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In Vitro Effect and Mechanism of Action of Ergot Alkaloid Dihydroergocristine in Chemoresistant Prostate Cancer Cells

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Abstract. *Background/Aim: Chemoresistance is a major obstacle in the treatment of prostate cancer (PCa). It is imperative to develop novel strategies for overcoming chemoresistance and improving clinical outcomes. We evaluated the in vitro activity and mechanism of action of dihydroergocristine (DHECS), an ergot alkaloid approved for the treatment of dementia, in PCa cells. Materials and Methods: The in vitro effects of DHECS on PCa cell cycle and viability were determined by flow cytometry and colorimetric assay. The effects of DHECS on PCa cell signaling were evaluated by quantitative PCR, western blot analysis and reporter assay. Results: DHECS was effective in inducing cell cycle arrest and apoptosis in human PCa cells. Of particular interest, DHECS demonstrated high potency against chemoresistant PCa cells. At the molecular level, DHECS affected multiple factors implicated in the regulation of cancer cell cycle and programmed cell death, including p53, mouse double minute 2 homolog (MDM2), retinoblastoma protein (RB), p21, E2F transcription factor 1 (E2F1), survivin, myeloid cell leukemia 1 (Mcl-1) and poly ADP ribose polymerase (PARP). Furthermore, DHECS may function through dopamine receptor-mediated effects on 5'-AMP-activated protein kinase (AMPK) and nuclear factor kappa B (NF-κB). Conclusion: DHECS has the potential to be repurposed as a novel anticancer agent for the management of chemoresistant PCa.*

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Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer-related death among men in the United States (1). Docetaxel, the first-line standard chemotherapy for metastatic PCa, initially prolongs overall survival by 3 to 4 months; however, most patients relapse and become chemoresistant without a cure (2, 3). It is imperative to develop novel strategies to overcome chemoresistance and improve clinical outcomes in PCa patients.

As a metabolite of tyrosine, dopamine acts as both a neurotransmitter and a hormone, and plays important roles in numerous physiological processes, such as voluntary movement and sympathetic regulation (4, 5). Dopaminergic dysfunction results in multiple human diseases including Parkinson's disease (6), Huntington's disease (7) and depression (8). The physiological and pathological functions of dopamine are thought to be mediated by at least five distinct dopamine receptors, which belong to the G protein-coupled receptors (GPCRs) superfamily. Based on their downstream effects on adenosine 3',5'-cyclic monophosphate (cAMP) signaling, dopamine receptors can be classified into D1-like (D1, D5) and D2-like (D2, D3, D4) classes (9). D1-like dopamine receptors stimulate cAMP production and protein kinase A (PKA) activity through coupling to $G\alpha_{s/olf}$ proteins, whereas D2-like dopamine receptors suppress cAMP production and PKA activity through coupling to $G\alpha_{i/o}$ proteins (10). In addition to their classical roles in cAMP signaling, dopamine receptors regulate diverse molecular pathways *via* G

Table I. *Sequences of PCR primers*.

Gene	Forward primer	Reverse primer
Survivin	TGCCCCGACGTTGCC	CAGTTCTTGAATGTAGAGATGCGGT
GAPDH	CGAGATCCCTCCAAAATCAA	TTCACACCCATGACGAACAT

protein-dependent or -independent mechanisms. For example, D1-like dopamine receptors or D1/D2 receptor heterodimers regulate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) signaling *via* Gαq proteins (11, 12). The formation of D2-like dopamine receptors/β-arrestin2/protein phosphatase 2A (PP2A)/Akt complex leads to PP2A-mediated Akt deactivation and glycogen synthase kinase-3 (GSK-3) activation (13). D2-like dopamine receptors are involved in the regulation of intracellular calcium levels by modulating $G_{\beta\gamma}$ signaling (14), and D1 and D2-like receptors differentially affect mitogen-activated protein kinases (MAPKs) signaling (15, 16).

Dihydroergocristine (DHECS) is a dihydrogenated alkaloid of ergot (17) that mainly affects several neurological processes, including memory (18-20), cerebral hypoxia (21), sleep-wakefulness cycle (22) and prolactin release (23, 24). DHECS has been used, alone or combined with other ergot alkaloids, in the treatment of Parkinson's disease (25), peripheral vascular diseases (26), hypertension (27), hyperprolactinemia (28) and depression (29). Although the exact mechanisms underlying these clinical benefits remain to be fully understood, it has been proposed that DHECS acts as an agonist or antagonist of two specific groups of GPCRs, *i.e.,* dopaminergic receptors and adrenergic receptors (30). For example, DHECS-induced inhibition of prolactin release and cAMP accumulation can be abolished by D2 receptor (DRD2) antagonists haloperidol and pimozide (23). In anterior pituitary cells, DHECS and bromocriptine, a DRD2 agonist, inhibit angiotensin II-medicated release of fatty acids, an effect that is completely blocked by the selective D2 receptor antagonist sulpiride (31).

Accumulating evidence from various experimental models has implicated a molecular connection between the dysregulation of dopamine receptor biology and human cancers (32-34). In a recent study, we provided the first evidence demonstrating that DRD2 expression is inversely associated with clinical PCa progression. We further identified bromocriptine, a semisynthetic ergot alkaloid, as a potential adjunct therapy to sensitize PCa cells to docetaxel chemotherapy (35). These observations supported the notion that DRD2 agonism may represent a novel strategy to overcome chemoresistance. In this study, we report that DHECS exhibits *in vitro* cytotoxicity in a panel of established PCa cell lines, and intriguingly, demonstrates high specificity against chemoresistant PCa cells by affecting multiple oncogenic signals involved in cancer cell survival and proliferation.

Materials and Methods

Cell culture and chemicals. Human PCa cell lines LNCaP and C4-2 were obtained from Dr. Leland WK Chung (Cedars-Sinai Medical Center, Los Angeles, CA, USA) and maintained in T-medium (Life Technologies, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA). PC-3 cell line was obtained from American Type Culture Collection (ATCC) and maintained in RPMI1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS. CWR22Rv1 cell line was obtained from Dr. Jin-Tang Dong (Emory University, Atlanta, GA, USA), and maintained in RPMI1640 medium supplemented with 10% FBS, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 10 mmol/l sodium pyruvate, and 10 mmol/l HEPES. $ARCaP_E$ cells stably expressing control short hairpin RNA (shRNA) ($ARCaP_E$-shCtrl) or human EPLIN shRNA ($ARCaP_E$-shEPLIN) were established and maintained as described in our previous publication (36). C4-2B and its docetaxel-resistant derivative C4-2B-TaxR subline (37) were originally provided by Dr. Allen C. Gao (University of California Davis) and cultured following the procedures described in (37), with the modification that C4-2B-TaxR cells were routinely maintained in the presence of 100 nM docetaxel (LC Laboratories, Woburn, MA, USA). The final concentration of docetaxel in culture medium was reduced to 5 nM before experimental assays. KB-3-1 and its vinblastine-resistant derivative KB-V1 were obtained from Zhuo G. Chen (Emory University) and maintained as described previously (38). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). DHECS was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

Cell viability assay. *In vitro* cytotoxicity was measured using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instruction. Half maximal inhibitory concentration (IC_{50}) was calculated using SigmaPlot program (Systat Software Inc., San Jose, CA, USA).

Apoptosis and cell cycle assays. For apoptosis assay, C4-2B-TaxR cells were incubated with DHECS at varying concentrations for 72 h, and then stained with an APC Annexin V apoptosis detection kit (BioLegend, San Diego, CA, USA) according to the manufacturer's instruction. For cell cycle assay, cells were serum-starved for 24 h and incubated with DHECS at varying concentrations for 48 h, then stained with propidium iodide (PI, Sigma-Aldrich) (50 μg/ml) according to standard procedures. Both apoptosis and the distribution of cells in the cell cycle were analysed by flow cytometry with FACSCanto II flow cytometer (BD Biosciences, Bedford, MA, USA). The results were analyzed by FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Quantitative PCR (qPCR). Total RNA was extracted using Qiagen RNeasy Kit (Valencia, CA, USA). cDNA was synthesized using SuperScript® III First-Strand Synthesis System (Life Technologies). qPCR was performed by the Stratagene Mx3005P system (Agilent technologies) using *Power*SYBR® Green PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instruction. Primers used for qPCR are listed in Table I.

Western blot analysis. Total cell lysates were extracted using radioimmunoprecipitation (RIPA) buffer (Santa Cruz Biotechnology). Halt™ protease inhibitor cocktail (Thermo Fisher Scientific) was added to the total cell lysates. Immunoblotting analyses were performed according to standard procedures (Bio-Rad Bulletin 6376, Bio-Rad, Hercules, CA, USA). Primary antibodies used in immunoblotting are listed in Table II.

Transfection and reporter assay. Human survivin promoter reporters pSurvivin-Luc1430 and pSurvivin-Luc230 (39) were provided by Dr. Allen C. Gao. pRL-TK was purchased from Promega (Madison, WI, USA). Lipofectamine 3000 reagent (Life Technologies) was used for cDNA transfection according to the manufacturer's instructions. Twenty-four hours after transfection, C4-2B-TaxR cells were treated with DHECS at the indicated concentrations for 48 h. Cell lysates were extracted and luciferase activities were measured using a Dual-Luciferase reporter assay system (Promega). Relative luciferase units were defined as firefly luciferase intensity normalized to Renilla luciferase activity.

Table II. *Antibodies*.

Antibody	Catalog number	Source
β-actin	4970	Cell Signaling Technology
Cleaved PARP	5625	Cell Signaling Technology
PARP	9542	Cell Signaling Technology
Survivin	NB500-201	Novus Biologicals
MCL-1	sc-819	Santa Cruz Biotechnology
E2F1	sc-56661	Santa Cruz Biotechnology
P53	sc-126	Santa Cruz Biotechnology
p21	556430	BD Medical Technology
p-RB/RB	sc-102	Santa Cruz Biotechnology
MDM2 (Ab-6)	OP146	EMD Millipore
p-MDM2	3521	Cell Signaling Technology
DRD1	720276	Invitrogen
DRD2	sc-5303	Santa Cruz Biotechnology
p-ERK	sc-7383	Santa Cruz Biotechnology
ERK	sc-135900	Santa Cruz Biotechnology
p-PP1α	2581	Cell Signaling Technology
PP1α	sc-271762	Santa Cruz Biotechnology
p-CREB	9191	Cell Signaling Technology
CREB	sc-377154	Santa Cruz Biotechnology
p-NF-κB	3033	Cell Signaling Technology
NF-κB	8242	Cell Signaling Technology
p-AKT	4060	Cell Signaling Technology
AKT	9272	Cell Signaling Technology
p-CaMKK2	12818	Cell Signaling Technology

Results

DHECS inhibits the in vitro viability of PCa cells and demonstrates selectivity against chemoresistant PCa cells. We first determined the *in vitro* cytotoxicity of DHECS in several established PCa cell lines, including LNCaP [androgen-dependent, androgen receptor (AR)-positive], C4-2 (androgen-independent, AR-positive), CWR22Rv1 [androgen-independent, AR- and AR variant 7 (AR-V7)-positive] and PC-3 (AR-negative). Although these cell lines have distinct molecular profiles and phenotypes, they are sensitive to docetaxel treatment in cellular cultures. As shown in Figure 1A, DHECS exhibited various degrees of cytotoxicities in these cells, with the IC_{50} value of 25.78 μM in LNCaP, 25.31 μM in C4-2, 13.44 μM in CWR22Rv1 and 10.63 μM in PC-3 cells.

We further examined the *in vitro* cytotoxicity of DHECS in two newly established models of chemoresistant PCa. Our previous work has demonstrated that the depletion of epithelial protein lost in neoplasm (EPLIN) in a low-invasive, epithelial-like PCa line $ARCaP_E$ promotes epithelial-to-mesenchymal transition (EMT), increases invasiveness and stemness, and confers chemoresistance (36, 40). These results allowed us to propose that EPLIN-depleted $ARCaP_E$ cells ($ARCaP_E$-shEPLIN) represent a subpopulation of inherently chemoresistant PCa cells. Interestingly, DHECS demonstrated potent cytotoxicity in $ARCaP_E$-shEPLIN cells (IC_{50}=3.28 μM) but showed much weaker activity in the chemosensitive counterpart $ARCaP_E$-shCtrl expressing control shRNAs (IC_{50}=17.19 μM). The selectivity index (SI) of DHECS, defined as the ratio of its IC_{50} in $ARCaP_E$-shCtrl *versus* that in $ARCaP_E$-shEPLIN cells, was determined as 5.24 (Figure 1B). These results suggested that DHECS appeared to be more potent against chemoresistant PCa cells. To confirm this observation, we determined the IC_{50} of DHECS in a highly docetaxel-resistant C4-2B-TaxR subline, which was established by incubating parental, chemosensitive C4-2B cells in the presence of gradually increasing doses of docetaxel (37), therefore representing a cellular model of acquired chemoresistance. Consistently, DHECS demonstrated a higher *in vitro* cytotoxicity in C4-2B-TaxR cells (IC_{50}=11.25 μM) than in C4-2B cells (IC_{50}>80 μM), with a SI of more than 7.11 (Figure 1C). The high potency of DHECS in chemoresistant cancer cells was also observed in other cancer types. For example, DHECS effectively inhibited the viability of KB-V-1 cells, a HeLa derivative that is highly resistant to vinblastine (41) (IC_{50}=17.19 μM) (Figure 1D). Taken together, these results indicated that DHECS has excellent *in vitro* activities in chemoresistant cancer cells.

DHECS induces cell cycle arrest and apoptosis in chemoresistant PCa cells. As a cellular model, C4-2B-TaxR cells closely recapitulated the clinical features of bone metastatic, chemoresistant PCa and were used to determine the effect and mechanism of action of DHECS in

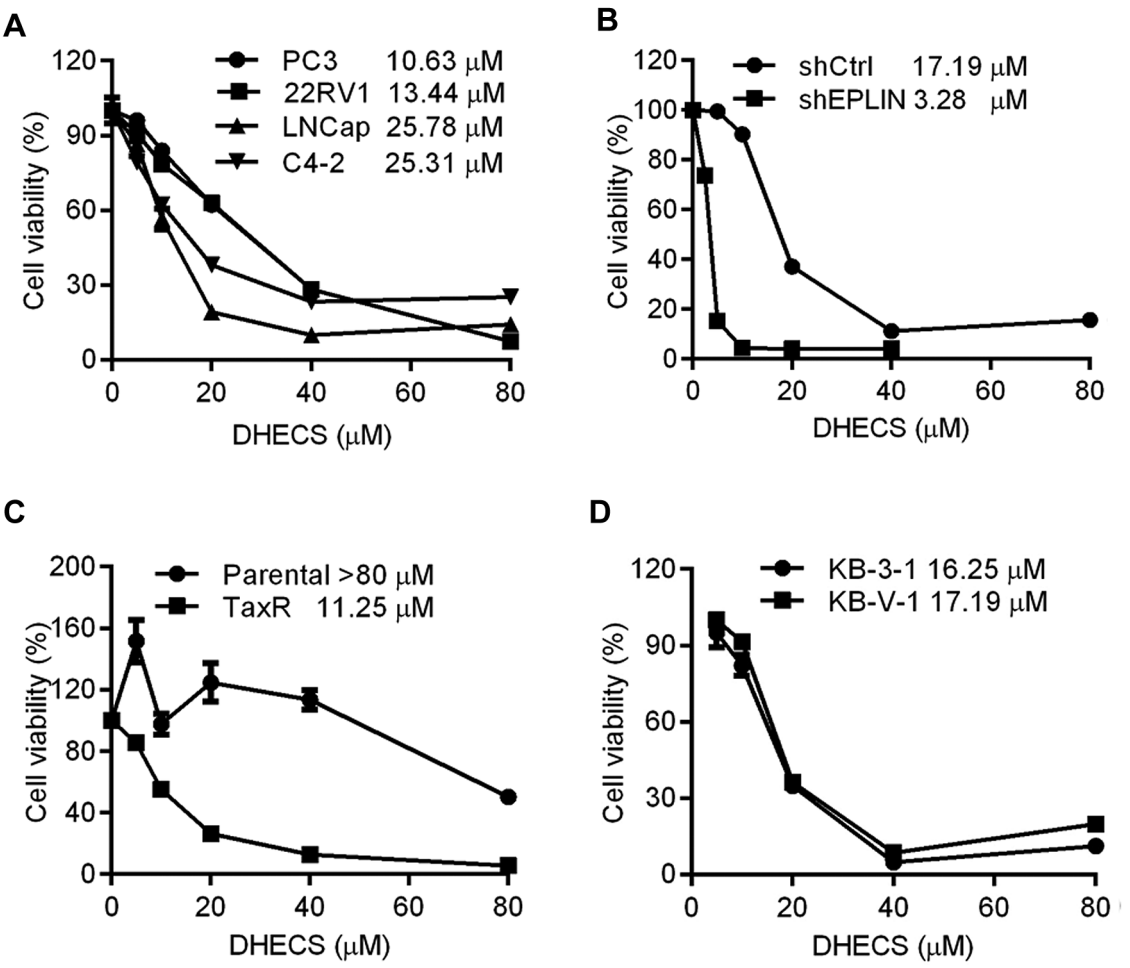

Figure 1. *In vitro cytotoxicity of DHECS in prostate cancer (PCa) cells. (A) In vitro cytotoxicity of DHECS in established PCa cell lines (72 h). (B) In vitro cytotoxicity of DHECS in the ARCaPE chemoresistant PCa model (72 h). ARCaPE cells expressing EPLIN shRNAs (ARCaPE-shEPLIN) are resistant to docetaxel. (C) In vitro cytotoxicity of DHECS in the C4-2B chemoresistant model (72 h). C4-2B-TaxR cells are highly resistant to docetaxel. (D) In vitro cytotoxicity of DHECS in the KB-3-1 and its chemoresistant derivative KB-V1 cells (72 h).*

chemoresistant PCa cells. Flow cytometry showed that DHECS significantly induced cell cycle arrest at the G_1/S checkpoint and to a lesser degree, at the G_2/M checkpoint, in a dose-dependent manner (Figure 2A). On the other hand, DHECS only markedly caused cell death at high concentrations (15 and 30 μM; Figure 2B).

Multiple factors are involved in the aberrant regulation of cell cycle in cancer cells (42). During G_1/S transition, E2F transcription factor 1 (E2F1) activates the transcription of numerous S-phase proteins. Hypophosphorylated retinoblastoma protein (RB) inhibits E2F1 function by binding to E2F1, whereas hyperphosphorylated RB (p-RB) releases E2F1 to active downstream genes (43, 44). The p53-p21 axis is responsible for the G_1/S and G_2/M arrest after DNA damage (45). Mouse double minute 2 homolog (MDM2), an ubiquitin ligase, promotes p53 ubiquitination and degradation (46). Since DHECS mainly affected cell cycle at the G_1/S checkpoint in chemoresistant PCa cells, we performed western blot analyses to examine the effect of DHECS on protein expression of the above cell cycle regulators. As summarized in Figure 2C, DHECS markedly suppressed the expression of E2F1 and p-RB, and increased p53 and p21 in a time-dependent manner. MDM2 phosphorylation (p-MDM2) was moderately inhibited by DHECS treatment, which might partially contribute to p53 upregulation (46). Taken together, these molecular changes are consistent with the inhibitory effect of DHECS on G_1/S transition.

Survivin is the smallest member of the inhibitor of apoptosis (IAP) family and plays an essential role in cell survival. Myeloid cell leukemia 1 (Mcl-1) belongs to the BCL-2 family and is a critical anti-apoptotic protein. Previous studies from us and others have shown that overexpression of

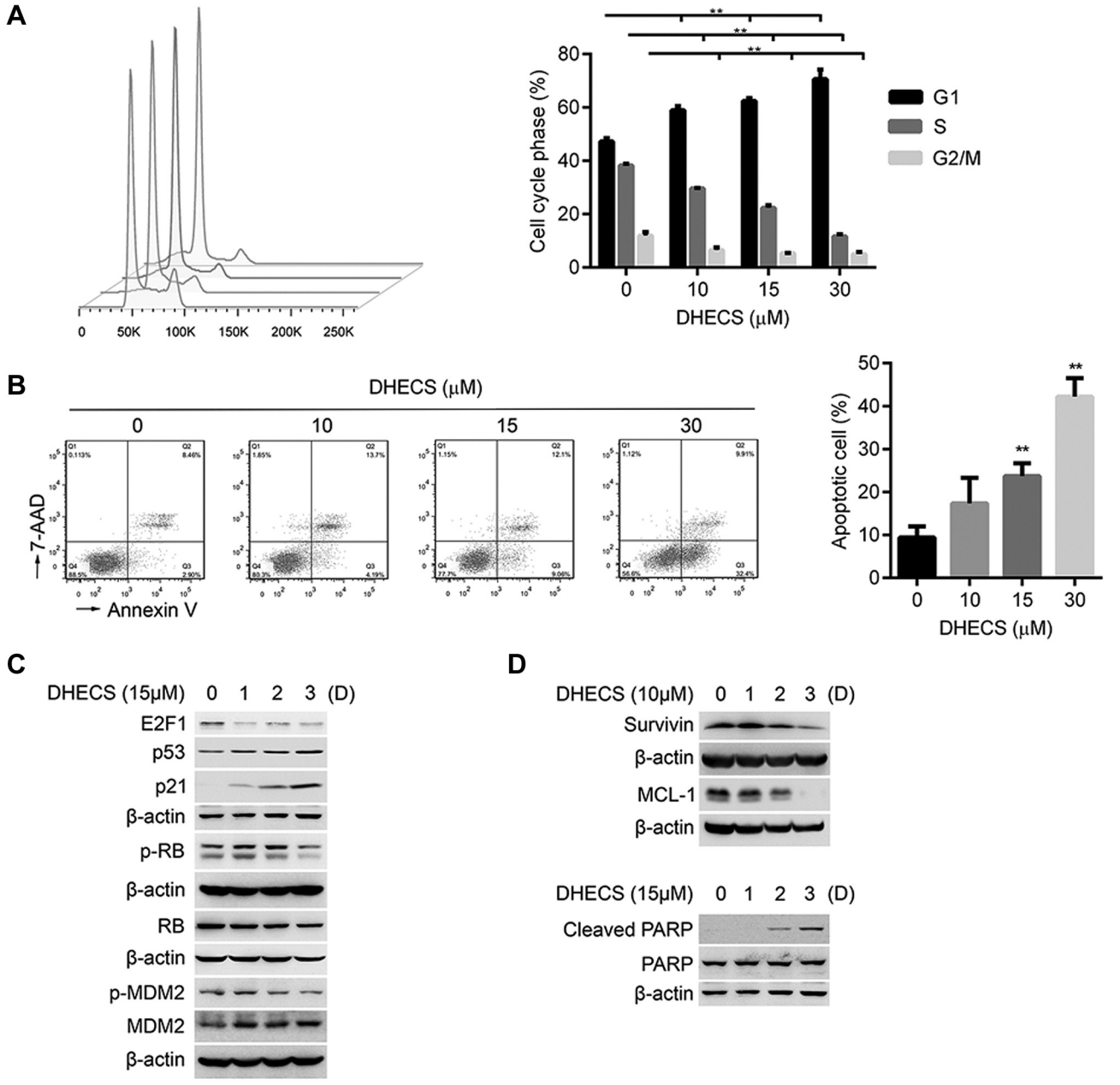

Figure 2. *Effects of DHECS on apoptosis and cell cycle in chemoresistant C4-2B-TaxR cells. (A) Flow cytometry analysis of the cell cycle in the docetaxel resistant C4-2B-TaxR cells treated with varying concentrations of DHECS (48 h). **p<0.01 for all pairwise comparisons between the percentages of cells in each cell cycle phase from the control and DHECSS treatment groups. (B) Flow cytometry assay of Annexin V staining in C4-2B-TaxR cells treated with varying concentrations of DHECS (72 h). **p<0.01. (C) Western bot analysis on the expression of E2F1, p53, p21, p-RB, RB, p-MDM2, and MDM2 1 in C4-2B-TaxR cells treated with DHECS (15 μM) at the indicated time points. (D) Western bot analysis on the expression of survivin, MCL-1, cleaved PARP, and PARP in C4-2B-TaxR cells treated with DHECS at the indicated concentrations and time points.*

both survivin and Mcl-1 is associated with clinical PCa metastasis and therapeutic resistance (47-50). As shown in Figure 2D, DHECS significantly reduced the expression of survivin and Mcl-1. Consistently, DHECS increased the cleavage of poly ADP ribose polymerase (PARP) in a time-dependent manner. These results suggested that the inhibition of survivin and Mcl-1 may contribute to the pro-apoptosis effect of DHECS in chemoresistant PCa cells.

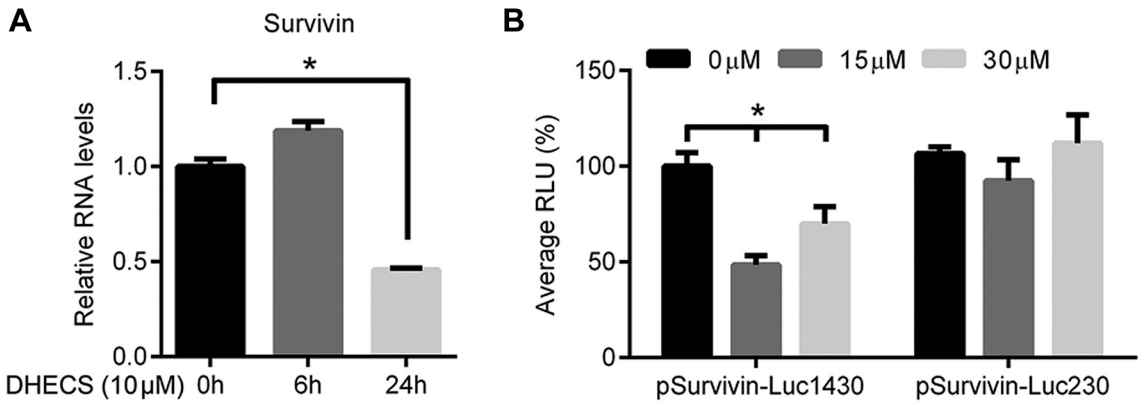

Figure 3. *Effect of DHECS on survivin transcription in chemoresistant PCa cells. (A) qPCR analysis on the mRNA expression of survivin in C4-2B-TaxR cells treated with DHECS (10 μM) at the indicated time points. (B) Relative luciferase activities of pSurvivin-Luc1430 and pSurvivin-Luc230 in the docetaxel resistant C4-2B-TaxR cells treated with DHECS (48 h) at the indicated concentrations.*

DHECS inhibits survivin expression at the transcriptional level. Given the important role of survivin in the control of cancer cell viability, we investigated whether DHECS regulates survivin expression at the transcriptional level. Quantitative PCR analyses showed that survivin mRNA was significantly downregulated at 24 h following DHECS treatment (Figure 3A). We further determined the effect of DHECS on the luciferase activity of two human survivin reporters, *i.e.*, pSurvivin-Luc1430 that contains a 1,430-bp region of survivin promoter and pSurvivin-Luc230 that only contains a 230-bp truncated fragment of pSurvivin-Luc1430 (39). Interestingly, it appeared that DHECS significantly inhibited the luciferase activity of pSurvivin-Luc1430 reporter but not that of pSurvivin-Luc230 reporter (Figure 3B), indicating that DHECS may suppress survivin transcription *via* certain *cis* elements located within the 1,430-bp region and upstream of the 230-bp fragment of the survivin promoter (51).

DHECS affects the expression and downstream signaling of dopamine receptors. Since DHECS is a known activator of dopamine receptor signaling, we examined the effect of DHECS on the expression and signaling of two representative dopamine receptors in chemoresistant PCa cells. As shown in Figure 4A, DHECS slightly decreased DRD1 expression whereas moderately increased DRD2 expression in a time-dependent manner in C4-2B-TaxR cells, indicating that DHECS may differentially affect the expression of these two dopamine receptors in PCa cells.

To identify potential signaling pathways involved in the observed DHECS effects in chemoresistant PCa cells, we examined several major DRD1/DRD2 downstream signaling factors, *i.e.*, cAMP response element-binding protein (CREB), extracellular signal-regulated protein kinase 1/2 (ERK1/2) and serine/threonine-protein phosphatase 1 (PP1α)/AKT (52). As shown in Figure 4B, CREB phosphorylation at serine 133 was increased at a time point as early as 24 h following DHECS treatment. Phosphorylation of both ERK1/2 and PP1α were markedly increased at 24 h but subsequently decreased between 48 h and 72 h, with a slight reduction at 72 h when compared with the vehicle control. The endogenous level of total ERK1/2 kinases was moderately decreased from 24 h, whereas total PP1α expression was not affected by DHECS treatment. These results indicated that DHECS could activate cAMP signaling, inhibit MAPK activity on ERK1/2, and suppress PP1α phosphorylation that may be partially responsible for the increased AKT phosphorylation.

The effect of DHECS on other putative dopamine receptor-mediated signaling pathways was also investigated (53-55). DHECS reduced the phosphorylation of calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) at Ser511 and increased the phosphorylation of 5'-AMP-activated protein kinase (AMPK) at Thr172 in a time-dependent manner (Figure 4A), indicating that DHECS may also activate AMPK and augment its function as a tumor suppressor in chemoresistant PCa cells. Phosphorylation of nuclear factor kappa B (NF-κB), a transcriptional factor playing a crucial role in cancer progression (56), was significantly inhibited by DHECS (Figure 4B). Taken together, these results suggested that DHECS might exert its functions in PCa cells by affecting several dopamine receptor-related signaling pathways.

Discussion

Although our understanding of the biology of dopamine receptor signaling in cancer progression is still rudimentary and sometimes controversial, mounting experimental and clinical evidence has indicated that the dysregulation of

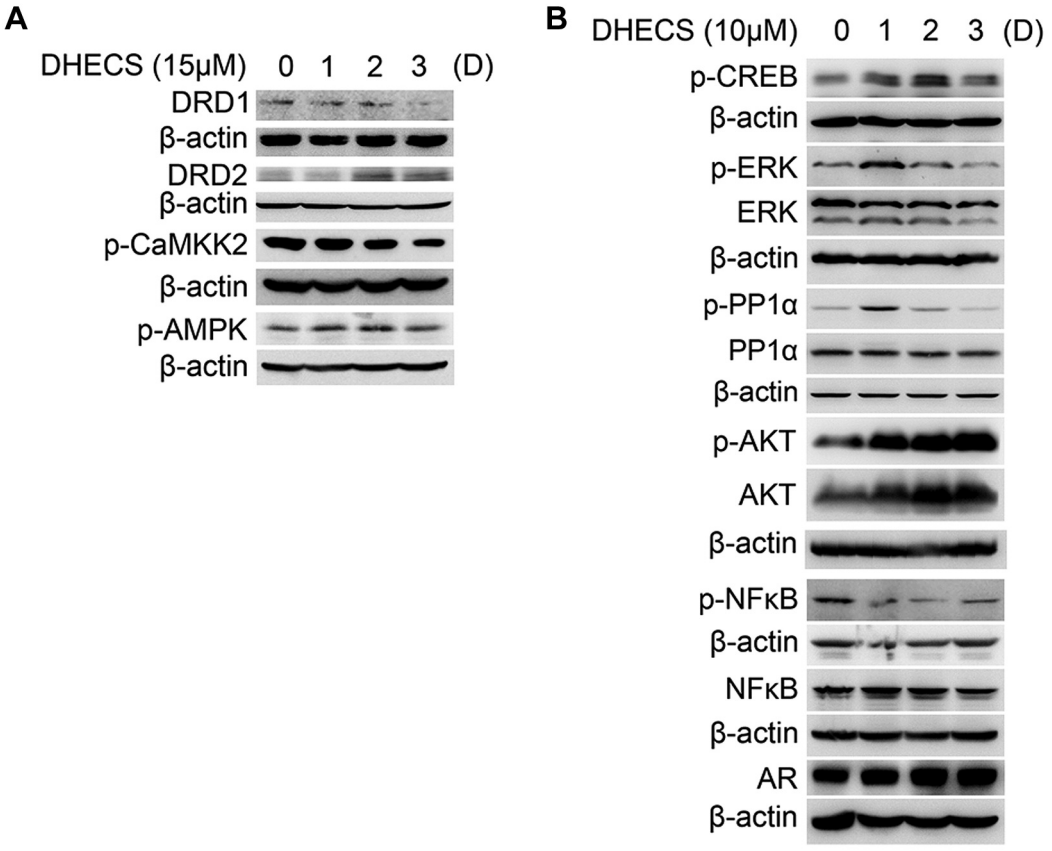

Figure 4. *Effects of DHECSS on the expression of dopamine receptors and downstream signaling molecules. (A) Western bot analysis on the expression of DRD1, DRD2, p-CaMKK2, and p-AMPK in the docetaxel resistant C4-2B-TaxR cells treated with DHECS (15 μM) at the indicated time points. (B) Western bot analysis on the expression of p-CREB, p-ERK, ERK, p-PP1α, PP1α, p-AKT, AKT, p -NFκB, NFκB, and AR in C4-2B-TaxR cells treated with DHECS (10 μM) at the indicated time points.*

dopamine receptor signaling is associated with the progression of human cancers, including PCa (35, 57-60). Some epidemiologic studies further suggested a correlation between psychotic disorders in which dopaminergic drugs are applied and the risk of cancer, of particular note, a reduced risk of PCa (61, 62). These observations have led to a hypothesis that certain dopaminergic drugs for neurological diseases can be repurposed to treat cancers (63). A previous report examined the *in vitro* effects of DHECS and several other ergot alkaloids in established human cancer cell lines and found that DHECS has a moderate cytotoxicity in PCa cell lines ($Log_{10}IC_{50}$=–5 ~ –4.5 M) (64). In current study, we demonstrated a novel feature of DHECS, that is, DHECS is highly selective against chemoresistant PCa cells *via* the induction of cell cycle and programmed cell death. We further revealed that DHECS affects the expression of several key regulators of the cancer cell cycle and apoptosis, such as p53, RB, E2F1, survivin, Mcl-1, in chemoresistant PCa cells (Figure 5). To our knowledge, this is the first study investigating the cellular effect and mechanism of action of an ergot alkaloid in chemoresistant PCa cells.

A major challenge in drug repurposing for cancer therapy is to identify novel mechanisms of action and in many situations new target(s), of non-oncology drugs in cancer cells. Given the excellent *in vitro* cytotoxicity of DHECS in PCa cells and its high selectivity against chemoresistant cancer cells, we explored the underlying mechanism of DHECS. Several key regulators of the cancer cell cycle and apoptosis were found to be significantly affected by DHECS treatment, which may account for the observed cytotoxicity of DHECS in these cancer cells. For example, DHECS increased the expression of p53 and p21 and inhibited the expression of E2F1 and p-RB, which may subsequently result in the arrest at the G_1/S checkpoint of the cell cycle. The suppression of survivin and Mcl-1, two critical survival factors implicated in therapeutic resistance, may contribute to DHECS-induced apoptosis. These molecular studies elucidated several new downstream effectors of DHECS in chemoresistant PCa cells.

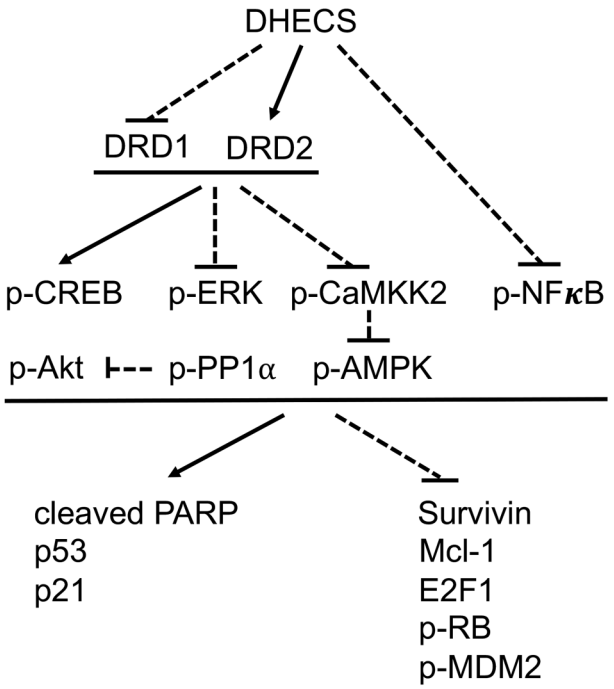

Figure 5. *A schematic diagram of DHECS function in PCa cells. DHECS affects the expression of several key regulators of apoptosis and cell cycle through dopamine receptor-related pathways, thereby inducing apoptosis and cell cycle arrest in PCa cells. Solid lines represent the pathways potentially activated by DHECS, and dotted lines represent the pathways potentially inhibited by DHECS.*

We further attempted to identify the signaling factors that directly mediate the effects of DHECS in chemoresistant PCa cells. In nerve cells, D1-like dopamine receptors stimulate whereas D2-like dopamine receptors suppress the activation of cAMP signaling, as demonstrated by the phosphorylation of CREB protein (10, 15, 65). Our previous work has shown that the activation of CREB signaling is associated with invasive phenotypes of PCa and clinical bone metastasis, which indicated that CREB phosphorylation is a survival signal in PCa cells. Since DHECS is a known agonist of DRD2, we examined the effect of DHECS on DRD2 expression and CREB phosphorylation. DHECS increased DRD2 expression and reduced DRD1 expression in chemoresistant C4-2B-TaxR cells, suggesting that DHECS treatment may lead to the activation of DRD2 signaling and inactivation of DRD1 signaling. Interestingly, in contrast to our anticipations, DHECS treatment led to increased CREB phosphorylation instead of p-CREB inhibition. These results suggested that the effects of DHECS on the expression of DRD1/DRD2 and CREB phosphorylation may be partially mediated by independent mechanisms. In other words, DHECS may activate cAMP-CREB signaling *via* a dopamine receptor-independent manner. However, given the excellent cytotoxicity of DHECS in chemoresistant PCa cells and the pro-survival role of CREB, it is plausible that the cAMP-CREB pathway may not be a major mediator of DHECS's cellular effects, and DHECS may act through other signaling molecules to exert its functions.

A possible mediator of DHECS functions in PCa cells could be CaMKK2, a serine/threonine protein kinase that is frequently upregulated in PCa and further overexpressed in metastatic castration-resistant tumors. Recent studies have proposed CaMKK2 as a direct target of androgen receptor (AR) and a potential driver of PCa progression, and the depletion of CaMKK2 suppresses cell proliferation, migration, invasion and tumor growth in preclinical models (66, 67). In prostate cells, CaMKK2 appeared to be the dominant kinase of AMPK, a master regulator of cellular homeostasis and tumor suppressor, by directly phosphorylating AMPK at Thr172 and activating AMPK (68, 69). Phosphorylation of CaMKK2 at Ser155 by death-associated protein kinase (DAPK) inhibits CaMKK2 activity (70, 71). Considering the effects of dopaminergic signaling on intracellular calcium levels and that the activation of CaMKK2 is dependent on calcium-mediated calmodulin (14), we investigated whether the CaMKK2-AMPK axis is responsive to DHECS treatment in PCa cells. Our data showed that DHECS simultaneously down-regulated p-CaMKK2 (Ser511) and increased p-AMPK(Thr172) (Figure 4A), which may subsequently activate AMPK and elicit its function as a tumor suppressor. Supporting this notion, the DRD2 agonist aripiprazole induced AMPK phosphorylation and sensitized MCF-7 breast cancer cells to radiation therapy (53). Future mechanistical studies are needed to elucidate the role of DRD2, CaMKK2 and AMPK in mediating the effects of DHECS in chemoresistant PCa cells.

NF-κB plays essential roles in diverse biological processes, including inflammation and cancer (56). Activation of NF-κB signaling pathway promotes PCa progression and significantly correlates with advanced diseases (72). It has been shown that DRD2 activation suppresses nuclear translocation of NF-κB in the intracerebral hemorrhage mouse model, and DRD2 depletion retards non-small cell lung cancer progression through the inhibition of NF-κB (54, 55). NF-κB transcriptionally activates the expression of multiple oncogenic factors, including anti-apoptotic proteins survivin and Mcl-1 (73, 74). In chemoresistant PCa cells, DHECS treatment significantly inhibited NF-κB phosphorylation, an indicator of NF-κB-dependent transcriptional activity, which may contribute to the suppression of survivin and Mcl-1 and increased cell death.

AR plays a fundamental role in tumorigenesis and progression of PCa (75). Increased expression of AR and its variants, such as AR-V7, has been associated with therapeutic resistance and poor prognosis in patients receiving androgen-

deprivation therapy (ADT) (76, 77). Loss of AR expression in PCa cells, possibly mediated by neuroendocrine differentiation, also promotes the progression to castration resistance (78, 79). Although DHECS did not significantly affect AR expression in PCa cells (Figure 4B), it is interesting to notice that DHECS had lower IC_{50} values in CWR22RV1 (AR- and AR-V7-positive, ADT-resistant) and PC-3 (AR-negative, ADT-resistant, neuroendocrine) cells than in LNCaP, C4-2 and C4-2B cells (expressing full-length AR). Considering DHECS also exhibited a high potency against chemoresistant C4-2B-TaxR cells, it is plausible to propose that DHECS could be an excellent drug candidate to specifically treat advanced PCa, which is usually associated with castration-resistant and neuroendocrine phenotypes.

This study demonstrated that DHECS has excellent cytotoxicity in established PCa cells and intriguingly, more selectively targets PCa cells with aggressive phenotypes. Mechanistic studies indicated that DHECS may exert its inhibitory effect in chemoresistant PCa cells by affecting multiple signaling factors implicated in PCa progression. These results provide the first preclinical evidence supporting the hypothesis that DHECS is a novel inhibitor of aggressive PCa. Further preclinical and clinical investigation could allow the repositioning of DHECS as a safe and effective treatment for advanced PCa to overcome therapeutic resistance and improve clinical outcomes in PCa patients.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this study.

Authors' Contributions

LB and DW conceived and designed the project, LB, XL, XM and RZ acquired the data, LB and XL analyzed and interpreted the data, XL and DW wrote the paper.

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