The Mechanisms of the Growth Inhibitory Effects of Paclitaxel on Gefitinib-resistant Non-small Cell Lung Cancer Cells

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Abstract. Background/Aim: Coronavirus disease 2019 (COVID-19) poses a great challenge for the treatment of cancer patients. It presents as a severe respiratory infection in aged individuals, including some lung cancer patients. COVID-19 may be linked to the progression of aggressive lung cancer. In addition, the side effects of chemotherapy, such as chemotherapy resistance and the acceleration of cellular senescence, can worsen COVID-19. Given this situation, we investigated the role of paclitaxel (a chemotherapy drug) in the cell proliferation, apoptosis, and cellular senescence of gefitinib-resistant non-small-cell lung cancer (NSCLC) cells (PC9-MET) to clarify the underlying mechanisms. Materials and Methods: PC9-MET cells were treated with paclitaxel for 72 h and then evaluated by a cell viability assay, DAPI staining, Giemsa staining, apoptosis assay, a reactive oxygen species (ROS) assay, SA-β-Gal staining, a terminal deoxynucleotidyl transferase dUTP nick-end labeling assay and Western blotting. Results: Paclitaxel significantly reduced the viability of PC9-MET cells and induced morphological signs of apoptosis. The apoptotic effects of paclitaxel were observed by increased levels of cleaved caspase-3 (Asp 175), cleaved caspase-9 (Asp 330) and cleaved PARP (Asp 214). In addition, paclitaxel increased ROS production, leading to DNA damage. Inhibition of ROS production by N-acetylcysteine attenuates paclitaxel-induced DNA damage. Importantly, paclitaxel eliminated cellular senescence, as observed by SA-β-Gal staining. Cellular senescence elimination was associated with p53/p21 and p16/pRb signaling inactivation. Conclusion: Paclitaxel may be a promising anticancer drug and offer a new therapeutic strategy for managing gefitinib-resistant NSCLC during the COVID-19 pandemic.

Recent studies have reported that cancer patients have a higher mortality rate than usual due to the 2019 coronavirus disease (COVID-19), especially those patients with lung cancer (1-3). Consequently, COVID-19 has altered lung cancer treatment provision, but the type and extent of these variations are still unknown. Chemotherapy side effects, such as resistance to chemotherapy (e.g. resistance to gefitinib) and cellular senescence, may also make treatment more challenging, demanding promising drug therapy during the COVID-19 pandemic.

In the United States, lung cancer accounts for nearly a quarter of all cancer deaths (4). Non-small-cell lung cancer (NSCLC) is the most common subtype and accounts for 85% of lung cancer cases. Most NSCLC patients are diagnosed in the advanced stage, so surgery is no longer possible, despite significant advances in the early detection and prevention of lung cancer (5-8). Paclitaxel, the most widely used anticancer drug, is used to treat various cancers (9-12). However, the mechanisms underlying its anticancer activities on PC9-MET cells have not been fully clarified. Targeted cancer therapies may be more effective than traditional chemotherapy, as standard chemotherapy interferes with all dividing cells.

Reactive oxygen species (ROS) production is increased by the effects of chemotherapy or radiotherapy on the downstream signaling cascade that regulates the cell survival or death (13-15). ROS production may therefore be targeted to prevent cancer or enhance the treatment response (15, 16). ROS production is recognized as hallmark of DNA damage (17). Chemotherapy drugs enhance the ROS production, thereby increasing genotoxicity (18). The primary source of endogenous DNA damage and double-strand break (DSB) production in cancer is oncogene-induced replication stress (19). ATM/ATR kinases and a network of sensory proteins play an essential role in the DNA damage response (20). H2AX phosphorylation at Ser 139 (γH2AX) is an early cellular response to the induction of DNA DSBs (21) and
represents the most well-established chromatin modification related to DNA repair and damage response (22).

Depending on the type and extent of damage, DNA damage can trigger apoptotic cell death (23). Caspases, a family of protease enzymes, play an essential role in achieving apoptosis. There are two main signaling pathways that activate the caspase cascade: intrinsic (mitochondrial pathway) and extrinsic (death receptor pathway) (24). Following DNA damage, these pathways activate caspase-3, which plays a central role in apoptotic signaling, while caspase-9 and caspase-8 are the initiators of the intrinsic and extrinsic pathways, respectively (25).

Cancer cells can undergo senescence in response to chemotherapeutic agents (9). Due to the shortening of telomeres at the ends of chromosomes, this replicable senescence can occur in cancer cells (26). Therapy-induced cellular senescence may have a short-term benefit against the tumor, but it can also activate invasion-related genes and exacerbate tumor progression (27).

Many researchers are now focusing on suitable chemotherapy drugs for repurposing to treat COVID-19 (28, 29). Additionally, preliminary findings suggest that chemoresistance and cellular senescence are associated with COVID-19 complications (30-32). In the current study, we hypothesized that paclitaxel might exert growth inhibitory and cellular senescence-suppressing effects on gefitinib-resistant NSCLC cells, which might be a novel treatment that can reduce COVID-19 severity. To evaluate our hypothesis, this study investigated the molecular mechanisms by which paclitaxel inhibits the proliferation of PC9-MET cells and the mechanisms underlying the senescence system.

Materials and Methods

Cell line and cell culture. We established the gefitinib-resistant the PC9-MET subline as previously described (33). PC9-MET 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% CO₂.

Drug preparation. Paclitaxel [C₄₂H₇₄NO₁₄] was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). This drug was dissolved in 0.1% dimethyl sulfoxide (DMSO) for in vitro experiments.

Cell proliferation assay. The cytotoxicity of different paclitaxel concentrations in PC9-MET cells was assessed using a watersoluble 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 (4x10^3 cells/well) was seeded, and 100 μL of paclitaxel solution at concentrations of 50 and 100 nM (or 0.1% DMSO as a control) was added to each well (33). After incubation for 72 h at 37°C in 5% CO₂ atmosphere, 10 μL of WST-1 solution was added to each well, and the plates were incubated at 37°C for an additional 4 h (33). The absorbance was measured at 450 nm with a microplate enzyme-linked immunosorbent assay reader (Multiskan FC; Thermo Scientific, Tokyo, Japan).

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

Intracellular ROS assay. We used the Cellular Reactive Oxygen Species Detection Assay Kit (Deep Red Fluorescence, ab186029; Abcam, Tokyo, Japan) to determine the intracellular ROS level. In brief, after treatment with paclitaxel (50 and 100 nM) or with 0.1% DMSO as a control for 72 h, cells were harvested and incubated with ROS Deep Red Dye Working Solution at 37°C for 60 min. Subsequently, cells were subjected to a flow cytometry analysis. Fluorescent intensities were measured with a FACScanto II (BD Biosciences) flow cytometer using APC channel.

A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. For 72 h, PC9-MET cells were treated with different concentrations of paclitaxel (50 and 100 nM) or with 0.1% DMSO as a control (25). 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.

Apoptosis assay. PC9-MET cells were treated with different concentrations of paclitaxel (50 and 100 nM) or with DMSO as a control for 72 h. Apoptotic cell death was quantified by flow cytometry using the FITC Annexin V Apoptosis Detection Kit with propidium iodide (BioLegend, San Diego, CA, USA).

Western blotting. For 72 h, PC9-MET cells were treated with 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. Whole cell lysates (40 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: cleaved caspase-3 (Asp 175), cleaved caspase-9 (Asp 330), cleaved PARP (Asp 214), p53, p21, p16, pRB, and γH2AX (ser 139) and then with the appropriate horseradish peroxidase-conjugated secondary...
antibodies. All antibodies were obtained from Cell Signaling Technology, Beverly, MA, USA, except for anti-γH2AX that was obtained from Abcam. Proteins of interest were revealed 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). Bands were quantified with the densitometric program of iBright Imaging System and normalized against β-actin.

Senescence-associated β-galactosidase staining. For 72 h, PC9-MET cells (1x10^6) were cultured in 25-cm² flasks (Falcon) and treated with paclitaxel (50 and 100 nM) or with 0.1% DMSO as a control. To detect senescent cells, the Senescence-β-gal Staining Kit (Cell Signaling Technology, Beverly, MA, USA) was used according to the manufacturer's instructions. The cells were washed twice with PBS and incubated in 1 ml of fixative solution at room temperature for 15 min. After removing the fixative solution and

Figure 1. Effect of paclitaxel on the viability and morphology of PC9-MET cells. (A) After 72 h of treatment with different concentrations of paclitaxel (50 and 100 nM), the cell viability was analyzed by the WST-1 cell proliferation assay. From three independent experiments, data were acquired and presented as the mean±SD. A one-way ANOVA with Dunnett's multiple comparison test determined the significance: *p<0.05 compared with the DMSO-treated group. (B) DAPI and (C) Giemsa staining of PC9-MET cells treated with different paclitaxel concentrations for 72 h.
washing the fixed cells twice, the cells were stained with 1 ml of β-galactosidase staining working solution and incubated at 37˚C for 12 h under light protection. After staining, the cells were washed, and the senescent cells were identified using a light microscope (Olympus, Tokyo, Japan).

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. Differences between the groups with \( p<0.05 \) were considered statistically significant. All graphs were created using the GraphPad PRISM 7.0 software program (GraphPad Software Inc., San Diego, CA, USA).

Results

Paclitaxel inhibits the growth of PC9-MET cells. Previous studies suggested that paclitaxel might inhibit the proliferation of cancer cells by inducing apoptosis (34, 35). Therefore, it was crucial to determine whether or not paclitaxel affected the growth of PC9-MET cells. As shown in Figure 1A, paclitaxel inhibited the growth of PC9-MET cells in a concentration-dependent manner. Conversely, paclitaxel-treated cells were stained with Giemsa and DAPI to investigate morphological changes. As indicated in Figure 1B and C, treatment of PC9-MET cells with paclitaxel produced apoptotic features, such as nuclear fragmentation.

Paclitaxel treatment causes ROS-mediated DNA damage. Many chemotherapy drugs induce apoptosis through ROS-mediated cell damage (36, 37). Compared to the DMSO-treated cells, paclitaxel increased the ROS level in a concentration-dependent manner (Figure 2A). To prove that the cell death caused by paclitaxel was due to DNA fragmentation, we conducted a TUNEL assay. As presented in Figure 3A, after 72 h of paclitaxel treatment, the proportion of fragmented cells increased from 1.24% to 74.4%.

Although our data revealed that paclitaxel-induced DNA damage was associated with increased ROS production in PC9-MET cells, it remains unclear whether or not paclitaxel inhibits ROS-mediated damage to DNA using NAC (ROS scavenger). To this end, we pre-incubated PC9-MET cells with NAC before paclitaxel treatment. As indicated in Figure 2B and Figure 3B, our data showed that NAC pretreatment significantly inhibited ROS production and DNA damage. Taken together, these results strongly support the hypothesis that paclitaxel induces ROS-mediated DNA damage in PC9-MET cells.

DNA damage caused by paclitaxel leads to γH2AX formation. Previous studies have suggested that many chemotherapy agents can kill cancer cells by inducing DNA damage (38, 39). Phosphorylated H2AX (γH2AX) is a strong marker of DNA DSBs due to its early appearance and essential role in the DSB response (40). As shown in Figure 4, paclitaxel markedly increased the expression of γH2AX protein compared to DMSO-treated group. These outcomes show for the first time that the anticancer effect of paclitaxel is, at least in part, due to paclitaxel-induced DNA damage in PC9-MET cells.

Paclitaxel induces apoptosis in PC9-MET cells. To quantify the percentage of cells undergoing apoptosis, we conducted...
Figure 3. The impact of paclitaxel on DNA fragmentation. (A) PC9-MET cells were stained with fluorescein isothiocyanate-dUTP dye after incubation with paclitaxel at different concentrations for 72 h. (B) The bar diagram shows the quantification of DNA fragmentation. Results are the mean±SD of three independent experiments. Comparisons were made between the control group and experimental group using a one-way ANOVA followed by Sidak’s multiple comparison test. **p<0.01 and ***p<0.001.
Figure 4. Generation of γH2AX after treatment with paclitaxel. The generation of γH2AX after 72 h treatment with paclitaxel was analyzed by Western blotting. β-actin was used as a standard for the equal loading of protein for SDS-PAGE. The data (mean±SD) are representative of three independent experiments. A one-way ANOVA followed by Dunnett's multiple comparison test determined the significance. NS, Not significant.

Figure 5. Paclitaxel induced apoptosis in PC9-MET cells. (A) Flow cytometry of apoptosis in PC9-MET cells by Annexin V/PI dual staining. 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<0.01 and ***p<0.001 compared with the DMSO-treated group.
Figure 6. The effects of paclitaxel on the expression of caspase cascade proteins. PC9-MET cells were incubated for 72 h with paclitaxel, and the expression of cleaved caspase-3 (Asp 175), cleaved caspase-9 (Asp 330) and cleaved PARP (Asp 214) was assessed via immunoblotting. β-actin was used as a loading control. Results are the mean±SD of three independent experiments. A one-way ANOVA with Dunnett's multiple comparison test determined the significance: *p<0.05, **p<0.01 and ***p<0.001 compared with the DMSO-treated group. NS, not significant.
Annexin V-FITC and PI fluorescence staining. As presented in Figure 5A, after 72 h of exposure to paclitaxel, the proportion of necrotic cells was significantly increased, while that of viable cells was significantly decreased compared to the DMSO-treated control group. The flow cytometry results showed that paclitaxel treatment increased the percentage of necrotic cells in a dose-dependent manner (Figure 5B).

Previous studies have reported that caspases can serve as the primary mediators of apoptosis (25, 41). To further validate our annexin data, we checked the expression of cleaved caspase-3 (Asp 175), cleaved caspase-9 (Asp 330) and cleaved PARP (Asp 214). Western blot analysis showed an increase in the expression of cleaved caspase-3 (Asp 175), cleaved caspase-9 (Asp 330), and cleaved PARP (Asp 214) in a dose-dependent manner (Figure 6), suggesting that these proteins are involved in paclitaxel-induced apoptosis.

Paclitaxel eliminated cellular senescence of PC9-MET cells. Cancer cells can undergo cellular senescence in response to clinically used chemotherapeutic agents (42, 43). Recent studies have provided evidence that some drugs can selectively eradicate senescent cells (44, 45). Therefore, it is essential to characterize senescent cells and recognize them correctly, especially when it comes to cancer. To this end, we investigated the senescence status of PC9-MET cells. To determine whether paclitaxel eliminated or induced cellular senescence in PC9-MET cells, we analyzed the effect of paclitaxel on the SA-β-Gal activity. The number of SA-β-Gal-positive cells was significantly decreased after paclitaxel treatment, compared to the control group (Figure 7A and B), suggesting that paclitaxel has an effect of eliminating cellular senescence in PC9-MET cells.

Previous studies have suggested that the suppression of the p53/p21 and p16/pRb signaling pathways can inhibit cellular senescence (46-48). Herein, western blot analysis showed a decrease in the expression of p53, p21, hypo-phosphorylated pRb and p16 (Figure 7C), suggesting that these proteins may be involved in the elimination of cellular senescence of PC9-MET cells.

Discussion
This current study showed that paclitaxel inhibits PC9-MET cell proliferation, enhances ROS production, and triggers DNA damage. We found that caspase cascade activation by paclitaxel plays a vital role in apoptotic cell death. Paclitaxel eliminated cellular senescence, which was linked with the suppression of p53/p21 and p16/pRb signaling. To our
Figure 7. Elimination of cellular senescence in PC9-MET cells. (A) Representative bright-field microscopy images of SA-β-Gal staining in PC9-MET cells treated with DMSO or paclitaxel (50 nM and 100 nM) for 72 h. Cells show SA-β-Gal activity stained in blue. (B) The bar graph shows the percentage of senescent cells. The data (mean±SD) are representative of three independent experiments. Significance was determined by a one-way ANOVA followed by Dunnett’s multiple comparison test: ***p<0.001 compared with the DMSO-treated group. (C) The effects of paclitaxel on the expression of cellular senescence regulatory proteins. The expression of p53, p21, pRb and p16 was assessed via Western blotting. β-actin was used as a loading control. Results are the mean±SD of three independent experiments. A one-way ANOVA with Dunnett’s multiple comparison test determined the significance: *p<0.05, **p<0.01 and ***p<0.001 compared with the DMSO-treated group. NS, not significant.
knowledge, the anticancer effect of paclitaxel on PC9-MET cells has not been well documented, and this is the first study to investigate this point.

ROS are generated as natural by-products of normal cellular activity, playing a vital role in cell signaling (49). The elevated ROS production leads to apoptosis pathways mediated by death receptors, mitochondria, and endoplasmic reticulum (ER) (50). Furthermore, excessive ROS generation can damage lipids, cellular proteins and DNA (51). The present study demonstrated the paclitaxel-induced ROS-mediated DNA damage in PC9-MET cells (Figures 2 and 3). ATM and ATR kinases are activated in response to DNA damage, where ATM is principally triggered by DSBs, but ATR acts in response to a wide range of DNA damage (52). A previous study reported that γH2AX is an early indicator of DNA damage caused by replication stress (53), with other studies supporting this finding. One such study indicated that γH2AX is a sensitive indicator of DNA replication stress and DNA damage (54). Our present study demonstrated elevated levels of γH2AX during paclitaxel treatment in PC9-MET cells (Figure 4).

DNA fragmentation is a sign of apoptosis triggered by multiple apoptotic stimuli (55). After DNA damage, the release of cytochrome c from the mitochondria and the subsequent activation of procaspase-9 is crucial for activating subsequent apoptotic effectors (25). Thus, activated caspase-9 can cleave and directly activate other effector caspases, such as caspase-3 (25). Finally, activated caspase-3 enhances the proteolytic cleavage of PARP, which is a hallmark of apoptosis (56, 57). The present study showed that paclitaxel-activated caspase-9 propagates apoptotic signaling by activating the downstream effector caspase-3 resulting in PARP cleavage (a hallmark of apoptosis) (Figures 5 and 6).

Cellular senescence is a phenomenon in which cells stop dividing and undergo many distinctive phenotypic alterations, including chromatin rearrangement, metabolic reprogramming, and activation of tumor suppressors (58). Many studies have reported that p53/p21 pathway activation is responsible for inducing cellular senescence (58-60). Furthermore, the activation of p16/pRb signaling is a vital indicator of senescence (58, 61). This study demonstrated that paclitaxel suppressed the expression of p53, p21, hypophosphorylated pRb and p16, which might be linked to the elimination of cellular senescence of PC9-MET cells (Figure 7). We have proposed a model of the major mechanisms of induction of apoptosis through ROS-mediated DNA damage and the elimination of cellular senescence through the inactivation of the p53/p21 and p16/pRb signaling by paclitaxel in PC9-MET cells (Figure 8).

Lung cancer patients are at an increased risk of contracting COVID-19 and related diseases as well as dying (62). The COVID-19 pandemic has increased the difficulty
of treating lung cancer (63). The most important risk factors for patient death with COVID-19 were pre-existing lung cancer, an elderly age, and resistance to chemotherapy (64-66). Several studies have claimed that cellular senescence is a potential mediator of COVID-19 severity in the elderly (67-69). Therefore, there is an urgent need to develop new therapies targeting cellular senescence and chemotherapy resistance during the COVID-19 pandemic. The current study showed that paclitaxel suppressed cellular senescence and induced apoptosis in gefitinib-resistant NSCLC cells, which may aid in the development of a new therapeutic approach, reducing the severity of COVID-19 in NSCLC cancer patients resistant to gefitinib treatment. These findings may also improve the protection of lung cancer patients, although more preclinical and clinical studies will be needed to confirm our results.

Conclusion

Our results revealed novel mechanisms of action of paclitaxel, indicating a potential anticancer effect in PC9-MET cells. Moreover, our findings highlight a promising direction for the development of a novel therapeutic strategy for gefitinib-resistant NSCLC during the COVID-19 pandemic. Further studies are needed to explore how COVID-19 interacts with lung cancer and characterize the possibility of adverse events in COVID-19 patients undergoing chemotherapy treatment.

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.

Acknowledgements

We would like to thank Ms. Miki Kashiwano (Department of Respiratory Medicine, Graduate School of Medical Sciences, Kanazawa University) for her technical assistance. This work was supported by Grant-in-Aid for Scientific Research (C) (JSPS KAKENHI Grant Number 17K09606) to K.K. The funders had no role in the study design, data collection, or interpretation or decision to submit the work for publication.

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