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Co-treatment with the anti-malarial drugs mefloquine and primaquine highly sensitizes drug-resistant cancer cells by increasing P-gp inhibition

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ABSTRACT

The purpose of this study was to identify conditions that will increase the sensitivity of resistant cancer cells to anti-mitotic drugs. Currently, atovaquine (ATO), chloroquine (CHL), primaquine (PRI), mefloquine (MEF), artesunate (ART), and doxycycline (DOY) are the most commonly used anti-malarial drugs. Herein, we tested whether anti-malarial drugs can sensitize drug-resistant KBV20C cancer cells. None of the six tested anti-malarial drugs was found to better sensitize the drug-resistant cells compared to the sensitive KB cells. With an exception of DOY, all other anti-malarial drugs tested could sensitize both KB and KBV20C cells to a similar extent, suggesting that anti-malarial drugs could be used for sensitive as well as resistant cancer cells.

Furthermore, we examined the effects of anti-malarial drugs in combination with an antimitotic drug, vinblastine (VIN) on the sensitisation of resistant KBV20C cells. Using viability assay, microscopic observation, assessment of cleaved PARP, and Hoechst staining, we identified that two anti-malarial drugs, PRI and MEF, highly sensitized KBV20C-resistant cells to VIN treatment. Moreover, PRI- or MEF-induced sensitisation was not observed in VIN-treated sensitive KB parent cells, suggesting that the observed effect is specific to resistant cancer cells. We demonstrated that the PRI and MEF sensitisation mechanism mainly depends on the inhibition of p-glycoprotein (P-gp). Our findings may contribute to the development of anti-malarial drug-based combination therapies for patients resistant to anti-mitotic drugs.

1. Introduction

Anti-mitotic drugs are widely used to treat numerous types of cancers [1,2]. These compounds inhibit mitosis by targeting microtubules and preventing their polymerization or depolymerization [1–3]. However, patients develop resistance to these drugs [4–7]. Thus, in order to improve the efficacy of treatment, research has focused on increasing anti-mitotic-associated apoptosis.

Various anti-malarial drugs have been developed to effectively combat malaria in humans. Atovaquine (ATO), chloroquine (CHL), primaquine (PRI), mefloquine (MEF), artesunate (ART), and doxycycline (DOY) are the most commonly used anti-malarial drugs [8–10]. These anti-malarial drugs are also shown to be potentially useful in the treatment of cancer [11–21]. These drugs have been investigated in the treatment of numerous types of cancers, sometimes in combination with chemotherapy [22–24]. Since the toxicity of these drugs is already known, these drugs would be readily available for clinical use once their anti-cancer activities are better understood.

Since a better understanding of the mechanism governing the sensitisation effect of anti-malarial drugs in cancer patients could facilitate their therapeutic use, we tried to identify the mechanism involved in the sensitizing effect of anti-malarial drugs on cancer cells. In the present study, we found that co-treatment with either PRI or MEF in combination with vinblastine (VIN) highly sensitized drug-resistant KBV20C cancer cells. We also found that PRI and MEF sensitisation effect involved p-glycoprotein (P-gp) inhibition, which prevented the efflux of VIN. Our results may contribute to the development of PRI- or MEF-based therapy for drug-resistant cancer patients.

2. Materials and methods

2.1. Reagents and Cell culture

Reagents and cell lines [25,26], in this study are provided in the Supporting Information. The cell lines were cultured in RPMI1640

Abbreviations: VIN, vinblasitne; ATO, atovaquone; CHL, chloroquine; PRL, primaquine; MEF, mefloquine; ART, artesunate; DOY, doxycycline; VER, verapamil; P-gp, p-glycoprotein; DMSO, dimethylsulfoxide; C-PARP, cleaved ploy ADP ribose polymerase; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; TCA, trichloroacetic acid; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT, room temperature.

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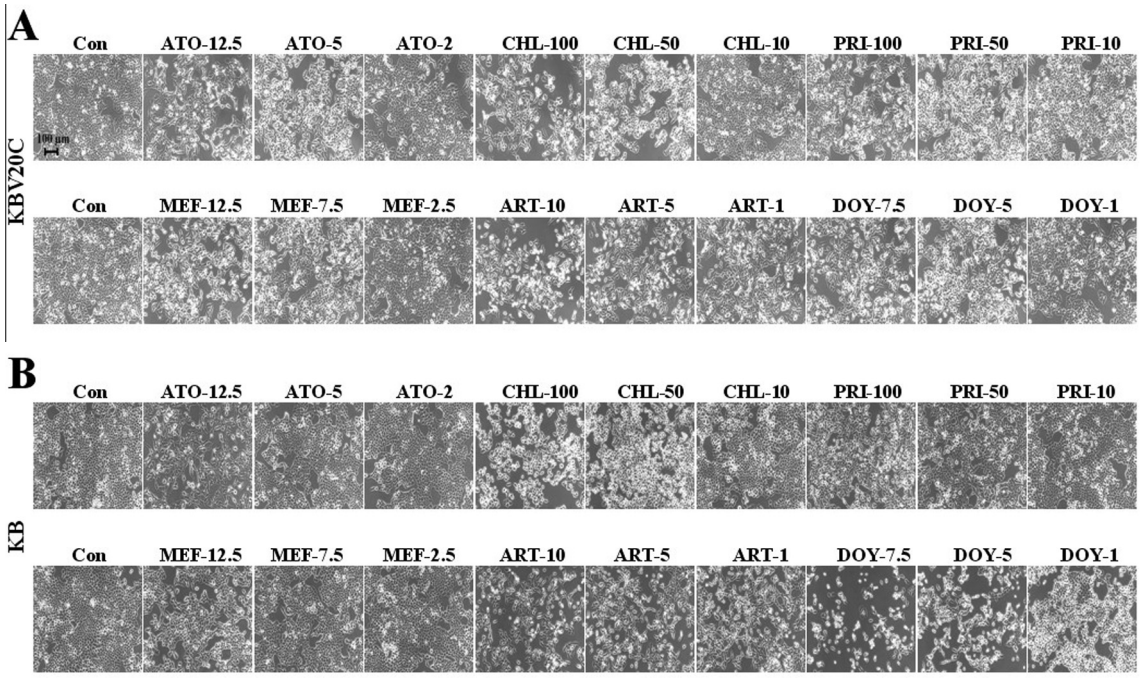

Fig. 1. Anti-malaria drugs have similar sensitisation effect on drug-sensitive KB and drug-resistant KBV20C cells, except DOY. (A) KBV20C cells and (B) KB cells were grown on 6-well plates and treated with 2 μM ATO (ATO-2), 5 μM ATO (ATO-5), 12.5 μM ATO (ATO-12.5), 10 μM CHL (CHL-10), 50 μM CHL (CHL-50), 100 μM CHL (CHL-100), 10 μM PRI (PRI-10), 50 μM PRI (PRI-50), 100 μM PRI (PRI-100), 2.5 μM MEF (MEF-2.5), 7.5 μM MEF (MEF-7.5), 12.5 μM MEF (MEF-12.5), 1 μg/ml ART (ART-1), 5 μg/ml ART (ART-5), 10 μg/ml ART (ART-10), 1 μg/ml DOY (DOY-1), 5 μg/ml DOY (DOY-5), 7.5 μg/ml DOY (DOY-7.5), or DMSO (Con). After 48 h, all cells were observed using an inverted microscope with a 10X objective lens.

containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (WelGENE, Daegu, South Korea).

2.2. Cellular viability assay

The detailed method is described in the Supporting Information.

2.3. Calcein-AM uptake tests

The detailed method [27–29] is described in the Supporting Information.

2.4. Western blot analysis

The detailed method [27–31] is described in the Supporting Information.

2.5. Hoechst staining

The detailed method is described in the Supporting Information.

3. Results

3.1. Anti-malarial drugs have similar sensitisation effect on drug-sensitive KB and drug-resistant KBV20C cancer cells, except DOY

To identify anti-malarial drugs that can sensitize cancer cells resistant to anti-mitotic drugs, ATO, CHL, PRI, MEF, ART, and DOY were tested. The oral squamous cancer cell line KB and its sub-line KBV20C, which show multi-drug resistance to the microtubule-targeting drugs [25,26], were used to test the sensitisation effects of anti-malarial drugs. Drug concentration ranges previously used by other groups for in vitro studies were chosen. We assume that positive results obtained with the selected drug concentrations may be easily applied in clinical settings.

Microscopic observations for cellular growth were performed after treatment with anti-malarial drugs. KB and KBV20C cells were treated for 48 h in the presence of increasing concentrations of anti-malarial drugs, and their cellular growth was compared. Three different concentrations of each drug were used. As shown in Fig. 1A–B, cellular growth was similar between KB and KBV20C cells treated with ATO, CHL, PRI, MEF, or ART. KBV20C cells offered resistance to DOY. In fact, treatment with high concentrations of DOY only reduced growth in drug-sensitive KB cells. While none of the anti-malarial drugs alone had a stronger effect on KBV20C cells compared to KB cells, our results indicate that anti-mitotic drug-resistant cancer cells can be sensitized by most of the anti-malarial drugs to a similar level as the sensitive cells.

3.2. Co-treatment with either PRI or MEF sensitizes VIN-treated KBV20C resistant cancer cells

We next tested whether any anti-malarial drug can sensitize the KBV20C drug-resistant cells to an anti-mitotic drug, VIN. We performed a cellular viability assay to determine whether ATO, CHL, PRI, MEF, ART, or DOY sensitizes VIN-treated KBV20C cells. As shown in Fig. 2A–F, we found that co-treatment with PRI or MEF and VIN reduced viability at the three different concentrations tested. However, the other drugs tested did not have any sensitizing effects. Comparison of sensitisation levels between PRI and MEF showed that MEF reduced viability at lower concentrations than PRI, suggesting a higher sensitizing effect of MEF on VIN-treated KBV20C cells. Microscopic observation for cellular growth after co-treatment with anti-malarial drugs and VIN were performed. Fig. 3A shows that cellular growth was highly reduced in VIN-treated KBV20C cells co-treated with PRI or MEF. In addition, microscopic observation showed that co-treatment with PRI or MEF

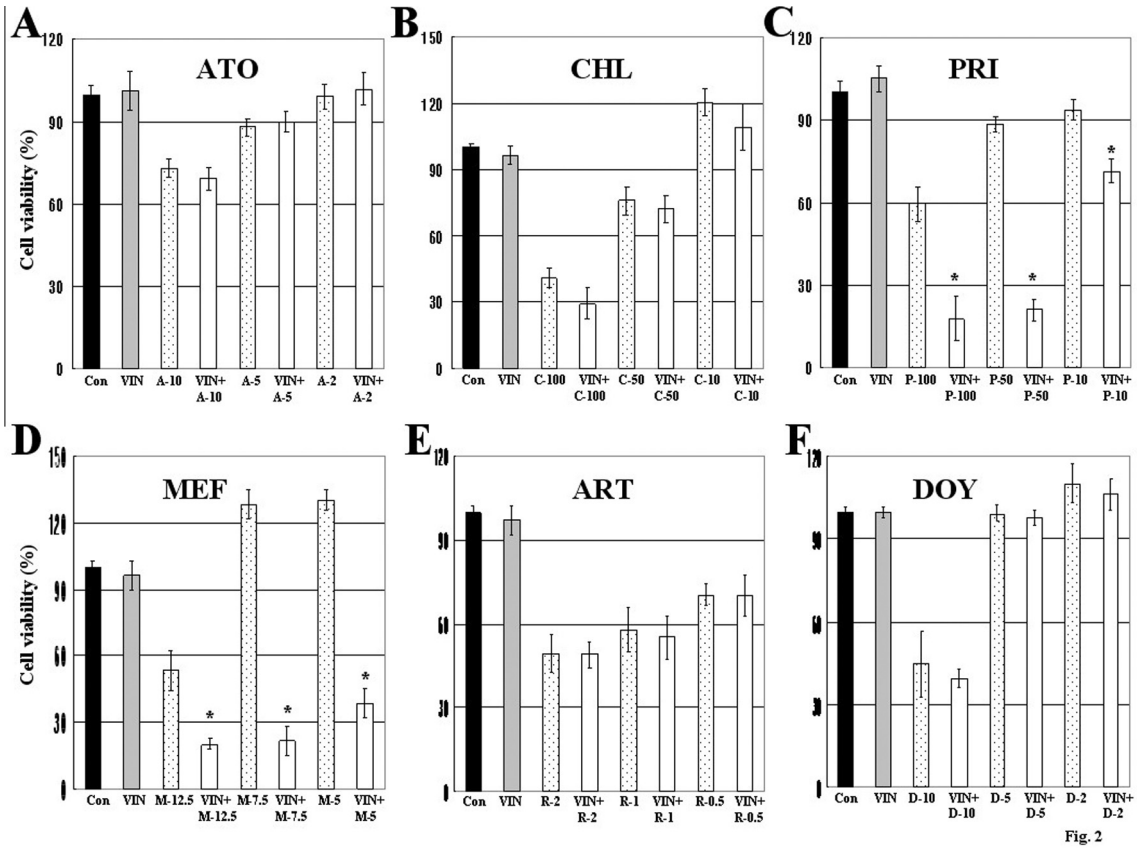

Fig. 2

Fig. 2. Co-treatment of either PRI or MEF sensitizes VIN-treated KBV20C resistant cancer cells. (A–F) KBV20C cells were plated on 96-well plates and grown to 30–40% confluence. The cells were then stimulated for 48 h with 5 nM VIN (VIN), 2 μM ATO (A-2), 5 μM ATO (A-5), 10 μM ATO (A-10), 10 μM CHL (C-10), 50 μM CHL (C-50), 100 μM CHL (C-100), 10 μM PRI (P-10), 50 μM PRI (P-50), 100 μM PRI (P-100), 5 μM MEF (M-5), 7.5 μM MEF (M-7.5), 12.5 μM MEF (M-12.5), 0.5 μg/ml ART (R-0.5), 1 μg/ml ART (R-1), 2 μg/ml ART (R-2), 2 μg/ml DOY (D-2), 5 μg/ml DOY (D-5), 10 μg/ml DOY (D-10), 5 nM VIN with 2 μM ATO (VIN+A-2), 5 nM VIN with 5 μM ATO (VIN+A-5), 5 nM VIN with 10 μM ATO (VIN+A-10), 5 nM VIN with 10 μM CHL (VIN+C-10), 5 nM VIN with 50 μM CHL (VIN+C-50), 5 nM VIN with 100 μM CHL (VIN+C-100), 5 nM VIN with 10 μM PRI (VIN+P-10), 5 nM VIN with 50 μM PRI (VIN+P-50), 5 nM VIN with 100 μM PRI (VIN+P-100), 5 nM VIN with 5 μM MEF (VIN+M-5), 5 nM VIN with 7.5 μM MEF (VIN+M-7.5), 5 nM VIN with 12.5 μM MEF (VIN+M-12.5), 5 nM VIN with 0.5 μg/ml ART (VIN+R-0.5), 5 nM VIN with 1 μg/ml ART (VIN+R-1), 5 nM VIN with 2 μg/ml ART (VIN+R-2), 5 nM VIN with 2 μg/ml DOY (VIN+D-2), 5 nM VIN with 5 μg/ml DOY (VIN+D-5), 5 nM VIN with 10 μg/ml DOY (VIN+D-10), or DMSO (Con). A cell viability assay was performed as described in "Section1". The data are represented by 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; $^{*}P < 0.05$ compared to the corresponding control.

and VIN increased the extent of cell detachment and the number of circular cells. Our results indicate that co-treatment with PRI or MEF could overcome the resistance of KBV20C cancer cells to VIN.

Furthermore, we tested whether PRI or MEF could also sensitize VIN-treated KB parent cancer cells. As presented in Supplementary data Fig. 1A–F, co-treatment with any anti-malarial drug and VIN did not reduce the viability of KB cancer cells. Although we observed a small decrease in KB cells co-treated with PRI and VIN, the effect was negligible compared to that noted in KBV20C cells under identical conditions. Collectively, our co-treatment experiments allowed us to identify two anti-malarial drugs, namely, PRI and MEF, which sensitize KBV20C drug-resistant cancer cells to VIN.

3.3. Co-treatment with PRI or MEF sensitizes VIN-treated KBV20C cells by increasing apoptosis

Co-treatment with PRI or MEF led to apoptosis of VIN-treated KBV20C cells, as indicated by the increase in C-PARP protein levels (Fig. 3C–D). In the co-treated cells, C-PARP expression was higher at 24 h than at 12 h (Fig. 3C–D), suggesting that PRI- or MEF-induced apoptosis is sustained in VIN-treated KBV20C cells. As expected, we did not observe any effect on C-PARP levels in CHL-VIN co-treated cells (Fig. 3C–D), in agreement with our viability assays and microscopic observations (Fig. 2B and Fig. 3A). To confirm these results, we performed Hoechst staining, which revealed marked morphological changes in PRI or MEF-VIN co-treated cells consistent with cellular apoptosis such as condensation of chromatin and nuclear fragmentation (Supplementary Fig. 2(A–B)). Collectively, these data indicate that co-treatment with PRI or MEF increased apoptosis in VIN-treated KBV20C cells.

3.4. PRI and MEF highly increase p-glycoprotein (P-gp) inhibition

Next, we examined the mechanisms underlying the PRI- and MEF-mediated sensitisation of VIN-treated KBV20C cells. Inhibition of membrane efflux of anti-cancer drugs is an important sensitisation mechanism for KB-resistant cancer cells [25,26]. Therefore, we compared the ability of the six anti-malarial drugs included in this study to inhibit this mechanism. We measured whether anti-malarial drugs increased the inhibition of P-gp substrate efflux. Calcein-AM, a well-known P-gp substrate, was used to measure P-gp inhibition [32]. In this experiment, cellular accumulation of green fluorescence was indicative of Calcein-AM intracellular accumulation. Using different concentrations of anti-malarial drugs, we assessed whether P-gp inhibition increased in a dose-dependent manner.

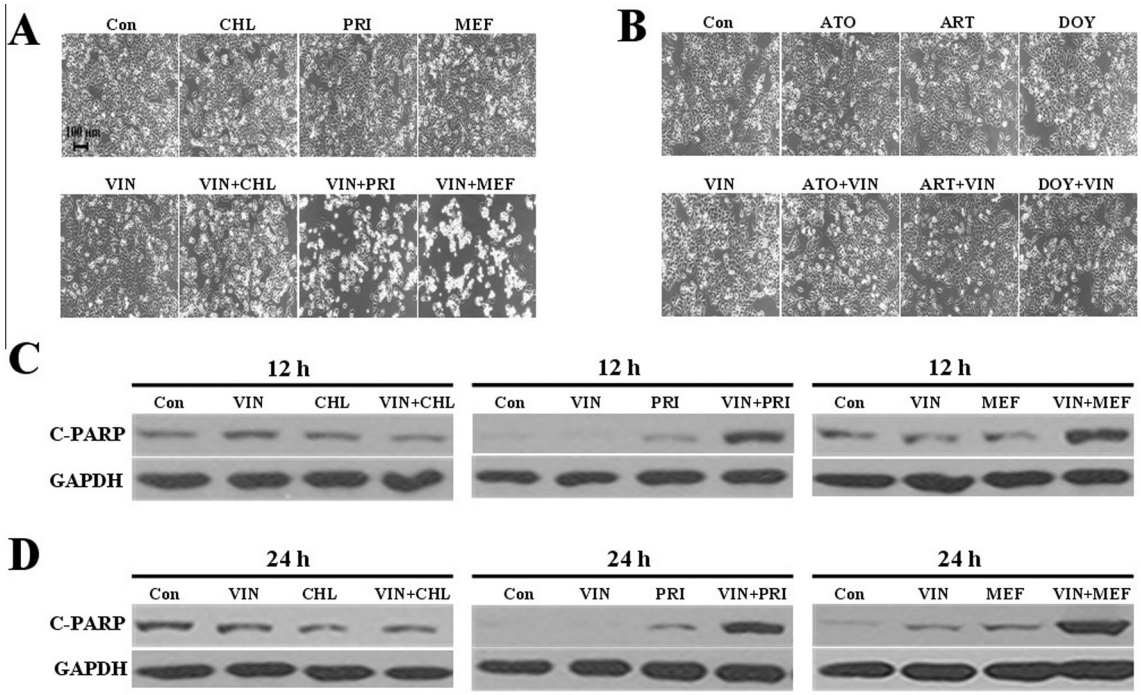

Fig. 3. Co-treatment of PRI or MEF sensitizes VIN-treated KBV20C cells by increasing apoptosis. (A–B) KBV20C cells were grown on 6-well and treated with 5 nM VIN (VIN), 50 μM CHL (CHL), 50 μM PRI (PRI), 7.5 μM MEF (MEF), 5 μM ATO (ATO), 1 μg/ml ART (ART), 5 μg/ml DOY (DOY), 5 nM VIN with 50 μM CHL (VIN+CHL), 5 nM VIN with 50 μM PRI (VIN+PRI), 5 nM VIN with 7.5 μM MEF (VIN+MEF), 5 nM VIN with 5 μM ATO (VIN+ATO), 5 nM VIN with 1 μg/ml ART (VIN+ART), 5 nM VIN with 5 μg/ml DOY (VIN+DOY), or DMSO (Con). After 24 h, all cells were observed using an inverted microscope with a 10× objective lens. (C–D) KBV20C cells were plated on 60 mm-diameter dishes and treated with 5 nM VIN (VIN), 50 μM CHL (CHL), 50 μM PRI (PRI), 7.5 μM MEF (MEF), 5 nM VIN with 50 μM CHL (VIN+CHL), 5 nM VIN with 50 μM PRI (VIN+PRI), 5 nM VIN with 7.5 μM MEF (VIN+MEF), or DMSO (Con). After (C) 12 h or (D) 24 h, Western blot analysis was performed using antibodies against C-PARP and GAPDH.

As shown in Fig. 4A–B, CHL, PRI, and MEF showed increased P-gp inhibition, which was both dose- and time-dependent. Compared to PRI and CHL, MEF presented the strongest inhibitory effect on P-gp. Verapamil, a well-known P-gp inhibitor [27,29], also showed a high increase in P-gp inhibition, while VIN, when used as a negative control, did not inhibit P-gp. Altogether, we found that PRI and MEF induce increased inhibition of P-gp compared to other anti-malarial drugs, suggesting that the sensitisation mechanism for resistant cells involves P-gp inhibition. Interestingly, CHL, which also presented high levels of P-gp inhibition, did not sensitize VIN-co-treated KBV20C cells.

3.5. Verapamil sensitizes VIN-treated resistant cancer cells, to the same extent as PRI and MEF

We tested whether co-treatment with verapamil sensitizes VIN-treated KBV20C cells. In order to confirm the role of P-gp inhibition in the sensitisation mechanism, we compared cellular growth at a microscopic level. As presented in Supplementary Fig. 3(A–C), verapamil largely reduced cellular growth and increased apoptosis in VIN-treated resistant KBV20C cancer cells, suggesting that P-gp inhibition is important for sensitizing KBV20C cells. However, no sensitisation effect was observed in VIN-treated sensitive KB cells (Supplementary Fig. 4A), suggesting that verapamil is also specific for resistant cancer cells.

We tested the efficacy of sensitisation by comparing verapamil, PRI, and MEF co-treatments with VIN. As shown in Supplementary Fig. 3(A–C), verapamil increased apoptosis to the same extent as PRI and MEF co-treatments. These results further confirmed that P-gp inhibition is involved in the sensitisation of VIN-resistant cancer cells by MEF and PRI. Because of its high toxicity, verapamil has not been used in clinical settings. Our results suggest that PRI and MEF could be used as alternatives for verapamil.

3.6. Co-treatment with MEF increases P-gp inhibition in VIN-treated resistant cells

As shown in Figs. 2C–D, 3A, and 3C–D, MEF co-treatment can sensitize VIN-treated resistant cancer cells better than PRI. Since the sensitisation mechanism for both PRI and MEF involves P-gp inhibition, we tested whether MEF was more effective in inhibiting P-gp than PRI. As shown in Supplementary Fig. 4B, P-gp inhibition presented a 1.5-fold increase in resistant cancer cells co-treated with MEF and VIN compared to MEF-treated cells. PRI/VIN co-treatment did not increase P-gp inhibition in resistant cancer cells compared to PRI treatment alone. Similarly, other anti-malarial drugs, namely, ATO, CHL, ART, and DOY, did not increase P-gp inhibition in VIN-co-treated resistant cancer cells (Supplementary Fig. 4B). Collectively, our results showed that P-gp inhibition by MEF is very important to sensitize VIN-treated cancer cells and to overcome the resistance to anti-mitotic drugs.

4. Discussion

Recently, the cancer-sensitizing ability of anti-malarial drugs was demonstrated in various cancer models [11–24], suggesting that anti-malarial drugs could be used as anti-cancer drugs. Here, we tested the sensitizing effects of six anti-malarial drugs, namely, ATO, CHL, PRI, MEF, ART, and DOY, on anti-mitotic resistant cancer cells. These drugs are already used in clinical settings, and therefore, once their mechanism of action on cancer is known, these drugs would be readily available for use without further toxicity studies. A recent study showed that drug-resistant malarial parasites could be sensitized with anti-mitotic drugs such as docetaxel and paclitaxel [33], we hypothesized that anti-malarial drugs might increase the sensitisation of cancer cells resistant to anti-mitotic drugs.

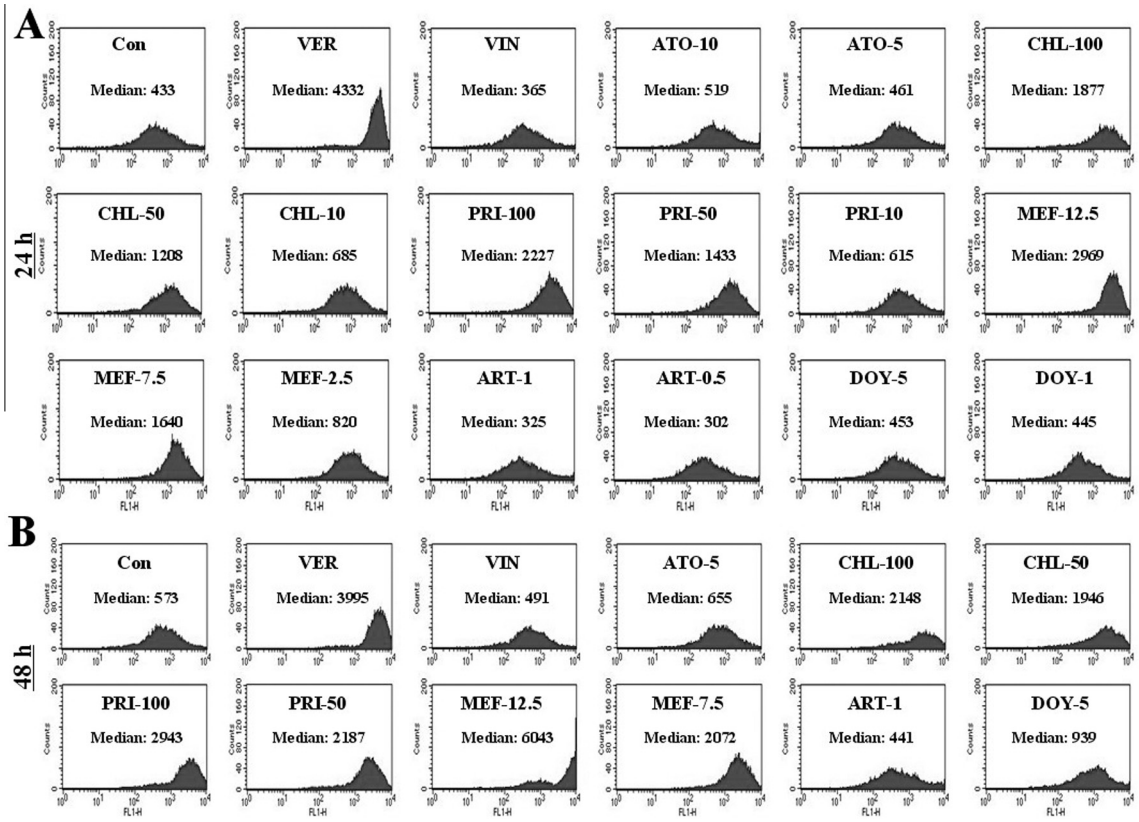

Fig. 4. PRI and MEF highly increase P-gp inhibition or MDR inhibition. (A) KBV20C cells were grown on 6-well plates and treated with 5 nM VIN (VIN), 20 μM VER (VER), 5 μM ATO (ATO-5), 10 μM ATO (ATO-10), 10 μM CHL (CHL-10), 50 μM CHL (CHL-50), 100 μM CHL (CHL-100), 10 μM PRI (PRI-10), 50 μM PRI (PRI-50), 100 μM PRI (PRI-100), 2.5 μM MEF (MEF-2.5), 7.5 μM MEF (MEF-7.5), 12.5 μM MEF (MEF-12.5), 0.5 μg/ml ART (ART-0.5), 1 μg/ml ART (ART-1), 1 μg/ml DOY (DOY-1), 5 μg/ml DOY (DOY-5), or DMSO (Con). (B) KBV20C cells were grown on 6-well plates and treated with 5 nM VIN (VIN), 20 μM VER (VER), 5 μM ATO (ATO-5), 50 μM CHL (CHL-50), 100 μM CHL (CHL-100), 50 μM PRI (PRI-50), 100 μM PRI (PRI-100), 7.5 μM MEF (MEF-7.5), 12.5 μM MEF (MEF-12.5), 1 μg/ml ART (ART-1), 5 μg/ml DOY (DOY-5), or DMSO (Con). After (A) 24 h or (B) 48 h, all cells were then stained with Calcein-AM the stained cells were subsequently examined using FACS analysis as described in "Section 2".

Two drugs, PRI and MEF, which sensitized drug-resistant cancer cells when co-treated with VIN. CHL, ART, and DOY were studied and their sensitisation effects in cancer cells was shown [11,13,14,19,20,22–24]. Recently, CHL has been extensively investigated as an autophagy inhibitor for application in cancer cell sensitisation [20]. However, only a few studies have studied the cancer sensitisation potential of MEF and PRI [12,18]. CHL, PRI, and MEF belong to the quinolone family [8–10]. CHL did not present any sensitizing effects, suggesting that the sensitisation mechanism of PRI and MEF is peculiar to these drugs, making our results quite novel. Our data showed that MEF presented more sensitizing ability than PRI. In fact, co-treatment with MEF increased P-gp inhibition, while co-treatment with PRI did not. A possible explanation for the increased effect of MEF could be its involvement in reducing P-gp expression or increasing P-gp degradation.

Treatment with PRI or MEF alone did not show similar sensitizing effects on sensitive KB and resistant KBV20C cells. In addition, VIN-treated drug-sensitive KB cells were not sensitized by co-treatment with PRI or MEF. Thus, non-resistant cells do not seem to be affected. It also indicates that PRI and MEF can specifically sensitize drug-resistant cancer cells when applied in clinical settings. Although PRI and MEF are already known to be safe for humans and non-toxic to normal cells, we showed that low concentrations of PRI or MEF can effectively sensitize VIN-treated resistant cancer cells, indicating that PRI or MEF can be used in combination with anti-mitotic drugs at concentrations for which toxicity is controlled. PRI and MEF could also be used for stem cells, which present drug-resistant phenotypes. PRI or MEF could alternatively be chosen for clinical use, based on the resistance of cancer cells to one or the other drug. The ability of MEF and PRI to sensitize drug-resistant cancer cells offers chemotherapeutic choices for co-treatment with anti-mitotic drugs.

Analysis of possible mechanisms demonstrated that PRI and MEF increased P-gp inhibition in resistant cancer cells, increasing the anti-mitotic effects of VIN by blocking its efflux through a P-gp based mechanism. Three possible mechanisms exist for P-gp inhibition: down-regulation of P-gp mRNA expression or protein expression, increase of P-gp degradation, or blocking of the binding of P-gp substrate. No sensitisation by MEF and PRI was observed in the sensitive cell line KB when co-treated with VIN, suggesting that mechanisms of sensitisation employed by PRI and MEF involve P-gp inhibition. Our data showed that the other three anti-malarial drugs tested did not present any P-gp inhibitory effect. Using verapamil as a positive control for P-gp inhibition, we found that verapamil, MEF, and PRI have similar apoptotic effects on VIN-treated resistant cells, indicating a similar mechanism of action through P-gp inhibition for all three drugs. Because of its high toxicity in normal cells, the clinical use of verapamil seems difficult; however, our finding suggest that PRI and MEF, two relatively non-toxic drugs, can be used as substitutes in co-treatment with anti-mitotic drugs to sensitize resistant cancer cells. We also found that CHL increased P-gp inhibition; however, no sensitisation effect was observed in VIN-treated resistant cells. This lack of sensitizing effect could be attributed to the fact that CHL, a strong autophagy inhibitor, might not be a suitable sensitizing drug when combined with anti-cancer drugs. It suggests that a strong ability to inhibit autophagy reversibly correlates with P-gp inhibition. Second, as several mechanisms of drug efflux involving different membrane

proteins exist, MEF and PRI could be inhibiting several proteins compared to CHL. Further studies are required to answer this hypothesis.

In summary, our study demonstrated that PRI or MEF sensitizes resistant cancer cells to VIN by increasing apoptosis. Future studies using an in vivo mouse model are warranted to assess the sensitisation effect and toxicity of these drug combinations. Our study may help improve various combination chemotherapeutic treatments for cancer patients who develop resistance to anti-cancer drugs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.095.

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