Cellular Pharmacology of N,N-Dimethyl Daunorubicin and N,N-Dimethyl Adriamycin

Merrill J. Egorin,1 Ronald E. Clawson, Louis A. Ross, and Nicholas R. Bachur

Laboratory of Clinical Biochemistry, Baltimore Cancer Research Program, Division of Cancer Treatment, National Cancer Institute, NIH, Baltimore, Maryland 21201

ABSTRACT

The cellular accumulation and disposition of the anthracycline antibiotics daunorubicin (DNR) and Adriamycin (ADR) were compared to those of their N,N-dimethyl derivatives. The cellular accumulation of N,N-dimethyl daunorubicin (DMD) was greater than that of N,N-dimethyl adriamycin (DMA) which was greater than the accumulation of DNR or ADR. DNR- and ADR-resistant P388 murine leukemia cells accumulated less of each drug than did DNR- and ADR-sensitive P388 murine leukemia cells. The presence of 15% fetal bovine serum in the incubation medium did not affect the accumulation of DNR, increased the accumulation of DMD by 20 to 25%, and reduced that of ADR and DMA by 20 to 25%. Lowered temperature (0°C) reduced the intracellular content of DMD and DMA to a lesser extent than that of DMA. The loss of all four drugs from cells placed in drug-free buffer, in drug-free medium did not affect the accumulation of DNR, increased the accumulation of DMD by 20 to 25%, and reduced that of ADR and DMA by 20 to 25%. Lowered temperature (0°C) reduced the intracellular accumulation of all four drugs. Sodium azide (10 mM) did not alter the cellular accumulation of DNR or ADR but reduced the intracellular content of DMD and DMA to a lesser extent than that of DMA. The loss of all four drugs from cells placed in drug-free medium was greatly reduced at 0°C compared to 37°C. Sodium azide (10 mM) increased the efflux of DMD but did not affect that of DNR, ADR, or DMA. Unlike intact L1210 cells, isolated nuclei accumulated more ADR than DNR and more DMA than DMD. The nuclear accumulation of DMA > ADR > DMD > DNR. Nuclear accumulation of all four drugs reached equilibrium by 10 to 30 min and was the same at 0°C and 37°C. All four drugs were lost from nuclei placed into drug-free buffer, and this loss was reduced at 0°C. DMD and DMA were neculearly localized as were DNR and ADR. All four drugs produced dose-dependent inhibition of [3H]uridine incorporation, and DMA and DMD inhibited DNR- and ADR-resistant P388 cells to the same degree as did DNR- and ADR-sensitive P388 cells. DNR and ADR inhibited [3H]thymidine incorporation more than [3H]uridine incorporation, whereas DMA and DMD inhibited these processes to the same degree.

INTRODUCTION

A major research endeavor in drug development for the past 10 years has been the search for natural anthracycline antibiotics and the synthesis of analogs that possess antitumor activity but lack myocardial toxicity (1–3, 7, 10, 16, 19, 23, 25, 33, 34). Recent studies indicate that N,N-dimethyl substitution of the daunosamine residue of DNR and ADR produces compounds which are active against a number of animal tumors (17) and are less cardiotoxic in rabbits than are DNR and ADR. Although pharmacological studies of N-acetylated, N-formylated, and N-trifluoroacetylated anthracycline antibiotics have been described, N-alkylated compounds have not been investigated. Since the cellular pharmacology of these compounds was undefined, we compared them to each other as well as to their parent compounds with regard to cellular drug accumulation, metabolism, disposition, and in vitro inhibition of macromolecular biosynthesis.

MATERIALS AND METHODS

Cell Lines. L1210 murine leukemia cells were maintained in vitro by serial culture in RPMI Medium 1630 (NIH Media Unit, Bethesda, Md.) containing penicillin (50 units/ml), streptomycin (50 μg/ml), and L-glutamine (2 μmol/ml) (Flow Laboratories, Inc., Rockville, Md.) and 15% FBS (Flow Laboratories) (Medium A). Under these conditions, cells had a population-doubling time of 14 to 18 hr and reached a maximum cell density of 1.5 to 2.0 x 10^6 cells/ml.

Two strains of P388 murine leukemia, P388/S and P388/ADR (18, 21), were obtained from Dr. Randall Johnson, National Cancer Institute, Bethesda, Md., and maintained in vitro by serial culture in RPMI Medium 1630:RPMI Medium 1640 (1:1), containing 0.01 mm mercaptoethanol, 15% FBS, and penicillin, streptomycin, and L-glutamine as above (Medium B). Under these conditions, both the P388/S and P388/ADR cells had population-doubling times of 12 to 14 hr and achieved maximum cell densities of 2.0 to 2.6 x 10^6 cells/ml. Except where noted, all experiments with L1210 cells were performed in Medium A, and all experiments with P388 cells were performed in Medium B.

L929 mouse fibroblasts, obtained from the American Type Culture Collection (Rockville, Md.) were maintained in vitro by serial culture as described previously (27).

Isolation of Human Neutrophils. Peripheral venous blood was collected on the day of the experiment in a syringe containing heparin (10 units/ml) and centrifuged at 100 x 9 for 5 min. The platelet-rich plasma supernatant was discarded, and the remaining cells were sedimented by centrifugation at 3400 x g for 5 min. The cells were resuspended in an equal volume of hydroxyethyl starch (6 g/100 ml) in 0.154 M NaCl (Voxel; McGaw Labs, Miledgeville, Ga.) and allowed to sediment at 1 x 9 for 60 min. The resulting supernatant was collected, and unsedimented erythrocytes were osmotically lysed by addition of 2 volumes of 0.15 M NH₄Cl at 4°C. Leukocytes, sedimented by centrifugation at 175 x 9 for 8 min at 4°C, were resuspended in 36 ml 0.154 M NaCl, layered on a Ficoll (Sigma Chemical Co., St. Louis, Mo.):Hypaque (Winthrop Laboratories, New

1 To whom requests for reprints should be addressed.

2 The abbreviations used are: DNR, daunorubicin; ADR, Adriamycin; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; P388/S, P388 murine leukemia sensitive to Adriamycin and daunorubicin; P388/ADR, P388 murine leukemia resistant to Adriamycin and daunorubicin; DMD, N,N-dimethyl daunorubicin; DMA, N,N-dimethyl adriamycin; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.

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York, N. Y.) gradient (8), and centrifuged at 400 × g for 40 min at room temperature. The final pellet, consisting of greater than 95% neutrophils, was resuspended in RPMI Medium 1640 and used in drug uptake studies.

Drugs. DNR (NSC 82151), ADR (NSC 123127), DMD (NSC 258812), and DMA (NSC 261045) were obtained from the Developmental Therapeutics Program, National Cancer Institute, Bethesda, Md. The purity of these materials was confirmed by TLC (9) and HPLC. The HPLC system consisted of a Spectra Physics Model 3500B HPLC (Spectra Physics, Santa Clara, Calif.), fitted with a µBondapak phenyl column (3.9 mm × 30 cm) (Waters Associates, Milford, Mass.) and a 10-min gradient of 15 to 50% tetrahydrofuran in 0.1 g per 100 ml ammonium formate buffer, pH 4.0, at a flow rate of 2 ml/min.

Fluorescence Spectra. Fluorescence excitation and emission spectra were obtained with a Model SPF-100CS absolute spectrofluorometer (American Instrument Co., Silver Spring, Md.).

Incubation Conditions. Tumor cells were washed twice with 0.154 M NaCl and resuspended in fresh medium, and 10⁶ cells were preincubated for 60 min prior to addition of drugs. The final incubation volume was 1 ml and, unless indicated, all incubations were performed at 37°, with 5% CO₂, and 95% humidity.

Assay of Drug Uptake and Metabolism. After incubation, 10 ml of iced 0.154 M NaCl were added to the incubation mixture, and the cells were centrifuged at 2500 × g for 5 min at 4°. The resulting cell pellets were washed with another 10 ml of iced 0.154 M NaCl before being resuspended in 2 ml of 0.3 N HCl in 50% ethanol. Fluorescence was determined with excitation at 470 nm and with emission at 585 nm, and drug content was calculated by comparison with simultaneously performed DNR, ADR, DMD, and DMA standards (5). In drug metabolism experiments, cell pellets were extracted with iced chloroform:methanol (2:1) (9). The extracts were evaporated to dryness under a nitrogen stream, redissolved in small volumes of methanol, and analyzed with TLC (9) and HPLC.

Efflux Experiments. After incubation with 10 µM drug for 120 min, cells were washed twice with 10 ml of iced 0.154 M NaCl and resuspended in 10 ml of drug-free medium at either 0° or 37° with or without 10 mM sodium azide. After varying periods of incubation at 0° or 37°, cells were sedimented, and the remaining drug content was determined fluorometrically.

Drug Uptake and Efflux from Isolated Nuclei. Nuclei were isolated by homogenization of L1210 cells after swelling in hypotonic buffer (28). Studies of drug uptake and export by nuclei were performed as described above for whole cells except a buffer (50 mM Tris, 92 mM NaCl, 5 mM KCl, 1.3 mM MgSO₄, and 4 mM CaCl₂, pH 7.4) was used in place of medium and 0.154 M NaCl.

Fluorescence Microscopy. Cells incubated with DNR, ADR, DMD, or DMA were washed once or twice with 0.154 M NaCl, and the pellet was resuspended in a small volume of 0.154 M NaCl. Cell suspensions were examined immediately with an American Optical Model 10 microscope fitted with an HBO 50 mercury arc lamp, an FITC interference filter for incident illumination, and 500 and 515 nm secondary filters. Photomicro-

RESULTS

Fluorescence Spectra. DNR, ADR, DMD, and DMA all had fluorescence spectra identical to that described previously for DNR (5) with activation maximum at 470 nm and 2 emission maxima at 554 and 585 nm.

Drug Purity. On Silica Gel G thin-layer plates, 1-nmol samples of all 4 compounds chromatographed as single spots (Table 1). Similarly, HPLC of 1-nmol samples of each compound showed all fluorescence eluting as a single peak for each drug.

Drug Accumulation by Whole Cells. The time course of accumulation was similar for all 4 drugs (Chart 1). As described previously, the cellular accumulation of DNR exceeded that of ADR [p < 0.05 (Chart 1; Refs. 6, 24, 26, and 31)]. Not only did the accumulation of both DMD and DMA greatly exceed that of their parent compounds (p < 0.05), but the accumulation of DMA was 2 to 3 times that of DNR [p < 0.05 (Chart 1)].

The presence of FBS in the incubation medium affected the cellular accumulation of these 4 drugs in different ways (Chart 1). Although the accumulation of DNR was not altered by FBS, that of DMA was enhanced by 20 to 25% (p < 0.05), while that of DMD was reduced by 20 to 25% (p < 0.05).

The uptake of all 4 anthracyclines was dose dependent.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>TLC system 1</th>
<th>TLC system 2</th>
<th>K' (HPLC system)</th>
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</thead>
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<tr>
<td>DNR aglycone</td>
<td>1.00</td>
<td>0.32</td>
<td>8.88</td>
</tr>
<tr>
<td>Daunorubicinol aglycone</td>
<td>1.00</td>
<td>0.32</td>
<td>7.25</td>
</tr>
<tr>
<td>DNR</td>
<td>0.64</td>
<td>0.00</td>
<td>7.88</td>
</tr>
<tr>
<td>DMD</td>
<td>0.59</td>
<td>0.00</td>
<td>7.07</td>
</tr>
<tr>
<td>Daunorubicinol</td>
<td>0.48</td>
<td>0.00</td>
<td>6.75</td>
</tr>
<tr>
<td>DMD metabolite</td>
<td>0.44</td>
<td>0.00</td>
<td>5.73</td>
</tr>
<tr>
<td>N,N-Dimethyl daunorubicinol</td>
<td>0.34</td>
<td>0.00</td>
<td>5.07</td>
</tr>
<tr>
<td>ADR</td>
<td>0.45</td>
<td>0.00</td>
<td>6.63</td>
</tr>
<tr>
<td>DMA</td>
<td>0.31</td>
<td>0.00</td>
<td>4.70</td>
</tr>
</tbody>
</table>

(Chart 2) and, at any given nonsaturating drug concentration, the uptake of DMD and DMA was 3 to 5 times that of their respective parent compounds. Although the accumulation of DMD > DMA > DNR > ADR in each cell type studied, P388/ADR cells accumulated less of each drug than did P388/S cells (p < 0.05). The cellular uptake of DNR and ADR has previously been reported to be temperature sensitive (22, 24). As with DNR and ADR, the accumulation of DMD and DMA was also temperature dependent, the uptake at 0° being much less than that at 37° (p < 0.05 (Chart 3)). As reported previously (12, 31), 10 mM sodium azide had no effect on the intracellular accumulation of DNR or ADR (Chart 4). On the other hand, sodium azide reduced the intracellular content of both DMD and DMA, although only the reduction in DMD was statistically significant (p < 0.05 (Chart 4)).

All 4 compounds exited the cells by a process that was reduced at 0° compared to 37° (p < 0.05 (Chart 5)). Sodium azide (10 mM) did not alter the efflux of DNR, ADR, or DMA from cells; however, it significantly hastened the efflux of DMD (p < 0.05) at 180 and 240 min (Chart 5).

Fluorescence microscopy allowed evaluation of the intracellular distribution of DNR, ADR, DMD, and DMA. DNR and ADR, as described previously (13, 30), were concentrated in cell nuclei with minor cytoplasmic localization (Fig. 1, A and B). DMD and DMA behaved as did their respective parent compounds (Fig. 1, C and D). These distribution patterns were apparent after 5 min of incubation of cells with drugs, and the pattern did not change during incubations of up to 120 min. These characteristic intracellular drug localization patterns were most easily defined in human neutrophils but were also observed in L1210, P388/S, and P388/ADR leukemic cells and L929 fibroblasts.

**Drug Uptake by Isolated Nuclei.** Unlike intact L1210 cells, L1210 cell nuclei accumulated more ADR than DNR (p < 0.05) and more DMA than DMD [p < 0.05 (Chart 6)]. Whereas the
uptake of DNR and DMA by nuclei was essentially the same as the uptake of these drugs by an identical number of whole cells, nuclei accumulated much more ADR (p < 0.05) and much less DMD (p < 0.05) than did the equal number of intact cells. Similar to intact cells, L1210 nuclei accumulated far more of each N,N-dimethyl derivative than its respective parent compound; the intranuclear accumulation of DMA > DNR > DMD > DNR (Chart 6). Nuclear accumulation of each drug reached equilibrium after 10 to 30 min as opposed to 120 to 180 min for whole cells (Chart 6). These patterns of nuclear accumulation were not affected by temperature, being identical at 0° (data not shown) and 37°. All 4 drugs were lost from drug-loaded nuclei which were placed into drug-free buffer. Although lowering the temperature to 0° reduced the loss of drug from nuclei (p < 0.05), this reduction was much less than that seen when whole cells were treated similarly (Charts 5 and 6).

Metabolism of Anthracyclines. TLC and HPLC of drug extracted from L1210 cells incubated with ADA and DMA showed all cellular fluorescent material to be either ADA or DMA, respectively. More specifically, there was no conversion of DMA to ADA and no conversion of either drug to aglycone or to more polar metabolites such as adriamycinol. Extraction of drug from L1210 cells incubated with DNA or DMD showed less than 5% conversion of both drugs to more polar metabolites. Whereas the DNA metabolite cochromatographed with daunorubicinol, the known principal metabolite of this drug (Table 1; Ref. 4) DMD was metabolized to a material more polar than daunorubicinol but less polar than N,N-dimethyl daunorubicinol which is produced by incubating DMD with daunorubicin reductase (Table 1; Ref. 14). No aglycone me-
DISCUSSION

Recognition of the cardiotoxicity associated with DNR and ADR therapy has prompted a concerted effort to develop active analogs of these compounds which would spare the myocardium. As a result, numerous naturally occurring and chemically modified anthracyclines have been produced and studied to various degrees on preclinical and clinical levels (1–3, 7, 10, 11, 16, 19, 23, 25, 29, 34). From these studies, it is apparent that relatively minor molecular alterations may produce major differences in cellular and organismic drug metabolism and disposition. Our studies with the N,N-dimethyl derivatives of DNR and ADR are consistent with this concept. Conversion of daunosamine from a primary to a tertiary amine by N,N-dimethylation greatly enhances the cellular accumulation of the resultant DMD and DMA. However, closer inspection reveals 4 patterns of relationships in the cellular pharmacology of DNR and ADR and their N,N-dimethyl derivatives.

In some respects, all 4 anthracyclines behave similarly regardless of the chemical nature of the amino sugar. For example, the cellular accumulation and efflux of all 4 drugs are temperature dependent, being much less at 0° than at 37°. Also, there is a similar handling of all 4 compounds by nuclei. Nuclear drug accumulation of DNR, ADR, DMD, and DMA is the same at 0° as at 37°. In addition, P388/ADR cells accumulate less of each anthracycline than do P388/S cells. Finally, fluorescence microscopy shows that parent drugs and N,N-dimethyl derivatives are all restricted to cell nuclei and that the increased intracellular amounts of DMD and DMA do not represent additional cytoplasmic localization of these compounds.

In other aspects of cellular metabolism and disposition, the behavior of DMD or DMA shows more similarity to that of its parent compound than to that of the other N,N-dimethyl derivative. More specifically, cellular accumulation of DNR is greater than ADR and that of DMA exceeds that of DNR and DMD. Conversely, nuclei accumulate more ADR and DMA than DNR and DMD. FBS reduces the cellular accumulation of both ADR and DMA but enhances that of DMD. Finally, DMA, like its parent drug, is metabolized to a more polar metabolite, while ADR and DMA undergo no such transformation in these cell preparations.

In certain cellular processes, both DMD and DMA demonstrated properties not found in either parent compound. The addition of 2 methyl groups to the amino sugar of DNR or ADR caused a great increase in intracellular drug content. The cellular accumulation of DMD and DMA is reduced by 10 mM sodium azide, an agent that does not alter the cellular accumulation of DNR or ADR (12, 32). DMD and DMA differ from DNR and ADR in their ability to inhibit tumor cell incorporation of [3H]thymidine and [3H]uridine into trichloroacetic acid-precipitable material. The most dramatic differences are the sensitivity of P388/ADR cells to DMD and DMA (Table 2). This is interesting in view of the reduced accumulation of each drug by P388/ADR compared to P388/S cells. On the other hand, this in vitro sensitivity must be considered in light of the reports

Inhibition of Cellular Macromolecular Synthesis. All 4 anthracyclines produced dose-dependent inhibition of [3H]thymidine and [3H]uridine incorporation by L1210 and P388 cells. Despite the much greater intracellular concentrations of DMD and DMA (Chart 2), there was little difference in the ability of all 4 drugs to inhibit [3H]thymidine incorporation by L1210 cells (Table 2). Although [3H]thymidine incorporation in P388/S cells proved more susceptible to DMA than to ADR, the most striking finding was that DMA and DMD inhibited [3H]thymidine incorporation by P388/ADR and P388/S cells to the same degree (Table 2).

DMD and DMA differed from DNR and ADR in several ways with respect to inhibiting [3H]uridine incorporation. Unlike DNR and ADR, which inhibited [3H]thymidine incorporation more effectively than [3H]uridine incorporation, DMD and DMA inhibited these processes to the same degree (Table 2). In addition, [3H]uridine incorporation in P388/ADR cells appeared less resistant to DMD and DMA than to DNR and ADR (Table 2).

In conclusion, the N,N-dimethyl derivatives of DNR and ADR are more active and specific cytoplasmic localization of these compounds.


of Johnson et al. (20, 21), who demonstrated no improved survival over control of DMD- and DMA-treated mice bearing ascitic P388/ADR tumor. N,N-Dimethylation of DNR and ADR produces derivatives that are equally effective in inhibiting [3H]thymidine and [3H]uridine incorporation. This is in contrast to the parent compounds which inhibit the former process more effectively than the latter (Table 2; Refs. 6, 24, and 33).

The final type of relationship evident in the interaction of DNR, ADR, DMD, and DMA with cells concerns DMD. This compound had properties not shared either with its parent compound or the other N,N-dimethyl derivative studied. Only DMD has its cellular accumulation increased by FBS and its cellular egress enhanced by 10 mM sodium azide. The only anthracycline that we find that behaves similarly to DMD in these respects is 7-[(R)]-O-methyl nogarol, a semisynthetic derivative of the naturally occurring anthracycline, nogalamic (12). Although its structure includes an N,N-dimethyl sugar, it has little other structural similarity with DMD. Moreover, 7-[(R)]-O-methyl nogarol is cytoplasmically localized in contrast to DMD (12).

In summary, we have characterized the cellular pharmacology of DMD and DMA, 2 promising new anthracycline antitumor agents with several novel properties. Further studies in our laboratory have been directed at defining the tissue distribution, excretion patterns, and plasma pharmacokinetics of these 2 drugs when administered to animals.

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