Attenuation of Colchicine Toxicity in Drug-resistant Cancer Cells by Co-treatment with Anti-malarial Drugs

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Abstract. Background/Aim: Colchicine (COL) is a well-known and potent microtubule targeting anticancer agent. The purpose of our study was to identify conditions that increase sensitization of COL-resistant cancer cells that overexpress P-glycoprotein (P-gp). Materials and Methods: The anti-malarial drugs chloroquine (CHL), mefloquine (MEF) and primaquine (PRI) have been shown to increase sensitization in drug-resistant KBV20C cells via P-gp inhibition. Therefore, we tested whether co-treatment of COL with PRI, CHL or MEF increases sensitivity in COL-resistant KBV20C cells over that of cells treated with COL alone and whether these effects are attributable to P-gp activity. Results: Interestingly, we found that both CHL and PRI, but not MEF, reduced cytotoxicity in KBV20C cells receiving high concentrations of COL, suggesting that the effects of CHL and PRI have specific mechanisms among the anti-malarial drugs. The effects of CHL and PRI were specific to COL-resistant cells, since we did not detect a reduction in cytotoxicity in drug-sensitive parent KB cells. These data suggest that CHL and PRI inhibit the signaling pathways of COL-treated-resistant cells without P-gp inhibition. Furthermore, we studied the molecular mechanisms underlying the effects of COL-CHL co-treatment in KBV20C cells. FACS analysis, annexin V staining and western blot analysis revealed that G2 arrest and apoptosis were lower in cells co-treated with COL and CHL than in cells treated with COL alone. We also found that pH2AX, pHistone H3 and pRb expression was highly reduced in COL-CHL co-treated cells but not in COL-VIB co-treated cells. In addition, expression of the p21 protein, which correlates with drug-resistant phenotypes, increased in cells receiving COL-CHL co-treatment over that of COL-treated cells. Conclusion: These results suggest that reduced G2 arrest and apoptosis resulting from COL-CHL co-treatment was attributable to DNA damage and reduced cell cycle progression. These findings provide important information regarding the prevention of COL toxicity in COL-resistant cells and indicate that CHL, PRI and MEF may contribute to sensitization in COL-resistant cells.

Anti-mitotic drugs target specific domains in microtubules to prevent polymerization or depolymerization and are, therefore, widely used for treating numerous types of cancers (1). The anti-mitotic drugs paclitaxel (PAC) and docetaxel (DOC) target the taxane-binding site of microtubules, whereas vinblastine (VIB), vinorelbine (VIO) and vincristine (VIC) target the vinca domain. Colchicine (COL) targets the colchicine domain (1-3). COL is a well-known and potent microtubule-targeting chemotherapeutic agent (4-7). However, COL is not commonly used to treat cancer because of its toxicity in normal cells. In order to overcome this issue, many studies have been conducted using modified COL compounds for the treatment of cancer (8, 9). Since patients develop resistance to these drugs (10-12), research to identify the mechanism(s) underlying cell sensitization to anti-mitotic drugs is considered to be an important step in the development of new pharmacological cancer treatments.

The anti-malarial drugs, chloroquine (CHL), primaquine (PRI) and mefloquine (MEF) have been shown to be potentially useful in the treatment of cancer (13-16). In particular, CHL has been investigated for the treatment of numerous types of cancers, sometimes in combination with other chemotherapeutics (13, 17). Previously, we reported that CHL, PRI and MEF have P-glycoprotein (P-gp) inhibitory activity in resistant cells (18, 19). Since these drugs have already been approved for the treatment of malaria, they could be readily available for clinical use once their anticancer activities are better understood.

In the present study, we tested whether co-treatment of COL with PRI, CHL or MEF increases sensitivity in COL-resistant
KBV20C cells over that of cells treated with COL alone and whether these effects are attributable to P-gp activity. Our results indicate that CHL and PRI attenuate toxicity in COL-treated KBV20C cells. We further investigated molecular signatures to determine the factors involved in the effects observed in cells co-treated with COL-CHL and COL-PRI. These results will contribute to the development of CHL-, PRI- and MEF-based therapy for the treatment of cancer in COL-resistant patients.

Materials and Methods

Reagents. CHL, PRI, MEF and Verapamil (VER) were purchased from Sigma-Aldrich (St.Louis, MO, USA). VIB and COL were purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Antibodies. Antibodies against pGSK3β, pp70S6K, Cdc2, Cdc2, Chk1, Chk2, Histone H3, p21, PCNA and cleaved poly ADP ribose polymerase (C-PARP) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), survivin and pRb were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against pH2AX was from Abcam (Cambridge, UK).

Cell culturing. Human oral squamous carcinoma cell lines, KB and its multidrug-resistant subline, KBV20C, were obtained from Dr. Yong Kee Kim and have been previously described (20-22). All cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (WelGENE, Daegu, Korea)

Fluorescence-activated cell sorting (FACS) analysis. FACS analysis was performed as previously described (21, 22). Cells were grown in 60-mm diameter dishes and treated with the indicated drugs for the prescribed times. The cells were then dislodged by trypsin and pelleted by centrifugation. The pelleted cells were washed thoroughly with PBS, suspended in 75% ethanol for at least 1 h at 4°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 for relative DNA content using a FACSCalibur flow cytometry system (BD Bioscience, Franklin Lakes, NJ, USA). We performed two independent experiments.

Annexin V analysis. Annexin V analysis was conducted using the annexin V-fluorescein isothiocyanate (FITC) staining kit (BD Bioscience) as previously described (21, 23). Cells were grown in 60-mm diameter dishes and treated with the indicated drugs for the prescribed times. The cells were then dislodged by trypsin and pelleted by centrifugation. The pelleted cells were washed with PBS. Cells in 100 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 using a FACSCalibur flow cytometry system (BD Bioscience). We performed two independent experiments.

Western blot analysis. Total cellular proteins were extracted using a previously described trichloroacetic acid (TCA) method (23, 24). Briefly, cells grown in 60-mm dishes were washed three times with 5 ml PBS. Next, 500 μl of 20% trichloroacetic acid (TCA) were added to each plate. The cells were then dislodged by scraping and transferred to Eppendorf tubes. Proteins were pelleted by centrifugation for 5 min at 3,000 rpm and re-suspended in 1M Tris- HCl (pH 8.0) buffer. The total protein concentrations were estimated. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to Western blot analysis as previously described (21, 22, 24).

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

Results

Co-treatment of CHL attenuates toxicity of COL-treated KBV20C-resistant cancer cells. We tested whether KBV20C cells, which have an anti-mitotic drug-resistant phenotype, are resistant to COL. Our results demonstrate that KBV20C cells have increased resistance to COL at higher concentrations than that by the anti-mitotic drug-sensitive KB cells (Figure 1A).

However, with increasing COL concentrations (approximately 4-fold), we determined that sensitization is similar between KB and KBV20C cells. These data suggest that differences in the half-maximal inhibitory concentration (IC50) between sensitive KB and resistant KBV20C cells are approximately 4-fold of the COL concentration (Figure 1A) and that COL sensitization can result from P-gp-independent mechanisms at high COL concentrations. We also determined whether drug efflux attributable to P-gp activity is involved in COL-resistance of KBV20C cells. As seen in Figure 1B, co-treatment of COL with the P-gp inhibitor (VER) increased sensitization of COL in KBV20C cells, suggesting that KBV20C P-gp activity contributes to COL resistance in KBV20C cells. Furthermore, we investigated mechanisms involved in COL sensitization in KBV20C cells. Previously, we demonstrated that CHL has P-gp activity (18); therefore, we first tested whether CHL treatment results in the recovery of COL-resistant phenotypes in KBV20C cancer cells. Surprisingly, we found that CHL attenuates toxicity of COL at higher concentrations in KBV20C-resistant cancer cells (Figure 1B), suggesting that CHL is not a good candidate for increasing the efficacy of COL in COL-resistant cancer cells (Figure 1B).
microscopy at high (Figure 1B-C) and low magnifications (Figure 1D-E). Furthermore, we tested whether the reduced toxicity observed with CHL and COL co-treatment occurs in parent KB-sensitive cells. However, these effects were not observed in KB cells (Figure 1F), suggesting that CHL-associated attenuation of COL toxicity is specific for COL-resistant KBV20C cells independent of P-gp activity.

Co-treatment of COL with CHL reduces COL-induced G2 arrest and apoptosis in KBV20C-resistant cells. Next, we examined the mechanisms underlying attenuation of COL-induced toxicity by the addition of CHL in KBV20C cells. We performed FACS analysis to compare cell-cycle arrest between single treatments with COL and co-treatment with COL and CHL. As shown in Figure 2A, G2-phase cell cycle arrest was lower in KBV20C cells co-treated with COL and CHL than in cells treated with COL only, suggesting that CHL attenuates the effects of COL in KBV20C-resistant cells via reduced G2 arrest. These results also indicate that reduced G2 arrest results in reduced sensitization to COL-CHL co-treatment in KBV20C cells.

To determine if co-treatment of COL and CHL affects apoptosis in KBV20C cells, we analyzed the pre-G1 region using FACS analysis. As seen in Figure 2A, the pre-G1 region
was much lower in cells receiving COL-CHL co-treatment than in cells treated with COL alone, suggesting that reduced G₂ arrest by COL-CHL co-treatment results in reduced apoptosis. Analysis of annexin V staining demonstrated that COL-CHL co-treatments attenuate the increase in early apoptotic regions seen in cells treated with COL alone (Figure 2B-2C). These data suggest that, in addition to G₂ arrest, CHL attenuates the effects of COL by reducing early apoptotic events. We compared these results to VIB, an anti-mitotic drug that does not have P-gp inhibitory activity. As seen in Figure 3A, the results of FACS analysis did not show an increase in sensitization of KBV20C cells co-treated with COL and VIB over that of cells treated with COL alone. As expected, the G₂ and pre-G₁ region was lower in cells receiving COL-CHL co-treatment than in COL alone (Figure 3A). The increase in G₂ arrest observed in cells receiving treatment with COL alone was unaffected by the addition of VIB, suggesting that increased sensitization requires P-gp inhibitory activity.

Increased expression of p21 and decreased expression of pH2AX and pHistone H3 contribute to CHL-associated reductions in toxicity in COL-treated-resistant cells. To determine the molecular mechanisms underlying the CHL-associated reductions in COL-induced toxicity in KBV20C
cells, we tested whether co-treatment with COL and CHL influences the activation or expression of signaling proteins. In particular, we examined signaling pathways upstream and downstream of growth regulators, such as damage response-, cell cycle- and proliferation-related proteins (21, 24-26). In addition, we analyzed the phosphorylated forms of the major proteins Gsk3β, P70S6K, Cdc2, Rb, Chk1, H2AX, Histone H3 and Chk2. We also checked for any alterations in pathway-related proteins (PCNA, p21, Cdc2 and survivin) and differences between cells co-treated with COL and CHL together or alone in order to identify significant changes in protein levels and/or activation. It is likely that the proteins identified are involved in reduced G2 arrest and early apoptotic events observed in cells co-treated with COL and CHL. As expected, C-PARP levels were lower in cells co-treated with COL-CHL than in COL alone (Figure 3B), thus confirming that CHL is involved in the anti-apoptotic effects in COL-treated KBV20C cells.

In addition to involvement of anti-apoptosis, we determined that pH2AX expression is increased in KBV20 cells treated with COL, an effect that is reduced by the concomitant treatment of CHL, suggesting that the prevention of DNA damage is likely an underlying mechanism of CHL-associated reductions in COL toxicity (Figure 3B). Expression of pRb and pHistone H3 was lower in cells co-treated with COL-CHL than in cells treated with COL alone (Figure 3B), suggesting that

Figure 3. Increased p21 expression and decreased pH2AX and pHistone H3 expressions are associated with reduced toxicity in KBV20C-resistant cancer cells co-treated with chloroquine (CHL) and colchicine (COL). (A) KBV20C cells were plated on 60 mm-diameter dishes and treated with 30 nM COL (COL-30), 50 nM COL (COL-50), 5 nM VIB (VIB-5), 50 μM CHL (CHL-50), 30 nM COL with 5 nM VIB (COL-30+VIB), 30 nM COL with 50 μM CHL (COL-30+CHL), 50 nM COL with 5 nM VIB (COL-50+VIB), 50 nM COL with 50 μM CHL (COL-50+CHL) or 0.1% DMSO (control (Con)). After 24 h, FACS analysis was performed as described in Materials and Methods. (B) KBV20C cells were plated on 60 mm-diameter dishes and treated with 40 nM COL (COL), 5 nM VIB (VIB), 50 μM CHL (CHL-50), 40 nM COL+ 5 nM VIB (COL+VIB), 40 nM COL+50 μM CHL (COL+CHL) or 0.1% DMSO (Con). After 24 h, Western blot analysis was performed using antibodies against C-PARP, pGSK3β, pRb, pP70S6K, PCNA, Cdc2, Survivin, pCdc2, p21, pChk1, pH2AX, pHistone H3, pChk2 and GAPDH.
activation of those proteins is involved in CHL-associated reductions in COL toxicity. In addition, p21 levels were much higher in cells co-treated with COL-CHL than in cells treated with COL alone (Figure 3B), suggesting that resistant phenotypes of KBV20C cells are increased. The expression levels of the remaining proteins examined were marginally changed, suggesting that they are not involved in CHL-associated reductions in COL toxicity. These results provide important information about signaling pathways involved in the attenuation of COL toxicity by the addition of CHL. Collectively, we conclude that pH2AX, pRb, pHistone H3 and p21 are the primary factors involved in the effects observed in KBV20C-resistant cells co-treated with COL and CHL.

Co-treatment with the anti-malarial drug PRI attenuates the toxicity of COL in a manner similar to CHL. Previously, we examined P-gp activity for the anti-malarial drugs PRI, MEF and CHL (18, 19). PRI and MEF were both found to have P-gp inhibiting activity. Therefore, we tested whether co-treatment of KBV20C cells with COL and PRI or MEF would result in a reduction of toxicity similar to that observed with COL and CHL co-treatments. As shown in Figure 1C and E, there were no reductions in toxicity observed in cells co-treated with COL and MEF. In contrast, COL-induced toxicity was attenuated by the addition of PRI in a similar manner to that observed in cells co-treated with COL and CHL (Figure 4A), suggesting that CHL and PRI have similar effects on
signaling pathways in KBV20C-resistant cells. Since a reduction in G2 arrest was observed in cells treated with CHL (Figure 2A and A), we determined if PRI could have a similar effect. Therefore, we used FACS analysis to measure G2 arrest in cells co-treated with COL and PRI. As seen in Figure 4B, co-treatment of COL and PRI attenuated the increase in G2 arrest observed in KBV20C cells treated with COL alone. These results suggest that PRI and CHL reduce COL-induced toxicity in a similar manner in KBV20C-resistant cells. Taken together, the described co-treatment experiments allowed the identification of two anti-malarial drugs, namely PRI and CHL, able to attenuate COL-induced toxicity in KBV20C drug-resistant cancer cells.

Discussion

Despite its toxicity to normal cells, COL has been studied as an anti-cancer agent in various cancer models (4-7). COL derivatives have also been developed to reduce the toxicity to normal cells (8, 9). Additional studies focusing on the mechanisms of COL sensitization and toxicity would be beneficial for future studies examining the utility of COL derivatives as chemotherapeutic agents. Therefore, we aimed to increase our understanding of the mechanisms associated with sensitization of COL-resistant cancer cells that have increased expression of P-gp. We found that COL-resistant KBV20C cells can be sensitized at COL concentrations 4-fold higher than that required for parent sensitive KB cells, suggesting that P-gp activity is saturated at high COL concentrations. The cancer-sensitizing ability of anti-malarial drugs has been demonstrated in various cancer models (13-17), suggesting that anti-malarial drugs have potential as chemotherapeutic agents. CHL, MEF and PRI treatment may increase the toxicity of other chemotherapeutics through their P-gp inhibitory activity (18, 19), which increases their clinical potential. Therefore, we tested whether CHL, MEF and PRI increase sensitization in COL-resistant KBV20C cells.

Here, we demonstrated that CHL, PRI and MEF do not increase sensitization in COL-treated-resistant cancer cells, despite having P-gp inhibitory activity. Interestingly, co-treatment of CHL or PRI attenuated the cytotoxicity observed in COL-treated-resistant cancer cells. Attenuation of COL-induced toxicity by CHL likely involves a reduction in apoptosis, based on a reduced pre-G1 region as determined by FACS analysis, reduced early apoptotic events as determined by annexin V staining and reduced C-PARP levels as determined by Western blot analysis. These data suggest that CHL prevents the initiation of apoptosis in COL-treated-resistant cancer cells. Since CHL has been extensively investigated as an inhibitor of autophagy for application in cancer cell sensitization (17), our findings contribute to further clinical applications of CHL in combination treatments with anti-mitotic drugs. COL derivatives that are less toxic to normal cells have been developed (8, 9). The effects of CHL in combination with COL in resistant cells may significantly affect COL-related cancer therapy research.

CHL-associated attenuation of COL-induced toxicity is independent of P-gp activity in KBV20C-resistant cells; therefore, we assumed that a similar effect could be observed in the sensitive parent KB cells. However, CHL treatment had no impact on COL-induced toxicity in KB cells, suggesting that this phenomenon is specific for resistant KBV20C cells with active P-gp proteins.

These results suggest that some signaling molecules or pathways involved in COL-induced toxicity are negatively regulated by CHL co-treatment in P-gp overexpressing resistant cancer cells. As such, we identified specific mechanisms involved in the effects observed in COL-CHL co-treated KBV20C cells by examining pathways involved in cell cycle arrest, activation and deactivation. We found that G2 arrest was lower in cells co-treated with COL and CHL than in cells treated with COL alone, indicating that CHL treatment prevents COL sensitization via G2 arrest. In addition, we showed that CHL-induced reductions in pRb and pHistone H3 expression are important factors in the attenuation of COL-induced toxicity in KBV20C-resistant cells. Considering that increased pRb and pHistone H3 expression negatively correlates with growth and proliferation (27, 28), it was hypothesized that reduced expression of activated Rb and Histone H3 is an underlying mechanism of CHL attenuation of COL toxicity. We also found increased expression of the resistant-related protein p21 in cells co-treated with COL and CHL over that of cells treated with COL alone. In addition, expression of pH2AX was lower in cells co-treated with COL and CHL than in cells treated with COL alone. These results provide important information regarding the signaling pathways involved in CHL attenuation of COL toxicity in the absence of increased P-gp activity. We suggest that CHL attenuates COL-induced toxicity by reducing DNA damage and G2 cycle arrest in resistant cells.

Further studies will determine how the activated proteins or signaling pathways modulate the effects observed in COL-CHL co-treated cells and the mechanisms underlying the switch from increased chemo-sensitivity to reduced toxicity.

In summary, this study demonstrated that the anti-malarial drugs CHL and PRI, but not MEF, have specificity for attenuating COL-induced toxicity in resistant cancer cells. The results obtained are relevant to the use of the anti-malarial drugs CHL, PRI and MEF in COL-resistant cancer. In addition, our results can contribute to our understanding of the COL-resistant phenotype with P-gp activity and reduced COL-toxicity. Since these anti-malarial drugs are already used in clinical settings, the urgent need for pharmacological treatments of resistant cancers can be efficiently addressed and applied to human patients at a relatively faster pace.
Conflicts of Interest

The Authors declare no conflicts of interest.

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