Involvement of everolimus-induced ABCB1 downregulation in drug-drug interactions

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Abstract. Everolimus is an oral mammalian target of rapamycin (mTOR) inhibitor used in cancer chemotherapy and transplantation. Due to its therapeutic properties, everolimus has been used long-term in clinical practice. Drug interactions with everolimus during gastrointestinal absorption can alter the oral bioavailability of everolimus and/or concomitant drugs. However, the effects of everolimus on gastrointestinal absorption remain unknown. The present study assessed the impact of continuous exposure to everolimus on expression and function of the ATP-binding cassette (ABC) transporter ABCB1 and ABCG2 using a Caco-2 intestinal cell model. Caco-2 subline, Caco/EV, was established by continuously exposing Caco-2 cells to 1 μM everolimus. Cell viability was evaluated using WST-1 assay. mRNA levels were measured by reverse transcription-quantitative PCR. Transport activity of ABCB1 was evaluated through the cellular accumulation of Rhodamin 123, a substrate for ABCB1. The half-maximal inhibitory concentration (IC_{50}) values for everolimus in Caco-2 and Caco/EV cells were 0.31 and 4.33 μM, respectively, indicating 14-fold resistance in Caco/EV cells. Sensitivity to paclitaxel and 7-ethyl-10-hydroxycamptothecin, which are substrates for ABCB1 and ABCG2, respectively, was enhanced in Caco/EV, but not in Caco-2 cells. The IC_{50} values of cisplatin were comparable in both cell lines. Furthermore, mRNA expression levels of ABCB1 and ABCG2 were lower in Caco/EV cells than in Caco-2 cells, and the cellular accumulation of Rhodamine 123 was significantly higher in Caco/EV cells. These findings demonstrated that continuous exposure to everolimus suppressed the expression and function of ABCB1 and ABCG2, suggesting potential drug-drug interactions via the suppression of ABCB1 and ABCG2 in the intestinal tract.

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Introduction

Overexpression of ATP-binding cassette (ABC) transporters, particularly ABCB1 (also known as P-glycoprotein or MDR1), is a key factor in multidrug resistance in cancer cells (1-3). ABCB1 transports substances from the intracellular to extracellular space using energy derived from ATP hydrolysis (1-3), leading to resistance against anticancer drugs in cancer cells. Additionally, this membrane protein is widely expressed in numerous types of normal tissue, including the placenta, kidney, liver, intestine, adrenal gland, heart and small blood vessels, as well as lymphocytes and the blood-brain barrier (1-3). Thus, ABCB1 serves a crucial role in drug pharmacokinetics as well as in resistance to anticancer drugs. As with ABCB1, ABCG2 (also known as the breast cancer resistance protein) is also one of the ABC transporters, making it important to monitor its role in drug metabolism and cancer drug resistance (4).

Numerous mechanisms regulating ABCB1 and ABCG2 expression have been reported, indicating that numerous substances induce upregulation or downregulation (3-6). For example, digoxin, a cardiac glycoside, has been shown to upregulate ABCB1 mRNA, leading to enhanced ABCB1-mediated transport (7,8). Conversely, simvastatin, an inhibitor of 3-hydroxymethyl-3-methylglutaryl-Coenzyme A reductase, has been found to downregulate microRNA (miRNA)-33a expression, resulting in downregulation of ABCB1 (9). Thus, the factors regulating ABCB1 and ABCG2 expression are diverse (4,10,11).

The oral mammalian target of rapamycin (mTOR) inhibitor everolimus is used in both cancer chemotherapy and transplantation therapy, with its long-term use common in clinical practice (12,13). Everolimus may interact with other drugs, potentially affecting therapeutic outcomes and/or causing toxicity. Studies have been conducted on the interactions of everolimus with numerous types of drug and food, focusing on the drug-metabolizing enzyme cytochrome P450 (CYP)3A4 and ABCB1 (14-16). Recently, everolimus has been reported to inhibit ABCG2-mediated transport (17). However, effects of long-term exposure to everolimus on the function and

expression of ABCB1 and ABCG2 in the intestine are unclear both *in vitro* and *in vivo*, as there is limited information on how everolimus affects ABCB1 and ABCG2 expression (16,18).

The present study examined the effects of long-term exposure to everolimus on expression and function of ABCB1 and ABCG2 using a Caco-2 human intestinal cell model, exploring a novel potential mechanism of drug-drug interaction in the intestinal tract.

Materials and methods

Chemicals. Everolimus was purchased from Selleck Chemicals. 7-Ethyl-10-hydroxycamptothecin (SN-38) was purchased from LKT Laboratories, Inc. Cisplatin and paclitaxel were purchased from FUJIFILM Wako Pure Chemical Corporation. Rhodamine 123 was purchased from Molecular Probes (Thermo Fisher Scientific, Inc.). WST-1 and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were obtained from Dojindo Laboratories, Inc.

Cell culture. The human colon carcinoma cell line Caco-2 (research resource identifiers:CVCL_0025) was obtained from the American Type Culture Collection. Caco-2 cells were cultured in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G, 100 μg/l streptomycin sulfate and 0.1 mM non-essential amino acids (Gibco; Thermo Fisher Scientific, Inc.). Cells were seeded at a density of $2x10^4$ cells/cm^2 in culture dishes, incubated in a humidified atmosphere of 5% CO_2 at 37˚C and passaged using 0.05% trypsin-0.02% EDTA (Gibco; Thermo Fisher Scientific, Inc.).

Continuous exposure of Caco-2 cells to everolimus. The cells were treated with 1 μM (958.22 μg/ml) everolimus. To establish a subline capable of tolerating 1 μM everolimus, cells were cultured at 37˚C in complete DMEM with 1 μM everolimus for ~3 months. Cells displaying stable growth were isolated and cloned using the limiting dilution method, where cells were serially diluted in 24-well plates to achieve an estimated concentration of <1 cell per well. In preliminary experiments, the proliferation activity and drug sensitivity of several clones were assessed and the chosen clone was named Caco/EV. Caco/EV cells were maintained as Caco-2 cells without the addition of 1 μM everolimus to the DMEM.

Assay for cell proliferation activity. The proliferation of Caco-2 and Caco/EV cells was assessed through proliferation curves. On day 0, cells (1,000 cells/well) were seeded in 96-well plates using complete DMEM without everolimus and the cell count was monitored from day 0 to 9. Cell count was determined using the WST-1 assay based on the MTT assay (19-22). The culture medium was substituted with DMEM containing WST-1 reagent solution (10 μl WST-1 including 0.2 mM 1-methoxy PMS + 100 μl culture medium) and after 2 h, absorbance was measured at 450 nm using a microplate reader (Spectra Fluor; Tecan Group Ltd.) with a reference wavelength of 630 nm. The relationship between absorbance and cell count was confirmed through preliminary experiments. Specifically, both cell types were seeded in 96-well plates at a starting density of $2x10^4$ cells/100 μl/well, followed by a 10-step, 2-fold serial dilution. Thirty minutes after seeding, the WST-1 assay was performed as described above, resulting in a calibration curve correlating absorbance with cell count. The doubling time of each cell during the logarithmic phase was calculated as follows: Doubling time=$(t_2-t_1) \times \log 2/(\log N_2-\log N_1)$ where t_1 and t_2 represent the time of cell counting and N_1 and N_2 represent the cell count at t_1 and t_2, respectively (19).

Growth inhibition assay. The growth inhibitory effects of everolimus, paclitaxel, SN-38 and cisplatin were evaluated in Caco-2 and Caco/EV cells using WST-1 assay (19-22). Both cell lines were seeded at a density of 1,000 (everolimus) or 5,000 (other drugs) cells/well in 96-well plates with DMEM without any drugs on day 0. On day 1, the medium was replaced with fresh DMEM containing the test drugs. The maximum concentration used was 10 for everolimus, 4 μM for paclitaxel, 1 μM for SN-38, and 1024 μM for cisplatin. Following 72 h (paclitaxel, SN-38 and cisplatin) or 144 h (everolimus) incubation at 37˚C, cell viability was assessed using the WST-1 assay as aforementioned. The half-maximal inhibitory concentration (IC_{50}) of the test drugs in both cell lines was calculated using the sigmoid inhibitory effect model (19-22): $E=E_{max} \times [1-C^{\gamma}/(C^{\gamma} + IC_{50}^{\gamma})]$, where E and E_{max} represent the surviving fraction (% of control) and its maximum, respectively, C is concentration in the medium and γ represents sigmoidicity factor. This calculation was performed using the non-linear least-squares fitting method (Solver, Microsoft® Excel 2019). The relative resistance (RR) was obtained by dividing IC_{50} value for Caco/EV cells by that for Caco-2 cells.

Reverse transcription-quantitative (RT-q)PCR. Cells ($2x10^6$ cells/60 mm culture dish) were pre-cultured at 37˚C for 48 h, after which total RNA was extracted using GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich; Merck KGaA). mRNA expression levels were quantified using RT-qPCR, employing three independent samples (22-24). RT of 500 ng total RNA was performed using the PrimeScript RT reagent kit (Takara Bio, Inc.) and a thermal cycler (i-Cycler, Bio-Rad Laboratories, Inc.). RT was conducted in 20 μl reaction buffer at 37˚C for 15 min and terminated by heating at 85˚C for 5 sec followed by cooling at 4˚C qPCR was conducted using the 7500 Fast Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc.) and SYBR® Premix Ex Taq (Takara Bio, Inc.). PCR reaction was performed at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec; dissociation curve analysis was initiated at 95˚C for 15 sec, followed by 60˚C for 1 min and 95˚C for 15 sec. β-actin served as the internal standard. Primer sequences and were synthesized by GeneDesign, Inc. (Table I). The comparative Cq method was employed to compare relative expression levels of target mRNAs (22-25).

Cell accumulation of Rhodamine 123. Caco-2 and Caco/EV cells ($5x10^4$ cells/well) were seeded in 24-well plates (7,8). The plates were incubated for 10 days in a humidified atmosphere of 5% CO_2 air at 37˚C with the complete DMEM being replaced with fresh medium every 2 days. Cells were

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Forward primer, 5'→3'	Reverse primer, 5'→3'
β-actin	TCATGAAGTGTGACGTGGACATC	TGCATCCTGTCGGCAATG
ABCB1	TTCCTTCACCCAGGCAATG	ATGAGTTTATGTGCCACCAAGTAG
ABCG2	TGACGGTGAGAGAAAACTTAC	TGCCACTTTATCCAGACCT

ABC, ATP-binding cassette.

washed three times with warmed Hanks' balanced salt solution (HBSS) containing phenol red and HEPES. Fresh HBSS containing 3 μM Rhodamine 123 was added, followed by further incubation for 5, 15, 30, 60, 90 and 120 min at 37˚C. The accumulation process was terminated by removing HBSS from the wells, followed by three washes with ice-cold phosphate-buffered saline. Cells were solubilized with 0.3 M NaOH and aliquots were neutralized using 0.3 M HCl. A total of 100 μl neutralized cell-solubilized solution were transferred to 96-well black plates and the fluorescence intensity of Rhodamine 123 was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using Spectra Fluor. Protein content was determined using the Lowry method (26), with bovine serum albumin (FUJIFILM Wako Pure Chemical Corporation) serving as the standard.

Statistical analysis. Data are presented as the mean ± SD of ≥3 independent experimental repeats. Comparisons between groups were conducted using the unpaired Student's t test. Statistical analysis was performed using JMP® Pro 15.2.0 (SAS Institute Japan Ltd., Tokyo). A two-tailed P<0.05 was considered to indicate a statistically significant difference.

Results

Establishment of everolimus-resistant Caco/EV cell. Despite the clinically achievable plasma concentration of everolimus being ~60 ng/ml (equivalent to 0.06 μM) at the clinical dose of 10 mg (27), Caoc-2 cells were culture in DMEM supplemented with 1 μM everolimus, because exposure to lower concentrations did not affect cell proliferation. After 3 months, cells displaying stable growth were cloned and named Caco/EV. Preliminary experiments confirmed a strong correlation between absorbance in the WST assay and cell count (data not shown).

Caco-2 and Caco/EV cell proliferation. Growth curves of Caco-2 and Caco/EV cells displayed a logarithmic phase starting within 2 days of cell seeding; this phase persisted for ≥7 days (Fig. 1). On day 4, the proliferation rates of both cell types were comparable, with a doubling time of 28.4 h and 32.8 h for Caco-2 and Caco/EV cells, respectively.

Sensitivity to everolimus. IC_{50} for everolimus in Caco/EV cells was significantly lower than that in Caco-2 cells, showing 14-fold resistance compared with that in Caco-2 cells (Table II). IC_{50} for paclitaxel, a substrate of ABCB1, were significantly lower in Caco/EV than in Caco-2 cells, indicating enhanced sensitivity to paclitaxel. IC_{50} value of SN-38, a substrate of ABCG2, was significantly lower in Caco/EV cells than in Caco-2 cells. The IC_{50} of cisplatin in Caco-2 cells was comparable to that in Caco/EV cells.

Table II. Caco-2 and Caco/EV cell sensitivity.

	Mean half-maximal inhibitory concentration		
Drug	Caco-2	Caco/EV	RR
Everolimus	0.31±0.11 μM	4.33±1.56 μM[a]	14.0
Paclitaxel	146.4±32.8 nM	38.0±28.8 nM[a]	0.26
SN-38	0.30±0.06 nM	0.10±0.15 nM[a]	0.33
Cisplatin	5.81±0.70 μM	5.37±1.92 μM	0.92

n=6-8. [a]P<0.01 vs. Caco-2. RR, relative resistance; SN-38, 7-Ethyl-10-hydroxycamptothecin.

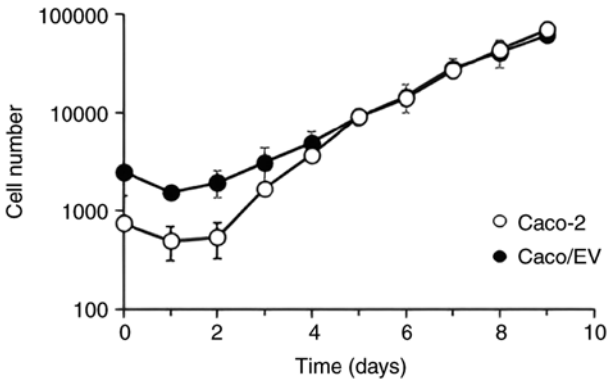

Figure 1. Growth curves for Caco-2 and Caco/EV cells. n=24.

mRNA expression of ABCB1 and ABCG2. mRNA expression of ABCB1 in Caco/EV cells was significantly lower than that in Caco-2 cells, with an estimated 72% decrease (Fig. 2). mRNA expression of ABCG2 was also significantly reduced in Caco/EV cells.

Cell accumulation of Rhodamine 123. Rhodamine 123 is a substrate of ABCB1 (7,8,20). The cellular accumulation of Rhodamine 123 in both cell lines was time-dependent and reached steady state after 90 min (Fig. 3). The accumulation of Rhodamine 123 in Caco/EV cells was significantly higher than that in Caco-2 cells from 15 min.

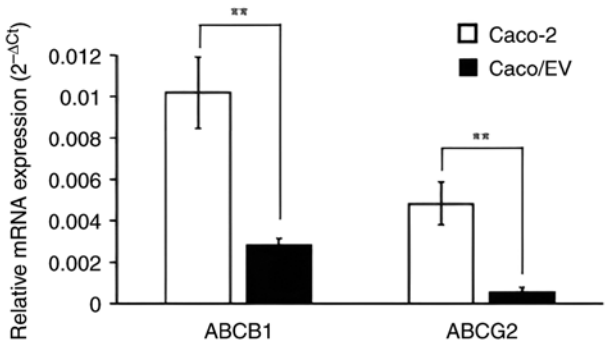

Figure 2. mRNA expression of ABCB1 and ABCG2 in Caco-2 and Caco/EV cells. n=3. **P<0.01 vs. Caco-2. ABC, ATP-binding cassette.

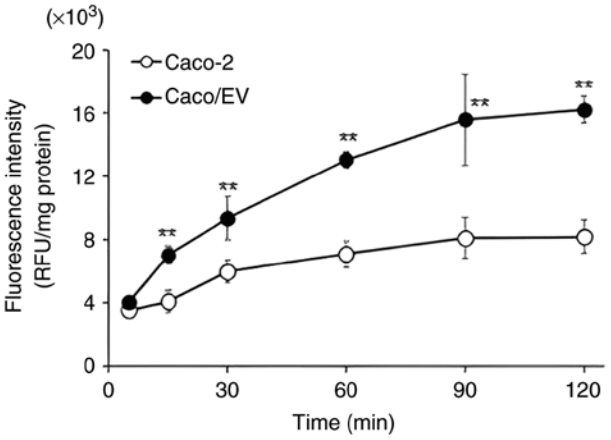

Figure 3. Rhodamine 123 accumulation in Caco-2 and Caco/EV cells. n=4. **P<0.01 vs. Caco-2. RFU, relative fluorescence unit.

Discussion

Everolimus is metabolized by CYP3A4 and transported by ABCB1 (14-16). Everolimus has potent inhibitory activity against ABCG2-mediated transport (17). Since CYP3A4 and ABC transporters are responsible for the pharmacokinetics of many drugs (28), the pharmacokinetic interactions between everolimus and drugs or food are hypothesized to be a major problem in clinical practice. The present study aimed to clarify the effect of continuous everolimus exposure on expression and function of ABCB1 and ABCG2 in a Caco-2 human intestinal cell model.

Although cell growth rates were comparable in Caco-2 and Caco/EV cells, Caco/EV cells showed a 14-fold resistance to everolimus. Continuous exposure to everolimus was induced resistance to everolimus, similar to previous findings in renal cell carcinoma Caki-2 and 786-O cells (29,30). However, the sensitivity to paclitaxel and SN-38, which are substrates for ABCB1 and ABCG2, respectively, was enhanced by continuous exposure to everolimus. The IC_{50} value of cisplatin in Caco-2 cells was comparable with that in Caco/EV cells. Furthermore, mRNA expression levels of ABCB1 and ABCG2 were lower in Caco/EV than in Caco-2 cells and the cellular accumulation of Rhodamine 123, a substrate for ABCB1, was significantly higher in Caco/EV cells. This may be explained by the decreased efflux transport activity of ABCB1 in Caco/EV cells due to downregulation of ABCB1 (30), resulting in higher accumulation of Rhodamine 123 in Caco/EV cells compared with Caco-2 cells. Shihab *et al* (14) reviewed drug interactions between mTOR inhibitors and tacrolimus, both of which are substrates for CYP3A4 and ABCB1. Competition for CYP3A4-mediated metabolism and ABCB1-mediated drug efflux may disrupt drug absorption or elimination, potentially causing notable changes in drug exposure when these agents are administered together. Based on the present findings, the mechanism underlying this drug interaction may include downregulation of ABCB1. In our previous study of renal cell carcinoma Caki-2 and 786-O cells, ABCB1 and ABCG2 mRNA expression levels were lower in everolimus-resistant Caki/EV and 786/EV cells compared with their parental cells (30), consistent with the present findings. Additionally, the aforementioned resistant cells showed increased sensitivity to paclitaxel and doxorubicin, but not to SN-38, which aligns with the present findings (30). Therefore, continuous exposure to everolimus may suppress expression and function of ABCB1, indicating potential drug interactions via the suppression of ABCB1 in the intestinal tract. As everolimus was recently reported to inhibit ABCG2-mediated transport (17) and sensitivity to SN-38 was enhanced in Caco/EV cells in the present study, only the expression of ABCG2 mRNA was examined. Although the transport function of ABCG2 was not examined, similar drug interactions via its suppression may be elicited by long-term clinical use of everolimus. Additionally, other mechanisms such as decrease in mTOR activity, downregulation of DNA-damage-inducible transcript 4, DEP domain-containing mTOR-interacting protein (DEPTOR), hypoxia inducible factor 1, alpha subunit (HIF1A) and phospholipase D1 (PLD1), may contribute to resistance to everolimus in Caco/EV cells, as supported by previous reports (29,30).

The mechanism underlying downregulation of ABCB1 and ABCG2 mRNA following everolimus exposure remains unclear. Transcriptional regulation of ABCB1 gene expression is complex (5) and nuclear receptors SXR and PXR, inverted CCAAT element (Y-Box), P13K/AKT, MAPK, NF-κB, AP-1 play a key role in modulating ABCB1 gene expression (6). Downregulation of ABCB1 via inhibition of transcriptional factors and aforementioned cell signaling pathways has been reported (5,6). Therefore, everolimus may affect these transcriptional factors and cell signaling pathways related to ABCB1 downregulation (5,6). miRNAs are short, non-coding, single-stranded RNA molecules. By binding complementarily to the 3' untranslated region of their target mRNAs, miRNAs have the ability to silence genes and are key in regulating cell functions (31). ABCB1 is regulated by numerous miRNAs (miR-27a, miR-451 and miR-495) in different tumor types (32,33) and 34 miRNAs have been predicted to target ABCB1 based on online database for miRNA target prediction and functional annotation miRDB (mirdb.org/mirdb/index.html). miR-200c and miR-21-5p have been shown to sensitize multidrug-resistant cells to anticancer drugs that target ABCB1 substrates (34,35). On the other hand, everolimus modulates expression of miR-145, miR-15a, and miR-4328 (36,37). mTOR dual inhibitor BEZ235 sensitizes acute myeloid leukemia cells to chemotherapy by upregulating miR-1-3p and reducing the expression of ABCB1 (38). Collectively, it is hypothesized that fluctuation in the expression of some miRNAs following everolimus exposure

may lead to downregulation of ABCB1. ABCG2 levels are modulated by transcription factors (NF-κB and HIF), nuclear receptors (PXR, CAR), kinase signaling (PI3K/AKT, MAPK, and Wnt/β-catenin), inflammatory mediators (TNF-α, IL-6, and IL-1β), growth factors (TGF-β, HGF, EGF and so on)and miRNAs (miR-519c, miR-520h, miR203 and so on) (4).

The present study demonstrated that continuous exposure to everolimus suppressed function of ABCB1 by downregulating ABCB1 mRNA; however, these findings may be limited as they were based solely on *in vitro* studies and further animal experiments are necessary. Potent interactions between everolimus and drugs may be observed in clinical settings. Consequently, it is crucial to comprehend the effects of extended everolimus usage in cancer chemotherapy and post-transplant care, as notable interactions between concurrent medications, mediated by the suppression of ABCB1 function, could modify blood concentrations of everolimus and/or concomitant drugs.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YN performed experiments, constructed figures and wrote the manuscript. AI and KY analyzed and interpreted data, constructed figures and edited the manuscript. KT conceived the study and edited the manuscript. YN and KT confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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