The Novel Anthracycline Annamycin Is Not Affected by P-Glycoprotein–Related Multidrug Resistance: Comparison With Idarubicin and Doxorubicin in HL-60 Leukemia Cell Lines

By Ugo Consoli, Waldemar Priebe, Yi-He Ling, Radha Mahadevia, Mark Griffin, Shourong Zhao, Roman Perez-Soler, and Michael Andreeff

A major factor in limiting the efficacy of anthracyclines is overexpression of the MDR1-encoded p-glycoprotein (p-gp). A new analogue less affected by p-gp is annamycin (ANN), an anthracycline antibiotic with high affinity for lipid membranes and significantly more activity than doxorubicin (DOX). We investigated whether ANN was affected by p-gp–mediated multidrug resistance (MDR) by comparing the cellular accumulation and retention of ANN, idarubicin (IDR), and DOX in the p-gp–negative human leukemia cell lines (HL-60S) and its DOX-selected p-gp–positive subline (HL-60/DOX) with and without verapamil (VER). As expected, HL-60/DOX cells showed lower DOX uptake than HL-60S cells; coincubation with VER (10 mmol/L) increased uptake 2.6-fold, restoring it to 100% of uptake in HL-60S cells. IDR uptake increased 1.5-fold in the presence of VER, but ANN was not affected. Coincubation with VER increased DOX retention in HL-60/DOX cells 2.8-fold and IDR retention 1.4-fold; unchanged ANN retention indicated that ANN may overcome p-gp. In the cytotoxicity assay to correlate intracellular anthracycline content with antitumor activity, we found ANN to be less potent than DOX and IDR in sensitive cells, ID_{50} being the drug concentration that inhibits cell growth by 50%, but its resistance index (RI; ID_{50} resistant cells divided by ID_{50} sensitive cells) was lower than that of IDR and DOX (2.6 vs 40 and 117.5). Coincubation in the presence of VER resulted in 4.5-fold and 2-fold RI decreases of DOX and IDR, respectively, whereas ANN did not change, further confirming ANN’s ability to circumvent p-gp–mediated MDR. Confocal microscopy studies of IDR, ANN, and DOX showed higher intracellular drug compartmentalization for DOX in HL-60/DOX cells incubated in the presence of VER. This study provided evidence that, unlike DOX and IDR, ANN is not affected by p-gp–mediated MDR.

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DRUG RESISTANCE OF HUMAN cancer, which has been shown to be mediated by multiple molecular mechanisms, is a major problem in chemotherapy. Despite the fact that 80% to 90% of patients with adult acute myeloid or lymphoid leukemia respond to intensive chemotherapy, more than 50% of the patients relapse, often because the recurrence of disease is associated with clinical drug resistance. One well-characterized mechanism of multidrug resistance (MDR) is an energy-dependent transport system for such natural-product cytotoxic drugs as anthracycline antibiotics, vinca alkaloids, epipodophyllotoxins, and dactinomycin. This mechanism of action is often related to overexpression of the 170-kD membrane p-glycoprotein (p-gp), and the cellular pharmacological consequence of MDR seems to be reduced accumulation of drugs. Recently p-gp was shown not to be a pump itself but a regulator for the activity of an endogenous volume-activated chloride channel. The gene (MDR1) for p-gp has been cloned, sequenced, and located on human chromosome 7. Transfection with MDR1 cDNA confers the MDR phenotype on drug-sensitive cells. To overcome this type of resistance, agents that block p-gp have been used in chemotherapy. Verapamil (VER), a calcium channel blocker, was the first agent shown to reverse MDR in several resistant cell lines by interfering with p-gp function, but many other p-gp–blocking agents have been identified. In these in vitro and in vivo systems, MDR is overcome by increased intracellular accumulation of cytotoxic drugs. Because intrinsic and acquired drug resistance remains a major problem in cancer, great interest has been generated by studies showing that a variety of agents can successfully overcome resistance in MDR1–positive cells. This has led to clinical trials of combinations of chemotherapy and p-gp blockers. Although some studies have shown positive results, the p-gp blockers’ toxicity has aroused controversies regarding their clinical use. Moreover, their evaluation is further complicated by the multifactorial nature of drug-resistance mechanisms.

Another potentially effective way to circumvent the MDR phenotype is by using new compounds that are not p-gp substrates. Idarubicin (IDR; 4-demethoxydaunorubicin; see Fig 1), a daunorubicin (DNR) analogue that has been reported to be more potent than DNR or doxorubicin (DOX) and less cardiotoxic as well, also has been shown to be less affected than DNR. The latter observation could partly account for the improved results obtained in some clinical trials. In structural formula IDR differs from DNR by the absence of a methoxy group at the 4 position of the anthracycline molecule; its greater lipophilicity explains, in part, its higher uptake compared with those of DNR and DOX.

Another anthracycline antibiotic recently developed at The M.D. Anderson Cancer Center (Houston, TX) is annamycin (ANN; see Fig 1). ANN is, in addition to other structural changes, deaminated at the 3’ position of the sugar ring and has a high affinity for lipid membranes. In DOX,
Interestingly, removal of the amino group seems to reduce its cardiotoxicity without altering its antitumor properties. The amino group, known to be a common structural feature of various chemotherapeutic drugs that show cross-resistance in p-gp–positive cells, also determines the affinity of anthracyclines for negatively charged phospholipids, including cardiolipin. ANN has been shown to be significantly more active than DOX in several in vitro and in vivo tumor models including L-1210 leukemia and subcutaneous B-16 tumor. Unlike DOX, ANN was also found to be capable of inducing different types of DNA lesions and drug-induced apoptosis in resistant cells. Recently, ANN was shown to be effective in treating KB-V1 xenografts in vivo. Phase I clinical studies with ANN formulated in a liposomal carrier in solid tumors have just been started at The M.D. Anderson Cancer Center.

In this study, we compared the cellular pharmacology of ANN and IDR with that of DOX, because both IDR and ANN have been shown to overcome MDR1 partially in different systems. We studied uptake and retention in the presence of VER using laser flow cytometry to quantitate intracellular anthracycline content in the p-gp–negative human myeloid leukemia cell line (HL-60S) and its DOX-derived p-gp–positive cell line (HL-60/DOX). We also performed cytotoxicity assays to determine whether increased intracellular content of anthracycline was associated with increased cytotoxicity. Finally, to better understand the cellular pharmacology and distribution of anthracyclines, we studied the different intracellular distribution patterns using confocal laser microscopy (CLM) with available optical sectioning. The results of these experiments suggested that, in our system,
ANN was effective in overcoming p-gp-related MDR and more efficient than IDR and DOX in circumventing it.

MATERIALS AND METHODS

Drugs. ANN was synthesized and purified as described previously. Free ANN was suspended in 10% dimethyl sulfoxide and 90% normal saline (1 mg/mL of stock solution). The final dimethyl sulfoxide concentration was 0.05% for an ANN concentration of 5 mg/mL. DOX (Cetus Corp, Emeryville, CA) and IDR (Adria Laboratories, Columbus, OH) were dissolved in sterile water. All anthracyclines were diluted with RPMI 1640 containing 10% fetal calf serum (FCS) before each experiment. VER (Sigma Chemical Co, St Louis, MO), cyclosporine-A (CyA; Sandoz, Basel, Switzerland), and PSC 833 (Sandoz) were prepared as stock solution in absolute ethanol at 5 mg/mL and stored at 4°C. Dilutions were made in medium immediately before use.

Cell lines. The human acute myeloid leukemia parent cell line HL-60 and its DOX-resistant mutants HL-60/DOX were kindly provided by Dr M. Beran (M.D. Anderson Cancer Center). The multiple myeloma parent cell line 8226 and its DOX-resistant mutants 8226/DOX6 and 8226/DOX40 were kindly provided by Dr W.S. Dalton (University of Arizona, Tucson, AZ). All cells were grown in RPMI 1640 supplemented with 10% FCS, 1% penicillin, 1% streptomycin, and 1% L-glutamine (all from GIBCO-BRL, Gaithersburg, MD). HL-60/DOX cells were grown in Iscove's modified Dul-
becco’s medium (GIBCO-BRL) supplemented with 10% FCS, 1% penicillin, 1% streptomycin, and 1% L-glutamine. Cell cultures were maintained at 37°C in a humified atmosphere of 5% CO2/95% air.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was isolated according to the acid-guanidinium-phenol-chloroform method. RT-PCR was performed as previously reported. Briefly, 1 µg of total RNA was used for cDNA production with a CDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN); 1/10 of the reaction was used for the PCR. The MDR1 and β2-microglobulin genes were amplified simultaneously in different aliquots. The PCR (total volume, 25 µL) contained 1 mmol/L of each primer, 1.25 U of Taq-polymerase, 2.5 µL of 10× PCR buffer (Perkin Elmer-Cetus, Norwalk, CT), 0.2 µL of 25-mmol deoxynucleotide triphosphate, and 2 µCi of [32P]-deoxycytidine triphosphate (Dupont, Wilmington, DE). Each cycle included a denaturation step at 94°C for 30 seconds, an annealing step at 47°C for 30 seconds, and an elongation step at 65°C for 45 seconds. Twenty-eight cycles were performed, followed by a final extension at 72°C for 10 minutes using a PCR processor (Model 9600; Perkin Elmer-Cetus). PCR products (3 µL) were analyzed through 8% polyacrylamide gel (Bio-Rad, Richmond, CA). Electrophoresis was performed at 300 V for 3.5 hours. [32P]-incorporation was quantitated to Betascope 603 (Bata-gen, Waltham, MA) and subsequently exposed for 8 hours at −70°C to a Kodak X-Omat film (Eastman-Kodak, Rochester, NY) without an intensification screen.

Antibody staining. For the study of p-gp, monoclonal antibody (MoAb) 4E3 (generously supplied by Dr R. Arceci, Dana-Farber Cancer Center, Boston, MA) was used as follows: 2 × 10^6 cells/tube in 100 µL of phosphate-buffered saline (PBS), without Ca2+ or Mg2+ (Sigma) and containing a 1:1 dilution of human serum with PBS, were incubated into Falcon tubes (Becton Dickinson, San Jose, CA) at 4°C for 30 minutes to inhibit nonspecific binding of antibodies to Fc receptors. PBS (2 mL) was then added to the cells, which were collected by centrifugation at 600 × g for 5 minutes. Pelleted cells were resuspended in 100 µL of PBS containing 1% bovine serum albumin and 10 µg/mL of the anti-p-gp MoAb or isotype-matched nonreactive controls. This mixture was incubated for 30 minutes at 4°C; then, cells were washed twice with cold PBS and resuspended in 100 µL of PBS containing 1% bovine serum albumin and a secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat-antimouse IgG (Becton Dickinson). Cells were incubated with the second antibody for 30 minutes at 4°C in the dark and then washed twice in cold PBS.

Flow cytometry. Flow cytometric analysis was done with a FACSCAN flow cytometer (Becton Dickenson) equipped with an argon ion laser (Spectra Physics, Mountain View, CA) operating at 488 nm and 15 mW. Light was collected through 585/42 band-pass filters for anthraclycline uptake and retention studies and a 530-30-nm filter (FITC) for 4E3 MoAb detection. Data acquisition and analysis were performed with the Lysys II software (Becton Dickenson). Forward and side scatter signal were collected using a linear scale, and fluorescence signals were collected on a logarithmic scale. Kolmogorov-Smirnov analysis of data was performed, and the results are expressed as D-values.

Cell uptake and retention studies. Cells were incubated at the concentration of 2 × 10^6/mL in medium plus 5% FCS with or without VER (10 µmol/L) for 2 hours at 37°C with ANN, IDR, or DOX at the concentration of 5 µg/mL. For uptake studies, aliquots of 5 × 10^6 cells were obtained after 15, 30, 60, 90, and 120 minutes of incubation. Cells were washed in ice-cold PBS without Ca2+ or Mg2+ (Sigma) and resuspended in 0.5 mL of ice-cold PBS, and anthraclycline fluorescence was measured within 5 minutes. For retention studies, at the end of 2 hours of exposure, every sample was washed twice in ice-cold PBS and resuspended for 2 hours in fresh medium with or without VER, at the same concentration used for uptake studies. Cells were incubated at 37°C, and aliquots of cells were analyzed at 15, 30, 60, 90, and 120 minutes, under the same conditions as those used for uptake studies. Each experiment was performed in triplicate, and representative experiments are shown in Figs 4 and 5. Increased uptake and retention were calculated by dividing the median level of fluorescence after 2 hours of incubation with VER by the fluorescent signal of the drug alone measured at the same time point. The level of increase was calculated after correcting each value for autofluorescence of the blank and is expressed as the mean of three experiments. Similar experiments were also conducted using CyA (5 mg/mL) and PSC833 (1 mg/mL) as p-gp blockers.

Cytotoxicity assay. HL-60s and HL-60/DOX cells were plated in 96-microwell plates and incubated at 37°C overnight. Cultures were exposed to various concentrations of DOX, IDR, and ANN at 37°C for 4 hours; the drugs were removed; the cells were rinsed 3 times with Ca2+ and Mg2+-free PBS (pH, 7.4) and were recultured in drug-free fresh medium for 3 days. Cell survival was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide (MTT) as described by Hansen et al.27 The ID50 value was calculated by extrapolating from the graphic results the drug concentration that causes a 50% cell death.

CLM. We used a Zeiss (New York, NY) confocal laser scanning microscope, upright version with LSM/2.0 software, equipped with an external Argon laser (488 nm, 10 mW). Signals were collected by a photomultiplier after passing a 540/20 band-pass filter. Digitized images were transmitted to an Macintosh-based image analysis system through a general purpose interface board (GPIB) interface using BDS-LSM software (Biological Detection Systems, Pittsburgh, PA). For image processing, we used IPLab software (Signal Analytics, Vienna, VA). Teflon-coated glass microscopy slides (Cel-line Associates, Newfield, NJ) with 10 wells (each 7 mm in diameter and containing 10 mL) were used. Cells adhering to the microscope slide were analyzed for ANN, IDR, and DOX distribution under oil-immersion microscopy. HL-60s and HL-60/DOX were incubated in presence of ANN, IDR, and DOX either with or without VER as described above for uptake studies. Drug distribution was recorded within 5 minutes after 30 and 90 minutes of incubation with the drugs. The images showed the distribution of anthraclycline fluorescence after confocal sectioning of interphase cells.

RESULTS

Flow cytometric analysis of p-gp expression. Figure 2 shows strong fluorescence of HL-60/DOX cells stained with MoAb 4E3, indicating the presence of p-gp. Kolmogorov-Smirnov analysis showed D-values of .98 for 4E3. Background fluorescence was determined by incubation with an isotype control. No p-gp-related fluorescence was detected in the parent cell line HL-60 (D-value, .02).

<table>
<thead>
<tr>
<th>Table 1. Fold increase in DOX, IDR, and ANN Relative Fluorescence Levels During Uptake and Retention Studies in HL-60/DOX Cells in Presence of VER (10 µmol/L), PSC833 (1 µg/mL), or CyA (5 µg/mL)</th>
<th>P-gp Blockers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake studies</td>
<td></td>
</tr>
<tr>
<td>VER</td>
<td>2.5</td>
</tr>
<tr>
<td>PSC 833</td>
<td>2.0</td>
</tr>
<tr>
<td>CyA</td>
<td>2.3</td>
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<tr>
<td>Retention studies</td>
<td></td>
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<tr>
<td>VER</td>
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</tr>
<tr>
<td>PSC 833</td>
<td>2.8</td>
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<tr>
<td>CyA</td>
<td>2.5</td>
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A

Fig 5. In vitro cellular uptake and efflux of ANN (A), IDR (B), and DOX (A and B) in human acute leukemia cell line HL-60S. A total of 10⁶ cells/ml was incubated for 120 minutes with 5 μg/ml of ANN, IDR, or DOX with (filled symbols and lines) and without (open symbols and dotted lines) VER. After 120 minutes of uptake, every sample was re-incubated in drug-free medium with (filled symbols and lines) and without (open symbols and dotted lines) VER for 120 minutes. Control experiments using medium (M) alone and VER show curve overlap.

B

Analysis of MDR1 mRNA by PCR amplification using MDR1-specific primers. To ensure that our cell lines did not change their MDR1 expression status on prolonged culture, we directly measured mdr-1 by RT-PCR. Results are shown in Fig 3. A sensitive (8226/S) and two multidrug-resistant (8226/DOX-6 and 8226/DOX40) cell lines were included as controls. MDR1 expression was undetectable in 8226/S and elevated in 8226/DOX6 and 8226/DOX40 cell lines. A strong band was detected for MDR1 mRNA in HL-60/DOX cells, and no band was present in HL-60S cells.

DOX cells and effect of VER. Figure 4 shows uptake and retention of ANN (Fig 4A) and IDR (Fig 4B) compared with those of DOX in HL-60/DOX cells exposed to 5 μg/ml of anthracyclines either alone or in combination with 10 μmol/l of VER. Incubation with DOX resulted in minimal drug uptake that increased 2.5-fold in the presence of VER. Uptake of ANN (Fig 4A) was faster than that of DOX and similar to that of IDR (Fig 4B), with the plateau phase having already been reached at the first measurement and with no uptake increase being observed in the presence of VER.

After 2 hours of incubation in the continuous presence of
Table 2. Flow Cytometric S/R Index of DOX, IDR, and ANN Uptake With and Without VER in HL-60S and HL-60/DOX Cells

<table>
<thead>
<tr>
<th>Drugs</th>
<th>HL-60S Cells</th>
<th>HL-60/DOX Cells</th>
<th>S/R Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANN</td>
<td>202 ± 4.5</td>
<td>196 ± 14.3</td>
<td>1.03</td>
</tr>
<tr>
<td>ANN + VER</td>
<td>219 ± 8.5</td>
<td>210 ± 16.2</td>
<td>1.04</td>
</tr>
<tr>
<td>IDR</td>
<td>208.5 ± 3.2</td>
<td>108.8 ± 10.8</td>
<td>1.9</td>
</tr>
<tr>
<td>IDR + VER</td>
<td>211.6 ± 2.5</td>
<td>162 ± 14.2</td>
<td>1.3</td>
</tr>
<tr>
<td>DOX</td>
<td>14.2 ± 0.8</td>
<td>5.4 ± 1.13</td>
<td>2.6</td>
</tr>
<tr>
<td>DOX + VER</td>
<td>13.9 ± 1.1</td>
<td>13.6 ± 4.2</td>
<td>1.02</td>
</tr>
</tbody>
</table>

*Median channel fluorescence was determined by dividing the median level of fluorescence after 2-hour incubation with drugs by the median fluorescence signal of cells incubated in medium at the same time point. All data are mean ± SD of three independent experiments.

different anthracyclines, cells were washed twice in cold medium and reincubated for 2 hours in fresh medium at 37°C with or without VER. HL-60/DOX cells showed a decrease in intracellular concentration of DOX that was almost completely reversed by VER. The HL-60/DOX retention of DOX was increased 2.8-fold by VER. No change in the intracellular content of ANN was observed in presence of VER during the retention studies. As shown in Fig 4B, the curve for IDR was similar to that of ANN, with rapid achievement of the plateau phase and a stable level of intracellular drug concentration during the 2 hour-incubation with the drug. Incubation with VER increased IDR uptake and retention 1.5- and 1.4-fold, respectively.

CyA and PSC 833 were assayed under the same experimental conditions for their ability to increase anthracycline uptake and retention in HL-60/DOX cells, and their efficacy was compared with that of VER. Results shown in Table 1 showed a similar efficacy for the three p-gp blockers at the concentration used. The rapid drop in ANN and IDR fluorescence levels observed within the first few minutes of efflux was mainly determined by the washing steps required for removing the drug from the medium after the uptake studies (data not shown). This phenomenon was observed in both HL-60/DOX and HL-60 cells, and we believe it may be related to higher ANN and IDR lipophilicity and affinity for external cytoplasmic membrane.

Cellular uptake and retention of anthracyclines in HL-60S and effect of VER. Figure 5 shows the uptake and retention studies of ANN (Fig 5A) and IDR (Fig 5B) compared with those of DOX in HL-60S as described above. Again, IDR and ANN uptakes were faster than DOX uptake. Cells were then washed twice and reincubated in fresh medium for the efflux studies, as described above, and resuspended in fresh medium. In HL-60S, no DOX efflux was
Table 3. In Vitro Cytotoxicity of DOX, IDR, and ANN With and Without VER in HL-60S and HL-60/DOX Cells

<table>
<thead>
<tr>
<th>Drugs</th>
<th>ID50 (µg/mL)*</th>
<th>HL-60S Cells</th>
<th>HL-60/DOX Cells</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANN</td>
<td>0.30 ± 0.17</td>
<td>0.80 ± 0.04</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>ANN + VER</td>
<td>0.29 ± 0.18</td>
<td>0.77 ± 0.06</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>IDR</td>
<td>0.004 ± 0.003</td>
<td>0.16 ± 0.16</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>IDR + VER</td>
<td>0.004 ± 0.002</td>
<td>0.08 ± 0.02</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>DOX</td>
<td>0.078 ± 0.003</td>
<td>9.13 ± 0.55</td>
<td>117.5</td>
<td></td>
</tr>
<tr>
<td>DOX + VER</td>
<td>0.057 ± 0.006</td>
<td>1.46 ± 0.68</td>
<td>25.6</td>
<td></td>
</tr>
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</table>

Cytotoxicity was determined after 4-hour exposure of cells to different concentrations of drugs, using the MTT assay as described in Materials and Methods. All data are mean ± SD of three independent experiments.

* Values for ID50 were determined from plot of cell survival fractions versus drug concentrations.

related slow pattern of efflux is probably related to the higher lipophilicity of ANN and IDR.

Cellular accumulation in sensitive cells was higher than that in resistant cells; the resistance uptake factor, S/R (S/R, the uptake by sensitive cells divided by uptake by resistant cells), was 1.03 for ANN, 1.9 for IDR, and 2.6 for DOX. Incubation with VER markedly reduced the S/R for IDR (from 1.9 without VER to 1.3 with VER) and for DOX (from 2.6 without VER to 1.02 with VER). No changes were observed for ANN (1.03 without VER and 1.04 with VER; see Table 2).

In vitro cytotoxicity against HL-60S and HL-60/DOX. Results of the cytotoxicity studies with ANN, IDR, and DOX with or without VER against HL-60S and HL-60/DOX cells are shown in Fig 6 and Table 3. In these studies, cells were exposed to different concentrations of the agents for 4 hours, and cytotoxicity was determined by an MTT assay after a 72-hour incubation period. In HL-60S, IDR was about 100 times more cytotoxic than ANN (ID50, 0.004 ± 0.003 v 0.30 ± 0.17, respectively) and 10 times more cytotoxic than DOX (ID50, 0.004 ± 0.003 v 0.078 ± 0.003, respectively). How-
ever, ANN was almost equally cytotoxic for the parental and MDR-positive cells (ID$_{50}$, 0.30 ± 0.17 for HL-60S v 0.80 ± 0.04 for HL-60/DOX), whereas IDR required a 40-fold increase (ID$_{50}$, 0.004 ± 0.003 for HL-60S v 0.16 ± 0.16 for HL-60/DOX) and DOX required a 117-fold increase in concentration to reach the ID$_{50}$ (ID$_{50}$, 0.078 ± 0.003 for HL-60S v 9.13 ± 0.55 for HL-60/DOX). In accordance with these results, the calculated resistance index (RI; ID$_{50}$ HL-60DOX/ID$_{50}$ HL-60S) was 2.6 for ANN, 40 for IDR, and 117.5 for DOX.

We also evaluated cytotoxicity in the presence of VER to learn whether an increase in intracellular drug content was associated with increased cytotoxicity. In HL-60/DOX cells, incubation with VER increased the cytotoxicity of DOX and IDR, with a 6.2-fold reduction of ID$_{50}$ for DOX (ID$_{50}$, 1.46 ± 0.68 with VER v 9.13 ± 0.55 without VER) and a 2-fold reduction for IDR (ID$_{50}$, 0.08 ± 0.02 with VER v 0.16 ± 0.16 without VER), whereas no increase in cytotoxicity was observed for ANN (ID$_{50}$, 0.77 ± 0.06 with VER v 0.80 ± 0.04 without VER). We observed no change in HL-60S cells for DOX (ID$_{50}$, 0.078 ± 0.003 without VER v 0.057 ± 0.006 with VER), IDR (ID$_{50}$, 0.004 ± 0.003 without VER v 0.004 ± 0.002 with VER), or ANN (ID$_{50}$, 0.30 ± 0.17 without VER v 0.29 ± 0.18 with VER). When we compared the RI of the different anthracyclines incubated alone or with VER, the addition of VER decreased the RI 4.6-fold for DOX and 2-fold for IDR and was unchanged for ANN.

*Intracellular anthracycline distribution in HL-60S and HL-60/DOX as determined by CLM.* After 30 minutes, HL-60S cells incubated in the presence of DOX showed a pattern of DOX-related fluorescence predominantly in the cytoplasm and perinuclear regions (Fig 7A). After 90 minutes, an increased distribution of the drug was observed within the nucleus (Fig 7B). This intracellular distribution was not modified by the presence of VER (Figs 7C and D). In HL-60/DOX cells, DOX uptake was limited mainly to the intracytoplasmic region after 30 minutes, and the fluorescence intensity was lower than that in HL-60S cells (Fig 8A). No...
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Fig 9. IDR intracellular fluorescence distribution detected by CLM in HL-60S (A and B) and HL-60/DOX (C and D) cells after 90 minutes of incubation with the drug in the presence (B and D) or absence (A and C) of verapamil. In HL-60S cells, IDR was distributed in the cytoplasmic region, perinuclear zone, and nuclear membrane (A) and was not modified by VER (B). After 90 minutes, HL-60/DOX cells showed a similar but slightly reduced intensity in IDR-related fluorescence (C), which was only partially increased by VER (D).

Further increase was noted after 90 minutes (Fig 8B). In these cells, incubation of DOX in the presence of VER resulted in a consistent increase of DOX-related fluorescence after 30 and 90 minutes (Figs 8C and D), with the drug distributed mainly within intracytoplasmic structures. In contrast to DOX, no significant changes were observed for IDR and ANN when sensitive and resistant cell lines were compared with or without VER. No differences were noted during the different time points (data not shown), probably indicating a faster uptake and intracellular distribution related to higher IDR and ANN lipophilicity. IDR-related fluorescence (Fig 9A) was distributed mainly in the cytoplasmic region, the perinuclear zone, and nuclear membrane (Fig 9A). The intracellular distribution was similar in HL-60S (Fig 9A) and HL-60/DOX cells (Fig 9C), and only a small increase in IDR fluorescence was noted in HL-60/DOX cells when incubated in the presence of VER (Figs 9C and D). Subcellular distribution of ANN (Fig 10) was observed mainly in the cellular membrane and cytoplasm region. The pattern was similar for the two cell lines and was not influenced by VER.

DISCUSSION

The main objective of our study was to assess the ability of the new anthracycline ANN to overcome MDR1 in a human leukemia cell line and to compare it with IDR and DOX, which are widely used in front-line protocols for treating patients with acute leukemia. For that purpose, we compared the cellular pharmacology and in vitro cytotoxicity of the three agents against HL-60S and HL-60/DOX cells in the presence and absence of the resistance-modifier VER. Although the total intracellular concentration of each anthracycline could not be directly compared by flow cytometry and CML, because of each drug's different fluorescent emission pattern, our results indicated the following:

1) The intracellular accumulation of ANN is similar in sensitive and resistant cells (S/R index, 1.03), whereas the cellular accumulation of DOX and IDR is lower in resistant than in sensitive cells. The difference was more pronounced for DOX (S/R index, 2.6) than for IDR (S/R index, 1.9). It is noteworthy that measurement of the anthracycline's relative fluorescence level by flow cytometry or microscopy may not
represents precisely the total amount of drug in cell compartments because fluorescence is partially quenched, especially in the nucleus. However, we assumed that these phenomena take place to the same relative extent in both sensitive and resistant cells and should not influence evaluation of the S/R index.

(2) For each drug we found that differences in cellular drug accumulation and cytotoxicity correlated between the two cell lines; ANN showed almost unimpaired cytotoxicity against resistant cells (RI 2.6), whereas the resistance indices for IDR and DOX were 40 and 117, respectively. Previous studies showed anthracycline fluorescence to correlate well with external, intracellular (measured by extraction methods), and cytotoxic effects. As in our results, however, the relationship was not always linear. We believe that the divergence may be related to differences in subcellular drug localization. Higher drug concentration may first saturate the nonmatrix compartment and then lead to increased affinity for the nuclear matrix where DNA synthesis occurs. Simultaneous action of alternative mechanisms of resistance is another possible explanation; however, when we tested HL-60S and HL-60/DOX, we observed no differences in expression of the MDR-associated protein and the M, 110,000 protein (p110) recognized by MoAb LRP-56 (data not shown). Recently, we suggested that expression of genes related to the bcl-2 family in HL-60 is altered during development of anthracycline chemoresistance, which implicates multiple distinct mechanisms acting at various points to confer cell resistance to chemotherapy.

(3) VER affected neither ANN’s cellular accumulation nor cytotoxicity against resistant cells (no change in RI and S/R index), whereas VER restored almost completely the cellular accumulation of IDR and DOX in resistant cells, with a corresponding, although modest, decrease in the RI. This effect was greater for DOX than for IDR (4-fold reduction in RI for DOX versus 2-fold for IDA). Similar results have been obtained with other MDR1 cell lines selected for resistance to DOX or vinblastine in studies comparing ANN and DOX. None of the p-gp modulators tested significantly increased the accumulation or retention of ANN.
in either HL-60/DOX or KB-V1.49 Taken together, the results of this and previous studies strongly indicate that ANN may overcome p-gp–mediated resistance and that it is a potentially effective agent for treatment of human neoplasms, such as acute leukemia, malignant lymphoma, or breast carcinoma, in which expression of the MDR1 phenotype may be a clinically relevant mechanism of constitutive or acquired resistance.50,51

As we have said, the data suggested that additional mechanisms of resistance may be involved in the HL-60/DOX cell line. Although the same fluorescence levels were achieved for DOX and IDR in HL-60S and HL-60/DOX cells by using VER and for ANN even without VER, MDR was not completely circumvented. ANN was much more effective than DOX or IDR in overcoming resistance (Table 3). Therefore, ANN seemed to overcome such non-MDR1 mechanisms more efficiently than DOX or IDR. Ling et al52 have shown that DOX localizes predominantly in the nucleus of KB-3-1 cells, in contrast to ANN, which locates preferentially in the perinuclear region, Golgi apparatus, endoplasmic reticulum, and endosomes. However, Ling et al52 also showed by cell fractionation that nuclear ANN tends to localize preferentially in the nuclear matrix, unlike DOX, which distributes preferentially in the nonmatrix. Because topoisomerase (topo II) locates mainly in the nuclear matrix, and topo II–mediated mechanisms of anthracycline resistance have been described, ANN may be able to overcome such topo II–mediated mechanisms of resistance as a result of its different subnuclear distribution.

ANN differs from DOX at four positions in its chemical structure: (1) substitution at C-2′ with iodine, (2) hydroxylation at C-3′, (3) epimerization at C-4′, and (4) demethylation at C-4′. Previous studies showed that the replacement of the basic amino group at C-3′ by a hydroxyl is one of the primary reasons for ANN’s high in vivo and in vitro activity against MDR1 tumors.27,28,35 Information regarding the contribution of 4-demethylation at C-4 on the biological properties of this class of compounds can be derived from this study because we compared ANN and DOX with IDR (4-demethyldaunorubicin). IDR, which is currently approved for the treatment of acute leukemia, differs from DOX not only at position C-4 (4-demethylation) but also at C-14 (14-hydrogen versus 14-hydroxy). The lower RI and lower resistance uptake factor (S/R) of IDR, compared with those of DOX, can most likely be attributed to the 4-demethylation, because the 14-hydroxy modification that distinguishes DOX and DNR has not been consistently reported to confer those properties.22,52 This indicates that 4-demethylation might also contribute to ANN’s ability to partially overcome MDR1 and suggests that ANN may be useful in treating DOX-resistant leukemias.

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The novel anthracycline annamycin is not affected by P-glycoprotein-related multidrug resistance: comparison with idarubicin and doxorubicin in HL-60 leukemia cell lines

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