Cordycepin resensitizes T24R2, cisplatin-resistant human bladder cancer cell, to cisplatin by inhibiting the AKT-mediated Ets-1 activation

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Running head: Cordycepin-mediated resensitization of T24R2 bladder cancer cell to cisplatin

Abstract

Resistance of tumor cells to anticancer drugs is a major obstacle in tumor therapy. In this study, we investigated the mechanism of cordycepin-mediated resensitization to cisplatin inT24R2, a derived T24 cell line. Treatment with cordycepin or cisplatin (2 μg/ml) alone could not induce cell death of T24R2, but combination treatment of these drugs significantly induced apoptosis of the cells through mitochondrial pathway including depolarization of mitochondrial membrane, decrease of anti-apoptotic proteins, Bcl-2, Bcl-xL, and Mcl-1, and increase of pro-apoptotic proteins, Bak and Bax. . High expression of MDR1 was the cause of cisplatin resistance in T24R2, and cordycepin significantly reduced MDR1 expression through inhibition of MDR1 promoter activity. MDR1 promoter activity was dependent on a transcription factor, Ets-1, in T24R2 cells. Although there is a correlation between MDR1 and Ets-1 expression in bladder cancer patients, active Ets-1, Thr-38 phosphorylated form (pThr-38), was critical to induce MDR1 expression. Cordycepin decreased pThr-38 Ets-1 level through inhibition of AKT, which reduced MDR1 transcription and induced the resensitization of T24R2 to cisplatin. The results suggest that cordycepin effectively resensitizes cisplatin-resistant bladder cancer cells to cisplatin, thus serving as a potential strategy for treatment of anti-cancer drug resistant patients.

Keywords: Cordycepin, Cisplatin-resistance, Resensitization, MDR1, Ets-1, AKT

1. Introduction

Although chemotherapy is the most effective treatment, the patient's clinical response to this treatment varies widely, and survival is often unsatisfactory. The resistance to the anti-cancer drugs often hampers the therapeutic effect of chemotherapy. Conventional anti-cancer drugs actually have different targets and action mechanisms, and most of them kill most cancer cells by inducing apoptosis. However, due to the imbalanced regulation of the apoptotic machinery, a small subset of cancer cells can acquire resistance to anti-cancer drugs [1].

Resistance to anti-cancer drugs is conferred on a multitude of mechanisms, including the decreased drug influx [2], the increased drug efflux [3], the activation of DNA repair mechanism [4], among others. The most studied mechanism for drug resistance in tumor cells is an increased efflux of anti-cancer drugs. This mechanism is very effective in preserving intracellular drug concentrations below the apoptosis-triggering threshold by using a variety of ATP-dependent active drug transporters, such as multi-drug resistant protein 1 (MDR1), breast cancer resistant protein (BCRP), and multi-drug resistance-related protein (MRP1) [5,6]. These active transporters in cellular membranes play an important role in not only recognizing anti-cancer drugs but also detoxifying intracellular compartments by pumping these drugs out of intracellular compartments. Novel pharmacologic approaches to overcome the drug resistance in cancer have been established in recent decades. For instance, targeting active drug transporters such as MDR1 can resensitize drug-resistant tumor cells to anti-cancer drugs [7,8].

Cordycepin (3'-deoxyadenosine) is a major bioactive substance extracted from a mushroom, *Cordyceps militaris*, that is traditionally used in the oriental medicine [9,10]. Cordycepin exhibits anti-tumor activities, including anti-angiogenic, anti-metastatic, anti-proliferative, or pro-apoptotic activity in cancer cells [11-13].

In this study, we investigated the mechanism of cordycepin-mediated resensitization to cisplatin in

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T24R2, a cisplatin-resistant derivative cell line of T24 human bladder cancer cell line [14], suggesting that cordycepin may be developed as a candidate of drug combinations in patients with cisplatin resistance.

2. Results

2.1. Cordycepin resensitize T24R2 to cisplatin

The MTT assay was used to confirm the resistance of T24R2 cells to cisplatin. Cell viability was quantified 24 h after the cisplatin treatment of T24 and T24R2 at the concentrations of 1μg/mL and 2μg/mL. As shown in figure 1A, although cell death of T24 cells was increased in a concentration-dependent manner, no significant effect has been observed in T24R2 cells, which displayed clear resistant to cisplatin. To investigate the effect of cordycepin on T24R2, we treated T24R2 cells with various concentration of cordycepin only or mixture of cordycepin and cisplatin, and measured the cell viability using MTT assay (Figure 1B). Whereas cordycepin-induced cytotoxicity in T24R2 was slightly increased at a high dose of cordycepin (50μg/mL), the combined treatment of cordycepin and cisplatin significantly induced cell death starting at 20μg/mL of cordycepin. Cytotoxicity caused by apoptosis was confirmed by sub-$G_{1/0}$ (Figure 1C) and TUNEL assay (Figure 1D). These data suggest that cordycepin resensitizes T24R2 to cisplatin.

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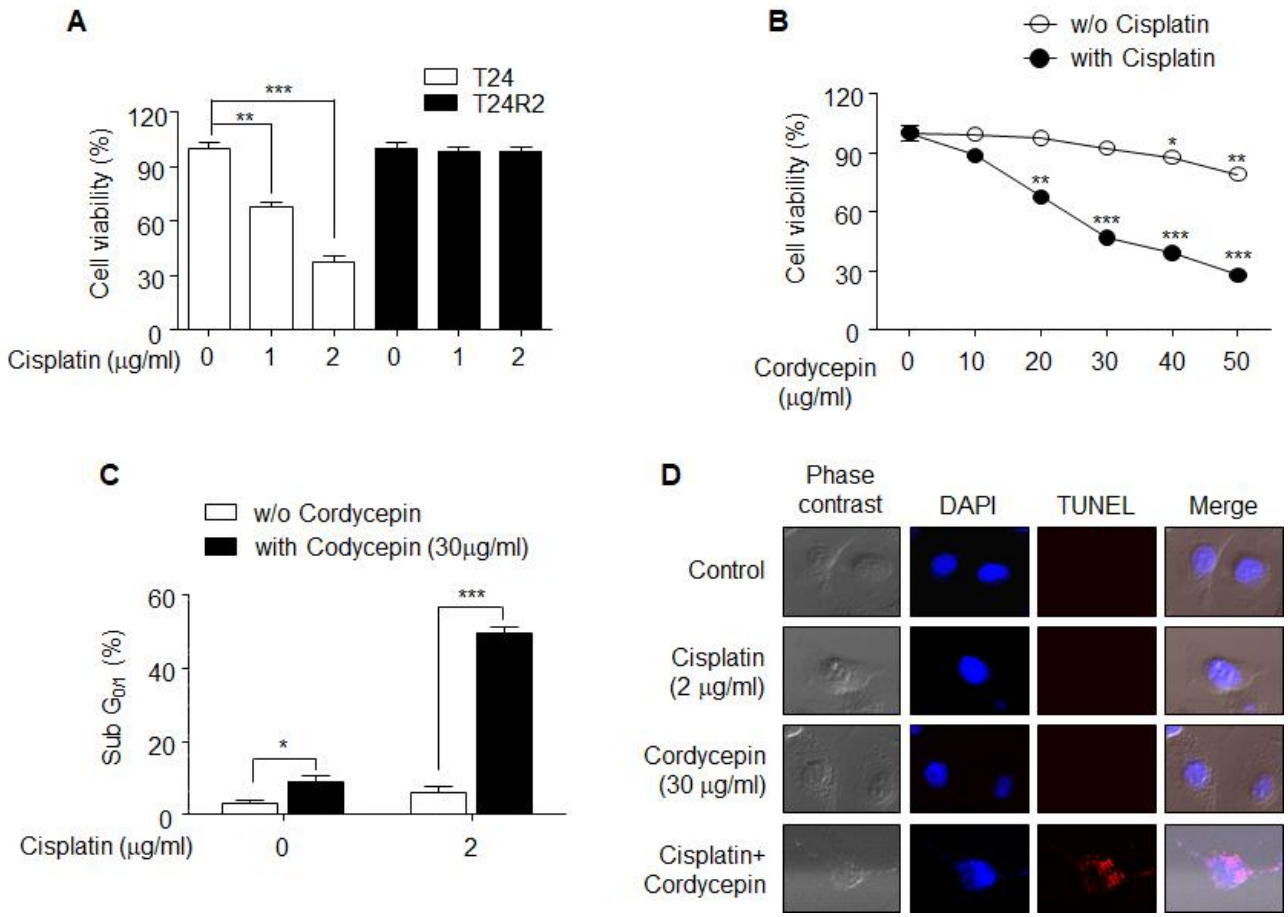

Figure 1. Effect on sensitivity of T24R2 to cisplatin by treatment of cordycepin or/and cisplatin

(A) T24 and T24R2 cells were incubated for 24h with different concentrations of cisplatin (0, 1, 2μg/ml). Cell viability was determined by MTT assay. (B and C) Cordycepin-mediated resensitization of T24R2 to cisplatin. T24R2 cells were treated with cordycepin in presence or absence of cisplatin and their viability was measured using the MTT assay (B). The determination of sub-$G_{1/0}$ using propidium iodide staining (C). (D) Apoptosis of T24R2 induced by co-treatment of 20 μg/ml cordycepin and 2 μg/ml cisplatin was analyzed by TUNEL and DAPI staining. $^{*}P < 0.05$, $^{**}P < 0.01$. $^{***}P < 0.001$ by *t*-test.

2.2. Cordycepin-mediated resensitization of T24R2 to cisplatin is induced by apoptosis via the mitochondrial pathway

To investigate the mechanism of cordycepin-mediated resensitization of T24R2 to cisplatin, we assessed the protein expression levels involved in caspase pathway activation. In brief, T24R2 cells were treated with cisplatin (2μg/mL) and cordycepin (20μg/mL) for 24h, and subsequently the levels of caspase-3, -9 and PARP were evaluated by western blotting. Interestingly, the combined treatment of cisplatin and cordycepin induced cleavage of caspase-3, -9, and PARP (Figure 2A). As shown in Figure 2B, whereas the anti-apoptotic proteins, Mcl-1, Bcl-2, and Bcl-xl were significantly reduced, the pro-apoptotic proteins, Bax and Bak, were considerably increased in T24R2 cotreated with cordycepin and cisplatin. Moreover, to confirm that the combination of cordycepin and cisplatin activated the caspase cascade in T24R2 cells via the mitochondrial pathway, we examined active Bax redistribution. The combined treatment induced active Bax translocation to the mitochondrial membrane as shown in Figure 2C. In addition, the effect of the combined treatment induced a significant loss of the mitochondrial potential, which is considered as a hallmark of apoptosis (Figure 2D). These data clearly demonstrate that cordycepin combined with cisplatin induces apoptosis in T24R2 cells via the mitochondrial pathway-dependent caspase activation cascade.

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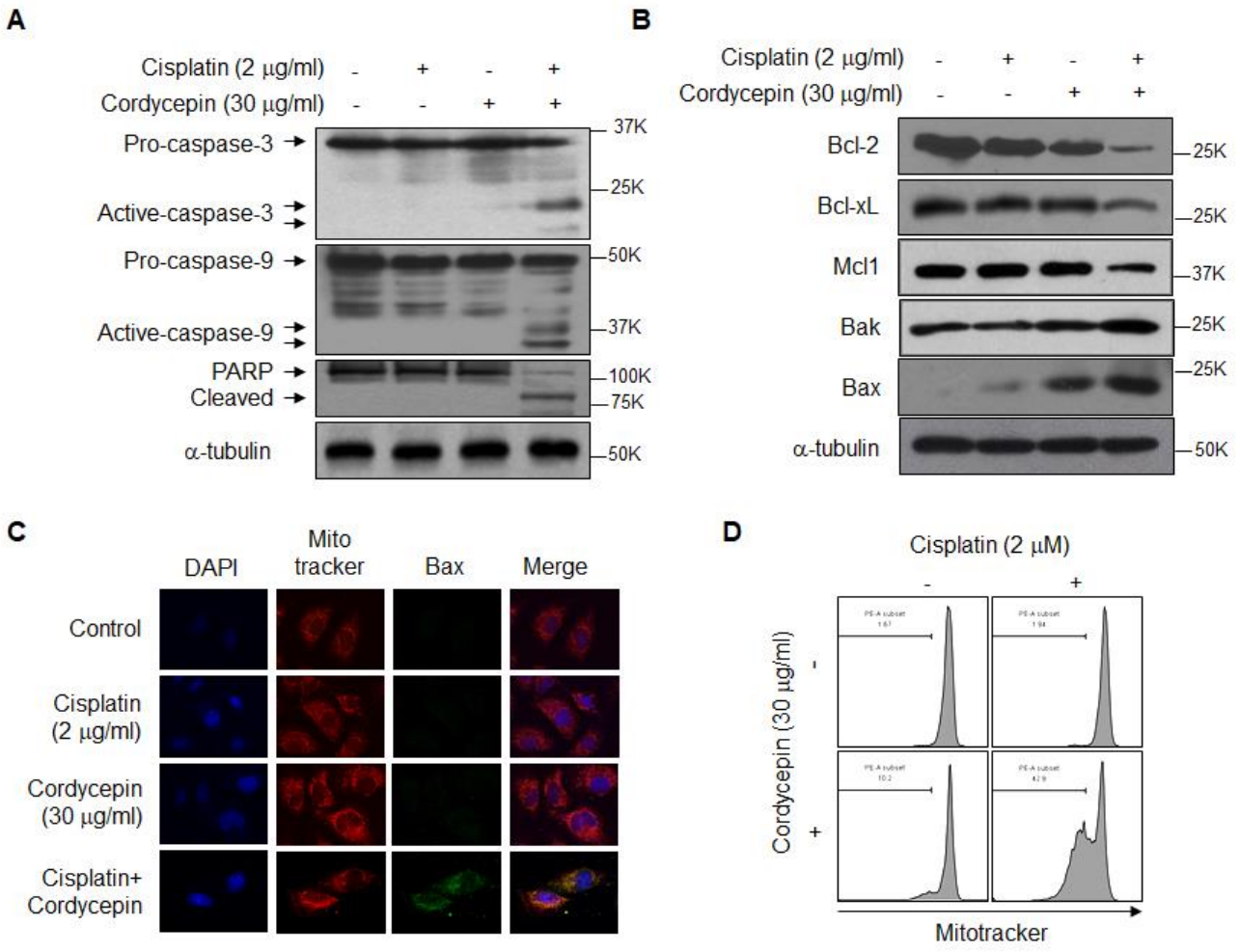

Figure 2. Induction of cordycepin-mediated apoptosis via mitochondrial pathway in cisplatin-treated T24R2.

T24R2 cells were treated with 20 μg/mL cordycepin and/or 2 μg/mL cisplatin for 24h, and then the whole cell extracts were analyzed by western blotting using the each indicated antibody against caspase-3, -9, PARP cleavage (A), anti-apoptotic, and pro-apoptotic proteins (B). α-tubulin was used as an internal control to confirm equal loading of proteins. T24R2 cells treated with cisplatin and/or cordycepin were immunostained with anti-active Bax and analyzed using a confocal microscope. (C) Active Bax was labeled with a FITC-conjugated secondary antibody (green), and the mitochondria and nuclei were stained with Mitotracker CMXRos (red) and DAPI (blue), respectively. (D) The cells were stained with MitoTracker Red CMXRos, and the depolarization of mitochondria was analyzed

by flow cytometry.

2.3. Reduction of MDR1 expression is involved in cordycepin-mediated resenstization of T24R2

MDR1 expression was more increased in T24R2 compared with T24 (Figure 3A) and was down-regulated by cordycepin at the transcriptional level (Figure 3B and C). Moreover, to confirm that MDR1 is directly associated with resistance of T24R2 cells to cisplatin, we proceeded to knockdown MDR1 in T24R2. After treatment of 2μg/mL cisplatin for 24h, siMDR1-transfected T24R2 showed very higher cell death rate compared with siGFP-transfected T24R2 (Figure 3D), reduction of anti-apoptotic proteins, Bcl-2 and Mcl-1, and induction of pro-apoptotic proteins, Bax and Bak (Figure 3E). To confirm whether cordycepin could inhibit the drug efflux mediated through decrease of MDR1 expression, we assayed the intracellular level of rhodamine in T24, T24R2, cordycepin-treated T24R2, and siMDR1 T24R2. T24R2 cells exhibited very low level of cytosolic rhodamine accumulation, whereas T24 cells contained high levels of rhodamine. As expected, cordycepin-treated T24R2 and siMDR1 T24R2 contained high level of rhodamine (Figure 3F). Taken together, these suggest that cordycepin-mediated MDR1 reduction is a major mechanism inducing resensitization of T24R2 to cisplatin.

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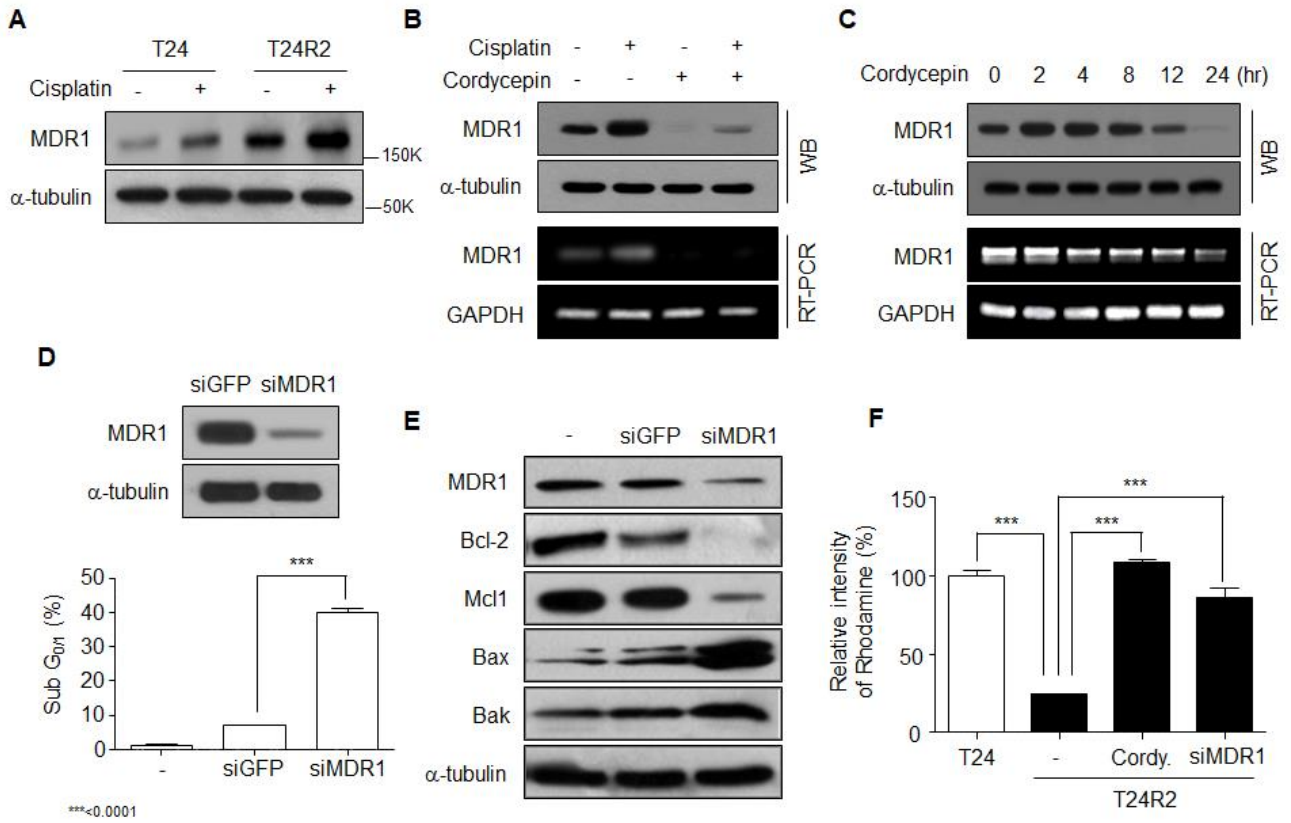

Figure 3. Cordycepin-mediated downregulation of MDR1 associated with the resistance of T24R2 to cisplatin

(A) Expression of multi-drug resistance protein 1 (MDR1) in T24 and T24R2 cells. (B) MDR1 mRNA and protein levels in T24R2 treated with cordycepin and/or cisplatin. (C) MDR1 expression levels were analyzed after cordycepin-treatment for the indicated times. (D) T24R2 cells were transduced with a control siGFP or siMDR1 for knockdown of MDR1 expression. A representative flow cytometric analysis on DNA content in T24R2 cells treated with cisplatin under MDR1 knockdown (siRNA MDR1). (E) After transfection with MDR1 siRNA, the T24R2 cells were treated for 24h with 2 μg/ml of cisplatin. Anti-apoptotic proteins (Mcl-1 and Bcl-2) and pro-apoptotic proteins (Bax and Bak) were assessed by western blotting. (F) Cells were transduced with siMDR1, and then treated with cordycepin (20 μg/mL) for 24h. The cells were incubated for 1h in the presence of Rhodamine123. The amount of intracellular Rhodamine 123 was analyzed using flow cytometry and determined as the MFI (Mean Fluorescence

Intensity). $^{***}P < 0.001$ by *t*-test.

2.4. Cordycepin regulates MDR1 promoter activity

Because the level of MDR1 mRNA was significantly decreased in T24R2 cells treated with cordycepin (Figure 3B), we investigated whether cordycepin suppressed MDR1 expression by down-regulating MDR1 promoter activity. Using the dual luciferase reporter gene assay system, MDR1 promoter (from -751 to +122 bp) activity was compared in T24 and T24R2 cells. As shown in figure 4A, T24R2 cells showed a higher luciferase activity than T24 cells. Furthermore, cordycepin treatment reduced the promoter activity in T24R2 to the same level as in T24 (Figure 4B). A previous report suggested that Ets-1 activated the human MDR1 promoter in the human osteosarcoma, Saos-2 [15]. To investigate whether Ets-1 is necessary for MDR1 expression in T24R2 cells, we constructed Ets1 mt-1, Ets-1 mt-2, and Ets-1 mt-1&2 with mutations in the Ets-1 binding sequence of the MDR1 promoter, as shown in Figure 4C. Both Ets-1 binding sequences are necessary for MDR1 promoter activation in T24R2. To confirm whether Ets-1 binded to MDR1 promoter in T24R2 and whether the binding inhibited by cordycepin treatment, we performed ChiP assay with an anti-Ets-1 antibody. Ets-1 did not bind to the promoter in T24, whereas Ets-1 bound directly to the MDR1 promoter in T24R2. Cordycepin effectively inhibited the binding of Ets-1 on MDR1 promoter (Figure 4D). Therefore, we suggest that cordycepin downregulates MDR1 expression via inhibition of transcription factor Ets-1 activity, which makes T24R2 sensitize to cisplatin.

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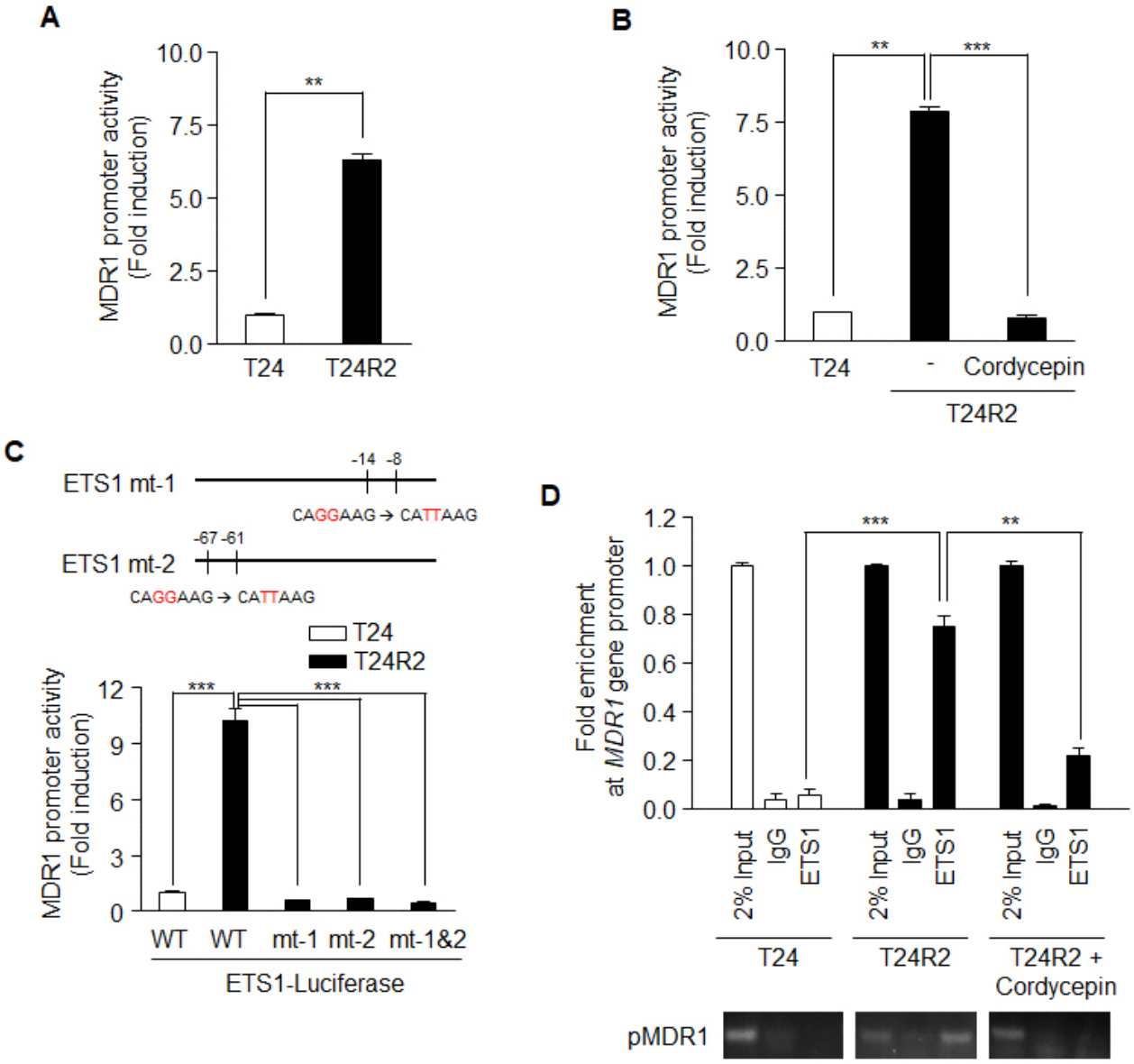

Figure 4. Ets-1 activity on MDR1 promoter is attenuated by cordycepin treatment in T24R2

T24 and T24R2 cells were transiently transfected with pGL3-MDR1 luciferase vector which contained the 5`-promoter region of the MDR1 gene (873 bps (from -751 to +122)), and then luciferase activity was determined from cell lysates treated without (A) or with cordycepin (B). (C) Site-directed mutagenesis was performed at Ets-1 biding sites (mt-1, mt-2, or mt-1&2). pGL3 containing wild type of MDR1 promoter (WT) or one of the site mutated promoters was transfected into T24 or T24R2, and luciferase activity was determined and normalized. All values were normalized to the renilla luciferase

activity. (D) Using genomic DNA from T24, T24R2, and cordycepin-treated T24R2 cells, we performed chromatin immunoprecipitation (ChIP)-PCR. $^{**}P < 0.01$. $^{***}P < 0.001$ by *t*-test.

*2.5. Cordycepin inhibits AKT activation that phosphorylates Ets-***1**

To determine whether a correlation exists between the expression of Ets-1 and that of MDR1, the cBioPortal database for cancer genomics (http://www.cbioportal.org) was utilized in January 2018. When the correlation was analyzed with the mRNA levels of Ets-1 and MDR1 from 413 bladder cancer patients, there was a positive correlation between mRNA expression levels of two genes (Figure 5A). Although cordycepin reduced MDR1 expression in transcription level (Figure 3B and C), transcript level and protein level of Ets-1 were unaffected by cordycepin (Figure 5B). These data suggested that cordycepin might reduce MDR1 expression through inhibiting Ets-1 activity but not its expression. Accordingly, we investigated the post-translational modification of Ets-1 affected by cordycepin. The phosphorylation of Ets-1 (T308) was known to induce its activation as a transcription factor [16]. As shown in Figure 5C, the phosphorylation of Ets-1 was attenuated in cordycepin-treated T24R2, which was followed by a reduction of MDR1 expression. In particular, the activation of AKT was significantly inhibited in cordycepin-treated T24R2 (Figure 5D), and inhibition of AKT using wortmannin, a specific inhibitor of PI3K, also attenuated Ets-1 phosphorylation and reduced MDR1 expression as observed in cordycepin-treated T24R2 (Figure 5E). To confirm whether AKT inhibition conferred the improved sensitivity to cisplatin in T24R2, these cells were treated with cordycepin or wortmannin in the presence or absence of cisplatin. Wortmannin treatment induced resensitization of T24R2 to cisplatin similar to cordycepin treatment (Figure 5F). Taken together, inhibition of AKT activation by cordycepin attenuates Ets-1 activity, which leads to resensitization of T24R2 to cisplatin through the reduction of pEts-1.

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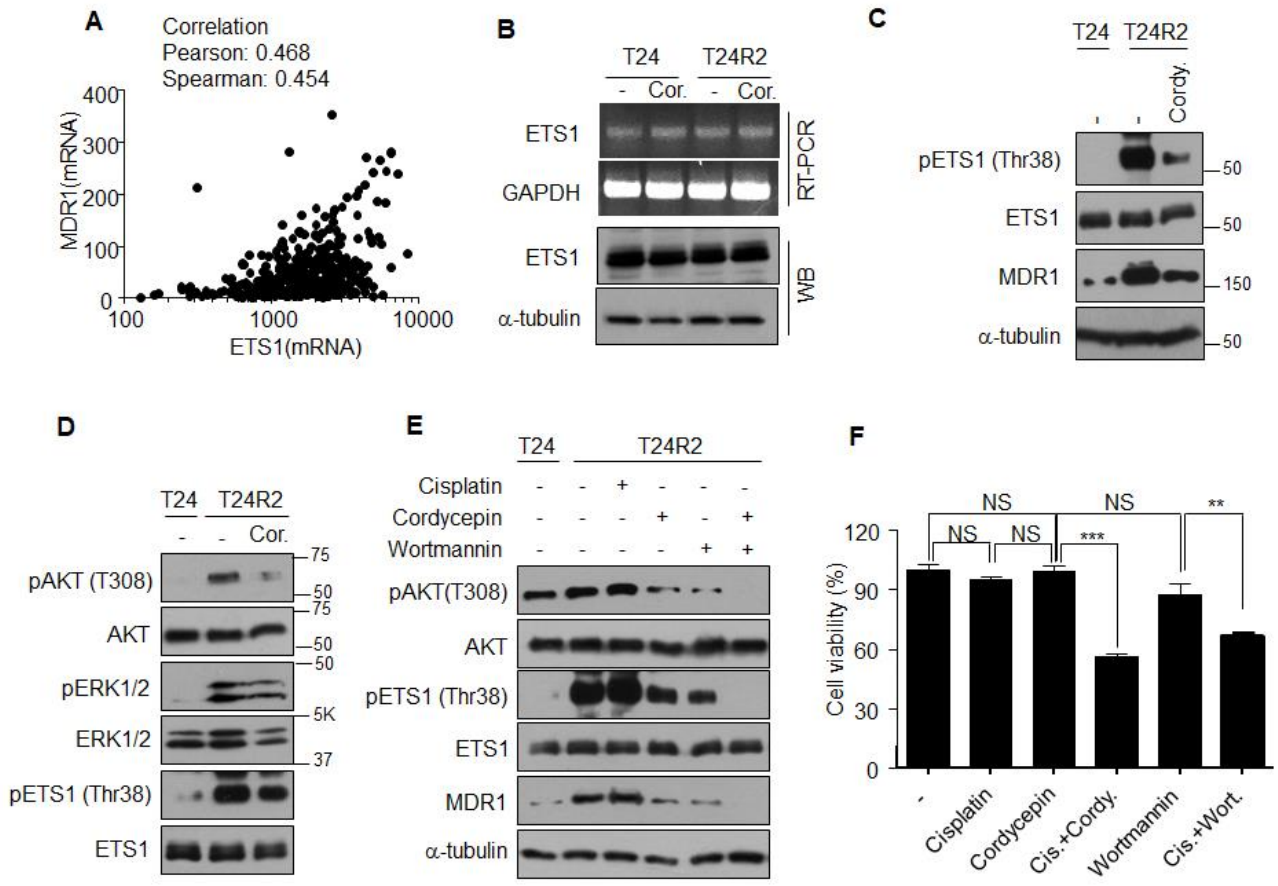

Figure 5. Cordycepin reduces Ets-1 phosphorylation through inhibition of AKT, followed by reduction of MDR1 expression.

(A) Correlation between Ets-1 and MDR1 mRNA expression levels was determined using the cBioPortal (http://www.cbioportal.org). Pearson correlation = 0.468, Spearman correlation = 0.454. (B) T24 or T24R2 was treated with cordycepin for 24 h, and Ets-1 mRNA or protein level was determined. (C) Cordycepin-mediated reduction of Ets-1 phosphorylation. Whole lysates from the indicated experimental groups were analyzed by western blotting using the indicated antibodies. (D) Cordycepin inhibits active phosphorylation of AKT. (E) T24R2 was treated with cordycepin and/or wortmannin, and lysates were analyzed by western blotting to determine correlation among active phosphorylation of AKT(T308), Ets-1 phosphorylation (Thr38), and MDR1 expression. (F) T24R2 was treated with the indicated agent for 24h, and the cell viability was determined by PI staining and

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flow cytometry analysis. $^{***}P < 0.001$ by *t*-test.

3. Discussion

Although chemotherapy is one of the representative and effective methods of tumor therapy, the acquisition of drug resistance in tumor cells is one of the major obstacles for improvement of therapy efficacy. Therefore, the development of strategies to overcome anti-cancer drug resistance should be considered as an important field in chemotherapy. One of these strategies is to combine two or more drugs to the patient, and the establishment of combination therapy strategies and drug selection are considered very important.

Cordyceps militaris is a fungus used as a natural tonic agent in oriental traditional medicine, and is reported to have many biological functions, including anti-inflammation [17], immune modulation [18,19], anti-angiogenesis [11], and having anti-tumor properties [12,13]. Cordycepin, also known as 3'-deoxyadenosine, is known to be a primary bioactive component of C. *militaris*. The tumor-suppressive effect of cordycepin has been reported in various carcinoma cells [20-22].

In this study, we found that cordycepin attenuated Ets-1 activity through inhibition of AKT, which played an important role in increasing the sensitivity of cells to cisplatin by reducing MDR1 promoter activity. To investigate the effect of cordycepin on the cisplatin-resensitization in cisplatin-resistant tumor cells, we used the T24R2 cell line, which was established by stepwise increments of exposure to cisplatin (Figure 1) [14]. Whereas the cytotoxicity of cordycepin to T24R2 was shown to be mild in 50 μg/ml cordycepin-treated group, the group cotreated with cordycepin and cisplatin showed significantly high cytotoxicity starting at 20 μg/ml cordycepin. The cytotoxicity induced by the cotreatment with cordycepin and cisplatin was induced by caspase activation through loss of mitochondrial outer membrane potential, which was induced through a decrease of anti-apoptotic proteins and the increase of pro-apoptotic proteins. MDR-1, a P-glycoprotein, is a well-known ATP-

binding cassette (ABC) transporter, which is an integral plasma membrane protein [23]. MDR-1 reduces the efficacy of chemotherapy by shortening the retention time of intracellular drugs. In fact, high expression of MDR-1 induces drug resistance in many tumors. Thus the inhibition of MDR-1 is a potential strategy for resensitizing anti-cancer drug resistant cancer cells to the drug [24]. T24R2 cell line showed higher expression of MDR1 compared with T24, and cordycepin inhibited MDR1 expression at the transcriptional level (Figure 3). The inhibition of MDR1 expression using siMDR1 eliminated the resistance of T24R2 to cisplatin and prolonged the retention of intracellular drug as in the case of cordycepin-treated T24R2 cells. Therefore, cordycepin might resensitize T24R2 cells to cisplatin via reduction of MDR1 expression. MDR1 promoter activity was actually higher in T24R2 compared to T24, and the activity was decreased in cordycepin-treated T24R2. Ets-1 is an essential transcription factor regulating the expression of MDR1 at the transcriptional level and promotes anti-cancer drug resistance in various tumors [15,25]. The activity of Ets-1 was much higher in T24R2 than in T24, and the was eliminated by mutation of the Ets-1 binding sites on the MDR1 promote activity in T24R2. In addition, the binding of Ets-1 to the MDR1 promoter disappeared in cordycepin-treated T24R2. Therefore, cordycepin inhibits MDR1 expression via inhibition of Ets-1 activity.

Various growth factors, such as hepatocyte growth factor, basic fibroblast growth factor, and vascular endothelial growth factor, increase the mRNA level of Ets-1 through the Ras/Raf/MEK/ERK1/2 pathway. MDR1 expression showed a quantitative linear correlation with Ets-1 expression at transcript level in human bladder cancer patients analysis using cBioPortal for cancer genomics (http://www.cbioportal.org). Although T24 and T24R2 showed similar Ets-1 expression levels of mRNA and protein, MDR1 expression was higher in T24R2 than in T24. Therefore, cordycepin-mediated reduction of MDR1 expression should not be dependent on Ets-1 expression. Various post-translational modifications, such as phosphorylation, ubiquitination, sumoylation, acetylation, and parylation, are known to regulate Ets-1 activity [26]. Among the modifications of Ets-1, Thr-38

phosphorylation of Est-1 by ERK1/2 was well defined [27-29]. Strangely, no significant decrease in ERK activation was observed in T24R2 treated with cordycepin, while a significant inhibition in PI3K-mediated AKT activation was observed. PI3K/AKT inhibition using wortmannin significantly inhibited the phosphorylation of Ets-1 and MDR1 expression in T24R2 (Figure 5E), and also resensitized T24R2 to cisplatin (Figure 5F). Although it was known that AKT can affect Ets-1 expression levels [30], cordycepin treatment did not affect total Ets-1 expression levels.

These results suggest that cordycepin inhibits MDR1 expression and resensitizes T24R2 to cisplatin through inhibition of PI3K/AKT pathway. Active PI3K/AKT pathway induces transcriptional upregulation of MDR1 through activation of Ets-1. Therefore, cordycepin may be a candidate for combination therapy with cisplatin in the treatment of patients exhibiting cisplatin resistance.

4. Material and Methods

Cell lines and Cultures

T24 cell line was purchased from the American Type Culture Collection (VA, USA). T24R2, a cisplatin-resistant derivative cell line of T24, was established through serial desensitization of T24 cells and showed resistance to 2 μg/ml cisplatin [14]. The cells were cultured as monolayers in Dulbecco's Modified Eagle's medium (Sigma, MO, USA) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (Sigma Aldrich, MO, USA) and 1 % penicillin/streptomycin (Lonza, Basel, Switzerland) and maintained at 37 ℃ in a humidified chamber with 5 % CO_2.

Antibodies and reagents

Cordycepin purified from *Cordyceps militaris* was purchased from Sigma, and cisplatin was purchased from ILDONG Pharmaceutical (Korea). Anti-Bcl-2, anti-Bax, anti-Bak, anti-MDR1, anti-Mcl-1, anti-Caspase 3, anti-Caspase 9, anti-cleaved poly ADP ribose polymerase (PARP), anti-phospho-

AKT(Thr308), and anti-AKT antibodies were purchased from Cell Signaling Technology (MA, USA). Anti-α-tubulin, anti-Bcl-xl, and anti-Ets-1 (C-4) antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-pEts-1 (T38) antibody was purchased from Invitrogen (CA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit-IgG, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), propidium iodide (PI), cordycepin, and 4′, 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich.

MTT assay

An MTT assay was performed on the T24 and T24R2 cells to determine cell viability. Cells were plated in 96-well plate (5×10^3 cells/well) and incubated for 24 h in the presence or absence of cisplatin and/or cordycepin. MTT solution (5 mg/mL) was added to the wells and then the cells were incubated for another 3 h. Following incubation, the medium was removed and 200 μl DMSO was added to each well to extract the formazan products produced by viable cells. The absorbance of the solutions was measured on a Bio-Rad 550 microplate reader at 595 nm. The relative cell viability (%) was determined by comparing the absorbance at 595 nm with control, which was treated with DMSO.

PI staining

The cells were fixed with 70 % ethanol and stored at -20 ℃ overnight. The cells were washed with PBS, treated with RNase A (Sigma, MO, USA), and then stained with PI (Sigma). The cells were then subjected to flow cytometric analysis using FACSVerse™ (BD Bioscience, NJ, USA) and the data was analyzed using the Flow Jo software (FLOW JO LLC, USA).

RNA isolation and real-time RT-PCR analysis

Total RNA was extracted from cells using the total RNA isolation solution (RiboEx™; GeneAll, Seoul,

Korea) according to the manufacturer protocol. RNA was quantified using the NanoDrop™ spectrophotometer (ThermoFisher Scientific Inc., TN, USA). cDNA was synthesized with oligo-dT primers and reverse transcriptase (Fermentas, St. Leon-Rot, Germany). Reverse transcription polymerase chain reaction (RT-PCR) was performed using a PCR detection kit (Solgent Co. LTD, Daejeon, Korea). The following primers were used for amplification: *GAPDH* 5′-CCATCACCATCTTCCAGGAG-3′, and 5′-ACAGTCTTCTGGGTGGCAGT-3′, *MDR1* 5′-GACACCACTGGAGGGTGACT-3′, and 5′-GGCGTTTGGAGTGGTAGAAA-3′, and *Ets-1* 5′-ACCCAGATGAGGTGGCCAGG-3′, and 5′-TCAGGGGTGTACCCCAGCAG-3′..

Immunoblotting analysis

Total proteins extracted from cells were quantified by using the Bradford method. Equal amounts of protein were separated on 6 %−15 % SDS-PAGE. After cold transfer onto a PVDF membrane, nonspecific binding sites were blocked for 1 h with 5 % skim milk (Difco Laboratories, Surrey, UK) in Tris-buffered saline containing 0.1 % Tween20. The antibodies were treated at 4°C for O/N, and then HRP-conjugated antibodies were treated at RT for 1h. The HRP signal was visualized using Clarity™ ECL Western Blotting Substrate (Bio-Rad, CA, USA).

Confocal microscopy

T24R2 cells were seeded and incubated overnight onto 25 mm round glass coverslips. Following induction of apoptosis by a co-treatment of cordycepin and cisplatin, the cells were fixed with 4 % paraformaldehyde. The fixed cells were treated with active-Bax antibody (1:50 in PBS) overnight at 4°C and then α-Mouse-FITC (1:100 in PBS) for 2 h at room temperature. Coverslips were mounted on glass slides with mounting solution, and fluorescence was visualized with an Olympus FluoView™ FV1000 Confocal Microscope (Olympus, Tokyo, Japan). For terminal deoxynucleotidyl transferase

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(TdT-mediated biotinylated UTP nick and labeling (TUNEL) analysis, T24R2 cells were treated with cisplatin (2 μg/ml), cordycepin (20 μg/ml) or a combination of them for 24 h. DNA fragmentation was determined by TUNEL staining kit (Promega, USA) according to the manufacturer's instructions.

Rhodamine123 uptake/retention assay

Rhodamine123 (Rh123) uptake/retention experiments were performed by adding 0.25 μM of Rh123 following cell harvest. After 1 h of incubation in the presence of Rh123, cells were washed with PBS two times. Cellular efflux of Rh123 was measured using flow cytometry (FACSVerse^{TM}).

Construction of luciferase reporter plasmid and dual luciferase assay

The 873 bp fragment (from -751 to +122) was excised from a pMDR1-1202 cloning recombinant vector purchased from Addgene (Cambridge, USA) and ligated into the equivalent site of a pGL3-basic vector (Promega, USA) to form pGL3-MDR1 construct. The site-directed mutagenesis was performed using the KOD-Plus-Mutagenesis kit (TOYOBO, Osaka, Japan) with the templates pGL3-MDR1. The primers used for the different mutation were, Ets-1 mt-1, 5′-TTAACAGCGCCGGGGCGTGGGCTGA-3′ and 5′- TGCCCAGCCAATCAGCCTCACCACA-3′ , and Ets-1 mt-2, 5′-TTAAGC CTGAGCTCATTCGAGTAGC-3′ and 5′-TGTGGCAAAGAGAGCGAAGCGGCTG-3′. T24R2 cells were grown at 37 ℃ in 5 % CO_2 and transfected with FuGENE^{TM} HD in 24-well plates with 7×10^4 cells. All cells were analyzed for dual-luciferase reporter gene expression 48 h after completion of the transfection procedure. The activities of firefly luciferase and renilla luciferase in the pGL3-MDR1 vector were determined following the dual luciferase reporter assay protocol recommended by Promega.

Chromatin immunoprecipitation (ChIP) assay

T24, T24R2, and cordycepin-treated T24R2 cells were fixed with 1 % formaldehyde solution. Chromatins was prepared from the fixed cells and fragmented by sonication. Immunoprecipitation of cross-linked protein/DNA was performed by using the Ets-1 (C-4) mouse monoclonal antibody. Briefly, 2 μg of the Ets-1 antibody was bound to chromatin mixture at 4 ℃ overnight with rotation, and then 30 μl of protein A magnetic beads (Merck Millipore) was added and additionally incubated at 4 ℃ overnight with rotation. Then real-time PCR analysis using precipitated DNA was performed. The following primers were used for amplification: MDR1 promoter, 5′-CGTTTCTCTACT TGCCCTTTCT-3′ and 5′-CCGGATTGACTGAATGCTGAT-3′.

Statistical analysis

The obtained data were expressed as the means ± SD from three experiments. Differences among means were analyzed using a one-way ANOVA or student's *t*-test. $P < 0.05$ was significantly different.

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Conflict of interest

The authors declare that there are no conflict of interests.

Author Contributions

K.K conceptualized the study, supervised experiments, supported the study and wrote paper. S.O. and K. L. helped design and performed experiments. H.M., J.J., S.P., M.K., Y.L., and H.H helped various experiments. C.H. and J.Y. shared reagents, gave variable input, and helped in editing the paper.

Disclosure statement

The authors have nothing to disclose.

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