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Idarubicin overcomes *P*-glycoprotein-related multidrug resistance: comparison with doxorubicin and daunorubicin in human multiple myeloma cell lines

Dick J. Roovers [a], Marja van Vliet [a], Andries C. Bloem [b], Henk M. Lokhorst [a,*]

[a] *Department of Hematology (G03.647), University Hospital Utrecht, PO Box 85500, 3508 GA Utrecht, Netherlands*
[b] *Department of Immunology, University Hospital Utrecht, PO Box 85500, 3508 GA Utrecht, Netherlands*

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Abstract

The clinical utility of anthracyclines like doxorubicin (DOX) and daunorubicin (DNR) for treatment of multiple myeloma (MM) is limited by the occurrence of multidrug resistance (MDR). Highly lipophilic anthracyclines like idarubicin (IDA) might circumvent MDR and thereby enhance chemotherapeutic efficacy. To determine the efficacy of IDA in myeloma cells, the pharmacokinetics and cytotoxicity of IDA and its major metabolite idarubicinol (IDAol) were compared with those of DNR, DOX, and doxorubicinol (DOXol) in the cell line RPMI 8226-S and two MDR sublines (8226-R7 and 8226-Dox40) that overexpress the drug transporter *P*-glycoprotein (*P*gp). Cytotoxicity assays using MTT (viability) or annexin V (apoptosis) showed a 10–50-fold higher potency of IDA compared with DNR or DOX in the MDR variant cell lines. The difference in cytotoxicity was lower in the sensitive parental cell line (3-fold). These results are explained by a better intracellular uptake of IDA compared to DNR in resistant 8226 cell lines. The *P*gp-inhibitor verapamil affected IDA uptake only in the most resistant cell line 8226-Dox40. This indicates that IDA is less sensitive than DNR to transport-mediated MDR. IDAol was at least 32-fold more cytotoxic than DOXol, and more susceptible to *P*gp transport than IDA. These studies demonstrate that the efficacy of IDA in MDR MM cell lines is superior to that of DOX or DNR, and that IDA may become an important drug in the treatment of MM, especially in refractory disease.

Keywords: Idarubicin; Multidrug resistance; *P*-glycoprotein; Apoptosis; Multiple myeloma

1. Introduction

Anthracyclines (e.g. doxorubicin (DOX) and daunorubicin (DNR)) are important antineoplastic drugs for the treatment of hematologic malignancies. In the treatment of MM, DOX is active as a single agent [1] and is now mainly used in a combination chemotherapy regimen including vincristine and dexamethasone (VAD) [2]. However, their clinical utility is limited due to the appearance of cell populations resistant to multidrug-based chemotherapy.

Drug resistance to anthracyclines may be partly attributed to the action of *P*-glycoprotein (*P*gp), an active plasma membrane drug efflux pump, which decreases the intracellular accumulation of cytotoxic drugs. Several studies have implicated *P*gp in multidrug resistance (MDR) in MM [3–5]. In addition, other MDR-related proteins may be involved in refractory MM, like the transporter proteins LRP (lung resistance protein) and MRP (MDR-related protein) [6–8] or the apoptosis-inhibiting protein Bcl-2 [9,10].

Current strategies to reverse MDR are based on (1) chemosensitization of resistant cells by MDR-reversing agents like verapamil or cyclosporin A; and (2) the development of non-cross-resistant analogues of cytotoxic drugs. An example of the second approach is the use of the DNR analogue idarubicin (IDA; 4-demethoxydaunorubicin). IDA is a highly lipophilic anthracycline drug that has been shown to be more potent than DNR in MDR human leukemic cell lines that overexpress *P*gp [11–13] and in leukemia patients

* Corresponding author. Tel.: +31-30-2507230; fax: +31-30-2511893.
E-mail address: h.lokhokst@digd.azu.nl (H.M. Lokhorst)

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[14]. In addition, its major circulating metabolite, idarubicinol (IDAol) is described to have a relatively high cytotoxicity, compared with doxorubicinol (DOXol) or daunorubicinol, the metabolites of DOX and DNR, and a long plasma half-life in vivo.

In the present study, the anthracyclines DOX, DNR, and IDA, and two metabolites, IDAol and DOXol, were analyzed for their ability to induce cytotoxicity and their pharmacokinetics in vitro in sensitive- and drugresistant RPMI 8226 myeloma cell lines. Our data establish that the kinetics of influx of IDA differs from that of DNR or DOX, which is reflected in their cytotoxicity.

2. Materials and methods

2.1. Cell culture

The myeloma RPMI 8226 parental cell line [15] (RPMI 8226-S) was obtained from the American Type Culture Collection (Rockville, MD). The DOX-resistant cell line RPMI 8226-Dox40 was a kind gift of Dr Dalton. The RPMI 8226 subline R7 was selected at our laboratory by single dose exposure of the parental line RPMI 8226 S with 32 μM DNR for 24 h. After ten months in culture, no change in its phenotype was observed. The cell lines were cultured in suspension in a CO_2 incubator at 37°C in RPMI 1640 medium (Gibco, Grand Island, NY) with the usual supplements, including 10% fetal calf serum. MDR sublines RPMI 8226 R7 and RPMI 8226 Dox40 were cultured in the presence of 16 and 400 nM DNR, respectively, once every 2 weeks. Cells were maintained in drug-free medium for 2 weeks prior to drug sensitivity assays or drug accumulation studies. The cell cultures were free of Mycoplasma contamination (Gen Probe, San Diego, CA). Viability of the cells was controlled by trypan blue dye exclusion.

2.2. Drugs and chemicals

Purified DOX, IDA, IDAol, and DOXol were provided by Pharmacia and UpJohn. Daunorubicin (DNR) was obtained as daunorubicin.HCl (Cerubine) from Rhône-Poulenc Pharma, Amstelveen, the Netherlands. Concentrations of the anthracyclines were determined by diluting stock solutions in distilled water to 10^{-5} M and using $\epsilon 480 = 11\,500\ M^{-1}\ cm^{-1}$. Stocks were stored at −20°C. Verapamil was purchased from Sigma Chemical Company (St. Louis, MO).

2.3. Expression of MDR-related proteins

For determination of *P*gp expression at the plasma membrane by flow cytometry, 4×10^5 cells were incubated with 10 μl of 50 μg/ml mouse MoAb MRK16 (IgG2a), for 60 min at RT. Cells were washed with 1 × PBS, 10% FCS, 0.02% NaN_3 (PBS-FCSa) and then incubated for 15 min at RT with 0.5 μg of GAM IgG2a-FITC (SBA, Birmingham, AL). Expression of intracellular LRP, MRP, and Bcl-2 in RPMI 8226 cells was determined as described earlier [16]. Mouse monoclonal antibodies (MoAbs) MRPm6 (IgG1) or LRP56 (IgG2b) were a kind gift of Dr R. Scheper, Free University Amsterdam. MoAb 124 (Dako, Glostrup, Denmark) was used for detection of Bcl-2. After staining, the cells were washed and resuspended in 250 μl of PBS-FCSa for analysis by flow cytometry.

2.4. Rh123 efflux assay

A flow cytometric assay that detects efflux of the fluorescent dye rhodamine 123 (Rh123) (Molecular Probes, Eugene, OR) was used for the analysis of *P*gp function. Cells were washed, resuspended in culture medium (8×10^5 cells/ml) containing 125 nM Rh123 and incubated at 37°C for 10 min. The cells were washed twice, resuspended in culture medium and placed in 24-well plates (0.5 ml/well) in a CO_2-incubator. After incubation for 20 min, cells were pelleted, washed, and resuspended in 500 μl of PBS-FCSa and analyzed by flow cytometry. Verapamil (50 μM) was used in control incubations as an inhibitor of *P*gp function.

2.5. Cytotoxicity of anthracyclines

Drug sensitivity of the RPMI 8226 cell lines was determined in the absence or presence of anthracyclines and 50 μM of the *P*gp modulator verapamil. The anthracyclines and verapamil were diluted in RPMI-1640 medium prior to usage. To quantitate the number of viable cells, MTT assays were performed by the method of Hansen et al. [20]. Additionally, cell viability was assayed by trypan blue exclusion.

2.6. Measurement of apoptosis

Induction of apoptosis was analyzed by incubating the cells in a 6-well plate (Nunc, Roskilde, Denmark) for 72 h with the chosen concentrations of drugs. Cells were washed once with culture medium and resuspended in 250 μl culture medium containing 150 ng of FITC-labeled annexin V (NeXins Research Hoeven, the Netherlands), a protein that binds to apoptotic cells [21,22]. Incubation for 15 min on ice was directly followed by flow cytometric analysis of the cells with a FACScan.

2.7. Cellular accumulation/retention studies

Intracellular accumulation of anthracyclines was measured by flow cytometry as described before [17,18]. The uptake of anthracyclines in cells was followed by monitoring the increase of cellular fluorescence. Intracellular IDA, DOX, DNR, IDAol, and DOXol content was quantitated by flow cytometry using a FACScan flow cytometer (Becton Dickinson) with excitation at 488 nm. The anthracycline drugs of interest were added to MM cell line cultures containing 10^6 cells/ml in 6-well flat bottom tissue culture plates (Nunc, Roskilde, Denmark) at a final concentration of 1.0 μM. Cell aliquots were removed from the cultures at 15, 30, 45, 60, 90, 120, and 150 min after the addition of drug and kept on ice until analysis. The intracellular steady-state drug concentration (C_{ss}, fluorescence units/cell) was calculated by averaging the intracellular drug content during the plateau phase of drug accumulation for a given experiment. The AUC (area under the curve, fluorescence units/cell × min) values were calculated by estimating the total surface of the accumulation curves.

Detection of anthracycline fluorescence was performed through a 540 nm long pass interference filter. Intracellular drug content was expressed in relative fluorescence units (mean fluorescence channel number divided by 1024). Fluorescent beads (Dako) were used to ensure day-to-day reproducibility of fluorescence measurements.

Drug retention studies were performed in separate experiments. Cells were incubated for 150 min with 1.0 μM of anthracycline, washed twice with ice-cold PBS (10 ml wash) to remove extracellular drug and resuspended in pre-warmed drug-free medium at the same cell concentration. Flow cytometric analysis was performed at specified time points; immediately after washing and at 15, 30, 45, 60, 90, and 120 min following washing and reincubation in drugfree medium.

To control for binding of anthracycline to the outside of the plasma membrane of the cells, the cells were exposed to 1.0 μM drug at 4°C for 3 h, and then analyzed as before. This is sufficient for steady-state membrane binding to occur at 4°C [19].

3. Results

3.1. Analysis of MDR-related proteins in the RPMI 8226 cell lines

Both MDR 8226 variants R7 and Dox40 express *P*gp present on the cell surface as analyzed by flow cytometry, whereas their parental cell line 8226-S does not (Fig. 1a). Levels of *P*gp on 8226-R7 are two to four times higher than those seen on plasma cells in MM patients (Roovers et al., unpublished observations), while the levels of *P*gp on 8226-Dox40 are much higher than on 8226-R7. The *P*gp expression on these cell lines correlates with the efflux of Rh123, which indicates that their *P*gp is functional as transporter protein (Fig. 1b). The two MDR 8226 variants used here do not differ from the parental cell line 8226-S in their expression of other MDR-related proteins, like LRP, MRP, or Bcl-2 (Fig. 1c).

3.2. Cytotoxicity of IDA, DOX, DNR, IDAol and DOXol

Cytotoxicity of these anthracycline drugs in the three RPMI 8226 cell lines was determined by quantitation of living and apoptotic cells using the MTT assay and the annexin V flow cytometric assay, respectively. Both tests gave comparable results when differences in cytotoxicity between drugs are considered. For comparison, annexin V- and MTT dose-response curves for RPMI-8226-S treated with IDA, DNR, and DOX are depicted in Fig. 2, showing that the annexin V assay is about a factor 16 more sensitive than the MTT assay.

The concentrations of drug causing a 50% decrease in viability as determined with the MTT assay (ID50, 50% MTT reaction inhibiting dose) for these cell lines was calculated from the dose-response curves in Fig. 3 and are displayed in Table 1. From these results, it can be inferred that the MDR variants 8226-R7 and 8226-Dox40 are more resistant to DNR (6- and 46-fold, respectively) and DOX (14- and 28-fold, respectively) than the parental cell line. IDA was more effective in inducing cytotoxicity than DOX and DNR in both the 8226 parental and MDR variant cell lines. The difference in efficacy is most evident in the most resistant cell line 8226-Dox40. The IDA-metabolite IDAol had at least a factor 32 higher cytotoxicity than comparable concentations of DOXol, whereas its cytotoxicity was even higher (2–10-fold) than DOX.

The effects of verapamil on cytotoxicity are shown in the right half of Fig. 3. Verapamil itself had a slight toxic effect on 8226-S. Cytotoxicity of DNR/DOX in the MDR cell lines was clearly enhanced by verapamil, while cytotoxicity of IDA was only slightly affected. In general, verapamil increased the cytotoxicity of the different drugs in the MDR cell lines to the level seen in 8226-S.

3.3. Accumulation of IDA and DNR

Fig. 4 shows IDA and DNR uptake in the 8226 cell lines when exposed at a concentration of 1.0 μM of anthracycline. IDA and DNR-related fluorescence was linear in relation to concentration for the range of concentrations used here (16 nM–2.0 μM, results not shown). RPMI 8226-S showed prompt DNR uptake, while incubation of 8226-R7 resulted in clearly lower

DNR accumulation and 8226-Dox40 showed minimal DNR uptake. IDA accumulates much faster than DNR in both the sensitive and MDR 8226 variants. The IDA accumulation of the MDR subline 8226-R7 was about 60% of that of the drug-sensitive parental cell line. In the highly resistant variant 8226-Dox40 a clearly lower IDA accumulation was found, compared with 8226-S and 8226-R7. Accumulation of IDAol showed a behavior intermediate between IDA and DOX (shown in Fig. 5). Transfer of cells to drugfree medium (at the 150 min timepoint) resulted in different retention curves for DNR and IDA; DNR showed a slow decrease in fluorescence, while for IDA the fall in fluorescence was more markedly.

Our data show that the kinetics of uptake vary for the drugs tested here. The rate of IDA uptake was about seven times that of DNR (calculated by dividing the change in fluorescent signal by elapsed time in the first 30 min). For all RPMI 8226 cell lines the steady state level (C_{ss}) was reached sooner for IDA (60–90 min) than for DNR and IDAol (90–150 min). This results in much higher AUC values for IDA (see Table 2).

From Table 2 we can infer that the cellular accumulation of IDA in MDR cells is relatively higher than for DNR and IDAol. When expressed in resistance factor (the drug uptake by the 8226-R7 cell line divided by uptake of drug by the sensitive 8226-S cells), it was 0.21 for DNR, 0.34 for IDAol and 0.51 for IDA in 8226-R7.

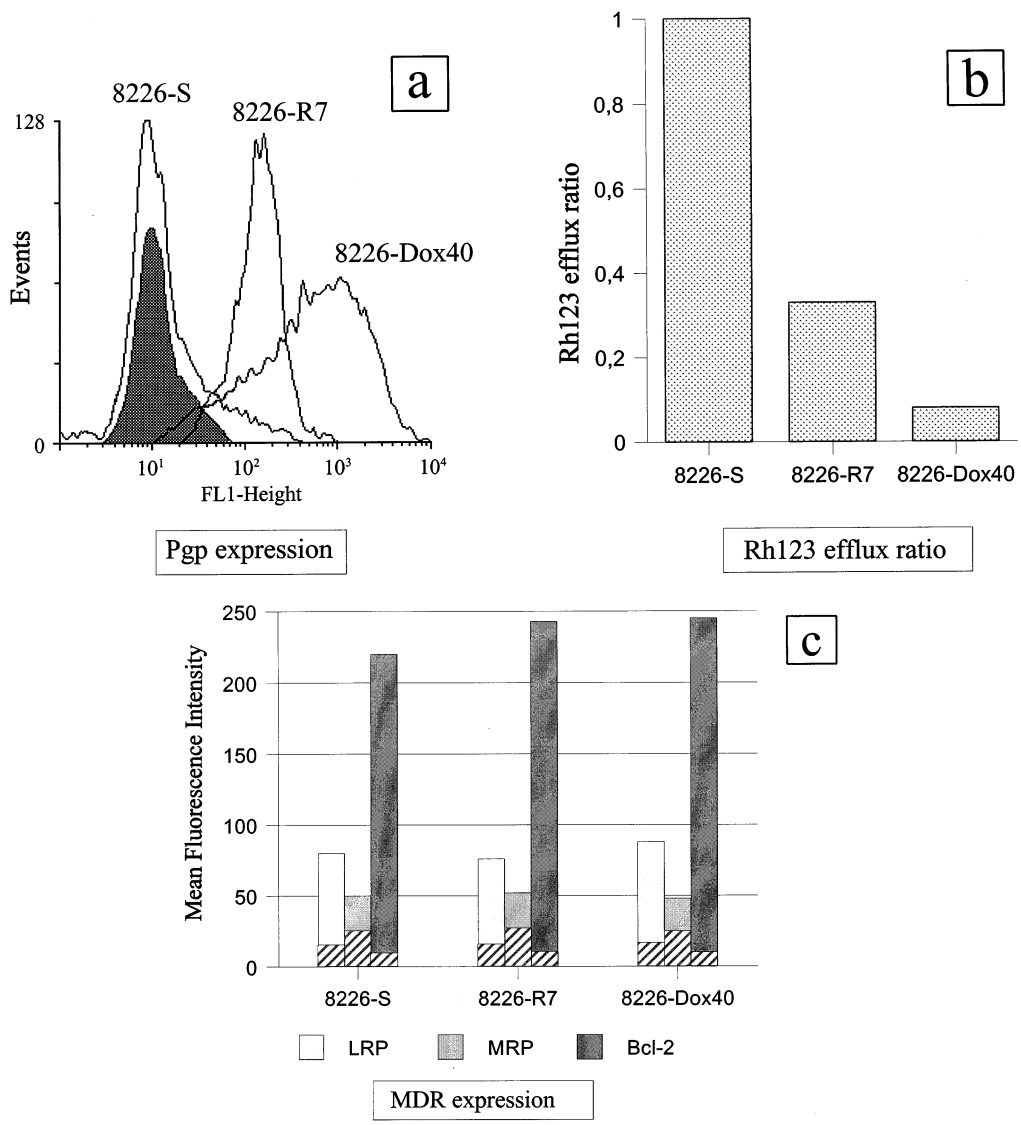

Fig. 1. (a)*P*gp staining of sensitive (8226-S) and DOX/DNR-resistant (8226-R7 and 8226-Dox40) MM cell lines with MoAb MRK16. Isotype-matched control for 8226-S (overlapping with controls for 8226-R7 and 8226-Dox40) is depicted as a shaded peak. (b)Rh123 efflux in the three RPMI 8226 cell lines. Depicted is the Rh123 efflux ratio: Rh123 fluorescence after efflux without verapamil, divided by Rh123 fluorescence after efflux in the presence of verapamil. (c)LRP-, MRP-, and Bcl-2-staining of the used MM cell lines with MoAbs LRP56, MRPm6, and 124, respectively. Isotype-matched controls are shown as a shaded area. MFI-mean fluorescence intensity.

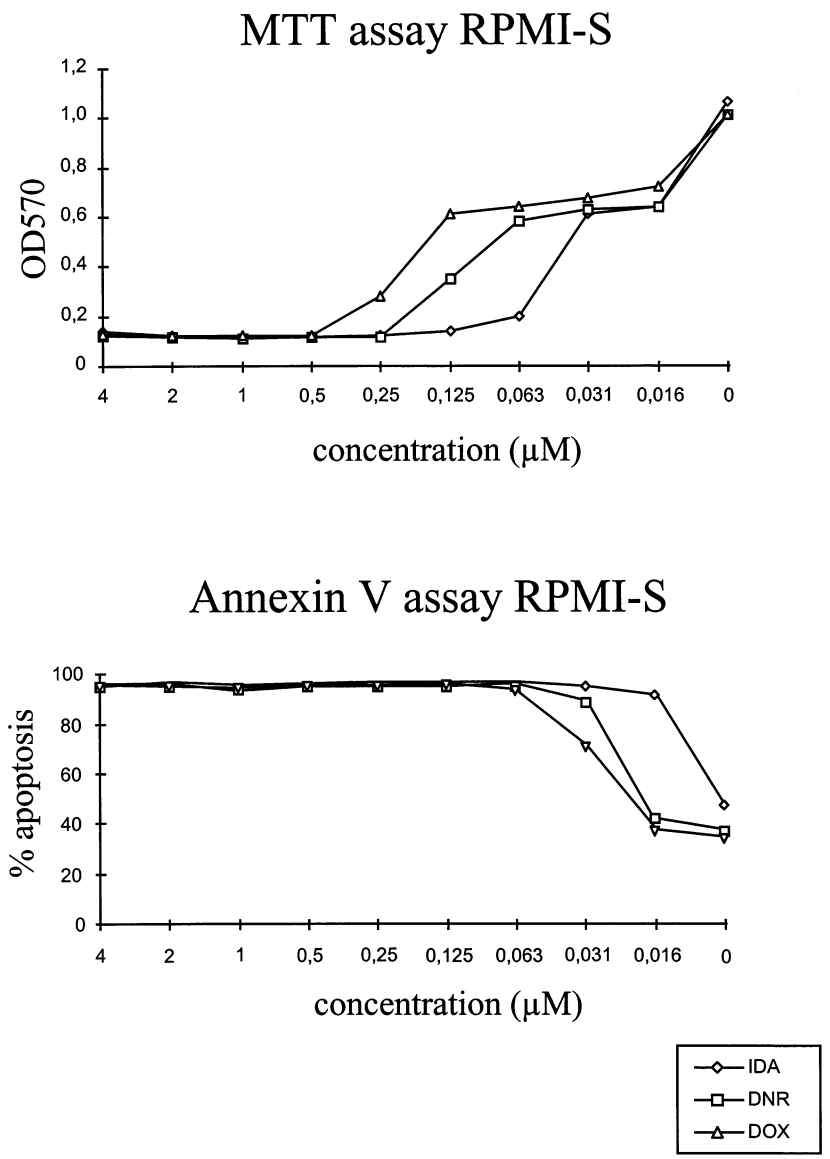

Fig. 2. Comparison of assays to measure cytotoxicity of IDA, DNR, and DOX. RPMI 8226-S cells were exposed to various concentrations of anthracycline. After a 3-day incubation, survival of cells was determined by the MTT assay or the annexin V assay as described in Section 2.

This trend is even more clear in 8226-Dox40 (see Table 2). From Table 2 we can see that the AUC value of IDA is about 1.5 times that of DNR in drug-sensitive 8226-S cells and about 4–5-fold higher in the MDR cells. IDAol showed a behavior intermediate between IDA and DNR.

3.4. Effect of verapamil on drug accumulation in RPMI 8226 cells

The MDR cell line 8226-R7 demonstrated a clearly slower DNR uptake than 8226-S, while simultaneous incubation with the *P*gp-inhibitor verapamil increased intracellular DNR uptake to the level in the sensitive parental cell line.

Fig. 5 shows the effects of the *P*gp-inhibitor verapamil on accumulation of IDA (Fig. 5a), IDAol (Fig. 5b), and DNR (Fig. 5c) in the different cell lines. RPMI 8226-S showed a rapid DNR uptake and verapamil did not increase the intracellular DNR concentration. In addition, verapamil did not enhance accumulation of any of the other drugs in RPMI 8226-S cells, as could be expected for a *P*gp-negative cell line. The addition of verapamil to 8226-R7 increased DNR uptake approximately 4-fold, to levels approaching that of the drug-sensitive parental cell line. 8226-R7 cells exposed to IDA showed no significant increase in uptake after incubation with verapamil. However, in the 8226-Dox40 highly resistant variant, a clearly lower IDA accumulation was enhanced by the addition of verapamil (uptake increased about 3.5-fold).

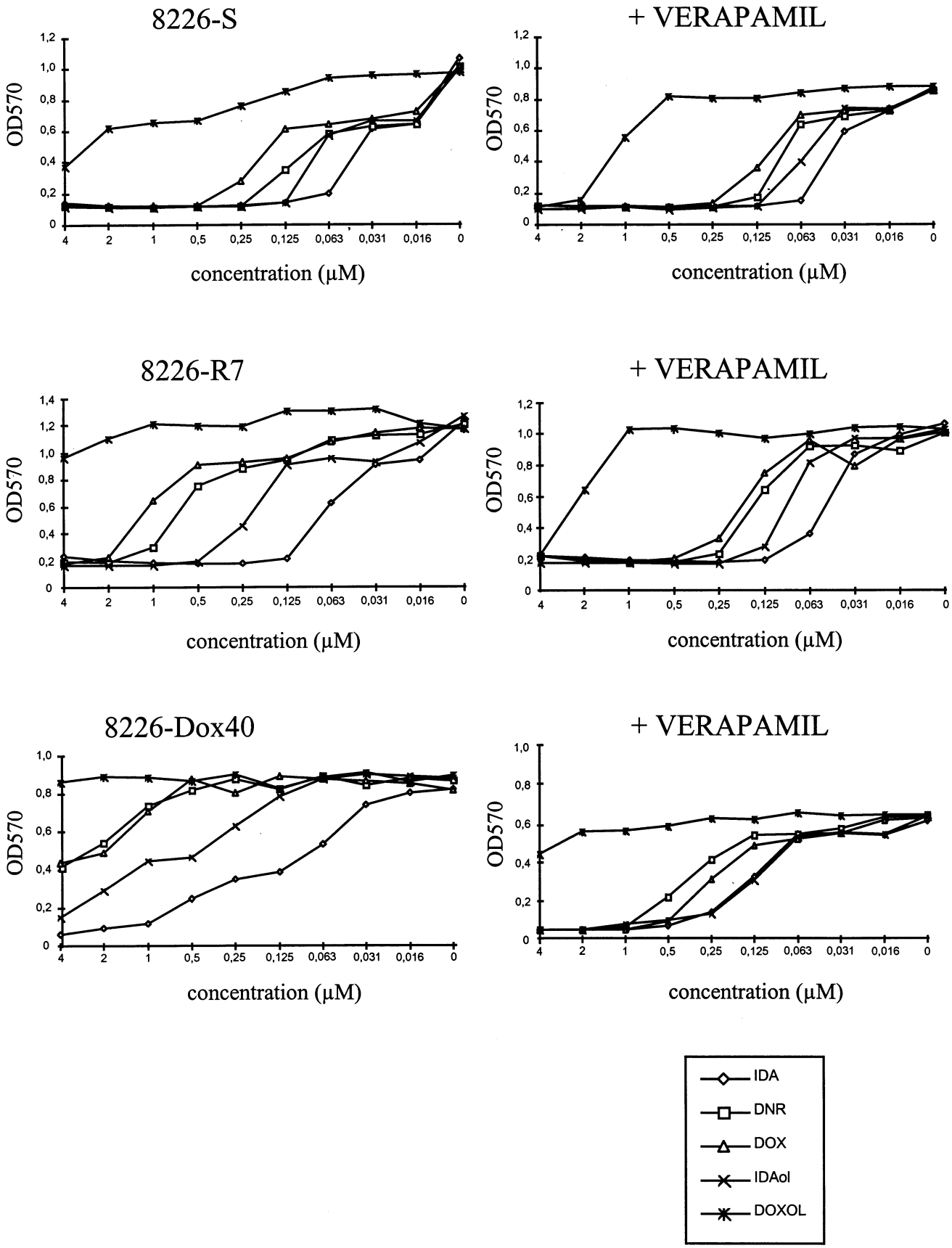

Fig. 3. Cytotoxicity of IDA, DNR and DOX in RPMI 8226 cell lines. Cells were exposed to various concentrations of anthracycline alone (left side of the figure) or in combination with verapamil (right side). After a 3-day incubation, survival of cells was determined by the MTT assay as described in Section 2.

Table 1
In vitro cytotoxicity of DNR, DOX, IDA, DOXol, and IDAol in 8226-S, 8226- R7 and 8226-Dox40[a]

	DNR		DOX		IDA		DOXol		IDAol	
RPMI 8226 cell line:	ID50	RF	ID50	RF	ID50	RF	ID50	RF	ID50	RF
8226-S	108	1.0	164	1.0	36	1.0	2800	1.0	94	1.0
8226-R7	615	5.7	2290	14.0	62	1.7	ND	ND	204	2.2
8226-Dox40	4925	45.6	4590	28.0	94	2.6	ND	ND	434	4.6

[a] Cytotoxicity was determined after 3-days exposure of cells to different concentrations of drugs, using the MTT assay as described Section 2. All data are the mean of three independent experiments. Values (in nM) for ID50 (50% MTT reaction inhibiting dose) were determined from the plots of MTT versus drug concentrations (Fig. 3). Resistance factor (RF) value for each drug in each cell line was calculated as ID50 measured = 8226-S ID50. ND, not determined.

4. Discussion

The work presented here provides multiple lines of evidence that IDA has a higher efficacy than DOX or DNR in killing myeloma plasma cells, especially in *P*gp-expressing MDR cells. In addition, our data confirm that the major IDA metabolite IDAol has a marked cytotoxicity compared with other metabolic derivatives of anthracyclines, like DOXol. Myeloma cells required at least 32-fold lower extracellular IDAol concentrations than DOXol for the same cytotoxic effect, whereas cytotoxicity of IDAol was 2–10-fold higher than DOX. The ability of verapamil to enhance the cytotoxicity of IDAol in *P*gp-expressing cells suggests that *P*gp modulators may improve the overall treatment response to IDA. These results confirm that IDAol, which has a long in vivo half-life, may significantly contribute to the clinical effects of IDA.

The two MDR 8226 variants used here do not differ from the parental cell line 8226-S in their expression of other MDR-related proteins, like LRP, MRP, or Bcl-2 (data not shown). This suggests that their difference in resistance is mainly due to the different levels of expression of *P*gp.

We used flow cytometry to study DNR, IDA, and IDAol uptake in different variants of the RPMI 8226 MM cell line and showed that IDA had more advantageous pharmacokinetics in the MDR variants, relative to their parental cell line. DNR accumulation was clearly impaired in MDR 8226-R7, while the accumulation rate of IDA in 8226-R7 was comparable to that in the drug-sensitive parental cell line. This indicates that IDA is less susceptible to *P*gp-mediated MDR. In the highly resistant variant 8226-Dox40 however, a clearly lower IDA accumulation could be reversed by the addition of verapamil, suggesting that *P*gp was responsible for the lower IDA accumulation in this cell line. For each drug tested, we found a good correlation between intracellular accumulation, as expressed in AUC values (see Section 3, Table 2) and cytotoxicity (ID50, Table 1), suggesting that the differences in accumulation for a large part can explain the different sensitivities towards the different anthracyclines used here: DOX, DNR, IDA, and IDAol. This does not apply to DOXol because of its lower toxicity. The AUC values shown here do not include anthracyclines intercalated in the DNA of the nucleus, because their fluorescence is quenced for 98%. Other studies have shown that the maximum intercalation in the nucleus is reached 5 min after first exposure to the drug and depends on the cytoplasmatic concentration [23].

4.1. IDA induces apoptosis

After incubation with IDA, apoptotic annexin V-positive myeloma cells were identified by flow cytometry. Binding of annexin V to phosphatidylserine in the outer

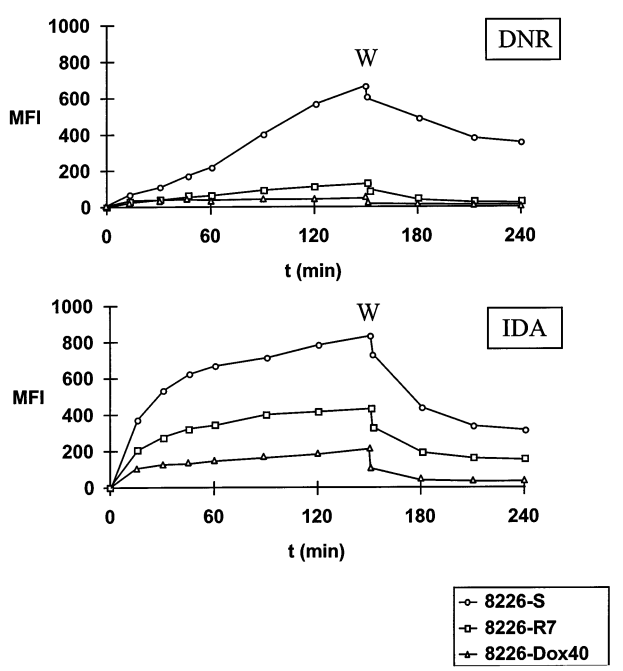

Fig. 4. Timecourse of accumulation and retention of IDA and DNR in 8226-S, 8226-R7, and 8226-Dox40 cells exposed at a concentration of 1.0 μM of anthracycline. Mean fluorescence intensity (MFI) was plotted as a function of the time of incubation. W, cells were washed and resuspended in drug-free medium.

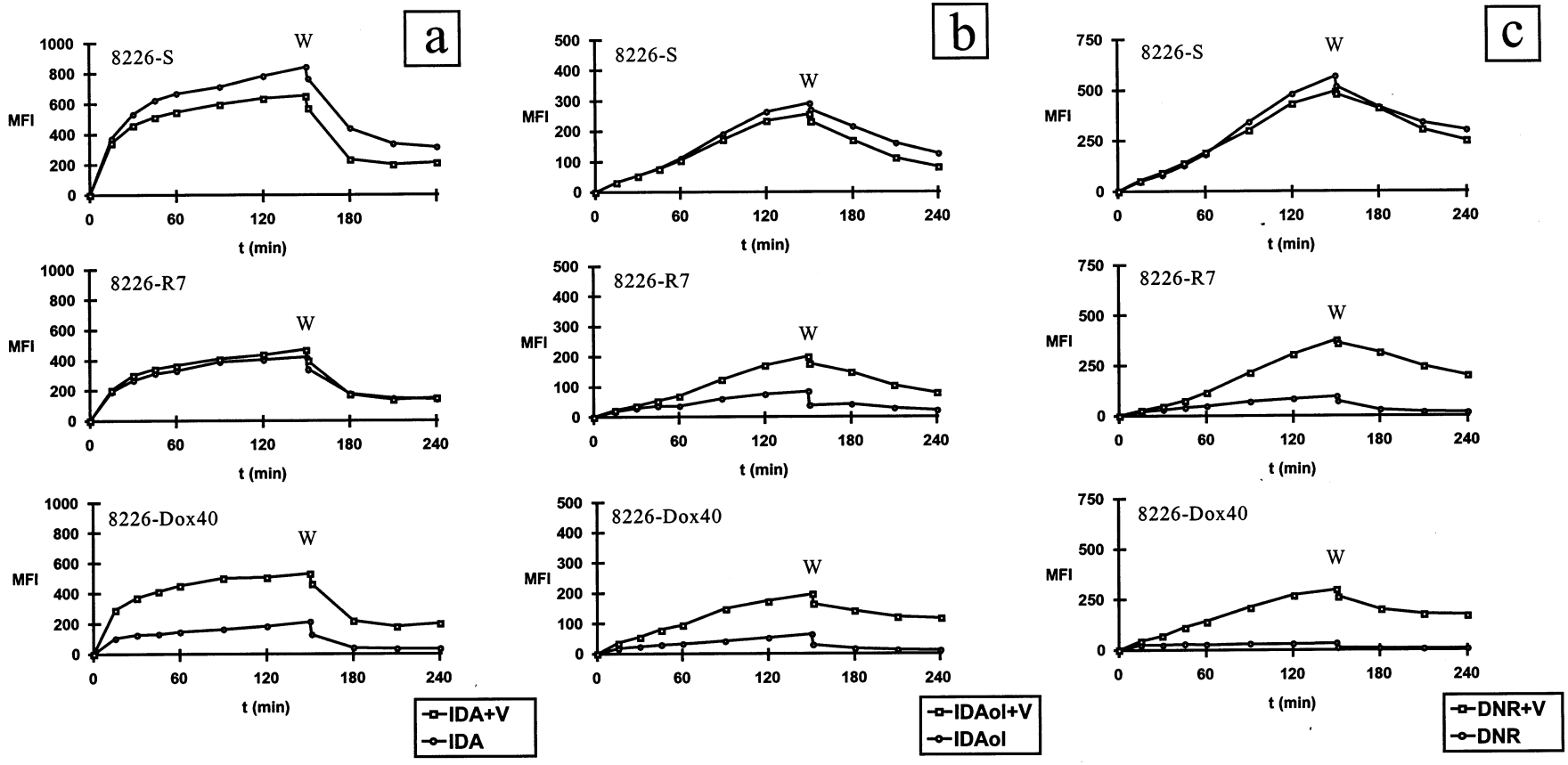

Fig. 5. Intracellular uptake and efflux of IDA, IDAol, and DNR in human MM cell lines 8226-S, 8226-R7, and 8226-Dox40 in the presence and absence of verapamil at 50 μM l^{-1}. A total of 10^6 cells ml^{-1} were incubated for 150 min with 1.0 μM of anthracycline drug, with (□) or without (○) 50 μM of verapamil. Mean fluorescence intensity (MFI) was plotted as a function of the time of incubation. After uptake, cells were washed and reincubated in drugfree medium with or without verapamil for 120 min. All uptake and retention experiments were performed in duplicate and a representative experiment is shown for each. W, cells were washed and resuspended in drug-free medium.

Table 2
Steady state level (C_{ss}) and area under the curve (AUC) for DNR, IDA, and IDAol in the different RPMI 8226 variants[a]

	DNR		IDA		IDAol	
RPMI 8226 cell line:	C_{ss}	AUC	C_{ss}	AUC	C_{ss}	AUC
8226-S	610	1472	850	2569	300	1200
8226-R7	90	310	480	1314	95	405
8226-Dox40	10	152	180	624	50	308

[a] The AUC ($10^3 \times$ fluorescence units cell$^{-1} \times$ min) was calculated by estimating the total surface of the accumulation curves of Fig. 4 to the left of the 150 min point.

leaflet of the plasma membrane indicates an early stage of apoptosis [22]. For the tested anthracycline drugs, we found an excellent correlation between cytotoxicity as determined with the MTT assay and apoptosis as measured with the annexin V assay. The same results were obtained with both assays, the annexin V assay being 16–32-fold more sensitive than the MTT assay. These results imply that flow cytometric analysis of annexin V positive cells is a quantitative and sensitive assay for the evaluation of drug induced cytotoxicity in cell lines and also in primary tumor material of myeloma patients.

4.2. Better efficacy of IDA in MDR cells

The explanation for the high biological activity of IDA compared to DNR or DOX can be attributed either to a more favorable cellular pharmacokinetics or to a higher interaction of IDA with its targets [24]. The ability of IDA to overcome MDR can be explained, at least in part, by its high level of accumulation, both in sensitive and MDR myeloma cells. It has previously been shown that the transport of anthracycline over the plasma membrane is the rate-limiting step [25,26]. The three drugs used here, DOX, DNR, and IDA, have comparable affinity for *P*gp and are extruded at comparable rates by *P*gp [27,28]. With the efficiency of *P*gp in pumping out the three drugs being almost the same, it follows that the differences in accumulation found mainly depend on their kinetics of uptake. Consequently, the high potency of IDA is not due to a lack of activity of *P*gp on IDA, but rather to an enhanced influx of this lipophilic drug compared to DOX or DNR, which may overwhelm the *P*gp pump. A close correlation between the lipid solubility of anthracyclines and their ability to accumulate in MDR cells has been described earlier [29–31]. In addition, IDA may act as a self-modulator by binding to *P*gp and thus interfering with the efficiency of the pump [32].

These studies demonstrate that the pharmacokinetics and cytotoxicity of IDA in *P*gp-overexpressing MDR cell lines are superior to those of DOX and DNR and indicate that IDA is less sensitive to *P*gp-mediated MDR. Because of this characteristic, this suggests that IDA may become an important drug in treatment of MM, especially in refractory disease.

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