Tamoxifen and 5-Fluorouracil in Breast Cancer: Cytotoxic Synergism

in Vitro

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ABSTRACT

The cytokinetic and cytotoxic interactions involved in combining tamoxifen, methotrexate, and 5-fluorouracil were studied in two hormone-dependent human breast cancer cell lines, 47-DN and MCF-7. These cells had measurable cytosol and nuclear estrogen receptor and cytosol progesterone receptor. Growth of the MCF-7 cells in medium containing gelding serum was stimulated maximally by addition of 10 pm estradiol. Both MCF-7 and 47-DN cells showed dose-dependent in vitro growth inhibition on exposure to tamoxifen, and toxicity from tamoxifen at concentrations up to 10 µM could be prevented by 1 nM estradiol. After exposure of 47-DN cells to 10 µM tamoxifen, cytosol progesterone and nuclear estrogen receptor levels were still detectable at 30 and 60% of control values. With this same concentration of tamoxifen, 47-DN cells in S phase declined 50% in association with a buildup of G2 cells. By clonogenic assay, tamoxifen enhanced 47-DN and MCF-7 cytotoxicity to 5-fluorouracil and 5-fluorouridine, but not to methotrexate alone. When given either concurrently or using a pretreatment-synchronizing schedule, tamoxifen enhanced markedly the growth inhibition of sequentially combined methotrexate and 5-fluorouracil. Isobologram analysis was used to prove that the cytotoxic interaction between tamoxifen and 5-fluorouracil was synergistic.

INTRODUCTION

More than one-half of all women diagnosed with breast cancer require systemic treatment with either chemo- or endocrine therapy. It is thus reasonable to wonder whether combining these modalities might improve upon the current 5 to 15% complete response rates and negligible cure rates achieved by either modality alone (12). Early clinical trials testing empiric combinations of chemo- and endocrine therapies reported equivocal benefit over single-modality therapy (13). It was even proposed that chemo- and endocrine therapies might be antagonistic in combination (31). Recently, however, 2 well-controlled studies reported that combining TAM with FUra-containing chemotherapy improved objective response rates over chemotherapy alone in postmenopausal women with ER-positive breast cancer (16, 30). Using 2 hormone-dependent human breast cancer cell lines, we have studied the cytokinetic and cytotoxic interactions involved in combinations of TAM, MTX, and FUra.

MATERIALS AND METHODS

Cell Lines, Drugs, and Growth Assays. The human mammary carcinomas 47-DN (a subline of T-47D) and MCF-7 are 2 well-characterized, continuously growing monolayer cell lines that double in 27 and 35 hr, respectively, when grown in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with insulin (0.2 IU/ml) and 10% fetal calf serum (Grand Island Biological Co.) (24, 34). Stock cultures and clonogenic assays were grown in 75-sq cm sterile plastic culture flasks (Costar Data Packaging, Cambridge, Mass.) with 25 ml of supplemented medium in 5% CO2 incubators at 37°C. Growth curves for the determination of tumor cell doubling times and growth-promoting effects of estradiol were performed by plating 104 cells into triplicate 35-mm culture wells (Costar) and counting single-cell suspensions at 3, 5, 7, and 9 days. Single-cell suspensions were prepared from cultures using a trypsin (0.05%):EDTA (0.02%) solution. All cell counts were performed on a Model ZBI Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). In medium not supplemented with insulin, 47-DN doubling time increased to ≥37 hr, and sensitivity to FUra (5 µM for 6 hr) decreased 40%. The estradiol content of commercial fetal calf serum used was measured by radioimmunoassay and found to be ≤100 pm; thus, cultures not supplemented with estradiol contained approximately 10 pm endogenous estradiol. Removing endogenous estradiol from serum by dextran-coated charcoal may alter cell growth by adsorption of other essential growth factors (25). Use of medium with 10% gelding serum (Sterile Systems, Inc., Logan, Utah; containing ≤30 pm endogenous estradiol by radioimmunoassay) enabled us to determine that approximately 10 pm estradiol was necessary for optimal in vitro growth of MCF-7 cells (Chart 1). TAM treatment in vivo can increase serum estradiol (28), and since postmenopausal women also have residual levels of estradiol (29), studying TAM effects in medium containing endogenous estradiol was considered to be physiologically and clinically relevant.

Clonal growth and drug cytotoxicity assays were performed using a monolayer technique described in detail elsewhere (5–7). All drugs were purchased from Sigma Chemical Co. (St. Louis, Mo.) except the antiestrogens, tamoxifen citrate (Stuart Pharmaceuticals, Wilmington, Del.) and clomiphene citrate (Sigma Chemical Co., Inc., St. Louis, Mo.). Methotrexate and 5-fluorouracil were generously donated by Merck Sharp & Dohme Research Laboratories, West Point, Pa. Epidermal growth factor (Parklane Biomedical, Inc., San Diego, Calif.) and insulin (IP products, Inc., Springfield, N. J.) were added at 0.2 µg/ml. Control cells were grown in medium with 10% gelding serum (Sterile Systems, Inc., Logan, Utah; containing ≤30 pm endogenous estradiol by radioimmunoassay) and 1% horse serum (Sterile Systems, Inc., Logan, Utah; containing ≤30 pm endogenous estradiol by radioimmunoassay) to determine the optimal estradiol concentration for clonogenic growth.

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6The abbreviations used are: TAM, tamoxifen; FUra, 5-fluorouracil; ER, estrogen receptor; MTX, methotrexate; PBS, phosphate-buffered saline [CaCl2 (100 mg):KCl (20 mg):K2HPO4 (20 mg):MgSO4 (59 mg):NaCl (800 mg):Na2HPO4 (1150 mg) in 1 liter of sterile aqueous solution]; FCCM, flow cytofluorometry; O:E, observed:expected values; ERa, cytosol estrogen receptor; ERb, nuclear estrogen receptor; PGR, progesterone receptor; FdUMP, 5-fluorodeoxyuridylate; FUDP, 5-fluorouridine diphosphate; FUTP, 5-fluorouridine triphosphate.

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FCM. Monolayer cultures of 47-DN cells, the growth of which had been inhibited by hormone deprivation or treatment with TAM, were prepared for FCM by PBS wash and trypsinization. Cells analyzed for DNA content were fixed in 10% formalin and stained with acriflavin as described previously (4). After washing formalin-fixed cells in PBS, cells were resuspended in 4 N HCl for 20 min, centrifuged, washed in distilled water, and recentrifuged to a pellet containing approximately 10^7 cells. Cells were then suspended in 2 ml of a filtered acriflavin solution (20 g/100 ml of acriflavin in 0.05 N HCl) for 20 min, washed in acid-alcohol (0.1 N HCl in 70% ethanol) twice, and resuspended in distilled water. Stained cells were filtered through a 37-mm nylon mesh, wrapped in foil, and stored refrigerated until analysis by FCM. Cell cycle analysis by FCM was performed on a Becton-Dickinson (Mountain View, Calif.) FACS IV instrument. Two-dimensional DNA histograms were recorded on an X-Y chart recorder. Histograms were analyzed graphically by time sequencing DNA content through "windows" selected to monitor the fraction (percentage of control untreated cells) of cells flowing through G0, S, and G2-M phases, a technique described by Zietz (38).

A 488-nm argon laser line (310 milliwatts, with 650-V photomultiplier tube) was used to excite fluorescence, and filters were used to permit only emitted wavelengths ±530 nm to be analyzed for pulse-height distribution over 255 channels. Coefficients of variation of the G0, peaks were usually ±10%. Flow rates were maintained between 500 and 1000 cells/sec, and calibration of the instrument with chicken RBC and Chinese hamster ovary cells was performed to optimize light scatter and fluorescence.

Steroid Receptor Assay. After extensive rinsing and trypsin harvesting, cells were pelleted in PBS at 4° and stored for less than 30 min in an ice bath before homogenization. Cell fractionation and receptor assays followed standard procedures as described by Horwitz et al. (22). The cells were resuspended in phosphate buffer (5 mM, pH 7.4, 4°) with 1 mM thioglycerol and 10% glycerol. Homogenization in a glass Teflon homogenizer was monitored by light microscopy with trypan blue staining until more than 90% of the cells were disrupted. The crude nuclear pellet was obtained by centrifuging the lysed cells at 800 x g for 10 min and then twice washing in cold PBS. The pellet was suspended in Tris-HCl (pH 8.5, 0°), and cold KCl was added to a final concentration of 0.6 M. After 1 hr of cold incubation with vortexing every 15 min, the pellet was centrifuged in a microfuge. The KCl supernatant was diluted with phosphate buffer to approximately 1 mg of protein per ml (Bradford assay), and 0.5 ml was mixed with 0.25 ml of protamine sulfate (1 mg/ml of phosphate buffer). The protamine precipitates were incubated for 5 hr with 5 nM [3H]estradiol (100 Ci/mmol) in the presence and absence of a 100-fold excess of nonradioactive diethylstilbestrol to determine specific binding. The protamine pellet-containing tubes were incubated at 30° for 5 hr to allow [3H]estradiol to attach to free receptor and to exchange with occupied nuclear receptor. Supernatants were decanted, and the pellets were washed twice with 2 ml of PBS containing 1% Tween 80 which improved the ratio of receptor to nonspecific binding levels. The pellets were then extracted with ethanol, and bound radioactivity was measured. Cytosol receptors were determined from the original lysed cell supernatant following centrifugation at 100,000 x g for 1 hr. Cytosol was incubated with 5 nM [3H]estradiol ± 100-fold excess of diethylstilbestrol in replicate tubes for 2 hr at 0° and then 1 hr at 30° (occupied receptor). Small Sephadex LH-20 columns were used to separate macromolecular bound from free radioactivity. For cytosol progesterone receptor assay, the 100,000 x g supernatant was incubated with 20 nM [3H]progesterone (50020, New England Nuclear, Boston, Mass.) and 1,000 nM dexamethasone (to prevent binding to glucocorticoid receptors) with and without 2,000 nM nonradioactive R5020 for 3 hr at 0°. Receptor bound was separated from free with the Sephadex LH-20 columns, and the radioactivity in the macromolecular fraction was determined. DNA in the nuclear pellet was measured using the modified Burton assay, while cytosol protein was measured by Bradford assay using bovine albumin as standard. Determination of specific receptor binding by single high concentration of tritiated steroid affords limited information about receptor characteristics. There was insufficient 47-DN sample material for Scatchard analysis and determination of receptor affinity constants (Kd).

Statistics and Analysis of Synergy. All growth and receptor assays were performed on at least triplicate conditions, and mean values (± S.D.) were recorded; FCM determinations were made on duplicate conditions. In clonogenic assays measuring interactive effects between toxic agents, synergistic cytotoxicity was often determined quantitatively by the O:E ratio of the percentage of clonal growth, lower ratios (≤ 1) indicating greater synergism. This specific criterion for synergy is correct for cell populations demonstrating homogeneous sensitivity to the individual toxic agents and for agents showing simple exponential dose-response curves (6). With the use of isoeffect (isobologram) analysis, as utilized in Chart 4, these assumptions are not required. Given the doses of 2 cytotoxic agents that produce independently a specified clonogenic survival equal to [A] and [B], and doses in combination equal to [A] and [B] that also produce this same effect, then the family of isoeffect curves (for growth, i = 90, 80, 70, 60, 50, and 40%) must have the following algebraic relationships (8).

\[
\begin{align*}
[A] + [B] & = 1 & \text{for zero interaction (additivity)} \\
& < 1 & \text{for synergy} \\
& > 1 & \text{for antagonism}
\end{align*}
\]

To determine the family of predicted isoeffect curves shown in Chart 4, 25 pairs of dose-response data points measured in a single experiment (A = TAM; B = FUra) were fitted by linear regression to the following general formula.

\[
\]

Using an IBM 370/Model 158 computer with SAS software package (Yale Computer Center), a predicted fit (F = 20.54; p > F = 0.0001) was obtained with the observed data points using the following parameters: \(Y_0 = (4.579); a = (-0.052); b = (-0.060); a' = (-0.002); b' = (-0.007); ab = (-0.077); a'b = (0.005);\) and ab' = (0.008). As shown in Chart 4, the entire family of isoeffect curves was concave and fulfilled the synergistic condition,

\[
\frac{[A] + [B]}{[A][B]} < 1
\]
Studied the effect of estradiol on 47-DN growth inhibition by TAM. Whether growth inhibition was measured by prolonged culture doubling time or by reduced clonogenic growth, the toxic effects of 0.001 to 10 μM TAM were prevented by 1 nm estradiol. Thus, it is probable that the endogenous amounts of estradiol in fetal calf serum competed with and reduced TAM toxicity, especially at lower TAM doses. It is also possible that toxicity observed with >10 μM TAM was nonspecific and not mediated by ER; however, the effects of TAM (≤10 μM) measured throughout most of this study would be preventable by 1 nm estradiol and consistent with previous reports, suggesting dependency on functional ER (19, 26, 27).

Levels of ERα, ERβ, and cytosol PGR were determined in the absence of exogenous estradiol and were reduced following TAM exposure. For untreated MCF-7, ERα = 44 (fmol/mg of protein), ERβ = 527 (fmol/mg of DNA), and PGR = 11 (fmol/mg of protein). Table 2 shows that, for untreated 47-DN, ERα and ERβ were less than the measured levels in MCF-7. When 47-DN cells were treated with 10 μM TAM for 72 hr and adherent cells were rinsed extensively to diminish free TAM concentration, ER, and ERβ were reduced to 14 and 57%, while PGR was reduced to 28% of control levels. These results were consistent with previous reports (18, 20, 22). The incubation of TAM-pretreated cells for 1 hr in hormone-free medium prior to extensive washing and subsequent receptor determination did not necessarily assure complete removal of intracellular TAM. The detection of specific [17βestradiol nuclear binding at 30° in TAM-pretreated 47-DN did not prove, but was consistent with, the nuclear presence of TAM:ER complex and ER mediation of TAM effects.

DNA histograms, obtained by FCM and analyzed by the method of Zietz (38), were used to monitor the growth-arresting effects of TAM exposure on 47-DN (Chart 3). After 24 hr of TAM, the number of cells traversing S phase was reduced 50%, resulting in a buildup of G0, cells. These results agree with those of Sutherland et al. (35) in MCF-7. Clonogenic growth of 47-DN was reduced 50% by lack of exogenous insulin; and by FCM analysis, a similar reduction in S-phase cells occurred by 24 hr, as with TAM exposure, but without concomitant buildup in G0, cells. These patterns of cell cycle arrest suggested that both insulin deprivation and antiestrogen treatment of 47-DN might be antagonistic with administration of cell cycle-active antimitabolites, such as MTX and FUra (36).

When 47-DN were exposed to combinations of TAM, MTX, and FUra, synergistic cytotoxicity was apparent (Table 3). FUra was added during the last 6-hr interval of a 24-hr exposure to MTX, as this sequencing schedule had been shown previously
to produce optimal synergy between the 2 antimitabolites (6, 7). TAM was added 48 hr prior to MTX exposure and continued for 72 hr, at which time all drugs were washed out. Table 3 shows that sequenced MTX: FUra had greater than additive toxicity. In addition, cells pretreated with TAM showed greater than additive cytotoxicity with FUra and MTX: FUra combined sequentially. Although TAM and MTX: FUra produced the greatest synergism, TAM did not appear to have significant synergy with MTX alone. Further experiments with 47-DN showed that a variety of TAM pretreatment exposures were synergistic with FUra and MTX: FUra combined sequentially (Table 4). When MCF-7 cells were exposed continuously to varying concentrations of TAM and a fixed concentration of FUra, synergism was also measured (Table 5). These cells were also tested against an investigational antiestrogen, LY117018. This compound represents a new class of potent antiestrogens, the mechanism of action of which may differ from that of TAM (9, 10). Continuous exposure to 0.01 to 1.0 μM LY117018 inhibited MCF-7 growth from 4 to 60% and resulted in synergistic (O:E) ratios with FUra (5 μM for 6 hr) ranging from 0.43 to 0.63.

Further studies were performed to determine whether cytokininetic sequencing of TAM, MTX, and FUra altered these synergistic interactions in MCF-7 and 47-DN and also whether FdUrd or FdUrd was as synergistic with TAM as was FUra. TAM (10 μM) was administered for 48 hr either in combination with MTX and MTX: FUra or sequenced and washed our prior to these antimitabolites. For this TAM dose, sequential TAM → MTX and TAM → MTX: FUra schedules were more cytotoxic than the combination schedules, while combination TAM: FUra was more potent than was sequenced TAM → FUra. When 2-hr equitoxic doses of FdUrd (20 μM), FdUrd (1 μM), and FUra (20 μM) were administered in combination with continuous exposure to 1 μM TAM, significant differences in synergy were also observed. In both cell lines, FdUrd yielded the greatest synergistic interaction with TAM, with O:E ratios 50 to 100% lower than FdUrd-treated cells.

The use of O:E growth ratios as a valid measure of TAM: FUra synergism assumed homogeneity in cell culture sensitivity to both TAM and FUra, as well as an exponential dose responsiveness to each of these agents (8). Thus, a more rigorous proof of synergy required isobologram analysis. Chart 4 shows that when 47-DN cells were exposed to varying concentrations of TAM and FUra, the resulting data points could be described accurately by a family of isoeffect curves (isobols). These concave curves fulfilled the generally defined criteria for synergistic interaction between TAM and FUra at all concentrations tested (see "Materials and Methods").

**Table 3**

Clonal growth of 47-DN cells treated with MTX, FUra, and TAM

<table>
<thead>
<tr>
<th>Condition</th>
<th>−TAM</th>
<th>+TAM</th>
<th>O:E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 3</td>
<td>58 ± 6</td>
<td>0.81</td>
</tr>
<tr>
<td>MTX (0.5 μM for 24 hr)</td>
<td>47 ± 10</td>
<td>22 ± 6</td>
<td>0.70</td>
</tr>
<tr>
<td>FUra (5 μM for 6 hr)</td>
<td>93 ± 12</td>
<td>26 ± 6</td>
<td>0.48</td>
</tr>
<tr>
<td>MTX:FUra</td>
<td>11 ± 5</td>
<td>2 ± 0</td>
<td>0.33</td>
</tr>
</tbody>
</table>

*The percentage of clonal growth was used as an index of drug interaction as defined in "Materials and Methods": O:E < 1.0 (±0.2) = synergism; > 1.0 (±0.2) = antagonism.

1 Mean ± S.D.

**Table 4**

Effect of TAM exposure on MTX: FUra toxicity in 47-DN

<table>
<thead>
<tr>
<th>TAM (μM)</th>
<th>MTX (μM)</th>
<th>MTX:FUra (μM)</th>
<th>O:E</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1</td>
<td>0.10</td>
<td>0.68</td>
</tr>
<tr>
<td>10</td>
<td>0.88</td>
<td>0.01</td>
<td>0.50</td>
</tr>
<tr>
<td>10</td>
<td>0.60</td>
<td>0.01</td>
<td>0.50</td>
</tr>
<tr>
<td>0.1</td>
<td>0.50</td>
<td>0.01</td>
<td>0.32</td>
</tr>
<tr>
<td>1.0</td>
<td>0.63</td>
<td>0.01</td>
<td>0.20</td>
</tr>
</tbody>
</table>

**Table 5**

MCF-7 cells treated with TAM and FUra

<table>
<thead>
<tr>
<th>TAM (μM)</th>
<th>−FUra</th>
<th>+FUra</th>
<th>O:E</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 3</td>
<td>91 ± 15</td>
<td>0.55</td>
</tr>
<tr>
<td>1</td>
<td>54 ± 16</td>
<td>27 ± 7</td>
<td>0.55</td>
</tr>
<tr>
<td>5</td>
<td>32 ± 6</td>
<td>17 ± 4</td>
<td>0.58</td>
</tr>
<tr>
<td>10</td>
<td>21 ± 5</td>
<td>6 ± 2</td>
<td>0.31</td>
</tr>
<tr>
<td>25</td>
<td>1 ± 0</td>
<td>&lt;0.2 ± 0</td>
<td>&lt;0.25</td>
</tr>
</tbody>
</table>

*Mean ± S.D.*
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These curves define the interaction between TAM and FUra as true synergism (8). The concave appearance of the indicated growth range encompasses -4% to +5% of the indicated growth. Using a linear regression equation described in "Materials and Methods," a predicted fit was achieved (p = 0.0001) for each isoeffect range, resulting in the family of concave curves as shown. The concave appearance of these curves defines the interaction between TAM and FUra as true synergism (8).

FUra is a cycle-active, phase-nonspecific chemotoxin by virtue of its multiple intracellular metabolites: FdUMP; FUDP:glucose; and FUTP (2, 14, 23). A reduction in S-phase cells produced by lack of insulin or during TAM administration might lead to decreased toxicity from S-phase specific antimetabolites, such as FdUMP and MTX (36). If TAM is washed out prior to MTX or FUra exposure, G1-arrested cells enter S phase in a synchronized pulse (35), possibly enhancing sensitivity to S-phase antimetabolites. This study confirmed that sequential TAM → MTX and TAM → MTX:FUra schedules were more cytotoxic than their respective combination schedules. Toxicity from FUra alone was not appreciably enhanced by TAM synchronization; on the contrary, combination TAM:FUra showed greater than additive cytotoxicity over a wide range of doses and exposure times. This cytotoxic interaction with concurrent administration of TAM and FUra was shown by isobologram analysis to be true synergism. The clinical significance of these in vitro findings may be apparent from the study of Allegra et al. (1) who have achieved approximately 50% complete responses in Stage IV breast cancer by treating patients with TAM, attempting to synchronize their tumor cells before administering MTX:FUra.

It is possible that the RNA-directed toxicity of FUra accounted for synergy with TAM. Unlike DNA synthesis which is an S phase-specific event, synthesis of different RNA species occurs throughout the cell cycle, and G1 arrest by various means is known to alter cellular RNA more than 3-fold (4, 17, 37). Of interest, the measured differences in FUra toxicity with TAM treatment and lack of insulin corresponded to opposing effects on the G1 cell population, suggesting that RNA incorporation of FUTP rather than FdUMP inhibition of DNA synthesis mediated these toxicity differences. Furthermore, MTX alone was not synergistic with TAM; it was, however, able to enhance the synergy between TAM and FUra. We have shown in several malignant cell lines that MTX pretreatment increased FUra toxicity by increasing intracellular formation of FUra nucleotides. The increased amounts of FUTP and incorporation into RNA (5, 7, 11) were associated with enhanced cytotoxicity. It therefore seems reasonable to postulate that TAM:FUra synergy resulted from the ability of TAM to enhance the RNA-directed toxicity of FUra. Two other fluoropyrimidines were tested at equitoxic doses for cytotoxic synergism with TAM: FUrd, which produces more RNA-directed toxicity (via FUTP), and FdUrd, which over short exposure intervals generally produces more DNA-directed toxicity (via FdUMP). These experiments revealed that TAM was more synergistic with FUrd than either FdUrd or FUra, consistent with a putative interaction involving RNA.

Sartorelli first introduced the concept of "complementary inhibition" to describe the synergistic effects observed between such drugs as actinomycin D, that bind and inactivate DNA or RNA, and others such as FUra, which enter the biosynthetic pathways leading to these macromolecules (32, 33). TAM and other antiestrogens are known to bind to nuclear chromatin before exerting their cytotoxic effects (3, 20, 21). Furthermore, actinomycin D has been shown to mimic some antiestrogenic effects of TAM on ER processing (21). Thus, it is possible that TAM:FUra synergy is another example of complementary inhibition. Alternatively, since ER half-life has been estimated at 6 hr (15), it is also possible that FUra incorporation into RNA alters ER synthesis or function in such a fashion as to enhance the receptor-mediated toxicity of TAM. Biochemical studies now under way may uncover the mechanism(s) underlying TAM:FUra synergism and provide more information for rationally combining these agents in treatment of breast cancer.

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