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Cisplatin and Pemetrexed Have Distinctive Growth-inhibitory Effects in Monotherapy and Combination Therapy on *KRAS*-dependent A549 Lung Cancer Cells

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Abstract. *Background/Aim: Cisplatin combined with pemetrexed disodium heptahydrate (pemetrexed) is considered the standard treatment for patients with advanced, non-squamous, non-small-cell lung cancer. However, its growth-inhibitory effects on KRAS-dependent lung cancer as monotherapy and combination therapy are not well understood. The aim of this study was to compare the effects of cisplatin and pemetrexed on A549 cells as mono- and combination therapies and elucidate the underlying mechanisms. Materials and Methods: For in vitro studies, A549 cells were exposed to cisplatin with/without pemetrexed for 72 h. The results were then evaluated by cell viability, apoptosis, reactive oxygen species, terminal deoxynucleotidyl transferase dUTP nick-end labeling, and western blotting assays. Results: Our results revealed that cisplatin monotherapy was most cytotoxic to A549 cells, while cisplatin plus pemetrexed combination had an intermediate effect, and pemetrexed monotherapy induced a minimal cytotoxic effect on A549 cells. This effect was evidenced by cell viability results, inhibition of KRAS proto-oncogene, GTPase (KRAS)/Raf proto-oncogene, serine/threonine kinase/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathways and apoptosis induction triggered by reactive oxygen species-mediated DNA damage. The immunoblotting result of conversion of microtubule-associated protein 1 light chain 3 alpha (LC3)-I to -II indicated that the greatest inducer of autophagy was combined treatment with cisplatin plus pemetrexed, while pemetrexed monotherapy had the lowest effect on autophagy induction, with cisplatin monotherapy having an intermediate effect. We found that the AKT serine/threonine kinase 1/mechanistic target of rapamycin kinase (mTOR) and AMP-activated protein kinase/mTOR signaling pathways were associated with autophagy activation. Interestingly, combination therapy with cisplatin plus pemetrexed was the primary eliminator of cellular senescence; cisplatin monotherapy had an intermediate effect, while pemetrexed monotherapy increased cellular senescence of A549 cells, as assessed by the expression of β-galactosidase protein. Conclusion: Cisplatin monotherapy may be more effective than pemetrexed monotherapy or cisplatin plus pemetrexed combination therapy against KRAS-dependent lung cancer.*

In terms of incidence and mortality, lung cancer is the most common cancer globally (1). Lung cancer is a leading cause of death, with non-small-cell lung cancer being the predominant form of the disease (2), accounting for nearly 80% of all lung cancer cases (3).

Mutations in the KRAS proto-oncogene, GTPase (*KRAS*) gene are frequently found in various types of human cancer, including of the lung, pancreas, and large intestine (4, 5). Approximately 15-25% of patients with non-small cell lung cancer reportedly have *KRAS* mutations (6, 7). These mutations alter the intrinsic GTPase activity of RAS and confer resistance to GTPase activators, which causes the accumulation of RAS in its active GTP union state, supporting the activation of KRAS (8, 9). Constitutive activation of KRAS triggers stimulation of downstream signaling pathways, including the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT serine/threonine kinase 1 (AKT)/mechanistic target of rapamycin kinase (mTOR) and Raf proto-oncogene, serine/threonine kinase (RAF)/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling cascades (10, 11).

In multicellular organisms, apoptosis can occur in the form of programmed cell death (12). Apoptosis can be initiated *via* two crucial pathways: the extrinsic and the intrinsic (13).

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Various types of stress can induce programmed cell death (14). The generation of reactive oxygen species (ROS) is a crucial stressor that can trigger DNA damage (15). DNA damage can activate members of the caspase family, leading to cleavage of poly (ADP-ribose) polymerase (PARP), a hallmark of apoptosis (16). It has been reported that ROS-mediated DNA damage is related to cellular senescence (17). The production of DNA damage can lead to permanent arrest of the cell cycle (18). Under irreversible conditions, damaged cells remain alive but unable to proliferate, a phenomenon known as cellular senescence (19, 20).

Several studies have reported the interaction between ROS generation and autophagy (21, 22). Autophagy is considered a double-edged sword in cancer cells (23). Under nutrient starvation conditions, autophagy can promote cell survival by providing the energy required for cellular metabolism (24). On the other hand, autophagy can lead to cell death by consuming essential cellular components (25, 26). Various studies have claimed that the PI3K/AKT/mTOR and AMP-activated protein kinase (AMPK)/mTOR pathways regulate autophagy to induce apoptosis (27, 28).

Herein, we compared the anticancer effects of cisplatin and pemetrexed on *KRAS*-dependent A549 cells as mono- and combination therapies and clarified the underlying mechanisms.

Materials and Methods

Cell line and cell culture. A *KRAS*-mutated A549 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). A549 cells were cultured in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Drug preparation. Cisplatin [$PtCl_2(NH_3)_2$] and pemetrexed disodium heptahydrate [$C_{20}H_{19}N_5Na_2O_6{\cdot}7H_2O$] were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). These drugs were dissolved in dimethyl sulfoxide for *in vitro* experiments.

Cell viability assay. The cytotoxicity of cisplatin and pemetrexed as single-drug or combined therapies in A549 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 (200 nM) or pemetrexed (100 nM) solution as single-drug or combined treatments was added to each well (29). 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 (29). The absorbance was then measured at 450 nm with a microplate enzyme-linked immunosorbent assay reader (Multiskan FC; Thermo Scientific, Tokyo, Japan). Data are presented as relative viability (%) by comparing drug-treated cells with DMSO-treated cells; the viability of DMSO-treated cells was assumed to be 100%.

Intracellular ROS assay. The intracellular ROS level was determined using a Reactive Oxygen Species Detection Assay Kit (ab186029; Abcam, Tokyo, Japan). In brief, after treatment with cisplatin and pemetrexed as single-drug or combined therapy for 72 h, cells were harvested for staining with a working solution of deep red ROS dye. Subsequently, the cells were incubated at 37˚C for 60 min before flow cytometric (FCM) analysis.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. For 72 h, A549 cells were treated with cisplatin or pemetrexed as single-drug therapy or in combination. An *in situ* Direct DNA Fragmentation (TUNEL) Assay Kit (ab66108; Abcam) was used to measure DNA fragmentation in apoptotic cells. In brief, cells were fixed with 1% paraformaldehyde in phosphate-buffered saline 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 the addition of rinse buffer, cells were resuspended in propidium iodide/RNase A solution and incubated at room temperature for 30 min for the FCM analysis.

Apoptosis assay. A549 cells were treated with cisplatin and pemetrexed as single-drug or combined therapy for 72 h. Apoptotic cell death was quantified by FCM using fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit with propidium iodide (PI) (BioLegend, San Diego, CA, USA).

Western blotting. For 72 h, A549 cells were treated with cisplatin and pemetrexed as single-drug or combined therapy. Whole protein lysates were isolated using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific), which included a phosphatase inhibitor cocktail and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were assessed using BCA protein assay reagent (Thermo Scientific). Total cellular protein (40 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Milk-blocked blots were then incubated at 4˚C overnight with primary antibodies against the following proteins: KRAS, RAF, MEK, phospho (p)-MEK (Ser 217/221), ERK, p-ERK (Thr 202/Tyr 204), AKT, p-AKT (Ser 473), mTOR, p-mTOR (Ser 2448), AMPKα, p-AMPKα (Thr 172), microtubule-associated protein 1 light chain 3 alpha (LC3), β-galactosidase, β-actin, and cleaved PARP (Asp 214) (all from Cell Signaling Technology, Danvers MA, USA). They were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Proteins of interest were revealed using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA) and viewed using an Invitrogen iBright FL1000 Imaging System (Thermo Fisher Scientific). The bands were quantified with the densitometric program of iBright Imaging System and normalized against β-actin.

Statistical analysis. Statistical analysis was conducted using GraphPad PRISM software, v. 7.0 (GraphPad Software Inc., San Diego, CA, USA). Results are presented as the mean±standard deviation of three independent experiments and were analyzed by a one-way analysis of variance followed by Dunnett's multiple comparison test. Values of $p<0.05$ were considered statistically significant.

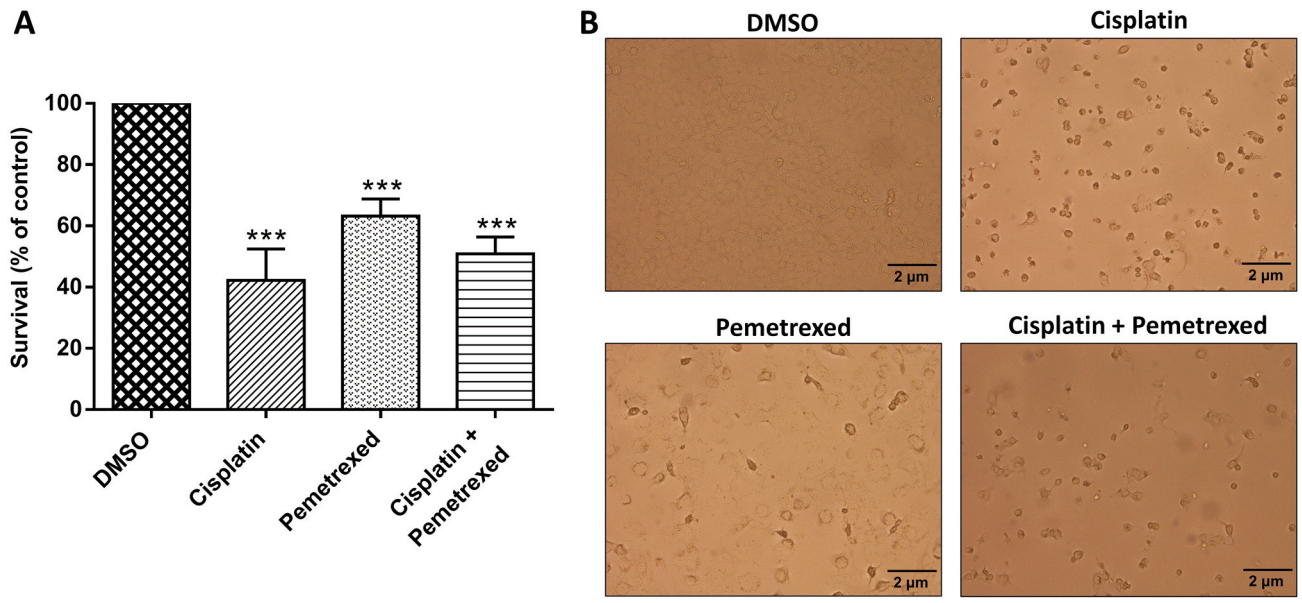

Figure 1. *Impact of cisplatin and pemetrexed on the viability of A549 cells. A: A549 cells were treated with cisplatin (200 nM) and pemetrexed (100 nM) alone and in combination for 72 h, and the cell survival rate was measured by the WST-1 assay. Data are presented as the mean±SD from three independent experiments. ***Significantly different at p<0.001 compared with the dimethyl sulfoxide (DMSO)-treated group using one-way analysis of variance with Dunnett's multiple comparison test. B: After 72-h treatment with cisplatin (200 nM) and pemetrexed (100 nM) as single-agents and combined, the morphological changes of A549 cells were captured under optical microscopy.*

Results

Effects of cisplatin and pemetrexed on the viability of A549 cells, alone and in combination. The effect of cisplatin and pemetrexed on the viability of A549 cells was determined using a WST-1 assay. As shown in Figure 1A, cisplatin monotherapy was most cytotoxic to A549 cells, whilst cisplatin and pemetrexed combination therapy had an intermediate effect; pemetrexed monotherapy had the least effect on A549 cells. As illustrated in Figure 1B, after treatment with cisplatin and pemetrexed, both as monotherapy and combination therapy, we noted that a proportion of A549 cells had grown round and become detached from the culture dish, features typical of apoptotic cells. Cisplatin monotherapy was more potent in inducing these apoptotic characteristics than pemetrexed monotherapy and the combination therapy.

Effects on inducing ROS-mediated DNA damage. Previous studies reported that many chemotherapy drugs induce cytotoxic effects *via* ROS-mediated DNA damage (30-32). Therefore, we hypothesized that cisplatin and pemetrexed might cause DNA damage through the generation of ROS. The intracellular ROS level was determined by FCM after treatment with cisplatin and pemetrexed alone and in combination to investigate ROS generation. As indicated in Figure 2, cisplatin monotherapy was more potent in increasing ROS production than pemetrexed monotherapy. In contrast, cisplatin plus pemetrexed combination therapy had an intermediate effect on increasing ROS generation.

To determine whether cell death caused by cisplatin and pemetrexed was due to DNA fragmentation, we performed a TUNEL assay. As shown in Figure 3A, after 72 h of cisplatin monotherapy, the proportion of cells with fragmented DNA increased from 0.18% to 78%. In contrast, the combination therapy had an intermediate effect, resulting in a rate of 71%, while pemetrexed monotherapy showed a fragmented cell rate of 20.4%. These results are consistent with the findings of the ROS-generation assay.

Effect on apoptosis of A549 cells. Previous studies reported that ROS-mediated DNA damage triggered growth inhibition *via* activation of apoptosis signaling (32-34). To investigate whether or not growth inhibition due to apoptosis occurred, we conducted annexin V-FITC and propidium iodide fluorescence staining. As illustrated in Figure 4A and B, combined treatment with cisplatin and pemetrexed resulted in an intermediate increase in the apoptotic population, while cisplatin monotherapy was the most potent apoptosis-inducing agent. By contrast, pemetrexed monotherapy induced the lowest proportion of apoptotic cells.

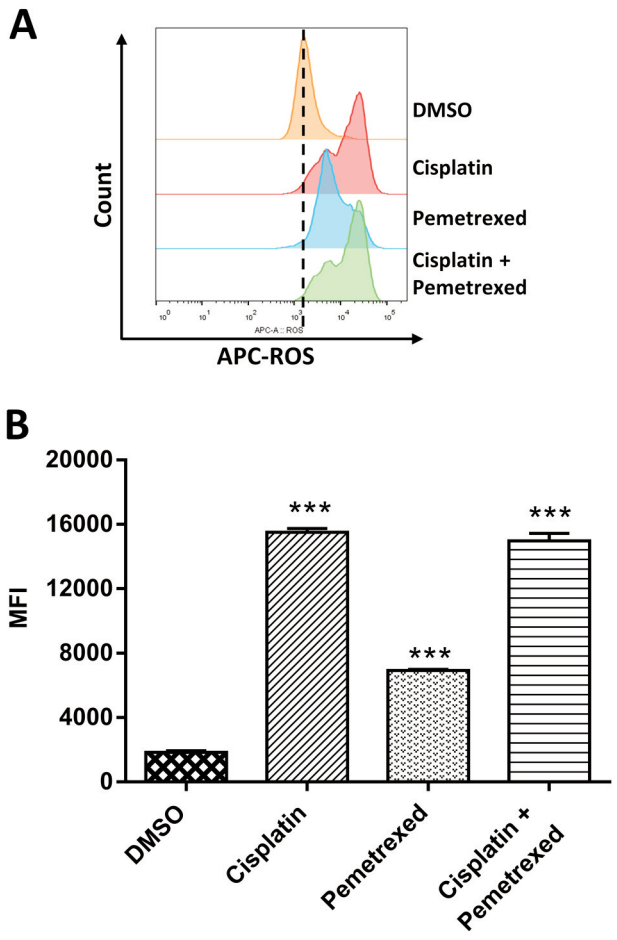

Figure 2. *The effect of cisplatin and pemetrexed on reactive oxygen species (ROS) production. A: A549 cells were incubated with cisplatin (200 nM) and pemetrexed (100 nM) alone and in combination for 72 h and then stained with ROS deep-red dye. The fluorescent signal was evaluated by flow cytometry, and the median fluorescence intensity (MFI) was determined. The histogram indicates the ROS levels after drug treatment. B: The bar diagram shows the quantification of ROS production. The results are presented as the mean±SD from three independent experiments. ***Significantly different at p<0.001 vs. control by one-way analysis of variance followed by Dunnett's multiple comparison test.*

Several studies have claimed that ROS-mediated DNA damage can activate a caspase cascade (33, 35, 36). The cleavage of PARP, a DNA-repair enzyme, by activated caspases (16, 37) is a hallmark of apoptosis (38, 39). To examine the expression of cleaved PARP, we performed immunoblotting. As shown in Figure 4C, the strongest inducer of cleaved PARP was cisplatin monotherapy, while combination therapy with cisplatin and pemetrexed had an intermediate effect on increasing cleaved PARP. Pemetrexed monotherapy induced the lowest amount of cleaved PARP. This result is consistent with the findings from the apoptosis assay.

Effects on the RAF/MEK/ERK signaling pathway. Previous studies have revealed that the RAF/MEK/ERK signaling pathway plays a vital role in cell growth, cell-cycle arrest, and in prevention of apoptosis and drug resistance in various cancer cell lines (40-42). The effects of cisplatin and pemetrexed on RAF/MEK/ERK signaling were evaluated by immunoblotting. As indicated in Figure 5, combined treatment with cisplatin and pemetrexed had an intermediate inhibitory effect on the RAF/MEK/ERK signaling cascade, whereas cisplatin monotherapy induced a maximum inhibitory effect. In contrast, when A549 cells were treated with pemetrexed alone, we observed activation of the RAF/MEK/ERK signaling cascade. These results suggest that cisplatin monotherapy is more effective at inhibiting RAF/MEK/ERK signaling in A549 cells than pemetrexed monotherapy and cisplatin plus pemetrexed combination therapy.

Effects on autophagy. mTOR is a signaling molecule of the PI3K/AKT/mTOR signaling pathway closely connected with the inhibition of autophagy (43, 44). Several studies have reported that inhibition of the PI3K/AKT/mTOR signaling pathway can induce autophagy (45, 46). To determine whether or not cisplatin and pemetrexed induce autophagy, A549 cells were treated for 72 h. The levels of total and phosphorylated mTOR and AKT were examined by immunoblotting. As indicated in Figure 6, cisplatin and pemetrexed exerted a distinctive inhibitory effect on the AKT/mTOR signaling pathway both as monotherapies and in combination.

In contrast, AMPK is a key regulator of cellular metabolism and energy balance (47). Previous studies have reported that mTOR is a sensor of changes in the cellular energy state through AMPK (48, 49). Activation of AMPK can inhibit mTOR-dependent signaling, which can trigger protein synthesis inhibition (49, 50). Several studies have reported that AMPK/mTOR signaling is associated with autophagy, and AMPK can enhance autophagy initiation (49, 51). As shown in Figure 6, combined treatment with cisplatin plus pemetrexed increased the expression of p-AMPKα (Thr 172), triggering inhibition of mTOR-dependent signaling. Importantly, when A549 cells were treated with cisplatin and pemetrexed as monotherapies, AMPK activation was not observed.

LC3 is currently broadly used to study autophagy (52, 53). During autophagy, conversion of LC3 type II from LC3 type I occurs; therefore, an increase in the ratio of LC3-II/LC3-I expression is considered an autophagy marker (54, 55). As shown in Figure 6, cisplatin plus pemetrexed treatment was most potent for increasing the LC3-II/LC3-I ratio, whereas cisplatin monotherapy had an intermediate effect and pemetrexed monotherapy had the least autophagy induction activity.

Effects on cellular senescence. Previous studies have reported that chemotherapy drugs can induce cellular senescence of cancer cells (56-59). Some claim that some

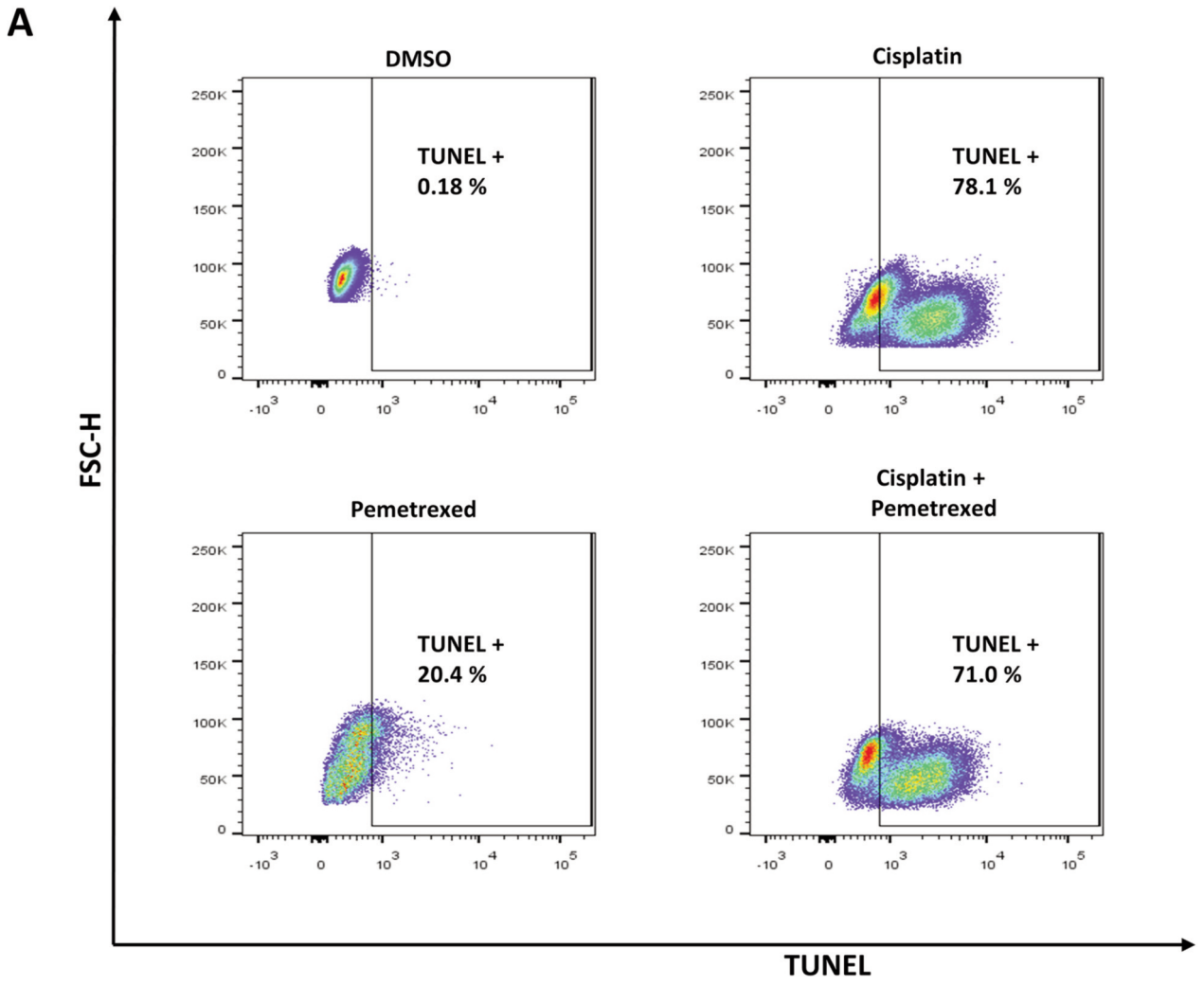

Figure 3. *The effect of cisplatin and pemetrexed on DNA fragmentation. A: A549 cells were stained with fluorescein isothiocyanate-dUTP dye after incubation with cisplatin (200 nM) and pemetrexed (100 nM) as single-agent therapy and combined therapy for 72 h. The fluorescence signal was measured by flow cytometry. (B) The bar diagram shows the quantification of DNA fragmentation. The results were expressed as the mean±SD from three independent experiments. ***Significantly different at p<0.001 vs. control by one-way analysis of variance followed by Dunnett's multiple comparison test.*

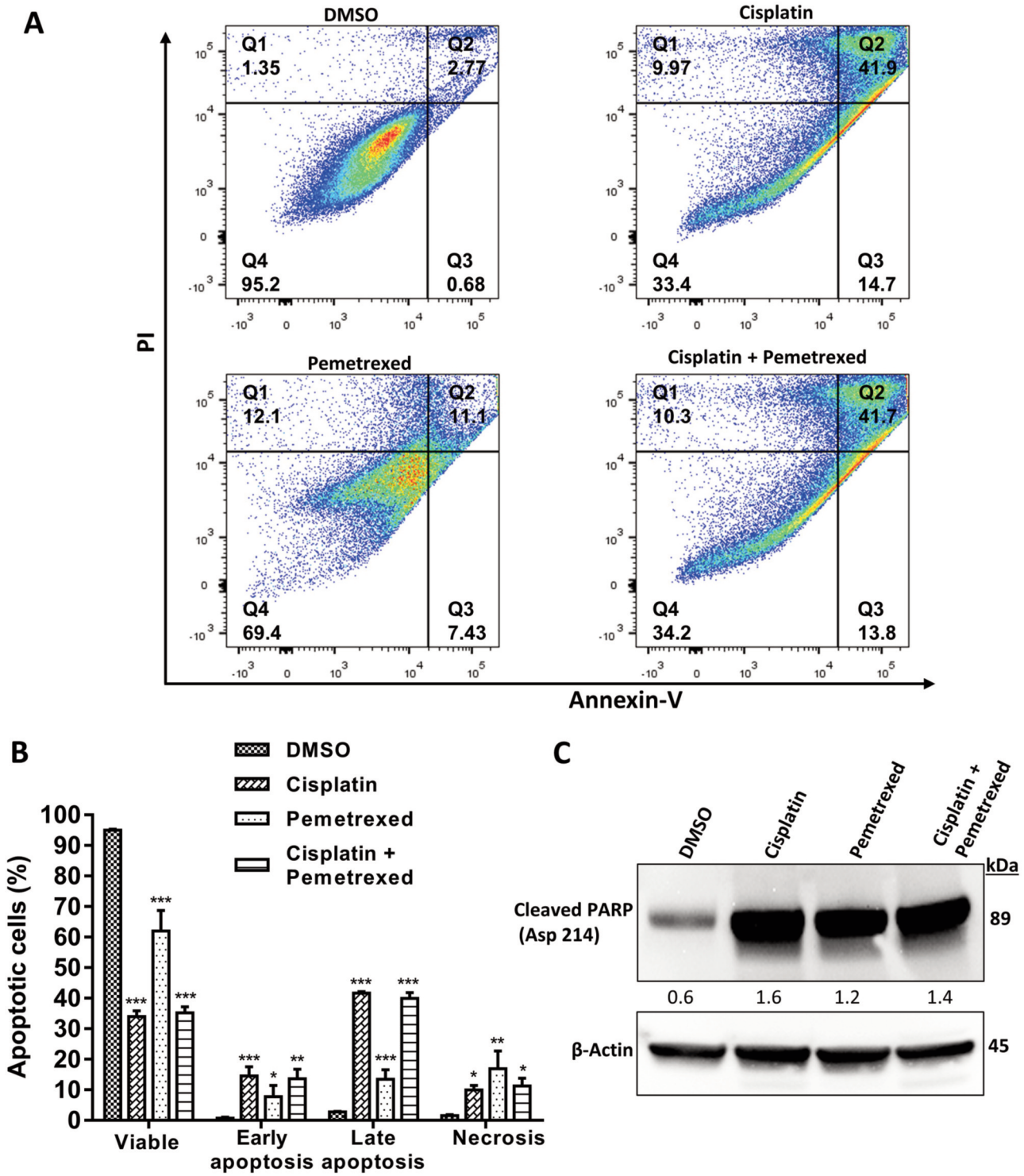

Figure 4. *Effect of cisplatin and pemetrexed on the induction of apoptosis in A549 cells using an annexin V-fluorescein isothiocyanate/propidium iodide (PI) apoptosis detection kit. A: A549 cells were treated with cisplatin (200 nM), and pemetrexed (100 nM) alone and in combination for 72 h and then 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: Quantification of apoptotic cells. The results were expressed as the mean±SD from three independent experiments. Significance was determined by one-way analysis of variance followed by Dunnett's multiple comparison test at: *p<0.05, **p<0.01 and ***p<0.001 when compared with the control. C: To evaluate the expression of cleaved poly (ADP-ribose) polymerase (PARP) (Asp 214), western blotting was performed. β-Actin served as a loading control. Representative immunoblots with quantification relative to β-actin from three independent experiments are shown.*

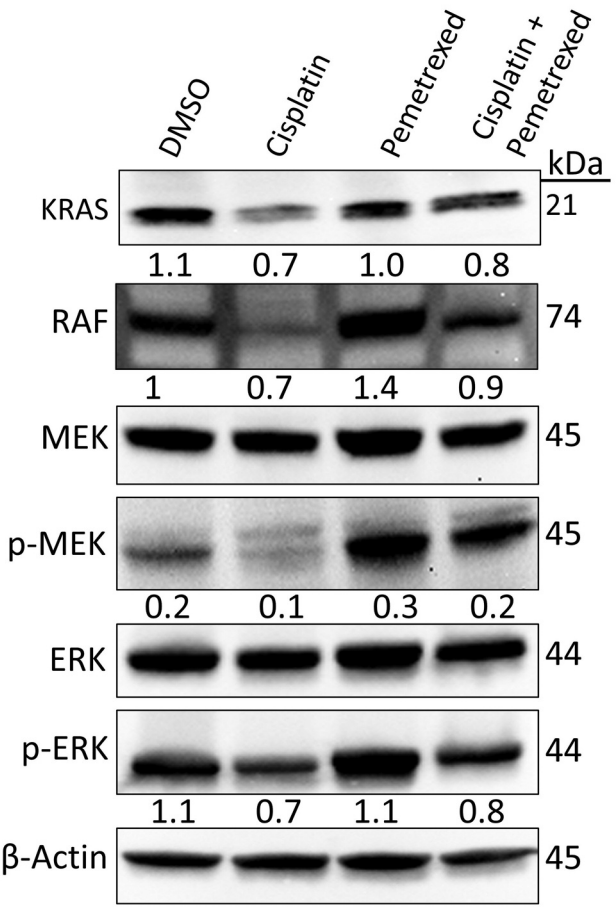

Figure 5. *Effect of cisplatin and pemetrexed on KRAS proto-oncogene, GTPase (KRAS) signaling in KRAS-dependent A549 cells. Representative western blot data of the levels of KRAS proto-oncogene, GTPase (KRAS), Raf proto-oncogene, serine/threonine kinase (RAF), mitogen-activated protein kinase kinase (MEK), phospho (p)-MEK (Ser 217/221), ERK and p-ERK (Thr 202/Tyr 204) after 72-h treatment with cisplatin (200 nM) and pemetrexed (100 nM) alone and in combination. β-Actin was used as a loading control. Representative immunoblots with quantification relative to β-actin or non-phosphorylated forms from three independent experiments are shown.*

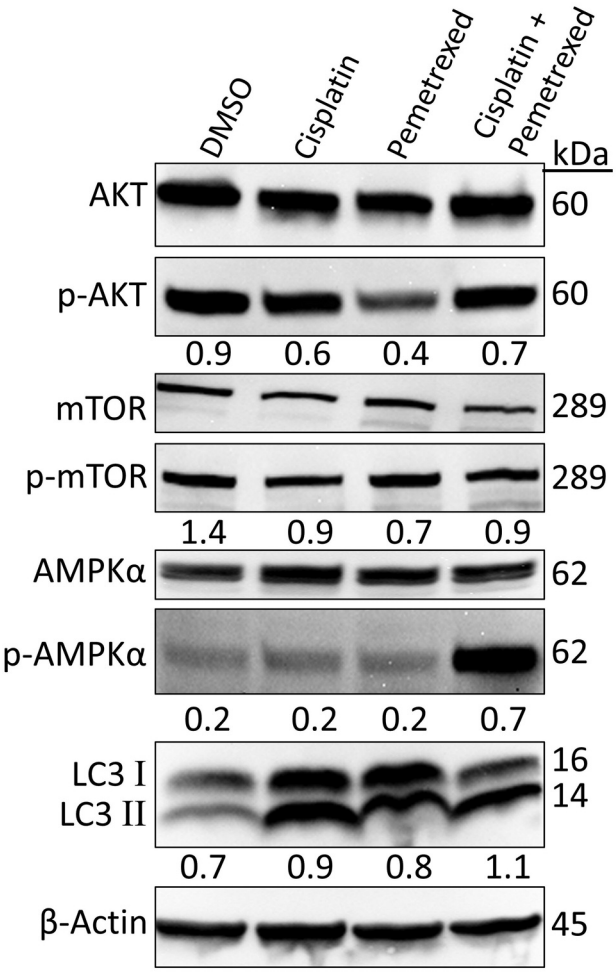

Figure 6. *Effect of cisplatin and pemetrexed on autophagy induction in A549 cells. Representative western blot data of the levels of AKT serine/threonine kinase 1 (AKT), phospho (p)-AKT (Ser 473), mechanistic target of rapamycin kinase (mTOR), p-mTOR (Ser 2448), protein kinase AMP-activated catalytic subunit alpha (AMPKα), p-AMPKα (Thr 172) and microtubule-associated protein 1 light chain 3 alpha (LC3) after 72-h treatment with cisplatin (200 nM) and pemetrexed (100 nM) alone and in combination. β-Actin served as a loading control. Representative immunoblots with quantification relative to β-actin, non-phosphorylated forms, or LC3-I from three independent experiments are shown.*

chemotherapy drugs can remove cellular senescence (60, 61). Therefore, it is crucial to characterize the senescence status correctly when managing patients with cancer.

To examine the effect of cisplatin and pemetrexed treatment on cellular senescence in A549 cells, the level of β-galactosidase, a biomarker for senescent cells, was determined by immunoblotting. A549 cells were exposed to drugs for 72 h. As shown in Figure 7, cisplatin plus pemetrexed combined treatment most potently reduced expression of β-galactosidase, whereas cisplatin monotherapy had a milder effect. In contrast, in A549 cells that were exposed to pemetrexed monotherapy, β-galactosidase expression was induced, suggesting that pemetrexed monotherapy may promote adverse effects (62).

Discussion

Cisplatin and pemetrexed are well-known anticancer drugs but to our knowledge, this is the first study to elucidate the anticancer effects of cisplatin and pemetrexed as monotherapy or combination therapy and to propose potential mechanisms of action in A549 cells.

ROS have been reported to promote or slow the progression of cancer cells (63, 64). Furthermore, ROS play a fundamental role in pathological and physiological processes (65, 66). Many studies have claimed that excessive ROS generation by chemotherapy drugs can trigger DNA damage and lead to the onset of apoptosis (63, 67). In the present study, cisplatin monotherapy most potently increased ROS-mediated DNA damage, causing apoptosis of A549 cells, as evidenced by PARP cleavage (Figure 2-4). In contrast, we found that pemetrexed monotherapy induced greater expression of β-galactosidase, a biomarker for senescent cells, than cisplatin monotherapy or its combination treatment (Figure 7).

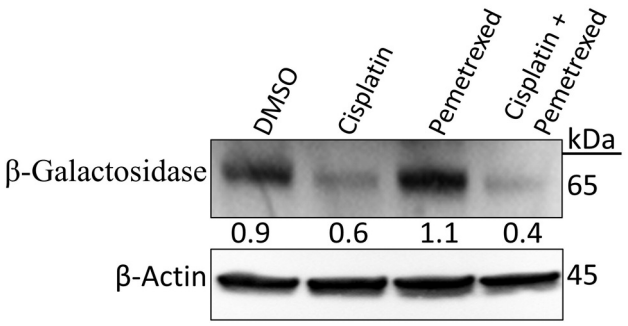

Figure 7. *Effect of cisplatin and pemetrexed on cellular senescence in A549 cells. A549 cells were treated with cisplatin (200 nM) and pemetrexed (100 nM) alone and in combination for 72 h. The expression level of cellular senescence marker β-galactosidase was determined by western blotting. β-Actin was used as a loading control. Representative immunoblots with quantification relative to β-actin from three independent experiments are shown.*

The RAS/RAF/MEK/ERK pathway (also known as the mitogen-activated protein kinase signal transmission pathway) has been reported to be crucial in the regulation of several physiological processes, including cell division, growth, development, and death (68, 69). This pathway is the core of the signaling network involved in cell division, growth, and development (70, 71). RAS acts as a key upstream molecule of the RAF/MEK/ERK signaling pathway (71, 72). In the present study, we found that cisplatin monotherapy was the most effective agent at impeding RAF/MEK/ERK signaling in *KRAS*-dependent A549 cells, suggesting cell growth inhibition (Figure 5).

Previous studies have reported many vital molecules and signaling pathways responsible for regulating autophagy (73-75). The PI3K/AKT/mTOR signaling pathway is well-studied, and critical in regulating the cell cycle, apoptosis, and autophagy (43, 76). Drugs that suppress the PI3K/AKT/mTOR signaling pathway have been reported to induce autophagy (77, 78). In our study, we found that cisplatin and pemetrexed inhibited AKT/mTOR signaling, triggered activation of autophagy (Figure 6). Various studies have reported that AMPK positively regulates autophagy and inhibits the mTOR-dependent signaling pathway (28, 79, 80). The AKT/mTOR signaling pathway is involved in regulating autophagy (81). AKT controls autophagy mainly through the alteration of mTOR activity (82-84). Our results showed that cisplatin plus pemetrexed therapy induced AMPK activation, thereby stimulating autophagy through the blockade of the mTOR-dependent signaling pathway (Figure 6). In addition, several studies have claimed that LC3 is an autophagosome marker and can be used for monitoring autophagy (85-87). In actuality, the LC3-II/LC3-I ratio is a hallmark of the degree of activation of autophagy (88, 89). In the present study, our approach to detect the conversion of LC3 (LC3-I to LC3-II) by western blotting revealed cisplatin plus pemetrexed combination therapy to be more potent than both cisplatin and pemetrexed monotherapies for inducing autophagy in A549 cells (Figure 6).

Finally, this study revealed that cisplatin and pemetrexed induced growth inhibition in A549 cells in four explicit systems (Figure 8). Firstly, these drugs enhance ROS-mediated DNA damage, which triggers apoptosis. Secondly, they induced autophagy by regulating the AMPK/mTOR and PI3K/AKT/ mTOR signaling pathways. Thirdly, the generation of fragmented DNA may regulate cellular senescence in A549 cells. Fourthly, these drugs impede the RAS/RAF/MEK/ERK signaling pathway, thereby inhibiting the growth of A549 cells. Overall, our findings suggest that cisplatin monotherapy is the most potent against A549 cells, as evidenced by the cell viability outcome, RAS/RAF/MEK/ERK pathway inhibition, and induction of apoptosis triggered by excessive generated ROS.

Conclusion

Our results revealed the mechanisms of action of cisplatin and pemetrexed alone and in combination against A549 cells. We conclude that cisplatin monotherapy may be more efficient than pemetrexed monotherapy or cisplatin plus pemetrexed combination therapy in eliminating *KRAS*-dependent cells.

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 article; Kazuo Kasahara supervised the experiments and project.

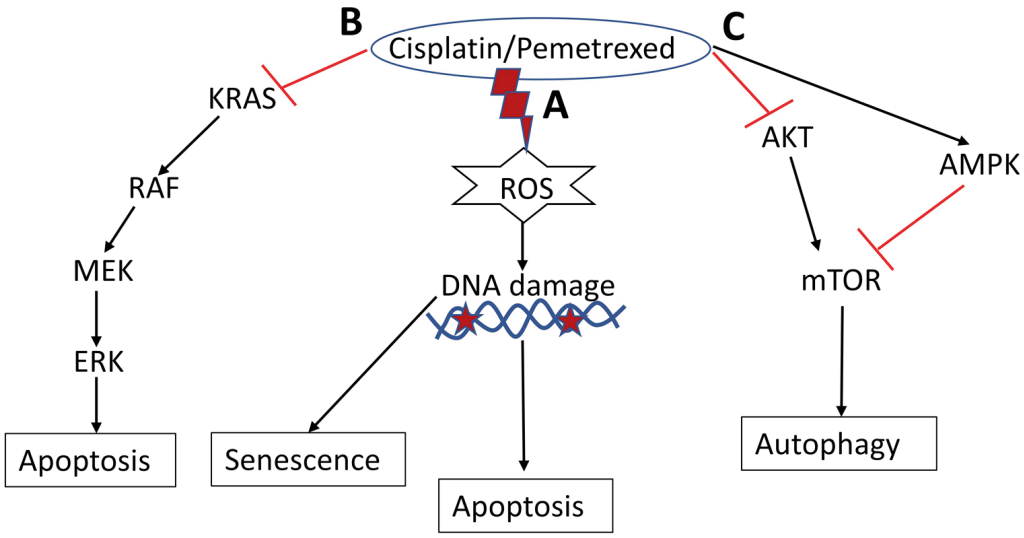

Figure 8. *The hypothetical mechanism underlying cisplatin- and pemetrexed-induced autophagy, cellular senescence, and apoptosis in A549 cells. A: Drug treatment enhances reactive oxygen species (ROS)-mediated DNA damage to trigger apoptosis and induce cellular senescence. B: Drug treatment suppresses the KRAS proto-oncogene, GTPase (KRAS)/Raf proto-oncogene, serine/threonine kinase (RAF)/ mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway to induce apoptosis. C: Cisplatin and pemetrexed induce autophagy in A549 cells through the regulation of the AKT serine/threonine kinase 1 (AKT)/mechanistic target of rapamycin kinase (mTOR) and AMP-activated protein kinase (AMPK)/mTOR signaling pathways.*

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