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Tyrosine Kinase Inhibitors Imatinib and Erlotinib Increase Apoptosis of Antimitotic Drug-resistant KBV20C Cells Without Inhibiting P-gp

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Abstract. *Background/Aim: This study investigated drugs able to sensitize P-glycoprotein (P-gp)-overexpressing resistant KBV20C cancer cells to vincristine or eribulin treatment and assessed their associated mechanisms of action. Materials and Methods: Eight tyrosine kinase inhibitors (lapatinib, gefitinib, imatinib, erlotinib, nilotinib, pazopanib, cediranib, and vandetanib) and one serine/threonine kinase inhibitor (selumetinib) were evaluated for their sensitizing effects on vincristine-resistant KBV20C cells at relatively low doses. Fluorescence-activated cell sorting, annexin V analyses, and rhodamine uptake tests were further performed to investigate their mechanisms of action. Results: Co-treatment of KBV20C cells with lapatinib, gefitinib, imatinib, or erlotinib at low doses highly sensitized them to vincristine treatment. These drugs reduced cellular viability, increased G_2 arrest, and up-regulated apoptosis when co-administered with vincristine. In a detailed quantitative analysis using lower doses, we demonstrated that lapatinib, with high P-gp inhibitory activity, yielded the best pairing for sensitizing P-gp-overexpressing KBV20C cells to vincristine. Co-treatment with eribulin and lapatinib, gefitinib, or erlotinib also increased the sensitivity of KBV20C cells, suggesting that they can be combined with other antimitotic drugs to sensitize resistant cancer cells. Lapatinib was shown to have a higher P-gp-inhibitory activity than verapamil, even at lower doses, indicating that its sensitizing of cells to vincristine involves its P-gp-inhibitory effects. However, interestingly, imatinib- and erlotinib-sensitizing of cells to vincristine appears to be independent of their P-gp inhibition. Conclusion: These findings provide valuable information regarding the sensitizing of drug-resistant cells and indicate that imatinib and erlotinib may be used in patients with potentially resistant cancer without any toxic effects from P-gp inhibition.*

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Key Words: Lapatinib, gefitinib, erlotinib, tyrosine kinase inhibitors, cancer, P-gp, drug resistance.

Antimitotic drugs inhibit mitosis by targeting microtubules and preventing their polymerization or depolymerization. Paclitaxel, docetaxel, vincristine vinorelbine, vinblastine, and eribulin are examples of antimitotic drugs (1-4). Although antimitotic drugs are widely used to treat cancer, cancer cells can develop resistance to these drugs in various ways. P-glycoprotein (P-gp) overexpression is a well-known mechanism of resistance to antimitotic drugs. P-gp is a membrane channel that can pump out antimitotic drugs, thus enabling cells to avoid drug-induced toxicity (5-8). Identifying mechanisms to sensitize cancer cells that overexpress P-gp can lead to better treatment in patients who develop resistance to antimitotic drugs. Although P-gp inhibitors have been developed, their toxicity in normal cells leads to treatment failure. Therefore, it is important to investigate novel therapeutic options without P-gp inhibition for P-gp-overexpressing drug-resistant cancer cells.

Thus, the urgent need for pharmacological treatments for P-gp-overexpressing resistant cancer can be effectively addressed if novel mechanisms of approved anti-cancer drugs are identified because these drugs can be used without further toxicity evaluation (9-11).

Tyrosine kinase inhibitors (TKIs) generally target the epidermal growth factor receptor (EGFR) family and have been developed as a cancer therapy for preventing growth factor signaling in various cancer models (12-14). They are reversible competitors of ATP for binding at the intracellular catalytic domain of the EGFRs. In addition, TKIs act as inhibitors of P-gp in cancer cells (15, 16). These drugs have also been reported to sensitize drug-resistant cancer cells (17, 18). However, the exact mechanisms of action of individual TKIs have not yet been investigated.

Based on a literature search, we identified eight TKIs (lapatinib, gefitinib, imatinib, erlotinib, nilotinib, pazopanib, cediranib, and vandetanib) and one serine/threonine kinase

inhibitor (selumetinib) (12-14, 19) which might have sensitizing effects on P-gp-overexpressing drug-resistant KBV20C cancer cells. We also investigated the mechanisms involved in the sensitizing of resistant cancer cells. As these agents are already in clinical use as targeting anticancer drugs, these results can contribute to the development of therapies using co-treatment with TKIs for highly drug-resistant tumors.

Materials and Methods

Reagents and cell culture. Rhodamine123 (Rhodamine) and verapamil were purchased from Sigma–Aldrich (St. Louis, MO, USA). Vincristine was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Lapatinib, gefitinib, imatinib, erlotinib, nilotinib, pazopanib, cediranib, vandetanib, and selumetinib 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, South Korea) and have been described elsewhere (20-25). 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 to 40%-50% confluence in 60-mm diameter dishes and treated with 5 μM lapatinib, gefitinib, imatinib, erlotinib, nilotinib or pazopanib, or 10 μM verapamil alone and in combination with 50 ng/ml (60 nM) eribulin or 5 nM vincristine for 24 h. The medium was removed, and phosphate-buffered saline (PBS) was added into each dish. Attached cells were examined immediately in two independent experiments using an ECLIPSE Ts2 inverted routine microscope (Nikon, Tokyo, Japan) with a 4× or a 10× objective lens (Nikon's Microscopy U).

Rhodamine uptake tests. The tests used to assess the ability of a drug to inhibit P-gp were based on a previously described method (20-25). Briefly, cells were grown to 40- 50% confluence in 60-mm diameter dishes and treated with 5 μM lapatinib, gefitinib, imatinib, erlotinib, nilotinib, pazopanib, cediranib, vandetanib or selumetinib, or 10 μM verapamil for 4 h or 24 h at 37˚C. Cells were then incubated with 2 μg/ml 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 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 (20-25). Cells were grown to 40%-50% confluence in 60-mm diameter dishes and treated 5 μM lapatinib, gefitinib or erlotinib, or 10 μM verapamil alone and 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 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 in two independent experiments for relative DNA content using a Guava EasyCyte Plus Flow Cytometer (Merck Millipore).

Annexin V analysis. Annexin V analysis was conducted using annexin V-fluorescein isothiocyanate (FITC) staining kit (BD Bioscience, Franklin, NJ, USA) as previously described (20-25). Cells were grown to 40-50% confluence in 60-mm diameter dishes and treated with 5 μM lapatinib, gefitinib or erlotinib, or 10 μM verapamil alone and 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 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 15 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, South Korea) according to the manufacturer's instructions. Briefly, cells were grown to 30-40% confluence in wells of 96-well plates and treated with 5 μM of lapatinib, gefitinib, imatinib, erlotinib, nilotinib, pazopanib, cediranib, vandetanib or selumetinib, or 10 μM verapamil alone and in combination with 5 nM vincristine for 48 h. They were then incubated with 10 μl of EZ-CyTox solution for 1-2 h at 37˚C. Absorbance at 450 nm was determined immediately using VERSA MAX Microplate Reader (Molecular Devices Corp., Sunnyvale, CA, USA). All experiments were performed at least in triplicate and repeated twice.

Statistical analysis. Data are presented as mean±standard deviation (S.D.). Statistical analysis was performed using Student's *t*-test and one-way analysis of variance (ANOVA) followed by a multiple-comparison test. Results were considered statistically significant compared to those of the control when $p<0.05$.

Results

Lapatinib, gefitinib, imatinib, and erlotinib sensitize resistant KBV20C cancer cells to vincristine treatment better than other TKIs. We planned to identify specific TKIs for sensitizing resistant cancer cells at relatively low doses. Previously it was shown that TKIs enhanced the sensitivity of P-gp-overexpressing resistant cancer (15-18). We considered TKIs that are already in clinical use because they can be readily used without the need for further toxicity studies after elucidation of their mechanism of action on resistant cancer cells. Therefore, we performed further detailed analysis with eight TKIs: Lapatinib, gefitinib, imatinib, erlotinib, nilotinib, pazopanib, cediranib, and vandetanib. In addition, we tested one serine/threonine kinase inhibitor, selumetinib (19). We focused on TKIs that sensitize P-gp-overexpressing resistant cancer cells at low doses. We evaluated the TKIs in combination with vincristine, an antimitotic drug that is routinely used as a chemotherapeutic agent in cancer (1, 2), using KBV20C resistant cancer cells, which present a vincristine-resistant phenotype due to P-gp overexpression (26-28).

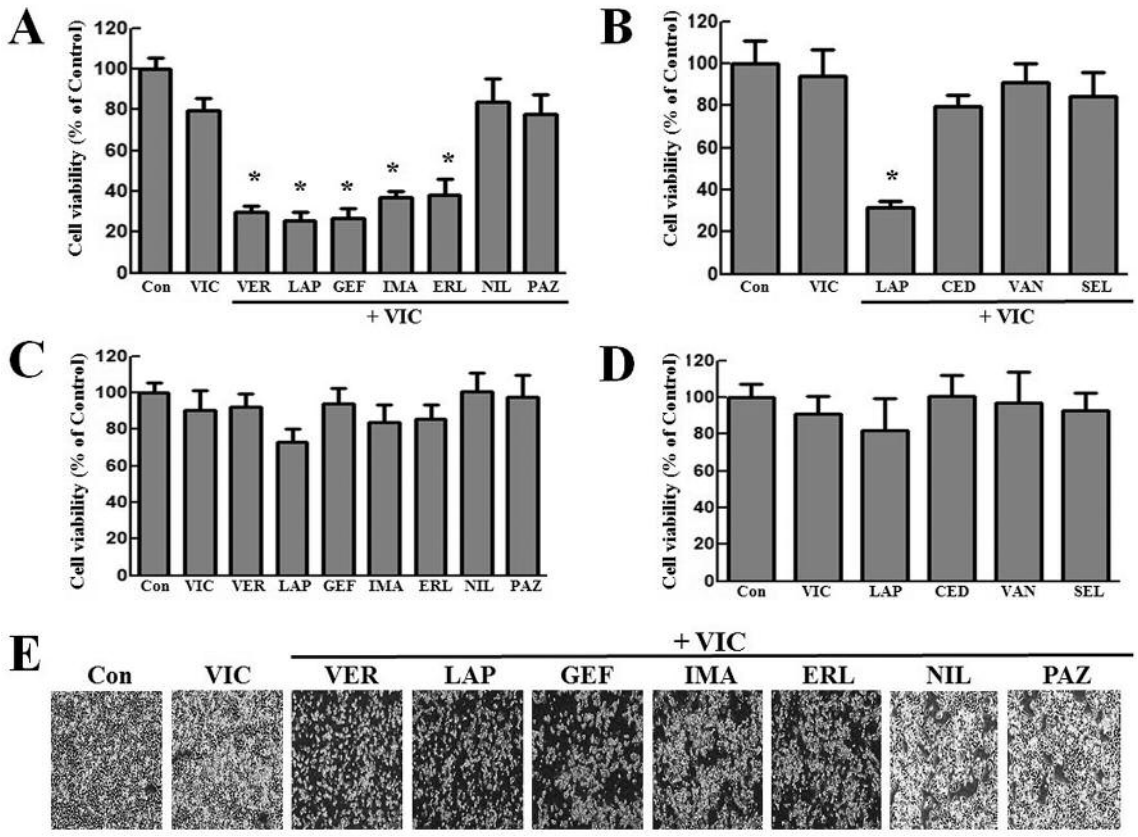

Figure 1. *Lapatinib (LAP), gefitinib (GEF), imatinib (IMA), erlotinib (ERL) sensitize vincristine (VIC)-treated resistant KBV20C cancer cells better than other TKIs. A-D: KBV20C cells were plated on 96-well plates and grown to 30-40% confluence. The cells were then stimulated for 48 h with 10 μM verapamil, or 5 μM lapatinib, gefitinib, imatinib, erlotinib, nilotinib (NIL), pazopanib (PAZ), cediranib (CED), vandetanib (VAN), or selumetinib (SEL) alone and in combination with 5 nM vincristine, or with 0.1% dimethyl sulfoxide (Con). Cell viability assay was performed as described in the Materials and Methods. The data are presented as the mean±S.D. of at least two experiments repeated in triplicate experiments. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by multiple-comparison test; *Significantly different at $p<0.05$ compared to the corresponding control. E: KBV20C cells were grown on 60 mm-diameter dishes and treated as described above. After 1 day, cells were examined using an inverted microscope at ×4 magnification (scale bar=100 μm).*

Firstly, we tested whether co-treatment with TKIs increased sensitivity of KBV20C cells to vincristine treatment using a quantitative cell viability test. As seen in Figure 1A and B, lapatinib, gefitinib, imatinib, and erlotinib highly reduced viability of vincristine-treated resistant KBV20C cells. The viability of cells treated with the vincristine with lapatinib, gefitinib, imatinib, and erlotinib combinations was >60% as compared to that of the control. We compared the effects of TKIs with those of the well-known P-gp inhibitor verapamil. Lapatinib (5 μM), gefitinib (5 μM), and verapamil (10 μM) were similarly effective when applied with vincristine in reducing KBV20C cell viability (Figure 1A and B). There was no difference between the control and monotherapy with TKIs (Figure 1C and D), suggesting that there were synergistic effects of vincristine in combination with lapatinib, gefitinib, imatinib, and erlotinib in the VIC-resistant cancer cells. We confirmed the results of the viability tests by microscopic observation. As shown in Figure 1E, when combined with vincristine, 5 μM of lapatinib, gefitinib, imatinib, or erlotinib showed sensitizing effects similar to those of verapamil.

Altogether, when we analyzed eight known TKIs to identify those sensitizing P-gp-overexpressing resistant cancer cells, we observed that lapatinib, gefitinib, imatinib, and erlotinib at low doses elicited highly sensitizing effects with greater reduction of viability than the other TKIs. We conclude that low doses of lapatinib, gefitinib, imatinib, or erlotinib can be used to reduce drug toxicity and sensitize resistant cancer cells to vincristine.

Co-treatment with vincristine and TKIs sensitizes resistant KBV20C cells via apoptosis through induction of G_2 arrest. To further clarify the mechanism of action of co-treatment

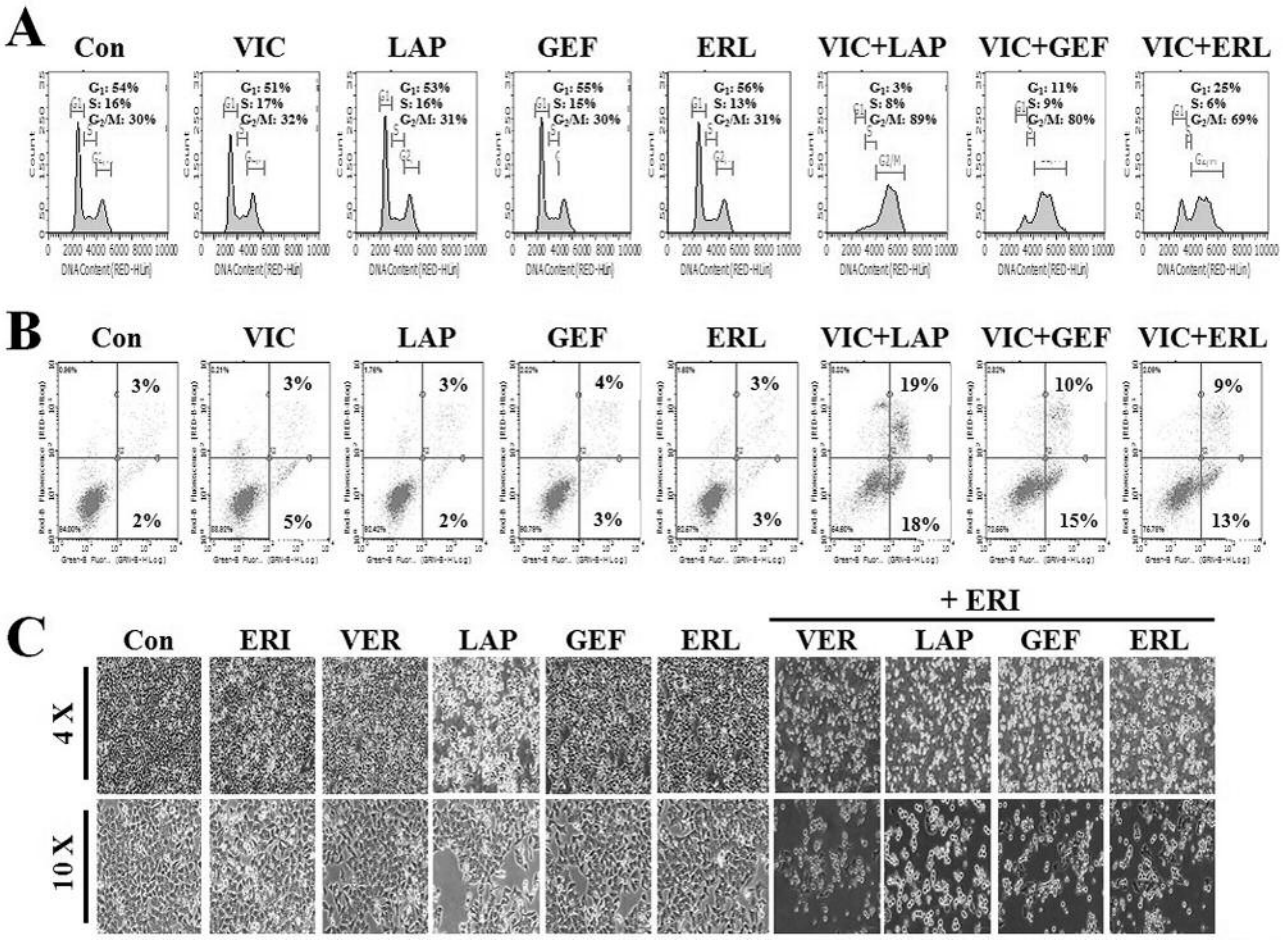

Figure 2. *Vincristine (VIC) with tyrosine kinase inhibitors sensitize resistant KBV20C cells via apoptosis through induction of G_2 arrest. A: KBV20C cells were grown on 60 mm-diameter dishes and treated with 5 μM lapatinib (LAP), 5 μM gefitinib (GEF), or 2.5 μM erlotinib (ERL) alone and in combination with 5 nM vincristine, or 0.1% dimethyl sulfoxide (DMSO; Con). After 24 h, fluorescence-activated cell sorting analyses were performed as described in the Materials and Methods. B: KBV20C cells were grown on 60 mm-diameter dishes and stimulated with 5 μM lapatinib, gefitinib or erlotinib alone and in combination with 5 nM vincristine, or with 0.1% DMSO (Con). After 24 h, annexin V analyses were performed as described in the Materials and Methods. C: KBV20C cells were grown on 60 mm-diameter dishes and treated with 10 μM verapamil, or 5 μM lapatinib, gefitinib, or erlotinib alone and in combination with 50 ng/ml eribulin, or with 0.1% DMSO (Con). After 24 h, cells were examined using an inverted microscope at ×4 or x10 magnification (scale bar=100 μm).*

with vincristine and TKIs, we performed FACS analyses. As shown in Figure 2A, co-treatments using vincristine with lapatinib, gefitinib, and erlotinib considerably increased the number of cells in G_2 arrest compared to that observed after monotherapy with either agent. This indicates that cell-cycle arrest resulted in the reduction of cellular viability.

Using annexin V analysis, we also tested whether combinations increased cell death by apoptosis. As seen in Figure 2B, apoptotic cell death greatly increased after co-treatment with vincristine with lapatinib, gefitinib, and erlotinib. This indicates that reduced G_2 arrest contributed to increased apoptotic death. Annexin V staining was also analyzed in detail. As seen in Figure 2B, the proportion of apoptotic cells (in both early and late phases) after vincristine treatment with lapatinib was about 37%, 25% when combined with gefitinib, and 22% with erlotinib, suggesting that lapatinib sensitized cells to vincristine much better than did gefitinib and erlotinib. Overall, we demonstrated that co-treatments with lapatinib, gefitinib, and erlotinib sensitized vincristine-treated resistant KBV20C cells *via* G_2 cell-cycle arrest and apoptosis.

TKIs increase sensitivity of KBV20C cells to eribulin when used at low doses. We also investigated whether lapatinib, gefitinib, or erlotinib could be used in combination with other antimitotic drugs. We tested eribulin, an antimitotic drug which has been recently developed and used in the

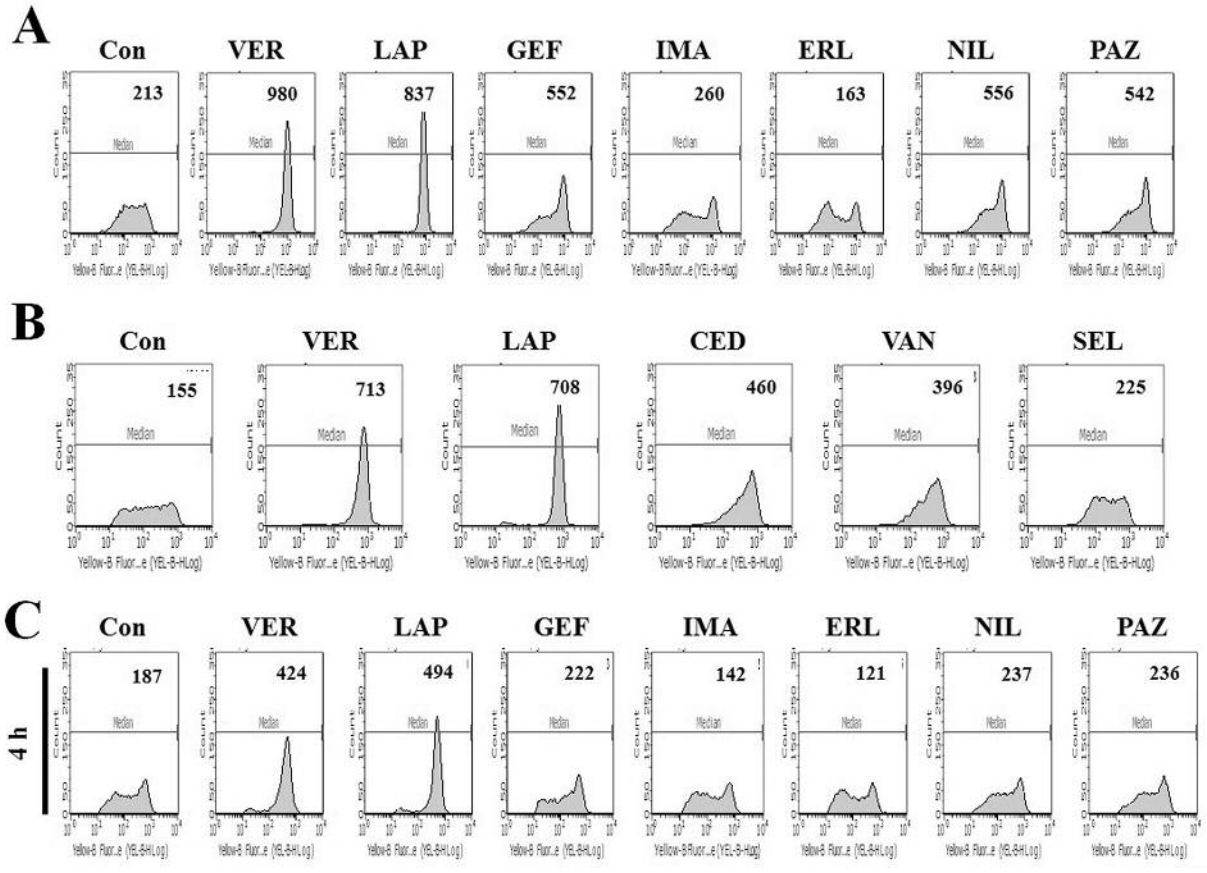

Figure 3. *Only lapatinib (LAP) has a high P-glycoprotein (P-gp)-inhibitory activity, whereas other tyrosine kinase inhibitors have low P-gp-inhibitory activity. A-C: KBV20C cells were grown on 60 mm-diameter dishes and treated with 10 μM verapamil (VER), or 5 μM lapatinib, gefitinib (GEF), imatinib (IMA), erlotinib (ERL), nilotinib (NIL), pazopanib (PAZ), cediranib (CED), vandetanib (VAN), selumetinib (SEL), or 0.1% dimethyl sulfoxide (DMSO; Con). After 4 h and 24 h, all cells were stained with rhodamine and examined using fluorescence-activated cell sorting analysis, as described in the Materials and Methods. The value in each graph is the median of cellular distribution.*

treatment of metastatic cancer (29-31). Previously, we found that the KBV20C cell line is a very useful model to study highly eribulin-resistant cancer (32).

As seen in Figure 2C, eribulin combined with 5 μM of lapatinib, gefitinib, or erlotinib was similar to their effects when combined with vincristine treatments with regard to sensitizing effectiveness (Figure 1E). Eribulin–verapamil co-treatment at the same dose also had effects similar to those of vincristine–verapamil co-treatment (Figures 1E and 2C). These results demonstrate that treatment with lapatinib, gefitinib, or erlotinib is as effective in sensitizing resistant cancer cells to eribulin as well as vincristine. This finding also suggests that at low doses, the TKIs lapatinib, gefitinib, and erlotinib can be combined with other antimitotic drugs to sensitize P-gp-overexpressing cancer cells. We conclude that lapatinib, gefitinib, or erlotinib can be used in patients with various types of drug-resistant cancer.

Lapatinib has high P-gp-inhibitory activity, whereas other TKIs have low P-gp-inhibitory activity. In the next phase of our investigation, we evaluated the P-gp-inhibitory activity of TKIs in P-gp-overexpressing KBV20C cells since TKIs have been suggested for sensitizing of resistant cancer cells with high P-gp inhibitory activity (15, 16, 18). We also expected that variation in the degree of P-gp inhibition among TKIs would be responsible for the difference in their sensitizing effects on VIC-treated KBV20C cells. We tested whether TKIs increased the inhibition of P-gp substrate efflux. Rhodamine 123, a well-known P-gp substrate, was used to measure P-gp inhibition (22-24). In this experiment, green fluorescence in the cell was indicative of intracellular accumulation of rhodamine 123.

As shown in Figure 3A and B, lapatinib exhibited high P-gp-inhibitory activity, whereas other TKIs had much lower P-gp inhibitory activity. In a detailed quantitative analysis, the known P-gp inhibitor verapamil was used as a positive control (5, 7), and upon comparison we found that lapatinib at half the dose required for verapamil induced equivalent P-gp-inhibitory activity. This suggests that P-gp inhibition

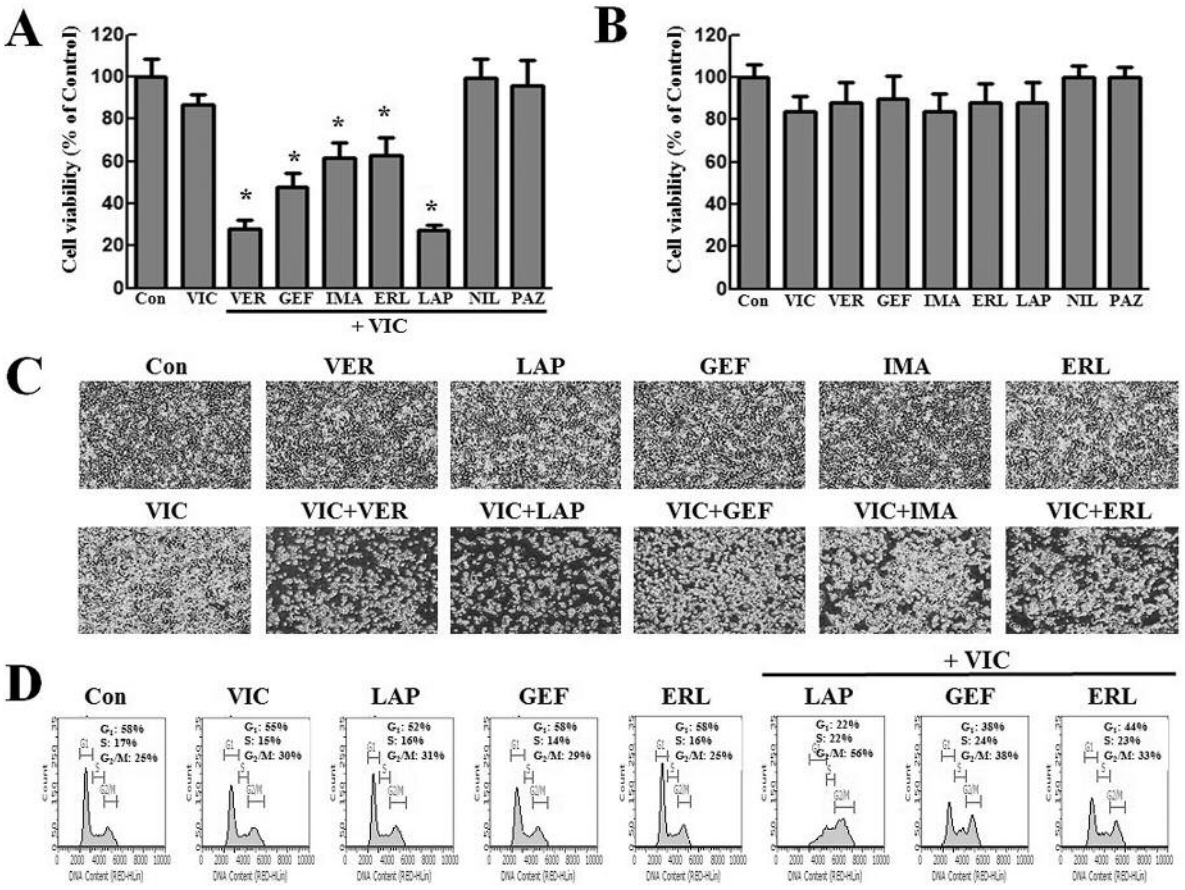

Figure 4. *Lapatinib (LAP) at a low dose has a much greater vincristine (VIC)-sensitizing effect on KBV20C cells than other tyrosine kinase inhibitors. A and B: KBV20C cells were plated on 96-well plates and grown to 30-40% confluence. The cells were then stimulated for 48 h with 10 μM verapamil (VER), or 2.5 μM lapatinib, gefitinib (GEF), imatinib (IMA), erlotinib (ERL), nilotinib (NIL), or pazopanib (PAZ) and in combination with 5 nM vincristine, or 0.1% dimethyl sulfoxide (DMSO; Con). Cell viability assay was performed as described in the Materials and Methods. C: KBV20C cells were grown on 60 mm-diameter dishes and treated as described above. After 1 day, cells were examined using an inverted microscope at ×4 magnification (scale bar=100 μm). D: KBV20C cells were grown on 60 mm-diameter dishes and treated with 2.5 μM lapatinib, gefitinib, or erlotinib alone and in combination with 5 nM vincristine, or 0.1% DMSO (Con). After 24 h, fluorescence-activated cell sorting analyses were performed as described in Materials and Methods.*

by lapatinib plays a key role in sensitizing cells to vincristine–lapatinib co-treatment. However, P-gp inhibition by other TKIs was much lower than that of verapamil or slightly higher than that of the control (Figure 3A and B). When we tested selumetinib, a serine/threonine kinase (19), we also found low P-gp-inhibitory activity (Figure 3B). As shown in Figure 3C, treatment with lapatinib or verapamil for 4 h led to results similar to those obtained after 24 h of treatment. This suggests that lapatinib inhibits P-gp by direct binding, similarly to the mechanism of verapamil. Considering that previously developed P-gp inhibitors, including verapamil, are toxic to normal cells (5, 7, 8), we believe that lapatinib is a suitable alternative for sensitizing P-gp-overexpressing resistant cancer cells in clinical treatment.

The results also indicate that sensitizing KBV20C cells with low doses of vincristine–gefitinib is minimally affected by the P-gp-inhibitory effects of gefitinib. Notably, the level of P-gp inhibition by imatinib, and erlotinib was similar to that of the blank control (Figure 3A and C), suggesting that sensitizivity induced by vincristine–imatinib and vincristine–erlotinib in KBV20C cells was independent of their P-gp-inhibitory effects. Considering that even with very low or no P-gp-inhibitory activity, gefitinib, imatinib, or erlotinib still sensitized vincristine-treated KBV20C cells (Figure 3A and C), they might be useful in clinical settings due to minimal toxic P-gp-inhibitory effects in normal cells.

Altogether, we found that these TKIs have different levels of P-gp inhibition and they function differently in sensitizing resistant KBV20C cells, which overexpress P-gp, to

vincristine. Although co-treatments of vincristine with lapatinib, gefitinib, imatinib, and erlotinib had similarly high sensitizing effects on KBV20C cells, it is interesting that only lapatinib did so with very high P-gp-inhibitory activity, whereas the other TKIs induced sensitivity with little or no P-gp-inhibitory effect. Therefore, we conclude that these co-treatments have different mechanisms of sensitizing KBV20C cells.

At a low dose, lapatinib has much greater vincristine-sensitizing effect on KBV20C cells than other TKIs. Our apoptosis results showed vincristine–lapatinib had a greater sensitizing effect than other co-treatments with TKIs (Figure 2B), suggesting that lapatinib is an ideal candidate in co-treatment for sensitizing resistant cancer cells. In order to demonstrate that lapatinib is better than other TKIs, we tested a lower dose (2.5 μM) of TKIs for sensitizing KBV20C cells to vincristine. We believed that this lower dose along with VIC might better distinguish any variation in sensitizing effects among co-treatments with vincristine and TKIs.

As seen in Figure 4A, 2.5 μM of lapatinib highly reduced viability in vincristine-treated KBV20C cells, whereas 2.5 μM of gefitinib, imatinib, or erlotinib led to about half that effect. Viability of cells treated with with vincristine–lapatinib co-treatment was reduced by >80% as compared to the control. When we compared the effects of lapatinib with the well-known P-gp inhibitor verapamil, 2.5 μM lapatinib or 10 μM verapamil had similar effectiveness when applied with vincristine to reduce KBV20C cell viability (Figure 4A). The results suggest that lapatinib is ideal, with a low half-maximal inhibitory concentration (IC_{50}), for sensitizing antimitotic drug-resistant cancer cells. There was no difference in viability between the control and treatment with individual TKIs (Figure 4B), suggesting that sensitizing by vincristine–lapatinib co-treatment resulted in synergistic effects in vincristine -resistant cancer cells.

We confirmed the results of these viability tests by microscopic observation. As shown in Figure 4C, 2.5 μM of lapatinib was more effective than 2.5 μM of gefitinib, imatinib, or erlotinib and had sensitizing effects similar to those of verapamil when combined with vincristine.

When cells in G_2 arrest after treatment with low doses (2.5 μM) of TKIs were quantitatively estimated (Figure 4D), we found that the proportion of G_2-arrested cells comprised about 56% in cells treated with vincristine combined with lapatinib, 38% with gefitinib, and 33% with erlotinib. The results suggest that the effect of vincristine–lapatinib co-treatment in causing G_2 arrest was much greater than that of vincristine–gefitinib or vincristine–erlotinib.

Altogether, when we analyzed eight known TKIs to identify sensitizing of P-gp-overexpressing resistant cancer cells, we observed that lapatinib, gefitinib, imatinib, and erlotinib at low doses have greater sensitizing effects than the other drugs. Interestingly, gefitinib, imatinib, and erlotinib sensitized with little or no P-gp-inhibitory activity. In a detailed analysis with lower doses, we concluded that lapatinib, with high P-gp-inhibitory activity, may be a better and more potent agent in co-treatment for vincristine-treated resistant cancer cells than gefitinib, imatinib, or erlotinib. Therefore, lapatinib can be used to reduce drug toxicity and effectively sensitize cancer cells resistant to vincristine.

Discussion

Drug repositioning or repurposing is the application of known drugs for new indications. It has been used for the treatment of various diseases and has advantages, such as low cost and avoidance of many toxicity tests, which saves time (9-11). The urgent need for pharmacological treatments for resistant cancer can be efficiently addressed with drug repositioning and these drugs can be administered to patients sooner. In the current study, we investigated the novel application of some anticancer drugs (TKIs), repositioned for sensitizing P-gp-overexpressing resistant cancer cells.

Most importantly, we identified four TKIs, lapatinib, gefitinib, imatinib, and erlotinib, which can sensitize resistant KBV20C cells at a lower dose than other TKIs. Considering that half of the TKIs studied had vincristine-sensitizing effects on resistant cancer cells at low doses, we conclude that TKIs generally have the ability to overcome resistance to antimitotic drugs. As EGFR or growth signaling receptor kinases are located in the cellular membrane (12-14), we hypothesized that TKIs play a role in reducing or modifying increased P-gp activity in the membranes of resistant cancer cells. Although the resistant cancer-sensitizing ability of TKIs has been demonstrated (15-18), our research is pioneering in using selective TKIs as P-gp targeting drugs against cancer resistant to antimitotic drug. Our findings might also assist in selecting TKIs for preventing or reducing the occurrence of resistant cancer.

Our results were not limited to vincristine co-treatment because we confirmed that lapatinib, gefitinib, or erlotinib had sensitizing effects similar to those observed with eribulin in P-gp-overexpressing KBV20C cells. Eribulin was recently developed and is a promising drug for the treatment of resistant cancer (29-31). We previously reported that P-gp-overexpressing KBV20C cells are highly eribulin-resistant (32). Therefore, KBV20C cells are useful as models to study highly eribulin-resistant cancer. We found that that lapatinib, gefitinib, and erlotinib can sensitize KBV20C cells to eribulin-treated, suggesting that that lapatinib, gefitinib, and erlotinib can sensitize other antimitotic drug-resistant cancer cells similar to vincristine-treated KBV20C cells.

As the efflux of vincristine by P-gp is the main mechanism for resistance of KBV20C cells to vincristine, we hypothesized that the different vincristine-sensitizing effects of lapatinib,

gefitinib, imatinib, and erlotinib co-treatment resulted from their P-gp-inhibitory effects. We demonstrated that lapatinib has a stronger P-gp-inhibitory effect than the well-known P-gp inhibitor verapamil, suggesting that vincristine–lapatinib sensitizing results from the inhibitory effects of lapatinib to prevent pumping out of vincristine. However, interestingly, we detected low levels of P-gp inhibition with gefitinib and no P-gp inhibition with imatinib and erlotinib, suggesting that gefitinib, imatinib, and erlotinib remove or inhibit factors that block vincristine effects in drug-resistant cancer cells and that their combination with vincristine thus has a synergistic effect on cells. Further investigation with gefitinib, imatinib, and erlotinib may be needed for determining the molecular targets for sensitizing resistant cancer cells without P-gp inhibition. As little or no P-gp-inhibitory activity was detected with gefitinib, imatinib, and erlotinib, an improved combination of chemotherapeutic agents can be developed for patients with cancer resistant to antimitotic drugs. As P-gp inhibitors have shown toxicity to normal cells (5, 7, 8), we believe that gefitinib, imatinib, and erlotinib may be considered as combination drugs with little or no P-gp inhibition to sensitize the P-gp-overexpressing resistant cancer cells. As personalized medicines are currently gaining popularity, our findings with the TKIs lapatinib, gefitinib, imatinib, or erlotinib might contribute to effective prescriptions in patients with drug-resistant cancer who are allergic or sensitive to P-gp-inhibitory effects in normal tissues.

To conclude, our results highlight the novel selective sensitizing ability of TKIs. Furthermore, drug-resistant KBV20C cells that overexpress P-gp can be sensitized to the antimitotic drugs vincristine or eribulin by co-treatment with the cancer-targeting drugs lapatinib, gefitinib, imatinib, or erlotinib at low doses. Notably, gefitinib, imatinib, or erlotinib can sensitize the drug-resistant cancer cells with low P-gp-inhibitory activity. In addition, lapatinib, with a high P-gp inhibitory activity, is the best choice for increasing sensitizing of P gp-overexpressing KBV20C cells. As the toxicities of these drugs are already documented, they are readily available for use to treat patients with cancer. Our results could contribute to improvement in the efficacy of various chemotherapeutic agents used in combination for the treatment of patients with cancer which becomes resistant to chemotherapeutic drugs *via* P-gp-overexpression.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Ji Yeong Kim: Collected the data, contributed data or analysis tools, wrote the article. Hyung Sik Kim: Contributed data or analysis tools. Sungpil Yoon: Conceived and designed the analysis, collected the data, contributed data or analysis tools, wrote the article.

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