Reversal Effects of Ca²⁺ Antagonists on Multidrug Resistance *via* Down-regulation of MDR1 mRNA

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   In previous reports, the effects of 12 Ca²⁺ antagonists on a multidrug resistant transporter, P-glycoprotein/MDR1, were evaluated in terms of those on MDR1-mediated transport of [³H]digoxin and the sensitivity of vinblastine sulfate or paclitaxel, and they were able to be classified into 4 subgroups based on their actions, as those with transport inhibition and sensitivity recovery, those with or without transport inhibition but marginal sensitivity recovery, and those without both.   In this study, our previous findings were confirmed by the resistance against doxorubicin hydrochloride and daunorubicin hydrochloride, and by the recovery of [³H] vinblastine sulfate accumulation.   Furthermore, it was found that the effects of 12 Ca²⁺ antagonists on the sensitivity recovery were also explained by the down-regulation of MDR1 mRNA, suggesting a novel mechanism to reverse the MDR1-mediated multidrug resistance.

   The intrinsic or acquired resistance to anticancer drugs remains one of the most serious problems responsible for the failure of cancer chemotherapy [20, 21].   The phenomenon often involves simultaneous resistance to other anticancer drugs, even those that have not been used in the patient, and differ in chemical structure and mode of action.   This phenotype is called multidrug resistance (MDR).   The cellular basis underlying MDR is not fully understood, and a number of mechanisms have been proposed.   The best characterized one is a multidrug resistant transporter, P-glycoprotein/MDR1, with clinical data showing that its overexpression in the tumor results in poor clinical outcome after chemotherapy [21].   MDR1 is a glycosylated membrane protein of 170 kDa, consisting of two similar regions containing six putative transmembrane segments and intracellular binding sites for ATP [13,

15, 17, 18, 20].   MDR1 has been understood to act as an efflux pump to remove anticancer drugs from the cells, and to decrease their intracellular concentrations to below the lethal levels.   It has been an important clinical issue to establish the strategy to reverse MDR1-mediated MDR for the improvement of cancer chemotherapy.

In our previous study, the effects of 12 Ca^{2+} antagonists on MDR1 were evaluated by two independent models in terms of; 1) the inhibitory effect on MDR1-mediated transport of [3H]digoxin, a non-cytotoxic typical MDR1 substrate, using the MDR1-overexpressing cell line LLC-GA5-COL150, established by transfection of human *MDR1* cDNA into porcine kidney epithelial LLC-PK_1, and 2) the effect on vinblastine sulfate (VLB) or paclitaxel (TXL) sensitivities using the MDR1-overexpressing subline Hvr100-6, established from human cervical carcinoma HeLa-Ohio (HeLa) [19, 22].   The Ca^{2+} antagonists could be classified into 4 subgroups based on their action on MDR1; Group I: transport inhibition / sensitivity recovery, including barnidipine, benidipine, manidipine, nicardipine and verapamil; Group II: transport inhibition / marginal sensitivity recovery, including bepridil, efonidipine and nilvadipine; Group III: no transport inhibition / marginal sensitivity recovery, including diltiazem; and Group IV: no transport inhibition / no sensitivity recovery, including nifedipine, nisoldipine and nitrendipine.   For Groups II and III, the difference in the affinity of substrates for MDR1 might be a key factor; however diltiazem of Group III showed about 3 times the recovery of resistance for VLB and TXL, suggesting the mechanisms other than the inhibition of MDR1-mediated efflux transport resulting in the recovery of sensitivity.

This study was conducted to assess the effects of Ca^{2+} antagonists on MDR1 mRNA expression using HeLa and Hvr100-6 cells in order to find a novel strategy to reverse MDR1-mediated MDR.   In advance, the reversal effects on the resistance to doxorubicin hydrochloride (DXR) and daunorubicin hydrochloride (DNR), and the effects on [3H]VLB accumulation were also assessed to confirm our previous findings [19, 22].

MATERIALS AND METHODS

Materials

Barnidipine was a kind gift from Astellas Pharma Inc. (Tokyo, Japan), benidipine was from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), bepridil was from Organon Co., Ltd. (Tokyo, Japan), efonidipine was from Nissan Chemical Industries, Ltd. (Tokyo, Japan), manidipine was from Takeda Chemical Industries, Ltd. (Osaka, Japan), nilvadipine was from Astellas Pharma Inc. (Tokyo, Japan), nisoldipine was from Bayer Yakuhin, Ltd. (Tokyo, Japan), and nitrendipine was from Astellas Pharma Inc. (Tokyo, Japan).   Diltiazem hydrochloride, nifedipine, verapamil hydrochloride, VLB, TXL, DXR and DNR were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).   Nicardipine was purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).   [3H]VLB (422 GBq/mmol) was obtained from Amersham International, plc (Buckinghamshire, UK).   All other chemicals were of the highest purity available.

Cell culture

HeLa cells (396-401 passage), obtained from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan), were maintained in culture medium consisting of Dulbecco's modified Eagle's medium (D-MEM with glucose (4.5 g/l), L-glutamine (4 mM) and sodium pyruvate (1 mM); Cat. No. 12800-017, Invitrogen Corp., Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Lot. No. AGM7413, HyClone, UT, USA) and 100 mg/l kanamycin sulfate (Invitrogen Corp.).   The MDR1-overexpressing subline, Hvr100-6, was established by stepwise increases in the concentration of VLB in the culture medium [4, 23].

FACS and RT-PCR analysis confirmed the induction of MDR1 in Hvr100-6 cells without alterations of the related transporters, including the multidrug resistance-associated protein 1 (MRP1), MRP2, and breast cancer resistance protein (BCRP) [4, 16, 23].   Hvr100-6 cells (82-98 passages) were maintained with 100 nM VLB.   Both of these cell lines (4 and 12 x 10^4 cells/cm^2, respectively) were seeded into culture flasks (Nalge Nunc International, NY, USA), grown in a humidified atmosphere of 5% CO_2-95% air at 37°C, and subcultured every 3 or 4 d with 0.05% trypsin-0.02% EDTA (Invitrogen Corp.).

Effects of 12 Ca^{2+} antagonists on the antiproliferative effects of anticancer drugs in HeLa and Hvr100-6 cells

The antiproliferative effects of VLB, TXL, DXR and DNR were assessed in HeLa and Hvr100-6 cells by the WST-1 (tetrazolium salts) colorimetric assay using a Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan) [4, 8, 23].   Cells (1,000 cells/well) were seeded on 96-well plates (Nunclon^{TM}, Nalge Nunc International) in 100 µl of culture medium without any anticancer drug on Day 0, and the culture medium was exchanged to that containing a test anticancer drug at various concentrations on Day 1.   After incubation for 3 days at 37°C (on Day 4), the culture medium was exchanged in to 110 µl of that containing WST-1 reagent solution (10 µl WST-1 solution and 100 µl of culture medium), and 3 h later, the absorbance was determined at 450 nm with a reference wavelength of 630 nm using a microplate reader (Sjeia Auto Reader II; Sanko Junyaku Co. Ltd., Tokyo, Japan).   The 50% growth inhibitory concentration (IC$_{50}$) of the anticancer drugs in HeLa and Hvr100-6 cells was calculated according to the sigmoid inhibitory effect model as follows using the nonlinear least-squares fitting method (WinNonlin$^®$, ver. 2.1, Pharsight Corp., CA, USA): E $= E_{max} \times [1 - C^\gamma / (C^\gamma + IC_{50}^\gamma)]$.   E and E_{max} represent the surviving fraction (% of control) and its maximum, respectively, and C and γ represent the drug concentration in the medium and the sigmoidicity factor, respectively.   The reversal effects of Ca^{2+} antagonists on antiproliferative effects of anticancer drugs were also assessed by the WST-1 colorimetric assay.   A Ca^{2+} antagonist (1 µM) was added simultaneously with an anticancer drug on Day 1.   This concentration had no antiproliferative effects (data not shown).

Effects of 6 Ca^{2+} antagonists on [^{3}H]VLB accumulation in HeLa and Hvr100-6 cells

Among 12 Ca^{2+} antagonists, barnidipine, manidipine, nicardipine and verapamil of Group I, diltiazem of Group III and nifedipine of Group IV were selected for evaluation, and their accumulation was assessed as described previously [23].   Briefly, the cells (2 × 10^5 cells/ml/well) were seeded into 24-well plates (Nalge Nunc International) in culture medium without VLB, and cultured for 2 days in a humidified atmosphere of 5% CO_2-95% air at 37°C.   The cells were washed twice with warmed complete Hanks' balanced salt solution (HBSS, Cat. No. H-6136, Sigma-Aldrich Chemical).   The accumulation studies were immediately started by the addition of HBSS, including [^{3}H]VLB (1 µM, 18.5 kBq/ml/well) with or without a Ca^{2+} antagonist (1 or 10 µM).   A high concentration of [^{3}H]VLB was necessary for the accurate determination of cellular radioactivity.   Two hours later, the experiment was terminated by the aspiration of HBSS from each well.   Preliminary experiments had demonstrated that on amount of [^{3}H]VLB in the cells was attained at steady-state by the incubation for 90 minutes (data not shown).   The cells were then quickly washed three times with ice-cold PBS (-) on ice, and subsequently lysed in 1 ml of 0.3 N NaOH.   It was confirmed that there was no detectable radioactivity in the ice-cold PBS (-) used for washing.   The radioactivity of the lysed cells was counted in 3 ml of ACS II (Amersham Biosciences Corp., Piscataway, NJ, USA) by liquid scintillation counting (LS6000TA, Beckman Instruments, Inc., CA, USA).   The % recovery of [^{3}H]VLB accumulation was calculated as ([radioactivity in Hvr100-6 with a Ca^{2+} antagonist] -

[radioactivity in Hvr100-6 without Ca^{2+} antagonist]) / ([radioactivity in HeLa without Ca^{2+} antagonist] - [radioactivity in Hvr100-6 without Ca^{2+} antagonist]).

Protein assay

The protein content was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, CA, USA) [2]. Bovine γ-globulin was used as the standard.

Effects of 6 Ca^{2+} antagonists on MDR1 mRNA levels in HeLa and Hvr100-6 cells

Barnidipine, manidipine, nicardipine and verapamil of Group I, diltiazem of Group III and nifedipine of Group IV were selected for evaluation, and the expression levels of MDR1 mRNA were measured by real-time quantitative RT-PCR analysis as described previously [11, 12, 16]. Briefly, HeLa and Hvr100-6 cells were seeded on dishes at a density of 2×10^5 cells/100 mm dish and 6×10^5 cells/100 mm dishes, respectively, in 10 ml of VLB-free culture medium on Day 0. The culture medium was exchanged to that with or without a Ca^{2+} antagonist (1 or 10 μM) on Day 1. After incubation for 3 days (on Day 4), the cells were scraped off from the subconfluent monolayers and the cell pellet was washed twice with ice-cold PBS (-). Total RNA was extracted using an RNeasy Mini kit (QIAGEN, Hilden, Germany) and an RNase-Free DNase Set (QIAGEN). In each run of the assay, mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and MDR1 was analyzed in 5-fold serially diluted samples from an authentic colorectal adenocarcinoma cell line, Caco-2, and the mRNA levels of MDR1 were expressed relative to the concentration of GAPDH mRNA. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. The primer pairs and TaqMan probes for MDR1 mRNA were designed using Primer Express 1.0 (Applied Biosystems, Foster City, CA, USA). Primers and the TaqMan probe for GAPDH were purchased from Applied Biosystems (TaqMan GAPDH Control Reagent Kit).

Statistical analysis

Values are given as the mean ± S.D. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Sheffé's test, providing the variance of groups was similar. *p* values of less than 0.05 (two-tailed) were considered significant.

RESULTS

Effects of 12 Ca^{2+} antagonists on antiproliferative effects of anticancer drugs in HeLa and Hvr100-6 cells

Table 1 summarizes the antiproliferative effects of VLB, TXL, DXR and DNR in HeLa and Hvr100-6 cells, and the alterations in Hvr100-6 cells by 12 Ca^{2+} antagonists. Resistance to VLB, TXL, DXR and DNR were found in Hvr100-6 cells with relative resistances of about 500, 4000, 50 and 200, respectively. The Ca^{2+} antagonists of Group I showed the reversal of resistance to DXR and DNR in Hvr100-6 cells, whereas those of Group IV showed no reversal effects, similar to the cases of VLB and TXL published previously [22]. Group II Ca^{2+} antagonists also showed no reversal, but efonidipine had marginal effects. Diltiazem, belonging to Group III, showed a slight reversal.

Effects of 6 Ca^{2+} antagonists on [³H]VLB accumulation in HeLa and Hvr100-6 cells

Figure 1 shows the cellular accumulation of [³H]VLB in HeLa and Hvr100-6 cells, and the alterations in Hvr100-6 cells by the addition of barnidipine (Group I), diltiazem (III), manidipine (I), nicardipine (I), nifedipine (IV) and verapamil (I). The cellular accumulation of [³H]VLB in Hvr100-6 cells was one-fifth of that of HeLa cells, and this was significantly restored in a concentration dependent manner. With the presence of 1 μM antagonists, the % recovery of the [³H]VLB accumulation reached 31.1±1.4%, 49.9±3.9%, 36.7±2.7% and 24.1±1.3% for the 4 antagonists of Group I, respectively, and at 10 μM, they were

60.8±0.9%, 81.6±1.8%, 79.6±5.7% and 43.7±1.6%, respectively.   However, no effects were observed for diltiazem, and nifedipine showed a slight recovery only at 10 µM.

Effects of 6 Ca^{2+} antagonists on MDR1 mRNA levels in HeLa and Hvr100-6 cells

Figure 2 shows the expression level of MDR1 mRNA in HeLa and Hvr100-6 cells, and their alterations by the addition of barnidipine (Group I), diltiazem (III), manidipine (I), nicardipine (I), nifedipine (IV) and verapamil (I).   In HeLa cells, the expression of MDR1 mRNA was decreased only by 10 µM of manidipine.   In Hvr100-6 cells, 4 antagonists of Group I, barnidipine, manidipine, nicardipine and verapamil, decreased at 1 µM to 96.4±14.5%, 108.6±21.0%, 99.3±7.1% and 71.5±3.8% of the control, respectively, and at 10 µM, the values were 86.8±21.4%, 46.6±19.7%, 92.6±21.7% and 89.8±14.3% of the control, respectively.   Diltiazem also showed a significant reduction of the expression of MDR1 mRNA, but nifedipine had a slight effect only at 10 µM.

Table 1. Effects of 12 Ca^{2+} antagonists (1 µM) on the antiproliferative effects of anticancer drugs in HeLa and Hvr100-6 cells

	VLB		TXL		DXR		DNR	
	IC$_{50}$ (nM)	RR	IC$_{50}$ (nM)	RR	IC$_{50}$ (nM)	RR	IC$_{50}$ (nM)	RR
HeLa cells	0.135 ± 0.036	—	0.136 ± 0.238	—	2.94 ± 0.54	—	1.09 ± 0.31	—
Hvr100-6 cells	67.0 ± 30.1	496	562 ± 255	4145	153 ± 57	52.1	216 ± 87	198
+ Barnidipine (I)	8.03 ± 2.60	59.5	72.5 ± 16.9	533	51.5 ± 62.2	17.5	49.8 ± 34.4	45.7
+ Benidipine (I)	5.48 ± 2.69	40.6	72.1 ± 30.3	530	15.3 ± 13.0	5.2	29.3 ± 17.0	26.9
+ Bepridil (II)	39.5 ± 22.6	293	298 ± 241	2191	180 ± 79	61.2	240 ± 84	220
+ Diltiazem (III)	81.1 ± 54.2	601	190 ± 112	1397	73.5 ± 99.0	25.0	52.8 ± 27.9	48.4
+ Efonidipine (II)	56.9 ± 21.3	421	497 ± 274	3657	55.8 ± 50.5	19.0	78.7 ± 61.8	72.2
+ Manidipine (I)	2.68 ± 1.22	19.9	4.00 ± 1.21	29.4	27.5 ± 18.0	9.35	7.45 ± 3.80	6.83
+ Nicardipine (I)	11.1 ± 8.6	82.2	96.5 ± 37.6	710	57.2 ± 18.3	19.5	67.6 ± 57.8	62.0
+ Nifedipine (IV)	49.2 ± 33.1	364	659 ± 345	4846	152 ± 109	51.7	137 ± 78	126
+ Nilvadipine (II)	22.8 ±13.5	169	264 ± 210	1941	160 ± 79	54.5	55.4 ± 38.5	50.8
+ Nisoldipine (IV)	104 ± 29	770	461 ± 355	3390	151 ± 136	51.4	68.9 ± 48.4	63.2
+ Nitrendipine (IV)	49.9 ± 31.5	370	427 ± 192	3140	155 ± 75	52.7	38.6 ± 46.1	35.4
+ Verapamil (I)	4.57 ± 2.33	33.9	68.3 ± 37.2	502	41.4 ± 21.1	14.1	120 ± 98	110

Previously, it has been suggested that 12 Ca^{2+} antagonists can be classified into Group I-IV based on the effects on MDR1-mediated efflux transport and the recovery of sensitivity, as transport inhibition/sensitivity recovery = yes/yes (I), yes/marginal (II), no/marginal (III) and no/no (IV) [19, 22]. The values are the means ± S.D. of four-eight independent experiments.   "RR" means relative resistance, the ratio of IC$_{50}$ values for Hvr100-6 cells in the absence or presence Ca2+ antagonists to that for HeLa cells.   The data on VLB and TXL were already published [19, 22] and presented to compare with those on DXR and DNR obtained in this study.
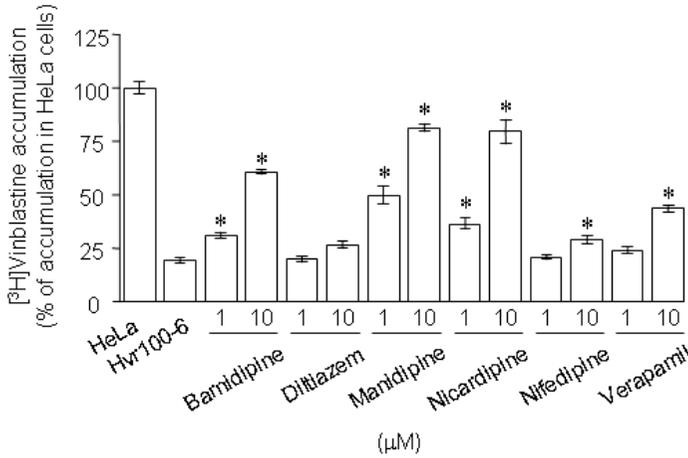
Figure 1: Effects of 6 Ca^{2+} antagonists on [^{3}H]VLB accumulation in HeLa and Hvr100-6 cells. In previous reports, the effects of 12 Ca^{2+} antagonists were evaluated in terms of those on MDR1-mediated transport of [^{3}H]digoxin and the sensitivity of VLB or TXL, and they could be classified into 4 subgroups based on their actions. Barnidipine, manidipine, nicardipine and verapamil belong to Group I, and diltiazem and nifedipine belong to Group III and IV, respectively. Hvr100-6 cells were incubated in HBSS containing [^{3}H]VLB (1 µM, 18.5 kBq) for 2 h at 37°C in the absence and presence of Ca^{2+} antagonists (1 or 10 µM). The accumulation of [^{3}H]VLB in HeLa cells in the absence of Ca^{2+} antagonists is shown as a reference. Each bar represents the mean ± S.D. of 3 experiments.
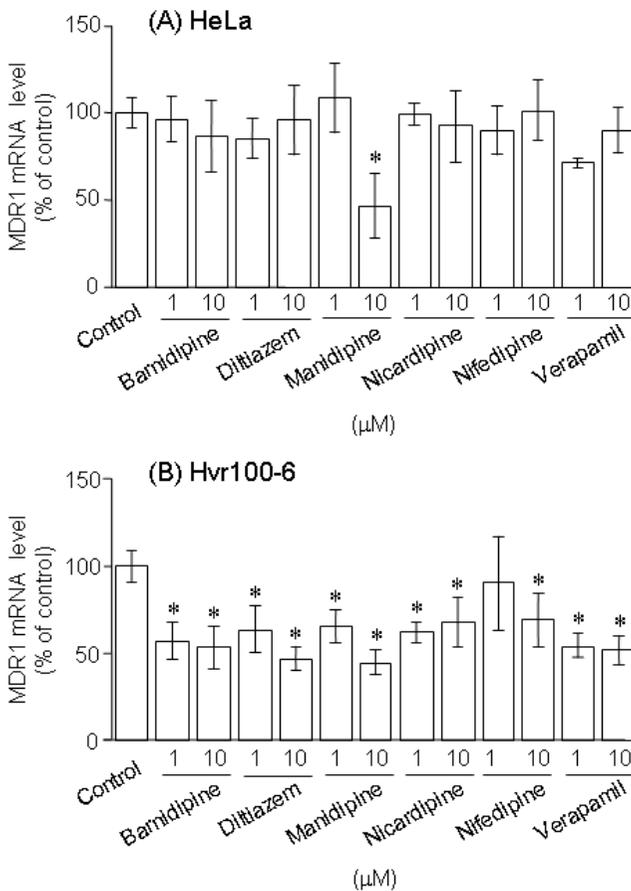
Figure 2: Effects of 6 Ca^{2+} antagonists on MDR1 mRNA levels in HeLa and Hvr100-6 cells. (A) HeLa cells, (B) Hvr100-6 cells. In previous reports, the effects of 12 Ca^{2+} antagonists were evaluated in terms of those on MDR1-mediated transport of [^{3}H]digoxin and the sensitivity of VLB or TXL, and they could be classified into 4 subgroups based on their actions. Barnidipine, manidipine, nicardipine and verapamil belong to Group I, and diltiazem and nifedipine belong to Group III and IV, respectively. Cells were incubated in culture medium with or without Ca^{2+} antagonists (1 or 10 µM) for 3 d at 37°C. MDR1 mRNA levels were determined by real-time quantitative RT-PCR. Data were calculated as the ratios relative to those without Ca^{2+} antagonists. The expression levels of MDR1 mRNA (/GAPDH mRNA) were 4.22±0.35 and 439.36±36.98 in HeLa and Hvr100-6 cells, respectively. Each bar represents the mean ± SD. of 3 experiments.

DISCUSSION

In 1981, Tsuruo et al. reported that verapamil overcame the resistance to vincristine in vitro and in vivo [24]. They and Rogan et al. demonstrated that this effect was via the inhibition of the efflux of anticancer drugs in the resistant cells [14]. After then, we and Katoh et al. indicated that other Ca^{2+} antagonists also had the inhibitory effects on the MDR1-mediated efflux transport of anticancer drugs, and thereby their co-administration resulted in the recovery of MDR [5, 19, 22]. However, during substantial investigations in vitro, it has been suggested that 12 Ca^{2+} antagonists can be classified into Group I-IV based on the effects on MDR1-mediated efflux transport and the recovery of sensitivity, as transport inhibition/sensitivity recovery = yes/yes (I), yes/marginal (II), no/marginal (III) and no/no (IV). The difference in the affinity of substrates for MDR1 might be a key factor; however the data for Group III suggested the mechanisms other than the inhibition of MDR1-mediated efflux transport resulting in the recovery of sensitivity. This study was focused on the effects of Ca^{2+} antagonists on MDR1 mRNA expression using HeLa and Hvr100-6 cells. In advance, the reversal effects on the resistance to DXR, DNR and 5-FU, and the effects on [^{3}H]VLB accumulation were also assessed to confirm our previous findings on VLB and TXL [19, 22].

As summarized in Table 1, the Ca^{2+} antagonists of Group I recovered the resistance to DXR and DNR. Those of Group II and III had no or marginal effects on sensitivity against DXR and DNR. Group IV antagonists had no effects. Thus, our previous findings on the recovery of sensitivity against VLB and TXL [19, 22] were confirmed herein using anthracyclines. The effects of Ca^{2+} antagonists on MDR1-mediated efflux transport was also evaluated herein in terms of the effects on [^{3}H]VLB accumulation (Figure 1). Those of Group I inhibited the transport of [^{3}H]VLB in Hvr100-6 cells, and diltiazem of Group III and nifedipine of Group IV had no effects. These data were consistent with their inhibitory effects on MDR1-mediated [^{3}H]digoxin transport across the cell monolayers of a human MDR1 transfectant, LLC-GA5-COL150 [19, 22]. Collectively, our previous findings resulting in Group classification of 12 Ca^{2+} antagonists were confirmed.

Herein, the effects of Ca^{2+} antagonists on MDR1 mRNA expression were assessed in order to find a novel strategy to reverse MDR1-mediated MDR. As shown in Figure 2, it was demonstrated that diltiazem of Group III showed a significant reduction of MDR1 mRNA expression. Additionally, it was found that 4 agonists of Group I also had the effects. About 10 years after the pioneering findings on the reversal of MDR by verapamil by Tsuruo et al. [24], Muller et al. [9, 10] reported that verapamil treatment decreased MDR1 expression in human leukemic cell lines possibly through the down-regulation of MDR1 expression. Our findings support the data on verapamil, and moreover indicate the possibility that these effects will be found non-specifically on the Ca^{2+} antagonists, not specifically on verapamil, indicating a novel strategy to reverse MDR1-mediated MDR. Although the mechanisms of mRNA reduction of MDR1 by Ca^{2+} antagonists should be resolved, we recently have found that sorcin, a 22 kDa calcium binding protein, could regulate MDR1 expression [6]. Thus, the reduction of intracellular calcium concentration by Ca^{2+} antagonists may induce down-regulation of MDR1. The down-regulation of MDR1 mRNA expression must contribute to the recovery of resistance via increases of intracellular concentrations of anticancer drugs, in addition to inhibition of the transport of anticancer drugs.

In conclusion, the Ca^{2+} antagonists could be classified into 4 subgroups based on their action on MDR1-mediated efflux transport of and resistance against anticancer drugs. The Ca^{2+} antagonists with no inhibitory effects on MDR1-mediated transport, but with recovery

of resistance will show a down-regulation of MDR1 mRNA. The Ca^{2+} antagonists with the recovery of resistance also show a down-regulation of MDR1 mRNA in addition to the inhibition of MDR1-mediated transport.

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