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Cisplatin Activates the Growth Inhibitory Signaling Pathways by Enhancing the Production of Reactive Oxygen Species in Non-small Cell Lung Cancer Carrying an EGFR Exon 19 Deletion

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Abstract. *Background/Aim: Cisplatin is a potent anticancer drug for treating several types of cancer, including non-small-cell lung cancer (NSCLC). In this study, we investigated the cytotoxicity and mechanism of action of cisplatin in the human NSCLC cell line PC9. Materials and Methods: PC9 cells were treated with cisplatin for 72 h and then evaluated by a cell viability assay, DAPI staining, Giemsa staining, apoptosis assay, membrane permeability assay, cell cycle assay, ROS assay, SA-β-gal staining, TUNEL assay and Western blotting. Results: Our findings revealed that the cytotoxic activity was associated with an apoptotic signaling pathway in response to DNA damage. Cisplatin exerted a significant concentration-dependent antiproliferative effect on PC9 cells. Cells subjected to cisplatin treatment showed morphological indications of apoptosis. Cell cycle arrest was related to the restriction of E2F-1 action by the cyclin-dependent protein kinase inhibitor $p21^{WAF1/CIP1}$. Cisplatin induced apoptosis of PC9 cells by upregulating Fas, FasL, Bak, and tBID expression and PARP proteolytic cleavage. Cisplatin also reduced the mitochondrial membrane potential (MMP) and initiated a caspase cascade. Furthermore, the apoptotic impact of cisplatin depended on reactive oxygen species (ROS), as confirmed by ROS generation. Conclusion: Cisplatin induced anticancer effects through cell cycle arrest, ROS generation and caspase activation, resulting in cell apoptosis. Overall, the results show the mechanism by which cisplatin works as an anticancer drug in the treatment of NSCLC.*

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Lung cancer is a leading cause of death worldwide (1, 2). EGFR mutations have been identified (3, 4) in 10%-30% of non-small-cell lung cancers (NSCLCs). Cisplatin is a standard drug for first-line treatment after surgery (5). Indeed, cisplatin chemotherapy was found to have reduced the rate of lung cancer-related death by 6.9% after 5 years compared with an untreated control group (6). However, the precise antineoplastic mechanism of cisplatin is not fully understood (7).

In multicellular organisms, apoptosis is a vital process of modified cell death. This cellular process acts as a barrier to cancer by removing damaged or unwanted cells (8, 9). Various stressors, including DNA damage, reactive oxygen species (ROS), viral disease, serum deprivation, hypoxia, and heat shock, initiate apoptosis. ROS are considered toxic to cell metabolism and regulate many physiological processes (10). In recent years, numerous studies have shown that oxidative stress can cause cell apoptosis through intrinsic (mitochondria) and extrinsic (death receptor) pathways (11, 12).

The essential molecular sensors of DNA damage incorporate the kinase ataxia telangiectasia mutated (ATM) and Rad3-related (ATR) checkpoint kinase (13-15). Due to genotoxic stress or DNA damage, these protein kinases accumulate at the site of DNA damage, forming nuclear foci (13, 16, 17). This process is supported by the enrollment and activation of other checkpoint molecules, including checkpoint kinase 1 (Chk1) and Chk2, triggering cell cycle arrest or apoptosis (13, 18, 19). Importantly, these checkpoint kinases can phosphorylate p53 at various serine sites (13, 18). Despite the general understanding of the DNA damage response, the molecular mechanisms underlying DNA damage and cell death caused by platinum-based cancer drugs is unclear (20, 21).

The p53-encoding TP53 gene is the most frequently mutated gene in human cancer (22, 23). The two main routes by which p53 hinders tumor cell development are growth arrest and apoptosis induction. p53 plays a crucial role in genotoxic stress-induced apoptosis following exposure to DNA-damaging agents (24). However, the mechanism by which p53 causes apoptosis is unclear. It has been proposed that p53 can control the expression of some proteins that participate in apoptosis and cell cycle arrest, such as Bax, Bak, and $p21^{WAF1/CIP1}$ (25, 26). At the same time, senescence appears to play a central role in the anticancer impact of various anticancer agents and ionizing radiation (27-29). Another prominent event during apoptosis is the proteolytic cleavage of PARP into 89- and 24-kDa fragments by caspases (30-32). PARP breakdown induced by DNA damage contributes to DNA repair and participates in cell cycle arrest, cell death, and cell survival signaling mechanisms (33, 34).

In the present study, we investigated the mechanism underlying the anticancer effects of cisplatin on PC9 cells. We herein report the impact of cisplatin on growth inhibition in PC9 cells.

Materials and Methods

Cell line and cell culture. The PC9 human NSCLC cell line (EGFR exon 19 deletion), derived from a previously untreated adenocarcinoma patient (35) was kindly provided by Professor K. Hayata (Tokyo Medical College, Tokyo, Japan). PC9 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), in 5% CO_2 at 37˚C.

Drug formulation and administration. Cisplatin [$PtCl_2(NH_3)_2$] was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The drug was dissolved in DMSO for the *in vitro* study.

Cell proliferation assay. The cytotoxicity of various cisplatin concentrations in PC9 cells was assessed using a water-soluble tetrazolium salt (WST-1) assay (Cell Proliferation Reagent WST-1; Roche, Tokyo, Japan). Into each well of a 96-well microtiter plate, 100 μl of a growing cell suspension (4×10^3 cells/well) was seeded, and 100 μl of cisplatin solution at various concentrations was added to each well (35). After incubation for 72 h at 37˚C in 5% CO_2, 10 μl of WST-1 solution was added to each well, and the plates were incubated at 37˚C for an additional 4 h (35). The absorbance was measured at 450 nm with a microplate enzyme-linked immunosorbent assay reader (Multiskan FC; Thermo Scientific, Tokyo, Japan).

Giemsa staining. Cells were treated with various cisplatin concentrations or DMSO (as control) for 72 h. After collection, the cells were resuspended in phosphate buffer solution and centrifuged in a Cytospin at 800 rpm for 8 min to adhere to a microscopic slide. After air-drying, the cells were fixed with methanol, stained with Giemsa (Merck KGaA, Darmstadt, Germany) for 20 min, rinsed in water, and air-dried again. Under a light microscope, morphological changes were observed to assess apoptosis.

Fluorescent staining of nuclei. PC9 cells from exponentially growing cultures were seeded in 12-well plates. After cisplatin treatment for 72 h, cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde phosphate buffer solution (FUJIFILM Wako Pure Chemical Corporation) for 7 min at room temperature. Subsequently, cells were permeabilized using 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 10 min at room temperature. Cells were then stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) for 10 min at room temperature and examined using a BZ-X710 All-in-One Fluorescence Microscope (Keyence, Osaka, Japan).

ROS assay. To measure ROS in cells by flow cytometry, we used an ab186029 (Abcam, Tokyo, Japan) Cellular Reactive Oxygen Species Detection Assay Kit (Deep Red Fluorescence), following the manufacturer's instructions. 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.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNL) assay. To quantify DNA fragmentation in apoptotic cells, we used the in situ Direct DNA Fragmentation (TUNEL) Assay Kit (ab66108, Abcam), following the manufacturer's instructions. In brief, cells were fixed with 1% paraformaldehyde in PBS and placed on ice for 15 min. Following this, the samples were treated with a staining solution and incubated at 37˚C for 60 min. After addition of rinse buffer in propidium iodide/RNase A solution, cells were resuspended and incubated at room temperature for 30 min for a flow cytometry analysis.

Western blotting. Cells were treated with several concentrations of cisplatin (or DMSO for control) for 72 h. After treatment, whole-cell lysates were prepared using a mammalian protein extraction reagent M-PER; Thermo Scientific) accompanied by a phosphatase inhibitor cocktail and protease inhibitor cocktail (Sigma-Aldrich) for 30 min at 4˚C. The BCA protein assay (Thermo Scientific) was used to evaluate protein concentrations. Total cellular protein (40 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Bio-Rad, USA). Milk-blocked blots were incubated at 4˚C overnight with primary antibodies against the following proteins: ATR (Cell Signaling Technology, Beverly, MA, USA), p-ATR (Ser 428) (Cell Signaling Technology), ATM (Cell Signaling Technology), p-ATM (Ser 1981) (Cell Signaling Technology), Chk1 (Cell Signaling Technology), p-Chk1 (Ser 345) (Cell Signaling Technology), Chk2 (Cell Signaling Technology), p-Chk2 (Thr 68) (Cell Signaling Technology), p-p53 (Ser 15) (Cell Signaling Technology), p-p53 (Ser 46) (Cell Signaling Technology), γ-H2AX (Phospho S140) (Abcam, Tokyo, Japan), Fas (Cell Signaling Technology), FasL (Cell Signaling Technology), Bak (Cell Signaling Technology), caspase-9 (Cell Signaling Technology), caspase-8 (Cell Signaling Technology), Bax (Cell Signaling Technology), BID (Cell Signaling Technology), cleaved caspase-7 (Asp 198) (Cell Signaling Technology), cleaved caspase-3 (Asp 175) (Cell Signaling Technology), cleaved caspase-6 (Asp 162) (Cell Signaling Technology), PARP (Cell Signaling Technology), cyclin D1 (BioLegend, San Diego, CA, USA), CDK4 (Cell Signaling Technology), E2F-1 (Cell Signaling Technology), $p21^{WAF1/CIP1}$ (Cell Signaling Technology), p53 (Cell Signaling Technology), Rb (Cell Signaling Technology), p-Rb (Ser 807/811) (Cell Signaling

Technology) and β-actin (Cell Signaling Technology), followed by the appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Proteins of interest were visualized using the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA) and the Invitrogen iBright FL1000 Imaging System (Thermo Fisher Scientific).

MMP evaluation. The MMP was evaluated using the JC-10 Mitochondrial Membrane Potential Assay Kit-Flow Cytometry (ab112133; Abcam). Cells were seeded in 75-cm^2 flasks (Falcon, USA), treated as indicated in the results, 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 FACSCanto™ II (BD Biosciences, San Jose, CA, USA).

Apoptosis assay. For 72 h, PC9 cells were treated with cisplatin at various concentrations (or DMSO as a control). FITC Annexin V Apoptosis Detection Kit with Pl Protocol (BioLegend) was used to evaluate the apoptotic cell percentage by flow cytometry analysis.

Flow cytometry for cell cycle analysis. For 72 h, PC9 cells were treated with cisplatin at several concentrations (or DMSO as a control). A cell cycle analysis was conducted following the Propidium Iodide Cell Cycle Staining Protocol (BioLegend). The DNA content was determined with a FACSCanto™ II. The data were analyzed using an FCS Express 7 (De Novo Software, Pasadena, CA, USA).

Senescence-associated β-galactosidase staining. Cells (1×10^6) were cultured in 25-cm^2 flasks (Falcon) and treated with various concentrations of cisplatin (or DMSO as a control) for 72 h. For senescence-activated β-galactosidase (SA-β-gal) staining, we used the Senescence-β-gal Staining Kit (Cell Signaling Technology) following the manufacturer's instructions.

Statistical analysis. Graphs were generated using the GraphPad PRISM software, v. 7.0 (GraphPad Software Inc., San Diego, CA, USA). Results are presented as the mean±standard deviation (SD) of three independent experiments and were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. Results were considered to be statistically significant when $p<0.05$.

Results

Impact of cisplatin on the viability of PC9 cells: morphological and nuclear changes. The water-soluble tetrazolium salt (WST-1) assay was performed to examine the effects of cisplatin on the viability of PC9 cells. Cisplatin treatment reduced the cell viability in a concentration-dependent manner (Figure 1A). Cells were stained with Giemsa and DAPI to investigate morphological changes induced by cisplatin. Cells going through cisplatin treatment showed morphological indications of apoptosis, such as cytoplasmic vacuolization (black arrows) and membrane blebbing (green arrows) (Figure 1B). These alterations were combined with nuclear shrinkage (red arrows) and nuclear fragmentation (yellow arrows) (Figure 1C).

Impact of cisplatin on ROS generation and DNA fragmentation. Increased ROS production can lead to mitochondrial dysfunction through depolarization of the mitochondrial membrane potential (MMP) (36-39). We examined whether cisplatin increased ROS production, which can trigger apoptosis. Indeed, compared to cells treated with the vehicle alone, cisplatin increased ROS production in a dose-dependent manner (Figure 2). These outcomes indicate that ROS might be involved in apoptosis of cisplatin-treated PC9 cells. These changes are related to DNA fragmentation, a key indicator of apoptosis (Figure 3A, 3B).

Impact of cisplatin on the DNA damage signaling pathway. After treatment with cisplatin, we analyzed the expression of DNA damage-related proteins. Oxidative DNA damage causes ATM phosphorylation at Ser 1981 and ATR phosphorylation at Ser 428 (40). The phosphorylation activation of these proteins promotes the phosphorylation of different downstream proteins (including H2AX, p53, Chk1, Chk2, *etc*.) and ultimately promotes cell cycle arrest and apoptosis (41, 42). ATR can phosphorylate a broad scope of protein substrates during genotoxic stress, bringing about the activation of complex signaling cascades (14, 16). A key sign of ATR activation during genotoxic stress is ATR accumulation in nuclear foci, where signaling proteins aggregate and connect due to DNA damage (17, 43). Genotoxic stress incited by camptothecin brings about the decay of Chk1 through the proteasomal pathway (44). As presented in Figure 3C, cisplatin treatment increased the protein expression of p-ATR (Ser 428), p-Chk2 (Thr 68), γ-H2AX (Phospho S140), p-p53 (Ser 15) and p-p53 (Ser 46) but decreased the protein expression of p-ATM (Ser 1981) and p-Chk1 (Ser 345). These results suggest that ATR-Chk2 signaling plays a crucial role in p53 activation and the DNA damage response during cisplatin treatment in PC9 cells.

Impact of cisplatin on MMP. Once generated, ROS can cause mitochondrial membrane permeabilization (45). Using the fluorescent cation dye JC-10, we evaluated the depolarization of the MMP. The intact MMP of non-apoptotic cells allows JC-10 to accumulate in the mitochondria, forming red fluorescent "J aggregates." In contrast, the MMP is broken down in apoptotic cells, resulting in JC-10 staying in the cytoplasm with a green fluorescent monomer structure (37). In cisplatin-treated PC9 cells, at low MMP (lower-right quadrant, Q3), JC-10 is predominantly a monomer that produces green fluorescence, indicating that cisplatin impairs mitochondrial integrity (as assessed by MMP loss) (Figure 4).

The impact of cisplatin on the induction of apoptosis in PC9 cells. To confirm that cisplatin-induced cell death was due to apoptosis, we used annexin V and PI staining to conduct a

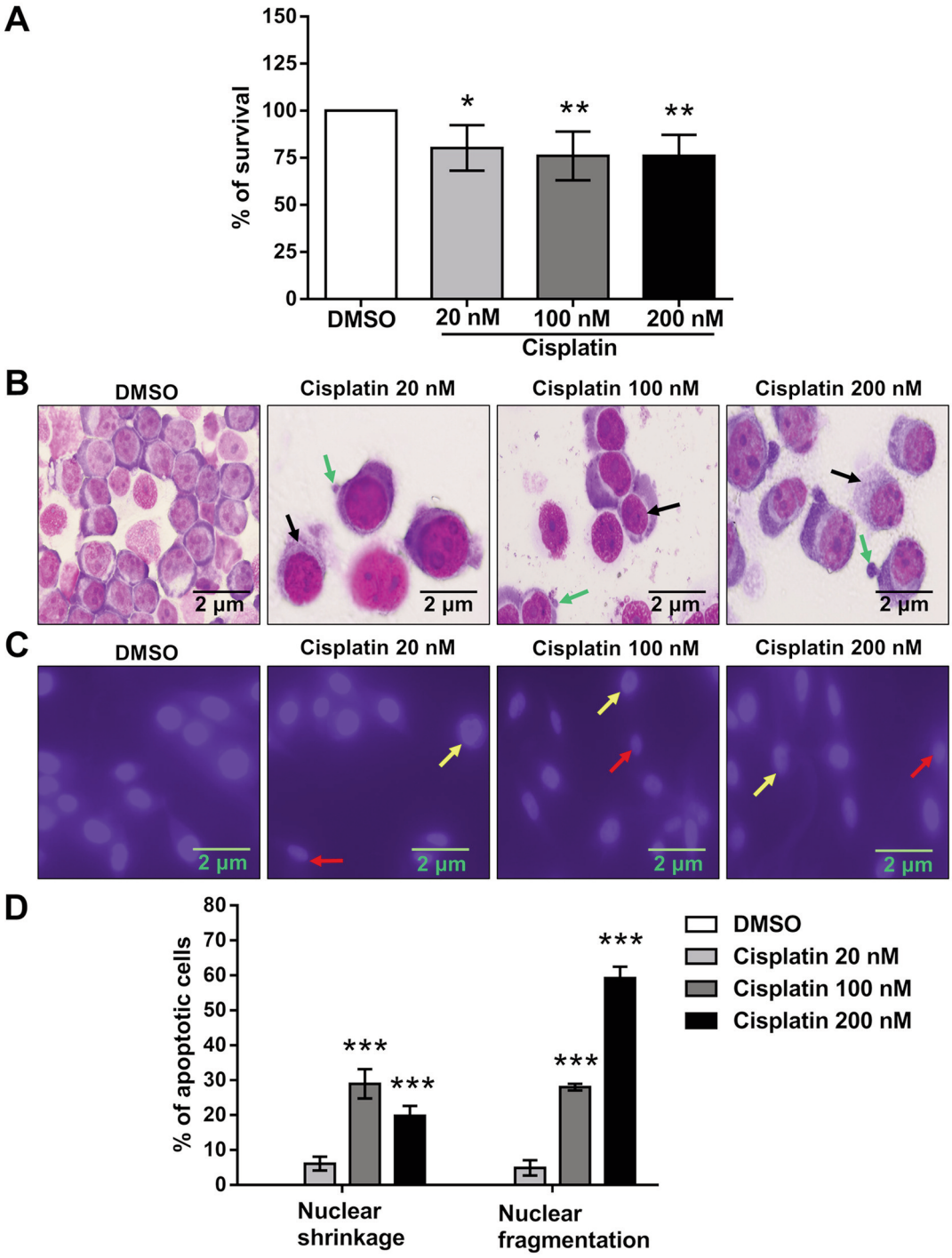

Figure 1. *Impact of cisplatin on the viability and morphology of PC9 cells. (A) After 72 h of treatment with various cisplatin concentrations, the cell viability was analyzed by the WST-1 cell proliferation assay. Data were acquired and presented as the mean±SD from six independent experiments. *p<0.05 and **p<0.01 compared with the DMSO-treated group using one-way ANOVA with Bonferroni's multiple comparison test. (B) Cells were treated with different concentrations of cisplatin (or DMSO as control) for 72 h and stained with Giemsa to detect morphological changes. Cells with cytoplasmic vacuolization (black arrows) and membrane blebbing (green arrows) were found among the cisplatin-treated cells but not the DMSO-treated controls. The images are representative results of three independent experiments. (C) Cells were treated at different concentrations of cisplatin (or with DMSO alone) for 72 h. Cells with condensed (red arrows) and fragmented (yellow arrows) fluorescent nuclei are seen among the cisplatin-treated cells but not the DMSO-treated control group. (D) The percentage of cells with nuclear shrinkage and nuclear fragmentation was increased in the cisplatin-treated groups but not in the DMSO-treated control cohort. Data were analyzed by one-way ANOVA with Bonferroni's multiple comparison test. ***p<0.001 compared with the DMSO-treated group.*

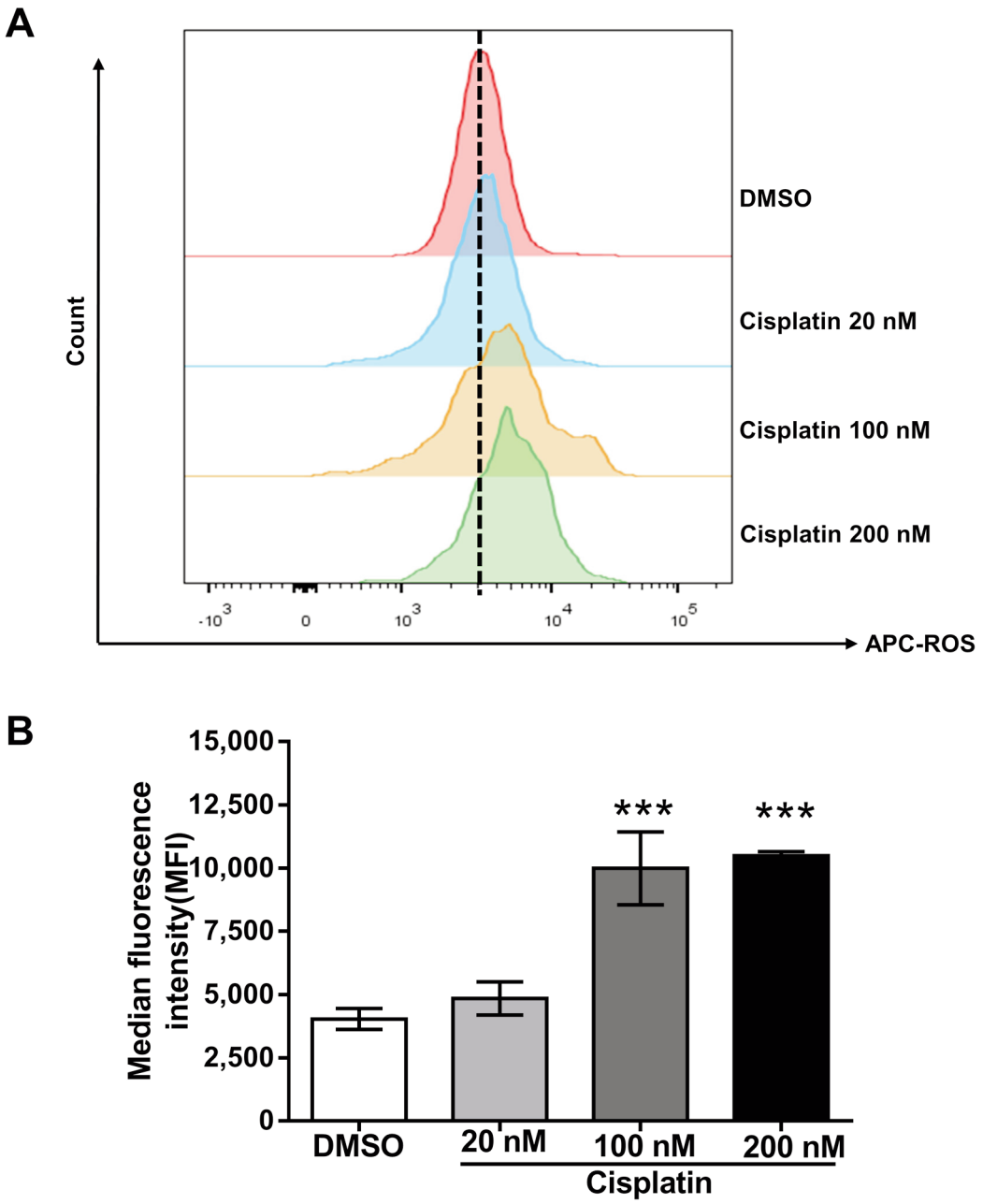

Figure 2. *The impact of cisplatin on ROS generation. (A) PC9 cells incubated with cisplatin at different concentrations (or DMSO as control) for 72 h were stained with ROS deep red dye. The fluorescent signal was measured by flow cytometry, and the median fluorescence intensity (MFI) was determined. 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. Data (mean±SD) are representative of three technical replicates. Data were analyzed by one-way ANOVA with Bonferroni's multiple comparison test. ***p<0.001 compared with the DMSO-treated group.*

flow cytometry analysis. As shown (Figure 5A), after 72 h of exposure to cisplatin, the proportion of late-apoptotic cells (upper right quadrant 2, Annexin V/PI positive) increased from 3.15% to 22.6%. Flow cytometry results showed that cisplatin-induced apoptosis was dose-dependent (Figure 5B).

Western blotting experiments targeting apoptosis marker proteins were performed to examine the molecular mechanism underlying cell death due to apoptosis following cisplatin treatment. As shown in Figure 5C, the expression of Fas and FasL increased with cisplatin treatment in a dose-dependent

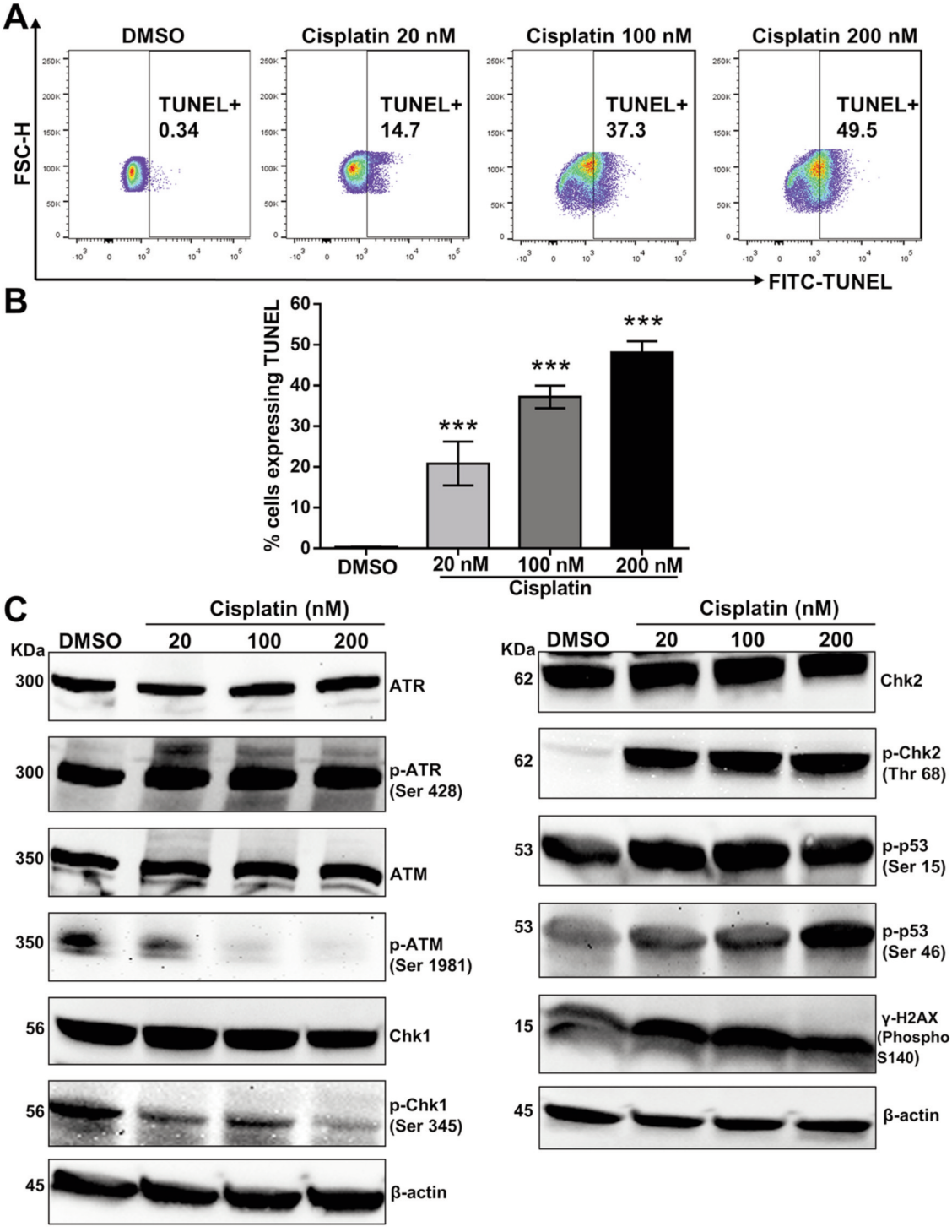

Figure 3. *The impact of cisplatin on DNA fragmentation and the DNA damage signaling pathway. (A) PC9 cells were stained with fluorescein isothiocyanate-dUTP dye after incubation with cisplatin at different concentrations for 72 h. Fluorescence was measured by flow cytometry. DNA fragmentation increased in a concentration-dependent manner, as represented by the dot plot. (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. ***p<0.001 compared with the DMSO-treated group, using one-way ANOVA with Bonferroni's multiple comparison test. (C) Total cell lysates from cisplatin-treated PC9 cells for 72 h were subjected to a Western blot analysis with the following antibodies: ATR, p-ATR (Ser 428), ATM, p-ATM (Ser 1981), Chk1, p-Chk1 (Ser 345), Chk2, p-Chk2 (Thr 68), p-p53 (Ser 15), p-p53 (Ser 46), and γ-H2AX (Phospho S140). β-actin served as a loading control. Representative Western blots are shown from three independent experiments.*

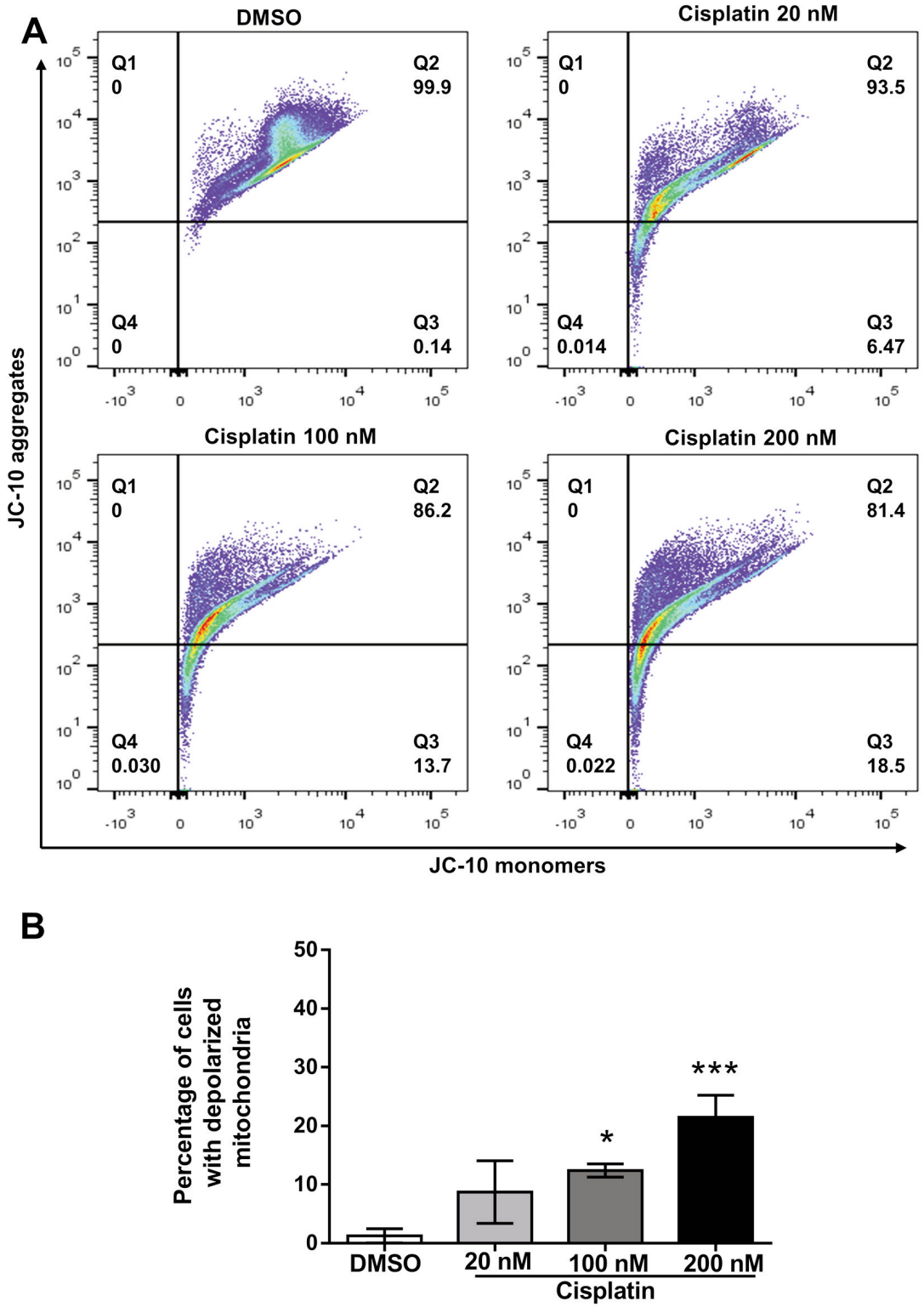

Figure 4. *The impact of cisplatin on the MMP. (A) PC9 cells were incubated with various cisplatin concentrations for 72 h to examine the MMP, stained with JC-10 dye, and analyzed by flow cytometry. Representative results are shown from three independent experiments. (B) Quantitative data are presented by green fluorescence (depolarized MMP) of JC-10 monomer. Results are presented as the mean±SD of three independent measurements. *p<0.05 and ***p<0.001 compared with the DMSO-treated group, using one-way ANOVA with Bonferroni's multiple comparison test.*

manner. Compared to the control group, the expression of the proapoptotic Bak protein was also increased.

Compared with the control group, cisplatin treatment increased the expression of truncated BID (tBID). tBID is located in the mitochondrial membrane, where it promotes the release of cytochrome c and enhances the process of apoptosis (11, 12). Compared to the untreated control, cisplatin also increased the levels of cleaved caspase-3 (Asp 175), caspase-9, caspase-8, caspase-6 (Asp 162) and caspase-7 (Asp 198), in a dose-dependent manner. Exposure of PC9 cells to cisplatin also resulted in cleavage of PARP (Figure 5C), a standard substrate of caspase-3; PARP cleavage is considered an indicator of cell apoptosis. These results suggested that cisplatin induced PC9 cell apoptosis.

Impact of cisplatin on cell cycle regulation and senescence induction in PC9 cells. Cell cycle arrest is an important cause of cell death. To study the cell cycle arrest induced by cisplatin, we used flow cytometry to analyze the cell cycle distribution in PC9 cells in order to evaluate the DNA content in each cell cycle phase. As shown (Figure 6A, 6B), compared to DMSO-treated control, cisplatin treatment resulted in a dose-dependent increase in the proportion of G1 phase cells, while the proportions of S and G2/M phase cells decreased. The data show that cisplatin induced G1 phase arrest.

Having shown that cisplatin induced cell cycle arrest, we endeavored to characterize the mechanisms by which this outcome was achieved. Activated cyclin D1, CDK4, and CDK6 enable cells to enter S phase from G1, during cell cycle progression (46, 47). The cyclin D1-CDK4/6 complex is responsible for DNA synthesis, while CKIs, such as $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, negatively regulate this process (48, 49). Western blotting (Figure 6C) revealed that cisplatin treatment significantly reduced cyclin D1, CDK4, and E2F-1 protein expression and led to the upregulation of the $p21^{WAF1/CIP1}$ expression, accompanied by p53 activation.

Since senescence is considered irreversible cell cycle arrest, especially in the G1 phase (50, 51), we observed the induction of senescence-associated beta-galactosidase (SA-β-gal) activity by cisplatin treatment. We found that, at a relatively high dose (200 nM) of cisplatin, the proportion of SA-β-gal-positive cells (black arrows indicate senescent cells) was lower and the staining weaker than in cells treated with 100 nM cisplatin (Figure 7). At this high dose, cellular damage may be too high for the cells to respond actively, resulting in significant growth inhibition and decreased cell viability.

Discussion

In the present study, we evaluated the cytotoxic impacts of cisplatin in PC9 cells. The results demonstrated that cisplatin caused intense growth inhibitory effects against PC9 cells. Cisplatin induced G1 phase arrest via an increase in $p21^{WAF1/CIP1}$ and decrease in cyclin D1, CDK4, and E2F-1 activities in PC9 cells. Further analysis showed that an upregulated Bak, tBID, Fas, and FasL expression, increased ROS production, and activated caspase cascade are associated with cisplatin-induced apoptosis.

ROS are constantly produced and eliminated. In both homeostasis and infection, ROS play a vital role. In mitochondria, increased ROS generation plays an essential role in apoptosis (52, 53). The oxidative pressure created by ROS can damage parts of the cell, including DNA and proteins (54). Many studies have confirmed that ROS can disrupt cell cycle progression and induce apoptosis by activating the ATM and ATR pathways (55). The present study showed that exposure to cisplatin causes ROS production, enhancing the cytotoxic effects associated with DNA damage.

The DNA damage response (DDR) is a complex signaling pathway (17). It includes sensor proteins that detect DNA damage and activate signal transducer proteins including ATM and ATR, resulting in the activation of effector proteins (Chk1 and Chk2) that control DNA repair, cell cycle arrest, and apoptosis (13, 14, 17). In this study, we show that ATR but not ATM was activated during cisplatin treatment (Figure 3C).

Activated ATR can phosphorylate H2AX in response to single-stranded DNA breaks. The increase in ATR activity during cisplatin therapy is an essential factor in evaluating therapeutic efficacy (16). The ATR-mediated Chk1 phosphorylation at serine 345 is necessary to achieve maximum kinase activity (14). Chk2 is not homologous to Chk1 but participates in DNA structure modification (56). In response to genotoxic stress, Chk2 is phosphorylated at threonine 68 (56). After DNA damage, the monomeric Chk2 undergoes dimerization through phosphorylation, promoting kinase activity (56). DNA double-strand breaks induced ATM-Chk2 pathway activation (56, 57). However, ATR-dependent Chk2 activation has also been reported (58). Our present results showed ATR-dependent Chk2 phosphorylation at threonine 68 during cisplatin treatment (Figure 3C).

Mitochondria react to numerous death stimuli, including those related to the Bcl-2 group of proapoptotic proteins, such as Bax and Bak, which cause permeabilization of the mitochondrial membrane and release of apoptotic molecules (59-62). Furthermore, Bak can induce cell apoptosis in a Bax-dependent or independent manner (60, 63, 64). Conversely, Fas receptor and tumor necrosis factor receptor 1 (TNFR1) can activate caspase-8 and release the active sections of caspase-8 (p18 and p10) (65-67). Activated caspase-8 cleaves and activates downstream effectors like BID, caspase-3, 6, 7 and 9, eventually prompting the morphological signs of apoptosis. In the present study, cisplatin increased the Bak, Fas, and FasL expression as well as the proteolysis of BID protein in PC9 cells (Figure 5C).

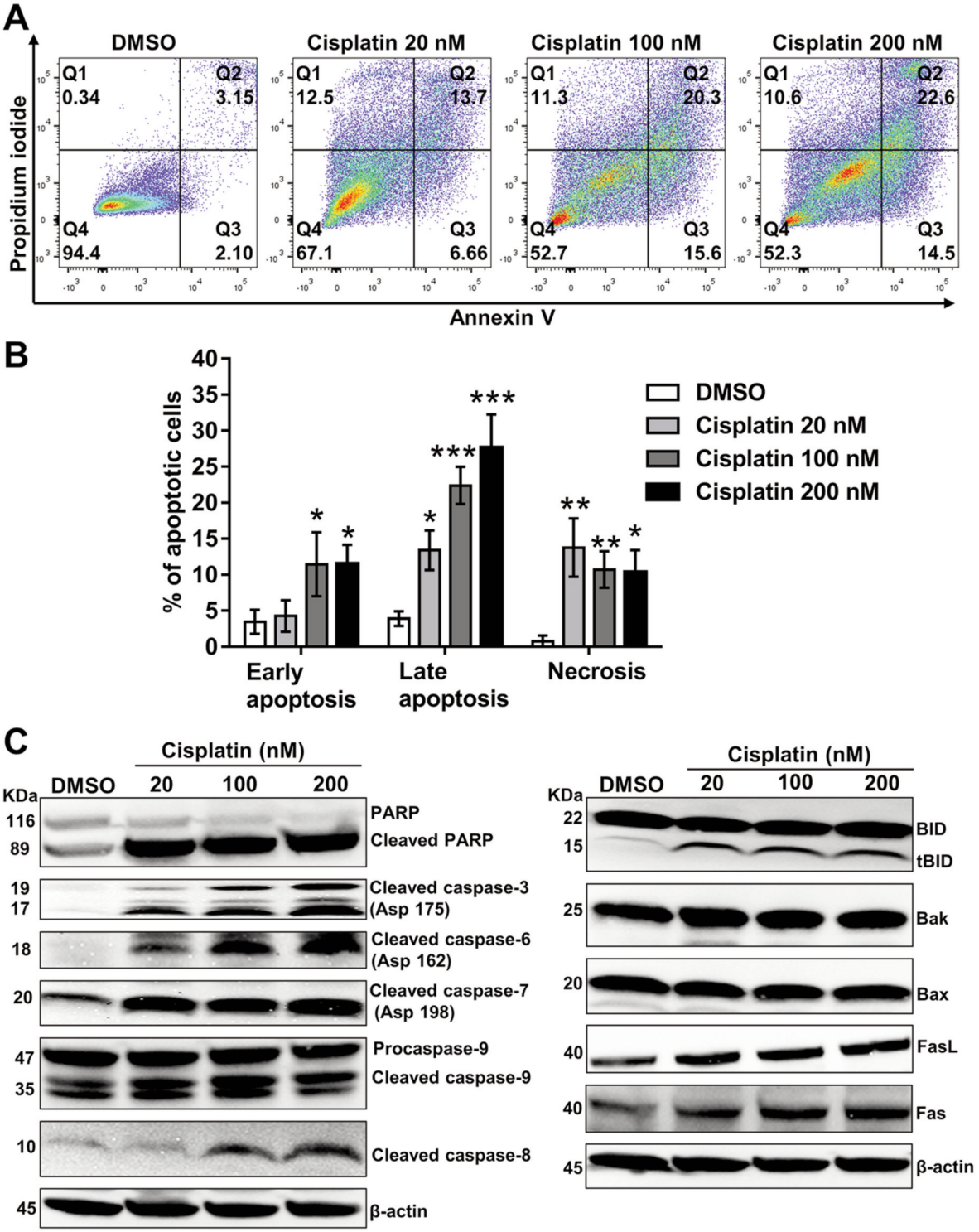

Figure 5. *The impact of cisplatin on the induction of apoptosis in PC9 cells. (A) Apoptosis was assessed by Annexin V/PI dual staining. Flow cytometry was used to evaluate the number of Annexin V- and PI-positive cells. Quadrant 1 shows necrotic cells, quadrant 2 late-apoptotic cells, quadrant 3 early-apoptotic cells and quadrant 4 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 one-way ANOVA with Bonferroni'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 Fas, FasL, Bak, caspase-9, caspase-8, Bax, BID, cleaved caspase-7 (Asp 198), cleaved caspase-3 (Asp 175), cleaved caspase-6 (Asp 162), and PARP proteins, Western blotting was performed. β-actin served as a loading control. Representative immunoblots from three independent experiments are shown.*

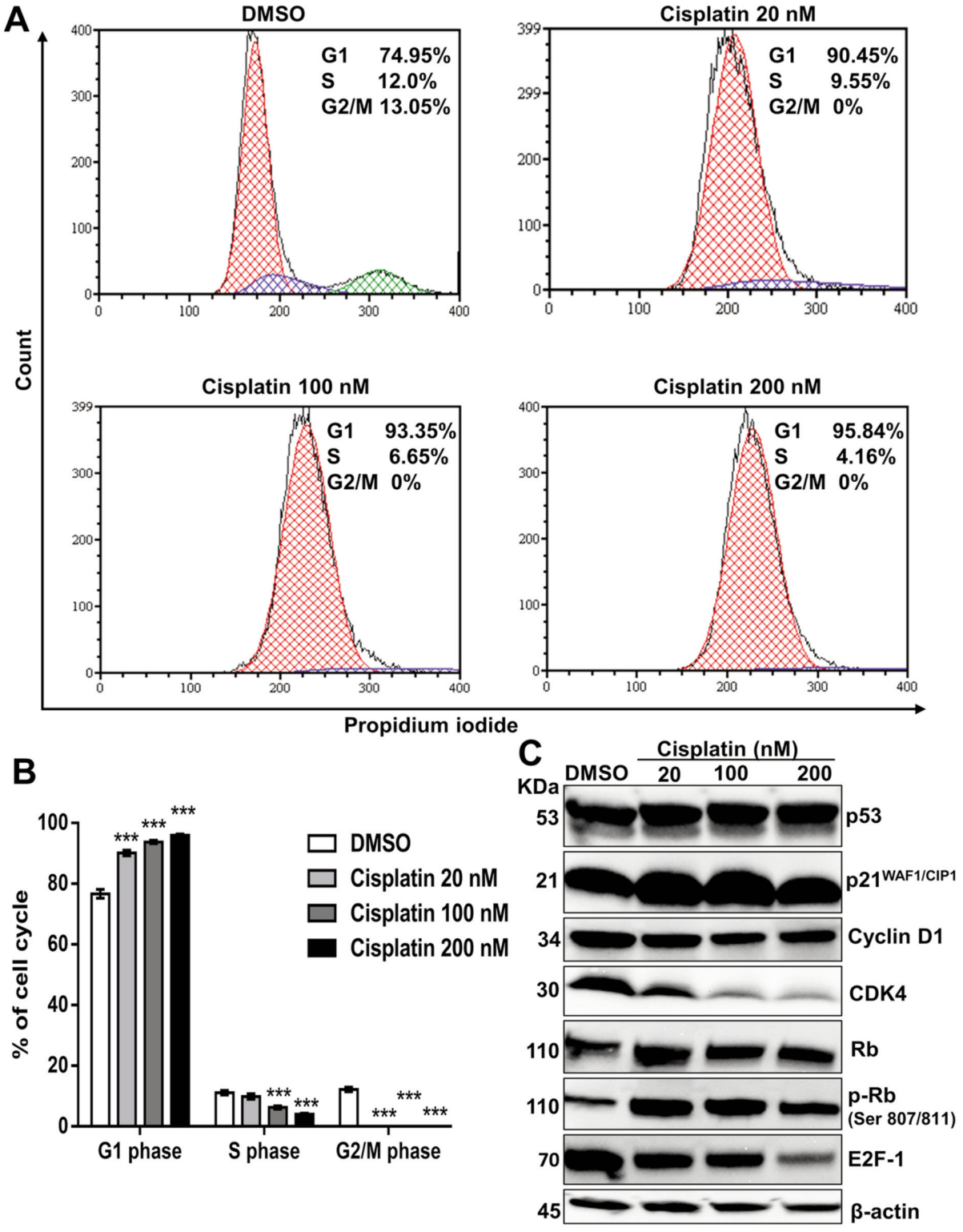

Figure 6. *The impact of cisplatin on cell cycle regulation in PC9 cells. (A) PC9 cells were treated with DMSO or cisplatin (20 nM, 100 nM and 200 nM) for 72 h and stained with propidium iodide (PI) before a flow cytometric analysis. Representative results from three independent experiments are shown. (B) The results are presented as the mean±SD of three independent experiments. ***p<0.001 compared with the DMSO-treated group, using one-way ANOVA with Bonferroni's multiple comparison test. (C) For the Western blot analysis, PC9 cells were treated with DMSO or cisplatin (20 nM, 100 nM, 200 nM) for 72 h. β-actin served as a loading control. Representative immunoblots from three independent trials are shown.*

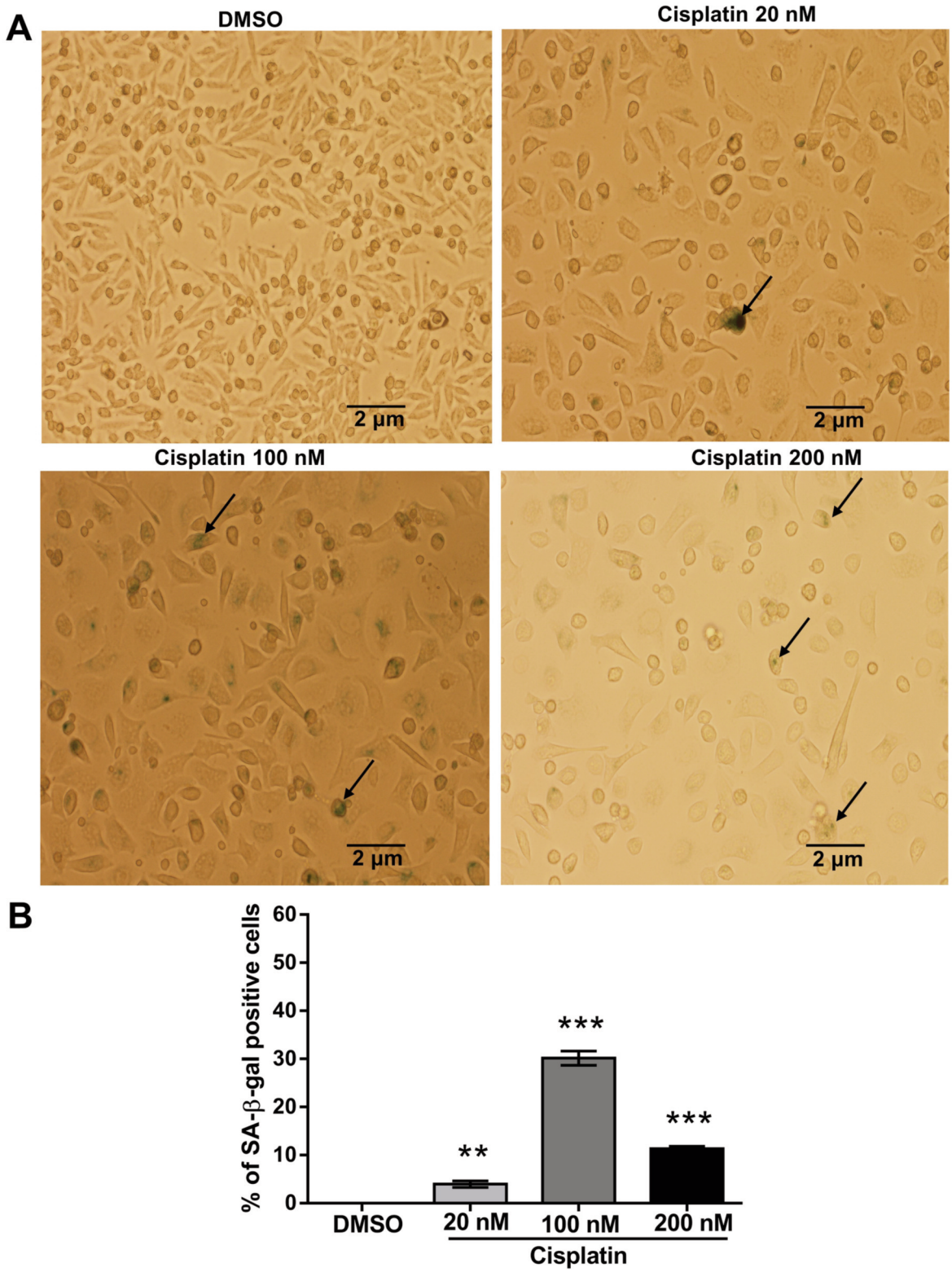

Figure 7. *Impact of cisplatin on senescence induction in PC9 cells. (A) Before staining, PC9 cells were treated with different concentrations of cisplatin (DMSO-treated control) for 72 h. The cells showing SA-β-gal activity were stained green (black arrows). (B) The bar diagram represents the percentage of senescent cells. Values are the mean±SD of three independent experiments. Significance was determined by one-way ANOVA with Bonferroni's multiple comparison test: **p<0.01 and ***p<0.001 compared with the DMSO-treated group.*

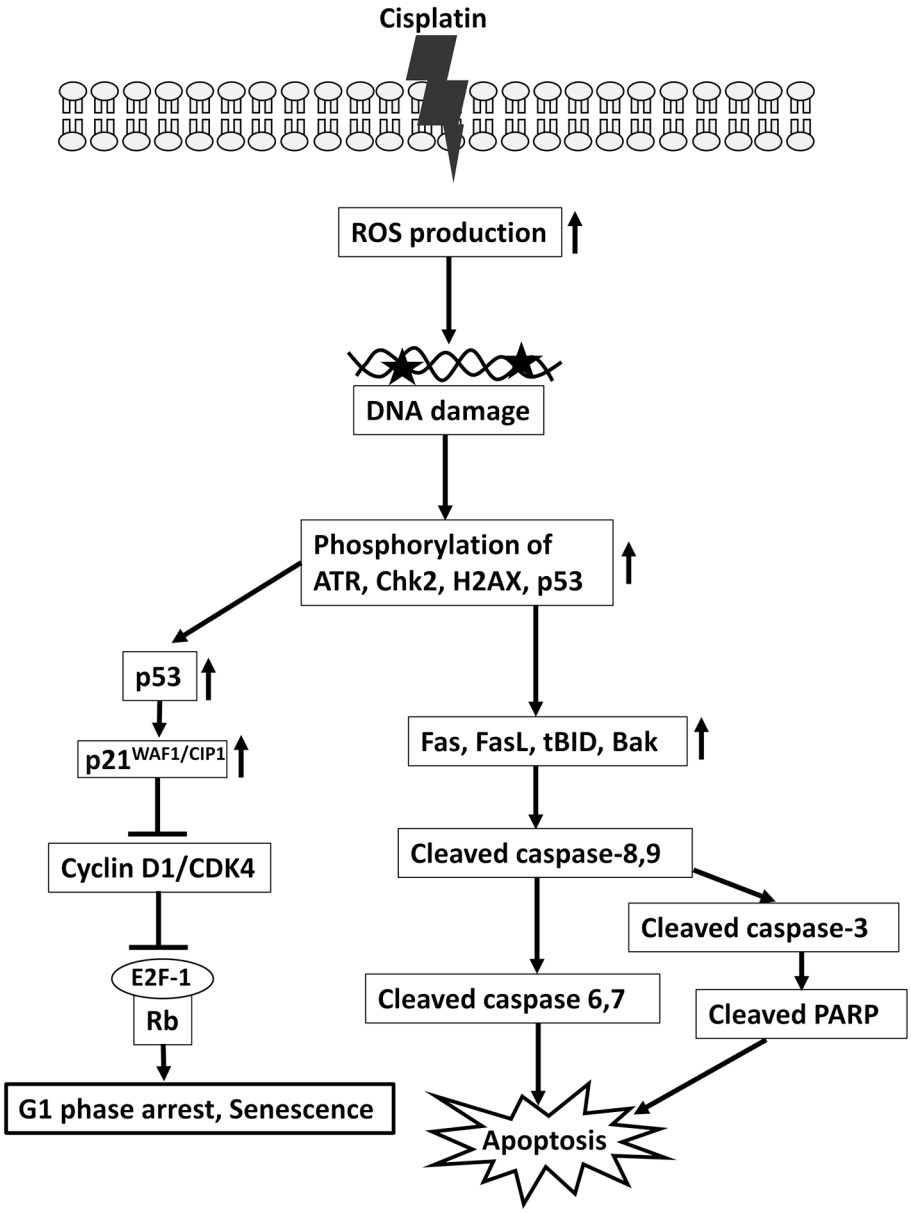

Figure 8. *A schematic illustration demonstrates the effects and mechanism of cisplatin on apoptosis and cell cycle distribution in PC9 cells. Cisplatin enhances ROS production, triggers DNA damage response signaling, and activates a caspase cascade to induce apoptosis. It also demonstrates that cisplatin up-regulates p21$^{WAF1/CIP1}$ and inhibits the activities of cyclin D1, CDK4, and E2F-1 to induce G1 phase arrest in PC9 cells.*

Previous studies have shown that mitochondrial phosphorylation of p53 to Ser 15 can mediate Bak activation (68). The phosphorylation of p53 to Ser 46 controls the ability of p53 to cause apoptosis (69). Activated caspase-9 prompts other caspases to activate, including caspase-3, 6 and 7, and start a caspase cascade, resulting in apoptosis (70-73). Caspase-3 is essential because it contributes to the proteolytic cleavage of many key proteins, similar to the nuclear PARP enzyme (74). Our study provides evidence in support of this conclusion.

After cisplatin treatment, the viability of PC9 cells decreased in a concentration-dependent manner, demonstrating the strong cytotoxic activity of cisplatin, which was related to an increase in the proportion of cells in G1. This indicated a cell cycle block. Cell cycle phases are controlled through various cyclins and their complex

development with CDKs (75). The activity of cyclin D-CDK4/6 drives cells through the early G1 phase of the cell cycle, whereas cyclin E-Cdk2 complexes control the transition from the G1 phase into the S phase (76). In the present study, a flow cytometry analysis showed that cisplatin induced PC9 cell arrest in G1 phase by inhibiting the activity of CDK4, cyclin D1, and E2F-1. Members of the CIP/KIP family ($p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) and INK4 family ($p15^{INK4b}$, $p16^{INK4a}$, $p19^{INK4d}$ and $p18^{INK4c}$) can inhibit the activities of cyclin-CDK complexes (77). $p21^{WAF1/CIP1}$ negatively regulates cyclin-CDK complexes for G1 phase arrest. Previous studies have shown that anticancer drugs can cause cell cycle arrest through a p53-dependent or independent manner (78, 79). In the present study, cisplatin upregulated the $p21^{WAF1/CIP1}$ expression in PC9 cells, accompanied by an increase in the p53 protein level. $p16^{INK4a}$ (80) and $p21^{WAF1/CIP1}$ (81) cause cellular senescence (82-84). The retinoblastoma (Rb) tumor suppressor protein plays an important role at decision points in the G1 phase of the cell cycle (85). E2F-1 is an essential transcription factor that controls cell cycle progression and cell proliferation. E2F-1, 2, and 3 bind to pRb, and these connections are under the control of the cell cycle (86, 87). The present findings suggest a possible mechanism by which cisplatin upregulates the $p21^{WAF1/CIP1}$ expression, leading to G1 arrest by inhibiting the E2F-1 activity.

Conclusion

Cisplatin can inhibit PC9 cell proliferation through oxidative damage caused by ROS generation, cell cycle arrest at the G1 phase, and activation of a caspase cascade to induce apoptosis. Overall, our outcomes clarify how cisplatin can be used as a therapeutic agent against PC9 cells, a representative NSCLC cell line. We have proposed a model of the major mechanism of the induction of apoptosis and cell cycle arrest by cisplatin in PC9 cells (Figure 8).

Conflicts of Interest

The Authors declare no competing financial interests.

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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