

Ivermectin Enhances Paclitaxel Efficacy by Overcoming Resistance Through Modulation of *ABCB1* in Non-small Cell Lung Cancer

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Abstract. *Background/Aim:* Chemoresistance to paclitaxel (PTX) significantly ameliorates therapeutic efficacy in patients with non-small cell lung cancer (NSCLC), especially in advanced stages, deteriorating the progression free and overall survival rates. One of the critical mechanisms contributing to drug resistance is the excretion of PTX from target cells via efflux pumps. Ivermectin was developed as a bactericidal agent against parasites; however, it has recently been shown to inhibit the proliferation of human cancer cells. Hence, we aimed to evaluate the therapeutic potential of ivermectin in combination with PTX and investigate the molecular mechanisms by which ivermectin overcomes PTX resistance. *Materials and Methods:* We assessed the antitumor effects of ivermectin in A549 cells treated with or without PTX. We also established PTX-resistant cells using this cell line and explored the underlying mechanisms. Additionally, we evaluated whether ivermectin attenuates PTX-resistance with the retrieval of drug sensitivity. *Results:* Combined treatment of A549 cells with PTX

and ivermectin inhibited cell growth. These cells acquired chemoresistance upon long-term exposure to gradually increasing PTX concentrations, which was accompanied by *ABCB1* mRNA up-regulation, and subsequent overproduction of P-glycoprotein (P-gp). Consistent with this, P-gp over-expression resulted in a PTX-resistant phenotype. Notably, the simultaneous ivermectin treatment during the gradual exposure completely abolished P-gp expression, leading to an increased intracellular PTX concentration and sustained PTX sensitivity. Ivermectin was found to regulate P-gp expression via the EGFR/ERK/Akt/NF- κ B pathway. *Conclusion:* Combined treatment of PTX-resistant A549 cells with ivermectin and PTX may circumvent PTX resistance caused by P-gp induction, highlighting a novel therapeutic avenue for drug repurposing.

As one of the most prevalent malignant tumors, lung cancer is associated with high mortality and morbidity. According to cancer statistics, there were approximately 2.48 million newly diagnosed cases and 1.81 million deaths worldwide in 2022 (1). Chemotherapy remains the primary treatment option for patients with lung cancer who are ineligible for surgery or radiotherapy. Despite extensive advances in targeted molecular therapy and immunotherapy, cytotoxic agents continue to play a pivotal role in the clinical management of non-small cell lung cancer (NSCLC), a major subtype of lung cancer, especially in patients with advanced stages (2-4). However, the acquisition of chemoresistance toward these agents severely reduces therapeutic efficacy.

Cancer chemotherapy is impeded by drug resistance, which is derived from either the intrinsic resistance of tumor cells to drug action or the selection of cells capable of circumventing the drug action despite the initial response (5). The latter comprises several mechanisms, including decreased drug uptake into cells, increased drug efflux, activation of detoxifying

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enzymes (e.g., cytochrome P450), activation of the DNA repair system, and inhibition of apoptotic signaling pathways (6). Notably, drug efflux is a crucial strategy employed by cancer cells for survival and ATP-binding cassette (ABC) transporters function as efflux pumps, enabling cancer cells to excrete substrates through cellular membranes (7, 8). ABC transporter proteins, encoded by seven ABC genes (*ABCA-ABCG*), contain two domains: the transmembrane domain (TMD) and the nucleotide-binding domain (9). P-glycoprotein (P-gp) is translated from the *ABCB1* gene, consisting of 14 targeted binding sites, including 12 TMDs and two ATP-binding sites (10). In several different cancer cells, P-gp actively pumps drugs out of the cells, decreasing their efficacy, thereby inducing cancer resistance. Therefore, modulating P-gp expression using potent inhibitors appears to be a promising therapeutic strategy for circumventing multidrug resistance (MDR).

Ivermectin belongs to the group of avermectins, which is a group of 16-membered macrocyclic lactone compounds discovered in 1967 in the Kitasato Institute in Japan (11). Ivermectin was found to be especially effective against a variety of diseases caused by parasites, including onchocerciasis, lymphatic filariasis, and malaria (12-14). Besides these bactericidal effects, ivermectin has recently been reported to inhibit the growth of human cancer cells (11, 15-17). In addition, ivermectin has been suggested to inhibit P-gp, implicating its inherent properties in overcoming MDR (18-20). However, whether these findings are applicable to lung cancer cells has yet to be determined.

We herein hypothesized that ivermectin regulates lung cancer growth by modulating P-gp expression and cell growth. To validate this hypothesis, we assessed the effect of ivermectin on cell proliferation using the adenocarcinoma cell line A549 in combination with the cytotoxic agent paclitaxel (PTX), a member of the taxane family that binds to microtubules and interferes with mitotic processes (21). We also established PTX-resistant cells using this cell line, and evaluated the effect of ivermectin on P-gp expression and recovery from PTX resistance. Furthermore, we investigated the regulatory mechanisms by which ivermectin controls P-gp expression. Based on our extensive research experiences on *ABCB1* and lung cancer chemoresistance (22, 23), elucidating the pivotal role of ivermectin in lung cancer cells with an MDR phenotype could provide novel insights into chemoresistance and warrants further exploration towards identifying potential therapeutic targets for this refractory disorder.

Materials and Methods

Cell culture. We obtained the A549 human lung adenocarcinoma cell line from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). This cell line was cultured in RPMI-1640 (FUJIFILM, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Biosera, Cholet, France) and 1% penicillin and

streptomycin (FUJIFILM) at 37°C in a 5% CO₂ incubator. This sample was obtained in 2021 and routinely examined for the absence of mycoplasma.

Drugs and cell viability assay. Ivermectin was purchased from FUJIFILM. PTX, U0126, SC75741, and tariquidar were purchased from Selleck Chemicals (Houston, TX, USA). Elacridar was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The effects of PTX and ivermectin on A549 cells were assessed with a tetrazolium (MTS) assay as previously described (22, 24-26). Briefly, cell suspensions (5,000 cells/well) were seeded into 96-well plates, and increasing concentrations of drugs or vehicle (dimethyl sulfoxide) were added. After incubation at 37°C for 72 h, 10% Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was added to each well and cells were further incubated at 37°C for 60 min. After shaking the plate, absorbance was measured at a test wavelength of 450 nm using a microplate reader (Infinite M200 PRO; Tecan Group Ltd., Männedorf, Switzerland). The IC₅₀ value was defined as the concentration of PTX or ivermectin required for 50% reduction in growth. All experiments were independently repeated at least thrice.

Apoptosis assay. A549 cells (2×10⁵ cells/well) were seeded onto six-well plates in medium containing PTX with or without ivermectin, and incubated at 37°C in 5% CO₂. After 96 h of incubation, cells were trypsinized, collected, and stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) using an apoptosis detection kit (Nacalai Tesque Inc., Kyoto, Japan) according to the manufacturer's protocol. A total of 10,000 cells were acquired with the BD FACSVerser flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Analyses were performed with Flow Jo software ver.10.2 (Becton Dickinson). The percentage of total apoptotic cells, which were both Annexin V positive and Annexin V/PI double positive cells, was calculated (23).

Western blot analysis. Protein samples were lysed in a buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, and 0.5% sodium deoxycholate. Western blotting was performed as previously described (25, 27). The membranes were incubated with the following primary antibodies. Antibodies against P-gp (#22336-1-AP) were purchased from Proteintech (Rosemont, IL, USA). Antibodies against epidermal growth factor (EGF) Receptor (EGFR; #2232), phospho-EGF Receptor (Tyr1068) (p-EGFR; #2234), Akt (Akt; #9272), phospho-Akt (Ser473) (p-Akt; #9271), p44/42 MAPK (Erk1/2) (Erk; #9102), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (p-Erk; #9101), nuclear factor-κB (NF-κB) p65 (D14E12) XP (NF-κB; #8242), and phospho-NF-κB p65 (Ser536) (95H1) (p-NF-κB; #3033) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #sc-47724) were purchased from Santa Cruz Biotechnology. Goat Anti-Rabbit IgG-HRP (#4030-05) or Goat Anti-Mouse IgG₁-HRP (#1071-05) were used as secondary antibodies and purchased from Southern Biotech (Birmingham, AL, USA). Quantification was performed by densitometry using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Preparation of cell-line stably overexpressing *ABCB1* using A549 cells. To prepare a targeting plasmid against Adeno-Associated Virus Integration Site 1 (*AAVS1*), a safe harbor locus in humans, pX330-U6-Chimeric_BB-CBh-hSpCas9 (pX330, #42230; Addgene,

Watertown, MA, USA) was used. After digestion of pX330 with BbsI (R3539; NEB, Ipswich, MA, USA), the following sense and anti-sense strands of oligo ssDNA were annealed and inserted as spacer sequences targeting the *AAVS1* locus (sense strand: CACC GTCACCAATCCTGTCCCTAG, anti-sense strand: AACCTAG GGACAGATTGGTGAC) (28). This vector was treated as pX330-AAVS1. For the donor vector of the genomic integration of *ABCB1* into the *AAVS1* locus, the *ABCB1* protein-coding sequence was obtained from the A549 cell line. Total RNA was extracted from A549 cells using Trizol reagent (#15596026; Thermo Fisher Scientific, Waltham, MA, USA), and the protein-coding sequence of *ABCB1* mRNA was cloned by RT-PCR using the SuperScript IV One-Step RT-PCR System (#12595025; Thermo Fisher Scientific) with the following primers (*ABCB1* sequences are underlined): *ABCB1_CDS_F*: TCTGCAGTCGACGCCACCATGGATCTTGAA GGGGACCG, *ABCB1_CDS_R*: AGCGGGTTTAAACTCGAGTC ACTGGCGCTTTGTTC. The PCR product was seamlessly inserted into the *AAVS1* hPGK-PuroR-pA donor (#22072; Addgene) using the NEBuilder HiFi DNA Assembly Kit (E2621; NEB). This vector was treated as pAAVS1-*ABCB1*. For integration of the *ABCB1* transgene into the *AAVS1* locus, 10^5 cells of A549 were transfected with 0.15 μ g of pX330-AAVS1 and 0.45 μ g of pAAVS1-*ABCB1* using the Neon Transfection System (MPK5000; Thermo Fisher Scientific). Two days after transfection, the cells were continuously cultured in medium with puromycin (3.0 μ g/ml) for one week, and then maintained in normal medium. After cloning the cells by limiting dilution, the genotype of *AAVS1* locus in each clone was determined by PCR using the following primers: *AAVS1_Geno_F1*: CGGTCCTGGACTTTGTCTCC, *AAVS1_Geno_R1*: CGAACGGA CGTGAAGAATGTG, *AAVS1_Geno_F2*: GATGAAGCCACGTC AGCTCT, *AAVS1_Geno_R2*: GGCCACG TAACCTGAGAAGG.

RNA extraction and quantitative real-time reverse transcription-PCR. Total RNA was extracted from cultured cells using the Isogen reagent (Nippon Gene, Tokyo, Japan), and its concentration was determined using a NanoDrop 2000 (Thermo Fisher Scientific). RNA was reverse-transcribed to cDNA using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The primers, cDNA, and THUNDERBIRD Probe qPCR Mix (Toyobo) were mixed, and qPCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, San Francisco, CA, USA). *ABCB1* (Hs00184500) gene expression was measured using the TaqMan Gene Expression Assay (Thermo Fisher Scientific) and compared to *GAPDH* (Hs02786624) gene expression as an internal control. The gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method.

Establishment of PTX-resistant cells. The establishment of PTX-resistant A549 cells were performed using a stepwise method, as previously described (29, 30). Briefly, the cells were treated with gradient PTX concentrations (1, 1.5, 3, 5, 7.5, 10, 20, 40, 60, 80, 100, and 120 nM) for 16 weeks (wks). We named the PTX-resistant cells A549/PTXR.

Measurement of the intracellular PTX concentration using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples were separated chromatographically on an ACQUITY Premier UPLC system (Waters Corporation, Milford, MA, USA). The separation was performed at 40°C with an Acquity BEH C18 (1.7 μ m, 2.1×50 mm, Waters Corporation). The mobile phase had a flow of 0.4 ml/min and the injection volume was 5 μ l. Mass analysis was performed using a

QTRAP 6500+ (SCIEX, Tokyo, Japan) in the multiple reaction monitoring (MRM) mode (31, 32).

Statistical analysis. Data were expressed as the mean±standard error (SE) of three independent experiments, unless otherwise stated. Differences between the mean values of two groups were evaluated using an unpaired Student's *t*-test. Differences between three or more groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's test. A *p*-value <0.05 was considered statistically significant. Analyses were performed using SPSS statistical software (version 29, IBM, Chicago, IL, USA).

Results

The combination of PTX and ivermectin synergistically induces cell death in NSCLC cells. Initially, we examined the effects of PTX and ivermectin on the proliferation of A549 cells using the MTS assay. This cell line was highly sensitive to PTX with an IC_{50} value of 0.0027 ± 0.00023 μ M. Ivermectin was also a potent inhibitor of cell proliferation, with an IC_{50} value of 4.29 ± 0.768 μ M. Subsequently, we compared the anti-proliferative effects of the combination treatment with PTX and ivermectin. As expected, PTX in combination with ivermectin exhibited a significant anti-proliferative effect on A549 cells when compared with either treatment alone (Figure 1A). Furthermore, this was corroborated by enhanced apoptotic activity as documented by increased proportions of annexin V-positive cells (Figure 1B).

Ivermectin attenuates P-gp expression in A549 cells via the inhibition of EGFR/ERK/Akt/NF- κ B pathway. Besides an anti-microbial effect, ivermectin has been shown to inhibit P-gp expression in several solid and hematologic tumor cell lines, including colorectal and breast cancer, and chronic myeloid leukemia cells (20). Based on these observations, we tested whether ivermectin similarly decreases P-gp expression in A549 cells. As illustrated in Figure 2A, P-gp expression was hindered in response to ivermectin treatment in a concentration-dependent manner. Previous studies have demonstrated that EGFR signaling regulates P-gp expression (33, 34). Accordingly, to evaluate whether EGFR signaling is involved in P-gp expression and ivermectin-induced P-gp down-regulation, we assessed the changes in EGFR phosphorylation using western blotting. As shown in Figure 2B, EGFR activation was inhibited by ivermectin and this was highlighted when co-treated with PTX. We then evaluated the molecules located downstream of EGFR. The levels of phosphorylated ERK and Akt were found to be markedly reduced by ivermectin treatment as determined by western blotting (Figure 2B). NF- κ B is also known to be involved in the regulation of P-gp expression and the drug resistance in tumor cells (35). Consistently, NF- κ B phosphorylation was also attenuated by ivermectin treatment (Figure 2B). Notably, U0126 and SC75741, an MEK inhibitor that hinders ERK activity and an NF- κ B inhibitor, respectively, attenuated P-gp expression (Figure 2C).

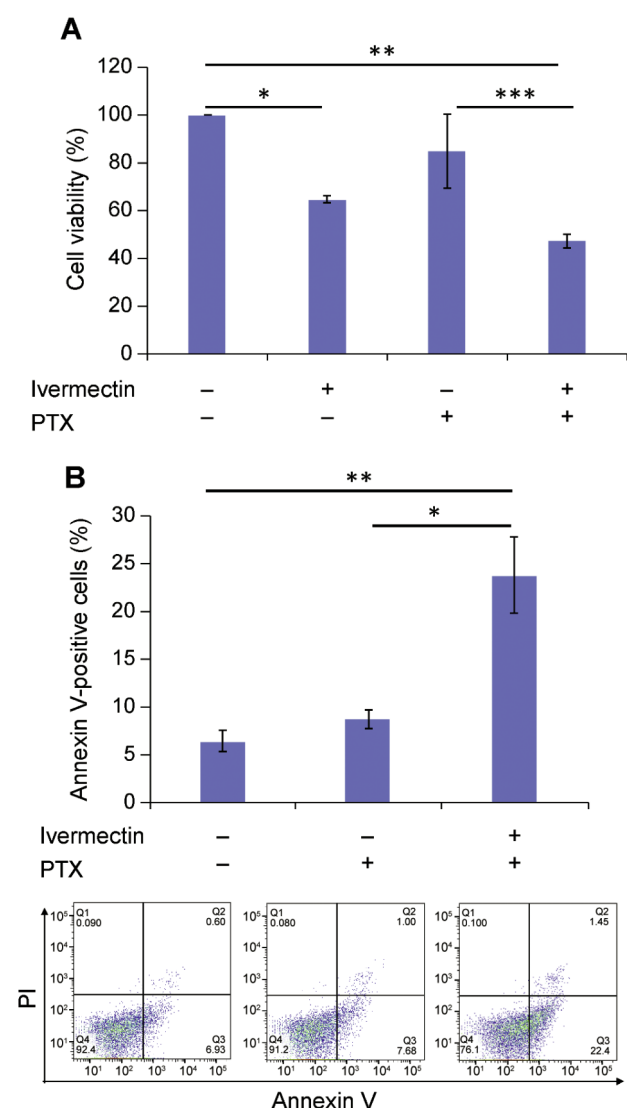


Figure 1. Effect of the combination of paclitaxel (PTX) and ivermectin on cell viability in A549 cells. (A) A549 cells were incubated with PTX (0.001 μM) or ivermectin (3 μM), or the combination for 72 h. The anti-proliferative effect of the combination treatment was increased relative to PTX or ivermectin alone. * $p < 0.001$, ** $p = 0.008$, and *** $p = 0.006$ by one-way ANOVA followed by Tukey-Kramer's test. (B) Apoptosis assay performed using an Annexin V-FITC Apoptosis Detection Kit. The percentages of apoptotic cells were higher with combination treatment. * $p = 0.002$ and ** $p = 0.001$ by one-way ANOVA followed by Tukey-Kramer's test.

Effect of inhibition of ABC transporters on PTX-induced NSCLC cell death. Subsequently, we assessed whether the ABCB1 and ABCG2 inhibitors, elacridar and tariquidar, enhance the therapeutic effects of PTX. A549 cells were cultured with either elacridar or tariquidar along with PTX. Elacridar partially accelerated the effects of PTX (Figure 3A), whereas tariquidar did not affect cell viability (Figure 3B).

Long-term exposure to PTX and up-regulation of ABCB1 expression in A549 cells. Since chemoresistance generally develops after long-term exposure to agents, we continuously exposed A549 cells to gradually increasing concentrations of PTX for up to 16 weeks (wks). As illustrated in Figure 4A, the A549/PTXR cells exhibited an apparent chemoresistant phenotype compared to the parental cells. These changes were accompanied by a robust increase in ABCB1 gene expression, reaching up to 80 times the baseline levels (Figure 4B). Notably, this was followed by a decrease in PTX concentration in PTX-resistant cells (Figure 4C).

Effect of over-expression of ABCB1 in A549 cells on PTX-induced cell death. To explore the roles of P-gp, we generated a cell line that could stably over-express ABCB1 with genome editing. The structure of the vector pAAVS1-ABCB1 is shown in Figure 5A. The exogenous expression of ABCB1 is confirmed at mRNA levels by qRT-PCR (Figure 5B). Using this cell line, we compared the cell viability in response to PTX between parental and three clones of ABCB1 over-expressing A549 cells. As shown in Figure 5C-E, A549 cells over-expressing ABCB1 exhibited PTX resistance.

Simultaneous ivermectin treatment hinders ABCB1 up-regulation induced by PTX prolonged exposure. The aforementioned data prompted us to investigate whether simultaneous administration of ivermectin during long-term PTX exposure could inhibit the increase in P-gp expression, thus preventing the development of chemoresistance to PTX. To test this hypothesis, A549 cells were continuously treated with ivermectin during the induction of PTX-resistance using a stepwise method. As depicted in Figure 6A, the attenuation of P-gp expression by concurrent ivermectin treatment was sustained until 16 weeks of PTX exposure. Consistent with this, PTX-sensitivity was maintained in A549 cells via simultaneous ivermectin treatment with significantly lower IC₅₀ values compared to cells cultured with PTX alone by 16 weeks (4 wks; 0.004±0.0001 vs. 0.130±0.068 μM , 8 wks; 0.010±0.0003 vs. 0.132±0.025 μM , 12 wks; 0.007±0.001 vs. 0.266±0.047 μM , 16 wks; 0.013±0.006 vs. 0.331±0.011 μM) (Figure 6B). As expected, the intracellular PTX concentration was significantly increased in A549 cells co-treated with ivermectin (Figure 6C). EGFR activation was inhibited by ivermectin co-treatment in A549/PTXR cells, followed by ERK and Akt inactivation (Figure 6D). The same was observed for NF- κ B phosphorylation induced by long-term PTX exposure (Figure 6D).

Discussion

In the current study, we demonstrated that chemoresistance upon long-term exposure to a stepwise increase in PTX was attributed to ABCB1 up-regulation accompanied by P-gp

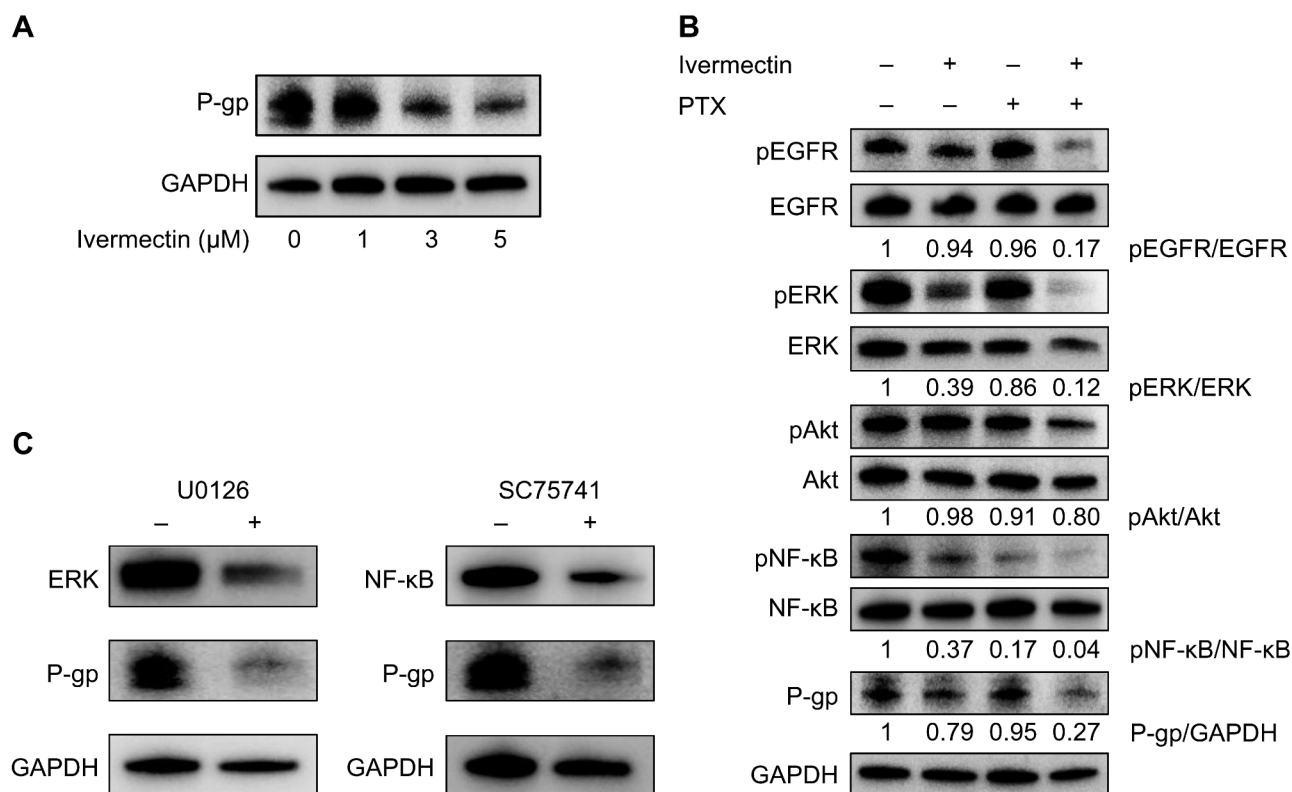


Figure 2. P-glycoprotein (P-gp) down-regulation by ivermectin and the relevant signaling pathways. (A) A549 cells were incubated with ivermectin with the concentrations indicated. Ivermectin attenuated P-gp expression in a concentration dependent manner. (B) Ivermectin (3 μM) hindered the EGFR, ERK, Akt, and NF-κB phosphorylation in A549 cells and this was emphasized when co-treated with PTX (0.001 μM). (C) A549 cells were treated with either U0126 (10 μM) and SC75741 (10 μM), an MEK inhibitor that inhibits ERK activity and an NF-κB inhibitor, respectively, for 72 h. These inhibitors attenuated P-gp expression.

over-expression, which was ameliorated by concurrent ivermectin treatment, sustaining sensitivity to PTX with increased intracellular PTX concentration. Additionally, ivermectin itself showed antitumor effects on A549 cells. P-gp expression was regulated by ivermectin *via* the inhibition of the EGFR/ERK/Akt/NF-κB pathway, and its over-expression led to PTX resistance. Our findings support the use of ivermectin in patients with NSCLC to overcome PTX resistance attributed to an excessive P-gp induction.

Lung cancer treatment was initially restricted to cytotoxic chemotherapy with platinum compounds associated with 3rd generation cytotoxic agents, such as PTX, gemcitabine, and pemetrexed. Among them, PTX is one of the key drugs used as a platinum doublet and has been positioned as a standard regimen for advanced NSCLC in several past trials (36, 37). Moreover, the widely used nanoparticle albumin-bound form of PTX (nab-PTX) is less toxic, with promising pharmacological improvements (38), providing efficacy in patients with previously treated advanced NSCLC (39) or in the elderly population with NSCLC (40).

Despite these treatment benefits, resistance to PTX or nab-PTX is developed and acquired after several courses of administration *via* various mechanisms, which include the alterations of the target molecule β-tubulin of mitosis (41), changes in molecular mechanisms that activate drug efflux out of cells (8), and over-expression of Bcl-2 (42). Overproduction of mutant p53 (43) and spontaneous mutations (44) have also been noted as mechanisms of resistance to the taxane family. Among these, we observed a robust increase in P-gp levels in A549 cells as a result of stepwise exposure to PTX, followed by accelerated drug efflux, thereby highlighting the acquisition of the PTX-resistant phenotype. Therefore, targeting P-gp is considered a therapeutic strategy for retrieving sensitivity to chemotherapeutic agents. This is supported by an observation that PTX sensitivity is associated with *ABCB1* down-regulation induced by long-term exposure to everolimus in renal cell carcinoma cells (45).

Based on this concept, many inhibitors of P-gp have been developed to test in clinical trials in patients with resistant cancers. The first studies on *ABCB1* were performed using

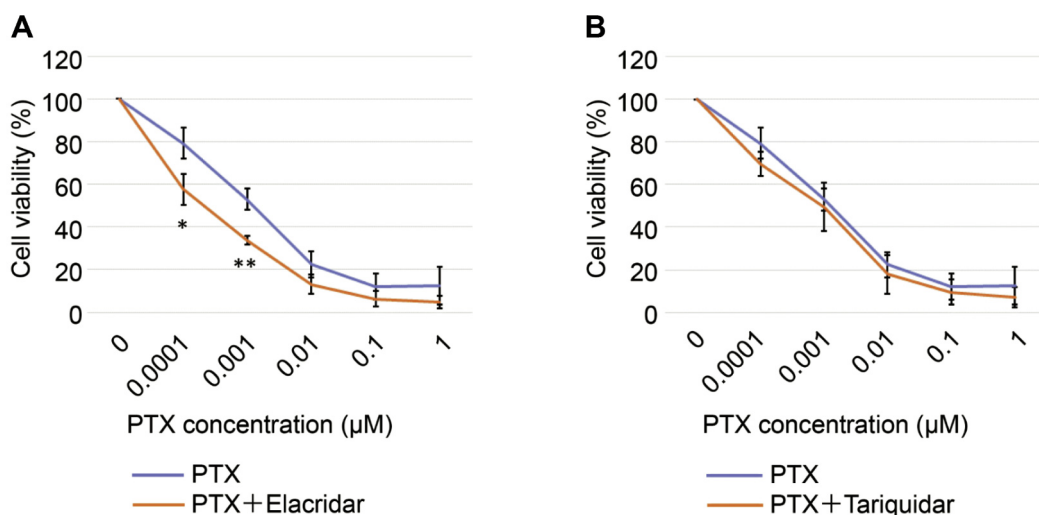


Figure 3. Effect of elacridar and tariquidar on cell viability in A549 cells treated with paclitaxel (PTX). A549 cells were incubated with PTX in combination with either elacridar (0.5 µM) (A) or tariquidar (1 µM) (B) for 72 h. Elacridar partially accelerated the effect of PTX; however, tariquidar had no effect on cell viability. * $p=0.04$, ** $p=0.008$ compared to control cells.

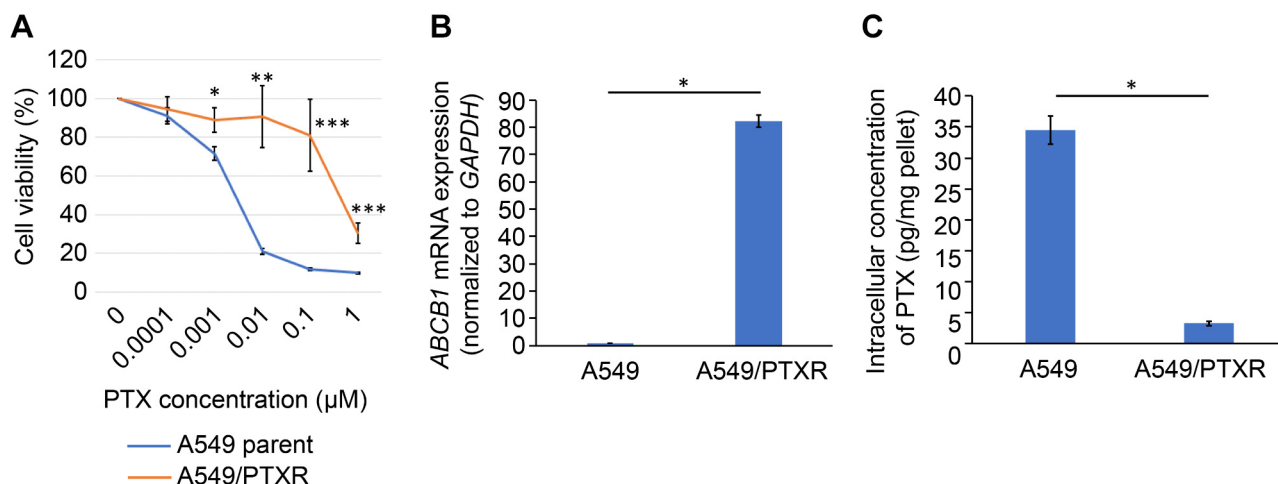


Figure 4. P-glycoprotein (P-gp) up-regulation is implicated in acquired resistance to paclitaxel (PTX) in A549 cells. (A) MTS assays showing that A549 cells exposed to PTX for 16 weeks in a step-wise method developed a PTX-resistant phenotype. * $p=0.027$, ** $p=0.004$, and *** $p=0.006$ compared to control cells. (B) ABCB1 mRNA expression was up-regulated in A549/PTXR cells compared with parental A549 cells, from 1.0 to 82.24. * $p<0.001$ by Student's t-test. (C) Intracellular PTX concentration was determined using LC-MS/MS method both in parental A549 and A549/PTXR cells after cultured for 72 h. * $p<0.001$ by Student's t-test. A549/PTXR; PTX-resistant A549 cells.

first-generation drugs, such as verapamil (46, 47). However, high doses were required to inhibit ABC transporters, which was associated with serious adverse events. Second- and third-generation ABC transporter inhibitors (e.g., elacridar and tariquidar) are significantly more potent than first-generation agents and are therefore used at much lower doses. Phase I studies demonstrated that the combination of elacridar and oral topotecan resulted in complete oral bioavailability of

topotecan (48). Studies on tariquidar in combination with vinorelbine or docetaxel showed no significant adverse events or pharmacokinetic interactions (49, 50). However, two large phase III trials evaluating tariquidar combined with first-line chemotherapy for patients with NSCLC were terminated early because of toxicity (47, 51, 52). This was, in part, due to increased adverse events caused by the chemotherapeutic agents, whose concentration in normal cells was increased by

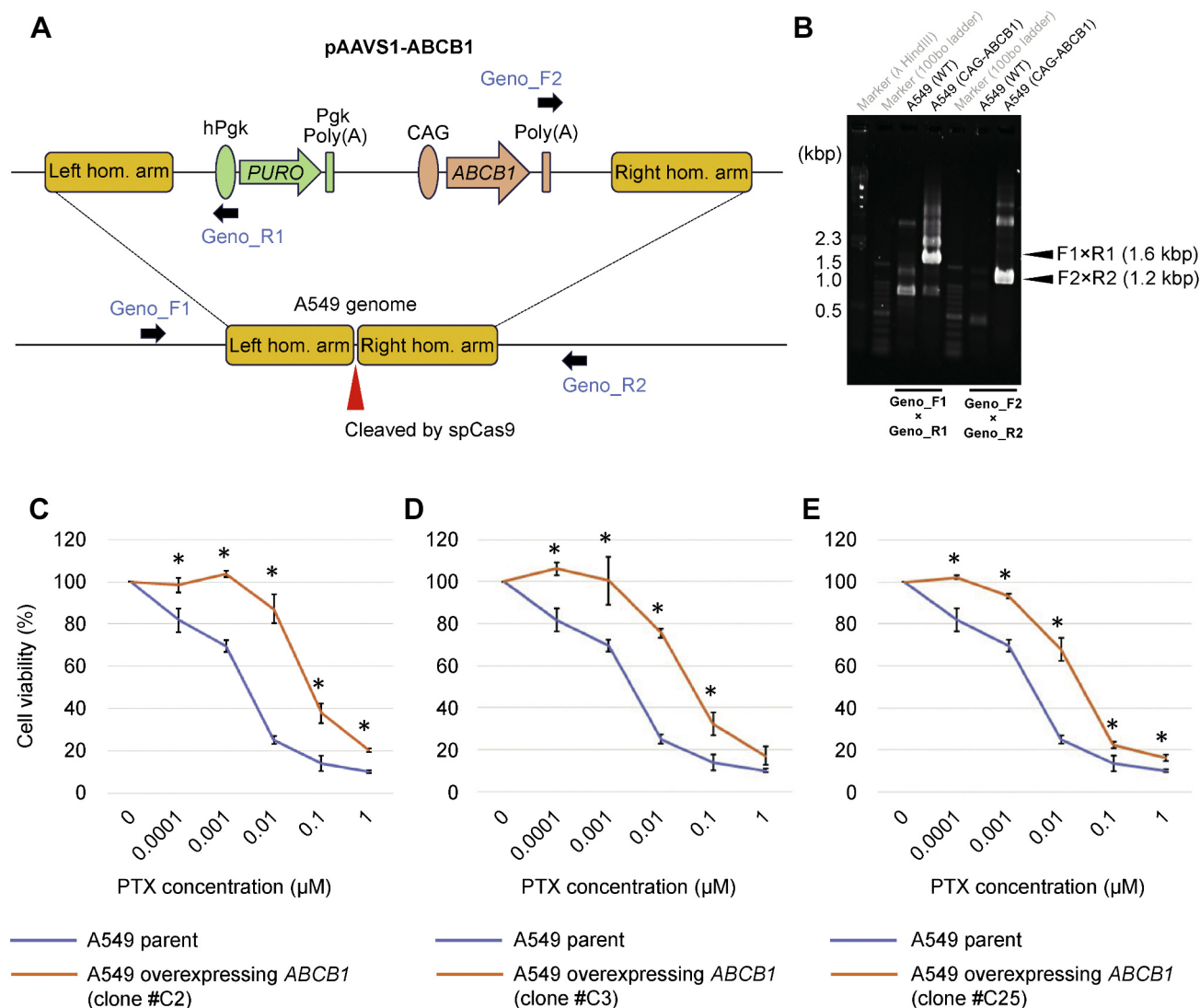


Figure 5. Effect of *ABCB1* over-expression on paclitaxel (PTX)-induced cell death. (A) The structure of the final construct in pAAVS1-*ABCB1* donor vector. The positions of binding sites of primers for genotyping the AAVS1 locus, are also indicated. (B) The representative data of genotyping of the AAVS1 locus by qRT-PCR with AAVS1_Geno primers. (C-E) Parental and *ABCB1* over-expressing A549 cells were treated with PTX and the cell viability was assessed. *ABCB1* over-expression exhibited PTX-resistant phenotype. * $p < 0.05$ compared to parental cells.

the blockade of physiological drug efflux (51, 53, 54). Therefore, there is an urgent need to develop P-gp inhibitors for the clinical treatment of drug-resistant cancers. Ivermectin has been reported as an anti-cancer agent (11, 15, 16), and we also observed this effect in a lung adenocarcinoma cell line, as well as sustained inhibition of P-gp in PTX-resistant cells. Taking this dual action of ivermectin into account, a clinical trial can be designed in which ivermectin is administered both therapeutically and prophylactically to patients with NSCLC treated with PTX regimens.

In this study, we found that NF- κ B is involved in the regulation of *ABCB1* and that its activation is inhibited by

ivermectin in both parental and PTX-resistant A549 cells. This finding was supported by an NF- κ B inhibitor, SC75741, which down-regulated P-gp expression. NF- κ B signaling has critical roles in various cellular processes, including apoptosis, proliferation, response to DNA damage, and immune function. Hence, numerous human diseases, such as autoimmune or inflammatory disorders and tumors, are closely linked to NF- κ B activation (55). Thus, targeting NF- κ B signaling contributes to the alleviation of these pathological conditions. When considering the involvement of NF- κ B in tumorigenesis, it is plausible expecting ivermectin not only to inhibit tumor proliferation by directly targeting NF- κ B but also to down-

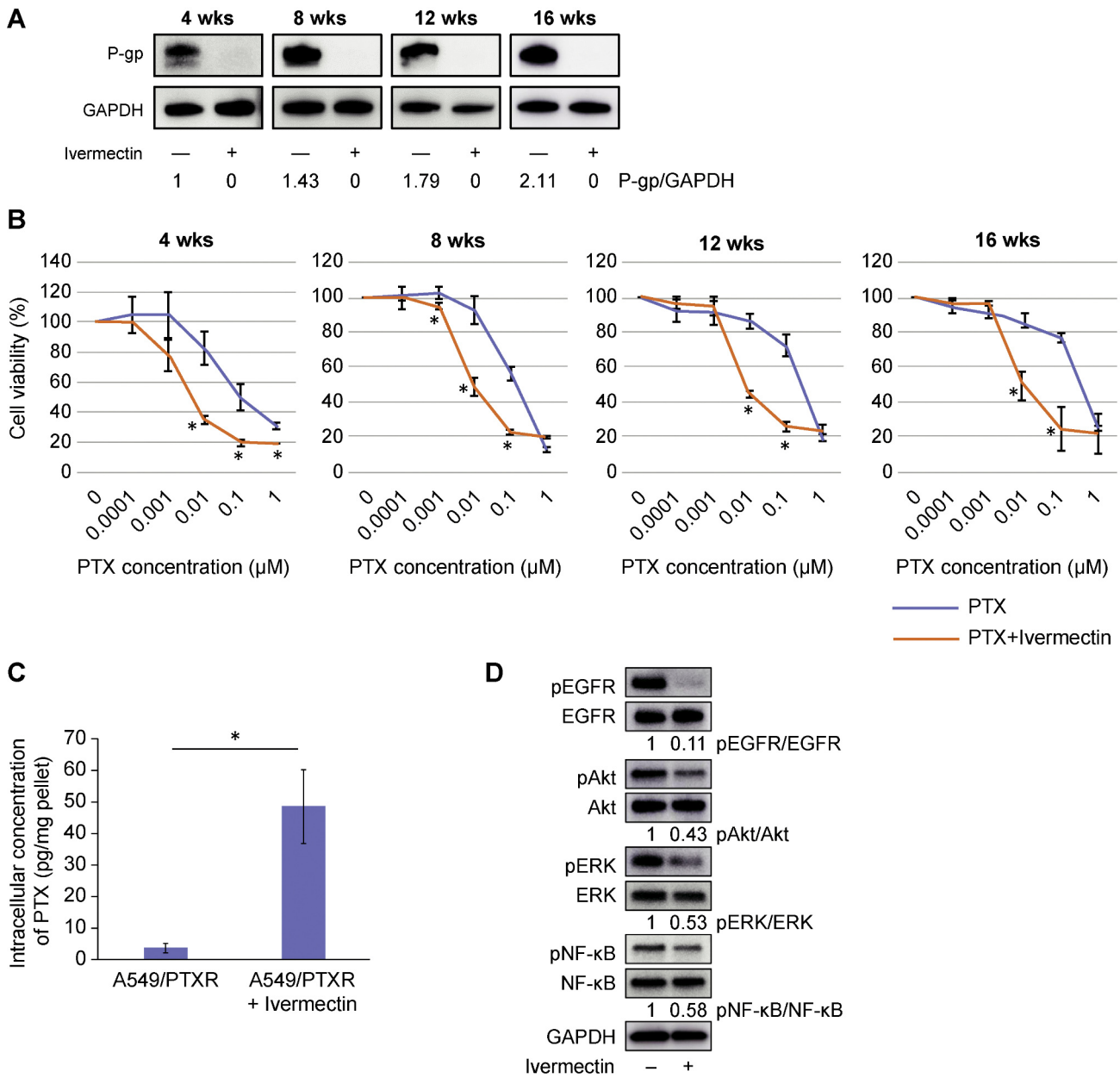


Figure 6. Effect of ivermectin on P-glycoprotein (P-gp) expression induced by paclitaxel (PTX) long-term exposure. (A) A549 cells were cultured with ivermectin in a constant concentration (3 µM) during the induction of PTX-resistance. Ivermectin treatment almost completely abolished P-gp induction. (B) MTS assays showed that A549 cells co-cultured with ivermectin during the establishment of PTX-resistant cells were kept significantly sensitive to PTX at every time point examined until 16 weeks (wks). *p < 0.05 when compared to A549 cells cultured with PTX alone. (C) A549 cells cultured with PTX using a step-wise method, with or without ivermectin, were treated with PTX (0.001 µM) for 72 h. Then, intracellular concentration of PTX was measured by the LC-MS/MS method. Intracellular PTX concentration significantly increased with ivermectin co-treatment. *p = 0.006 by Student's t-test. (D) Ivermectin inhibited the EGFR, ERK, Akt, and NF-κB phosphorylation in A549/PTXR cells. A549/PTXR; PTX-resistant A549 cells.

regulate *ABCB1* as a result of NF-κB inhibition, providing novel therapeutics for patients with advanced NSCLC.

Moreover, the inherent anti-inflammatory properties of ivermectin (56-58) support its use in interstitial lung diseases (ILDs). Idiopathic pulmonary fibrosis (IPF) is a devastating

form of ILD, and is associated with an increased risk of lung cancer, with cumulative incidence rates of 3.3% and 15.4% at 1 and 5 years of follow-up, respectively (59). Patients with IPF complicated by lung cancer exhibit a worse prognosis than those with IPF alone because of the progression of lung

cancer and complications arising from cancer treatment (60-64). However, the potency of ivermectin in lung injuries (65-67) together with the data presented in this article, provide insights toward the novel application of this agent in patients with IPF and lung cancer.

Study limitations. First, we examined the effects of PTX using only one lung adenocarcinoma cell line, A549. Therefore, further studies using other lung adenocarcinoma or squamous cell carcinoma cell lines should be conducted in order to evaluate whether these observations apply to a broader array of patients with lung cancer. Likewise, given the numerous anticancer drugs available for lung cancer, agents other than PTX should be tested. Secondly, this study was conducted only *in vitro*. Further studies using animal models or clinical samples are required to validate ivermectin as a novel therapeutic option for patients with lung cancer.

Conclusion

In conclusion, our findings provide a novel therapeutic approach for patients with advanced-stage NSCLC, avoiding the development of chemoresistance with the concurrent administration of ivermectin. This will enhance the efficacy of cytotoxic agents, thus warranting further investigations, including clinical challenges.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

Conception and design: A. Hayashi, K. Kamio, A. Miyana, M. Seike; Acquisition of data: A. Hayashi, K. Kamio, K. Yoshida, T. Amano, K. Matsuda, M. Hirao; Analysis and interpretation of data: All Authors; Drafting the manuscript or revising it critically for important intellectual content: A. Hayashi, K. Kamio, K. Yoshida, M. Seike; Final approval of the version to be published: All Authors; Agreement to be accountable for all aspects of the work: All Authors.

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