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P-gp Inhibition by the Anti-psychotic Drug Pimozide Increases Apoptosis, as well as Expression of pRb and pH2AX in Highly Drug-resistant KBV20C Cells

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Abstract. *Background/Aim: The present study was designed to identify drugs that could sensitize P-glycoprotein (P-gp)-overexpressing resistant KBV20C cancer cells to treatment with Halaven (HAL) or vincristine (VIC). Materials and Methods: Using relatively low doses or IC_{50} concentrations of drugs to sensitize anti-mitotic drug-resistant KBV20C cells, pimozide (PIM) sensitized HAL-resistant KBV20C cancer cells, with higher P-gp inhibitory activity than another anti-psychotic drug, fluphenazine (FLU). Results: The first-generation P-gp inhibitor verapamil required a dose that was similar to that of PIM for P-gp inhibition, suggesting that PIM has a similar specificity for binding P-gp to prevent efflux of anti-mitotic drugs. Furthermore, co-treatment with PIM and another anti-mitotic drug, VIC, also increased sensitization of KBV20C cells, suggesting that PIM can be combined with other anti-mitotic drugs to sensitize resistant cancer cells. PIM caused a reduction in cell viability and an increase in the number of cells arresting at the G_2 phase of the cell cycle. PIM induced both early and late apoptosis in KBV20C cells in response to HAL treatment. Furthermore, the DNA damage marker pH2AX, the cell-cycle protein pRb, and the pro-apoptotic protein C-PARP levels increased after HAL-PIM co-treatment, indicative of a mechanism involving G_2 phase arrest and an increase in the number of cells undergoing apoptosis. Conclusion: PIM may be a promising therapeutic agent for the treatment of cancers that are resistant to anti-mitotic drugs.*

Anti-mitotic drugs inhibit mitosis by targeting microtubules and preventing their polymerization or depolymerization. Paclitaxel, docetaxel, vincristine (VIC), vinorelbine, vinblastine, and halaven (HAL) are some examples of anti-mitotic drugs (1-3). Eribulin, also called Halaven®, was recently developed and used in the clinical treatment of resistant or metastatic cancer. HAL has been developed to overcome the resistance of cancer cells to routinely used anti-mitotic drugs (4, 5). It targets the depolymerization of microtubules. HAL is considered a promising drug for triple-negative breast cancer and certain resistant cancer types (5, 6). Since patients are expected to develop resistance to HAL, identifying the mechanism(s) that underlie cell sensitization would be an important step in the development of more effective treatments by designing approaches to increase HAL-associated apoptosis. While anti-mitotic drugs are widely used to treat cancer, cancer cells can develop resistance in various ways. One such mechanism is *via* overexpression of P-glycoprotein (P-gp) on cell membranes. P-gp is a membrane channel that can pump out anti-mitotic drugs and thus prevent drug-induced toxicity (7-10). Identifying sensitization mechanisms or drugs for cancer cells that overexpress P-gp would lead to better treatment for patients who develop resistance to anti-mitotic drugs.

Drug repositioning or drug repurposing is the application of known drugs to new indications (11-13). This approach has been used for various diseases and is inexpensive. The time-consuming process of performing a significant number of toxicity tests can also be avoided and this is one of the advantages of drug repositioning. This is a powerful tool to identify effective treatment options for new diseases in a timely manner. For example, the urgent need for pharmacological treatments for resistant cancer can be efficiently addressed with drug repositioning and can be applied to human patients in a relatively short time frame. Successful application of the antiangiogenic drug thalidomide in cancer treatment is an example of drug repositioning (12, 13).

This study aimed to identify novel mechanisms of repositioned drugs, sensitize resistant cells, inhibit P-gp expression, and improve the efficacy of repositioned drugs

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when used in combination with chemotherapeutic drugs. Based on experimental candidate approaches found in the literature, we tried to identify novel repositioned drugs to be used in order to increase sensitization when used in single treatment or in combination with anti-mitotic drugs and overcome resistance in P-gp-overexpressing cancer.

Previously, the anti-psychotic drug fluphenazine (FLU) demonstrated inhibitory activity against P-gp and an ability to sensitize HAL-resistant cancer cells to treatment (14). Here, we found another anti-psychotic drug, pimozide (PIM), for the sensitization of KBV20C cells. We found that PIM, when used at low doses, had high inhibitory activity against P-gp and sensitized HAL- or VIC-treated resistant cancer cells. As anti-psychotic drugs have already been used in humans, these results will contribute to the development of P-gp inhibitor-based therapies for the co-treatment of highly drug-resistant tumors.

Materials and Methods

Reagents and cell culture. Rhodamine 123 (Rhodamine), FLU, and verapamil (VER) were purchased from Sigma-Aldrich (St. Louis, MO, USA). VIC was purchased from Enzo Life Sciences (Farmingdale, NY, USA). PIM was purchased from Selleckchem (Houston, TX, USA) and Abcam (Cambridge, UK). Aqueous solutions of HAL (Eisai Korea, Seoul, South Korea) were obtained from the National Cancer Center in South Korea.

Antibodies against p21 and cleaved poly ADP ribose polymerase (C-PARP) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β-actin, cyclin-dependent kinase 4 (CDK4), retinoblastoma protein (pRb), cyclin D1, cyclin B1, cell division cyclin protein 2 (CDC2), cyclin E, survivin, pAkt, pJnk, and p38 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against phosphated-H2A histone family member X (pH2AX) was obtained from Abcam (Cambridge, UK). Human oral squamous carcinoma cell line 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 (14-18). All cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (WelGENE, Daegu, South Korea).

Microscopic observation. Cells were grown in 30-40% confluency in 60-mm diameter dishes and treated for 24 or 48 h with HAL, FLU, verapamil, or PIM, alone and in combination or with 0.1% dimethylsulfoxide (DMSO; Control). The medium was removed, and phosphate-buffered saline (PBS) was added to each dish. Cells were examined immediately in two independent experiments using an ECLIPSE Ts2 inverted microscope (Nikon, Tokyo, Japan) with a 4× or a 10× objective lens.

Rhodamine uptake tests. The tests used to assess the ability of a drug to inhibit P-gp were based on a previously described method (19-21). Briefly, cells grown at 30-40% confluency in 60-mm diameter dishes were treated for 4 or 24 h with verapamil, FLU, PIM, or 0.1% DMSO. Cells were then incubated with 2 μg/mI rhodamine for 1 h 30 min at 37°C. The medium was removed, and the cells were washed with PBS. The stained cells were analyzed for P-gp inhibition in two independent experiments using a Guava EasyCyte Plus Flow Cytometer (Merck Millipore, Billerica, MA, USA).

Fluorescence-activated cell sorting (FACS) analysis. FACS analysis was performed as previously described (19-21). Cells grown at 30-40% confluency in 60-mm diameter dishes were treated for 24 h with HAL, or PIM, alone and in combination or with 0.1% DMSO. 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°C, washed with PBS, and re-suspended in a cold propidium iodide (PI) staining solution (100 μg/ml RNase A and 50 μg/ml PI in PBS) for 30 min at 37°C. The stained cells were analyzed to determine the distribution of cells in the different cell cycle phase in two independent experiments using a Guava EasyCyte Plus Flow Cytometer (Merck Millipore, Billerica, MA, USA).

Annexin V analysis. Annexin V analysis was conducted using annexin V-fluorescein isothiocyanate (FITC) staining kit (BD Bioscience, Franklin, NJ, USA) as previously described (19-21). Cells grown at 30-40% confluency in 60-mm diameter dishes were treated for 24 h with HAL, verapamil, or PIM, alone and in combination or with 0.1% DMSO. The cells were then dislodged by trypsin and pelleted by centrifugation. The pelleted cells were washed with PBS. Cells in 100 μl of binding buffer received 5 μI of Annexin V-FITC and 5 μI of PI and were then incubated for 15 min at room temperature. The stained cells were analyzed for apoptosis in two independent experiments using a Guava EasyCyte Plus Flow Cytometer (Merck Millipore, Burlington, MA, USA).

Western blot analysis. KBV20C cells grown at 30-40% confluency on 60 mm-diameter dishes were treated with HAL, or PIM, alone and in combination or with 0.1% DMSO (Con). Total cellular proteins were then extracted as previously described (16, 19, 20). Briefly, cells were washed twice with cold PBS and detached with scrapers. For total protein isolation, cells were suspended in PRO-PREP™ protein extract solution (iNtRON, Seongnam, South Korea) and placed on ice for 30 min. The suspension was collected after centrifugation at 15,000 × *g* for 5 min at 4˚C. Protein concentrations were measured by using a protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to western blot analysis as previously described (16, 19, 20).

Results

Co-treatment with PIM sensitized HAL-treated KBV20C cells more effectively than treatment with FLU. The KBV20C cell line is a very useful model of highly HAL-resistant cancer cells. We previously showed that the concentration of HAL required for the induction of a similar rate of apoptosis was approximately 500-fold higher than that required by the parental drug-sensitive KB cells (14, 21).

Previously, the anti-psychotic drug FLU was shown to sensitize HAL-treated KBV20C cells (14). Investigating whether other anti-psychotic drugs could also sensitize HAL-treated KBV20C cells, we discovered that PIM can also

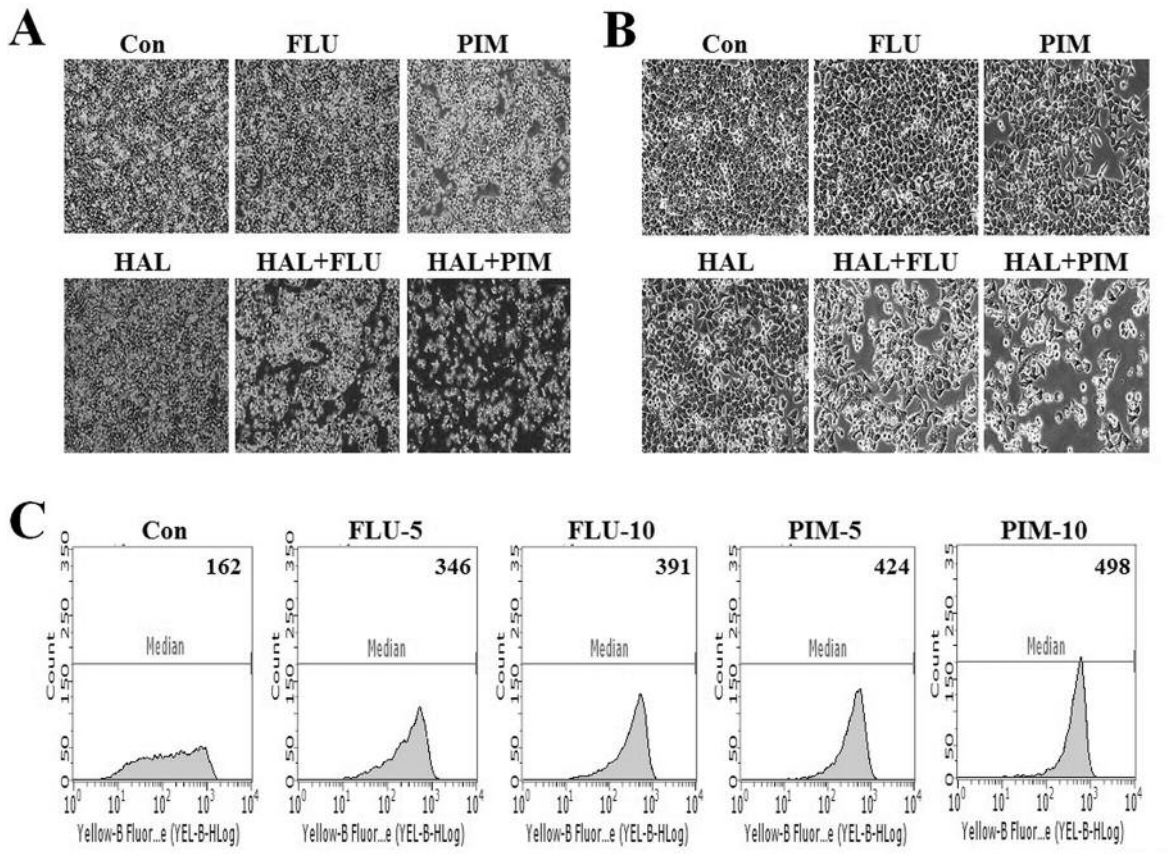

Figure 1. *Co-treatment with another anti-psychotic drug, PIM, sensitized HAL-treated KBV20C cells to a greater extent than FLU. (A-B) KBV20C cells were grown on 60 mm-diameter dishes and treated with 50 ng/ml HAL (HAL), 5 μM fluphenazine (FLU), 5 μM pimozide (PIM), 50 ng/ml HAL with 5 μM fluphenazine (HAL+FLU), 50 ng/ml HAL with 5 μM pimozide (HAL+PIM), or 0.1% DMSO (Con). After 1 day, all cells were observed using an inverted microscope at (A) ×4 magnification or (B) ×10 magnification (scale bar=100 μm). (C) KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 μM fluphenazine (FLU-5), 10 μM fluphenazine (FLU-10), 5 μM pimozide (PIM-5), 10 μM pimozide (PIM-10), or 0.1% DMSO (Con). After 24 h, all cells were stained with rhodamine 123 and examined by using FACS analysis, as described in Materials and Methods.*

sensitize these cells. Therefore, a more detailed analysis of the effects of PIM was performed in order to provide further evidence for its clinical application in anti-mitotic drug-resistant cancer cells.

First, the sensitizing effects of the two anti-psychotic drugs FLU and PIM on HAL-treated KBV20C cells were compared. Microscopic observations showed that PIM exerts a sensitization effect approximately 2-fold greater than FLU at the same concentration (Figure 1A and B). These results demonstrated that PIM has greater effectiveness in sensitizing HAL-resistant cancer cells than FLU. This suggests that PIM can be used to sensitize HAL-resistant cancer at a low dose and with reduced drug toxicity.

KBV20C cells are HAL-resistant because they overexpress P-gp (14, 17, 21). Since the anti-psychotic drug FLU sensitized HAL-treated KBV20C cells *via* P-gp inhibition (14), it was also tested whether increased sensitization by PIM could be achieved by inhibiting P-gp activity. Rhodamine 123, a well-known P-gp substrate, was used to measure P-gp inhibition (21, 22). In this experiment, cellular accumulation of green fluorescence was indicative of rhodamine 123 intracellular accumulation. As shown in Figure 1C, PIM had better P-gp inhibitory activity than FLU, suggesting that PIM binds to P-gp with higher specificity than FLU does. Considering that KBV20C cells are sensitized to a greater extent by PIM than by FLU, P-gp inhibition for HAL efflux may be the main mechanism by which PIM sensitizes HAL-treated KBV20C cancer cells. In conclusion, P-gp inhibition by PIM resulted in the sensitization of HAL-treated KBV20C cells.

The anti-psychotic drug PIM had similar P-gp inhibitory activity to verapamil. In order to investigate P-gp inhibition

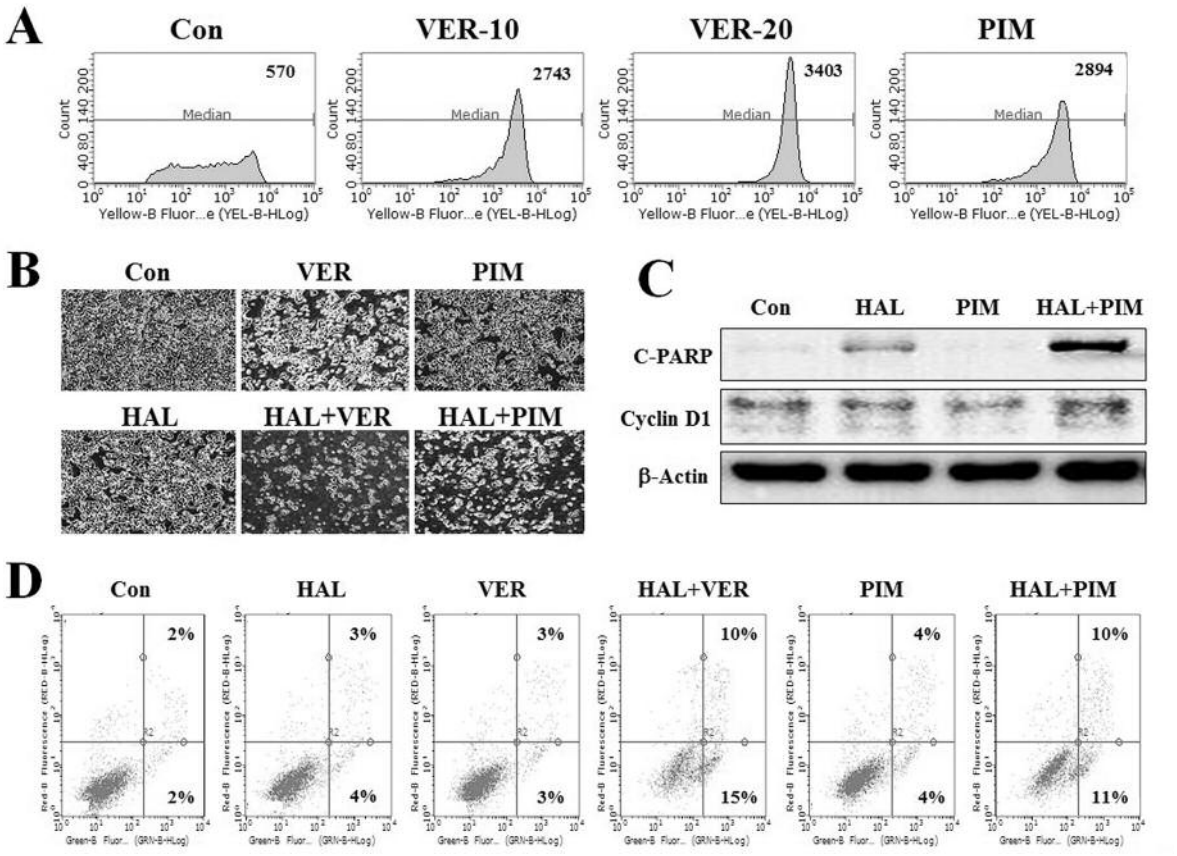

Figure 2. *The anti-psychotic drug PIM showed similar sensitizing effects and P-gp inhibitory activity to verapamil. (A) KBV20C cells were grown on 60 mm-diameter dishes and treated with 10 μM verapamil (VER-10), 20 μM verapamil (VER-20), 5 μM pimozide (PIM), or 0.1% DMSO (Con). After 24 h, all cells were stained with rhodamine 123 and examined by FACS analysis, as described in Materials and Methods. (B) KBV20C cells were grown on 60 mm-diameter dishes and treated with 50 ng/ml HAL (HAL), 10 μM verapamil (VER-10), 10 μM pimozide (PIM), 50 ng/ml HAL with 10 μM verapamil (HAL+VER), 50 ng/ml HAL with 10 μM pimozide (HAL+PIM), or 0.1% DMSO (Con). After 1 day, all cells were observed using an inverted microscope at ×4 magnification (scale bar=100 μm). (C) KBV20C cells were plated on 60 mm-diameter dishes and treated with 50 ng/ml HAL (HAL), 5 μM pimozide (PIM), 50 ng/ml HAL with 5 μM pimozide (HAL+PIM), or 0.1% DMSO (Con). After 24 h, western blot analysis was performed using antibodies against C-PARP, cyclin D1, and β-actin. (D) KBV20C cells were grown on 60 mm-diameter dishes and stimulated with 50 ng/ml HAL (HAL), 10 μM verapamil (VER-10), 10 μM pimozide (PIM), 50 ng/ml HAL with 10 μM verapamil (HAL+VER), 50 ng/ml HAL with 10 μM pimozide (HAL+PIM), or 0.1% DMSO (Con). After 24 h, Annexin V analyses were performed as described in Materials and Methods.*

by PIM, its inhibitory activity was compared to that of the well-known P-gp inhibitor verapamil (21, 22). As seen in Figure 2A, PIM had similar P-gp inhibitory activity to verapamil when used at the same dose. This indicates that PIM and verapamil have similar P-gp binding specificity, suggesting that PIM can be used to minimize toxicity, instead of the well-known first-generation P-gp inhibitor verapamil. Also, a shorter treatment time (4 h treatment with PIM or verapamil) resulted in similar P-gp inhibitory activity as that obtained after 24 h treatment (data not shown). This suggests that PIM inhibited P-gp directly *via* binding, an inhibitory mechanism that is similar to that of verapamil. It can be suggested that PIM can replace well-known P-gp inhibitors in clinical applications, while maintaining P-gp inhibiting specificity and providing reduced toxicity.

Co-treatment with PIM increased apoptosis in HAL-treated KBV20C cells similarly to verapamil treatment. Next, the sensitizing effects of verapamil were compared to that of PIM on HAL-treated KBV20C cells. As shown in Figure 2B, 10 μM PIM and 10 μM verapamil produced similar sensitization effects on cells treated with HAL, and suggesting that a low dose of PIM is sufficient and as effective as the P-gp inhibitor verapamil in sensitizing P-gp-overexpressing resistant cancer cells.

Apoptotic analysis using annexin V staining was also used to confirm these results (Figure 2D). The results demonstrated that PIM is as effective as verapamil in sensitizing HAL-resistant cancer cells. This also suggests that PIM can be used at a low dose and with reduced drug toxicity to sensitize HAL-resistant cancer.

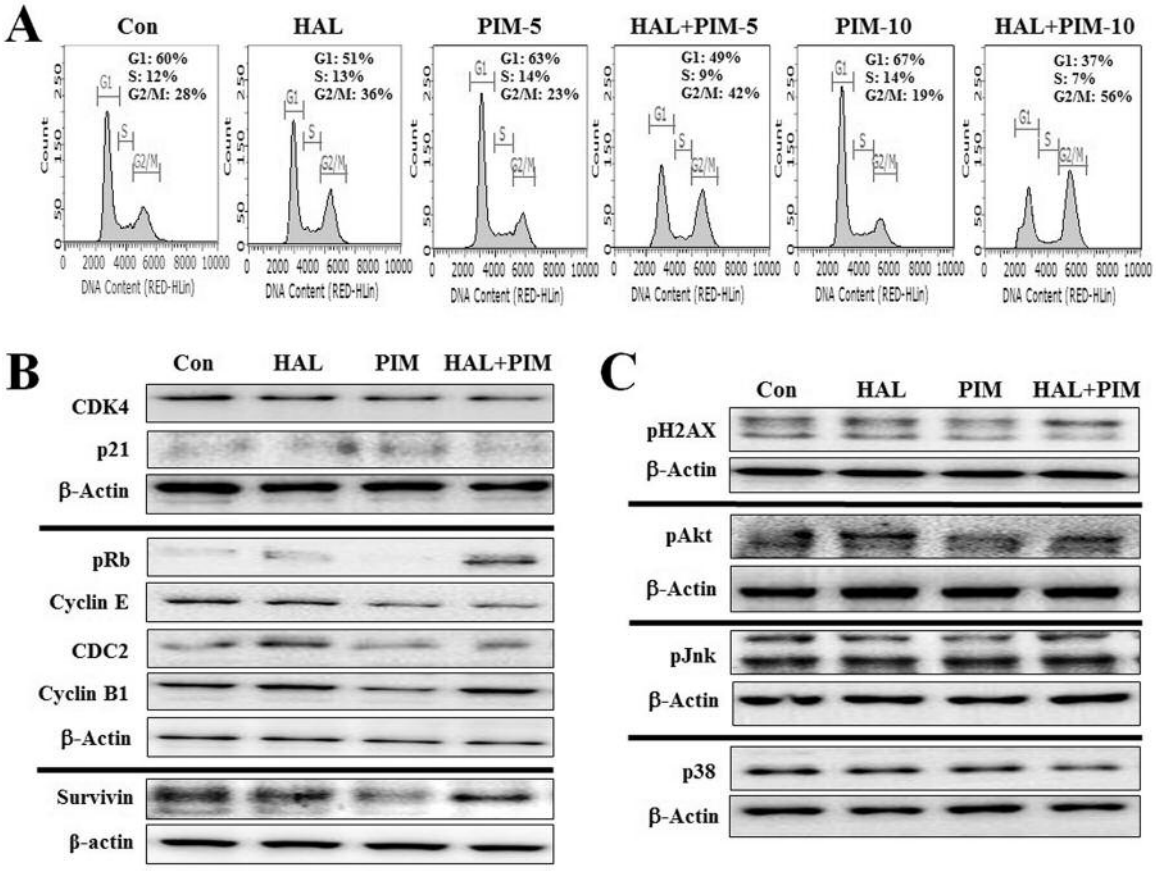

Figure 3. *Co-treatment of KBV20C cells with PIM and HAL induced G_2 arrest and increased expression of DNA damage proteins. (A) KBV20C cells were grown on 60 mm-diameter dishes and treated with 50 ng/ml HAL (HAL), 5 μM pimozide (PIM-5), 10 μM pimozide (PIM-10), 50 ng/ml HAL with 5 μM pimozide (HAL+PIM-5), 50 ng/ml HAL with 10 μM pimozide (HAL+PIM-10), or 0.1% DMSO (Con). After 24 h, FACS analyses were performed as described in Materials and Methods. (B-C) KBV20C cells were plated on 60 mm-diameter dishes and treated with 50 ng/ml HAL (HAL), 5 μM pimozide (PIM), 50 ng/ml HAL with 5 μM pimozide (HAL+PIM), or 0.1% DMSO (Con). After 24 h, western blot analysis was performed using antibodies against CDK4, p21, pRb, Cyclin E, CDC2, Cyclin B1, Survivin, pH2AX, pAkt, pJnk, p38, and β-actin.*

In order to confirm the increased apoptosis seen in co-treated cells, the levels of C-PARP were measured (18, 23). As seen in Figure 2C, C-PARP expression increased in HAL-PIM co-treated cells, indicating that co-treatment with PIM and HAL increased apoptosis of drug-resistant KBV20C cells. In addition, co-treatment with HAL and PIM increased the number of cells in both early and late phases of apoptosis, when compared to single treatment with PIM (Figure 2D). Altogether, these data indicate that co-treatment with the anti-psychotic drug PIM sensitized KBV20C cells to HAL treatment, with similar efficacy to that of verapamil.

Co-treatment of KBV20C cells with PIM and HAL induced G2-arrest. In order to further clarify the mechanism of action of HAL-PIM co-treatment, FACS analysis was performed. As shown in Figure 3A, co-treatment with HAL and PIM increased the number of cells in G_2 compared with that observed after single treatment with either agent. A positive relationship was also detected between the dose of PIM and the proportion of HAL-treated cells arrested in G2 (Figure 3A), suggesting that increased P-gp inhibition prevented continuous efflux of HAL from KBV20C cells. This also indicates that an increase in cell cycle arrest stimulated apoptosis.

In order to further investigate the expression of proteins involved in G_2 arrest (18, 24), western blot analysis was performed. As shown in Figure 2C and Figure 3B, when the production of the apoptotic marker C-PARP greatly increased, there were no significant differences in the expression of major cell cyclin-related proteins, such as CDK4, CDC2, cyclin E, cyclin D1, and p21.

Although a small increase in the levels of cyclin B1 and survivin was observed after co-treatment, these changes

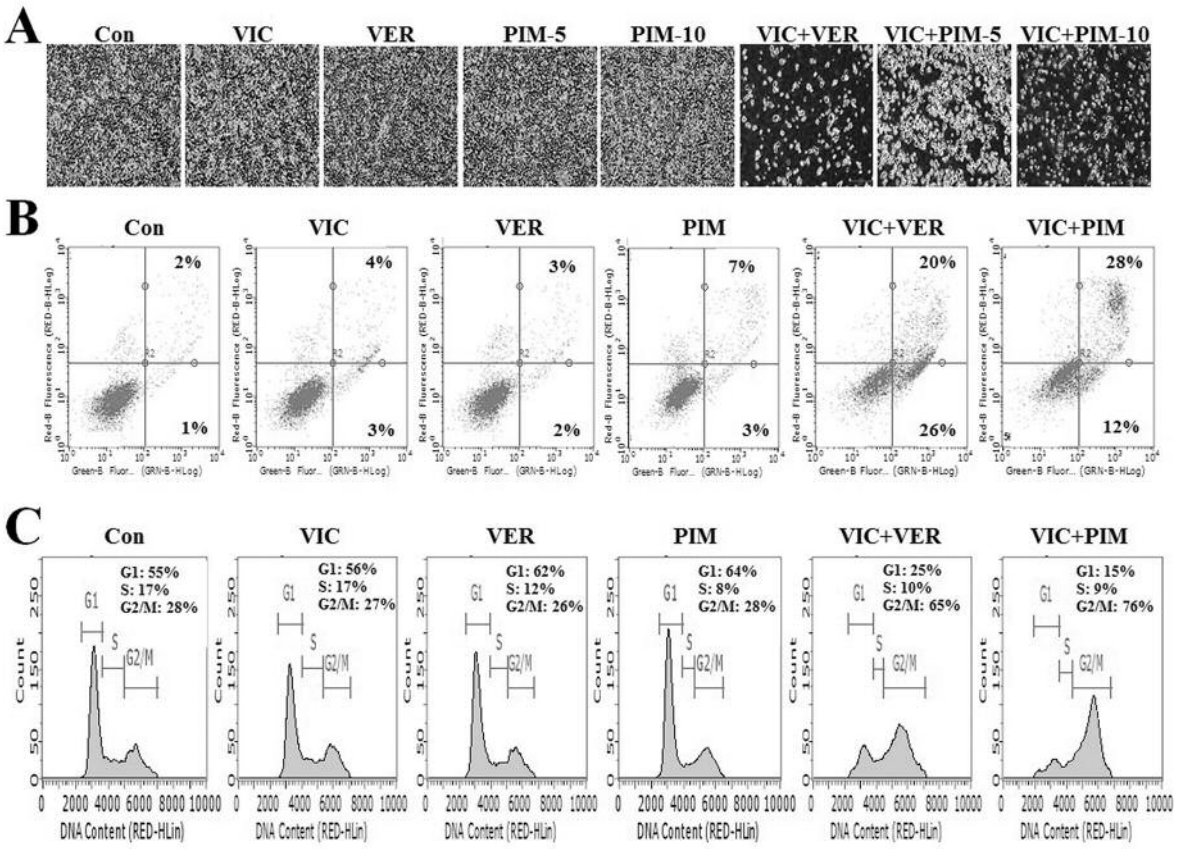

Figure 4. *Co-treatment with VIC-PIM sensitized drug-resistant KBV20C cells to the same extent as co-treatment with HAL-PIM. (A) KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 nM vincristine (VIC), 10 μM verapamil (VER), 5 μM pimozide (PIM-5), 10 μM pimozide (PIM-10), 5 nM vincristine with 10 μM verapamil (VIC+VER), 5 nM vincristine with 5 μM pimozide (VIC+PIM-5), 5 nM vincristine with 10 μM pimozide (VIC+PIM-10), or 0.1% DMSO (Con). After 1 day, all cells were observed using an inverted microscope at ×4 magnification (scale bar= 100 μm). (B) KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 nM vincristine (VIC), 10 μM verapamil (VER), 10 μM pimozide (PIM), 5 nM vincristine with 10 μM verapamil (VIC+VER), 5 nM vincristine with 10 μM pimozide (VIC+PIM), or 0.1% DMSO (Con). After 24 h, Annexin V analyses were performed as described in Materials and Methods. (C) KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 nM vincristine (VIC), 10 μM verapamil (VER), 10 μM pimozide (PIM), 5 nM vincristine with 10 μM verapamil (VIC+VER), 5 nM vincristine with 10 μM pimozide (VIC+PIM), or 0.1% DMSO (Con). After 24 h, FACS analyses were performed as described in Materials and Methods.*

were similar to control and negligible, suggesting that they did not contribute significantly to sensitization by HAL-PIM co-treatment. Importantly, pRB levels were largely changed following co-treatments, suggesting that it may increase G2 arrest in HAL-PIM co-treated KBV20C cells. Overall, co-treatment with PIM and HAL increased apoptosis of drug-resistant KBV20C cells *via* G_2 cell-cycle arrest.

In the next phase of our investigation, we investigated the expression of proteins involved in DNA damage and cellular signaling pathways. As seen in Figure 3C, the expression of the DNA damage protein marker pH2AX increased significantly following co-treatment. However, there were no significant differences in the expression of major signaling related proteins such as pAkt, pJnk, and p38. Thus, DNA damage and cell-cycle arrest ultimately increased apoptosis in HAL-PIM co-treated KBV20C cells.

PIM increased sensitization of VIC-treated KBV20C cells when used at a low dose. VIC is an anti-mitotic drug that is routinely used as a chemotherapeutic agent in patients with cancer (15, 20, 24). As seen in Figure 4A, low doses of PIM produced similar sensitizing effects when combined with VIC, as seen in comparison with PIM-HAL co-treatments. A positive relationship was also detected between the dose of PIM and the proportion of VIC-treated cells in sensitization, indicating that increased P-gp inhibition prevents continuous efflux of VIC from inside KBV20C cells. Annexin V staining was also used to confirm the results (Figure 4B). These results demonstrated that PIM was also effective in sensitizing VIC-

co-treated resistant cancer cells. As shown in Figure 4C, co-treatment with HAL and PIM increased the number of cells in G_2 arrest compared with that observed after single treatment with either agent. This suggests that co-treatment with VIC-PIM has similar mechanisms of action to HAL-PIM in sensitizing resistant cancer cells. PIM can be combined with other anti-mitotic drugs to prevent their efflux. PIM can be used in patients with various drug-resistant cancers.

Discussion

P-gp-overexpressing KBV20C cells were highly resistant to HAL (14, 21). KBV20C cells are, therefore, very useful as models for highly HAL-resistant cancer. We assumed that our studies using KBV20C cancer cells could provide insights in the treatment of patients with HAL-resistant cancer. HAL was recently developed and is especially promising in the treatment of patients who were not responsive to anticancer drugs (4-6). Patients with cancer who are treated with HAL will ultimately develop resistance to HAL, thus there is need to identify agents that sensitize HAL-resistant cancers.

We previously observed the sensitization effects of the anti-psychotic drug FLU in HAL-treated KBV20C cells (14). The cancer-sensitizing ability of another anti-psychotic drug, PIM, has been demonstrated in various cancer models (25-28), suggesting that it has potential as a chemotherapeutic agent. In this study, the effects of HAL-PIM co-treatment were analyzed for the first time. Interestingly, PIM exerted greater sensitizing effects than FLU in HAL-treated KBV20C cancer cells, suggesting that PIM can be more effectively used in combination with HAL at low dose and with reduced toxicity. Since the efflux of HAL by P-gp is the main mechanism of the resistance of KBV20C cells to HAL, we tested whether sensitization by HAL-PIM resulted from the P-gp inhibitory activity of PIM. We demonstrated that PIM has P-gp inhibitory activity and prevents the efflux of HAL.

Because PIM has higher P-gp inhibitory activity than FLU, we suggest that the greater sensitizing effects observed after HAL-PIM co-treatment resulted from the increased P-gp inhibitory activity by PIM. These results also indicate that the main sensitizing mechanism by HAL-PIM resulted from the specific P-gp inhibitory activity of PIM.

Furthermore, the P-gp inhibitory activity of PIM was compared to the first-generation P-gp inhibitor verapamil (7, 9, 22). Treatment with PIM required a similar dose to verapamil to obtain similar inhibitory activities in KBV20C cells. These results suggest that PIM can specifically inhibit P-gp members in HAL-resistant cancer cells. Our results also demonstrated that P-gp inhibition by PIM treatment can be achieved in a shorter treatment time, suggesting that PIM inhibited P-gp activity *via* direct binding to P-gp, thus preventing P-gp-mediated drug efflux. Considering that verapamil is a representative and well-known P-gp inhibitor (7, 8, 22), PIM should be regarded as a P-gp inhibitor. Since P-gp inhibitors have shown toxicity in normal cells (7-9, 22), our findings suggest that PIM can be applied as a low-dose P-gp inhibitor in combination with HAL for the sensitization of HAL-resistant cancer cells. Thus, repositioned drugs such as PIM may easily be given to patients with HAL-resistant cancer.

More importantly, our results were not limited to HAL co-treatment. PIM could exert similar sensitizing effects when used in combination with VIC in P-gp-overexpressing KBV20C cells. These results also indicated that PIM can sensitize other anti-mitotic drug-resistant cancer cells by inhibiting drug efflux. We propose that PIM may be used in combination with other anticancer drugs to sensitize resistant cancer cells. However, further *in vivo* studies using animal models are required to assess the efficacy of the combination therapy of anti-mitotic drugs and PIM before investigating its possible use in clinical trials.

In addition, HAL-PIM co-treatment reduced cellular proliferation and increased G_2 arrest in highly HAL-resistant KBV20C cells. Based on the microscopic analysis, FACS analysis, annexin V analysis, and western blot analysis, we concluded that apoptosis was increased by HAL-PIM co-treatment through an increase in G_2 arrest and reduced proliferation. Furthermore, the DNA damage marker pH2AX, the cell cycle protein pRb, and the pro-apoptotic protein C-PARP levels increased after HAL-PIM co-treatment. This is indicative of a mechanism involving G_2 phase arrest *via* DNA damage, and of an increase in the number of cells undergoing apoptosis. We hypothesize that PIM prevented efflux of HAL, thereby allowing HAL to stimulate G_2 arrest by increasing DNA damage, reducing cellular proliferation, and finally increasing apoptosis of HAL-treated resistant KBV20C cells.

Altogether, our results showed that drug-resistant KBV20C cells that overexpress P-gp can be sensitized to treatment with anti-mitotic drugs (HAL or VIC) by anti-psychotic drugs (FLU or PIM). Most importantly, it was found that PIM selectively sensitizes anti-mitotic drug-resistant cancer cells *via* P-gp inhibition. Since these repositioned drugs are already used in clinical settings, the urgent need for pharmacological treatments of drug-resistant cancers can be effectively addressed.

Conflicts of Interest

The Authors declare no conflict of interests.

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