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Treatment of Multidrug Resistant (*MDR*1) Murine Leukemia with P-Glycoprotein Substrates Accelerates the Course of the Disease

Jin-Ming Yang,[1] Guang-Yu Yang, Daniel J. Medina, Andrew D. Vassil, Jie Liao, and William N. Hait[1]

Departments of Medicine and Pharmacology, The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, 195 Little Albany Street, New Brunswick, New Jersey 08901; and Laboratory for Cancer Research, College of Pharmacy, Rutgers University, Piscataway, New Jersey 08855

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The prognosis of patients with tumors expressing P-glycoprotein (P-gp), the *MDR*1 gene product, is generally poor. It is assumed that this is due to decreased tumor responsiveness that results from decreased drug accumulation. We observed that treatment of animals bearing *MDR*1-transfected leukemic cells with P-gp substrates (i.e., drugs that are transported by P-gp) significantly worsened host survival compared to treatment with vehicle or non-P-gp substrates. This effect was seen with cancer chemotherapeutic agents (paclitaxel and vincristine) and with the MDR modulator, *trans*-flupenthixol. To determine the mechanism(s) underlying this observation, we studied alterations in pharmacokinetics, pharmacodynamics, and metastasis. We found that the drug-induced acceleration of disease was associated with increased metastases. P-gp(+) cells treated with P-gp substrates demonstrated several pro-metastatic features, including membrane ruffling and invasion through a hepatocyte monolayer. These results suggest that the treatment of MDR tumors with P-gp substrates may produce changes in malignant behavior that could adversely affect therapeutic outcomes. © 1999 Academic Press

Key Words: **multidrug resistance; P-glycoprotein; P-glycoprotein substrates; invasion; metastasis.**

Drug resistance remains a formidable obstacle to the effective treatment of human cancer. The discovery of P-gp (1–3), the *MDR*1 gene product that functions as an energy-dependent drug transporter, revealed a previously unknown mechanism of resistance to anticancer drugs. It was established that P-gp is expressed in human cancers (4), and imparts a poor prognosis. Although it was generally assumed that this effect on prognosis was due to the inability of chemotherapeutic drugs to reach therapeutic concentrations at intracellular targets, we observed experimental results that did not easily fit this paradigm. We now describe experiments demonstrating that treatment of MDR leukemia with drugs that are effluxed by P-gp decreases survival compared to treatment with vehicle. In addition, we uncovered a potential mechanism to explain these results, which appears unrelated to drug efflux.

Abbreviations used: P-gp, P-glycoprotein; MDR, multidrug resistant or multidrug resistance; VCR, vincristine; TAX, paclitaxel; tFPT, *trans*-flupenthixol; MEM, mechlorethamine; i.p., intraperitoneal.

[1] To whom correspondence may be addressed. Fax: 732-235-8098. E-mail: jyang@rwja.umdnj.edu.

MATERIALS AND METHODS

Animal studies. Cells from log-phase cultures of P388/S or P388/VMDRC.04 (5) were washed in PBS by centrifugation at 1000×g for 10 min, then resuspended in sterile saline at a concentration of 5×10^6 cells/ml. One million cells in 0.2 ml of PBS were inoculated into groups of five 20- to 22-g female $CD2F_1$ mice (Charles River Laboratories, Wilmington, MA) via the peritoneal cavities on day 0. Vehicles or drugs were given i.p. on days 1, 5, and 9 for VCR (0.2–1.0 mg/kg), tFPT (40 mg/kg) or MEM (0.5 mg/kg), and on days 1–5 for TAX (10–15 mg/kg). Animals were given food and water *ad libitum* and were checked daily for weight, signs of tumor growth, or illness. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

Pharmacokinetics. P388/S or P388/VMDRC.04 cells were inoculated into mice as described above. On day 10, mice were given an i.p. injection of TAX (15 mg/kg). [^{3}H]TAX (5 μCi/mouse) was used as a tracer to determine drug content in various organs or tissues. The ^{3}H content of tissues was determined as described by Teicher *et al.* (6). At various time points after drug administration, mice were sacrificed and known amounts of liver, kidney, spleen, heart, lung, brain, blood, and ascites were collected and dissolved in SOLVABLE (Packard Instrument Co., CT), then counted by liquid scintillation.

Histopathology. Tumor-bearing mice treated as described above were sacrificed on day 12 and the livers, kidneys, brains, spleens, hearts, and lungs were removed, fixed in Buffered Formalde-Fresh (Fisher Scientific, NJ) and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin for general histopatholog-

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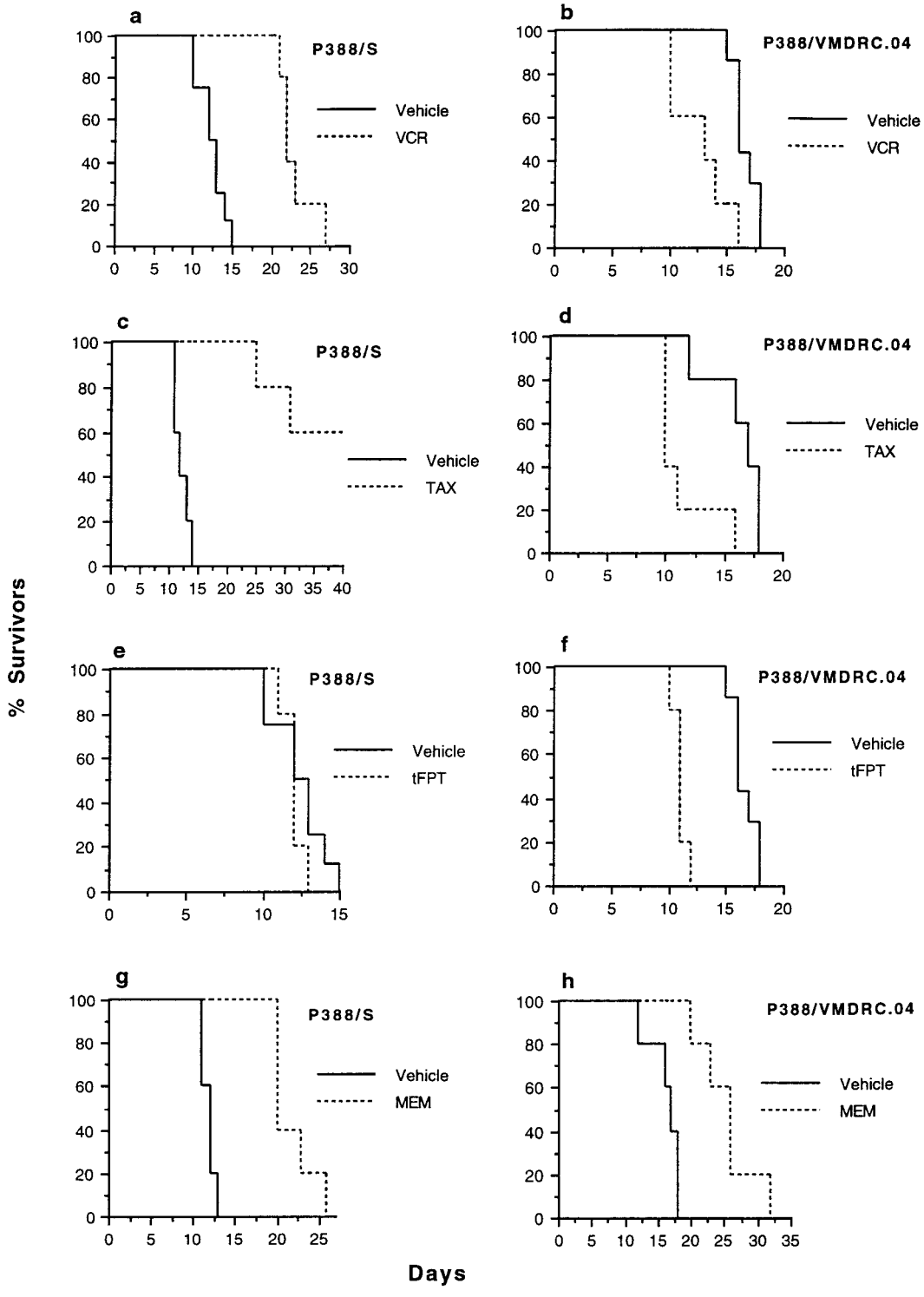

FIG. 1. Effects of VCR (a, b), TAX (c, d), tFPT (e, f), and MEM (g, h) on survival of mice bearing sensitive P388/S or multidrug resistant P388/VMDRC.04 leukemia. Mice were inoculated with 1×10^6 cells into the peritoneal cavities on day 0 and treated on days 1, 5, and 9 with VCR (1 mg/kg), MEM (0.5 mg/kg), or tFPT (40 mg/kg) or on days 1–5 with TAX (15 mg/kg), as described under Materials and Methods. Data shown are the results of a representative of three separate experiments with five mice per group. Points, animals surviving at the time indicated on the *abscissa* divided by the initial number of animals treated.

ical analysis. The stained slides were examined with a NIKON microscope with a PC Image-Pro Plus system (Media Cybernetics, Silver Spring, MD).

Invasion assay. The invasive capacity of sensitive and MDR P388 cells were assessed using a modification of the methods of Janiak *et al.* (7) and Habets *et al.* (8). Briefly, normal mouse embryonic liver

TABLE 1

AUC of TAX in the Livers, Kidneys, Lungs, Hearts, Spleens, Brains, and Blood of Mice Bearing P388/S or P388/VMDRC.04 Tumors

	AUC (μg.h/g or μg.h/ml)[a]							
	Liver	Kidney	Lung	Heart	Spleen	Brain	Blood	Tumor cells
P388/S	174.4 ± 20.1	135.1 ± 15.8	63.4 ± 5.6	22.0 ± 4.2	108.0 ± 11.3	3.39 ± 1.2	10.0 ± 5.8	592.5 ± 35.2
P388/VMDRC.04	170.9 ± 32.1	79.1 ± 9.8	38.2 ± 2.9	16.4 ± 3.2	75.1 ± 12.5	2.31 ± 1.5	4.18 ± 4.7	452.3 ± 30.1

[a] Mean ± SD of 4 mice from 2 separate experiments.

cells BNL CL.2 (American Type Culture Collection, Rockville, MD) were seeded on the upper surface of an 8.0 μm polyethylene terephthalate membrane filter in a cell culture insert within a blind-well chamber (Becton Dickinson, Franklin Lakes, NJ). Forty-eight hours later, 2×10^5 P388/S or P388/VMDRC.04 cells in log-phase of growth were layered on the hepatocyte monolayer in the upper chamber of the assay vessel and incubated at 37°C in 95% O_2/5% CO_2, in the presence or absence of drugs. Drug concentrations were chosen that produced <10% cell killing and had no effect on the hepatocyte monolayer during the 24 h-incubation. Cells traversing the hepatocyte layer through the porous membrane were collected in the lower chamber and counted.

Measurement of intracellular pH. P388/S or P388/VMDRC.04 cells (2×10^5 cells/tube) in log-phase of growth were treated with drugs or vehicle for 24 h at 37°C in 95% O_2/5% CO_2. Drug concentrations were chosen which produce <10% cell killing. Intracellular pH was measured using flow cytometry (EPICS ELITE flow cytometer, Beckman Coulter, Inc., FL) with carboxy-SNARF-1, as previously described by Wieder *et al.* (9).

Membrane ruffling assay. Membrane ruffling was determined using fluorescently labeled phalloidin as previously described (10). Briefly, sensitive MCF-7 or MDR MCF-7/BC-19 cells were plated on glass coverslips in 35-mm cell culture dishes and grown for 24 h, then treated with drugs or vehicle overnight. The glass coverslips were then rinsed twice with pre-warmed PBS, and the cells were fixed in 3.7% formaldehyde in PBS for 15 min. Cells were permeabilized with 0.2% Triton-X 100 in PBS for 5 min, then treated with 50 mM ammonium chloride in PBS for 10 min. Membrane ruffling was visualized by staining with 5 μM phalloidin-tetramethyl-rhodamine isothiocyanate (TRITC) (Sigma Chemical Co.) for 1 h at room temperature. The slides were viewed using a fluorescence microscope with the PC Image-Pro Plus system (Media Cybernetics, Silver Spring, MD).

Statistical analysis. For analysis of between-group differences, *P* values were determined using Student's *t*-test. A *P* value of 0.05 was considered significant.

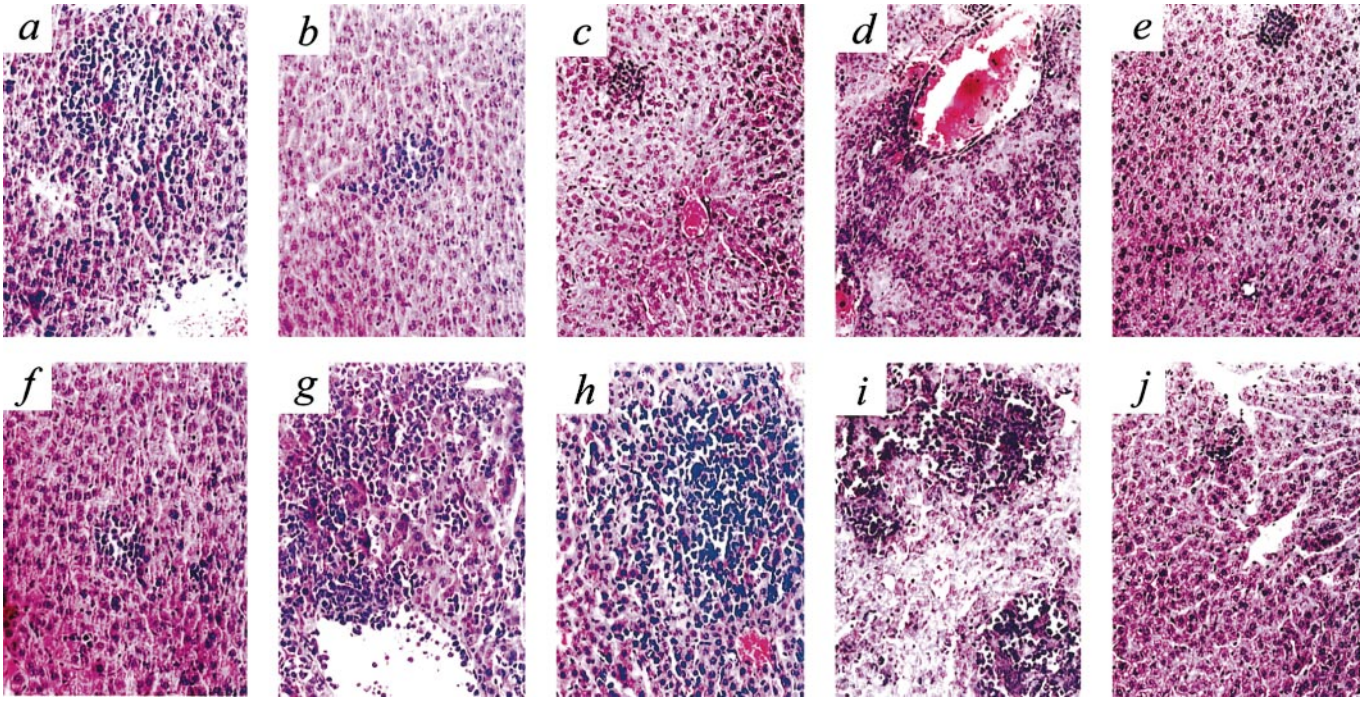

FIG. 2. Photomicrographs of livers from sensitive P388/S (a–e) and multidrug resistant P388/VMDRC.04 (f–j) tumor-bearing mice treated with vehicle (a, f), TAX (b, c), VCR (c, h), tFPT (d, i), or MEM (e, j). Mice were inoculated with 1×10^6 cells into the peritoneal cavities on day 0 and treated on days 1, 5, and 9 with VCR (1 mg/kg), MEM (0.5 mg/kg), or tFPT (40 mg/kg) or on days 1–5 with TAX (15 mg/kg), as described under Materials and Methods. Mice were sacrificed on day 12 and the livers removed, fixed in Buffered Formalde-Fresh, and embedded in paraffin. The samples were stained with hematoxylin and eosin and examined with a NIKON microscope (20× objective lens) with a PC Image Pro Plus system.

RESULTS AND DISCUSSION

Using a *MDR*1-transfected mouse leukemic cell line P388/VMDRC.04 (5), we compared the survival of CDF_1 mice bearing these tumor cells treated with the P-gp substrates, VCR or TAX, to those treated with vehicle. This *MDR*1-transfected cell line displayed a full MDR phenotype, and was about 20-fold resistant to VCR and TAX (5). Figure 1 demonstrates that P388/S (Fig. 1a, c, e and g) and P388/VMDRC.04 (Fig. 1b, d, f and h) tumor cells were lethal to mice in 12.1 ± 0.3 (S.E.) and 16.3 ± 0.2 (S.E.) days, respectively. VCR (1 mg/kg; Fig. 1a) and TAX (15 mg/kg; Fig. 1c) significantly increased the survival of mice bearing sensitive P388/S cells ($P < 0.01$; increased life span ≥100%). In contrast, the same doses of VCR and TAX significantly decreased the survival of mice bearing P388/VMDRC.04 cells as compared with vehicle treatment (Fig. 1b and 1d; $P < 0.01$; decreased life span = 20 ~ 25%). Treatment of the P-gp(+) tumor-bearing mice with tFPT (40 mg/kg), a prototype modulator of MDR (11), also decreased survival compared to vehicle treatment (Fig. 1f; $P < 0.01$; decreased life span = 31%). tFPT did not affect the survival of mice inoculated with P388/S cells (Fig. 1e). In contrast, MEM (0.5 mg/kg), which is not a P-gp substrate, increased the survival of mice bearing either sensitive (Fig. 1g; $P < 0.01$; increased life span = 83%) or MDR (Fig. 1h; $P < 0.01$; increased life span = 56%) P388 cells.

A possible explanation for the decreased survival of treated animals was a change in pharmacokinetics or pharmacodynamics of drug in animals harboring a mass of P-gp(+) cells capable of drug efflux rather than drug binding. Therefore, we compared the pharmacokinetics and pharmacodynamics of TAX and VCR in sensitive and MDR tumor-bearing animals. After injection of TAX (15 mg/kg), the levels of the drug in blood and various organs were not increased in animals bearing the P-gp(+) cell line as compared to animals bearing the P-gp(−) cell line (Table 1). As expected, the content of TAX in MDR cells was lower than that of sensitive cells (Table 1). Furthermore, we found no differences in neutrophil, platelet or red blood cell counts between treated mice harboring the sensitive or MDR cell line. In addition, there was no histological organ damage in mice regardless of the cell line they carried (data not shown).

Unexpectedly, histopathological examination (Fig. 2 and Table 2) revealed that animals bearing P-gp(+) tumors treated with P-gp substrates (TAX, VCR or tFPT) had greater hepatic metastases (Fig. 2g, h and i) than animals treated with vehicle (Fig. 2f) or the non-P-gp substrate, MEM (Fig. 2j). The increased hepatic metastasis is consistent with the liver being the preferred site of metastasis of P388 tumor cells (12). In contrast, animals bearing P388/S cells treated with TAX, VCR or MEM had fewer metastases (Fig. 2b, c and e) than animals treated with vehicle (Fig. 2a). The MDR modulator, tFPT, had no effect on the dissemination of P388/S cells to the liver (Fig. 2d). Untreated P388/VMDRC.04 cells (Fig. 2f) were less metastatic than parental cells (Fig. 2a), as demonstrated with other P-gp(+) cancer cells (13–17).

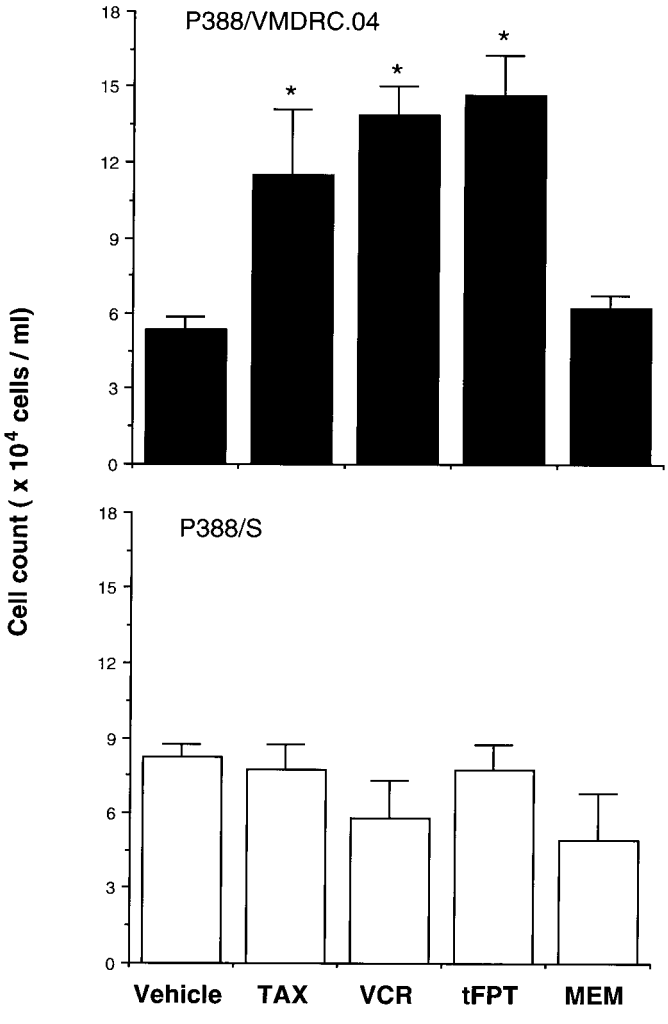

FIG. 3. Effects of drugs on invasiveness of P388/VMDRC.04 and P388/S cells. Two hundred thousand cells suspended in medium supplemented with 10% serum were plated on a murine hepatocyte layer in a cell culture insert contained within a blind-well chamber. After 24 h of incubation at 37°C in the absence or presence of drugs, the invasive cells were recovered from the lower compartment and counted. Drug concentrations were chosen that produce <10% cell killing and had no effect on the hepatocyte monolayer during the 24-h incubation. P388/VMDRC.04: TAX (20 nM), VCR (20 nM), tFPT (5 μM), or MEM (0.5 μM); P388/S: TAX (1 nM), VCR (1 nM), tFPT (1 μM), or MEM (0.5 μM). Each bar represents the mean ± SD of quadruplicate determinations from a representative of three separate experiments. *$P < 0.01$ vs vehicle or MEM.

We next measured the capacity of sensitive and MDR P388 cells to invade a monolayer of hepatic cells grown on polyethylene terephthalate membranes. Figure 3 demonstrates that P388/VMDRC.04 cells were more invasive after treatment with P-gp substrates

TABLE 2

Effects of P-gp Substrates and Non-P-gp Substrate on the Liver Metastases of Sensitive and MDR P388 Leukemic Cells in Mice

	Vehicle				TAX				VCR				tFPT				MEM			
	−	+	++	+++	−	+	++	+++	−	+	++	+++	−	+	++	+++	−	+	++	+++
P388/S			3/5	2/5		5/5			1/5	4/5						5/5	4/5	1/5		
P388/VMDRC.04	1/5	4/5					1/5	4/5				5/5				5/5	3/5	2/5		

Note. Three slides from each block of paraffin-embedded livers were analyzed for tumor metastases. Histopathological grading of liver metastasis was categorized into: −, no metastasis by reviewing all of three slides (under microscope with 20× objective lens); +, 0 to 1 metastatic tumor focus per microscope field; ++, 1–2 metastatic tumor focus per microscope field; and +++, more than 2 metastatic tumor focus per microscope field. Each group consisted of 5 mice.

than when treated with vehicle or MEM. In contrast, the invasiveness of P388/S cells was not affected by drug treatment (Fig. 3). These results suggest that the alteration of cellular motility and invasiveness of P-gp(+) cells by P-gp substrates may contribute to the enhanced metastases of MDR cells and earlier death seen in P-gp(+) tumor bearing-mice treated with P-gp substrates. A physiological role for P-gp in cell migration has been suggested by Randolph *et al.* who found that antibodies to P-gp blocked migration of mononuclear phagocytes and dendritic cells (18). In addition, Weinstein *et al.* reported a possible association of the expression of P-gp with tumor invasion in human colon carcinomas (19). Recently, Emanuel *et al.* also reported that antimitotic drugs caused increased tumorigenicity of multidrug resistant cancer cells (20).

Changes in intracellular pH of tumor cells are known to alter invasiveness (21, 22). To determine whether expression of P-gp in P388/VMDRC.04 cells and drug treatments changed intracellular pH, we analyzed both sensitive and MDR P388 cells treated with P-gp substrates or non-P-gp substrate. Figure 4 demonstrates that the intracellular pH of P388/S and P388/VMDRC.04 cells were identical and that treatment of the cells either with P-gp substrates or non-P-gp substrate did not affect intracellular pH. These results are consistent with those of Litman *et al.* who demonstrated that in Ehrlich ascites tumor cells expressing P-gp, intracellular pH was unaffected by P-gp modulators and found no correlation between the amount of P-gp and changes in cellular pH (23). These results exclude the possibility that the increased motility

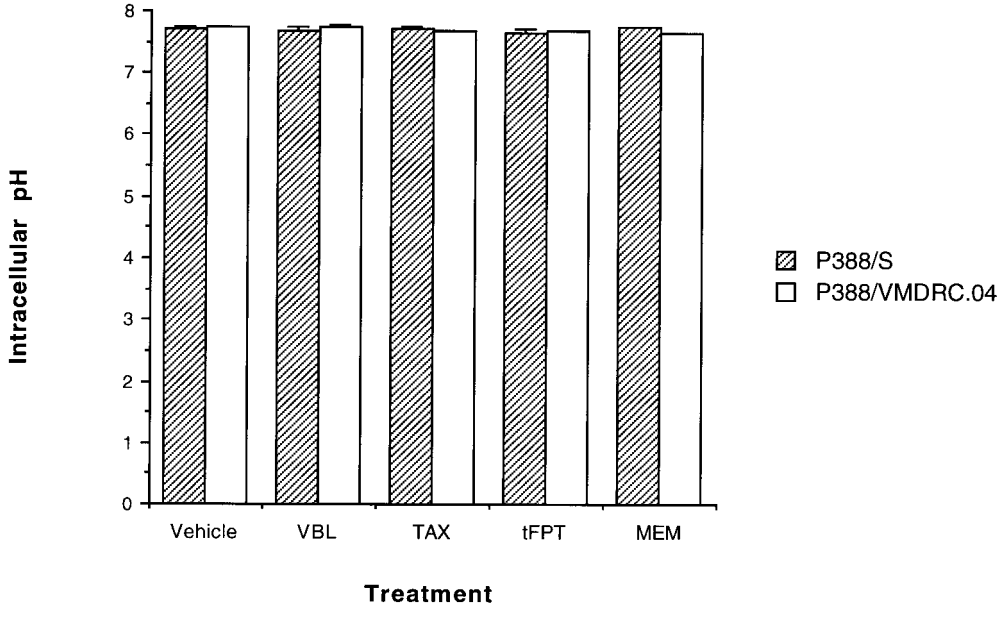

FIG. 4. Measurements of intracellular pH of P388/S and P388/VMDRC.04 cells in the presence or absence of drugs. Two hundred thousand cells in log-phase of growth were treated with drugs or vehicle for 24 h at 37°C in 95% O_2/5% CO_2. Drug concentrations were chosen that produce <10% cell killing. Intracellular pH was measured by flow cytometry with carboxy-SNARF-1 as described under Materials and Methods.

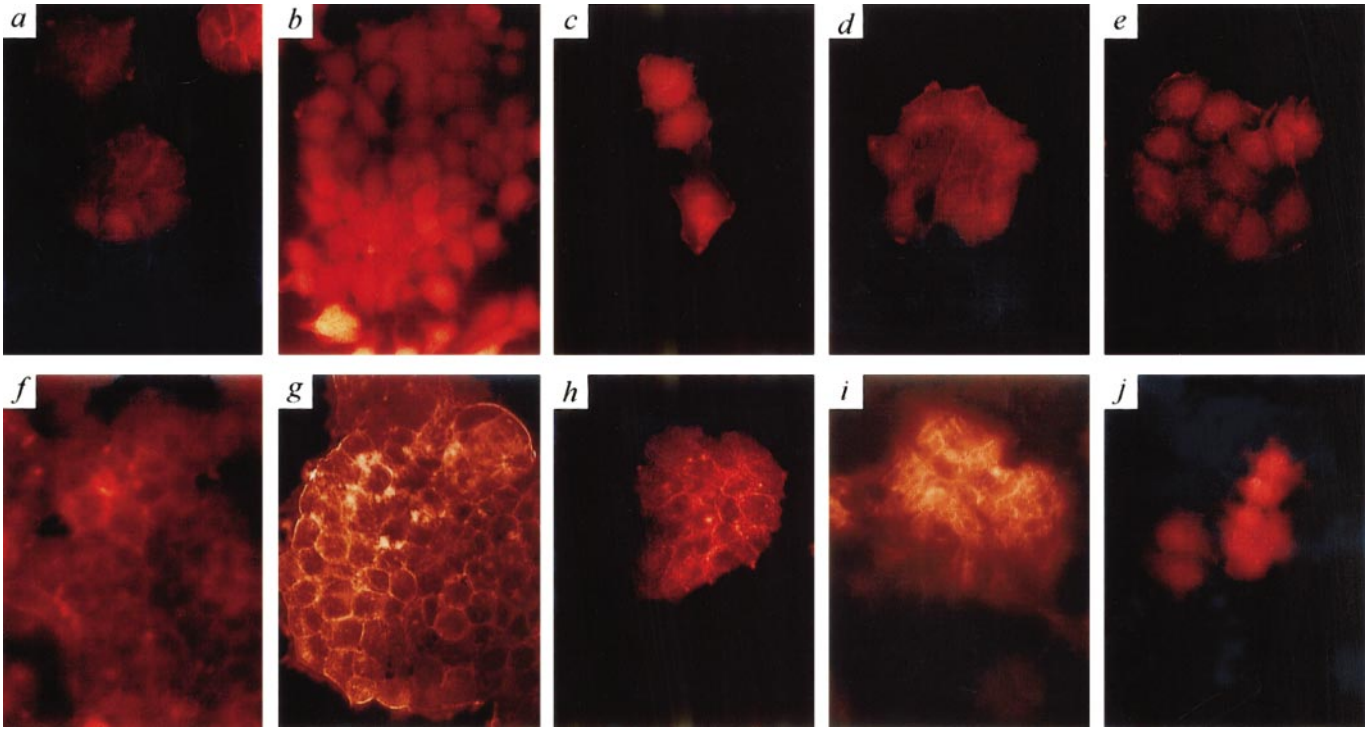

FIG. 5. Membrane ruffling of sensitive MCF-7 (a–e) and MDR MCF-7/BC-19 (f–j) cells treated with vehicle (a and f), TAX (b and g), VCR (c and h), tFPT (d and i), or MEM (e and j). Cells grown on glass coverslips in 35-mm cell culture dishes were treated with drugs or vehicle, fixed in 3.7% formaldehyde in PBS, and permeabilized with 0.2% Triton-X 100 in PBS. Membrane ruffling was visualized by staining with 5 μM phalloidin-tetramethyl-rhodamine isothiocyanate (TRITC) as described under Materials and Methods.

and invasiveness of P388/VMDRC.04 cells treated with P-gp substrates result from changes in intracellular pH.

The ability of cancer cells to invade and metastasize is characteristic of malignancy and is responsible for the majority of cancer deaths. Since P388/VMDRC.04 cells do not express other well-characterized drug resistance mechanisms (5), and since the effects on metastasis were seen only with P-gp substrates, these data suggest a causal interaction between the drugs and the transporter. Therefore, we postulated that the interaction of P-gp substrates with P-gp could lead to membrane changes favoring metastases. To explore this possibility, we examined the effect of drugs on membrane ruffling, an early indicator of enhanced cellular motility and metastatic potential of cancer cells (24). Measurement of membrane ruffling in small lymphoid cells grown in suspension culture is not technically feasible. Therefore, we tested this hypothesis using models of MDR that grow as monolayers. In MCF-7/BC-19, a *MDR*1-transfected human breast carcinoma cell line, we observed an increased membrane ruffling when cells were treated with P-gp substrates (Fig. 5g, h and i). This effect was not seen with MEM (Fig. 5j) or in sensitive cell line, MCF-7 (Fig. 5b, c and d), which does not express P-gp. Identical results were found in P-gp(+) and P-gp(−) human epidermoid carcinoma cell lines, KB3-1 and KBV-1 (data not shown).

In summary, our studies reveal that P-gp substrates accelerate the progression of a transplantable leukemia that overexpresses P-gp, and that this effect is associated with increased invasiveness and metastasis of P-gp(+) cancer cells. The clinical significance of this disquieting result remains to be determined.

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