0026-895X/91/010001-08$03.00/0

MOLECULAR PHARMACOLOGY, 39:1-8

ACCELERATED COMMUNICATION

Relationship Between Expression of P-Glycoprotein and Efficacy of Trifluoperazine in Multidrug-Resistant Cells

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Received May 15, 1990; Accepted October 16, 1990

SUMMARY

Tumor cell resistance due to enhanced efflux of drugs with diverse structures and/or mechanisms of action is termed multidrug resistance (MDR), and modulation of the MDR phenotype by calcium blockers or calmodulin inhibitors is suggested to involve P-glycoprotein. In drug-sensitive (S) and 5-fold doxorubicin (DOX)-resistant (R0) L1210 mouse leukemia cells, no obvious differences in *mdr* mRNA or P-glycoprotein expression or alterations in cellular uptake, retention, or cytotoxicity of vincristine (VCR) were observed. However, in the 10-fold (R1) and 40-fold (R2) DOX-resistant sublines, expression of P-glycoprotein was correlated with the level of resistance (R2 > R1). An RNase protection assay revealed that elevated levels of *mdr1* and *mdr2* mRNA were detected in R1 and R2 cells, with an additional increase in *mdr3* mRNA in the R2 subline. Further, in the R1 and R2 sublines, no VCR dose-dependent cytotoxicity was apparent, and cell kill of >40% was not achievable following a 3-hr drug exposure. Cellular uptake and retention of VCR were 2- to 4-fold lower in the R1 and R2 sublines, compared with similarly treated S or R0 cells. Potentiation of VCR cytotoxicity by a noncytotoxic concentration of 5 μM trifluoperazine (TFP) was >2-fold in S and R0 cells and <1.3-fold in the R1 and R2 sublines. Modulation of VCR uptake by 5 μM TFP in the S and R0 cells was 2-fold and it was 4- to 7-fold in the R1 and R2 sublines. The presence of 5 μM TFP, by competing for efflux, enhanced VCR retention 1.5-fold in S and R0 cells and 2- to 4-fold in the R1 and R2 sublines. In contrast to these results with VCR, dose-dependent cytotoxicity of DOX was apparent in all the resistant sublines, and modulation of DOX cytotoxicity by 5 μM TFP was dependent on the level of resistance. Cellular accumulation of DOX was 20 and 50% lower in the R1 and R2 sublines, respectively, compared with similarly treated S or R0 cells. Marked increases (>1.5-fold) in cellular accumulation of DOX by TFP were apparent only in the R2 subline. Results suggest that a relationship between overexpression of P-glycoprotein isoforms and their role in affecting cellular drug levels and consequent cytotoxicity in MDR L1210 cells determines resistance to VCR but not DOX. Further, the differential effects of TFP on cellular levels versus cytotoxicity of VCR and DOX suggest that targets involved in modulation of resistance are possibly different and not solely related to the expression of P-glycoprotein.

Treatment failure in cancer chemotherapy is often due to the emergence of drug-resistant cells (1, 2). Although the mechanisms that determine expression of resistance to antitumor drugs of various classes are distinctly different (2), a common mechanism related to drug efflux has been suggested for the anthracyclines and *Vinca* alkaloids (3, 4). The role of drug efflux as a mechanism governing expression of resistance to antitumor drugs of diverse structure and/or mechanism of action is based on the occurrence of a high molecular weight membrane glycoprotein (M_r150,000–180,000) (P-glycoprotein) with homology to bacterial active transport proteins (3–5).

This work was supported by Public Health Service Grant R01CA35531 and Grant R01CA43621 awarded by the National Cancer Institute, Department of Health and Human Services.

The identification of P-glycoprotein in tumor cells selected for resistance to anthracyclines or *Vinca* alkaloids in experimental model systems and its expression relative to the level of resistance have provided compelling evidence suggesting drug efflux as the major mechanism governing resistance (3, 4). However, although alterations in drug uptake and/or efflux of the anthracycline DOX are apparent in MDR cells, expression of resistance to lipophilic anthracyclines is unrelated to alterations in cellular pharmacokinetics (6–8). Further, in MDR cells it appears that alterations in uptake and/or efflux of VCR or vinblastine (9, 10) versus DOX or daunorubicin (9, 11, 12) are related to the expression of resistance.

Calcium channel blockers and calmodulin inhibitors (12–14) have been demonstrated to be capable of significantly sensitizing MDR cells by possibly interfering with cellular drug accu-

ABBREVIATIONS: DOX, doxorubicin; TFP, trifluoperazine dihydrochloride; VCR, vincristine sulfate; FBS, fetal bovine serum; MDR, multidrug resistance (resistant); *m*-AMSA, *N*-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulfonamide; VP-16,4′-demethylepipodophyllotoxin 9-[4,6-O-ethylidene-β-D-glucopyranoside]

mulation (13, 14). Further, modulation of drug accumulation, notably by calcium blockers, in resistant cells is suggested to involve direct binding of these hydrophobic agents to P-glycoprotein (4). The role of the calmodulin inhibitor TFP in modulating vinblastine resistance by altering drug uptake/efflux is readily apparent, but in the case of DOX marked increases in cytotoxicity are unrelated to restoration of cellular drug levels (10, 12).

In a series of progressively DOX-resistant tumor cells, we have demonstrated that expression of resistance is not related to corresponding alterations in DOX efflux or glutathione and glutathione-*S*-transferases involved in xenobiotic metabolism and, for equivalent cytotoxicity, cellular DOX levels in the absence versus presence of TFP are markedly higher (12). Further, in these DOX-resistant sublines, marked reductions in *m*-AMSA- and VP-16-induced topoisomerase II-mediated cleavage were found to exceed decreases in the catalytic activity of the enzyme (15). Because these progressively DOX-resistant (5- to 40-fold) cells express the MDR phenotype (15), in the present study we have determined the relationship between expression of P-glycoprotein and efficacy of TFP in modulating cellular pharmacokinetics versus cytotoxicity of VCR and DOX. The choice of evaluating VCR and DOX is based on the premise that, in a cell population sensitive to the cytotoxic effects of these drugs, cell kill is observed following treatment for 1–3 hr and can be related to cellular levels of the drug (11, 16, 17).

Materials and Methods

The sensitive and progressively DOX-resistant sublines of L1210 mouse leukemia were cultured *in vitro*, using RPMI 1640 supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid buffer (M. A. Bioproducts, Walkersville, MD), 10% FBS (Sterile Systems, Logan, UT), and 10 μM 2-mercaptoethanol (12).

Tumor from ascites of a mouse with L1210 leukemia was used to establish an *in vitro* culture of parental sensitive cells (12). The progressively DOX-resistant L1210 mouse leukemia cells were developed *in vitro* by sequential exposure of the parental sensitive (L1210/S) cells to increasing concentrations of DOX (12). Briefly, cells adapted to grow in 0.043, 0.086, and 0.43 μM DOX were identified as L1210/DOX0.025 (R0), L1210/DOX0.05 (R1), and L1210/DOX0.25 (R2), respectively. Based on *in vitro* cytotoxicity studies using a soft agar colony assay and 3-hr exposure to DOX, the R0, R1, and R2 sublines were 5-, 10-, and 40-fold DOX-resistant, respectively, compared with the L1210/S cells. The doubling time (mean ± standard error) *in vitro* of the sensitive and progressively DOX-resistant sublines was 10.7 ± 0.1 hr. Further, the DOX-resistant sublines maintained in the absence of DOX were stably resistant for at least 3 months (about 200 doublings) during *in vitro* culture. VCR sulfate was a gift from Eli Lilly Co. (Indianapolis, IN). [^{3}H]VCR sulfate (specific activity, 259 GBq/mmol; purity, >98% by high performance liquid chromatography) was obtained from Amersham Corporation (Arlington Heights, IL). The calmodulin inhibitor TFP was a gift from SmithKline Beecham Pharmaceuticals (King of Prussia, PA).

VCR or DOX cytotoxicity *in vitro*. Logarithmic phase cultures of sensitive (S) and progressively DOX-resistant sublines (R0, R1, and R2) of L1210 mouse leukemia cells in RPMI 1640 with *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid (buffered RPMI 1640) and 10% FBS were treated (1×10^6 cells/ml) with 0.01–2.17 μM VCR or 0.02–3.45 μM DOX, in the absence or presence of 5 μM TFP, for 3 hr at 37° in a humidified 5% CO_2 plus 95% air atmosphere. Control and treated cells were centrifuged ($80 \times g$), washed twice with drug-free buffered RPMI 1640, and plated in triplicate at a density of 5×10^3 cells/35- × 10-mm Petri dish. Details of the plating medium, incubation conditions, and analysis of colony formation are similar to those previously reported (12). The colony count (mean ± standard error) in untreated controls of the sensitive and progressively DOX-resistant sublines was 1385 ± 50, corresponding to a plating efficiency of approximately 28%.

Cellular accumulation of VCR. Logarithmic phase cultures of S, R0, R1, and R2 L1210 mouse leukemia cells, in buffered RPMI 1640 supplemented with 10% FBS, were treated (1×10^6 cells/ml) with 0.054 or 0.54 μM VCR ([^{3}H]VCR plus unlabeled VCR), in the absence or presence of 5 μM TFP, at 37° in a humidified 5% CO_2 plus 95% air atmosphere. Duplicate aliquots (1×10^6 cells) retrieved at 1 hr and 3 hr were washed three times with 7 ml of cold (4°) 0.85% sodium chloride solution, and cells were recovered by centrifugation ($350 \times g$). The cell pellet was digested in Protosol (New England Nuclear Research Products, Boston, MA) at room temperature for 18 hr, followed by 2 hr at 54°, and counted in a Beckman LS-3801 liquid scintillation counter (Beckman Instruments, Fullerton, CA), using Econofluor (New England Nuclear Research Products) as the scintillation cocktail. Counting efficiency for tritium in the Protosol-treated samples was 55%, and cellular levels of VCR were expressed as pmol of [^{3}H]VCR/10^6 cells.

Cellular retention of VCR. Logarithmic phase cultures of S, R0, R1, and R2 sublines of L1210 mouse leukemia cells were treated (1×10^6 cells/ml) with 0.54 μM VCR ([^{3}H]VCR plus unlabeled VCR), in the absence or presence of 5 μM TFP, for 3 hr at 37° in a humidified 5% CO_2 plus 95% air atmosphere. Duplicate aliquots (1×10^6 cells) removed at the end of the 3-hr accumulation phase were processed for quantification of cellular levels of [^{3}H]VCR, as described in cellular accumulation of VCR. The remainder of the cells were centrifuged ($80 \times g$), washed once with buffered RPMI 1640 or buffered RPMI 1640 supplemented with 5 μM TFP, and resuspended in 1) buffered RPMI 1640 for cells treated with VCR alone or 2) buffered RPMI 1640 or buffered RPMI 1640 supplemented with 5 μM TFP for cells treated with VCR plus 5 μM TFP. Cells were incubated at 37° in a humidified 5% CO_2 plus 95% air atmosphere, and aliquots were retrieved at 15, 30, 60, 90, and 120 min and then processed for quantification of cellular [^{3}H]VCR levels, as described in cellular accumulation of VCR. Additionally, in order to relate cellular VCR retention to a cytotoxic response, S, R0, R1, and R2 cells were treated with an IC_{50} concentration of 0.054, 0.108, 5.4, or 5.4 μM VCR, respectively, for 3 hr, and retention was measured as described above. Cellular [^{3}H]VCR levels were expressed as pmol/10^6 cells and as a percentage of the initial accumulation at 3 hr (100%).

Cellular accumulation of DOX. Logarithmic phase cultures of S, R0, R1 and R2 sublines were treated (1×10^6 cells/ml), at 37° in a humidified 5% CO_2 plus 95% air atmosphere, with 0.086, 0.86, or 3.45 μM DOX, in the absence or presence of 5 μM TFP. Aliquots (1×10^6 cells) retrieved at the end of 1 and 3 hr were washed three times with 7 ml of ice-cold 0.85% sodium chloride solution, and the cell pellet obtained following centrifugation was vortexed in 50% ethanol/0.3 N hydrochloric acid. The supernatant after centrifugation was analyzed for DOX content fluorimetrically, as described earlier (12), and cellular DOX levels were expressed in ng/10^6 cells. Previous studies (12) have established that DOX is not metabolized by the sensitive or DOX-resistant cells in the absence or presence of calmodulin inhibitors.

Southern blot hybridization. Ten micrograms of *Eco*RI (Boehringer Mannheim)-digested genomic DNA were electrophoresed on 1% agarose gels, transferred to nitrocellulose filters (Millipore), and hybridized to a probe containing the DNA sequence from the *BglI* site (nucleotide 3444 from the transaction start codon) to the 3′ end of the *mdr1* cDNA. This cDNA clone was isolated from a mouse B cell cDNA library using the Chinese hamster *mdr* DNA sequence (pDR2-4,1) as probe. Hybridization conditions have been described previously (18).

RNase protection analyses of the *mdr* gene expression. Methods of RNA extraction from mouse liver, intestine, kidney, and cultured mouse cells are similar to those described by Chirgwin *et al.* (19). The method of RNase protection using *mdr1* and *mdr2* probes is described elsewhere (19A). Briefly, a ^{32}P-labeled antisense RNA probe specifying the nucleotide sequence 2561 to 2772 from the translation start codon

of the *mdr1* mRNA was synthesized from a linearized pSP19 vector containing a *mdr1* cDNA insert. This probe would detect a 211-nucleotide fragment of the *mdr1* transcript in the RNase protection assay. Within these 211 nucleotides, there is an uninterrupted 56-nucleotide sequence that is identical to that in the *mdr3* transcript (20, 21). Therefore, this probe can simultaneously detect the *mdr3* transcript and yield a 56-nucleotide protected fragment. For detection of the *mdr2* transcript, an antisense probe spanning nucleotides 1875 to 2168 was synthesized from a pGEM3Z vector containing a *mdr2* cDNA insert. Detailed methods for preparation of the RNA probes, annealing of these probes to total RNA, RNase digestion, and analysis of the protection fragments by polyacrylamide gel electrophoresis are described elsewhere.[1]

Immunochemical detection of P-glycoprotein. Plasma membrane-enriched microsomal fractions from the control standard cell lines CH^RC5 (colchicine-resistant Chinese hamster ovary cells) and its parent drug-sensitive counterpart AUXB1 (22), as well as the L1210 sensitive and DOX-resistant sublines (R0, R1, and R2), were prepared as described earlier (15). The membrane samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as originally described by Laemmli (23) and modified by Greenberger *et al.* (24). Proteins were transferred onto nitrocellulose (Western blotted), essentially as described by Towbin *et al.* (25), and probed with C219 monoclonal antibody against P-glycoprotein (26), followed by ^{125}I-labeled goat anti-mouse IgG (ICN Radiochemicals, Irvine, CA), as described earlier (15).

Results

Effect of TFP on VCR cytotoxicity. The effect of TFP on VCR cytotoxicity in S, R0, R1, and R2 L1210 cells is presented in Fig. 1. Survival in the S and R0 cells was VCR dose dependent and, in the presence of a noncytotoxic concentration of 5 μM TFP, cell kill was enhanced >2-fold. Modulation of cytotoxicity in the S and R0 cells by TFP was not VCR dose dependent. In contrast, cytotoxic effects in the R1 and R2 cells were not VCR concentration dependent over the range of 0.1–2.17 μM. Further, except for a 1.3-fold increase in cytotoxicity at 0.1 μM VCR with 5 μM TFP, cell kill with VCR in the absence or presence of 5 μM TFP was <40% over the entire dose range of 0.54–2.17 μM.

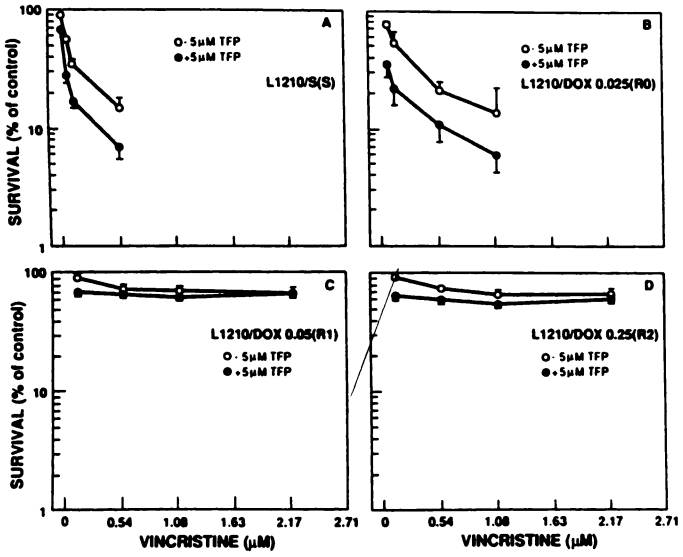

Fig. 1. Cytotoxic effects of VCR in the absence (○) or presence (●) of 5 μM TFP in sensitive (L1210/S) and progressively DOX-resistant L1210/DOX0.025 (R0), L1210/DOX0.05 (R1), and L1210/DOX0.25 (R2) L1210 mouse leukemia cells treated for 3 hr. Survival is based on colony counts. Cells were plated at a density of 5×10^3 cells/35 × 10-mm Petri dish, and the colony count (mean ± standard error) in the untreated control was 1385 ± 50, corresponding to a colony-forming efficiency of 28%. Each *point* is the mean ± standard error of triplicate experiments.

Effect of TFP on VCR accumulation. Reduced drug uptake is a notable characteristic of MDR cells (3), and results characterizing the uptake of VCR in the absence or presence of 5 μM TFP in sensitive and progressively DOX-resistant L1210 cells are outlined in Fig. 2. At 1 and 3 hr, the uptake of VCR in the R0 resistant subline was similar to that in the parental sensitive S cells. Cellular drug levels were dependent on both the extracellular concentration of VCR (0.54 μM > 0.054 μM) and time (3 hr > 1 hr). In the presence of 5 μM TFP, a 2-fold increase in cellular VCR levels apparent in S and R0 cells was not dependent on the exposure time or VCR concentration. In contrast, compared with similarly treated S cells, at both 0.054 and 0.54 μM VCR cellular drug levels at both 1 and 3 hr were 3-fold and 5-fold lower in the R1 and R2 cells, respectively. Further, in the R1 and R2 sublines, although the effect of 5 μM TFP on VCR uptake was not dependent on extracellular VCR concentration or time, cellular drug levels were 4- to 7-fold higher in the presence versus the absence of 5 μM TFP. Overall, compared with S or R0 cells, remarkable differences in VCR uptake and efficacy of TFP in increasing cellular drug levels were apparent in the R1 and R2 sublines, which are 10-fold and 40-fold DOX resistant, respectively.

Effect of TFP on VCR retention. Because reduced drug retention is also a key determinant of resistance (3), the characteristics of VCR retention in the absence or presence of 5 μM

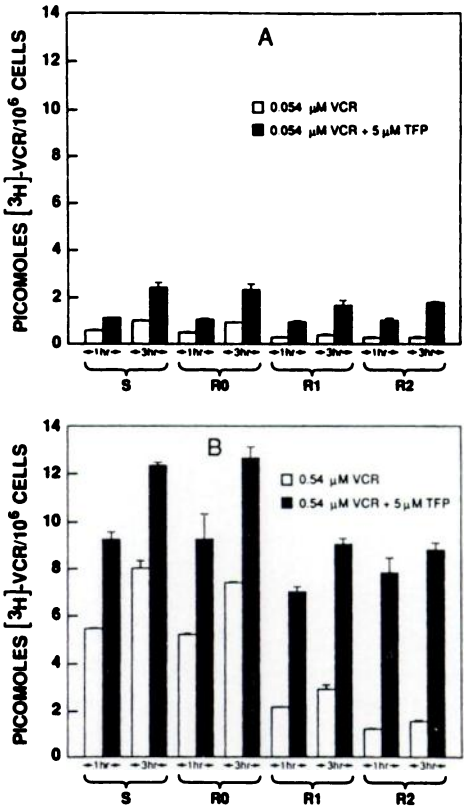

Fig. 2. Effect of TFP on cellular accumulation of VCR at 1 and 3 hr in S, R0, R1, and R2 L1210 mouse leukemia cells treated with 0.054 μM VCR (A) or 0.54 μM VCR (B). Values are means of duplicate determinations from triplicate experiments; *bars*, standard error.

TFP were determined in S, R0, R1, and R2 sublines; and results are presented in Fig. 3. Accumulation of VCR for 3 hr before measurement of retention in VCR-free medium was chosen in order to relate cellular drug levels to cytotoxic response, and the inclusion of 5 μM TFP during the accumulation and/or retention phase was utilized to determine a possible role of competition in the drug efflux process. In both S and R0 cells treated with VCR alone, about 45% of the drug initially accumulated was retained at the end of 120 min. The presence of 5 μM TFP during the uptake phase enhanced the absolute amount (pmol) of VCR during the initial accumulation in the S or R0 cells, but the percentages of VCR retained at 120 min were similar (45%). However, the inclusion of 5 μM TFP during the retention phase markedly (1.5-fold) increased both the percentage (60%) and the absolute amount (pmol) of VCR in the S and R0 cells, suggesting that TFP can compete for efflux of VCR. The retention of VCR in the R1 and R2 sublines demonstrated that, unlike in the S and R0 cells, only 20% of the drug was retained in cells treated with VCR alone. Although the use of 5 μM TFP only during the uptake phase markedly increased VCR accumulation, cellular retention of drug was similar to that of cells treated with VCR alone. In contrast, the inclusion of 5 μM TFP during the retention phase as well had a remarkable effect on VCR retention, and cellular levels in the R1 and R2 sublines were enhanced 2- to 4-fold. Overall, VCR retention in R1 and R2 sublines was significantly ($p < 0.05$) lower than in similarly treated S or R0 cells. The presence of 5 μM TFP in the extracellular medium increased cellular retention of VCR, and its efficacy in the R1 and R2 sublines was remarkably greater than in S or R0 cells.

Cellular drug retention following treatment for 3 hr with a concentration of VCR capable of producing 50% cell kill in a soft agar colony assay is presented in Fig. 4. The retention of

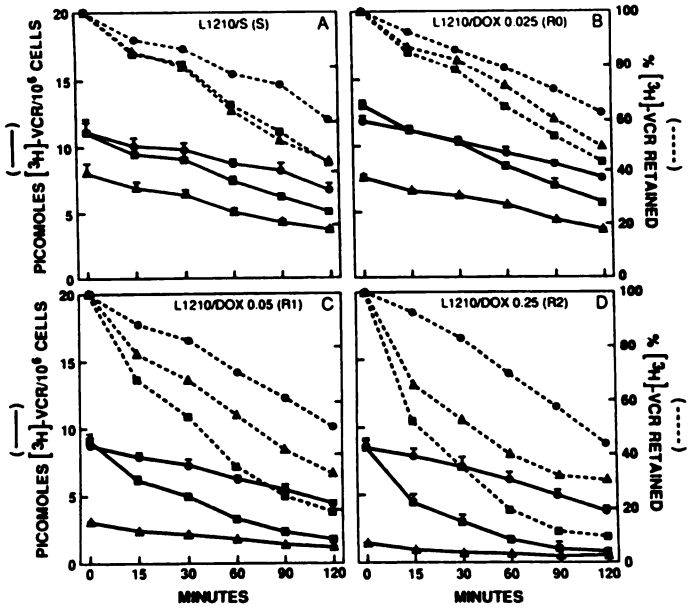

Fig. 3. Effect of TFP on cellular retention of VCR in sensitive and progressively DOX-resistant L1210 mouse leukemia cells. Each *point* is the mean value from triplicate experiments; *bars*, standard error. Cells were treated for 3 hr with 0.54 μM VCR in the absence (▲) or presence (■, ●) of 5 μM TFP and subsequently incubated in drug-free medium in the absence (▲, ■) or presence (●) of 5 μM TFP. Cellular levels of VCR during retention are expressed in pmol/10^6 cells (——) and as a percentage of drug initially accumulated (– – –).

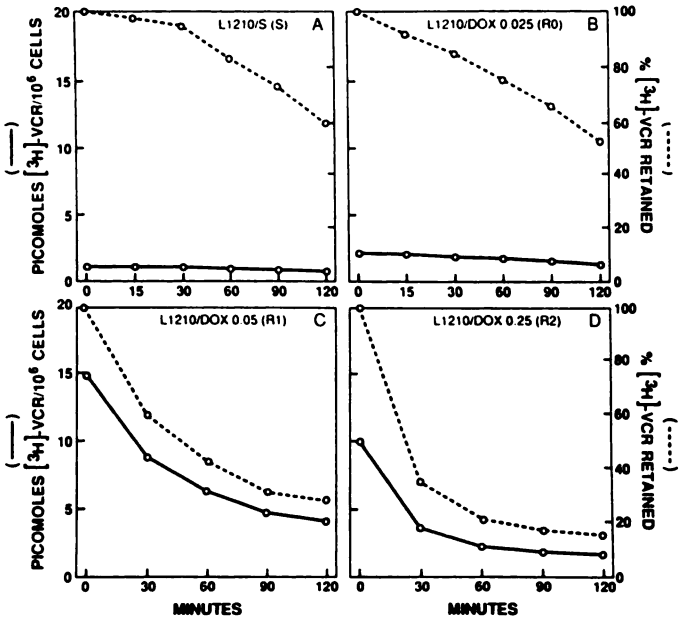

Fig. 4. Cellular retention of VCR in sensitive and progressively DOX-resistant L1210 mouse leukemia cells treated with the IC_{50} concentration of VCR. Cells were initially treated with following concentrations of VCR for 3 hr: L1210/S, 0.054 μM VCR; L1210/DOX0.025, 0.108 μM VCR; L1210/DOX0.05 and L1210/DOX0.25, 5.4 μM VCR; retention was subsequently determined in drug-free medium. Each *point* is the mean value of replicate determinations from at least duplicate experiments. Cellular VCR levels are expressed in pmol/10^6 cells (——) and as a percentage (– – –) of drug initially accumulated.

VCR in the S and R0 cells treated for 3 hr with 0.054 and 0.108 μM VCR, respectively, was 50–60% of the drug initially accumulated. Absolute cellular VCR levels at 120 min during retention in the S and R0 cells were 0.61 and 1.1 pmol of [^{3}H]VCR/10^6 cells, respectively. In contrast, drug retention in the R1 and R2 cells treated for 3 hr with 5.4 μM VCR was 15–20% of that initially accumulated. Cellular levels at 120 min during the retention phase in R1 and R2 sublines were 4 and 1.6 pmol of [^{3}H]VCR/10^6 cells, respectively.

Effect of TFP on cellular levels and cytotoxicity of VCR. Results summarizing the effect of TFP on accumulation and cytotoxicity of VCR in sensitive and progressively DOX-resistant L1210 cells are outlined in Table 1. In the S and R0 cells, accumulation of VCR was enhanced 2-fold in the presence of 5 μM TFP, and cell kill was enhanced by a similar magnitude. In contrast, although cellular accumulation of VCR in the R1 and R2 sublines was enhanced 4- to 7-fold in the presence of 5 μM TFP, cell kill was increased <15%. Thus, a relationship between cellular levels of VCR and cytotoxic response without or with 5 μM TFP was apparent in the S and R0 cells but not in the more resistant R1 and R2 sublines.

Effect of TFP on cellular levels and cytotoxicity of DOX. The effect of TFP on the cellular accumulation and cytotoxicity of DOX in S, R0, R1, and R2 L1210 cells is outlined in Table 2. Cellular DOX levels in the absence or presence of 5 μM TFP were similar in S and R0 cells, and modulation of DOX cytotoxicity by TFP was apparent in the R0 but not S cells. In contrast, remarkable differences in cellular accumulation of DOX, compared with that in S cells, was apparent in the R1 and R2 cells, and in the presence of 5 μM TFP the increase in cellular levels of DOX was dependent on the level of resistance (R2 > R1). Modulation of DOX cytotoxicity by

TABLE 1

Effect of TFP on cellular accumulation and cytotoxicity of VCR in sensitive and DOX-resistant L1210 mouse leukemia cells

Cell line	Extracellular VCR concentration	VCR accumulation[a]				Survival (3 hr)[b]	
		1 hr		3 hr			
		−5 μM TFP	+5 μM TFP	−5 μM TFP	+5 μM TFP	−5 μM TFP	+5 μM TFP
	μM	pmol of [^{3}H]VCR/10^6 cells				% of control	
L1210/S (S)	0.054	0.5	1.04	0.92	2.34	56	28
	0.54	5.33	9.16	7.95	12.30	15	7
L1210/DOX0.025 (R0)	0.054	0.45	0.96	0.81	2.22	73	35
	0.54	5.06	9.12	7.26	12.56	22	11
L1210/DOX0.05 (R1)	0.054	0.2	0.84	0.3	1.51	ND[c]	ND
	0.54	2.01	6.90	2.79	8.92	72	65
L1210/DOX0.25 (R2)	0.054	0.17	0.89	0.22	1.62	ND	ND
	0.54	1.1	7.7	1.4	8.67	72	59

[a] Cells were treated with the indicated extracellular concentrations of VCR for 1 or 3 hr, and cellular VCR levels were quantified as outlined in Materials and Methods. Values are means of duplicate determinations from triplicate experiments; standard deviations, <15%.

[b] Survival is based on colony counts following exposure to VCR, in the absence or presence of 5 μM TFP, for 3 hr. Colony-forming efficiency and other details were similar to those outlined in the legend to Fig. 1.

[c] ND, not determined.

TABLE 2

Effect of TFP on the cellular accumulation and cytotoxicity of DOX in sensitive and progressively DOX-resistant L1210 mouse leukemia cells

Cell line	Extracellular DOX concentration	DOX accumulation[a]				DOX IC$_{50}$[b]	
		1 hr		3 hr			
		−5 μM TFP	+5 μM TFP	−5 μM TFP	+5 μM TFP	−5 μM TFP	+5 μM TFP
	μM	pmol of DOX/10^6 cells				μM	
L1210/S (S)	0.086	8.1	8.57	11.17	13.00	0.09	0.09
	0.86	56.50	60.81	88.94	97.80		
L1210/DOX0.025 (R0)	0.086	8.15	8.79	10.93	12.46	0.39	0.14
	0.86	60.98	66.48	85.41	105.06		
L1210/DOX0.05 (R1)	0.086	6.45	8.36	8.28	11.79	1.14	0.23
	0.86	47.65	62.01	60.67	89.24		
L1210/DOX0.25 (R2)	0.86	28.45	45.36	39.10	67.13	3.45	0.43
	3.45	84.22	135.66	115.41	224.55		

[a] Cells were treated with the indicated extracellular concentrations of DOX for 1 or 3 hr, and cellular DOX levels were quantified as outlined in Materials and Methods. Values are means from replicate determinations from at least duplicate experiments; standard deviations, <15%.

[b] Concentration of DOX, computed from dose-response curves (12), that produces a 50% reduction in colony formation compared with the untreated control, in the absence or presence of 5 μM TFP.

TFP was evident in the R0, R1, and R2 cells, and in the presence versus absence of 5 μM TFP the IC$_{50}$ concentration of DOX for equivalent cell kill was about 3-fold, 5-fold, and 8-fold lower in the R0, R1, and R2 sublines, respectively.

P-glycoprotein mRNA expression. Recent studies have identified three *mdr* genes in the mouse genome (21, 27, 28). These genes are expressed in a tissue-specific manner (19),[1] with *mdr 1, -2,* and *-3* transcripts being the predominant *mdr* species in kidney, liver, and intestine, respectively. A RNase protection protocol was used to investigate the expression of these three *mdr* genes in the progressively DOX-resistant L1210 mouse leukemia cells (Fig. 5). In L1210/S cells (Fig. 5, *lanes 1* and *8*), only the *mdr1* transcript was detectable, at a level comparable to that in the mouse kidney (Fig. 5, *lane 13*), whereas the levels of *mdr2* and *mdr3* transcripts were too low to be detected. In L1210/DOX0.025 (R0), the levels of all three *mdr* gene transcripts were not different from those in the drug-sensitive L1210/S cells (Fig. 5, *lanes 2* and *9*). However, significant increases in *mdr1* and *mdr2* transcripts were detected in the L1210/DOX0.05 (R1) line (Fig. 5, *lanes 3* and *10*), with the level of the *mdr2* transcript being comparable to that in the normal mouse liver (Fig. 5, *lane 5*). The level of the *mdr3* transcript was also increased in the R1 line but to a lesser extent than that of the *mdr1* transcript. The cell line established in the subsequent selection, i.e., L1210/DOX0.25 (R2), exhibited no significant additional increase in the *mdr1* and *mdr2* transcripts, but the level of the *mdr3* transcript was significantly higher than that in the R1 line. The level of the *mdr3* transcript in the R2 line was about 50% of that in the normal mouse intestine (Fig. 5, *lane 14*). These results indicate that, in the progressively DOX-resistant L1210 cells established in a stepwise selection protocol, different members of the *mdr* gene family are expressed at different stages during selection.

P-glycoprotein gene amplification. Fig. 6 shows a Southern blot hybridization detection of the *mdr* gene in sensitive and progressively DOX-resistant L1210 cells. The probe used here is from the 3′ end of the *mdr1* gene. The nucleotide sequence in this probe shares 96% and 90% identity with those in the corresponding regions of the *mdr3* (*mdr1a*) (28) and *mdr2* genes (21), respectively. The annealing conditions used here would allow cross-hybridization to the *mdr2* and *mdr3* genes. This probe has been used to isolate three *mdr* genes from a mouse genomic DNA library under the same hybridization conditions.[1] As shown in Fig. 6B, significant DNA amplification was observed in the R1 but not R0 subline. It is not known at this time which of the bands seen correspond to the

[1] M. Ikeguchi and M. T. Kuo, unpublished observations.

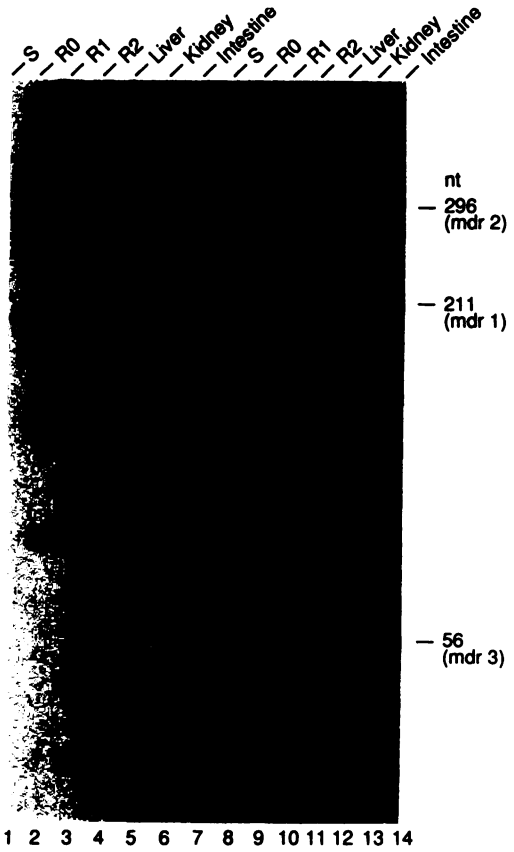

Fig. 5. RNase protection assay of *mdr* gene expression in progressively DOX-resistant L1210 mouse leukemia cells and mouse tissues. Forty micrograms of total RNA from L1210/S (S), L1210/DOX0.025 (R0), L1210/DOX0.05 (R1), and L1210/DOX0.25 (R2) cells and mouse liver, kidney, and intestine were annealed to ^{32}P-labeled antisense RNA probes specifying the *mdr2* transcript (*lanes 1–7*) or the *mdr1/mdr3* transcripts (*lanes 8–14*). The RNA duplex was protected from subsequent RNase A + T1 digestions. After denaturation, the size, in number of nucleotides (*nt*), was analyzed by polyacrylamide gel electrophoresis.

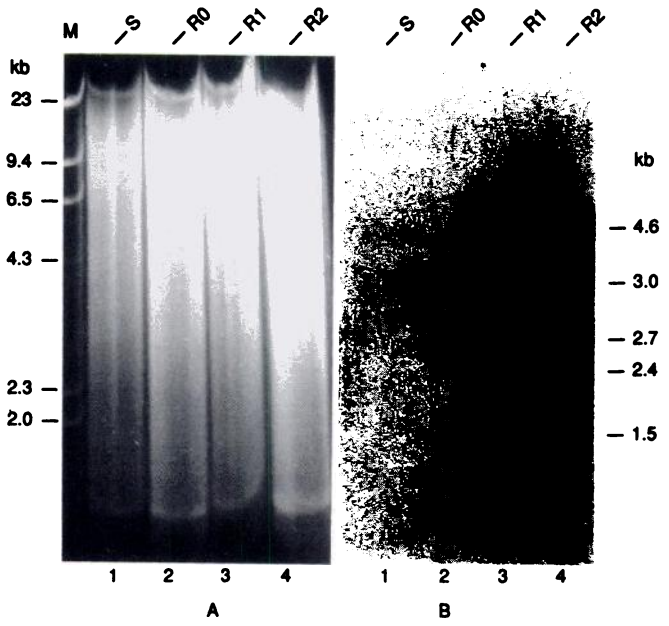

Fig. 6. Southern blot hybridization detection of *mdr* gene amplification in mouse L1210 MDR cell lines. Ten micrograms of DNA from L1210/S (S), L1210/DOX0.025 (R0), L1210/DOX0.05 (R1), and L1210/DOX0.25 (R2) were digested by restriction endonuclease *Eco*RI. DNA was separated by agarose gel electrophoresis (1%) and blot-hybridized to the 3′ end of the *mdr1* cDNA. A, Ethidium bromide-stained DNA in agarose gel; B, autoradiograph of the blot. *kb*, kilobases.

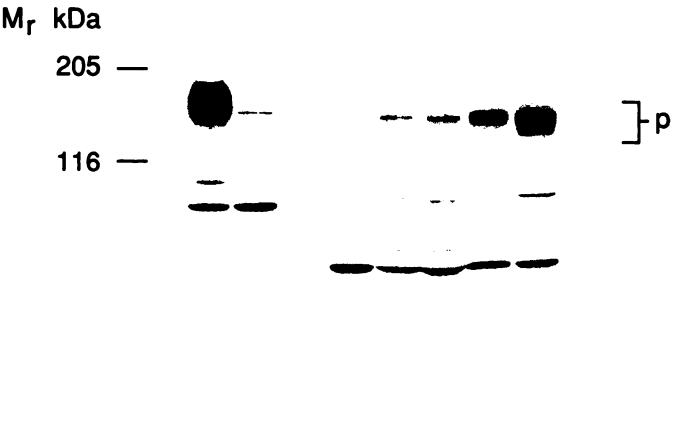

Fig. 7. Detection of P-glycoprotein (*P*) in progressively DOX-resistant L1210 mouse leukemia cells. *Lane 1*, CH^{R}C5 (colchicine-resistant Chinese hamster ovary cells); *lane 2*, AUXB1 (parental sensitive Chinese hamster ovary cells); *lane 3*, L1210/S (S); *lanes 4* and *5*, L1210/DOX0.025 (R0); *lane 6*, L1210/DOX0.05 (R1); *lane 7*, L1210/DOX0.25 (R2). Protein (50 μg, except *lane 5*, 100 μg) from a plasma membrane-enriched microsomal fraction was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with C219 monoclonal antibody (24).

three *mdr* genes. Results are consistent with data in Fig. 5 showing no difference in the steady state level of *mdr* mRNA in the R0 lane, as compared with that in the S lane. No significant additional DNA amplification is seen in the R2 lane, however, suggesting that overexpression of *mdr3* gene in the R2 lane may be due to transcriptional activation of the *mdr3* gene. Overexpression of *mdr* mRNA without accompanying gene amplification has been reported in other MDR cells (20, 30).

Expression of P-glycoprotein. The expression of P-glycoprotein, determined by immunochemical detection, in the standard sensitive (AUXB1) and colchicine-resistant (CH^{R}C5) Chinese hamster ovary cells (22) and the S, R0, R1, and R2 L1210 cells is shown in Fig. 7. Under these experimental conditions, P-glycoprotein was not detectable in the AUXB1 (Fig. 7, *lane 2*) and parental sensitive L1210 (Fig. 7, *lane 3*) cells. As expected, expression of the P-glycoprotein antigen was apparent in the CH^{R}C5 (Fig. 7, *lane 1*) cells. Although an antigen similar to P-glycoprotein in CH^{R}C5 cells (Fig. 7, *lane 1*) was not overexpressed in the R0 subline, compared with S cells, by using 50 μg (Fig. 7, *lane 4*) or 100 μg (Fig. 7, *lane 5*) of protein increased expression of P-glycoprotein in R1 (Fig. 7, *lane 6*) and R2 (Fig. 7, *lane 7*) sublines was evident and dependent on the level of DOX resistance [R2 (Fig. 7, *lane 7*) > R1 (Fig. 7, lane 6)]. The sensitivity of detection of P-glycoprotein in the DOX-resistant sublines demonstrated that, using ^{125}I-C219, increased expression can be observed using 6.25 and 3.13 μg of protein from a plasma membrane-enriched microsomal fraction in the R1 and R2 sublines, respectively.[2]

[2] R. Ganapathi and R. Baker, unpublished observations.

Discussion

Expression of resistance to drugs of diverse structure and/or mechanism of action, termed the MDR phenotype, has been suggested to be primarily due to reduced cellular drug accumulation mediated by P-glycoprotein (3, 4). Identification of agents capable of modulating the MDR phenotype has been actively pursued in a number of laboratories and, in general, the effects of these agents have been suggested to be due to increased drug accumulation as a consequence of interaction with P-glycoprotein (31–35). Although the alteration in cellular drug levels and subsequent increase in cytotoxic response induced by various hydrophobic agents including TFP form an attractive hypothesis, the modulation of resistance by a common mechanism for *Vinca* alkaloids and anthracyclines is not apparent (10, 12). The present results suggest that relationships between expression of P-glycoprotein and efficacy of TFP in modulating resistance to VCR versus DOX are notably different. Although a differential efficacy of TFP in modulating VCR versus DOX resistance has been difficult to identify in cells with high levels of resistance, the present results suggest that, in a model system with low levels of progressive resistance, which is possibly more clinically relevant, the relationship between cellular drug levels, their modulation by TFP, and a cytotoxic response can be characterized.

The results on *in vitro* cell kill (Fig. 1) demonstrate that TFP significantly ($p < 0.05$) enhances the cytotoxicity of VCR in the S and R0 cells over the entire dose range (0.01–1.08 μM) and in R1 and R2 sublines only at 0.108 μM VCR. Although VCR dose-dependent cytotoxicity in the absence or presence of TFP was apparent in the S and R0 cells, a 10-fold increase in VCR concentration, with or without TFP, did not enhance kill > 40% in the R1 and R2 sublines (Table 1 and Fig. 1). In contrast to these results with VCR, dose-dependent cell kill with or without TFP is apparent in the sensitive or resistant sublines treated with DOX (12). Further, as outlined in Table 2, the efficacy of TFP in modulating DOX cytotoxicity is dependent on the level of resistance, with R2 > R1 > R0 > S.

A 3- to 5-fold decrease in VCR uptake in the R1 and R2 sublines, compared with S or R0 cells (Fig. 2), suggests that reduced drug uptake could be a major factor determining expression of resistance to VCR. However, a 4- to 7-fold increase in cellular VCR uptake by TFP in the R1 and R2 sublines does not result in cell kill comparable to that of the S or R0 cells (Fig. 1 versus Fig. 2 and Table 1). Further, in contrast to the R1 and R2 sublines, a 2-fold increase in VCR uptake by TFP in the S and R0 cells results in potentiation of cytotoxicity by a similar magnitude (Table 1). It thus appears that, in contrast to results with DOX (Table 2), cellular levels of VCR in the absence or presence of TFP are related to a comparable cytotoxic response in the S and R0 cells but not R1 or R2 sublines.

Because uptake *per se* may be inadequate to correlate with a cytotoxic response, reduced retention has been suggested to be a major determinant in MDR cells (3, 4). An analysis of cellular VCR retention patterns and the effects of TFP demonstrates that reduced drug retention is a prominent characteristic of the R1 and R2 sublines but not the S or R0 cells (Fig. 3 and 4). A key finding is that the extracellular presence of TFP during retention can markedly (>1.5-fold) enhance cellular VCR levels in sensitive or resistant sublines, by competing for efflux (Fig. 3). Previous studies with TFP and DOX in the L1210 cells have revealed that competition for DOX efflux by TFP is not apparent in the S or R1 cells, but only in the R2 cells (12). Overall, the results suggest that competition for DOX but not VCR efflux by TFP is dependent on the level of resistance.

A relationship between cellular VCR levels producing equivalent cytotoxicity in the sensitive and resistant sublines is apparent from the results on VCR retention following drug uptake at IC_{50} concentrations (Fig. 4). In contrast to these results with VCR, cellular levels of DOX required for equivalent cytotoxicity in the resistant sublines are significantly greater than in the sensitive cells and are dependent on the level of resistance (12). The requirement of higher cellular DOX levels for equivalent cytotoxicity in the resistant versus sensitive cells is also consistent with observations on the altered DNA cleavage activity of topoisomerase II in the DOX-resistant L1210 cells (15). Further, whereas cellular VCR levels for equivalent cell kill in the absence or presence of TFP were comparable in the sensitive and resistant sublines, with DOX the resistant sublines required significantly lower levels of DOX in the presence versus absence of TFP for similar cell kill (Table 2).

It is widely accepted that P-glycoprotein-mediated drug efflux governs resistance and that interaction of the calcium modifiers with P-glycoprotein may be responsible for modulation of cytotoxicity (4, 34, 35). The modulation of cellular uptake and/or retention of VCR in the sensitive and resistant sublines (Fig. 3 and 4, Table 1) suggest that efficacy of TFP may not be exclusively due to interaction with P-glycoprotein. Further, competition for efflux of VCR during retention by TFP, with a concomitant increase in cell kill, was also observed in the sensitive and resistant sublines (data not shown).

Results in Figs. 5 and 6 complement data in Fig. 7 demonstrating that overexpression of P-glycoprotein in R1 and R2 sublines parallels the occurrence of *mdr* transcripts and amplification of *mdr* DNA sequences. The data on *mdr* transcripts also suggest that with increasing resistance there is a switching in the expression of *mdr* genes, based on overproduction of *mdr3* in the R2 subline. This is consistent with the overproduction of P-glycoprotein in the R2 versus R1 subline (Fig. 7, *lane 7* versus *lane 6*). The increase in *mdr3* mRNA level in R2 versus R1 (Fig. 5, *lane 11* versus *lane 10*) without apparent increase in gene copy number (Fig. 6B, *lane 4* versus *lane 3*) suggests that transcriptional regulation may be responsible (20). Thus, multiple mechanisms may be involved in the overexpression of P-glycoprotein isoforms in these DOX-resistant L1210 sublines.

A significant increase in *mdr1*/*mdr3* gene expression is detected in the R1 and R2 sublines. Although the function of the *mdr2* gene product is still not known, it has been demonstrated recently that *mdr1* and *mdr3* gene products are capable of conferring the MDR phenotype in animal cells (20). Cells expressing the *mdr1* gene product exhibited preferential resistance to DOX, whereas those expressing *mdr3* gene products displayed preferential resistance to vinblastine (20). Our present results demonstrating differences in uptake and retention between DOX and VCR in the progressively DOX-resistant L1210 sublines are consistent with the notion that expression of different P-glycoprotein genes may result in alterations in the cytotoxicity profiles to antitumor drugs involved in MDR.

In summary, the results from this study using MDR L1210 cells demonstrate that overexpression of P-glycoprotein and the consequent reduction in drug uptake/retention may corre-

late better with cellular resistance to VCR versus DOX. The role of TFP in modulating cellular VCR levels appears to be not solely due to the presence of P-glycoprotein, because significant ($p < 0.05$) effects on VCR accumulation and cytotoxicity also occur in parental sensitive (S) and 5-fold DOX-resistant (R0) cells. The differential efficacy of TFP suggests that targets involved in modulation of VCR versus DOX resistance are possibly different and this could influence the choice of agents for overcoming resistance in an *in vivo* setting.

Acknowledgments

The authors gratefully acknowledge Dr. George Williams, Sharon VanderBrug Mendendorp, and Lata Paranandi for statistical analysis of data, Ms. Frances Adjei for her excellent secretarial assistance, and Jim Reed and Ken Celebucki of the Art-Medical Illustrations and Photography Department for skillful preparation of the art work.

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