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SUSCEPTIBILITY OF IDARUBICIN, DAUNORUBICIN, AND THEIR C-13 ALCOHOL METABOLITES TO TRANSPORT-MEDIATED MULTIDRUG RESISTANCE*

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Abstract—The intracellular pharmacokinetics and cytotoxicity of idarubicin (IDA), daunorubicin (DNR), and their corresponding C-13 alcohol metabolites, idarubicinol (IDAol) and daunorubicinol (DNRol), were studied in drug-sensitive HL-60/W human leukemia cells, and in two multidrug-resistant (MDR) sublines, HL-60/Vinc (overexpress P-glycoprotein, Pgp) and HL-60/Adr (overexpress multidrug resistance-associated protein, MRP). Intracellular drug accumulation (1 μg/mL) and retention were measured by flow cytometry. Mean intracellular steady-state concentration (C_{ss}, fluorescence units/cell) and area under the intracellular drug concentration × time curve (AUC, Fl.U./cell · min) were calculated. Relative to the values for the respective drugs in HL-60/W cells, the C_{ss} and AUC of IDA were much higher than those of DNR in the MDR cell lines, with C_{ss} and AUC of IDAol intermediate between IDA and DNR. In the MDR cell lines, the MDR modulator cyclosporine A (CsA), in concentrations of 0.3 to 30 μmol/L, caused minimal effects on 3-hr IDA accumulation, intermediate enhancement of IDAol accumulation, and greatest enhancement of DNR accumulation. The MDR cell lines were much less resistant to IDA (3- to 16-fold) than to DNR (65- to 117-fold). This difference was not the result of IDA being more potent than DNR, since the sensitivity of HL-60/W cells to IDA differed from their sensitivity to DNR by <2-fold. The cellular pharmacokinetics and cytotoxicity of IDA in MDR human breast carcinoma cells MCF-7/AdrVp, which overexpress the putative MDR transporter P-95, were far superior to those of DNR, and were comparable to these parameters for IDA in parental MCF-7/W cells. These studies demonstrate that the cellular pharmacology and cytotoxicity of IDA in MDR cell lines that overexpress MRP, Pgp, or P-95 are more advantageous than those of DNR, suggesting that IDA is less susceptible to the transport-mediated MDR mechanism manifested. IDA is not completely invulnerable to MDR, however, since the MDR sublines studied did display a demonstrable level of resistance to IDA, compared with their drug-sensitive counterparts. IDAol, the major plasma metabolite of IDA, demonstrated behavior intermediate between the MDR-susceptible drug DNR and its parent compound, suggesting that its cytotoxic action is subject to transport-mediated cellular defenses. The ability of CsA to enhance the cytotoxicity of IDAol suggests that the expression of the MDR transporters studied may have an adverse influence on the overall treatment response to IDA, and that regimens containing IDA should be considered in the context of clinical trials that investigate the effects of MDR modulators such as CsA on therapeutic outcome in acute myeloid leukemia.

Key words: idarubicin; multidrug resistance; P-glycoprotein; multidrug resistance-associated protein

MDR‡ mediated by the plasma membrane transporter Pgp has come to the forefront of research efforts directed at improving survival in AML [1–4]. This is due, in part, to the discovery of Pgp expression or the demonstration of an MDR phenotype in blast cells from 20 to 30% of *de novo* AML patients, with higher levels of expression of Pgp in relapsed or refractory AML patients. Furthermore, there is the potential for the clinical use of inhibitors of Pgp, such as CsA, to aid in the eradication of Pgp-overexpressing cells and thus enhance the response to therapy and possibly the rate of cure [5, 6]. In addition to Pgp, other membrane-resident proteins such as P-95 [7, 8] and MRP [9] have been identified that may function as MDR transporters. In blast cells from a cohort of AML patients, we have detected P-95 expression in approximately 30% of the patients, and have correlated this expression with reduced accumulation and retention of DNR [10]. MRP, which has been recently cloned and sequenced, is overexpressed in two MDR sublines of the human AML cell line HL-60, HL-60/AR [11] and HL-60/Adr [12], neither of which overexpress Pgp.

Among the anticancer drugs susceptible to Pgp-mediated MDR is the anthracycline DNR, which is one of the most effective agents available for the treatment of AML. The 4-demethoxy derivative of DNR, IDA, has been shown in recent clinical trials to be at least as effective as DNR in the treatment of AML (see Ref. 13 for a review). IDA has also been reported to be more effective than DNR against HL-60/Vinc cells, an MDR subline of HL-60 that overexpresses Pgp [14], based on

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‡ Abbreviations: MDR, multidrug resistance; IDA, idarubicin; IDAol, idarubicinol; DNR, daunorubicin; DNRol, daunorubicinol; Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; C_{ss}, intracellular steady-state concentration of drug; AUC, area under the concentration · time curve; AML, acute myeloid leukemia; FBS, fetal bovine serum; Fl.U., fluorescence units; FDA, fluorescein diacetate; PI, propidium iodide; LC_{50}, concentration causing lethality to 50% of the cells; CsA, cyclosporine A; and R_F, distance traveled by the compound of interest divided by the distance traveled by the solvent front.

observations of a lack of enhancement of IDA accumulation, retention, or cytotoxicity by the MDR modulator verapamil, and a greater sensitivity of Pgp-overexpressing MDR cells to IDA than to DNR.

IDA differs from DNR because its major plasma metabolite, IDAol, is also a potent cytotoxic agent [15]. IDAol is produced in the process of clearance of IDA by a class of enzymes called aldoketoreductases, that are found in many tissues of the body [16]. Like DNRol, the major metabolite of DNR, IDAol has a long plasma half-life [17–19]; however, the cytotoxic potency of IDAol is much greater than that of DNRol [15]. Because of the long plasma half-life, the plasma AUC of IDAol is more than 2 times larger than that of IDA following an intravenous dose of IDA [17–19]. Hence, administration of IDA to a cancer patient actually results in much greater overall exposure of the tumor to IDAol than to the parent compound.

The goal of our studies was to test the hypothesis that IDA is not susceptible to transport-mediated MDR. In view of the above considerations, work aimed at testing this hypothesis must include studies of IDAol, in order to have clinical relevance. For this reason, and because previous investigations [14] did not answer the issue of potency as opposed to the selective cytotoxic advantage of IDA against MDR cells, we studied IDA, IDAol, DNR, and DNRol with respect to cytotoxicity, intracellular pharmacokinetics, and response to the MDR modulator CsA, in the Pgp-overexpressing MDR cell line HL-60/Vinc, in the MRP-overexpressing MDR cell line HL-60/Adr, and in the P-95-overexpressing cell line MCF-7/AdrVp.

MATERIALS AND METHODS

Materials

IDA, IDAol and DNRol were obtained as pure crystalline compounds from Pharmacia Adria, Dublin, OH. DNR was obtained as daunorubicin · HCl (Cerubidine) from Wyeth Laboratories, Philadelphia, PA. CsA was obtained as "Sandimmune Injectable™" from the Sandoz Corp. (East Hanover, NJ).

Cell culture

HL-60/W, HL-60/Adr and HL-60/Vinc cells were obtained and maintained in suspension culture in RPMI 1640 medium, 10% (v/v) FBS (Biofluids Inc., Rockville, MD), precisely as described previously [4]. HL-60/Vinc cells [12] are identical to the cells identified as HL-60/RV$^+$ cells used by Berman and McBride [14] (Melvin Center, personal communication, cited with permission). Cells were used in logarithmic growth phase, with cell viability routinely >95% by trypan blue dye exclusion. These cell cultures were tested regularly to assure the absence of contamination by Mycoplasma (Gen Probe, San Diego, CA).

Human breast carcinoma cells MCF-7/W and the P-95-overexpressing subline MCF-7/AdrVp were obtained from Dr. Antonio Fojo (National Cancer Institute, Medicine Branch) [7], and were cultured as monolayers in Iscove's modification of Eagle's medium with 25 μg/mL gentamicin, 2 mmol/L L-glutamine (Biofluids, Inc.), and 10% FBS (Hyclone, Logan, UT). The MCF-7/AdrVp subline was cultured continuously in 100 ng/mL doxorubicin and 5 μg/mL verapamil until 7–10 days before the experiments were performed.

Accumulation and retention studies

The anthracycline drugs of interest were added to HL-60 cell cultures containing 500,000 cells/mL or MCF-7 cells growing in 6-well flat bottom Multiwell™ tissue culture plates (Falcon 3046, Becton Dickinson Labware, Lincoln Park, NJ), at a final concentration of 1 μg/mL. For HL-60 cells, aliquots of the cell culture were removed 10, 20, 30, 60, 90, and 120 min after the addition of drug, immediately before and after washing (which was done 150 min after the addition of drug) and at 30-min intervals following washing for the determination of intracellular drug content by flow cytometry, as described below. Following removal from the cultures, the cell culture aliquots were kept on ice until the time of flow cytometric analysis. To remove extracellular drug (after 150 min of drug accumulation), HL-60 cells were washed twice (10 mL/wash) with ice-cold PBS, then resuspended in pre-warmed drug-free culture medium at a cell concentration equal to that prior to the wash. For MCF-7 cells, following exposure to drug using an exposure scheme similar to that for HL-60 cells, the cells were trypsinized (0.25% trypsin, 1 mmol/L EDTA, Gibco, Grand Island, NY) for 5 min at 37°, and then washed with ice-cold PBS immediately before the flow cytometric analysis. This trypsinization procedure was observed, with trituration, to create a single cell suspension in these breast cancer sublines, and did not alter DNR accumulation or retention in free-floating HL-60/W or HL-60/Vinc cells.

To control for binding of anthracycline to the plasma membrane or external surface of the cells, HL-60 or MCF-7 cells in culture medium were exposed to 1 μg/mL drug at 4° for 3 hr (a time found to be sufficient for steady-state membrane binding to occur at 4°), and then were analyzed for cellular fluorescence by flow cytometry. This fluorescence value was subtracted from those obtained for cells incubated with drug at 37°. For each drug tested, the degree of membrane binding was approximately equal in parental and drug-resistant cell lines. In relation to accumulation of drug in HL-60/W or MCF-7/W cells in culture, membrane binding was small, and was <10% that of the intracellular steady-state concentration of drug attained at 37°.

Intracellular IDA, IDAol, DNR, or DNRol content or binding of the drug to the plasma membranes was quantified in HL-60 cells by flow cytometry, using the same basic procedure described previously [4]. The excitation wavelength used was 488 nm. In preliminary studies of emission spectra of these anthracyclines, using 488 nm wavelength excitation, it was determined that detection of >540 nm wavelength was suitable for IDA, IDAol, DNR, or DNRol fluorescence; to accomplish this, a 540-LP filter (Omega Optical, Brattleboro, VT) was used. Logarithmic amplification of fluorescence signals was used throughout. Fluorescent beads (Propidium Iodide Alignment Microbead Standards, Flow Cytometry Standards Corp., Research Triangle, NC) were used to ensure day-to-day reproducibility of fluorescence measurements. Intracellular drug content was expressed in relative terms as Fl.U. Fl.U are numbers between 1 and 10,000, and are obtained by dividing the channel number that represents mean fluorescence for a given sample by 256 (the number of channels per log decade), and then obtaining the antilog of this value.

The AUC, in units of Fl.U./cell · min, was calculated

from intracellular accumulation and retention data using the LAGRAN software program [20], version 2.1. The C_{ss} (Fl.U./cell) was calculated by averaging the intracellular drug content during the plateau phase of drug accumulation for a given experiment. The mean C_{ss} values displayed in Table 1 were obtained by calculating the mean of the C_{ss} values obtained in each replicate experiment, as indicated in the table.

Cell survival assays

The cell survival assay used for HL-60 cells has been described previously [21]. Cells (500,000/mL) in culture were exposed to drug (and, in some cases, with and without 5 μmol/L CsA) for 4 hr, washed, and then placed in anthracycline-free medium for 72 hr. Exposure to CsA was continued in the case of cells that had been preexposed to CsA and an anthracyline. After 72 hr, the number of surviving cells was counted. Briefly, at the time of analysis, FDA (final concentration 0.5 μg/mL) was added to the cell cultures that were then incubated in the dark at room temperature for 30 min. Next PI was added to a final concentration of 50 μg/mL, and the cultures were placed on ice. The number of surviving cells/mL culture was determined by counting the number of FDA/PI viable cells (bright green fluorescence, low red fluorescence) acquired over a fixed period of time by a flow cytometer. For the surface-adherent MCF-7 breast cancer cell cytotoxicity studies, 4000 cells in 2 mL of culture medium were seeded per well of a 6-well flat bottom Multiwell™ tissue culture plate (Falcon). The cells were placed in the incubator, allowed to adhere to the plastic surface overnight, and then exposed to drug for 4 hr. Following exposure to drug, the cells were incubated for an additional 10 days; then the colonies adherent to the culture plates were washed with ice-cold PBS and stained with 1 mL crystal violet solution (1.25 g crystal violet dissolved in 450 mL methanol, 50 mL formaldehyde). The number of colonies per well was counted with an automatic colony counter (Artek model 982, Dynatech, Inc., Farmingdale, NY). This method reproducibly produces 200–500 colonies per well in control (drug-free) cultures.

TLC assay of DNR, DNRol, IDA, and IDAol

HL-60 cells (10^6/mL) in RPMI 1640 culture medium containing 10% FBS were exposed to 1 μg/mL drug for 3 hr, placed on ice, washed with ice-cold PBS, and collected by sedimentation (1000 *g*, 2 min). The cell pellet was extracted with 20 vol. of ice-cold $CHCl_3$:methanol (2:1), then subjected to silica-gel TLC using a mobile phase of chloroform:methanol:acetic acid:water (80:20:14:6) as described previously [22]. RPMI 1640 medium containing 10% FBS (alone, or the supernatant after incubation with 10^6 HL-60 cells/mL) was obtained after a 3-hr incubation with 1 μg/mL of drug. Two volumes of $CHCl_3$:isopropanol (1:1, v/v) were then added along with sufficient ammonium sulfate to saturate the aqueous layer. This mixture was vortexed, and then the phases were separated by centrifugation (31,000 *g*, 30 min, 4°). The upper (organic layer) was collected, dried under nitrogen, and then redissolved in a small volume of $CHCl_3$:methanol (2:1) for TLC analysis. Standard curves were generated by adding a concentration range of authentic drug to medium, followed by extraction and TLC as described above. Following chromatography, the TLC plates were inverted on a UV light box and photographed; then the intensity of fluorescence for each drug in the chromatogram was determined by densitometric measurements of the photographic negative. Standard curves showed a linear relationship between log drug concentration (0.01 to 1 μg/mL) and densitometric reading, with linear regression R^2 values typically >0.98. The R_F values (±SD) were: 0.65 ± 0.02 for DNR, 0.51 ± 0.01 for DNRol, 0.64 ± 0.04 for IDA, and 0.49 ± 0.02 for IDAol.

RESULTS

Intracellular metabolism and stability in culture medium of IDA, IDAol, DNR, and DNRol

The stability in culture medium and the intracellular metabolism of IDA, IDAol, DNR, and DNRol in HL-60 leukemia cells were investigated by the use of TLC. HL-60/W, HL-60/Vinc, or HL-60/Adr cells were exposed to 1 μg/mL of drug for periods up to 4 hr. This exposure time was chosen because it is sufficient for each drug to achieve C_{ss} (see below), and because it is equal to the 4-hr exposure time used in the cytotoxicity studies (see below). Each drug was stable in culture medium containing cells (10^6/mL) during this time interval. For cultures containing IDA or DNR, the accumulation of IDAol or DNRol in the culture medium over the 4-hr time period was <5% of the parent compound. In terms of intracellular drug, little metabolism of any drug occurred during a 3-hr interval of observation, with >95% of intracellular drug remaining as the parent compound. Hence, the fluorescence that we measured as representing intracellular drug (Table 1, Figs. 1 and 2) or the cytotoxicity measured in response to a particular drug (Table 2, Fig. 3) represented predominantly the intracellular concentration or lethality, respectively, of the original drug that was added to the culture, not that of any metabolite(s).

Intracellular pharmacokinetics in HL-60 cells

The kinetics of intracellular accumulation and retention of IDA, IDAol, DNR, or DNRol are presented in Fig. 1. Inspection of the curves in Fig. 1 revealed that, in general, a plateau in intracellular accumulation occurred for all drugs and cell lines tested after 90 min. For IDA accumulation in HL-60/W or HL-60/Adr, this plateau was reached after only 30 min in culture. The plateau represents the C_{ss}, which is a point at which influx and efflux of drug are equal. For IDA or DNR, we found that C_{ss} varied in proportion to the extracellular concentration of drug. We have studied the C_{ss} obtained in response to extracellular concentrations of IDA or DNR of 0.03, 0.1, 0.3, 1, and 3 μg/mL in HL-60/W, HL-60/Adr, and HL-60/Vinc cells. The log of C_{ss} was found to vary in direct proportion to the log of extracellular drug concentration with no evidence of plateau or saturability in any cell line. For IDA or DNR, linear regression analysis of log extracellular drug concentration (independent variable) versus log C_{ss} (dependent variable) revealed R^2 values >0.94 for each analysis, with no significant differences between the regression coefficients (slopes) obtained for IDA or DNR in the HL-60/W, HL-60/Vinc, or HL-60/Adr cell line by Student's *t*-test. For DNR or IDA, the antilog of the intercepts of the regression lines or the actual C_{ss} values obtained at each extracellular concentration of drug tested in this study did differ for each cell line in the proportion illustrated in Fig. 1,

Table 1. Intracellular pharmacokinetics of IDA, IDAol, DNR, and DNRol

(A) IDA and IDAol				
	IDA		IDAol	
HL-60 cell type	C_{ss}	AUC	C_{ss}	AUC
W	1,142 ± 283 (100)*	411,787 (100)	799 ± 56 (100)	338,353 (100)
Vinc (Pgp+)	681 ± 140 (60)	251,845 (61)	354 ± 43 (44)	103,196 (31)
Adr (MRP+)	1,092 ± 77 (96)	418,402 (102)	426 ± 17 (53)	112,556 (33)
(B) DNR and DNRol				
	DNR		DNRol	
HL-60 cell type	C_{ss}	AUC	C_{ss}	AUC
W	813 ± 152 (100)*	312,917 (100)	162 ± 1 (100)	44,584 (100)
Vinc (Pgp+)	80 ± 44 (15)	10,802 (4)	15 ± 7 (9)	1,862 (4)
Adr (MRP+)	382 ± 73 (47)	87,545 (28)	39 ± 5 (24)	5,393 (12)

HL-60 cells were exposed to drug as described in Materials and Methods and in the legend to Fig. 1. The intracellular steady-state concentration of drug (C_{ss}, fluorescence units/cell) was calculated from the plateau of the drug accumulation curve; the total area under the intracellular drug concentration · time curve (AUC, fluorescence units/cell · min) values were calculated using the LAGRAN software program, version 2.1 [20]. The data in this table represent the following number of replicate sets of experiments (done on different days): IDA, N = 4; IDAol, N = 3; DNR, N = 4, DNRol, N = 2. For C_{ss}, SDs are shown, except in the case of N = 2, where the range is given.

* Numbers in parentheses represent the parameter as a percentage of the value obtained for HL-60/W cells.

where an extracellular concentration of 1 μg/mL was used.

To control for possible differences in fluorescence quenching among the HL-60 cell lines as a cause of differences in intracellular drug content measured by flow cytometry, the HL-60 cell lines were exposed to IDA or DNR (1 μg/mL culture medium) for 3 hr; then the anthracycline was extracted from the cells with chloroform/methanol, as described in Materials and Methods. TLC analysis of these extracts revealed differences in the cellular accumulation of IDA or DNR in agreement with the differences observed in C_{ss} as measured by flow cytometry.

The total AUC and C_{ss} derived from the data points shown in Fig. 1 are listed in Table 1. Relative to the values for the respective drugs in HL-60/W cells, the C_{ss} and AUC of IDA were much higher in the MDR cell lines than those of IDAol, DNR, and DNRol (Fig. 1, Table 1). For MRP-overexpressing HL-60/Adr cells, the intracellular pharmacokinetics of IDA were almost identical to those of HL-60/W. For Pgp-overexpressing HL-60/Vinc cells, the AUC and C_{ss} of IDA were approximately 60% of these values for IDA in HL-60/W. IDAol clearly displayed cellular pharmacokinetic behavior intermediate between IDA and DNR. If, relative to drug-sensitive parental cells, one regards diminution of intracellular C_{ss} or AUC of a given drug in a resistant cell line as evidence of susceptibility of that drug to an MDR transporter, then in HL-60/Vinc cells, the order of susceptibility is DNRol ≥ DNR > IDAol > IDA. For HL-60/Adr cells, the order of susceptibility is DNRol > DNR = IDAol > IDA.

Cytotoxicity to HL-60 cells

The concentrations of IDA, IDAol, or DNR causing 50% lethality (LC_{50}) to these HL-60 cell lines are displayed in Table 2. Analysis of the data in the columns of Table 2 (single drug effect on the three cell lines) by the Kruskal–Wallis test revealed highly significant differences in the median LC_{50} values shown for each drug ($P \leq 0.006$). Similarly, Kruskal–Wallis analysis of the rows in Table 2 (effects of each drug on a single cell line) showed highly significant differences in the median LC_{50} values of the three anthracyclines tested against the MDR cell lines ($P = 0.001$ for HL-60/Vinc, $P = 0.018$ for HL-60/Adr), but only marginal significance for HL-60/W ($P = 0.049$). Hence, the difference in potency of IDA, IDAol, and DNR in HL-60/W cells is small and borders on statistical significance, despite the relatively large sample size in this group; numerically, the LC_{50} values for IDA and IDAol differed from DNR only by approximately 2-fold. In a previous work [23], we found the LC_{50} of DNRol against HL-60/W to be 21 times greater than that of DNR. For this reason, further studies of DNRol cytotoxicity were not pursued in the present investigations.

Scanning down the columns in Table 2, it can be seen that HL-60/Vinc and HL-60/Adr were much more resistant to DNR (65- and 117-fold) than to IDA (3- and 16-fold). IDAol also had a cytotoxic advantage compared with DNR against HL-60/Vinc cells, which were 29- and 65-fold resistant to IDAol and DNR, respectively; IDAol had no cytotoxic advantage compared with DNR against HL-60/Adr. Scanning across the columns, it can be observed that the toxicity of IDAol to HL-60/Vinc was less than that of IDA but greater than that of DNR. Despite the observation that relative to HL-60/W, the AUC and C_{ss} of IDA or IDAol in HL-60/Adr cells were greater than those in HL-60/Vinc cells (Fig. 1A, Table 1), HL-60/Adr cells were more resistant to IDA or IDAol than were HL-60/Vinc cells (Table 2).

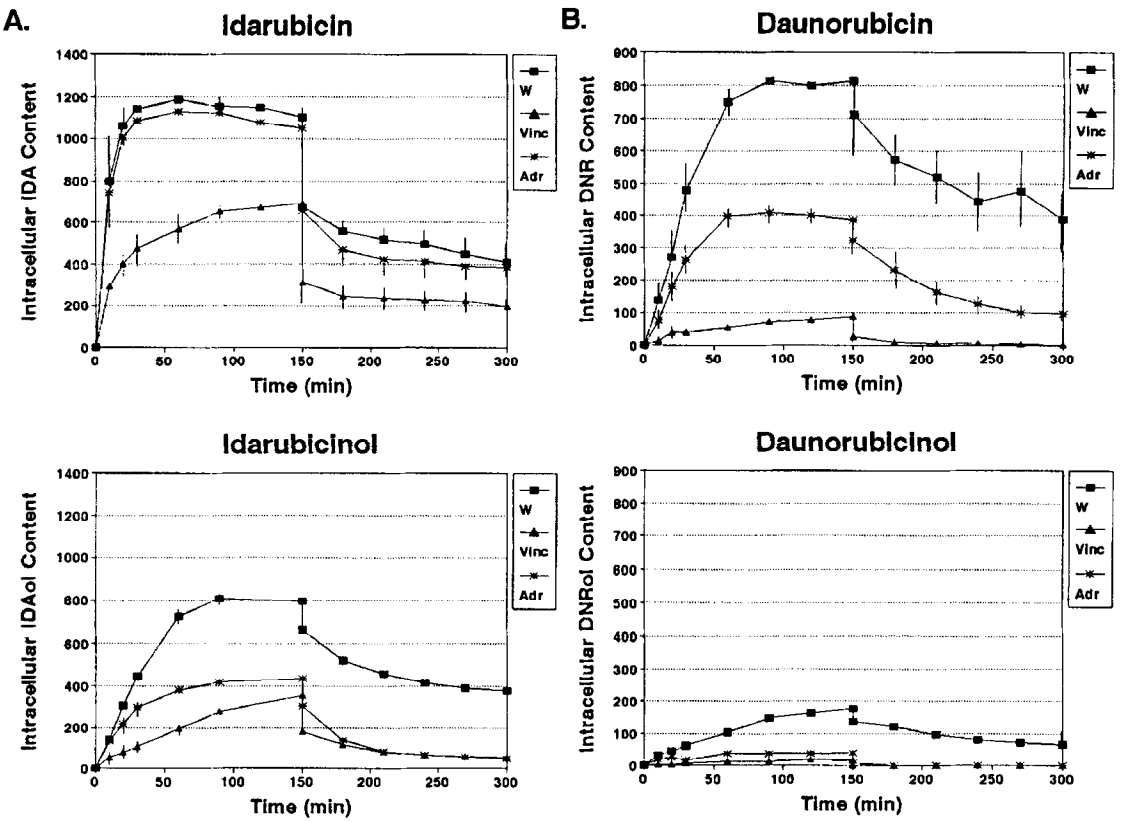

Fig. 1. Accumulation (150 min) and retention of IDA or IDAol (Fig. 1A) and DNR or DNRol (Fig. 1B) by HL-60/W, HL-60/Vinc, or HL-60/Adr cells. Cells were exposed to 1 μg/mL drug; after 150 min, cells were washed free of drug and then placed in drug-free medium. Aliquots of cells were removed at the times indicated for flow cytometric determination of intracellular drug content. The units for the ordinate are F.U. per cell, as described in Materials and Methods. The points in the figure represent the means of the following number of replicate sets of experiments (done on different days): IDA, N = 4; IDAol, N = 3; DNR, N = 4, DNRol, N = 2. The vertical lines represent SD, or in the case where N = 2, the range.

Effects of CsA on drug accumulation and cytotoxicity in HL-60 cells

CsA, given in the form of Sandimmune Injectable™, is an effective inhibitor of certain MDR transporters of DNR, including those manifested in HL-60/Vinc and HL-60/AR cells [4, 24]. Figure 2 shows the effects of CsA on the 3-hr accumulation of IDA, IDAol, and DNR in these HL-60/W, HL-60/Vinc, and HL-60/Adr cells, both in terms of intracellular drug content (Fig. 2A), and percent change (Fig. 2B—see legend to Fig. 2). CsA caused no enhancement in the accumulation of any of the drugs studied in HL-60/W cells. Similarly, CsA did not enhance IDA accumulation in HL-60/Adr cells, but did effect a small increase in IDA accumulation in HL-60/Vinc cells to bring intracellular drug to levels equal to those of HL-60/W. For IDAol and DNR, CsA concentrations of 3 μmol/L or greater enhanced drug accumulation in HL-60/Vinc or HL-60/Adr cells to levels equal to those of HL-60/W. In HL-60/Vinc cells, CsA caused the greatest enhancement in DNR accumulation, followed by IDAol and IDA accumulation (Fig. 2B).

Previous studies [4, 24] have shown that, relative to HL-60/W cells, CsA (5 μmol/L) can completely sensitize HL-60/Vinc cells and partially sensitize HL-60/AR cells to the cytotoxic effects of DNR. To determine whether the enhancement of IDAol accumulation observed translates into reversal of the resistance manifested by these MDR cells to IDAol, the effects of 5 μmol/L CsA on IDAol toxicity were studied (Fig. 3). The LC_{50} values calculated from the survival curves shown are given in the legend to Fig. 3. These LC_{50} values were within the range of values for IDAol reported in Table 2. CsA caused significant sensitization of the MDR cells to IDAol, reducing the LC_{50} by 7-fold in HL-60/Vinc cells, and by 4-fold in HL-60/Adr. CsA had no effect on the cytotoxicity of IDAol in HL-60/W cells. In analogous studies of CsA effects on IDA cytotoxicity (data not shown), no enhancement of toxicity against HL-60/W or HL-60/Adr was observed. However, CsA did cause a 3-fold reduction in the LC_{50} of IDA against HL-60/Vinc cells, which may be a reflection of the small degree of enhancement of IDA accumulation seen in response to CsA in this cell line (Fig. 2B).

Intracellular pharmacokinetics and cytotoxicity of IDA and DNR in MCF-7 cells

Recently, we reported the detection of a newly described putative MDR transporter, P-95, in blast cells from AML patients [10]. No leukemia cell line model for resistance manifested by P-95 overexpression exists. The cells in which multidrug resistance associated with P-95 expression was first described are MCF-7/AdrVp, a subline of the human breast carcinoma cell line MCF-7/W that was selected for resistance to doxorubicin in the

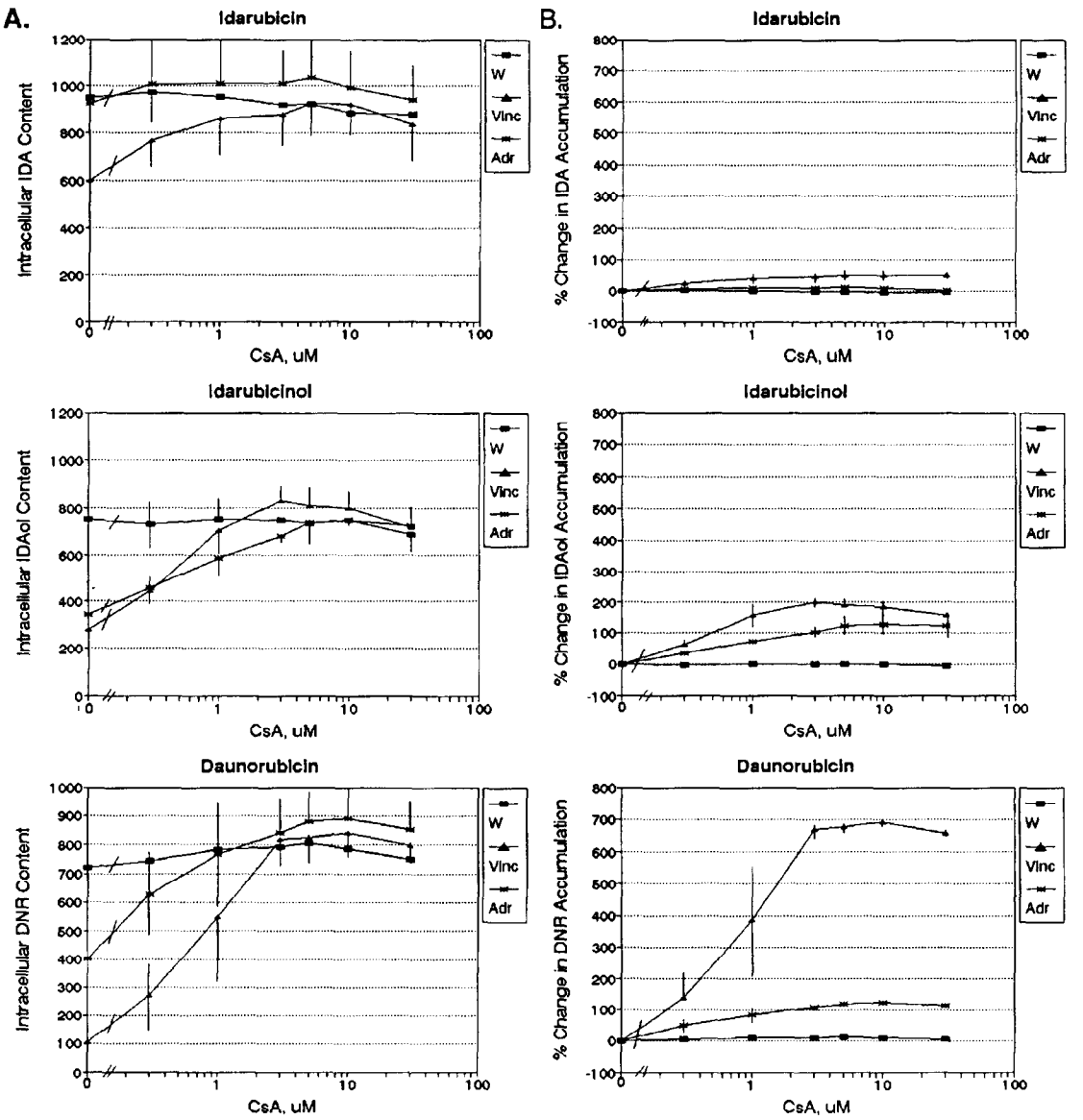

Fig. 2. Effects of various concentrations of CsA (Sandimmune Injectable™) on a 3-hr accumulation of IDA, IDAol, or DNR (1 μg/mL each) in HL-60 cells. Cells were co-incubated with anthracycline and the indicated concentration of CsA for 3 hr. Units for the ordinate are Fl.U. per cell, which are derived as described in Materials and Methods. The data shown are the means of three replicate experiments, done on different days. Vertical lines represent SD. (A) Data are expressed as intracellular drug content. (B) Data are expressed as percent change. Percent change was calculated as follows:

$$\% \text{ Change} = 10 \cdot (A_{CsA} - A)/A$$

where A_{CsA} = a 3-hr intracellular accumulation of drug (IDA, IDAol, or DNR) in the presence of CsA, and A = accumulation of drug in the absence of CsA.

presence of verapamil [7]. We find that MCF-7/AdrVp cells display a reduction in the intracellular accumulation and retention of DNR [10], with AUC and C_{ss} of DNR in the MCF-7/AdrVp cells being 17 and 22%, respectively, of these values in the drug-sensitive MCF-7/W cells. To test whether IDA is also a substrate for the transporter manifested in these cells, we studied the accumulation and retention of IDA in MCF-7/AdrVp and MCF-7/W cells, respectively. The kinetics of IDA accumulation and retention were identical in both cell lines (Fig. 4). These differences in DNR and IDA intracellular pharmacokinetics translated directly in terms of the cytotoxicity of these agents, where, in comparison to parental MCF-7 cells, MCF-7/AdrVp cells displayed approximately 30-fold resistance to DNR, but were minimally resistant to IDA (Fig. 5, see legend for LC_{50} values). For MCF-7/W cells, the potencies of DNR and IDA were similar (LC_{50} values were 60 and 40 nmol/L, for DNR and IDA, respectively).

DISCUSSION

The work presented here offers multiple lines of evidence that IDA is more effective than DNR against transport-mediated MDR. First, in terms of cytotoxicity, the MDR cell lines tested were more sensitive to IDA

Table 2. Cytotoxic effects of IDA, IDAol, and DNR on HL-60 cells

	IDA		IDAol		DNR	
Cells	LC_{50} (nmol/L)	Fold-resistant	LC_{50} (nmol/L)	Fold-resistant	LC_{50} (nmol/L)	Fold-resistant
W	18 (5–55) N = 11	1	14 (7–69) N = 8	1	35 (16–68) N = 7	1
Vinc (Pgp+)	62 (32–200) N = 7	3	410 (210–520) N = 5	29	2,300 (1200–3800) N = 5	65
Adr (MRP+)	280 (170–580) N = 5	16	1950 (1200–3900) N = 4	139	4100 (3100–5100) N = 2	117

Cells were exposed to drug for 4 hr, and then were washed free of drug and returned to culture in drug-free medium. Increments in concentration of 1x, 3x and 10x were used over a 4 log range encompassing the LC_{50} value. After 72 hr, cell survival was determined by counting the number of FDA/PI viable cells by flow cytometry, as described in Materials and Methods. The concentrations causing 50% lethality (LC_{50}) were then calculated. See Fig. 3 for typical cytotoxicity curves. The LC_{50} values in this table represent the median of LC_{50} values obtained from replicate experiments done on different days. N = the number of replicate experiments performed; the number in parentheses below the median LC_{50} value is the range.

than to DNR. This effect was not due simply to IDA being more potent than DNR, since HL-60/W cells and MCF-7/W cells differed only slightly in their sensitivity to IDA compared with DNR. Furthermore, IDA had more advantageous pharmacokinetics in the MDR cell lines than did DNR, relative to HL-60/W or MCF-7/W cells. Finally, CsA exerted much less of an enhancing effect on the accumulation and cytotoxicity of IDA than it did in this regard for DNR. IDAol, on the other hand, displayed more vulnerability to MDR than IDA. Both MDR HL-60 cell lines exhibited greater resistance to IDAol than to IDA, and CsA caused greater enhancement of the accumulation, retention, and cytotoxicity of IDAol than was observed for IDA. Both IDA and IDAol caused significant cytotoxicity to Pgp-overexpressing HL-60/Vinc cells at exposures (concentration · time) that are obtainable with clinical dosing [18, 25]; In contrast, exposures of DNR and DNRol that are clinically achievable would be considerably less effective against these MDR cells [23, 26].

Other reports of IDA evading Pgp-associated MDR are beginning to emerge; ours is the first to report in detail the effects of IDA in MRP- or P-95-overexpressing cell lines. IDA was observed recently to have superior intracellular pharmacokinetics, cytotoxicity, and inhibition of DNA synthesis in Pgp-expressing MDR cell lines [27–29] or in mice bearing MDR P388 tumors [30] than DNR or doxorubicin. A number of studies of blast cells obtained from AML patients also report superior intracellular pharmacokinetics and/or less enhancement of intracellular accumulation or cytotoxicity by verapamil for IDA compared with DNR [29, 31, 32]. Müller *et al.* [32] measured the cytotoxicity of IDA, vincristine, or doxorubicin in combination with verapamil in blast cells from thirty-seven AML patients. Specimens that overexpressed Pgp demonstrated verapamil-induced chemosensitization to vincristine or doxorubicin, but not to IDA. Scheulen *et al.* [33] reported the intracellular pharmacokinetics of IDA and IDAol in blast cells from AML patients, and found the AUC of IDAol but not IDA to be lower in Pgp-overexpressing specimens compared with those that do not overexpress Pgp, leading them to conclude that unlike IDA, IDAol is an "MDR-dependent" anthracycline. Michieli *et al.* [34] also noted that IDA was more effective than DNR against Pgp-overexpressing CEM cells; the "residual" resistance of these MDR cells to IDA could be overcome by MDR modulators. In contrast, some investigators do not report superiority of IDA compared with doxorubicin against MDR cells. Lacayo *et al.* [35] studied MDR K562 cells, human sarcoma MES-SA and *mdr*1-transfected NIH-3T3 cells, and found that IDA was much more potent than doxorubicin (10- to 20-fold) against the corresponding drug-sensitive parental or non-transfected cells. Moreover, the MDR cells demonstrated considerable resistance to IDA (70- to 180-fold), with the resistance modulated by known inhibitors of MDR such as CsA or its analogue, PSC 833. List *et al.* [36] found IDA to be more potent than DNR against Pgp-overexpressing MDR K562 cells, with enhancement of IDA cytotoxicity by CsA. Furthermore, they found that IDA exhibits concentration-dependent inhibition of the binding of [^{3}H]azidopine to Pgp in a photoaffinity-labeling assay, suggesting that IDA is a substrate for Pgp. Interestingly, DNR binds only weakly to Pgp in this photoaffinity-labeling system [36].

In the studies depicted in Fig. 1, the cells were washed extensively at 4° (to prevent active efflux of drug during the wash), and then were returned to culture in drug-free medium. Intracellular drug content measurements were made immediately before and after this wash, and these values are displayed in Fig. 1 by the two data points shown for each cell line at 150 min. For all drugs studied, there was a fall in intracellular drug content immediately after the wash; for IDA in all cell lines tested, this decrement was marked compared with that of the other drugs tested, and amounted to a fall of intracellular drug content to approximately 50% of the measured C_{ss} (Fig. 1A). This suggests that, in contrast to the other anthracyclines tested, a major component of the mechanism by which IDA is conveyed into and out of the cell may involve non-active transport processes. This may be related to the greater lipophilicity of IDA compared with the other anthracyclines tested. One possible cause of non-active accumulation or efflux of an anthracycline is diffusion into or out of cells that have lost membrane integrity. In these studies, the cells used were >95% viable by trypan blue dye exclusion; no change in trypan blue exclusion was observed after a 150-min exposure to drug. Furthermore, our preliminary work with HL-60 cells and trypan blue dye exclusion enables us to distinguish live from dead cells by means of gating forward and side scatter of laser light, and thus to ignore dead cells in the analysis of intracellular drug content.

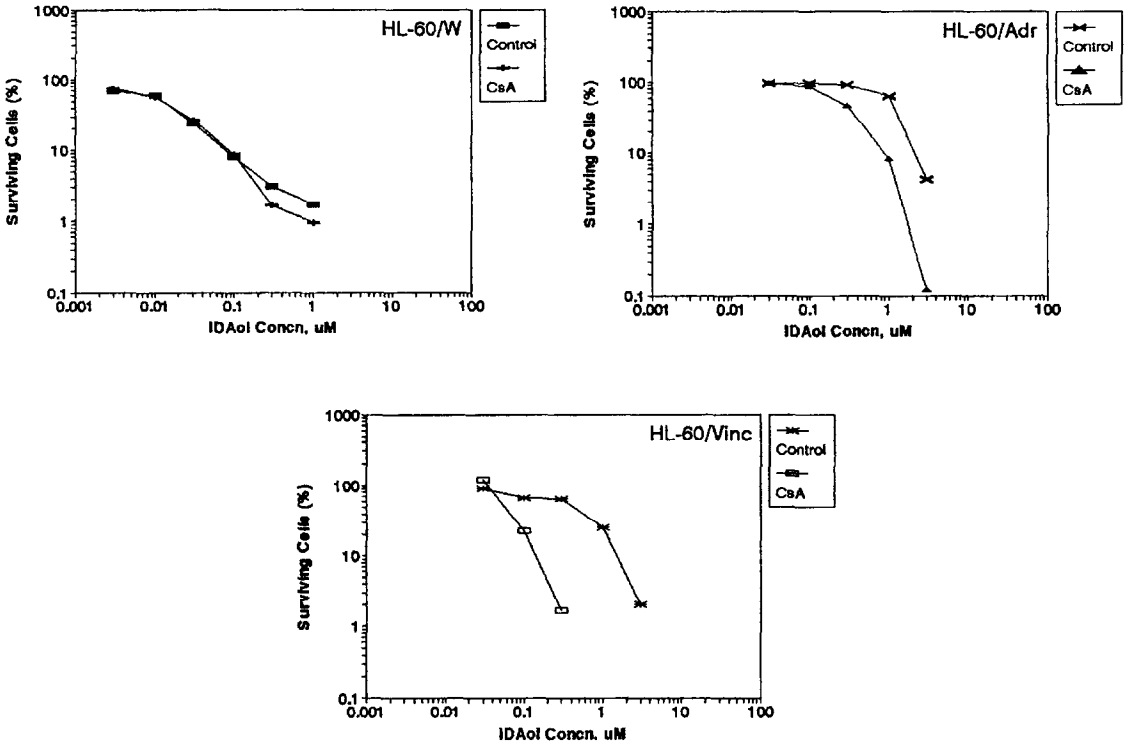

Fig. 3. Effects of 5 μmol/L CsA on the cytotoxicity of IDAol to HL-60/W, HL-60/Vinc or HL-60/Adr cells. Cells (500,000/mL cell culture) were exposed to the indicated concentrations of IDAol for 4 hr with and without CsA, and then placed in IDAol-free culture medium. Exposure to CsA was continued in the case of cells that had been exposed to CsA prior to washing. After 72 hr in culture, the number of surviving cells was determined by flow cytometry, as described in Materials and Methods. For each point shown, the standard error was less than 10% of the mean value. The LC_{50} values (nmol/L) calculated from these data for control and CsA-treated cultures, respectively, were as follows: HL-60/W, 13 and 13; HL-60/Vinc, 430 and 70; and HL-60/Adr, 1200 and 290.

The appearance of alcohol metabolites in the plasma following the administration of an anthracycline is the result of metabolism of the drug by aldoketo (carbonyl) reductases, which are found in many tissues of the intact organism [16]. High activity of carbonyl reductase capable of converting IDA to IDAol has been detected recently in human liver and brain [37]. In contrast, our studies indicated that the intracellular conversion of IDA to IDAol or DNR or DNRol was minimal in HL-60 human leukemia cells during the 3-hr period of observation. This finding is in agreement with studies of the expression of cloned human carbonyl reductase in human K562 leukemia cells by Forrest *et al.* [38], who observed only 2% conversion of DNR to DNRol in control (non-transfected) cells. The low degree of metabolism of IDA, IDAol, DNR, or DNRol in the culture medium or by the cells themselves is fortuitous for our studies, since the effects we observed (intracellular drug fluorescence and cytotoxicity) can thus be attributed primarily to those of the anthracycline or alcohol metabolite originally added to the culture.

Relative to HL-60/W cells, the cytotoxic advantage of IDA or IDAol compared with DNR against the MDR HL-60 sublines was most apparent in Pgp-overexpressing HL-60/Vinc cells. The degree of enhancement of IDA or DNR accumulation produced by CsA in HL-60/Vinc cells that we observed is in agreement with that seen in response to verapamil in this cell line as reported by Berman and McBride [14]. In contrast to the report of Berman and McBride, we did detect a relatively small but definite enhancing effect of CsA on IDA cytotoxicity in HL-60/Vinc cells in terms of a 3-fold reduction in the LC_{50} of IDA in the presence of CsA. The lack of enhancement of IDA cytotoxicity by verapamil observed by Berman and McBride may have been a result of the IDA concentration used (1 μg/mL or 2000 nmol/L), which caused a >95% decrease in colonies in the absence of verapamil, and was considerably greater than the LC_{50} that we observed for this line (62 nmol/L—see Table 2). In these Pgp-overexpressing HL-60/Vinc cells, IDAol was clearly more susceptible to the MDR mechanism operative than IDA, with greater resistance to IDAol manifested, and greater enhancement by CsA of IDAol accumulation and cytotoxicity.

MRP-overexpressing HL-60/Adr cells had a C_{ss} and AUC of IDA equal to those of IDA in HL-60/W cells, and clearly greater than those of IDA in HL-60/Vinc cells. Despite this, the MRP-overexpressing cells were more resistant to IDA than were HL-60/Vinc cells. A possible reason for this may be the cytoplasmic sequestration of drug reported for MRP-overexpressing cells [9, 39], which may prevent the drug from reaching presumed targets in the cell nucleus. Future studies of MDR cells exposed to IDA with fluorescence and/or confocal microscopy may be helpful. The MRP-overexpressing HL-60/Adr cells also manifested much greater resistance to IDAol than did HL-60/Vinc cells. Both lines (Adr and Vinc) were found to be >60-fold resistant to DNR. The effects of CsA on DNR accumulation in HL-60/Adr cells were similar to those we observed previously for another

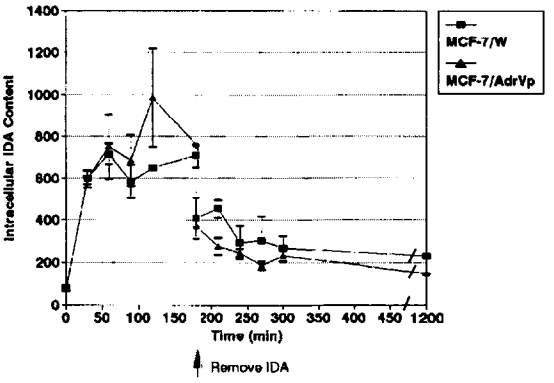

Fig. 4. Accumulation (3 hr) and retention of IDA by MCF-7/W or P-95-overexpressing multidrug resistant MCF-7/AdrVp cells. Cells were exposed to 1 μg/mL IDA, after which they were washed free of drug, and then placed in drug-free medium. Aliquots were removed, at the times indicated in the figure, for flow cytometric determination of intracellular IDA content. The units for the ordinate are Fl.U per cell, which were derived as described in Materials and Methods. The points in the figure represent the mean of two replicate sets of experiments, performed on different days. The vertical lines represent the range. The value for cellular IDA content given at time zero is that for cells exposed to IDA for 3 hr at 4°.

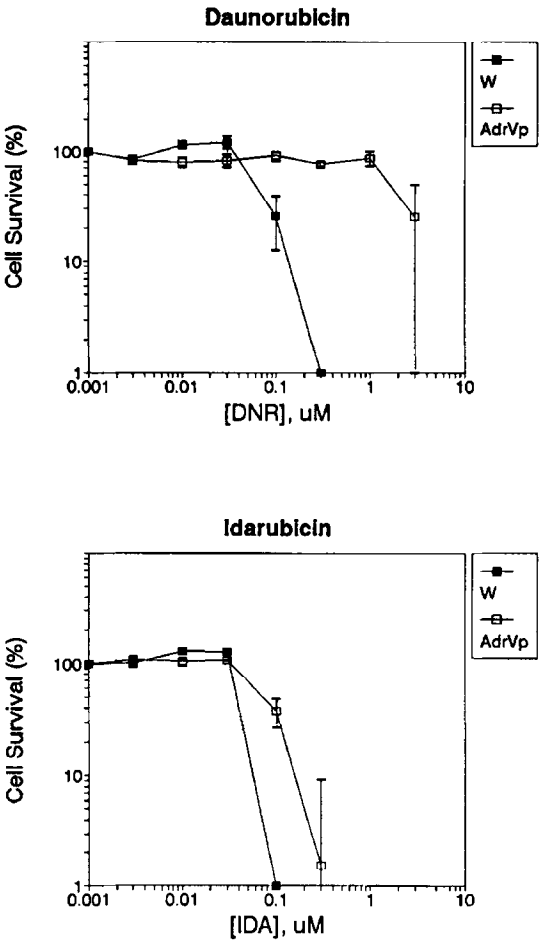

Fig. 5. Sensitivity of human breast carcinoma cells MCF-7/W and multidrug resistant MCF-7/AdrVp cells to DNR or IDA. Cells were exposed to the indicated concentrations of drug for 4 hr; then clonogenic survival was determined as described in Materials and Methods. Each point is the mean ± SEM of triplicate determinations. The mean number of colonies per well (±SEM) in control cultures (no drug exposure) was: 227 ± 9 for MCF-7/W, and 289 ± 5 for MCF-7/AdrVp cells. The LC_{50} values (nmol/L) calculated from these data for MCF-7/W and MCF-7/AdrVp, respectively, were as follows: DNR, 60 and 1700; IDA, 40 and 70.

MRP-overexpressing subline of HL-60/W, HL-60/AR cells [24].

P-95-overexpressing MDR MCF-7/AdrVp cells have reduced intracellular accumulation of DNR, but not IDA. Hence, the resistance of these cells to DNR appears to correlate with the ability of the cells to facilitate its efflux. Using western blots, we have detected P-95 expression in 30% of bone marrow specimens from a cohort of patients with AML, and have correlated P-95 expression with diminished accumulation, retention, and cytotoxicity of DNR in the marrow specimen [10]. If ongoing cloning and transfection studies demonstrate that P-95 is a cause of transport-mediated drug resistance, then IDA may be a very useful drug for tumors manifesting this form of MDR.

CsA enhanced the accumulation and cytotoxicity of both IDA and IDAol in HL-60/Vinc (Pgp-overexpressing) cells, although the greatest enhancement was observed for IDAol. IDAol (but not IDA) accumulation and cytotoxicity were also enhanced measurably in the MRP-overexpressing HL-60/Adr cells. Hence, the observed effects of CsA on IDAol and IDA accumulation and cytotoxicity suggest that clinical trials with MDR modulators such as CsA in combination with IDA are reasonable.

One powerful method to gain insights into mechanisms of cellular resistance to a particular drug is to develop cell lines that are resistant to that drug. Presently, no cell lines resistant to IDA have been reported. Work in this area is ongoing in our laboratory. To date we have produced, by stepwise exposure of HL-60/W to IDA, cells that are approximately 10-fold resistant to IDA; these cells do not manifest a defect in IDA or DNR accumulation and do not overexpress Pgp, MRP or P-95 [40]. The cells are cross-resistant to DNR and etoposide, but sensitive to paclitaxel and vincristine. These data suggest that at this point in the selective process, the pressure exerted by IDA does not yet muster transport-mediated defenses, and further strengthens our conclusions from the data presented in this paper that IDA is less vulnerable to transport-mediated MDR.

In consideration of the data presented here, and of the present literature, we conclude that despite the superiority of IDA compared with DNR demonstrated against the MDR cell lines studied, IDA itself and, to a greater extent its major metabolite IDAol, are measurably susceptible to the various MDR transporters studied. Hence, the influence of the expression of Pgp, MRP, or P-95 should be considered in clinical studies that relate IDA treatment with clinical outcome. Furthermore, regimens containing IDA should be considered in the context of clinical trials that investigate the effects of Pgp modulators such as CsA on therapeutic outcome in AML.

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