Paclitaxel Impedes EGFR-mutated PC9 Cell Growth *via* Reactive Oxygen Species-mediated DNA Damage and EGFR/PI3K/AKT/mTOR Signaling Pathway Suppression

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Abstract. *Background/Aim: Paclitaxel is used as a first-line and subsequent therapy for the treatment of various cancers. However, the function and mechanisms of action of paclitaxel in non-small-cell lung cancer (NSCLC) remain unknown. In this study, the molecular mechanism underlying the anticancer activity of paclitaxel was investigated in vitro in a human NSCLC cell line carrying the EGFR exon 19 deletion (PC9). Materials and Methods: PC9 cells were treated with paclitaxel and then evaluated with a cell viability assay, DAPI staining, Giemsa staining, apoptosis assay, reactive oxygen species (ROS) assay, terminal deoxynucleotidyl transferase dUTP nick-end labeling assay and Western blotting. Results: Paclitaxel markedly decreased the viability of PC9 cells and induced morphological signs of apoptosis. The apoptotic effects of paclitaxel were observed through caspase cascade activation, along with ROS generation and loss of mitochondrial membrane potential (MMP). Furthermore, paclitaxel induced ROS-mediated DNA damage that triggered the activation of the extrinsic pathway of apoptosis via the up-regulation of death receptor (DR5) and caspase-8 activation. In addition, we found that paclitaxel effectively suppressed the EGFR/PI3K/AKT/mTOR signaling pathway to impede PC9 cell growth. Paclitaxel induced cell cycle arrest at the G1 phase in response to DNA damage, in association with the suppression of CDC25A, Cdk2 and Cyclin E1 protein expression. Conclusion: Paclitaxel showed anticancer effects against NSCLC by*

activating extrinsic and intrinsic apoptotic pathways through enhancing ROS generation, inducing cell cycle arrest, and suppressing EGFR/PI3K/AKT/mTOR signaling pathway.

In the United States, nearly a quarter of all cancer deaths are due to lung cancer (1). Non-small-cell lung cancer (NSCLC) is the most common type and accounts for approximately 85% of all lung cancer cases in the United States (2). Paclitaxel is an antimicrotubule agent that effectively treats various cancers as first-line chemotherapy (3, 4). However, its molecular mechanism of action in NSCLC is still not fully understood.

In cell death regulation, reactive oxygen species (ROS) play a fundamental role (5). It has been reported that elevated ROS production can expedite cancer treatment response (6). As mediators, ROS are well known to cause oxidative DNA damage (7). ATM serine/threonine kinase (ATM)/ATR serine/threonine kinase (ATR) protein kinases and a network of sensory proteins play an essential role in the DNA damage response (8). Activated ATR and ATM phosphorylate the checkpoint kinases Chk1 and Chk2, respectively, and regulate the proteins involved in the DNA damage response (DDR), DNA repair, and cell cycle arrest progression (9). Typically, stable cell cycle arrest occurs in response to DNA damage, called cellular senescence, thereby limiting cell proliferation (10). In addition, increased ROS production is associated with the loss of mitochondrial membrane potential (MMP) (11). In response to DNA damage, Bax/Bak can be activated to induce mitochondrial membrane permeabilization through the inhibition of anti-apoptotic molecules, such as Bcl-2 and Bcl-xL (12).

In numerous cancer cells, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) enhances apoptosis (13). In the extrinsic apoptosis pathway, TRAIL binds to the death receptors DR4 and DR5 to induce apoptosis by recruiting the death-inducing signaling complex (DISC) (14-16). Through the death domain (DD) and death effector domain (DRD), Fas-associated protein death domain (FADD) corporates with death receptors (DRs) and

procaspase-8 (17). The formation of DISC triggers the activation of caspase-8, leading to the activation of downstream effector caspases (*e.g.* caspase-3, caspase-7) and thereby inducing TRAIL-mediated apoptosis (17, 18). In addition, the activation of caspase-8 cleaves Bid into tBid, which triggers the mitochondrial apoptotic pathway, thus releasing cytochrome c (19). Once cytochrome c is released from the mitochondria into the cytoplasm, it can initiate the caspase cascade's activation by interacting with apoptotic protease-activating factors (Apafs) (20).

Epidermal growth factor receptor (EGFR) is a transmembrane protein and receptor for members of the epidermal growth factor (EGF) family of extracellular protein ligands (21). Binding of the ligand to the extracellular domain results in EGFR activation. Activated EGFR then homodimerizes, leading to intracellular tyrosine kinase phosphorylation and triggering the PI3K/Akt/mTOR intracellular signaling pathway (22). EGFR plays a crucial role in repairing DNA damage (23). However, the anticancer effects and mechanisms of action of paclitaxel in human EGFR-mutated PC9 cells remain unclear. Therefore, the major purpose of the present study was to evaluate the impact of paclitaxel on growth inhibition in EGFR-mutated PC9 cells and clarify the underlying mechanisms.

Materials and Methods

Cell line and cell culture. Professor K. Hayata (Tokyo Medical College, Tokyo, Japan) provided the PC9 (EGFR exon 19 deletion) human NSCLC cell line derived from a previously untreated adenocarcinoma patient (24). PC9 cells were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Invitrogen) and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Drug preparation. Paclitaxel [$C_{47}H_{51}NO_{14}$] was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). This drug was dissolved in DMSO for in vitro experiments.

Cell proliferation assay. The water-soluble tetrazolium salt (WST-1) assay (Cell Proliferation Reagent WST-1; Roche, Tokyo, Japan) was used to evaluate the cytotoxicity of different paclitaxel concentrations (50 and 100 nM). A 100-μl volume of a growing cell suspension was seeded into each well (4×10^3 cells/well) of a 96-well microtiter plate, and 100 μl of a solution of paclitaxel at different concentrations was added to each well. After incubation for 72 h at 37°C in a 5% carbon dioxide atmosphere, 10 μl of WST-1 solution was added to each well, and the plates were incubated at 37°C for a further 4 h. Sample absorbance was measured at 450 nm using a microplate enzyme-linked immunosorbent assay reader (Multiskan FC; Thermo Scientific, Tokyo, Japan).

Giemsa and DAPI staining. For 72 h, PC9 cells were treated in 12-well plates with 2 concentrations of paclitaxel (50 and 100 nM) or with 0.1% DMSO as a control. DAPI (Invitrogen) was used to stain cells fixed in 4% paraformaldehyde in phosphate-buffered solution (FUJIFILM Wako Pure Chemical Corporation) and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Tokyo, Japan). The morphology of cell nuclei was observed using a BZ-X710 All-in-

One Fluorescence Microscope (Keyence, Osaka, Japan). Giemsa staining (Merck KGaA, Darmstadt, Germany) was performed with methanol-fixed cells, and morphological changes were evaluated under a light microscope.

ROS assay. To measure the ROS in cells by flow cytometry, we used an ab186029 (Abcam, Tokyo, Japan) Cellular Reactive Oxygen Species Detection Assay Kit (Deep Red Fluorescence), as described previously (25). In brief, after 72 h of drug treatment, cells were collected to stain with ROS deep red dye working solution. Subsequently, cells were incubated at 37°C for 60 min before being subjected to a flow cytometry analysis. The signal was calculated in terms of the median fluorescence intensity (MFI).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. For 72 h, PC9 cells were treated with different concentrations of paclitaxel (50 and 100 nM) or with 0.1% DMSO as a control. We used the in situ Direct DNA Fragmentation (TUNEL) Assay Kit (ab66108; Abcam) to measure DNA fragmentation in apoptotic cells. In brief, cells were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) and placed on ice for 15 min. Subsequently, the samples were treated with a staining solution and incubated at 37°C for 60 min. After rinse buffer addition, cells were resuspended in propidium iodide/RNase A solution and incubated at room temperature for 30 min for a flow cytometry analysis.

MMP evaluation. The MMP was evaluated using the JC-10 Mitochondrial Membrane Potential Assay Kit-Flow Cytometry (ab112133; Abcam). Cells were seeded in 75-cm² flasks (Falcon, Tewksbury, MA, USA), treated with paclitaxel (50 and 100 nM) or with 0.1% DMSO as a control for 72 h, trypsinized, washed with PBS and incubated with 1X JC-10 dye-loading solution at room temperature for 30 min. Cell fluorescence was measured using a BD FACSCantoTM II (BD Biosciences, San Jose, CA, USA).

Annexin V-FITC/PI apoptosis assay. Using an Annexin V-FITC/PI apoptosis detection kit (BioLegend, San Diego, CA, USA), cell apoptosis was detected. In brief, after treatment with paclitaxel (50 and 100 nM) or with 0.1% DMSO as a control for 72 h, cells were stained with Annexin V-FITC and PI. They were then analyzed by flow cytometry (FACSCanto™ II), and their fluorescence intensity was detected. Data were analyzed using the FlowJo software program, version 10.2 (FLOWJO, LLC, Ashland, OR, USA).

Cell cycle analysis. The cells were treated with different concentrations of paclitaxel (50 and 100 nM) or with 0.1% DMSO as a control for 72 h. A cell cycle analysis was performed following the Propidium Iodide Cell Cycle Staining Protocol (BioLegend). DNA content was determined with a FACSCanto™ II. Data were analyzed using the FCS Express 7 Flow software program (De Novo Software, Pasadena, CA, USA).

Western blotting. For 72 h, PC9 cells were treated with different concentrations of paclitaxel (50 and 100 nM) or with 0.1% DMSO as a control. Whole protein lysates were isolated using the M-PER mammalian protein extraction reagent (Thermo Scientific), which included a phosphatase inhibitor cocktail and a protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were assessed

using the BCA protein assay reagent (Thermo Scientific). Total cellular protein (50 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Milk-blocked blots were incubated at 4°C overnight with primary antibodies against the following proteins: Bak, Bcl-2, cleaved caspase-3 (Asp 175), cleaved caspase-9 (Asp 315), PARP, DR5, FADD, Bid, cleaved caspase-8 (Asp 391), Cdk2, Cyclin E1, CDC25A, EGFR, p-EGFR (Thy 1068), PI3K p110α, Akt, p-Akt (Ser 473), mTOR, p-mTOR (Ser 2448), ATR, p-ATR (Ser 428), Chk1, p-Chk1 (Ser 345), β-actin and γ-H2AX (ser 139), followed by appropriate horseradish peroxidase-conjugated secondary antibodies. All primary and secondary antibodies were purchased from Cell Signaling Technology, Beverly, MA, USA, except for anti-γ-H2AX that was obtained from Abcam. Proteins of interest were detected using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA) and viewed using the Invitrogen iBright FL1000 Imaging System (Thermo Fisher Scientific).

Statistical analysis. All data are expressed as the means±standard deviation (SD) of three independent experiments and analyzed by a one-way analysis of variance with Dunnett's multiple comparison test or with Sidak's multiple comparison test. All *p*-values <0.05 were considered statistically significant. Graphs were created using the GraphPad PRISM 7.0 software program (GraphPad Software Inc., San Diego, CA, USA).

Results

Cytotoxic effects of paclitaxel in PC9 cells. PC9 cells were treated with 2 concentrations of paclitaxel (50 and 100 nM) for 72 h, and the WST-1 assay was used to examine the cell viability. The viability of PC9 cells was significantly reduced after paclitaxel exposure compared to the DMSO group (Figure 1A). Paclitaxel-treated cells were stained with Giemsa and DAPI to investigate the morphological changes. As indicated in Figure 1B and C, treating PC9 cells with paclitaxel produced apoptotic features, such as nuclear fragmentation.

Effects of paclitaxel on ROS generation. Many chemotherapy drugs cause an increase in intracellular ROS production, leading to growth inhibition (26-28). Therefore, we hypothesized that paclitaxel would enhance ROS generation. Compared to the DMSO group, paclitaxel increased the ROS level in a concentration-dependent manner (Figure 2A and B). To determine whether or not ROS production plays a role in the anticancer effects of paclitaxel against PC9 cells, ROS inhibitor N-acetyl-L-cysteine (NAC) was added alone or in combination with paclitaxel (50 nM and 100 nM). NAC suppressed ROS production levels, however, not statistically significant, and significantly increased cell proliferation (Figure 2C-E). Taken together, these results suggest that paclitaxel enhances ROS generation, leading to reduced cell proliferation.

Effects of paclitaxel on DNA fragmentation and the DNA damage signaling pathway. Previous studies reported that increased amounts of intracellular ROS could enhance DNA damage (9, 29). Therefore, we hypothesized that paclitaxel administration would result in increased DNA fragmentation. To prove that DNA fragmentation was caused by paclitaxel treatment, we conducted a TUNEL assay. As shown (Figure 3A), after 72 h of paclitaxel treatment, the proportion of fragmented cells increased from 0.27% to 81.8%, in a dose-dependent manner (Figure 3B).

Oxidative DNA damage causes ATR phosphorylation at Ser 428 (30, 31). Activation of this protein promotes phosphorylation of different downstream proteins (including H2AX and Chk1) and ultimately promotes growth inhibition (32, 33). To clarify the precise pathway of the DNA damage response after paclitaxel treatment, we examined the expression of γ-H2AX, p-Chk1 (Ser 345) and p-ATR (Ser 428), as these proteins are responsible for activating the DNA damage pathway. As shown (Figure 3C), the expression of γ-H2AX, p-Chk1 (Ser 345) and p-ATR (Ser 428) was markedly increased after paclitaxel treatment compared with the DMSO group. These results suggest that paclitaxel induced DNA damage in PC9 cells *via* ATR-Chk1 signaling axis activation.

Effects of paclitaxel on MMP. Previous studies have reported that ROS production can alter the MMP and structure (34, 35). To determine whether or not paclitaxel affected the MMP, we performed an MMP assay. As shown in Figure 4, the loss of MMP increased from 1.67% to 36.1% (lower right quadrant 3) in PC9 cells after paclitaxel treatment, indicating that the anticancer effect of paclitaxel is, at least in part, due to paclitaxel-induced MMP loss.

Effects of paclitaxel on apoptosis and the apoptosis signaling pathways in PC9 cells. Chemotherapy drugs can inhibit cell proliferation and induce apoptosis in human cancer cells (36-38). To confirm that the cell death caused by paclitaxel was indeed apoptosis, we performed Annexin V and PI staining for a flow cytometry analysis. As shown (Figure 5A), after 72 h of exposure to paclitaxel, a significant increase in early apoptotic and necrotic cells as well as an increase in the proportion of late apoptotic cells from 3.72% to 60.6%. Flow cytometry results showed that the paclitaxel-induced apoptosis was concentration-dependent (Figure 5B).

Chemotherapeutic agents induce apoptosis through extrinsic/intrinsic apoptotic pathway activation (39, 40). To examine whether or not the extrinsic apoptotic pathway regulates the apoptotic impact of paclitaxel, we evaluated several extrinsic apoptotic proteins. As shown in Figure 5C, the expression of tBid, DR5 and cleaved caspase-8 (Asp 391) was increased by paclitaxel treatment in PC9 cells. In contrast, paclitaxel induced intrinsic mitochondria-dependent
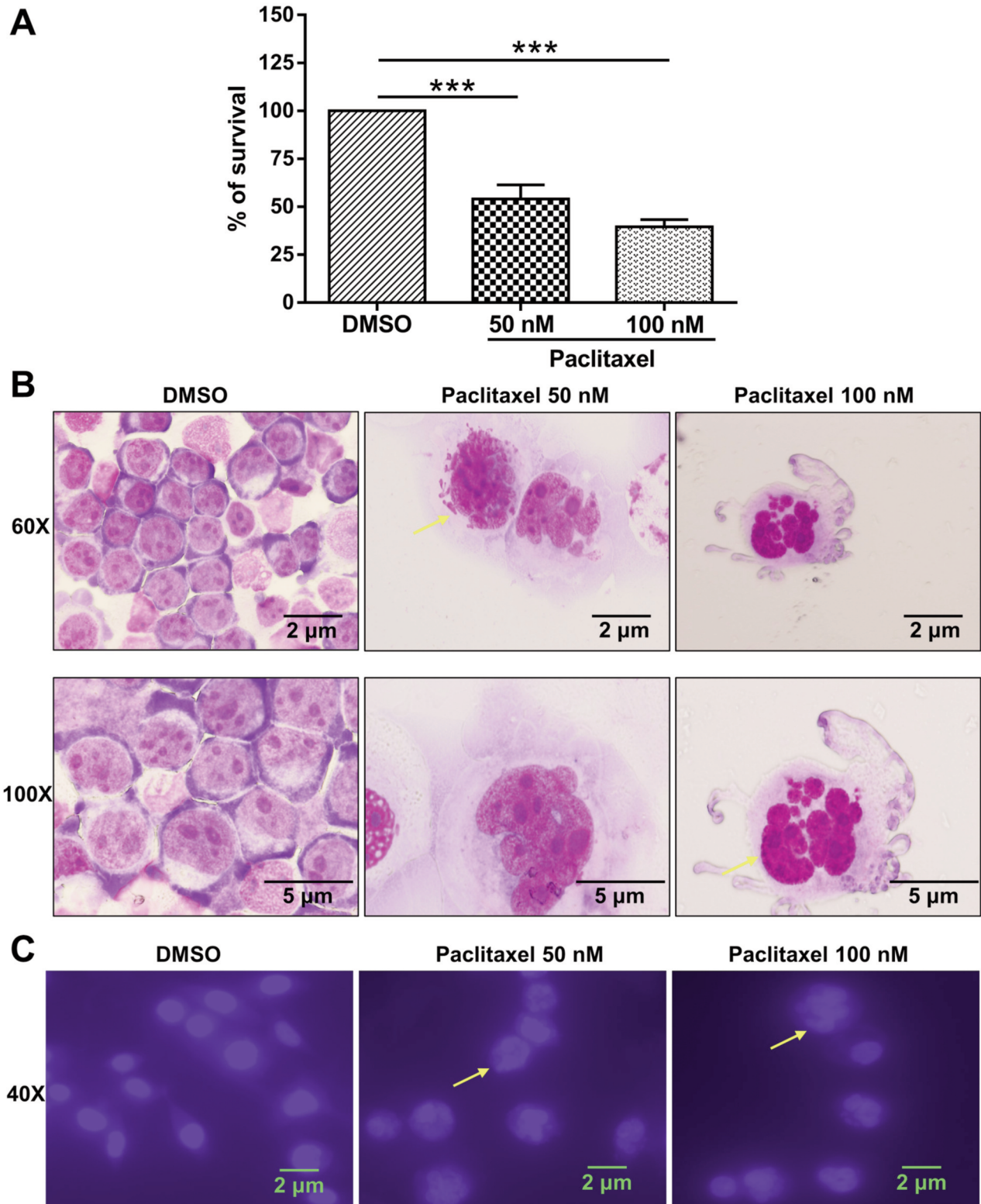
Figure 1. *Effects of paclitaxel on the viability and morphology of PC9 cells. (A) The WST-1 cell proliferation assay was used to evaluate the viability of PC9 cells treated with different concentrations of paclitaxel for 72 h. A one-way ANOVA with Dunnett's multiple comparison test determined the significance: ***p<0.001 compared with the DMSO-treated group. (B) DAPI and (C) Giemsa staining of PC9 cells were treated with different paclitaxel concentrations for 72 h. The yellow arrows in paclitaxel-treated cells indicated nuclear fragmentation.*
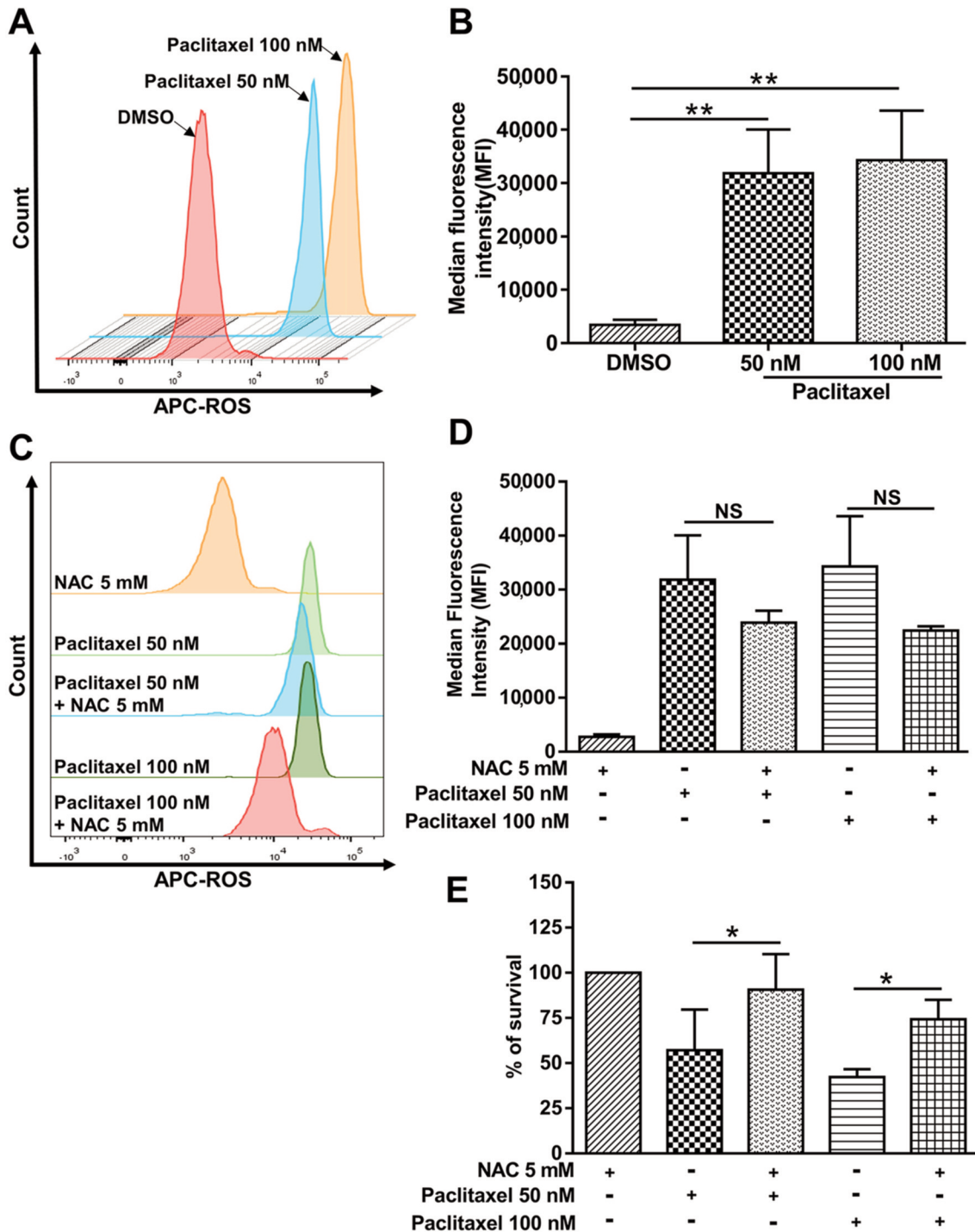
Figure 2. *Effects of paclitaxel on ROS generation. (A) PC9 cells incubated with paclitaxel at different concentrations (or 0.1% DMSO as a control) for 72 h were stained with ROS deep red dye. The histogram shows that the ROS levels increased in a concentration-dependent manner. (B) The bar diagram shows that the ROS production increased in a dose-dependent manner. A one-way ANOVA with Dunnett's multiple comparison test determined the significance: **p<0.01 compared with the DMSO-treated group. (C) After treatment with paclitaxel for 72 h in the absence or presence of NAC, cells were stained with ROS deep red dye. (D) The bar diagram shows the quantification of mitochondrial ROS production. Comparisons were made between the control group and experimental group using a one-way ANOVA followed by Sidak's multiple comparison test. NS, not significant. (E) After 72 h of treatment with paclitaxel in the absence or presence of NAC, the cell viability was analyzed by the WST-1 cell proliferation assay. Comparisons were made between the control group and experimental group using a one-way ANOVA followed by Sidak's multiple comparison test. Data are presented as mean±SD from three technical replicates.*p<0.05.*
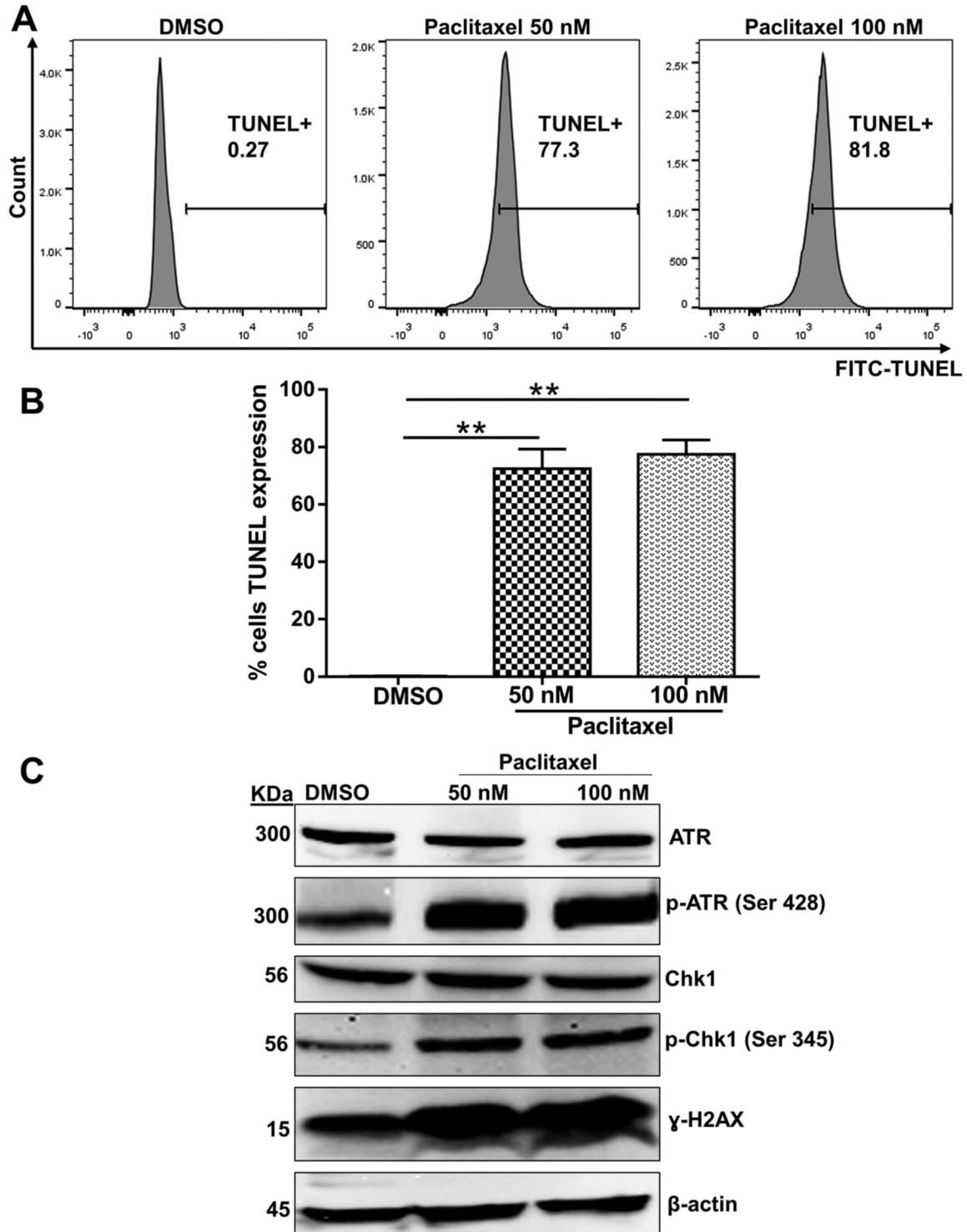
Figure 3. *Effects of paclitaxel on DNA fragmentation and the DNA damage signaling pathway. (A) PC9 cells were stained with fluorescein isothiocyanate-dUTP dye after incubation with paclitaxel at different concentrations for 72 h. Flow cytometry measured the fluorescence signal. Histograms show that DNA fragmentation increased in a concentration-dependent manner. (B) The percentage of cells expressing TUNEL increased in a dose-dependent manner, as represented in the bar graph. Results are the mean±SD of three independent experiments. Significance was determined by a one-way ANOVA followed by Dunnett's multiple comparison test. **p<0.01. (C) Total cell lysates from control and paclitaxel-treated PC9 cells for 72 h were subjected to Western blotting with the antibodies against ATR, p-ATR (Ser 428), Chk1, p-ChK1 (Ser 345) and γ-H2AX. β-actin served as a loading control. All images shown here are representative of three independent experiments with similar results.*
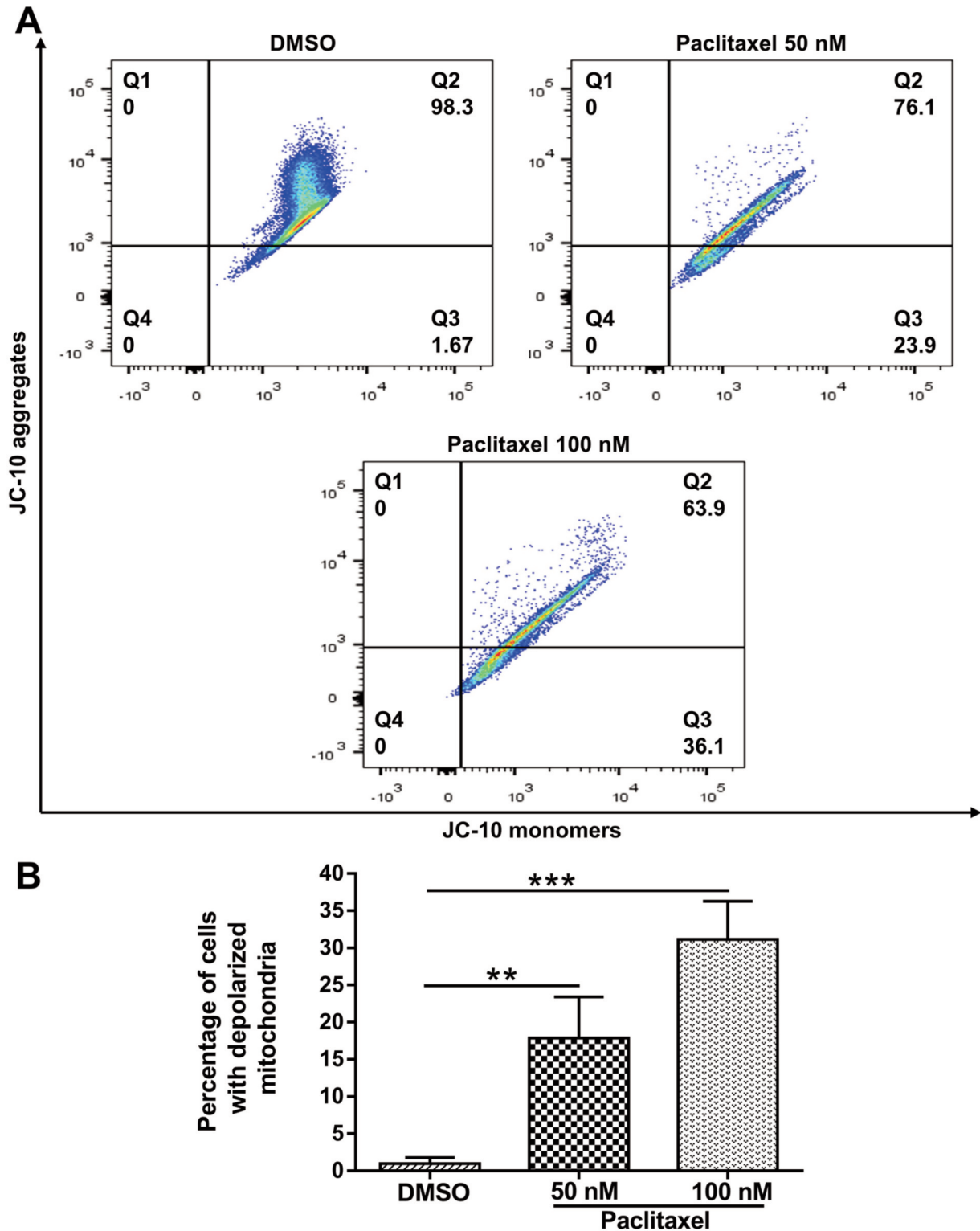
Figure 4. *Effects of paclitaxel on the mitochondrial membrane potential (MMP). (A) For 72 h, PC9 cells were treated with different concentrations of paclitaxel. Before the flow cytometry analysis, cells were stained with JC-10 dye. Representative results from three independent experiments. (B) Quantitative data are used from the green monomer fluorescence (depolarized MMP) of JC-10. Results are presented as the mean ± SD of three independent measurements. A one-way ANOVA with Dunnett's multiple comparison test determined the significance: **p<0.01 and ***p<0.001 compared with the DMSO-treated group.*
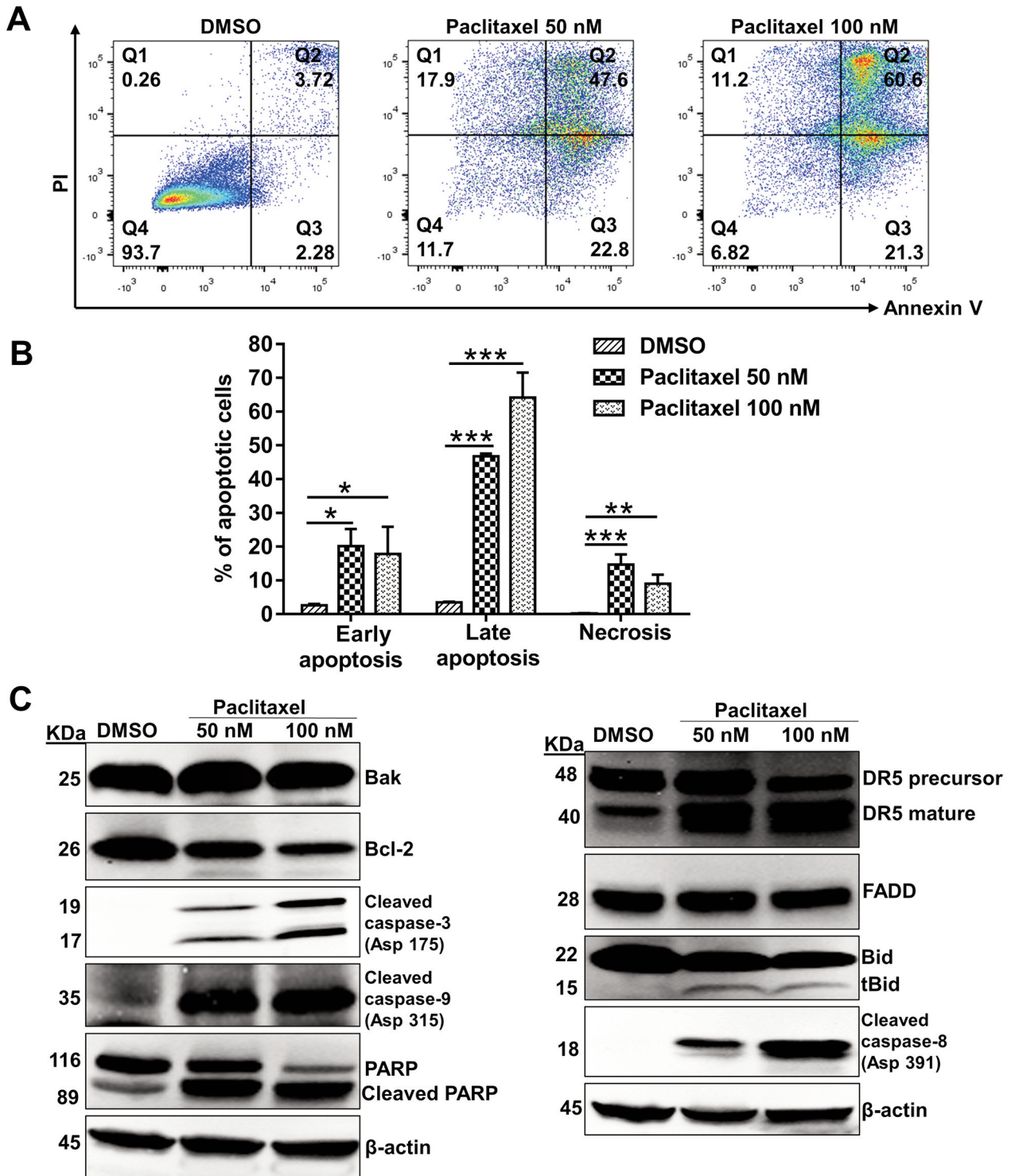
Figure 5. *Effects of paclitaxel on the induction of apoptosis in PC9 cells. (A) PC9 cells were treated with 2 concentrations of paclitaxel (50 and 100 nM) for 72 h, and flow cytometry was used to evaluate apoptosis. Quadrant 1 shows necrotic cells; Quadrant 2 shows late-apoptotic cells; Quadrant 3 shows early apoptotic cells; Quadrant 4 shows viable cells. (B) A bar diagram illustrates the percentage of apoptotic cells. The values were calculated as the mean±SD from three independent experiments. Significance was determined by a one-way ANOVA followed by Dunnett's multiple comparison test: *p<0.05, **p<0.01 and ***p<0.001 compared with the DMSO-treated group. (C) To assess the expression of Bak, Bcl-2, cleaved caspase-3 (Asp 175), cleaved caspase-9 (Asp 315), PARP, DR5, FADD, Bid and cleaved caspase-8 (Asp 391) proteins, Western blotting was performed. β-actin served as a loading control. All images shown here are representative of three independent experiments with similar results.*

apoptosis, evidenced by Bak up-regulation and Bcl-2 down-regulation (Figure 5C). Taken together, these results suggest that paclitaxel activates extrinsic and intrinsic apoptotic pathways to impede PC9 cell proliferation.

In the intrinsic and extrinsic pathways of apoptosis, signaling determines the activation of caspases that act on the proteolytic cleavage of PARP, which is a hallmark of apoptosis (41, 42). To clarify the apoptotic mechanism induced by paclitaxel treatment in PC9 cells, the expression of cleaved caspase-9 (Asp 315), cleaved caspase-3 (Asp 175) and cleaved PARP was evaluated. As shown in Figure 5C, PC9 cells treated with paclitaxel showed a higher expression of cleaved caspase-9 (Asp 315), cleaved caspase-3 (Asp 175) and cleaved PARP than the control. These results suggest that paclitaxel activates a caspase cascade to induce apoptosis in PC9 cells.

Effects of paclitaxel on cell cycle arrest. Previous studies reported that DNA damage could induce cell cycle arrest (43, 44). As indicated in Figure 6A, treatment with paclitaxel resulted in G_1 phase block in 96.37% of the population, suggesting that paclitaxel caused cell cycle arrest in the G_1 phase.

The G_1/S checkpoint confirms that all DNA damage has been repaired, and the cell is ready to start replication (43, 45). DNA damage activates the ATM/ATR pathway, leading to inhibition of the CDC25A phosphatase and thereby preventing Cdk2 activity and disrupting DNA replication (46-48). In addition, CDC25A phosphatase plays a vital role in activating the Cyclin E/Cdk2 complex, which is crucial for initiating G_1 to S phase transition (49). To further evaluate our cell cycle analysis data, we examined the expression of CDC25A, Cyclin E1 and Cdk2. Western blotting showed a decreased expression of CDC25A, Cyclin E1 and Cdk2 (Figure 6C), suggesting that these proteins may be involved in the cell cycle arrest of PC9 cells.

Effects of paclitaxel on the EGFR/PI3K/Akt/mTOR signaling pathway. Activated homodimerized EGFR leads to the activation of intracellular cascades, such as PI3K/Akt/mTOR (50). Consequently, the activated PI3K/AKT/mTOR signaling pathway triggers protein synthesis (51). The EGFR/PI3K/Akt/mTOR signaling cascade plays a crucial role in numerous cellular processes, including metabolism and cell proliferation (52). To evaluate whether or not paclitaxel impedes EGFR/PI3K/AKT/mTOR signaling pathway, we checked the expression of EGFR, p-EGFR (Thy 1068), PI3K p110α, Akt, p-Akt (Ser 473), mTOR and p-mTOR (Ser 2448). Western blotting showed a decrease in the expression of EGFR, p-EGFR (Thy 1068), PI3K p110α, Akt, p-Akt (Ser 473), mTOR and p-mTOR (Ser 2448) (Figure 7). These results suggest that inhibition of the EGFR/PI3K/Akt/mTOR signaling pathway is involved in the anticancer effects of paclitaxel in EGFR-mutated PC9 cells.

Discussion

Paclitaxel is a well-known anticancer drug, but the mechanism underlying its effects on PC9 cells remains unknown. We therefore evaluated the extent of PC9 cell death mediated by paclitaxel and clarified the mechanisms of actions. Based on the findings of this study, we proposed a model of the major mechanism of activation of intrinsic and extrinsic pathways of apoptosis, G1 phase arrest, and inhibition of the EGFR/PI3K/AKT/mTOR signaling pathway by paclitaxel in EGFR-mutated PC9 cells (Figure 8).

Pro-apoptotic proteins (such as Bak, Bax) and anti-apoptotic proteins (such as Bcl-2, Bcl-xL) are important upstream molecules that respond to intrinsic apoptosis (53, 54). A reduced Bak/Bcl-2 ratio is closely associated with the destruction of the MMP (55). An increase in the intracellular ROS level leads to the loss of MMP, resulting in the release of cytochrome c from the mitochondria into the cytosol (34). Cytochrome c binds with Apaf-1 to form an apoptosome and activates apoptosis, initiating procaspase-9 (56). Thus, activated caspase-9 can cleave and directly activate other effector caspases, such as caspase-3 (57). The initiation of a caspase cascade plays a vital role in apoptosis (42). Activated caspase-3 is the final step in the cascade that triggers apoptosis through the proteolytic cleavage of PARP (58). This current study demonstrated that paclitaxel enhanced MMP loss and caspase cascade activation to induce apoptosis (Figures 4 and 5).

The oxidative pressure created by ROS can damage parts of the cell, including DNA and proteins (59). Failure to repair cellular DNA damage by an adequate repair mechanism can cause cell death due to double-stranded DNA breakdown (60). ROS can induce cell death by initiating various DNA damage response pathways, such as ATM/Chk2 and ATR/Chk1 (9). Several studies reported that γ-H2AX formation is a sensitive indicator of DNA replication stress or DNA damage (61, 62). Some studies have reported that during apoptosis, the ATR/Chk1 pathway is activated by caspase-dependent cleavage (63, 64). The present study showed that paclitaxel increased ROS production, resulting in the activation of the ATR/Chk1 pathway as well as that of mitochondria-dependent caspase (Figures 2 and 3).

The TNF receptor, DR4 [TRAILR1] and DR5 [TRAILR2] receptors, Fas receptor and FADD adaptor constitute the DISC (65, 66). Several studies have reported that DNA damage leads to the activation of the extrinsic pathway by activating the DR4 and DR5 receptors (67-69). The pro-apoptotic cytoplasmic protein Bid connects the intrinsic and extrinsic pathway, which is cleaved by the activated caspase-8 generated by the DISC (42, 70). Activated caspase-8 can also directly cleave caspase-3 to enhance downstream apoptotic signals (57, 71). Once the generation of truncated Bid (tBid) is induced by activated caspase-8, the tBid advances until the mitochondrial membrane interacts with the Bax, Bak and Bad
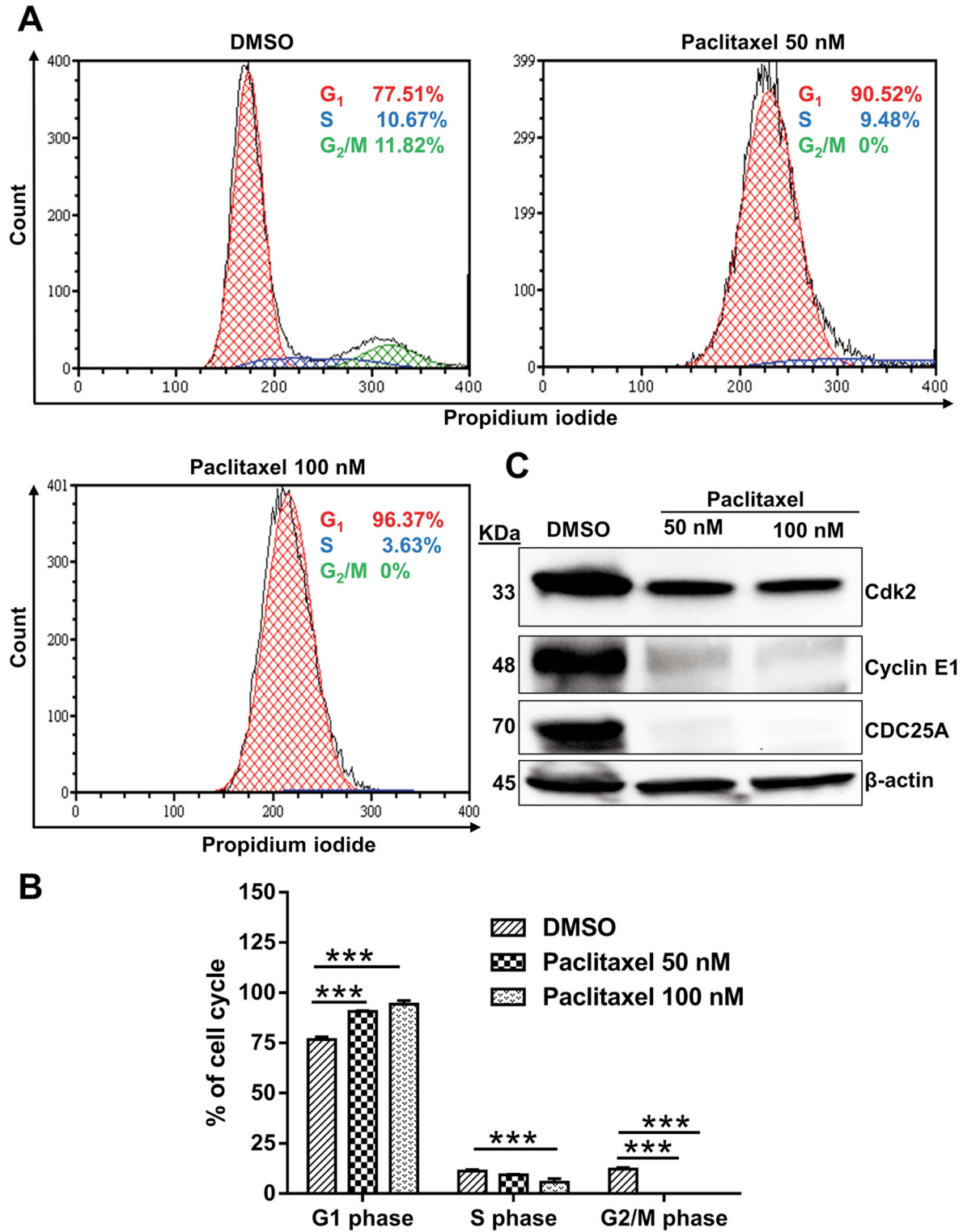
Figure 6. *Effects of paclitaxel on cell cycle regulation in PC9 cells. (A) Flow cytometry showed that paclitaxel induced cell cycle arrest in PC9 cells. (B) The bar graph represents the cell cycle distribution of PC9 cells. In the G1 phase, the proportion of PC9 cells increased after 72 h of treatment with paclitaxel. Results are presented as the mean±SD of three independent experiments. A one-way ANOVA with Dunnett's multiple comparison test determined the significance: ***p<0.001 compared with the DMSO-treated group. (C) PC9 cells were treated with paclitaxel for 72 h. The expression of CDC25A, Cyclin E1 and Cdk2 proteins was evaluated by Western blotting. β-actin was used as a loading control. All images shown here are representative of three independent experiments with similar results.*

proteins and induces mitochondrial permeabilization, resulting in the release of cytochrome c (72-74). The present study showed that ROS-mediated DNA damage triggered the activation of the apoptotic death receptor pathway *via* the up-regulation of DR5, resulting in the activation of caspase-8 to induce mitochondrial permeabilization.

DNA damage leads to the activation of the ATM/Chk2 and ATR/Chk1 pathway, resulting in the inhibition of CDC25A (75, 76). CDC25A is a phosphatase that can activate the cyclin E/Cdk2 complex, resulting in G1 to S phase transition (49). The present study showed that paclitaxel induced G1 phase arrest in response to DNA damage (Figure 6).

EGFR activation in response to ligands triggers several intracellular cascades, such as PI3K/AKT/mTOR and RAS/MAPK(ERK) (77, 78). The EGFR/PI3K/Akt/mTOR signaling cascade is crucial for protein synthesis and cell proliferation (77, 79). The present study revealed that paclitaxel effectively inhibited the EGFR/PI3K/AKT/mTOR signaling pathway and impeded cell proliferation (Figure 7).

Paclitaxel can bind to microtubules and prevent the normal dynamic reorganization of the microtubule network, which plays a crucial role in inhibiting cell proliferation (80). Various studies indicated that the binding site for paclitaxel is the beta-tubulin subunit (81, 82). Paclitaxel has been reported to arrest the cell cycle in the G_1 and G_2/M phases and induce cell death in cancer by stabilizing the microtubule polymer and preventing depolymerization (83, 84). This mechanism of action may also be involved in the effect of paclitaxel on cell cycle, in our study. However, further studies are needed to clarify the entire mechanism of microtubule stabilization by paclitaxel in EGFR-mutated PC9 cells.

Taken together, our results provide a basis for selecting paclitaxel chemotherapy for patients with EGFR-mutated NSCLC. We suggest that our current findings have broad implications for a clear understanding of ROS-mediated growth inhibition not only in EGFR mutant tumors but also in other oncogene-driven tumors.

Conclusion

Paclitaxel impeded the proliferation of PC9 cells in three explicit systems. First, paclitaxel increased ROS-mediated DNA damage, which triggered the activation of the extrinsic and intrinsic apoptotic signaling pathways. Second, paclitaxel-induced G_1 phase arrest was associated with the activation of ATR/Chk1 signaling. Third, paclitaxel inhibited the EGFR/PI3K/AKT/mTOR signaling pathway to prevent cell proliferation. Therefore, paclitaxel exerted a significant anticancer effect on PC9 NSCLC cells.

Conflicts of Interest

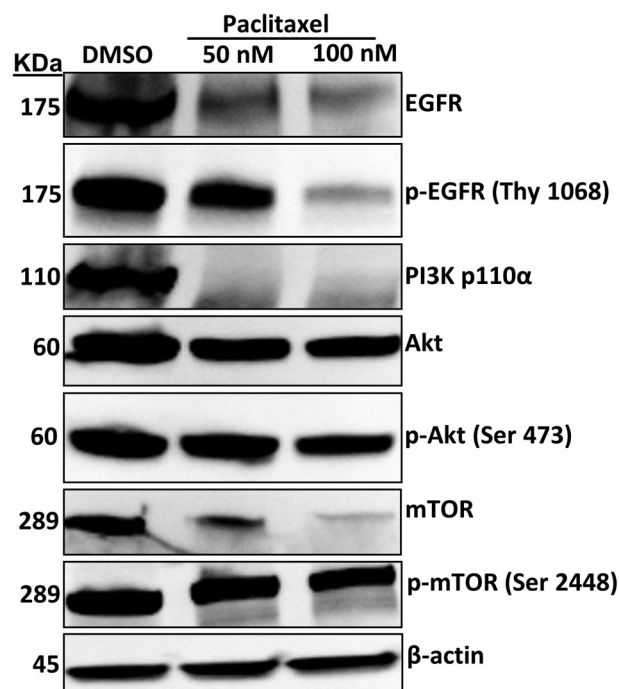The Authors declare no competing financial interests.

Figure 7. *Effects of paclitaxel on EGFR/PI3K/Akt/mTOR signaling regulation. PC9 cells were treated with different concentrations of paclitaxel for 72 h, and the expression of EGFR, p-EGFR (Thy 1068), PI3K p110α, Akt, p-Akt (Ser 473), mTOR and p-mTOR (Ser 2448) was evaluated by Western blotting. β-actin was used as a loading control. All images shown here are representative of three independent experiments with similar results.*

Authors' Contributions

Md Mohiuddin and Kazuo Kasahara conceived this study; Md Mohiuddin carried out the experiments; Md Mohiuddin and Kazuo Kasahara discussed and interpreted the results; Md Mohiuddin wrote the manuscript; Kazuo Kasahara supervised the experiments and project.

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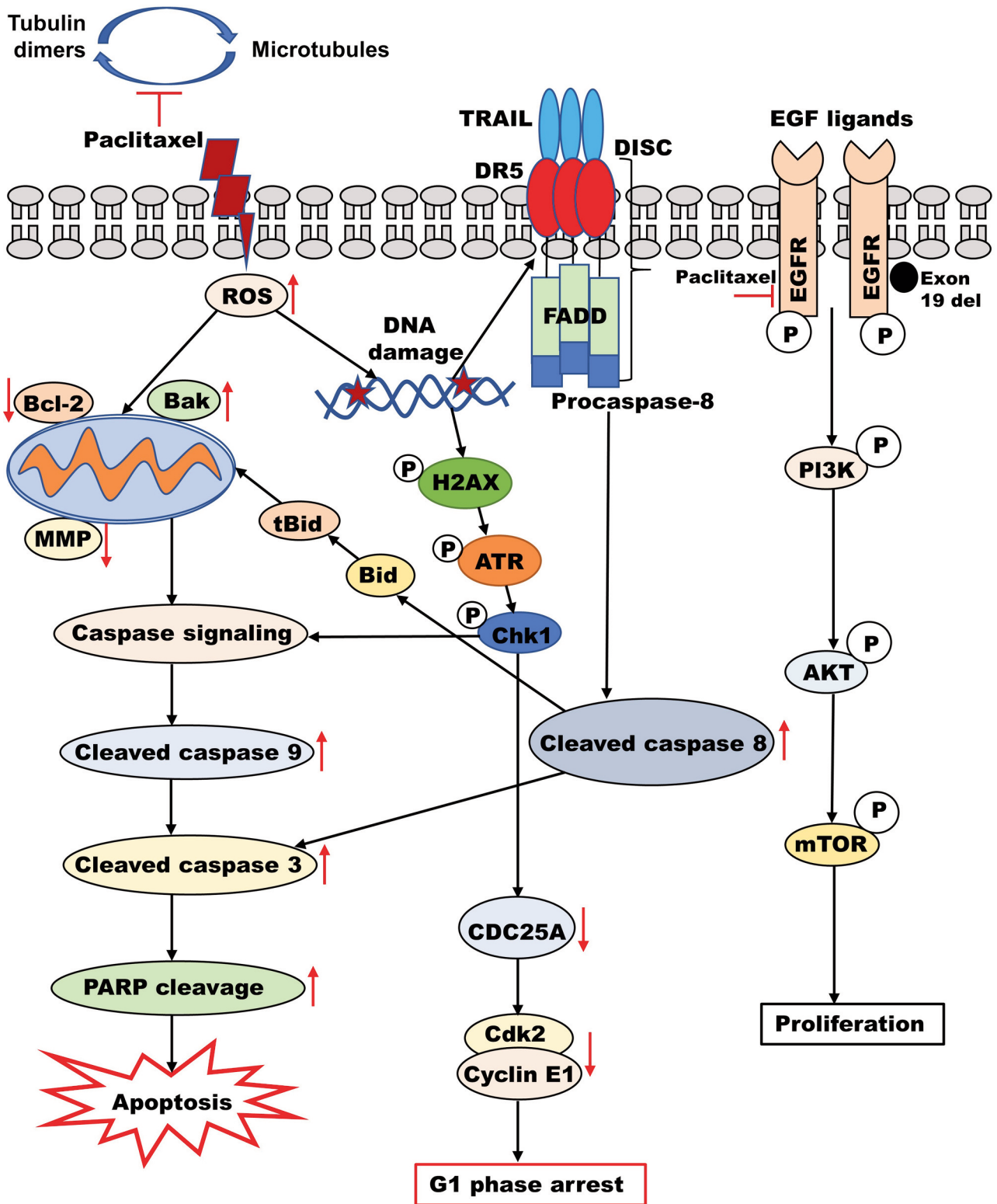
Figure 8. *A schematic illustration of the proposed mechanisms for paclitaxel-induced growth inhibition in PC9 cells.*

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