

Co-treatment with Celecoxib or NS398 Strongly Sensitizes Resistant Cancer Cells to Antimitotic Drugs Independent of P-gp Inhibition

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Abstract. *Background/Aim:* Inhibition of cyclooxygenase-2 (COX-2) has been investigated in clinical trials. Currently, NS398 and celecoxib are the most commonly used COX-2 inhibitors. The purpose of this study was to identify conditions that would increase the sensitivity of resistant cancer cells to antimitotic drugs. *Materials and Methods:* We tested whether COX-2 inhibitors can sensitize drug-resistant KBV20C cancer cells. We also compared the efficacy of NS398 with that of celecoxib. *Results:* Both NS398 and celecoxib could sensitize KB and KBV20C cells to a similar extent, suggesting that COX-2 inhibitors could be used for sensitive, as well as resistant, cancer cells. We demonstrated that the NS398 and celecoxib sensitization mechanism is independent of the inhibition of p-glycoprotein (P-gp), suggesting that resistant KBV20C cells are sensitized through targeting of signaling pathways by both drugs. Furthermore, through using microscopic observation, assessment of cleaved poly ADP ribose polymerase (C-PARP) and annexin V staining we determined that both COX-2 inhibitors strongly sensitized resistant KBV20C cells to vinblastine (VIB) or paclitaxel (PAC) treatment. These results suggest that antimitotic drug-resistant cancer cells can be strongly sensitized by co-treatment with COX-2 inhibitors, without P-gp inhibitory activity. *Conclusion:* These findings provide important information regarding the sensitization of drug-resistant cells and indicate that COX-2 inhibitors may be used for potentially resistant cancer patients, without the toxic effects of P-gp inhibition.

Antimitotic drugs are widely used to treat numerous types of cancers (1, 2). These compounds inhibit mitosis by targeting microtubules and preventing their polymerization or depolymerization (1-4). However, patients develop resistance to these drugs (5). Thus, to improve the efficacy of treatment, research has been focused on increasing antimitotic drug-associated apoptosis.

Cyclooxygenase-2 (COX-2) is an enzyme whose expression increases in response to inflammation and mitotic stimuli (6-8). COX-2 expression is also positively correlated with cancer cell proliferation and growth (9). Increased COX-2 expression is also observed in P-glycoprotein (P-gp)-mediated drug resistance (10, 11). There are reports that the COX-2 inhibitors NS398 and celecoxib can suppress cancer by COX-2-dependent and COX-2-independent mechanisms (12, 13). In addition, co-treatment with COX-2 inhibitors showed increased sensitization to anti-cancer drugs in various models (14-17). Adverse effects, such as cardiovascular events, have been reported in clinical trials involving celecoxib-treated cancer patients (18, 19). Better understanding of the mechanism governing the sensitization effect of COX-2 inhibitors in cancer patients could facilitate their safe therapeutic use.

In the present study, we compared the sensitization efficacy of two well-known COX-2 inhibitors, NS398 and celecoxib, in antimitotic drug-resistant KBV20C cancer cells. We also tested whether co-treatment with COX-2 inhibitors increases sensitization in antimitotic drug-treated KBV20C resistant cancer cells. Since we have already demonstrated their strong inhibitory effects in drug-resistant cancer, the current study supports the use of COX-2 inhibitors in combinatorial treatment of antimitotic drug-resistant patients.

Materials and Methods

Reagents. Paclitaxel (PAC) and verapamil (VER) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Vinblastine (VIB) and vincristine (VIC) were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Celecoxib and rhodamine123 (rhodamine) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

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NS398 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Calcein-AM was obtained from Invitrogen (Carlsbad, CA, USA).

Antibodies. Antibodies against pGSK3 β , pHistone H3, Cdc2, p21, PCNA and cleaved poly ADP ribose polymerase (C-PARP) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), p27 and survivin were from Santa Cruz Biotechnology. Antibody against pH2AX was from Abcam (Cambridge, UK).

Cell culturing. Human oral squamous carcinoma cell lines, KB and its multidrug-resistant subline, KBV20C, were obtained from Dr. Yong Kee Kim (College of Pharmacy, Sookmyung Women's University, Seoul, Republic of Korea) and have been previously described (20-22). All cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin (WelGENE, Daegu, South Korea).

Microscopic observation. Cells grown in 6-well plates were treated with the indicated drugs for the indicated times. The medium was removed and PBS was added in each dish. Cells were examined immediately in two independent experiments using an Axio observer.Z1 fluorescence inverted microscope (Carl Zeiss, Oberkochen, Germany) with a 5 \times or 10 \times objective lens (Carl Zeiss EC Plan-Neofluar).

Fluorescence-activated cell sorting (FACS) analysis. FACS analysis was performed as previously described (23-25). Cells were grown in 60-mm diameter dishes and treated with the indicated drugs for the prescribed times. The cells were then dislodged by trypsin and pelleted by centrifugation. The pelleted cells were washed thoroughly with PBS, suspended in 75% ethanol for at least 1 h at 4 $^{\circ}$ C, washed with PBS and re-suspended in a cold propidium iodide (PI) staining solution (100 μ g/ml RNase A and 50 μ g/ml PI in PBS) for 30 min at 37 $^{\circ}$ C. The stained cells were analyzed in two independent experiments for relative DNA content using a FACSCalibur flow cytometry system (BD Bioscience, Franklin Lakes, NJ, USA).

Annexin V analysis. Annexin V analysis was conducted using the annexin V-fluorescein isothiocyanate (FITC) staining kit (BD Bioscience) as previously described (23-25). Cells were grown in 60-mm diameter dishes and treated with the indicated drugs for the prescribed times. The cells were then dislodged by trypsin and pelleted by centrifugation. The pelleted cells were washed with PBS. Cells in 100 μ l of binding buffer received 5 μ l of Annexin V-FITC and 5 μ l of PI and were, then, incubated for 15 min at room temperature. The stained cells were analyzed in two independent experiments using a FACSCalibur flow cytometry system (BD Bioscience).

Rhodamine and calcein-AM uptake tests. The tests used for determination of ability for inhibition of P-gp are based on a previously described method (23-25). Briefly, cells grown in 6-well plates were treated with indicated drugs and incubated for 24 h at 37 $^{\circ}$ C. Cells were then incubated with and 1 μ g/ml rhodamine or 0.1 μ g/ml calcein-AM for 1 h 30 min at 37 $^{\circ}$ C. The medium were removed and the cells were washed with PBS. The stained cells were analyzed in two independent experiments using a FACSCalibur flow cytometry system (BD Bioscience).

Western blot analysis. Total cellular proteins were extracted using a previously described trichloroacetic acid (TCA) method (23-25). Briefly, cells grown in 60-mm dishes were washed three times with 5 ml PBS. Next, 500 μ l of 20% TCA was added to each plate. The cells were then dislodged by scraping and transferred to Eppendorf tubes. Proteins were pelleted by centrifugation for 5 min at 3,000 rpm and re-suspended in 1M Tris-HCl (pH 8.0) buffer. The total protein concentrations were estimated. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to Western blot analysis as previously described (23-25).

Results

COX-2 inhibitors are not substrates for P-gp in drug-resistant KBV20C cells. The importance of COX-2 inhibitors in cancer therapy has been documented (6, 7). However, the relative efficacy of different COX-2 inhibitors in cancer treatment has not been compared. We, thus, tested whether the COX-2 inhibitors NS398 and celecoxib could sensitize KBV20C cells, that have an antimitotic drug-resistant phenotype. NS398 and celecoxib have been widely studied for clinical applications in cancer therapy (11, 12, 14). We also tested whether drug-resistant KBV20C cells have resistance to these drugs. Cellular growth was microscopically observed after 48 h of treatment with each drug in both sensitive parent KB and resistant KBV20C cells. As seen in Figure 1A-B, KBV20C cells were highly resistant to the anticancer drug VIC, while both KB and KBV20C cells exhibited a similar level of sensitization by NS398 or celecoxib. This result suggests that NS398 and celecoxib are not substrates of P-gp in drug-resistant KBV20C cells. It also suggests that COX-2 inhibitors can be used as an anticancer drug in resistant cells. NS398 and celecoxib have different structures, suggesting that our findings may be evident with other COX-2 inhibitors.

We also assessed whether NS398 and celecoxib inhibit P-gp. Our results showed that NS398 and celecoxib did not inhibit P-gp activity, as seen with anticancer drugs VIB and PAC (Figure 1C). However, P-gp inhibition was observed when using a well-known P-gp inhibitor, verapamil (Figure 1C). Thus, we conclude that COX-2 inhibitors sensitize resistant KBV20C cells *via* cellular signaling pathways.

Co-treatment with COX-2 inhibitors strongly increases toxicity of antimitotic drugs in resistant KBV20C cells without P-gp inhibition. Although we did not observe P-gp inhibitory activity by COX-2 inhibitors (Figure 1C), we tested whether co-treatment with NS398 or celecoxib could increase sensitization of antimitotic drug-treated KBV20C cells. As seen in Figure 2A, co-treatment with NS398 or celecoxib reduced proliferation of both VIB- and PAC-treated KBV20C cells. Considering that these antimitotic drugs have different modes of action, with VIB targeting the

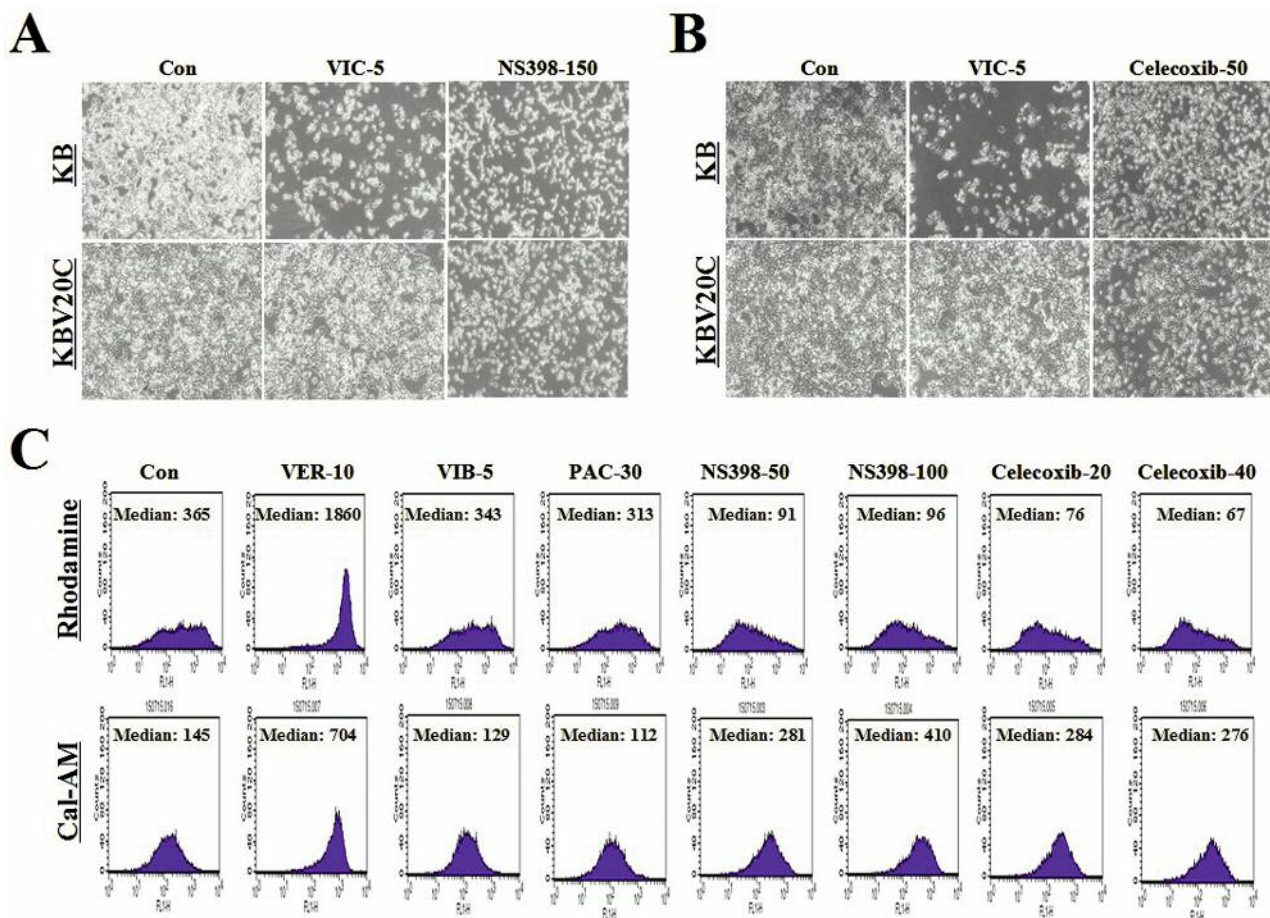


Figure 1. COX-2 inhibitors are not substrates for P-gp in drug-resistant KBV20C cells. (A-B) KB and KBV20C cells were grown on 6-well plates and treated with 5 nM vincristine (VIC-5), 150 μ M NS398 (NS398-150), 50 μ M celecoxib (Celecoxib-50) or 0.1% DMSO (Con). After 2 days, all cells were observed using an inverted microscope with a 50 magnification. (C) KBV20C cells were grown on 60 mm-diameter dishes and treated with 10 μ M verapamil (VER-10), 5 nM vinblastine (VIB-5), 30 nM paclitaxel (PAC-30), 50 μ M NS398 (NS398-50), 100 μ M NS398 (NS398-100), 20 μ M celecoxib (Celecoxib-20), 40 μ M celecoxib (Celecoxib-40) or 0.1% DMSO (Con). After 24 h, all cells were stained with rhodamine or calcein-AM, as described in the Materials and Methods section. The stained cells were subsequently examined by FACS analysis.

vinca domain and PAC targeting the taxane-binding site of microtubules, we hypothesized that these results could be observed with various kinds of antimetabolic drugs.

In a more detailed analysis using fluorescence-activated cell sorting, all co-treatments (VIB-NS398, VIB-celecoxib, PAC-NS398 and PAC-celecoxib) showed increased G2 arrest when compared with that observed upon monotherapy with each drug (Figure 2B). The finding suggests that the reduced proliferation observed upon the use of COX-2 inhibitors resulted from increased G2 cell cycle arrests. Because we observed an increased pre-G1 region in fluorescence-activated cell sorting (Figure 2B), we also performed annexin V staining. As seen in Figure 2C, early apoptotic events were increased by co-treatment. We confirmed our results with western blot analysis,

wherein C-PARP production greatly increased due to VIB co-treatments (Figure 3A). We conclude that COX-2 inhibitors increased apoptosis in VIB- and PAC-treated KBV20C cells.

We also observed that the number of apoptotic events increased with an increase in treatment duration. As seen in Figure 3B-C, the pre-G₁ region and early apoptotic events were greatly increased when drug treatment was continued for 2 days. The finding suggests that co-treatment can maintain toxicity over a longer period, with greater numbers of cells being irreversibly damaged.

We assumed that co-treatment with NS398 or celecoxib could restore cytotoxicity in antimetabolic drug-treated KBV20C cells. Since COX-2 inhibitors are not involved in inhibition of P-gp activity, it is evident, from Figure 1C, that

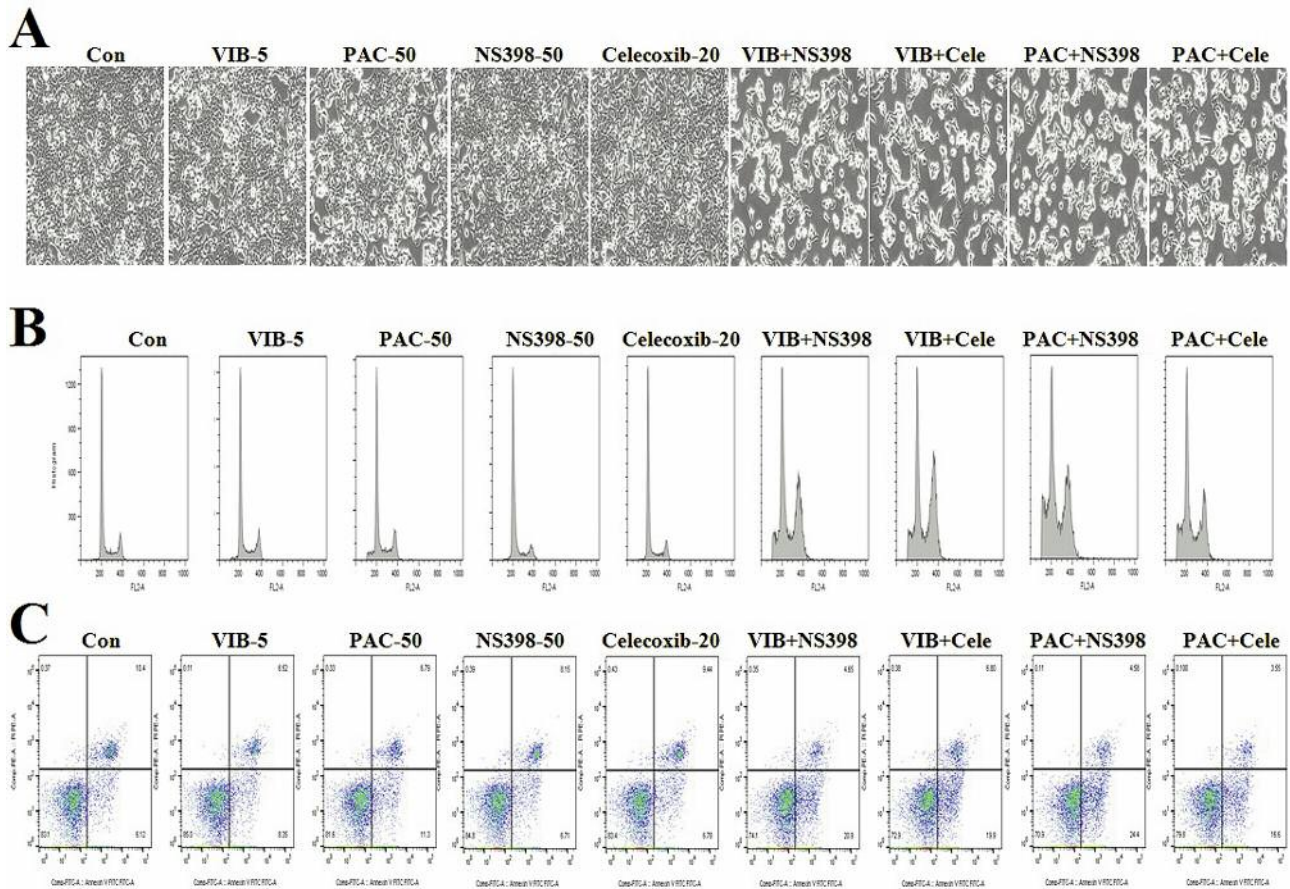


Figure 2. Co-treatment with COX-2 inhibitors strongly recovers the toxicity of antimetabolic drugs in resistant KBV20C cells. (A-C) KBV20C cells were grown on 6-well plates or 60 mm-diameter dishes and treated with 5 nM vinblastine (VIB-5), 50 nM paclitaxel (PAC-50), 50 μ M NS398 (NS398-50), 20 μ M celecoxib (Celecoxib-20), 5 nM vinblastine with 50 μ M NS398 (VIB+NS398), 5 nM vinblastine with 20 μ M celecoxib (VIB+Cele), 50 nM paclitaxel with 50 μ M NS398 (PAC+NS398), 50 nM paclitaxel with 20 μ M celecoxib (PAC+Cele) or 0.1% DMSO (Con). After 2 days, all cells were observed using an inverted microscope with a 100 magnification (A). After 24 h, FACS (B) and Annexin V (C) analyses were performed as described in the Materials and Methods section.

VIB-NS398 and VIB-celecoxib sensitization in KBV20C cells involves intracellular pathways to recover NS398 and celecoxib to increase sensitization. We have also determined the most effective combination of COX-2 inhibitors and antimetabolic drugs. Using various techniques, such as microscopic observations, fluorescence-activated cell sorting and C-PARP production analysis (Figure 2A-3C), we could identify effective co-treatments. However, after analyzing the obtained data, we conclude that there was no significant difference in efficacy between any of the treatment combinations. Considering that both NS398 and celecoxib have different molecular structures for targeting COX-2 inhibition, we conclude that co-treatment with other COX-2 inhibitors to sensitize drug-resistant cancer cells may be similarly effective.

Celecoxib has more sensitization activity than NS398 in co-treated sensitive KB cells. To determine the molecular mechanisms underlying the NS398- or celecoxib-associated increase in VIB-induced toxicity in KBV20C cells, we tested whether co-treatment with VIB and COX-2 inhibitors influences the activation or expression of signaling proteins. When we assessed the levels of important proliferation-related proteins or phosphorylation levels (26, 27), we did not identify major signal changes (Figure 4A), suggesting that a more detailed analysis may be needed for further identification of important signaling proteins in co-treatments.

Sensitive KB and resistant KBV20C cells exhibit similar sensitization by monotherapy with COX-2 inhibitors (Figure 1A-B). Additionally, we tested whether KB cells also showed increased sensitization upon combination

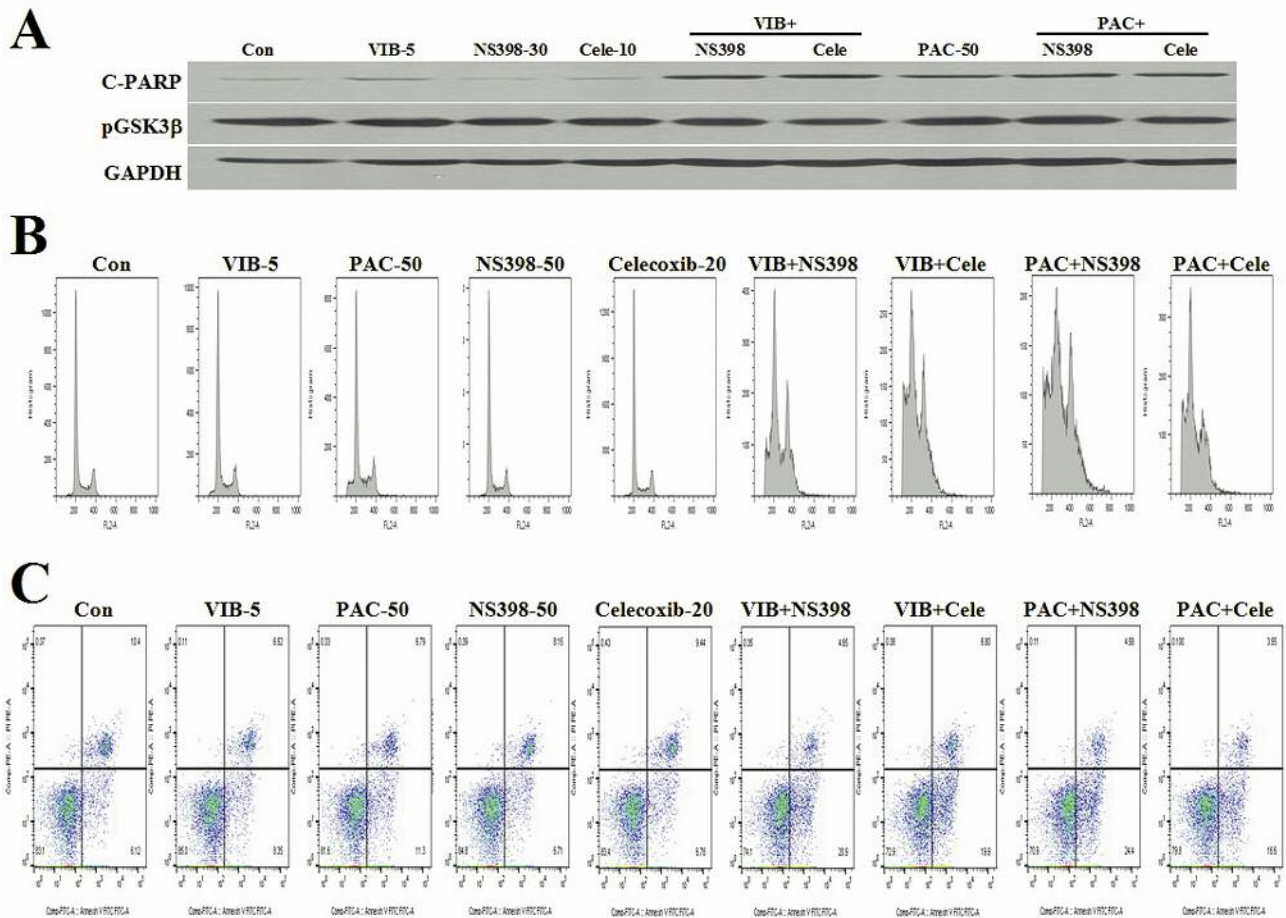


Figure 3. Co-treatment with COX-2 inhibitors greatly increases apoptosis of antimitotic drug-treated resistant KBV20C cells. (A) KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 nM vinblastine (VIB-5), 50 nM paclitaxel (PAC-50), 30 μM NS398 (NS398-30), 10 μM celecoxib (Celecoxib-10), 5 nM vinblastine with 30 μM NS398 (VIB+NS398), 5 nM vinblastine with 10 μM celecoxib (VIB+Cele), 50 nM paclitaxel with 30 μM NS398 (PAC+NS398), 50 nM paclitaxel with 10 μM celecoxib (PAC+Cele) or 0.1% DMSO (Con). After 24 h, Western blot analysis was performed using antibodies against C-PARP and GAPDH. (B-C) KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 nM vinblastine (VIB-5), 50 nM paclitaxel (PAC-50), 50 μM NS398 (NS398-50), 20 μM celecoxib (Celecoxib-20), 5 nM vinblastine with 50 μM NS398 (VIB+NS398), 5 nM vinblastine with 20 μM celecoxib (VIB+Cele), 50 nM paclitaxel with 50 μM NS398 (PAC+NS398), 50 nM paclitaxel with 20 μM celecoxib (PAC+Cele) or 0.1% DMSO (Con). After 2 days, FACS (B) and Annexin V (C) analyses were performed as described in the Materials and Methods section.

treatment with VIB-NS398 or VIB-celecoxib. As seen in Figure 4B, VIB-celecoxib exhibited more sensitization than VIB-NS398, suggesting that celecoxib is more effective than NS398 in treating sensitive KB cells when used in combination with antimitotic drugs. We also confirmed that this increased sensitization occurs upon co-treatment with VIC (Figure 4C). This finding implies that cell line specificity for different COX-2 inhibitors is possible but resistant cancer cells have similar sensitization specificity for both NS398 and celecoxib, thus suggesting that COX-2 inhibitors have common sensitization mechanisms for resistant cancer cells.

Discussion

The importance of COX-2 inhibitors in cancer treatment has been previously demonstrated (6, 7, 18, 19). There are two well-known inhibitors, NS398 and celecoxib, that have been investigated in various cancer models, including drug-resistant cancer cells, as potential cancer treatments (11, 12, 14). However, studies comparing different COX-2 inhibitors in drug-resistant cancer models have not been conducted. It is important to test which type of drug can sensitize specific cell types for their optimal application in the clinical setting. In this study, we investigated the sensitization efficacy of

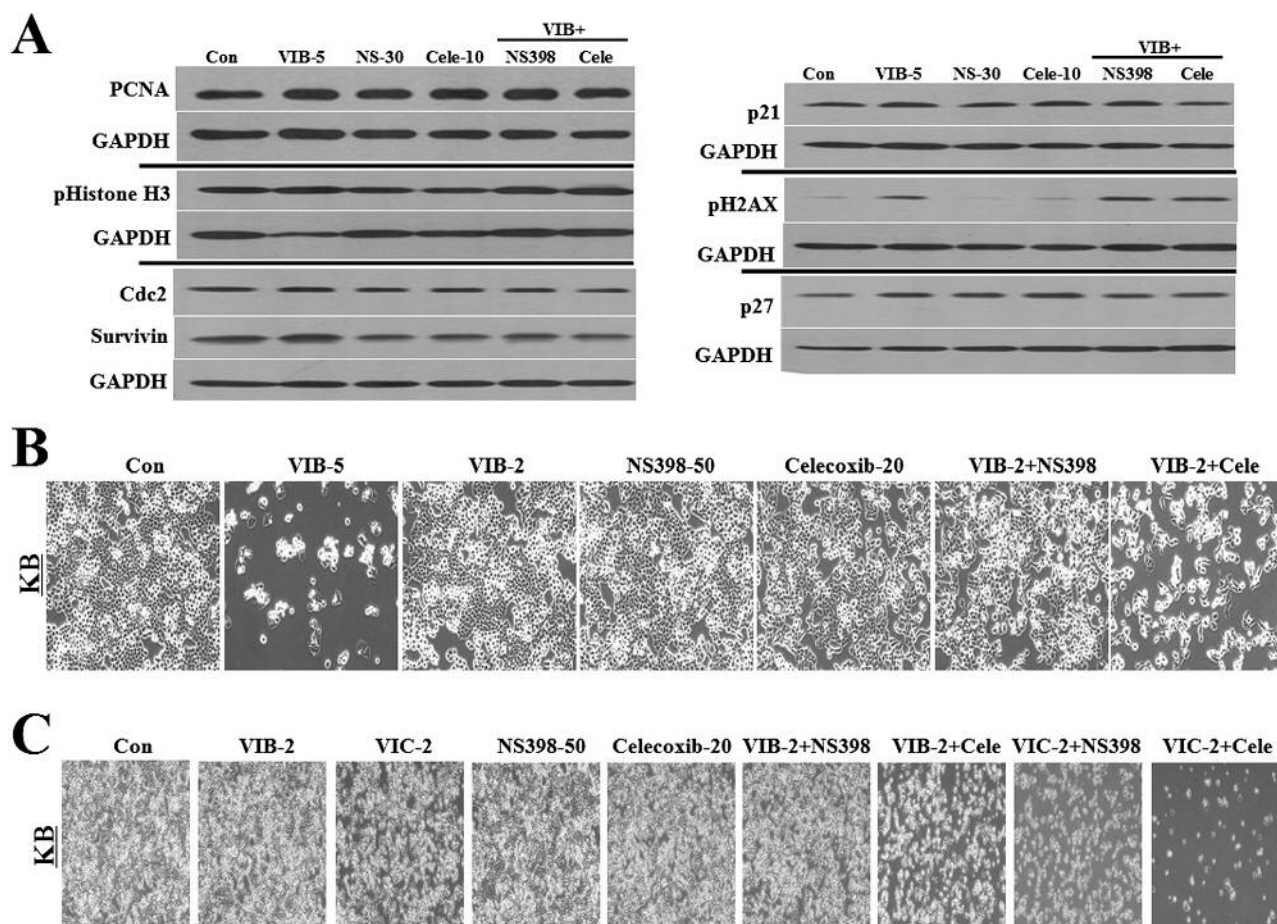


Figure 4. Celecoxib has greater sensitization activity than that of NS398 in co-treated sensitive KB cells. (A) KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 nM vinblastine (VIB-5), 30 μ M NS398 (NS-30), 10 μ M celecoxib (Cele-10), 5 nM vinblastine with 30 μ M NS398 (VIB+NS398), 5 nM vinblastine with 10 μ M celecoxib (VIB+Cele) or 0.1% DMSO (Con). After 24 h, western blot analysis was performed using antibodies against PCNA, pHistone H3, Cdc2, survivin, p21, pH2AX, p27 and GAPDH. (B-C) KB cells were grown on 6-well plates and treated with 5 nM vinblastine (VIB-5), 2 nM vinblastine (VIB-2), 2 nM vincristine (VIC-2), 50 μ M NS398 (NS398-50), 20 μ M celecoxib (Celecoxib-20), 2 nM vinblastine with 50 μ M NS398 (VIB-2+NS398), 2 nM vinblastine with 20 μ M celecoxib (VIB-2+Cele), 2 nM vincristine with 50 μ M NS398 (VIC-2+NS398), 2 nM vincristine with 20 μ M celecoxib (VIC-2+Cele) or 0.1% DMSO (Con). After 1 day, all cells were observed using an inverted microscope with a x100 magnification (B). After 2 days, all cells were observed using an inverted microscope with a x50 magnification (C).

NS398 and celecoxib in antimitotic drug-resistant KBV20C cancer cells. These drugs are already used in clinical settings. Thus, once their mechanism of action in cancer cells is known, these drugs could be readily available for use without further toxicity studies.

We found that NS398 and celecoxib have similar sensitization effects in drug-resistant cancer cells. KBV20C cells are highly resistant to anticancer drugs, such as VIC. However, both COX-2 inhibitors sensitized drug-resistant KBV20C cells and parent sensitive KB cells to a similar degree. These results suggest that COX-2 inhibitors are not substrates for P-gp-mediated efflux pumping. Our data also

confirmed that neither of the two drugs affected P-gp activity in KBV20C cells.

Next, we tested whether co-treatment with NS398 or celecoxib could increase sensitization in combination with VIB or PAC in resistant KBV20C cells. We found that co-treatment with NS398 or celecoxib sensitized both VIB- and PAC-treated KBV20C cells. We confirmed that the KBV20C-specific sensitization was independent of P-gp inhibition when using two different P-gp substrates, calcein-AM and rhodamine. The results suggest that NS398 and celecoxib target cellular signaling pathways specific to antimitotic drug-resistant cancer cells. The sensitization

mechanism involves an increase in G₂-phase cell-cycle arrest in KBV20C cells. It is assumed that patients who are resistant to antimetabolic drugs could be treated with NS398 or celecoxib to effectively increase G₂-phase cell-cycle arrest. COX-2 inhibitors can be considered and categorized as drugs that enhance the activity of antimetabolic drugs in resistant cancer cells without P-gp inhibition. The increase in G₂-phase cell-cycle arrest by COX2 inhibitors finally resulted in increased apoptosis, which was confirmed by an increase in pre-G₁ phase cell-cycle arrest, C-PARP production and annexin V staining. Additionally, the COX-2 inhibitor sensitization effect on KBV20C cells is time- and dose-dependent showing that co-treatment can maintain toxicity over a prolonged period, with greater numbers of cells being irreversibly damaged. We also demonstrated that two different antimetabolic drugs, VIB and PAC, have similar efficacy when combined with either NS398 or celecoxib for sensitization. Considering that the antimetabolic drug VIB targets the vinca domain and PAC targets the taxane-binding site of microtubules, we assumed that the results could be replicated for various kinds of antimetabolic drugs.

Since both NS398 and celecoxib have different structures and similar functional activity to target COX-2 inhibition, we conclude that our findings are conserved in COX-2 inhibitors and can be applied to other COX-2 inhibitors. In the future, it will be important to determine whether other resistant cancer cell lines, including different organ-derived cancer cells, also exhibit the same sensitivity to COX-2 inhibitors. The molecular targets for the strong sensitization effects of co-treatment with COX-2 inhibitors and antimetabolic drugs could be one of the important issues to address in further studies. Future studies using an *in vivo* mouse model are warranted for assessing the sensitization effect and toxicity of COX-2 inhibitors.

Our work demonstrated that two COX-2 inhibitors could sensitize both resistant and sensitive cancer cells without P-gp inhibition. We also provided evidence that both COX-2 inhibitors have applications in the treatment of drug-resistant cancer patients. We found that both NS398 and celecoxib, as strong sensitizing drugs in antimetabolic drug-treated resistant cancer cells, increased apoptosis *via* G₂-phase cell-cycle arrest. Since COX-2 inhibitors have been shown to have a sensitization effect on cancer independent of COX-2 inhibition (12, 13), we propose that our results can be applied to other drugs targeting resistant cancer cells, irrespective of whether they are COX-2 inhibitors or not. Our study may help improve COX-2 inhibitor-based chemotherapeutic treatments for cancer patients who develop resistance to anticancer drugs.

Conflicts of Interest

The Authors declare that no conflicts of interest exist.

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