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Anti-cancer effects of statins on castration- and chemotherapy-resistant prostate cancer cells

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Anti-cancer effects of statins on castration- and chemotherapy-resistant prostate cancer cells

1. Introduction

1.1 Epidemiology, etiology and progression of prostate cancer

1.11 Morbidity and mortality of prostate cancer

Prostate cancer (PCa) is currently the most prevalent cancer in men globally, and its morbidity and mortality continue to rise in some regions and populations [1,2]. PCa accounts for the highest number of cancer diagnoses (29%) and ranks as the second leading cause of cancer-related deaths in men [1]. Incidence and mortality rates vary considerably based on geographic location and race. The highest morbidity rates were found in Northern and Western Europe, the Caribbean, Australia/New Zealand, Northern America, and Southern Africa while the lowest rates were observed in Asia and Northern Africa, ranging from 6.3 to 83.4 per 100,000 men across regions. The mortality rates remained stable, ranging from 5.9 to 8.1 per 100,000 men. In contrast, developing countries shows a higher mortality rate [1,3]. Localized PCa exhibits a promising 5-year cancer-specific survival rate of 97% following appropriate treatment. However, metastatic PCa (bone or lymph node metastasis) only has an approximately 30% 5-year survival rate, which is the main cause of PCa-related deaths [4,5].

1.12 Etiology of prostate cancer

Identification of risk factors for PCa is key to targeting primary and secondary prevention. Well-established non-modifiable risk factors for PCa include age, race, family history, and genetic predispositions. Meanwhile, metabolic syndrome and lifestyle factors such as weight, physical activity, smoking, and diet have been suggested as potential modifiable risk factors [4,6,7]. Among these, age is a well-recognized and prominent risk factor for PCa. Recent statistics indicate that the age-specific incidence of PCa is the highest of all cancers, steeply increasing from 1.8% for men aged 60-69 to 9.0% for men aged ≥70 [1].

PCa is also characterized by substantial genetic-ethnic heterogeneity. PCa-specific mortality rates among individuals of African descent are approximately 2 to 4 times higher than those of other races and ethnicities [8]. Conversely, individuals of Asian

descent have been observed to have lower rates of PCa incidence and mortality compared to their African and Caucasian counterparts [1,2]. This disparity is further compounded by genetic alterations, which play a crucial role in the risk and progression of PCa. The most common genomic alterations associated with PCa are found in speckle-type pox virus and zinc finger protein (SPOP), tumor protein P53 (TP53), phosphatase and tensin homolog (PTEN), and forkhead box A1 (FOXA1) [9,10]. SPOP mutations lead to dysregulated proteasome degradation of the androgen receptor (AR) and the E-26 transformation-specific-related gene (ERG), a pioneering factor of AR signaling [11]. TP53, known as a tumor suppressor, plays a critical role in regulating cell division and determining cellular destiny through TP53-dependent cell cycle and apoptosis [12]. PTEN, another tumor suppressor gene, often loses its function in many types of cancer, including metastatic PCa. The loss of PTEN function was observed in metastatic PCa. PTEN plays a crucial role in regulating the protein kinase B (PKB/AKT) pathway through negative feedback mechanisms. The loss of PTEN can lead to excessive activation of the AKT pathway, promoting cell growth, cell survival, and tumorigenesis. Loss or dysfunction of PTEN is also associated with the biochemical recurrence (BCR) of PCa, indicating a return of the cancer following an apparently successful treatment in PCa [13,14]. FOXA1 is a transcription factor that directly interacts with AR and shapes AR signaling, this interaction is essential for regulating gene expression necessary for the growth and survival of PCa cells. Alterations in FOXA1 expression or mutations are associated with a more aggressive form of PCa, impacting the response to androgen deprivation therapies [15].

1.13 Progression of prostate cancer

Early-stage localized PCa shows a promising overall survival rate with curative management, including but not limited to surveillance, radical prostatectomy, or radiation therapy [16]. Despite early intervention, 20-50% of men with PCa will experience BCR within 10 years of initial definitive therapy, characterized by an increase in serum prostate-specific antigen (PSA) [17,18]. Approximately 20% of PCa patients have metastatic disease at the time of diagnosis [19], with the most frequent site of metastasis being bone [20]. Charles Huggins and colleagues pioneered the application of androgen deprivation therapy (ADT) for advanced PCa management, increasing the perception that blocking the AR in PCa presents a clinical benefit [21]. However, PCa inevitably progresses to castration-resistant prostate cancer (CRPC)

from months to years, posing a lethal and enduring therapeutic challenge [22]. Additionally, 10-20% of patients with metastatic PCa develop CRPC within five years of follow-up, and average overall survival from the occurrence of castration resistance is approximately 1.5 years [23,24].

PSA in combination with the Gleason score are currently the strongest conventional risk and subclassification biomarkers for PCa. The discovery of serum PSA has revolutionized the diagnostic process and disease management of PCa. To date, PSA is the only universally accepted biomarker in urological practice worldwide for screening, early and definitive diagnosis, prognosis, and therapeutic decisions [25]. Progression is accompanied by increasing PSA levels, indicating AR activity owing to proliferation of luminal epithelial cells [4]. However, the PSA has limited performance in clinically significant PC (Gleason score ≥ 7). Therefore, PSA screening carries a substantial risk of overdiagnosis and overtreatment of clinically indolent cases. PCa aggressiveness has been assessed using the Gleason system clinically, which categorizes tumor tissue as well-differentiated (the lowest grade) or poor-differentiated (the highest grade) based on characteristics of cancer architecture determined by histological features [25]. The Gleason score is the sum of the most prominent and second most prominent lesion pattern numbers, resulting in a low (≤6), intermediate (7) or high (8-10) Gleason grade. What`s more, advance molecular profiling techniques, such as next-generation sequencing and bioinformatics analysis tools, have enabled surgeons to stratify and risk PCa patients based on factors associated with clinical outcomes and response to therapy.

1.2 Docetaxel-based palliative chemotherapy in prostate cancer

Docetaxel ($C_{43}H_{53}NO_{14}$) is a well-established anti-mitotic chemotherapeutic agent with hydrophobic nature. It is a semi-synthetic analog of paclitaxel and is synthesized from extracts of the European yew tree needles (Taxus baccata). It has high cellular retention and inhibits microtubule assembly, resulting in cell cycle arrest at the G2/M phase. This further causes initiation of apoptosis and cytotoxicity in tumor cells [26]. Docetaxel suppresses AR nuclear translocation by bundling microtubules, resulting in cytoplasmic accumulation of the AR [26]. In 2004, the US Food and Drug Administration (FDA) approved docetaxel as clinical therapy for metastatic CRPC (mCRPC) based on the results of the TAX-327 trials [27].

Subsequently, docetaxel has become the first-line treatment of mCRPC according to guidelines for over a decade [25]. Docetaxel-based chemotherapy significantly prolongs median survival by 2.0-2.9 months compared to mitoxantrone plus prednisone [27,28]. Interestingly, one recent review noted that docetaxel remains a moderate effect and activity in treating mCRPC patients before or after abiraterone [29]. However, most patients are unable to tolerate the standard dose (75 mg/m^2) and schedule (3-weekly up to 10 cycles) [30]. Docetaxel treatment was related to an increase in adverse events and systemic toxicity due to its higher cellular retention/concentration in cells [27,31]. Additionally, resistance to docetaxel invariably emerges, either intrinsically or via various acquired mechanisms [26]. Docetaxel resistance has been a clinical challenge since its inception as a front-line therapy for mCRPC. Newly developed chemotherapy agents for the treatment of docetaxel-resistant PCa exhibit considerable systemic toxicity and may provide side effects that surpass their benefits. Therefore, emerging approaches and future directions to overcome docetaxel resistance for PCa treatment are urgently needed.

1.3 Castration and Chemotherapy resistance in prostate cancer

The mechanisms of castration- or chemotherapy-resistant is complex and multifactorial. Prominently, the androgen signaling axis in PCa derived multiple adaptive mechanisms in response to ADT [32,33]. Adaptations occur to supporting the growth and development to CRPC and involve conventional and novel intracrine/intratumoral androgen synthesis pathways [34], androgen transport as well as AR overexpression, mutation, and splice variation [35–37]. Patients with mCRPC or CRPC have several approved treatment options, such as poly (ADP-ribose) polymerase (PARP) inhibitors (olaparib), chemotherapies (docetaxel, cabazitaxel), AR signaling inhibitors (enzalutamide, abiraterone), and radioisotope (radium-223). Unfortunately, acquired resistance following the initial response remains an eternal and inevitable problem.

Mechanisms of chemotherapy resistance are distinct and include the following: increased expression of multidrug resistance genes [38,39], impaired drug delivery [40,41], upregulation of oncogenic signaling pathways [42], tumor microenvironment alteration [43], and epithelial-mesenchymal transition [43]. Studies suggest that the two oncogenic pathways AR signaling and AKT signaling cross-regulate each other

through reciprocal feedback loops [44,45]. Combined inhibition of Phosphoinositide 3-kinase (PI3K)/AKT and AR signaling led to near-complete PCa regressions in the xenograft model and resulted in synergistic inhibition of cell proliferation and induction of apoptosis in vitro [44,45]. Cancer cells can evade apoptosis (programmed cell death) leading to uncontrolled tumor progression, therapeutic resistance and recurrence. The dysregulation of anti-apoptotic and de-regulated apoptotic signaling in PCa therapy resistance has been extensively studied. In particular, B-cell lymphoma 2 (Bcl-2) overexpression has been shown link to androgen ablation resistance, metastatic and advanced phenotype [46]. Myeloid cell leukemia-1 (Mcl-1) is a member of the prosurvival Bcl-2 family, and is a potent anti-apoptotic protein that acts as a critical survival factor in a wide range of tumors [46]. It is noteworthy that the interaction and crossover between castration-, AR signaling inhibitors - and chemotherapy-resistant have been exist, making it provides a promising therapeutic target.

1.4 Statin application in prostate cancer

Statins, potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), are widely prescribed cholesterol-lowering medications applied extensively in the elderly population for several decades [47]. Statins can be categorized as either hydrophilic or lipophilic, depending on their solubility in water or lipid-containing solvents. Due to their outstanding tolerance and economic advantages, statins are globally utilized in both primary and secondary cardiovascular prevention. Notably, statins present low cytotoxicity towards non-transformed cells while exhibit high tumoricidal activity, resulting in the survival of normal cells while elimination of transformed cells [48]. In contrast to other molecular targeted chemotherapeutic agents that necessitate local delivery and have toxic side effects, statins can be administered systemically to patients owing to their tumor-specific apoptosis properties and minimal general toxicities [48,49]. Additionally, clinical trials have shown that long-term used of low doses or short-term use of high doses of statin is well-tolerated and associated with minimal toxicity [50,51].

Increasing clinical evidence suggests that statin administration might improve the outcome of PCa. A large cohort study involving 31,790 patients indicated that post-diagnosis statin use was associated with decreased PCa mortality [52]. Statin use at the initiation of ADT has also been correlated with a longer time to progression in

patients with hormone-sensitive PCa [53,54]. Consequently, some preclinical studies have shed light on the potential protective effects of statins in PCa [55–57]. However, comprehensive and in-depth research is required to uncover the underlying mechanisms of statin use in PCa patients.

1.5 AKT signaling pathway in prostate cancer

AKT signaling is the most prominent non-AR pathways in PCa. More than 40% of primary and up to 70% of metastatic PCa exhibit genomic alterations in the PI3K/AKT-signaling pathway [9,44]. AKT functions as an oncogene and its phosphorylated form (pAKT) regulates cell survival, proliferation, growth, apoptosis, and glycogen metabolism [58,59]. Activated AKT phosphorylates several targets including Bcl-2, mammalian target of rapamycin (mTOR), glycogen synthase kinase 3 (GSK3), P21, and cyclin-dependent kinases (CDKs), which are implicated in multiple cellular processes [60,61]. The activation of AKT pathway contributes to the tumorigenesis, progression [62,63] and castration-resistant of PCa [63,64]. In addition, the up-regulation of phosphorylated AKT (pAKT) is involved in docetaxel resistance [65]. Thus far, promising outcomes have been shown in recent phase II and III clinical trials for mCRPC patients treated with AKT inhibitors (Ipatasertib and capivasertib) in combination with ADT or docetaxel [66,67]. Multiple ongoing trials are evaluating various AKT inhibitors in PCa, employing different combination at various stages. In this context, focusing on AKT inhibition might emerge as a key approach for both preventing and treating PCa.

2. Hypothesis and study aims

Despite of potential anti-cancer properties and preventive role suggested by various studies, the differential efficacy of statins, the sensitivity of castration- and chemotherapy-resistant PCa to statins and the underlying biological mechanisms still remain unclear.

We hypothesize that statins exert potent tumor-inhibitory effects on both CRPC and docetaxel-resistant PCa cells, showing promise as either primary or complementary therapeutic agents. This project, utilizing different types of androgen-insensitive PCa cell lines with castration- and chemotherapy-resistant features, aims to

i) elucidate the anti-cancer activities of statins.
ii) identify the substance class having stronger potency in inhibiting tumor growth.
iii) investigate the involved signaling pathway responsible for these antitumor activities.
iv) determine sensitivities of castration- and chemotherapy-resistant PCa models to statins.

By comparing the effects on the parental androgen-insensitive and docetaxel-resistant sublines treated with different statins, we aim to clarify the optimal treatment settings and to identify the molecular subgroups of men who might benefit more from statin use than others. Figure 1 provides an overview of the initial study design.

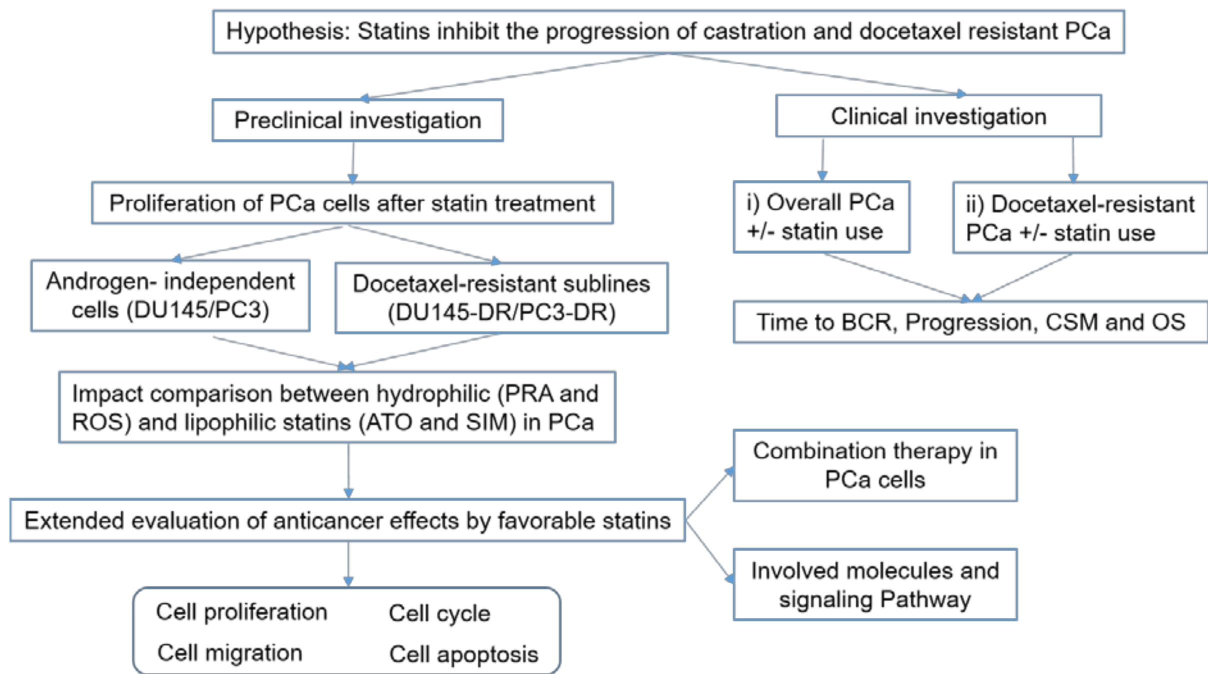

Figure 1. An overview of the study.

3. Materials and Methods

3.1 Cell culture and reagents

Androgen-insensitive cell lines PC3 and DU145 were obtained from the American Type Culture Collection (ATCC) and authenticated by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The PC3 cells were derived from bone metastasis of grade IV of PCa, they do not respond to androgens and have high metastatic potential [68]. DU145 was isolated from the brain metastasis of primary prostate adenocarcinoma origin, these cells are androgen-insensitive and with moderate metastatic potential [69]. Cells were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, USA) and 50 U/ml of penicillin/streptomycin (Gibco) at 37 °C in a humidified incubator with 5% CO_2. Chemical atorvastatin (S5715), simvastatin (S1792), pravastatin (S5713) and rosuvastatin-calcium (S2169) were purchased from selleckchem (USA) and it was dissolved in solvent dimethylsulfoxid (DMSO. Serva, Heidelberg, Germany).

3.2 Culture of docetaxel-resistant cells

The used docetaxel-resistant sublines PC3-DR and DU145-DR were friendly provided by Prof. Z.Culig (Urology Laboratory, Innsbruck Medical University, Austria). PC3-DR and DU145-DR cells were derived from corresponding parental cells by long-term exposure to docetaxel and finally cultured in the presence of 12.5 nM docetaxel. Establishment of stable docetaxel-resistant cell lines in accordance with a previous study [70]. Docetaxel was purchased from Sigma-Aldrich (St. Louis, MO, USA) and it was dissolved in 99.9% ethanol (EtOH. Geyer, Renningen, Germany).

3.3 Cell proliferation assay

Cell proliferation was evaluated using the Cell proliferation Kit II (XTT. Roche, Mannheim, Germany) following the manufacturer’s instructions. Briefly, the optimal cell number required for each well was determined. Parental cells (PC3 and DU145) and corresponding docetaxel-resistant cells (PC3-DR and DU145-DR) maintained in RPMI 1640 medium were seeded in a 96-well plate. The cells were then treated with different concentrations of chemicals (atorvastatin, simvastatin, pravastatin, rosuvastatin or docetaxel) after 24 h incubation. The XTT mixture (50 μL) was added to per well after

72 h treatment. After 4 h of incubation under culture conditions, the absorbance was measured at 490 nm with a reference wavelength of 630 nm utilizing ELISA reader (DIAS MAX 002, Dynex Technologies, Chantilly, USA).

3.4 Wound healing assay

Parental androgen-insensitive cells (PC3 and DU145) and corresponding docetaxel-resistant cells (PC3-DR and DU145-DR) were seeded in 6-well plates and maintained under culture conditions. The appropriate number of cells for each well was determined based on the cells reaching approximately 95% confluence after incubation for 24 h. Wounds were then created by scratching the cell monolayer with a 200 µL sterile pipette tip. This was followed by a gentle phosphate buffered saline (PBS) washing to remove the suspended cells and debris. Next, the cells were treated with atorvastatin (5 and 10 µM) or simvastatin (5 and 10 µM) maintained in serum-free medium (3%) and incubated for 24h. The wound was photographed under an inverted microscope (Axiocam 105 color, Zeiss) at 10 × magnification at 0 and 24 h intervals using ZEN lite software (Zeiss, Oberkochen, Germany). The relative migration rate was calculated by comparison of the residual cell-free area to the original scratch area. ImageJ software was employed for the area measurement.

3.5 Caspase 3/7 apoptosis assay

The parental androgen-insensitive cells (PC3 and DU145) and corresponding docetaxel-resistant cells (PC3-DR and DU145-DR) were seeded in T25 flasks with optimal density. The cells were incubated for 24h under culture conditions and then treated with various concentrations (5 and 10 µM) of lipophilic statins (atorvastatin and simvastatin) for 48h. The Caspase-Glo® 3/7 assay was performed followed the manufacturer instructions (Promega, Madison, USA). Briefly, 20 µL PBS and 5 µL cell lysates were added to each well of a white polystyrene plate (Thermo Fisher, USA). Subsequently, 50 µL Caspase-Glo reagent was added to each well, the plate was gently shaken and then the cells were incubated in the dark for 30 min at room temperature. Luminescence was detected using a microplate reader (TECAN GENios, Switzerland) and the luminescence intensity was normalized to protein content of the cell lysates.

3.6 Cell Cycle Analysis

The parental cells (PC3 and DU145) and the corresponding docetaxel-resistant cells (PC3-DR and DU145-DR) were seeded in T25 flask with suitable density. The cells were cultured for 24h and then treated with different concentrations (2.5 and 5 μM) of lipophilic statins (atorvastatin and simvastatin) for 48h to reach a confluence of 70-80%. Subsequently, the cells were washed with pre-cooled PBS, harvested with Trypsin and fixed with 4mL pre-cold 80% EtOh. Following this, the cells were stained with 10 μg/mL of propidium iodide solution containing 1 μg/mL RNaseA solution for 30 min in the dark. Finally, the cell cycle distribution was monitored by flow cytometry (FACS CANTO 2, BD Bioscience Systems, Heidelberg, Germany) and analyzed using Mod-Fit software (Verity Software House).

3.7 Western blot

Cell pellets were harvested from parental androgen-insensitive cells (PC3 and DU145) and respective docetaxel-resistant cells (PC3-DR and DU145-DR) that were treated with different concentrations (5 μM and 10 μM) of lipophilic statins (atorvastatin and simvastatin) for 48h in T25 flasks. Proteins were extracted using cold radio-immunoprecipitation assay (RIPA) lysis buffer supplemented with protease and phosphatase inhibitors (EMD biosciences, Darmstadt, Germany). Cell lysate was centrifuged at 14,000 rpm/min for 30 min at 4°C. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Thermo, Rockford, USA). Protein samples were equilibrated in RIPA and lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) and then boiled at 70 °C for 10 min.

Equal amounts of protein (30 μg) were separated on 4%-12% precast NuPAGE Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membranes (Whatman). The membranes were blocked with blocking buffer (Thermo, Rockford, USA) at room temperature and incubated with primary antibodies overnight at 4 °C, then incubated with corresponding secondary antibodies at room temperature for 1h. Followed by incubation with the enhanced chemiluminescence reagent kit (ECL. Thermo, Rockford, USA) for 5min. The target protein bands were detected using the Fusion solo S and VisionCatp Image system (v16.13a) from Vilber (France).

The following specific primary antibodies were obtained from Cell Signaling Technology: AKT (1:1000, 9272), p-AKT (Ser473) (1:1000, 4060), p-GSK-3β (Ser9) (1:1000, 9336), p21 Waf1/Cip1 (12D1) (1:1000, 2947), and CDK2 (78B2) (1:1000, 2546). The Mcl-1 (S-19) (1:500, sc-819) primary antibody was purchased from Santa Cruz. The Cyclin D1 (1:150, MA1-39546) and GAPDH (1:4000, MA5-15738-HRP) loading control primary antibodies were purchased from Invitrogen. The CDK1 antibody [A17] (1:1500, ab18) primary antibody was obtained from Abcam. The goat anti-mouse (1:1500, P0447) and swine anti-rabbit (1:1700, P0217) immunoglobulins/HRP secondary antibodies were supplied by Dako.

3.8 Statistical Analysis

Statistic Package for Social Science (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was utilized for data analysis. GraphPad Prism 8.0 software (La Jolla, CA, USA) was used for graphing. Densities of the western blot's protein bands were quantified by Image J software. Two-tailed unpaired Student`s t- test was employed for comparison between two groups. Data are presented as the Mean ± standard error of the mean (SEM). A P-value of < 0.05 was considered statistically significant (*$P< 0.05$, **$P< 0.01$ and ***$P< 0.001$), NS indicates no statistical difference.

4. Results

4.1 Verification of the docetaxel resistance in PC3-DR and DU145-DR cell lines

The used docetaxel-resistant sublines PC3-DR and DU145-DR were kindly provided by Prof. Z. Culig from the Urology Laboratory at Innsbruck Medical University, Austria. Resistance to docetaxel was verified using the XTT assay, which measured the cell proliferation response to increasing concentrations of docetaxel. While a concentration of 6.25 nM of docetaxel induced more than a 50% inhibition of cell proliferation (IC_{50} < 6.25 nM) in the parental androgen-independent cell lines (PC3 and DU145), both PC3-DR and DU145-DR cells didn´t show any significant alternations in cell viability and proliferation after treatment with up to 12.5 nM of docetaxel (Figure 2A and 2B).

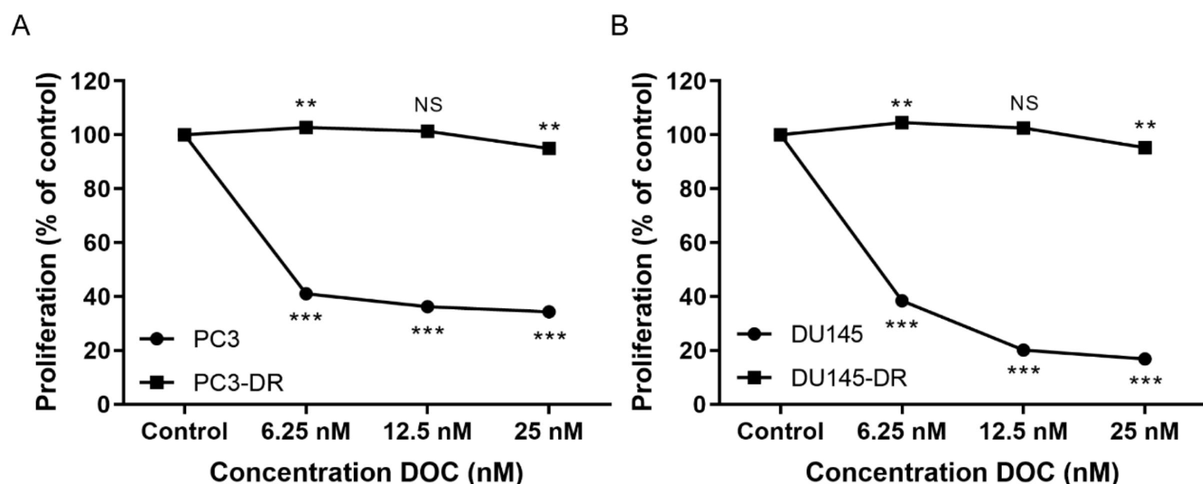

Figure 2. Proliferation of both parental and resistant cell lines treated with docetaxel (DOC), as measured by the XTT assay (A) PC3 and PC3-DR (B) DU145 and DU145-DR. The data are displayed as the Mean ± SEM from at least three independent experiments. NS indicates no statistical difference. Statistical significance is noted as follows: *P< 0.05, **P< 0.01 and *P< 0.001 compared to the control.

4.2 Impact of commonly used statins on androgen-independent PCa cell proliferation and superiority of lipophilic agents

Given the uncertain anti-cancer activity of statins against PCa, we conducted a comprehensive evaluation using the XTT assay. This was to measure the effects of two hydrophilic (Pravastatin (PRA) and Rosuvastatin (ROS)) and two lipophilic (Atorvastatin (ATO) and Simvastatin (SIM)) statins which are widely prescribed to men.

The study initially focused on androgen-independent (castration-resistant) PC3 cells and their docetaxel-resistant counterparts PC3-DR, treating them with varying concentrations (2.5, 5, and 10 µM) of the selected statins over a 72-hour period. The cell viability and proliferation after the treatment of each group were determined by XTT assay.

As shown in Figure 3, both lipophilic statins (ATO and SIM) significantly inhibited the PCa cell proliferation, whereas the hydrophilic statins (PRA and ROS) had no substantial impact. Among them, SIM proved to be the most potent inhibitor of proliferation, reducing it by more than 60% in both PC3 (64.5%) and PC3-DR (64.6%) cell lines at the concentration of 10 µM (Figure 3A and 3B).

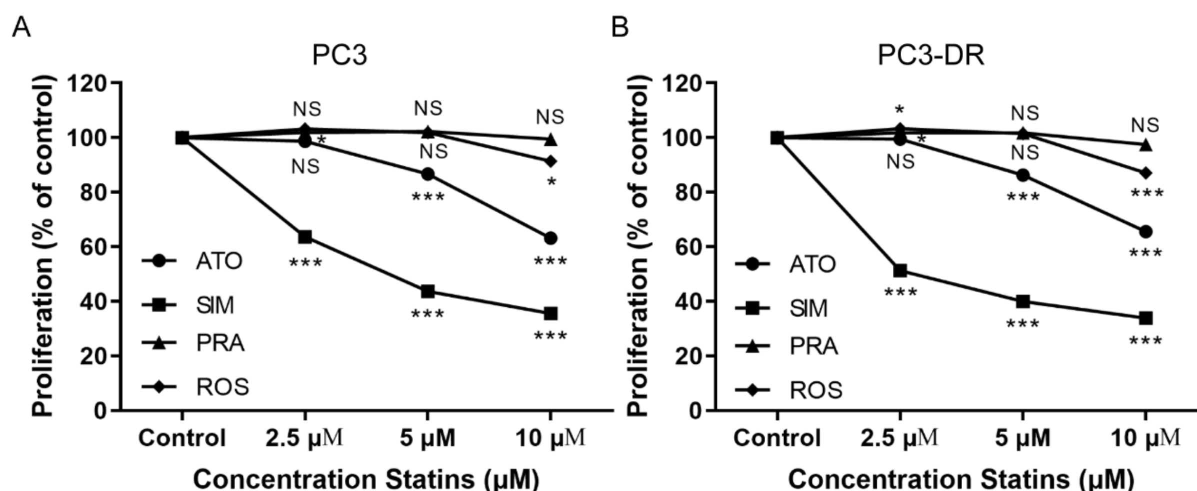

Figure 3. Determination of cell proliferation in (A) PC3 and (B) PC3-DR cell lines following treatment with hydrophilic (PRA and ROS) or lipophilic (ATO and SIM) statins using the XTT assay. The data are displayed as the Mean ± SEM from at least three independent experiments. NS indicates no statistical difference. Statistical significance is noted as follows: *P< 0.05, **P< 0.01 and *P< 0.001 compared to the control.

4.3 Enhanced sensitivity of docetaxel-resistant PCa cells to lipophilic statins

Since observations revealed a more favorable anti-proliferative effect of the lipophilic statins (ATO and SIM) on cell growth, further experiments were carried out to validate this activity of the lipophilic statins in other cell lines. For this purpose, two additional androgen-insensitive cell lines, DU145 and DU145-DR, were treated with the

increasing concentrations (2.5, 5 and 10 μM) of ATO and SIM, similar to the treatments applied to PC3 and PC3-DR. The inhibitory effects on cell growth in DU145 and DU145-DR varied significantly based on the type of statins (ATO vs SIM) and the cell´s therapy resistance (parental CRPC cells vs docetaxel-resistant sublines). Figure 4 compares the inhibitor effects of ATO and SIM on the proliferation of androgen-independent PC3 and DU145 cells, with SIM showing a markedly stronger effect than ATO in both cell lines (Figure 4A and 4B).

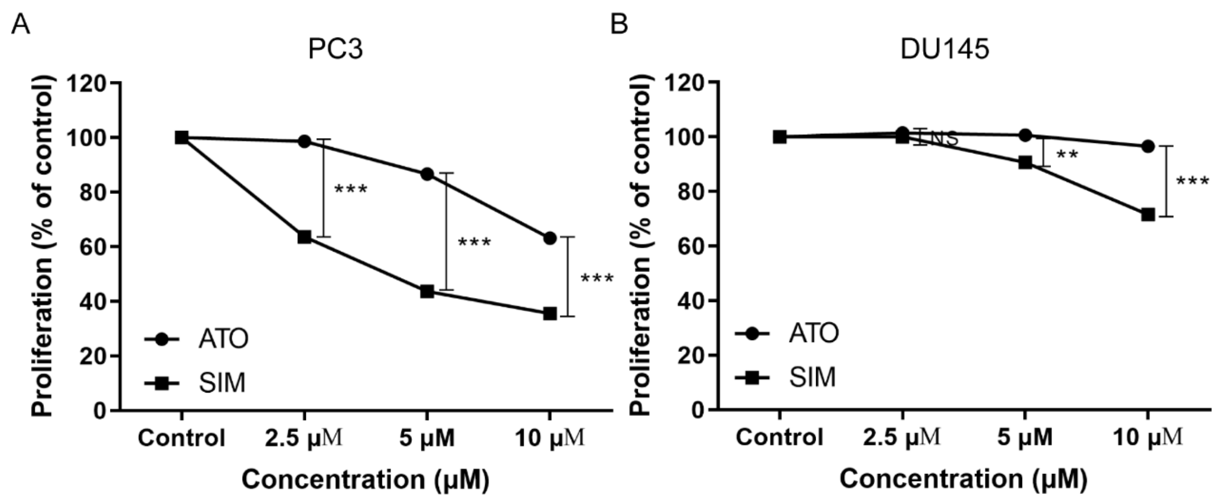

Figure 4. Differential efficacy of ATO and SIM in inhibiting proliferation of (A) PC3 and (B) DU145 cells. Treatment involved exposure to increasing concentrations of ATO and SIM (2.5, 5, and 10 μM) for 72 hours, which highlighted significant differences in their inhibitory effectiveness at these concentrations. The data are displayed as the Mean ± SEM from at least three independent experiments. NS indicates no statistical difference. Statistical significance is noted as follows: *P< 0.05, **P< 0.01 and *P< 0.001 between ATO and SIM at the same concentrations.

As in the PC3 cell lines, SIM induced equal inhibition of proliferation in both parental and docetaxel-resistant cell lines as ATO (Figures 5A and 5B). Unlike the similar response exhibited by the parental PC3 and docetaxel-resistant PC3-DR cells to both lipophilic statins, the DU145 cell lines showed a clear difference in sensitivity based on the presence of docetaxel resistance as illustrated in Figure 5C and 5D. Notably, DU145-DR cells demonstrated greater sensitivity to both statins compared to their non-resistant, parental DU145 counterpart at the same concentrations (Figures 5C and 5D). Reflecting these findings, doses of drugs (5 and 10 μM) were identified as the most

effective for subsequent phases of the study. Taken together, the lipophilic statins either demonstrated comparable inhibitory effects between the parental and its docetaxel-resistant counterpart, as observed in PC3 and PC3-DR, or showed even enhanced activity in the docetaxel-resistant subline as seen in DU145-DR. This was despite the aggressive and multiple therapy-refractory features of the latter (docetaxel resistant), as previously reported [71,72].

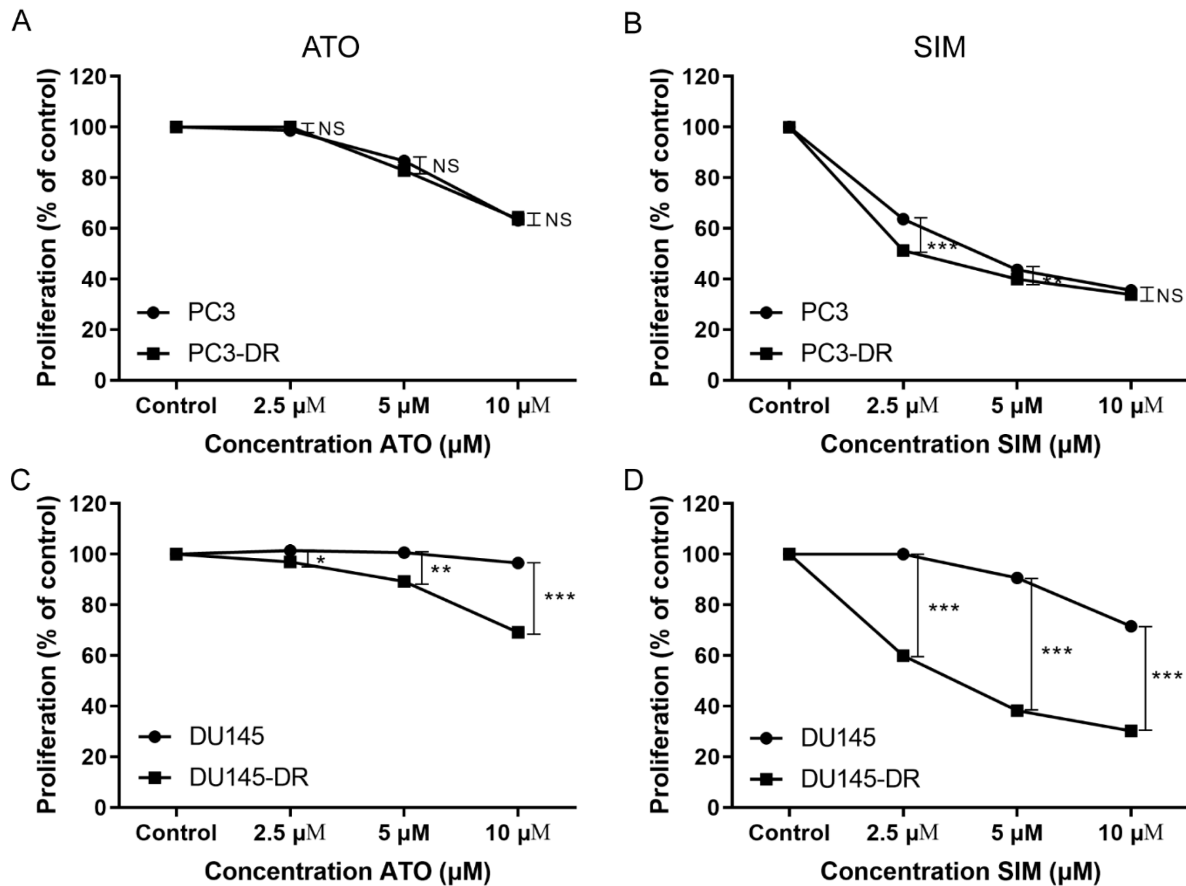

Figure 5. Difference in sensitivity of PCa cells to lipophilic statins (ATO and SIM) depending on the presence of docetaxel resistance. Cells were subjected to treatment with ATO and SIM at same increasing concentrations (2.5, 5, and 10 μM) for 72 hours. This facilitated a comparative analysis of sensitivity across cell lines between PC3 and its docetaxel-resistant counterpart PC3-DR, at the identical concentrations of (A) ATO and (B) SIM. Similarly, comparisons were made between DU145 and its docetaxel-resistant DU145-DR counterpart at the identical concentrations of (C) ATO and (D) SIM. Data represent the Mean ± SEM of at least three independent experiments. Statistical significance (Student's t-test) is noted as follows: *$P < 0.05$, **$P < 0.01$ and

*P< 0.001 between parental and docetaxel-resistant cell lines at the same concentrations, NS indicates no statistical difference.

4.4 Reduced migration of PCa cells following ATO and SIM treatment

To further explore the effects of lipophilic statins (ATO and SIM) on the migration of PCa cells in vitro, wound healing assays were conducted (Figure 6 and 7). Both ATO and SIM significantly reduced the mobility of both parental and docetaxel-resistant cells. Interestingly, a concentration of 5 μM SIM was already sufficient to dramatically limit the migration in both PC3 and PC3-DR cells (Figure 6A and 6B), where motility was reduced to 43.1% and 13.0% in PC3 and PC3-DR cells, respectively (Figure 6C and 6D). In contrast, the control group of PC3 and PC3-DR cells exhibited high motility, achieving 100% wound closure within 24 hours. Similar to the observed proliferation inhibition, SIM outperformed ATO in curtailing migration across a spectrum of concentrations for both DU145 and DU145-DR cells (Figure 7A and 7B). The most significant reduction in migration was observed at a 10 μM concentration of SIM, with DU145 and DU145-DR cells showing relative migration rates of 13% and 4.9%, respectively (Figure 7C and 7D). These findings support the significant inhibitory influence of lipophilic statins on PCa migration.

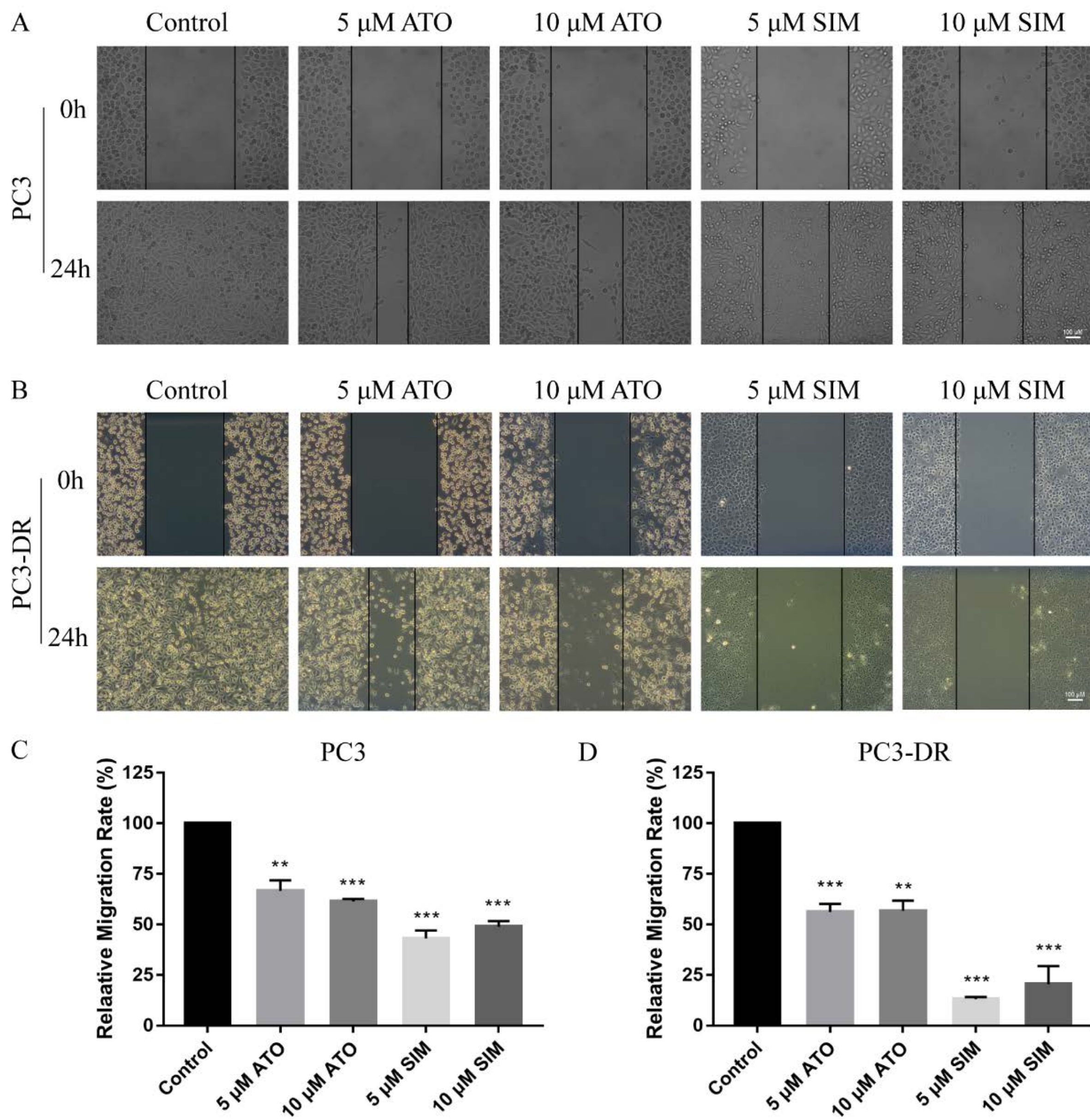

Figure 6. Inhibition of PC3 and PC3-DR cell migration by lipophilic statins (ATO and SIM). Representative images of scratch wound healing assays in (A) PC3 and (B) PC3-DR cells, following treatment with ATO and SIM treatments at concentrations of 5 or 10μM for designated time intervals. The corresponding quantification of relative migration rates in (C) PC3 and (D) PC3-DR. Images were captured at 10 × magnification and evaluated in ImageJ software. A scale bar of 100 μm is provided for reference. Data represent the Mean ± SEM of at least three independent experiments. Statistical significance was determined using Student’s t-test compared to control: *P < 0.05, **P < 0.01, ***P < 0.001, NS = not significant.

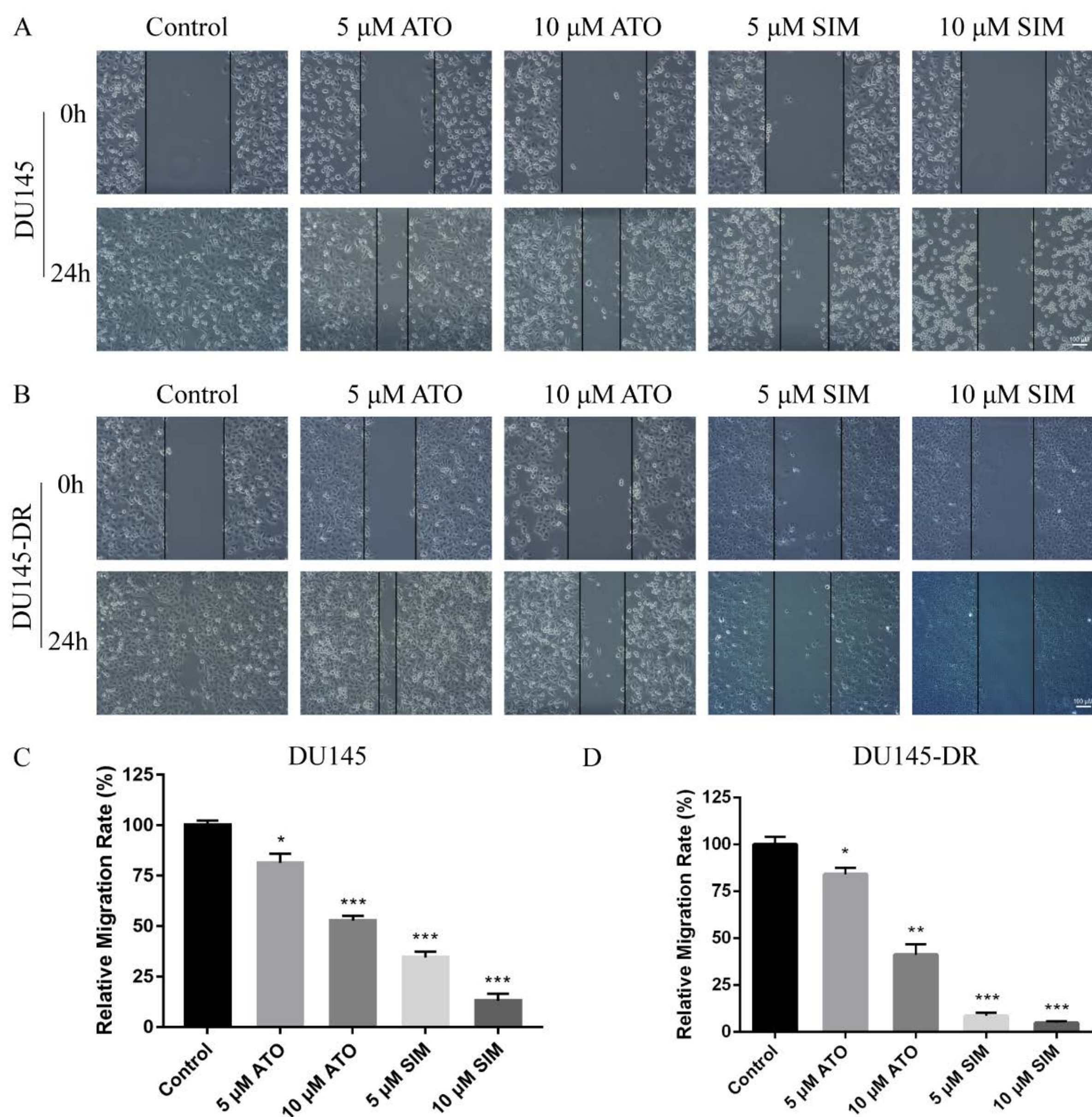

Figure 7. Inhibition of DU145 and DU145-DR cell migration by lipophilic statins (ATO and SIM). Representative images of scratch wound healing assays in (A) DU145 and (B) DU145-DR cells, following treatment with ATO and SIM at concentrations of 5 or 10μM for designated time intervals. The corresponding quantification of relative migration rates for (C) DU145 and (D) DU145-DR. Images were captured at 10 × magnification and evaluated in ImageJ software. A scale bar of 100 μm is provided for reference. Error bars indicate the Mean ± SEM of at least three independent experiments. Statistical significance was assessed by Student's t-test compare to the control group; *P < 0.05, **P < 0.01, ***P < 0.001, NS = significant.

4.5 Induction of apoptosis in PCa cells following treatment with ATO and SIM

Apoptosis is mediated by caspases, with caspase 3/7 serving as the primary executioners. Thus, changes in caspase 3/7 activity were assessed to determine whether used PCa cells exhibited an apoptotic response following treatment with lipophilic statins (ATO and SIM) at concentrations of 5 μM and 10 μM. Treatment with ATO and SIM notably enhanced caspase 3/7 activity in both androgen-insensitive PC3/DU145 and their corresponding docetaxel-resistant cells, as illustrated in Figure 8. A similar dramatic increase in apoptosis was observed in PC3, PC3-DR and DU145, with the most significant effects seen following treatment with 5 μM SIM (Figure 8A and 8B). These dramatic changes in caspase-3/7 activity of PC3, PC3-DR and DU145 cells were 3.1-fold, 8.6-fold and 2.4-fold increase, respectively, compared to control induced by 5 μM SIM. DU145-DR cells showed a concentration-dependent increase in caspase 3/7 activity, with 10 μM SIM leading to a notable 2.7-fold increase (2967.0 ± 199.4 RUL, $P < 0.001$) compared to the control group (Figure 8B).

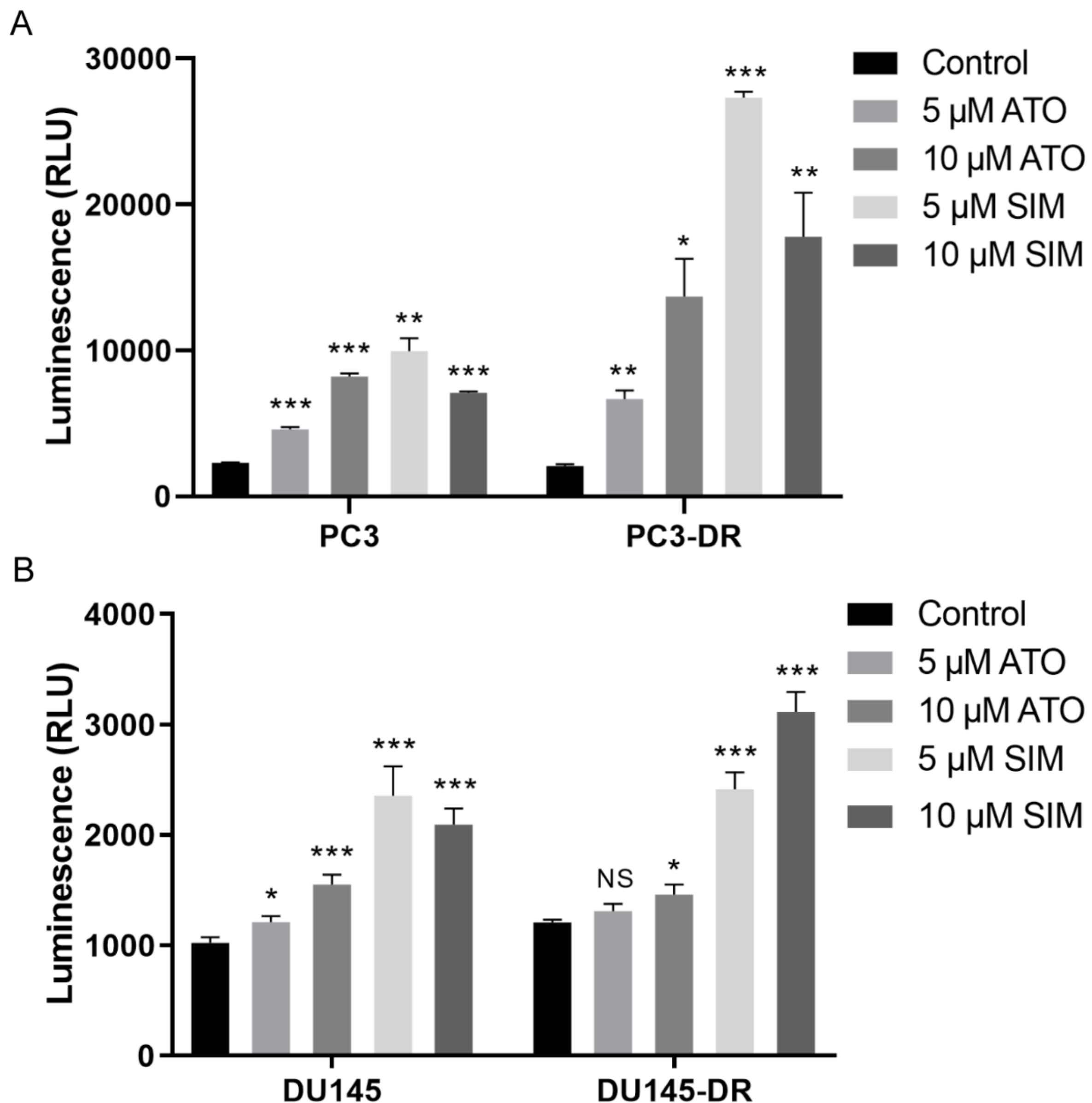

Figure 8. Impact of lipophilic statins (ATO and SIM) on caspase 3/7 activity in PC3/-DR and DU145/-DR cells after 48 hours of treatment. Caspase 3/7 activity of (A) PC3 and PC3-DR cells (B) DU145 and DU145-DR cells was quantified using luminescence (RUL). Data represent the Mean ± SEM of at least three independent experiments. Statistical significance was determined using Student's t-test compared to control: *P < 0.05, **P < 0.01, ***P < 0.001, NS indicates not significant.

4.6 Blocking the transition from G1 to S phase in the cell cycle by ATO and SIM treatments

Flow cytometry was conducted to investigate the changes in cell cycle distribution after treatment with lipophilic statins (ATO and SIM). Collectively, both parental and

docetaxel-resistant cells exhibited a more pronounced response to SIM in suppressing cell division through blocking the transition from G1 to S Phase than ATO (Figure 9 and 10). In PC3 and PC3-DR cells, ATO and SIM treatments led to a dose-dependent accumulation of cells in G1 phase (Figure 9A), with SIM exhibiting a superior ability to regulate the cell cycle in comparison to ATO. The application of low dose SIM (2.5 μM and 5 μM) already induced the cell cycle blocking effects in both cell lines. The proportion of PC3 cells in the G1 phase increased from 48.6% in the control to 63.3% ($P < 0.001$) with 5 μM ATO and to 70.0% ($P < 0.001$) with 5 μM SIM. These alterations were accompanied by reduced populations in the S phase to 23.4% ($P < 0.001$) with 5 μM ATO and to 16.8% ($P < 0.001$) with 5 μM SIM, compared to 35.4% in the control group (Figure 9B). A similar influence of ATO and SIM on cell cycle progression was observed in the PC3-DR cell line (Figure 9A). At a 5 μM concentration, both ATO and SIM induced a G1 phase arrest at 48 hours, increasing the G1 cell population from 37.2% in control to 45.9% with ATO ($P < 0.01$), and to 49.1% with SIM ($P < 0.001$, Figure 9C).

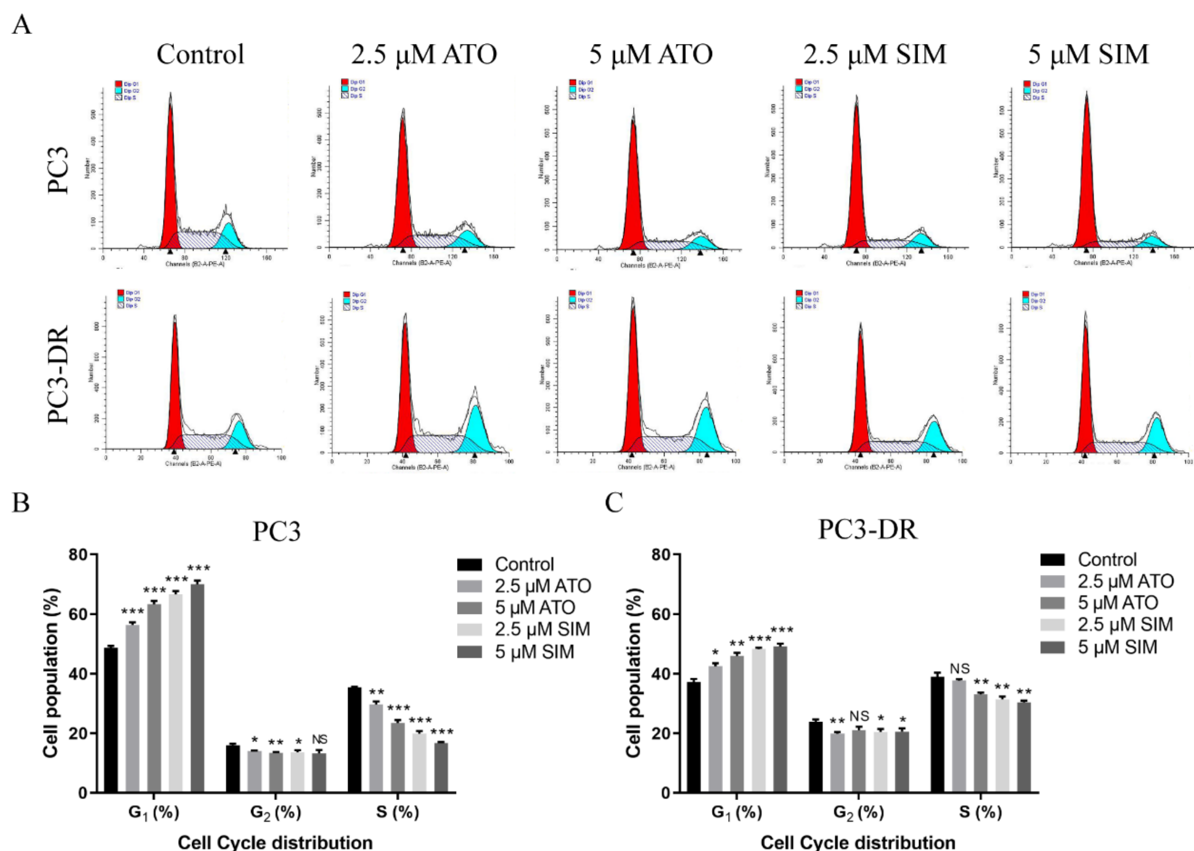

Figure 9. Cell cycle distribution in PC3 and PC3-DR after statin treatment (PI-staining). (A) Representative images of PC3 and PC3-DR cell populations are displayed after 48 hours of treatment with ATO and SIM. Statistical outcomes for (B) PC3 and (C) PC3-DR cells. Data represent the Mean ± SEM of at least three independent experiments. Statistical significance was determined using Student's t-test compared to control: *P < 0.05, **P < 0.01, ***P < 0.001, NS = not significant.

Similarly, treating DU145 cells with 5 μM SIM for 48 hours also led to increase in the G1 cells population (62.2%, P < 0.01) and a reduction in the S phase cells population (24.6%, P < 0.001) compared to control group (53.7% and 32.1%, respectively) as detailed in Figure 10A and 10B. The application of 5μM SIM caused a significant G1 arrest in the DU145-DR cells (52.1% vs 42.9%, P < 0.001) and a decrease in S phase (36.9% vs 45.5%, P < 0.001) compared to control group (Figure 10A and 10C). The observed inhibition of the transition from the G1 to the S phase correlates with the previously evaluated reduction of proliferation in PCa cells and indicates suppressed cell division due to G1 arrest.

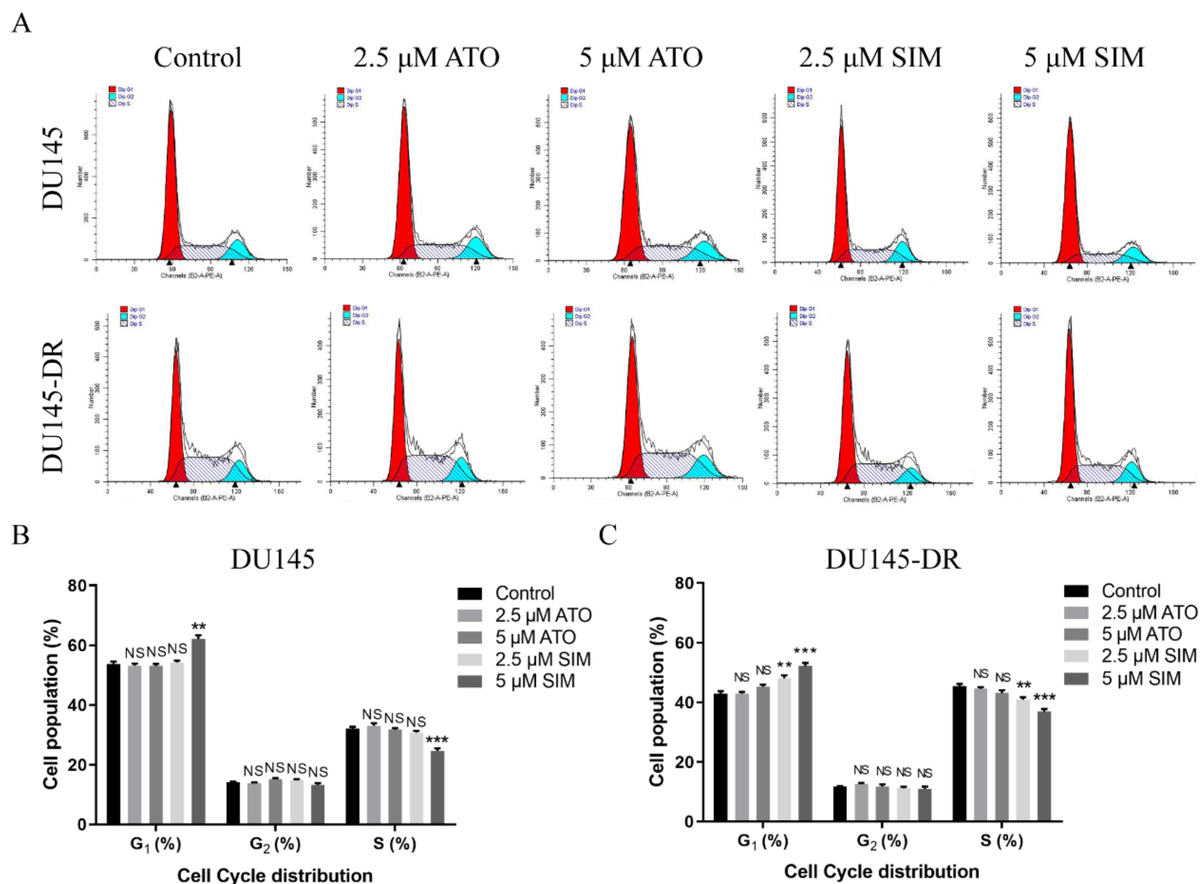

Figure 10. Cell cycle distribution after statin treatment (PI-staining). (A) Representative images of DU145 and DU145-DR cell populations are displayed after 48 hours of treatment with ATO and SIM. Statistical outcomes for (B) DU145 and (C) DU145-DR cells. Data represent the Mean ± SEM of at least three independent experiments. Statistical significance was determined using Student's t-test compared to control: *P < 0.05, **P < 0.01, ***P < 0.001, NS = not significant.

4.7 Impact of ATO and SIM on cell cycle regulating proteins

To elucidate the molecules contributing to the anticancer effects of statins, we analyzed the expression of cell cycle and apoptosis-regulating proteins, alongside the associated signaling pathways. In terms of cell cycle regulation, the expression of p21, CDK1, CDK2, and Cyclin D1 in all tested cell lines was assessed after 48 hours of treatment with ATO and SIM using Western Blot (WB).

Subsequent WB analysis revealed that ATO and SIM exerted comparable effects in PC3/-DR cells (Figure 11), significantly increasing p21 levels in both PC3 and PC3-DR cells (Figures 11A and 11B) and decreasing Cyclin D1, CDK1, and CDK2 expressions (Figures 11C-11E). These findings suggest that lipophilic statins may trigger G1 phase arrest in the cell cycle through the p21-Cyclin D1/CDK1/CDK2 pathway, thereby halting the progression of PCa.

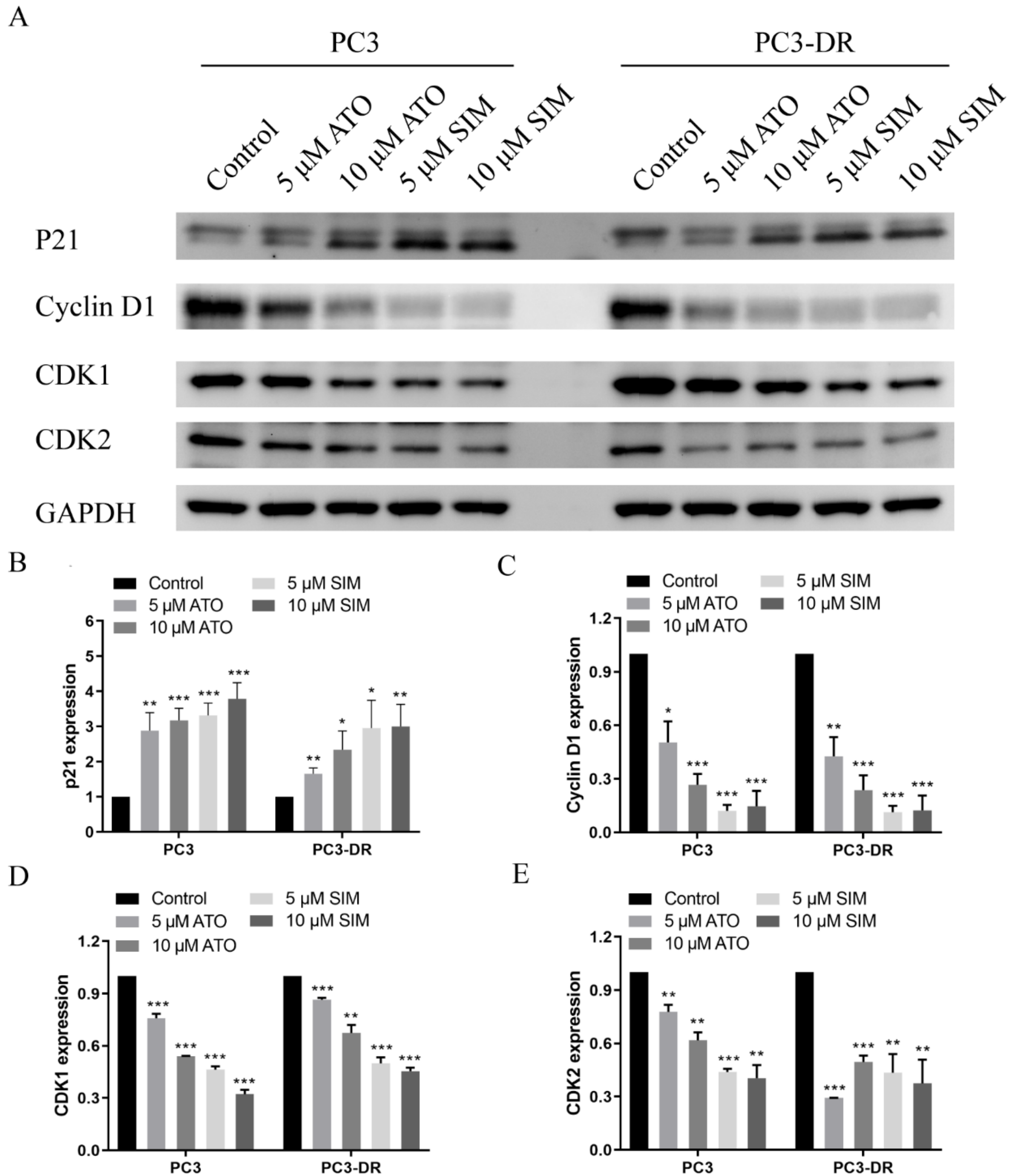

Figure 11. Regulation of cell cycle-related proteins in DU145 and DU145-DR cells after lipophilic statin treatment. (A) Representative Western blot images for p21, Cyclin D1, CDK1, and CDK2. (B-E) Quantitative analysis of their expression level. GAPDH served as loading control. Data represent the Mean ± SEM of at least three independent experiments. Statistical significance was determined using Student's t-test compared to control: *P < 0.05, **P < 0.01, ***P < 0.001, NS = not significant.

In DU145 and DU145-DR cells, WB results confirmed that ATO and SIM significantly affected cell cycle proteins (p21, Cyclin D1, CDK1, CDK2) (Figure 12A). Both statins increased p21 expression in DU145/-DR cells notably (Figures 12A and 12B). However, the Cyclin D1 expression rose in DU145 cells treated with ATO and SIM, while it was markedly reduced in cells harboring docetaxel resistance (DU145-DR) as shown in Figures 12A and 12C. CDK1 was also inhibited by ATO (10 μM) and SIM (5 μM and 10 μM) in both cell types (Figure 12A and 12D). Moreover, SIM significantly reduced CDK2 in both DU145/-DR cells, unlike ATO, which only affected DU145 cells.

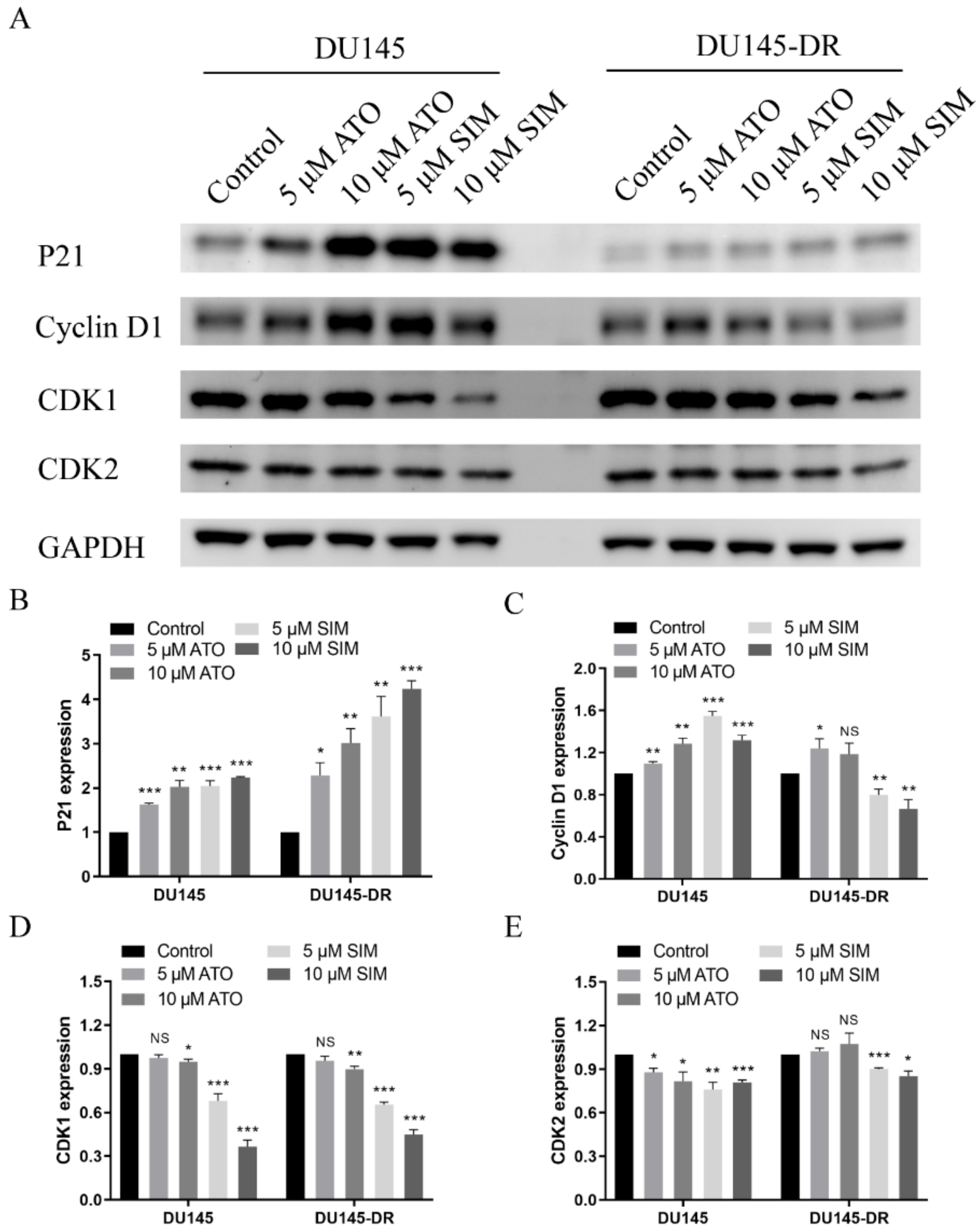

Figure 12. Regulation of cell cycle-related proteins in DU145 and DU145-DR cells after lipophilic statin treatment. Protein levels of key cell cycle-regulators were detected by Western blotting. (A) the representative Western blot images for p21, Cyclin D1, CDK1, and CDK2. (B-E) quantitative analysis of their expression level. GAPDH served as loading control. Data represent the Mean ± SEM of at least three independent

experiments. Statistical significance was determined using Student's t-test compared to control: *P < 0.05, **P < 0.01, ***P < 0.001, NS = not significant.

4.8 Impact of statins on AKT signaling and apoptosis regulating downstream targets in androgen-independent and docetaxel-resistant PCa cells.

To explore mechanisms behind enhanced apoptosis and reduced cell division, we examined the AKT signaling pathway. AKT activation plays a crucial role in PCa cell survival and progression, primarily through phosphorylation, impacting downstream proteins such as GSK-3β and MCL-1 splice variants (Mcl-1L and Mcl-1S). Particularly, Mcl-1 is a key apoptosis regulating member of Bcl-2 family in PCa. Thus, we analyzed the expression and phosphorylation of AKT, pGSK-3β, Mcl-1L, and Mcl-1S following statin treatment using WB.

In PC3/-DR cells, a significant reduction in the p-AKT/AKT ratio indicated AKT dephosphorylation and thereby decreased activation (Figures 13A, 13B and 13D) of the signaling. This led to decreased levels of downstream targets like p-GSK-3β and Mcl-1L, while Mcl-1S expression varied, indicating that statins may modulate the AKT-GSK-3β-MCL-1 pathway (Figures 13A and 13E-13G). These results suggest lipophilic statins can impair cell viability through disrupting the AKT-GSK-3β-MCL-1 signaling axis.

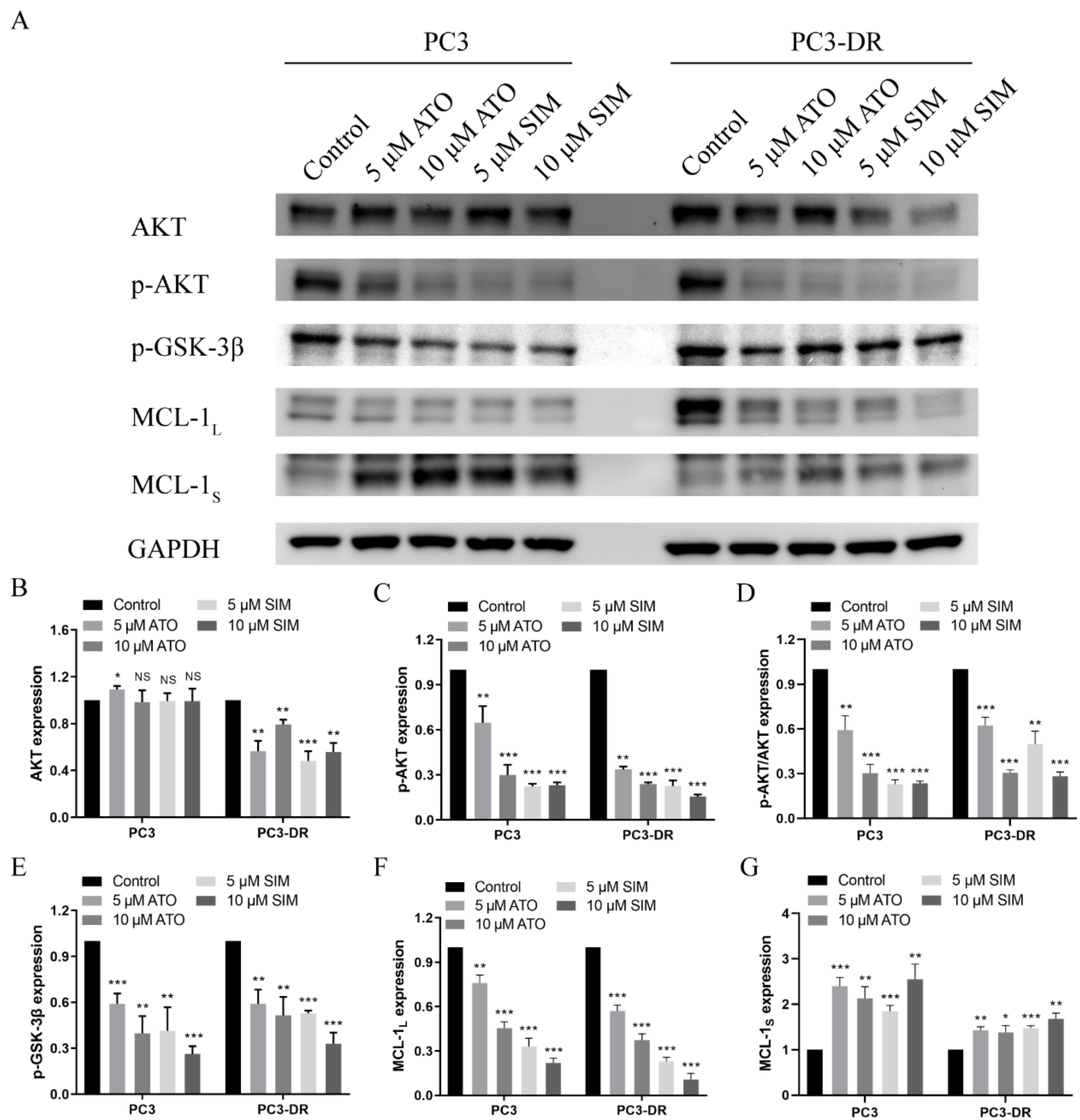

Figure 13. Impact of ATO and SIM on apoptosis-relevant proteins and AKT signaling in PC3/-DR cells. (A) Representative Western blot images for AKT, p-AKT, p-GSK-3β, Mcl-1L and Mcl-1S. (B-H) Quantitative analysis of their expression levels. GAPDH served as loading control. Data represent the Mean ± SEM of at least three independent experiments. Statistical significance was determined using Student's t-test compared to control: *P < 0.05, **P < 0.01, ***P < 0.001, NS = not significant.

SIM (5 and 10 μM) significantly decreased both basal AKT and phosphorylated AKT levels in DU145/-DR cells, as illustrated in Figures 14A, 14B, and 14C, without altering the p-AKT/AKT ratio (Figure 14D). Notably, 10 μM SIM significantly downregulated the

anti-apoptotic Mcl-1L and upregulated the pro-apoptotic Mcl-1S in both cell lines (Figures 14A, 14F, 14G). This modulation coincides with increased caspase 3/7 activity, suggesting apoptosis, as previously shown (Figure 8B).

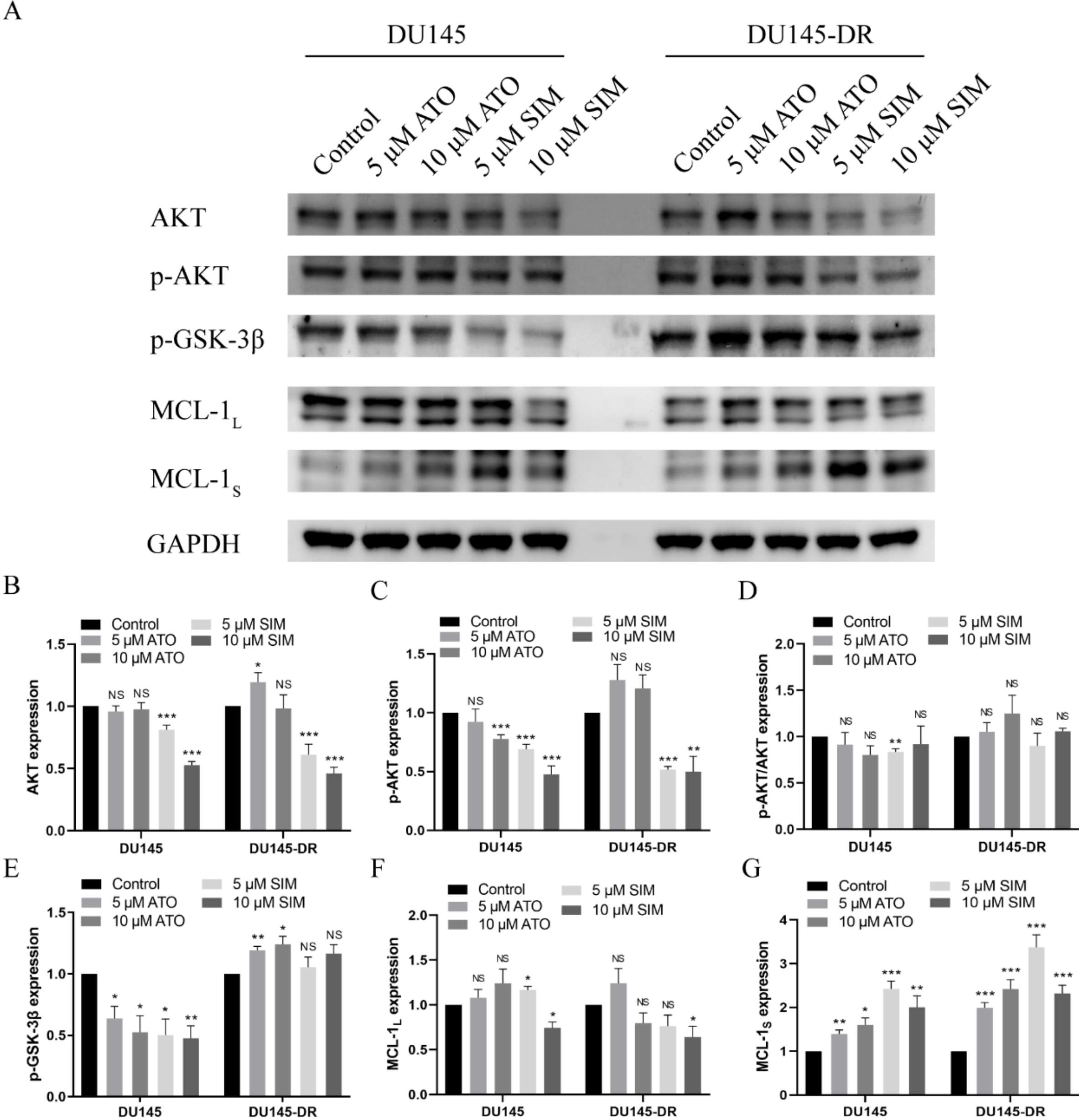

Figure 14. Impact of ATO and SIM on apoptosis-relevant proteins and AKT signaling in DU145/-DR cells. (A) Representative Western blot images for AKT, p-AKT, p-GSK-3β, Mcl-1L and Mcl-1S. (B-H) Quantitative analysis of their expression levels. GAPDH served as loading control. Data represent the Mean ± SEM of at least three independent experiments. Statistical significance was determined using Student's t-test compared to control: *P < 0.05, **P < 0.01, ***P < 0.001, NS = not significant.

4.9 ATO and SIM do not re-sensitize the docetaxel-resistant cells to docetaxel

Encouraged by the favorable effects of statins on PCa cells, we were curious whether the lipophilic statin (ATO and SIM) would re-sensitize the docetaxel-resistant cells to docetaxel. A proliferation assay was conducted to investigate the combination therapy effects using androgen-independent (PC3 and DU145) and corresponding DR cells (Figure 15). Unfortunately, ATO and SIM do not re-sensitized the docetaxel-resistant cells to docetaxel (Figure 15C, and 15G). We then proceeded to verify our findings at protein level in PC3 and PC3-DR cells, focusing on the apoptosis-related proteins (AKT, p-AKT, p-GSK-3β, Mcl-1L and Mcl-1S) and cell cycle-relevant proteins (P21, Cyclin D1, CDK1 and CDK2) (Figure 16). Similar protein regulation was observed in combination therapy as in the case of monotherapy of lipophilic statins (ATO and SIM).

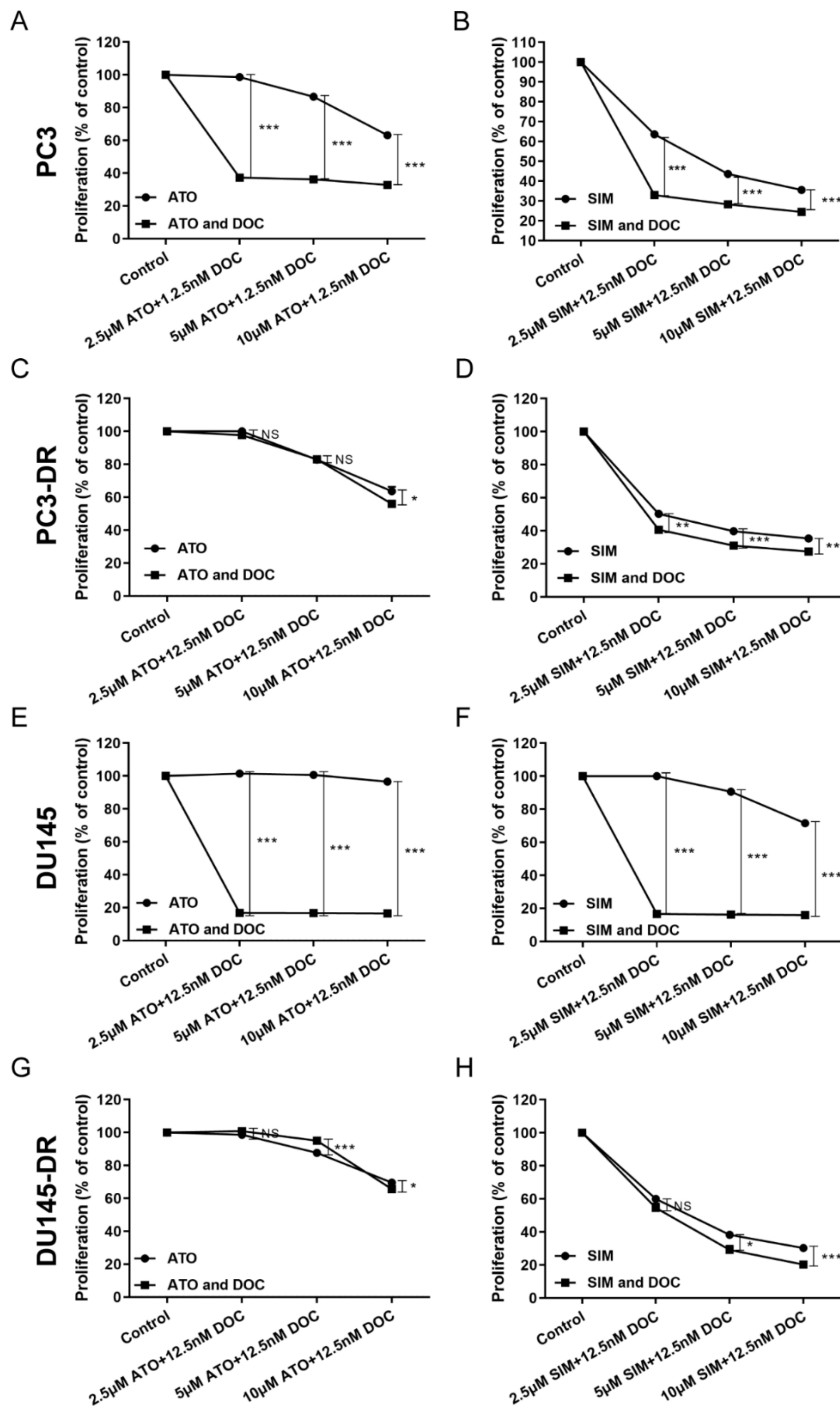

Figure 15. Differences in inhibitory effects between lipophilic statin (ATO or SIM) and combination therapy (lipophilic statin and docetaxel) treatment in (A) PC3 (B) PC3-DR (C) DU145 and (D) DU145-DR cells. All values are displayed as Mean ± SEM from triplicate experiments. NS indicates not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

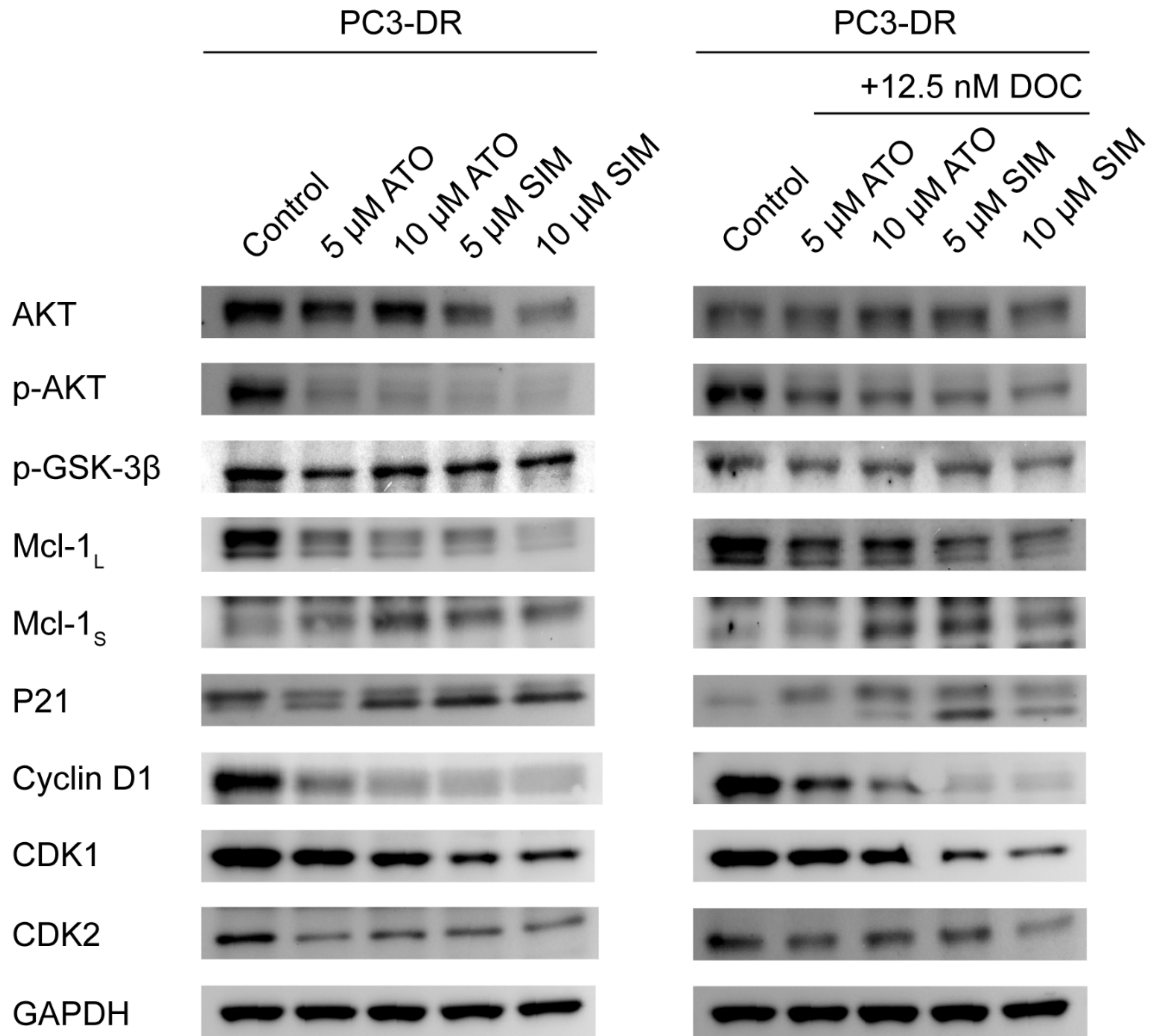

Figure 16. The expression levels of cell cycle and apoptosis regulators in PC3 and PC3-DR after the combination therapy. Representative bands for apoptosis-related proteins (AKT, p-AKT, p-GSK-3β, Mcl-1L and Mcl-1S) and cell cycle-related proteins (P21, Cyclin D1, CDK1 and CDK2). GAPDH served as the loading control. Data from one experiment.

4.10 Conclusions

Lipophilic statins demonstrate anti-cancer effects in both androgen-independent and docetaxel-resistant CRPC cells via the AKT pathway. SIM proves to be more effective than ATO in targeting both types of CRPC, with docetaxel-resistant cells exhibiting higher sensitivity to statins compared to their parental counterparts. Our study provides evidence of the significant anticancer capability of lipophilic statins in treatment-

resistant PCa, supporting their use as complementary therapy for CRPC patients undergoing hormone and chemotherapy interventions.

5. Discussions

PCa boasts a 97% 5-year cancer-specific survival rate when diagnosed and treated at localized stages, in contrast to 30% in the metastatic setting [4,5]. Advanced-stage of disease is present in approximately 10-20% of PCa patients at diagnosis. This includes both locally advanced and metastatic cases that often require ADT therapy [73,74]. However, PCa eventually progresses to CRPC following ADT strategies [22], and the prevalence of CRPC has been estimated at 17.8% among patients with PCa in the US population [23]. CRPC diminishes patient`s quality of life and reduces the life expectancy, while only a limited number of therapeutic approaches are capable of improving the survival. A docetaxel-based regimen has demonstrated a 2.4 months survival benefit in patients with advanced PCa [27]. However, an inevitable emergence of resistance to docetaxel eventually develops in PCa patients, presenting a significant challenge in modern oncology [26].

Since 2011, the therapeutic spectrum for CRPC has rapidly expanded, and new agents include drugs that target the androgen axis, such as abiraterone and enzalutamide [75]. These novel drugs provide survival benefits to CRPC patients, however they also impose a significant economic burden, serious side effects and acquired resistance. [75,76].

Accumulating epidemiologic evidence has shown that statin use reduces the risk of advanced PCa [77–79] and statin users have significantly lower PCa-specific mortality compared to non-users [80–82]. Statins are among the most frequently prescribed medications in the US [83], with simvastatin and atorvastatin being the two most commonly used agents, accounting for 42% and 20% of all statin users, respectively [84]. A prospective meta-analysis of 90056 participants in 14 randomized trials of statins demonstrated the long-term (5-year) safety and tolerability of statin treatment [85]. Additionally, the cost of statin use is negligible compared to the expensive chemotherapy drugs and AR signaling inhibitors that contribute considerable economic burden of PCa. A population-based study revealed that PCa is related to high economic costs (€8.43 billion) in the European Union, including €3.12 billion medication costs [86].

In the present study, we provided preclinical evidence regarding the variable anti-cancer effects of statins on androgen-insensitive PCa cells representing CRPC and docetaxel-resistant PCa cells. We observed that lipophilic statins (atorvastatin and simvastatin) have a higher cytotoxic effect than hydrophilic statins (pravastatin and rosuvastatin) in both castration- and chemotherapy-resistant PCa cells. The treatment with lipophilic statins inhibited the proliferation, migration, cell cycle and induced apoptosis in PCa cells via the AKT pathway. Furthermore, our data revealed that simvastatin has a superior cytotoxic effect and induces apoptosis more effectively in both androgen-insensitive and docetaxel-resistant CRPC cells than atorvastatin. To our knowledge, this is the first study to report that simvastatin displays greater efficacy in docetaxel-resistant cells than parental androgen-insensitive cells.

Interestingly, several studies have also suggested that lipophilic statins have a promising potential in PCa treatment, alongside a variety of anticancer mechanisms. The nuclear factor-κB (NF-κB) has been suggested to play an important role in inhibiting cell growth and inducing apoptosis in CRPC following simvastatin treatment [56,87]. Additionally, simvastatin has been proven to enhance anti-cancer effects when combined with various drugs in different androgen-sensitive PCa cell lines (LNCaP and VCaP) [88,89]. Furthermore, both simvastatin and atorvastatin have been shown to enhance radiosensitivity or radiotherapy on both PCa cells and Xenograft models [90–92]. These studies underscore the superior anti-cancer effects of lipophilic statins in PCa, as investigated in our study. The consistent research also provides molecular biological rationales for the clinical investigations that have revealed an association of lipophilic statins with decreased incidence, improved prognosis [93], and decreased PCa risk [94]. Similarly, a population-based case-control study demonstrated that statin use, the majority of prescriptions (93%) were for lipophilic statins, is related to risk reduction of advanced PCa [95].

Regarding the biological mechanisms underlying anticancer-action of statins are not clearly defined yet. In this project, we more focused on apoptosis and cell cycle regulating activity of statins as well as involved signaling pathway than on the biochemical explanation about the cholesterol metabolisms and cancer progression.

The evasion from programmed cell death via apoptosis is a hallmark of cancer and throughout cancer development and progression [96]. Members of Bcl-2 protein family regulate intrinsic apoptosis and are stratified into two subgroups based on their structural and sequence homology domains: anti-apoptotic members (Bcl-2, Bcl-XL, Bcl-W and Mcl-1) and pro-apoptotic members (Bak, Bax and Bim) [46,97]. Among others, the human Mcl-1 gene is located on chromosome 1q21 and consists of three exons [98]. The Mcl-1 gene generates three splice variants, comprising of anti-apoptotic protein Mcl-1L (full length), the pro-apoptotic proteins Mcl-1S (short) and Mcl-1ES (extra short) [99,100]. Our results demonstrated that lipophilic statins treatment could induce apoptosis in CRPC and docetaxel-resistant CRPC by targeting Mcl-1L and Mcl-1S through AKT pathway for the first time (Figure 8, 13 and 14). Moreover, Mcl-1 has been shown to be associated with metastasis in PCa [101,102], consistent with our data showing that the migration rate are reduced following statin treatment (Figure 6 and 7) and the Mcl-1 expression (Mcl-1L and Mcl-1S). A similar study discovered that simvastatin promotes irinotecan-induced apoptosis in PCa via inhibition of Mcl-1 [103]. However, this study failed to elucidate the underlying signaling pathway and the effect was based on the combination therapy of two therapeutic agents, simvastatin and irinotecan. Mcl-1 expression is high in hormone-insensitive, metastatic phenotype of advanced stage PCa, emphasizing it`s significance in PCa progression [104]. In this context, several studies indicated that Mcl-1 is a promising target for PCa treatment independent of the AR pathway [101,105,106] and multiple clinical trials with selective Mcl-1 inhibitors are currently ongoing [107].

As shown in Figure 17, the AKT signaling pathway is recognized for its involvement in cell cycle progression, apoptosis, migration as well as cell survival. P21 belongs to cyclin-dependent kinase inhibitor (CDI) family that modulates cell cycle through binding to cyclin/CDK complexes and inhibiting their activity [108]. In the context of cell cycle regulation in PCa, previous research has highlighted the significant role of AKT, CDK1, CDK2 and p21 play in antitumor and cell cycle regulatory activity of CD44+ PCa stem cells as well as in PCa cells resistant to docetaxel or cabazitaxel [109,110]. In our study, we revealed that lipophilic statins treatment in the both CRPC and docetaxel-resistant CRPC cells inducing substantial cell cycle arrest at the G1 phase and downregulation of pAKT with upregulated p21 and decreased Cyclin D1, CDK1 and CDK2 (Figure 9,

10, 11 and 12). Lipophilic statins targets cell cycle by G1 arrest through the AKT pathway in castration- and chemotherapy-resistant PCa.

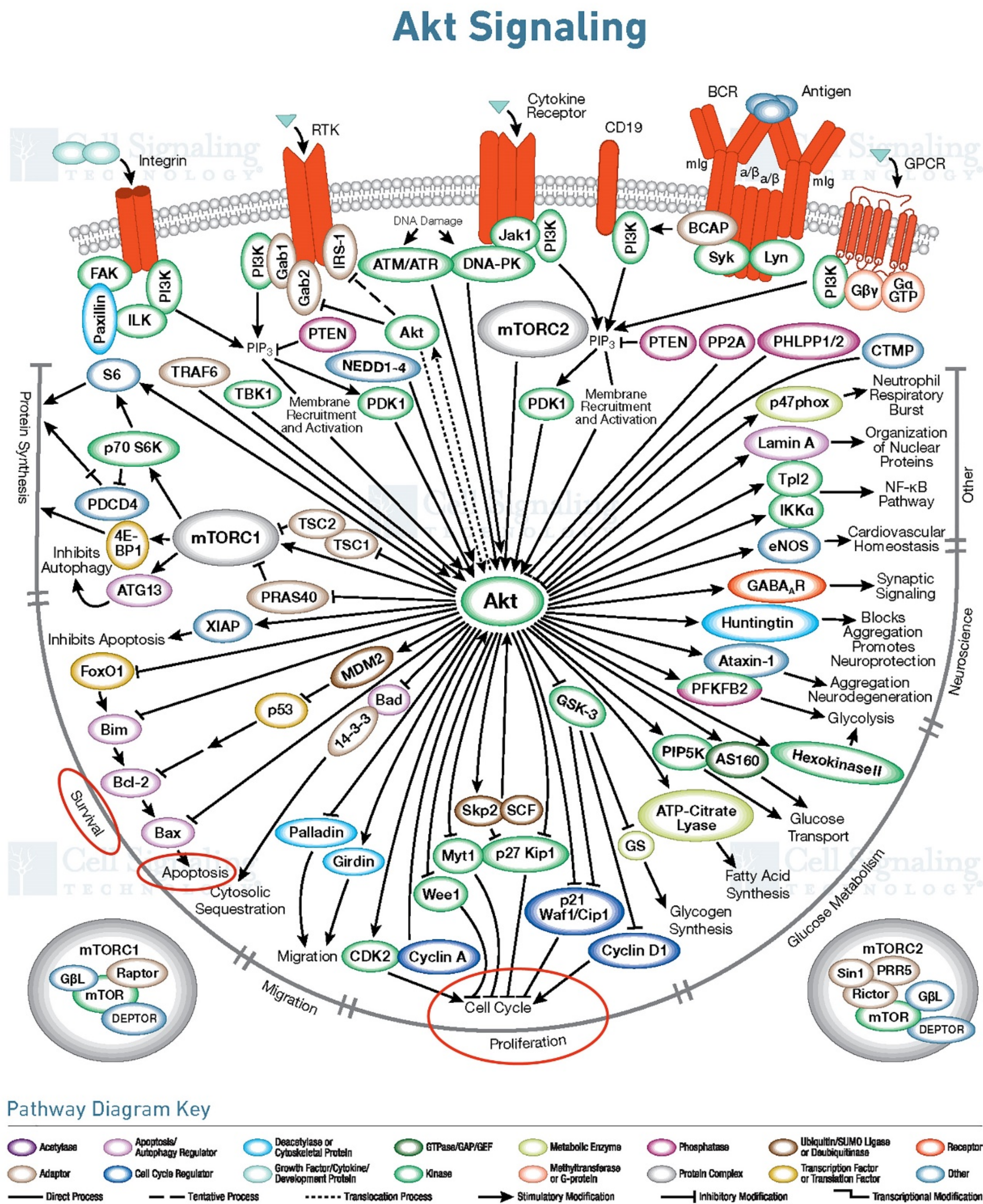

Figure 17. AKT signaling pathway. AKT plays a critical role in regulating diverse cellular functions including metabolism, proliferation, migration, survival, transcription and protein synthesis (illustration reproduced courtesy of Cell Signaling Technology, Inc.).

Our study is the first to reveal the anticancer effects of lipophilic statins in docetaxel-resistant PCa via the AKT signaling pathway. Several mechanisms have been proposed regarding how statins might impact distinct pathways essential for cancer formation and progression through both cholesterol-mediated or non-cholesterol-mediated mechanisms [111]. Previous studies demonstrated that atorvastatin and simvastatin could inhibit the development of PCa by attenuating epithelial-mesenchymal transition [112,113], whereas only a single PCa cell line (PC3 or DU145) was selected for research. Another study showed that statins decrease castration-induced bone marrow adiposity to inhibit PCa progression in bone through reducing BMSC-to-adipocyte transition [57]. In addition, multiple mechanisms and proteins have been investigated to show that atorvastatin or simvastatin could enhance radiosensitivity [90–92]. However, no specific molecular mechanisms were investigated in the study. Our study could provide strong evidence and complements these findings thereby support the therapeutic value of statin.

Despite the findings mentioned above, our study has several limitations. First, it is an in vitro investigation based on cell lines, lacking animal experiments and patient cohorts. Furthermore, the absence of supportive data from patients with castration- and chemotherapy-resistant PCa is another limitation. Most patients at our institution undergo surgery at the localized stage, which offers an excellent cure rate. Consequently, clinical data on chemotherapy-resistant PCa patients are limited, and even fewer patients receive statins during palliative therapy to avoid medication-induced interactions or synergistic side effects. Therefore, collecting comprehensive data across departments poses a significant challenge. In future studies, the anticancer effects of lipophilic statins should be confirmed in animal models and through large-scale prospective or retrospective research across multiple oncology centers.

We acknowledge that the treatment concentration (5-10 μM) adopted in our research is supratherapeutic compared to the commonly prescribed patient doses. The oral administration dosage range for patients of atorvastatin and simvastatin is 10-80 mg/day and 5-40 mg/day, respectively. The bioavailability of them has been reported as 12% and 5%, respectively [114]. Clinically relevant plasma concentrations of statins fall within the range of 10-100 nM [115]. Studies reported that a 20 mg simvastatin pill

in humans will be plasma concentration of 3.2-8.7 nM and a time to maximum concentration will be 1.3-2.4 hr [116,117]. However, the vast majority of in vitro anticancer studies use 5-10 μM dose or higher, which exceed clinical pharmacologic conditions. It is worth noting that cells are incubated around hyperglycemic (glucose) and overabundant nutrient (FBS and glutamine) medium conditions, which resulting in excessive growth stimulation [118]. This definitely is a valid explanation for why higher doses are needed to see the effects of statins in vitro cell experiments than what is typically used in patients. Furthermore, a phase I study reported that oral doses of lovastatin ranging from 2 to 45 mg/kg/day can reach concentrations of up to 3.9 μM, which is link to anti-proliferative activity in vitro [50]. Their team also demonstrated that simvastatin at a high but clinically achievable concentration by dose-escalation when combined with a low concentration of enzalutamide [50,89]. Another potential strategy to enhance the anticancer effects of statins in PCa is to develop a targeted drug delivery system to overcome the low bioavailability and concentration. Hybrid nanocarrier systems can guide and augment statins cytotoxic activity against PCa and these smart carriers are continuously being implemented to improve the drug bioavailability and effectiveness, most importantly, allow for programmed sustained drug release. What`s more, nanotechnology can be specifically targeted at PCa, improving the curative effect and reducing side effects [119–122]. Considering the above reasons combined with our findings, we therefore recommend lipophilic statins (atorvastatin and simvastatin) as adjuvant therapy against castration-resistant and chemotherapy-resistant PCa and not the main therapeutic agents. The use of the lipophilic statins for treatment of castration-resistant and chemotherapy-resistant PCa could be achieved with the assistance of a targeted drug delivery system, but not available yet. Further research is needed to explore this perspective in the future.

In summary, our study demonstrate that lipophilic statins (atorvastatin and simvastatin) inhibit growth and migration of CRPC cells and induce apoptosis and cell cycle arrest in a variety of androgen-insensitive and docetaxel-resistant cell lines through blocking the AKT pathway. Notably, we observed pronounced anticancer effects of simvastatin and significantly enhanced sensitivity of docetaxel-resistant cells to lipophilic statins compared to the parental cells. These findings indicate that lipophilic statins could serve as a potential adjunctive therapeutic medication for both CRPC and docetaxel-resistant CRPC alongside hormonal- and chemotherapeutic intervention. Nevertheless,

further research is necessary to better understand the synergistic anticancer effect of lipophilic statins and novel agents (docetaxel, abiraterone, enzalutamide) in CRPC and chemotherapy-resistant PCa.

6. Summary

6.1 Summary

In recent years, several clinical evidences have demonstrated that statin use improves the outcome of PCa. While various investigations suggested a potential preventive and therapeutic role for statins in PCa, limited evidence exists regarding their activity in aggressive PCa characterized by diverse therapy resistances. Furthermore, statins offer a therapeutic advantage of the confirmed safety and acceptable expenses by extensive clinical use in the general population over decades.

In order to evaluate the therapeutic potential of statins in CRPC, we investigated the efficacy and mechanisms of various statins in the androgen-insensitive PCa cell lines (PC3 and DU145) and their docetaxel-resistant sublines (PC3-DR and DU145-DR) mimicking castration-resistant and chemotherapy-resistant PCa. We observed that lipophilic statins (atorvastatin and simvastatin) effectively inhibited cell proliferation and migration in both castration- and chemotherapy- resistant PCa cells, which exhibit comparable drug sensitivity to statins. They also induced apoptosis and blocked cell cycle by arresting G1 phase through upregulating proteins p21 and downregulating Cyclin D1 and CDK1/CDK2 causing cell growth arrest. Statins strongly dephosphorylated AKT, which is able to promote apoptosis by inhibition of antiapoptotic proteins (Mcl-1L and p-GSK-3β) and upregulation of proapoptotic proteins (Mcl-1S). The most significant effects were consistently elicited by simvastatin in both androgen-insensitive and docetaxel-resistant sublines, while the docetaxel-resistant cells exhibited heightened sensitivity to simvastatin compared to the parental cells.

Our study provides evidence for the substantial anti-cancer potential of lipophilic statins in therapy-resistant PCa. Furthermore, it elucidates a remarkable amplification in treatment response of docetaxel-resistant cells, with a notable emphasis on the efficacy of simvastatin. Given the outcomes of our investigations, coupled with the economic advantages and established safety profile of these drugs, the adjunctive use of lipophilic statins for patients with CRPC should be carefully considered during hormone and chemotherapy interventions.

6.2 Zusammenfassung

Statine, cholesterinsenkende Medikamente, zeichnen sich durch ihre nachgewiesene Verträglichkeit und akzeptable Kosten aus, begründet durch ihren breiten klinischen Einsatz in der Allgemeinbevölkerung über Jahrzehnte. In jüngster Zeit wurde beobachtet, dass die Einnahme von Statinen sowohl das Risiko für Prostatakrebs (PCa) verringert als auch das Überleben der Patienten verbessert. Obwohl nachfolgende Studien auf eine potenzielle präventive und therapeutische Rolle der Statine bei PCa hindeuten, besteht nur begrenzte Evidenz für ihre Wirksamkeit bei aggressiven PCa-Formen, die durch verschiedene Therapieresistenzen charakterisiert sind.

Um das therapeutische Potenzial von Statinen bei aggressivem, kastrationsresistentem PCa (CRPC) zu evaluieren, untersuchten wir die Wirksamkeit und die Mechanismen verschiedener Statine in androgen-unabhängigen PCa Zelllinien (PC3 und DU145) und ihren Docetaxel-resistenten Sublinien (PC3-DR und DU145-DR), die kastrations- und chemotherapieresistentes PCa repräsentieren.

Es wurde festgestellt, dass lipophile Statine, wie Atorvastatin und Simvastatin, die Zellproliferation und -migration in kastrations- und chemoresistenten PCa-Zellen effektiv hemmten, während hydrophile Wirkstoffe nur begrenzte Effekte zeigten. Diese Statine blockierten den Zellzyklus, indem sie die G1-Phase durch Hochregulierung von p21 und Herunterregulierung von Cyclin D1 und CDK1/CDK2 stoppten, was zu einem vermehrten Zellwachstumsstillstand führte. Die signifikantesten krebshemmende Effekte wurden konsistent durch Simvastatin erzielt. Überraschenderweise zeigten die aggressiven, Docetaxel-resistenten Sublinien eine deutlich erhöhte Empfindlichkeit gegenüber Simvastatin im Vergleich zu den parentalen Zellen, insbesondere in Bezug auf verringerte Migration und verstärkte Apoptose.

Neben der Induktion von Apoptose durch deutlich erhöhte Caspase-3/7-Aktivität reduzierten die Statine auch die Expression und Phosphorylierung von AKT, was die Hemmung anti-apoptotischer Mcl-1L und die Hochregulierung pro-apoptotischer Mcl-1S förderte. Diese Blockade des AKT-Signalwegs trug vermutlich zu den beobachteten pro-apoptotischen und anti-proliferativen Effekten der Statine bei.

Unsere Studie belegt das antikarzinogene Potenzial lipophiler Statine bei PCa. Insbesondere die gesteigerte Sensibilität von Docetaxel-resistenten Zellen gegenüber lipophilen Statinen könnte therapeutisch bedeutsam für die Behandlung des chemoresistenten CRPC sein. Angesichts der Ergebnisse unserer Untersuchungen, gekoppelt mit den wirtschaftlichen Vorteilen und dem etablierten Sicherheitsprofil dieser Medikamente, sollte der Einsatz von lipophilen Statinen, insbesondere Simvastatin, als unterstützende Therapieoption bei Patienten mit CRPC in Erwägung gezogen werden, auch im Rahmen von Hormon- und Chemotherapieinterventionen.

7. Abbreviations

PCa	Prostate cancer
SPOP	Speckle-type pox virus and zinc finger protein
TP53	Tumor protein P53
PTEN	phosphatase and tensin homolog
FOXA1	Forkhead box A1
AR	Androgen receptor
ERG	E-26 transformation-specific-related gene
AKT	Protein kinase B
BCR	Biochemical recurrence
PSA	Prostate-specific antigen
ADT	Androgen deprivation therapy
CRPC	Castration-resistant prostate cancer
FDA	Food and Drug Administration
mCRPC	Metastatic castration-resistant prostate cancer
PARP	Poly (ADP-ribose) polymerase
PI3K	Hosphoinositide 3-kinase
Bcl-2	B-cell lymphoma 2
Mcl-1	Myeloid cell leukemia-1
HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase
pAKT	Phosphorylated AKT
mTOR	Mammalian target of rapamycin
GSK3	Glycogen synthase kinase 3
CDKS	Cyclin-dependent kinases
ATCC	American Type Culture Collection
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DMSO	Dimethylsulfoxid
FBS	Fetal bovine serum
PBS	Phosphate suffered saline
RIPA	Radio-immunoprecipitation assay
BCA	Bicinchoninic acid
LDS	Lithium dodecyl sulfate
ECL	Enhanced chemiluminescence
SPSS	Statistic package for social science

SEM	Standard error of the mean
NF-κB	Nuclear factor-κB
p-CHK1	Phospho-checkpoint kinase 1
CDI	Cyclin-dependent kinase inhibitor
CDKs	Cyclin-dependent kinases

8. Reference

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Since 10.2019	Martini-Klinik Prostatakrebszentrum Hamburg, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
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Training

2016-2019	Resident training. Xiangya Hospital Central South University, Hunan Province, P.R. China.
2014-2015	Clinical internship. The First Affiliated Hospital of University of South China, P.R. China.
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Research experiences

02.2021-Present	Anti-cancer effects of statins on castration- and chemotherapy-resistant prostate cancer cells.
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Scholarship

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CN208823593U Continuous Irrigation Device for Urology (2nd)

CN210956172U Erectile Dysfunction Rapid Diagnostic Card (2nd)

CN210521459U Clinical urinary catheter for urology (3rd)

11. Publications

Original Articles

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Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

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Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: ....