ANTICANCER RESEARCH *41*: 687-697 (2021)
doi:10.21873/anticanres.14820

A Low Dose of Aripiprazole Has the Strongest Sensitization Effect Among 19 Repositioned Bipolar Drugs in P-gp-overexpressing Drug-resistant Cancer Cells

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Abstract. *Background/Aim: We investigated drugs that could sensitize P-glycoprotein (P-gp)-overexpressing drug-resistant cancer cells to vincristine (VIC) or eribulin treatment and assessed their associated mechanisms of action. Materials and Methods: We investigated 15 bipolar drugs (quetiapine, risperidone, clozapine, asenapine, iloperidone, paliperidone, ziprasidone, trifluoperazine, loxapine succinate, pilocarpine, valproic acid, carbamazepine, levetiracetam, topiramate, and felbamate) to identify drugs with a sensitizing effect on VIC-resistant KBV20C cells at relatively low doses. Fluorescence-activated cell sorting (FACS), annexin V analyses, and rhodamine uptake tests were performed to further investigate the mechanism of action. Results: We found that co-treatment with half the tested drugs (quetiapine, iloperidone, trifluoperazine, loxapine, risperidone, ziprasidone, or felbamate) at low doses could highly sensitize VIC-resistant KBV20C cells. With lower amounts of the bipolar drugs or VIC, we found that among the 15 bipolar drugs tested, 2 combinations (VIC-quetiapine and VIC-trifluoperazine) had much higher sensitization effects, suggesting that lower effective doses were sufficient for sensitizing P-gp-overexpressing resistant cells compared to those required with the other drugs. Furthermore, when we compared quetiapine and trifluoperazine to previously known bipolar drugs (fluphenazine, thioridazine, pimozide, or aripiprazole), we found that aripiprazole, administered at lower doses, had a much higher sensitization effect. We also demonstrated that co-treatment with another anti-mitotic drug (eribulin) increased the sensitization of KBV20C cells similar to VIC. We also found that aripiprazole had higher P-gp-inhibitory activity than the other bipolar drugs, indicating that this activity was involved in the higher level of VIC-aripiprazole sensitization. Conclusion: Co-treatment of anti-mitotic drug-resistant cancer cells with a low dose of aripiprazole had the strongest sensitization effect and is highly dependent on P-gp-inhibitory activity.*

Anti-mitotic drugs inhibit mitosis by targeting microtubules and preventing their polymerization or depolymerization. Paclitaxel, docetaxel, vincristine (VIC), vinorelbine, vinblastine, and eribulin are examples of anti-mitotic drugs (1-3). Although anti-mitotic drugs are widely used to treat cancer, cancer cells can develop resistance to these drugs in various ways. P-glycoprotein (P-gp) overexpression is one well-known resistance mechanism in which P-gp, a membrane channel that can pump anti-mitotic drugs out of the cell, can counteract drug-induced toxicity (2, 4, 5). By identifying mechanisms that sensitize P-gp-overexpressing cancer cells to anti-mitotic drugs, better treatments can be developed for patients who develop resistance. We aimed to identify novel repositioned drugs that sensitize P-gp-overexpressing resistant cancer cells to anti-mitotic drugs, to characterize the sensitizing efficacy, and investigate the mechanisms involved. The urgent need for pharmacological treatments of P-gp-overexpressing resistant cancers can be effectively addressed through the identification of novel mechanisms of repositioned drugs, as these drugs can be used without further evaluation of toxicity (6-8). We demonstrated that drugs for bipolar diseases or schizophrenia, including fluphenazine, thioridazine, pimozide, and aripiprazole, have P-gp-inhibitory activities and drug-sensitization effects in P-gp-overexpressing drug-resistant cancer cells (9-12). In addition, bipolar drugs have been shown to possess P-gp-

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Key Words: Aripiprazole, quetiapine, trifluoperazine, bipolar drugs, repositioning drug, resistant cancer, P-gp.

inhibitory activities (9-12), though comparisons of individual bipolar drugs and their exact mechanisms of action have not yet been conducted. In this study, we investigated different bipolar drugs for their sensitizing effects in drug-resistant cancer cells. We identified 15 bipolar drugs from the literature (13-17) and then screened for individual bipolar drugs that could sensitize P-gp-overexpressing drug-resistant KBV20C cancer cells to treatment with anti-mitotic drugs at relatively low doses. We also investigated the mechanisms involved in the sensitization of these resistant cancer cells. We found that low doses of quetiapine and trifluoperazine sensitized KBV20C cells best among the 15 drugs tested. Notably, we also found that VIC-aripiprazole co-treatment had the lowest dose with anti-mitotic drugs among the 19 bipolar drugs tested. As these drugs are used in clinical settings to treat bipolar disorder, these results contribute to the development of bipolar drug-based therapies in co-treatment strategies for highly drug-resistant tumors.

Materials and Methods

Reagents and cell culture. Rhodamine123 (Rhodamine), fluphenazine, and verapamil were purchased from Sigma-Aldrich (St. Louis, MO, USA). VIC was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Quetiapine fumarate, risperidone, clozapine, asenapine maleate, iloperidone, paliperidone, ziprasidone hydrochloride, trifluoperazine-HCl, loxapine succinate, pilocarpine-HCl, valproic acid, carbamazepine, levetiracetam, topiramate, and felbamate were purchased from Selleckchem (Houston, TX, USA). Aqueous solutions of eribulin (Eisai Korea, Seoul, South Korea) were obtained from the National Cancer Center in South Korea.

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 (18-21). All cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (WelGENE, Daegu, Republic of Korea).

Microscopic observation. Cells grown in 60-mm diameter dishes were treated with 10 μM of verapamil, 5 μM quetiapine, 5 μM iloperidone, 5 μM trifluoperazine, 5 μM loxapine, 5 μM risperidone, 5 μM ziprasidone, 5 μM felbamate, 5 μM fluphenazine, 5 μM thioridazine, 5 μM pimozide, 2.5 μM aripiprazole or 0.1% DMSO (Control), alone and in combination with 10nM eribulin or 2.5 nM vincristine for 24 h. The medium was removed, and phosphate-buffered saline (PBS) was added into each dish. Cells were examined immediately in two independent experiments using an ECLIPSETs2 inverted routine 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 (22-25). Briefly, cells grown in 60-mm diameter dishes were treated with 10 μM verapamil, 5 μM trifluoperazine, 5 μM quetiapine, 5 μM thioridazine, or 5 μM aripiprazole and then incubated for 24 h or 4 h at 37˚C. Cells were then incubated with 2 μg/ml rhodamine for 30 min at 37˚C. The medium was removed, and the cells were washed with PBS. The stained cells were analyzed in two independent experiments using a Guava EasyCyte Plus Flow Cytometer (Merck Millipore, Burlington, MA, USA).

Fluorescence-activated cell sorting (FACS) analysis. FACS analysis was performed as previously described (22-25). Cells were grown in 60-mm diameter dishes and treated 5 μM quetiapine, 5 μM iloperidone, 5 μM trifluoperazine, 5 μM loxapine, 5 μM fluphenazine, 5 μM thioridazine, 5 μM pimozide, 2.5 μM aripiprazole or 10 μM verapamil in combination with 5 nM vincristine for 24 h. 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 8 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 in two independent experiments for relative DNA content using a Guava EasyCyte Plus Flow Cytometer (Merck Millipore, Burlington, MA, USA).

Annexin V analysis. Annexin V analysis was conducted by using the annexin V-fluorescein isothiocyanate (FITC) staining kit (BD Bioscience, Franklin, NJ, USA) as previously described (22-25). Cells were grown in 60-mm diameter dishes and treated with 10 μM verapamil, 5 μM quetiapine, 5 μM iloperidone, 5 μM trifluoperazine, 5 μM fluphenazine, 5 μM thioridazine, 5 μM pimozide, 2.5 μM aripiprazole in combination with 2.5 nM vincristine or 10nM eribulin for 24 h. 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 μl of Annexin V-FITC and 5 μl of PI and were, then, incubated for 30 min at room temperature. The stained cells were analyzed in two independent experiments using a Guava EasyCyte Plus Flow Cytometer (Merck Millipore).

Cell viability assay. Cell proliferation was measured by a colorimetric assay using the EZ-CyTox cell viability assay kit (Daeillab, Republic of Korea) according to the manufacturer's instructions. Briefly, cells grown in wells of 96-well plates were incubated with 10 μl of EZ-CyTox solution for 1-2 h at 37˚C. Absorbance at 450 nm was determined immediately using the VERSA MAX Microplate Reader (Molecular Devices Corp., Sunnyvale, CA, USA). All experiments were performed at least in triplicate and repeated twice.

Results

Among 15 repositioned bipolar drugs, 7 sensitize VIC-treated resistant KBV20C cancer cells at low doses. We aimed to identify repositioned drugs that sensitize P-gp-overexpressing resistant cancer cells to treatment with chemotherapeutic drugs. Previously, we identified that bipolar drugs, including fluphenazine, thioridazine, pimozide, and aripiprazole, have drug-sensitization effects in P-gp-overexpressing drug-resistant cancer cells (9-12), though comparisons of the individual bipolar drugs and their exact mechanisms of action were not investigated in detail. We assume that additional findings for repositioned bipolar drugs can increase potential applications in personalized medicine. Therefore, we performed further detailed analysis with 15 known bipolar drugs, including quetiapine,

risperidone, clozapine, asenapine, iloperidone, paliperidone, ziprasidone, trifluoperazine, loxapine, pilocarpine, valproic acid, carbamazepine, levetiracetam, topiramate, and felbamate.

In this study, we focused on bipolar drugs that sensitize P-gp-overexpressing resistant cancer cells at low doses and investigated their mechanism of sensitization. We tested sensitization with VIC, an anti-mitotic drug that is routinely used as a chemotherapeutic agent in cancer (26, 27), using KBV20C resistant cancer cells, which present with a VIC-resistant phenotype through P-gp overexpression (27, 28). We compared the sensitizing effects of these bipolar drugs to verapamil (positive control), which is a P-gp inhibitor and is known to increase the sensitization of VIC-treated KBV20C cells (28-30).

First, we performed a quantitative analysis with a cell viability test. As seen in Figure 1A and B, seven drugs (quetiapine, risperidone, iloperidone, ziprasidone, trifluoperazine, loxapine, and felbamate) showed highly reduced viability in VIC-treated KBV20C cells, with VIC co-treatments leading to cultures with >50% viability compared to the control. Treatment with an equivalent dose of verapamil (as a positive control) produced similar sensitization effects on cells co-treated with VIC (Figure 1A and B), suggesting that a lower dose of the seven drugs is sufficient and as effective as verapamil in sensitizing P-gp-overexpressing resistant cancer cells. There was no viability difference between the control cells and those receiving individual treatment with the seven bipolar drugs (Figure 1C and D), suggesting that sensitization by VIC co-treatments of the seven bipolar drugs resulted in synergistic effects in the VIC-resistant cancer cells.

In summary, we observed that 7 of the 15 known bipolar drugs produced sensitization effects (reduced cell viability with VIC co-treatment) at a low dose. We conclude that the seven bipolar drugs can be used to reduce drug toxicity and sensitize VIC-resistant cancer cells. Seven out of the 15 tested bipolar drugs showed a sensitization effect in P-gp-overexpressing resistant cancer cells, suggesting that most bipolar drugs have similar sensitization effects.

Quetiapine, iloperidone, trifluoperazine, and loxapine result in higher sensitization of VIC-treated resistant cancer cells than other bipolar drugs. It is important to identify which drugs among the seven bipolar drugs identified produce better sensitization effects with low doses. We conducted further detailed analysis of both viability and microscopy with lower concentrations of the seven identified drugs to distinguish which one was most effective in the VIC-treated resistant KBV20C cells. As seen in Figure 1E and F, further detailed analysis of both the viability tests and microscopy results showed that VIC-quetiapine VIC-iloperidone, VIC-trifluoperazine, and VIC-loxapine co-treatments produced greater sensitization effects than VIC-risperidone, VIC-ziprasidone, and VIC-felbamate.

Co-treatment with VIC and the identified bipolar drugs sensitize resistant KBV20C cells to apoptosis through induction of G_2 arrest. To further clarify the mechanism of action for co-treatment combining VIC and the identified bipolar drugs, we performed fluorescence-activated cell sorting (FACS) analyses. As shown in Figure 2A, VIC-quetiapine, VIC-iloperidone, VIC- trifluoperazine, and VIC-loxapine co-treatments considerably increased the number of cells in G_2 arrest compared to control cells. This indicates that the reduction of cellular viability resulted from cell-cycle arrest.

Using annexin V analysis, we also tested whether VIC combined with the identified bipolar drugs increased cell death by apoptosis. As seen in Figure 2B, apoptotic cell death greatly increased after VIC-quetiapine, VIC-iloperidone, and VIC-trifluoperazine co-treatments. This indicates that reduced G_2 arrest contributes to increased apoptotic death.

Quetiapine and trifluoperazine strongly induce both G_2 arrest and apoptosis in VIC-treated resistant KBV20C cells. When the G_2-arrest phase was quantitatively estimated, we found that the proportions of the G_2-arrested cells were approximately 46% with VIC-quetiapine, 35% with VIC-iloperidone, 39% with VIC-trifluoperazine, and 27% with VIC-loxapine (Figure 2A). These results suggest that the effects of the VIC-quetiapine and VIC-trifluoperazine co-treatments leading to G_2 arrest were greater than those of the VIC-iloperidone or VIC-loxapine co-treatments. As seen in Figure 2B, annexin V staining also showed that the proportion of apoptotic cells (in both early and late phases) was about 14% after treatment with VIC-quetiapine, 9% with VIC-iloperidone, and 17% with VIC-trifluoperazine, suggesting that the VIC-quetiapine and VIC-trifluoperazine co-treatments sensitized the cells much better than the VIC-iloperidone co-treatment. Detailed microscopic observations with a low dose of VIC also confirmed that quetiapine and trifluoperazine sensitized the cells much better than iloperidone or loxapine in VIC-treated KBV20C cells (Figure 2C and D).

Overall, we demonstrated that among the 15 bipolar drugs tested, both VIC-quetiapine and VIC-trifluoperazine co-treatments led to high sensitization of VIC-treated resistant KBV20C cells via G_2 cell-cycle arrest and apoptosis. Considering that these drugs sensitize VIC-treated KBV20C cells with very low doses (Figure 2A-D), they may be useful in clinical settings, given these minimal toxic concentrations in normal cells.

Low doses of quetiapine and trifluoperazine also increase the sensitization of KBV20C cells treated with other anti-mitotic drugs. We also investigated whether quetiapine, iloperidone, and trifluoperazine were effective in combination with other anti-mitotic drugs. We tested eribulin (or halaven), an anti-mitotic drug that has been recently

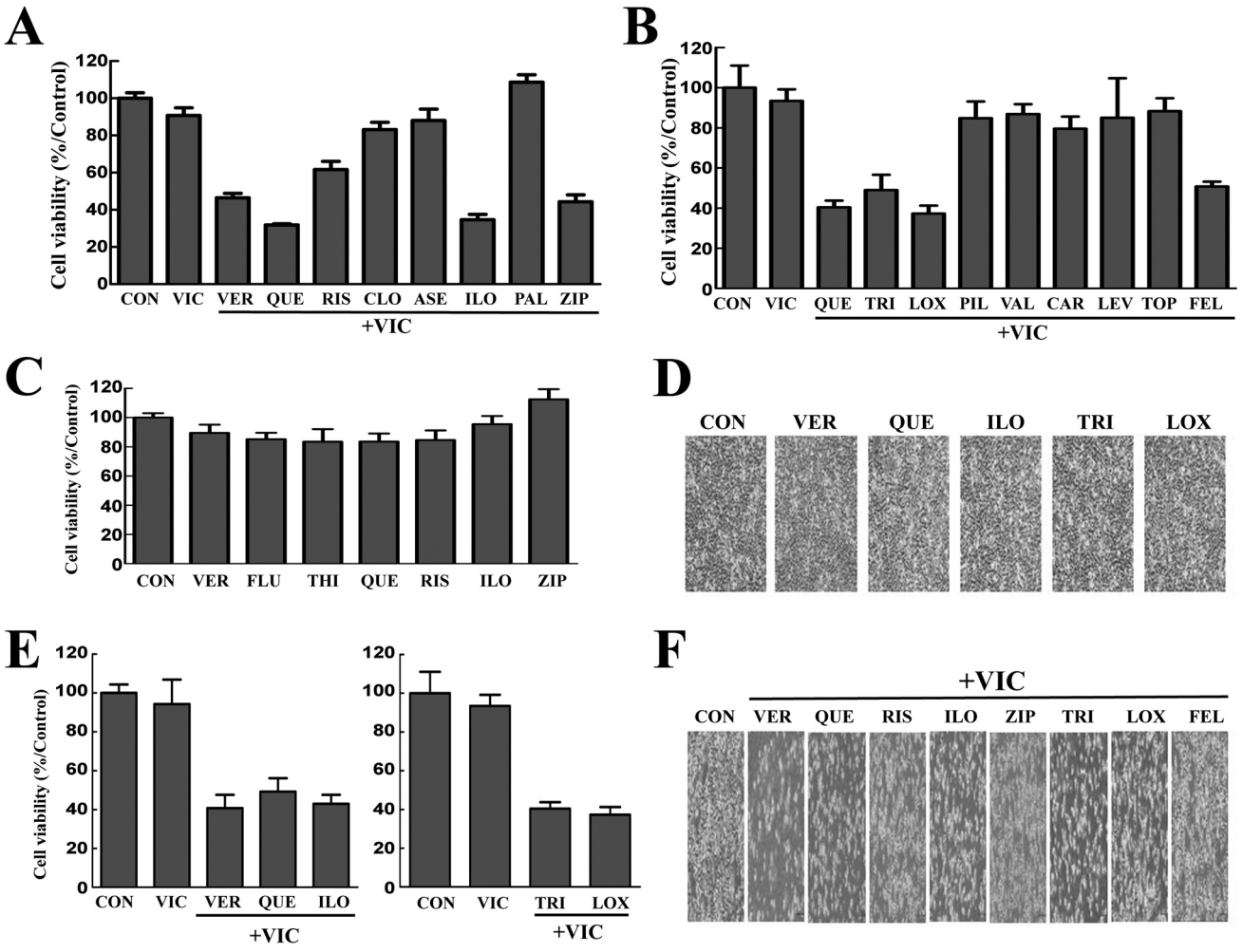

Figure 1. *Quetiapine, iloperidone, trifluoperazine, and loxapine result in higher sensitization of VIC-treated resistant cancer cells than other bipolar drugs. (A-B) KBV20C cells were plated on 96-well plates and grown to 30-40% confluence. The cells were then stimulated for 48 h with 10 μM of verapamil (VER), quetiapine (QUE), risperidone (RIS), clozapine (CLO), asenapine (ASE), iloperidone (ILO), paliperidone (PAL), ziprasidone (ZIP), trifluoperazine (TRI), loxapine (LOX), pilocarpine (PIL), valproic acid (VAL), carbamazepine (CAR), levetiracetam (LEV), topiramate (TOP), felbamate (FEL) and in combination with 5 nM VIC or alone, or 0.1% DMSO (CON). Cell viability assay was performed as described in Materials and Methods. The data are presented as the mean±SD of at least two experiments repeated in triplicate. (C) KBV20C cells were plated on 96-well plates and grown to 30-40% confluence. The cells were then stimulated for 48 h with 10 μM of verapamil (VER), fluphenazine (FLU), thioridazine (THI), quetiapine (QUE), risperidone (RIS), iloperidone (ILO), ziprasidone (ZIP), or 0.1% DMSO (CON). Cell viability assay was performed as described in Materials and Methods. The data are presented as the mean±SD of at least two experiments repeated in triplicate experiments. (D) KBV20C cells were grown on 60 mm-diameter dishes and treated with 10 μM of verapamil (VER), quetiapine (QUE), iloperidone (ILO), trifluoperazine (TRI), loxapine (LOX), or 0.1% DMSO (CON). After 1 day, all cells were observed using an inverted microscope at ×4 magnification. (E) KBV20C cells were plated on 96-well plates and grown to 30-40% confluence. The cells were then stimulated for 48 h with 10 μM of verapamil (VER), 5 μM of quetiapine (QUE), 5 μM of iloperidone (ILO), 5 μM of trifluoperazine (TRI), 5 μM of loxapine (LOX) and in combination with 5 nM VIC or alone, or 0.1% DMSO (CON). Cell viability assay was performed as described in Materials and Methods. The data are presented as the mean±SD of at least two experiments repeated in triplicate. (F) KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 nM VIC with 10 μM verapamil (VER), 5 nM VIC with 5 μM quetiapine (QUE), 5 nM VIC with risperidone (RIS), 5 nM VIC with 5 μM iloperidone (ILO), 5 nM VIC with ziprasidone (ZIP), 5 nM VIC with 5 μM trifluoperazine (TRI), 5 nM VIC with 5 μM loxapine (LOX), 5 nM VIC with felbamate (FEL), or 0.1% DMSO (CON). After 1 day, all cells were observed using an inverted microscope at ×4 magnification.*

developed and used in the treatment of metastatic cancers (31, 32). Previously, we found that the KBV20C cell line is a very useful model of highly eribulin-resistant cancer. As seen in microscopic observations of Figure 2E, 5 μM of quetiapine, iloperidone, or trifluoperazine produces similar sensitizing effects when combined with eribulin. In a detailed quantitative analysis of annexin V staining, eribulin-iloperidone co-treatment had a lower sensitization effect than

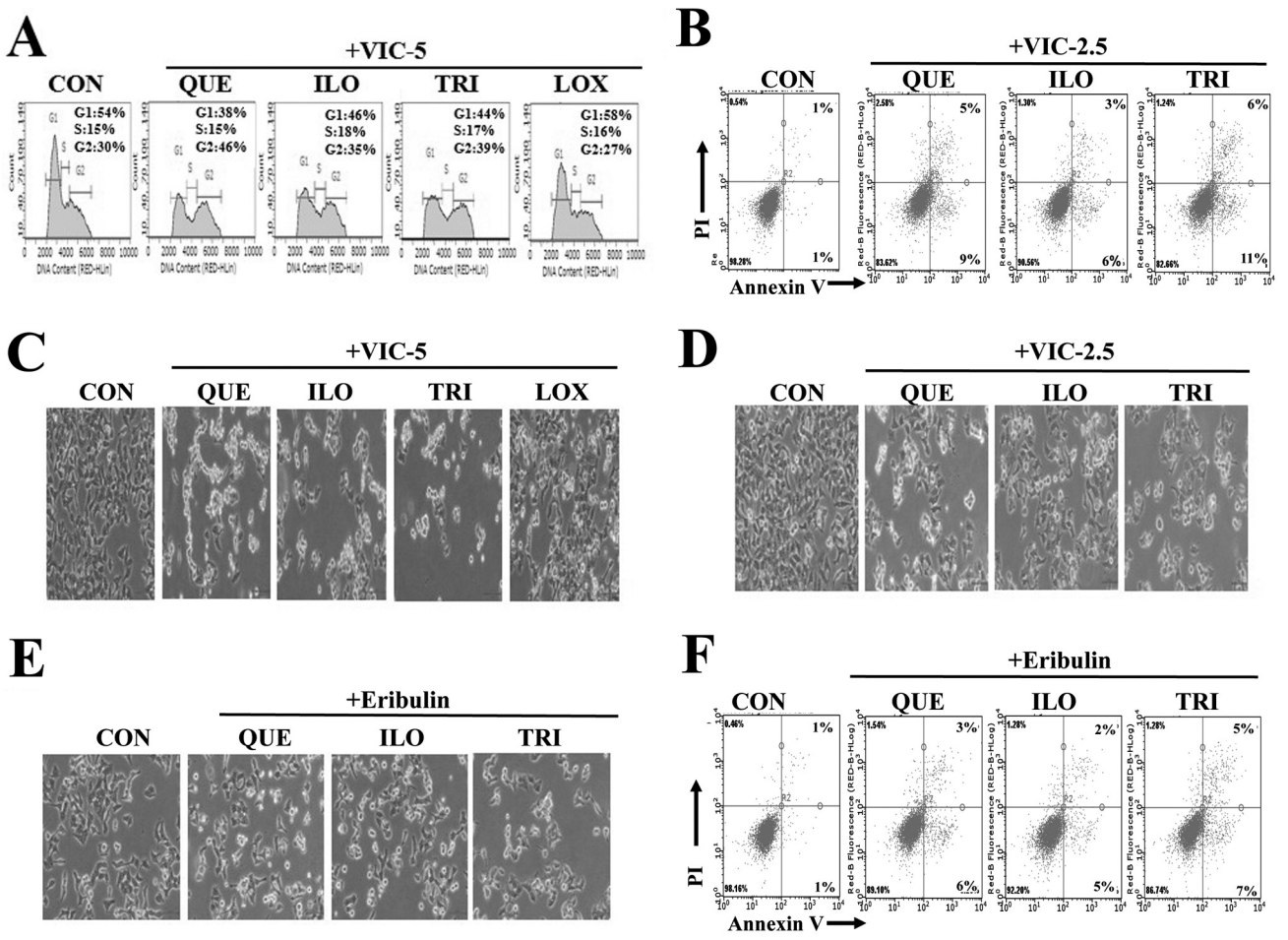

Figure 2. *Quetiapine and trifluoperazine strongly induce both G_2 arrest and apoptosis in VIC-treated resistant KBV20C cells. (A-D) KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 nM VIC with 5 μM quetiapine (QUE), 5 nM VIC with 5 μM iloperidone (ILO), 5 nM VIC with 5 μM trifluoperazine (TRI), 5 nM VIC with 5 μM loxapine (LOX), 2.5 nM VIC-2.5 with 5 μM quetiapine (QUE), 2.5 nM VIC-2.5 with 5 μM iloperidone (ILO-5), 2.5 nM VIC with 5 μM trifluoperazine (TRI-5), or 0.1% DMSO (CON). After 24 h, FACS analyses (A), annexin V analyses (B), or microscopic observation at ×4 magnification (C-D) were performed as described in Materials and Methods. (E-F) KBV20C cells were grown on 60 mm-diameter dishes and treated with 10 nM eribulin with 5 μM quetiapine (QUE), 10 nM eribulin with 5 μM iloperidone (ILO), 10 nM eribulin with 5 μM trifluoperazine (TRI) or 0.1% DMSO (CON). After 24 h, microscopic observation at ×4 magnification (E) or annexin V analyses (F) were performed as described in Materials and Methods.*

eribulin-quetiapine or eribulin-trifluoperazine co-treatment (Figure 2F). These results demonstrate that quetiapine and trifluoperazine are also very effective in sensitizing eribulin-co-treated resistant cancer cells. This finding also suggests that low doses of quetiapine or trifluoperazine could be combined with other anti-mitotic drugs to sensitize P-gp-overexpressing cancer cells. We conclude that quetiapine or trifluoperazine could be applied to various drug-resistant cancer patients.

A low dose of aripiprazole produces much higher sensitization in VIC-treated KBV20C cells than 18 other bipolar drugs. Previously, we reported that bipolar drugs, including fluphenazine, thioridazine, pimozide, and aripiprazole, produced high drug-sensitization effects in P-gp-overexpressing drug-resistant cancer cells (9-12), though detailed comparisons were not conducted. In this study, we found that both quetiapine and trifluoperazine had the highest sensitization effects among the 15 bipolar drugs tested. Next, we tested whether the combination of quetiapine or trifluoperazine with VIC was more effective than co-treatments with other previously identified bipolar drugs, namely fluphenazine, thioridazine, pimozide, and aripiprazole (9-12). To better compare the identified bipolar drugs, a reduced amount of VIC (2.5 nM) was used in the co-treatment.

Microscopic observations indicated that low doses of aripiprazole produced much better sensitization effects than quetiapine, iloperidone, trifluoperazine, fluphenazine, thioridazine, or pimozide in VIC-treated KBV20C cells (Figure 3A), suggesting that a low dose of aripiprazole has the highest sensitization effects, compared to the 18 bipolar drugs tested at similarly low doses. The VIC-aripiprazole sensitization effect is much higher than that of 10 μM verapamil, a positive control (Figure 3A).

Collectively, VIC-aripiprazole showed a higher sensitization effect than co-treatments with VIC and other 18 bipolar drugs, suggesting that aripiprazole is the ideal co-treatment partner among the various bipolar drugs for the sensitization of resistant cancer cells. Aripiprazole could be used with reduced drug toxicity for resistant cancer in the clinic as an anti-cancer drug-combination therapy.

High sensitization by low doses of VIC-aripiprazole results from high increases of both G_2 arrest and apoptosis. We next tested how a low dose of aripiprazole could increase sensitization in VIC-treated resistant KBV20C cells. As seen in Figure 3B and C, 2.5 μM aripiprazole increased both G_2 arrest and apoptosis in 2.5 nM VIC-treated resistant KBV20C cells to a much greater extent than in cells receiving 5 μM quetiapine, trifluoperazine, fluphenazine, thioridazine, or pimozide co-treatments. The results suggest that the higher sensitization effect of VIC-aripiprazole co-treatment resulted from large numbers of G_2-arrested cells, ultimately leading to apoptotic death. The G_2 arrest and apoptosis resulting from the VIC-aripiprazole sensitization effect is much higher than that of 10 μM verapamil, the positive control (Figure 3B and C).

In summary, we observed that low doses of aripiprazole have higher sensitization effects in P-gp-overexpressing resistant cancer cells than the other 18 bipolar drugs. In a detailed analysis with lower doses, we concluded that aripiprazole, with both high G_2 arrest and apoptotic effects, can increase sensitization for VIC-treated resistant cancer cells. Therefore, aripiprazole could be used to reduce drug toxicity and effectively sensitize VIC-resistant cancer cells.

VIC-quetiapine and VIC-trifluoperazine co-treatments have slightly higher sensitization effects than VIC-fluphenazine, VIC-thioridazine, and VIC-pimozide co-treatments. As shown in Figure 3A and C, a low dose of aripiprazole produces the best sensitization drug for VIC-treated resistant cancer cells. Next, we tried to identify better sensitizing co-treatments among the remaining bipolar drugs, namely quetiapine, trifluoperazine, fluphenazine, thioridazine, or pimozide.

In a detailed quantitative analysis of G_2 arrest (Figure 3B), we found that VIC-quetiapine co-treatment led to >30% G_2 arrest, compared to lower arrest levels with trifluoperazine, fluphenazine, thioridazine, or pimozide. We also found that both the early and late apoptotic portions of cells receiving VIC-trifluoperazine co-treatment were approximately 22%, higher than those of VIC-quetiapine, VIC-fluphenazine, VIC-thioridazine, or VIC-pimozide.

This suggests that low doses of quetiapine and trifluoperazine have slightly better sensitization effects on VIC-treated resistant cancer cells than the other identified bipolar drugs, whereas fluphenazine, thioridazine, and pimozide have similar sensitization effects.

The high sensitization resulting from a low dose of VIC-aripiprazole results from its high P-gp-inhibitory activity. In the next phase of our investigation, we evaluated the P-gp-inhibitory activities of bipolar drugs fluphenazine, thioridazine, pimozide, and aripiprazole in P-gp-overexpressing KBV20C cells, since these drugs have drug-sensitization effects (9-12). We also expected that variations in the degree of P-gp inhibition among these bipolar drugs may be responsible for their different sensitizing effects on VIC-treated KBV20C cells. We first tested whether quetiapine, trifluoperazine, thioridazine, and aripiprazole increased the inhibition of the P gp substrate efflux. Rhodamine 123, a well-known P-gp substrate, was used to measure P-gp inhibition (18, 22). In this experiment, yellow fluorescence in the cell was indicative of intracellular accumulation of rhodamine 123.

As shown in Figure 4A and B, aripiprazole showed the highest P-gp inhibitory activity, whereas the other bipolar drugs had much lower activities. This suggests that P-gp inhibition by aripiprazole plays a key role in the sensitization by VIC-aripiprazole co-treatment. This also suggests that aripiprazole strongly inhibits P-gp by direct binding, similar to the mechanism of verapamil. Considering that previously developed P-gp inhibitors, including verapamil, are toxic to normal cells (5, 30, 33), we believe that aripiprazole could be an adequate replacement for sensitizing P-gp-overexpressing resistant cancer cells in clinical treatments. However, P-gp inhibition by quetiapine, trifluoperazine, or thioridazine was much lower than that by verapamil, being slightly higher than (or similar to) that of the control (Figure 4A and B).

Low doses of aripiprazole also greatly increase the sensitization of eribulin-treated KBV20C cells. We also investigated whether aripiprazole provided greater sensitization than quetiapine, trifluoperazine, fluphenazine, thioridazine, or pimozide in combination with other anti-mitotic drugs. We tested the anti-mitotic drug eribulin, which has been recently developed and used in the treatment of metastatic cancers, using the KBV20C cell line, which we found to be a very useful model for studying highly eribulin-resistant cancer (9, 19, 30).

As seen in Figure 4C, eribulin combined with 2.5 μM of aripiprazole provided much higher sensitization than co-

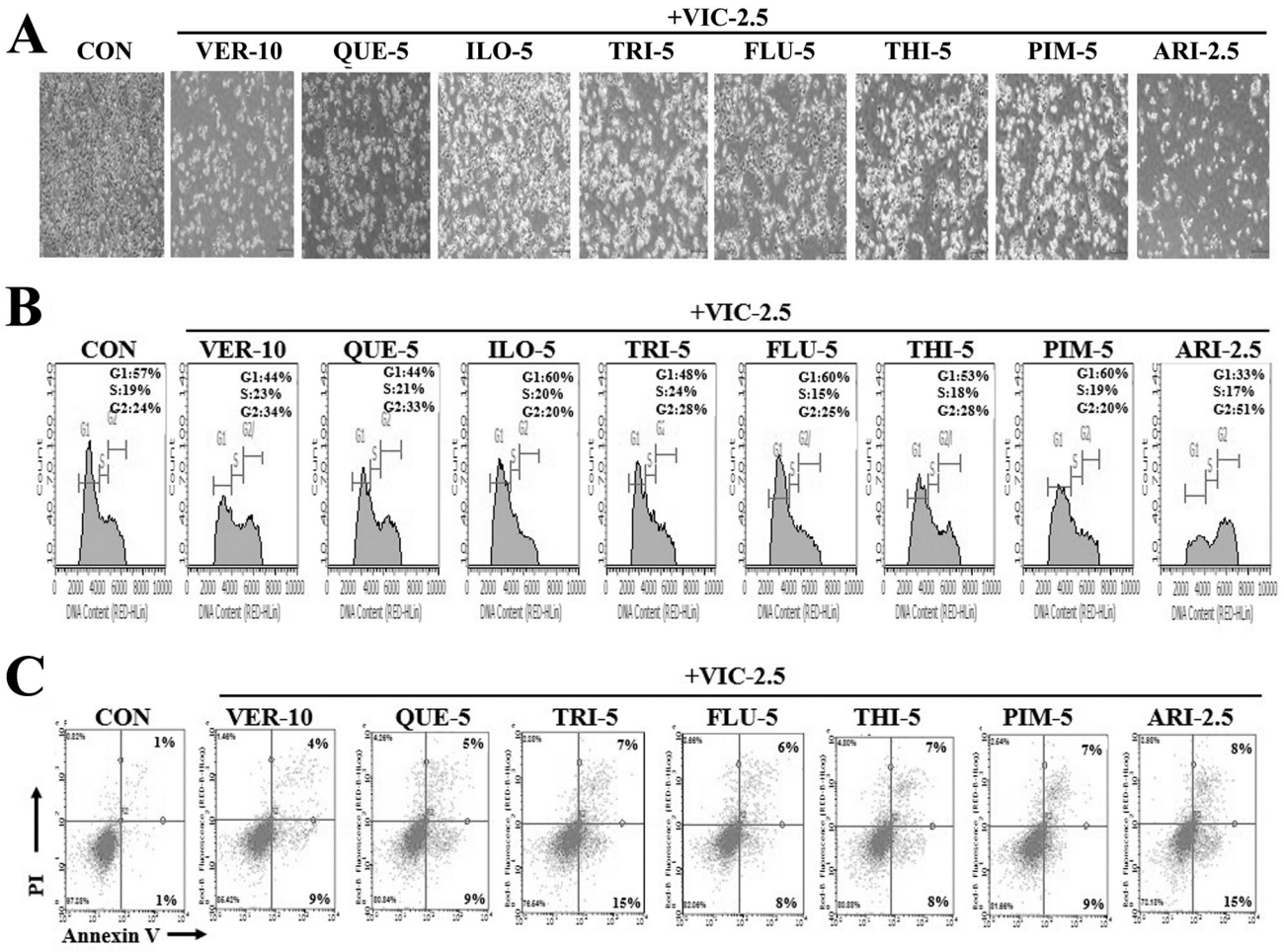

Figure 3. *A low dose of aripiprazole produces much higher sensitization in VIC-treated KBV20C cells than 18 other bipolar drugs. (A) KBV20C cells were grown on 60 mm-diameter dishes and treated with 2.5 nM VIC with 10 μM verapamil (VER-10), 2.5 nM VIC with 5 μM quetiapine (QUE-5), 2.5 nM VIC with 5 μM iloperidone (ILO-5), 2.5 nM VIC with 5 μM trifluoperazine (TRI-5), 2.5 nM VIC with 5 μM fluphenazine (FLU-5), 2.5 nM VIC with 5 μM thioridazine (THI-5), 2.5 nM VIC with 5 μM pimozide (PIM-5), 2.5 nM VIC with 2.5 μM aripiprazole (ARI-2.5), or 0.1% DMSO (CON). After 1 day, all cells were observed using an inverted microscope at ×10 magnification. (B) KBV20C cells were grown on 60 mm-diameter dishes and treated with 2.5 nM VIC with 10 μM verapamil (VER-10), 2.5 nM VIC with 5 μM quetiapine (QUE-5), 2.5 nM VIC with 5 μM iloperidone (ILO-5), 2.5 nM VIC with 5 μM trifluoperazine (TRI-5), 2.5 nM VIC with 5 μM fluphenazine (FLU-5), 2.5 nM VIC with 5 μM thioridazine (THI-5), 2.5 nM VIC with 5 μM pimozide (PIM-5), 2.5 nM VIC with 2.5 μM aripiprazole (ARI-2.5), or 0.1% DMSO (CON). After 24 h, FACS analyses were performed as described in Materials and Methods. (C) KBV20C cells were grown on 60 mm-diameter dishes and treated with 2.5 nM VIC with 10 μM verapamil (VER-10), 2.5 nM VIC with 5 μM quetiapine (QUE-5), 2.5 nM VIC with 5 μM trifluoperazine (TRI-5), 2.5 nM VIC with 5 μM fluphenazine (FLU-5), 2.5 nM VIC with 5 μM thioridazine (THI-5), 2.5 nM VIC with 5 μM pimozide (PIM-5), 2.5 nM VIC with 2.5 μM aripiprazole (ARI-2.5), or 0.1% DMSO (CON). After 24 h, annexin V analyses were performed as described in Materials and Methods.*

treatments combining eribulin with 5 μM quetiapine, trifluoperazine, fluphenazine, thioridazine, or pimozide. These results demonstrate that aripiprazole is as effective in sensitizing resistant cancer cells as eribulin as it is with VIC. This finding also suggests that low doses of aripiprazole could sensitize P-gp-overexpressing cancer cells through combinations with other anti-mitotic drugs for use in patients with various drug-resistant cancers. Quetiapine, trifluoperazine, fluphenazine, thioridazine, and pimozide also had similar sensitization effects with eribulin, similar to those found in VIC co-treatments (Figure 3A-C).

In summary, among the 15 bipolar drugs tested, we identified 2 compounds (quetiapine and trifluoperazine) that provide high sensitization of P-gp-overexpressing resistant cancer cells to treatment with anti-mitotic drugs. When these drugs were compared to previously identified compounds, namely fluphenazine, thioridazine, pimozide, or aripiprazole (9-12), we found that low doses of aripiprazole had the best sensitization

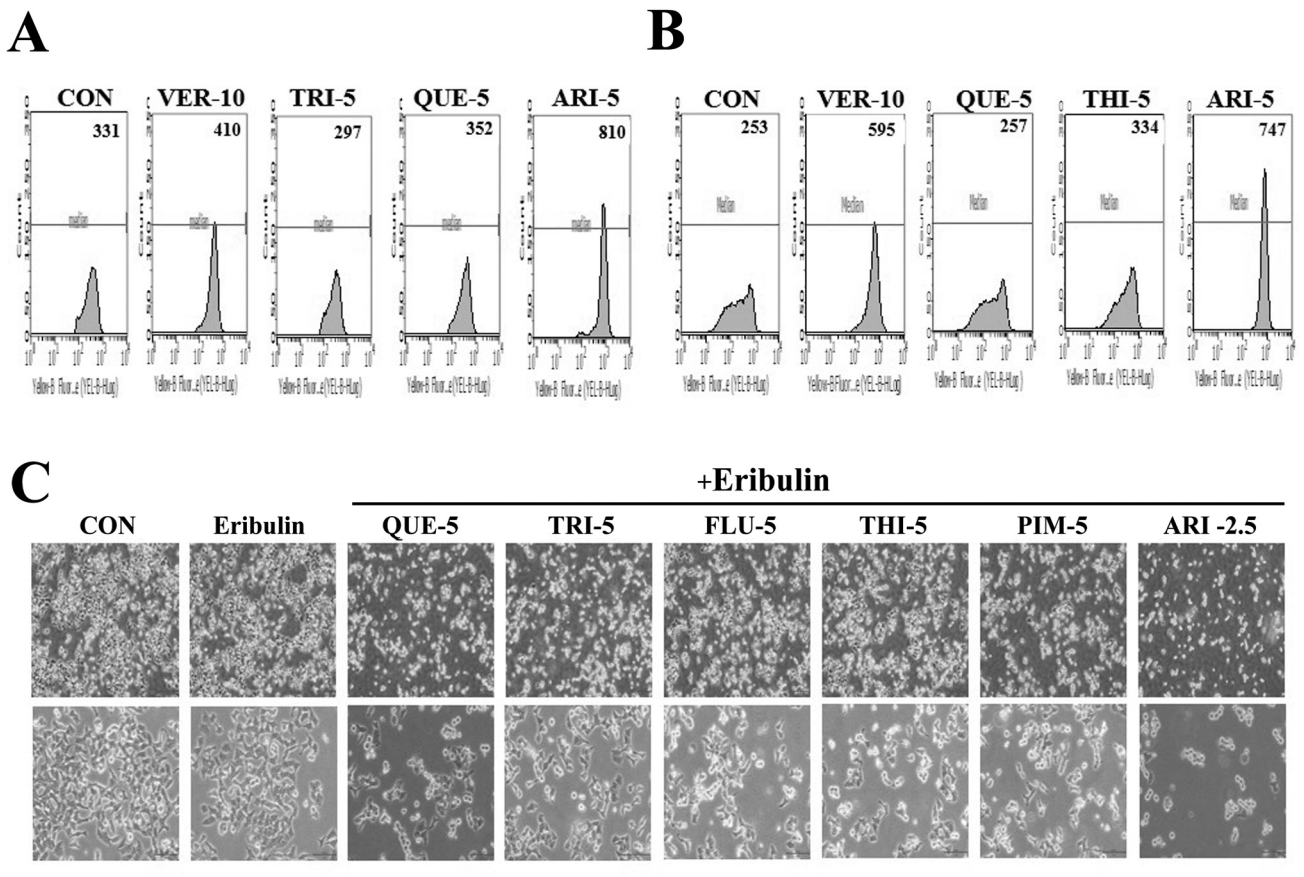

Figure 4. *The high sensitization resulting from a low dose of VIC-aripiprazole results from its high P-gp-inhibitory activity. (A-B) KBV20C cells were grown on 60 mm-diameter dishes and treated with 10 μM verapamil (VER-10), 5 μM trifluoperazine (TRI-5), 5 μM quetiapine (QUE-5), 5 μM thioridazine (THI-5), 5 μM aripiprazole (ARI-5), or 0.1% DMSO (CON). After 24 h, all cells were stained with rhodamine and examined by using FACS analysis, as described in Materials and Methods. (C) KBV20C cells were grown on 60 mm-diameter dishes and treated with 10 nM eribulin alone, 10 nM eribulin with 5 μM quetiapine (QUE-5), 10 nM eribulin with 5 μM trifluoperazine (TRI-5), 10 nM eribulin with 5 μM fluphenazine (FLU-5), 10 nM eribulin with 5 μM thioridazine (THI-5), 10 nM eribulin with 5 μM pimozide (PIM-5), 10 nM eribulin with 2.5 μM aripiprazole (ARI-2.5), or 0.1% DMSO (CON). After 1 day, all cells were observed using an inverted microscope at ×4 or ×10 magnification.*

effect in VIC- or eribulin-treated resistant cells. This sensitization results from increased G_2 arrest and apoptosis, driven by aripiprazole's very high P-gp inhibitory activity.

Discussion

Drug repositioning or drug repurposing is the application of known drugs for new indications. It has been used for the treatment of various diseases and has several advantages, including low cost and minimal requirements for toxicity testing, which is a time-consuming process (6-8). In this study, we investigated several repositioned drugs in a novel application for sensitizing P-gp-overexpressing resistant cancer cells to chemotherapeutic drugs. The urgent need for pharmacological treatments of resistant cancers can be efficiently addressed with drug repositioning, where these drugs can be applied to patients at a relatively faster pace. We have also investigated and reported the applications of these repositioned drugs in drug-resistant cancer patients. For example, we also suggest the application of anti-malarial or anti-psychotic drugs for the treatment of P-gp-overexpressing resistant cancer (9-12, 20, 24).

Previously, we found that P-gp-overexpressing KBV20C cancer cells highly resistant to anti-mitotic drugs were sensitized by co-treatment with repositioned drugs developed for the treatment of psychotic disease, namely fluphenazine, thioridazine, pimozide, and aripiprazole (9-12). Many anti-psychotic drugs used for the treatment of bipolar or schizophrenic disorders have been found to have anticancer effects and have been tested as repositioned drugs in patients with cancer (10, 12-16). Most of these drugs target the dopamine or histamine receptors on the cell membrane and

block the extracellular signals that are responsible for abnormal responses in brain cells. Considering that mutations in these membrane-related receptors or kinases correlate with cancer growth, it is assumed that drugs that target these cellular membrane receptors may also possess anticancer effects. A literature search identified 15 additional dopamine or histamine receptor antagonists already in use for the treatment of bipolar disease in the clinic. As these drugs have anticancer effects, several clinical trials have been conducted to test their efficacy in various types of solid tumors as well as in combination therapy with other chemotherapeutic drugs (13-16). However, comparisons of the individual bipolar drugs and their exact mechanisms of action have not been investigated in detail with P-gp-overexpressing resistant cancer.

In this study by testing 15 bipolar drugs, we observed that co-treatments with 7 drugs (quetiapine, risperidone, iloperidone, ziprasidone, trifluoperazine, loxapine, and felbamate) sensitized anti-mitotic drug-resistant KBV20C cells at relatively low doses. Considering that half of the bipolar drugs studied have sensitizing effects on VIC-treated resistant cancer cells, we conclude that bipolar drugs generally have the ability to overcome resistance to anti-mitotic drugs. As the dopamine or histamine receptors are located in the cellular membrane and transport external signals (15, 17, 24), we assume that bipolar drugs play a role in decreasing or modifying overexpressed P-gp activity in the membrane of resistant cancer cells. Although the resistant cancer-sensitizing abilities of bipolar drugs have been previously demonstrated (13-16), our findings represent a pioneering application of selective bipolar drugs as repositioned drugs. Considering that patients with mental or psychotic diseases show much higher incidence of cancer (34-36), our finding might also contribute to the use of select bipolar drugs for preventing or decreasing cancer occurrence in patients with bipolar disorder.

In particular, we identified two bipolar drugs, quetiapine and trifluoperazine, that can sensitize the resistant KBV20C cells at relatively lower doses than those required with other bipolar drugs, namely risperidone, iloperidone, ziprasidone, loxapine, and felbamate, all of which required higher doses than those of quetiapine and trifluoperazine to mediate sensitizing effects in VIC-treated KBV20C cells. Using more detailed quantitative FACS and annexin V analysis, we demonstrated that VIC-quetiapine or VIC- trifluoperazine co-treatments reduced cellular proliferation and increased G_2 arrest in the P-gp-overexpressing resistant KBV20C cells. Based on the microscopic features, FACS results, and annexin V analyses, we conclude that apoptosis was increased by VIC-quetiapine or VIC-trifluoperazine co-treatments via increased G_2 arrest and reduced proliferation. Future studies may determine the molecular mechanisms underlying these sensitization effects so that quetiapine and trifluoperazine can be quickly applied to patients, especially in patients resistant to combination therapy with anti-mitotic drugs.

Most importantly, we compared previously identified bipolar drugs to identify those providing the best sensitization of P-gp-overexpressing resistant cancer. Herein, we found that both quetiapine and trifluoperazine have the highest sensitization effects of the 15 bipolar drugs tested. We also found that fluphenazine, thioridazine, pimozide, and aripiprazole provide high drug-sensitization effects in P-gp-overexpressing drug-resistant cancer cells (9-12), though we have not compared their activities directly. Next, we tested whether the combination of quetiapine or trifluoperazine with VIC would be more effective than co-treatment with previously identified bipolar drugs, namely fluphenazine, thioridazine, pimozide, and aripiprazole. We demonstrated that VIC-aripiprazole showed a higher sensitization effect than co-treatments combining VIC and other 18 bipolar drugs, suggesting that aripiprazole is the ideal co-treatment partner for the sensitization of resistant cancer cells. Aripiprazole can be used as anti-cancer drug-combination therapy for resistant cancer with reduced drug toxicity in the clinic.

As the efflux of VIC by P-gp is the main mechanism for the resistance of KBV20C cells, we tested whether the sensitization by VIC-aripiprazole co-treatment results from the P-gp inhibitory effects of aripiprazole. We demonstrated that aripiprazole has high P-gp-inhibitory activity, much higher than that of the well-known P-gp inhibitor verapamil, suggesting that VIC-aripiprazole sensitization results from aripiprazole's inhibitory effects, which prevent the removal of VIC from the cell. Interestingly, we did not detect any substantial P-gp-inhibitory activity (or little inhibitory activity) from quetiapine, trifluoperazine, fluphenazine, thioridazine, and pimozide, suggesting that they remove or inhibit factors that block VIC in drug-resistant cancer cells and that they then exerts a synergistic effect in co-treated cells. Further investigations with quetiapine, trifluoperazine, fluphenazine, thioridazine, and pimozide may be needed to determine the molecular targets that allow sensitization without P-gp inhibition. As no increased P-gp inhibition was detected for these drugs, an improved combination of chemotherapeutic agents may be developed for cancer patients who develop resistance to anti-mitotic drugs. As P-gp inhibitors have shown toxicity to normal cells (5, 33, 37), we assume that quetiapine, trifluoperazine, fluphenazine, thioridazine, pimozide, or aripiprazole might be considered combination drugs with or without P-gp inhibitory activity to sensitize P-gp-overexpressing resistant cancer cells. As personalized medicines are gaining popularity, our findings with the bipolar drugs quetiapine, trifluoperazine, fluphenazine, thioridazine, pimozide, or aripiprazole might contribute to effective prescriptions in drug-resistant cancer patients who are allergic or sensitive to the P-gp-inhibitory effect in normal tissues.

Our results were not limited to VIC co-treatment, as we confirmed that aripiprazole provided much better sensitization than quetiapine, trifluoperazine, fluphenazine, thioridazine, or pimozide in combination with other anti-mitotic drugs. Co-treatment of bipolar drugs with eribulin, which was recently developed and is a promising drug for the treatment of resistant cancers (31, 32, 38), had sensitization effects similar to those observed with VIC in P-gp-overexpressing KBV20C cells. We previously reported that P-gp-overexpressing KBV20C cells are highly eribulin-resistant (9, 30), making them useful as models to study highly eribulin-resistant cancer. We also found that quetiapine, trifluoperazine, fluphenazine, thioridazine, or pimozide can sensitize eribulin-treated KBV20C cells. We hypothesize that these drugs (or aripiprazole) can be used in combination with other anticancer drugs for sensitizing resistant cancer cells.

Conclusion

Our results highlight the novel selective sensitization of bipolar drugs. Furthermore, drug-resistant KBV20C cells that overexpress P-gp can be sensitized to the anti-mitotic drugs VIC or eribulin by co-treatment with low doses of the repositioned drugs quetiapine, fluphenazine, trifluoperazine, thioridazine, pimozide, or aripiprazole. Notably, aripiprazole, which has very high P-gp inhibitory activity, provides the best sensitization of drug-resistant cancer cells among the 19 bipolar drugs tested. As the toxicities of these drugs are already documented, they are readily available for clinical use. Our results could contribute to the improved efficacy of various chemotherapeutic agents, when used in combination with these sensitizing agents, for the treatment of cancer patients who develop resistance to chemotherapeutic drugs via P-gp overexpression.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Chunxue Jiang, and Su Hyun Lee: Collected the data, contributed data or analysis tools, wrote the article. Jae Hyeon Park, Jin-Sol Lee, Ji Won Park, Ju Ri Kim, and Song Hee Lee: Contributed data or analysis tools. Hyung Sik Kim and Sungpil Yoon: Contributed data or analysis tools, conceived and designed the analysis, collected the data, contributed data or analysis tools, wrote the article.

Acknowledgements

The Authors thank Ji Young Kim and In Hwan Tae for their technical support and preparation of the manuscript. This work was supported by National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2019R1A2C2002923).

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Received December 3, 2020
Revised December 22, 2020
Accepted January 14, 2021