

Reversal of Acquired Resistance to Doxorubicin in P388 Murine Leukemia Cells by Tamoxifen and Other Triparanol Analogues¹

A. Ramu,² D. Glaubiger, and Z. Fuks

Department of Radiation and Clinical Oncology, Hadassah University Hospital, P. O. Box 12000, Jerusalem, Israel 91120 [A. R., Z. F.], and The Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205 [D. G.]

ABSTRACT

The effects of the triparanol analogues chlorotrianisene, clomiphene, tamoxifen, 5-[*p*-(fluoren-9-ylidenemethyl)phenyl]-2-piperidineethanol (MDL 10393), MDL 8917v, nafoxidine, 2-[*p*-(6-methoxy-2-phenylinden-3-yl)phenoxy]triethylamine (U-11555A), 2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]triethylamine (U-10520A), and nitromifene, as well as triparanol itself, were studied in the P388 murine leukemia cell line and in a doxorubicin-resistant subline (P388/ADR). At noninhibitory concentrations, all the analogues increased the sensitivity of P388/ADR cells to doxorubicin but did not have such an effect on the doxorubicin-sensitive cells.

Diethylstilbestrol, deacetylated cyclofenil (F6060), hexestrol, and 17 β -estradiol did not have such an activity. The effects of tamoxifen on doxorubicin sensitivity of P388/ADR cells could not be reversed by 17 β -estradiol. Estrogen receptors could not be demonstrated in either cell line. It is therefore suggested that the reversal of the doxorubicin-acquired resistance by the triparanol analogues is unrelated to their estrogenic or antiestrogenic activities. The possible clinical implications of these findings are discussed.

INTRODUCTION

In many patients, cancer chemotherapy fails after an initial response due to the development of resistance to anticancer drugs. The greater effectiveness of combination chemotherapy over single agents is attributed at least in part to the lower probability of selecting tumor cells with multiple acquired drug resistance. However, it has been shown in cancer patients and in experimental systems that tumors which acquire resistance to a plant alkaloid or antibiotic are often cross-resistant to other natural products (2). Therefore, treatment strategies designed to circumvent acquired drug resistance are a pressing need. Reduction in acquired resistance to anthracyclines was first obtained in the presence of the non-ionic detergent Tween 80 (13, 15). Similar results were recently reported in the presence of verapamil, carboxipine, prenylamine, trifluoperazine, and clomipramine (19). Tsuruo *et al.* (19) have speculated that these compounds reduce the resistance to doxorubicin by interfering with a calcium-dependent drug extrusion mechanism. We have recently reported that doxorubicin sensitivity of a doxorubicin-resistant subline of P388 murine leukemia could be restored in the presence of perhexiline maleate (9). We suggested that this effect did not involve calcium antagonism but rather was caused by an interaction of the drug with the membrane lipid domain of the cell that results in increased doxorubicin accumulation (9, 12).

We now report on the reduction of doxorubicin resistance by a different group of tricyclic compounds, tamoxifen and other triparanol analogues, and bring evidence that this effect is not a result of the antiestrogenic activity of these compounds.

MATERIALS AND METHODS

Cell Culture and Determination of Drug Sensitivity. These were carried out as described previously (9). Briefly, P388 murine leukemia cells and a doxorubicin-resistant subline (P388/ADR)³ were maintained in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (Grand Island Biological Co.), 10 μ M 2-mercaptoethanol, penicillin base (50 units/ml), and streptomycin (50 μ g/ml).

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

The sensitivity of both cell lines to doxorubicin, actinomycin D, triparanol, triparanol analogues, diethylstilbestrol, F6060, hexestrol, 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 used to determine the ED₅₀.

The effect of tamoxifen on the accumulation of [³H]daunorubicin (3.9 Ci/mmol) was studied as described previously (9). In brief, cells from both lines at a density of 1.5 \times 10⁶/ml were preincubated with or without 3 \times 10⁻⁶ M tamoxifen for 2.5 hr at 37°. [³H]Daunorubicin was then added to the medium (final concentration, 3.3 \times 10⁻⁹ M). After 40 min, 0.8-ml aliquots (in triplicates) of the cell suspension were transferred into capillary tubes containing 0.3 ml of diluted lymphocyte separation medium (Bionetics, Kensington, MD). The tubes were centrifuged for 90 sec at 1800 \times g and then frozen in liquid nitrogen; the tube tips were clipped into scintillation vials; scintillation fluid was added, and the radioactivity was counted.

Estrogen Receptor Assay. Washed cells (5 \times 10⁷) of both lines were homogenized with a Polytron PT 10/35 (Kinematica, Lucerne, Switzerland) in 2 ml of 10 mM Tris-HCl, pH 7.4, containing 1.5 mM EDTA, 1 mM dithiothreitol, 0.3 M sucrose, and 10% glycerol. The homogenate was centrifuged at 125 \times g for 5 min at 4°, and the resulting supernatant fluid was centrifuged at 48,000 \times g for 30 min at 4°. Then the supernatant fluid was assayed for protein by the method of Lowry *et al.* (6) and for estrogen receptors by the method of McGuire and DeLaGarza (7). In brief, 0.2 ml of cytosol sample was incubated with 5 \times 10⁻⁹ M [2,4,6,7-³H]estradiol (94 Ci/mmol; New England Nuclear, Boston, MA) overnight at 4°. After incubation, the nonbound estradiol was removed by treatment with dextran-coated charcoal, and the radioactivity of the supernatant was counted in a liquid scintillation counter. Specific binding was

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² To whom requests for reprints should be addressed.
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³ The abbreviations used are: P388/ADR, doxorubicin-resistant P388 cells; ED₅₀, concentration of drug effective in inhibiting the growth rate by 50%; MDL 10393, 5-[*p*-(fluoren-9-ylidenemethyl)phenyl]-2-piperidineethanol; U-10520A, 2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]triethylamine; U-11555A, 2-[*p*-(6-methoxy-2-phenylinden-3-yl)phenoxy]triethylamine; F6060, deacetylated cyclofenil.

calculated from the difference in tritium counts between samples that were preincubated for 15 min at 4° with and without 5×10^{-7} M diethylstilbestrol.

Drugs. Received as gifts were: triparanol, MDL 8917v, and MDL 10393 from Dr. W. J. Hudak, Merrell Dow Pharmaceuticals, Cincinnati, OH; clomiphene and tamoxifen from Dr. D. Ladkani, Teva Pharmaceutical Industries, Jerusalem, Israel; nafoxidine, U-10520A, and U-11555A from Dr. S. J. Stein, The Upjohn Co., Kalamazoo, MI; nitromifene from Dr. M. L. Black, Warner-Lambert Co., Ann Arbor, MI; F6060 from Dr. T. Leide-man, AB Ferrosan, Malmo, Sweden; and hexestrol from Dr. K. Thiele, Siegfried AG, Zofingen, Switzerland. Chlorotrianisene was supplied by the Investigation Drug Branch, National Cancer Institute, Bethesda, MD; diethylstilbestrol and 17β -estradiol were purchased from Sigma Chemical Co., St. Louis, MO. [3 H]Daunorubicin was purchased from New England Nuclear, Boston, MA.

RESULTS

The analogues of triparanol that were tested, chlorotrianisene, tamoxifen, clomiphene, MDL 10393, MDL 8917v, nafoxidine, U-10520A, U-11555A, and nitromifene as well as triparanol itself and diethylstilbestrol, F6060, and hexestrol, inhibited the growth of both P388 and P388/ADR cells in a concentration-dependent manner (data not shown).

The effects of doxorubicin on the growth rate of both cell lines, in the presence of noninhibitory concentration of tamoxifen (3×10^{-6} M) are shown in Chart 1. In the presence of tamoxifen, there was a marked increase in the sensitivity of P388/ADR cells to doxorubicin. The ED_{50} for doxorubicin was reduced from 7.6×10^{-7} M in the absence of tamoxifen to 7.9×10^{-8} M in its presence. The sensitivity of P388 cells to doxorubicin was only minimally increased by the presence of tamoxifen (ED_{50} reduced from 1.85×10^{-8} M to 1.1×10^{-8} M).

In order to characterize further the enhancement of doxorubicin inhibition of growth of P388/ADR cells by tamoxifen, we measured the effect of increasing concentrations of tamoxifen on the growth of P388/ADR cells incubated in the presence of a low concentration (3×10^{-7} M) of doxorubicin (Chart 2). In the absence of tamoxifen, doxorubicin at this concentration failed to inhibit the growth of P388/ADR cells. However, when tamoxifen was added to P388/ADR cells incubated with the low concentration of doxorubicin, a clear dose-dependent effect was observed. This enhancement of doxorubicin effect was caused by tamoxifen in concentrations well below those having independent growth-inhibitory effects of their own. In a similar experiment (Chart 3), where P388 cells were incubated with a concentration

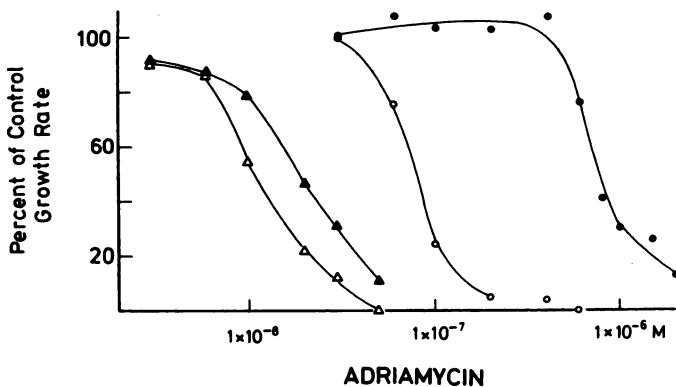


Chart 1. Sensitivity of P388 (Δ , \triangle) and P388/ADR (\bullet , \circ) cells to doxorubicin in the absence (Δ , \bullet) and presence (\triangle , \circ) of 3×10^{-6} M tamoxifen.

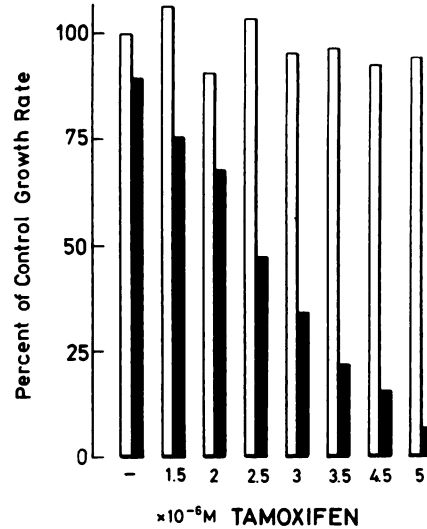


Chart 2. Effects of tamoxifen on growth rate of P388/ADR cells in the absence (\square) and presence (\blacksquare) of 3×10^{-7} M doxorubicin.

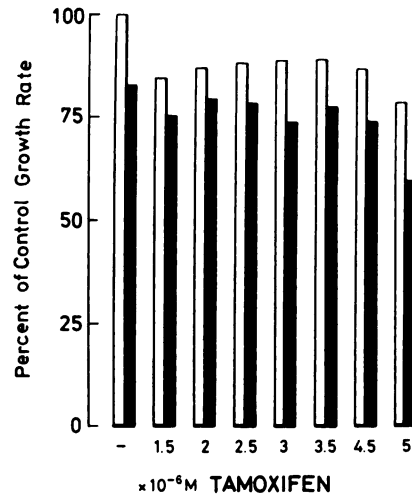


Chart 3. Effects of tamoxifen on the growth rate of P388 cells in the absence (\square) and presence (\blacksquare) of 1×10^{-8} M doxorubicin.

of doxorubicin just below that needed to demonstrate growth inhibition (1×10^{-8} M), no enhancement of growth inhibition could be obtained by adding tamoxifen at concentrations up to those having growth-inhibitory effects of their own.

To evaluate whether tamoxifen could also enhance the sensitivity of these cell lines to the cross-resistant drugs, we measured the effects of tamoxifen at a noninhibitory concentration (3×10^{-6} M) on the sensitivity of P388 and P388/ADR cells to actinomycin D. While tamoxifen had only a minor effect on the sensitivity of P388 cells to actinomycin D (ED_{50} was reduced from 4.5×10^{-10} M to 2.8×10^{-10} M), it had a marked effect on the sensitivity of P388/ADR cells to actinomycin D (Table 1).

Similar results were obtained with other triparanol analogues. In Table 2 are shown the effects of certain analogues, at concentrations below those having growth-inhibitory effects of their own, on the doxorubicin ED_{50} in both cell lines. While chlorotrianisene, tamoxifen, MDL 10393, triparanol, and MDL 8917v have lowered considerably the doxorubicin ED_{50} in P388/ADR cells,

they had only a minimal effect on the doxorubicin sensitivity of P388 cells. In this experiment, chlorotrianisene was considerably less effective than were the other analogues. Reduction in the doxorubicin ED₅₀ in P388/ADR was also obtained in the presence of clomiphene, nitromifene, U-11555A, U-10520A, and nafoxidine.

To further evaluate the relative potency of these compounds in lowering the resistance to doxorubicin, their effect on growth rate of P388/ADR cells was studied in the presence of a low noninhibitory concentration (3 × 10⁻⁷ M) of doxorubicin (Table 3). The ED₅₀ of diethylstilbestrol, F6060, or hexestrol were not affected by the presence of doxorubicin. However, the ED₅₀ of clomiphene, U-11555A, chlorotrianisene, MDL 10393, triparanol, tamoxifen, MDL 8917v, nafoxidine, nitromifene, and U-10520A were lowered by 2.9-, 3.2-, 3.9-, 4.4-, 4.7-, 6.0-, 6.6-, 12.8-, 15.0-, and 19.0-fold, respectively. In a similar experiment with P388 cells, no significant reduction in the ED₅₀ of these compounds was obtained by adding doxorubicin, at subinhibitory concentration (1 × 10⁻⁸ M). Previously, we have shown that the

Table 1
Effect of 3 × 10⁻⁸ M tamoxifen on the sensitivity of doxorubicin-resistant P388 cells to actinomycin D

Actinomycin D concentration (M)	% of control growth rate	
	No addition	+ tamoxifen
	100	116.0
4 × 10 ⁻⁹	108.9	44.0
6 × 10 ⁻⁹	102.6	42.0
1.2 × 10 ⁻⁸	72.9	23.9
4 × 10 ⁻⁸	30.3	13.8
6 × 10 ⁻⁸	22.8	0

Table 2
Effects of subinhibitory concentrations of triparanol analogues on the sensitivity of P388 and P388/ADR cells to doxorubicin

Compound	Concentration (M)	Doxorubicin ED ₅₀ (M)	
		P388	P388/ADR
No addition		2.9 × 10 ⁻⁸	9.4 × 10 ⁻⁷
Chlorotrianisene	1 × 10 ⁻⁵	2.0 × 10 ⁻⁸	2.5 × 10 ⁻⁷
Tamoxifen	3 × 10 ⁻⁶	1.1 × 10 ⁻⁸	7.9 × 10 ⁻⁸
MDL 10393	2 × 10 ⁻⁶	2.0 × 10 ⁻⁸	3.5 × 10 ⁻⁷
Triparanol	3 × 10 ⁻⁶	1.2 × 10 ⁻⁸	9.4 × 10 ⁻⁸
MDL 8917v	1 × 10 ⁻⁶	2.1 × 10 ⁻⁸	2.1 × 10 ⁻⁷
Clomiphene	1 × 10 ⁻⁶		1.7 × 10 ⁻⁷
U-11555A	6 × 10 ⁻⁷		1.3 × 10 ⁻⁷
Nitromifene	6 × 10 ⁻⁷		1.0 × 10 ⁻⁷
Nafoxidine	6 × 10 ⁻⁷		9.4 × 10 ⁻⁸
U-10520A	6 × 10 ⁻⁷		7.0 × 10 ⁻⁸

Table 3
Effect of 3 × 10⁻⁷ M doxorubicin on the sensitivity of P388/ADR cells to triparanol analogues

	ED ₅₀ (M)	
	No addition	+ doxorubicin
Diethylstilbestrol	1.2 × 10 ⁻⁶	1.2 × 10 ⁻⁶
Hexestrol	2.5 × 10 ⁻⁶	2.5 × 10 ⁻⁶
F6060	2.5 × 10 ⁻⁶	2.5 × 10 ⁻⁶
Chlorotrianisene	3.5 × 10 ⁻⁶	9.0 × 10 ⁻⁶
Tamoxifen	1.5 × 10 ⁻⁶	2.5 × 10 ⁻⁶
MDL 10393	8.0 × 10 ⁻⁶	1.8 × 10 ⁻⁶
Triparanol	8.0 × 10 ⁻⁶	1.7 × 10 ⁻⁶
Clomiphene	4.0 × 10 ⁻⁶	1.4 × 10 ⁻⁶
MDL 8917v	5.5 × 10 ⁻⁶	8.3 × 10 ⁻⁷
Nafoxidine	2.3 × 10 ⁻⁶	1.8 × 10 ⁻⁷
U-10520A	1.9 × 10 ⁻⁶	1.0 × 10 ⁻⁷
U-11555A	1.7 × 10 ⁻⁶	5.3 × 10 ⁻⁷
Nitromifene	1.5 × 10 ⁻⁶	1.0 × 10 ⁻⁷

Table 4
Effect of 3 × 10⁻⁶ M tamoxifen on the accumulation of [³H]daunorubicin by P388 and P388/ADR cells that were incubated (40 min, 37°C) with 3.3 × 10⁻⁸ M [³H]daunorubicin

	pmol/10 ⁶ cells	
	No addition	+ tamoxifen
P388	2.81 ± 0.26 ^a	2.67 ± 0.12
P388/ADR	1.11 ± 0.16	1.89 ± 0.01

^a Mean ± S.D.

Table 5
Lack of effect of 17β-estradiol on the synergism between subinhibitory concentrations of tamoxifen and doxorubicin in P33/ADR cells

Estradiol (M)	Tamoxifen (3 × 10 ⁻⁶ M)	Doxorubicin (2 × 10 ⁻⁷ M)	% of control growth rate
	-	+	97.1
	+	-	94.0
3 × 10 ⁻⁶	-	+	86.3
	+	+	12.8
1 × 10 ⁻⁶	+	+	15.9
3 × 10 ⁻⁶	+	+	16.5
1 × 10 ⁻⁶	+	+	14.9
3 × 10 ⁻⁶	+	+	17.5
1 × 10 ⁻⁷	+	+	18.7
3 × 10 ⁻⁷	+	+	15.8
1 × 10 ⁻⁶	+	+	15.5
3 × 10 ⁻⁶	+	+	17.7

enhancement of anthracycline sensitivity in P388/ADR cells by perhexiline maleate was associated with an increase in drug accumulation (9). We have therefore tested whether the enhancement in doxorubicin sensitivity that occurred in the presence of tamoxifen was also associated with an increase in drug accumulation. Cells of both lines were preincubated with 3 × 10⁻⁶ M tamoxifen for 2.5 hr, and then the accumulation of [³H]-daunorubicin was studied. The results are presented in Table 4. While tamoxifen did not affect the [³H]daunorubicin accumulation in P388 cells, it did increase the drug accumulation in P388/ADR cells by 70.3%.

Because it was reported that the effects of tamoxifen and similar analogues on macromolecular synthesis and DNA polymerase activity in a human breast cancer cell line can be prevented by simultaneous addition of as little as 1000-fold less estradiol (3, 5), we have studied whether adding 17β-estradiol can inhibit the reduction in doxorubicin resistance caused by tamoxifen. The results are shown in Table 5. 17β-Estradiol, in concentrations from 3000-fold lower than that of tamoxifen up to a concentration equal to the concentration of tamoxifen, did not prevent the enhancement of doxorubicin sensitivity caused by tamoxifen. The question whether these cell lines carry receptors for estrogen was also examined, using the well-established radio-receptor assay (7). The specific binding was less than 0.1 fmol/mg cytosol protein, and the difference between the lines was not significant.

DISCUSSION

In this study, we have shown, in a subline of P388 cells that have acquired resistance to doxorubicin, that certain triparanol analogues can increase the sensitivity of the cells to doxorubicin, as well as to actinomycin D. The effect was observed at low concentrations of the analogues which did not inhibit the growth of these cells on their own. The increase in drug sensitivity by the triparanol analogues was limited to the resistant subline and could not be obtained in the doxorubicin-sensitive parent cell

line. Although these compounds have shown in other systems estrogenic and antiestrogenic activities (4, 17), we suggest that their ability to lower doxorubicin resistance is unrelated to these activities for several reasons. Diethylstilbestrol, F6060, and hexoestrol, which have estrogenic activity and are relatively weak antiestrogens, did not affect the sensitivity of either cell line to doxorubicin. Furthermore, 17β -estradiol up to a concentration of 3×10^{-6} M did not increase the sensitivity of P388/ADR cells to doxorubicin (Table 5). Although triparanol has considerably less antiestrogenic activity than tamoxifen, both drugs have a similar potency to reduce the resistance to doxorubicin. Estradiol did not antagonize the effect of tamoxifen on doxorubicin resistance. Finally, although estrogen receptors were reported recently in a number of lymphomas and leukemias (8, 14), no significant levels of such receptors were found in the present study in the P388 murine leukemia cell line or in its doxorubicin-resistant subline. Others (16) have also observed that certain effects of tamoxifen in cultured cells could not be reversed by estradiol and have suggested that they involve mechanisms independent of the estrogen receptor system.

We have recently reported that the lipid phase of the plasma membranes of P388/ADR cells has a higher degree of structural order compared to that of doxorubicin-sensitive P388 cells, and we have suggested that it may account for the decreased rate of accumulation of anthracycline drugs seen in these cells (10). We speculate that tamoxifen and the other triparanol analogues which have a lipophilic tricyclic structure and a cationic group located at some distance from the lipophilic moiety act in this system by interacting with the cell membrane lipid domain like many other amphiphathic compounds (1). This interaction results in decreased packing density; therefore, compounds like doxorubicin can now enter the cells at an increased diffusion rate. We further suggest that the distinct cell membrane lipid composition of P388/ADR cells (11) is responsible for the effect of these compounds in this cell line, while they have much weaker effect on the sensitive parent cell line.

The possible clinical implications of these findings cannot be underestimated. However, the possibility of increased toxicity of combinations of triparanol analogues with either an anthracycline drug or any of the cross-resistant drugs should be considered first. There is at least one report on patients treated concurrently with tamoxifen and doxorubicin (18). The authors indicated that the addition of tamoxifen to chemotherapy regimen that contained doxorubicin did not cause increased side effects. Therefore, we suggest that a chemotherapy regimen containing tamoxifen and doxorubicin may be beneficial to patients with tumors which have acquired resistance to this drug, regardless of their estrogen receptor status.

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