Cytofluorescence Localization of Anthracycline Antibiotics

Merrill J. Egorin, Ronald E. Clawson, Jeffery L. Cohen, Louis A. Ross, and Nicholas R. Bachur

ABSTRACT

Previous fluorescence microscopic studies have shown daunorubicin (DNR) and Adriamycin (ADR) are localized in cell nuclei, whereas trifluorocetyladracyramycin-14-valerate is localized in the cytoplasm. Using cultured cells, we correlated structural characteristics of a series of anthracycline antibiotics with cellular disposition and metabolic response. When observed under fluorescence microscopy, DNR, ADR, N,N-dimethyladriamycin, N,N-dimethyladriamycin, and 4'-epiadiamycin localized in cell nuclei. When observed under fluorescence microscopy, N-acetyl daunorubicin, N,N-dimethyladriamycin, 3',4'-diacetyldeaminodaunorubicin, NSC 200681, triferric ADR, aclacinomycin A, marcellomycin, noxamycin, carminomycin, 4-demethoxydaunorubicin, nogalamycin, nogamycin, and 7-con- and 7-dis-O-methyl nogarol were localized in the cytoplasm. 3'-Deaminodaunorubicin, N-formyladriamycin, and 13-amidonaunorubicin were observed in both nucleus and cytoplasm. Therefore, alterations at ring positions 4 and 9 and on the glycoside amino group were critical in determining intracellular drug localization as assessed by fluorescence microscopy. Total cellular drug accumulation was not related to microscopically determined intracellular location. For nuclearily localized drugs, accumulation of N,N-dimethyladriamycin > N,N-dimethyladriamycin > DNR > 4'-epiadiamycin > ADR. For cytoplasmically and nonspecifically localized compounds, accumulation of musetamyacin > carminomycin > 7-con-O-methyl nogarol > 4'-demethoxy-daunorubicin > NSC 200681 > marcellomycin > aclacinomycin A > nogalamycin = 13-aminoadracyramycin > nogamycin > 7-dis-O-methyl nogarol > N-formyladriamycin > N-acetyladiamycin. The accumulation of these drugs by isolated L1210 cell nuclei did not correlate with their cellular accumulation or cytofluorescence localization. Although the compounds the fluorescence of which was most severely quenched by DNA, RNA, or isolated L1210 nuclei were among those localized by microscopy to the cytoplasm, the quenching of many cytoplasmic drugs was comparable to that of nuclear ones. Both nuclear and cytoplasmically localized drugs inhibited L1210 cell [3H]thymidine and [3H]uridine incorporation without relationship to intracellular disposition or total accumulation. Thus, modification of specific sites on the anthracycline molecule are correlated with intracellular drug localization as defined by fluorescence microscopy, but the effects of these modifications on other aspects of drug accumulation and cell macromolecular biosynthesis are much less predictable.

INTRODUCTION

The anthracycline antibiotics represent a major group of useful antineoplastic agents (7, 10, 11, 14, 16, 26) (Chart 1). Major research efforts have been directed toward elucidating the mechanism of action and clinical pharmacology of known anthracycline compounds, as well as toward discovering new natural and semisynthetic anthracyclines with enhanced therapeutic efficacy or reduced toxicity (2-4, 9, 18, 24, 25, 30, 31, 33, 34, 38) (Chart 1). It is apparent that minor molecular alterations may produce major differences in antitumor activity and toxicities as well as in cellular and animal pharmacology. Another characteristic which is not constant for all drugs in this family is the localization within cells of drug-related fluorescence. When assessed by fluorescence microscopy and radioautography, DNR and ADR are nuclearily localized (21, 36); but trifluorocetyladracyramycin-14-valerate (28), 7-con-O-methyl nogarol (19), and aclacinomycin A (20) are cytoplasmically localized. Since this property of the anthracyclines had not been thoroughly investigated, we studied an array of anthracyclines with known structural modifications in order to define the relationship of structure to intracellular fluorescence location. If this were accomplished, we hoped to define what relationship, if any, the intracellular location of a drug might have to its accumulation by intact cells or isolated nuclei and to the ability of the drug to inhibit cellular incorporation of radiolabeled nucleosides.

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, Rockville, Md.), and 15% fetal bovine serum (Medium A: Flow Laboratories). Under these conditions, cells had a population-doubling time of 14 to 18 hr and achieved a maximum cell density of 1.5 to 2.0 x 10⁶ cells/ml.

Two strains of P388 murine leukemia, one sensitive (P388/S) and one resistant (P388/ADR) to DNR and ADR (18, 21), were obtained from Dr. Randall Johnson, National Cancer Institute, NIH, Bethesda, Md., and maintained in vitro by serial culture in RPMI Medium 1630:RPMI Medium 1640 (1:1) containing 0.01 mM mercaptoethanol, 15% fetal bovine serum, 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 10⁶ 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 (32).

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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 × g for 5 min. The platelet-rich plasma supernatant was discarded, and the remaining cells were sedimented by centrifugation at 3400 × g for 5 min. The cells were resuspended in an equal volume of hydroxyethyl starch solution [0.154 M NaCl (6 g/100 ml); Volex, McGaw Labs, Milledgeville, Ga.] and allowed to sediment at 1 × g for 60 min. The resulting supernatant was collected and mixed with 2 volumes of 0.15 M NH₄Cl at 4° to lyse unsedimented erythrocytes. Leukocytes, sedimented by centrifugation at 175 × g for 8 min at 4°, were resuspended in 36 ml 0.154 M NaCl, layered on a Ficoll (Sigma Chemical Co., St. Louis, Mo.):Hypaque (Winthrop Laboratories, New York, N. Y.) gradient (12), and centrifuged at 400 × g for 40 min at room temperature. The final sediment, consisting of greater than 95% neutrophils, was resuspended in RPMI Medium 1640 and used in drug uptake studies.

Drugs. All drugs, except nogalamycin and its semisynthetic derivatives, were obtained from the Developmental Therapeutics Program, National Cancer Institute, NIH, Bethesda, Md. The nogalamycin-related family of anthracyclines were kindly provided by The Upjohn Company, Kalamazoo, Mich. Drug purity of 1-nmol aliquots was confirmed by thin-layer chromatography (13), and by high-performance liquid chromatography (1). The high-performance liquid chromatographic system consisted of a Spectra-Physics (Santa Clara, Calif.) Model 3500B high-performance liquid chromatograph, fitted with a μBondapak column (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 of solutions of drugs in 0.154 M NaCl and 0.3 N HCl in 50% ethanol were obtained with an absolute spectrofluorometer (Model SPF 1000; 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°, 5% CO₂, and 95% humidity.

Fluorescence Microscopy. Cells, incubated with drug for varying periods, were washed once or twice with 0.154 M NaCl...
and were resuspended in a small volume of 0.154 M NaCl. Cell suspensions were examined immediately on an American Optical Model 10 microscope fitted with an HBO 50 mercury arc lamp and FITC interference filter for incident illumination and 500 and 515-nm secondary filters. Photomicrographs were made on Kodak Ektachrome film (ET 135, ASA 160).

**Assay of Drug Content.** After incubation in media containing 10 μM drug, 10 ml of iced 0.154 M NaCl were added to the incubation mixture, and the cells were centrifuged at 2500 x g for 5 min at 4°C. 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 at the appropriate excitation and emission maxima for each drug. Drug content was calculated by comparison with simultaneously performed drug standards and drug-free cell controls (6).

**Drug Uptake by Isolated L1210 Nuclei.** Nuclei were isolated by homogenization of L1210 cells after swelling in hypotonic buffer (15). Studies of drug accumulation by nuclei were performed as described above for whole cells, except that 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.

**Incorporation of Radioactive Macromolecular Precursors.** After incubation for 120 min with drug, cells were pulsed with 0.1 ml of medium, containing either 1 μCi [methyl-3H]thymidine (2.0 Ci/mmol; New England Nuclear, Boston, Mass.) or 2 μCi [G-3H]uridine (8 Ci/mmol; New England Nuclear). After incubation for 60 min with radiolabeled nucleosides, cells were assayed for incorporation of 3H into trichloroacetic acid-precipitable material as described previously (22).

**Fluorescence Quenching by Nucleic Acids and Isolated Nuclei.** Ten μM solutions of various anthracyclines were prepared in 0.154 M NaCl. Using the appropriate excitation and emission maxima for each drug, the fluorescence of 1 ml of solution was determined before and at various times after addition of either 1 ml 0.154 M NaCl containing no nucleic acid, or 10 μg calf thymus DNA (approximately 16.7 nmol of base pairs) (Sigma), or 100 μg yeast RNA (approximately 167 nmol of base pairs) (Sigma). To study the quenching by nuclei, 0.1 ml of nuclei buffer containing 0 or 106 L1210 nuclei was added to 1 ml of a 10 μM solution of drug in nuclei buffer.

**RESULTS**

**Cytofluorescence Localization.** The anthracyclines exhibited 3 basic patterns of cytofluorescence localization, nuclear, cytoplasmic, and general (Table 1; Figs. 1 and 2). DNR, ADR, their N,N-dimethyl analogs, and 4'-epiadriamycin were localized primarily in cell nuclei. In contrast, a large number of anthracyclines appeared to be localized in the cytoplasm, often in discrete, brilliantly fluorescent granules. Included in this group of drugs were the N-acetyl and N,N-dibenzy derivatives of DNR and 3',4'-diacetyldeaminodaunorubicin, NSC 200681, 4-demethoxy-ADR, and carminomycin. In addition, aclacinomycin, musettamycin, and marcellomycin were cytoplasmically localized as were nogalamycin and its semisynthetic derivatives, nogamycin, 7-con-O-methyl nogarol and 7-dis-O-methyl nogarol. 3'-Deaminodaunorubicin, N-formyl-ADR, and 13-amino-daunorubicin were not preferentially localized in either nucleus or cytoplasm but rather appeared distributed about equally in both intracellular regions. These patterns were visible after as little as 3 min of incubation of cells with drugs and did not change during the course of observation for as long as 180 min. Discrete localization patterns were detectable after incubation of 106 cells with as little as 0.01 nmol/ml of drug and were consistently observed in all of the cell types studied.

**Cellular Accumulation of Drugs.** The cellular accumulation of anthracyclines bore no relationship to their cytofluorescence localization (Table 2). Both nuclear and cytoplasmic localized groups of compounds included drugs such as N,N-dimethyldaunorubicin, carminomycin, and musettamycin which were avidly accumulated by cells. Similarly, both groups of compounds included drugs such as ADR, N-acetyldaunorubicin, and 7-dis-O-methyl nogarol, of which less than 1 nmol/106 cells were accumulated.

**Nuclear Accumulation of Drugs.** As with drug accumulation by whole cells, the accumulation of anthracyclines by isolated L1210 nuclei had no obvious relationship to the cellular localization of drug fluorescence (Table 2). Of the nuclear localized drugs, DNR was accumulated by 106 nuclei to the same degree as by 10⁶ intact cells. However, N,N-dimethyldaframycin, 4'-epiadriamycin, and ADR were accumulated to a greater extent by nuclei than by whole cells, whereas N,N-dimethyldaunorubicin was accumulated to a much lesser degree by nuclei than

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**Table 1**

<table>
<thead>
<tr>
<th>Nuclear</th>
<th>Cytoplasmic</th>
<th>Nuclear and cytoplasmic</th>
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</thead>
<tbody>
<tr>
<td>DNR</td>
<td>N-Acetyl-daunorubicin</td>
<td>3'-Deaminodaunorubicin</td>
</tr>
<tr>
<td>ADR</td>
<td>N,N-Dibenzyldaunorubicin</td>
<td>N-Formyl-ADR</td>
</tr>
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<td>N,N-Dimethyldaunorubicin</td>
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<td>13-Amino-DNR</td>
</tr>
<tr>
<td>N,N-Dimethyladriamycin</td>
<td>NSC 200681</td>
<td>4-Demethoxyaunaorubicin</td>
</tr>
<tr>
<td>4'-Epidaurubicin</td>
<td>Carminomycin</td>
<td>Triferric ADR</td>
</tr>
<tr>
<td></td>
<td>Aclacinomycin</td>
<td>Marcellomycin</td>
</tr>
<tr>
<td></td>
<td>Musettamycin</td>
<td>Nogalamycin</td>
</tr>
<tr>
<td></td>
<td>Nogamycin</td>
<td>7-dis-O-Methyl nogarol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-con-O-Methyl nogarol</td>
</tr>
</tbody>
</table>
Fig. 1. Fluorescence microscopy of human neutrophils incubated with DNR (A), N,N-di-methyldaunorubicin (B), N,N-dimethyladriamycin (C), 4'-epi-adriamycin (D), 13-aminodaunorubicin (E), or ADR (F). Human neutrophils were isolated as described in "Materials and Methods" and incubated with 10 μM drug. After 60 to 120 min, cells were washed once with iced 0.154 M NaCl and examined under fluorescence microscopy as described in "Materials and Methods." G and H, light micrographs of human neutrophils in Wright-stained blood smears. × 500.

by intact cells. As might be expected, a number of cytoplasmically localized drugs were accumulated less by isolated nuclei than by intact cells. However, the nuclear accumulation of 2 cytoplasmic anthracyclines, 4-demethoxy-DNR and mar-cellomycin, was approximately the same and 3 times greater than their respective cellular accumulation. The nuclear accumulation of the generally localized drugs 13-aminodaunorubi-

Inhibition of Radiolabeled Nucleoside Incorporation. The ability of various anthracycline antibiotics to inhibit cellular incorporation of [3H]thymidine and [3H]uridine into trichloroacet-

acid-precipitable material did not depend on the cellular fluorescent localization of the compound in that a large number
Anthracycline Cytofluorescence Localization

Fig. 2. Fluorescence microscopy of human neutrophils incubated with 7-con-O-methyl nogarol (A), aclacinomycin (B), musettamycin (C), nogamycin (D), N,N-dibenzyladunorubicin (E), carminomycin (F), triferric ADR (G), or NSC 200681 (H). Human neutrophils were isolated as described in "Materials and Methods" and incubated with 10 μM drug. After 60 to 180 min, cells were washed once with iced 0.154 M NaCl and examined under fluorescence microscopy as described in "Materials and Methods." × 500.

of cytoplasmic drugs were very effective inhibitors of apparent nuclear activities (Table 2). In addition, there was no correlation between the cellular or nuclear accumulation of these drugs and their 50% inhibiting dose for either [3H]thymidine or [3H]uridine incorporation. As shown in Table 2, almost all of the compounds which inhibit [3H]uridine incorporation more effectively than [3H]thymidine incorporation were cytoplasmically localized.

Fluorescence Quenching by Nucleic Acids and Nuclei. The fluorescence of solutions of various anthracyclines remained essentially constant during the first 15 min, during which intermittent measurements were made, and then declined to ap-
nuclei were isolated as described in "Materials and Methods," and 10
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beled nucleoside incorporation into trichloroacetic acid-precipitable material was
varying concentrations of various anthracyclines, and the inhibition of radiola
determined as described in "Materials and Methods." l0 nuclei were incubated for 120 to 180 min at 37°C in 1 ml of buffer containing 10 nmol of various anthracyclines; then, drug accumulation was determined fluorometrically as described in "Materials and Methods." L1210 nuclei (106) were incubated with varying concentrations of various anthracyclines, and the inhibition of radiola-
beled nucleoside incorporation into trichloroacetic acid-precipitable material was
determined as described in "Materials and Methods." 

Addition of 106 L1210 nuclei to solutions of anthracyclines did DNA (Table 3). Other cytoplasmic drugs, like their nuclear
counterparts, were much less affected by RNA than by DNA. Addition of RNA to solutions of nuclearly localized anthracyclines caused a
rapid reduction in fluorescence. Addition of DNA quenched the fluorescence of some anthracyclines more than that of others (Table 3). Although the most severely quenched compounds were among those that appeared cytoplasmically localized under fluorescent microscopy, a large number of cytoplasmically localized drugs were quenched no more than were the 5 nuclearly situated compounds. In most cases, addition of RNA to solutions of nuclearly localized anthracyclines caused a much smaller reduction in fluorescence than did DNA (Table 3). On the other hand, RNA quenched the fluorescence of some cytoplasmically localized drugs to the same degree as did DNA (Table 3). Other cytoplasmic drugs, like their nuclear counterparts, were much less affected by RNA than by DNA. Addition of 106 L1210 nuclei to solutions of anthracyclines quenched some drugs more than others (Table 3), which to some extent correlated with the effect of DNA, in that the cytoplasmic drugs most severely quenched by DNA were also

DISCUSSION

Fluorescence of anthracycline antibodies is one of the most striking physico-chemical features of this class of antineoplastic
agents. This property has been utilized in developing convenient, accurate, and sensitive assays for anthracyclines in tissue and biological fluids (6, 8), as well as in elucidating their metabolic pathways (5). In addition, earlier studies used the characteristic orange-red fluorescence of DNR and ADR to localize these compounds in cells and showed them to be restricted to the nucleus (21, 36). These findings were not considered surprising in view of the known ability of DNR and ADR to bind tightly to DNA and chromosomes (17, 23, 27, 29, 35, 37). However, subsequent studies with trifluoroacetadriamycin-14-valerate showed this anthracycline to be localized to the cytoplasm (20). The studies presented here show that both nuclear and cytoplasmic patterns are observed when a large number of anthracyclines are systematically examined by fluorescence microscopy.

Comparison of the structures of those compounds in which fluorescence is localized to the nucleus with those drugs observed
in the cytoplasm reveals certain structure-disposition relationships. The methoxy group at carbon 4 appears to be
critical for nuclear localization since its absence on carminomycin and 4-demethoxy-DNR is the only difference between
those most affected by nuclei. Again, the fluorescence of a number of cytoplasmically localized drugs was no more
quenched by nuclei than was that of nuclear localized compounds.

The fluorescence of 10 μM solutions of drugs was determined before and after
addition of DNA, RNA, or nuclei as described in "Materials and Methods."

Table 3

Quenching of anthracycline fluorescence by DNA, RNA, and isolated L1210

Fluorescence of control solution as % of control fluorescence

<table>
<thead>
<tr>
<th></th>
<th>Without DNA</th>
<th>With DNA</th>
<th>With RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNR</td>
<td>79.3</td>
<td>95.3</td>
<td>94.0</td>
</tr>
<tr>
<td>ADR</td>
<td>81.5</td>
<td>97.5</td>
<td>89.0</td>
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<tr>
<td>N,N-Dimethyl-daunorubicin</td>
<td>65.0</td>
<td>88.0</td>
<td>81.0</td>
</tr>
<tr>
<td>N,N-Dimethyl-adriamycin</td>
<td>88.0</td>
<td>100.0</td>
<td>79.0</td>
</tr>
<tr>
<td>4'-Epiadriamycin</td>
<td>72.0</td>
<td>94.0</td>
<td>ND</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-con-O-Methyl nogarol</td>
<td>72.0</td>
<td>75.0</td>
<td>122.0</td>
</tr>
<tr>
<td>7-dis-O-Methyl nogarol</td>
<td>86.0</td>
<td>100.0</td>
<td>107.0</td>
</tr>
<tr>
<td>Nogalamycin</td>
<td>74.0</td>
<td>78.0</td>
<td>98.3</td>
</tr>
<tr>
<td>N-Acetyldaunorubicin</td>
<td>80.0</td>
<td>88.0</td>
<td>100.0</td>
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<tr>
<td>NSC 200681</td>
<td>96.0</td>
<td>93.0</td>
<td>ND</td>
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<td>Aclacinomycin</td>
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<td>4-Demethoxydaunorubicin</td>
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<td>Carminomycin</td>
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<tr>
<td>Marcellomycin</td>
<td>16.0</td>
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<td>73.3</td>
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<tr>
<td>Musettamycin</td>
<td>43.0</td>
<td>100.0</td>
<td>72.7</td>
</tr>
</tbody>
</table>

a Arbitrary fluorescence units.
Values are expressed as the percentage of control solution fluorescence 30 min after addition of quenching material. S.D. of 3 determinations were <25% of the mean in all cases.
ND, not determined.


28. Lin, C. C., and Van De Sande, J. H. Differential fluorescent staining of human
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