Effect of Verapamil and Other Agents on the Distribution of Anthracyclines and on Reversal of Drug Resistance


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ABSTRACT

We studied the intracellular distribution of drugs within anthracycline-sensitive and -resistant cells by computer-assisted digitized video fluorescence microscopy. We found that the antitumor antibiotic, daunorubicin, distributes differently in anthracycline-sensitive and -resistant human leukemia cells (HL-60). Verapamil and other agents known to circumvent drug-resistant cell lines were able to change the pattern of distribution of daunorubicin in the anthracycline-resistant HL-60 cells back to the distribution found in anthracycline-sensitive HL-60 cells.

To investigate the biochemical basis for this effect, we studied the distribution of daunorubicin and doxorubicin in a hydrophobic/hydrophilic (membrane/cyttoplasmic) environment using the two-compartment cell-free system of Folch. Our results demonstrate that various unrelated drugs known to overcome resistance will also change the distribution of the anthracyclines in the hydrophobic/hydrophilic compartments.

Our data allow the hypothesis that various unrelated agents known to circumvent resistance may act by altering the hydrophobic/hydrophilic solubility of anthracyclines in the resistant cell.

INTRODUCTION

Many tumor cell mutants selected for resistance to single agents are cross-resistant to multiple unrelated chemotherapeutic agents (1). This phenomenon, known as multidrug resistance, is usually associated with changes in composition of the cell membranes (2).

The ability of multidrug-resistant cultured cells to withstand the injurious effects of anthracycline drugs such as doxorubicin is believed to be due to the decreased capacity of the resistant cells to retain the drug (3, 4) which, in turn, has been ascribed to (a) energy-dependent enhanced drug efflux (5); or (b) reduced drug binding and a consequently greater fraction of releasable drug in the resistant cells (6).

Multidrug resistance can be reversed in vitro by calcium channel blockers [e.g., verapamil (7)], calmodulin inhibitors [e.g., trifluoperazine (8)], and other amphipathic compounds [e.g., chloroquine (9) and cyclosporine (10)].

The biochemical basis for reversal of drug resistance by these agents remains unknown. Some investigators have proposed that these drugs may act by modulating intracellular calcium exchange inhibiting the active efflux of drug (11); others have suggested that they may directly interact with the cell membranes by changing their permeability to anthracyclines (12).

An alternative hypothesis which correlates with the similar amphipathic nature of these agents despite their heterogeneous biological function is that they may alter the solubility of anthracyclines in various intracellular compartments in the resistant cell. The ability of verapamil to displace the anthracyclines from a rapidly releasable compartment and into a slowly releasable compartment would lead to an increased retention of the drug.

To determine the effect of these agents on the distribution of the anthracyclines doxorubicin and daunorubicin within various intracellular compartments, we studied the distribution of the anthracyclines in viable HL-60 and HL-60/AR3 cells 100- and 50-fold resistant to doxorubicin and daunorubicin, respectively (13), and in the hydrophobic/hydrophilic compartments of the cell-free system of Folch (14).

MATERIALS AND METHODS

Cells. HL-60 cells were obtained from the original line initially described by Collins (15). The isolation of the anthracycline-resistant subline HL-60/AR was previously described (13). Both cell lines were maintained in suspension culture in RPMI 1640 medium, supplemented with 10% fetal calf serum. Sterile 75-cm² tissue culture flasks containing the cells are kept at 37°C, in a 5% CO₂ controlled atmosphere incubator. Cells were passed twice weekly and routinely examined for mycoplasma contamination. The HL-60/AR cells were routinely grown in the presence of 1 × 10⁻⁴ M doxorubicin. Cells grown for more than 1 week in media without phenol red and without doxorubicin, and that exhibited logarithmic growth, were utilized for all experiments.

Prior to each experiment, the cells were washed twice with Dulbecco’s PBS and resuspended in RPMI 1640 medium without phenol red at the density of 10⁶ cells/ml.

Drugs. Daunorubicin and doxorubicin were purchased from Ives Laboratory, NY, and Adria Labs, Columbus, OH, respectively. Verapamil was obtained from Searle Laboratories, Chicago, IL. Perhexiline maleate and tamoxifen citrate were gifts from Merrill Dow Chemical Company, Cincinnati, OH, and Stuart Pharmaceuticals, respectively. Cyclosporine (Sandimmune) was obtained from Sandoz, East Hanover, NJ. Dulbecco’s PBS was purchased from GIBCO Laboratories, Chagrin Falls, OH. All other drugs were purchased from Sigma Chemical Company, St. Louis, MO. The PBS used for Folch partitioning contained 1.47 mM of KH₂PO₄, 2.68 mM of KCl, 1.37 mM of NaCl, and 8.0 mM of Na₂HPO₄.

Studies of Intracellular Localization of Anthracyclines by DVFM. The apparatus used for the DVFM has been previously described (16). The system consists of a Leitz Orthoplan microscope with phase/fluorescence objectives equipped with a 50-W tungsten substage illuminator and a 50-W mercury source vertical fluorescence illuminator (Ploem) with appropriate filters for different fluorescent compounds. For daunorubicin and acridine orange, we have found the optimal excitation and emission filters are 540 and 580 nm, respectively.

Using this system, it is possible to monitor cellular changes in fluorescence alternating with phase contrast microscopy in rapid sequence, allowing correlation of changes in anthracycline distribution with changes in cell morphology. The intracellular distribution of anthracyclines can be correlated to the localization of acridine orange, a localization marker of lysosomes (17).

In a prototypic experiment, an aliquot containing 5 × 10⁶ HL-60 or HL-60/AR cells incubated in media (pH 7.4 at 37°C) in the presence...
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of daunorubicin and doxorubicin was removed, centrifuged for 7 s, and resuspended in media (pH 7.4 at 4°C). It was then placed on a slide (ER 312; Erie Scientific Co., Portsmouth, NH). Fluorescent and phase contrast images magnification ×1000 were transcribed onto a videotape.

Studies of Intracellular Distribution of Daunorubicin and Doxorubicin in the Two-Compartment System of Folch. For the present studies, we used the Folch partitioning method previously employed by Duarte-Karim et al. (14) to demonstrate the high avidity of doxorubicin to lipids. The proportions of chloroform, methanol, and water used to prepare both phases were 3:48:47 by volume for the upper hydrophilic phase and 86:14:1 for the lower lipophilic phase. Each assay tube contained a total of 6 ml (3 ml of each phase) and contained a total of 0.1 µmol of doxorubicin or daunorubicin.

To redistribute the anthracyclines from the upper phase to the lower phase, the ionic strength was increased by adding 0.3% PBS to each assay tube. The ability of the verapamil and other agents to redistribute the anthracycline back to the upper hydrophilic phase was then tested. Because concentrations of 2–10 µM of verapamil and other amphophilic agents are typically needed to circumvent resistance in the various multidrug-resistant cell lines, we added 0.36 µmol of these agents to each assay tube to approximate a final concentration of 6 µM. The water-insoluble cyclosporine was used in higher concentrations than previously reported to circumvent resistance. After the addition of the drugs, the assay tubes were shaken and then incubated at 37°C for 30 min prior to reading the absorbance of the anthracyclines at 490 nm in upper and lower phases using a spectronic 20 Bausch and Lomb spectrophotometer. The various agents tested do not absorb at 490 nm and thus did not interfere with the study. Each agent that failed to alter the distribution of anthracycline was also tested at a 10-fold higher concentration.

RESULTS

Distribution of the Anthracyclines Daunorubicin and Doxorubicin in HL-60 and HL-60/AR Cells. The distribution of daunorubicin within HL-60 and HL-60/AR cells was monitored using DVFM. Photographs of the digitized video images of intracellular anthracycline fluorescence are shown in Fig. 1. HL-60 cells incubated with free daunorubicin 2.5 µM for 60 min in media (pH 7.4) demonstrate a diffuse pattern with an area of central pallor in viable cells (Fig. 1A). When the same cells are viewed under phase contrast microscopy, the area of central pallor corresponds to the nucleus (Fig. 1B). In contrast to the HL-60 cells, the HL-60/AR cells incubated with equimolar concentrations of free daunorubicin at pH 7.4 demonstrate a punctate pattern of fluorescence (Fig. 2A). This pattern of fluorescence is similar to the lysosomal fluorescence seen in both HL-60 and HL-60/AR cells exposed to acridine orange (Fig. 3). The punctate pattern of fluorescence is changed to the diffuse pattern if the HL-60/AR cells are incubated with both daunorubicin and verapamil (Fig. 4A). The distribution of doxorubicin (data not included) is similar to daunorubicin in HL-60/AR cells. Verapamil also changes the distribution of doxorubicin from a punctate to a more diffuse pattern. In HL-60 cells, verapamil does not change the distribution of daunorubicin (Fig. 5A).

Modification of the Distribution of Doxorubicin and Daunorubicin in the Two-Compartment System of Folch by Verapamil and Other Agents. Our results (Table 1) demonstrate that in

![Fig. 1. Distribution of daunorubicin in HL-60 cells. Logarithmically growing HL-60 cells were incubated with 2.5 µM daunorubicin for 1 h in media (pH 7.4). A, daunorubicin fluorescence; B, phase contrast image of the same cells incubated with daunorubicin.](cancerres.aacrjournals.org)
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Fig. 2. Distribution of daunorubicin in HL-60/AR cells. Logarithmically growing viable HL-60/AR cells incubated with 2.5 \( \mu \text{M} \) daunorubicin for 1 h in media (pH 7.4). A, daunorubicin fluorescence; B, phase contrast image of the same cells incubated with daunorubicin demonstrate a punctate pattern of fluorescence. The cell with the bright nuclear fluorescence is most likely a nonviable cell.

Fig. 3. Distribution of acridine orange in HL-60 cells. HL-60 cells were incubated for 1 h with acridine orange, 0.1 mg/ml, in media (pH 7.4).

Table 1 Modification of the distribution of doxorubicin in the two-phase system of Folch by various drugs

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Doxorubicin</th>
<th>Daunorubicin</th>
<th>Effect of drug on circumvention of multidrug resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without drug, without PBS</td>
<td>27</td>
<td>37</td>
<td>-</td>
</tr>
<tr>
<td>Without drug, with PBS</td>
<td>71</td>
<td>56</td>
<td>-</td>
</tr>
<tr>
<td>Verapamil</td>
<td>36</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Perhexilene maleate</td>
<td>31</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>29</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Clomiphene</td>
<td>27</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>36</td>
<td>47</td>
<td>+</td>
</tr>
<tr>
<td>Tamoxifen citrate</td>
<td>29</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Cyclosporine(^a)</td>
<td>31</td>
<td>21</td>
<td>+</td>
</tr>
<tr>
<td>Calcium ionophore</td>
<td>81</td>
<td>66</td>
<td>-</td>
</tr>
<tr>
<td>A23187</td>
<td>71</td>
<td>70</td>
<td>-</td>
</tr>
<tr>
<td>Estradiol</td>
<td>83</td>
<td>73</td>
<td>-</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>83</td>
<td>51</td>
<td>-</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>96</td>
<td>57</td>
<td>-</td>
</tr>
<tr>
<td>EDTA(^b)</td>
<td>96</td>
<td>56</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) \( \mu \text{mol} \) of cyclosporine was not calculated; 60 mg was used.

The two-compartment system of Folch, most of the anthracyclines are found in the hydrophilic compartment. This distribution is reversed by the presence of PBS in the chloroform, methanol, and water mixture. The addition of verapamil and other amphophilic agents in concentrations used to overcome drug resistance causes the anthracyclines to redistribute back to the hydrophilic Folch compartment. Higher concentrations of the more hydrophobic cyclosporine are required to change the distribution of anthracyclines. Other agents which do not circumvent resistance fail to modify the distribution of anthracyclines. Use of these agents in 10-fold higher concentrations also failed to change the distribution of anthracyclines (data not shown).
Fig. 4. Effect of verapamil on the distribution of daunorubicin in HL-60/AR cells. Logarithmically growing viable HL-60/AR cells incubated with 2.5 μM daunorubicin for 1 h in media (pH 7.4). A, daunorubicin fluorescence; B, phase contrast image of the same cells incubated with daunorubicin. HL-60/AR cells incubated with daunorubicin in the presence of verapamil demonstrate a diffuse pattern of fluorescence.

Fig. 5. Effect of verapamil on the distribution of daunorubicin in HL-60 cells. Logarithmically growing HL-60/AR cells incubated with 2.5 μM daunorubicin and 3 μM verapamil for 1 h in media (pH 7.4). A, daunorubicin fluorescence; B, phase contrast image of the same cells incubated with daunorubicin.
DISCUSSION

This is the first report demonstrating that daunorubicin and doxorubicin distribute differently in the cytoplasm of viable anthracycline-sensitive and -resistant HL-60 cells. In contrast to the HL-60 cells, in which the distribution of daunorubicin and doxorubicin appears to be homogeneous throughout the membranes and cytoplasm, the HL-60/AR cells localize these drugs in a distinct pattern within the cytoplasm corresponding to the intralysosomal distribution of acridine orange (17). There is increasing evidence demonstrating an association between drug resistance and alterations in both the glycoprotein and lipid composition of the membranes (18). Because of these alterations, the solubility of anthracyclines in various intracellular compartments may be changed, leading to the different distribution of doxorubicin in anthracycline-sensitive and -resistant HL-60 cells. The alteration of the intercompartmental distribution of daunorubicin and doxorubicin may enhance the ability of these drugs to be lost from the resistant cells, accounting for their decreased net accumulation and retention in HL-60/AR cells (13). It may also reduce the accessibility of doxorubicin and daunorubicin to intracellular targets, impairing the ability of these anthracyclines to exert a cytotoxic effect. It is thus possible that the different distribution of doxorubicin and daunorubicin in HL-60/AR cells may account for all the observed changes in their pharmacokinetics and cytotoxicity in HL-60/AR cells.

In the present study, we were also able to demonstrate that verapamil, an agent capable of increasing retention of daunorubicin and circumventing drug resistance in the HL-60/AR cell line (13) and in other multidrug-resistant cell lines, changed the distribution of daunorubicin and doxorubicin in the HL-60/AR cells from a punctate cytoplasmic distribution to the diffuse pattern found in HL-60 cells.

Verapamil and other agents known to circumvent drug resistance are very dissimilar in their biological function. Some affect intracellular calcium (11); others have hormonal properties (19). However, they do share one common characteristic: they all have an amphiphilic structure. Intracellularly, they may concentrate in the lysosomes (20) or interact with the membranes (12, 21) modifying the hydrophilic or hydrophobic character of the various intracellular compartments. They may thus change the distribution of daunorubicin or doxorubicin in the cell by altering their solubility in the various intracellular compartments. To test this hypothesis, we investigated the effect of these agents on anthracycline distribution within the two-compartment system of Folch. Verapamil, perhexiline maleate, trifluoperazine, clomiphene, chloroquine, and tamoxifen citrate, agents previously found to circumvent drug resistance in various multidrug-resistant cell lines (7–10, 12, 19) were all able to increase the distribution of doxorubicin in the hydrophilic compartment. Cyclosporine, also reported to circumvent drug resistance (10) is more hydrophobic than the other agents and may distribute differently in the cells. In the two-phase system of Folch, it had to be used in higher concentrations in order to shift the anthracyclines into the upper compartment. The calcium ionophore A23184, estradiol, diethylstilbesterol, indomethacin, EDTA, and CaCl₂, agents known to have hormonal activity or to affect intracellular calcium but previously reported to be incapable of reversing drug resistance in multidrug-resistant cell lines (12, 21), failed to modify the distribution of anthracyclines. Our results suggest that the ability of various drugs to circumvent resistance to anthracyclines in multidrug-resistant cell lines is related to their effect on the hydrophilic/hydrophilic interaction of anthracyclines and may be independent of their other biological effects.

By virtue of their amphiphilic structure, these drugs may circumvent resistance in multidrug-resistant cell lines by altering the hydrophilic/hydrophilic solubility of anthracyclines in the resistant cell, causing the drug to redistribute and to be more cytotoxic.

The two-phase system of Folch may be used in a screening test: to select drugs that may have potential in circumventing drug resistance. It may also provide us with clues for one mechanism of drug resistance and with an explanation as to how it is circumvented.

REFERENCES

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