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Relationship between multidrug resistant gene expression and multidrug resistant-reversing effect of MS-209 in various tumor cells

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Abstract MS-209 is a novel quinoline compound which can overcome multidrug resistance (MDR) both in vitro and in vivo, while having a low level of side effects, and is now being evaluated in a clinical phase II study. Reverse transcription–polymerase chain reaction (RT-PCR) was used to quantitate the expression levels of *MDR* genes in various mouse and human tumor cell lines. The *MDR* gene and the ß *actin* gene, as the internal reference standard, were coamplified separately, and the relative expression of the *MDR* gene was represented by the MDR/ß actin ratio. The in vitro MDR-reversing effect of MS-209 was then compared with the *MDR* gene expression (MDR/ß actin ratio). We found a significant correlation between these two parameters. Moreover, a significant correlation was also observed between the level of expression of the *MDR1* gene and that of P-glycoprotein in human cell lines. Therefore, the efficacy of MS-209 seems to specifically depend on the level of *MDR* gene expression (P-glycoprotein). From these observations, it is suggested that RT-PCR assays of *MDR1* gene in tumor biopsy specimens might be an effective means to predict the response of tumor cells to combination therapy with MS-209.

Key words MS-209 · MDR · RT-PCR

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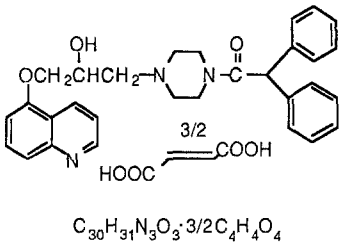
Abbreviations *MDR* multidrug resistance, *ADM* adriamycin, *VCR* vincristine, *MTT* 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, *RT* reverse transcription, *PCR* polymerase chain reaction, *SDS* sodium dodecyl sulfate, *PMSF* phenylmethylsulfonyl fluoride, *EtBr* ethidium bromide, IC_{50} concentration producing 50% inhibition

Introduction

Multidrug-resistant mammalian cells are characterized by cross-resistance to a wide variety of structurally and mechanistically unrelated antineoplastic agents. A major mechanism of this type of resistance is the reduced accumulation of antitumor agents in resistant cells. P-glycoprotein, encoded by the *MDR* gene, is considered to confer drug resistance to the cells by acting as a membrane-bound ATP-consuming drug efflux pump [reviewed in reference 8).

In human and mouse, *MDR* genes comprise a small gene family of two (*MDR1* and *MDR2*) and three (*mdr-1a*, *mdr-1b* and *mdr-2*) members, respectively [6, 18, 22]. Transfection studies, however, have demonstrated that only the expression of mouse *mdr-1a* and *mdr-1b* genes and human *MDR1* gene is sufficient to confer the MDR phenotype to drug-sensitive cells [11, 36]. Recently, the functions of the protein encoded by *mdr-2* have been shown to be a phosphatidylcholine translocase [25], and those of the protein encoded by *MDR2* have been suggested to be a phosphatidylcholine flippase in bile canalicular membranes [25, 28].

Herzog et al. [14] evaluated the expression of *MDR1*/P-glycoprotein in human cell lines using various methods, such as Northern blot, slot blot, PCR, in situ hybridization, immunofluorescence, immunohistochemistry, fluorescence-activated cell sorting, and immunoblotting analyses. A comparison of these methods shows none to be clearly superior. They reported however, that PCR analysis offers the



$C_{30}H_{31}N_3O_3 \cdot 3/2C_4H_4O_4$

Fig. 1 Chemical structure of MS-209

advantage of being the most sensitive, giving convincing quantifiable results at the lowest level. In addition, since the amount of biopsy specimens is often limited, the RNA from such samples has frequently suffered significant degradation. For these reasons, we selected the PCR method for the quantitative analysis of the expression of the *MDR* genes.

Since the finding that verapamil can reverse MDR, many MDR-reversing agents have been reported ([30–32] reviewed in 34). MS-209 is a newly synthesized quinoline derivative which can overcome MDR both in vitro and in vivo Nakanishi et al. (in preparation) [29], and is now being evaluated in a phase II study. In this study, we measured the levels of *mdr-1a*, *mdr-1b* and *MDR1* mRNA in various cell lines and compared them with the results of an in vitro cytotoxicity assay. We found a significant correlation between the expression of the *MDR* gene (P-glycoprotein) and the MDR-reversing effect of MS-209.

Materials and methods

Drugs

MS-209 (Fig. 1) was synthesized by Mitsui Toatsu Chemicals, Tokyo, Japan [9]. ADM and VCR were purchased from Kyowa Hakko Co., Tokyo, Japan, and Shionogi Co., Osaka, Japan, respectively.

Cell lines

Mouse P388 leukemia, ADM-resistant P388 (P388/ADM) and VCR-resistant P388 (P388/VCR) were supplied by the National Cancer Institute, NIH, Bethesda, Md. IMC carcinoma was provided by Dr. M. Ishizuka, Institute of Microbial Chemistry, Tokyo, Japan. Meth A fibrosarcoma was provided by Professor Y. Hashimoto, Pharmaceutical Institute, Tohoku University, Sendai, Japan. L1210 leukemia, Colon 26 adenocarcinoma and B16 melanoma were provided by the National Cancer Institute and maintained at the laboratories of the Japanese Foundation for Cancer Research, Tokyo, Japan. K562, a human myelogenous leukemia cell line, was provided by Dr. Ezaki, Cancer Chemotherapy Center, Tokyo, Japan. Its ADM-resistant (K562/ADM) and VCR-resistant (K562/VCR) sublines were established in our laboratory [33, 35]. Human gastric cancer lines 4-1St and St-4, were established in our laboratory. HCT-15, a human colorectal adenocarcinoma cell line, was purchased from ATCC. A2780, a human ovarian cancer cell line, and its ADM-resistant subline (2780AD) were provided by Drs. R. Ozols and T. Hamilton, Medicine Branch, National Cancer Institute, NIH. A cloned human epidermoid carcinoma cell line (KB-3-1) and its colchicine-resistant sublimes (KB-8-5, KB-C1, and KB-C4) were provided by Dr. I. Pastan, National Cancer Institute, NIH.

Cell culture and drug treatment

Murine tumor cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and streptomycin (100 μg/ml). For P388 and its sublines, 20 μ*M* 2-mercaptoethanol was added to the above medium. The KB cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and streptomycin. The other human cells were maintained in RPMI-1640 supplemented with 5% fetal bovine serum and streptomycin. For the in vitro drug treatment experiments, tumor cells ($1–3 \times 10^3$) were seeded in 0.2 ml culture medium/well in 96-well plates (Corning, N.Y.). Cells were treated in triplicate with graded concentrations of anticancer agents in the absence or presence of MS-209 and incubated in a CO_2 incubator at 37°C for 72 h (mouse cell lines) or 96 h (human cell lines). The MTT cytotoxicity assay was used to measure the cytotoxic effect [4]. The IC_{50} was determined by plotting the logarithm of the drug concentrations against cell survival. The IC_{50} of cells cultured without MS-209 was divided by that of cells cultured with 3 μ*M* MS-209, and this value was used to quantify the MDR-reversing effect.

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method [7]. Oligonucleotides for the PCR were synthesized by the standard phosphoramidite method using an automatic DNA synthesizer (Table 1). The *GeneAmp* RNA PCR Kit (Perkin-Elmer Cetus) was employed for the RT-PCR assays which were performed according to the instructions provided by the manufacturer. The RT reaction mixture consisted of 0.5 μg RNA, 2.5 μ*M* random hexamers and Master Mix to a total volume

Table 1 Oligonucleotides used in RT-PCR

Transcript	Primer sequence (5′-3′)	Nucleotide position in cDNA sequence	Reference
MDR, mouse	TACAGAAAGTGAAGCTGTGG	1674–1693 (*mdr-1a*)	19
		1683–1702 (*mdr-1b*)	12
MDR, mouse	ATCCTGTCTCAGCATGGATT	2388–2369 (*mdr-1a*)	19
		2394–2375 (*mdr-1b*)	12
MDR, human	AAGCGAAGCAGTGGTTCAGG	1692–1711 (*MDR1*)	5
MDR, human	ACCAACTCACATCCTGTCTG	2410–2391 (*MDR1*)	5
ß actin, mouse	CACACTGTGCCCATCTACGA	484–503	1
ß actin, mouse	CACAGGATTCCATACCCAAG	817–798	1
ß actin, human	CACACTGTGCCCATCTACGA	522–541	24
ß actin, human	ACAGGACTCCATGCCCAGGA	858–839	24

Table 2 MDR-reversing effect of MS-209.

Cell line	MDR-reversing effect	
	ADM	VCR
Mouse		
P388	2.2	11
P388/ADM	19	82
P388/VCR	20	270
L1210	2.7	10
IMC	2.6	11
Colon26	4.3	8.4
Meth A	6.9	8.4
B16	1.1	2.7
Human		
K562	1.3	2.0
K562/ADM	110	280
K562/VCR	10	110
4-1St	88	350
St-4	2.2	2.4
A2780	2.1	2.1
2780AD	36	180
HCT-15	18	79
KB-3-1	2.4	2.6
KB-8	2.1	3.4
KB-8-5	5.8	47
KB-C1	91	270
KB-C4	190	390

Tumor cells ($1–3 \times 10^3$) were seeded in 0.2 ml culture medium and then treated with graded concentrations of antitumor agents in the absence or presence of MS-209 at a concentration of 3 μ*M*. After 72–96 h of continuous drug exposure, the cytotoxic effect was measured using an MTT assay. The values for MDR-reversing effect were determined by dividing the IC_{50} of cells cultured without MS-209 by the IC_{50} of cells cultured with 3 μ*M* MS-209. The values are the mean value of triplicate determinations. SD was usually within 10% of the mean value

of 20 μl. The mixture was incubated for 10 min at room temperature, 15 min at 42°C, followed by 5 min at 99°C. The PCR mixture consisted of 2 μl 25 m*M* $MgCl_2$ solution, 4 μl 10 × PCR Buffer II, 0.3 μ*M* of each primer, 1.25 units *AmpliTaq* DNA polymerase, 10 μl RT product, in a total volume of 50 μl. Mineral oil was placed on top of the aqueous layer. The mixture was heated to dissociate the aggregated RT products for 2 min at 95°C. The PCR reaction was performed by repeating the cycle with 1 min at 95°C for denaturing and 1 min at 60°C for annealing and extension, and finally, extension was completed by 7 min at 60°C.

Quantitation of the RT-PCR products

The RT-PCR products were quantified by a method based on that of Higuchi et al. [15]. The RT-PCR mixture was diluted 40-fold with water and EtBr was added to a final concentration of 250 ng/ml. Fluorescence was measured in a spectrofluorometer (FP-770, Jasco, Tokyo, Japan). Excitation was at 280 nm and emitted light was detected at 590 nm. The amount of both *MDR* mRNA and *ß actin* mRNA products was measured, and the ratio of these measurements was calculated (MDR/ß actin ratio), and this ratio was taken to represent the *MDR* gene expression.

Detection of P-glycoprotein by Western blot analysis

Membrane fractions were prepared by a method based on that of Naito et al. [21]. Washed cells were homogenized in 1 ml of a solution containing 10 m*M* Tris-HCl, pH 7.4, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$ and 0.02 m*M* PMSF. The lysates were centrifuged at 1000 *g* for 10 min. The supernatants were applied to 1 ml 35% sucrose containing 10 m*M* Tris-HCl, pH 7.4, and centrifuged at 18,000 *g* (TLS-100, Beckman) for 1 h at 4°C. The band at the interphase was recovered, diluted two-fold with a solution of 10 m*M* Tris-HCl, pH 7.4 and 0.25 *M* sucrose and centrifuged at 100,000 *g* for 30 min at 4°C. The precipitates were resuspended in 100 μl 10 m*M* Tris-HCl, pH 7.4 and 0.25 *M* sucrose and used as the membrane fraction. Aliquots of membrane fraction (50 μg protein) were electrophoresed on a SDS-polyacrylamide gel (5%) and then electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose membrane was probed with a monoclonal antibody to human P-glycoprotein JSB-1 (0.5 μg/ml; Nichirei Co., Tokyo, Japan), and horseradish peroxidase-conjugated antimouse Ig (Amersham) was used as the second antibody. Expression of human P-glycoprotein was visualized using an ECL Western Blotting Kit (Amersham). Intensities of the bands corresponding to P-glycoprotein were quantified by densitometry using a digital scanner (TIAS-2000, ACI Japan, Tokyo, Japan).

Results

Effect of MS-209 on drug cytotoxicity in various tumor cells

The effect of MS-209 on the cytotoxicity of ADM and VCR was examined using various mouse and human tumor cell lines in vitro (Table 2). MS-209 at a concentration of 3 μ*M* strongly increased the cytotoxicity of both ADM and VCR against P388/ADM, P388/VCR, K562/ADM, K562/VCR, 4-1St, 2780AD, KB-8-5, KB-C1 and KB-C4. In addition, MS-209 also augmented the cytotoxicity of both ADM and VCR against so-called drug-sensitive cells.

Quantification of *MDR* gene expression

From the known sequences of *MDR* genes and those of the *ß actin* genes [1, 5, 12, 19, 24], oligonucleotides for PCR (Table 1) were designed to bind specifically to exon regions separated by an intron in the genomic DNA, and to have a G-C content as close as possible to 50%. In the case of mouse cells, since two genes (*mdr-1a* and *mdr-1b*) participate in the MDR phenotype, the primer sequences were designed to bind to both the *mdr-1a* gene and the *mdr-1b* gene. Using the primers described, a major band of the predicted size was obtained after PCR amplification (data not shown).

Figure 2 shows the results of the amplification quantified by fluorescence with varying numbers of PCR cycles. Clear differences were observed between MDR-cells (P388/ADM and P388/VCR) and drug-sensitive P388 cells in *MDR* gene amplification (Fig. 2A), but not in *ß actin* gene amplification (Fig. 2B). As shown in this figure, the PCR products of P388, P388/ADM and P388/VCR were all amplified exponentially at around 27 cycles. Furthermore, the differences in the MDR/ß

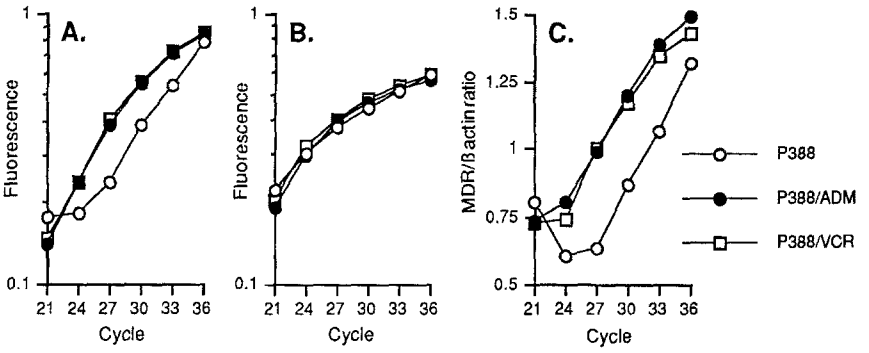


Fig. 2A–C Kinetics of PCR amplification of (**A**) *mdr* gene, (**B**) *ßactin* gene, and (**C**) MDR/ß actin ratio with varying numbers of PCR cycles. RNAs were extracted from mouse leukemia cells, P388, P388/ADM and P388/VCR. PCR products were quantified by fluorescence intensity of EtBr

Table 3 Expression of *MDR* gene

Cell line	MDR/ß actin ratio
Mouse	
P388	0.63
P388/ADM	0.99
P388/VCR	1.00
L1210	0.60
IMC	0.52
Colon26	0.82
MethA	0.73
B16	0.43
Human	
K562	0.32
K562/ADM	1.10
K562/VCR	0.96
4-1 St	0.68
St-4	0.30
A2780	0.27
2780AD	0.87
HCT-15	0.81
KB-3-1	0.39
KB-8	0.31
KB-8-5	0.46
KB-C1	0.86
KB-C4	0.99

Total RNA (0.5 µg) extracted from tumor cells was amplified by the RT-PCR method. The amount of both *MDR* mRNA and *ßactin* mRNA products was determined, and the *MDR* gene expression was represented by MDR/ß actin ratio. The PCR reaction was performed for 27 cycles for the mouse cell lines and for 30 cycles for the human cell lines. Values are the mean of triplicate determinations. SD was usually within 10% of the mean value

actin ratio (Fig. 2C) between MDR-cells and sensitive cells were greatest after 27 cycles of amplification. Therefore, in the case of mouse tumor cells, 27 amplification cycles were performed for quantitation; whereas for human tumor cells, 30 PCR cycles were necessary (data not shown).

To confirm the reliability of the quantitative method employed above, we calculated the MDR/ß actin ratio in various mouse cells by using both the radioactivity of the excised bands and fluorescence after PCR for 27 cycles. Since there was a significant correlation between them (data not shown), quantitation of the PCR products by using fluorescence was considered to be reliable. In addition, quantitative analyses by fluorescence are more easy to perform, so we used this method.

The *MDR* gene expression in various tumor cells is summarized in Table 3. As shown in Table 3, the *MDR* gene was expressed not only in MDR cells but also in various drug-sensitive cells.

Relationship between *MDR* gene (P-glycoprotein) expression and MDR reversing effect of MS-209

The MDR/ß actin ratio of mouse cell lines (Table 3) was plotted against the MDR-reversing effect of MS-209 (Table 2) at a concentration of 3 µ*M*. There was a significant correlation between the MDR/ß actin ratio and the logarithm of the reversing effect on ADM-resistant (Fig. 3A) or VCR-resistant (Fig. 3B) mouse cell lines. The correlation coefficient was 0.953 ($P < 0.001$) for ADM and 0.856 ($P < 0.01$) for VCR.

Figure 4 shows the level of *MDR* gene expression obtained with serial dilutions of K562/ADM RNA, amplified for 30 cycles. There was a significant

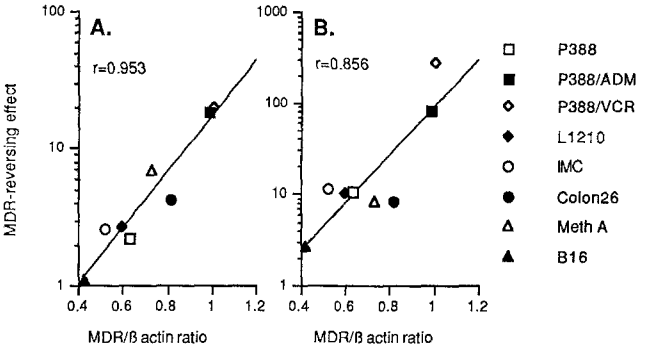


Fig. 3A, B, Relationship between the MDR/ß actin ratio and the reversing effect of 3 µ*M* MS-209 on (**A**) ADM-resistant or (**B**) VCR-resistant mouse tumor cell lines. PCR was performed for 27 cycles. MDR/ß actin ratios were calculated from the fluorescence intensity of EtBr. The MDR-reversing effect of MS-209 was calculated from the IC_{50} obtained in the MTT assay

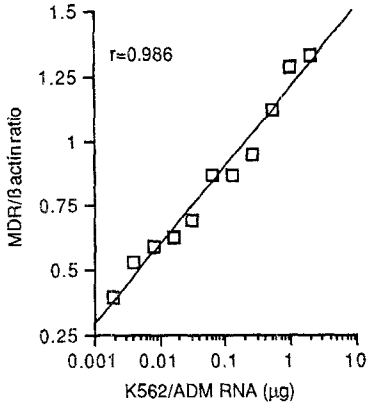


Fig. 4 Measurement of the MDR/ß actin ratio by serial dilution of RNA from K562/ADM cells. After serial dilution, RT and PCR for 30 cycles were performed. The MDR/ß actin ratio was calculated from the fluorescence intensity of EtBr

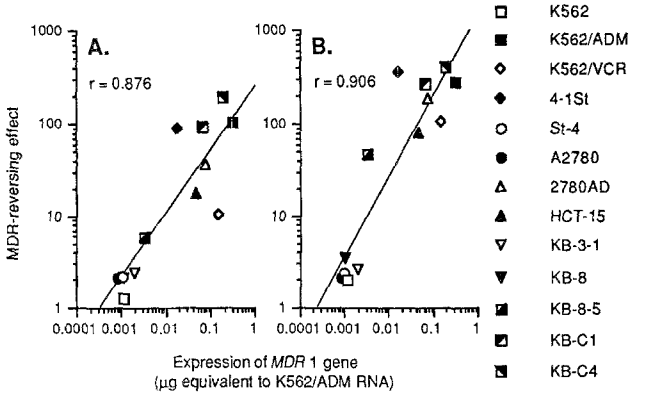


Fig. 5A, B Relationship between the expression of the *MDR*1 gene and the reversing effect of 3 µ*M* MS-209 on (**A**) ADM-resistant or (B) VCR-resistant human tumor cell lines. PCR was performed for 30 cycles. And the MDR/ß actin ratio was then calculated from the fluorescence intensity of EtBr. Then, the MDR/ß actin ratio (expression of *MDR1* gene) was converted into the equivalent amount of K562/ADM RNA. The MDR-reversing effect of MS-209 was calculated from the IC_{50} obtained in the MTT assay

correlation between the MDR/ß actin ratio and the logarithm of the amount of RNA ($r = 0.986$, $P < 0.001$). Therefore, the MDR/ß actin ratio (Table 3) of various human cell lines could be converted into the equivalent amount of K562/ADM RNA.

After the conversion, the values were plotted against the MDR-reversing effect of MS-209 at 3 µ*M* (Fig. 5A, B). Great differences (up to 400-fold) in the amount of *MDR1* mRNA were shown among these cell lines. There was also a significant correlation between *MDR1* gene expression and the reversing effect of MS-209 on ADM-resistant (Fig. 5A; $r = 0.876$, $P < 0.001$) or VCR-resistant (Fig. 5B; $r = 0.906$, $P < 0.001$) human cell lines.

Western blot analysis demonstrated high levels of P-glycoprotein in MDR cell lines, while in drug-sensitive cells this protein was not detectable (Fig. 6A). The band corresponding to P-glycoprotein was quantified by densitometric scanning, and compared with *MDR1* gene expression, which was converted into the equivalent amount of K562/ADM RNA from the MDR/ß actin ratio after 30 cycles of PCR (Fig. 6B). There was also a significant correlation between them ($r = 0.910$, $P < 0.001$). In addition, expression of P-glycoprotein showed a good correlation with the reversing effect of MS-209 on ADM-resistant (Fig. 6C; $r = 0.945$, $P < 0.001$) or VCR-resistant (Fig. 6D; $r = 0.892$, $P < 0.001$) cell lines. Consequently, apparent correlations were observed among *MDR* gene expression, P-glycoprotein expression and the MDR-reversing effect of MS-209.

Discussion

In order to analyze gene expression, a number of quantitative PCR methods have been developed. A common strategy has been to coamplify the added DNA segment [2, 10, 37] or internal standard gene [3, 16] in the same tube. However, when the target and the reference genes are amplified together in the same tube, the yield of PCR products is often low due to competition or reduced efficiency of amplification [20]. The relative levels of gene expression have therefore been determined by comparing the PCR products of the target DNA and endogenous internal standard gene in separate reactions [17, 23].

In this study, we used a quantitative PCR method to measure the expression of *MDR* genes. The key aspects of this method were that the *MDR* gene and the *ßactin* gene were amplified separately, and that the fluorescence intensity of EtBr was employed for quantitation. A small difference of initial quantity of *MDR* mRNA from various cells may lead to a large difference in the quantity of the PCR products. In addition, RNAs from biopsy specimens have frequently suffered significant degradation. Therefore, it is necessary to normalize the PCR products by an adequate internal standard. Since the amount of *ßactin* mRNA is constant in different cell types [13], the expression of the *MDR* gene was normalized by the *ßactin* gene expression. Moreover, the fluorescence intensity of EtBr increases in the presence of double-stranded DNA such as PCR products, and thus, gene amplification can be continuously monitored [15]. As shown in Fig. 2, fluorescence certainly increased as the numbers of cycles was increased.

MS-209 at a concentration of 3 µ*M* markedly enhanced the cytotoxicity of both ADM and VCR in various mouse and human cell lines in vitro. We have previously reported that the novel quinoline compound MS-073 can overcome MDR by inhibiting drug binding to P-glycoprotein [26]. However, high doses of MS-073 are necessary to obtain the effects in combination chemotherapy with VCR given orally in vivo.

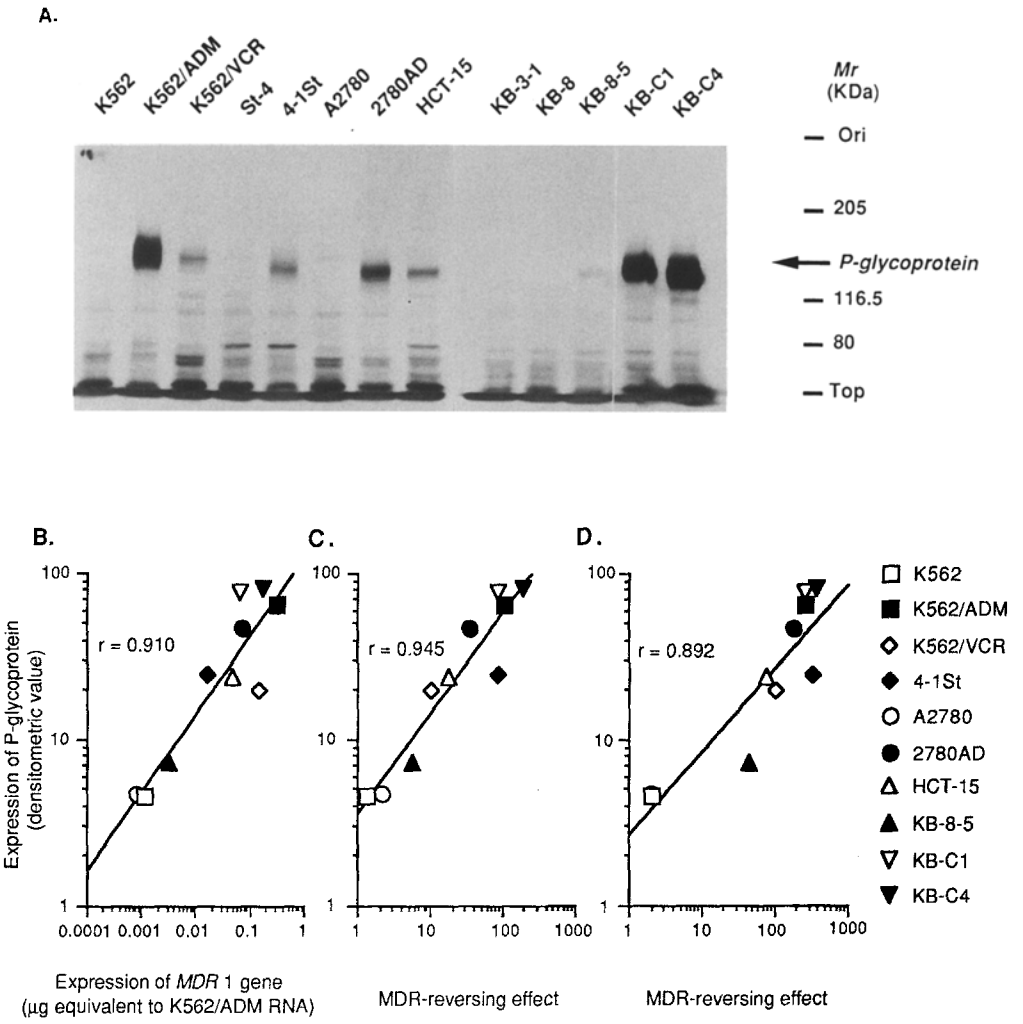


Fig. 6A Western blot analysis of P-glycoprotein in various human tumor cell lines. Membrane fraction (50 μg) was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred to a nitrocellulose filter. The blot was probed with the monoclonal antibody to human P-glycoprotein JSB-1. *Right ordinate* position of molecular mass markers in thousands of daltons (kDa). **B** Relationship between the expression of *MDR1* gene and that of P-glycoprotein. The MDR/ß actin ratio (expression of the *MDR1* gene) after 30 cycles of PCR was converted into the equivalent amount of K562/ADM RNA. The expression of P-glycoprotein was measured by densitometry. **C, D** Relationship between the expression of P-glycoprotein and the reversing effect of 3 μ*M* MS-209 on (**C**) ADM-resistant or (**D**) VCR-resistant human tumor cell lines. The MDR-reversing effect of MS-209 was calculated from the IC_{50} obtained in the MTT assay

A more effective compound, MS-209, was therefore selected from newly synthesized derivatives of MS-073 [27]. MS-209 is a more effective MDR-reversing agent for oral administration.

In this study, we assumed that there exists a good correlation between the level of expression of the 3MDR gene (amount of P-glycoprotein) and the efficacy of the MDR-reversing effect of MS-209. As expected, the results of PCR-based measurements of the *MDR* gene expression showed a significant correlation with the MDR-reversing effect of MS-209 both in the mouse cell lines (Fig. 3) and in the human cell lines (Fig. 5). This correlation indicates that all the examined cell lines, even the so-called drug-sensitive tumor cells, expressed the *MDR* gene, and the greater the expression of this gene, the greater the MDR-reversing effect of MS-209. Furthermore, *MDR1* gene expression and the expression of P-glycoprotein also showed a significant correlation in the human cell lines (Fig. 6B). Consequently, the MDR-reversing effect of MS-209 is specifically correlated with the *MDR* gene product, P-glycoprotein (Figs. 6C, D).

MDR1 mRNA has been reported to be present at low levels in most clinical samples, detectable almost exclusively by PCR [23]. Furthermore, PCR analysis is the most sensitive method available, giving convincing quantifiable results at the lowest levels [14]. We have demonstrated a significant correlation between the levels of *MDR1* gene expression, P-glycoprotein expression and the response to combination treatment with MS-209 as reported here. Therefore, RT-PCR assays of *MDR1* gene expression in tumor biopsy specimens could be an effective predictor of the tumor response to reversing agents, including MS-209, in clinical trials.

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