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Research paper

The *bis*benzylisoquinoline alkaloids, tetrandine and fangchinoline, enhance the cytotoxicity of multidrug resistance-related drugs via modulation of P-glycoprotein

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The occurrence of resistance to chemotherapeutic drugs is a major problem for successful cancer treatment and reducing drug accumulation by P-glycoprotein (P-gp) is one of the major mechanisms of multidrug resistance (MDR). The present study was performed to evaluate the MDR-reversal abilities of two *bis*benzylisoquinoline alkaloids, tetrandine (TET) and fangchinoline (FAN), compared with verapamil (VER), a well-known P-gp modulator. TET (3.0 μM), FAN (3.0 μM) and VER (10.0 μM) reduced the paclitaxel (TAX) concentration required to achieve 50% inhibition of cell growth (EC_{50}) to HCT15 (P-gp-positive) cells about 3100-, 1900- and 410-fold, and these compounds also reduced the EC_{50} value of actinomycin D (AMD) about 36.0-, 45.9- and 18.2-fold in the cells, respectively. Meanwhile, TET, FAN and VER had no effect on the cytotoxicity of the drugs to SK-OV-3 (P-gp-negative) cells. On the other hand, TET (3.0 μM), FAN (3.0 μM) and VER (10.0 μM) similarly enhanced the accumulation rates of rhodamine 123, a well known P-gp substrate, in HCT15 cells (200–250%). After efflux for 2 h with fresh medium, TET and FAN also enhanced the residual rate of rhodamine 123 about 5.0- and 2.6-fold in comparison with control, respectively. TET, FAN and VER could not affect the accumulation and residual rate of rhodamine 123 in SK-OV-3 cells. From the result, we conclude that TET and FAN enhanced the cytotoxicity of MDR-related drugs via modulation of P-gp. [© 1998 Rapid Science Ltd.]

Key words: Fangchinoline, multidrug resistance, MDR reversal, P-glycoprotein, tetrandrine.

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Introduction

Drug resistance is one of the most significant impediments to successful chemotherapy of cancer. Intrinsic or acquired drug resistance refers to the simultaneous development of resistance in tumor cells to mechanistically and/or structurally diverse agents, and this phenomenon has been termed multidrug resistance (MDR). A major form of MDR is a consequence of an overexpression of P-glycoprotein (P-gp), the 170 kDa transmembrane glycoprotein that is encoded by the *mdr1* gene in humans. P-gp consists of 1280 amino acids forming two homologous halves which contain a hydrophobic area with six membrane-spanning regions and an ATP-binding site each. It is thought that the hydrophobic loops span the cell membrane and are involved in drug transport, and that the ATP-binding site provides energy for the active pump process.[1]

Unfortunately, various human tumors such as colon and renal cell carcinoma express P-gp, and the rates of P-gp expression are increased in the relapsed tumors.[1] The mechanism of resistance in cancer cells that overexpress P-gp is due to increased transport of various classes of anticancer drugs out of cells, which results in decreased cellular accumulation and thus decreases the efficacy of the drugs.[2] Anticancer chemotherapeutic drugs associated with P-gp-mediated MDR include anthracyclines such as daunorubicin and doxorubucin (DOX), epipodophyllotoxins such as etoposide and teniposide, vinca alkaloids such as vincristine and vinblastine, and other compounds such as tamoxifen (TAX), actinomycin D (AMD), colchicine and trimetrexate.[1,3,4]

Tetradine (TET) and fangchinoline (FAN) are *bis*benzylisoquinoline alkaloids derived from the Chinese herb *Radix Stephania tetranda*. These *bis*benzylisoquinoline alkaloids have been characterized pharmacologically to exhibit Ca^{2+} antagonistic, hypotensive, immunosuppressive, anti-inflammatory properties, etc. In China, TET has been used in the clinical treatment of silicosis, high blood pressure and tumors.[5,6] As for the effects of those compounds on the lowering of blood pressure, TET was reported to inhibit the voltage-dependent Ca^{2+} channels,[7,8] or to act as a receptor-operated Ca^{2+} channel blocker[9] and/or an inhibitor of Ca^{2+} release from intracellular pools.[10]

In this study, on the basis of the fact that some Ca^{2+} channel blockers have modulating activity of P-gp,[1,11] we investigated the MDR modulating activities of those two *bis*benzylisoquinoline alkaloids, TET and FAN. To determine the reversal activity of each compound, we tested the cytotoxicities of some MDR-associated anticancer agents such as TAX and AMD against P-gp-negative or -positive human cancer cells in the presence or absence of those compounds. We also tested the effect of each compound on the cytotoxicities of 5-fluorouracil (5-FU), a non-MDR-associated agent, for control.[1] To identify the mechanism(s) of the sensitizing effects on the cancer cells by those compounds, we also compared the accumulation rates of rhodamine 123, a well known substrate of P-gp, in the presence or absence of each compound. Then, we assumed the mechanism(s) of their sensitizing activities in comparison with the results of verapamil (VER), a well known P-gp modulator.

Materials and methods

Chemicals

TET and FAN were isolated from the creeper *Stephania tetrandra* S. Moore (or *fenfangji*) and were confirmed by comparing the physical chemical properties and ^{1}H-NMR spectra with those in the previous reports.[12] The structure of those compounds is shown in Figure 1. The anticancer agents, TAX, AMD, 5-FU and VER were purchased from Sigma (St Louis, MO). RPMI 1640 cell growth medium, trypsin and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Other cell culture agents such as gentamycin, amphotericin, 1,2-cyclohexanediaminetetraacetic acid (CDTA), sodium bicarbonate, and agents for cytotoxicity tests such as trichloroacetic acid (TCA), sulforhodamine B (SRB), Tris base and rhodamine 123 were purchased from Sigma.

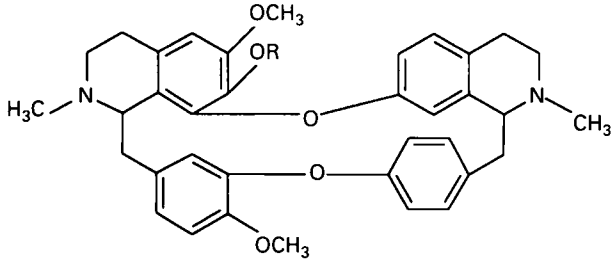

Figure 1. Chemical structures of TET and FAN.

Cells

Human ovarian cancer cell line SK-OV-3 and colorectal cancer cell line HCT15 cells were provided by the National Cancer Institute (NCI) and maintained in Korea Research Institute of Chemical Technology (KRICT) continuously. HCT15 cells were established from a colorectal cancer after surgical resection before chemotherapeutic treatment and it revealed a high level of P-gp expression. HCT15 cells were reported to have the highest rhodamine efflux activity among the cancer cells used for drug screening in the NCI.[13] On the other hand, human ovarian carcinoma cell line SK-OV-3 has been reported as a P-gp-negative cancer cell.[14] Stock cell cultures were conducted in Falcon T-25 (Becton Dickinson, Lincoln park, NJ) flasks containing 10 ml of RPMI 1640 medium with glutamine, sodium bicarbonate, gentamycin, amphotericin and 5% FBS. The cells were dissociated with 0.25% trypsin and 3 mM CDTA solution in the case of transferring or dispensing before experiment. The cells were maintained in the incubator at 37 C in a humidified atmosphere of 5% CO_2 in air continuously, except when adding drugs.

Cytotoxicity assay *in vitro*

All experimental procedures followed the NCI's protocol based on the SRB method as described previously.[15,16] Briefly, tumor cells were inoculated over a series of standard 96-well flat-bottom microplates (Falcon) on day 0. These cells were then preincubated for attachment on the microtiter plate for 24 h. For the study on the effects of TET, FAN and VER on the cytotoxicities of anticancer drugs, attached cells were incubated with serial dilutions of drugs in the absence or presence of TET, FAN (0.3, 1.0 and 3.0 μM) or VER (1.0, 3.0 and 10.0 μM). After 72 h of continuous drug exposure, the culture

medium in each well was removed and the cells were fixed with 10% cold TCA at 4°C for 1 h. After washing the TCA with tap water, 0.4% SRB solution was added and incubated for 30 min at room temperature. The cells were washed again and the bound stains were solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance of each well was measured spectrophotometrically at 520 and 690 nm in a microtiter plate reader (E-max; Molecular Devices, Sunnyvale, CA). The absorbance measured at 690 nm was subtracted from the absorbance at 520 nm so as to eliminate the effects of non-specific absorbance.

The data were transferred and transformed into Micro Excel format, and survival fractions were calculated by comparing the drug-treated wells with control. In the test of MDR reversal activities by TET, FAN and VER, the control meant the wells that contained each corresponding concentration of these compounds without the anticancer drugs. All data represented the average values of three wells in each experiment.

Rhodamine accumulation assay

Rhodamine accumulation assay was performed in 24-well plates using a fluorescence measurement system with minor modifications to that previously described in our reports.[16] Cells were seeded in 24-well flat-bottom plates at a same volume of cells per well in 1.5 ml of growth medium. The plates were incubated in 5% CO_2 at 37°C for 2-3 days. At the semiconfluent logarithmic growth phase of cells, the culture medium was removed and 4 μM rhodamine 123 in 1 ml growth medium with or without TET, FAN (1.0 and 3.0 μM) or VER (3.0 and 10.0 μM) was added to the cells. After incubation at 37°C in 5% CO_2 for 40 min, the rhodamine-containing medium was removed and the cells were washed twice with cold potassium-buffered saline (PBS, NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; KH_2PO_4, 0.2 g per liter in double-distilled water). Then, fresh growth medium which did not contain rhodamine nor reversal agents was added to one set of plates for the assay of rhodamine efflux. These cells were incubated for an additional 2 h at 37°C in 5% CO_2, followed by washing with cold PBS. At the end of both procedures for accumulation and efflux of rhodamine, cells were burst by adding 1.5 ml distilled water per well. The plates were kept under dark and cold conditions until quantification of fluorescence of each well. The green fluorescence of rhodamine 123 was measured at 485/20 nm excitation and 530/25 nm emission by a fluorescence microplate reader system (Cytofluor 2300; Millipore, Bedford, MA).

Each plate included a blank control which had no cells (BC_{acc} or BC_{eff}; the subscript 'acc' and 'eff' means accumulation and efflux, respectively), a cell control which contained cells without reversal agent (CC_{acc} or CC_{eff}) and tested wells which contained cells with reversal agent (T_{acc} or T_{eff}). The comparative rate as percent of rhodamine accumulation rate in accumulation experiments and residual rate in efflux experiments was calculated by $(T_{acc}-BC_{acc})/(CC_{acc}-BC_{acc})$ and $(CC_{eff}$ or $T_{eff}-BC_{eff})/(CC_{acc}-BC_{acc})$, respectively.

Statistical analysis

All values are expressed as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons (Sigma Stat®; Jandel, San Rafael, CA). In all the comparisons, the difference was considered to be statistically significant at $p<0.05$.

Results

Cytotoxicity assay

The effects of TET, FAN and VER on the cytotoxicity of TAX, AMD and 5-FU to HCT15 and SK-OV-3 human

Table 1. Effects of TET, FAN and VER on the cytotoxicities of TAX, AMD and 5-FU *in vitro*

Reversal agents (μM)	TAX (EC_{50}; nM)		AMD (EC_{50}; nM)		5-FU (EC_{50}; μM)	
	HCT15	SK-OV-3	HCT15	SK-OV-3	HCT15	SK-OV-3
None	28.502 ± 3.281[a]	0.013 ± 0.002	0.583 ± 0.082	0.034 ± 0.006	9.8 ± 2.2	5.3 ± 1.0
TET (3.0)	0.009 ± 0.001[b]	0.010 ± 0.002	0.016 ± 0.004[b]	0.039 ± 0.007	13.2 ± 1.9	4.9 ± 0.6
FAN (3.0)	0.015 ± 0.001[b]	0.010 ± 0.001	0.013 ± 0.003[b]	0.025 ± 0.007	11.9 ± 3.5	6.0 ± 0.8
VER (10.0)	0.073 ± 0.008[b]	0.014 ± 0.003	0.032 ± 0.005[b]	0.031 ± 0.004	8.5 ± 2.0	4.4 ± 0.6

[a]Data are presented as the mean ± SEM of at least distinct three experiments.
[b]$p<0.05$ (significantly different from control).

cancer cells are summarized in Table 1. The cytotoxicities of TAX to HCT15 and SK-OV-3 cells were increased concentration dependently, and the EC_{50} values were 28.5 and 0.013 nM, respectively, demonstrating that HCT15 cells were more resistant to the drug than SK-OV-3 cells by about 2200 times. TET, FAN and VER concentration-dependently potentiated TAX-induced cytotoxicity to HCT15 cells. At the concentration of 0.3, 1.0 and 3.0 μM, TET potentiated TAX-induced cytotoxicity to HCT15 cells about 40-, 1300- and 3100-fold (EC_{50}: 0.75, 0.022 and 0.009 nM, respectively; $p<0.05$) in comparison with control, respectively. On the other hand, FAN could not potentiate the TAX-induced cytotoxicity with statistical significance ($p>0.05$) to HCT15 cells at 0.3 μM in comparison with control. At 1.0 and 3.0 μM, FAN increased the cytotoxicity of TAX to HCT15 cells about 45- and 1900-fold (EC_{50}: 0.62 and 0.015 nM, respectively; $p<0.05$) compared with control, respectively. In the case of VER, it potentiated TAX-induced cytotoxicity to HCT15 cells about 12- and 410-fold (EC_{50}: 2.38 and 0.070 nM, respectively; $p<0.05$) at 3.0 and 10.0 μM in comparison with control, respectively (Figure 2). TET, FAN and VER could not potentiate the TAX-induced cytotoxicity to SK-OV-3 cells at all concentrations tested ($p>0.05$).

For AMD, the cytotoxicity of the drug to HCT15 and SK-OV-3 cells was increased concentration dependently, and the EC_{50} values in the cells were 0.583 and 0.034 nM, respectively. TET and FAN potentiated AMD-induced cytotoxicity to HCT15 cells about 36.0- and 45.9-fold (EC_{50}: 16.2 and 12.7 nM, respectively; $p<0.05$) at 3.0 μM, respectively. VER also potentiated AMD-induced cytotoxicity to HCT15 cells about 18.2-fold (EC_{50}: 32.0 μM) at 10 μM. TET, FAN and VER could also reverse the MDR phenotype of HCT15 cells at 1.0 (TET and FAN) and 3.0 (VER) μM (data not shown), but these compounds could not affect the AMD-induced cytotoxicity to SK-OV-3 cells up to the maximum concentration tested of each compound ($p>0.05$).

For 5-FU, EC_{50} values of the drug to HCT15 and SK-OV-3 cells were 9.82 and 5.28 μM, respectively. TET, FAN and VER had no effect on 5-FU-induced cytotoxicity to the cells up to the maximum concentration tested of each compound.

Rhodamine accumulation and efflux

In HCT15 cells, TET increased the rhodamine accumulation about 2.1- and 2.5-fold ($p>0.05$), and FAN increased it about 2.3- and 2.4-fold ($p<0.05$) at 1.0 and 3.0 μM in comparison with control, respectively. VER also increased the rhodamine accumulation about 2.0- and 2.2-fold ($p<0.05$) at 3.0 and 10.0 μM compared with control, respectively. There were statistical

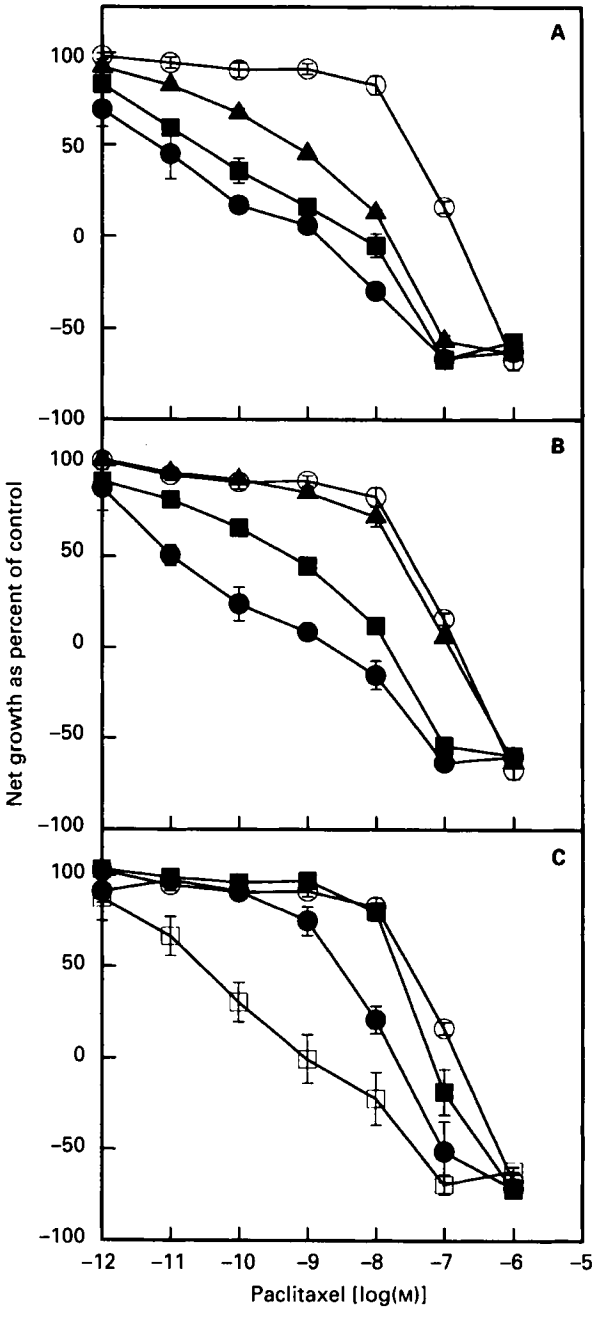

Figure 2. Effects of TET (A), FAN (B) and VER (C) on the cytotoxicity of TAX to HCT15 human cancer cells *in vitro*. The cells were cultured with serial dilution of TAX in the absence (○) or presence of each MDR-reversal agent at the concentration of 0.3 (▲), 1.0 (■), 3.0 (●) and 10.0 (□) μM. Cell survival fractions were assessed after continuous drug exposure for 3 days by SRB assay. Each data point represents the mean of at least three experiments and bar reveals SEM.

differences ($p > 0.05$) in the effects on rhodamine accumulation between TET, FAN and VER at all concentrations tested. In SK-OV-3 cells, TET, FAN and VER had no effect on the rhodamine accumulation at all concentrations tested (Figure 3).

After additional incubation with fresh medium for 2 h (efflux time), TET increased the residual rate of rhodamine to HCT15 cells about 3.1- and 4.8-fold ($p < 0.05$) in comparison with efflux control at 1.0 and 3.0 μM, respectively. FAN also increased the residual rate of rhodamine about 2.6-fold ($p < 0.05$) at 3.0 μM than that of efflux control. Meanwhile, after efflux time, VER could not increase the residual rate of rhodamine at 3.0 and 10.0 μM with statistical significance ($p > 0.05$) to HCT15 cells in comparison with efflux control. The residual rate of rhodamine at 1.0 and 3.0 μM of TET was significantly different ($p < 0.05$) from that of VER (10.0 μM). On the other hand, in SK-OV-3 cells, after efflux time, rhodamine was effluxed about 44% in comparison with the starting amounts in the cells in the absence of those reversal agents, and TET, FAN and VER had no effect on the efflux rate of rhodamine in the cells (Figure 4).

Discussion

There is considerable interest in strategies for minimizing drug resistance in cancer treatment protocols. Nearly 50% of all patients with malignant cancer are intrinsically resistant to chemotherapy and it is estimated that drug resistance is responsible for more

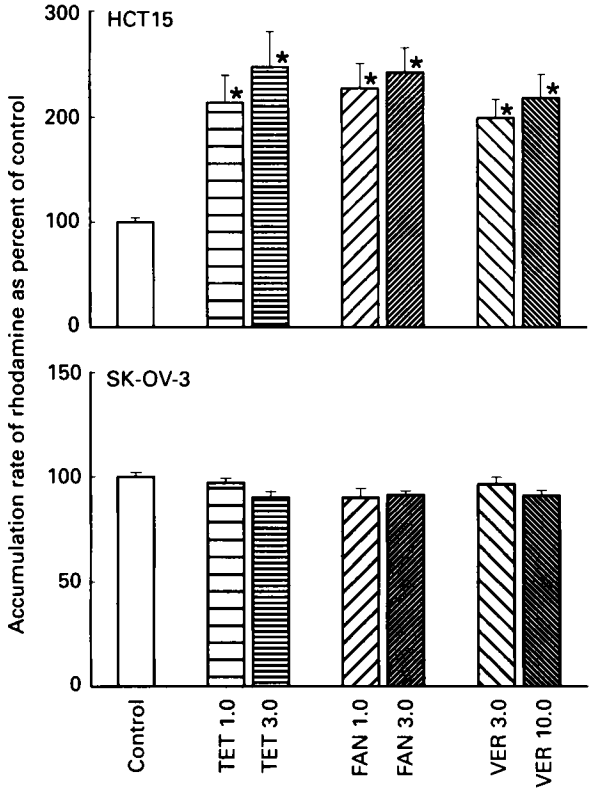

Figure 3. Effects of TET, FAN and VER on the accumulation rate of rhodamine 123 in HCT15 and SK-OV-3 human cancer cells. The cells were incubated with 4 μM rhodamine 123 in the absence or presence of TET (1.0 and 3.0 μM), FAN (1.0 and 3.0 μM) and VER (3.0 and 10.0 μM) for 40 min. Then the cells were washed twice with cold PBS, and the intracellular rhodamines were measured by a fluorescence microplate reader at 485/20 nm excitation and 530/25 nm emission. Data are presented as the mean of four distinct experiments and bar reveals SEM. *Significantly different from control ($p < 0.05$).

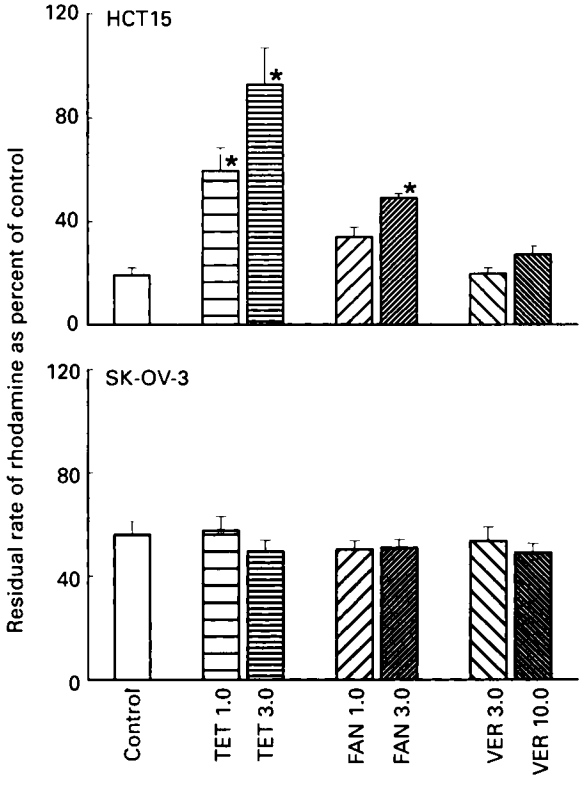

Figure 4. Effects of TET, FAN and VER on the residual rate of rhodamine 123 in HCT15 and SK-OV-3 human cancer cells. The cells were incubated with 4 μM rhodamine 123 in the absence or presence of TET (1.0 and 3.0 μM), FAN (1.0 and 3.0 μM) and VER (3.0 and 10.0 μM) for 40 min. Then extracellular rhodamines were removed by washing with cold PBS twice, followed by incubation with fresh medium for an additional 2 h. After 2 h efflux time, the cells were washed again with cold PBS, and the intracellular rhodamines were measured by a fluorescence microplate reader at 485/20 nm excitation and 530/25 nm emission. Data are presented as the mean of four distinct experiments and bar reveals SEM. *Significantly different from control ($p < 0.05$).

than 90% of all cancer deaths.[17] A major mechanism of this resistance is the enhanced efflux of chemotherapeutic agents due to the overexpression of P-gp. We previously tested the cytotoxicity of TET and FAN to the cells used in this experiment, and these compounds had no effect on the cell growth up to 5 μM (data not shown). TET and FAN concentration-dependently enhanced the TAX-induced cytotoxicity to HCT15 cells that express P-gp, but not to SK-OV-3 cells that do not express P-gp. In our unpublished data, these compounds also did not influence the cytotoxicity of TAX to other P-gp-negative cells such as human non-small cell lung cancer cell line A549. These observations are in line with recently published reports suggesting that some agents enhance cytotoxicity in P-gp-expressing cells via modulation of P-gp, but not in P-gp-negative cells.[16,18] These MDR-reversal agents include calcium channel blockers such as VER and nifedipine, calmodulin inhibitors such as fluphenazine and *cis*-flupenthixol, steroid derivatives such as tamoxifen and progesterone, antibiotics such as cefoperazone and erythromycin, cardiovascular drugs such as dipryidamole and quinidine, cyclosporins such as cyclosporin A and SDZ PSC-833, and miscellaneous compounds such as terfenadine and retinoids. Each of these compounds successfully reversed the MDR phenotype *in vitro* and some of them revealed good efficacy in laboratory animal models.[1] However, clinical trials of MDR inhibitors have been largely disappointing because of dose-limiting cytotoxicity. VER, the first MDR-reversal agent that reached clinical trial, has been disappointing due to its severe cardiotoxicity at subtherapeutic doses in clinics. Accordingly, much effort is currently being expended toward identifying MDR modulators which have no or less toxicological effects. In this experiment, TET and FAN reduced the EC_{50} values about 80- and 30-fold than VER at 1.0 μM in enhancing the TAX-induced cytotoxicity to HCT15 cells, respectively. TET and FAN at 3.0 μM also reduced the EC_{50} values of TAX to HCT15 cells about 260- and 160-fold than VER at 3.0 μM, and 8- and 5-fold than 10.0 μM VER, respectively ($p<0.05$). Furthermore, TET at 0.3 μM was more potent in enhancing the TAX-induced cytotoxicity to HCT15 cells in comparison with a 10-fold higher concentration of VER (3-fold; $p<0.05$). TET and FAN at 3.0 μM also enhanced the AMD-induced cytotoxicity to HCT15 cells more potently than 10.0 μM VER. We also tested the effects of these compounds on the TAX- and AMD-induced cytotoxicities to another MDR cancer cell line, HCT15/CL02 cells which were established from parental HCT15 cells by stepwise and continuous DOX exposure in KRICT, and similar results were obtained in that of HCT15 cells (unpublished data). Recently, cepharanthin, another *bis*benzylisoquinoline alkaloid, has been reported to circumvent MDR in P-gp-expressing cell lines, and it has been also reported that there was a significant correlation between the effect of cepharanthin on the cytotoxicity of DOX and P-gp expression in highly purified fresh human tumor cells obtained from cancer patients.[19] On the other hand, investigators have tried to clarify the chemical attributes of compounds that modulate MDR for a better understanding of their structure-activity relationships, and many potent MDR-inhibiting compounds share some common physical characteristics such as cyclicity, lipophilicity and a positive or neutral charge at physiological pH.[20] Accordingly, our results suggest strongly that TET and FAN enhance the TAX- and AMD-induced cytotoxicities to HCT15 cells via modulation of P-gp.

Rhodamine 123 is a fluorescent dye that is accumulated in mitochondria, specifically. Rhodamine was initially considered a potential anticancer agent because of its selective cytotoxicity to carcinoma cells in comparison with normal epithelial cells, but it was shown that the selectivity was due to P-gp expression in normal kidney cells and its absence in the particular carcinoma cell lines studied.[13] In this experiment, we confirmed previously that TET, FAN and VER reduced the EC_{50} values of rhodamine 123 to HCT15 cells, but not to SK-OV-3 cells (data not shown). On the other hand, recent studies have established a good correlation between rhodamine efflux and *mdr1* expression.[13] On the basis of this report, to investigate differential MDR-reversal activities of TET, FAN and VER, the accumulation and residual rates (after efflux for 2 h) of rhodamine were measured, TET, FAN and VER enhanced rhodamine accumulation in HCT15 cells about 200–250% in comparison with control at all concentrations tested, and there was no statistical difference between each other ($p>0.05$). On the other hand, after additional incubation with fresh medium for 2 h (efflux time), the residual rates of rhodamine accumulation in HCT15 cells were revealed in some different manner between those compounds. TET enhanced the residual rate of rhodamine about 3- and 5-fold at 1.0 and 3.0 M, respectively, and FAN enhanced the accumulation rate of rhodamine about 2.6-fold at 3.0 μM in HCT15 cells in comparison with control. Meanwhile, VER could not enhance the residual rate of rhodamine in HCT15 cells at the concentrations tested in comparison with control after the efflux time. These results were strongly correlated with those of the enhancing effects of TET, FAN and VER on TAX-induced cytotoxicity. Therefore, we suggest that TET and FAN can inhibit P-gp more efficiently and/or strongly than VER at the concentra-

tions tested, and that the differential MDR-reversal activities of those compounds to HCT15 cells are due to the different retardation efficacy of drug efflux between those compounds. TET, FAN and VER had no effect on the accumulation and efflux rate of rhodamine in SK-OV-3 cells. We also measured the residual rate of rhodamine after 1 h efflux time. We obtained slightly lower values from the mean values of the rhodamine accumulation and residual rate after 2 h, and the residual pattern of rhodamine after 1 h efflux time was similar to that after 2 h efflux time in the presence or absence of TET, FAN and VER (data not shown). It was shown that TET, FAN and VER also enhanced DOX accumulation in HCT15 cells but not in SK-OV-3 cells by flow cytometric analysis (data not shown).

Conclusion

Our results suggest that TET and FAN have MDR-reversal activities via modulation of P-gp, and in general these compounds revealed more potent activities than VER. These findings strongly suggest that these *bis*benzylisoquinoline alkaloids may have potential as forerunners of cancer-sensitizing agents for the treatment of P-gp-expressing MDR cancer. In addition, these results also suggest that the structural characteristics of these compounds could be helpful for designing more potent and non-toxic MDR-reversal agents.

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