Reversal of Acquired Resistance to Doxorubicin in P388 Murine Leukemia Cells by Perhexiline Maleate

A. Ramu, Z. Fuks, S. Gatt, and D. Glaubiger

Department of Radiation and Clinical Oncology, Hadassah University Hospital, P. O. Box 12000, Jerusalem, Israel 91120 [A. R., Z. F.]; Laboratory of Neurochemistry, Department of Biochemistry, The Hebrew University-Hadassah Medical School, Jerusalem, Israel 91010 [S. G.]; and Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205 [D. G.]

ABSTRACT

The effects of perhexiline maleate on growth and drug sensitivity were studied in the P388 murine leukemia cell line and in an anthracycline-resistant subline (P388/ADR). At noninhibitory concentrations, perhexiline maleate markedly increased the sensitivity of P388/ADR cells to doxorubicin but did not have such an effect on anthracycline-sensitive cells.

The effects of perhexiline maleate on P388/ADR cells were reversible. Perhexiline maleate also increased the accumulation of another anthracycline, daunorubicin, in P388/ADR cells but did not increase its accumulation in the anthracycline-sensitive cells.

Perhexiline maleate did not affect the sensitivity of either cell line to methotrexate or to 6-mercaptopurine. However, its effects on the sensitivity and on drug accumulation of vinblastine, a drug to which P388/ADR cells are cross-resistant, were similar to those observed for the anthracyclines.

Although perhexiline maleate has been reported to be a calcium antagonist in other systems, our data do not suggest that this mechanism is involved in its enhancement of the sensitivity of P388/ADR cells to doxorubicin. We suggest instead that this effect might be associated with alterations of cell lipid metabolism induced by perhexiline maleate.

INTRODUCTION

Initial responses to chemotherapy in cancer patients are often followed by relapses which are associated with resistance to the original agents used. Acquired resistance to anthracyclines, a group of potent anticancer drugs, has been studied in several experimental tumor systems (10, 20, 26). In all systems studied, resistance to anthracyclines was associated with resistance to other chemically unrelated drugs, including Vinca alkaloids and actinomycin D (10, 11, 16, 19, 20, 26). The mechanism of this cross-resistance is not known, but a number of investigators have noted decreased accumulation of these drugs in resistant cells (4, 12, 17, 18, 26, 30, 31). It was suggested that selection of cells with altered membrane properties, resulting in retarded drug uptake, might be the mechanism by which a cell population resistant to these drugs could emerge from sensitive cells that are exposed to them (5).

In early studies, anthracycline- and Vinca alkaloid-resistant cells were found to have increased glycoprotein content compared to sensitive parent cells (3, 5, 21, 23). However, inhibition of glycoprotein synthesis in these cells does not affect drug accumulation (6) or drug sensitivity (2). Therefore, it is unlikely that an increase in glycoprotein content is involved in the mechanism of acquired anthracycline resistance.

We recently found that anthracycline-sensitive and -resistant cell lines of P388 murine leukemia could be distinguished on the basis of their lipid composition.3 These observations led us to consider whether drugs known to modify lipid metabolism might diminish acquired resistance of P388 cells to anthracyclines.

We now report that perhexiline maleate, a drug known to raise the phospholipid content of fibroblasts (1), indeed reverses the resistance of these cells to anthracyclines and to vinblastine.

MATERIALS AND METHODS

Cell Culture. P388 murine leukemia cells and an anthracycline (doxorubicin)-resistant subline (P388/ADR), obtained from ascites fluids of tumor-bearing BALB/c x DBA/2 F₁ mice, were maintained continuously in suspension culture. The cells were maintained in RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co.), 10 μM 2-mercaptoethanol, penicillin base (50 units/ml), and streptomycin (50 μg/ml).

Cell growth was assessed by measurement of cell density in a Coulter Counter (Coulter Electronics, Harpenden, Hertfordshire, England). An inoculum of cells was transferred to fresh medium once every 4 days to maintain them in exponential growth. Initial cell density was 10⁶ cells/ml; after 4 days in culture, the density was 1 to 2 × 10⁷ cells/ml. Growth rates were calculated from the culture densities measured once a day for 4 days.

The sensitivity to doxorubicin of both cell lines was routinely assessed once every 5 transfers. No change in the drug sensitivity of either subline was observed during 3 years of continuous in vitro culture.

Determination of Drug Sensitivity. The sensitivity of both cell lines to perhexiline maleate, doxorubicin, vinblastine, and various combinations of these drugs was assessed as follows. Cells were cultured in the presence of various drug concentrations, and the slope of the log cell density versus time plot was calculated by linear regression analysis. The growth rate at each drug concentration was expressed as the percentage of the control growth rate. Dose-effect curves were thus produced and were used to determine the ED₅₀.

Drug Accumulation Studies. Cells from both lines at a density of 1 to 2 × 10⁶ cells/ml were preincubated with or without 1 × 10⁻⁶ M perhexiline maleate for 2 hr at 37°C. [³H]Daunorubicin (3.27 Ci/mmol) or [³H]vinblastine (8.26 Ci/mmol) was then added. Their concentrations in the medium were 7.64 × 10⁻⁸ and 3.03 × 10⁻⁹ M, respectively. At various time intervals after drug addition, 0.8-ml aliquots (in triplicate) of the cell suspension were transferred into 1-ml capillary tubes (Biotips with sealed 1 This work was supported by grants from the Joint Research Fund of the Hebrew University and Hadassah and from the Israel Cancer Research Fund.

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The abbreviations used are: RPMI 1640, Roswell Park Memorial Institute Medium 1640; ED₅₀, concentration of drug effective in inhibiting the growth rate by 50%; EGTA, ethyleneglycol bis[β-aminoethyl ether]-N,N'-tetraacetic acid.


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Perhexiline maleate inhibited the growth of both P388 and P388/ADR cells in a concentration-dependent manner. However, no effect on the growth rate of P388 or P388/ADR cells was observed at perhexiline maleate concentrations lower than 8 × 10⁻⁶ and 1.5 × 10⁻⁵ M, respectively. The effects of doxorubicin on the growth rate of P388 and P388/ADR cells in the presence of noninhibitory concentrations of perhexiline maleate (5 × 10⁻⁶ M for P388 cells and 1 × 10⁻⁵ M for P388/ADR cells) are shown in Chart 1. In the presence of perhexiline maleate, there is a marked increase in the sensitivity of P388/ADR cells to doxorubicin. The ED₅₀ for doxorubicin was reduced from 1.5 × 10⁻⁶ M in the absence of perhexiline maleate to 1.3 × 10⁻⁷ M in its presence. The sensitivity of P388 cells to doxorubicin was not affected by the presence of 5 × 10⁻⁶ M perhexiline maleate. At higher concentrations of perhexiline maleate, the inhibitory effects on growth of P388 cells were additive to those of doxorubicin. When the doxorubicin dose-response curves for P388 cells obtained at these high concentrations of perhexiline maleate were corrected for the inhibitory effects of perhexiline maleate alone, no effect of perhexiline maleate on the sensitivity of P388 cells to doxorubicin is observed.

In order to characterize further the enhancement of doxorubicin inhibition of the growth of P388/ADR cells by perhexiline maleate, we measured the effect of increasing concentrations of perhexiline maleate on the growth of P388/ADR cells incubated in the presence of a low concentration (5 × 10⁻⁷ M) of doxorubicin (Chart 2). In the absence of perhexiline maleate, doxorubicin at this concentration failed to inhibit the growth of P388/ADR cells.

However, if perhexiline maleate was added to P388/ADR cells incubated with this low concentration of doxorubicin, a clear dose-dependent effect was observed. This enhancement of doxorubicin effect was caused by perhexiline maleate in concentrations well below those having independent growth-inhibitory effects of their own. In a similar experiment, where P388 cells were incubated with a concentration of doxorubicin just below that needed to demonstrate growth inhibition (1 × 10⁻⁸ M), no enhancement of growth inhibition could be obtained by adding perhexiline maleate at concentrations up to 1 × 10⁻⁵ M.

The enhancement of doxorubicin sensitivity of P388/ADR cells by perhexiline maleate was found to be concentration-dependent. The effects of perhexiline maleate on the growth rate of P388/ADR cells are shown in Chart 2. In the presence of perhexiline maleate, the effects of doxorubicin were additive to those of perhexiline maleate alone.
by perhexiline maleate is reversible. When cells that had been preincubated with perhexiline maleate (1 × 10^{-5} M) for 4 days were washed and transferred to perhexiline-free medium and their sensitivity to doxorubicin was tested, it was found that the sensitivity returned to the level observed prior to the exposure to perhexiline maleate, while P388/ADR cells that were maintained in medium with perhexiline maleate continued to demonstrate decreased resistance to doxorubicin.

To evaluate whether perhexiline maleate could also enhance the sensitivity of these cells to the cross-resistance drugs, we measured the effects of perhexiline maleate at noninhibitory concentrations on the sensitivity of P388 and P388 cells to vinblastine (Chart 3). While perhexiline maleate had only a minor effect on the sensitivity of P388 cells to vinblastine (ED_{50} was reduced from 5.3 × 10^{-9} M to 2.5 × 10^{-9} M), it had a marked effect on the sensitivity of P388/ADR cells to vinblastine (ED_{50} was reduced from 1.3 × 10^{-7} M to 9.5 × 10^{-9} M). In contrast to these results, dose-effect curves of both cell lines to drugs that are not cross-resistant, methotrexate and 6-mercaptopurine, were not affected by the presence of perhexiline maleate. To test whether the enhancement of anthracycline sensitivity in P388/ADR cells by perhexiline maleate is associated with an increase in drug accumulation, we studied their ability to accumulate [3H]daunorubicin. Cells from both lines were preincubated with perhexiline maleate (1 × 10^{-5} M) for 2 hr and then further incubated with 7.64 × 10^{-8} M [3H]daunorubicin. The time course of [3H]daunorubicin accumulation by the cells is shown in Chart 4. The accumulation of labeled drug in the P388/ADR cells reached a plateau after 30 min, at a level that was 34.9% of the drug accumulated in P388 cells at the same point in time. Drug accumulation in P388 cells continued to increase beyond this time and did not level off during the 90 min of incubation. Perhexiline maleate did not affect the time course of drug accumulation in these cells. In contrast, in anthracycline-resistant P388 cells, perhexiline maleate induced increased accumulation of [3H]daunorubicin. In the presence of perhexiline maleate, the initial rate of labeled drug accumulation and the plateau level were more than doubled when compared to the accumulation of the drug measured in the absence of perhexiline maleate.

We have further tested whether the enhancement of vinblastine inhibitory effect on P388/ADR cells by perhexiline maleate is also associated with increased vinblastine accumulation. Cells from both lines were preincubated with 1 × 10^{-5} M perhexiline maleate for 2 hr and then further incubated with 3.03 × 10^{-8} M [3H]vinblastine. As shown in Chart 5, in the presence of perhexiline maleate, [3H]vinblastine accumulation at 30 min was increased by a factor of 2.5, and the plateau level was achieved markedly later than that observed in the absence of perhexiline maleate. [3H]Vinblastine plateau levels in P388 cells were 12.7 times higher than those measured in P388/ADR cells, and these levels were not affected by perhexiline maleate.

Perhexiline maleate has been shown to be a calcium antagonist (14). It is therefore possible that its effects on anthracycline sensitivity are calcium dependent. Cells from both lines were cultured in medium containing only 10% of the standard concentration of calcium in the growth medium (made of calcium-free RPMI 1640 plus 10% fetal calf serum), and their sensitivity to doxorubicin was tested. No changes in sensitivity to doxorubicin were observed in either cell line. Furthermore, the effects of perhexiline maleate on the sensitivity of both cell lines to doxorubicin were similar to those measured in standard growth medium. Likewise, the sensitivity of both cell lines to doxorubicin was not affected when EGTA (1 × 10^{-5} M) was added to standard growth medium. No changes were observed in the sensitivity of

![Chart 4](chart4.png)

![Chart 3](chart3.png)

![Chart 5](chart5.png)
either cell line in the presence of $1 \times 10^{-2}$ M calcium or in the presence of $6 \times 10^{-8}$ M calcium ionophore A23187 which was the maximal concentration of A23187 having no measurable effect on the growth of either cell line. In addition, neither indomethacin, another calcium antagonist (22), nor lanthanum chloride (in concentrations of up to 1 mm), a competitive blocker of calcium uptake, altered the sensitivity of either cell line to doxorubicin.

**DISCUSSION**

In the present study, we have demonstrated that the inhibition of growth of an anthracycline-resistant P388 cell subline by doxorubicin is greatly enhanced in the presence of perhexiline maleate. Perhexiline maleate has no such effect on the anthracycline-sensitive parent cell line. The effects of perhexiline maleate are reversible. This suggests that selection of anthracycline-sensitive cells from the anthracycline-resistant cell population has not occurred. Rather, perhexiline maleate must be present concurrently with doxorubicin to exert its enhancement of the inhibitory effect.

We have demonstrated further that perhexiline maleate increases the accumulation of another anthracycline, daunorubicin, in anthracycline-resistant cells but not in the anthracycline-sensitive cell line. It is therefore suggested that perhexiline maleate lowers the resistance of P388/ADR cells by increasing their ability to accumulate anthracyclines. As mentioned previously, anthracycline-resistant cells are known to be cross-resistant to Vinca alkaloids and some other drugs, but not to methotrexate or 6-mercaptopurine. We have found that perhexiline maleate did not affect P388 and P388/ADR cells sensitivities to methotrexate or 6-mercaptopurine. In contrast, it greatly enhances the sensitivity of P388/ADR cells to vinblastine, without having the same effect on anthracycline-sensitive cells. Again, this effect of perhexiline maleate on P388/ADR cells is associated with increased drug accumulation, while it has no effect on uptake of vinblastine into anthracycline-sensitive cells. The difference between the effects of perhexiline maleate on the sensitivities to doxorubicin and vinblastine in one hand and on the sensitivities to methotrexate and 6-mercaptopurine on the other may indicate a fundamental difference in the way these drugs are transported into cells. While a relatively well-defined carrier-mediated transport has been shown for methotrexate and 6-mercaptopurine (15, 24), the nature of the cellular transport processes for anthracyclines and Vinca alkaloids is not known. In early studies (12, 18, 28, 29), based mainly upon saturation kinetics, it was suggested that these drugs are taken up via a passive carrier-mediated transport system. However, a recent study has shown that the saturation kinetics may have resulted from concentration-dependent self-association of anthracycline molecules in the medium (9). Furthermore, it was suggested that the transport of anthracyclines takes place by simple Fickian diffusion through the lipid domain of the cell membrane (8). Therefore, it is plausible that a compound like perhexiline maleate, known to affect the content of phospholipids in cells (1), may affect the transport (and thus sensitivity) of drugs that enter cells by diffusion through the lipid domain of the plasma membrane of the cell. These compounds would not be expected to affect the cellular uptake of drugs, like methotrexate or 6-mercaptopurine, which enter cells via carrier-mediated transport systems.

One must also consider the differential effect of perhexiline maleate on the inhibitory activity of doxorubicin and vinblastine in anthracycline-sensitive and -resistant cells. In our previous studies, it was demonstrated that anthracycline-resistant cells have a higher intracellular content of triglycerides and a lower phosphatidyicholine:sphingomyelin ratio when compared to anthracycline-sensitive cells. These changes apparently result in the observed increase in the structural order of lipid domain of the plasma membrane of resistant cells (25) and lead to a reduced rate of inward diffusion of anthracyclines into these cells. We suggest that the observed differences in the effects of perhexiline maleate on drug uptake and sensitivity between these cell lines are the result of differences in its ability to interact with cell plasma membrane lipids. A similar differential effect on anthracycline-sensitive and -resistant cells was reported to occur in the presence of Tween 80, a surface active non-ionic detergent (17, 27). This further supports our suggestion that differences in the composition of plasma membrane lipids between anthracycline-sensitive and -resistant cells allow for different effects of compounds on the lipid domains of the membranes and lead to differential effects on anthracycline uptake and sensitivity in these cell lines.

Finally, we note that perhexiline maleate, a drug used as a coronary vasodilator, has been shown to act as a calcium antagonist in other systems (14). Tsuru et al. (32) have reported that verapamil, another calcium antagonist, also enhanced the accumulation and cytotoxicity of vincristine in vincristine-resistant P388 cells. However, in contrast to the results reported in the present study, these effects of verapamil were also obtained in vincristine-sensitive P388 cells. These workers speculated that verapamil blocked a calcium-dependent vincristine efflux mechanism in a manner similar to the effect of verapamil on hormonal secretion (13). Hormonal secretion is also suppressed by lowering the extracellular calcium concentration (7), and under these conditions the inhibitory effect of verapamil is markedly increased (13). We therefore studied whether the effects of perhexiline maleate are related to extracellular calcium concentration. Reduction of the calcium concentration in the medium to one-tenth of its standard level, or chelation of calcium ions using EGTA, or blocking calcium uptake with lanthanum chloride did not result in changes in the sensitivity of either cell line to doxorubicin. There was also no change in the enhancement of sensitivity of resistant cells to doxorubicin by perhexiline maleate. Furthermore, if calcium antagonism could lower anthracycline resistance, one would expect that, by increasing the concentration of calcium in the medium or by adding a calcium ionophore, one would increase the level of resistance to anthracyclines. However, no change in doxorubicin sensitivity of either cell line was observed in the presence of high calcium concentrations or in the presence of the calcium ionophore A23187. Finally, indomethacin, another drug reported to be a calcium antagonist (22), did not affect the sensitivity of either cell line to doxorubicin.

The present results indicate that perhexiline maleate enhances the effectiveness of anthracyclines against resistant cells in vitro. They imply that concomitant administration of these drugs in patients may result in enhanced chemotherapeutic activity of anthracyclines in refractory patients. Prior to such trials, in vivo studies demonstrating no decrease in the therapeutic index of anthracyclines in the presence of perhexiline maleate are needed. Such studies are presently in progress.
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REFERENCES

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