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EVIDENCE FOR IMPAIRED MITOXANTRONE AND VINBLASTINE BINDING IN P388 MURINE LEUKEMIA CELLS WITH MULTIDRUG RESISTANCE

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Abstract—Multidrug resistance is associated with a P170 glycoprotein efflux pump that limits net drug accumulation in resistant cell lines. Other evidence has suggested that diminished net drug uptake in multidrug resistant (MDR) cells is due to decreased drug binding as well. To assess the contribution of binding differences to net drug accumulation and retention in MDR cells, mitoxantrone and vinblastine, two agents commonly associated with the MDR phenotype but with different mechanisms of action and intracellular binding sites, were studied in P388 murine leukemia cells. For both drugs, resistance was associated with a *marked reduction* in tightly bound drug which can account for the diminished net drug accumulation in this cell line; even at 1 μM vinblastine when the exchangeable component was one-half that of the sensitive cells, the nonexchangeable component was only one-seventh. For mitoxantrone, the exchangeable drug component was greater in resistant cells at low drug levels (1 μM) and similar at high drug levels (10 μM). For vinblastine, the exchangeable drug component was decreased in the resistant cells at 1 μM, but the difference compared to sensitive cells became neglible at 10 μM. The data indicate that diminished net drug uptake in the P388 MDR cell line was associated with a marked decrease in tightly bound, i.e. nonexchangeable, drug fractions for both mitoxantrone and vinblastine. Therefore, alterations in intracellular binding are an important factor in the decreased cellular uptake and retention of drugs in the multidrug resistance phenomenon. The relationship between these changes and the P170 efflux pump requires further clarification.

Development of resistance to cytotoxic agents by tumor cells is considered to be one of the major causes for treatment failure during clinical chemotherapy of cancer [1]. Tumors initially responsive to chemotherapy often are unresponsive in later stages of treatment; other tumors have been characterized as "intrinsically" resistant to anticancer drugs [2]. Several models of resistance to heterocyclic agents have been developed by exposure of tumor cells to increasing concentrations of these drugs. The cell lines which have emerged display resistance to the selecting drug as well as cross-resistance to other large, heterocyclic agents with unrelated structures or mechanisms of action including the anthracyclines, epipodophyllotoxins, vinca alkaloids, and actinomycin D. The development of this form of drug resistance, often referred to as "multidrug resistance", has been used as a model to understand the genetic, biochemical, and pharmacological basis for drug resistance to these agents [for reviews, see Refs. 3–6].

Multidrug resistant (MDR)† lines typically achieve lower intracellular steady-state drug levels than drug-sensitive cells [3–6]. While apparent diminished passive diffusion of these lipid soluble drugs into cells has been observed [7–9], the major common element is enhanced drug efflux due to an "active efflux pump" of "broad specificity" that is blocked by metabolic poisons [10–13]. MDR cells overexpress a membrane glycoprotein of 170 kD (P-glycoprotein or P170) [14, 15]. Drug resistance correlates with the level of membrane P170 glycoprotein [14, 15] and the degree of amplification and expression of the MDR gene [16, 17]. Further, the MDR phenotype and the P170 glycoprotein are expressed in drug-sensitive lines by the transfection of the MDR gene [18, 19]. Membrane vesicles prepared from MDR cells bind a variety of drugs that are associated with the resistance phenotype [20], also bind ATP [21], and demonstrate ATP-dependent transport of vinblastine [22]. The MDR protein appears to have the properties of a transport carrier that spans the cell membrane with potential ATP binding sites within the cell interior [23, 24] and homology to a bacterial exporter for proteins [23, 24].

While the P170 protein is a critical factor in the multidrug resistance phenomenon, there is also evidence for differences in drug binding in resistant cells [25, 26]. However, the contribution of these binding differences to net drug accumulation in MDR cell lines has not been quantitated carefully. This paper addresses the role of binding in the multidrug resistance phenomenon using two drugs associated with multidrug resistance but with entirely different mechanisms of action and intracellular targets: (i) mitoxantrone, a potent intercalating agent with few other biological effects, i.e. no free

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† Abbreviations: MDR, multidrug resistant, MITOX, mitoxantrone, 1,4-dihydroxy-5,8-bis((2-[(2-hydroxyethyl)-amino]ethyl)amino)-9,10-anthracenedione dihydrochloride, and VBL, vinblastine.

radical production [27], and (ii) vinblastine, a potent inhibitor of microtubule assembly [28]. The data indicate that diminished net drug accumulation and retention in the resistant cell line is associated with a marked decrease in intracellular tight binding. The relationship between this finding, the P170 glycoprotein, and drug efflux is unclear, but it is clear that alterations in intracellular binding are an important factor in the decreased cellular uptake and retention of mitoxantrone and vinblastine in the multidrug resistance phenomenon.

MATERIALS AND METHODS

Chemicals. [^{14}C]Mitoxantrone (24 mCi/mmol) and unlabeled mitoxantrone were supplied by Lederle Laboratories (Pearl River, NY). [^{3}H]Vinblastine sulfate (17 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Purity of both agents was determined to be >98% as determined by HPLC (see below). Adriamycin® was obtained from Aldrich Chemicals (Milwaukee, WI), and vinblastine, daunorubicin, and colchicine were purchased from the Sigma Chemical Co. (St. Louis, MO). Methotrexate was obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD, and teniposide from Bristol Laboratories (Syracuse, NY). All other chemicals were obtained from commercial sources.

Cells. P388 murine leukemia cells resistant to Adriamycin (P388/R) were developed by *in vivo* treatment of parental P388 cells (P388/S) with Adriamycin [29]. Cells were grown in suspension culture in Roswell Park Memorial Institute Medium 1640 (RPMI) supplemented with 10% heat inactivated undialyzed fetal calf serum, 2 mM L-glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL) (Gibco Laboratories, Grand Island, NY) with 20 μM 2-mercaptoethanol. Cells were grown in a humidified atmosphere of 95% air–5% CO_2 at 37°, harvested during exponential growth (10^6 cells/mL) and washed twice with 0° 0.85% NaCl before use for cellular pharmacologic analysis.

Uptake studies. Cells washed in 0° 0.85% NaCl were suspended into incubation flasks stirred with Teflon paddles and maintained at 37° in a water bath [30]. For uptake measurements at 0°, the incubation flask was placed in an ice bucket. The cells were suspended at a density of 3×10^6 cells/mL (unless otherwise specified) in a buffer consisting of 135 mM NaCl, 4.4 mM KCl, 16 mM $NaHCO_3$, 1.1 mM KH_2PO_4, 1.0 mM $MgCl_2$, and 1.9 mM $CaCl_2$ or into the complete RPMI medium described above. The pH of the medium was maintained (throughout the course of the experiments) at 7.4 by passing warm and humidified 95% air–5% CO_2 over the cell suspension. Uptake was initiated with the addition of sufficient [^{14}C]mitoxantrone or [^{3}H]vinblastine to achieve an extracellular concentration of 0.2–10 or 1–10 μM respectively. At appropriate times, 4-mL samples of the suspensions were injected into conical tubes containing 10 mL of the 0° saline solution and then centrifuged at 3000 *g* for 3 min to terminate the uptake process. The cells were washed twice with this solution and analyzed for radioactivity. Viability of both sensitive and resistant cells (as measured by trypan blue dye exclusion) was >80% after incubation with mitoxantrone or vinblastine for 75 min.

Initial assessment of mitoxantrone accumulation in P388 murine leukemia cells sensitive and resistant to anthracyclines. There was extensive cellular accumulation of mitoxantrone resulting in rapid and marked depletion of extracellular drug when the cell suspension was at a density of 2.4×10^7 cells/mL (a 4% cytocrit) and the initial mitoxantrone concentration was 1 μM. This caused a rapid decline in the net drug uptake rate. To minimize changes in uptake related to the depletion of extracellular drug, suspensions of cells with low cytocrits, 3×10^6 cells/mL (0.5% cytocrit), were employed. Accurate initial uptake rates could not be measured because of a component of radiolabel that very rapidly associates with cells at both 37° and 0° and may represent surface adsorption or, to some extent, a very small contaminant in the radiolabeled material that rapidly equilibrates within the cell water and/or other cell compartments. This was further complicated by the very low levels of radioactivity within cells over uptake intervals of 2–20 sec which resulted in considerable variability in the data.

Efflux studies. At designated times, cells loaded with radiolabeled mitoxantrone or vinblastine were collected by centrifugation at 4°, washed twice with the 0° saline solution, and resuspended at 37° in incubation flasks containing a large volume of medium. Samples of the cell suspension were obtained over the next hour and then processed as described below. Studies demonstrated that after 30 min the intracellular drug level was constant and further efflux of intracellular mitoxantrone or vinblastine was negligible for at least 1 hr.

Analytical methods. The washed cell pellet was aspirated into the tip of a Pasteur pipet, extruded onto a polyethylene tare, dried overnight at 70°, and weighed on a Cahn 4700 electrobalance (Cahn Instruments, Paramount, CA). The weighed pellet was transferred to a scintillation vial and digested in 0.25 mL of 1 N KOH for 1 hr at 70°. After neutralization with 0.3 mL of 1 N HCl, 4 mL of Ready Solv (Beckman Instruments, Inc., Irvine, CA) was added and radioactivity was determined in a liquid scintillation spectrometer. Intracellular water was calculated from the difference between the wet and dry weights of cell pellets, less the [^{14}C]inulin space [30]. Intracellular water/dry weight (μL/mg) was determined to be 3.96 ± 0.08 and 3.85 ± 0.08 for resistant and sensitive cells respectively. Data are expressed as nanomoles of mitoxantrone or vinblastine per gram dry weight or micromoles of mitoxantrone or vinblastine per liter cell water.

Effects of drugs on cell growth. P388/S and P388/R cells in log phase were diluted to 2×10^5 cells/mL and grown for 48 hr in the continuous presence of mitoxantrone, Adriamycin, daunorubicin, methotrexate, vinblastine, colchicine or teniposide. Cell growth was determined by counting cells either with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) or by direct microscopic examination. The IC_{50} value is defined as the drug concentration that produces 50% inhibition of cell growth.

HPLC analysis of intracellular [^{14}C]*mitoxantrone*

and [^{3}H]*vinblastine*. Cells were incubated with 1 μM [^{14}C]mitoxantrone, separated by centrifugation, washed twice in 0° saline, and extracted with 0.3 mL of chloroform/methanol (2:1) for 2 hr at 4°. The cells were then disrupted with a model 300 probe sonicator (Artek, Farmingdale, NY) until over 95% of the cells were broken. Following this the suspension was centrifuged, and 0.2-mL portions of the supernatants were dried under N_2, resuspended in 50 μL of methanol, and analyzed by HPLC on a Gilson model 302 chromatograph equipped with a Rheodyne model 7125 injector and a 4.5 × 250 mm 5-μm Spherisorb ODS column (IBM Instruments, Inc., Danbury, CT). Radioactivity was eluted isocratically with a buffer composed of 50% 2.1 M ammonium formate (pH 4.3), 23% acetonitrile, and 27% water [31]. The flow rate was 1 mL/min. The effluent was monitored by absorbance detection at 608 nm using a Knauer variable wavelength monitor, and ^{14}C was determined in 0.5-mL fractions. By this technique >98% of the extracted radiolabel (90% recovery) represents unchanged mitoxantrone after exposure of cells to the drug for 45 min.

Cells were incubated with 5 μM [^{3}H]vinblastine, separated by centrifugation, washed twice in 0° saline, and extracted with 200 μL of ice-cold 95% ethanol (pH 4.9 with glacial acetic acid) for 60 min at 4°. The cells were then centrifuged at 12,000 *g* for 5 min, and the supernatants were analyzed by HPLC by a modification of the method of Houghton *et al.* [32]. Vinblastine was eluted from a reverse-phase C18 column (IBM Instruments, Inc.) using a linear gradient of from 20% MeOH (containing 10 mM KH_2PO_4, pH 4.9) to 80% MeOH over a period of 30 min and continuing at 80% MeOH for an additional 5 min. The flow rate was 2 mL/min, and absorbance was monitored at 254 nm. Fractions were collected at 30-sec intervals and ^{3}H was assessed. By this technique >90% of the extracted radiolabel recovered (95%) represents unchanged vinblastine after exposure of cells to the drug for 45 min.

RESULTS

Comparison of mitoxantrone cytotoxicity with that of other heterocyclic agents in P388 murine leukemia cells sensitive to, and with primary resistance to, Adriamycin. These cells, originally selected for resistance to Adriamycin, are cross-resistant to a variety of other agents. The IC_{50} for Adriamycin and mitoxantrone in the resistant P388 line was 40- to 50-fold greater than the sensitive line after continuous exposure to either drug for 48 hr (Table 1). However, within a cell line, the IC_{50} for mitoxantrone was one-fifteenth that of Adriamycin. Hence, mitoxantrone is a much more potent agent in both lines, but resistance is associated with quantitatively similar cross-resistance to both drugs.

The pattern of cross-resistance to continuous exposure to a variety of agents in P388 cells is also indicated in Table 1. Resistance to teniposide was about 4-fold greater than, and resistance to daunorubicin and colchicine about one-half, that observed with Adriamycin and mitoxantrone. There was only 7-fold resistance to vinblastine and possible slight collateral sensitivity to methotrexate.

Table 1. Comparison of inhibition of growth of P388 murine leukemia cells by heterocyclic agents

	IC_{50} (μM)		
	P388/S	P388/R	R/S
Teniposide	0.015	2.47	164.7
Adriamycin	0.041	2.08	50.7
Mitoxantrone	0.0035	0.14	40.0
Colchicine	0.024	0.521	21.71
Daunorubicin	0.028	0.52	18.6
Vinblastine	0.024	0.177	7.38
Methotrexate	0.0081	0.0051	0.63

Cells were grown in the continuous presence of drugs for 48 hr following which cell numbers were quantitated on a Coulter Counter. The data are the average of three experiments performed on different days. R/S is the ratio of the IC_{50} in resistant versus sensitive cells.

Analysis of mitoxantrone accumulation and retention as a function of time of drug exposure and concentration in sensitive and resistant cells. Cellular accumulation and retention of mitoxantrone were studied in cells incubated over an extracellular concentration range of 0.2 to 10 μM. As the extracellular level of drug increased, drug accumulation in both the sensitive and resistant cell lines increased (Fig. 1 and Table 2). Total drug accumulation was always higher in sensitive cells at all extracellular drug levels examined.

Exchangeability of drug was assessed after incubation of cells with mitoxantrone for 45 min. The exchangeable drug component was greater in the resistant as compared to sensitive cells at the lowest drug levels and similar at the higher drug levels examined (Table 2). The basis for the reduced net accumulation of drug in the resistant line was the *marked reduction in the nonexchangeable drug component.*

When drug in the nonexchangeable and exchangeable compartments was expressed as the percentage of total drug accumulated at a particular drug concentration, it can be seen that exchangeable drug in the resistant cell line was much greater than in the sensitive line (Table 2). This was attributed to the same amount or slightly higher amount of exchangeable drug in the resistant line associated with lower total uptake due to a marked decrease in nonexchangeable drug.

This was further quantitated in Fig. 2. In both cell lines, as the extracellular drug level was increased, total drug accumulation, and the nonexchangeable and exchangeable compartments increased linearly as a function of drug concentration. No drug component could be saturated over the concentration range studied. Analysis of the slope of drug accumulation as a function of extracellular drug concentration indicated that the increase in total drug accumulation was 1.60x ($P < 0.005$) greater in sensitive than resistant cells over a mitoxantrone range of 0.2 to 10 μM. This was due entirely to increasing accumulation of drug in the nonexchangeable compartment (2.58x, $P < 0.0005$) while the increase in uptake into the exchangeable

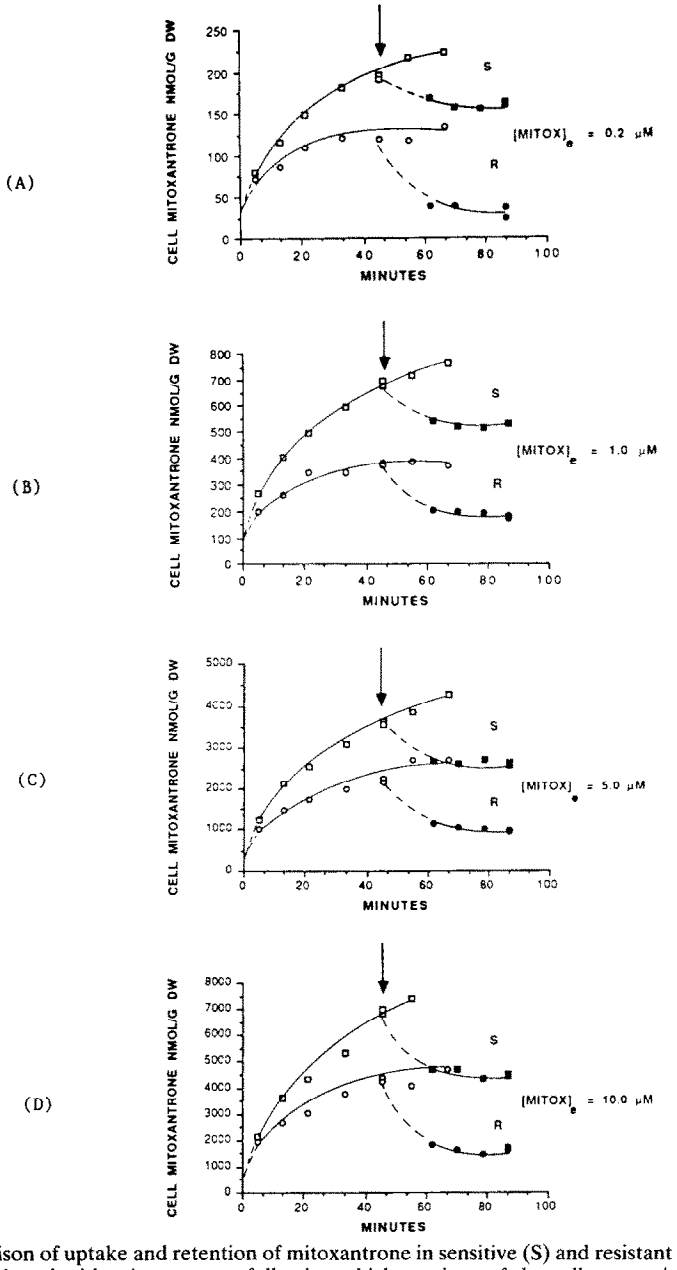

Fig. 1. Comparison of uptake and retention of mitoxantrone in sensitive (S) and resistant (R) P388 cells. Cells were incubated with mitoxantrone following which portions of the cell suspensions (at 45 min) were washed twice in ice-cold saline solution and resuspended into drug-free medium for 30 min to assess nonexchangeable and exchangeable components. By 30 min the nonexchangeable intracellular level was essentially constant. Extracellular drug [MITOX] levels were: (A) 0.2 μM, (B) 1.0 μM, (C) 5.0 μM and (D) 10.0 μM. The data are the average of four separate experiments performed on different days. Key: uptake [(□) Sens, (○) Res]; retention [(■) Sens, (●) Res].

compartment as a function of drug concentration was essentially the same (0.94x, $P < 0.2$). Therefore, total uptake in sensitive cells increased to a greater extent than in resistant cells due exclusively to the increase in the nonexchangeable component.

HPLC analyses were undertaken to determine whether metabolism of mitoxantrone could account for any of the differences in exchangeable or nonexchangeable radiolabel in sensitive and resistant cell lines. However, no metabolites were detected after incubation of either cell line with 1 μM mitoxantrone for 45 min.

Analysis of vinblastine accumulation and retention as a function of time and drug concentration in sensitive and resistant cells. Cellular accumulation and retention of vinblastine were studied in cells incubated over an extracellular concentration range of 1–10 μM. As the extracellular level of drug

Table 2. Total, nonexchangeable, and exchangeable mitoxantrone levels as a function of drug concentration

Extracellular drug concn (μM)	P388/S			P388/R			
		nmol/g dry wt	% of Total		nmol/g dry wt	% of Total	R/S
0.2	Total	193.4		Total	119.6		0.618
	Nonexch	161.3	83.4	Nonexch	37.4	31.3	0.232
	Exch	32.1	16.6	Exch	82.2	68.7	2.561
1.0	Total	689.3		Total	374.0		0.543
	Nonexch	532.8	77.3	Nonexch	177.4	47.4	0.333
	Exch	156.5	22.7	Exch	196.6	52.6	1.256
5.0	Total	3594.7		Total	2204.4		0.613
	Nonexch	2600.4	72.3	Nonexch	965.7	43.8	0.371
	Exch	994.3	27.7	Exch	1238.7	56.2	1.246
10.0	Total	6929.6		Total	4318.5		0.623
	Nonexch	4469.6	64.5	Nonexch	1673.5	38.8	0.374
	Exch	2460.0	35.5	Exch	2645.0	61.2	1.075

Data represent a composite analysis of mitoxantrone accumulation and retention in sensitive and resistant P388 cells. Cells were incubated with mitoxantrone for 45 min, and total drug accumulation was determined on a portion of the cell pellet. The remaining cells were washed twice in 0° saline and resuspended into drug-free medium at 37° for an additional 30 min to assess exchangeable and nonexchangeable components. The data are based upon four separate experiments performed on different days. R/S is the ratio of each cellular component in resistant to sensitive cells.

increased, increased net levels of drug accumulated in both the sensitive and resistant lines (Fig. 3 and Table 3). Total drug accumulation was always higher in sensitive cells, but the difference decreased as the extracellular drug level increased.

Exchangeability of drug was determined after incubation of cells with vinblastine for 45 min. The total, nonexchangeable, and exchangeable components were decreased in the resistant cells at the lowest concentration but the differences between sensitive and resistant cells decreased as the extracellular concentration increased (Table 3). Of interest is that at 1 μM vinblastine the nonexchangeable compartment was only one-seventh that of the sensitive cells.

The nonexchangeable and exchangeable drug components are further characterized in Fig. 4. In both sensitive and resistant cells, as the extracellular drug level was increased, total drug accumulation and drug in the nonexchangeable and exchangeable compartments increased linearly as a function of drug concentration. Again, no drug component could be saturated over the concentration range studied. Analysis of the slope of drug accumulation as a function of extracellular drug concentration showed that the change in total drug accumulation was slightly greater (1.11x, $P < 0.1$) in sensitive as compared to resistant cells over the vinblastine concentration range of 1 to 10 μM. This small difference was attributable to the small increase in accumulation of drug in the nonexchangeable compartment (1.52x, $P < 0.01$) while the slope of drug uptake into the exchangeable compartment as a function of extracellular drug level was essentially the same in both cell lines (1.04x, $P < 0.2$). Thus, as observed for mitoxantrone, total vinblastine uptake increased with increasing drug concentration. For mitoxantrone, the increase in sensitive cells was much greater due to the much greater increase in the nonexchangeable component.

When exchangeable vinblastine was expressed as a percentage of total drug accumulated at each extracellular drug level examined, the exchangeable component in the resistant line was again much greater than in the sensitive line at 1 μM VBL and slightly greater at the higher concentration, even though there was much less exchangeable drug at the low drug concentration (Table 3). In both lines, the percent exchangeable vinblastine level was considerably greater than the percent exchangeable mitoxantrone level at all concentrations.

No metabolites were detected by HPLC analyses after incubation of either cell line with 5 μM vinblastine for 45 min.

DISCUSSION

While diminished net drug accumulation in the multidrug resistance phenotype is related to the amplification of a 170 kD glycoprotein (P170) energy-dependent efflux mechanism, there is evidence that diminished drug accumulation and retention in MDR cells are associated with decreased drug binding as well [25, 26]. In an attempt to better discriminate between binding and transport phenomena in P388 cells with multidrug resistance, studies were undertaken with mitoxantrone and vinblastine, two agents commonly associated with multidrug resistance but with two entirely different mechanisms of action and different intracellular binding sites. The results of these studies demonstrated that for both agents multidrug resistance is associated with diminished drug accumulation and drug retention that are due to a marked decrease in nonexchangeable (bound) intracellular drug.

Studies to define membrane transport of heterocyclic agents are complicated by the rapid association of these drugs with cell membranes and extensive binding to intracellular constituents. Initial uptake rates are very difficult to measure because

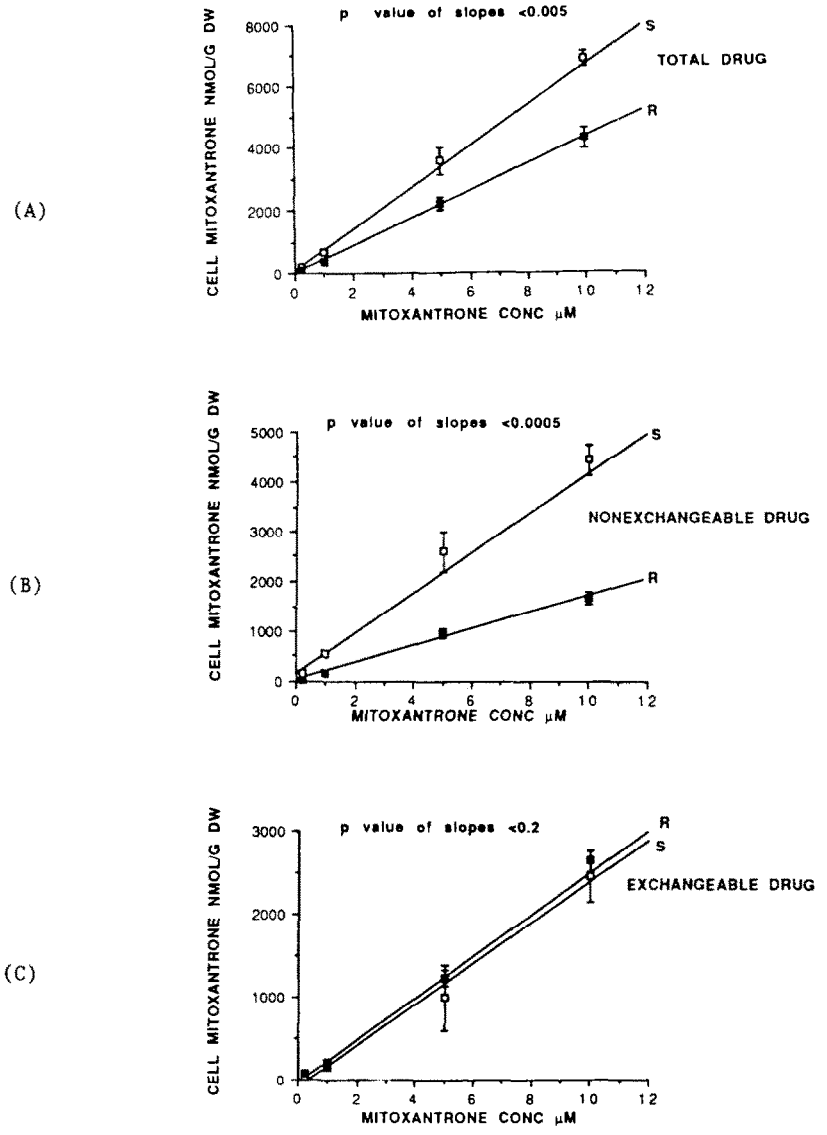

Fig. 2. Analysis of total, nonexchangeable, and exchangeable mitoxantrone as a function of extracellular drug concentration. Cells were incubated with a range of mitoxantrone concentrations (0.2 to 10 μM) for 45 min. Total drug accumulation and the nonexchangeable and exchangeable drug components were determined as described in Materials and Methods and the legend to Fig. 1 in sensitive (S) (□) and resistant (R) (■) lines. The data are the average ± SD of four separate experiments performed on different days. The P values of the slopes of the lines are shown in each panel.

of surface adsorption and low specific activity of drug [33, 34]. Within a few minutes the free drug component in cells becomes a trivial fraction of total cellular drug. For instance, within 1 min the component of intracellular mitoxantrone that represents an equilibrium level (1 μM) represented less than 4% of total cell drug. By 10 min this fraction was less than 1% of total cellular drug—a cell component that cannot be discriminated from the larger bound fractions. Likewise, enhanced drug "efflux" in resistant lines, in which *net* loss of drug is monitored rather than initial *rates* of efflux of free drug, while compatible with increased transport, can also be accounted for by decreased intracellular binding with the availability of more drug to exit the cell.

Studies reported in this paper implicate differences in tight binding of mitoxantrone and vinblastine as the critical factors that quantitatively account for the diminished net uptake of these drugs in the resistant line. For mitoxantrone, in fact, exchangeable drug was *greater* in resistant than sensitive cells at the low drug concentrations and similar at the higher drug levels examined. For vinblastine, resistant cells had lower levels of exchangeable and nonexchangeable drug than sensitive cells, but the more prominent difference was observed in the nonexchangeable component. In fact, at the highest levels studied,

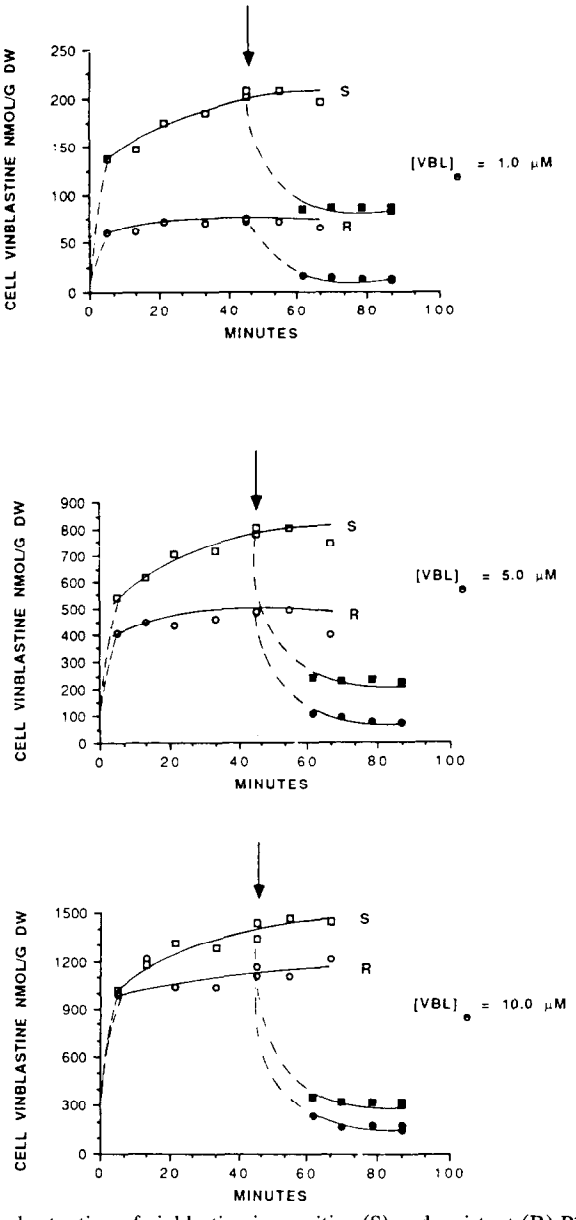

Fig. 3. Comparison of uptake and retention of vinblastine in sensitive (S) and resistant (R) P388 cells. Cells were incubated with vinblastine following which portions of the cell suspensions (at 45 min) were washed twice in ice-cold saline solution and resuspended into drug-free medium for 30 min to assess nonexchangeable and exchangeable components. By 30 min the nonexchangeable intracellular level was essentially constant. Extracellular drug levels $[VBL]_e$ were: (A) 1.0 μM, (B) 5.0 μM and (C) 10.0 μM. The data are the average of three separate experiments performed on different days. Key: uptake [(□) Sens, (○) Res]; retention [(■) Sens, (●) Res].

the exchangeable components were comparable in the sensitive and resistant lines. This is consistent with observations by other investigators in these P388 cell lines [25, 35], as well as for these and other agents in other MDR cell lines [36–38].

Diminished drug retention in the resistant line is due, at least in part, to diminished tight binding of drug within cells. Beck *et al.* [26] reported that resistant human lymphoblasts had a greater fraction of releasable vinblastine than sensitive cells and proposed that reduced drug binding in the resistant cells could account for the decreased drug retention. Similarly, for a series of KB multidrug resistant cell lines, as the degree of resistance increased, levels of membrane P170 increased, net drug accumulation decreased, and in fact a greater percentage of drug was released although the absolute amount of drug released was less [39]. However, in the more resistant cell lines, while net drug accumulation decreased, the amount of nonexchangeable drug in the cells also decreased at each stage of greater drug resistance; this observation could equally well explain

Table 3. Total, nonexchangeable, and exchangeable vinblastine levels as a function of drug concentration

Extracellular drug concn (μM)	P388/S			P388/R			
		nmol/g dry wt	% of Total		nmol/g dry wt	% of Total	R/S
1.0	Total	204.2		Total	73.3		0.359
	Nonexch	85.0	41.6	Nonexch	12.6	17.2	0.148
	Exch	119.2	58.4	Exch	60.7	82.8	0.509
5.0	Total	794.3		Total	487.4		0.614
	Nonexch	220.9	27.8	Nonexch	69.5	14.3	0.315
	Exch	573.4	72.2	Exch	417.9	85.7	0.729
10.0	Total	1392.0		Total	1138.4		0.818
	Nonexch	318.4	22.9	Nonexch	163.6	14.4	0.514
	Exch	1073.6	77.1	Exch	974.8	85.6	0.908

Data represent a composite analysis of vinblastine accumulation and retention in sensitive and resistant P388 cells. Cells were incubated with vinblastine for 45 min, and total drug accumulation was determined on a portion of the cell pellet. The remaining cells were washed twice in 0° saline and resuspended into drug-free medium at 37° for an additional 30 min to assess exchangeable and nonexchangeable components. The data are based upon three separate experiments performed on different days. R/S is the ratio of each cellular component in resistant to sensitive cells.

the greater percentage of exchangeable drug in the more resistant cell lines. Further, an HL-60 drug-resistant cell line which does not overexpress the P170 glycoprotein also demonstrated a decrease in the nonexchangeable drug compartment compared to sensitive cells; the amount of exchangeable drug was identical in the two cell lines [37, 40]. Finally, studies of two different MDR tumor cell lines, one which overexpresses P170 and one which does not, both showed marked decreases in the non-exchangeable drug components [38].

The basis for the nonexchangeable and exchangeable drug components and differences in these compartments in sensitive and resistant P388 murine leukemia cells is not clear. It is likely that the nonexchangeable fractions represent, at least in part, mitoxantrone tightly intercalated into DNA [27], or vinblastine tightly bound to microtubule protein [28]. However, these drugs bind to other cellular components (i.e. RNA, protein, and lipid) [41, 42], and it is probable that the nonexchangeable fraction represents drug distributed throughout the entire cell [40, 43, 44]. While decreased binding associated with multidrug resistance could be related to altered binding of drugs to a variety of sites, it is possible that one or more of these sites, distinct from the primary targets (i.e. DNA and microtubules), has a high affinity for both agents and is decreased in MDR cells. Further studies will be necessary to identify the nature of such a putative binding site and its location within the cell. Recent studies indicate that anthracycline resistance in MDR cells is associated with decreased drug binding to the nucleus with a shift of drug to other cellular structures (i.e. cytoplasm, Golgi, and lysosomes [40, 43, 44]. Other studies suggest that anthracycline resistance may be multifactorial and associated with alterations in intracellular drug levels, levels of P170, rates of DNA damage/repair and glutathione transferase activity [45, 46]. Hence, decreased binding associated with multidrug resistance could be related to altered binding of drugs to a variety of, as yet, unidentified cellular proteins or organelles [25, 26, 47]. However it is not known which subcellular drug fraction(s) might contribute to cytotoxicity.

Exchangeable drug, when expressed in units of concentration in the intracellular water, exceeds that of extracellular drug for both mitoxantrone and vinblastine. For example, when the extracellular mitoxantrone level was 1 μM, exchangeable drug levels in sensitive and resistant cells were 39 and 49 μM respectively. Similarly, for 1 μM vinblastine, exchangeable drug was greater than the extracellular drug level for both cell lines (30 μM for sensitive and 15 μM for resistant cells). At higher drug concentrations the exchangeable components tended to become comparable in sensitive and resistant cell lines but were still much greater than the extracellular drug level. Since there is no evidence for an energy-dependent accumulation of drug within these cells, this high apparent drug gradient can only be accounted for on the basis of considerable loose binding to intracellular sites, or electrostatic or pH factors that may influence the distribution of free drug across the plasma membrane or other intracellular membrane-bound compartments [40, 44]. The subcellular distribution of the exchangeable component is unknown, but could represent drug concentrated in subcellular organelles such as the lysosome [3, 48], or, alternatively, could represent rapidly reversible binding of drug to a cellular component such as DNA or protein.

It is possible that decreased tight binding of drug in MDR cells may be due solely to the energy-driven efflux pump which is so potent that it results in the rapid net efflux of drugs that have high on–off rates from tight binding sites and in the absence of the pump do not diffuse away from their binding site(s). Alternatively, the efflux pump may, in fact, transport a portion of these drugs, nonspecifically bound to a common site, out of the cells—the bound form being

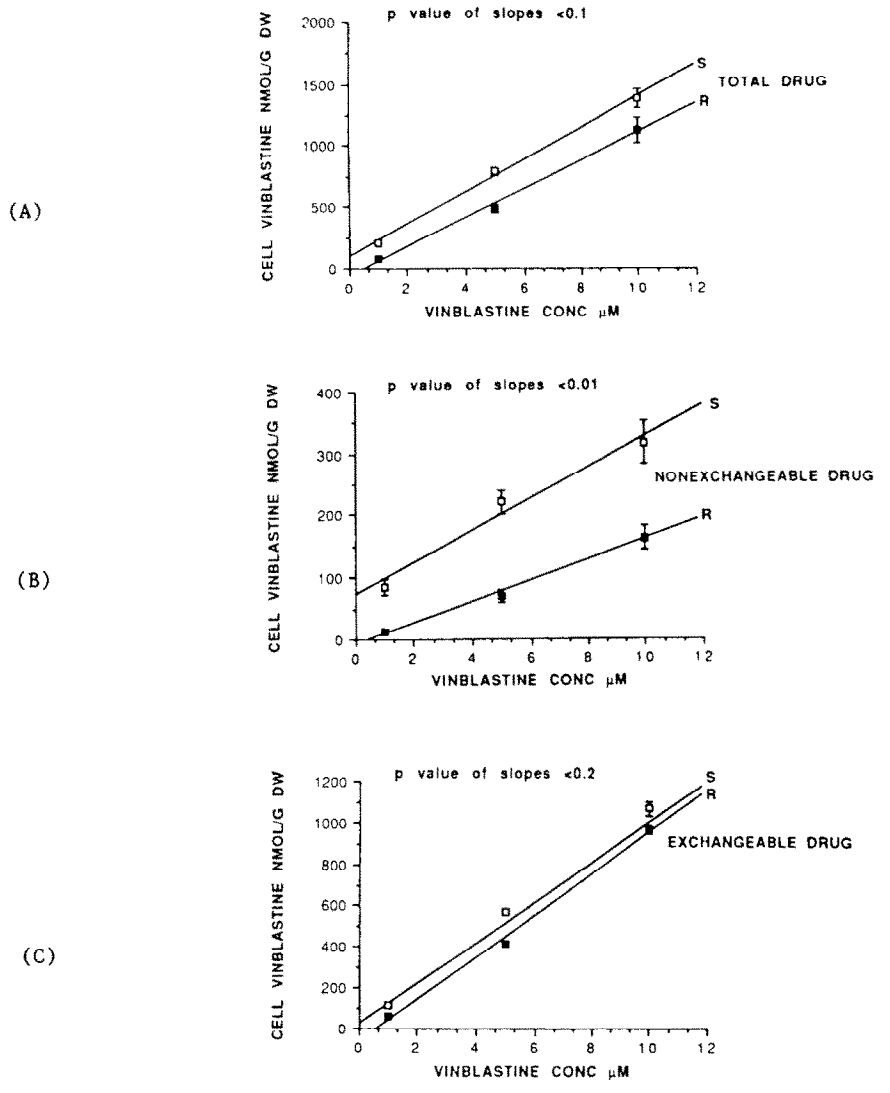

Fig. 4. Analysis of total, nonexchangeable, and exchangeable vinblastine as a function of extracellular drug concentration. Cells were incubated with a range of vinblastine concentrations (1–10 μM) for 45 min. Total drug accumulation and the nonexchangeable and exchangeable drug components were determined as described in Materials and Methods and the legend to Fig. 3 in sensitive (S) (□) and resistant (R) (■) lines. The data are the average ± SD of three separate experiments performed on different days. The P values of the slopes of the lines are shown in each panel.

a substrate for the exporter as occurs in the efflux of hemolysins from bacteria which have an exporter with homology to the MDR pump in mammalian cells.

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