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P-glycoprotein overexpression in mouse cells does not correlate with resistance to *N*-benzyladriamycin-14-valerate (AD 198)

Leonard Lothstein, Yoshihiro Koseki and Trevor W Sweatman
Department of Pharmacology, University of Tennessee College of Medicine, Memphis, TN 38163, USA.
Tel: (+1) 901 448 5156; Fax: (+1) 901 448 7300.

The novel anthracycline *N*-benzyladriamycin-14-valerate (AD 198) circumvents P-glycoprotein (P-gp)- and altered topoisomerase II-mediated drug resistance. Nevertheless, AD 198-resistant (AD 198^R) murine J774.2 cells overexpressed P-gp, were cross-resistant to other drugs through reduced accumulation and were rendered sensitive by continuous exposure to verapamil. Intracellular AD 198 was, however, similar in sensitive and resistant cells. Consequently, the ability of P-gp to confer AD 198 resistance was examined. It was observed that (i) AD 198 resistance in AD 198^R cells grown without drug for 15 months declined by 60% with only a 10–15% loss of vinblastine cross-resistance and P-gp expression; (ii) a cloned AD 198^R P388 mouse leukemic cell line did not express P-gp; and (iii) verapamil did not attenuate resistance against high-dose, short-term exposure to AD 198. Therefore, AD 198 resistance appeared to be P-gp-independent despite P-gp overexpression. Antioxidant enzyme and topoisomerase II activities remained unchanged between sensitive and resistant cells. These results suggest that AD 198 resistance was conferred by a novel mechanism.

Key words: Anthracycline antibiotics, AD 198, adriamycin, multidrug resistance, P-glycoprotein.

Introduction

Chemotherapeutic failure during the treatment of malignancies is often due to the emergence of cellular drug resistance.[1] The overexpression of the multidrug transporter P-glycoprotein (P-gp) has been associated with many chemotherapeutically-refractory tumors,[2] and shown to confer multidrug resistance (MDR) to a variety of cultured mammalian cell lines.[3,4] Circumvention of MDR, as well as some systemic adverse effects of antineoplastic agents, particularly adriamycin (ADR; doxorubicin), has been achieved to varying extents with semi-synthetic congeners.[5,11] *N*-benzyladriamycin-14-valerate (AD 198), a highly lipophilic, non-cardiotoxic ADR congener exhibiting novel cytotoxic mechanisms,[12,13] circumvented both MDR and altered topoisomerase II-mediated multidrug resistance (at-MDR).[10] Nevertheless, AD 198-resistant (AD 198^R) J774.2 mouse macrophage-like cells were selected by continuous exposure to increasing cytotoxic drug concentrations.[14] The resulting resistant variants overexpressed P-gp, were cross-resistant to ADR and vinblastine (VBL) through reduced drug accumulation, and were rendered sensitive to AD 198 by continuous exposure to 8 μM verapamil. These cells, however, exhibited similar intracellular AD 198 levels similar to sensitive cells. Fluorescence microscopy of cells exposed to AD 198 revealed some enhanced vesicular compartmentalization of drug in the cytoplasm that persisted in AD 198^R cells after conversion of AD 198 to its nuclear-localizing metabolite, *N*-benzyladriamycin (AD 288).[14,15] Cross-resistance in AD 198^R cells was detected against other anthracyclines that localized in the cytoplasm, but were converted to highly cytotoxic, nuclear-localizing metabolites.[16] Therefore, the presence of P-gp in AD 198^R cells without reduced AD 198 accumulation indicated either that P-gp played no significant role in AD 198 resistance or that P-gp conferred AD 198 resistance through activity at some other intracellular location. The significance of P-gp function in AD 198^R cells was the subject of this investigation.

Materials and methods

Materials

AD 198, AD 288 and adriamycin-14-valerate (AD 48) were prepared as described previously,[17,18] and were kindly provided by Dr M. Israel (University

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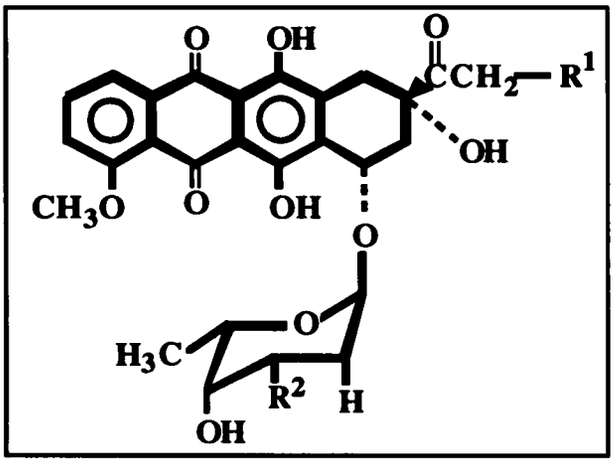
Correspondence to L Lothstein



	R^1	R^2
ADR	OH	NH_2
AD 198	$OCO(CH_2)_3CH_3$	$NHCH_2C_6H_5$
AD 288	OH	$NHCH_2C_6H_5$
AD 48	$OCO(CH_2)_3CH_3$	NH_2

Figure 1. Chemical structures of ADR, AD 198, AD 288 and AD 48.

of Tennessee, Memphis, TN). The chemical structures are shown in Figure 1. ADR was a generous gift from Farmitalia Carlo Erba (Milan, Italy). [^{3}H]Azidopine (56 Ci/mmol) was obtained from Amersham Radiochemicals (Arlington Heights, IL). Vinblastine sulfate and reagents for antioxidant enzyme activity assays (1-chloro-2,4,-dinitrobenzene, reduced glutathione, glutathione reductase, NADPH, oxidized glutathione and FAD) were obtained from Sigma (St Louis, MO).

Cell culture

J774.2 cells were selected for resistance to AD 198 and maintained in monolayer culture as previously described.[14] P388 mouse lymphocytic leukemia cells were maintained as suspension cultures in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 10 μM β-mercaptoethanol. Selection of AD 198-resistant P388 variants was performed as outlined in Figure 4. Resistant variants were cloned in soft agar.

Drug toxicity assay

The IC_{50} values (drug concentration inhibiting growth by 50% after 72 h of continuous exposure, unless otherwise defined) for J774.2 and P388 cell lines were quantitated by the MTT assay[19] under conditions described previously.[15] Values were the mean of three to five independent determinations, each performed in triplicate.

Net drug accumulation

Intracellular drug accumulation was quantitated by fluorescence HPLC.[14,20] Analyses were performed on 1.5×10^6 cell samples in duplicate for each time point. Mean total drug recovery (pellet, media and wash) was greater than 90%.

RNA purification and hybridization

RNA was purified from sub-confluent cells by guanidinium isothiocyanate-induced disruption followed by centrifugation of RNA through a CsCl buoyant density gradient.[21,22] Blotting and hybridization was performed as described previously[23] using random-primed[24] ^{32}P-labeled C1.5 cDNA representing the full-length *mdr 1b* coding region. C1.5 cross-hybridized with *mdr 1a* and *mdr 2*.[23] Autoradiographic signal intensity was quantitated by visible light densitometry (Discovery Series Image Analyzer, PDI, Huntington, NY) within the linear range of the densitometer according to prior calibration. Hybridization with ^{32}P-labeled murine β-actin cDNA probe pHFβA-1[25] provided quantitative normalization of *mdr* RNA hybridization.

Immunoblot analysis

Sub-confluent cells were allowed to swell in 9 volumes of cell lysis buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 3.6 mM $CaCl_2$, 30 μM leupeptin, 1 μg/ml pepstatin A and 100 units/ml aprotinin) for 10 min on ice, then lysed by 12 strokes in a metal homogenizer with a tight-fitting pestle. Nuclei were removed by centrifugation at 300 *g* for 5 min. Membrane and microsomal fractions were pelleted at 100 000 *g* for 60 min at 4°C. Pelleted protein was quantitated using a colorimetric protein assay (Bio-Rad, Oakland, CA) based on the procedure of Bradford,[26] size-fractioned by SDS–PAGE[27] and blotted onto nitrocellulose membrane as described previously.[14] The membrane was blocked with 10% (v/v) newborn calf serum in TTBS (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 0.2% Tween 20) for 1 h at 25°C,

incubated with the P-gp-specific monoclonal antibody C219 (Signet, Dedham, MA) at 2 μg/ml in blocking solution for 1 h, then incubated in goat anti-mouse IgG–horseradish peroxidase conjugate (Amersham) at a 1:1000 dilution for 1 h. Antibody–antigen conjugates were detected by treatment with a luminol solution (Amersham) and autoradiography. Signal intensity was quantitated by densitometry as described above.

Preparation and photoaffinity labeling of cell membranes

Membrane fragments from subconfluent cells were isolated, photoaffinity-labeled with [^{3}H]azidopine and analyzed by SDS–PAGE as described by Yang *et al.*[28] For competitive inhibition of photoaffinity labeling, membrane fragments were treated with 500-fold molar excesses of either ADR, VBL, AD 198, AD 48 or AD 288 simultaneously with 50 nM [^{3}H]azidopine. Autoradiographic images were quantitated as described above.

Topoisomerase II unknotting activity

Nuclear extracts of sensitive and AD 198-resistant J774.2 cells were prepared according to Danks *et al.*[29] Briefly, subconfluent cells were incubated in 5 mM KH_2PO_4, pH 7.0, 2 mM $MgCl_2$, 4 mM dithiothreitol, 0.1 mM EDTA, 30 μM leupeptin, 100 units/ml aprotinin, 1 μg/ml pepstatin A and 0.1 mM phenylmethylsulfonyl fluoride to achieve cell permeability in at least 80% of the cells, as determined by Trypan blue staining. Nuclei were purified by centrifugation at 2000 *g* for 20 min over 0.6 M sucrose in the previous buffer. Nuclei were extracted for 30 min at 4°C in 20 mM Tris–HCl, pH 7.5, 5 mM KH_2PO_4, 0.35 M NaCl, 4 mM dithiothreitol, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 10% glycerol, then subjected to centrifugation at 100 000 *g* for 70 min. Protein content of nuclear extracts in the supernatant was determined as described above. Topoisomerase II activity, as determined by unknotting of supercoiled P4 phage DNA, was analyzed by methods described previously.[29,30] Nuclear lysate was combined with 300 ng supercoiled P4 DNA, in reaction buffer with or without ATP. The reaction was stopped by the addition of 150 μg/ml proteinase K and 0.5% SDS for 2.5 h at 37°C. DNA was resolved in a 0.7% agarose gel in Tris–borate buffer and stained with 10 μg/ml ethidium bromide. P4 phage DNA was kindly provided by Dr MK Danks (St Jude Children's Research Hospital, Memphis, TN).

Measurements of antioxidant enzyme activities

Activities of superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase and glutathione reductase were measured in log-phase J774.2 and A300 whole cell lysates prepared as described[31] using the spectrophotometric methods of Beutler.[32] Enzyme activities were represented by the mean of three to five independent determinations performed in duplicate and were normalized based upon the amount of cell lysate protein added to each assay mixture.

Results

The inability of P-gp to transport AD 198 out of AD 198^R J774.2 cells, as observed previously,[14] was believed to be due to continued rapid penetration of the drug through the plasma membrane, and/or the inability of P-gp to bind and transport AD 198 due to incompatible drug structure. Therefore, as a measure of AD 198/P-gp interaction, the ability of AD 198 to competitively inhibit the photoaffinity labeling of P-gp by [^{3}H]azidopine was determined with enriched plasma membrane fragments from 15-fold AD 198-resistant A300 cells (Figure 2). In the absence of competing drugs, [^{3}H]azidopine exhibited preferential cross-linking to P-gp, as observed previously in MDR J774.2 cells.[28] In the presence of a 500-fold molar excess of AD 198, [^{3}H]azidopine binding was reduced to 12% of control levels (no competing drug). The principal intracellular metabolite of AD 198, *N*-benzyladriamycin (AD 288), reduced [^{3}H]azidopine binding to 33%, while adriamycin-14-valerate (AD 48) reduced binding to 60%. ADR exhibited the least efficient binding inhibition at 83% of control levels. VBL, a potent inhibitor of [^{3}H]azidopine binding to P-gp,[33] did not compete as effectively as AD 198.

Increasing P-gp expression was previously shown to coincide with selection of increasing levels of AD 198 resistance.[14] A300 cells were subsequently grown without drug to determine whether reversion of AD 198 resistance correlated with decreased P-gp expression (Figure 3). In revertant A300 cells, AD 198 resistance decreased by 60% after 15 months of growth without AD 198 (Figure

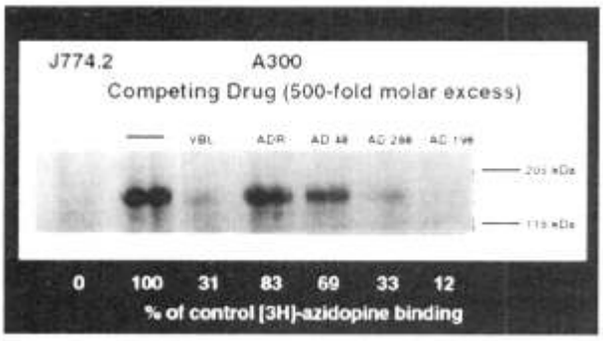
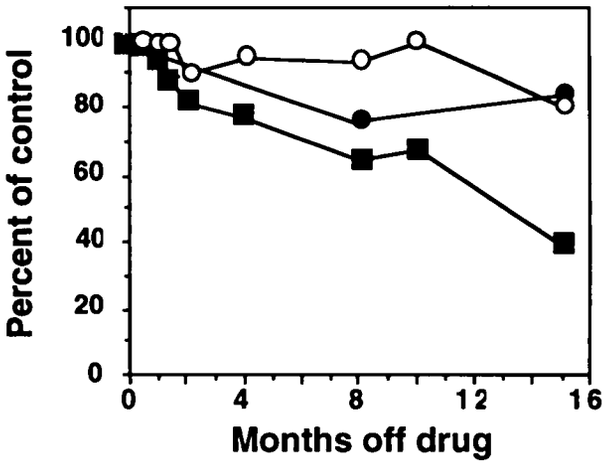


Figure 2. [^{3}H]Azidopine photoaffinity labeling of P-gp in A300 cells. Membrane fragments from either J774.2 and A300 were prepared by differential centrifugation of mechanically lysed cells.[28] Membrane protein (200 μg) was treated with 50 nM [^{3}H]azidopine (56 Ci/mmol) for 60 min at 25°C with or without a 500-fold molar excess of the drug indicated. Samples were UV cross-linked for 10 min[28] and subjected to SDS–PAGE through a 7.0% gel. The autoradiographic images were quantitated on a PDI Quantity One image analyzer. Control lane (—) indicates incubation with [^{3}H]azidopine in the absence of competing drug.



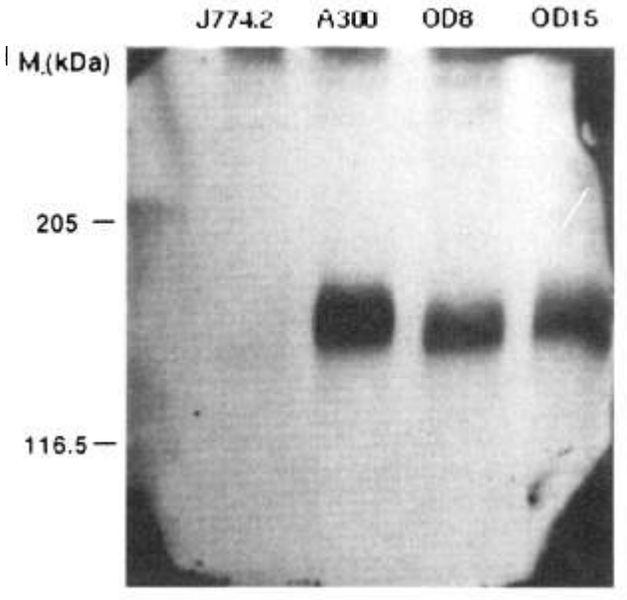


Figure 3. Correlation of P-gp expression and AD 198 resistance. AD 198-resistant cells were propagated without drug for up to 15 months. Upper panel: at times indicated, resistance to AD 198 and VBL was determined by the MTT colorimetric procedure[19] and expressed as a percentage relative to A300 cells maintained on AD 198 (control). ■, AD 198 resistance; ○, VBL resistance; ●, P-gp. Lower panel: 5 μg of non-nuclear protein from A300 cells maintained on AD 198 and A300 cells cultured without AD 198 for 8 (OD8) and 15 months (OD15) was resolved in a 7% polyacrylamide gel, then blotted onto nitrocellulose. P-gp levels were then determined by immunoblot analysis using mAb C219 as described previously.[14] Primary antibody was detected with goat anti-mouse antibody–horseradish peroxidase conjugate and luminol chemiluminescence reagent. Densitometry was performed with a PDI Quantity One image analyzer. The level of P-gp is expressed as a percentage relative to A300 cells maintained on AD 198.

3, upper panel). Cross-resistance to VBL, which was conferred by P-gp-mediated enhanced efflux,[14] did not change significantly until 15 months when a 15% decrease was observed. Similarly, AD 198 resistance determined by 3 h AD 198 exposure and subsequent growth for 69 h without drug revealed a 30% decrease, while no decrease was detected for VBL cross-resistance. During the 3 h exposure period, less than 20% of AD 198 was converted to AD 288. P-gp expression, based upon Western blot analysis, exhibited only a 15% quantitative decrease following 15 months off drug (Figure 3, lower panel). Hence, the decline in P-gp expression was more closely correlated with reversion of VBL resistance, than with AD 198 resistance.

AD 198^R J774.2 cells were selected by continuous exposure to AD 198, beginning with the IC_{50} concentration,[14] as outlined in Figure 4, and cloned by limiting dilutions of cell suspensions. Propagation of 15-fold resistant cells was achieved rapidly within 96 cell doublings after initial exposure. Initial selection of P388 lymphocytic leukemia cells by continuous exposure to up to 60 nM AD 198 resulted in progressive plasma membrane fragility and subsequent cell lysis. Consequently, cells were exposed to 80 nM AD 198 or greater for cycles of 12 days on drug followed by 5–7 days off drug. Using this schedule, 4-fold resistant P388/AD 198 cells were selected after 495 cell doublings. Three P388/AD 198 sublines were cloned in soft agar and maintained by intermittent exposure to either 80 nM AD 198 (clone D) or 200 nM (clones A and B)

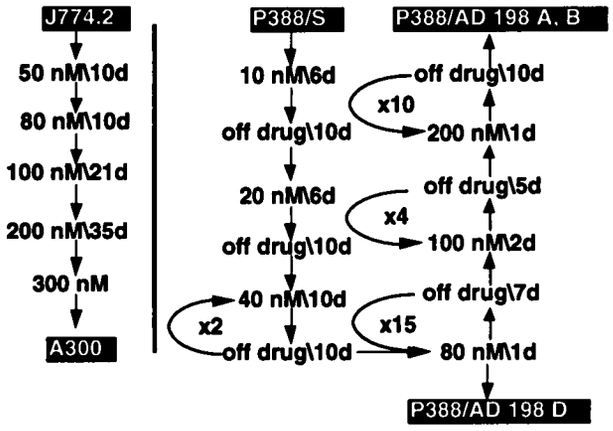


Figure 4. Selection of AD 198R J774.2 and P388 cells. The selection protocols for J774.2 A300 and P388/AD 198 clones A, B and D are outlined. Lengths of exposure to each drug concentration and of period off drug are indicated in days (d). For P388 variants, the number of on/off drug cycles greater than 1 are shown (×#).

or frozen for later use. As shown in Table 1, IC_{50} values for all three clones were below the selecting drug concentration due to differences in drug exposure times (24 h during selection versus 72 h during dose–response analyses). Clones A, B and D exhibited 2.5- to 4.0-fold AD 198 resistance, with resistance increasing slightly with higher amounts of selecting drug. Cross-resistance to VBL was observed only in clones A and B. Clone D, whose level of AD 198 resistance was approximately equal to that of clone B, remained sensitive to VBL. As observed previously with A300 cells,[14] no correlation was observed between intracellular accumulation of AD 198 and resistance to AD 198 in P388 variants (Table 1). Intracellular levels of AD 198 in clones A and B were similar to P388/S after 6 h of exposure to 900 nM AD 198. Clone D exhibited a 25% lower level of AD 198 accumulation compared with P388/S and clones A and B.

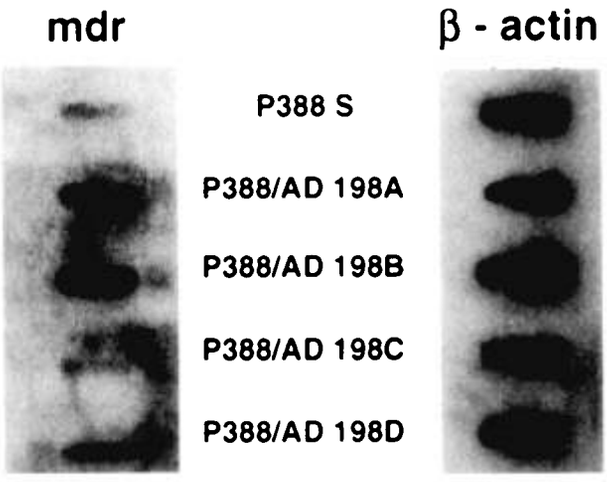
Analysis of P-gp expression in P388/AD 198 cells showed a direct correlation with VBL cross-resistance, but not with AD 198 resistance (Figure 5). Clones A and B both overexpressed 3- to 4-fold greater levels of P-gp mRNA than P388/S cells (Figure 5, upper panel). No overexpression was detected in clone D. Clone C, which was also selected at 80 nM AD 198, did not overexpress P-gp mRNA. This clone was lost before further analysis was possible. Likewise, immunoblot analysis of P388 plasma membranes detected P-gp in clones A and B (Figure 5, lower panel). Clone A overexpressed approximately 2-fold more P-gp than clone B and with an M_r of 130 kDa, compared with 145 kDa of clone B and A300 (not shown). P-gp expression was not detectable in clone D.

Continuous exposure of A300 cells to 2–8 μM verapamil over 72 h produced a dose-dependent potentiation of AD 198 cytotoxicity.[14] However, the extensive conversion of AD 198 to AD 288 during this time period and the ability of P-gp to transport AD 288 suggested that verapamil may have been potentiating AD 288 cytotoxicity under these conditions.[14,15] In order to assess the effect of verapamil against AD 198 cytotoxicity specifically, the cytotoxicity of AD 198 in A300 cells was determined by 3 h exposure of cells to either AD 198 or AD 288, alone, or in combination with 72 h exposure to 8 μM verapamil. Under these conditions, verapamil was ineffective in enhancing AD 198 cytotoxicity in A300 cells compared with J774.2 cells and with AD 288 (Table 2). A 72 h exposure of A300 to both AD 198 and 8 μM verapamil resulted in a 96% decrease in the IC_{50} value, while 3 h exposure with AD 198 and verapamil followed by 69 h exposure to 8 μM verapamil, alone, reduced the IC_{50} value by only 13%, compared with 37% in drug-sensitive cells. In contrast, 8 μM verapamil treatment of A300 cells decreased the 72 h IC_{50} value of AD 288 by 82 versus 64% for 3 h AD 288 exposure in A300 cells.

Table 1. Dose–response analysis and AD 198 accumulation in AD 198^R P388 cells

Cell line	IC_{50} (nM)[a] ± SE		Intracellular drug ($ng/10^6$ cells)[b]	
	AD 198	VBL	0.5 h	6 h
P388/S	24.9 ± 3.6 (1.0)[c]	5.7 ± 1.8 (1.0)	71.7 ± 8.9	62.7 ± 7.8
P388/AD 198A	100.7 ± 17.7 (4.0)	33.8 ± 10.7 (5.9)	55.1 ± 9.3	58.2 ± 7.1
P388/AD 198B	70.8 ± 9.8 (2.8)	14.8 ± 3.5 (2.6)	75.8 ± 3.6	57.8 ± 4.2
P388/AD 198D	61.1 ± 10.1 (2.5)	3.8 ± 1.0 (0.7)	52.5 ± 8.9	47.3 ± 3.4

[a] Drug concentration which inhibits cell growth by 50% after 72 h. Values are the mean of three to five independent determinations performed in triplicate.
[b] Cells were exposed to 900 nM AD 198.
[c] *N*-fold resistance: ratio of the IC_{50} of the resistant and parental lines.



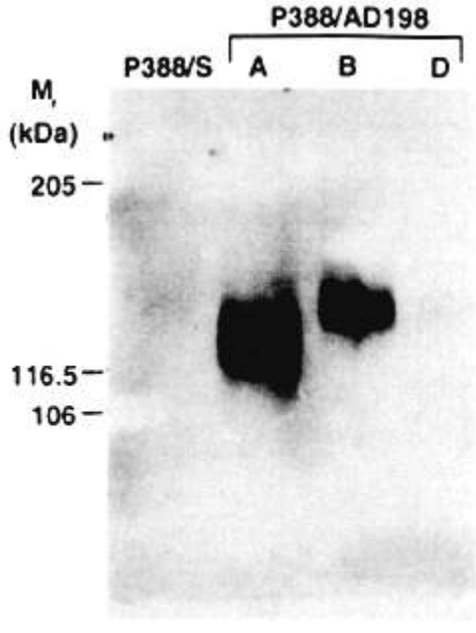


Figure 5. P-gp mRNA slot-blot and immunoblot analyses of AD 198^{R} P388 cells. Upper panel: 10 μg of total RNA was blotted on to nylon membrane and probed with ^{32}P-labeled full-length *mdr-1b* cDNA probe C1.5[23] or β-actin cDNA.[25] The membrane was subjected to autoradiography for 48 h at −70°C with pre-flashed Kodak XAR-1 film. Lower panel: 5 μg of non-nuclear protein were size fractioned by SDS–PAGE on a 5% gel, electroblotted onto nitrocellulose membrane and probed with mAb C219 for P-gp.[14] Primary antibody was detected with goat anti-mouse antibody–horseradish peroxidase conjugate and luminol chemiluminescence reagent.

P-gp activity appeared, then, to play no significant role in resistance to AD 198, based upon the absence of P-gp in P388/AD 198 clone D, the inability of P-gp overexpression to increase resistance to AD 198 in P388/AD 198 clone B, the dissociation of P-gp expression and VBL cross-resistance with declining AD 198 resistance in revertant A300 cells, and the inability of verapamil to significantly potentiate AD 198 cytotoxicity after brief exposure.

The absence of reduced accumulation of AD 198 or apparent involvement of P-gp in AD 198 resistance indicated the activation of an alternative mechanism(s) of resistance. AD 198 was metabolized at the same rate and to the same metabolite (AD 288) in A300 cells compared with J774.2.[14] In P388/AD 198 cells, no quantitative or qualitative changes in AD 198 metabolism were evident relative to P388/S (not shown). It was, therefore, determined whether AD 198 resistance correlated with other mechanisms associated with ADR resistance, including enhanced antioxidant enzyme activity involved in suppressing anthracycline-catalyzed free radical generation[31,34,35] or reduced topoisomerase II activity. Cytosolic extracts of J774.2 and A300 cells were assayed for changes in activities of catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione S-transferase (Table 3). No significant differences in activities were observed which might account for AD 198 resistance.

Topoisomerase II activity in nuclear lysates of J774.2 and A300 cells was measured by correlating P4 phage DNA unknotting activity with increasing amounts of nuclear lysate protein following resolution of topologically altered DNA by gel electrophoresis (Figure 6, upper panel). Supercoiled DNA, as represented by a broad pattern of ethidium bromide fluorescence, was converted to unknotted and nicked DNA of slower mobility and more discrete size following incubation with 0.25 μg of nuclear lysate from either J774.2 and A300. Catenated P4 DNA was formed in 1.5 μg of nuclear lysate from either cell line. The apparent absence of at-MDR in A300 cells was consistent with the poor topoisomerase II inhibitory activity of AD 198 (Figure 6, lower panel). Stabilization of cleaved DNA was achieved with 2 μM ADR, but required at least 35 μM AD 198. AD 288 stabilized the topo II–DNA complex at 5 μM, which was consistent with the comparable cytotoxic activity and mechanistic characteristics of AD 288 and ADR.[12] Similar topoisomerase II inhibitory drug concentrations were determined with both J774.2 and A300 nuclear lysates.

Table 2. Dose–response to AD 198 and AD 288 exposure ± verapamil

	J774.2	A300
3 h IC_{50} (μM) ± SE[a]		
AD 198	1.71 ± 0.17	3.86 ± 0.29
AD 198 + V	1.08 ± 0.09 (37%)[b]	3.36 ± 0.27 (13%)
AD 288	0.53 ± 0.11	1.91 ± 0.17
AD 288 + V	0.25 ± 0.04 (47%)	0.69 ± 0.10 (64%)
72 h IC_{50} (μM) ± SE		
AD 198	0.049 ± 0.007	0.750 ± 0.082
AD 198 + V	0.017 ± 0.005 (65%)	0.033 ± 0.001 (96%)
AD 288	0.033 ± 0.007	0.423 ± 0.094
AD 288 + V	0.021 ± 0.002 (46%)	0.074 ± 0.016 (82%)

[a] Cells were exposed to drug for 3 h and drug-free medium for 69 h or continuously for 72 h. Verapamil (V) exposure at 8 μM was continuous for 72 h. Values are the mean of three to five independent determinations performed in triplicate.
[b] Percent decrease in IC_{50} value.

Table 3. Antioxidant enzyme activities in J774.2 and A300 cells

Enzymes	Mean specific activity ± SE[a]	
	J774.2	A300
Catalase (μmol H_2O_2/min/mg)	3.0 ± 0.1	2.5 ± 0.2
Superoxide dismutase I_{50} (μg) of pyrogallol oxidation	495.5 ± 67.0	468.0 ± 50.6
Glutathione-S-transferase (nmol CDNB/min/mg)	12.5 ± 1.7	13.6 ± 1.1
Glutathione peroxidase (nmol NADPH/min/mg)	446.8 ± 72.2	389.6 ± 23.8
Glutathione reductase (nmol NADPH/min/mg)	22.8 ± 1.5	24.2 ± 1.2

[a] Values are the mean of three to four independent determinations performed in duplicate and normalized per mg of cellular protein.

Discussion

Cells selected for resistance to AD 198 presented an interesting model of multifactorial resistance to anthracyclines. As shown previously, AD 198^R J774.2 cells overexpressed P-gp, yet intracellular AD 198 levels in sensitive and resistance cells were identical. These cells exhibited cross-resistance to ADR, VBL and the AD 198 metabolite, AD 288, through reduced intracellular drug accumulation.[14,15] Therefore, from these observations it was concluded that P-gp either conferred resistance to AD 198 and cross-resistance to other drugs through two distinct mechanisms and/or at two different subcellular sites, or that P-gp was not involved in AD 198 resistance. High levels of P-gp have been observed in the Golgi stacks of normal and neoplastic human cells, suggesting an association between P-gp and enhanced vesicular accumulation of lipophilic drugs.[36,37] We have previously presented evidence of persistent cytoplasmic compartmentalization of AD 198 in A300 cells.[15] However, the results described in this report indicated that P-gp was not an essential component of the AD 198 resistance phenotype. This conclusion was based upon the observations that (i) P-gp expression and P-gp-mediated resistance to VBL could be dissociated from AD 198 resistance in revertant AD 198^R cells, (ii) the emergence of AD 198 resistance in P388 cells occurred without P-gp overexpression, (iii) P-gp overexpression did not augment AD 198 resistance, and (iv) AD 198 cytotoxicity was not significantly potentiated by verapamil relative to AD 288, for which cross-resistance was conferred by reduced accumulation.

In agreement with Friche *et al.*,[38] AD 198 was observed to effectively inhibit [^{3}H]azidopine photoaffinity labeling of P-gp, despite the absence of P-

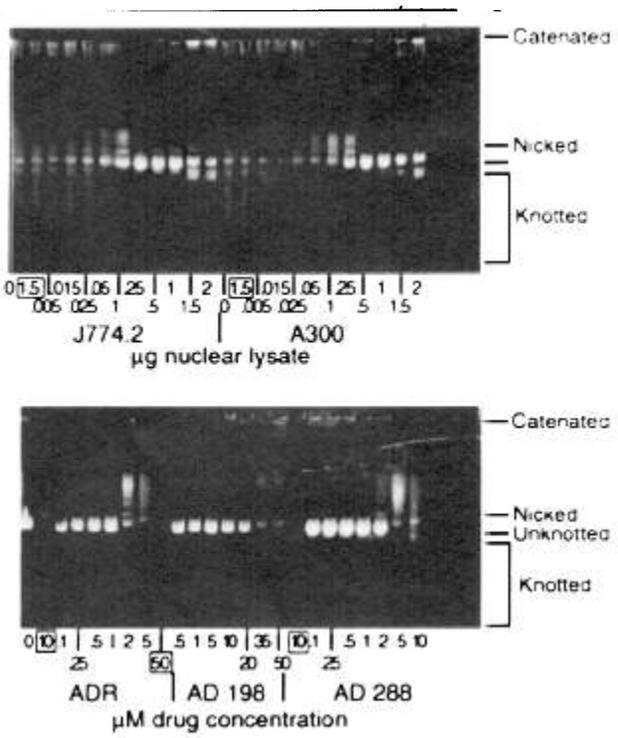


Figure 6. P4 DNA unknotting assay for topoisomerase II activity. Assays were performed as described previously.[29] Upper panel: 5–2 µg nuclear lysates were incubated with 300 ng P4 DNA for 30 min at 37°C. P4 DNA was then size-fractioned in a 0.7% agarose gel in Tris–borate buffer and stained with 10 µg/ml ethidium bromide. Position of topological forms of DNA are indicated at right. The lane designated '0' indicates P4 DNA incubated without nuclear lysate. The lane denoted by boxed 1.5 indicates DNA incubated with 1.5 µg lysate but without exogenous ATP. Lower panel: 500 ng A300 nuclear lysate and 300 ng P4 DNA were incubated with increasing concentrations of drugs, as indicated, for 30 min at 37°C. DNA was size-fractioned in a 0.7% agarose gel and stained with ethidium bromide. Box denotation indicates incubation in the absence of exogenous ATP. Quantitation of DNA was performed by image analysis.

gp-mediated transport of AD 198 from the AD 198^R cells. This was possibly due to an allosteric inhibition of photolabeling through structural perturbation of plasma membrane by AD 198 that, in turn, interfered with P-gp function. Perturbation and subsequent rapid deterioration of the plasma membrane of human CEM cells have been observed after 3 h of exposure to AD 198 at 5 µM,[12] compared with 25 µM AD 198 used in photoaffinity labeling studies. In this same study, membrane perturbations were not observed following treatment with ADR and AD 288. [^{3}H]Azidopine binding inhibition by AD 198 might then be more analogous to a non-specific detergent effect upon membrane rather than direct binding site interaction,[39] as has been shown with Cremophor EL and Tween 80.[38]

A direct correlation was observed between anthracycline affinity for negatively-charged lipid bilayers, as measured by Burke *et al.*,[39] and inhibition of [^{3}H]azidopine photolabeling of P-gp (Table 4). This was consistent with the more rapid intracellular accumulation of AD 198[20] and the probability of significantly higher amounts of AD 198 in plasma membrane fragments, compared with ADR. Also, we have described a correlation between increasing lipophilicity and the ability to bypass plasma membrane P-gp.[15] AD 48- and AD 288-induced inhibition might result from both direct binding site competition, as suggested by the ability of P-gp to transport these compounds,[15] and, to a limited extent, plasma membrane disruption, due to increased lipophilicity, particularly of AD 288. These results suggest that photoaffinity labeling of P-gp by [^{3}H]azidopine can be inhibited by anthracyclines that do not bind P-gp, but exert an effect by virtue of their increasing lipophilic nature. Thus, photoaffinity labeling studies may not accurately assess the P-gp binding potential of anthracyclines. It is not yet known whether this caveat applies to the use of anthracycline photoaffinity probes.[40]

Table 4. Cellular characteristics of 3'-*N*-alkylanthracyclines in A300 cells

	N-fold resistance[15]	[^{3}H]azidopine binding inhibition[a] (%)	Decrease in accumulation[b] (%)	Octanol/PBS partitioning[39]	$K_{(DMPG)}$ (M^{-1})[c]
ADR	24.8	17	42	1.2	3400
AD 48	19.9	31	47	35	8000
AD 288	16.5	67	29	>99	10000
AD 298	15.3	88	0	>99	45000

[a] See Figure 1.
[b] Intracellular drug accumulation levels were compared with drug sensitive J774.2 cells and measured by fluorescence HPLC.[15]
[c] Drug association constant for negatively-charged dimyristoyl phosphatidylglycerol bilayers.[39]

The steady decline in AD 198 resistance in A300 cells grown without drug indicated that the AD 198^R phenotype was much less stable than VBL cross-resistance based upon drug toxicity assays using both 72 h continuous drug exposure (Figure 3) and 3 h, high-dose exposure to AD 198 (not shown). We have previously observed that VBL cross-resistance in A300 cells was mediated by P-gp.[14] Thus, it was not surprising that after 15 months without AD 198, VBL resistance and P-gp levels exhibited a similar decrease. After the same time period, however, the decline in AD 198 resistance exceeded the decrease in P-gp levels, suggesting that AD 198 resistance either did not decrease coordinately with quantitative changes in P-gp or decreased in response to post-translational modifications of P-gp, such as phosphorylation.[41] Putative changes in P-gp phosphorylation did not appear to induce drug-specific modulation of P-gp activity.[42] Therefore, it was more likely that AD 198 resistance and P-gp levels in A300 cells varied independently of each other.

Overexpression of P-gp has been previously shown not to be sufficient to confer cross-resistance to AD 198 in CEM/VLB-100 or L1210/ADR cells *in vitro*.[10,43] Further, in 100-fold resistant P-gp-positive P388/ADR cells, no significant AD 198 resistance was detected (not shown). Analysis of AD 198^R P388 clones revealed not only AD 198 resistance without detectable P-gp (clone D), but also the inability of subsequent P-gp overexpression to further increase resistance to AD 198. The overexpression of P-gp in clone B resulted in a 4-fold increase in VBL cross-resistance relative to clone D, but had no effect on the level of AD 198 resistance. Thus, P-gp neither conferred nor increased the level of AD 198 resistance in cells selected for resistance to AD 198 or other drugs. We observed that while the levels of P-gp in membranes from clones A and B correlated with VBL cross-resistance, these levels were not consistent with the corresponding amounts of mRNA. The P-gp isoform found in clone B corresponds to *mdr 1b*, based upon M_r, while clone A appears to express the *mdr 1a* isoform.[14,44] It was likely that this discrepancy was due either to isoform and/or promoter-specific differences in translational efficiency of P-gp transcripts, as has been described previously for *mdr 1a*[23,44,45] or differential expression of *mdr 2* mRNA without subsequent synthesis of the corresponding P-gp.[23]

The inability of P-gp to confer AD 198 resistance raises the question of why was P-gp overexpressed in some, but not all AD 198^R lines. The emergence of the P-gp-mediated MDR phenotype was probably due to secondary selection of AD 198^R cells with AD 288, as has been observed for the 13-dihydro metabolite of 4′-deoxy-4′-iododoxorubicin, which, unlike the parent drug, selected for MDR.[46] AD 198 was hydrolyzed to AD 288 at a rate of approximately 50% of starting AD 198 per 24 h both intracellularly and in conditioned media.[14] Selection of AD 198^R cells by continuous exposure of up to 300 nM AD 198 resulted in extracellular AD 288 concentrations that exceeded the IC_{50} value for J774.2 following 72 h drug exposure (33 nM). Therefore, J774.2 cell underwent selection for both AD 198 and AD 288 resistance. This was consistent with the identification of MDR J774.2 variants following selection with AD 288, alone (L. Lothstein, unpublished results). P388/AD 198 clone D cells were selected by discontinuous 24 h exposures to 80 nM AD 198. At 24 h and just before replacement by drug-free medium, the AD 288 concentration in the medium was approximately 35 nM. This would have been insufficient to apply selective pressure for AD 288 resistance. Recently, AD 198-resistant J774.2 cells were selected by 1 h, high-dose AD 198 exposure to up to five times the IC_{50} of J774.2. Under these conditions, less than 10% conversion of AD 198 to AD 288 was detected and, consequently, P-gp was not overexpressed (unpublished results). These results further emphasize the concept that anthracycline metabolites can be distinct from the parent compound in the mechanism(s) of cytotoxicity and of cellular resistance that is selected.

Recognizing the inability of P-gp to confer AD 198 resistance has not, however, indicated the mechanism of resistance. Bodley *et al.*[47] have shown that AD 198 did not demonstrate any stimulation of topoisomerase II-mediated DNA cleavage and, therefore, might not be cytotoxic through topoisomerase II inhibition. Our studies confirmed the significantly reduced ability of AD 198 to stabilize the P4 DNA/topoisomerase II complexes, relative to ADR and AD 288. Consequently it was not surprising that AD 198 resistant cells did not appear to possess reduced levels of topoisomerase II activity, based upon comparative P4 DNA unwinding activity. In addition, the ability to sensitize A300 cells to ADR with verapamil further indicates that A300 cells did not confer resistance through reduced topoisomerase II activity. Since the topoisomerase II inhibitory activity of AD 288 was comparable to ADR, the previously demonstrated ability of AD 198 to circumvent at-MDR in CCFR-CEM human leukemic[10] further suggested that AD 198 exerts its cytotoxicity through a novel pathway.

ADR resistance has been shown to be associated with enhanced antioxidant and metabolic enzyme activities[31,34] in response to the quinone-catalyzed generation of free hydroxyl radicals.[35] AD 198-resistant J774.2 cells did not exhibit changes in the activities of these enzymes relative to total non-nuclear protein, suggesting either that AD 198 was a poor catalyst of free radical generation or that free radical-induced damage was not a significant cytotoxic mechanism in J774.2 cells. However, the preservation of the quinone ring in AD 198 suggests that AD 198 was capable of contributing to free-radical generation.

Hindenberg *et al.*[48] have described ADR-resistance HL-60 cells that exhibited cytoplasmic rather than nuclear localization of ADR. A 180 kDa microsomal protein, MRP, has recently been associated with this ADR resistance phenotype in H69 small cell lung carcinoma cells that do not overexpress P-gp.[49] As a member of the ABC superfamily of transmembrane transporters, it is thought that MRP might be involved in vesicular compartmentalization of ADR. Preliminary studies of MRP expression in AD 198^R cells revealed no difference in MRP levels in sensitive and AD 198^R J774.2 cells (L. Lothstein, unpublished results).

In summary, P-gp overexpression did not confer resistance to AD 198 in J774.2 and P388 cells, although its presence accounted for cross-resistance to VBL, ADR and AD 288. P-gp overexpression probably occurred in response to secondary selective pressure by AD 288. Further AD 198 resistance in J774.2 was not conferred either by at-MDR or enhanced anti-oxidant enzyme and glutathione-S-transferase activities. The apparent persistent cytoplasmic localization of AD 198 as a principal mechanism of AD 198 resistance is currentlty under investigation.

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