Enhancement of cisplatin cytotoxicity by Retigeric acid B involves blocking DNA repair and activating DR5 in prostate cancer cells

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Abstract. Retigeric acid B (RAB), a natural compound isolated from lichen, has been demonstrated to inhibit cell growth and promote apoptosis in prostate cancer (PCa) cells. The present study evaluated the function of RAB combined with clinical chemotherapeutic drugs in PCa cell lines by MTT assay, reverse transcription quantitative polymerase chain reaction and western blot analysis, and identified that RAB at low doses produced significant synergistic cytotoxicity in combination with cisplatin (CDDP); however, no marked synergism between RAB and the other chemotherapeutics was observed. Additional studies revealed that RAB exerted an inhibitory effect on DNA damage repair pathways, including the nucleotide excision repair and mismatch repair pathways, which are involved in the sensitivity to CDDP-based chemotherapy, as suggested by the significantly downregulated expression of certain associated repair proteins. Notably, Excision repair cross-complementing 1, a critical gene in the nucleotide excision repair pathway, exhibited the most significant decrease. When combined with CDDP, RAB-mediated impairment of DNA repair resulted in prolonged DNA damage, as demonstrated by the long-lasting appearance of phosphorylation of histone H2AX at Ser139, which potentially enhanced the chemosensitivity to CDDP. Concurrently, the proapoptotic protein death receptor 5 (DR5) was activated by RAB, which also enhanced the chemotherapeutic response of CDDP. Knockdown of DR5 partially blocked RAB-CDDP synergism, suggesting the crucial involvement of DR5 in this event. The results of the present study identified that RAB functioned synergistically with CDDP to increase the efficacy of CDDP by inhibiting DNA damage repair and activating DR5, suggesting the mechanistic basis for the antitumor effect of RAB in combination with current chemotherapeutics.

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Introduction

Prostate cancer (PCa) is one of the most common malignant tumors in males worldwide, representing a global public health problem (1). The development of PCa in humans presents as a multistage process, proceeding from a localized, androgen-dependent disease to invasive and metastatic hormone-refractory PCa. Chemotherapy is one of the primary treatment methods used in patients with PCa (2,3). However, the therapeutic strategies for this disease are limited as chemotherapy and radiation therapy are largely ineffective due to cross-resistance and metastatic disease frequently develops, even following potentially curative surgery (2-4). Therefore, the development of novel therapeutic options is urgently required.

Cisplatin (CDDP), one of the most widely used chemotherapy drugs, also known as cisplatinum or cis-diamminedichloridoplatinum (II), is a member of the most widely-used group of platinum-containing anticancer drugs (5,6). Platinum complexes exert antitumor activities via the formation of covalent adducts with cellular DNA, resulting in DNA damage, which in turn triggers apoptosis (6,7). CDDP is one part of the treatment modalities used for a variety of solid tumors, including ovarian, testicular, esophageal, non-small cell lung, and head and neck cancer, as well as PCa (8-10). In addition, alone or in combination with other chemotherapy agents, platinum compounds have been examined in the aforementioned clinical trials in patients with advanced PCa. Their antitumor activity as monotherapy in randomly selected patients was mostly moderate, and certain combination therapies resulted in significant toxicity. For example, in cisplatin/5-fluorouracil treatment as a chemo-radiotherapy regimen for the treatment of locally advanced squamous cell carcinoma of the head and neck, high doses (cisplatin 15 mg/m^2 + 5-fluorouracil 750 mg/m^2 per day) resulted in significantly higher level of neutropenia and a trend towards higher rate of mucositis (11). Cisplatin in combination

with bleomycin and vinca alkaloids may provoke even more chest pain presentations compared with cisplatin alone, at an incidence as high as 40% (12,13). The concurrent chemotherapy of cisplatin (30 mg/m^2) and docetaxel (40 mg/m^2) and external radical radiotherapy for transitional cell bladder carcinoma caused severe early and late side effects including acute gastrointestinal toxicity, myelotoxicity, stomatitis, skin toxicity and nephrotoxicity (14). The combination of docetaxel and cisplatin with radiotherapy was also demonstrated to be associated with a higher incidence of side effects compared with single-agent cisplatin with radiotherapy in high-risk early-stage cervical cancer (15).

Clinical resistance to CDDP remains a major obstacle to increasing its cytotoxic effects. The capacity for DNA repair is a crucial molecular pathway implicated in resistance to platinum-based chemotherapy (16,17). As the cytotoxicity of platinum drugs is principally attributable to the formation of platinum-DNA adducts (6,16), nucleotide excision repair (NER) is the primary DNA repair mechanism for the removal of bulky DNA lesions caused by CDDP from genomic DNA. The core proteins required for NER are xeroderma pigmentosum group A (XPA), replication protein A (RPA), XPC-UV excision repair protein RAD23 homolog B (RAD23B), transcription factor II human (TFIIH), excision repair cross-complementing 1 (ERCC1)/DNA excision repair protein ERCC-4 (XPF), ERCC excision repair 5 and endonuclease (XPG) (16,18). The downregulation of ERCC1, a critical gene in the NER pathway (16,19), was identified to increase the sensitivity of cancer cells to platinum-based chemotherapy (16,19,20). It is highly conserved during evolution and constitutively expressed in all tissues at relatively high levels. The ERCC1/XPF heterodimer is a structure-specific endonuclease and its function in NER is to create the 5'-incision on the damaged strand (20). Functional ERCC1 is essential for survival; knockdown of the ERCC1 gene in mice was observed to lead to an accelerated-aging phenotype, with brain damage, liver failure and mortality occurring following weaning (21). It has also been demonstrated that the downregulation of ERCC1 sensitized PCa cells to CDDP, and excision repair of CDDP adducts in PCa cells was attenuated to a similar extent by ERCC1 downregulation (20), suggesting that ERCC1 is a potential therapeutic target to sensitize cancer cells to chemotherapy.

With the previous advances in the identification of the mechanisms regulating CDDP-induced apoptosis, the pleiotropic effects of CDDP on the cell may lead to the development of novel targeted therapies (22-24). Death receptor 5 (DR5) is a cell surface receptor for tumor necrosis factor-related apoptosis-inducing ligand, and triggers apoptosis via mitochondria-dependent and independent pathways (25). Previous studies have also demonstrated that DR5 upregulation sensitizes cells to the cytotoxic effects of CDDP (26-29), suggesting that combining CDDP treatment with an inducer of DR5 has the potential to sensitize cells, leading to synergy.

In light of the aforementioned results, the present study aimed to identify novel natural agents that may enhance sensitivity of PCa cells to chemotherapeutic drugs. Our previous studies demonstrated the marked antitumor activity of Retigeric acid B (RAB), a natural pentacyclic triterpenic acid isolated from the *Lobaria kurokawae* Yoshim, suggesting it to be a promising anticancer agent in PCa cells (30-33). In the present study, RAB was identified as an enhancer of CDDP-induced cytotoxicity. Combining RAB with CDDP resulted in a synergistic effect via the suppression of DNA repair and the activation of DR5 following the induction of DNA damage.

Materials and methods

Cell culture and treatments. Human PCa cell lines, PC3 and DU145 [American Type Culture Collection (ATCC), Manassas, VA, USA], were cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) and 100 units/ml penicillin-streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Non-neoplastic prostate epithelial RWPE-1 cells (ATCC) were used as controls. RWPE-1 cells were maintained in keratinocyte-SFM medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with bovine pituitary extract (Gibco; Thermo Fisher Scientific, Inc.) and epidermal growth factor (Gibco, Thermo Fisher Scientific, Inc.). All the cells were maintained in a humidified incubator with 5% CO_2 at 37˚C.

RAB was isolated from the lichen *L. kurokawae* Yoshim, and its purity and structure was determined as described previously (30). RAB was prepared in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 10 mM as stock solutions and stored at -20˚C to be diluted to final concentrations of 2, 4, 5, 6, 8 and 10 μM, according to experimental requirements. Various chemotherapeutic agents including CDDP (Qilu Pharmaceutical Co., Ltd., Jinan, Shandong, China), docetaxel (DTX; Qilu Pharmaceutical Co., Ltd.), etoposide (VP-16; Qilu Pharmaceutical Co., Ltd.), doxorubicin (ADM; Shenzhen Main Luck Pharmaceuticals Inc., Shenzhen, Guangdong, China), vincristin (VCR; Shenzhen Main Luck Pharmaceuticals Inc.) were used in combination with RAB as described subsequently.

Viability assay. The effects of the indicated drugs on the viability of the human cell lines were evaluated by MTT assay (Sigma-Aldrich; Merck KGaA). PC3, DU145 and RWPE-1 cells (1x10^4 per well) were seeded into 96-well plates for 24 h. Different treatments were as follows: PC3 and DU145 cells were treated with different concentrations of RAB (2, 4, 6, 8 and 10 μM) for 48 h at 37˚C; PC3, DU145 and RWPE-1 cells were simultaneously treated with 4 μM of RAB and chemotherapeutic agents including CDDP (2 μg/ml), ADM (300 nM), VP-16 (20 μM), DTX (10 nM) and VCR (10 nM) for 48 h at 37˚C; PC3 and Du145 cells were treated with different concentrations of RAB (2, 4, 6 and 8 μM) and a fixed concentration of CDDP (2 μg/ml) for 48 h at 37˚C, or treated with different concentrations of CDDP (1, 2, 3 and 4 μg/ml) and a fixed concentration of RAB (4 μM) for 48 h at 37˚C; PC3 cells were treated with 2 μg/ml CDDP alone or simultaneously with 4 μM RAB for 48 h at 37˚C following siRNA transfection. Then, the RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) was removed and the cells were incubated with 10 μl MTT for 4 h. Subsequently, the formazan crystals were dissolved using 0.05% (v/v) DMSO. The cell growth response was detected by measuring the light absorbance at 570 nm using a Multiskan™

microplate reader (Thermo Fisher Scientific, Inc.). The viability assay was performed in triplicate.

Apoptosis assay. Following treatment with RAB (4 μM) and CDDP (2 μg/ml) alone or in combination for 48 h at 37˚C, PC3 cells were digested and centrifugalized at 120 x g for 5 min at 4˚C. Following 2 washes with PBS, levels of apoptosis were analyzed using an Annexin V-fluorescein isothiocyanate/propidium iodide Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Quantification of fluorescence was determined by flow cytometry (FACSCalibur™; BD Biosciences), and the data were analyzed by WinMDI Software 2.8 (Purdue University Cytometry Laboratories, West Lafayette, IN, USA).

Western blot analysis. Different treatments were as follows: PC3 and DU145 cells were treated with different concentrations of RAB (4, 6, 8 and 10 μM) for 48 h at 37˚C; PC3 and DU145 cells were treated with different concentrations of CDDP (1, 2, 3 and 4 μg/ml) and a fixed concentration of RAB (4 μM) for 48 h at 37˚C; PC3 cells were treated with RAB (4 and 8 μM) for 24 and 48 h at 37˚C; PC3 cells were treated with 2 μg/ml CDDP alone or simultaneously with 4 μM RAB and for 12, 24 or 48 h at 37˚C; PC3 cells treated with 2 μg/ml CDDP alone or simultaneously with 4 μM RAB for 48 h at 37˚C following siRNA transfection. Then PC3 and DU145 cell lysates were prepared using radioimmunoprecipitation assay lysis buffer according to the manufacturer's protocol (Beyotime Biotechnology Institute of Biotechnology, Inc., Haimen, Jiangsu, China). Proteins were quantified using the BCA protein assay (Beyotime Biotechnology Institute of Biotechnology, Inc.). Samples containing equal amounts of protein (60 μg) from the lysates were separated by 8, 10 and 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% Difco™ Skim Milk (cat. no. 232100; BD Biosciences) in TBST buffer (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween-20, pH 8.0) for 1 h at room temperature prior to incubation with specific antibodies. Then, the membranes were incubated overnight at 4˚C with the specific antibodies against poly adenosine 5'-adenosine diphosphate ribose polymerase (PARP; cat. no. sc-7150; rabbit polyclonal antibody; dilution 1:2,000), ERCC1 (cat. no. sc-10785; rabbit polyclonal antibody; dilution 1:200) from Santa Cruz Biotechnology, Inc., CA, USA, phosphorylation of histone H2AX at Ser139 (γH2AX; cat. no. 9718, mouse monoclonal antibody; dilution 1:1,000) and DR5 (cat. no. 3696; rabbit polyclonal antibody; dilution 1:1,000) from Cell Signaling Technology, Inc. Danvers, MA, USA, followed by peroxidase-conjugated appropriate secondary antibodies [anti-mouse IgG (H+L) peroxidase-labeled polyclonal antibody (cat. no. 074-1806; dilution 1:5,000); anti-rabbit IgG (H+L) peroxidase-labeled polyclonal antibody; (cat. no. 074-1506; dilution 1:5,000); both were purchased from Seracare Life Sciences Inc., Milford, MA, USA] for 1 h at room temperature. Immunocomplexes were visualized using chemiluminescence (EMD Millipore), according to the manufacturer's protocol. Membranes were stripped and re-probed with GAPDH (cat. no. sc-47724; mouse monoclonal antibody; dilution 1:2,000; Santa Cruz Biotechnology) as a protein loading control. Protein levels were quantified using densitometry of X-ray films by ImageJ 1.6 (National Institutes of Health, Bethesda, Maryland, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. PC3 and DU145 cells were treated with 0, 4 and 8 μM RAB for 24 and 48 h, or treated with 4 μM RAB and 2 μg/ml CDDP alone or simultaneously for 0, 6, 12, 18, 24 and 48 h at 37˚C. Total RNA of PC3 and DU145 cells were extracted using an RNAiso plus kit (Takara Bio, Inc., Otsu, Honshu, Japan). For the RT-qPCR assays, cDNA was synthesized using a PrimeScript™ RT reagent kit (Takara Bio, Inc.). RT-qPCR was performed using the QuantiNova SYBR-Green PCR kit (Qiagen China Co., Ltd., Shanghai, China) and an ABI Prism 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR reaction conditions for all assays were as follows: 95˚C for 30 sec, followed by 40 cycles of amplification (95˚C for 5 sec, 58˚C for 30 sec and 72˚C for 30 sec). Changes in the mRNA levels of desired genes were normalized to the level of GAPDH and calculated using the $2^{-\Delta\Delta Cq}$ method (34). The heat map was generated by The R Project for Statistical Computing (R version 3.4.1, The University of Auckland, Auckland, New Zealand). The sequences of primers selected are summarized in Table I.

siRNA transfection. PC3 cells were plated into 6-well plates at 20-30% confluency, and 24 h later, knockdown of DR5 was performed by transiently transfecting small interfering RNA (siRNA) targeting DR5 (GenePharma Co., Ltd, Shanghai, China) using Invitrogen Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol (DR5 siRNA sequence, 5'-AUCAGCAUCGUGUACAAG GUGUCCC; scramble siRNA sequence, 5'-UUCUCCGAA CGUGUCACGUTT). The final concentration of the siRNA was 50 nM. After 48 h of transfection, cells were treated with combinations of RAB (4 μM) and CDDP (2 μg/ml) for an additional 48 h as previously described (33,35), and the effects of different treatments on the conditioned cells were evaluated by western blot and cell viability assay, as aforementioned.

Microscopy. Morphological changes of apoptosis were determined by staining PC3 cell nuclei with DAPI. Following treatment with RAB (4 μM) and CDDP (2 μg/ml) alone or in combination for 48 h at 37˚C, PC3 cells were fixed with 90% ethanol/5% acetic acid for 1 h at room temperature. Following 2 washes with PBS, cells were incubated with DAPI solution (1.5 mg/ml in PBS) for 30 min at room temperature. Images of DAPI fluorescence were captured using a fluorescence microscope (magnification, x200; Nikon Corporation, Tokyo, Japan).

Statistical analysis. The data are presented as the mean ± standard deviation of at least 3 independent experiments and analyzed by GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance of the mean difference between the control and treated groups was determined with two-tailed Student's t-tests. Multiple group comparisons were performed with a one-way analysis of variance, followed by

Table I. Primers used for reverse transcription quantitative polymerase chain reaction analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ERCC1	GGCGACGTAATTCCCGACTA	AGTTCTTCCCCAGGCTCTGC
XPA	GGTCTCTTGAAGTTTGGGGTAGTC	TTCCACACGCTGCTTCTTACTG
XPB	CTAACTGCCTACTCCTTGTATGC	TCCATAGCTGACAGTACACAACT
XPC	CTTCGGAGGGCGATGAAAC	TTGAGAGGTAGTAGGTGTCCAC
XPD	GGAAGACAGTATCCCTGTTGGC	CAATCTCTGGCACAGTTCTTGA
XPF	CCTCTTTCGCCAGAAAAACAAAC	TTTACTGCTACATGGAACCTTGG
XPG	GACTTAGCGTCCAGTGACTCC	GGCAGTTTTGATGGCTTGTCTTT
RAD23B	TTCCACACCTGCATCCATCAC	TCAGTTGCTGTTGGGCTAGTA
TFB5	AAGACATTGATGACACTCACGTC	GGGAAAAAGCATTTTGGTCCATT
TFIIH	GACCTTGTTGTGAGTCAAGTGA	CCTGCTTATGATTGGATGTGGAA
RPA1	CTCGGGAATGGGTTCTACTGT	CACTTGGACTGGTAAGGAGTGA
MSH2	AAGCCCAGGATGCCATTG	CATTTGACACGTGAGCAAAGC
MSH3	GTGGCAAAAGGATATAAGGTGGG	AAAGGGCAGTCAATTTCCGGG
MSH6	AGCTTAAAGGATCACGCCATC	AAGCACACAATAGGCTTTGCC
MLH1	GCAAACCCCTGTCCAGTCAG	CTGGGAGTTCAAGCATCTCCT
PMS1	CCTATTGATCGGAAGTCAGTCCA	CTACTAACTCCTTTACCGCAGTG
GAPDH	TGGTCACCAGGGCTGCTT	AGCTTCCCGTTCTCAGCCTT

Dunnett's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

In vitro cytotoxic evaluation of RAB combined with conventional chemotherapeutic agents. As an initial screening approach to assess the antitumor activities of RAB combined with various conventional chemotherapeutic agents against PCa cells, a cell viability assay was performed. The effect of RAB treatments (2, 4, 6, 8 and 10 μM) on cell viability and PARP cleavage was first determined using MTT and western blot analysis in 2 hormone-refractory PCa cell lines, PC3 and DU145. The results revealed that low-dose RAB did not significantly inhibit cell viability in PC3 (2-4 μM; P>0.05) or DU145 (2-6 μM, P>0.05) cells (Fig. 1A). Concurrently, apoptosis was markedly activated in the PC3 and DU145 cells upon treatment with high doses of RAB (>8 μM) for 48 h, as suggested by the levels of PARP cleavage (Fig. 1B).

Subsequently, an *in vitro* drug combination analysis was performed to investigate whether RAB sensitized PCa cells to chemotherapeutic agents. As demonstrated in Fig. 1C and D, PC3 and DU145 cells were simultaneously exposed to 4 μM RAB and distinct drugs, including CDDP (2 μg/ml), ADM (300 nM), VP-16 (20 μM), DTX (10 nM) and VCR (10 nM). RAB in combination with CDDP produced the greatest significant inhibitory effect on PC3 and DU145 cells compared with the other drug combinations, decreasing cell viability rate by 35-40% (P<0.05; Fig. 1C and D) compared with CDDP alone. RAB also significantly upregulated the sensitivity of PC3 cells to ADM, with a decrease in cell viability of 8-10% compared with single ADM treatment (P<0.05; Fig. 1C) in the PC3 cell line. In contrast, the cytotoxicity of all other chemotherapeutic agents was not significantly enhanced by RAB (P>0.05) in the DU145 cell line. Therefore, these results revealed the antitumor effects of low-dose RAB in combination with distinct chemotherapeutic agents, and suggested that RAB significantly increased the apoptotic ability of CDDP in PCa cells.

RAB demonstrates synergistic antitumor effects in combination with CDDP in PCa cells. Based on the aforementioned results, it was important to additionally elucidate the potential of RAB to enhance cellular sensitivity to CDDP in PCa cells. As demonstrated in Fig. 2A, RAB at doses ranging from 2-8 μM significantly enhanced the cytotoxicity of CDDP (2 μg/ml) in a dose-dependent manner, as suggested by the more pronounced inhibition of cell viability in comparison to single CDDP treatment (Fig. 2A). As PC3 cells were exposed simultaneously to 4 μM RAB and increasing concentrations of CDDP (1-4 μg/ml), a significant decrease in cell viability was also observed, with a decreased half maximal inhibitory concentration (IC_{50}) of CDDP from 4.5 to ~2 μg/ml (Fig. 2B). Similar results were obtained in PC3 and DU145 cells under the same experimental conditions (Fig. 2A and B). Measurement of apoptosis revealed an increased level of cleaved PARP in the combination treatments compared with the CDDP-alone group (Fig. 2C). Additionally, co-treatment with RAB (4 μM) and CDDP (2 μg/ml) caused an increased percentage of apoptotic cells, as determined by the flow cytometry assay (Fig. 2D). The sum of early and late apoptotic cell death induced by CDDP alone at 48 h was 14.0%; when used in combination with RAB the level of apoptotic cells reached 23.37% in PC3 cells. In addition, the percentage of necrotic cells increased from 0.39 to 1.56% in PC3 cells following the combined treatment. The nucleic morphological changes of apoptosis were determined by staining nuclear DNA with DAPI. Correspondingly, combined treatment of RAB with CDDP resulted in a marked increase in the number of apoptotic cells with condensed and fragmented DNA, by a more marked blue fluorescence compared with the non-apoptotic cells as

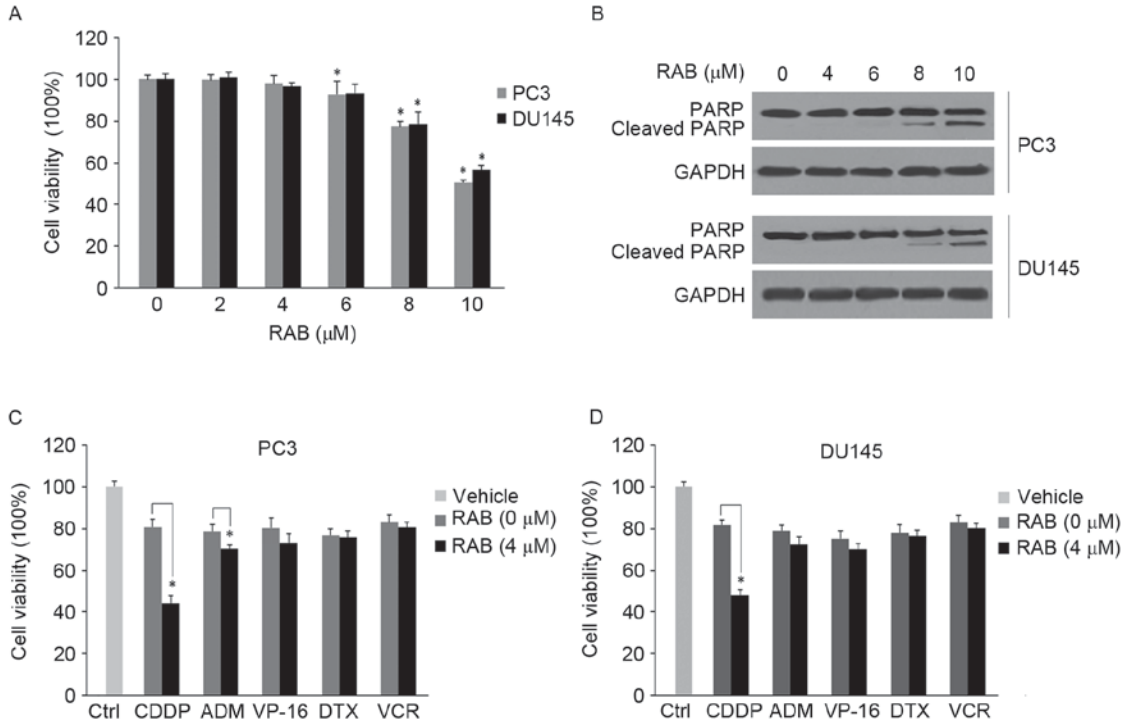

Figure 1. Combined effect of RAB and chemotherapeutic agents in PCa cells. (A) Anti-proliferative effect of RAB in PCa cell lines. PC3 and Du145 cells were treated with different concentrations of RAB and cell viability was measured by MTT assay. *P<0.05 vs. respective RAB-untreated control groups. (B) Western blot analysis of cleavage of PARP in response to RAB treatment. GAPDH served as an internal control. (C and D) Anti-proliferative activity of RAB combined with different chemotherapeutic agents; (C) PC3 and (D) Du145 cells were simultaneously treated with 4 μM of RAB and indicated chemotherapeutic agents including CDDP (2 μg/ml), ADM (300 nM), VP-16 (20 μM), DTX (10 nM) and VCR (10 nM) for 48 h at 37˚C. Cell viability was measured by a MTT assay. *P<0.05 vs. single treatment with different chemotherapeutic agents. PCa, prostate cancer; CDDP, cisplatin; ADM, doxorubicin; VP-16, etoposide; DTX, docetaxel; VCR, vincristine; PARP, poly adenosine 5'-adenosine diphosphate ribose polymerase; RAB, Retigeric acid B.

observed in the microscopic images (Fig. 2E). In contrast, the non-neoplastic prostate epithelial RWPE-1 cells, were resistant to the combination treatment compared with PCa cells (data not shown). Together, these results suggested that RAB synergistically increased CDDP-mediated cell growth inhibition and apoptosis.

RAB-induced DNA repair inhibition enhances chemotherapy sensitization of PCa cells to CDDP. As impairment of DNA repair may cause cell death following lethal DNA damage, and the NER pathway is particularly associated with the efficacy of CDDP (16,18), the present study focused on the effect of RAB on this process to reveal the RAB-induced sensitization mechanisms of CDDP cytotoxicity. RT-qPCR analysis identified the changes in the expression of genes associated with NER in response to RAB treatment (Fig. 3A). The expression of NER-associated genes including ERCC1, TFB5 and RPA1 in RAB-treated cells markedly decreased at 24 h treatment, and their expression levels decreased further at 48 h compared with at 24 h (Fig. 3A). The levels of XPB and XPD also decreased following RAB exposure, but in a moderate manner (Fig. 3A). In contrast, XPA, XPC, XPF, RAD23B and TFIIH expression remained unchanged or increased insignificantly following RAB treatment (Fig. 3A). Levels of XPG expression remained unchanged in PC3 cells, while they decreased over time in in a moderate manner in DU145 cells. The effect of RAB on the expression of NER-associated genes demonstrated a similar pattern in PC3 and DU145 cells, as indicated in Fig. 3A. In addition, RAB demonstrated an inhibitory effect on mismatch repair (MMR), another DNA repair pathway associated with sensitivity to CDDP (16), as indicated by the marked inhibition of the expression of MMR genes MSH2 and MSH6 following RAB treatment.

Notably, the expression of ERCC1 mRNA, a critical gene in the NER pathway, presented the most significant decrease among all the genes examined following treatment with RAB (Fig. 3A). RAB markedly downregulated the protein level of ERCC1 in a dose- and time-dependent manner (Fig. 3B); therefore, it was selected for additional combination study. As indicated in Fig. 3C and D, the expression of ERCC1 in response to co-treatment with RAB and CDDP was decreased in a time-dependent manner, particularly at the later time points (12-48 h, P<0.05) compared with CDDP treatment alone. Correspondingly, RAB markedly increased the level of γH2AX, an indicator of DNA damage elicited by CDDP (Fig. 3E). It was noted that the aggravated DNA damage caused by RAB became marked following 12 h co-treatment with the two drugs and persisted up to 48 h, suggesting that the RAB-evoked DNA repair blockade may serve a pivotal function in this event. Therefore, these data demonstrated that RAB was able to impair DNA repair gene expression, which may function together with the induction of lethal DNA damage to cause cell death when combined with CDDP.

DR5 overexpression mediated by RAB accelerates CDDP-induced apoptosis. Following the observation that RAB may also increase the mRNA level of DR5, according to the microarray data obtained from our previous study (24),

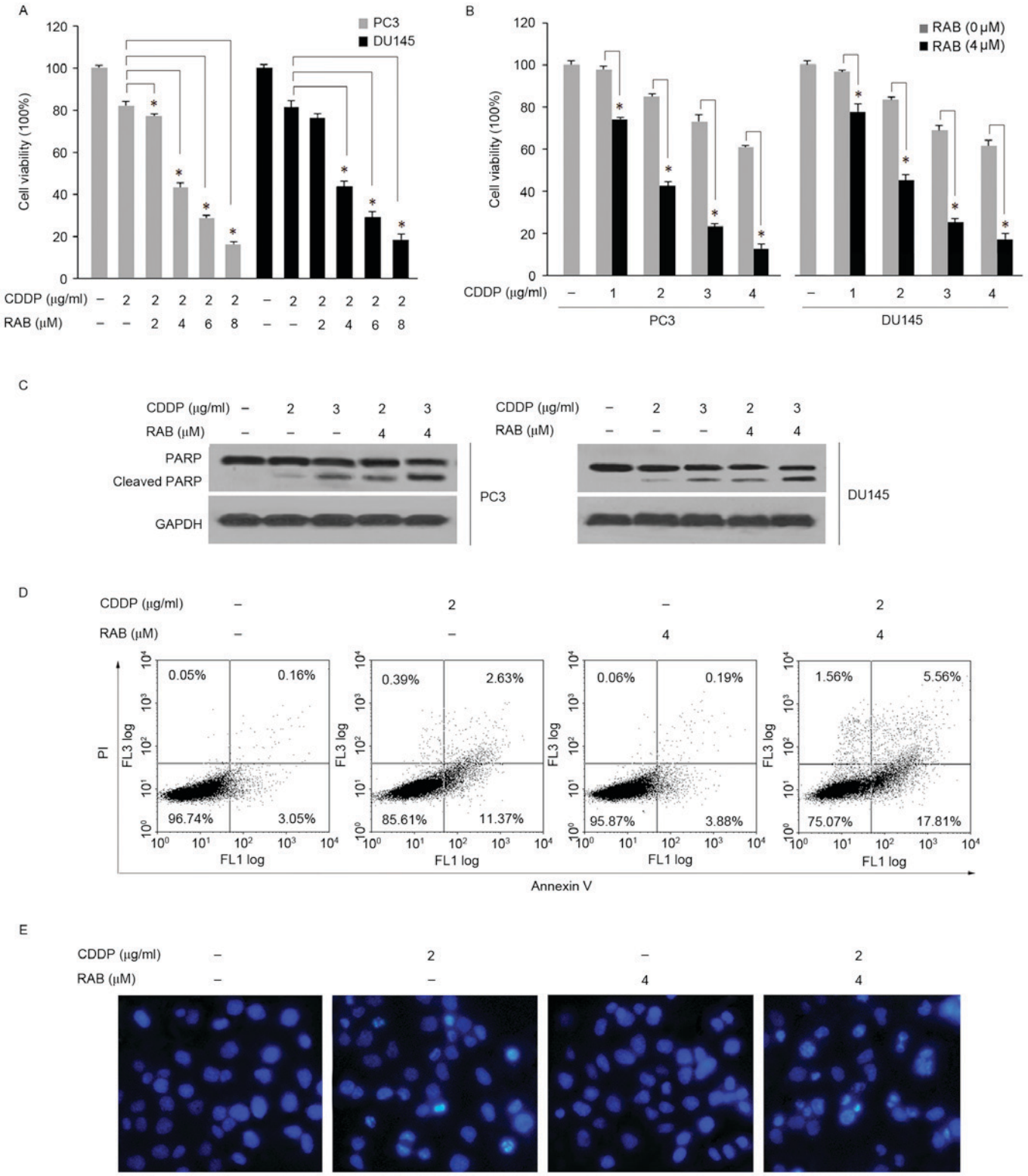

Figure 2. RAB enhances the cytotoxicity of CDDP. RAB sensitized PC3 and DU145 cells to CDDP-mediated anti-proliferation and apoptosis; (A) PC3 and DU145 cells were treated with different concentrations of RAB (2-8 μM) and a fixed concentration of CDDP (2 μg/ml) for 48 h at 37˚C; (B) PC3 and DU145 cells were treated with different concentrations of CDDP (1-4 μg/ml) and a fixed concentration of RAB (4 μM) for 48 h at 37˚C as measured by MTT assay. *P<0.05 vs. single treatment with the corresponding concentrations of CDDP. RAB sensitized PC3 and Du145 cells to CDDP-induced apoptosis as measured by (C) PARP cleavage and (D) flow cytometry. (E) Apoptosis in PC3 cells as visualized using DAPI staining. Cells were exposed for 48 h at 37˚C to the indicated treatments prior to staining with DAPI for 30 min. CDDP, cisplatin; RAB, Retigeric acid B; PI, propidium iodide; PARP, poly adenosine 5'-adenosine diphosphate ribose polymerase.

together with the aforementioned data suggesting that DR5 possesses the potential to promote the sensitivity of PCa cells to CDDP through DNA repair inhibition (26-29), the present study assessed whether RAB-mediated DR5 overexpression

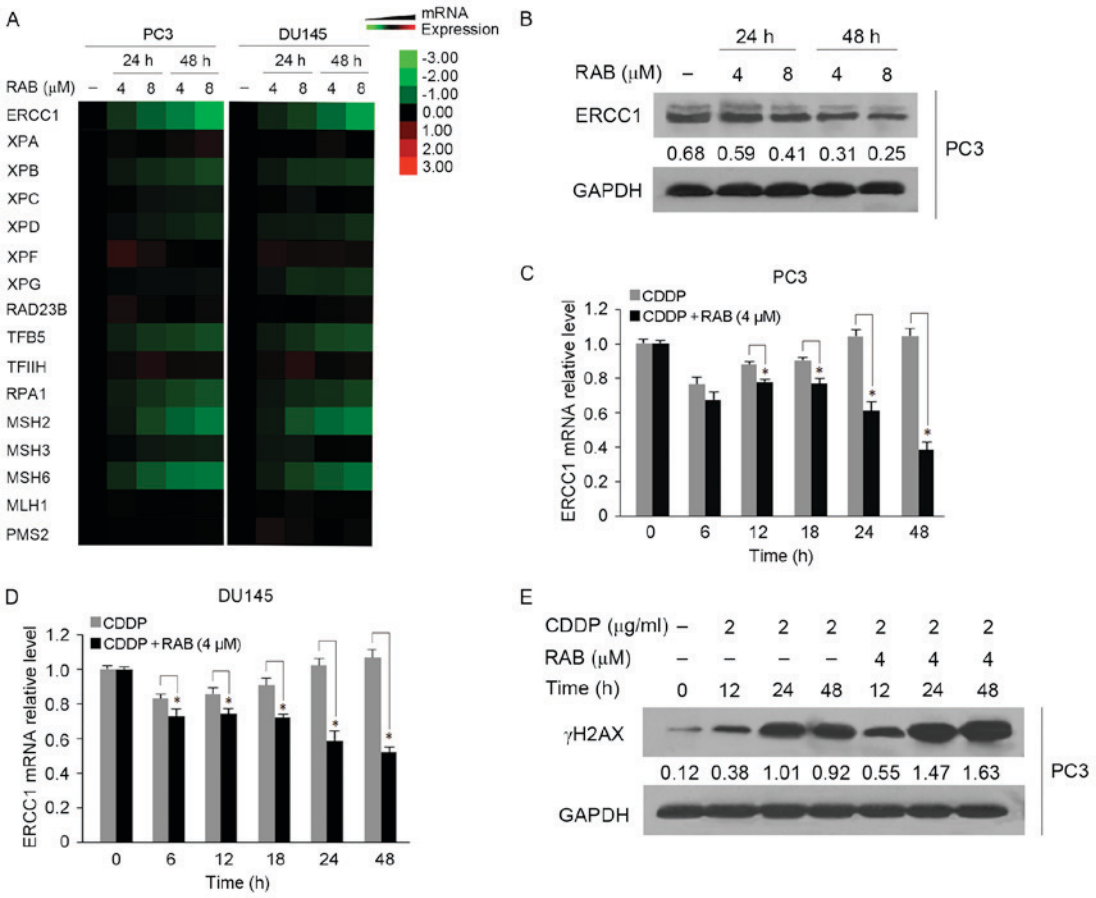

Figure 3. NER pathway is involved in the synergistic antitumor activity of combined CDDP and RAB treatment, and the effect of RAB on activities of DNA damage repair. (A) Heat map for mRNA levels of NER and MMR genes in RAB-treated cells that were determined by RT-qPCR. Red represents overexpression, green represents under-expression and black represents unchanged expression. (B) Western blot analysis of ERCC1 in PC3 cells following RAB treatment for different time intervals. Protein levels were normalized to GAPDH, and was quantified using densitometry of X-ray films. Changes in mRNA levels of ERCC1 in response to CDDP combined with RAB; (C) PC3 and (D) DU145 cells were treated with 4 μM RAB and 2 μg/ml CDDP simultaneously for the indicated times, and ERCC1 mRNA levels were detected by RT-qPCR assay. *P<0.05 vs. respective RAB-untreated groups. (E) Effect of co-treatment with RAB and CDDP on the γH2AX expression in PC3 cells, as determined by western blot analysis. GAPDH served as a loading control. Protein levels were quantified using densitometry of X-ray films. NER, nucleotide excision repair; MMR, mismatch repair; ERCC1, Excision repair cross-complementing 1; RT-qPCR, reverse transcription quantitative polymerase chain reaction; γH2AX, phosphorylation of histone H2AX at Ser139; RAB, RAB, Retigeric acid B; CDDP, cisplatin.

was involved in the synergistic mechanism of the increased cytotoxicity of CDDP. The upregulation of DR5 mRNA induced by RAB was validated by RT-qPCR assay (Fig. 4A and B). The results demonstrated that DR5 was elevated in a dose- and time-dependent manner in PC3 and DU145 cells. In these cell lines, the expression of DR5 mRNA significantly increased to between 1.3-1.5- and between 2 and 3-folds following 6 h treatment by 4 and 8 μM of RAB, respectively, and became more pronounced with increasing treatment durations, up to 48 h (between 2.5-3.0- and between 6-7-fold, respectively). Subsequently, the protein levels of DR5 in response to RAB were detected and revealed to be markedly increased after 24 and 48 h treatment, by ~3.0- and ~4.0-fold, respectively (Fig. 4C,D). Next, the changes in DR5 expression in response to combination application of CDDP and RAB were investigated. As indicated in Fig. 4E and F, CDDP increased the levels of DR5 at 6 h (~1.5-fold), which was maintained up to 18 h (between 3-3.5-fold), then gradually declined following 48 h treatment (~1.8-fold). In contrast, combined use with RAB enhanced CDDP-induced DR5 expression, particularly following prolonged RAB treatment (between 5-6-fold increase for 24-48 h). This effect was more marked in PC3 cells compared with DU145 cells.

To additionally examine the functional involvement of DR5 in RAB-mediated increased sensitivity of cells to CDDP, PC3 cells were transfected with DR5-targeting siRNA. As demonstrated in Fig. 4G and H, successful knockdown of DR5 expression was confirmed by western blot analysis. Cells transfected with non-target siRNA and treated with RAB exhibited DR5 expression, and the cell growth was synergistically inhibited by RAB and CDDP, while the synergistic effect was markedly decreased in PC3 and DU145 cells transfected with DR5-targeting siRNA, as evidenced by the attenuation of the levels of cleaved PARP (Fig. 4G and I). Concurrently, depletion of DR5 partially reversed the inhibitory effect of the combination treatment (RAB+CDDP) on cell proliferation by 30% (P<0.05), which additionally confirmed the role of DR5 in the mechanism of action of the RAB-CDDP treatment complex (Fig. 4J). Together, these data suggest that RAB-induced DR5 expression promoted CDDP cytotoxicity, leading to a contribution to the synergistic antitumor effect of co-treatment with RAB and CDDP.

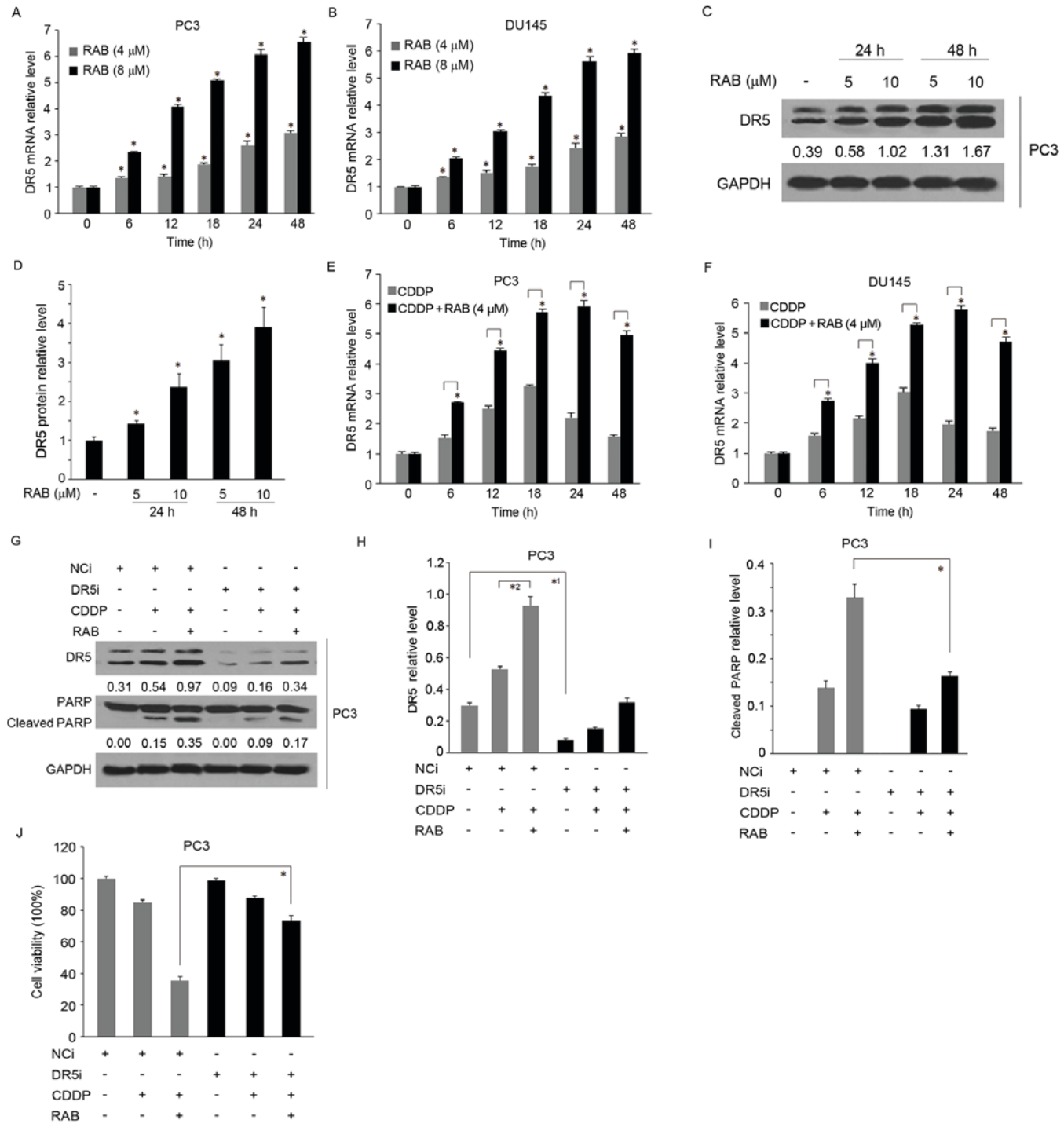

Figure 4. RAB-induced DR5 overexpression enhances chemotherapy sensitivity to CDDP. RT-qPCR analysis of DR5 in RAB-treated (A) PC3 and (B) DU145 cells. *P<0.05 vs. respective RAB-untreated groups in PC3 and DU145 cells. (C and D) Western blot analysis of protein expression of DR5 in PC3 cells. *P<0.05 vs. RAB-untreated group. (D and E) Combined effect of CDDP and RAB on the expression of DR5 as determined by RT-qPCR. (E) PC3 and (F) DU145 cells were treated with 4 μM RAB and 2 μg/ml CDDP simultaneously for the indicated times. *P<0.05 vs. respective RAB-untreated groups. Silencing of DR5 expression by siRNA attenuated the cytotoxicity induced by the combined use of CDDP and RAB; the silencing efficiency and expression of cleaved PARP were examined using (G-I) western blot analysis. Equal protein loading was evaluated by GAPDH and was quantified using densitometry analysis. *1P<0.05 vs. the control in the NCi group, *2P<0.05 vs. single CDDP treatment in the NCi group. *P<0.05 vs. the combined treatment of CDDP and RAB in the NCi group. (J) Cell viability was detected by MTT assay. *P<0.05 vs. the NCi group. RT-qPCR, reverse transcription quantitative polymerase chain reaction; RAB, Retigeric acid B; CDDP, cisplatin; si, small interfering; NCi, negative control siRNA; DR5i, Death receptor 5 siRNA; PARP, poly adenosine 5'-adenosine diphosphate ribose polymerase.

Discussion

RAB is a natural pentacyclic triterpenic acid that exhibits potential antitumor activity in PCa cells *in vitro* and *in vivo* (31,33). The present study aimed to investigate the potential of combination treatment of RAB with chemotherapeutic drugs to promote their anticancer effects. To the best of our knowledge, it was identified here for the first time that RAB exerted potent

chemotherapy sensitization to CDDP in PCa cells. To additionally elucidate the mode of action of this synergistic cytotoxicity, the function of DNA repair in the enhanced antitumor activity of CDDP by RAB-treatment was assessed. It was revealed that RAB blocked the NER pathway by inhibiting the expression of multiple NER-associated genes following prolonged treatment, which may inhibit the removal of CDDP-DNA adducts and accelerate the rate of cell death. Concurrently, treatment of tumor cells with RAB markedly promoted the expression of DR5. Regarded as a proapoptotic protein in cancer (25,27), DR5 overexpression also contributed to the cell death mediated by the combination treatment.

The development of resistance to chemotherapy remains one of the major challenges of curing advanced and metastatic PCa. Combined use of different chemotherapeutic drugs without cross resistance, with distinct mechanisms of action may decrease the risk of drug-resistant cell clone formation, and may also increase the tumor remission rate and the possibility of a cure (2,3). The hypothesis of the present study was that novel natural compounds that are able to induce cancer cell death through various signaling pathways may potentially improve the effectiveness of chemotherapy. RAB has gained attention for its potential antitumor activity in PCa. In the present study, among different treatment combinations, the combined use of CDDP and RAB exhibited the highest efficacy. As PCa cells were exposed simultaneously to low-dose RAB and increasing doses of CDDP, a significant decrease in cell viability and an induction of apoptosis were observed, with a decreased IC_{50} of CDDP. This treatment method may decrease the effective dose of CDDP, therapeutically, which may also contribute to decreased adverse effects of CCDP. For example, administration of epigallocatechin-3-gallate (50 mg/kg) together with CDDP (10 mg/kg) was identified to prevent CDDP-induced nephrotoxicity, ototoxicity and their consequences, including mortality (36). It was also suggested previously that reduced dosages of these two drugs achieved maximal cytotoxic effects by combining topotecan with a Checkpoint kinase 1 inhibitor (PF477736HEK1), which may potentially minimize side effects of the drugs (37).

It has been established that the formation of platinum-DNA adducts blocks replication and transcription and ultimately leads to G2 phase cell cycle arrest or cell death (5,6). However, previous studies have indicated that DNA-damaging agents only offer modest benefit for the majority of patients due to the proficient DNA repair processes available in cancer cells (6-10). Therefore, the inhibition of DNA repair remains an effective approach to improve the sensitivity to CDDP. Of the 4 major DNA repair pathways: NER, base excision repair, MMR and double-strand-break repair, NER is the major pathway regarded to remove CDDP lesions from DNA (16,18). The data from the present study revealed that γH2AX persisted at the later treatment time point (24-48 h) of the combination treatment of CDDP and RAB, while decreased accumulation of γH2AX was observed in samples treated with CDDP alone compared with the combined treatment of CDDP and RAB. The results may be due to significant NER impairment at the later time points by RAB, as supported by the decreased expression of multiple NER-associated genes. Therefore, the inhibitory effect of RAB on the NER pathway potentiated the cytotoxic activity of CDDP. Additionally, decreased sensitivity to CDDP-induced DNA damage may also occur through a loss of function of the MMR pathway (16). During MMR, CDDP-induced DNA adducts are recognized by the MMR proteins MSH2, MSH3 and MSH6, which are homologues of the bacterial protein MutS (38). Loss of MMR with respect to CDDP-DNA adducts may result in decreased apoptosis and drug resistance (16). RAB impaired the MMR pathway, as evidenced by the downregulation of MSH2 and MSH6, which function together with NER deficiency to accelerate DNA damage and cell death.

DNA damage-mediated apoptotic signals, however, may be attenuated (4,5). Alternative therapies with potential antitumor activity that are mediated by various mechanisms have been considered. Previous studies have suggested that targeting death receptors and their respective signaling pathways to trigger apoptosis promotes the sensitivity of tumor cells (29,39,40). A variety of agents such as delphinidin (39), ursolic acid (40), carnitine (41), salirasib (42), monensin (43) and 2-tellurium-bridged β-cyclodextrin (44), have been demonstrated to sensitize tumors to apoptosis by inducing DR5. In the present study, it was identified that RAB increased the mRNA and protein levels of DR5 in PCa cells. Depletion of DR5 effectively decreased the rate of cell death in the presence of CDDP in combination with RAB, confirming the functional significance of DR5 upregulation in the enhanced cytotoxicity of CDDP.

In summary, the present study proposed a mechanistic basis for the antitumor effect of RAB in combination with CDDP, a front-line treatment for a variety of neoplasms, used to enhance the efficacy of CDDP therapy. RAB sensitized PCa cells to CDDP at low IC_{50} values by a combination of mechanisms, including the impairment of DNA repair and the activation of DR5, which suggested the combined use of CDDP and RAB as a potential chemotherapeutic strategy.

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