Quantitative Determination of Factors Contributing to Doxorubicin Resistance in Multidrug-Resistant Cells

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There is a large discrepancy between the changes in drug accumulation and the changes in drug cytotoxicity that accompany development of anthracycline resistance in multidrug-resistant cells. In our study, a quantitative relationship has been established between reversal of multidrug resistance by resistance modifiers and a concomitant decrease in intracellular levels of doxorubicin measured at equitoxic concentrations (IC₅₀) in CHRC5 and 2780AD multidrug-resistant cells. (IC₅₀ = concentration required for 50% growth inhibition.) We have demonstrated that resistance modifiers like verapamil and Ro 11-2933/001 act by increasing the effectiveness of intracellular doxorubicin, apparently by inducing redistribution of the drug from the cytoplasm to the nucleus of a multidrug-resistant cell, as shown by quantitative fluorescence microscopy. At complete reversal of resistance, as measured directly or inferred by extrapolation, the amount of intracellular doxorubicin at the IC₅₀ as well as the ratio of nuclear doxorubicin to cytoplasmic doxorubicin were the same as those in sensitive cells. These results offer an explanation for the frequently observed discrepancies between drug accumulation and cytotoxicity and also show quantitatively that a decrease in drug accumulation and a change in intracellular drug distribution together are the only determinants of doxorubicin resistance in the multidrug-resistant cells studied. [J Natl Cancer Inst 81:1887-1892, 1989]

Many doxorubicin-resistant cells with the multidrug-resistance phenotype exhibit impaired doxorubicin accumulation, compared with their sensitive parent cells (1–17). This phenomenon has been attributed mainly to enhanced active efflux of the drug out of the cells (1,2,5–12,14), which is thought to occur via P-glycoprotein (18).

A discrepancy between the defect in drug accumulation and the coexisting difference in cellular sensitivity has repeatedly been demonstrated. In a number of studies, sensitive cells accumulated two to seven times more drug than multidrug-resistant cells, while the cytotoxicity of anthracyclines was 10-400 times greater in sensitive cells (1,6,19–23). Consequently, it has been speculated that factors in addition to differences in drug accumulation must be responsible for resistance to anthracyclines (22,24–29). One such factor could be an increased drug detoxification capacity in multidrug-resistant cells (30). In the present study with the multidrug-resistant cell lines CHRC5 and 2780AD, we established a quantitative relationship between the sensitivity of the cells to doxorubicin and the accumulation of the drug during exposure to resistance-modifying agents measured at equitoxic concentrations. The data show (a) that doxorubicin resistance in multidrug-resistant cells overexpressing P-glycoprotein is mainly due to the impaired ability of the drug to reach cellular targets critical for cytotoxicity, rather than to impairment of drug accumulation only, and (b) that resistance modifiers act on both processes. Preliminary data were presented at the 78th Annual Meeting of the American Association for Cancer Research (31).

Materials and Methods

Drugs

Verapamil and colchicine were obtained from Sigma Chemical Co. (St. Louis, MO). Bepridil was provided by Organon International (Oss, The Netherlands) as a solution containing 4 mg of bepridil and 48.4 mg of glu-

Received September 5, 1989; revised September 19, 1989; accepted September 19, 1989.

Supported in part by The Netherlands Cancer Foundation grant IKA VU 88-22, the Haak-Bastaanse Kuneman Foundation, and by a grant from Bristol-Myers Co.

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We thank Mrs. N. W. Schipper and Mr. T. K. ten Kate for their assistance with laser scan microscopy and image processing; Mr. Opheenhoffen, Mrs. Altena, and Mrs. Lilian for secretarial help; AKZO Pharma B.V. for bepridil monohydrochloride monohydrate; and Hoffmann-La Roche, Inc. for Ro 11-2933/001.

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cose/mL. Ro 11-2933/001 (N-[3,4-dimethoxyphenyl]-2-[2-naphthyl]-N-methyl-m-dithiane-2-propylamine), which is a tiapamil analog, was a gift from Hoffmann-La Roche, Inc. (Basel, Switzerland). Doxorubicin was obtained from Laboratoire Roger Bellon (Neuilly sur Seine, France), and [14-14C]doxorubicin (53.3 Ci/mol) was obtained from Amersham Radiochemical Center (Amersham, United Kingdom). All stock solutions were prepared in 0.9% NaCl, except Ro 11-2933/001, which was prepared in dimethyl sulfoxide.

Cells and Cell Culture

The Chinese hamster ovarian cell line AUXB1 and its colchicine-resistant subline CHRC5 were supplied by V. Ling (Ontario Cancer Institute, Toronto, Ontario, Canada); the doxorubicin-sensitive human ovarian carcinoma cell line A2780 and its resistant subline 2780AD were obtained from R. F. Ozols (National Cancer Institute, Bethesda, MD). Cells were cultured as described previously (32). The resistant cell lines were cultured in the presence of drugs until 1–2 weeks before the experiments. The CHRC5 cell line was cultured in the presence of 10 μg of colchicine/mL, and the 2780AD cell line was cultured in the presence of 2 μM doxorubicin. Both types of multidrug-resistant cells highly overexpress P-glycoprotein (33).

Drug Treatment

In cytotoxicity experiments, we used a 2-hour incubation period with doxorubicin either with or without resistance modifier (verapamil, bepridil, or Ro 11-2933/001) and a 2-hour postincubation period with or without modifier only, as described previously (32). In all cases, the synergistic effects of resistance modifiers on doxorubicin cytotoxicity were determined after correction for the growth inhibition exerted by the modifiers alone (maximum, 15%).

Doxorubicin Accumulation

For measurement of doxorubicin accumulation, trypsinized cells were suspended in growth medium (pH 7.4) containing 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) but lacking NaHCO3 and incubated for 90 minutes at 37 °C (32). Cellular accumulation was defined as the amount of cell-associated doxorubicin at 37 °C at the time studied minus the amount at time zero at 0 °C. Doxorubicin accumulation at any concentration of interest was calculated from the amount present after incubation of cells with 2 μM doxorubicin. Before making calculations, we confirmed two conditions at concentrations of 0.2–2.0 μM: (a) that cellular doxorubicin levels were linear with extracellular concentrations in all cell lines and (b) that the percentage of enhancement of drug accumulation produced by resistance modifiers in the multidrug-resistant cells was not significantly different at different doxorubicin concentrations. In some cases (see fig. 2 and 3 legends), doxorubicin accumulation at the IC50 was measured directly. (IC50 = concentration required for 50% growth inhibition.)

Microscopy

Conventional fluorescence microscopy. Adherent cells were trypsinized, seeded on sterile coverslips, and cultured for 1–2 days. The slides were then incubated in the medium for 1 hour at 37 °C in the presence of doxorubicin, either with or without resistance modifier. The coverslips were mounted carefully upside down on object slides with a thin film of medium. Cells were viewed with a Leitz Orthoplan fluorescence microscope equipped with a superpressure mercury lamp (model HBO 200W/4, Osram, Federal Republic of Germany); a 570-nm filter (Leitz, Wetzlar, Federal Republic of Germany) was attached. Fluorescence photographs were made at exposure times of 60 or 120 seconds with 400 Ektachrome film (Kodak Ltd., Hemel Hempstead, United Kingdom).

Laser scan microscopy. Cells were grown and incubated with drugs as described, except that the total incubation time was 2 hours. We chose doxorubicin concentrations that achieved approximately equal intracellular amounts of doxorubicin for each type of treatment: 6 μM doxorubicin for treatment of AUXB1 cells and the following concentrations for treatment of CHRC5 cells—6 μM doxorubicin plus 16, 32, or 64 μM verapamil; 10 μM doxorubicin plus 8 μM verapamil; 14 μM doxorubicin plus 4 μM verapamil; or 20 μM doxorubicin without verapamil. After incubation, we washed the cells with phosphate-buffered saline without glucose to prevent drug efflux and mounted them on object slides. For each concentration of verapamil in each experiment, we photographed 10–20 cells on a laser scan microscope (model LSM 41, Carl Zeiss, Oberkochen, Federal Republic of Germany) using a 488-nm-emission laser (34). Each image containing 500 × 500 pixels, digitized to 8 bits per pixel, was transferred to a digital image-processing and image-analyzing computer (Kontron Bildanalyse GmbH, Eching, Federal Republic of Germany). On this system, transmission indicates that there is fluorescence, and fluorescence is measured interactively by tracing of the nucleus and cytoplasm of each cell in the image.

Results

When the multidrug-resistant and sensitive cell lines used in this study were incubated with 2 μM doxorubicin, we observed that the cells from the parent lines AUXB1 and A2780 demonstrated greater accumulation of doxorubicin than cells from the corresponding multidrug-resistant sublines: AUXB1 cells contained 3.7-fold more doxorubicin than CHRC5 cells, and A2780 cells contained 2.9-fold more doxorubicin than 2780AD cells (table 1). In contrast, the differences in sensitivity to doxorubicin were much greater: 348-fold for Chinese hamster ovary cells and 72-fold for 2780 cells (table 1). When drug accumulation was measured at the IC50 of doxorubicin, however, multidrug-resistant cells appeared to accommodate more drug than sensitive cells (table 1), clearly showing that other mechanisms of resistance are operable in these cells. Exposure of CHRC5 cells to doxorubicin in the presence of the resistance modifier verapamil or bepridil resulted in strong potentiation of doxorubicin cytotoxicity to levels close to those for AUXB1 cells (32). Such effects are at least partly due to enhancement of doxorubicin accumulation, as illustrated in figure 1.

In an attempt to investigate the mechanism by which small
Table 1. Doxorubicin cytotoxicity and accumulation in two multidrug-resistant and two sensitive cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ (µM)</th>
<th>RI</th>
<th>Cellular Dx, pmol/10⁶ cells At 2 µM</th>
<th>Cellular Dx, pmol/10⁶ cells At IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHRC5</td>
<td>160 ± 50</td>
<td>348</td>
<td>36.4 ± 15.1 (1)</td>
<td>172 ± 37</td>
</tr>
<tr>
<td>AUXB1</td>
<td>0.46 ± 0.23</td>
<td>1</td>
<td>134 ± 41 (3.7)</td>
<td>30.0 ± 1.2</td>
</tr>
<tr>
<td>2780AD</td>
<td>17.4 ± 11.0</td>
<td>72</td>
<td>52.4 ± 14.8 (1)</td>
<td>128 ± 33</td>
</tr>
<tr>
<td>A2780</td>
<td>0.24 ± 0.16</td>
<td>1</td>
<td>154 ± 19 (2.9)</td>
<td>23.5 ± 7.8</td>
</tr>
</tbody>
</table>

*Doxorubicin (Dx) cytotoxicity and accumulation were measured in 2- and 1.5-hr incubation assays, respectively. Values = means ± SD for three to seven experiments. Values in parentheses indicate accumulation relative to level in resistant cells. RI (resistance index) = IC₅₀ in resistant cell line/IC₅₀ in sensitive cell line. CHRC5 cell line is colchicine-selected, multidrug-resistant subline of sensitive AUXB1 parent line, and 2780AD cell line is doxorubicin-selected, multidrug-resistant subline of sensitive A2780 parent line.

Increments in accumulation can result in relatively large increases in sensitivity to doxorubicin, we determined doxorubicin accumulation using various concentrations of verapamil at the IC₅₀ values determined with those verapamil concentrations. Accumulation values were calculated from the data shown in figure 1 as described in the Materials and Methods section. The results of the calculations and measurements are shown in figure 2. Clearly, the intracellular amount of doxorubicin at the IC₅₀ decreased with increasing concentrations of verapamil. Most remarkably, the intracellular doxorubicin levels in CHRC5 cells were equal to those observed at the IC₅₀ value for AUXB1 cells (0.46 µM) when resistance had been reversed completely. The inset in figure 2 shows that the relationship between log IC₅₀ and the corresponding doxorubicin level was linear.

We performed experiments similar to those described for CHRC5 and AUXB1 cells with the multidrug-resistant human ovarian cancer cell line 2780AD and its sensitive parent line A2780, using the potent resistance modifier Ro 11-2933/001 (28). Findings were similar to those for CHRC5 cells (fig. 3). The amounts of intracellular doxorubicin measured at the IC₅₀ dropped with increasing sensitivity (decreasing IC₅₀); both values approached those observed for the parent line (fig. 3).

From these results, we speculated that resistance modifiers mainly act by increasing the doxorubicin concentration at cellular sites critical for cytotoxicity. To further substantiate this hypothesis, we looked at the intracellular localization of doxorubicin in resistant and sensitive cells and the effects of a resistance modifier on this localization. It appears that doxorubicin was present almost exclusively in the nucleus of AUXB1 cells, but mainly in the cytoplasm of CHRC5 cells (fig. 4A–4D). The distribution was similar for AUXB1 cells exposed to 2, 6, 20, or 40 µM doxorubicin and CHRC5 cells exposed to 20, 40, or 100 µM doxorubicin (data not shown). However, when 40 µM doxorubicin was used with at least 16 µM verapamil, the drug localized mainly in the nucleus in CHRC5 cells (fig. 4E and 4F). The same results were observed with 6 µM doxorubicin in the presence of 16–64 µM verapamil. Qualitatively similar changes in fluorescence distribution patterns were obtained in CHRC5 cells with the resistance modifiers bepridil and Ro 11-2933/001 and in 2780AD cells with all three resistance modifiers (data not shown).

The results of laser scan microscopy demonstrated that the
Figure 3. Relationship between Ro 11-2933/001-induced decrease of IC_{50} and intracellular doxorubicin (Dx) present at IC_{50} in 2780AD cells. Intracellular doxorubicin at IC_{50} was calculated as described for CH_{R}C5 cells from data on doxorubicin accumulation at 2 μM doxorubicin. Each point represents data from two or three independent experiments on drug accumulation (data not shown) and cytotoxicity. Accumulation at IC_{50} in A2780 cells (open circles) and 2780AD cells (closed circles) without Ro 11-2933/001 (Ro) present was measured directly. In inset, data are plotted semilogarithmically.

Figure 4. Effect of verapamil on intracellular localization of doxorubicin in AUB1 and CH_{R}C5 cells. Logarithmically growing adherent cells were incubated with doxorubicin (40 μM) with or without verapamil (16 μM) for 60 min at 37 °C and then photographed with fluorescence microscopy. Arrows indicate nuclei; arrowheads indicate cytoplasm. A and B: AUB1 cells without verapamil. C and D: CH_{R}C5 cells without verapamil. E and F: CH_{R}C5 cells with verapamil. Bar indicates 50 μm in panels A, C, and E and 25 μm in panels B, D, and F.

ratio of nuclear fluorescence to cytoplasmic fluorescence was high for AUB1 cells and low for CH_{R}C5 cells. Furthermore, verapamil caused a dose-dependent increase of this ratio for CH_{R}C5 cells, up to the value for AUB1 cells, at verapamil concentrations that also maximally potentiated doxorubicin cytotoxicity close to the sensitive level (fig. 5).

Discussion

It has already been established that, among other drugs involved in multidrug resistance, anthracyclines show impaired accumulation in multidrug-resistant cells (1–17). To date, however, there has been poor correlation between the degree of accumulation impairment and the level of resistance in these cells (1,16,19–23). For this reason, it has been argued that mechanisms other than those relating to total cellular accumulation must be involved in the development of anthracycline resistance (22,24–29). We have now shown (a) that inefficient drug sequestering makes a major quantitative contribution to resistance to doxorubicin in multidrug-resistant cells and (b) that this factor, together with an accumulation defect, completely explains resistance in the two multidrug-resistant cell lines studied. Our results with fluorescence microscopy, including the quantitative determination of intracellular doxorubicin distribution, strongly suggest that this resistance-associated change in drug sequestering in the multidrug-resistant cells is due to a change in the localization of intracellular doxorubicin. Localization is mainly in the nucleus in sensitive cells and mainly in the cytoplasm in resistant cells.

Furthermore, the effects of a resistance modifier on multidrug-resistant cells are twofold. A resistance modifier stimulates drug accumulation (fig. 1), but most important, it increases the ability of a given amount of intracellular doxorubicin to inhibit cell growth (fig. 2 and 3) by changing intracellular drug localization to the sensitive phenotype (fig. 4 and 5).

We have demonstrated that our methods for study of multidrug resistance in the CH_{R}C5 cell line, in which resistance to doxorubicin was reversed completely, can also be applied to cell lines like 2780AD, in which complete reversal cannot be achieved—a common phenomenon in human
mulation in such multidrug-resistant cells, which has indeed been observed (19–21).

Further study is required to explain on a molecular level how resistance modifiers achieve reversal of resistance. Competition with the anthracycline for an efflux pump, presumably P-glycoprotein (18), is the most plausible explanation (48). Such competition for the pump might occur at the expense of adenosine triphosphate, as we have shown recently (49). Although it is likely that drug transport via the plasma membrane is affected by resistance modifiers, our results suggest that resistance modifiers also affect doxorubicin cytotoxicity by interaction with cytoplasmic doxorubicin binding sites or stores. In addition to membrane P-glycoprotein (18), cytoplasmic P-glycoprotein may be a binding site for doxorubicin. To date, cytoplasmic P-glycoprotein has been detected only in the Golgi apparatus (50). If P-glycoprotein is present on endocytic vesicles, it may be active in cellular drug sequestration and sensitive to modulation by resistance modifiers. Alternatively, resistance modifiers may change the distribution of anthracyclines over hydrophobic–hydrophilic phases (51) or may affect drug exocytosis involving acidic vesicles (52), which has been suggested as an alternative mechanism for multidrug resistance (52,53).

Conclusions

We have shown that doxorubicin resistance in two multidrug-resistant cell lines is mainly due to an impaired ability of doxorubicin to affect cellular targets critical for cytotoxicity, rather than to an impaired accumulation. This difference in effectiveness is likely due to a difference in intracellular drug localization. Resistance modifiers mainly act by increasing this effectiveness. Our quantitative approach to delineation of these phenomena could be applied to the study of anthracycline resistance in other multidrug-resistant cells.

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