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Construction of a model cell line for the assay of MDR1 (Multi Drug Resistance gene-1) substrates/inhibitors using HeLa cells

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Cancer cells often become resistant to chemotherapy, and induction of the ABC transporter Multi-drug Resistance gene-1 (MDR1) is a major cause. We established a tool for high-throughput screening of substrates and inhibitors of MDR1, using transformed HeLa cells that over-express MDR1. The cDNA for human MDR1 was subcloned into the eukaryotic expression vector pBK-CMV to produce an MDR1 expression vector, pBK-CMV/MDR1. HeLa cells were transfected with pBK-CMV/MDR1 or the empty vector pBK-CMV. Transfection of the vector sequence for MDR1 and its expression were evaluated by genomic PCR and western blotting, respectively. The efficiency of the MDR1 transporter for pumping a substrate out of the transformed cells was evaluated using rhodamine123 (R-123), a mitochondrial dye that is also an MDR1 substrate. After treatment of the MDR1-expressing HeLa cells with MDR1 substrate vinblastin or inhibitors cyclosporin A and verapamil, the amount of R-123 retained in the cells was increased to 2 to 2.3 times the level in untreated MDR1-expressing HeLa cells. The transfection of empty pBK-CMV had no effect on the R-123 retention in HeLa cells, regardless of drug treatment. In conclusion, we have established a model human carcinoma cell line that expresses functional MDR1 and can be used to screen for substrates and inhibitors of MDR1.

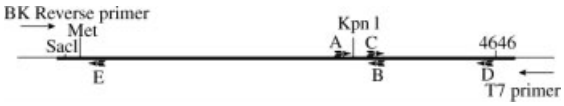
1. Introduction

It is well known that long-term treatment with anti-cancer drugs can lead to the acquisition of drug tolerance by patients' cancer cells, late in the therapeutic period. Although several mechanisms for this tolerance have been proposed (Hao et al. 1994; Kuzumich and Tew 1991), it is generally agreed that the expression of the MDR1 protein (the gene product of *Multi-Drug Resistance gene-1*) on the cancer cells is one of the main culpable factors.

MDR1 (also called P-glycoprotein or ABCB1) is the best understood of the ABC transporter proteins. The characteristic features of the ABC family transporters are an ATP Binding Cassette in the cytoplasmic region and, typically, a 12-pass transmembrane structure (Gottesman 1993). Since it was first cloned in 1986 (Gros et al. 1986), MDR1's characteristics as a membrane protein have been studied by many researchers; its most important feature, its function as a "pump", was revealed by Horio et al. in 1988, using partially purified membrane vesicles (Horio et al. 1988). In 1993, Gottesman and Pastan advocated the "hydrophobic vacuum cleaner model" for the MDR1 transporter mechanism, which explained MDR1's broad substrate specificity (Gottesman and Pastan 1993).

It is precisely MDR1's broad substrate specificity and transporter functions that sometimes cause serious problems in cancer chemotherapy (Noonan et al. 1990; Roninson 1992). Long-term exposure to chemotherapy drugs induces the transcriptional activation of the *MDR1* gene, and the increased expression of the MDR1 transporter protein on the cancer cells' surface causes significant amounts of the anticancer drugs to be pumped out of the cell (Yuen and Sikic 1994). Moreover, many anticancer drugs are substrates for MDR1, so multiple administrations of anticancer drugs can induce MDR1 activity in malignant cells rather easily (Marzolini et al. 2004). Therefore, it would be beneficial to find anticancer chemical compounds (either approved medicines or other chemical compounds) that are not suitable substrates for MDR1, or that inhibit it. Furthermore, such a compound that also had apoptosis-inducing activity in cancer cells would be an ideal candidate for cancer chemotherapy, because it would remain in the target cells long enough to induce apoptosis.

To cherry-pick the most useful small compounds that exert these biological effects from among the wide variety of available chemical libraries (Kugawa et al. 2007), a high-throughput screening assay is indispensable. Here, we created a biological assay to select molecules that are not MDRI substrates, i.e. that can remain in the cancer cells



Primers:	A	#3091-3111	GTA	GAA	GGT	GCT	GGG	AAG	AT
	B	#3402-3421	CAT	TTG	CTC	CTG	ACT	ATG	CC
	C	#3421-3402	GGC	ATA	GTC	AGG	AGC	AAA	TG
	D	#4568-4549	AAG	GCA	GTC	AGT	TAC	AGT	TC
	E	# 591- 571	AAA	GTT	CCC	ACC	ACC	ATA	TA

Fig. 1: PCR primer sets for detecting MDR1 cDNA, and primers annealing sites on the pBK-CMV plasmid
(Top) The thick horizontal line indicates the subcloned human MDR1 cDNA inserted into pBK-CMV (thin lines at the left and right ends). The annealing sites of primers are indicated as arrows on the MDR1 cDNA. The letters correspond to the sequences listed below. (Bottom) The primer positions according to the bp numbers of the human MDR-1 mRNA sequence (accession number M14758 at NCBI) and primer sequences are shown. The length of the entire MDR1 cDNA is 4646 bp. The ORF starts at bp 425 and ends at bp 4267

and would be expected to induce apoptosis, for use in high-throughput screening. To do this, we chose HeLa cells, because they are widely acknowledged to be a representative human cancer cell line, and we had already observed that HeLa cells are susceptible to apoptosis induced by an analgesic, buprenorphine hydrochloride (Kugawa et al. unpublished data). In this article, we report that our new, stable MDR1-expressing HeLa cell line is a suitable tool for the evaluation of MDR1 transporter activity.

2. Investigations, results and discussion

2.1. Confirmation of the HeLa/MDR1 and HeLa/vec lines

After introduction of the pBK-CMV/MDR1 or pBK-CMV plasmid into HeLa cells, G418 selection was conducted for about 4 weeks at a high concentration (2 mg/ml). To verify the integration of the *MDR1* cDNA, genomic DNA was purified from some of the candidate HeLa cell clones and used as the template for PCR. Fig. 1 shows the PCR primers and their predicted annealing positions on the pBK-CMV/MDR1 plasmid. The predicted PCR product for primers A and B was about 330 bp. The predicted product of primers C and D was about 1,170 bp. To confirm the integration of the control empty pBK-CMV vector into the HeLa cell genome, the BK-reverse and T7 primers were used to amplify a 250-bp product of the plasmid alone.

Although some nonspecific bands were observed, clones E2 and E3 clearly yielded PCR products of the expected sizes (Fig. 2-A and 2-B). Figure 2-C shows that at least three HeLa cell clones (VE2, VE4, and VF4) had integrated the pBK-CMV vector. Thus, we obtained at least two stably transfected lines of HeLa/MDR1 (E2 and E3) and three of HeLa/vec (VE2, VE4, and VF4).

We next analyzed the expression level of the MDR1 protein by western blotting. After routine cultivation of the HeLa/E2, HeLa/E3, and the HeLa/vec (VF4) lines, the cells were harvested and pelleted as described in the Experimental section. Twenty micrograms of protein from each HeLa cell lysate were fractionated by 7.5% SDS-PAGE and subjected to western blotting to detect MDR1 protein expression. Fig. 2-D shows a ca. 170-kD band, which is close to the predicted size for the MDR1 protein, in the HeLa/MDR1/E2 and HeLa/MDR1/E3 cells. Because of the slightly higher expression level of the MDR1 protein by the E2 clone, we chose to use it for the subsequent experiments, as "HeLa/MDR1 cells." Western blotting of control HeLa cell lines that had no pBK-CMV vector was performed to detect intrinsic MDR1 expression, but none was observed in the cell line we used in this study (data not shown).

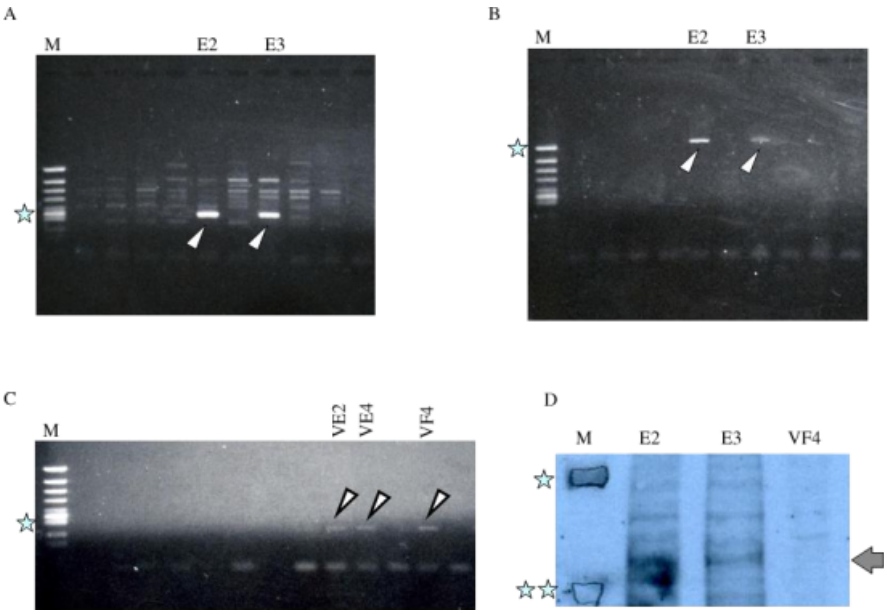


Fig. 2: MDR1 cDNA in transformed HeLa cell clones and MDR1 protein expression
A: Genomic DNA was purified from pBK-CMV/MDR1-transfected HeLa cells, and the integration of human MDR1 was examined by genomic PCR with primers A and B. Clones E2 and E3 showed an amplified band of ca. 300 bp (arrowheads). M, ϕX174-*Hinc* II-digested markers. Star indicates the position of the 335-bp marker. B: The integration of human MDR1 was examined by genomic PCR with primers C and D. Clones E2 and E3 showed an amplified band of ca. 1,170 bp (arrowheads). M, ϕX174-*Hinc* II-digested markers. Star indicates the 1,057-bp marker. C: Genomic DNA was purified from pBK-CMV-transfected HeLa cells, and the integration of the vector plasmid was examined by genomic PCR with the BK Reverse and T7 primers. Clones VE2, VE4, and VF4 showed an amplified band of ca. 250 bp (arrowheads). M, ϕX174-*Hinc* II-digested markers. Star indicates the 291-bp marker. Some bands that ran below 250 bp are from primer dimers. D: The over-expressed MDR1 protein in transformed HeLa cell clones was detected by western blotting. Lysates from HeLa cell clones E2 and E3 contained an MDR1 antibody−reactive band at about 170 kD (arrow), but no signal was detected in a HeLa cell clone bearing only the pBK-CMV vector (VF4). In lane M, the two bands marked with stars (traced with a pencil to make them more prominent) are the 247-kD and 127-kD standard markers. The arrow indicates the position of the 170-kD MDR1 protein

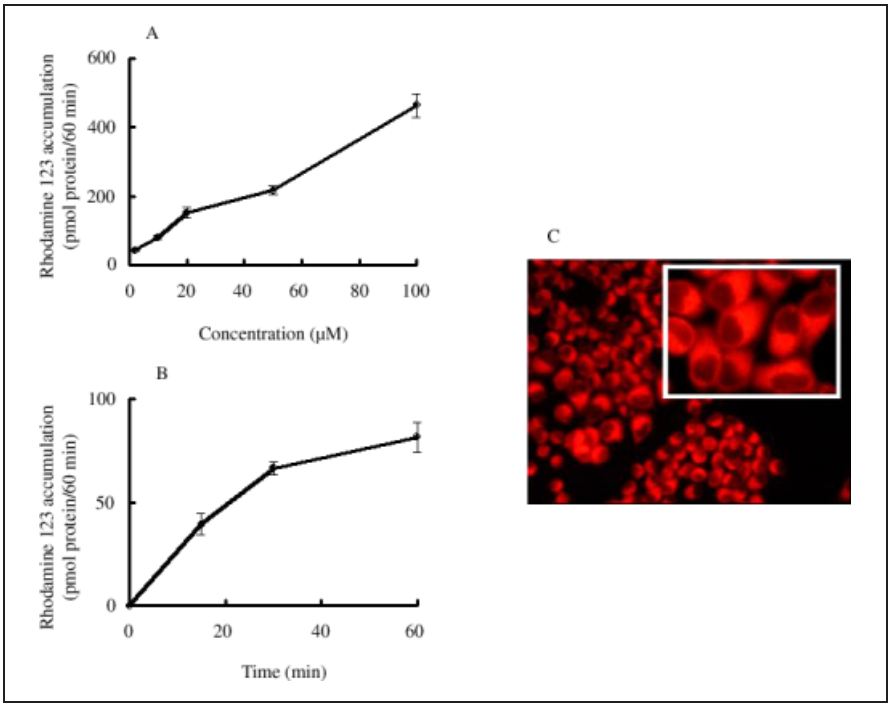


Fig. 3:
Rhodamine 123 (R-123) accumulation in HeLa/MDR1 cells
A: Concentration dependence of R-123 accumulation. HeLa/MDR1 cells were incubated in HBSS buffer with various concentrations of R-123 for 60 min at 37 °C, as described in the Experimental section. B: Time course of R-123 accumulation in HeLa/MDR1 cells. The concentration of R-123 was fixed at 10 μM. Cells were incubated in R-123 in HBSS buffer for up to 60 min at 37 °C, and the accumulated R-123 was measured. Each data point represents the mean ± S.E. (n = 6). C: Fluorescence micrograph of HeLa/MDR1 cells after treatment with R-123. HeLa/MDR1 cells were incubated in HBSS containing 10 μM R-123 for 30 min, then photographed as described in the Experimental section. The accumulated R-123 fluorescence in mitochondria is visible in the cytosol of the HeLa/MDR1 cells. Magnification × 200; Inset × 400

2.2. Rhodamine 123 accumulation in Hela/MDR1 cells

Rhodamine 123 (R-123), a fluorescent dye that accumulates in mitochondria, is also a known MDR1 substrate (Hisch-Ernest et al. 2001) and a well-established tool for studying drug transport by MDR1 (Woodahl et al. 2004). First, we evaluated the accumulation of R-123 in HeLa/MDR1 cells. Figure 3 shows the concentration and time dependency of R-123 accumulation in the HeLa/MDR1 cells. The accumulation was almost linear from 1 to 100 μM when incubated for 60 min at 37 °C (Fig. 3-A). To avoid the cytotoxicity of R-123, we used 10 μM R-123 in the subsequent experiments. This is a typical dosage of R-123 when it is used as an MDR1 substrate (Hirsch-Ernes et al. 2001; Woodahl et al. 2004). The time course of R-123 accumulation in HeLa/MDR1 cells was also evaluated. About 70% of the total accumulation at 60 min was observed by 30 min of exposure to R-123 (Fig. 3-B). From the data obtained in Fig. 3-A and -B, we determined that a 10-μM concentration and 30-min exposure period were suitable conditions for assessing the accumulation of R-123 in the HeLa cells. Fig. 3-C shows the examination by fluorescence microscopy of the HeLa/MDR1 cells under these experimental conditions. The red fluorescent signal could be seen in the cytosol of the HeLa/MDR1 cells, clearly demonstrating the accumulation of R-123 dye, which labels mitochondria. This result confirmed the suitability of our experimental conditions.

2.3. MDR1-dependent drug transport

Figure 4 summarizes our drug transport results. To assess the function of the over-expressed MDR1 protein in HeLa cells, we chose three medicines that are known substrates or inhibitors of the MDR1 transporter. HeLa/MDR1 cells were treated with each drug for 60 min before the R-123 treatment, to allow the drugs to diffuse passively into the cells. The medium was then changed to HBSS buffer containing R-123, and the cells were incubated for another 30 min at 37 °C, to allow the passive diffusion of R-123 into the cells. The amount of R-123 retained by the HeLa cells was then evaluated as described in the Experimental section.

None of the three drugs caused morphological changes to the HeLa/MDR1 cells, at least during the 90-min duration of this experiment (data not shown). However, in the HeLa/MDR1 cells, the amount of retained R-123 was about 1.5 times greater when the cells were treated with cyclosporin A, 2.3 times greater with verapamil, and 2 times greater with vinblastine, than in the untreated HeLa/MDR1 cells (control bar in Fig. 4). These results clearly showed that these three drugs inhibited the efflux of R-123 driven by the MDR1 protein expressed on the HeLa cell membrane. On the other hand, the amount of R-123

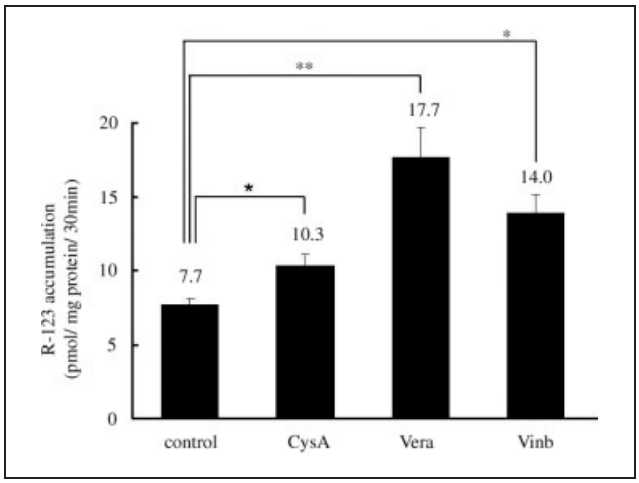


Fig. 4: Effect of MDR1 substrate and inhibitors on the R-123 accumulation in HeLa cells
HeLa/MDR1 cells were treated with an MDR1 substrate (vinblastine) or one of two inhibitors (cyclosporin A or verapamil). CysA, 10 μM cyclosporin A; Vera, 100 μM verapamil; Vinb, 50 μM vinblastine. Control shows the R-123 retained in HeLa/MDR1 cells without drug treatment. The numbers above each bar represent the actual concentration of R-123 that accumulated in the cells. Each bar represents the mean ± S.E. (n = 6). Statistical significance was determined by Student's t-test. Differences were considered statistically significant when the calculated p value was < 0.05 (*) and < 0.01 (**)

remaining in the HeLa/vec cells was almost unaffected by cyclosporin A, verapamil, or vinblastine treatment (data not shown).

Cyclosporin A and verapamil are well-known non-competitive MDR1 inhibitors (Chen et al. 1997; Matsuzaki et al. 1999). On the other hand, vinblastine, an anticancer drug derived from a *Vinca* alkaloid, acts as an MDR1 substrate at the concentration used in this study (Goldberg et al. 1988). However, the result shown in Fig. 4 clearly shows that the R-123 efflux was inhibited in the vinblastine-treated HeLa/MDR1 cells. Although we have not conducted a Scatchard plot analysis of the effects of adding vinblastine at various concentrations to the R-123-accumulated HeLa/MDR1 cells, the vinblastine probably inhibited the efflux of R-123 from HeLa/MDR1 cells in a competitive manner (Ernest and Bello-Reuss 1996), because R-123 and vinblastine are both good substrates of the MDR1 transporter protein.

Some MDR1 over-expressing cell lines have already been established by other researchers. For example, Horio et al. (1989) constructed MDCk cells and Ueda et al. (1992) established porcine-derived LLC-PK1 cells. These MDR1 over-expressing cell lines were derived from canine and porcine kidney epithelial cells, and are widely used for transport studies. However, importantly, our goal is to discover chemical compounds that are capable of inducing apoptosis in tumor cells while not being pumped out by the MDR1 protein. For this purpose, the HeLa cell line, a well-characterized representative human carcinoma-derived cell line, is much more appropriate.

In this report, we constructed a eukaryotic MDR1 expression plasmid, pBK-CMV/MDR1, and established its stable expression in HeLa cell transfectants. Using two well-known MDR1 inhibitors and one substrate, we confirmed that the MDR1 on the HeLa/MDR1 cell membrane functions as a drug efflux pump. Therefore, the HeLa/MDR1 cell line is a promising assay tool for our comprehensive goal of investigating small organic chemical compounds that could cause apoptosis in cancer cells (Kugawa and Aoki 2007). Even compounds that are simply inhibitors or competitive inhibitors of MDR1 might represent powerful new therapeutic agents for treating cancer patients. This report describes our first step toward developing this new chemotherapy.

3. Experimental

3.1. Construction of the MDR1 expression vector

The pMDRA-1 vector, which was originally made by cloning human MDR1 cDNA into pBluescript KS (+) (Kioka et al. 1989), was obtained from the RIKEN DNA Bank (RDB 1372). To construct the eukaryotic MDR1 expression vector pBK-CMV/MDR1, pMDRA-1 was digested with *Sac* I and *Kpn* I, resulting in a 2.5-kb *Sac* I/*Kpn* I fragment and a 1.6-kb *Kpn* I/*Kpn* I fragment. A eukaryotic expression vector, pBK-CMV (Stratagene, USA), was linearized by *Sac* I and *Kpn* I at its multiple cloning site (MCS) and ligated with the 2.5-kb *Sac* I/*Kpn* I fragment of the MDR1 cDNA, resulting in a 7.0-kb pBK-CMV/MDR1 Sac/Kpn construct. Finally, the 1.6-kb *Kpn* I/*Kpn* I fragment was inserted into the *Kpn* I-digested pBK-CMV/MDR1 Sac/Kpn construct, resulting in pBK-CMV/MDR1. The pBK-CMV/MDR1 was sequenced to confirm that it contained the entire ORF of human MDR1 in-frame under control of the CMV promoter.

3.2. Establishment of MDR1-expressing HeLa cells

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (Nissui, Japan) supplemented with 10% fetal bovine serum and the appropriate antibiotics under 5% CO_2 at 37 °C and 100% humidity. Cell morphology was observed using an Olympus IX-70 inverted phase-contrast microscope with an IX-FLA fluorescence observation device.

HeLa cells were transfected with the pBK-CMV/MDR1 plasmid using the TransIT-HeLaMONSTER Transfection Kit® (Mirus, USA), a high-efficacy, low-toxicity DNA transfection reagent optimized for HeLa cells. Transfectants were selected by incubation with 2.0 mg/ml G418 (Gibco BRL, USA) for more than 4 weeks. Three independent G-418-resistant colonies (E2, E3, and F1) were obtained. Clone F1 was so fragile that it was hard to keep the cells alive for routine cultivation, so the data in Fig. 2 are only for E2 and E3. Stably transfected control cell lines harboring the empty pBK-CMV plasmid were also established.

3.3. Confirmation of MDR1 expression in HeLa cells

MDR1 insertion into the HeLa cells was confirmed by examining both its DNA and protein expression levels. To detect the human *MDR1* cDNA in HeLa cells, two sets of PCR primers were constructed (Fig. 1). The total genomic DNA of the HeLa transfectants harboring either pBK-CMV/MDR1 or pBK-CMV was extracted using Qiagen's DNeasy Blood Tissue kit® (Qiagen, USA) and used as a template for PCR experiments. PCR was conducted using TaKaRa's Premix Taq® (EX Taq™ version) (TaKaRa Bio, Japan) under the following conditions: 40 cycles of denaturation at 94 °C for 30 sec, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min. The PCR products were analyzed by 2.0% agarose gel electrophoresis.

The expression of MDR1 protein was analyzed by western blotting. Transfectant HeLa cells were harvested at 70% confluence by trypsinization and washed twice with ice-cold PBS(−) including the appropriate protease inhibitors. After centrifugation, the HeLa cell pellet was solubilized in Laemmli sample buffer (BioRad, USA), and 20 μg of solubilized protein was fractionated by 7.5% SDS-PAGE (poly-acrylamide gel electrophoresis). Western blotting was performed using standard methods. The first antibody was Santa Cruz's Mdr(G-1) SC-13131 antibody (Santa Cruz Biotechnology, USA) diluted 1 : 200. The second antibody was GE Healthcare Amersham's Anti-mouse HRP antibody diluted 1 : 5000 (GE Healthcare, USA). The bands were developed using Cell Signaling's LumiGRO® System (Cell Signaling, USA). Finally, one of the HeLa stable cell lines (E2), which was confirmed to over-express human MDR1, was renamed "HeLa/MDR1." A pBK-CMV vector transfectant line (VF4) was renamed "HeLa/vec."

3.4. Rhodamine 123 accumulation in HeLa/MDR1 cells

To evaluate the function of MDR1 as a transporter protein, a Rhodamine 123 (R-123) uptake experiment was conducted. About 2.5×10^4 HeLa cells/well were seeded into 12-well culture plates (Corning, USA). When the cultures were about 70 to 80% confluent, the cells were washed twice with pre-warmed Hank's balanced salt solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 5.6 mM D-glucose, 0.06 mM phenol red, and 25 mM HEPES, pH 7.4). Next, R-123 at various concentrations (2 to 100 μM) in HBSS was added to the wells, and the cells were further incubated in a CO_2 incubator at 37 °C for 60 min. The influx of R-123 into the cells was halted by aspirating the medium. The cells were then washed three times with ice-cold PBS(−) and pelleted for the R-123 measurement.

The cell pellets were solubilized with 1 ml of 0.3 M NaOH for 60 min, and aliquots (500 μl) were neutralized by adding the same volume of 0.3 M HCl. Two-hundred microliters of the neutralized solution was transferred into 96-well black micro-titer plates (Corning, USA), and the resulting fluorescence intensity of the R-123 was measured under excitation at 485 nm and emission at 520 nm using a FLUOstar OPTIMA® fluorescence spectrophotometer (BMG Labtech, Germany). Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard reference protein.

3.5. Effect of MDR substrate and inhibitors on the accumulation of R-123 in the HeLa cells

To evaluate the effect of substrate and inhibitor compounds on the MDR1 transporter function, the HeLa transfectants were incubated with vinblastine (50 μM), a typical MDR substrate (Renes et al. 1999; Ushigome et al. 2003), or with either of two typical MDRI inhibitors cyclosporin A (1 μM) or verapamil (100 μM) (Horio et al. 1991; Lelong et al. 1991; Ushigome et al. 2003), in HBSS in a CO_2 incubator for 60 min. The drugs were dissolved in ethanol at a final concentration of 0.02%. Just before the R-123 was added, the medium was changed to fresh HBSS buffer.

Ten millimolar R-123 was then added to the HeLa cells, which were incubated for 30 min at 37 °C in the CO_2 incubator. The experiment was halted by the aspiration of HBSS buffer from the well. The cells were washed twice with ice-cold PBS(−), and the remaining R-123 in the HeLa cells was assayed as described in the section above.

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