Comparative Cellular Pharmacology of Daunorubicin and Idarubicin in Human Multidrug-Resistant Leukemia Cells

By Ellin Berman and Mary McBride

We examined the effect of daunorubicin (DNR), the new anthracycline derivative idarubicin (IDR), and verapamil on two leukemia cell lines that displayed the multidrug resistant (MDR) phenotype and used laser flow cytometry to quantitate intracellular anthracycline content. The vinblastineresistant human lymphoblastic leukemia cell line CEM-VBL demonstrated minimal DNR uptake; simultaneous incubation with verapamil and DNR increased intracellular DNR uptake fourfold. IDR uptake was 10 times more rapid in these cells and simultaneous incubation with IDR and verapamil resulted in only a 1.2-fold increase of intracellular IDR. Similar results were observed in the vincristine-resistant human myeloid leukemia cell line HL-60/RV+. Intracellular retention of DNR and IDR was also measured in each cell line. In CEM-VBL cells, 38% of the original DNR concentration remained after a 2-hour resuspension in fresh medium compared with 71% of the original IDR concentration. In HL-60/RV+ cells, 36% of the DNR concentration remained compared with 51% of the IDR concentration. After incubation of CEM-VBL and HL-60/RV+ cells with DNR for 1 hour followed by resuspension in fresh medium plus verapamil, intracellular DNR retention increased 5- and 5.2-fold, respectively. However, incubation of these cells for 1 hour with IDR followed by resuspension in fresh medium plus verapamil resulted in only a 1.6- and 2.4-fold increase in intracellular IDR retention. Lastly, clonogenic experiments were performed to correlate intracellular anthracycline content with cytotoxicity. DNR alone had a minimal effect on the clonogenic growth of CEM-VBL cells, whereas the combination of DNR plus verapamil resulted in ~80% growth inhibition. However, incubation of these cells with IDR alone resulted in greater than 95% growth inhibition. These results suggest that IDR may be more effective than DNR in leukemia cells that display the MDR phenotype.

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Materials and Methods

Cell lines. The drug-sensitive parent cell lines CEM and HL-60 were obtained from the American Type Culture Collection (Rockville, MD). The CEM vinblastine-resistant cell line, CEM-VBL, display the MDR phenotype. We performed drug uptake and retention experiments using laser flow cytometry to quantitate intracellular anthracycline concentration in both a myeloid and lymphoid MDR cell line and compared the uptake patterns with the drug-sensitive parent cell lines. We also examined the effect of verapamil on anthracycline uptake and retention and performed clonogenic assays to determine whether the intracellular concentration of anthracycline obtained after drug uptake correlated with cytotoxicity. Results from these studies show that the intracellular uptake pattern of IDR in the MDR cells is nearly identical to that seen in the drug-sensitive parent cell lines, whereas the MDR cells have minimal DNR uptake compared with the drug-sensitive parent cells. IDR is also retained in the MDR cells to a greater degree than is DNR. Lastly, clonogenic growth in the MDR cells is minimally inhibited by DNR, whereas IDR is markedly toxic to these cells. Taken together, these results suggest that IDR may not be transported out of MDR cells via Pgp; this may in part account for the drug's improved clinical efficacy compared with DNR.

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was originally developed by Beck et al.18 The HL-60 vincristine-resistant cell line HL-60/RV+ was a kind gift from Dr Melvin Center (Kansas State University, Manhattan, KS). Both the CEM-VBL and HL-60/RV+ cell lines have been shown previously to have Pgp present on their surface membranes as measured by the monoclonal antibody HYB-241,19 whereas their respective drug-sensitive parent cell lines do not.20 All cell lines were kept in RPMI 1640 (GIBCO Laboratories, Grand Island, NY), supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; HyClone, Salt Lake City, UT) and 1% L-glutamine (GIBCO) and incubated at 37°C with 5% CO2 and 95% humidified air.

Drugs. DNR (Wyeth Laboratories, Philadelphia, PA) and IDR (Adria Laboratories, Dublin, OH) were dissolved in sterile water, and diluted with RPMI containing 10% FCS before each experiment. Verapamil was purchased from Knoll Pharmaceuticals (Whippany, NJ) and dissolved in sterile NaCl with 1.57% dimethylsulfoxide (DMSO) before each experiment. The final concentration of DMSO in the verapamil suspension was 0.008% in each experiment. Solutions containing verapamil were protected from light at all times by tin foil wrapping.

Flow cytometry. For determination of flow cytometric intracellular anthracycline concentrations, a FACScan (Becton Dickinson, Mountain View, CA) equipped with an argon laser using a 488-nm filter for the DNR uptake and retention studies and a 545/20-nm filter for the IDR uptake and retention studies.

Cellular uptake and retention studies. For uptake studies, cells at a concentration of 2 × 106 cells/mL were incubated with either DNR or IDR at a concentration of 1 μg/mL with or without verapamil 10 μmol/L. After 5, 15, 30, 45, and 60 minutes of incubation, cells were washed twice in ice-cold phosphate-buffered saline (PBS) without Ca2+ or Mg2+ (GIBCO) and resuspended in 0.5 mL ice-cold PBS with 5% FCS. Anthracycline fluorescence was immediately measured using the FACScan with the appropriate filter.

Drug retention studies were performed in separate experiments. To determine whether one anthracycline retained higher intracellular anthracycline levels compared with the other over a 2-hour "washout" period, cells were incubated for 1 hour with IDR or DNR at a concentration of 1 μg/mL. Cells were then washed twice and resuspended in fresh medium and flow cytometric analysis was performed at specified time points over a 2-hour period. The percent of original drug concentration was calculated as the fluorescent signal measured after the 2-hour resuspension divided by the original fluorescent signal.

To determine whether the addition of verapamil significantly increased intracellular anthracycline retention, cells were incubated for 1 hour with either DNR or IDR at a concentration of 1 μg/mL plus verapamil 10 μmol/L/mL; cells were then washed twice and resuspended in fresh medium containing verapamil. Flow cytometric analysis was then performed as above using the appropriate filter. The fold increase in anthracycline retention was calculated by dividing the fluorescent signal after the 2-hour resuspension of anthracycline plus verapamil by the fluorescent signal of the anthracycline alone measured at the same time point.

All uptake and retention experiments were performed in duplicate and a representative experiment is shown for each. Controls were medium alone and medium with verapamil 10 μmol/L.

Clonogenic assays. Cells at a concentration of 2 × 105 cells/mL were incubated with either medium, DNR 1 μg/mL, IDR 1 μg/mL, verapamil 10 μmol/L or the combination of anthracycline plus verapamil for 1 hour. Aliquots of test samples were then washed twice and plated in triplicate at a concentration of 250 cells per plate.

Culture medium used for cloning consisted of 1 mL of Iscove’s modified Dulbecco’s medium (IMDM; GIBCO) containing 24% FCS, 0.8% deionized bovine serum albumin (BSA; Sigma Chemicals, St Louis, MO), 10-4 mol/L 2-mercaptoethanol (Sigma), and methylcellulose at a final concentration of 1.3% in 35-mm Lux tissue culture dishes (Miles Scientific, Naperville, IL). Culture plates were incubated in a humidified atmosphere of 5% CO2 in air for 10 to 12 days and scored using an inverted microscope. Aggregates ≥ 40 cells were considered colonies. All experiments were performed in triplicate.

Controls for the clonogenic experiments were as follows: medium alone served as controls for experiments using DNR and IDR and medium with 0.008% DMSO served as controls for experiments using verapamil.

RESULTS

Rate of anthracycline uptake and effect of verapamil on anthracycline uptake. Figure 1A shows DNR and IDR uptake in CEM-VBL cells exposed to either DNR or IDR.
alone at a concentration of 1 μg/mL or in combination with verapamil 10 μmol/L. The rate of anthracycline uptake during the first 10 minutes of incubation was calculated by dividing the change in fluorescent signal by elapsed time. The rate of IDR uptake was ~10 times that of DNR (96/10 vs 9/10) in these cells.

Incubation with DNR resulted in minimal drug uptake; however, in the presence of verapamil DNR uptake increased approximately fourfold (Table 1). Cells exposed to IDR had a 1.2-fold increase in uptake enhancement after coincubation with verapamil (Table 1).

Similar findings were noted in the HL-60/RV+ cell line (Fig 1B). In this instance, the rate of IDR uptake was four times that of DNR (60/10 vs 15/10). The addition of verapamil increased DNR uptake 5.3-fold, whereas IDR alone at a concentration of 1 pg/mL or in combination with verapamil increased DNR uptake 5.3-fold, whereas IDR alone at a concentration of 1 pg/mL or in combination with verapamil increased DNR uptake 5.3-fold, whereas IDR alone at a concentration of 1 pg/mL or in combination with verapamil (Table 1).

Comparative anthracycline retention after 2-hour resuspension. As shown in Fig 2A, CEM-VBL cells incubated with DNR for 1 hour and resuspended in fresh medium for 2 hours showed a rapid reduction in intracellular drug level; approximately 38% of the original DNR concentration remained in these cells at the end of 2 hours (Table 1). The addition of verapamil resulted in a fivefold increase in the intracellular DNR retention. In contrast, IDR retention remained at a more constant level in the CEM-VBL cells (Fig 2A), with 71% of the original intracellular IDR concentration remaining after a 2-hour resuspension in fresh medium (Table 1). Incubation of these cells with IDR and verapamil led to only a 1.6-fold increase in IDR retention (Table 1).

Similar studies were performed in HL-60/RV+ cells (Fig 2B). Thirty-six percent of the original DNR concentration remained in these cells after a 2-hour “washout” period in fresh medium, whereas 51% of the original IDR concentration remained (Table 1). Cells incubated with DNR and verapamil for 1 hour, washed, and resuspended in fresh medium with verapamil had a 5.2-fold greater DNR retention after a 2-hour resuspension, whereas verapamil increased IDR retention only 2.4-fold (Table 1).

Anthracycline uptake in the drug-sensitive parent cell lines. The parent cell lines CEM and HL-60 were also studied for DNR and IDR uptake, as described above. Figure 3A shows DNR uptake with and without verapamil in CEM cells and CEM-VBL cells. Compared with the CEM-VBL cells, CEM cells showed prompt DNR uptake and verapamil did not increase the intracellular DNR concentration over baseline. The addition of verapamil to CEM-VBL cells increased DNR uptake to levels approaching that of the drug-sensitive parent cell line as shown.

Figure 3B shows the same experiment performed with IDR in CEM and CEM-VBL cells. In this instance, the intracellular IDR concentration in the MDR subline was nearly identical to that of the drug-sensitive parent cell line. As shown earlier, the addition of verapamil did not increase the intracellular IDR concentration in the MDR cell line.

The same set of experiments was performed with HL-60 and HL-60/RV+ cells with very similar results observed (data not shown).

Clonogenic growth of CEM, CEM-VBL, HL-60, and HL-60/RV+ cells after exposure to anthracycline alone or in combination with verapamil. Clonogenic assays were performed to determine the relationship between anthracycline uptake and cytotoxicity after cells were exposed for 1 hour to either DNR, IDR, or each anthracycline with verapamil. Cells exposed to medium alone were designated as 100% colony growth as there was no difference in growth between medium alone and medium containing DMSO 0.008%, which served as control for cells incubated with verapamil (data not shown). As shown in Fig 4A, exposure of both CEM and CEM-VBL cells to verapamil alone did not change the percent of clonogenic growth (panels 3 and 4). Exposure of CEM-VBL cells to DNR resulted in minimal inhibition (panel 5), whereas CEM cells exposed to DNR displayed less than 5% growth (panel 6). In contrast, IDR was equally inhibitory to both CEM-VBL and CEM cells (panels 7 and 8). When CEM-VBL cells were exposed to the combination of DNR plus verapamil, clonogenic growth decreased to ~20% of control growth (panel 9); because IDR was by itself markedly inhibitory to CEM-VBL colony growth, no additive effect was seen with the addition of IDR plus verapamil (panel 11).

Figure 4B shows the results of identical experiments performed with HL-60 and HL-60/RV+ cells were very similar to those performed with the CEM and CEM-VBL cells. In this instance, DNR incubation alone in HL-60/RV+ cells led to ~50% growth inhibition (panel 5); significant inhibition in this cell line was only seen with the combination of DNR plus verapamil (panel 9). Similar to the CEM-VBL growth pattern, however, IDR alone had a marked inhibitory effect (panel 7).

**DISCUSSION**

In this report, we show that the anthracycline analogue IDR is more toxic in two leukemia cell lines that display the MDR phenotype than is the parent compound DNR. While the intracellular concentrations of each anthracycline could not be directly compared with each other because the different fluorescent emission pattern of each anthracycline

<table>
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<th>Table 1. Comparison of DNR and IDR Uptake and Retention as Measured by Fluorescence in CEM-VBL and HL-60/RV+ Cells</th>
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<td><strong>Anthracycline (1 μg/mL)</strong></td>
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*Calculated as fluorescent signal of anthracycline measured at 2 hours/anthracycline measured at time 0.
†Calculated as fluorescent signal of anthracycline + verapamil/anthracycline measured at 1 hour.
required a separate filter, some important observations can nonetheless be made.

First, incubation of CEM-VBL cells with IDR alone resulted in an uptake pattern almost identical to the drug-sensitive parent cell line CEM (Fig 3B). However, DNR by itself showed little uptake in the CEM-VBL cells and required the presence of the MDR blocking agent verapamil to increase intracellular concentration to levels that approached the drug-sensitive parent cell line (Fig 3A).

Second, the degree to which verapamil could maintain intracellular drug accumulation differed between the two anthracyclines (Table 1). The amount of retained DNR was increased approximately fivefold after a 2-hour resuspension in fresh medium containing verapamil compared with resuspension in fresh medium alone, whereas in intracellular IDR the retained concentration increased approximately twofold over baseline in the same experiment.

Lastly, cytotoxicity was closely correlated with anthracycline uptake in the clonogenic experiments, as shown in Fig 4A and B. CEM-VBL cells were impervious to the cytotoxic effects of DNR and required the presence of verapamil to effect a significant degree of growth inhibition. However, IDR was as toxic to CEM-VBL cells as it was to the drug-sensitive parent cells CEM, with clonogenic growth in each less than 5% of control. A similar pattern of inhibition to DNR, DNR plus verapamil, and IDR was seen in the HL-60 and HL-60/RV* cells.

That anthracycline analogues other than IDR are able to overcome MDR has been previously described with the morpholino derivative 3'-deamino-3'-morpholino-13-deoxy-10-hydroxycarminomycin (MX2) and the adriamycin derivatives 3'-deamino-3'-(4'-morpholinyl) adriamycin (MRA) and 3'-deamino-3'(3-cyano-4'-morpholinyl) adriamycin (MRA-CN). Watanabe et al performed uptake experi-
Fig 4. Clonogenic growth of (A) CEM-VBL and CEM and (B) HL-60/RV+ and HL-60 cells after incubation with medium (M), verapamil (V), DNR plus medium (DNR), IDR plus medium (IDR), or each anthracycline in combination with verapamil (+V). Control growth is designated as 100% in the bar graph. See text for details. Cells were incubated for 1 hour in medium or designated drug or drug combination. Aliquots were then washed twice and plated in triplicate at a concentration of 250 cells/plate. Colony growth was measured after 10 days of incubation and the percent of control growth is shown.

ments with MX2, Adriamycin, and each anthracycline with verapamil in the Adriamycin-resistant leukemia cell line K562. Similar to the results of the IDR experiments described here, verapamil markedly increased the Adriamycin intracellular concentration, whereas it had little effect on the intracellular MX2 concentration. Moreover, the steady-state concentration of MX2 was eight times higher than Adriamycin in these cells. Streeter et al. have shown that the morpholino derivatives MRA and MRA-CN have cytotoxicity comparable with Adriamycin in the drug-sensitive parent cell line P388, whereas Adriamycin showed little uptake in the drug-resistant subtype P388/ADR. Both morpholino derivatives, however, had increased cellular uptake and retention relative to Adriamycin in the P388/ADR cells; verapamil significantly increased the intracellular Adriamycin concentration, whereas it had little effect on drug uptake of the two derivatives.

Reasons why IDR, in addition to these three anthracycline derivatives, appears able to overcome the MDR phenotype remain speculative. However, one important factor may be the high lipophilic coefficient each of these analogues share. In this regard, Friche et al. have shown a close correlation between the lipid solubility of anthracycline analogues, including IDR, and the ability of these analogues to increase [3H]DNR accumulation in MDR-resistant cells. In addition, Sehested et al. have shown that DNR, unlike vincristine and verapamil, does not inhibit azidopine binding to Pgp in the MDR Erlich ascites tumor cell line. These studies suggest that Pgp may not be the only transport mechanism for anthracyclines, especially perhaps the more lipophilic analogues, in MDR cells. Alternatively, the more lipophilic anthracyclines may have a differential effect on topoisomerase II, low levels of which have recently been shown to coexist with overexpression of Pgp in MDR cells; however, this theory remains untested.

That the data presented in this current series of experiments with IDR may correlate with clinical activity is suggested by three recently reported prospectively randomized clinical trials in adult AML in which the efficacy of IDR combined with Ara-C was compared with that of DNR and Ara-C. The results of these trials are outlined in Table 2. More patients treated on the IDR/Ara-C arm achieved remission than did patients on the DNR arm; this was of variable statistical significance, as shown. Of note, however, is the consistent finding that fewer patients on the IDR/Ara-C arm had primary refractory disease compared with patients on the DNR/Ara-C arm; this proved highly significant in each study. In addition, three recently completed European trials that have randomized patients to either DNR or IDR or rubidazone and IDR have shown a lesser incidence of primary refractory disease in patients on the IDR arm.

However, it is also possible that the in vivo pharmacokinetic properties of IDR contribute to the difference in clinical response in these studies. Speth et al. have shown that IDR is metabolized to an active metabolite, idarubicin-ol, which has a prolonged plasma half-life compared with the main DNR metabolite, daunorubicin-ol. Idarubicin-ol has been shown to have in vitro and in vivo activity equal to the parent compound itself. However, the formation of

| Table 2. Primary Failure Rates in Patients Treated With IDR/Ara-C Compared With DNR/Ara-C |
|---|---|---|---|
| Patients | MSKCC12 | US Multicenter13 | SEG14 |
| No. entered | IDR | DNR | P |
| No. complete remission | 60 | 60 | 97 | 111 | .005 |
| No. persistent blasts | 48 (80) | 35 (58) | 68 (70) | 65 (59) | .098 |
| No. complete remission | 103 | 106 | .004 |
| No. persistent blasts | 13 (13) | 26 (25) | .03 |

Percentages in parentheses.
idarubicin-ol would not play a role in the in vitro experiments described here as the liver is the site of this biodegradation.

It is also important to note that the studies outlined here were performed at drug concentrations much higher than those achievable in vivo. However, preliminary data repeating these experiments using DNR and IDR plasma levels, 0.2 μg/mL and 0.05 μg/mL, respectively, suggest that the same phenomenon exists at these concentrations (E. Berman, unpublished data).

In summary, this report suggests that the clinical efficacy of IDR compared with DNR may be based in part on the differential effect of the former anthracycline on MDR cells. As this form of drug resistance may be a potential cause of primary drug resistance in acute leukemia, further exploration of drugs that appear to circumvent this phenomenon is warranted.

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