

Enhancement of paclitaxel transport and cytotoxicity by 7,3',4'-trimethoxyflavone, a P-glycoprotein inhibitor

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ABSTRACT

Purpose.: Paclitaxel has problems with respect to bioavailability and resistance. The aim of this study was to select a P-glycoprotein (Pgp)-inhibitory flavonoid to enhance paclitaxel bioavailability in the Caco-2 cell monolayer.

Methods. Cytotoxicity and chemosensitization were determined using MTT assay. Paclitaxel transport was examined in the Caco-2 cell monolayer, which mimics the intestinal barrier. Paclitaxel concentrations were quantitated by HPLC assay using the internal standard method.

Results. Chemosensitizing indexes of 7,3',4'-trimethoxyflavone (TMF) and verapamil was > 333 and 152, respectively. The basolateral (BL)-to-apical (AP) transport of paclitaxel was more than 10-fold greater than its AP-to-BL transport. TMF and verapamil increased the AP-to-BL transport of paclitaxel but decreased its BL-to-AP transport in a concentration-dependent manner. The net absorptive effect of 50 μ M TMF on paclitaxel transport was comparable to that of 50 μ M verapamil. In addition, AP loading of TMF increased the paclitaxel sensitivity of paclitaxel-resistant SK-MES-1/PT4000 cells overexpressing Pgp on the BL side. **Conclusions.** These results indicate that TMF with low toxicity can be used as an enhancer of oral paclitaxel bioavailability and as a Pgp inhibitor.

INTRODUCTION

The current clinical formulation of paclitaxel is an important anti-cancer agent against breast, ovary and non-small cell lung cancers (1). However, paclitaxel chemotherapy presents two major problems: its administration route and drug resistance. The current clinical paclitaxel formulation has significant side effects, including allergic shock, that are related to the use of Cremophor EL/ethanol as co-solvents in the intravenous formulation; these co-solvents are necessary due to its poor aqueous solubility (2).

To solve this problem, water-soluble paclitaxel prodrugs have been prepared and then tested in breast xenograft assays (3). Paclitaxel liposomal formulations were much better tolerated than paclitaxel after i.v. or i.p. administration in both healthy and tumor-bearing mice (4). Recently, phase I clinical and pharmacokinetic studies of a novel water-soluble polymer-conjugated paclitaxel prodrug were performed (5). Moreover, paclitaxel also has poor bioavailability because of its high affinity for the membrane transporter P-glycoprotein (Pgp) in the gastrointestinal tract (6-8). *Mdr1a* Pgp knock-out mice, which lack functional Pgp activity in the gut, have shown significant bioavailability of orally administered paclitaxel (9). Pgp blockers such as cyclosporin A or PSC 833 can drastically improve oral bioavailability of paclitaxel in mice and humans (7, 8, 10).

Emergence of resistance to paclitaxel is one of the major obstacles in clinical cancer chemotherapy. Paclitaxel is a substrate for Pgp whose overexpression is mainly responsible for paclitaxel resistance (11). Therefore, if a substance could not only reverse paclitaxel resistance in Pgp-overexpressing cancer cells, but also enhance paclitaxel bioavailability through Pgp inhibition, it would increase the efficiency of paclitaxel chemotherapy.

In this study, we screened flavonoids for Pgp inhibition and then tested whether they could not only enhance paclitaxel bioavailability in the Caco-2 system, but also increase the sensitivity of Pgp-overexpressing cancer cells to paclitaxel. Here, we report that 7,3',4'-trimethoxyflavone (TMF) can serve as an enhancer of paclitaxel bioavailability and as a Pgp inhibitor.

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MATERIALS AND METHODS

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), daunorubicin, vincristine, dimethyl sulfoxide (DMSO), triethylamine and ammonium acetate were obtained from Sigma Chemical Co. (ST. Louis, MO, USA). Flavonoids (5,7,3',4',5'-pentamethoxyflavone, 7, 3', 4'-trimethoxyflavone, 3', 4' - dimethoxyflavone, 3,6,3',4' - tetramethoxyflavone, 5, 6, 7, 3', 4'-pentamethoxyflavone, 5, 6, 7, 3', 4', 5' - hexamethoxyflavone, 5,7,4'-trimethoxyflavone, 3,5,7-trihydroxy-3',4',5' - trimethoxyflavone, 5,7,3',4'-tetrahydroxyflavone, 3,7-dihydroxy-3',4'-dimethoxyflavone and 3,5,7,3',4'-penta-hydroxyflavone) were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). Paclitaxel and docetaxel were obtained from Korea United Pharm Co. (Seoul, South Korea). Cell culture media, α -MEM and DMEM, were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) and penicillin were obtained from Cambrex (San Diego, CA, USA). TRIzol reagent LSTM was obtained from Molecular Research Center (Cincinnati, OH, USA). Moloney murine leukemia virus (MMLV) reverse transcriptase was obtained from Invitrogen (Carlsbad, CA, USA). Taq polymerase was purchased from Perkin-Elmer/Cetus (Norwalk, CT, USA). [α -³²P]dCTP was obtained from AgenBio Ltd. (Seoul, South Korea). Methanol, hexane and acetonitrile were obtained from Merck Co. (Darmstadt, Germany). All other reagents were of analytical grade.

Cell culture

The acute myelogenous leukemia cell line OCI-AML-2 (AML-2) from the Ontario Cancer Institute (Toronto, Canada) and its daunorubicin-resistant subline AML-2/D100 were cultured at 37°C in a 5% CO₂ and 95% air atmosphere using α -MEM with 10% FBS and penicillin (12). The human colon carcinoma cell line Caco-2 and lung cancer cell SK-MES-1 line from the American Type Culture Collection were cultured in the same culture conditions using DMEM. Caco-2 cells were used between passage number 24 and 32. The paclitaxel-resistant SK-MES-1 subline, SK-MES-1/PT4000, was selected from SK-MES-1/WT cells after chronic exposure to paclitaxel on an intermittent dosage schedule at sufficient time intervals to permit expression of the resistance phenotype. Paclitaxel was initially administered

from $1 \times IC_{50}$, increased at 50% increments, and then finally cultured at a fixed concentration of paclitaxel (4000 ng/ml).

Cytotoxicity and chemosensitization assay

The *in vitro* cytotoxic and chemosensitization activities of the drugs were determined by MTT assays with AML-2/D100 cells in the presence or absence of vincristine (13). The IC₅₀ was defined as the drug concentration that resulted in a 50% reduction in the number of cells compared with untreated control cells after a 3-day incubation. The IC₅₀ values were determined directly from the semi-logarithmic dose-response curves. The chemosensitizing index was defined as the ratio of the IC₅₀ value in the absence of vincristine to the IC₅₀ value in the presence of vincristine. Survival (%) = [(sample absorbance - media control absorbance) / (control absorbance - media control absorbance)] \times 100.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) assay

Total cellular RNA was isolated from cells using TRIzol[®] according to the manufacturer's protocol. RT-PCR was used to analyze the expression of mRNA for *MDR1* and β -*actin* (internal control). RNAs from each sample were reverse-transcribed using 200 units of MMLV and oligo (dT)₁₈ primer for 90 min at 37°C. The resulting cDNA was diluted 1:5 with water and then amplified using 2.5 units of Taq polymerase and 10 pmole of each primer in a GeneAmp 2720 Thermal Cycler (Applied Biosystems, Boston, MA, USA). PCR products were quantitated by incorporation of 5 μ Ci of [α -³²P] dCTP in each PCR reaction mixture. The PCR products were electrophoresed on 7% non-denaturing polyacrylamide gels. The amount of each mRNA transcript was then normalized against that of β -*actin* mRNA. Autoradiographic films of the RT-PCR assay were subjected to densitometric analyses using the Kodak Image Station 4000MM (Eastman Kodak, Rochester, NY, USA).

Bi-directional transport experiment using Caco-2 cells

Caco-2 cells were seeded at a density of 4×10^4 cells/cm² on top of Transwell[®] polycarbonate filters (pore size, 0.4 μ m; diameter, 24.5 mm; growth area, 4.71 cm²) (Corning Costar Corp., Cambridge, MA, USA) and grown in a CO₂

incubator (37°C, 5% CO₂); the media were changed every 3 to 5 days. Experiments were conducted 16 to 20 days after the cells were seeded according to a previous report (14). Trans-epithelial electrical resistance (TEER) was measured using a Millicell-ERS device (Millipore, Bedford, MA, USA) equipped with rod-shaped electrodes. The TEER data were corrected for background readings from the blank filter and culture medium. The experiments were started when TEER values reached 800-850 Ω/cm². Paclitaxel (100 μM) was added to the apical (AP) or basolateral (BL) chamber with verapamil or flavonoids. Cells were incubated for 3 hours to assure measurable concentrations in the receiving chamber.

Determination of paclitaxel by HPLC

The method for determining paclitaxel concentration in media was modified slightly from the previous HPLC method (15). Cyano Bond Elut columns (1 ml, Analytichem International, Harbor, CA, USA) as solid-phase extraction were used for purification of paclitaxel and docetaxel (internal control). The reconstituted samples were then transferred to auto-sampler vials (250 μl) containing limited-volume inserts, and 50 μl was injected from each sample onto the ODS C18 column (4.6 × 250 mm, 5 μm; Shimadzu, Japan). The mobile phase was passed through a 0.22 μm membrane filter and degassed by ultrasonication under vacuum before use. The flow rate was 1 ml/min, and the effluent was monitored for UV absorption at 230 nm.

Statistical analysis

The results are expressed as mean ± standard error of the mean. Statistical comparisons were made using Student's *t* test. P value < 0.05 was considered significant.

RESULTS

Since intravenous paclitaxel administration can induce allergic shock, oral paclitaxel would be preferred if the bioavailability can be increased by co-administration with a drug that has low toxicity. In this study, we have screened flavonoids for Pgp inhibition and toxicity and then tested whether they could increase paclitaxel bioavailability in the Caco-2 system.

Extraction and determination of paclitaxel

The extraction ratio of paclitaxel using the Cyano Bond Elut column was approximately 61%. The amount of paclitaxel was determined by HPLC using docetaxel as an internal standard. The standard curve was linear (R = 0.9985) and a chromatogram revealed good separation between paclitaxel (retention time = 9.867 min) and docetaxel (retention time = 10.867 min). The average coefficient of variance (CV) for triplicate samples was 15% for paclitaxel at 250 pmole. The detection limit was 50 ng of paclitaxel. These parameters guaranteed a good quantitative assay of paclitaxel.

Screening flavonoids for Pgp inhibition using MTT assay

Flavonoids interact bifunctionally with the ATP-binding site and a steroid-interacting hydrophobic sequence of Pgp (16). The cytotoxicity and chemosensitizing activities of various hydroxy and/or methoxy flavonoids were determined by MTT assay in the presence or absence of vincristine in AML-2/D100 cells overexpressing Pgp (Figure 1). Eleven flavonoids with various methoxy and/or hydroxyl groups had already been screened for Pgp inhibition (17). Among the flavonoids screened for Pgp inhibition, 5,7,3',4',5'-pentamethoxyflavone (PMF) and TMF exhibited the highest chemosensitizing indexes, > 1000 and > 333, respectively. In contrast, the chemosensitizing index of the well-known Pgp inhibitor verapamil was 152 (Figure 1). Thus, on the basis of their high chemosensitizing indexes, PMF and TMF were selected and further tested in paclitaxel transport in Caco-2 cells.

Effect of TMF on bi-directional transport of paclitaxel in the Caco-2 system

The transport of paclitaxel in the Caco-2 cell monolayer was linear over 3 hours (data not shown). This result is consistent with a report that paclitaxel flux across the Caco-2 cell monolayer was linear with time for up to 3 hours (18). In this study, paclitaxel transport across the Caco-2 cell monolayer was determined after incubation for 3 hours in the presence and absence of PMF, TMF and verapamil in both AP-to-BL and BL-to-AP directions.

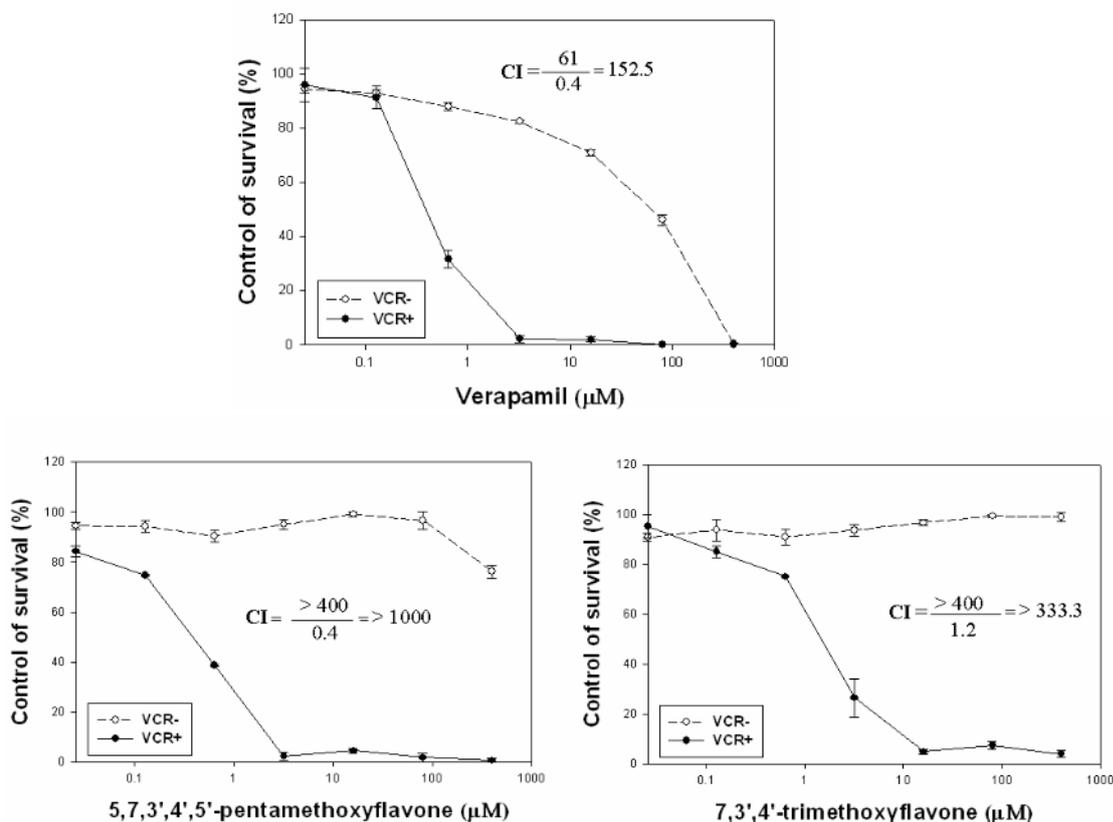


Figure 1. Cytotoxicity and chemosensitization of flavonoids and verapamil in AML-2/D100 cells. CI, chemosensitizing index = $IC_{50}(\text{VCR-}) / IC_{50}(\text{VCR+})$ in AML-2/D100 cells; VCR, vincristine (100 ng/ml).

Since PMF unexpectedly decreased AP-to-BL paclitaxel transport, only TMF was compared to verapamil in terms of paclitaxel transport in this study. The BL-to-AP transport of paclitaxel was more than 10-fold greater than its AP-to-BL transport (Figure 2). TMF and verapamil increased the AP-to-BL transport of paclitaxel and decreased its BL-to-AP transport in a concentration-dependent manner (Figure 2). However, the effect of TMF on absorptive (AP-to-BL) or secretory (BL-to-AP) paclitaxel transport was lower than that of verapamil. The net effect (BL-to-AP – AP-to-BL) of 50 μM TMF on paclitaxel transport was comparable to that of 50 μM verapamil, although the net effect of 400 μM TMF was equivalent to that of 100 μM verapamil.

Effect of TMF on the sensitivity of SK-MES-1/PT4000 to paclitaxel in the Caco-2 system

To further demonstrate the usefulness of TMF in increasing the chemosensitivity of paclitaxel-

resistant cancer cells, we selected a paclitaxel-resistant lung cancer cell subline. As shown in Figure 3A, SK-MES-1/PT4000 cells overexpressing Pgp are 87-fold more resistant to paclitaxel than SK-MES-1/WT that does not express Pgp (Figure 3A insert). We tested whether TMF could enhance the sensitivity of SK-MES-1/PT4000 cells to paclitaxel in the Caco-2 system. As shown in Figure 3B, aliquots of media were obtained from the BL chamber after both 50 μM paclitaxel and TMF were incubated in the AP chamber in order to simulate oral administration for 3 hours; these aliquots were then loaded on SK-MES-1/WT or /PT4000 cells in 96-well plates containing complete media. After a 3-day incubation, paclitaxel sensitivity was determined by MTT assay. TMF and verapamil both enhanced the sensitivity of SK-MES-1/PT4000 cells to paclitaxel compared with control (Figure 3C).

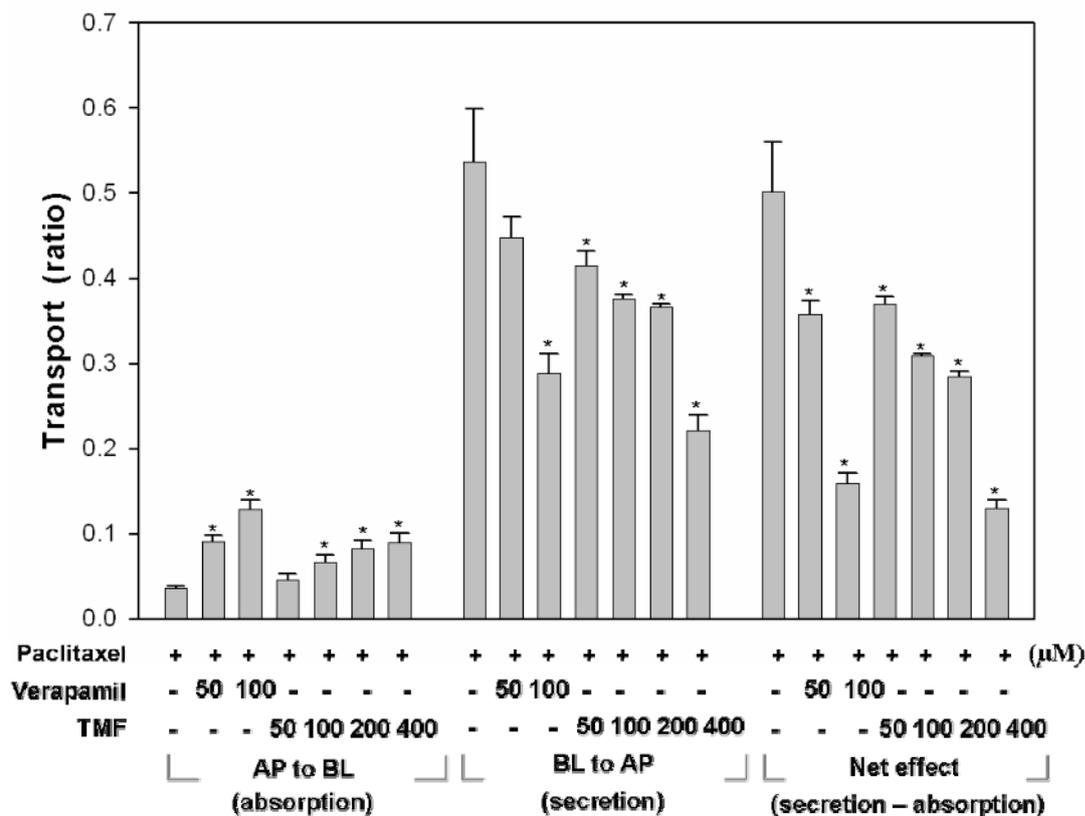


Figure 2. Effect of TMF on paclitaxel transport across the Caco-2 monolayer. Paclitaxel (50 μM) with and without Pgp inhibitors was added to the AP or BL chamber in DMEM (without serum). After incubating the cells with drugs for 3 hrs, paclitaxel in the opposite chamber (chamber not initially loaded with paclitaxel) was extracted, and the amount was determined by HPLC. AP, Apical load; BL, Basolateral load. * P < 0.05.

TMF (200 μM) loaded in the AP chamber for 3 hours increased the sensitivity of SK-MES-1/PT4000 cells to paclitaxel to that of SK-MES-1/WT cells. Since 50 μM paclitaxel decreased 50% in the growth of SK-MES-1/WT cells (Figure 3C), the concentration of paclitaxel in the BL chamber could be approximately estimated as 71 nM (IC₅₀ value) (Figure 3A).

DISCUSSION

Oral paclitaxel administration is preferred because this administration route is convenient to patients, reduces administration costs and facilitates the use of more chronic treatment regimens (9). Nevertheless, oral paclitaxel administration is not recommended due to low oral bioavailability. In this study, we searched for an ideal enhancer of paclitaxel bioavailability. Flavonoids are of

interest in this regard because of their pharmacological advantages including their Pgp inhibitory activity and low toxicity. We screened various hydroxyl and/or methoxy flavonoids for Pgp inhibition before selecting TMF as an enhancer of paclitaxel bioavailability. In this study, the BL-to-AP transport of paclitaxel was more than 10-fold greater than its AP-to-BL transport. This finding is consistent with the previous report that paclitaxel transport from the BL to the AP side was 4-10 times greater than that from the AP to the BL side (18). We observed that both TMF and verapamil increased AP-to-BL paclitaxel transport, and decreased its BL-to-AP transport; this decreased the net effect (secretory - absorptive transport) of paclitaxel transport. Pgp inhibition had previously been shown to decrease BL-to-AP paclitaxel transport and increase the AP-to-BL transport (19).

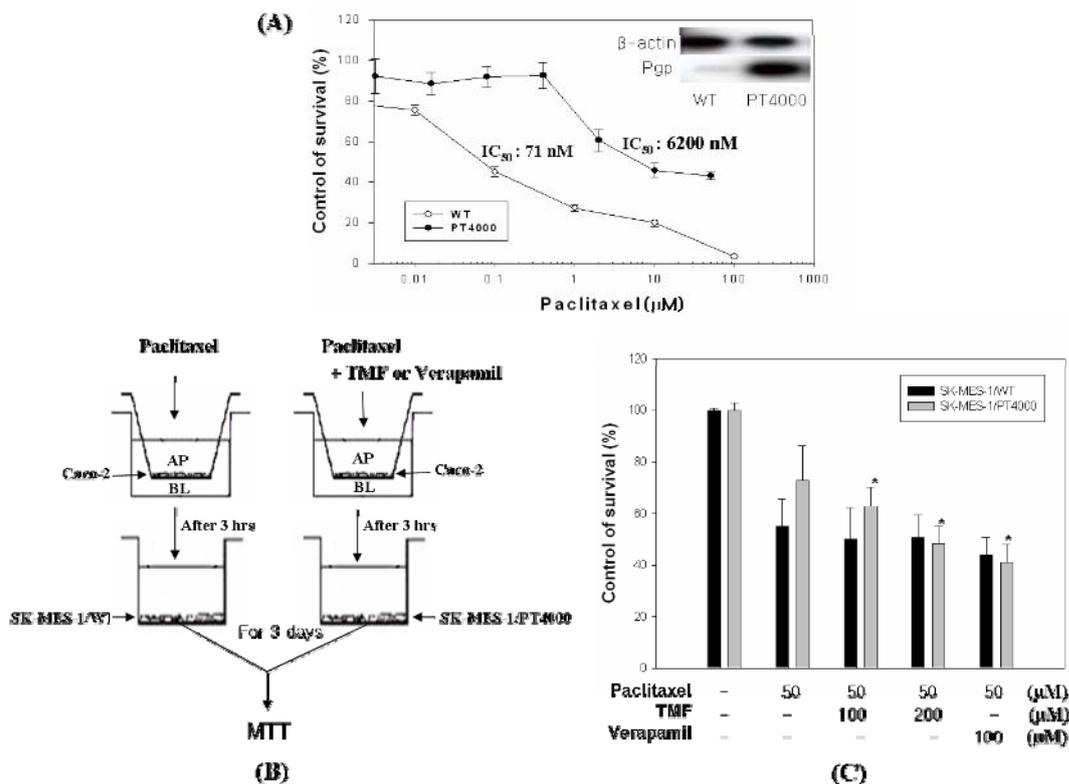


Figure 3. Effects of TMF and verapamil on the sensitivity of SK-MES-1/PT4000 cells to paclitaxel in the Caco-2 system. (A) Sensitivity of SK-MES-1/WT and /PT4000 cells to paclitaxel. Insert, mRNA levels; IC₅₀, Concentration of paclitaxel required for 50% inhibition of cell growth. (B) Experimental procedure: Aliquots of media were obtained from the BL chamber after incubation with 50 µM paclitaxel and TMF in the AP chamber for 3 hrs. (C) MTT assay data: Media samples were taken from the BL chamber after the AP loading and then loaded on SK-MES-1/WT or /PT4000 cells seeded in a 96-well plate containing complete media. After 3-day incubation, paclitaxel sensitivity was determined by MTT assay. The data are means ± SD, n=3. * P < 0.05, vs. 50 µM paclitaxel control.

TMF did not inhibit MRP1 (data not shown); it therefore enhances paclitaxel transport to the BL side, at least in part, by inhibiting Pgp, which is located in the brush border membrane. Although involvement of other transporters and cytochrome P-450 was not ruled out in this study, it is demonstrated that methylated flavones are superior to unmethylated analogues with respect to metabolic stability and transport (20). The net effect of TMF on paclitaxel transport was less than that of verapamil. However, considering the low toxicity of TMF compared with that of verapamil, we could increase TMF dose to further enhance paclitaxel bioavailability. Unexpectedly, PMF, which had the highest chemosensitizing effect, did not inhibit BL-to-AP paclitaxel transport (data not shown), whose mechanisms remain to be determined.

Taken together, these results indicate that TMF, with its low toxicity, can be used not only as a Pgp inhibitor in patients with cancer cells that

overexpress Pgp, but also as an enhancer of oral paclitaxel bioavailability.

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