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ANTAGONISM OF THE CYTOCIDAL ACTIVITY AND UPTAKE OF MELPHALAN BY TAMOXIFEN IN HUMAN BREAST CANCER CELLS *IN VITRO**

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Abstract—The effect of the antiestrogen tamoxifen on the cytocidal activity and uptake of melphalan in human breast cancer cells was investigated. A clonogenic assay was used to obtain dose–survival curves of estrogen receptor-positive MCF-7 cells and of estrogen receptor-negative Evsa T cells following treatment with melphalan and/or tamoxifen. Isobolograms derived from these dose–survival curves were concave downward, suggesting that the drug interaction was antagonistic. The effect of tamoxifen on melphalan uptake by breast cancer cells was evaluated at steady-state conditions. Thin-layer chromatography revealed that the intracellular level of free intact melphalan (mean ± S.E.) in control cells was 6.47 ± 1.21 fmoles/cell and that in cells treated with tamoxifen was 3.60 ± 0.35 fmoles/cell; this 44% reduction in cellular melphalan was statistically significant ($P = 0.006$). Thus, the antagonistic cytocidal effect of melphalan and tamoxifen against breast cancer cells appeared to be due to inhibition of melphalan uptake at the steady state by the antiestrogen. Further investigation revealed that tamoxifen inhibited unidirectional melphalan influx in human breast cancer cells both by the sodium-independent system L and by the sodium-dependent system ASC. Tamoxifen also appeared to stimulate melphalan efflux from human breast cancer cells. The first-order rate constant K for melphalan efflux from control cells was 0.085 ± 0.008 and that from cells treated with tamoxifen was 0.129 ± 0.005; the difference was highly significant ($P < 0.001$). Therefore, the antagonistic effect of tamoxifen on the uptake and cytocidal activity of melphalan in breast cancer cells appeared to be due to inhibition of melphalan influx and stimulation of drug efflux.

Recently, we reported the development of a clonogenic assay of long-term human breast cancer cell cultures that provided a reproducible method with which to quantitate tumor cell kill by hormones and/or cytotoxic chemotherapeutic agents [1]. The response of estrogen receptor (ER)-positive and -negative breast cancer cells to cytotoxic chemotherapeutic agents appeared to be independent of ER status [1]. The estrogen analog diethylstilbestrol (DES) and the antiestrogen tamoxifen appeared to kill human breast cancer cells by different mechanisms of action; the cytocidal activity of DES was independent of ER status, whereas tamoxifen was more active against ER-positive cells [1].

Over the past decade several clinical trials have been conducted in the United States and Canada [2, 3] and in Italy [4] to evaluate various regimens of adjuvant chemotherapy for women with primary breast cancer. The present report illustrates that the clonogenic assay may be used to investigate *in vitro* the relative activity of different drug combinations that may be used clinically in the treatment of breast cancer. Evidence is presented in this study that uptake and cytocidal activity of melphalan in human breast cancer cells may be inhibited by the antiestrogen tamoxifen; furthermore, the inhibition of melphalan uptake appears to be due to inhibition of unidirectional drug influx and stimulation of drug efflux.

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MATERIALS AND METHODS

Drugs and chemicals. Melphalan (Alkeran) was provided as a gift by Dr. M. J. Fletcher, Burroughs Wellcome & Co., Ltd., Lachine, Quebec, Canada. Tamoxifen citrate [*trans*-(*p*-dimethylaminoethoxyphenyl)1,2-diphenylbut-1-ene] was provided by Stuart Pharmaceuticals, Division of ICI United States, Inc., Wilmington, DE. [^{14}C]Melphalan (*p*-(di-2-chloroethyl)amino-L-[ring-U-^{14}C]phenylalanine), sp. act. 6.9 mCi/mmole, was prepared by Mr. M. Leaffer, Stanford Research Institute, Menlo Park, CA, and was provided as a gift by Dr. R. Engle, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. The radiochemical purity was 98% as determined by thin-layer chromatography on silica gel in *n*-butyl alcohol–acetonitrile–water (5:5:2). DL-β-2-Aminobicyclo[2,2,2]heptane-2-carboxylic acid (BCH) was obtained from the New England Nuclear Corp., Boston, MA.

Cell lines and cultures. MCF-7, a cloned ER-positive cell line originally established at the Michigan Cancer Foundation, Detroit, MI [5], was supplied by Dr. Robert Shiu, Department of Physiology, University of Manitoba. Evsa T, an ER-negative cell line established by Dr. Marc Lippman of the National

Cancer Institute, Bethesda, MD [6], was provided by Dr. Peter Lam, Manitoba Institute of Cell Biology. Both cell lines were grown in monolayer cultures in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS).

Assay of cytotoxic activity. Cytotoxicity was determined using a clonogenic assay described in detail previously [1]. Dose–survival curves of MCF-7 and Evsa T cells were obtained following treatment with melphalan and/or tamoxifen for 1 hr, at 37° in serum-free MEM. The plating efficiency of untreated MCF-7 cells ranged from 75 to 95% and that of Evsa T cells from 25 to 50%. The cloning efficiency of treated cells was determined at each drug concentration and surviving cell fraction (SCF) was calculated. Linear regression analysis of the dose–survival curves was performed, the regression equations being in the form $\log_e y = mx + b$, where y is SCF, x is dose of drug, m is slope of the regression line, and b is the y-intercept. D_0 (the dose of drug reducing survival to $1/e$, i.e. 37% of the initial cell population) was derived from the negative reciprocal of the slope of the regression line [7–10]. Statistical analysis of the dose–survival curves was performed by analysis of covariance comparing the significance of the difference of the slopes, and of elevation where no slope differences were noted.

Analysis of the nature of the drug interaction. Two approaches were used to analyze the nature of the pharmacologic interaction between melphalan and tamoxifen. The first involved a comparison of the slope and/or shoulder of dose–survival curves of cells treated with melphalan and tamoxifen alone and in combination as described previously [11–13]. The following formula was modified in order to calculate the additive pharmacologic response produced by the simultaneous administration of two agents each with independent cytocidal activity [13, 14]:

$$R_{A+B} = R_A + R_B\,(1 - R_A) \qquad (1)$$

where, R_A and R_B are the responses (fractional cell kill) produced by drugs A and B, respectively, and R_{A+B} is the additive cytocidal response produced by the simultaneous administration of two drugs each working independently.

$$R \text{ (fractional cell kill)} = 1 - SCF \qquad (2)$$

substituting these values in equation 1 we derive:

$$\begin{aligned} R_{A+B} &= (1 - SCF_A) + (1 - SCF_B)\,(SCF_A) \\ &= 1 - SCF_A + SCF_A - SCFA \cdot SCF_B \\ &= 1 - SCF_A \cdot SCF_B \\ SCF_{A+B} &= 1 - R_{A+B} \\ &= 1 - (1 - SCF_A \cdot SCF_B) \\ &= SCF_A \cdot SCF_B \end{aligned}$$

Thus, the combined additive effect of two drugs may be calculated from the product of the SCF attained for each drug acting individually; this relationship is known as the effect-multiplication criterion [13].

In the second method, isobolograms, which are graphs joining points representing equi-effective doses or dose combinations, were constructed to analyze the nature of the drug interaction as described in detail by Berenbaum [13]. For the interaction of two drugs A and B, let A_i and B_i represent the doses producing a level of cell kill for each drug acting independently, and let A_c and B_c be their doses in combination producing the same effect. Then a linear isobole indicative of zero interaction or a simple additive effect will be given by the equation:

$$\frac{A_c}{A_i} + \frac{B_c}{B_i} = 1$$

whereas this sum will be less than 1 for drug synergy, and greater than 1 for antagonism [13, 15].

Uptake of melphalan by breast cancer cells. [^{14}C]-Melphalan and tamoxifen were added to cells at a concentration of approximately 10^5 cells/well in Linbro multiwell dishes, incubated for 1 hr at 37°, and uptake was stopped by cooling on ice with immediate removal of the incubation medium. Cells were washed five times with cold phosphate-buffered saline (PBS), and the radioactivity was extracted with 10% trichloroacetic acid (TCA) and measured by liquid scintillation spectrometry.

Cell size was measured in a Coulter model Z_{BI} electronic particle counter (Coulter Electronics, Hialeah, FL); the cell volume (mean ± S.E.) of MCF-7 cells was 4600 ± 200 μ^3, and that for Evsa T cells was 4100 ± 400 μ^3. Drug uptake was expressed as cell-medium distribution ratio, which was based on the radioactivity calculated per cell volume relative to that of an equivalent volume of extracellular medium as described previously [8, 10, 16–20]; the results, which represent the mean ± S.E. of at least six determinations, were analyzed statistically by a two-tailed t-test.

The same techniques were used to measure melphalan influx with the important exception that incubations were terminated at 1 min to ensure that unidirectional drug influx conditions prevailed [8, 16–20]. Drug efflux was evaluated by methods reported previously [17].

RESULTS

Dose–survival curves of ER-positive human breast cancer cells treated with melphalan and tamoxifen. Dose–survival curves of ER-positive MCF-7 breast cancer cells treated with melphalan or with melphalan and tamoxifen are shown in Fig. 1. The dose–survival curve for cells treated with melphalan alone had little or no shoulder and cell kill followed first-order kinetics with a D_0 of 3.93 nmoles/ml. A biphasic dose–survival curve was observed for cells treated with melphalan and tamoxifen. A comparison of the actual and theoretical curves reveals that they were identical for melphalan concentrations up to 10 μM; the D_0 for the initial phase of the actual survival curve was 3.68 nmoles/ml, and this was not significantly different from the D_0 calculated for a putative additive effect. However, at melphalan concentrations above 10 μM the observed D_0 increased sharply to 34.77 nmoles/ml, approximately 9-fold greater than the predicted D_0, and this difference was highly significant ($P < 0.001$). Thus, combination therapy appeared to be additive up to a

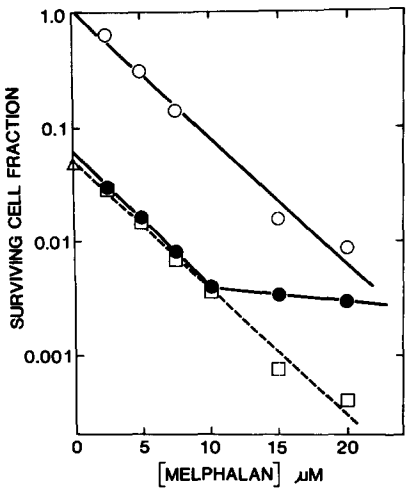

Fig. 1. Dose–survival curves of ER-positive MCF-7 breast cancer cells following treatment with the alkylating agent melphalan and the antiestrogen tamoxifen. Approximately 2×10^5 cells/ml were treated at 37°, for 1 hr, with melphalan alone (○), or with melphalan and 6 μM tamoxifen (●). The SCF (mean ± S.E.) of cells treated with 6 μM tamoxifen for 1 hr was $4.66 \pm 0.33 \times 10^{-2}$ (△); this value was used to calculate a theoretical dose–survival curve for cells treated with melphalan and 6 μM tamoxifen assuming such therapy was additive (□), as described in the text. Each experimental point represents the mean ± S.E. of at least six determinations; frequently the confidence intervals were too small to be illustrated. The linear regression equation for cells treated with melphalan alone was $\log_e y = -0.255x + 5.48 \times 10^{-2}$, with a correlation coefficient of −0.991. The regression equation for cells treated with melphalan and tamoxifen at melphalan concentrations of 10 μM and lower was $\log_e y = -0.272x - 2.80$ with a correlation coefficient of −0.999. An analysis of covariance comparing the significance of the difference of the slopes of these two curves was not statistically significant. The regression equation for cells treated with melphalan and tamoxifen at melphalan concentrations of 10 μM and greater was $\log_e y = -0.0288x - 5.25$ with a correlation coefficient of −0.999; an analysis of covariance comparing the significance of the difference of slopes between this curve and that of cells treated with melphalan alone was highly significant ($P < 0.001$).

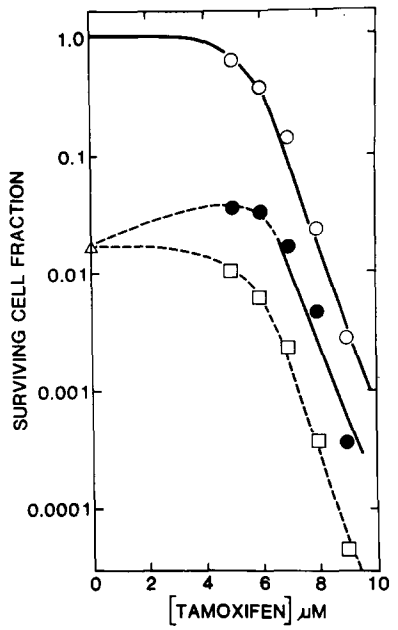

Fig. 2. Dose–survival curves of MCF-7 cells treated at 37°, for 1 hr, with tamoxifen alone (○), or with tamoxifen and 10 μM melphalan (●). The SCF (mean ± S.E.) of cells treated with 10 μM melphalan was $1.63 \pm 0.08 \times 10^{-2}$ (△); this value was used to generate a theoretical dose–survival curve for cells treated with tamoxifen and 10 μM melphalan assuming such therapy was additive (□), as described in the legend for Fig. 1 and in the text. The linear regression equation for cells treated with tamoxifen was $\log_e y = -1.66x + 9.28$ with a correlation coefficient of −0.987, and that for cells treated with tamoxifen and 10 μM melphalan was $\log_e y = -1.48x + 5.92$ with a correlation coefficient of −0.963. An analysis of covariance comparing the significance of the difference of slopes was not statistically significant; however, the dose–survival curve observed for cells treated with tamoxifen and melphalan was elevated significantly over the curve expected for an additive effect and that difference was statistically significant ($P < 0.005$).

melphalen concentration of 10 μM, but thereafter appeared to be antagonistic.

In a reciprocal experiment, the effect of adding melphalan as a second drug, to cells treated with a range of tamoxifen concentrations, was evaluated (Fig. 2). The dose–survival curves of MCF-7 cells treated with tamoxifen were characterized by a prominent shoulder followed by first-order cell kill. The D_0 for cells treated with tamoxifen alone was 0.604 nmole/ml, and that for cells treated with tamoxifen and melphalan was 0.675 nmole/ml; the difference was not statistically significant. However, the observed dose–survival curve appeared to be elevated over that expected for an additive effect; this finding suggested that combination therapy with tamoxifen and melphalan was antagonistic. In some experiments, SCF of cells treated with melphalan and tamoxifen was actually greater than that of control cells treated with either agent alone.

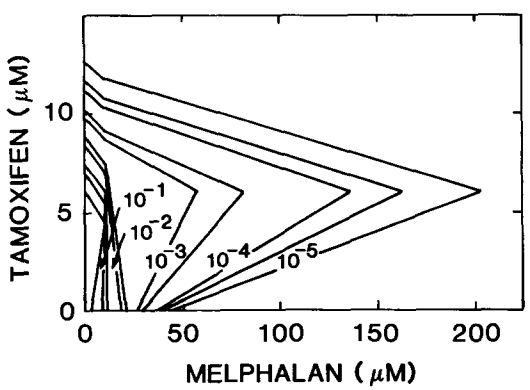

Fig. 3. Isobolograms for ER-positive MCF-7 human breast cancer cells treated with various combinations of melphalan and tamoxifen. The curves connect points representing doses of each drug used alone or in combination, which produce the same level of cell kill; the isobolograms were calculated from linear regression equations of dose–survival curves shown in Figs. 1 and 2 by methods described in the text and in the literature [13, 15].

A series of isoboles were derived from the dose–survival curves of MCF-7 cells treated with melphalan and tamoxifen alone and in combination (Fig. 3). The isoboles representing 0.5 to 5 logs of cell kill were concave towards the ordinate, suggesting that the interaction between melphalan and tamoxifen was antagonistic [13].

Dose-survival curves of ER-negative human breast cancer cells treated with melphalan and tamoxifen. Dose–survival curves of ER-negative Evsa T cells treated with melphalan or with melphalan and tamoxifen are shown in Fig. 4. The D_0 for cells treated with melphalan alone was 1.39 nmoles/ml. The D_0 for cells treated with melphalan and tamoxifen was 3.38 nmoles/ml, which was 2.5-fold greater than the D_0 calculated for an additive effect; this difference was highly significant ($P < 0.001$). Thus, combination therapy of Evsa T cells with melphalan and tamoxifen appeared to be antagonistic.

Dose–survival curves of Evsa T cells treated with tamoxifen alone, or with tamoxifen and a single dose of melphalan, are shown in Fig. 5. The D_0 for cells treated with tamoxifen alone was 1.66 nmoles/ml and a prominent shoulder was noted on the dose–survival curve. The D_0 for cells treated with tamoxifen and melphalan was 1.83 nmoles/ml, and that for the theoretical curve was 1.66 nmoles/ml, the difference not being statistically significant. However, the observed dose–survival curve was elevated over the theoretical curve, suggesting that combination therapy was antagonistic.

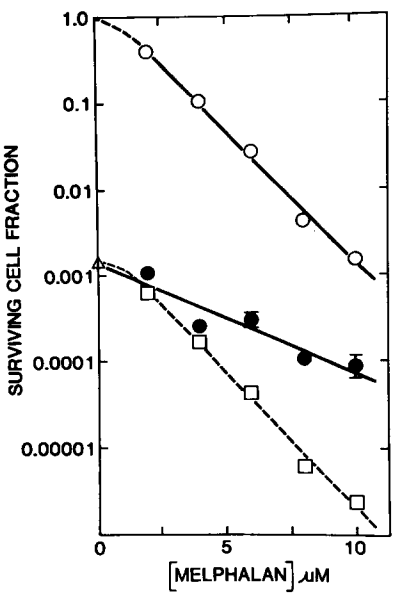

Fig. 4. Dose–survival curves of ER-negative Evsa T cells treated with melphalan alone (○), or with melphalan and 15 μM tamoxifen (●). The SCF of cells treated with 15 μM tamoxifen was $1.49 \pm 0.25 \times 10^{-3}$ (△); this value was used to generate a theoretical dose–survival curve for cells treated with melphalan and 15 μM tamoxifen assuming such therapy was additive (□), as described in the legend to Fig. 1 and in the text. The linear regression equation for Evsa T cells treated with melphalan alone was $\log_e y = -0.718x + 0.595$ with a correlation coefficient of −0.997, and that for cells treated with melphalan and 15 μM tamoxifen was $\log_e y = -0.296x - 6.54$ with a correlation coefficient of −0.933. An analysis of covariance comparing the significance of the difference of slopes was highly significant ($P < 0.001$).

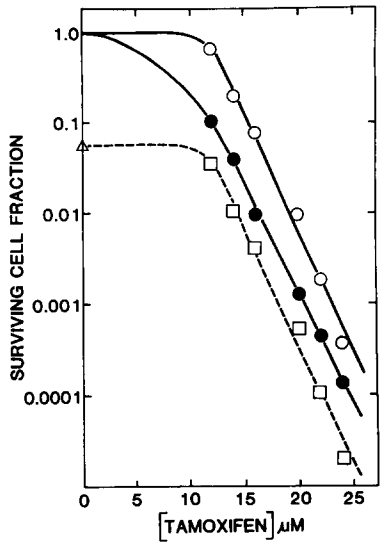

Fig. 5. Dose–survival curves of Evsa T cells treated with tamoxifen alone (○), or with tamoxifen and 5 μM melphalan (●). The SCF of cells treated with 5 μM melphalan was $5.52 \pm 0.44 \times 10^{-2}$ (△); this value was used to compute the theoretical dose–survival curve for cells treated with tamoxifen and 5 μM melphalan, assuming an additive effect (□), as described in the legend to Fig. 1 and in the text. The linear regression equation for cells treated with tamoxifen alone was $\log_e y = -0.602x + 6.92$ with a correlation coefficient of −0.994, and that for cells treated with tamoxifen and 5 μM melphalan was $\log_e y = -0.548x + 4.29$ with a correlation coefficient of −0.999. An analysis of covariance comparing the significance of the difference of slopes was not significant; however, the dose–survival curve observed for combination therapy was elevated over that expected for an additive effect, and this difference was highly significant ($P < 0.001$).

A series of isoboles were calculated from the dose–survival curves of Evsa T cells treated with various combinations of melphalan and tamoxifen (Fig. 6). The isoboles representing 0.5 to 5 logs of cell kill were concave downwards, once again suggesting that the interaction between melphalan and tamoxifen was antagonistic.

Inhibition of melphalan uptake by tamoxifen in ER-positive and -negative human breast cancer cells. The effect of tamoxifen on [^{14}C]melphalan uptake by MCF-7 cells followed a simple dose–response relationship. The cell/medium distribution ratio (mean ± S.E.) of radioactivity at 1 hr was 173 ± 4 in control cells, and it decreased progressively in the presence of higher concentrations of the antiestrogen to reach a minimal level of 140 ± 6 in cells treated with 15 μM tamoxifen; statistical analysis using a two-tailed *t*-test revealed that this 19% decrement in melphalan uptake was highly significant ($P < 0.001$).

The effect of tamoxifen on melphalan uptake by Evsa T cells was also evaluated; as with MCF-7 cells, a dose-dependent inhibition of drug uptake was observed with the greatest effect occurring at 15 μ

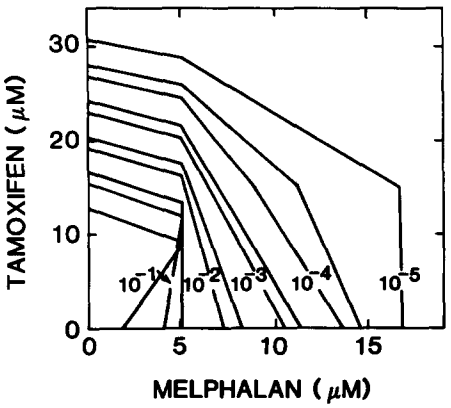

Fig. 6. Isobolograms for ER-negative Evsa T human breast cancer cells treated with various combinations of melphalan and tamoxifen. The data were derived from linear regression equations of dose–survival curves shown in Figs. 4 and 5, by methods outlined in the legend for Fig. 3, in the text, and in the literature [13, 15].

tamoxifen. The distribution ratio of radioactivity in control cells was 149 ± 3, and that in cells treated with 15 μM tamoxifen was 123 ± 6; this 17% inhibition of drug uptake was statistically significant ($P < 0.001$).

Effect of tamoxifen on the uptake of free intact melphalan and of bound drug in ER-positive human breast cancer cells. Thin-layer chromatography of cell lysates, medium and TCA-soluble cell extracts was undertaken to identify the radioactive constituents affected by tamoxifen (Table 1). Free intact drug accounted for 83% of radioactivity in the cells and 80% of that in the medium. The cell/medium ratio of free intact drug in control cells was 124 ± 19, and that in cells treated with tamoxifen was 80 ± 12; this 35% reduction was statistically significant ($P = 0.024$).

The intracellular level of free drug at the steady state in control cells was 6.47 fmoles/cell and that in tamoxifen-treated cells was 3.60 fmoles/cell; this 44% decrement was highly significant ($P = 0.006$). Similarly, tamoxifen treatment lowered the level of melphalan in the TCA-soluble and -insoluble fractions by 47 and 43% respectively. Free drug accounted for 93 ± 5% of the radioactivity in the TCA-soluble fraction, the balance being due to hydrolyzed drug and other polar metabolites. TLC analysis of the TCA-insoluble fraction, which accounted for approximately 6% of cellular drug, was not performed; all of this radioactivity was assumed to represent drug covalently bound to macromolecules. Tamoxifen also appeared to reduce the level of bound drug and this effect was statistically significant ($P = 0.045$, paired one-tailed *t*-test).

Inhibition of melphalan influx by tamoxifen in ER-positive human breast cancer cells. The observed inhibition of melphalan uptake at the steady state by tamoxifen could represent an effect on drug influx, drug binding and/or metabolism, or on drug efflux. Accordingly, an investigation of the effect of tamoxifen on unidirectional melphalan influx at 1 min was undertaken (Fig. 7). Drug influx in cells treated with 15 μM tamoxifen was 85.1 ± 2.7% of control; this 15% decrease in influx was highly significant ($P < 0.001$).

Since melphalan influx into human breast cancer cells is mediated by two separate amino acid transport systems [16, 18, 20], the effect of tamoxifen on melphalan transport by each amino acid carrier was evaluated. Drug influx by system L was determined in sodium-poor medium, in which transport by the sodium-dependent system ASC is inhibited [18, 20–22]. Influx by system L was 45.3 ± 5.2% of

Table 1. Effect of tamoxifen on the uptake of melphalan at the steady state by MCF-7 human breast cancer cells *in vitro**

Treatment	Cell/Medium ratio of free intact melphalan†‡	Intracellular melphalan (fmoles/cell) Total†	TCA-soluble§	TCA-insoluble‖
Control	124 ± 19	6.47 ± 1.21	5.97 ± 1.63	0.413 ± 0.082
Tamoxifen	80 ± 12 (5)	3.60 ± 0.35 (7)	3.19 ± 0.50 (6)	0.235 ± 0.024 (6)
P	0.024	0.006	0.032	0.090

* Monolayer cultures of MCF-7 cells were treated simultaneously with 15 μM [^{14}C]melphalan and 15 μM tamoxifen in PBS for 1 hr, at 37°. The supernatant fraction was removed and aliquots were obtained to determine total radioactivity and for TLC analysis. The cells were washed, removed with trypsin, and disrupted by osmotic lysis in deionized water to determine total cellular radioactivity and for TLC analysis. Cell aliquots were also extracted with equal volumes of cold 20% TCA; the TCA-soluble fraction was analyzed by TLC and radioactivity was measured. The TCA-insoluble fraction was separated by millipore filtration and radioactivity was determined. TLC analysis of cell contents, medium and the TCA-soluble fraction was performed on silica gel in a solvent system consisting of butanol–acetic acid–water (4:1:1) as previously described [20]. The solvent system used readily separated intact melphalan from hydrolyzed drug ($R_f = 0.02$) and from phenylalanine ($R_f = 0.38$). The data, which represent the mean ± S.E. of five to seven determinations (in parentheses), were normalized and analyzed statistically by a paired two-tailed *t*-test.

† On TLC analysis the percentage of radioactivity (mean ± S.E.) in the total cell extract that migrated as a single peak with an R_f of 0.46 identical to that of free intact melphalan was 83 ± 4%.

‡ The percentage of radioactivity attributable to free intact melphalan in the medium was 80 ± 2%.

§ The percentage of radioactivity due to free intact melphalan in the TCA-soluble extract of MCF-7 cells was 93 ± 5%.

‖ All of the radioactivity in the TCA-soluble fraction was assumed to represent covalently bound melphalan.

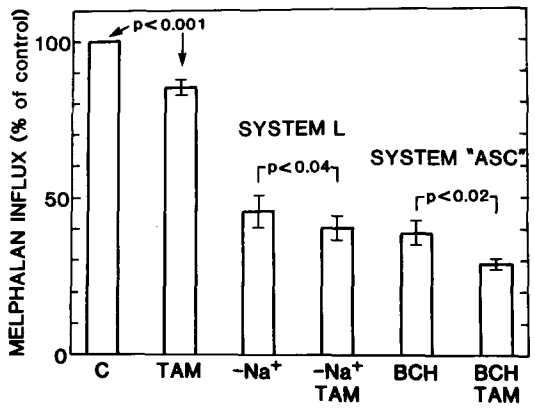

Fig. 7. Effect of tamoxifen on unidirectional melphalan influx in MCF-7 breast cancer cells. Cells at a density of approximately 10^5 cells/well were cultured in Linbro multiwell tissue culture plates and incubated with 15 μM [^{14}C]melphalan at 37°, for 1 min, in the presence and absence of 15 μM tamoxifen. The cell/medium distribution ratio (mean ± S.E.) of [^{14}C]melphalan in untreated control cells was 28.6 ± 1.8, or approximately 2 fmoles/cell. Drug influx by system L was determined in low sodium medium consisting of Hanks' Balanced Salt Solution (HBSS), in which sodium chloride was replaced by an isomolar amount of Tris with a final sodium concentration of 5 m-equiv/liter; influx by system ASC was measured in HBSS containing 25 mM BCH [16, 18, 20]. Influx by both amino acid transport systems was determined in HBSS. The data from four to ten experiments were pooled, normalized and evaluated statistically by a paired two-tailed *t*-test.

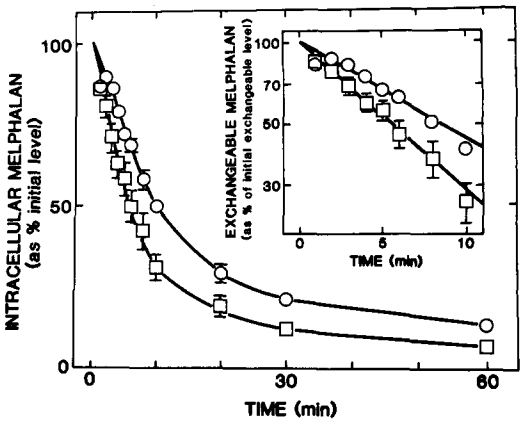

Fig. 8. A time course of melphalan efflux from MCF-7 breast cancer cells. Cells were preincubated with 10 μM [^{14}C]melphalan for 5 min and then resuspended in PBS at 37° either in the absence (○) or presence of 15 μM tamoxifen (□). The intracellular drug concentration was determined in cell aliquots removed at the times indicated, and the data are expressed as melphalan concentration as a percentage of the initial intracellular drug concentration plotted against the efflux time. The experimental points represent the mean ± S.E. of eleven determinations for the control curve, and of nine determinations for cells treated with tamoxifen. Inset: a decay time analysis of exchangeable drug over the first 10 min of efflux presented as a semilogarithmic plot of exchangeable intracellular melphalan concentration (expressed as a percentage of the initial exchangeable intracellular drug level) plotted against the efflux time. The exchangeable intracellular drug concentration was obtained by subtracting non-exchangeable drug from total intracellular melphalan; non-exchangeable drug was derived from the plateau region of the time course of drug efflux. The linear regression equation for melphalan efflux from control cells was $\log_e y = -0.085x + 4.62$ with a correlation coefficient of −0.977, and that from cells treated with tamoxifen was $\log_e y = -0.129x + 4.61$, with a correlation coefficient of −0.995. A *t*-test comparing the significance of the difference of the slopes was highly significant ($P < 0.001$).

control, and that in cells exposed to tamoxifen was 40.3 ± 3.8%; this 5% reduction was statistically significant ($P < 0.04$).

Melphalan influx by system ASC was measured in the presence of BCH, a synthetic amino acid that blocks drug transport by system L [18, 20, 22, 23]. Under these conditions, drug influx was 38.8 ± 3.9% of control, and that in cells treated with tamoxifen was 28.7 ± 1.7%; this 10% decrement in drug influx by system ASC was also statistically significant ($P < 0.02$).

Stimulation of melphalan efflux by tamoxifen in ER-positive human breast cancer cells. The effect of tamoxifen on melphalan efflux from MCF-7 cells was also investigated. A time course of melphalan efflux shows that efflux was augmented from cells suspended in medium containing tamoxifen (Fig. 8). A semilogarithmic plot of exchangeable intracellular melphalan against time was linear for 10 min, suggesting that initial efflux followed first-order kinetics (Fig. 8, inset). The first-order rate constant K and the half-time $T_{1/2}$ for drug efflux were obtained from the linear regression equations of these efflux curves, where K is the negative slope, and $T_{1/2} = \log_e 2/K$ [17]. The efflux rate constant K (mean ± S.E.) for control cells was 0.085 ± 0.008 with a $T_{1/2}$ of 8.2 min and in cells treated with tamoxifen K was 0.129 ± 0.005 with a $T_{1/2}$ of 5.4 min; this 52% increase in efflux K was highly significant ($P < 0.001$).

DISCUSSION

Using a previously described clonogenic assay of human breast cancer cells *in vitro* [1], treatment of ER-positive MCF-7 cells and of ER-negative Evsa T cells with combinations of melphalan and tamoxifen produced antagonistic cytocidal effects. Pharmacologic antagonism was considered to exist when the cytocidal effect achieved with combination therapy was less than the additive effect of each agent acting independently [13–15]. Antagonism was noted when cells were treated either with a range of melphalan concentrations and a constant dose of tamoxifen (Figs. 1 and 4), or in complementary studies, with a range of tamoxifen concentrations and a fixed dose of melphalan (Figs. 2 and 5).

Rather striking differences were noted not only in the nature of the dose–survival curves obtained for cells treated with melphalan or tamoxifen, but also with respect to the effect each agent exerted on the dose–survival curve generated with the opposite compound. The dose–survival curves of cells treated with melphalan exhibited little or no shoulder and followed a simple exponential relationship characteristic of first-order cell kill (Figs. 1 and 4). Conversely, the dose–survival curves of cells treated with tamoxifen demonstrated a very prominent shoulder

followed by an exponential decline (Figs. 2 and 5); the shoulder suggests that with tamoxifen therapy, as with ionizing radiation and some drugs, the cell must accumulate multiple lesions before cellular death ensues and/or that the cell is capable of repairing sub-lethal damage [7, 9, 24–26].

The nature of the pharmacologic antagonism also appeared to differ. The addition of a fixed dose of tamoxifen to cells treated with a range of melphalan concentrations resulted in a marked increase in the D_0 of dose–survival curves of both ER-positive and -negative breast cancer cells (Figs. 1 and 4). However, the antagonistic effect of a fixed dose of melphalan on cells exposed to a range of tamoxifen concentrations was quite different; such treatment produced no change in D_0 but the observed dose–survival curve was elevated significantly over the curve expected for an additive interaction (Figs. 2 and 5).

Berenbaum [13] in a careful review of the criteria for analyzing the nature of drug interactions was critical of the above approach, in which drug synergy or antagonism was based upon the detection of changes in the slopes and/or shoulders of dose–survival curves as described by others [11, 12]. Accordingly, isobolograms were calculated to analyze the nature of the interaction of melphalan and tamoxifen using the method of Berenbaum [13]; concave down isobolograms were obtained for both ER-positive and -negative breast cancer cells supporting the argument that the two agents were acting as pharmacologic antagonists (Figs. 3 and 6).

The concentration range of tamoxifen used in the cytotoxicity studies was similar to that used in previous studies of drug effect on cell proliferation [1] and on thymidine incorporation [27]. With 1 hr of tamoxifen treatment, the D_0 was 0.604 nmole/ml for MCF-7 cells and 1.66 nmoles/ml for Evsa T cells; these values are similar to those noted previously and reaffirm the observation that ER-positive cells are more sensitive to tamoxifen than ER-negative cells [1]. In evaluating pharmacological significance of dose–survival studies, the product of drug concentration × treatment time is particularly relevant. If one extrapolates and theoretically extends the treatment time from 1 to 24 hr, then the "corrected" D_0 for tamoxifen treatment of MCF-7 and Evsa T cells would be 0.025 and 0.069 nmole/ml respectively (or 25 and 69 nM). Continuous therapy of patients with breast cancer using tamoxifen 10 mg twice daily produced steady-state serum levels of 100–200 ng/ml or 168–336 nM [28]. Thus, the tamoxifen doses used here appear to be clinically attainable, and these results may have pharmacological relevance.

As part of an investigation into the mechanism of these drug interactions, the effect of tamoxifen on the uptake of [^{14}C]melphalan by human breast cancer cells was evaluated at steady-state conditions. Evidence that melphalan uptake was studied at the steady state comes from a previous report, in which a time course of drug uptake was linear for approximately 2 min and thereafter entered a plateau at which an apparent equilibrium was reached between drug influx and efflux [16]. Under such conditions, uptake of [^{14}C]melphalan by both MCF-7 and Evsa T cells was inhibited by tamoxifen. The concentrations of melphalan and tamoxifen and the experimental conditions used in the cytotoxicity studies were duplicated in the drug uptake experiments. The antagonistic cytocidal effects observed in the treatment of human breast cancer cells with the combination of melphalan and tamoxifen may be explained by inhibition of melphalan uptake at the steady state by the antiestrogen tamoxifen. TLC analysis of cell contents, medium and a TCA-soluble cellular extract of MCF-7 cells suggested that tamoxifen reduced both the level of free intact melphalan and of bound drug (Table 1). With respect to cell kill, the small pool of bound drug, which includes drug covalently bound to DNA, may be the most critical determinant of drug activity.

Further investigation was undertaken to determine if the effect of tamoxifen on melphalan uptake was due to alteration of the rates of drug influx and/or efflux. Unidirectional melphalan influx into MCF-7 cells at 1 min was inhibited by tamoxifen (Fig. 7). Further analysis, using sodium-poor medium to examine drug influx by amino acid transport system L [18, 20, 22, 23] and saturating concentrations of BCH to evaluate transport by system ASC [18, 20–22], revealed that melphalan influx by both amino acid transport systems was inhibited significantly by tamoxifen. The efflux constant K for melphalan efflux from cells suspended in medium containing tamoxifen was approximately 50% greater than that noted in untreated control cells ($P < 0.001$). Thus, the inhibitory effect of tamoxifen on melphalan uptake by human breast cancer cells appears to be due to inhibition of drug influx and stimulation of drug efflux.

These findings may be of interest clinically. In a trial of adjuvant chemotherapy of breast cancer, the addition of tamoxifen to a regimen containing melphalan resulted in an adverse effect on the survival of certain patients 49 years of age or younger [2, 3]. The present study provides a possible explanation of that observation since it would appear that tamoxifen may antagonize the cytocidal activity of melphalan by lowering melphalan influx and stimulating drug efflux in human breast cancer cells. The reason why such a drug interaction might be operative only in certain patients subsets, or why the antagonistic effect of tamoxifen was demonstrable in both ER-positive and -negative human breast cancer cell lines remain unanswered. Finally, other possible effects of tamoxifen on melphalan such as changes in the rates of metabolic inactivation or of drug binding have not been excluded by this study.

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