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Reversal of multidrug resistance by 4-chloro-*N*-(3-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl)phenyl)benzamide through the reversible inhibition of P-glycoprotein

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Abstract

Overexpression of P-glycoprotein (P-gp) is one of the major obstacles to successful cancer chemotherapy. In this study, we examined the ability of 4-chloro-*N*-(3-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl)phenyl)benzamide (C-4) to reverse multidrug resistance (MDR) in P-gp expressing KBV20C cells. Treatment of KBV20C cells with C-4 led to a dramatic increase in paclitaxel- or vincristine-induced cytotoxicity without any cytotoxicity by itself. In parallel, C-4 treatment caused an increase in apoptotic cell death by paclitaxel or vincristine. Furthermore, C-4 treatment significantly increases in intracellular accumulation of fluorescent P-gp substrate rhodamine 123, indicating that C-4 treatment leads to reversal of the MDR phenotype resulting from an increased accumulation of anticancer drugs by inhibiting drug efflux function of P-gp. This notion is further supported by the observation that C-4 treatment potentiates paclitaxel-induced G_2/M arrest of the cell cycle. In addition, the drug efflux function of P-gp was reversibly inhibited by C-4 treatment, while the expression level of P-gp was not affected. Collectively, our results describe the potential of C-4 to reverse the P-gp-mediated MDR phenotype through reversible inhibition of P-gp function, which may make it an attractive new agent for the chemosensitization of cancer cells.

Keywords: Multidrug resistance; P-glycoprotein; Curcumin; 4-Chloro-*N*-(3-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl)phenyl)benzamide

Multidrug resistance (MDR) is a significant impediment to providing effective cancer chemotherapy to many patients and a major mechanism for resistance of cancer cells to many structurally and functionally unrelated drugs [1]. The MDR phenotype is often associated with the overexpression of drug efflux pumps, known as ATP-binding cassette (ABC) transporters, in the plasma membrane of cancer cells. P-glycoprotein (P-gp), a 170-kDa transmembrane glycoprotein encoded by the *ABCB1* (*MDR1*) gene, is the best characterized drug efflux pump [2–4]. A wide range of anticancer drugs including anthracyclines, vinca alkaloids, and taxanes have been demonstrated to be substrates for P-gp [1]. Because overexpression of P-gp has been shown to confer MDR in cultured cells and has also been implicated in clinical MDR, P-gp overexpression appears to be closely correlated with poor prognosis for a number of human cancers [4,5]. Therefore, a successful inhibition of P-gp transporter function or its expression may overcome the MDR phenotype by increasing intracellular accumulation of anticancer drugs.

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The first P-gp inhibitor to be identified was the calcium channel blocker verapamil, which inhibits P-gp-mediated drug efflux and increases the accumulation and efficacy of anticancer drugs [6]. After this discovery, several MDR-reversing agents such as valspodar, tariquidar, LY335979, R101933, and ONT-093 have been studied for their inhibitory effect on P-gp [7–12]. Although these agents successfully reverse the MDR phenotype *in vitro*, these compounds have not been successful in animal studies or clinical trials due to dose-limiting toxicity and undesired adverse effects as well as pharmacokinetic intervention between the inhibiting agent and anticancer drugs [4,13–15]. Therefore, a great deal of effort is currently being expanded toward identifying novel compounds that inhibit P-gp function, reverse the MDR phenotype, and sensitize cancer cells to conventional anticancer drugs without undesired toxicological effects.

Curcumin (Fig. 1A), a natural phenolic coloring compound from the rhizome of *Curcuma longa* L, commonly known as turmeric, has been well known as a chemopreventive and chemotherapeutic agent [16–18]. Recent reports have demonstrated that curcumin treatment reverses the MDR phenotype by inhibiting P-gp function and expression, making it a possible candidate for the chemosensitization of cancer cells [19–21]. We thus synthesized and established a chemical library of curcumin analogs for the purpose of developing a novel MDR modulating agent and determined the effect of these compounds on the MDR phenotype. Among them, treatment of drug-resistant cells with compound C-4 (4-chloro-*N*-(3-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl)phenyl)benzamide, Fig. 1B) showed strong activity to reverse the MDR phenotype. In this study, we examined the ability of C-4 to modulate MDR in P-gp expressing KBV20C cells. Our data demonstrate that C-4 inhibits the function of P-gp in drug-resistant cancer cells and subsequently leads to an increase in the apoptotic potential of anticancer drugs resulting from their intracellular accumulation of drug.

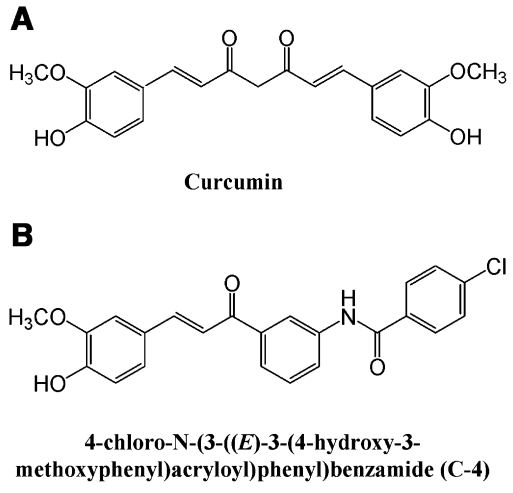

Fig. 1. Chemical structures of curcumin and its analog C-4.

Materials and methods

Cell culture. P-gp non-expressing KB cells and P-gp expressing KBV20C cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). KBV20C cells were grown in the presence of 20 nM vincristine (Sigma Chemical, St. Louis, MO) as described previously [22].

Reagents and cytotoxicity test. Paclitaxel, verapamil, and rhodamine 123 (Rh123) were obtained from Sigma. Compound C-4 (4-chloro-*N*-(3-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl)phenyl)benzamide) was synthesized and its structural identity was determined spectroscopically (^{1}H and ^{13}C NMR, and MS) as described previously [23]. Cytotoxicity was determined by the MTS assay (Promega, Madison, WI) according to the manufacturer's instruction.

Rh123 accumulation assay. Fluorescence intensity of intracellular Rh123 was determined by flow cytometry. Briefly, after treatment of KBV20C cells with 10 μM Rh123 for 3 h, cells were harvested (for detection of Rh123 accumulation) or cultured in Rh123-free RPMI for another 1 h followed by harvesting (for detection of Rh123 retention). Then, cells were washed with phosphate-buffered saline (PBS) and the mean fluorescence intensity of intracellular Rh123 was detected using flow cytometry. Finally, the Rh123 releasing index of KBV20C cells was calculated according to the formula: releasing index = (accumulation value − retention value)/accumulation value.

Cell morphology. After treatment with each combination of drugs, the cells were washed with PBS and fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The cells were washed twice more with PBS and analyzed using a fluorescence microscope.

Cell cycle analysis. KBV20C cells were treated with 50 nM paclitaxel alone or in combination with 10 μM C-4 for 24 h. Cells were harvested, washed twice with ice-cold PBS and fixed in 70% ethanol at −20 °C overnight. Cells were washed with PBS, incubated with 100 μg/ml RNase A at 37 °C for 30 min, stained with 25 μg/ml propidium iodide solution and analyzed with flow cytometry.

Immunoblot analysis. Cell lysates were boiled in Laemmli sample buffer for 3 min, and 30 μg of each protein were subjected to SDS–polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes, and the membranes were blocked for 30 min in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% (w/v) dry skimmed milk powder, and incubated overnight with primary antibodies to P-gp (Santa Cruz Biotechnology, CA), poly(ADP–ribose) polymerase (PARP, Santa Cruz Biotechnology), cleaved caspase-3 (Cell Signaling, Beverly, MA), and actin (Santa Cruz Biotechnology). The membranes were then washed with TBS–0.1% Tween 20 and incubated for 1 h with a secondary antibody, and were visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, IL).

RT-PCR. Total RNA was extracted, using the TRI_{ZOL} reagent (Invitrogen). The integrity of the RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as a template for each reverse transcriptase (RT)-mediated PCR (RT-PCR) by using an RNA PCR kit (Perkin Elmer, Wellesley, MA). The primer sets for P-gp were 5′-CCCATCATTGCAATAGCAGG-3′ and 5′-GTTCAAACTTCTGCTCCTGA-3′; and the primer sets for GAPDH were 5′-CTCATGACCACAGTCCATGCCATC-3′ and 5′-CTGCTT CACCACCTTCTTGATGTC-3′.

Results

C-4 treatment increases vincristine- or paclitaxel-induced cytotoxicity in drug-resistant KBV20C cells

In order to explore the molecular mechanism for the MDR modulating activity of curcumin and its analog C-

4, we used P-gp expressing KBV20C cells and its parental line of P-gp non-expressing KB cells. We first determined the cytotoxic response of both KBV20C and KB cells to vincristine or paclitaxel. As expected, KBV20C cells were more resistant than KB cells to paclitaxel and vincristine; IC_{50} in KB cells were 4.2 nM (paclitaxel) and 7.9 nM (vincristine) (data not shown), whereas those in KBV20C cells were 1.44 and 5.72 μM, respectively (Figs. 2A and B), suggesting that KBV20C cells are useful for experiments to develop MDR modulating agents. Treatment of KBV20C cells with verapamil which is a specific inhibitor of P-gp markedly increased vincristine- or paclitaxel-induced cytotoxicity (Figs. 2A and B). In addition, C-4 treatment dramatically enhanced the cytotoxicity of vincristine as well as paclitaxel: the IC_{50} shifted from 5.72 to 0.63 μM for vincristine and from 1.44 to 0.08 μM for paclitaxel, which was comparable to the shifts of verapamil (Figs. 2A and B). Alone C-4 treatment did not show cytotoxicity at the concentrations as high as 100 μM (data not shown). Unexpectedly, curcumin treatment did not affect the cytotoxicity of vincristine or paclitaxel (Figs. 2A and B). Taken together, these data suggest that C-4 treatment might be able to reverse P-gp-mediated MDR of KBV20C.

We next examined whether the C-4-increased cytotoxicity of vincristine or paclitaxel was mediated by apoptosis. Treatment with vincristine (100 nM) alone did not induce apoptosis of KBV20C, while co-treatment with verapamil led to an increase in the apoptotic potential of vincristine, as evidenced by the appearance of PARP cleavage and caspase-3 activation (Fig. 2C). In addition, similarly to verapamil, co-treatment with C-4 also caused a dramatic increase of PARP cleavage and caspase-3 activation in a dose-dependent manner (Fig. 2C). These results suggest that C-4 treatment might inhibit P-gp function and subsequently lead to an increase in its apoptotic potential resulting from the accumulation of vincristine. This notion was further supported by the observation that the apoptotic potential of paclitaxel was also enhanced by co-treatment with C-4 (Fig. 2D).

C-4 treatment potentiates G_2/M arrest in paclitaxel-treated KBV20C cells

It has been well known that vincristine and paclitaxel arrest the cell cycle at the G_2/M phase by disturbing microtubule function during cell cycle progression [24–26]. We first examined the effect of C-4 on the nuclear morphology and cell cycle of KBV20C cells. Treatment with paclitaxel at a low concentration (50 nM) did not affect the nuclear morphology of KBV20C cells as evidenced by nuclear staining with DAPI (Fig. 3A), while a high concentration of paclitaxel (1 μM) led to a dramatic decrease in cell

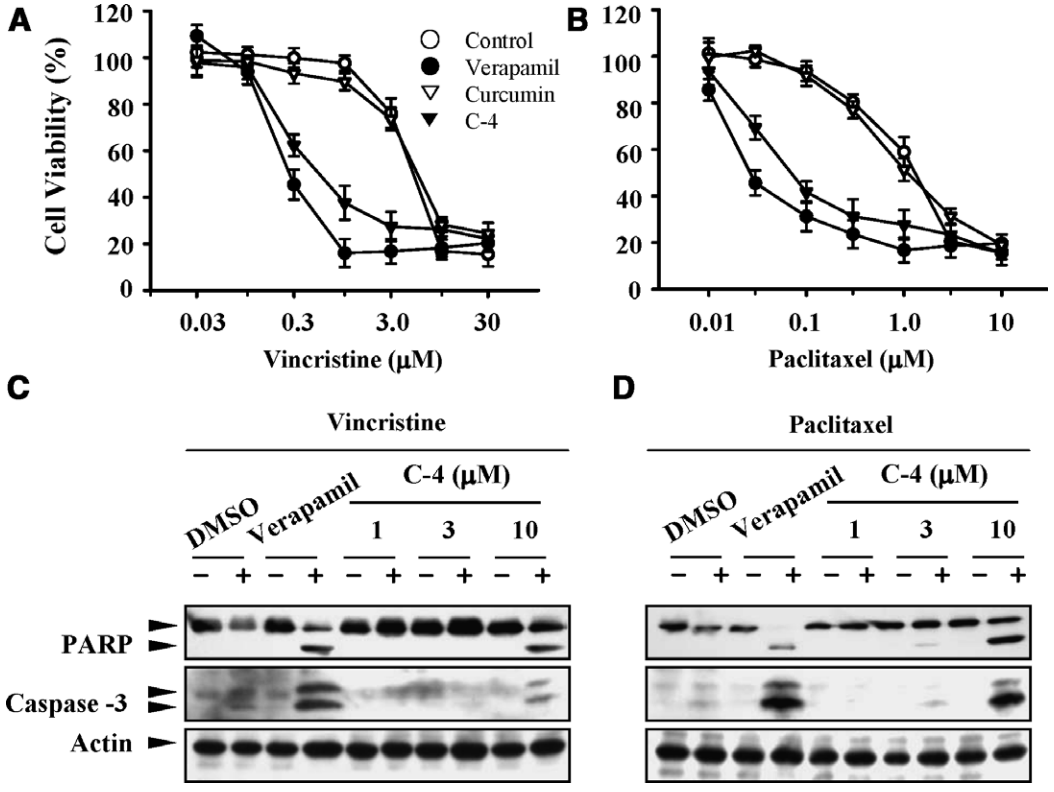

Fig. 2. C-4 treatment enhances the cytotoxicity by vincristine or paclitaxel in KBV20C cells. (A,B) KBV20C cells were seeded at a density of 1×10^4/well in 96-well plates and co-treated with various concentrations of vincristine or paclitaxel in the presence of 10 μM C-4 for 48 h. In some experiments, 10 μM verapamil or curcumin was added. Cell viability was determined using the MTS assay as described in Materials and methods. (C,D) KBV20C cells were co-treated with 0.1 μM vincristine or 0.05 μM paclitaxel and indicated concentrations of C-4 for 48 h, and the cleavage of PARP and caspase-3 activation were assessed by immunoblot analysis. Verapamil (10 μM) was used as a positive control.

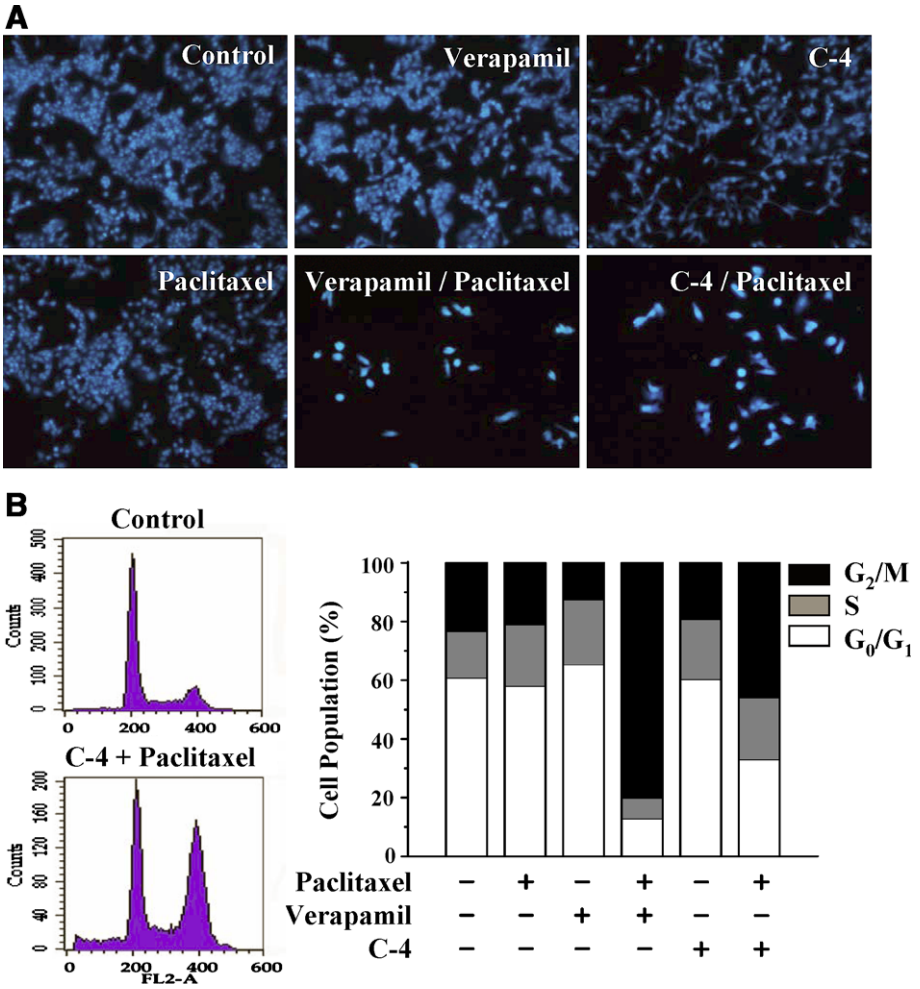

Fig. 3. C-4 treatment potentiates G_2/M arrest in paclitaxel-treated KBV20C cells. (A) KBV20C cells were treated with 0.05 μM paclitaxel alone or in combination with 10 μM C-4 or verapamil for 48 h. After staining with DAPI, nuclear morphology was analyzed using a fluorescence microscope. (B) KBV20C cells were treated with 0.05 μM paclitaxel alone or in combination with 10 μM C-4 or verapamil for 24 h. After staining with propidium iodide, cell cycle stage was analyzed using flow cytometry.

number and a significant increase in the size of the nucleus (data not shown), indicating that high intracellular concentration of paclitaxel caused enlargement of nuclear size and cell death by disturbing microtubule function. Inhibition of P-gp function with verapamil led to morphological change and cell death in KBV20C cells, even if the cells were treated with a low concentration of paclitaxel (Fig. 3A). Similarly to the effect of verapamil, C-4 treatment enhanced paclitaxel-induced morphological changes and cell death (Fig. 3A), indicating that C-4 might increase the potential of paclitaxel by allowing its intracellular accumulation due to the inhibition of P-gp function. In addition, the same effect was also observed with vincristine (data not shown). To demonstrate whether the enlargement of nucleus size is attributable to cell cycle arrest at the G_2/M phase, we examined the effect of C-4 on the cell cycle. As shown in Fig. 3B, 50 nM paclitaxel showed no effect on the cell cycle of KBV20C cells, while verapamil treatment led to a significant increase in cell population at the G_2/M phase from 23.2% to 80% and a decrease in the cells at G_1 and S phase. Furthermore, C-4 treatment also enhanced paclitaxel-induced arrest of the cell cycle at the G_2/M phase up to 45.8% (Fig. 3B). Similarly, we observed that vincristine-induced G_2/M arrest was increased by C-4 treatment (data not shown). Taken together, these results strongly suggest that C-4 treatment might potentiate the ability of paclitaxel to disturb microtubule function for G_2/M arrest resulting from its accumulation due to the inhibition of the drug efflux function of P-gp.

C-4 reversibly inhibits Rh123 efflux from KBV20C cells without affecting P-gp expression

To determine if the MDR-altering effect of C-4 was caused by inhibition of P-gp function, we examined the effect of C-4 on the intracellular accumulation of Rh123, a fluorescent P-gp substrate, using flow cytometry. As shown in Fig. 4A, inhibition of P-gp with verapamil caused an increase in the accumulation of Rh123 by about 3.7 fold in KBV20C cells. Treatment of KBV20C cells with C-4 led to an enhanced accumulation of Rh123, indicating that C-4 treatment was enough to inhibit P-gp function and lead to

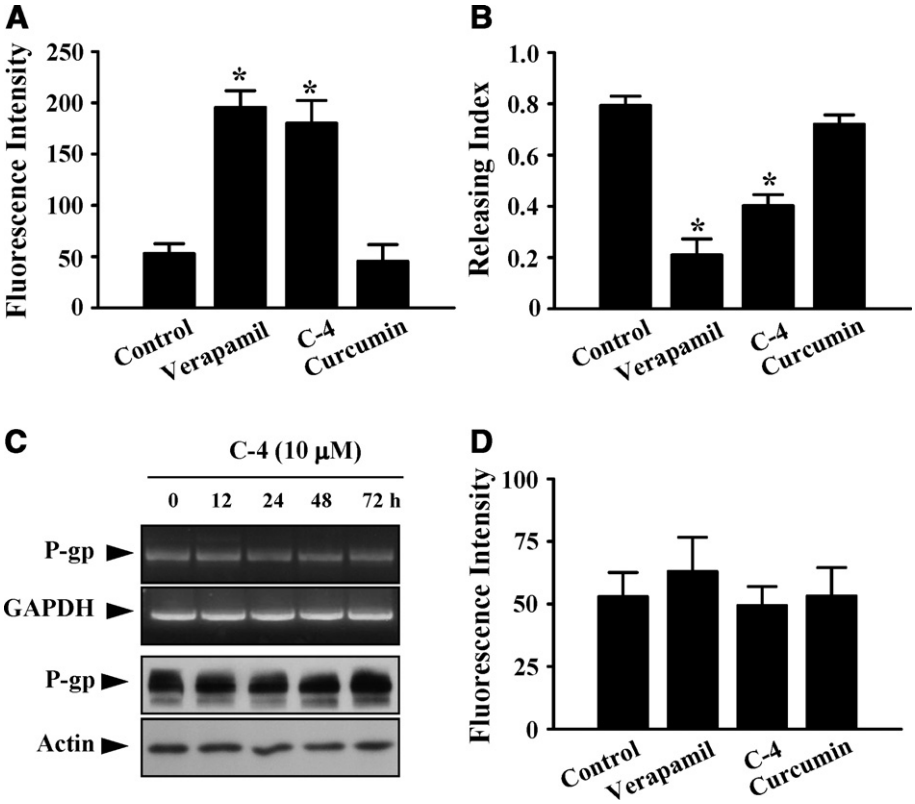

Fig. 4. C-4 reversibly inhibits Rh123 efflux from KBV20C cells without affecting P-gp expression. KBV20C cells were pre-treated with 10 μM verapamil, C-4 or curcumin for 1 h. Following a treatment with 10 μM Rh123, the mean fluorescence intensity of intracellular Rh123 was determined as described in Materials and methods. Relative fluorescence intensity (A) or releasing index of Rh123 (B) represent means ± SD of three independent experiments. $^{*}p < 0.05$, compared to control group with Student's t test. (C) After treatment of KBV20C cells with 10 μM C-4 for indicated time points, the expression level of P-gp was analyzed using RT-PCR and immunoblot analysis. (D) KBV20C cells were treated with 10 μM C-4 or verapamil for 48 h. Following a change with fresh media containing 10 μM Rh123 without C-4 or verapamil, the cells were further incubated for 3 h and then harvested for detection of Rh123 accumulation. Relative fluorescence intensity of Rh123 represents means ± SD of three independent experiments.

an accumulation of Rh123. In addition, treatment with verapamil or C-4 significantly decreased the release of Rh123 from KBV20C cells (Fig. 4B). However, curcumin treatment did not affect the accumulation or the releasing index of Rh123 (Figs. 4A and B). Collectively, these results imply that P-gp function might be sufficiently abrogated by treatment with C-4, but not curcumin, which leads to an accumulation of anticancer drugs enabling them to exert their cytotoxic effects.

Next we examined the effect of C-4 on P-gp expression in KBV20C cells. As shown in Fig. 4C, treatment of KBV20C cells with C-4 did not affect the expression level of P-gp mRNA or protein. Then, we tried to examine whether inhibition of P-gp function by C-4 was attributable to reversible or irreversible inhibition. After exposure of KBV20C cells to C-4 for 48 h, C-4 was withdrawn by exchanging the culture medium for fresh medium without C-4. Rh123 accumulation was then analyzed in the cells. As shown in Fig. 4D, exposure to C-4 for 48 h did not affect the accumulation of Rh123 in KBV20C cells, indicating that C-4 may reversibly inhibit P-gp function. Treatment with verapamil in the same manner also had no effect on the expression of P-gp (data now shown) or the accumulation of Rh123 (Fig. 4D). Altogether, these findings suggest that like verapamil, the drug efflux function of P-gp in drug-resistant cells can be reversibly abrogated by C-4 treatment without any effect on its expression.

Discussion

Chemotherapy is the most effective treatment for patients who suffer from metastatic cancers. The effectiveness of chemotherapy, however, is seriously limited by MDR which is mainly due to the overexpression of P-gp, an integral membrane protein. P-gp functions as a drug efflux pump which actively transports drugs from the inside to the outside of cancer cells and prevents the intracellular accumulation of anticancer drugs inside cancer cells necessary for cytotoxic activity. Therefore, novel agents which can inhibit the drug transporter function of P-gp or its expression have the potential to overcome the MDR phenotype by enhancing intracellular accumulation of anticancer drugs. The present study shows that treatment with C-4 can lead to a reversal of the MDR phenotype by reversibly inhibiting P-gp function, which allows for the intracellular accumulation of anticancer drugs.

It has been demonstrated that curcumin is an attractive biologically active compound, as it exerts diverse biological effects including anti-inflammatory, antioxidant, antiviral, anti-infectious, chemopreventive, and chemotherapeutic activities [16–18]. Furthermore, a recent report shows that treatment with curcumin leads to a significant increase in the cytotoxicity of vinblastine and a decrease in P-gp expression in drug-resistant cells [19,20]. Thus, to develop MDR reversing agents for more efficient cancer chemotherapy, we established a chemical library of curcumin analogs and searched for an active compound to reverse the MDR phenotype with the selection criteria that the active candidate compounds must significantly enhance the cytotoxicity of anticancer agents (vincristine and paclitaxel) in drug-resistant cancer cells without cytotoxic effect by itself. Among them, C-4 satisfied these criteria, as C-4 treatment showed not only no cytotoxic effect in KBV20C cells at the concentration in this study but also a significant enhancement of vincristine- or paclitaxel-induced cytotoxicity (Fig. 2). Although other compounds also displayed the ability to increase the cytotoxicity of anticancer drugs, they were less potent than C-4 and in some case showed cytotoxic effects by themselves (Y.K. Kim and S. Lee, unpublished data). In this study, we thus focused on the potential of C-4 to reverse the MDR phenotype. Unlike other reports demonstrating the MDR modulating effect of its parent molecule curcumin [19,20], our data showed that 10 μM curcumin did not affect the cytotoxicity of anticancer drugs. Although at the present time we do not know the exact reason for this discrepancy, it might be in part attributable to the difference in curcumin concentrations: while 10 μM curcumin was used in all our experiments, other studies used 20–30 μM [19,20]. Taken together, it seems clear that the ability of C-4 compound to reverse the MDR phenotype is more potent than that of curcumin.

Our data show that C-4 is very potent at reversing the accumulation deficit and at blocking the efflux of P-gp substrate Rh123 from P-gp overexpressing cell line KBV20C (Fig. 4). The finding that drug efflux and accumulation was not affected in the parental cell line KB (P-gp non-expressing) strongly indicates that the reversal of drug resistance by C-4 is probably attributable to the inhibition of P-gp-mediated efflux. Furthermore, the increased accumulation of anticancer drugs in response to C-4 dramatically enhances apoptotic potential, as evidenced by the observations that co-treatment with C-4 increases PARP cleavage and caspase-3 activation by vincristine or paclitaxel (Figs. 2C and D). Altogether, these findings indicate that C-4 may reverse the MDR phenotype in drug-resistant cells by reversibly inhibiting the drug efflux function of P-gp. This notion is further confirmed by our observation that C-4 treatment enhances the ability of paclitaxel to induce G_2/M arrest of KBV20C cells (Fig. 3).

In conclusion, we demonstrate the reversal of MDR phenotype by C-4 through the reversible inhibition of the drug efflux function of P-gp. In addition to this MDR reversing activity, a recent report shows that C-4 compound significantly inhibits the proliferation and tube formation of human umbilical vein endothelial cells at 5–10 μg/ml [23], implying anti-angiogenic potential of C-4 compound. It is noteworthy that C-4 compound has anti-angiogenic activity as well as MDR reversing activity, suggesting that C-4 compound could be a good candidate for treatment of malignant tumors which are hardly curable with conventional chemotherapy due to their MDR and angiogenic activities. Taken together, our studies suggest the potential of C-4 as an attractive chemotherapeutic agent for treating malignant tumors with P-gp-mediated MDR, and will help to better our understanding of the molecular mechanisms of C-4 for the purpose of further developing it as chemotherapeutic agent.

Acknowledgments

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