Cancer Chemother Pharmacol (1988) 22: 147–152

Cancer Chemotherapy and Pharmacology

Modulation of induced resistance to adriamycin in two human breast cancer cell lines with tamoxifen or perhexiline maleate

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Summary. The clinical utility of adriamycin in the treatment of patients with metastatic breast cancer is often limited by the development of drug resistance. It has been recognized that in addition to the development of primary resistance against adriamycin, malignant cells can simultaneously develop cross-resistance to other agents. An adriamycin-resistant human breast cancer cell line (MCF 7^{Ad}) was developed by exposing the parent line (MCF 7) to gradually increasing concentrations of adriamycin while the cells were being grown in monolayer. Using these lines in a clonogenic assay, the relative drug sensitivities to adriamycin, vinblastine, melphalan, 5-fluorouracil and methotrexate were studied. MCF 7^{Ad} was 12.5-fold more resistant to adriamycin than MCF 7 and 500-fold cross-resistant to vinblastine. There was no cross-resistance to melphalan, 5-fluorouracil or methotrexate. The resistance of MCF 7^{Ad} was decreased by simultaneous exposure to tamoxifen (by a factor of 3.33) or perhexiline maleate (by a factor of 7.50). This decreased resistance was evidenced by a shift to the left of the sensitivity curves. However, there was no consistent change in the sensitivity curves of MCF 7. At the selected concentration of tamoxifen and perhexiline maleate, the cloning efficiency of MCF 7 and MCF 7^{Ad} was 80%–90% of control values in medium without tamoxifen, perhexiline maleate or cytotoxic drugs. The resistance of MCF 7^{Ad} to adriamycin was associated with a lower accumulation of [^{14}C]adriamycin than exhibited by the sensitive MCF 7 line. There was no consistent change in [^{14}C]adriamycin accumulation in MCF 7 or MCF 7^{Ad} when tamoxifen was added, but when perhexiline maleate was added the [^{14}C] accumulation increased. These results suggest that the tamoxifen-induced change in MCF 7^{Ad} adriamycin resistance was not due to an increase in the amount of cell-associated adriamycin, but rather to some other mechanism that increased the cytotoxicity of the adriamycin.

Abbreviations: RPMI 1640 Roswell Park Memorial Institute medium 1640; FBS fetal bovine serum; PBS phosphate buffered saline; trypsin-EDTA trypsin (w/0.05%) plus ethylenediaminetetraacetic acid (w/0.02%); IC_{50} concentration of each drug, extrapolated from concentration versus colony formation curve, that inhibits colony formation by 50% as compared to controls without exposure to the drug

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Introduction

The development of resistance to cytotoxic chemotherapeutic agents imposes limitations on the ability of treatment regimens to eradicate malignant cells in tumors. Following the recognition of cancer cells' ability to develop selective resistance to a drug from treatment exposure to the drug (direct resistance), it was found that resistance to agents with different cytotoxic mechanisms, not used against the cells (cross-resistance), frequently occurred [7, 12, 28]. Joint direct resistance and cross-resistance (pleotropic resistance) is a frequent occurrence in human tumors [5]. The cellular mechanisms proposed for resistance have included (a) impaired transport of drugs across the cytoplasmic and/or nuclear membrane [1, 4, 6]; (b) rapid efflux of drug out of the cell before it has a cytotoxic effect [27]; (c) utilization of biosynthetic pathways which circumvent the drug-induced lesions [2]; (d) increased drug metabolism to form less active metabolites [24]; and (e) increased ability by the cell to directly bind and neutralize drugs [8].

Adriamycin resistance has been reported to be associated with a, b, d, and e. Indeed, maneuvers based on circumventing some of these proposed mechanisms has resulted in a relative restoration of adriamycin sensitivity in vitro [19, 20, 22, 27]. In addition, these approaches can in some cases decrease the level of cross-resistance [19, 27]. From a human breast cancer cell line (MCF 7) originally established from a pleural effusion [25], an adriamycin-resistent cell line was developed (MCF 7^{Ad}). These two lines were used to study the relative drug sensitivity and resistance to adriamycin, vinblastine, melphalan, 5-fluorouracil and methotrexate, cytotoxic drugs frequently used for the treatment of patients with breast cancer [10]. It has been reported by Ramu et al. that perhexiline maleate [19] and triparanol analogues like tamoxifen [20] reduce the level of induced resistance in a P388/ADR cell line. The effects of perhexiline maleate and tamoxifen on the sensitivity and resistance of MCF 7 and MCF 7^{Ad} to the cytotoxic agents listed in Fig. 1, and their effect on the accumulation of [^{14}C]adriamycin were studied. This is a report and discussion of the results obtained from these experiments.

Materials and methods

Reagents and drugs. RPMI 1640, FBS, trypsin-EDTA, penicillin, streptomycin and glutamine were obtained from Grand Island Biological Co., Chagrin Falls, Ohio.

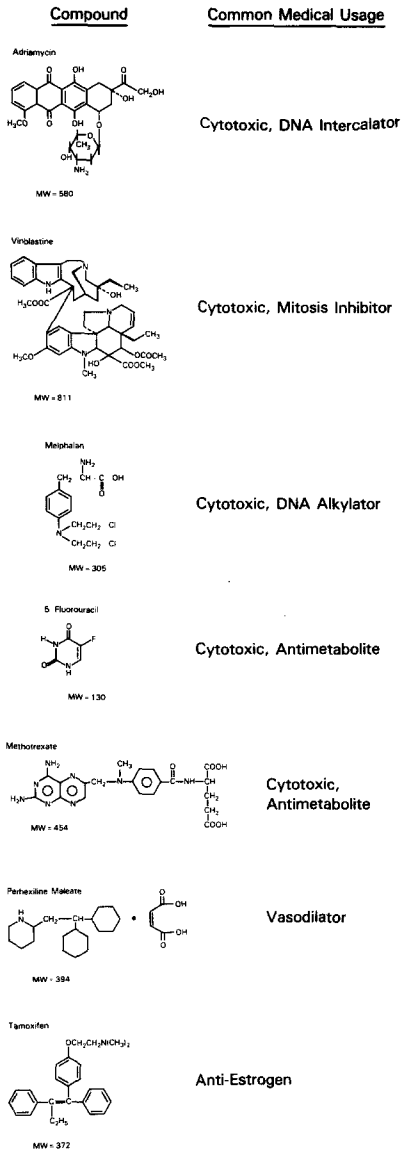

Fig. 1. Common names, biochemical structures, molecular weights and common medical uses of agents used

PBS was from Quality Biological, Inc., Gaithersburg, Md. Insulin (Iletin U-100) was from Eli Lilly and Co., Indianapolis, Ind. Adriamycin, vinblastine, melphalan, 5-fluorouracil and methotrexate were from the Developmental Therapeutics Program, National Cancer Institute. The [^{14}C]adriamycin was from Standard Research Institute, Palo Alto, Calif. Estradiol and type VII agarose were from Sigma Chemical Co., St. Louis, Mo. Perhexiline maleate was from Merrell Dow Pharmaceuticals, Inc., Cincinnati, Ohio. Tamoxifen was from Imperial Chemical Industries, Macclesfield, Cheshire, UK. Stock solutions of adriamycin were made in 0.9% saline and frozen at −20° C until immediately prior to use. Stock solutions of vinblastine, methotrexate, 5-fluorouracil and perhexiline maleate were made at appropriate dilutions and stored at 4° C. Stock solutions of estradiol and tamoxifen were made in 95% ethanol and stored at −20° C. Melphalan was reconstituted with appropriate diluents immediately prior to use.

Cell culture. The MCF 7 cell line was obtained from the laboratory of Dr. Marc Lippman, National Cancer Institute. The MCF 7^{Ad} line was developed by intermittent exposure of the parent line (MCF 7) to adriamycin in 0.1 *M* stepwise concentration increases. Adriamycin concentration varied from 10^{-9} *M* to 10^{-6} *M*. After 1 year of propagation the level of resistance of MCF 7^{Ad} has been maintained by exposure to 10^{-6} *M* adriamycin for 48–72 h every 2 months. Growth of MCF 7 and MCF 7^{Ad} was in RPMI 1640 supplemented with 10% FBS, insulin (0.25 units/ml), penicillin (100 u/ml), streptomycin (100 μg/ml) and glutamine (0.3 mg/ml). Cells were passaged by removing the growth medium and washing the monolayer in PBS. The washed monolayer of cells was detached with trypsin-EDTA at 37° C for 2–5 min. The cell suspension was then washed with growth medium and the suspension centrifuged at 200*g* for 10 min. The cell pellet was resuspended in maintenance growth media and placed in 250 ml tissue culture flasks (Costar, Cambridge, MA). Cells were incubated at 37° C in a humidified atmosphere with 5% CO_2. Medium was changed 24 h after passage, and if an adriamycin exposure cycle was scheduled, adriamycin was added to the culture. At 48–72 h following the addition of adriamycin, the media was removed, the cell monolayer washed with PBS and fresh drug-free maintenance growth medium added. Cells were passaged at 7- to 10-day intervals.

Clonogenic assay. Drug sensitivity determinations were performed by clonogenicity in a double-layer soft agarose system, as previously described for ovarian cancer cells [15] with some modifications. In brief, cells were harvested from culture with trypsin-EDTA solution and counted with a Coulter counter (Coulter Electronics, model ZBI). Trypsin-EDTA was removed following centrifugation at 200 *g*. A suspension of single cells was placed in a mixture of 0.3% (w/v) agarose, RPMI 1640 (supplemented as above except that insulin was omitted and 50 n*M* estradiol was added), saline or varying cytotoxic drug concentrations with and without 10^{-5} *M* tamoxifen or perhexiline maleate. The cytotoxic drug concentrations used were 1–500 n*M* adriamycin, 0.5–500 n*M* vinblastine, 0.5–500 μ*M* melphalan, 0.05–50 m*M* 5-fluorouracil and 0.01–10 m*M* methotrexate. The cell-containing mixture was plated in triplicate over a layer of solidified 0.6% (w/v) agarose in 10-sq-cm dishes. MCF 7 and MCF 7^{Ad} were plated at a concentration of 10^4 and 2×10^4 cells/dish, yielding average cloning efficiencies of 8% and 5%, respectively. Colonies of greater than 50 cells were counted using a Bausch and Lomb FAS II (Rochester, NY) after 7–10 days incubation at 37° C in a humidified atmosphere of 5% (v/v) CO_2. The ratios of the number of colonies formed in the drug-containing medium versus the non-drug-containing controls were plotted (number of colonies in each drug-containing plate/number of colonies in non-drug-containing control) to form a concentration versus colony inhibition graph. The IC_{50} for each sensitivity experiment was determined from these graphs. The minimum IC_{50} was the lowest IC_{50} value obtained from multiple (average 10) experiments for each cell line.

Statistical comparisons of all IC_{50} values were made by means of the paired *t*-test, and all *P* values are reported as two-sided. The statistical comparisons were all repeated using the logarithms of the IC_{50} values, with no appreciable changes in any of the conclusions.

Adriamycin accumulation. Cells were harvested with trypsin-EDTA, washed, resuspended in RPMI 1640 (supple-

mented as in the clonogenic assay) and plated as a monolayer in 30-sq-cm dishes at a concentration of approximately 10^6 cells/dish. Twenty-four hours later, 10^{-5} *M* tamoxifen or perhexiline maleate was added to the medium of half of the plates that would later have [^{14}C]adriamycin added. Forty-eight hours after plating, 0.15 μ*M* [^{14}C]adriamycin was added to the media and dishes were incubated at 37°C in an atmosphere of 5% (v/v) CO_2. Two hours after addition of radioactive drug, the dishes were washed 5 times with iced PBS. Cells were removed from each dish with trypsin-EDTA and the dishes were washed twice with iced PBS. Cells harvsted from the trypsin-EDTA and both iced PBS washes were placed in a scintillation vial. Twenty milliliters of Aquassure scintillation fluid was added and mixed. Samples were counted in a Beckman LS 2800 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif). The cell-associated [^{14}C]adiramycin at 2 h, in counts per million cells, was plotted and extrapolated to time zero for each cell line with and without perhexiline maleate or tamoxifen.

Results

MCF 7^{Ad} characteristics

The adriamycin-resistant cell line MCF 7^{Ad} developed 12.5-fold resistance to adriamycin and 500-fold cross-resistance to vinblastine, but maintained similar sensitivity to melphalan, 5-fluorouracil and methotrexate relative to the parent line MCF 7. The MCF 7^{Ad} doubling time (21.95 h) was nearly equal to that of MCF 7 (21.53 h). The cloning efficiency (number of colonies formed per number of cells plated × 100) of MCF 7^{Ad} was 5% ±2% and that of MCF 7 was 8% ±5% under the same conditions.

Drug sensitivity studies

The cloning efficiency of MCF 7 in the presence of estradiol and MCF 7^{Ad} in 10^{-5} *M* of tamoxifen or perhexiline maleate was 80% –100% of the controls that contained neither of the latter agents. However, at higher concentrations of tamoxifen or perhexiline maleate, there was a decline in the number and size of MCF 7 and MCF 7^{Ad} colonies. At 3×10^{-5} *M* tamoxifen there was 80% growth inhibition of both lines and at 6×10^{-5} *M* perhexiline maleate there was greater than 95% growth inhibition.

Cloned in the presence of 50 n*M* estradiol, the minimum IC_{50}s to the various cytotoxic drugs with and without tamoxifen or perhexiline maleate are shown in Table 1 (note: the two-sided *P* values were calculated from *all* adriamycin and vinblastine experiments, not the minimum IC_{50}s only). The respective minimum IC_{50}s for MCF 7 (with and without tamoxifen) and MCF 7^{Ad} (with and without tamoxifen) were: adriamycin, 0.006 μ*M* and 0.004 μ*M* ($P=0.49$ in favor of MCF 7 + tamoxifen), 0.015 μ*M* and 0.05 μ*M* ($P=0.02$ in favor of MCF 7^{Ad} + tamoxifen); vinblastine, 0.0003 μ*M* and 0.0006 μ*M* ($P=0.02$ in favor of MCF 7 + tamoxifen), 0.04 μ*M* and 0.3 μ*M* ($P=0.13$ in favor of MCF 7^{Ad} + tamoxifen); melphalan, 2.9 μ*M* and 1.7 μ*M*, 1.1 μ*M* and 1.2 μ*M*; 5-fluorouracil, 3.9 m*M* and 2.8 m*M*, 2.4 m*M* and 2.6 m*M*; methotrexate, 0.031 m*M* and 0.048 m*M*, 0.022 m*M* and 0.024 m*M*. Representative graphs from individual experiments with adriamycin or vinblastine, with and without tamoxifen, depicting the sensitivity of MCF 7, relative resistance of MCF 7^{Ad} and the tamoxifen effect are shown in Figs. 2 and 3. The respective minimum IC_{50}s for MCF 7 (with and without perhexiline maleate) and MCF 7^{Ad} (with and without perhexiline maleate) were: adriamycin, 0.006 μ*M* and 0.006 μ*M* ($P=0.10$ in favor of MCF 7 alone), 0.019 μ*M* and 0.06 μ*M* ($P=0.04$ in favor of MCF 7^{Ad} + perhexiline maleate); vinblastine, 0.0002 μ*M* and 0.003 μ*M* ($P=0.11$ in favor of MCF 7 + perhexiline maleate), 0.001 μ*M* and 0.009 μ*M* ($P=0.02$ in favor of MCF 7^{Ad} + perhexiline maleate). Representative graphs from individual experiments with adriamycin or vinblastine with

Table 1. The effects of tamoxifen and perhexiline maleate on the sensitivity and resistance of MCF 7 and MCF 7^{Ad} to various cytotoxic agents

Minimum 50% inhibitory concentrations

	MCF 7	MCF 7 + Tamoxifen	IC_{50} Δ^1 Factor	MCF 7^{Ad}	MCF 7^{Ad} + Tamoxifen	IC_{50} Δ^2 Factor
Adriamycin	0.004 μM	0.006 μM	<1	0.05 μM	0.015 μM	3.33
Vinblastine	0.0006 μM	0.0003 μM	2.00	0.3 μM	0.04 μM	7.50
Melphalan	1.7 μM	2.9 μM	<1	1.2 μM	1.1 μM	1.09
5-Fluorouracil	2.8 mM	3.9 mM	<1	2.6 mM	2.4 mM	1.08
Methotrexate	0.048 mM	0.031 mM	1.55	0.024 mM	0.022 mM	1.09
	MCF 7	MCF 7 + Perhexiline maleate	IC_{50} Δ^3 Factor	MCF 7^{Ad}	MCF 7^{Ad} + Perhexiline maleate	IC_{50} Δ^4 Factor
Adriamycin	0.006 μM	0.006 μM	1.00	0.06 μM	0.019 μM	3.16
Vinblastine	0.0003 μM	0.0002 μM	1.5	0.009 μM	0.001 μM	9.00

Tamoxifen or perhexiline maleate = 10^{-5} M

$$\Delta^1 = \frac{\text{MCF 7}}{\text{MCF 7 + Tamoxifen}} \qquad \Delta^2 = \frac{\text{MCF 7}^{Ad}}{\text{MCF 7}^{Ad} + \text{Tamoxifen}}$$

$$\Delta^3 = \frac{\text{MCF 7}}{\text{MCF 7 + Perhexiline maleate}} \qquad \Delta^4 = \frac{\text{MCF 7}^{Ad}}{\text{MCF 7}^{Ad} + \text{Perhexiline maleate}}$$

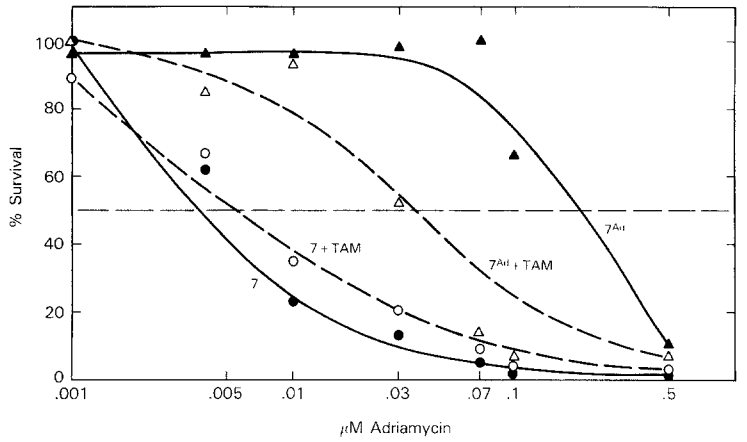

Fig. 2. A representative graft showing the effect of tamoxifen on the sensitivity of MCF 7 and MCF 7^{Ad} to adriamycin

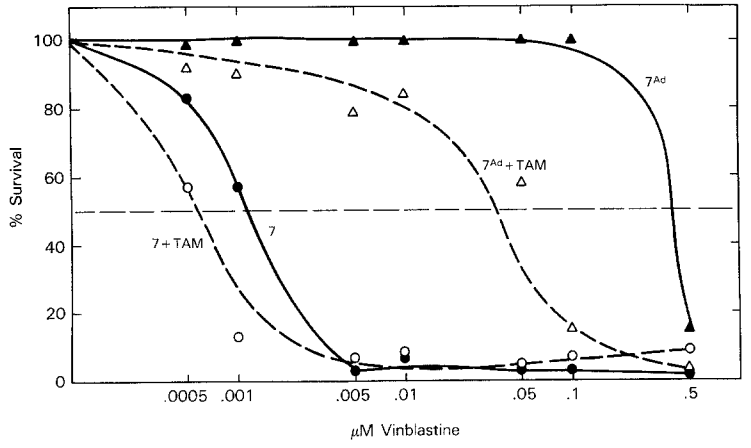

Fig. 3. A representative graft showing the effect of tamoxifen on the sensitivity of MCF 7 and MCF 7^{Ad} to vinblastine

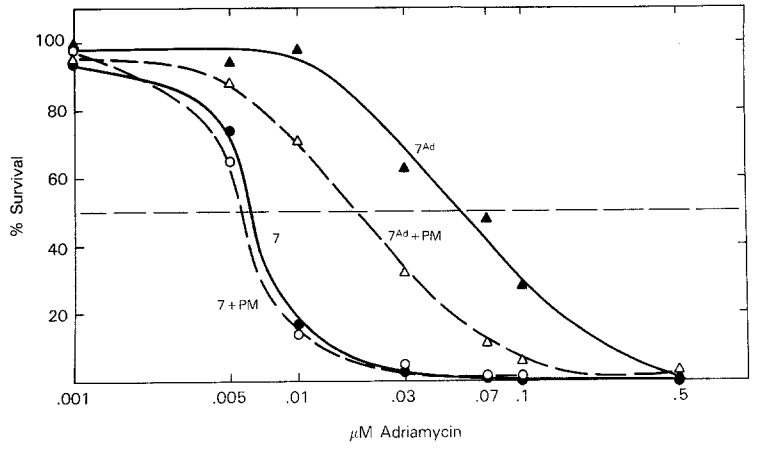

Fig. 4. A representative graft showing the effect of perhexiline maleate on the sensitivity of MCF 7 and MCF 7^{Ad} to adriamycin

and without perhexiline maleate, depicting the sensitivity of MCF 7, relative resistance of MCF 7^{Ad} and the effects of perhexiline maleate are shown in Figs. 4 and 5.

Accumulation of [^{14}C] *adriamycin*

To determine whether a change in cellular accumulation of adriamycin might account for the change in sensitivity, [^{14}C]adriamycin was used. Following the 2-h incubation, the counts per million cells with and without tamoxifen were 3190 and 2614 (MCF 7), 1069 and 1587 (MCF 7^{Ad}) respectively. The counts per million cells with and without perhexiline maleate were 820 and 791 (MCF 7), 709 and 512 (MCF 7^{Ad}) respectively. The results of the [^{14}C] accumulation experiments are shown in Figs. 6 and 7.

Discussion

Advanced metastatic breast cancer is responsive to agents with varying cytotoxic and cytostatic mechanisms [10]. Tamoxifen has been recommended as a standard of treatment for patients with estrogen-receptor-positive or postmenopausal metastatic breast cancer [11, 16]. Complete response rates of 20% and greater can be achieved with adriamycin-containing combinations [3, 13], but response durations are limited by the development of resistance.

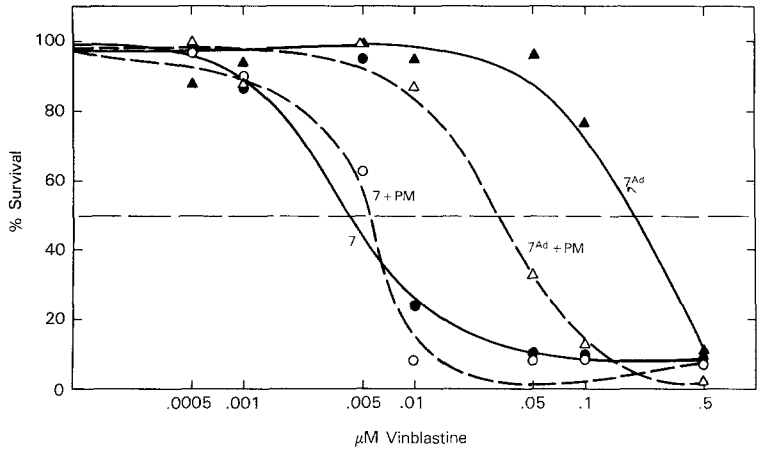

Fig. 5. A representative graft showing the effect of perhexiline maleate on the sensitivity of MCF 7 and MCF 7^{Ad} to vinblastine

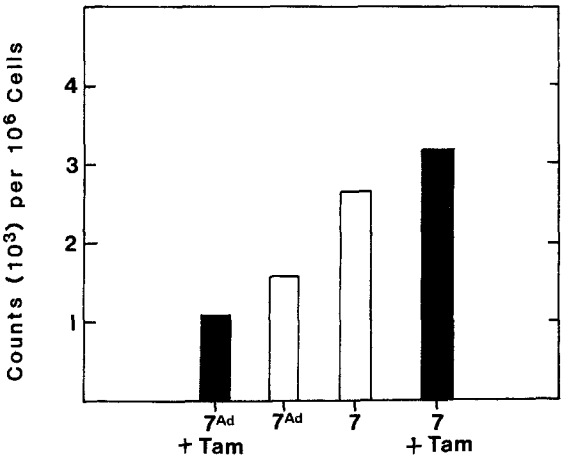

Fig. 6. The effect of tamoxifen on the accumulation of [^{14}C]adriamycin in MCF 7 and MCF 7^{Ad} following 2 h incubation

There have been reports of higher response rates and longer response durations in patients who received tamoxifen along with cytotoxic chemotherapy than in the group that received cytotoxic chemotherapy without tamoxifen [9, 23, 26]. The benefit from the addition of tamoxifen to chemoterhapy was related to estrogen receptor level and patient age [9].

MCF 7 is an estrogen-receptor-positive cell line originally established at the Michigan Cancer Foundation, Detroit, Mich. from a malignant pleural effusion in a female patient with metastatic breast cancer [25], and MCF 7^{Ad} is a variant we derived by in vitro exposure to increasing concentrations of adriamycin. Results from the sensitivity experiments showed that the parent-cell line, in the presence of estradiol and either 10^{-5} *M* tamoxifen or perhexiline maleate, cloned with an efficiency of 80%–100% of the cloning ability achieved in the presence of estradiol without tamoxifen or perhexiline maleate. MCF 7^{Ad} demonstrated 12.5-fold resistance to adriamycin relative to MCF 7 and 500-fold cross-resistance to vinblastine. However, MCF 7^{Ad} showed no cross-resistance to melphalan, 5-fluorouracil or methotrexate. Therefore, the development of adriamycin resistance did not confer cross-resistance to the majority of cytotoxic agents used for the treatment of advanced breast cancer.

It has been reported that adriamycin induces changes that result in a barrier to the ability of a drug to penetrate into the cell (drug uptake) [14, 18, 21]. The ability of tamoxifen or perhexiline maleate to increase the sensitivity of P388/ADR (a murine leukemia subline with acquired resistance to adriamycin [2]) was reported by Ramu et al. [19, 20] and correlated with increased intracellular adriamycin levels [17]. Ramu et al. were able to demonstrate a difference in the lipid composition of plasma membranes and a larger intracellular lipid content of the adriamycin-resistant subline [18, 21]. It was postulated that this change in membrane lipid composition effected a structural change that served to decrease the accumulation of some cytotoxic agents, e.g., adriamycin and vinblastine [18]. In addition, it was felt that the increase of intracellular lipid might serve as a storage reservoir for the cytotoxic drug, thus decreasing its cytotoxic activity. In our experiments, the resistance expressed by MCF 7^{Ad} relative to MCF 7 was decreased in the presence of a concentration of tamoxifen or perhexiline maleate that did not significantly inhibit colony formation. The minimum MCF 7^{Ad} IC_{50}s of adriamycin were changed by a factor of 3.33 with tamoxifen (in the presence of estradiol) and 3.16 with perhexiline maleate, while the change observed in MCF 7 was less than 1.0 (i.e., no change). There was a similar trend in the minimum MCF 7^{Ad} IC_{50}s of vinblastine, but the change was of a higher magnitude (MCF 7^{Ad}: tamoxifen factor = 7.50, perhexiline maleate factor = 9.00; MCF 7: tamoxifen factor = 2.00, perhexiline maleate factor = 1.50). The accumulation of [^{14}C]adriamycin in MCF 7^{Ad} was less than in MCF 7. Perhexiline maleate increased [^{14}C]adriamycin accumulation in both lines but tamoxifen did not. Therefore, the decrease in the degree of MCF 7^{Ad} resistance to adriamycin is probably not due to a disruptive change in the plasma membrane structure or to adriamycin storage in a drug-induced intracellular reservoir.

In conclusion, we have shown that induced resistance and cross-resistance in a human breast cancer cell line can be decreased by simultaneous exposure to tamoxifen or perhexiline maleate. The tamoxifen effect occurred in concentrations of estradiol that are slightly higher than physiologic and Strongly competitive for the estrogen receptor. Therefore, it is concluded that this tamoxifen effect is not mediated by estrogen receptors. The partial restoration of adriamycin sensitivity, particularly by tamoxifen, may have clinical implications. Clinical experience has shown that tamoxifen can safely be given with adriamycin-containing combinations, and it might be useful in producing

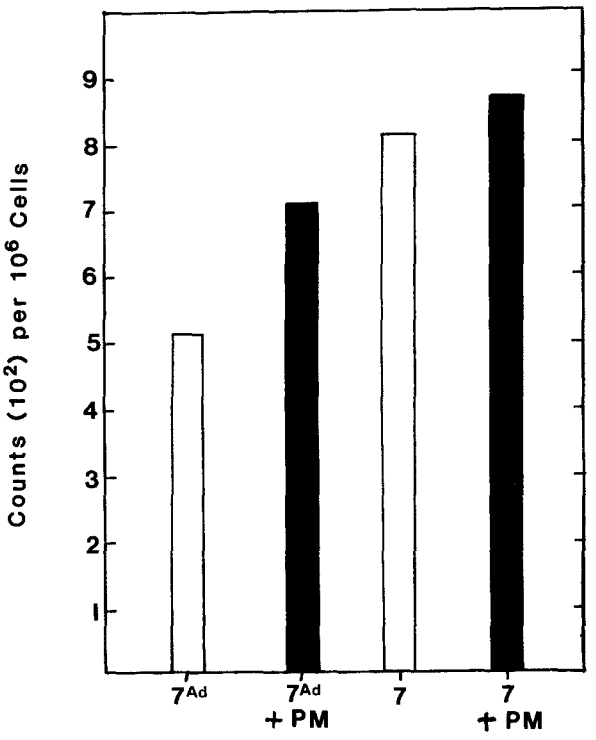

Fig. 7. The effect of perhexiline maleate on the accumulation of [^{14}C]adriamycin in MCF 7 and MCF 7^{Ad} following 2 h incubation

sensitivity in relatively resistant cells without increasing adverse effects.

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Received October 9, 1987/Accepted January 15, 1988