# Cellular Pharmacodynamics of Several Anthracycline Antibiotics

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Alterations in the C-9 side chain of the anthracycline antibiotics, adriamycin and daunorubicin, have a profound effect on antibiotic uptake and accumulation by cultured L1210 cells. The degree of inhibition of DNA and RNA biosynthesis in the L1210 cells is directly related to the cellular uptake and accumulation of the drug analogues. Polar drug metabolites, daunorubicinol and adriamycinol, retain inhibitory activity against nucleic acid metabolism but have a decreased membrane binding and permeability. Cellular uptake and accumulation of the C-9 analogues are inversely related to drug polarity. We propose that the polarity of the anthracycline analogues contributes heavily to the differences in therapeutic index and in vivo activity through fundamental effects on membrane permeability, metabolism, and macromolecular binding.

The clinical gains of the anthracycline antibiotics, adriamycin and daunorubicin (Figure 1), against neoplastic diseases in humans have aroused considerable interest in this class of compounds.[1-4] Consequently, natural and synthetic derivatives of these substances have been produced and studied for their anticancer activity. One recently developed synthetic derivative, the benzoyl hydrazone of daunorubicin, rubidazone (Figure 1), also is useful in the treatment of acute leukemia.[5]

In addition to synthetic analogues, metabolites of adriamycin and daunorubicin have biological activity. The major metabolites of daunorubicin and adriamycin are their reduced derivatives, daunorubicinol and adriamycinol, respectively[6-9] (Figure 1), which are found in plasma, urine, and tissues of treated patients and animals. These reduced products are formed by a cytoplasmic aldo–keto reductase native to all tissues.[10] Since adriamycinol and daunorubicinol are principal metabolites in treated patients[7,8] and since this metabolic alteration spares activity, the reduced metabolites probably contribute to the pharmacologic and toxicologic activities of anthracycline antibiotic therapy.[11]

Differences in the therapeutic indices and activity spectra of adriamycin and daunorubicin have provoked questions concerning their pharmacodynamics. In the present study we have investigated the inhibitory activities of several anthracycline analogues of the C-9 position (Figure 1) on nucleic acid synthesis in the murine leukemia L1210 cells. The cellular uptake of the anthracyclines was determined to account for major differences in activities of the compounds. These data were used to evaluate the sites on the antibiotic molecule which contribute to both the inhibitory activity and to membrane transport.

## Results

All the anthracycline derivatives inhibit both thymidine incorporation into DNA and uridine incorporation into RNA of the L1210 murine leukemia cells (Tables I and II). However, their inhibitory activities differ. Generally the inhibition of thymidine incorporation parallels the inhibition of uridine incorporation for each agent, and the inhibition is related to drug concentration from 1 to 5 $\mu$M.

Daunorubicin is the most active inhibitor of both thymidine and uridine incorporation at all drug concentrations, and adriamycin is the second most active inhibitor. Both drug metabolites, daunorubicinol and adriamycinol, retain inhibitory activity, but they are less active than their metabolic precursors. Rubidazone, the chemical derivative of daunorubicin, has significant activity as an inhibitor of nucleic acid biosynthesis.

Anthracycline antibiotic accumulation by the L1210 cells also differs among the compounds (Figure 2). An eightfold difference exists between the cellular accumulation of daunorubicin and adriamycinol after 120 min. Although the initial drug uptake is linear for about 10 min, uptake gradually plateaus by 60–120 min. The uptake rates of adriamycinol and daunorubicinol are quite low. However, in all cases, the final drug concentrations in the cells are greater than the medium concentration indicating an uptake of drug against a gradient. As previously reported,[11,15] the uptake of all the compounds is thermosensitive and is negligible at 0°, but binding of the drugs to the cell membrane is apparent at 0° (zero time) samples. Drug binding to the cells at 0°, zero time differs and generally reflects the same relationship as seen in cellular accumulation with daunorubicin having the greatest binding and adriamycinol the least. No decrease in cellular drug content occurs during the 2-h experiments.

Daunorubicin produced the highest degree of inhibition and had the highest rate and degree of accumulation over the 2-h period. Inhibitor accumulation into the L1210 cells bore a close relationship to degree of inhibition of nucleic acid biosynthesis (Figure 2). This relationship of inhibitory activity to the drug accumulation by the cells indicates that

Table I. Inhibition of Thymidine Incorporation in L1210 Cells[a]

| Inhibitor | % inhibn (mean + SE) | | |
|---|---|---|---|
| | 1.0 $\mu$M | 3.0 $\mu$M | 5.0 $\mu$M |
| Daunorubicin | 37.0 ± 3.2 | 68.8 ± 1.5 | 83.0 ± 1.4 |
| Daunorubicinol | 12.1 ± 4.6 | 30.8 ± 6.1 | 36.8 ± 7.9 |
| Adriamycin | 28.9 ± 2.7 | 34.5 ± 4.8 | 52.2 ± 3.3 |
| Adriamycinol | 23.7 ± 8.2 | 25.0 ± 4.0 | 32.0 ± 5.1 |
| Rubidazone | 12.7 ± 3.1 | 27.2 ± 3.1 | 41.0 ± 1.9 |

[a] After 1-h preincubation of $10^6$ cells with inhibitor, 1 $\mu$Ci of tritiated thymidine was added and the incubation was continued for an additional hour. Control L1210 cells incorporated from 10 000 to 60 000 cpm during the 1-h incubation. The percent inhibition is the mean ± SE of duplicate values from four to twelve experiments.

Table II. Inhibition of Uridine Incorporation in L1210 Cells[a]

| Inhibitor | % inhibn (mean + SE) | | |
|---|---|---|---|
| | 1.0 $\mu$M | 3.0 $\mu$M | 5.0 $\mu$M |
| Daunorubicin | 47.6 ± 5.9 | 69.8 ± 3.9 | 74.8 ± 2.3 |
| Daunorubicinol | 11.2 ± 2.6 | 28.9 ± 4.6 | 46.4 ± 0 |
| Adriamycin | 22.5 ± 6.8 | 50.8 ± 4.2 | 56.9 ± 3.9 |
| Adriamycinol | 10.1 ± 4.7 | 17.9 ± 3.7 | 22.4 ± 5.3 |
| Rubidazone | 11.6 ± 0.1 | 36.8 ± 3.4 | 58.2 ± 1.2 |

[a] After 1-h preincubation of $10^6$ cells with inhibitor, 0.33 $\mu$Ci of [$^{14}$C]uridine was added, and the incubation was continued for an additional hour. Control L1210 cells incorporated from 10 000 to 30 000 cpm during the 1-h incubation. The percent inhibition is the mean + SE of duplicate values from four to twelve experiments.

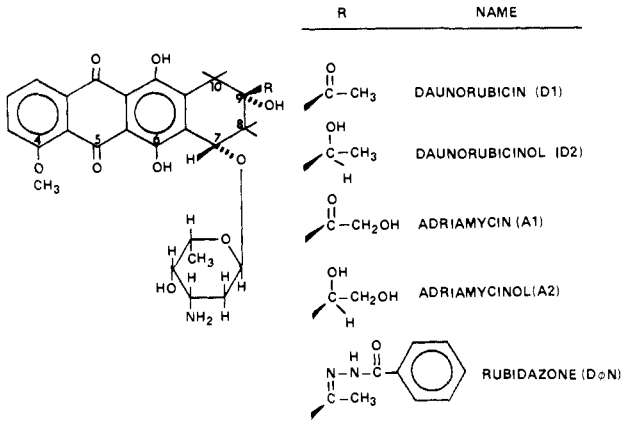


Figure 1. C-9 analogues of daunorubicin.

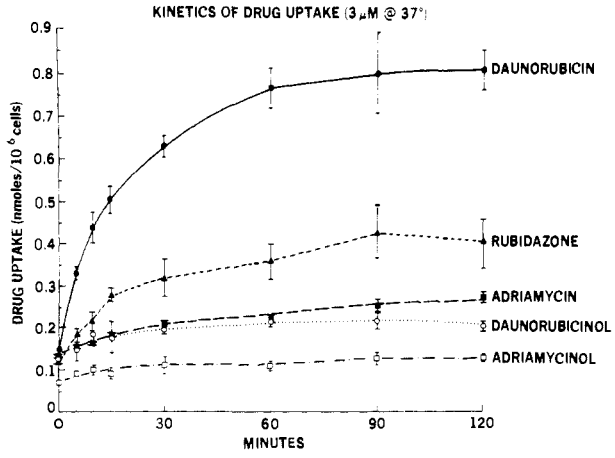


Figure 2. Uptake of anthracycline antibiotics by L1210 cells. L1210 cells were incubated with $3 \times 10^{-6}$ M drugs as described in Methods. The points are the means ± SEM of at least four experiments.

Table III. Specific Inhibitory Activity

| | Cellular drug content, nmol/ $10^6$ cells | % inhibn | | Specific inhibn[a] | |
|---|---|---|---|---|---|
| | | DNA | RNA | DNA | RNA |
| Daunorubicin | 0.31 | 37.0 | 47.6 | 119 | 155 |
| Daunorubicinol | 0.33 | 30.8 | 28.9 | 94 | 88 |
| Adriamycin | 0.19 | 28.9 | 22.5 | 152 | 121 |
| Adriamycinol | 0.16 | 32.0 | 22.4 | 200 | 137 |
| Rubidazone | 0.20 | 12.7 | 11.6 | 65 | 60 |

[a] Specific inhibition activity = percent inhibition/ cellular drug content.

drug action is modulated by cellular drug uptake.

To obtain an estimate of the specific activity of these agents, the inhibitory activities were compared at near-equal cellular drug concentrations (0.16–0.33 nmol/$10^6$ cells) and a calculation of specific inhibitory activity was made (Table III). For each drug the specific activity for thymidine inhibition is similar to uridine inhibition except adriamycinol which may have a predisposition for inhibition of thymidine incorporation. Adriamycin and adriamycinol had the highest specific activities as inhibitors of DNA synthesis. The low specific activity of rubidazone may reflect a decreased ability to interact with DNA despite the fact that the substance is accumulated rapidly by the cells.

When all of the cellular drug concentrations are examined in this way, at low drug concentrations the specific inhibitions are highest. With increasing cellular drug concentrations, the specific inhibitory activity decreases suggesting that all of the drug molecules are not effectively utilized as inhibitors of nucleic acid metabolism. This relationship of anthracycline concentration and inhibition of nucleic acid synthesis deserves further exploration.

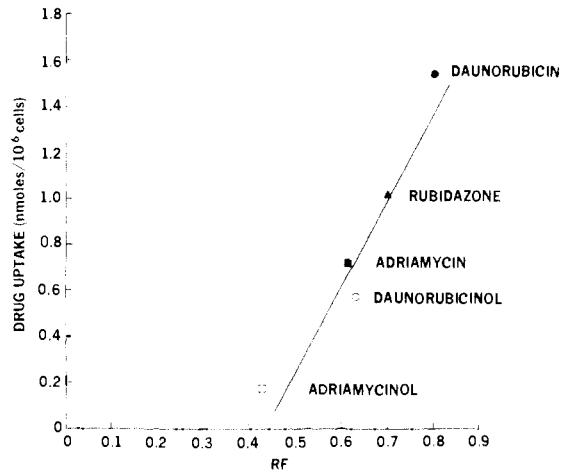


Figure 3. Relationship of antibiotic $R_f$ on silica gel chromatography to cellular antibiotic accumulation. L1210 cell uptake of $5 \times 10^{-6}$ M antibiotic after 120 min. Incubation is related to the antibiotic $R_f$. Details of the chromatographic analysis are in Methods.

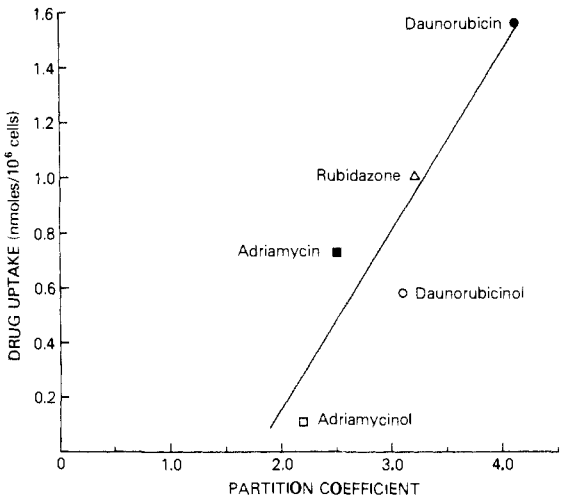


Figure 4. Relationship of antibiotic 1-butanol-$KPO_4$ buffer partition coefficient to cellular antibiotic accumulation. L1210 cell uptake of $5 \times 10^{-6}$ M antibiotic after 120 min. Incubation is plotted against the antibiotic's partition coefficient as described in Methods.

Since the anthracycline derivatives had different physical-chemical characteristics, we compared these differences to the drug accumlation by cells. Drug polarity was compared to the cellular accumulation of the drug. Drug polarity was estimated by both 1-butanol–phosphate buffer partitioning and by chromatographic mobility ($R_f$) of the drugs on silica gel thin-layer chromatography. The chromatographic mobility ($R_f$) of the agents bore a near-linear relationship to drug accumulation by the L1210 cell (Figure 3). Agents with greater chromatographic mobility had the higher accumulation in the L1210 cells, and agents with lower mobility had low cellular accumulation. Similarly the drug 1-butanol–phosphate buffer partition related to drug accumulation by the L1210 cells (Figure 4). This estimate of molecule polarity gave a similar near-linear relationship of low drug polarity to high

cellular uptake and high drug polarity to low cellular uptake.

**Confirmation of Radioisotope Incorporation into Nucleic Acids.** When cellular DNA and RNA were extracted and isolated from the L1210 cells, about 99% of the incorporated tritiated thymidine was in DNA, and 95% of the incorporated uridine was in RNA. Neither daunorubicin nor the other anthracycline agents significantly altered the amount of isotopically labeled nucleoside precursor present in the acid-soluble fraction of the L1210 cells. The action of all anthracyclines was limited to the incorporation of label into the acid precipitable material, and no effects by the anthracycline agents were discerned on the acid-soluble pool.

## Discussion

Several investigators have studied the structure–activity relationships of selected anthracycline antibiotics and their analogues. Those studies emphasized the action of the drugs on nucleic acid metabolism as well as the binding of the drugs to nucleic acids. When the binding affinities were compared with drug structures, a correlation existed between the drug–DNA binding and the state of the amino sugar moiety.[16-18] However, changes in the C-9 side chain of the anthracycline nucleus reflected little change in drug–DNA binding characteristics. Adriamycin, daunorubicin, and dihydrodaunomycin (a chemically reduced racemic mixture of daunorubicinol) had similar DNA binding characteristics as measured by changes in drug spectral absorption, DNA viscosity, and disruption of the methyl green–DNA complex.[17] Also the "apparent association constant" of these three analogues and DNA was similar.[18] When adriamycin or daunorubicin intercalated into double-stranded DNA, they unwound the DNA the same degree (5.2–5.3°).[19] None of the physical–chemical measurements of nucleic acid and anthracycline interaction adequately discerned between adriamycin and daunorubicin.

In their inhibition of DNA polymerase, the anthracycline antibiotics show relationship similar to their DNA binding; substitutions in the amino sugar moiety reduce the biochemical activity of the drug, but differences in the C-9 side chain of daunorubicin, adriamycin, and dihydrodaunomycin produce minimal effects.[20] With bacteriophage-induced DNA polymerases,[21] adriamycin is slightly more effective than daunorubicin both for stimulation at low concentrations and for inhibition at higher concentrations. Similarly, adriamycin is slightly more effective an inhibitor than daunorubicin with bacterial and mammalian DNA and RNA polymerases.[22,23] However, in all of these studies on modulation of nucleic acid metabolism, daunorubicin and adriamycin are nearly indistinguishable. Clearly, these findings do not explain the striking differences between adriamycin and daunorubicin seen clinically and in experimental animals.

Adriamycin and daunorubicin differ appreciably in their cellular pharmacodynamics.[11,24,25] Cellular uptake of adriamycin is slower and accumulation is lower than daunorubicin uptake and accumulation. In addition, adriamycin and daunorubicin are excreted differently in vivo.[9,26,27]

Certainly the differences on the C-9 side chain, although they only reflect small differences in nucleic acid interaction, cause major differences in the membrane transport, intracellular accumulation, and physiologic handling of the anthracycline antibiotics.

A relationship of physical–chemical characteristics of anthracycline C-9 analogues with cellular drug uptake is shown. Alteration of the anthracyclines at the C-9 position changes membrane permeability as a function of polarity. As the lipid solubility of the drug increases by modification of the C-9 side chain, so does the cellular drug uptake.

We can speculate that a membrane receptor is responsive to the polarity of the C-9 chain. Lower polarity (i.e., higher lipid solubility) of the drug (i.e., daunorubicin) increases the binding of the drug to the cell. As the C-9 group becomes more polar, the molecule is less receptive to membrane binding and membrane transport as exemplified by the very polar adriamycinol. This suggests that the membrane transport of the anthracycline occurs as a nonionized base which has a greater lipid solubility than an ionized molecule. It also suggests that the receptor for the ring system is in a lipid portion of the membrane. Steric factors seem less important since compounds such as the benzoyl hydrazone of daunorubicin which has a large steric contribution at C-9 are transported very well, in fact, better than the sterically smaller adriamycin, daunorubicinol, and adriamycinol.

In addition to functioning as a modulator of transport, the C-9 side chain is a major site for biotransformation and an important effector of other metabolic reactions. Enzymatic reduction of daunorubicin's carbonyl group occurs more readily than adriamycin reduction,[9] and the aldo–keto reductase catalyzing this reaction has a greater affinity for daunorubicin.[10] Although remote from other sites of enzymatic action, the C-9 group affects these substantially. Glycosidic cleavage of adriamycin is slower than cleavage of daunorubicin by both microsomes[9] and a solubilized and partially purified reductive glycosidase.[28] As determined from in vivo measurements, conjugation reactions of adriamycin and daunorubicin also differ.[8,9] Whether these enzymatic effects of C-9 variation are related to a steric factor or to polarity remain to be shown.

The clinical contrasts between adriamycin and daunorubicin are the composite of numerous factors: metabolism, macromolecular binding, membrane transport, immunologic suppression, etc. Certainly the differences in disposition and metabolism both qualitatively and quantitatively complicate the understanding of the therapeutic effects and activity spectra of the agents.

## Experimental Section

Daunorubicin hydrochloride was obtained from the Drug Development Branch, NCI. Adriamycin hydrochloride was provided by Farmitalia, Milan, Italy, and the benzoyl hydrazone derivative of daunorubicin, rubidazone, was supplied by the Rhône-Poulenc Co. of Paris, France. Adriamycinol and daunorubicinol were prepared enzymatically as previously described.[6] All of the anthracycline antibiotics were purified by column and thin-layer chromatography prior to use. The purified antibiotics were stored as lyophilized powders at −20° in the dark, and solutions of the drugs were prepared fresh for experiments to avoid decomposition. Equivalent molar concentrations were attained by use of spectral absorption at 485 nm.[12]

The L1210 murine leukemia cells were kindly provided by Dr. Richard Adamson of the NCI and were grown in spinner flask tissue culture. The cell harvesting, incubation procedure, assay of radioactive nucleosides, drug extraction and analysis, and extraction and isolation of RNA and DNA from the L1210 cells were carried out as previously described.[13]

Ascending thin-layer chromatography of the anthracycline antibiotics on 250-$\mu$ silica gel H thin-layer plates was done in chloroform–methanol–acetic acid–water (80:20:14:6). For $R_f$ determinations, the solvent front was allowed to ascend 15 cm.

Determinations of the antibiotic partition coefficient in butanol–0.1 M $KPO_4$, pH 7.4, buffer were made by shaking a 1-ml solution of $1 \times 10^{-6}$ M antibiotic in 0.1 M $KPO_4$, pH 7.4, with 1 ml of 1-butanol at 25° for at least 10 min. The suspension stood for 1 h and was centrifuged for separation of the phases. Antibiotic concentration was determined in the aqueous and organic phases by fluorescence quantitation.[14] Repeated analysis of these ex-

tractions established that equilibrium had been achieved under these conditions.

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# Reactions of 2-Acyloxyisobutyryl Halides with Nucleosides. 6.[1a] Synthesis and Biological Evaluation of Some 3'-Acyl Derivatives of 2,2'-Anhydro-1-(β-D-arabinofuranosyl)cytosine Hydrochloride[1b]


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The reactions of cytidine with 22 different 2-*O*-acyloxyisobutyryl chlorides lead to the isolation of the corresponding 2,2'-anhydro-1-(3'-*O*-acyl-β-D-arabinofuranosyl)cytosine hydrochlorides **9**. These compounds, which all show cytotoxicity against HeLa cells in tissue culture, have been examined for antiviral and antileukemic activity. Activity against DNA viruses (vaccinia and Herpes) in tissue culture is maximal in compounds containing acyl groups with 8–12 carbon atoms. Activity against L1210 leukemia in mice varies markedly according to the length of the acyl groups, and high activities were observed in the case of long-chain ($C_{16}$–$C_{22}$) esters. The reaction between cytidine and *O*-acetylsalicyloyl chloride provides an alternate route for the synthesis of 3'-*O*-Ac cycloC hydrochloride.


Of the myriad of nucleoside derivatives that have been examined for potential antitumor activity, 1-(β-D-arabinofuranosyl)cytosine (araC) remains the one that has found widest clinical use, particularly in the therapy of acute leukemias.[3] AraC is, however, rapidly degraded to the biologically inactive 1-(β-D-arabinofuranosyl)uracil by the ubiquitous enzyme cytidine deaminase, the plasma half-life in man being only 12 min.[4] Since the drug exerts its cytotoxic effect only during the late S phase and early $G_2$ phase of the cell cycle,[5] this short half-life has necessitated the clinical use of complex dosage schedules or, in particular, continuous intravenous infusion.[3]

The pronounced antileukemic,[3] immunosuppressive,[6] and anti-DNA viral[7] activities of araC have stimulated the synthesis of a considerable number of derivatives of the parent compound. In general, the addition of substituents on the cytosine ring has led to compounds of reduced activity or, at best, to compounds showing no marked advantage over araC itself.[7b,8] Also, certain biologically irreversible modifications of the sugar moiety (e.g., methyl ethers)[9] lead to a loss of activity although more subtle changes such as the formation of 4'-thio[10] or 2'-halogenated[11] derivatives can be tolerated. Of particular interest have been the observations by the Upjohn group that various 5'-esters of araC, as well as certain 2'- and 3'-esters,[12e] show high biological activity of a long duration since these substances are not substrates for cytidine deaminase[12] and slowly release araC following enzymatic hydrolysis. Also, recent work from Japan has reported that 2,2'-anhydro-1-(β-D-arabinofuranosyl)cytosine hydrochloride (cycloC),[13] a substance which is itself resistant to cytidine deaminase[14] but which is slowly hydrolyzed to araC under physiological conditions, is a highly effective antitumor agent[15] with toxicity somewhat less than that