Resistance to N-Benzyladriamycin-14-valerate in Mouse J774.2 Cells: P-Glycoprotein Expression without Reduced N-Benzyladriamycin-14-valerate Accumulation

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ABSTRACT

N-Benzyladriamycin-14-valerate (AD 198) is a highly lipophilic analogue of Adriamycin with novel cytotoxic mechanisms, greater in vivo antitumor activity, and the ability to circumvent multidrug resistance due to P-glycoprotein-mediated drug efflux or decreased topoisomerase II activity. To identify the mechanism(s) which may confer AD 198 resistance, J774.2 mouse macrophage-like cells were selected for growth in cytotoxic levels of AD 198 (AD 198R). AD 198 cells exhibited overexpression of the mdr1b (P-glycoprotein) gene, cross-resistance to Adriamycin and vinblastine, and potentiation of drug cytotoxicity by verapamil. However, net intracellular accumulation of AD 198 in AD 198R cells was unchanged compared to parental cells, while Adriamycin and vinblastine accumulations were reduced 40% and 95%, respectively. AD 198 was localized in the perinuclear region of the cytoplasm in both parental and AD 198R cells, with additional vesicular compartmentalization in AD 198R cells. Verapamil-induced reversal of AD 198 resistance coincided with some drug redistribution from cytoplasmic vesicles, but without redistribution of AD 198 into the nucleus. These results suggest that AD 198 resistance was not conferred through a P-glycoprotein-mediated reduction in intracellular drug accumulation but through other cytoplasmic mechanisms, including, but not limited to, drug compartmentalization.

INTRODUCTION

The anthracycline antibiotic ADR is an effective cancer chemotherapeutic agent that has been widely used against a variety of human malignancies (1). Concomitant with its antitumor activity, ADR can produce adverse systemic effects, including acute myelosuppression, cumulative cardiotoxicity, and gastrointestinal toxicity (2). At the cellular level, in both cultured mammalian cells and primary tumor cells, ADR can select for multiple mechanisms of drug resistance that decrease its chemotherapeutic efficacy. These mechanisms include P-gp-mediated MDR, characterized by the energy-dependent transport of drugs from the cell (3), and multidrug resistance conferred by decreased topoisomerase II activity, resulting in decreased ADR-induced DNA strand scission (4–6). Among the potential avenues of circumvention of systemic toxicity and cellular drug resistance is the development of semisynthetic ADR analogues which demonstrate greater tumor-specific toxicity and less susceptibility to various forms of resistance. One such highly hydrophobic analogue, AD 198, exhibits a variety of mechanistic differences compared with ADR, including weaker binding to purified DNA, preferential inhibition of RNA versus DNA synthesis, irreversible G2/M blockade, pronounced membrane lytic activity, and a lack of inhibition of purified mammalian topoisomerase II despite significant levels of protein-associated DNA strand breaks in alkaline elution assays (2, 7–9). When compared with ADR, AD 198 demonstrates enhanced cytotoxicity against cultured murine and human tumor cells and the ability to circumvent MDR in P388 and L1210 leukemic cells and B16-BL6 melanoma cells, and both MDR and resistance due to altered topoisomerase II activity in variant CCRF-CEM leukemic cells (10, 11). Despite the high degree of toxicity seen in vitro, AD 198 exhibits limited efficacy against transplanted MDR L1210 cells in vivo. This observation suggests that resistance to AD 198 may be conferred either systemically through enhanced drug metabolism or pharmacologic sanctuary of the neoplasia or through cellular resistance (10).

In an effort to identify the mechanism(s) by which mammalian cells may acquire resistance to AD 198, the selection of resistant variants of the mouse macrophage-like cell line J774.2 were attempted by continuous exposure to increasing concentrations of AD 198. J774.2 cells are amenable to the rapid selection of drug-resistant variants, a number of which have been extensively analyzed (12–17). Selection of variant J774.2 cells would represent the first reported selection of myeloid cells for resistance to AD 198. In this report, it is shown that (a) AD 198-resistant variants of J774.2 (AD 198R) can be rapidly selected; (b) AD 198R cells exhibit several characteristics of MDR, including P-gp overexpression, cross-resistance to other drugs, and attenuation of resistance by the calcium channel blocker VRP; and (c) unlike cross-resistance to ADR and VBL, AD 198 resistance is not associated with reduced net AD 198 accumulation.

MATERIALS AND METHODS

Materials. AD 198 (Fig. 1), [14C]AD 198 (3.8 mcCi/mmol), and [14C]ADR (5.05 mcCi/mmol) were prepared as described previously (8, 11). VBL and VRP were obtained from Sigma Chemical Co. (St. Louis, MO). ADR was a generous gift from Farmitalia Carlo Erba (Milan, Italy). [3H]VBL (11.2 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL), and [3H]dCTP (3000 Ci/mmol) was from NEN/DuPont (Boston, MA). Drug-sensitive J774.2 cells, VBL-resistant J774.2 cells (13, 17), and mdr cDNA probes (14) were generously supplied by Dr. Susan Horwitz (Albert Einstein College of Medicine, Bronx, NY).

Cell Culture. The propagation J774.2 cells and the selection of VBL-resistant J774.2 cells have been described previously (13, 14, 18). AD 198R variants were selected by stepwise, continuous exposure of J774.2 cells to increasing concentrations of AD 198, beginning with 50
cells/ml and incubated at 25°C for 2 h. Cell lysates were then digested with proteinase K (1.5 mg/10^6 cells) for 3 h at 65°C. Cell lysates were extracted twice with phenol:chloroform:isoamyl alcohol (first at 70:29:1 by volume, then at 50:49:1) followed by one extraction with chloroform:isoamyl alcohol (49:1). DNA was precipitated in 2 volumes of isopropanol and 0.3 mM sodium acetate (pH 5.5) at 25°C and then washed with 70% ethanol at −20°C. DNA was sheared by repeated passage through a 22-gauge needle, heat denatured in 0.3 N NaOH at 68°C for 1 h, then blotted directly onto Zeta-Probe nylon membrane in a slot blot manifold. Hybridization was performed with the random-primed 32P-labeled cDNA probe pC1.5 (14) and pHFR-A-1 under previously described conditions (26) and then quantitated as described above.

Net Drug Accumulation. AD 198^8 and J7.V3-0.04 cells were grown in the absence of drug for 24–48 h prior to analysis. Cells comprising each time point were washed once, resuspended at a density of 10^6 cells/ml in 1.5 ml of fresh media supplemented with 10% horse serum, and then incubated in suspension in either 900 nM [3H]AD 198 (3.0 mM in DMSO), 600 nM [3H]ADR (1.0 mM in DMSO), or 70 nM [3H] VBL (25.0 μM in methanol/sulfuric acid) for 6 h at 37°C with periodic agitation. At selected time intervals, cells were washed once with 1 ml PBS (170 mM NaCl, 5.0 mM Na2HPO4, 3.7 mM KCl, and 1.8 mM KH2PO4; pH 7.0) and pelleted. Drug content of the media, wash, and cell pellet was determined by scintillation counting and fluorescence HPLC as described previously (8). Analyses were performed on 1.5 × 10^6 cell samples in duplicate for each time point. I-4 and I-37 time points represented instantaneous drug exposures at 4°C and 37°C, respectively. More than 90% of each drug was recovered either in the cell pellet, media, or wash.

Drug Efflux. J774.2, AD 198^8, and J7.V3-0.04 cells, at a density of 4 × 10^6 cells/ml, were incubated in radiolabeled drug as described above for 2 h either in the absence or the presence of 8 μM VRP. Cells were washed once in 1.0 ml PBS and then either analyzed immediately for drug content or incubated further for 4 h at 37°C in 1.5 ml of fresh, drug-free media in the absence or presence of 8 μM VRP. Drug contents of the media, wash, and cell pellet were determined by scintillation counting and fluorescence HPLC as described above.

Flow Cytometry. Pelleted cells were washed once in PBS and then resuspended in Dulbecco’s Modified Eagle’s medium with 10% horse serum at a density of 1 × 10^6 cells/ml. AD 198 (0.5 mM in DMSO) was added to a final concentration of 900 nM for 1 h at 37°C. ADR (50 mM in DMSO) was added to a final concentration of 2.0 μM for 2 h. Where indicated, 8 μM VRP was added for the same duration of time at 37°C. Cell viability based upon trypan blue exclusion was determined by flow cytometry with an FACS 440 (Becton Dickinson, Mountain View, CA). Data (LFL2 in arbitrary units) were representative of at least three independent determinations, each derived from samples of 2.5 × 10^6 cells.

Fluorescence Microscopy. All cells were grown in monolayer on Lab Tek chamber slides (Nunc, Inc., Naperville, IL) in the absence of drug for 24 h prior to analysis. Cells were exposed to AD 198, ADR, and VRP under the same conditions as described for flow cytometry. The cells were washed briefly in PBS to remove detached dead cells and immediately observed with an Olympus BH-2 phase-contrast microscope with a mercury UV light source under UV illumination (excitation filter, 530-560 nm; barrier filter, 580 nm) at ×400 or ×1000 magnification. Fluorescence photographs were taken with Ektachrome 400 film (Eastman Kodak, Rochester, NY) at exposure times of 20–60 s.

RESULTS

Patterns of Drug Resistance in AD 198^8 Cells. AD 198^8 cells were selected by continuous exposure of J774.2 cells to 50 nM AD 198 for approximately 12 cell doublings, followed by successive stepwise selections in increasing drug concentrations at
analyses.

Cells were exposed continuously to VRP during the 72-h drug toxicity analyses. The MDR J774.2 cell line J7.V3-0.04 (subline C), which was selected for 120-fold resistance to VBL (13, 14, 15), demonstrated fluctuations in A750 and A1K cells which did not correlate with changes in resistance. No mdrla overexpression was observed in any AD 198R subline.

Overexpression of mdr mRNA corresponded to P-gp overproduction, as shown by immunoblot analysis of A300 using the P-gp monoclonal antibody C219 (Fig. 3). No P-gp was detectable in J774.2, as has been reported previously (13). A300 overexpressed a single species with a Mr of 150,000. By comparison, J7.V3-0.04 produced a Mr, 142,000 P-gp isofrom commensurate with the overexpression of the mdrla gene (13).

The overexpression of mdr mRNA was due at least in part to mdr gene amplification (Fig. 4). Hybridization of sheared total genomic DNA from each AD 198R subline with 32P-labeled nonspecific mdr gene probe pC1.5 (14) demonstrated a progressive increase in mdr gene copy up to 6.5-fold in A300 cells. No further increase in mdr amplification was observed in cells selected in higher levels of AD 198.

### Table 1 Levels of AD 198 resistance in AD 198R cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (nM)</th>
<th>R*</th>
</tr>
</thead>
<tbody>
<tr>
<td>J774.2</td>
<td>49±6</td>
<td>1</td>
</tr>
<tr>
<td>A100</td>
<td>355</td>
<td>7</td>
</tr>
<tr>
<td>A200</td>
<td>600</td>
<td>12</td>
</tr>
<tr>
<td>A300</td>
<td>750</td>
<td>15</td>
</tr>
<tr>
<td>A500</td>
<td>1200</td>
<td>25</td>
</tr>
<tr>
<td>A750</td>
<td>1380</td>
<td>28</td>
</tr>
<tr>
<td>A1K</td>
<td>1500</td>
<td>31</td>
</tr>
</tbody>
</table>

* Each value is the mean of at least three determinations.

### Table 2 Drug toxicity analyses of J774.2 and A300 cells ± VRP

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (nM) ± SD</th>
<th>ADR</th>
<th>VBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>No VRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J774.2</td>
<td>49 ± 6</td>
<td>24 ± 6</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>A300</td>
<td>750 ± 78 (15.3)*</td>
<td>595 ± 73 (24.8)</td>
<td>67 ± 9 (23.1)</td>
</tr>
<tr>
<td>J7.V3-0.04</td>
<td>663 ± 23 (13.5)</td>
<td>575 ± 60 (31.5)</td>
<td>352 ± 53 (121.4)</td>
</tr>
<tr>
<td>+2 µM VRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J774.2</td>
<td>25 ± 4</td>
<td>11 ± 3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>A300</td>
<td>123 ± 6 (4.9)</td>
<td>83 ± 20 (7.5)</td>
<td>26 ± 6 (21.6)</td>
</tr>
<tr>
<td>J7.V3-0.04</td>
<td>322 ± 14 (12.9)</td>
<td>353 ± 25 (32.1)</td>
<td>238 ± 77 (82.1)</td>
</tr>
<tr>
<td>+8 µM VRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J774.2</td>
<td>17 ± 5</td>
<td>15 ± 2</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>A300</td>
<td>33 ± 1 (1.9)</td>
<td>8 ± 1.0 (0.53)</td>
<td>3.3 ± 1 (4.1)</td>
</tr>
<tr>
<td>J7.V3-0.04</td>
<td>65 ± 7 (3.8)</td>
<td>75 ± 20 (5.0)</td>
<td>28 ± 7 (35)</td>
</tr>
</tbody>
</table>

* Each value is the mean of at least three determinations.

### Table 3 Analysis of mdr Gene Expression

<table>
<thead>
<tr>
<th>Probe</th>
<th>Amount of mdr mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>mdrla</td>
<td>1.5-fold overexpression</td>
</tr>
<tr>
<td>mdrb</td>
<td>2-fold overexpression</td>
</tr>
<tr>
<td>mdr2</td>
<td>3-fold overexpression</td>
</tr>
</tbody>
</table>

Specific for the three known murine mdr genes: mdrla, mdrb, and mdr2 (14) (Fig. 2). Expression of the mdrb gene increased commensurately with AD 198 resistance, exhibiting the greatest increments of overexpression during selection in lower levels of drug (100 and 200 nM), but with a subsequent plateau of expression following selection in higher concentrations of AD 198. Peak mdrb overexpression was 9.5-fold over J774.2 cells in the A1K subline. Overexpression of mdr2 generally increased with resistance, attaining a maximum 9.5-fold overexpression in the A500 subline. Expression of mdrl, however, demonstrated fluctuations in A750 and A1K cells which did not correlate with changes in resistance. No mdrla overexpression was observed in any AD 198R subline.

AD 198R cells displayed cross-resistance to both ADR and VBL at levels 1.5-fold higher than AD 198 resistance (Table 2). Interestingly, the MDR J774.2 cell line J7.V3-0.04 (subline C), which was selected for 120-fold resistance to VBL (13, 14, 15), demonstrated 13.5-fold resistance to AD 198. Resistance to all three compounds was attenuated by VRP. Continuous 72-h exposure of J774.2 and A300 cells to 2 µM VRP during drug toxicity analyses resulted in a 70% decrease in the levels of AD 198 and ADR resistance but a less than 10% decrease in VBL resistance. Continuous exposure to 8 µM VRP resulted in decreases of more than 90% in AD 198 and ADR resistance and 80% in VBL resistance. J774.2 cells exhibited an IC50 for VRP of 40 µM. Treatment with either 2 µM or 8 µM VRP alone did not inhibit proliferation of either J774.2 or AD 198R cells.

Analysis of mdr Gene Expression. Modulation of resistance to both anthracyclines and Vinca alkaloids by VRP is characteristic of either P-gp-mediated MDR (27-29) or, as observed for anthracyclines in some cultured cell lines, altered intracellular distribution of drug (30-32), which may be independent of P-gp. Accordingly, the overexpression of mdr mRNA in AD 198R cells was determined by slot blot hybridization of total RNA from each AD 198R subline with 32P-labeled cDNA probes specific for the three known murine mdr genes: mdrla, mdrb, and mdr2 (14).
RESISTANCE TO N-BENZYLADRIAMYCIN-14-VALERATE

Fig. 3. Immunoblot analysis of AD 198 sublines. Twenty-five μg of nonnuclear cellular protein were resolved on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis minigel system, transferred to nylon membrane, and probed with monoclonal antibody C219. Lane 1, J774.2; Lane 2, A300; Lane 3, J7.V3-0.04. Prestained proteins were run as M, standards.

![Immunoblot analysis](image)

Fig. 4. DNA slot blot analysis of AD 198 sublines. Total DNA was hybridized with 32P-labeled non-mdrl gene-specific cDNA probe pCl.5 (specific activity, 4 × 10⁸ cpm/μg). Exposure time, 44 h. Values represent n-fold amplification of the mdr gene family over drug-sensitive levels based on the quantitation of autoradiographic images by laser densitometry. The autoradiographic image was computer enhanced for visual clarity. However, densitometric quantitation was performed on the original image.

![DNA slot blot](image)

Net Drug Accumulation and Efflux Analyses. Based upon the presumed function of P-gp as a transmembrane drug transporter (33, 34), the expression of P-gp in AD 198 cells might be expected to coincide with a marked reduction in intracellular drug content due to enhanced drug efflux from the cell. However, following 2 h of incubation in 900 nM AD 198, the steady-state intracellular AD 198 concentrations in J774.2 and A300 were equal (Fig. 5). Further incubation (4–6 h) did not produce a significant difference in mean AD 198 accumulation between the two cell lines. AD 198 accumulated rapidly, attaining steady-state levels after 30 min of drug exposure. Approximately 50% of applied AD 198 was accumulated in the cells at steady state. J7.V3-0.04 cells demonstrated a 35% reduction in intracellular AD 198 after 6 h. AD 198 biotransformation to its principal metabolite in cultured cells, AD 288 (2), occurred at equal rates in all three cell lines, as did retention of AD 288 (Fig. 5, inset).

In contrast, A300 cells exhibited a 40% reduction in net accumulation of ADR compared to J774.2 following exposure to 600 nM for 6 h. ADR accumulation in the J7.V3-0.04 line was reduced 60% below the intracellular level of J774.2. Steady-state ADR accumulation was not attained during the 6-h time course, revealing a slower rate of ADR accumulation in A300 and J7.V3-0.04 compared with AD 198. After 6 h of VBL treatment, the steady-state levels in both A300 and J7.V3-0.04 was 95% lower than J774.2.

Consistent with net AD 198 accumulation in J774.2 and A300 cells, rates of AD 198 efflux were similar in the two cell lines (Fig. 6). J774.2 cells accumulated approximately 80 ng of AD 198/10⁶ cells, or 50% of the total applied drug after 2 h of exposure (L – VRP). Four h of incubation in drug-free media (E – VRP) reduced the intracellular level of AD 198 in J774.2 by approximately 20%. In A300 cells, AD 198 efflux resulted in a 25% decrease in the intracellular level of drug after 4 h, while J7.V3-0.04 cells exhibited a 30% reduction of intracellular drug. Treatment of all three cell lines with 8 μM VRP had no significant effect on either the net 2-h accumulation (L + VRP) or on the efflux rate (E + VRP) of AD 198.

A300 and J7.V3-0.04 exhibited significantly enhanced efflux of VBL compared to J774.2 cells. J774.2 cells accumulated 6.5 ng VBL/10⁶ cells (65% of the total applied VBL) after 2 h and exhibited a 50% reduction of accumulated VBL after 4 h. Neither accumulation nor efflux was affected by 8 μM VRP. VBL accumulation in A300 cells was 85% less than the level in
J774.2 in the absence of VRP. In the presence of 8 μM VRP, VBL accumulation in A300 cells was similar to that in J774.2 cells. A300 exhibited a 90% loss of drug during the efflux incubation but only a 30% loss in the presence of VRP. J7.V3-0.04 cells accumulated 95% less VBL than J774.2 but only 25% less in the presence of VRP. As with A300, VBL efflux in J7.V3-0.04 cells was attenuated by VRP, with 94% efflux in the absence of VRP. In the presence of 8 μM VRP, intracellular drug was quantitated as in Fig. 4.

In J774.2, A300, and J7.V3-0.04, AD 198 was distributed primarily in the Golgi-enriched perinuclear region of the cytoplasm, but with some diffuse fluorescence throughout the cytoplasm (Fig. 8). Fluorescence of either nuclei or mitotic chromosomes was not observed in any cell line. Cytoplasmic localization of AD 198 was observed in A300 cells in a more punctate array of heterogeneously sized structures as compared with J774.2 (Fig. 8, C and G). This localization was not evident either in J7.V3-0.04 (Fig. 8E) or J774.2 cells (Fig. 8A). Treatment of each cell line with 8 μM VRP for 1 h did not visibly alter the nuclear/cytoplasmic distribution (Fig. 8, B, D, and F). However, the punctate fluorescent localization in the cytoplasm of A300 cells was somewhat reduced (Fig. 8D).

ADR fluorescence in all three cell lines was primarily localized in the nucleus (Fig. 9). The granular fluorescence pattern was consistent with chromatin binding. Treatment with 8 μM VRP did not induce any observable redistribution of ADR.

**DISCUSSION**

AD 198 is one of a limited number of anthracycline analogues designed to circumvent MDR and thereby enhance chemotherapeutic efficacy against drug-resistant neoplastic cells. Circumvention of MDR by AD 198 appears to be due, at least in part, to the inability of P-gp to transport AD 198 from the cell (11). Despite difficulties in selecting lymphocytic sublines resistant to AD 198, we have demonstrated that cellular resistance to AD 198 could emerge rapidly within murine macrophage-like J774.2 cells and with characteristics that were similar to those of MDR. However, AD 198 cells differed from MDR cells in the persistence of high levels of intracellular AD 198 similar to that of drug-sensitive cells. Resistance to AD 198 could limit...
Fig. 8. Fluorescence microscopic analysis of J774.2, A300, and J7.V3-0.04 cells exposed to AD 198. Cells were grown without drug for 24 h prior to analysis and then attached to a chamber slide at 1 × 10^6 cells/ml. AD 198 was added to a final concentration of 900 nM for 1 h at 37°C. ADR was added to a final concentration of 2.0 μM for 2 h. As indicated, 8 μM VRP was added for the same duration of time at 37°C. × 400. n, nucleus; arrow, compartmentalization of AD 198 in cytoplasmic vesicles. G, × 1000. This figure is a computer-generated composite of photographic images.

The overexpression of the mdr1b isoform of P-gp in A300 conferred cross-resistance to VBL and ADR through reduced intracellular drug accumulation. Despite the overexpression of mdr2 mRNA in AD 198 cells, the fluctuations in mdr2 expression, as seen previously in VBL-resistant J774.2 cells (14), and the absence of mdr2 P-gp in this same cell line (14) suggest that mdr2 P-gp was not expressed in AD 198 cells. The absence of reduced AD 198 accumulation in A300 indicated that P-gp did not interact with AD 198 in a manner which resulted in AD 198 extrusion from the cell. Flow cytometric analysis suggested that P-gp overexpression resulting in reduced AD 198 accumulation did not constitute a subpopulation of A300 cells. Therefore, P-gp may have been induced in A300 cells exposed to AD 198 but not utilized in AD 198 resistance, similar to the situation in MDR DC-3F Chinese hamster cells selected for 9-hydroxyl ellipticine resistance (35). AD 198 may have avoided extrusion either through inhibition of drug interaction with P-gp by the N-benzyl and/or valerate moieties or through rapid intracellular penetration of subcellular membranes which would have rendered the drug spatially inaccessible to P-gp, as has been proposed for positively charged rhodamine analogues of increasing hydrophobicity (36). Both the
Fig. 9. Fluorescence microscopic analysis of J774.2, A300, and J7.V3-0.04 cells exposed to ADR. Cells were grown in the absence of drug for 24 h prior to analysis and then attached to a chamber slide at a density of 1 x 10^6 cells/ml. ADR was added to a final concentration of 2.0 μM for 2 h at 37°C. Where indicated, 8 μM VRP was added for the same duration of time at 37°C. x 400. This figure is a computer-generated composite of photographic images.

N-benzyl and 14-valerate side chain substitutions on AD 198 resulted in deeper membrane penetration relative to ADR (37), allowing for both rapid and extensive intracellular accumulation (8). In addition, the valerate moiety conferred a markedly greater affinity of AD 198 for uncharged (36,000 M^−1) and negatively charged (45,000 M^−1) unilamellar lipid vesicles compared to ADR (200 and 3,400 M^−1) and AD 288 (1,500 and 10,000 M^−1) and is, consequently, far more hydrophobic, as measured in these systems (37). The absence of the valerate side chain as in AD 288 resulted in a 60% reduction in net drug accumulation when A300 cells were exposed to extracellular AD 288, suggesting P-gp-mediated extrusion of AD 288. Yet, in both drug-sensitive and A300 cells, intracellular levels of AD 288 derived from intracellular AD 198 metabolism were equal. This suggests that intracellular AD 288 was sequestered from P-gp. Therefore, while it is not yet clear whether AD 198 is a potential substrate for P-gp, it appeared that AD 198 underwent intracellular compartmentalization and in this manner avoided P-gp interaction.

Fluorescence microscopic analysis of A300 cells treated with AD 198 also suggested that AD 198 was differentially compartmentalized. AD 198 exhibited preferential localization within the perinuclear region of the cytoplasm in both drug-sensitive and -resistant cells. This was attributed, at least in part, to the 14-valerate moiety, since ADR and AD 288 localized in the nuclei of both cells. The punctate fluorescence pattern observed in A300 cells following AD 198 treatment was similar to the lysosomal/mitochondrial distribution of daunorubicin observed in resistant HL-60 cells (38) but may have also reflected AD 198 distribution in larger phagosomes that are abundant in macrophages. J7.V3-0.04 cells did not exhibit the same punctate pattern of fluorescence as A300, despite a level of resistance similar to that of AD 198. However, preliminary time course analyses of intracellular AD 198 distribution suggest that vesicular localization of AD 198 in A300 but not J774.2 increased with time of drug exposure, suggesting a cytoplasmic redistribution of AD 198. This time course pattern of AD 198 distribution was evident in J7.V3-0.04 cells, but appeared to lag behind A300 by 2–3 h. The basis for this apparent delay is not known but may represent a difference in lysosomal and/or phagosomal formation between the resistant sublines, the latter being directly affected by changes in membrane fluidity (39).

The ability of chemosensitizing agents, such as VRP, to reverse resistance has been attributed to the ability of these agents to compete for the drug-binding sites on P-gp, resulting in increased net accumulation of cancer chemotherapeutic drugs. However, VRP decreased the IC_{50} of AD 198, ADR, and VBL in J774.2 cells in which P-gp was undetectable either by immunoblotting (Fig. 3) or by immunoprecipitation (16) and

[^1]: L. Lothstein and T. Sweatman, unpublished results.
at non-growth-inhibitory concentrations. This suggests that reversal of drug resistance in J774.2 cells occurred through a P-gp-independent mechanism (32, 38, 40). The attenuation of AD 198 resistance by VRP did not coincide with an inhibition of drug efflux but with some redistribution of AD 198 within the cytoplasm. It was observed that attenuation of cross-resistance to ADR by VRP did not coincide with increased net drug accumulation but resulted in increased intracellular ADR fluorescence. This probably reflected a change in drug binding within the nucleus, resulting either in decreased fluorescence quenching or an increase in the fluorescence lifetime of ADR, as has been observed for rhodamine 123 (41). While no shift in the wavelength of maximum absorbance has been detected (data not shown), other possibilities are currently under analysis.

It is not yet known whether VRP attenuates AD 198 resistance through interaction with P-gp. In ADR-resistant HL-60 cells which did not overexpress P-gp, resistance was attributed to the cytoplasmic sequestration of ADR in lysosomal vesicles of increased acidity through protonation of the glycosidic tertiary nitrogen (42-45). Attenuation of resistance by VRP was attributed to its lysosomotropic activity, leading to alterations in the lysosomal membrane and/or luminal milieu, with subsequent redistribution of ADR into more hydrophilic regions of the cytoplasm and into the nucleus (32). It is not yet known whether alkylation of the glycosidic nitrogen by a somewhat bulky benzyl group, as found in AD 198, would facilitate or inhibit protonation of the nitrogen, thus affecting potential lysosomal entrapment of this drug. Alternatively, entrapment of AD 198 in cytoplasmic vesicles may involve P-gp-mediated transport of drug into these vesicles. While immunohistochemical localization of P-gp in Golgi stacks has been observed in other MDR cells (46, 47), localization of P-gp in lysosomal and phagosomal membranes has yet to be confirmed. However, increased nonspecific adsorptive endocytosis has been observed in anthracycline- and Vinca-resistant cells (42). Consequently the presence of increased numbers of phagosomes in A300 cells, particularly if they are enriched in plasma membrane-derived P-gp, might contribute to AD 198 resistance.

The data presented here do not eliminate the possibility of multifactorial resistance against AD 198, as has been observed for ADR (30, 48). Other mechanisms may include enhanced activity of metabolic and antioxidant enzymes or altered topoisomerase II activity. Enhanced or differential metabolism of AD 198 in AD 198R cells is unlikely, since no anthracycline metabolites other than AD 288 were detected by HPLC analysis of cell lysates. It is also unlikely that altered topoisomerase II activity accounts for AD 198 resistance since (a) preliminary analysis revealed similar amounts of topoisomerase II in total cell lysates of J774.2 and A300; (b) AD 198 reportedly does not bind to purified topoisomerase II (9); (c) AD 198 was largely excluded from the nucleus in drug-sensitive cells, suggesting a cytoplasmic target for drug action; (d) AD 198 demonstrated the ability to circumvent resistance due to altered topoisomerase II activity in CEM human leukemic cells (11); (e) unlike A300 cells, VRP failed to potentiate the cytotoxic effects of ADR in human leukemic CEM/VM-1 cells (49); and (f) J7.V3-0.04 cells selected for resistance to VBL demonstrated cross-resistance to AD 198. Cell lines exhibiting MDR through altered topoisomerase II activity show no cross-resistance to the Vinca alkaloids (4). Consequently, VBL would not directly select for cells with altered topoisomerase II activity. The enhanced activity of antioxidant enzymes (50, 51) remains a possibility. However, unlike ADR, AD 198 is not cardiototoxic (52), suggesting that AD 198 does not initiate the cascade of intracellular free radical formation which has been implicated in anthracycline-induced cardiotoxicity (53). The ability of J7.V3-0.04 cells to exhibit cross-resistance to AD 198 while other MDR cells have not suggests that the emergence of AD 198 resistance may result from either a macrophe- or myeloid-specific characteristic, such as enhanced phagocytic or antioxidative activity.

In summary, J774.2 cells which were selected for resistance to AD 198 demonstrated intracellular drug levels and nuclear exclusion similar to those observed in drug-sensitive cells, yet AD 198R cells overexpressed P-glycoprotein and exhibited cross-resistance to ADR and VBL through reduced net drug accumulation. While increased cytoplasmic drug compartmentalization appears to contribute to AD 198 resistance, additional mechanisms may contribute to overall AD 198 resistance. Since altered topoisomerase activity and enhanced drug metabolism are not contributory mechanisms, resistance to AD 198 may encompass a novel mechanism of cellular resistance.

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