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A novel quinoline derivative, MS-209, overcomes drug resistance of human lung cancer cells expressing the multidrug resistance-associated protein (MRP) gene

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Abstract *Purpose and methods*: MS-209 is a newly synthesized quinoline compound used orally to overcome human P-glycoprotein (Pgp)-mediated multidrug resistance (MDR). The multidrug resistance-associated protein (MRP) gene is thought to play an important role in MDR in lung cancer. To investigate whether MS-209 could also overcome MRP-mediated MDR, we examined the effect of the compound using a cytotoxicity assay on MDR1 gene-negative drug-selected MDR and wildtype lung cancer cells with various levels of MRP gene expression. The effects of MS-209 were compared with those of verapamil (VER) and cyclosporin A (CsA). The level of MRP gene expression in the cells was evaluated semiquantitatively by RT-PCR. For vincristine (VCR), intracellular accumulation of [^{3}H]-VCR was measured with or without MS-209. *Results*: In MDR UMCC-1/VP small-cell lung carcinoma cell line, 5 μ*M* of MS-209 and VER enhanced the cytotoxicity of etoposide, doxorubicin (DOX) and VCR more than twofold, and completely reversed the resistance to VCR. The mean reversing effects of MS-209 on DOX and VCR were significantly stronger than those of VER and CsA. In wildtype non-small-cell lung carcinoma cells, the effects of MS-209 were almost equal to those of VER and CsA. The effect of these three agents correlated with the level of MRP gene expression. The MS-209-induced increase in intracellular accumulation of VCR was proportional to the level of MRP gene expression in these cells. *Conclusion*: Our results indicate that MS-209 is a potentially useful drug that can overcome MRP-mediated intrinsic and acquired MDR in human lung cancer.

Key words MS-209 · Reversal drug · Multidrug resistance · MRP · Lung cancer

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Introduction

Resistance of cancer cells to anticancer drugs is a serious obstacle in cancer chemotherapy [20, 34]. Most cancer cells have intrinsic resistance to the initial chemotherapy or acquired resistance at relapse, resulting in a poor prognosis. In lung cancer, non-small-cell lung carcinomas (NSCLC) usually show the former type of resistance, while the latter form is usually observed in small-cell lung carcinomas (SCLC) [23, 29]. The exact mechanisms of drug resistance are poorly understood. However, we have recently reported that the multidrug resistance-associated protein (MRP) gene, rather than the MDR1 gene encoding human P-glycoprotein (Pgp), may play an important role in multidrug resistance (MDR) in lung cancer [33]. MRP is a 190 kDa transmembrane drug-transporter protein, isolated from a doxorubicin (DOX)-selected MDR SCLC cell line [12], and in vitro MRP gene-transfectants show MDR phenotype [13, 22, 27, 46]. MRP is expressed in a large number of tumor cell lines [4, 15, 26, 38, 40, 45] and is a promising candidate in Pgp-negative cancers.

Several agents, including verapamil (VER) and cyclosporin A (CsA), have been reported to overcome Pgp-mediated MDR in cancer cells in vitro [7, 14, 18], and some of them have been investigated in clinical trials [5, 17, 28, 35, 41]. Recently, more effective Pgp-reversing agents with fewer side effects have been synthesized and used in clinical trials, such as dexverapamil, PSC833 and MS-209 [8, 37, 44]. MS-209 is a newly synthesized quinoline compound, and is administered orally with few side effects [19, 37]. It interacts directly with Pgp and effectively inhibits the active efflux of anticancer drugs in vitro and in vivo [42]. In addition, other Pgp-reversing agents, such as VER, CsA and PSC833, have also been reported to overcome MRP-mediated MDR [3, 9, 13, 15, 21].

In the present study, we evaluated the effect of MS-209 on MRP-mediated MDR in lung cancer by performing an in vitro cytotoxicity assay with MS-209 in MDR1 gene-negative drug-selected MDR and wildtype cancer cells with various levels of MRP gene expression. The effects of MS-209 were compared with those of VER and CsA. In addition, we also measured intracellular accumulation of [^{3}H]-vincristine ([^{3}H]-VCR) with or without MS-209.

Materials and methods

Chemical agents

RPMI-1640 cell culture medium and fetal calf serum (FCS) were obtained from Gibco BRL (Grand Island, N.Y.), etoposide (VP-16) from Nippon Kayaku Co. (Tokyo, Japan), DOX from Kyowa Hakko Kogyo Co. (Tokyo), VCR from Sigma Chemical Co. (St. Louis, Mo.), [^{3}H]-VCR from Amersham Co. (Tokyo), VER hydrochloride from Eisai Co. (Tokyo), and cyclosporin A from Sandoz (Tokyo). MS-209 was synthesized by Mitsui Toatsu Chemicals (Tokyo) [19].

Cell lines

MDR HL60R human promyelocytic leukemia and UMCC-1/VP human SCLC cell lines were selected by continuous exposure to DOX and VP-16, respectively, as reported previously [15, 33]. Adriamycin-resistant MCF7/ADR human breast cancer cell line was kindly provided by Dr. Kenneth H. Cowan (National Cancer Institute, Bethesda, Md.) [16]. The human NSCLC cell lines, NCI-H358 (H358), NCI-H520 (H520) and NCI-H460 (H460), were obtained from the American Type Culture Collection (Rockville, Md.). All cells were cultured at 37 °C in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 4% L-glutamine, and 80 mg/l kanamycin sulfate in a humidified atmosphere containing 5% CO_2. The UMCC-1/VP cell line was maintained in 4 μ*M* VP-16 until 7 to 14 days before the initiation of the individual experiments described below [15].

RNA extraction and semiquantitative reverse transcriptase polymerase chain reaction

The total RNA from the cells was obtained using the guanidine isothiocyanate method [10]. RT-PCR for MRP, MDR1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) genes, and the semiquantitative determination of the expression of each MDR gene was performed as previously described [1, 11, 12, 33, 43]. The relative mRNA expression of each MDR gene (relative MDR gene expression) in the cells was calculated according to the following formula [33]:

$$\text{Relative MDR gene expression} = \frac{\text{MRP or MDR1 gene in sample}}{\text{MRP or MDR1 gene in positive control}} \div \frac{\text{G3PDH gene in sample}}{\text{G3PDH gene in positive control}} \times 100$$

The HL60R and the MCF7/ADR cells were used as positive control for MRP and MDR1 gene, respectively.

Drug sensitivity assay

Sensitivity to anticancer drugs was determined using the tetrazolium dye (MTT) assay [31]. Prior to the execution of these assays, the linearity of the relationship between the cell number and absorbance was checked at 570 nm, followed by completion of the cell growth studies as described previously [21]. The cells (1500 cells/well for HL60, HL60R and H460; 3000 cells/well for H520; 11 250 cells/well for H358; 15 000/well for UMCC-1 and UMCC-1/VP) were seeded in 96-well plates (Costar Co., Cambridge, Mass.), at least in triplicate, with various concentrations of the anticancer test drug, with or without the reversal agents. In preliminary experiments, we measured the cytotoxicity of each agent in each cell line and determined the concentration of the drug to ensure that the cytotoxicity was less than 20%. These studies established that the concentrations were 5 μ*M* of MS-209 and VER and 3 μ*M* of CsA for HL60, HL60R, UMCC-1 and UMCC-1/VP, and 10 μ*M* of MS-209 and VER and 5 μ*M* of CsA for H358, H520 and H460. Following incubation for 4 days at 37 °C, 15 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to each well. The cells were lysed after incubation for 4 h at 37 °C by adding 100 μl 2-propanol 0.1 *N* HCl to each well. Absorbance was read on a microplate reader (MR 600; Dynatech Co., Alexandria, Va) with test and reference wavelengths of 570 and 630 nm, respectively. The IC_{50} was defined as the concentration of the anticancer drug that reduced the absorbance from the condition in reversal agent alone in each test by 50%. The reversing effects were expressed as IC_{50} values in the presence and absence of each agent. Each study was repeated three times.

Intracellular accumulation of [^{3}H]-VCR

For adhesion, cells (5000 cells/well) were seeded onto 12-well plates (Corning Glassworks, Corning, N.Y.) and cultured for 72 h before the assay. The cells (for suspension, 1×10^6 cells/ml) were incubated at 37 °C with 30 n*M* [^{3}H]-VCR in the presence or absence of each reversal agent. Aliquots (0.5 ml) of cells were removed at the appropriate times and ice-cold phosphate buffered saline (PBS) was added to stop accumulation. After two washes with ice-cold PBS, the cells were lysed with 1% sodium dodecyl sulfate, and the cell-associated radioactivity was determined using a liquid scintillation system. Each study was performed three times.

Statistical analysis

The reversing effects of the three agents were normalized logarithmically, followed by analysis using Tukey's multiple comparison method. The effects of these agents in NSCLC cells were analyzed to determine the correlation with the level of MRP gene expression. All statistical analyses were performed using the BMDP software (BMDP Statistical Software, Los Angeles, Calif.), and *P*-values < 0.05 were regarded as significant.

Results

Expressions of MRP and MDR1 mRNA in cell lines

The expression of MRP mRNA and MDR1 mRNA of the control cell lines have been reported previously [15, 33, 38]. All other cell lines, except MCF7/ADR cells, expressed no MDR1 gene, while the HL60R and UMCC-1/VP cells expressed abundant MRP gene (Fig. 1). The relative MRP gene expression was 15 in HL60, 118 in UMCC-1/VP, 8 in UMCC-1, 33 in H358, 52 in H520 and 77 in H460 cell lines.

Drug sensitivity and reversing effects of MS-209

The IC_{50} values of each drug in the cell lines used here are shown in Table 1 and Table 2. Compared with the sensitivity of the respective parental cells, the HL60R and UMCC-1/VP sublines showed more than a sixfold increase in resistance to VP-16, DOX and VCR (Fig. 2).

Table 1 and Fig. 3 show the reversing effects of these three agents in HL60 and UMCC-1 parental cells and sublines. In both parental cells, only a small effect (less than twofold reversing effect) was observed. However, in the two resistant sublines, 5 μ*M* of MS-209 and VER enhanced the cytotoxicity of the three anticancer drugs by more than twofold and completely reversed the VCR resistance of UMCC-1/VP cells (Table 1, Fig. 3). The reversing effect was significantly stronger against VCR resistance than against VP-16 and DOX resistance. The mean value of the reversing effect of MS-209 against resistance to each anticancer drug was higher than that of VER and CsA, and significantly higher than that of CsA against VP-16 and DOX resistance of HL60R cells ($P < 0.01$), and against DOX and VCR resistance of UMCC-1/VP cells ($P < 0.01$). However, the reversing effects of MS-209 and VER were only significantly different against DOX resistance of UMCC-1/VP cells

Fig. 1 RT-PCR for the MRP and MDR1 genes in parental and MDR sublines of HL60 and UMCC-1 cells, and wildtype NSCLC cells. Note the 430 bp MRP gene PCR product in all cells, while the 308 bp MDR1 gene PCR product is present in MCF7/ADR cells only. HL60R and MCF7/ADR cells were used as positive control for the MRP and MDR1 genes, respectively

Table 1 Effects of reversal agents MS-209, verapamil (VER) and cyclosporin A (CsA) on cytotoxicity of anticancer drugs in parental cells and MDR sublines overexpressing the MRP gene (concentrations: MS-209 5 μ*M*, VER 5 μ*M*, CsA 3 μ*M*). The reversing effects were calculated as the ratio of the IC_{50} value in the absence of the agent to that in the presence of the agent. Values are means ± SD of three separate experiments

Anticancer drug	Reversal agent	HL60		HL60R		UMCC-1		UMCC-1/VP	
		IC_{50}	Reversing effect	IC_{50}	Reversing effect	IC_{50}	Reversing effect	IC_{50}	Reversing effect
VP-16		0.15 ± 0.035 μ*M*		73.7 ± 8.5 μ*M*		3.72 ± 0.21 μ*M*		128 ± 48 μ*M*	
	MS-209		1.23 ± 0.27		5.30 ± 1.31*		1.23 ± 0.15		3.73 ± 1.99
	VER		1.42 ± 0.57		4.47 ± 0.82**		1.23 ± 0.17		2.25 ± 0.75
	CsA		0.71 ± 0.27		1.80 ± 0.63		1.09 ± 0.25		1.31 ± 0.60
DOX		34.8 ± 9.0 n*M*		4360 ± 550 n*M*		193 ± 21 n*M*		1740 ± 190 n*M*	
	MS-209		1.06 ± 0.15		8.19 ± 3.55*		0.76 ± 0.12		3.55 ± 0.33* ***
	VER		1.18 ± 0.06		4.65 ± 1.02		0.93 ± 0.25		2.12 ± 0.34*
	CsA		0.74 ± 0.33		2.20 ± 0.84		0.93 ± 0.30		1.12 ± 0.21
VCR		2.03 ± 0.49 n*M*		107 ± 49.3 n*M*		4.79 ± 2.39 n*M*		32.8 ± 10.2 n*M*	
	MS-209		1.74 ± 0.35		13.3 ± 10.2		1.18 ± 0.07		25.0 ± 16.1a*
	VER		1.36 ± 0.21		9.44 ± 5.90		1.41 ± 0.34		14.1 ± 8.6a*
	CsA		2.88 ± 0.63		4.03 ± 1.32		0.76 ± 0.03		1.77 ± 0.69

[a]Completely reversed by MS-209 and VER
*$P < 0.01$ vs CsA, **$P < 0.05$ vs CsA, ***$P < 0.05$ vs VER; Tukey's multiple comparison method

Table 2 Effects of reversal agents MS-209, verapamil (VER) and cyclosporin (CsA) on cytotoxicity of anticancer drugs in wildtype lung cancer cells (concentrations: MS-209 10 μ*M*, VER 10 μ*M*, CsA 5 μ*M*). The reversing effects were calculated as the ratio of the IC_{50} value in the absence of the agent to that in the presence of the agent. Values are means ± SD of three separate experiments

Anticancer drug	Reversal agent	H358		H520		H460		Correlation MRP gene expression vs reversing effect	
		IC_{50}	Reversing effect	IC_{50}	Reversing effect	IC_{50}	Reversing effect	*r*-value	*P*-value
VP-16		6.72 ± 0.79 μ*M*		8.92 ± 0.83 μ*M*		0.397 ± 0.072 μ*M*			
	MS-209		0.94 ± 0.05		2.11 ± 0.55		3.32 ± 0.64	0.943	< 0.001
	VER		1.05 ± 0.06		1.42 ± 0.32		2.27 ± 0.91	0.794	0.017
	CsA		0.94 ± 0.07		1.12 ± 0.39		2.27 ± 0.55	0.813	0.008
DOX		458 ± 46 n*M*		470 ± 48 n*M*		525 ± 94 n*M*			
	MS-209		1.57 ± 0.41		3.07 ± 0.56		2.95 ± 0.13	0.819	0.007
	VER		2.05 ± 1.10		2.22 ± 0.50		2.37 ± 0.41		
	CsA		0.91 ± 0.57		1.73 ± 0.54		2.26 ± 0.22	0.715	0.030
VCR		8.21 ± 2.68 n*M*		4.56 ± 1.36 n*M*		12.8 ± 4.2 n*M*			
	MS-209		2.92 ± 1.32		3.52 ± 0.20		5.75 ± 0.17	0.826	0.006
	VER		2.48 ± 0.89		3.11 ± 0.59		4.11 ± 0.38	0.722	0.028
	CsA		1.60 ± 0.95		3.77 ± 0.03		5.97 ± 1.12	0.885	0.002

Fig. 2 Relative resistance ratios of multidrug resistant HL60R and UMCC-1/VP cells overexpressing the MRP gene. The IC_{50} value of each drug was determined using the MTT assay. The resistance ratios represent the ratio of the IC_{50} of sublines to that of their parental cells. The HL60R cells were 491-, 125-, 52.5- and 1.9-fold resistant, and the UMCC-1/VP cells were 34-, 9.0-, 6.8- and 1.4-fold resistant to VP-16, DOX, VCR and CDDP, respectively (*VP-16* etoposide, *DOX* doxorubicin, *VCR* vincristine, *CDDP* *cis*-diamminedichloroplatinum(II)

($P < 0.05$). The mean values of the reversing effects of VER against resistance to each anticancer drug were higher than those of CsA, and the reversing effect of VER was significant in VP-16 resistance of HL60R cells ($P < 0.05$), and in DOX ($P < 0.01$) and VCR ($P < 0.01$) resistance of UMCC-1/VP cells.

The reversing effects of the agents in the three wildtype NSCLC cell lines are shown in Table 2 and Fig. 4. The mean values of the reversing effects of 10 μ*M* MS-209, 10 μ*M* VER and 5 μ*M* CsA in H460 cells were approximately two- to sixfold for the three anticancer drugs. They were less than approximately fourfold in H520 cells and less than threefold in H358 cells. Interestingly, significant positive correlations were noted between the level of MRP gene expression in the three NSCLC cells and the reversing effects of the three agents (Table 2). In general, higher levels of MRP gene expression in these cells were associated with higher reversing effects by MS-209, VER and CsA.

Effects of MS-209 on intracellular accumulation of [^{3}H]-VCR

Intracellular accumulation of [^{3}H]-VCR in HL60R cells increased by approximately 2.5 times at 120 and 180 min in the presence of 10 μ*M* MS-209, as shown in Fig. 5 ($P < 0.05$). Similarly, 10 μ*M* MS-209 increased by approximately twofold the intracellular accumulation of [^{3}H]-VCR in H460 cells compared with experiments when the agent was not used ($P < 0.05$), but increased it little in H358 cells (Fig. 6). The reversing effect of MS-209 on VCR resistance correlated with the level of MRP gene expression of and intracellular accumulation.

Discussion

The present study demonstrated that MS-209 enhances the in vitro cytotoxicity of VP-16, DOX and VCR in

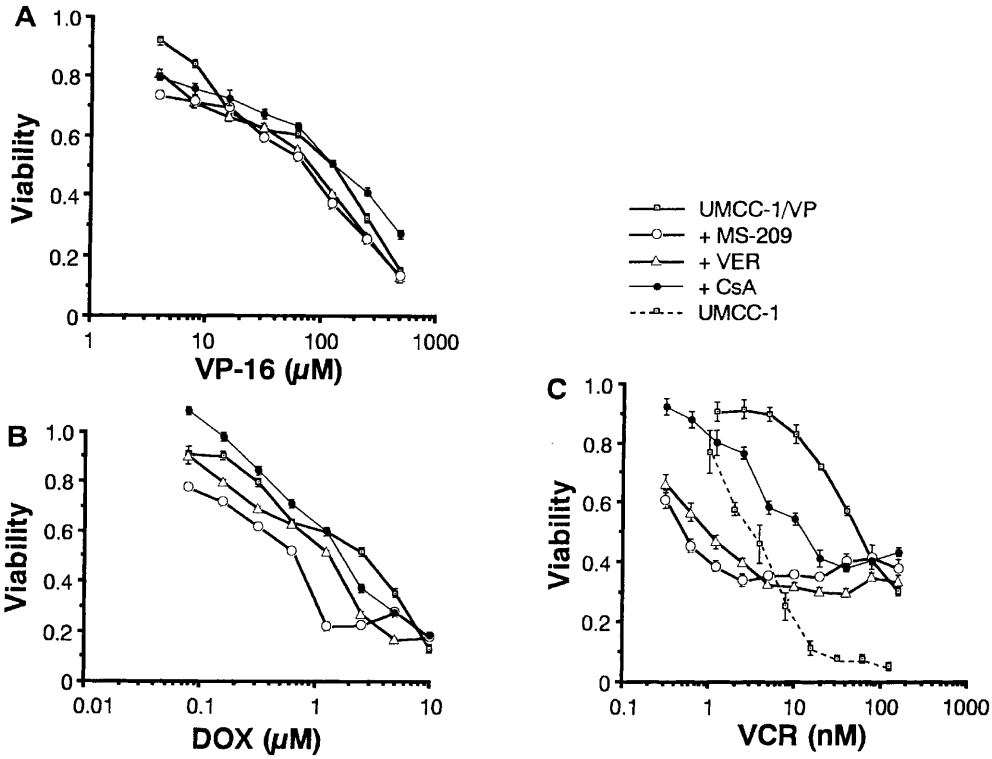

Fig. 3A–C Reversing effects of three agents on the cytotoxicity of anticancer drugs in multidrug resistant UMCC-1/VP SCLC cells. Values are the means ± standard error of triplicate determinations using the MTT assay in a single experiment; the standard error values were very small. MS-209 and VER at 5 μ*M* completely reversed the resistance to VCR (MS-209, 5 μ*M*; *VER* verapamil, 5 μ*M*; *CsA*, cyclosporin A, 3 μ*M*; *VP-16* etoposide; *DOX* doxorubicin; *VCR* vincristine)

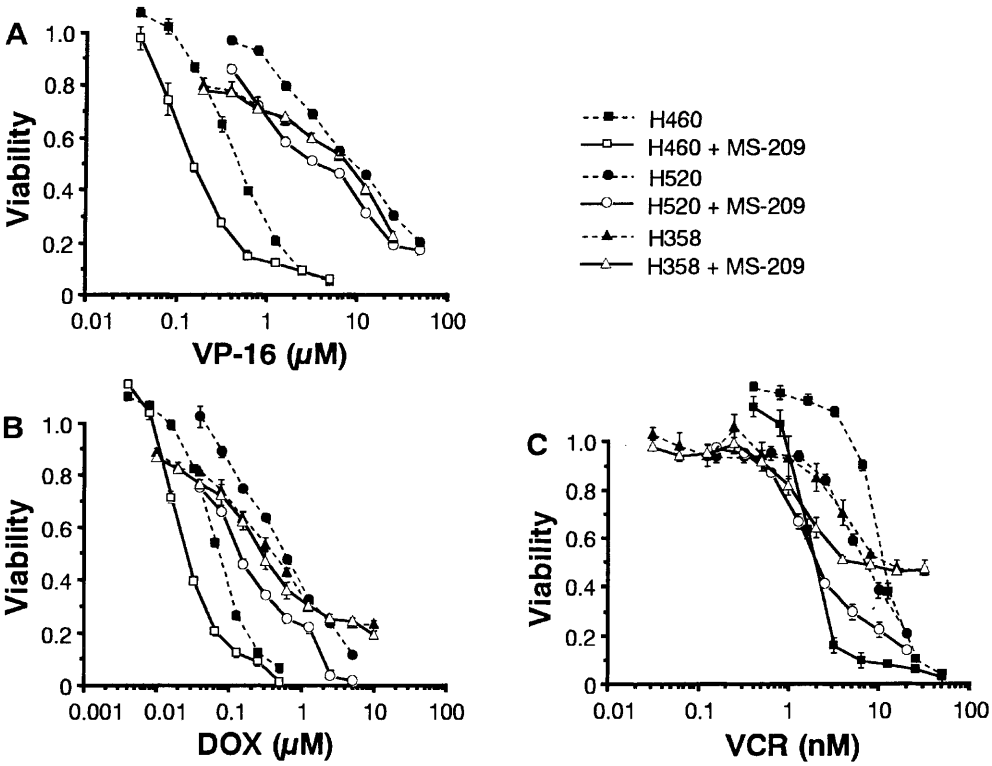

Fig. 4A–C Reversing effects of MS-209 (10 μ*M*) on cytotoxicity of anticancer drugs in wildtype NSCLC cells. Values are the means ± standard error of triplicate determinations using the MTT assay in a single experiment; the standard error values were very small. The effects correlated with the level of MRP gene expression in the three cell lines (*H460* large cell carcinoma cells, *H520* squamous cell carcinoma cells, *H358* adenocarcinoma cells, *VP-16* etoposide, *DOX* doxorubicin, *VCR* vincristine)

drug-selected MDR cells overexpressing the MRP gene as well as in wildtype NSCLC cells expressing the MRP gene. More importantly, the reversing effect of MS-209 correlated with the level of MRP gene expression in lung cancer cells. The effect of this agent was stronger than that of VER and CsA in MDR cells, but almost equal to their effect in wildtype cells. MS-209 increased the intracellular accumulation of VCR in these cells and completely reversed the VCR resistance in MDR UMCC-1/VP SCLC cells. Thus, our results indicate that MS-209 is

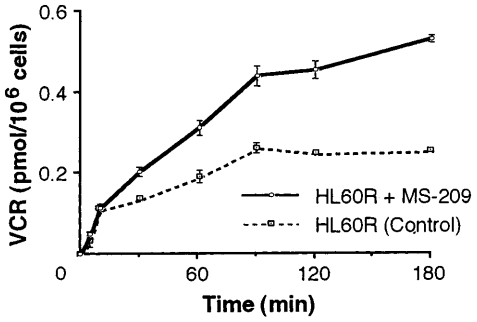

Fig. 5 Effect of MS-209 on intracellular accumulation of VCR in MDR HL60R cells.Accumulation of [^{3}H]-VCR was measured at 0 to 180 min after exposure to VCR alone (□) or to VCR with 10 μ*M* MS-209 (○). In preliminary experiments, incubation for 4 h with 10 μ*M* MS-209 did not affect the viability of HL60R cells. The values are the means ± standard error of triplicate determinations in a single experiment. Note the significant increase in the accumulation of [^{3}H]-VCR after 30 min induced by MS-209, compared with the control ($P < 0.01$, paired *t*-test)

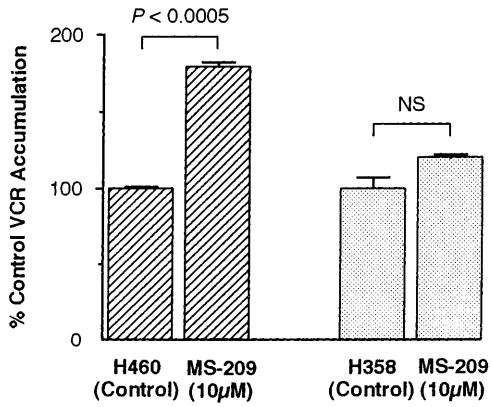

Fig. 6 Effect of MS-209 on intracellular accumulation of VCR in H460 and H358 cells. Accumulation of [^{3}H]-VCR was measured in H460 and H358 cells 180 min after exposure to VCR alone or to VCR with 10 μ*M* MS-209. The values are the means ± standard error of triplicate determinations in a single experiment. Note the significant increase in the accumulation of [^{3}H]-VCR induced by MS-209 in H460 cells ($P < 0.0005$, paired *t*-test; *NS* not significant)

a potentially useful drug for overcoming MRP-mediated intrinsic and acquired MDR in lung cancer.

MS-209, which has fewer side effects than VER and another quinoline analog MS-073, was synthesized to effectively overcome Pgp-mediated clinical MDR [36, 37]. At 1–10 μ*M*, MS-209 completely reverses VCR resistance of mouse and human Pgp-mediated MDR cells in vitro [37]. The antagonistic effect of MS-209 on calcium channels is approximately 30 times less than that of VER, resulting in negligible calcium channel blocking side effects [42]. Previous clinical studies using VER have shown that the plasma concentrations do not reach effective levels as a consequence of the development of side effects related to the drug itself [35, 39]. The achieved VER plasma concentrations (1–2 μ*M*) are lower than the optimal in vitro concentrations (6–10 μ*M*) that overcome Pgp-mediated MDR [35]. In the present study using MRP-mediated MDR cancer cells, the in vitro reversing effect of MS-209 was stronger than that of VER even at the same concentrations. Accordingly, MS-209 is a promising agent that reverses both Pgp- and MRP-mediated clinical MDR.

The reversing effects of MS-209, even at low concentrations, were stronger in MDR cells overexpressing the MRP gene than in wildtype cells. In this regard, Baba et al. [2] have reported a positive correlation between the level of expression of the MDR1 gene and the reversing effect of MS-209 on the in vitro cytotoxicity of DOX and VCR. In addition, in a clinical trial of dexverapamil for patients with chemotherapy-refractory lymphomas, Wilson et al. [44] observed a close association between the level of MDR1 gene expression and the response of the lymphomas to dexverapamil. The results of these in vitro and vivo studies suggest that MS-209, in conjunction with chemotherapy, may be more effective in relapsed tumors with high levels of MDR1 or MRP gene expression. However, the overall results are not very encouraging. Interestingly, Beketic-Oreskovic et al. [6] have shown recently that the early use of reversal agents in combination with anticancer drugs may suppress the activation of the MDR genes, thus preventing the emergence of resistant cancer cell clones. Further clinical studies of different designs are necessary to establish the clinical usefulness of reversal agents in cancer chemotherapy.

The exact mechanism producing the reversal of MRP-mediated MDR by the agents used in the present study remains undetermined. There is no evidence that these agents interact directly with MRP. MRP is a transporter of glutathione S-conjugates and does not interact directly with anticancer drugs [24, 25, 32, 47]. It is distributed in the plasma membrane and membrane vesicles, and is known to reduce intracellular accumulation and to alter the intracellular distribution of anticancer drugs [30, 46], mechanisms through which the MRP confers MDR on cancer cells. The MS-209-induced increase in intracellular accumulation of VCR was proportional to the level of MRP gene expression, suggesting that MS-209 may interact directly with and inhibit MRP. Moreover, the reversing effects of the agents on MRP-mediated VCR resistance were stronger than those on VP-16 and DOX resistances, indicating that DOX is a poor substrate for MRP, thus confirming the early results of Ishikawa et al. [24]. Therefore, the reversing effects of these agents on MRP may depend on the ability of individual cancer cells to conjugate the drug, on the change in intracellular glutathione levels, or on the ability of anticancer drugs to conjugate glutathione.

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