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# Microtubule-targeting anticancer drug eribulin induces drug efflux transporter P-glycoprotein

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ABSTRACT

This study examined the effects of microtubule-targeting anticancer drugs (paclitaxel, cabazitaxel, and eribulin) on the expression of drug efflux transporter P-glycoprotein, which is encoded by *MDR1*. Paclitaxel and eribulin induced *MDR1* promoter activity in a concentration-dependent manner, while cabazitaxel had little effect in human intestinal epithelial LS174T cells. Overexpression of the nuclear receptor pregnane X receptor (PXR) gene (*NR1I2*) enhanced paclitaxel- and eribulin-induced *MDR1* activation, but expression of the nuclear receptor co-repressor silencing mediator for retinoid and thyroid receptors (SMRT) gene (*NCOR2*) repressed *MDR1* activation. Eribulin increased the mRNA and protein expression of P-glycoprotein in LS174T cells. Cellular uptake of rhodamine 123 and calcein-acetoxymethyl ester (calcein-AM), P-glycoprotein substrates, decreased in paclitaxel- or eribulin-treated LS174T cells. Eribulin also increased *MDR1* promoter activity in human breast cancer MCF7 cells. The results suggest that the microtubule-targeting anticancer drug eribulin can induce the drug efflux transporter P-glycoprotein via PXR in human intestinal and breast cancer cells and thus influence the efficacy of anticancer drugs.

## 1. Introduction

Multidrug resistance—the resistance of tumors to chemically unrelated anticancer drugs—is one of the most formidable challenges in the field of cancer chemotherapy. Although many mechanisms mediate multidrug resistance, the first mediator of multidrug resistance to be recognized at the molecular level was P-glycoprotein, discovered in multidrug-resistant cancer cell membranes [1,2]. P-glycoprotein, encoded by the multidrug resistance 1 (*MDR1*, or *ABCB1*) gene, is the first member of the large adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily of membrane transport proteins. The molecular structure of P-glycoprotein comprises 12 transmembrane domains that form 1 drug-binding pore and 2 ATP-binding sites. P-glycoprotein mediates resistance to a variety of pharmacologically unrelated anticancer drugs, such as vinblastine, vincristine, daunorubicin, epirubicin, etoposide, imatinib, irinotecan, and paclitaxel, by actively transporting drugs from the cells, lowering intracellular concentrations [1,2].

Many drugs and steroid hormones induce P-glycoprotein expression. The nuclear receptor pregnane X receptor (PXR) plays a key role in regulating P-glycoprotein expression [3,4]. PXR is activated by a broad range of drugs and xenobiotics, including paclitaxel, vinblastine, tamoxifen, rifampicin, dexamethasone, and St. John's Wort component hyperforin [3–7]. Activated PXR forms a heterodimer with human retinoid X receptor α (RXRα) and binds to a cluster of deoxyribonucleic acid (DNA) response elements in a region between base pair (bp) −7975 to −7013 in *MDR1*, thereby inducing gene transcription [3].

The taxane paclitaxel (Taxol) suppresses microtubule dynamics by stabilizing microtubules, blocking mitosis in the G2-M phase and resulting in cell death. Paclitaxel is clinically important for chemotherapy of a number of cancers, including breast, ovarian, and lung cancer. Cabazitaxel (Jevtana, XRP6258) is a new semisynthetic taxane analog with microtubule-stabilizing activity [8]. Cabazitaxel was approved in 2010 in the United States and 2014 in Japan for prostate cancer treatment. Eribulin mesylate (Halaven, E7389) is a synthetic derivative of halichondrin B, a marine natural product, and a nontaxane microtubule dynamics inhibitor [9]. Eribulin destabilizes microtubules, leading to G2-M arrest, resulting in cell death. Eribulin was approved in 2010 in the United States and 2011 in Japan for breast cancer treatment. Paclitaxel is a known substrate and inducer of P-glycoprotein [1,2,5]. Cabazitaxel and eribulin are reportedly transported by P-glycoprotein, but their effects on expression have not been well studied [10–12].

In this study, we investigated the effects of two newly developed

*Abbreviations:* PXR, pregnane X receptor; RXRα, retinoid X receptor α; SMRT, silencing mediator for retinoid and thyroid receptors

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microtubule-targeting anticancer drugs—cabazitaxel and eribulin—on the expression of P-glycoprotein.

## 2. Materials and methods

### 2.1. Materials

Eagle's minimum essential medium (EMEM), rhodamine 123, and paclitaxel were obtained from Fujifilm Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum (FBS) and pEF6/V5-His A vector were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and calcein-acetoxymethyl ester (calcein-AM) from Dojindo Laboratories (Kumamoto, Japan). Cabazitaxel and eribulin mesylate were obtained from AdooQ BioScience (Irvine, CA, USA). All other chemicals used in the study were of the highest purity available.

The *MDR1* promoter construct (p-10224MDR) was provided by Dr. Oliver Burk (Dr. Margarete Fischer–Bosch Institute of Clinical Pharmacology, Stuttgart, Germany) [3]. The vector expressing human PXR (encoded by *NR1I2*), pEF-hPXR, was provided by Prof. Richard B. Kim (Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada) [13]. The vector expressing the silencing mediator for retinoid and thyroid receptors (SMRT) (encoded by *NCOR2*), pFN21A-hSMRT (pFN21AB0999), was obtained from Kazusa DNA Research Institute (Chiba, Japan).

### 2.2. Cell culture

Human intestinal epithelial LS174T cells (CL-188) and human breast cancer MCF7 cells (HTB-22) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in EMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were then incubated at 37 ˚C in a humidified atmosphere with 5% $CO_2$ and 95% air.

### 2.3. Luciferase assay

LS174T or MCF7 cells were seeded in 24-well plates ($1 \times 10^5$ cells/well) and cultured for 24 h. Then, the cells were transiently transfected with 300 ng of *MDR1* promoter firefly luciferase plasmid (p-10224MDR) and 200 ng of the control HSV-TK *Renilla* luciferase plasmid (pGL4.74) (Promega, Madison, WI, USA) in the absence or presence of 50 ng of pEF-hPXR or pEF6/V5 (empty vector), respectively, or 200 ng of pFN21A-hSMRT using Fugene HD transfection reagent (Promega) for 24 h. Next, the cells were incubated with 0.1, 0.5, 2, or 5 μM anticancer drugs for 48 h and lysed using Promega Reporter Lysis Buffer. Firefly and *Renilla* luciferase activity in cell lysates was measured using the Dual-Glo Luciferase Assay System (Promega) and a GloMax-20/20 luminometer according to the manufacturer's instructions; firefly luciferase activity was normalized to *Renilla* luciferase activity. *MDR1* luciferase activity was measured using cells incubated with ethanol (the vehicle) as the control.

### 2.4. Real-time PCR and western blot analysis

Reverse transcription real-time quantitative PCR assay was performed using a CellAmp Direct RNA Prep Kit for RT-PCR (Real Time), PrimeScript RT Master Mix (Perfect Real Time), TB Green Premix Ex Taq II (Tli RNaseH Plus), and a Thermal Cycler Dice Real Time System TP-800 (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The specific primer pairs used for human *MDR1* and β-actin (*ACTB*) were as follows: *MDR1*, 5′-ATGTCACCATGGATGAGATT GAGA-3′ and 5′- TGGCGATCCTCTGCTTCTG-3’; *ACTB*, 5′-ACCGAGCG CGGCTACA-3′ and 5′-CAGCCGTGGCCATCTCTT-3’. The threshold cycle ($C_T$) value for each mRNA was determined using the crossing point method. The relative mRNA levels of *MDR1* were normalized to *ACTB* as follows: $C_T$ (*MDR1*) - $C_T$ (*ACTB*) = $\Delta C_T$. Then, the relative mRNA levels of *MDR1* after anticancer drug treatment were calculated using the $\Delta\Delta C_T$ method: $\Delta\Delta C_T = \Delta C_T$ (anticancer drug) - $\Delta C_T$ (vehicle). The fold changes in mRNA levels of *MDR1* upon anticancer drug treatment were expressed as $2_T^{-\Delta\Delta C}$.

Cell lysates were prepared using Laemmli sample buffer without 2-mercaptoethanol and bromophenol blue. Protein concentrations were measured using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Then, 2-mercaptoethanol [final concentration 5% (v/v)] and bromophenol blue [final concentration 0.005% (w/v)] were added to the sample. The protein samples (3 μg protein/lane) were subjected to SDS-PAGE and immunoblotting using 4%–20% Mini-Protean TGX gels (Bio-Rad) and Can Get Signal immunoreaction enhancer solution (Toyobo, Osaka, Japan). The antibodies used were as follows: mouse monoclonal C219 (Enzo Life Sciences, Lausen, Switzerland) against human P-glycoprotein (1:100), horseradish peroxidase (HRP)-conjugated goat polyclonal anti-mouse IgG (1:5000; Jackson ImmunoResearch, Inc., West Grove, PA, USA), and HRP-conjugated mouse monoclonal AC-15 (ab49900; Abcam plc., Cambridge, UK) against human β-actin (1:200,000). Immunostar Zeta (Fujifilm Wako Pure Chemical Industries, Ltd.) was used for the chemiluminescent detection of proteins with an ImageQuant LAS 4000 (GE Healthcare UK, Ltd., Little Chalfont, UK). Protein levels of P-glycoprotein were determined using ImageQuant TL software (GE Healthcare UK, Ltd.) and normalized to β-actin.

### 2.5. Measuring the cellular uptake of fluorescent P-glycoprotein substrates

LS174T cells were seeded in 24-well plates and incubated with 0.5 μM anticancer drugs for 96 h. The medium was then aspirated and the cells were washed to remove the remaining drug. The drug-treated cells were incubated with 20 μM rhodamine 123 or 1 μM calcein-AM at 37 ˚C for 1 h, and the medium was subsequently aspirated. The cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with 0.1% Triton-X100 in PBS. The fluorescence intensity of rhodamine 123 and calcein-AM-derived calcein in the cells was measured using a DTX-880 microplate fluorometer (Beckman Coulter, Inc., Indianapolis, IN, USA), with excitation and emission wavelengths of 485 and 535 nm, respectively. Protein concentrations were measured using the detergent-compatible bicinchoninic acid (BCA) method and a TaKaRa BCA protein assay kit (Takara Bio Inc.). Bovine serum albumin was used as the standard. Fluorescence intensities were normalized to protein concentrations, and cellular uptake was calculated as a percentage of the ethanol (vehicle) control.

### 2.6. Statistical analysis

Data were expressed as mean ± standard deviation (SD). The statistical significance of differences was determined using one-way analysis of variance (ANOVA) followed by Dunnett's test, and $p < 0.01$ was considered statistically significant.

## 3. Results and discussion

### 3.1. Effects of microtubule-targeting anticancer drugs on MDR1 promoter activation, mRNA and protein expression of P-glycoprotein in LS174T cells

LS174T cells are a suitable model for investigating PXR-dependent *MDR1* transactivation [3,7]. We first tested the effects of paclitaxel, cabazitaxel, and eribulin on *MDR1* promoter activation in LS174T cells. The promoter construct containing the entire 10.2 kbp *MDR1* promoter region (p-10224MDR) was used [3]. We observed that paclitaxel and eribulin activated the *MDR1* promoter in a concentration-dependent manner, while cabazitaxel had little effect (Fig. 1A).

We examined the effects of PXR and SMRT overexpression to clarify PXR's role on *MDR1* transactivation via anticancer drugs. LS174T cells were treated with paclitaxel, cabazitaxel, and eribulin in the presence

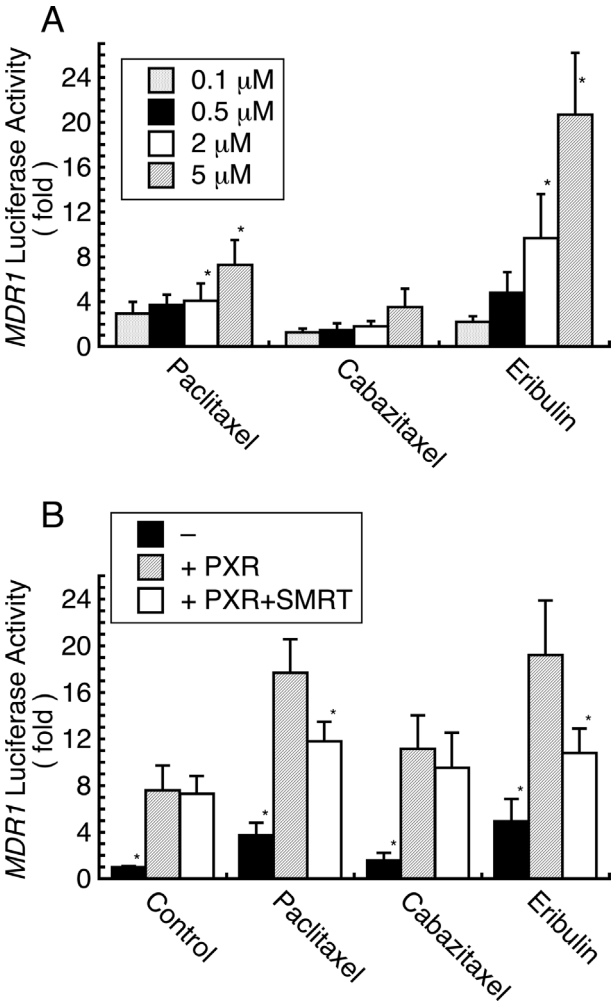


**Fig. 1.** Effects of microtubule-targeting anticancer drugs on *MDR1* promoter activation in LS174T cells. (A) LS174T cells transfected with the *MDR1* reporter vector were incubated with 0.1, 0.5, 2, or 5 μM anticancer drugs for 48 h, and luciferase activity was subsequently analyzed. *MDR1* luciferase activity was measured using cells incubated with the ethanol (vehicle) control. Dotted bars: 0.1 μM; closed bars: 0.5 μM; open bars: 2 μM; hatched bars: 5 μM. The data represent the mean ± SD of 6–9 measurements from 3 independent experiments. *$p < 0.01$ compared to the control. (B) Effects of PXR and SMRT on MDR1 promoter activation. LS174T cells were transfected with the MDR1 reporter vector in the presence of pEF6/V5 (empty vector), or of pEF-hPXR, and pFN21A-hSMRT. Transfected cells were incubated with 0.5 μM anticancer drugs for 48 h, and luciferase activity was subsequently analyzed. MDR1 luciferase activity in cells transfected in the absence of pEF-hPXR and pFN21A-hSMRT incubated in the absence of drugs was defined as 1. Closed bars: empty vector; open bars: human PXR; hatched bars: human PXR and SMRT. The data represent the mean ± SD of 9 measurements from 3 independent experiments. *$p < 0.01$ compared to cells transfected in the presence of pEF-hPXR ( + PXR).

of pEF6/V5 (empty vector), pEF-hPXR alone, and a combination of pEF-hPXR and pFN21A-hSMRT. PXR significantly enhanced paclitaxel- and eribulin-induced *MDR1* promoter activation, but these enhancements were reversed by nuclear co-repressor SMRT (Fig. 1B). Studies have shown that human PXR interacts with SMRT [5]. Hirooka-Masui *et al.* reported that SMRT repress PXR-mediated *MDR1* promoter activation in LS174T cells [7]. Our results suggest that both paclitaxel and eribulin activate PXR and induce *MDR1* transcription.

Fig. 2 shows the real-time PCR and Western blot analyses of P-glycoprotein in the LS174T cells incubated with 2 μM anticancer drugs for 48 h. *MDR1* mRNA levels were significantly increased by paclitaxel and eribulin (Fig. 2A). Eribulin also increased the protein expression

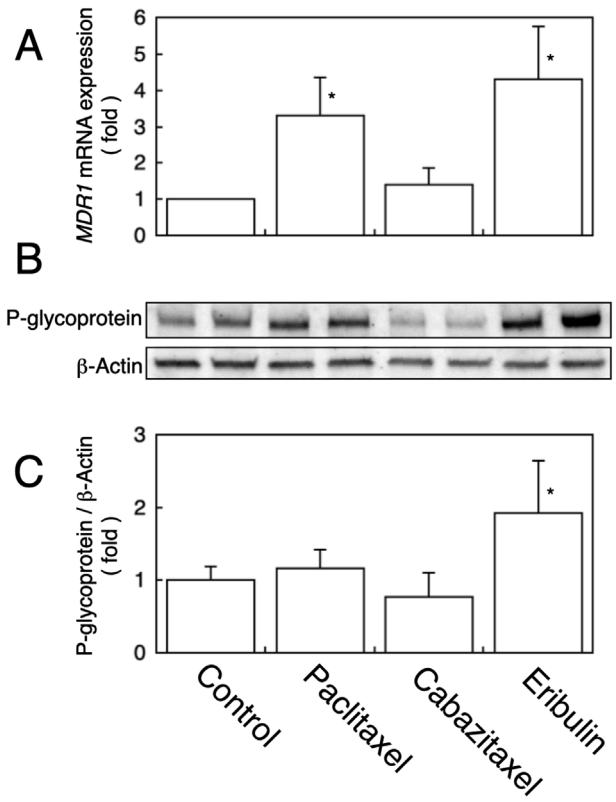


**Fig. 2.** Expression of mRNA and protein of P-glycoprotein in anticancer drug-treated LS174T cells. LS174T cells were incubated with 2 μM anticancer drugs for 48 h. (A) Real-time PCR analysis of *MDR1* mRNA levels. The data shown represent the mean ± SD of 3 independent experiments. (B) Representative Western blot of P-glycoprotein and β-actin. (C) Western blot analysis of protein levels of P-glycoprotein. The data shown represent the mean ± SD of 3 independent experiments. *$p < 0.01$ compared to the control.

(Fig. 2B and C).

### 3.2. Cellular uptake of P-glycoprotein substrates in anticancer drug-treated LS174T cells

Rhodamine 123 is a fluorescent P-glycoprotein substrate, and calcein-AM, an acetoxymethyl ester of calcein, is a nonfluorescent P-glycoprotein substrate [1]. When calcein-AM crosses the cell membrane, cytosolic nonspecific esterases immediately convert it to the highly fluorescent compound calcein. We observed that rhodamine 123 and calcein-AM accumulation decreased in cells treated with paclitaxel or eribulin, indicating an increase in the efflux of substrates from the cells (Fig. 3).

Posttranscriptional regulations, such as phosphorylation and acetylation, are not known for P-glycoprotein function [1,2]. P-glycoprotein has four serine residues that can be phosphorylated by protein kinases A and C. P-glycoprotein lacking all phosphorylation sites, however, exhibits normal transport function [14]. The level of protein in the plasma membrane is considered a sole determinant of the transport activity of P-glycoprotein in the absence of other substrates and/or inhibitors. Therefore, the decreased uptake of P-glycoprotein substrate in eribulin-treated cells indicates eribulin-induced expression of functional P-glycoprotein.

In addition to multidrug-resistant cancer cells, P-glycoprotein is expressed in normal tissues, such as the intestine, liver, kidney, placenta, and blood–brain barrier. P-glycoprotein mediates cellular elimination of clinically important drugs, including verapamil, digoxin, tacrolimus, clarithromycin, fexofenadine, and saquinavir. This shows that P-glycoprotein plays an important role in drug absorption, distribution,

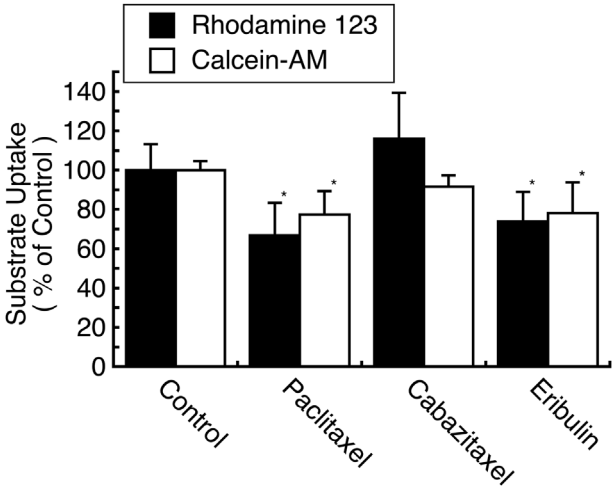


**Fig. 3.** Cellular uptake of rhodamine 123 or calcein-AM in anticancer drug-treated LS174T cells. The cells were incubated with 0.5 μM anticancer drugs for 96 h and then washed and further incubated with 20 μM rhodamine 123 or 1 μM calcein-AM at 37 °C for 1 h. Cellular uptake was calculated as the percentage of the ethanol (vehicle) control. Closed bars: rhodamine 123; open bars: calcein-AM. The data represent the mean ± SD of 6–9 measurements from 3 independent experiments. *$p < 0.01$ compared to the control.

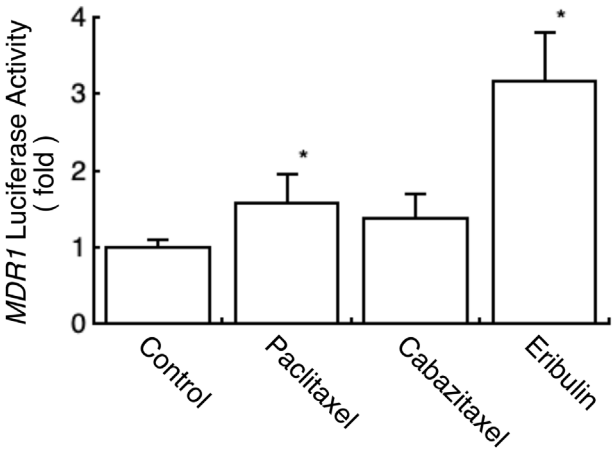


**Fig. 4.** Effects of microtubule-targeting anticancer drugs on *MDR1* promoter activation in MCF7 cells. The cells transfected with the *MDR1* reporter vector were incubated with 5 μM anticancer drugs for 48 h, and luciferase activity was subsequently analyzed. *MDR1* luciferase activity was measured using cells incubated with the ethanol (vehicle) control. The data represent the mean ± SD of 9 measurements from 3 independent experiments. *$p < 0.01$ compared to the control.

and elimination, and these effects ultimately determine drug efficacy and toxicity [1,2]. P-glycoprotein, for example, acts as a biochemical blood–brain barrier to restrict the entry of drugs into the brain. Therefore, we can presume that changes in P-glycoprotein expression by anticancer drugs modulate the pharmacokinetics of P-glycoprotein substrate drugs.

### *3.3. Effects of microtubule-targeting anticancer drugs on MDR1 promoter activation in MCF7 cells*

PXR is reported to be expressed in several types of cancers, including breast and prostate carcinomas. PXR is detected in breast tumor tissues and breast cancer cell line MCF7, the PXR ligand SR12813 induces P-glycoprotein expression in MCF7 cells, and eribulin is used to treat breast cancer [6].

We investigated the effects of anticancer drugs on *MDR1* promoter activation in MCF7 cells. At a concentration of 5 μM, paclitaxel and eribulin activated the *MDR1* promoter in MCF7 cells (Fig. 4). Product information of eribulin mesylate injection (Halaven, Eisai Co., Ltd., Tokyo, Japan) indicates that maximum plasma concentration (*C*max) of eribulin in Japanese cancer patients following administration of a 1.4 mg/m$^2$ dose was 519.4 ± 107.2 (ng/mL) (0.712 ± 0.147 (μM)), and half-life ($t_{1/2}$) was 39.4 ± 8.3 (h). Eisai reported that eribulin exposure increased 2.5-fold in patients with moderate hepatic impairment compared to patients with normal hepatic function. It is also reported that eribulin exposure increased 2-fold in patients with moderate renal impairment compared to patients with normal renal function. Therefore, it can be presumed that administration of eribulin can induce P-glycoprotein in cancer patients.

Newly developed microtubule-destabilizing anticancer drug eribulin activate the *MDR1* promoter in LS174T cells. PXR enhances this effect, and co-repressor SMRT reverses the enhancement. Cellular uptake of P-glycoprotein substrates decreases in paclitaxel- and eribulin-treated LS174T cells. Eribulin activate the *MDR1* promoter in MCF7 cells. Together, these results suggest that eribulin can induce P-glycoprotein and modulate the pharmacokinetics and efficacy of anticancer drugs.

## CRediT authorship contribution statement

**Tomohiro Nabekura:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Tatsuya Kawasaki:** Investigation, Writing - review & editing. **Misuzu Jimura:** Investigation. **Koichi Mizuno:** Investigation. **Yuichi Uwai:** Writing - review & editing.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100727.

## Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100727.

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