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Cross resistance to esters of methotrexate in a doxorubicin-resistant subline of P388 murine leukemia

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Summary. Resistance to methotrexate was developed by continuous exposure of P388 murine leukemia cells in vitro to increasing concentrations of methotrexate up to 1×10^{-7} M. Once established, the resistance to methotrexate was stable. This was also found in methotrexate-resistant cells that were maintained in methotrexate-free medium for more than 4 months.

The sensitivity of the methotrexate-resistant P388 cells to doxorubicin was comparable to the sensitivity measured in the parental cell line.

Another methotrexate-resistant cell line was developed, in a similar way, from doxorubicin-resistant P388 cells. This methotrexate-resistant cell line maintained its original resistance to doxorubicin.

In methotrexate-sensitive cells, the dimethyl and dibutyl esters of methotrexate were 18.3- and 2.7-fold less active, respectively, than the free methotrexate in inhibiting cell growth.

In methotrexate-resistant cells, the inhibitory effect of the methotrexate dimethyl ester was similar to its effect on the methotrexate-sensitive cell line. The activity of the methotrexate dibutyl ester was 3.3-fold lower than its activity in the parental cell line. However, both esters of methotrexate were much more active than free methotrexate in the methotrexate-resistant cell line.

In the doxorubicin-resistant cell line, the activity of free methotrexate was comparable to its activity in the doxorubicin-sensitive parent cell line. However, this cell line was remarkably resistant to the ester analogs of methotrexate.

The clinical implications of these findings are discussed.

Introduction

Several different mechanisms of resistance to methotrexate have been described in rodent cells [3, 4]. However, it was reported that methotrexate resistance in human cells was most often caused by defects in the drug transport [6, 16, 20, 21, 25].

Replacing methotrexate by other folate antagonists, which enter cells by diffusion rather than by the specific methotrexate transport system, is one of the approaches that have been suggested to overcome methotrexate transport resistance. Many studies have shown that the more lipophilic triazinate, trimethoxyquine, metoprine, and methotrexate esters can penetrate methotrexate-transport-deficient cells more readily than free methotrexate and are therefore more effective cytotoxic agents against these cells [1, 2, 6, 10, 19, 21, 22, 25–28].

Cancer patients are usually treated with drug combinations rather than a single cytotoxic agent. Thus, when these patients relapse after an initial response to the chemotherapy, it is most often because the tumor cells have acquired resistance to the cytotoxic drugs used in the treatment regimen.

We have recently shown that resistance to doxorubicin in P388 murine leukemia cells is associated with increased structural order of the lipid domain of the plasma membrane [23]. It is well documented that such a change may reduce the permeability of cells to different compounds that enter cells by passive diffusion [17, 18]. It is therefore not surprising that the doxorubicin-resistant P388 cells are indeed cross-resistant to many cytotoxic drugs [14, 29].

The present study was designed to find out whether these cells are also cross-resistant to certain lipophilic analogs of methotrexate prepared by esterification of the carboxyl groups of the drug.

The results indicate that doxorubicin-resistant P388 cells are also resistant to dimethyl and dibutyl esters of methotrexate. The sensitivity of these cells to free methotrexate is comparable to that of the parental doxorubicin-sensitive cell line. In contrast, P388 cells that have acquired resistance to low concentrations (1×10^{-7} *M*) of methotrexate are not cross-resistant to doxorubicin or to the esters of methotrexate.

Materials and methods

Cell culture. P388 murine leukemia cells [5] and a subline resistant to doxorubicin [15] were propagated continuously in suspension culture in Roswell Park Memorial Institute 1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co.), 10 μ*M* 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo), 50 units/ml penicillin base, and 50 μg/ml streptomycin base (both from Grand Island Biological Co.). Cell growth was assessed by measurement of cell density in a Coulter Counter (Coulter Electronics Ltd, Harpenden, Hertfordshire, England). An inoculum of cells was transferred to fresh medium once every 4 days to kepp growth in the exponen-

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tial phase. The initial cell density was 1×10^5 cells/ml; after 4 days in culture the density was $1-2 \times 10^6$ cells/ml. Cell growth rates were calculated from the culture densities measured once a day for 4 days.

Determination of drug sensitivity. The sensitivity of a cell line to a given drug 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 used to determine the concentration of drug effective in inhibiting the growth rate by 50% (ED_{50}). The doxorubicin ED_{50} for drug-sensitive and drug-resistant cells was $2-6 \times 10^{-8}$ *M* and $1-2 \times 10^{-6}$ *M*, respectively. No change in drug sensitivity of either cell line was observed during 4 years of continuous in vitro culture.

Development of methotrexate-resistant P388 cells. A methotrexate-resistant subline (P388/MTX) was developed by culturing P388 cells in the continuous presence of methotrexate. The initial methotrexate concentration of 1×10^{-8} *M* considerably inhibited the growth of these cells. However, after 2 weeks the growth rate gradually resumed its pace as obtained in drug-free medium. This process was continuously repeated, with a higher concentration of methotrexate each time. After 3 months the cells were growing in the presence of 1×10^{-7} *M* methotrexate at the same rate as in drug-free medium.

Preparation of dimethyl and dibutyl esters of methotrexate. Dimethyl and dibutyl esters of methotrexate were prepared by direct esterification as described by Rosowsky [24]. Purity, as established by thin-layer chromatography and high-pressure liquid chromatography (using a C-18 reversed-phase column), was over 98% for both esters of methotrexate.

Results

The methotrexate-resistant lines developed in the present study showed a stable level of resistance even after 4 months in methotrexate-free medium. The sensitivity of the methotrexate-resistant P388 subline to doxorubicin ($ED_{50} = 5.6 \times 10^{-8}$ *M*) was not significantly different from that measured in the parental cell line. In another methotrexate-resistant cell line, developed from P388/ADR cells in a similar way, the sensitivity to doxorubicin ($ED_{50} = 8.5 \times 10^{-7}$ *M*) was also not significantly different from that of its parent P388/ADR cell line.

As shown in Fig. 1, methotrexate is more potent in inhibiting the growth of P388 cells than its ester derivatives. In this cell line the ED_{50} measured for the dimethyl ester was 9.5×10^{-8} *M* and that for the dibutyl ester, 1.4×10^{-8} *M* (18.3- and 2.7-fold higher, respectively, than the ED_{50} of free methotrexate).

In the methotrexate-resistant cell line (Fig. 2) the inhibitory potency of the dimethyl ester of methotrexate is similar to that observed in the parental cells. In this cell line, the activity of the dibutyl analog is somewhat lower than its activity in the methotrexate-sensitive parent line. (The ED_{50} is 3.3-fold higher.) However, in this cell line both esters of methotrexate are much more active than free methotrexate, as its ED_{50} in this cell line is 8.5×10^{-7} *M* compared with 5.2×10^{-9} *M* measured in the parent cell line.

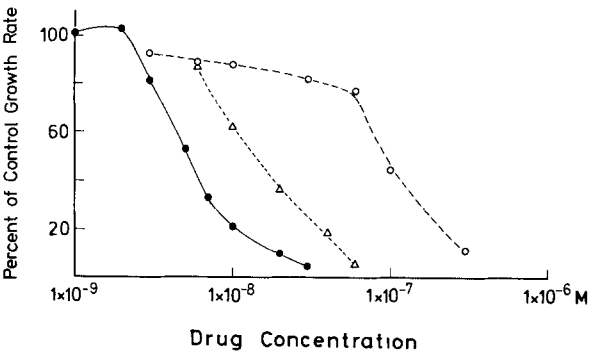

Fig. 1. The sensitivity of P388 cells to methotrexate (●), methotrexate dimethyl ester (○), and methotrexate di-butyl ester (△)

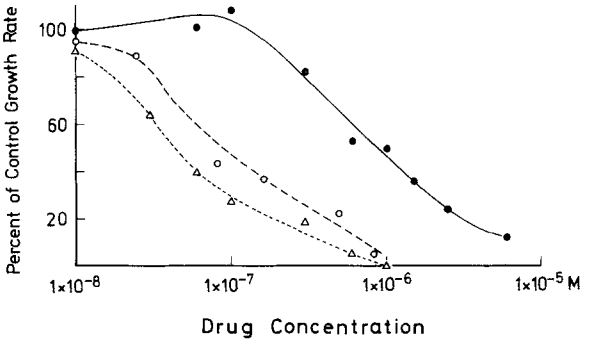

Fig. 2. The sensitivity of methotrexate-resistant P388 cells to methotrexate (●), methotrexate di-methyl ester (○), and methotrexate di-butyl ester (△)

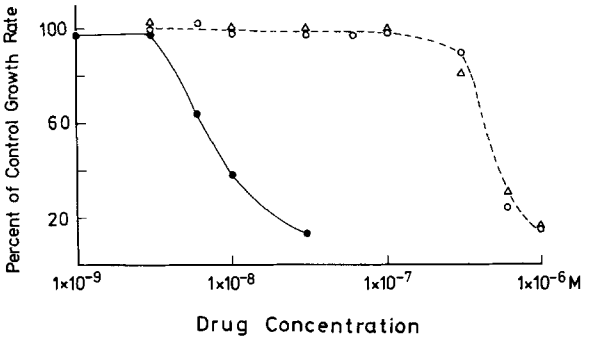

Fig. 3. The sensitivity of doxorubicin-resistant P388 cells to methotrexate (●), methotrexate di-methyl ester (○) and methotrexate di-butyl ester (△)

In the doxorubicin-resistant cells there is also a large difference in activity between the ester analogs and free methotrexate (Fig. 3). While the sensitivity of these cells to methotrexate is similar to that measured in the parent P388 cells ($ED_{50} = 8 \times 10^{-9}$ *M*), they show considerably lower sensitivity to the ester analogs (for both analogs the $ED_{50} = 4.8 \times 10^{-7}$ *M*).

The activity of dihydrofolate reductase was measured in the P388 cell line and its methotrexate- and doxorubicin-resistant sublines (by Dr. Frederika Mandelbaum-Shavit of the department of Bacteriology, The Hebrew University-Hadassah Medical School). The activity of this

enzyme in the methotrexate-resistant cells and in the doxorubicin-resistant cells was higher by 45% and 27%, respectively, than the activity measured in the parent P388 cells.

Discussion

In many studies it was shown that the methotrexate-resistant mutants that emerge from in vitro exposure of drug-sensitive cell population to low methotrexate concentrations (up to 1×10^{-6} *M*) are always methotrexate-transport-deficient mutants [7, 8, 20, 21, 25]. This is in contrast to mutants selected at higher methotrexate concentrations, where quantitative and qualitative changes in the dihydrofolate reductase activity were observed [12].

Diddens et al. [6] and others [21, 25, 27] have shown that the methotrexate-transport-deficient mutants but not the dihydrofolate reductase mutants retain their sensitivity to lipophilic folate antagonists.

In this study the methotrexate-resistant subline of P388 cells was developed in the presence of methotrexate concentrations up to 1×10^{-7} *M*. Its dihydrofolate reductase activity is only minimally increased compared with the parent P388 cell line. The sensitivity of the methotrexate-resistant cells to the ester analogs of methotrexate is comparable to that of the parental cells. Thus, it can be assumed that these cells are methotrexate-resistant primarily because of defects in their methotrexate transport system.

We have found that the methotrexate-resistant sublines of P388 and P388/ADR cells have the same sensitivity to doxorubicin as their methotrexate-sensitive parental lines. A similar finding was reported for L1210 cells [11] but not for the L5178Y cells, where a methotrexate-resistant subline showed increased sensitivity to doxorubicin [11], suggesting that this effect may depend on the cell line used.

Measurements of methotrexate ED_{50} in doxorubicin-resistant cells, show that the sensitivity of this cell line does not differ significantly from the methotrexate sensitivity observed in doxorubicin-sensitive P388 cells. These results are in contrast to the increase in methotrexate sensitivity found in hamster ovary cells selected for resistance to doxorubicin [9]. This discrepancy may merely reflect differences in the cell lines examined. However, the results of the present study suggest that the transport-related resistance to methotrexate and the resistance to drugs that enter cells by unmediated passive diffusion are independent phenomena, at least in some cell lines.

We have noted that the sensitivity to methotrexate dimethyl ester in methotrexate-resistant cells is similar to that measured in the methotrexate-sensitive parental line. The sensitivity to the methotrexate dibutyl ester was somewhat lower in methotrexate-resistant cells than in the methotrexate-sensitive cell line. This may be related to the modest increase in dihydrofolate reductase activity found in the methotrexate-resistant cells compared with the parent P388 cells. In contrast to these findings, it has been reported that methotrexate-resistant CEM cells that are only transport-devective showed increased sensitivity to the dibutyl ester of methotrexate [25]. However, our results are consistent with those obtained by Hill et al. [10]. These authors found that the lipophilic folate antagonist, metoprine, was taken up by methotrexate-sensitive L5178Y cells to a greater extent than by the methotrexate-resistant subline. We suggest that the discrepancy in the results of these studies, once again, is due to the use of different cell lines.

Johns et al.[13] have shown that 50% inhibition of isolated L1210 dihydrofolate reductase by the dibutyl ester of methotrexate is obtained at a concentration that is 70-fold lower than the concentration of the dimethyl ester analog needed to obtain the same level of enzyme inhibition. However, in P388 cells the ED_{50} of the dibutyl ester of methotrexate is only 6.8-fold lower than that of the dimethyl ester analog, and in P388/MTX and P388/ADR cells the differences are even smaller (2-fold and zero, respectively). The disparity between enzyme and cell growth inhibition by these alkyl esters of methotrexate suggests that the dimethyl ester analog is accumulated in the cells to higher levels than the dibutyl ester analog. However, at present we have no data to support this hypothesis.

The finding of the present study that doxorubicin-resistant P388 cells are cross-resistant to esters of methotrexate has an important clinical implication. Most current chemotherapy treatment regimens containing methotrexate also include drugs that enter cells by other means than carrier-mediated transport (e.g., anthracyclines or vinka alkaloids). It is therefore most probable that in addition to being selected for methotrexate transport resistance, the tumor cells of the relapsed patients are also selected for resistance to drugs that enter cells by passive diffusion. They are therefore also resistant to the lipophilic folate antagonists. Although there is experimental evidence that lipophilic folate antagnoists may overcome transport resistance to methotrexate, their beneficial effect is probably limited to a small number of patients who are treated with methotrexate as a single agent. However, they are not likely to help the majority of patients who are treated with methotrexate as a part of combination chemotherapy regimens that include other cytotoxic drugs such as anthracyclines or vinca alkaloids.

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