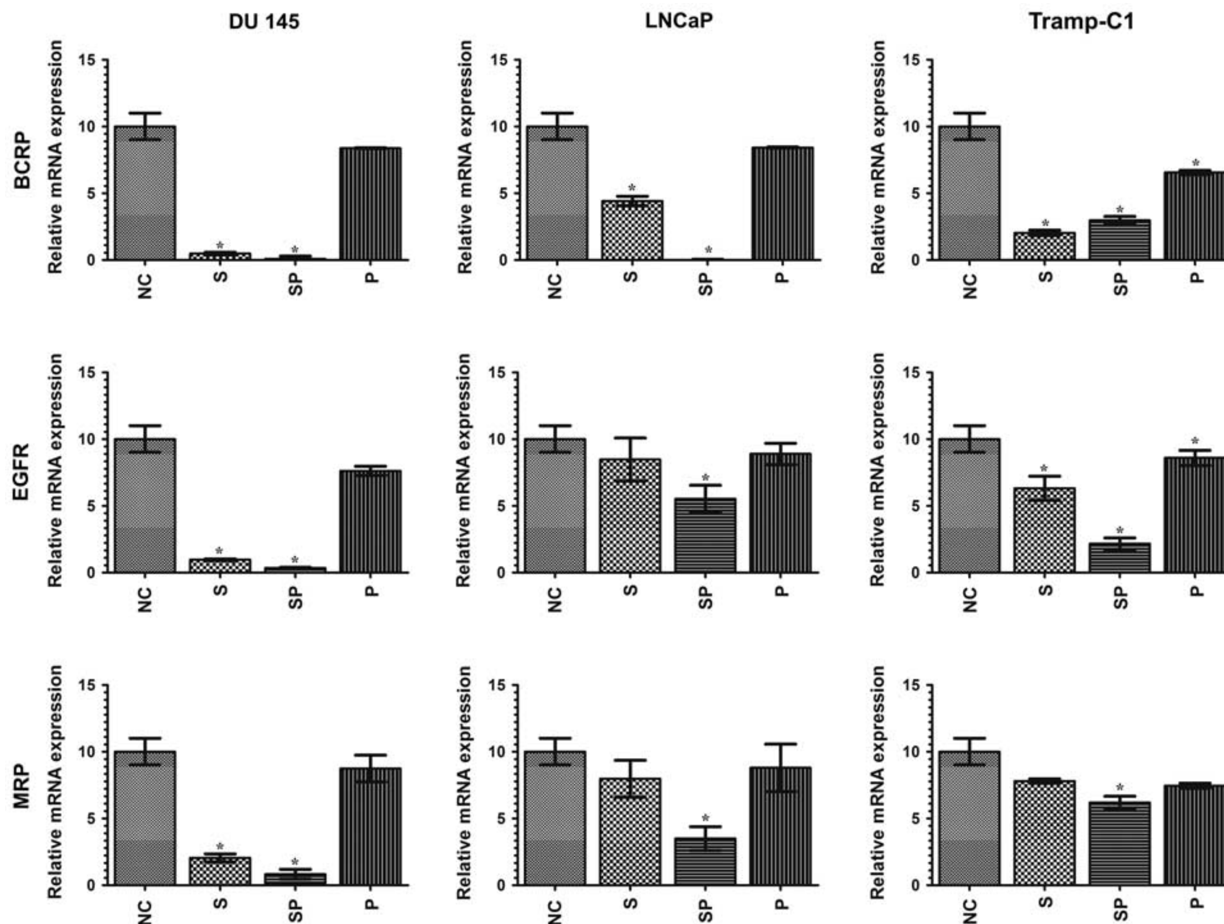
Fig. 3



Comparison of mRNA expression levels of BCRP, MRP, and EGFR in the experimental groups. Gene expression was evaluated by comparison with NC. EGFR, epidermal growth factor receptor; MRP, multidrug-resistance protein; NC, negative control (growth medium); P, Pluronic P85 (0.05% P85); S, Schiff base (2 μg/ml); SP, Schiff base–P85 complex (2 μg/ml S–0.05% P85). $^{*}P < 0.05$.

dose of the Schiff base and Pluronic combination was selected for animal experiments on the basis of our previously published toxicology analysis [18]. Schiff base (0.5 mg/ml) and P85 (0.05% P85) combination or vehicle (PBS) in 0.2 ml volume were applied intraperitoneally every 4 days during the experimental period. Weight changes were recorded periodically and observed daily to evaluate morbidity and mortality. Animals in each group were killed by cervical dislocation when they started to show tumor formation. The initial average weight was ~24 g for each experimental group. However, weights started to increase during the experimental period because of the rapidly growing tumors, except for the SP and P groups. The average weight was 32, 30, 26, and 24 g for the control, S, SP, and P, respectively (Fig. 5a). The mean life time during the experimental period was 58, 60, 85, and 70 days for the control, S, SP, and P groups, respectively (Fig. 5b). The animals treated with the SP combination lived 37 days after tumor formation was observable (drug application-day 48), whereas the mean life time was only 10 days for the control animals (Figs 5c and 6c). All animals in the experimental group had visible solid tumors. Figure 5d shows the exponential tumor growth for the control, S, and P groups. In SP animals, tumors started to grow after 16 days of drug application and increased slowly over the course of the experiment (Fig. 5d). Tumor volumes were ~5.4, 10, 3, and 5.7 cm^3 for the control, S, SP, and P groups, respectively, at the end of the experiment (Fig. 5e). Highly vascularized solid tumors and animals carrying these tumors on the dorsal site close to tail are shown in Fig. 6a.

Histopathological analysis

Gleason scores of tumors in the experimental groups were determined by histopathological analyses (Fig. 6b). Although high Gleason scores (5 + 5) and aggressive phenotypes were diagnosed in all experimental groups (Fig. 6b and Table 2), only the control and P groups showed tumor metastasis to the distant organs. Metastatic

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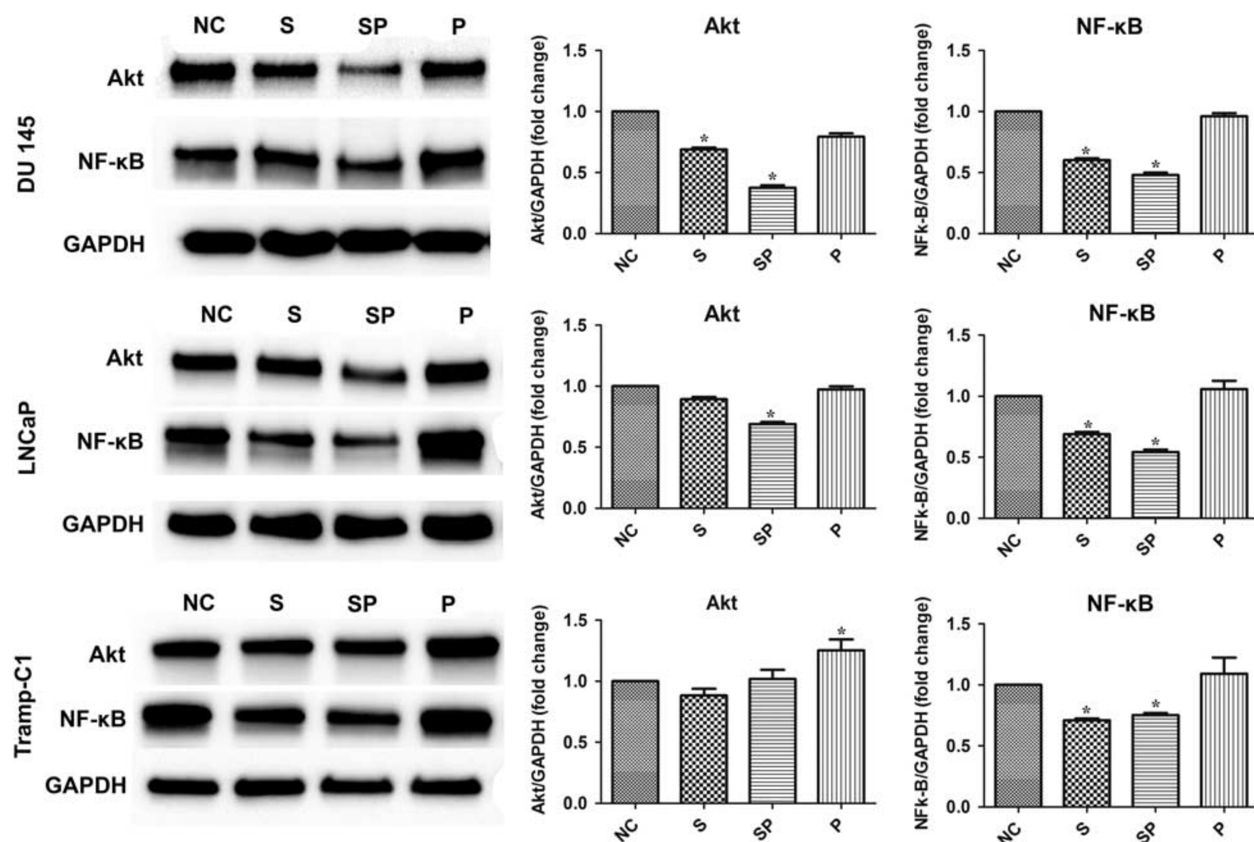
Fig. 4



Akt and NF-κB protein expression levels of DU 145, LNCaP, and Tramp-C1 cells. NC, negative control (growth medium); NF-κB, nuclear factor-κB; P, Pluronic P85 (0.05% P85); S, Schiff base (2 μg/ml); SP, Schiff base–P85 complex (2 μg/ml S–0.05% P85). *$P < 0.05$.

foci were observed in the kidney for control animals and the P group (Fig. 7a and b). In addition, metastasis was recorded in the testis of the P group (Fig. 7c and Table 2).

Discussion

Prostate cancer is a common malignancy among men, which can be responsive to chemotherapy before developing into androgen-independent CRPC and acquiring an aggressive phenotype [1,35]. Hormone-ablation therapy efficiency is similar to that of chemotherapy or radiotherapy for prostate cancer in the early stages; however, cancer cells can generally induce an androgen-independent stage and metastasize to other body parts, primarily to bone, which reduces the overall survival at the late stages [36]. Because treatment options are limited for the late-stage prostate cancer because of acquired resistance, new treatment modalities and pharmaceutical compounds are highly warranted. Although several chemotherapeutics have been approved for CRPC, high survival rates have not been observed so far, and the side effects of the chemicals prevent the usage in elderly patient population [37]. In the present study, we have investigated the anticancer activity of a Schiff base derivative and its P85 combination both in-vitro and in-vivo using a late-stage Tramp-C1 prostate cancer model. More recently, we have shown that the Schiff base and P85 combination inhibited tumor growth for an early-stage prostate cancer model in C57/B16 mice without exerting any tissue or organ toxicity, suggesting its potential use in metastatic cancer [18].

Using three different prostate cancer cell lines *in vitro* (DU 145, LNCaP, Tramp-C1), we showed that treatment with Schiff base and P85 suppressed the cell viability more significantly compared with the stand-alone Schiff base application. Similarly, the Schiff base and its P85 combination could induce the expression of apoptotic genes including *BAX*, *caspase 3*, and *p53*, while decreasing the antiapoptotic *Bcl-2* expression. To understand the possible molecular events occurring after the administration of Schiff base–P85, we carried out protein expression analyses. It was reported that the activation of Akt and its downstream pathways such as NF-κB are major regulators for cancer cell survival, growth, proliferation, and chemoresistance [38]. The combination of Schiff base and P85 decreased the Akt and NF-κB gene

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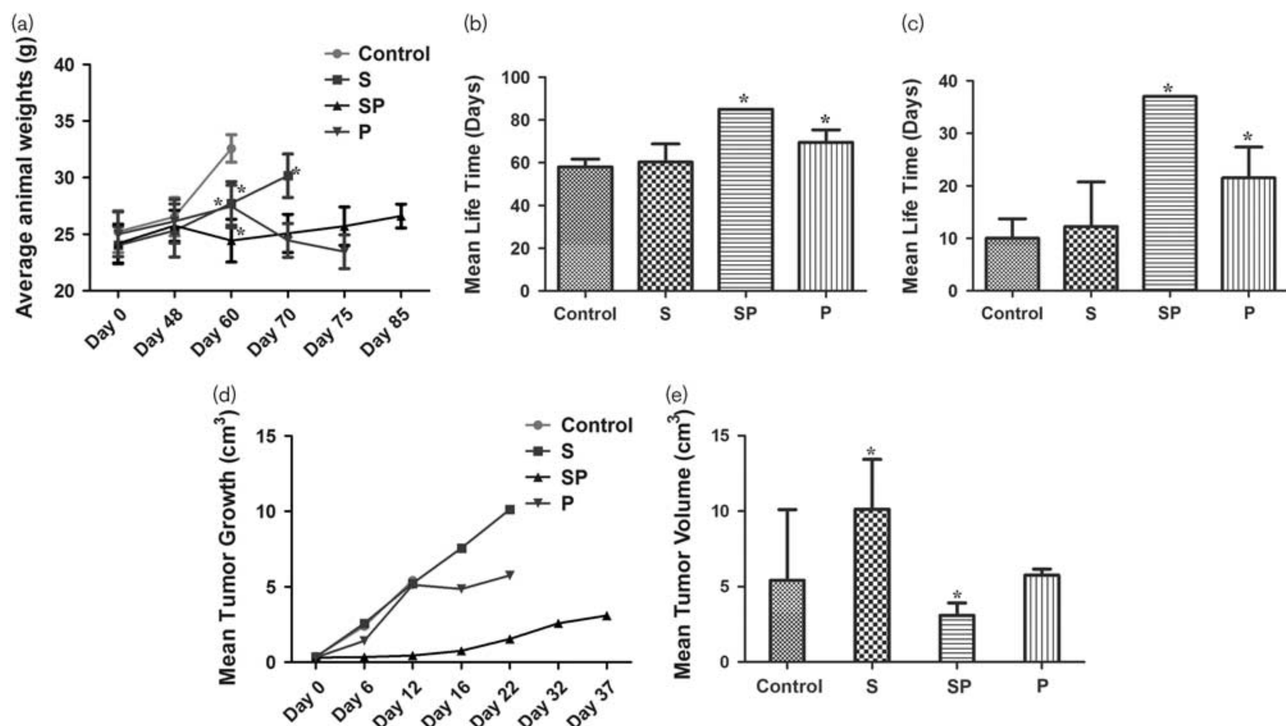
Fig. 5



Animal weight, mean life time, and tumor measurements of the experimental groups. (a) Average animal weights during the experiment, (b) mean life time from the beginning of the experiment, (c) mean life time after drug application, (d) tumor growth rate after drug application, (e) mean tumor volume at the end of the experiment. Control, animals received 0.2 ml PBS; P, Pluronic P85 (0.05% P85); S, Schiff base, administration of S at 0.5 mg/kg of body weight; SP, Schiff base–P85 complex, administration of the SP complex at 0.5 mg/kg S–0.05% P85 of body weight. *$P<0.05$.

and protein levels in the prostate cancer cells *in vitro*, which provides a possible explanation for the cytotoxic activity.

Drug efflux pumps (P-glycoprotein, BCRP, MRP-1, and MRP-2), 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 the cancer cell membranes are significantly high compared with that of normal cells [39]. The inhibitory role of P85 on these drug transporters could be an advantage for in-vitro and in-vivo drug-delivery approaches compared with conventional therapies. The gene expression analysis showed a marked decrease in *MRP* and *BCRP* gene levels for the prostate cancer cells treated with Schiff base–P85, indicating a strong correlation between Schiff base and P85. AR-dependent growth of prostate cancer cells is mediated by activating Elk-1-binding sites and promoter activation through phosphorylation and EGFR activation, which subsequently enables cell growth [40]. In the current study, the expression levels of *EGFR* were lower in the Schiff base–P85 group than the Schiff base or P85-alone groups, showing synergistic activity between the Schiff base and P85.

A prostate cancer model in subcutaneous Tramp-C1-injected C57/B16 mice that mimics the advanced stage of cancer was created by cell transplantation to the flank region and following cancer progression until tumor growth reached $\sim 0.34\,cm^3$ volume. Although control and Schiff base-administered animals gained weight throughout the course of the experiment possibly because of the development of large tumors, animal weights in the Schiff base and P85 combination or stand-alone P85 groups were normal. The Schiff base–P85 treatment improved the survival rate and increased the overall life time of tumor-bearing mice compared with single treatment or control groups. Tumor growth was markedly diminished and tumor volume was significantly reduced in the Schiff base–P85 group, suggesting a potential efficiency of this formulation in the treatment of late-stage prostate cancer tumors. Although the Schiff base–P85-treated animals had $3\,cm^3$ tumors at the end of the experiment, the animals that received Schiff base or P85 had 10 and $6\,cm^3$ visible tumors, respectively. Control group animals that were carrying $\sim 5.4\,cm^3$ tumors died rapidly after 10 days of saline application. All tumors derived from this prostate cancer model had high Gleason scores, explaining the aggressive tumor phenotype. Metastatic tumors in the distant organs were

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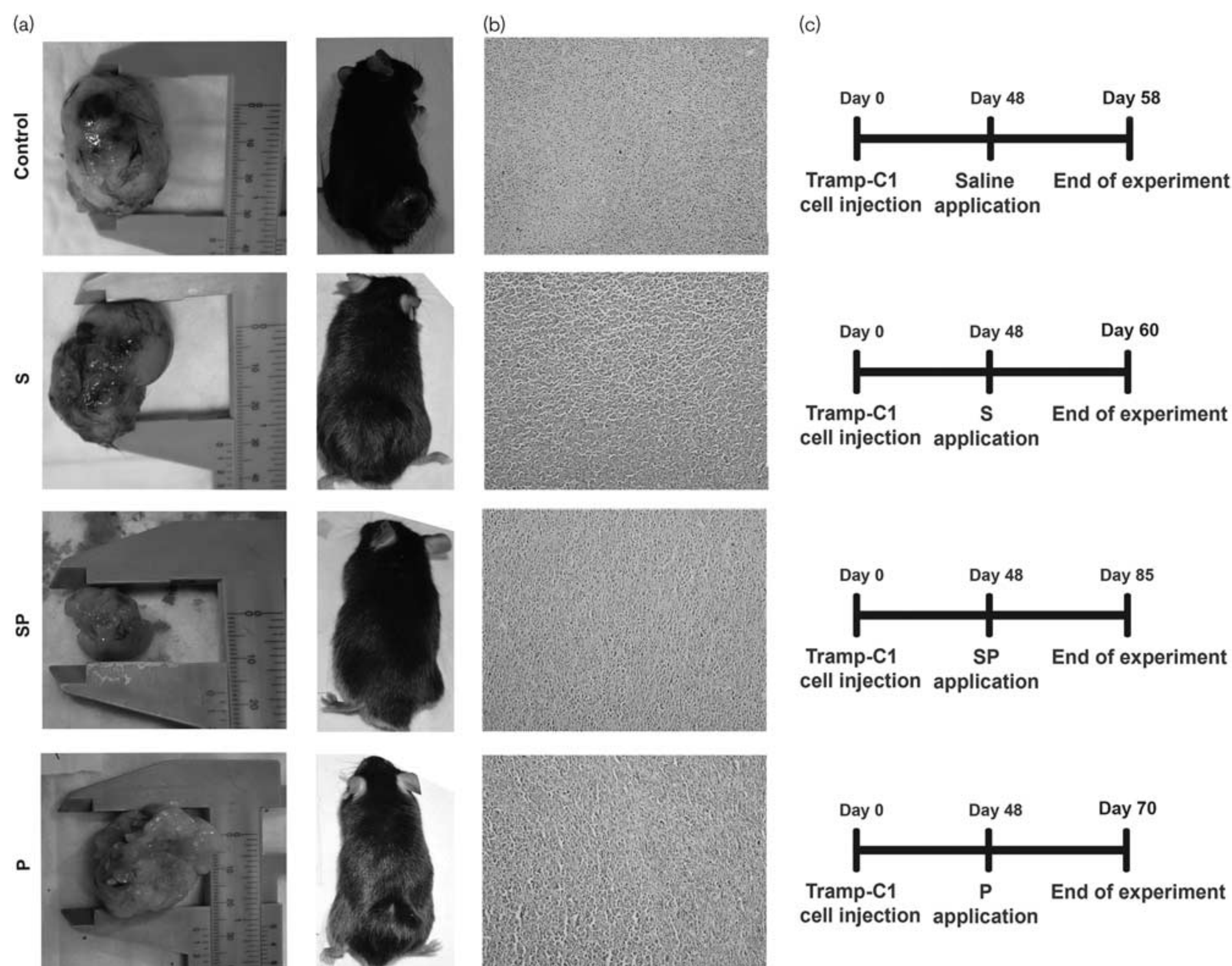
Fig. 6



Tumor gross appearance at the end of the experiments and histopathological analysis. (a) Tumors resected from animals and animals with large tumors on the flank region. (b) Histopathological appearance of tumor samples. All tumors have a high Gleason score, with a differentiated phenotype. (c) Timing plan for experimental groups of in-vivo analysis. Control, animals received 0.2 ml PBS; P, Pluronic P85 (0.05% P85); S, Schiff base, administration of S at 0.5 mg/kg of body weight; SP, Schiff base–P85 complex, administration of the SP complex at 0.5 mg/kg S–0.05% P85 of body weight; magnification $\times 100$.

Table 2 Gleason score analysis and organ metastasis after histopathologic examination

		Metastasis to organs					
Groups	Gleason score	Liver	Heart	Kidney	Testis	Intestine	Lung
Control	5 + 5	–	–	+	–	–	–
S (0.5 mg/kg)	5 + 5	–	–	–	–	–	–
SP (0.5 mg/kg S–0.5% P85)	5 + 5	–	–	–	–	–	–
P (0.5% P85)	5 + 5	–	–	+	+	–	–

S, Schiff base, administration of S at 0.5 mg/kg of body weight; SP, Schiff base–P85 complex, administration of the SP complex at 0.5 mg/kg S–0.05% P85 of body weight; P, Pluronic P85 (0.05% P85).

detected in kidney and testis tissues of control or Pluronic administered animals. Although the activity of stand-alone P85 has not been identified as yet, it is clear that P85 also played a protective role in the survival of animals by the stimulation of antigen-producing cell production in the immune system [41]. Moreover, the rapid degradation of Schiff base in the circulation and excretion through the kidney might be the reason for the unsatisfactory results observed in animals that received stand-alone Schiff base.

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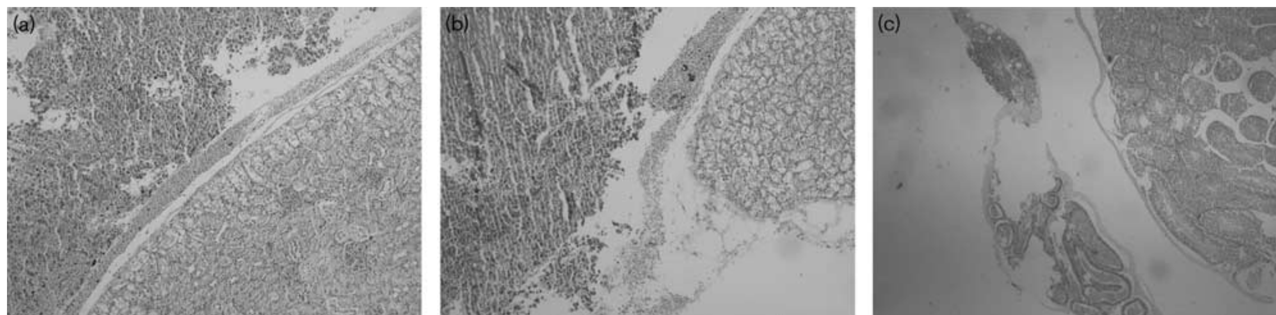
Fig. 7



Histopathological examinations of metastatic foci. (a) Metastasis in the kidney of the control group (b) and the P group. (c) Testis metastasis of the P group. Control, animals received 0.2 ml PBS; P, Pluronic P85 (0.05% P85); magnification (a, b) ×100, (c) ×40.

These preclinical data showed that the Schiff base–P85 combination functionally decreased tumor growth and, therefore, is a promising candidate for prostate cancer chemotherapy. In agreement with the published studies [23,24], the combination of P85 caused micellar encapsulation of the Schiff base, which enabled the easy transport of Schiff base through the membrane by blocking drug transporters. Transport of the Schiff base in P85 micelles plausibly provided stability in blood stream and prevented elimination [42]. P85, when used under a critical micelle concentration, forms unimers and interacts with the lipid membrane to enhance flexibility and block the ATP-dependent drug transporters [43]. The concentration of P85 (0.05%) used in in-vitro analysis alters the fatty acid content of the membrane and inhibits the drug transporters on cancer cells [44]. Schiff base, when combined with P85, could be protected in the circulation and could diffuse from the barriers (blood–brain barrier, testis–blood, and prostate–blood barriers) easily to obstruct tumor cell growth.

We have shown earlier that Schiff base–P85 application not only slows down the enlargement of grown tumors but also suppresses the tumor development of transplanted cells [18]. This study clearly showed that the Schiff base–P85 combination enhances the survival time and decreases the tumor growth in established tumors in C57/B16 mice, confirming our previous published studies [18]. These findings further validate that the Schiff base–P85 complex might be useful to suppress tumor growth and increase the life span of advanced-stage patient populations.

Conclusion

Taken together, our results in this study identified a potential chemotherapeutic formulation *in vitro* and *in vivo*. Thus, the Schiff base–P85, which is known for its inhibitory activity in tumor development without causing toxicity to animal tissues, holds promise with its therapeutic potential in late-stage prostate cancer.

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Conflicts of interest

There are no conflicts of interest.

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