Cancer Chemother Pharmacol (1996) 37: 222–228


ORIGINAL ARTICLE

Ting-Chao Chou · Glenys M. Otter
Francis M. Sirotnak

# Schedule-dependent synergism of taxol or taxotere with edatrexate against human breast cancer cells in vitro

Received: 7 March 1994/accepted: 21 March 1995

**Abstract** A new dihydrofolate reductase inhibitor, edatrexate (EDX), and the microtubule polymerization promotor, taxol (TXL) or taxotere (TXT), each have significant therapeutic activity against human breast cancer in clinical trials. Since they also have distinctly different mechanisms of actions and have mainly non-overlapping toxicities, they may be effective in combination in the treatment of this disorder. Schedule-dependent interactions between these taxanes and EDX against human breast adenocarcinoma cells (SK-Br-3) were quantitatively assessed in vitro to determine whether these interactions are synergistic or antagonistic. SK-Br-3 cells were grown as a monolayer in 96-well microplates. The dose-effect relationships of the drugs, singly and in combination, in inhibiting the growth over a 7-day period were determined by the SRB protein staining assays. Cell cultures were exposed to drug as a 3-h pulse at either 0–3 h or 24–27 h. Synergism or antagonism at different concentrations and at different effect levels were assessed with the medianeffect principle and the combination index-isobologram method using computer software. These methods were selected because they take into account both the potencies and the shape of the dose-effect curves. Exposure of cells to an equimolar combination of EDX + TXL (0–3 h) resulted in synergism at high effect levels. Pretreatment of cells with EDX (0–3 h) followed by TXL (24–27 h) showed even greater synergism in inhibiting cell growth. Moderate antagonism was observed with the reverse schedule. EDX + TXT (0–3 h) was additive, but pretreatment with EDX (0–3 hr) followed by TXT (24–27 h) showed synergism. However, the reverse order showed antagonism. Studies on another breast tumor cell line, ZR-57–1, also showed the schedule of EDX (0–3 h) + TXT or TXL (24–27 h) to be more synergistic than, the other two schedules examined. These results show potent schedule-dependent synergism of the combinations of TXL or TXT with EDX, and should form a rationale for designing clinical protocols utilizing these agents particularly for the treatment of breast cancer patients.

**Key words** Edatrexate · Taxol · Taxotere · Breast cancer · Combination treatment

**Abbreviations** *TXL* taxol · *TXT* taxotere · *EDX* 10-ethyl-10-deaza-aminopterin · *CI* combination index · *DRI* dose-reduction index

## Introduction

The taxane derivatives, taxol (TXL) and taxotere (TXT), have been shown to exhibit extremely encouraging therapeutic activity against human malignancies, particularly refractory ovarian and breast cancers [1–7]. These agents are unique in that they enhance the polymerization of tubulin to stable microtubules by interacting stoichiometrically with microtubules in the absence of any cofactors [1, 3, 4]. Consequently, tumor cells are blocked in the mitotic phase of the cell cycle and are unable to replicate. As with other chemotherapeutic agents, the clinical utility of these taxane derivatives will depend upon their optimum use in combination with other clinically useful agents. Ideally, agents used in such combinations should exhibit minimal overlapping toxicities and have confirmed effects that reflect at least additive, if not, synergistic interactions in the target tumor cells.

Other studies [8] have shown that the classical folate analogue, edatrexate (EDX, 10-ethyl-10-deazaaminopterin)

This work was supported in part by NCI grant CA18856 and by the Elsa U. Pardee Foundation

T.-C. Chou · G.M. Otter · F.M. Sirotnak (✉)
Molecular Pharmacology and Therapeutics Program, Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, 1275 York Avenue, New York, NY 10021, USA

is similar to methotrexate (MTX) as an inhibitor of dihydrofolate reductase, but is more effectively transported and polyglutamylated in tumor cells. More importantly, there is greater accumulation of cytotoxic EDX polyglutamates [8] in tumor cells than in normal proliferative tissues that are sites of limiting toxicity. In other studies EDX has been shown to be more efficacious than MTX in vivo against murine ascitic and solid tumors and against human tumor xenografts that are resistant to MTX [9, 10]. EDX has been evaluated as a single agent in phase I and phase II studies [11, 12] and has been shown to have therapeutic activity against a number of tumors greater than MTX, particularly non-small cell lung cancer [13, 14] and breast cancer [15, 16]. Toward the further clinical development of EDX, combination therapeutic studies have shown that EDX administered with alkylating agents or cisplatin [17, 18] or vinca alkaloids [19] is highly effective against a number of MTX-resistant tumors. Retrospective analysis by the median-effect principle and combination index methodology [20–23] of the effect of the combination of EDX and cisplatin against human leukemia [24] and human lung cancer [25, 26] cells has shown that this combination is potently synergistic, which would suggest a basis for the high degree of effectiveness of, at least, the EDX plus cisplatin combination in vivo. The results of these studies have led to a number of clinical trials evaluating EDX [11, 26] in combination with these other agents.

The dose-limiting toxicities of taxane derivatives in patients are leukopenia and peripheral neuropathy [5–7]. In contrast, mucositis is the dose-limiting toxicity of EDX in patients [11–16]. Other toxicities including myelosuppression, small bowel disturbances, rash and pneumonitis, are generally mild to moderate in severity [11–16]. In view of the distinctly different mechanisms of action and the dissimilarity in toxicity associated with these clinically active agents, we initiated a prospective study of their combined effects against human breast cancer cells (SK-Br-3 and ZR-75-1) in cell culture. These studies used the median-effect principle and the combination index method [20–23], and computerized data analysis [27–28] for quantitation of synergism or antagonism of the agents. The results of these studies may have utility in designing combination chemotherapy clinical protocols for these two types of agents that have non-overlapping toxicities, particularly in breast cancer patients.

## Materials and methods

### Drugs and drug solutions

EDX, originally synthesized at SRI International, Menlo Park, Calif., was made available by the Ciba Geigy Corporation, Summit, N.J. EDX stock solutions were prepared at 2 mg/ml in 5% sodium bicarbonate and the pH was adjusted to 7.2. TXL was obtained from the Drug Developmental Branch, National Cancer Institute, Bethesda, Md., and was first dissolved in 50% ethanol and 50% cremophor and stored at −20°C. A stock solution was prepared (< 0.6 mg/ml TXL) prior to use by dilution with 0.5% ethanol, 5% cremophor and 90% saline. Further dilutions were made in MEM/f12 medium. TXT (RP56976) was obtained as a gift from Rhone-Poulenc Pharmaceuticals, France. TXT was first dissolved at 50 mg/ml in ethanol and stored at −20°C. Immediately prior to use, 1 vol of the ethanolic stock solution was mixed with 1 vol of polysorbate 80 and then 18 vol of 5% glucose in aqueous solution was added to make a 2.5 mg/ml stock solution. Further dilutions were made in MEM/f12 medium.

### Cells and incubation

SK-Br-3 human breast adenocarcinoma and ZR-75-1 human breast carcinoma cells (obtained from American Type Culture Collection, Rockville, Md.) were used. They were grown as a monolayer in 96-well plates, in MEM/f12 supplemented with 10% fetal bovine serum, under an atmosphere containing 5% $CO_2$ in an incubator at 37°C. A suspension of $8 \times 10^3$ cells in 100 µl was added to each experimental well 24 h before drugs were added. Plates were read when control wells were almost confluent (7 days) using the SRB protein staining assay [29].

### Drug combinations and schedules

EDX combined with either TXL or TXT was explored with three different pulse treatment schedules: (a) simultaneous exposure: EDX, 0–3 h + another drug, 0–3 h; (b) sequential exposure: EDX, 0–3 h + another drug, 24–27 h; and (c) reverse exposure: another drug, 0–3 h + EDX, 24–27 h. For each drug combination, three 96-well plates were used, one for each pulse schedule. Each experiment was repeated several times as indicated in Tables 1–3.

Each plate included wells with the drugs combined, single-drug controls, solvent controls and cell controls. All single-drug wells as well as the control wells were handled in exactly the same way as the combination wells except for the concentration and timing of drug additions. Drugs were serially diluted twofold across the wells and any rows not getting drug at any time had medium passed from well to well. Similarly, when drugs were removed at the end of the pulse treatment (using a sterile pasteur pipette attached to vacuum) the medium of all control wells was similarly emptied and all were given fresh medium. Plates were read when control wells were almost confluent (7 days). The SRB protein staining assay [29] was used and absorbance was read on a microplate reader (Bio-Tek, model EL-340). Fractional inhibition ($f_a$) of cell growth (percent inhibition/100) as compared to untreated cell controls at the end of inhibition measurement was calculated.

### Quantitation of synergism or antagonism

For each of the individual drugs (EDX, TXL, TXT) and their two drug combinations, the dose-effect relationships obtained from two fold serial dilution and the SRB cytoxicity assay were subjected to a median-effect plot analysis [23, 28]. Briefly, the median-effect equation is expressed as:

$$(f_a)/(f_u) = (D/D_m)^m$$

or

$$\log (f_a/f_u) = m \log D - m \log D_m \qquad \text{Eq. 1}$$

For EDX, $D$ is the dose of EDX, $D_m$ is the median-effect dose ($ED_{50}$ or $IC_{50}$) of EDX, $f_a$ is the fractional inhibition (e.g. $f_a = 0.95$ for 95%

**Table 1** Dose-effect relationship parameters of EDX, TXL and TXT against the growth of SK-Br-3 and ZR-75–1 breast adenocarcinoma cells with different drug exposure times during a 7-day incubation period as described in Materials and methods. ($m$, $D_m$, and $r$ are the slope, antilog of the $x$-intercept, and the linear correlation coefficient of the median-effect plot [21–23] which signify the shape of the dose-effect curve, the potency ($IC_{50}$) and the conformity of the data to the mass-action law, respectively [27]; $n$ is the number of sets of dose-effect relationship experiments carried out). Values are mean ± SEM for SK-Br-3 cells, and mean ± variation for ZR-75-1 cells

| Drug | Breast cancer cells | $D_m$ ($\mu M$) | $m$ | $r$ | $n$ |
|---|---|---|---|---|---|
| EDX (0–3 h) | SK-Br-3 | 0.085 ± 0.022 | 0.802 ± 0.173 | 0.851 ± 0.050 | 11 |
| | ZR-75-1 | 0.273 ± 0.165 | 0.811 ± 0.281 | 0.880 ± 0.010 | 2 |
| TXL (0–3 h) | SK-Br-3 | 0.039 ± 0.020 | 0.648 ± 0.105 | 0.943 ± 0.013 | 8 |
| | ZR-75-1 | 0.020 ± 0.004 | 0.738 ± 0.157 | 0.919 ± 0.044 | 2 |
| TXT (0–3 h) | SK-Br-3 | 0.013 ± 0.004 | 1.039 ± 0.172 | 0.955 ± 0.017 | 5 |
| | ZR-75-1 | 0.043 ± 0.012 | 0.541 ± 0.111 | 0.875 ± 0.081 | 2 |
| EDX (24–27 h) | SK-Br-3 | 0.120 ± 0.043 | 0.964 ± 0.350 | 0.925 ± 0.020 | 6 |
| | ZR-75-1 | 0.435 ± 0.193 | 0.595 ± 0.046 | 0.853 ± 0.013 | 2 |
| TXL (24–27 h) | SK-Br-3 | 0.071 ± 0.027 | 0.734 ± 0.072 | 0.971 ± 0.014 | 4 |
| | ZR-75-1 | 0.047 ± 0.017 | 0.785 ± 0.359 | 0.960 ± 0.001 | 2 |
| TXT (24–27 h) | SK-Br-3 | 0.017 ± 0.006 | 1.248 ± 0.297 | 0.905 ± 0.053 | 4 |
| | ZR-75-1 | 0.049 ± 0.026 | 0.726 ± 0.299 | 0.886 ± 0.059 | 2 |

inhibition by EDX), $f_u$ is the fraction unaffected ($1 - f_a$), and $m$ is the coefficient signifying the slope of the dose-effect curve for EDX ($m = 1$, $> 1$, and $< 1$ indicate a hyperbolic, sigmoidal, and negatively sigmoidal curve, respectively). Based on Eq. 1, the median-effect plot of $x = \log D$ vs $y = \log[f_a/(1 - f_a)]$ yields a straight line with a slope ($m$) and an $x$-intercept of $\log D_m$. Thus, the $m$ and $D_m$ parameters of EDX can be easily determined quantitatively (Table 1). The same procedure was carried out for TXL, TXT and the combinations. The conformity of the experimental data to the median-effect principle of the mass-action law is indicated by the linear correlation coefficient ($r$) of the median-effect plot (Table 1).

From the $m$ and $D_m$ values, the isoeffective dose ($D_x$) for any effect level (e.g. $ED_{70}$ for $f_a = 0.7$, $ED_{90}$ for $f_a = 0.9$) for each drug or for the combinations can be easily determined by rearrangement of Eq. 1.

$$D_x = D_m[f_a/(1 - f_a)]^{1/m} \qquad \text{Eq. 2}$$

Synergism or antagonism for EDX plus TXL or TXT is determined by using $m$ and $D_m$ parameters and Eq. 2 by substituting $D_x$ values into the combination index (CI) equation of Chou and Talalay [21–23], where $CI < 1$, $> 1$ and $> 1$ indicate synergism, an additive effect, and antagonism, respectively. Based on the classical isobologram for mutually exclusive drug effects relative to the end-point of measurement, the CI value for $x$% inhibition is calculated as:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \qquad \text{Eq. 3}$$

For example, at 90% inhibition level, $(D_x)_1$ and $(D_x)_2$ are the doses for 90% inhibition by EDX and TXL, respectively, which can be obtained from Eq. 2; and $(D)_1$ and $(D)_2$ in the numerators are the doses of EDX and TXL in combination that also inhibit cell growth by 90% (i.e. isoeffective compared with the single drugs alone). If the calculation is based on the conservative isobologram equation, assuming that the effects of two drugs are mutually nonexclusive (i.e totally independent relative to the end-point of measurement), then an approximated third term designated as the product of the first two terms should be added to Eq. 3. For simplicity, CI values obtained from the classical isobologram equation (Eq. 3) have frequently been used, but the underlying assumption needs to be stated. When exclusivity is unknown, the CI value is routinely calculated in both ways [27, 28].

The dose-reduction index (DRI) defines the extent of dose reduction possible in a combination for a given degree of effect as compared with the dose of each drug alone [23, 28]: $(DRI)_1 = (D_x)_1/(D)_1$ and $(DRI)_2 = (D_x)_2/(D)_2$; therefore, the relationship between DRI and CI is expressed as

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{1}{(DRI)_1} + \frac{1}{(DRI)_2} \qquad \text{Eq. 4}$$

Computer softwares [27, 28] can be used for fully automated data analysis based on Eqs. 1–4. Example calculations on a set of crude data, using a pocket calculator, are demonstrated in references 22 and 24.

## Results

### Single-drug efficacy and parameters

As shown in Table 1, SK-Br-3 and ZR-75–1 breast cancer cells showed characteristic differences in their sensitivity to drugs ($D_m$ values or $IC_{50}$ values) and the shapes of the dose-effect curves ($m$ values) at both 0–3 h and 24–27 h drug exposure. EDX and TXT were about threefold more effective against SK-Br-3 cells than against ZR-75–1 cells. By contrast TXL was about twofold more effective against ZR-75–1 cells than against SK-Br-3 cells. In addition, ZR-75–1 cells showed shallower dose-effect curves (lower $m$ values) toward TXT and EDX, and somewhat steeper dose-effect curves (higher or similar $m$ values) for TXL, when compared with SK-Br-3 cells.

### Drug combination and scheduling studies

To avoid inter-experimental variations, each determination of synergism/antagonism was carried out in a large-scale experiment using a 96-well microplate reader so that the parameters ($D_m$, $m$ and $r$) and the CI values were calculated from the dose-effect relationships within each experiment. The experiments were repeated four or more times and the average CI and its

**Table 2** Combination effects of EDX + TXL during a 7-day incubation against SK-Br-3 and ZR-75-1 cell growth. CI < 1, = 1, and > 1 indicate synergism, an additive effect, and antagonism, respectively. Values are mean ± SEM for SK-Br-3 cells and mean ± variation for ZR-75-1 cells. Each data set for the CI calculations consisted of dose-effect relationships of two single drugs and their combinations obtained from individual experiments (*n* number of sets of dose-effect relationship experiments). Each dose-effect relationship consisted of six to eight drug concentrations in duplicate. $D_m$ and *m* values were used for calculating the CI values based on the CI equation of Chou and Talalay [21–23] using computer software [27]. Equations used were: $D_x = D_m\ [f_a/(1-f_a)]^{1/m}$ and $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$, where $D_x$ is the dose (concentration) for *x*% inhibition

| Drug Combination (1:1 molar ratio) | Breast cancer cells | CI value at $IC_{50}$ | $IC_{75}$ | $IC_{90}$ | $IC_{95}$ | *n* |
|---|---|---|---|---|---|---|
| EDX + TXL | SK-Br-3 | 3.324 ± 1.596 | 1.105 ± 0.154 | 0.740 ± 0.160 | 0.595 ± 0.167 | 4 |
| (0–3 h) (0–3 h) | ZR-75-1 | 1.644 ± 0.018 | 2.916 ± 1.551 | 6.791 ± 5.65 | 13.009 ± 12.00 | 2 |
| EDX + TXL | SK-Br-3 | 1.335 ± 0.289 | 0.684 ± 0.160 | 0.486 ± 0.173 | 0.446 ± 0.174 | 5 |
| (0–3 h) (24–27 h) | ZR-75-1 | 0.767 ± 0.133 | 0.693 ± 0.202 | 0.646 ± 0.251 | 0.621 ± 0.276 | 2 |
| EDX + TXL | SK-Br-3 | 1.088 ± 0.223 | 0.854 ± 0.145 | 1.004 ± 0.280 | 1.714 ± 0.822 | 5 |
| (24–27 h) (0–3 h) | ZR-75-1 | 0.507 ± 0.373 | 0.714 ± 0.032 | 2.160 ± 1.610 | 6.182 ± 5.690 | 2 |

**Table 3** Combination effects of EDX + TXT during a 7-day incubation against SK-Br-3 and ZR-75-1 cell growth. For an explanation of the CI values and their calculation, see Table 2

| Drug Combination (10:1 molar ratio) | Breast cancer cells | CI values at $IC_{50}$ | $IC_{75}$ | $IC_{90}$ | $IC_{95}$ | *n* |
|---|---|---|---|---|---|---|
| EDX + TXT | SK-Br-3 | 1.447 ± 0.174 | 1.457 ± 0.255 | 1.497 ± 0.330 | 1.540 ± 0.381 | 3 |
| (0–3 h) (0–3 h) | ZR-75-1 | 1.181 ± 0.146 | 0.783 ± 0.092 | 0.611 ± 0.006 | 0.552 ± 0.059 | 2 |
| EDX + TXT | SK-Br-3 | 1.233 ± 0.098 | 1.050 ± 0.056 | 0.900 ± 0.060 | 0.807 ± 0.070 | 3 |
| 0–3 h) (24–27 h) | ZR-75-1 | 0.865 ± 0.131 | 0.661 ± 0.129 | 0.509 ± 0.118 | 0.428 ± 0.108 | 2 |
| EDX + TXT | SK-Br-3 | 1.240 ± 0.040 | 1.278 ± 0.027 | 1.495 ± 0.135 | 1.680 ± 0.260 | 3 |
| (24–27 h) (0–3 h) | ZR-75-1 | 0.813 ± 0.304 | 1.639 ± 0.002 | 5.473 ± 2.845 | 15.48 ± 11.62 | 2 |

variations were then determined for EDX + TXL schedules (Table 2) and EDX + TXT schedules (Table 3). Since the experiments involved pulse drug exposure which includes washing of cells with fresh medium, this manipulation of cells would increase the variability of the data when compared with experiments involving continuous drug exposure without a washing, as has been described previously for EDX and cisplatin combinations [25].

Among the three treatment schedules for EDX + TXL given in Table 2, EDX (0–3 h) + TXL (24–27 h) in both SK-Br-3 and ZR-75-1 cells showed a greater synergism than the (0–3 h) simultaneous exposure schedule or the reverse schedule with EDX (24–27 h). For the EDX (24–27 h) + TXL (0–3 h) schedule, antagonism was observed for both SK-Br-3 and ZR-75-1 cell lines. The EDX (0–3 h) + TXL (0–3 h) simultaneous schedule showed mixed results with SK-Br-3 cells showing antagonism at low effect levels and synergism at high effect levels, and ZR-75-1 cells showing antagonism.

As shown in Table 3, among the three schedules for EDX + TXT, EDX (0–3 h) + TXT (24–27 h) was consistently synergistic in both SK-Br-3 and ZR-75-1 cells, EDX (0–3 h) + TXT (0–3 h) showed mixed results, and EDX (24–27 h) + TXT (0–3 h) showed various degrees of antagonism.

A set of data for an EDX (0–3 h) + TXL (24–27 h) experiment were used to illustrate the actual data analysis using the computerized simulation. For the $f_a$–CI plot (Fig. 1), the open circles are the actual experimental data points and the curve was obtained from computer simulation using Eqs. 2 and 3. In order to condense the *y*-axis for all CI values, Fig. 1 was plotted in $F_a$-log(CI) format. This format made the CI scale symmetrical at log(CI = 1) = 0. The results indicate that synergism (i.e. CI < 1) occured at high $f_a$ values. It should be noted that in actual cancer chemotherapy, $f_a > 0.95$ or $f_a > 0.99$ is considered to be more relevant to actual practice. Therefore, synergism at high effect levels in these experiments is more important than that at low effect levels.

Figure 1 is the effect-oriented plot (i.e. $f_a$-CI plot) for indicating synergism or antagonism. The same set of data can be presented in a dose-oriented format [21, 28] as shown in Fig. 2. Figure 2 is the computer-generated isobologram at $ED_{50}$, $ED_{70}$ and $ED_{90}$ effect levels for both EDX and TXL. Combination data points that fall to the lower left of the diagonal line of each effect level indicate synergism, those that fall on the diagonal line indicate an additive effect, and those that fall to the upper right indicate antagonism [21, 23]. In Fig. 2, the combination effect at $ED_{50}$, $ED_{70}$, and $ED_{90}$ shows an additive effect, synergism

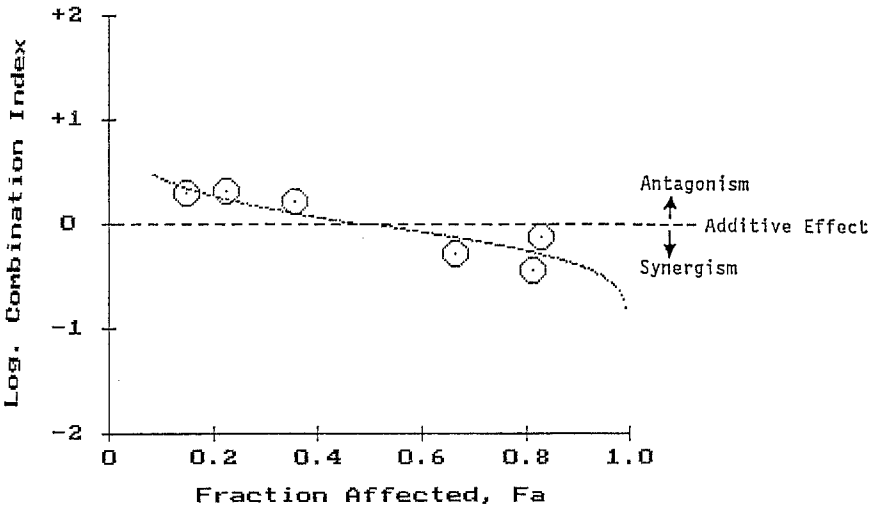


**Fig. 1.** Computer-simulated CI at various effect levels ($f_a$) for the combination of EDX (0–3 h) with TXL (24–27 h) at an equimolar ratio. The experiment was carried out on SK-Br-3 cells. Since CI $< 1$, $= 1$, and $> 1$ indicate synergism, an additive effect, and antagonism, respectively, in the $f_a$–log(CI) plot, log(CI) $< 0$, $= 0$, and $> 0$ indicate synergism, an additive effect, and antagonism, respectively. *Open circles* are the actual combination data points, and the curve was obtained from computer simulation

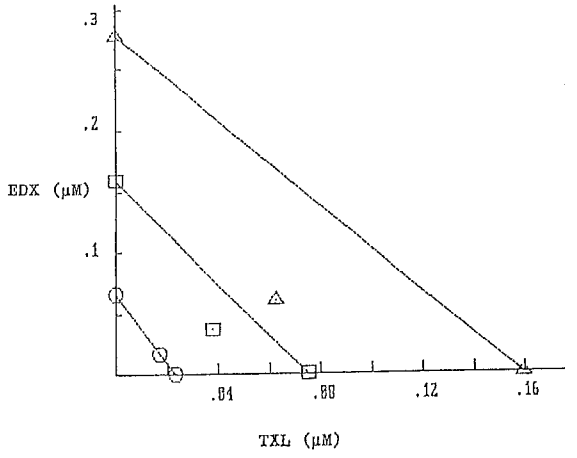


**Fig. 2.** The computer-generated classical isobologram for the combination of EDX (0–3 h) and TXL (24–27 h) at $ED_{50}$ (○), $ED_{70}$ (□) and $ED_{90}$ (△) dose levels. The same data as those in Fig. 1 were used for the analysis. *x*- and *y*-axes show the $ED_{50}$, $ED_{70}$ and $ED_{90}$ values for taxol and EDX, respectively. The combination data points that fall on the diagonal line, to the lower left and to the upper right, represent an additive effect, synergism, and antagonism, respectively. Note that the $ED_{50}$ isobologram shows an additive effect, whereas the $ED_{70}$ and $ED_{90}$ isobolograms show synergism

and synergism, respectively. Isobolograms at any other effect levels for EDX and TXL (e.g. $ED_{95}$ or $ED_{99}$ isobologram) can be readily constructed using computer software [27, 28]. The method for step-by-step calculation with a pocket calculator has been demonstrated previously [23, 24]. Figures 1 and 2 should give numerically identical conclusions since they are based on the same set of data and have used the same CI equation of Chou and Talalay [21–23]. It should be noted that isobolograms in Fig. 2 cannot show too many effect levels since they tend to be congested and become difficult to read whereas $F_a$–CI or $F_a$–log(CI) plots do not have such a restriction (Fig. 1).

## Discussion

The combination of TXL or TXT with EDX would appear to offer considerable potential in the treatment of breast and other human cancers that are responsive to these agents. In view of the non-overlapping toxicities associated with these agents [6, 12], the tolerance of patients to these combinations is a reasonable expectation. Moreover, the present studies showed synergism between both taxanes and EDX in the context of the two breast tumor cell culture systems. The synergism observed appeared to be schedule-dependent. There were mixed results of moderate synergism or antagonism following simultaneous exposure of SK-Br-3 or ZR-57-1 cells to taxane and EDX at 0–3 h, but synergism was consistently observed when EDX was given 24 h before either taxane. Most importantly, the synergism seen with those schedules was greatest at high-effect levels, a situation most relevant to the clinical use of these agents. It is not uncommon that drug combinations tend to be antagonistic at low doses and synergistic at high doses [21–24]. Interesting, as well, were other results showing that sequential administration of these agents in the reverse order was antagonistic. The causes for the schedule dependence of these effects is unknown. We also noted that the synergism attained for the combination of TXL with EDX was greater than for TXT with EDX, while TXT was threefold more cytotoxic than TXL. However, this result may only reflect the cytotoxic properties of these agents against these two particular cell lines and not necessarily others, and no general conclusion is offered in this regard.

These results may have particular relevance to the treatment of metastatic breast cancer. Standard combination therapy for this disorder, although achieving relatively high response rates (reviewed in reference 16), has had little impact on survival of these patients.

Although doxorubicin is the most active single agent in this disorder, its use is limited by acute toxicities and irreversible cardiotoxicity with high cumulative doses. TXL, TXT and EDX are among the new promising antitumor agents that are under active investigation in breast cancer and have shown promise as single agents against metastatic diseases [15, 16, 30]. The administration of recombinant human granulocyte colony stimulating factor reduces the incidence, depth and duration of neutropenia induced by TXL [31, 32] compared with previously reported experience. These findings, and others pertaining to the non-overlapping toxicities of these agents discussed above, prompted our investigation in vitro as to whether these two categories of agents might show a synergistic dose reduction in their effects on human breast cancer cells in the absence of granulocyte colony stimulating factor. These results have prompted the initiation of phase I clinical trials of TXL with EDX and planning for upcoming phase II trials at our institution and elsewhere.

## References


1. Horwitz SB (1992) Mechanism of action of taxol. Trends Pharmacol Sci 13: 134–136
2. Bissery MC, Guenard D, Voegelein-Gueritte F, Lavelle F (1989) Experimental antitumor activity of taxotere (RP 56976, NSC 628503), a taxol analogue. Cancer Res 51: 4845–4852
3. Schiff PB, Horwitz SB (1980) Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci USA 77: 1561–1565
4. Horwitz SB, Lothstein L, Manfredi JJ, et al (1986) Taxol: mechanisms of action and resistance. Ann NY Acad Sci 466: 733–744
5. Kris MG, O'Connell JP, Gralla RJ, et al (1986) Phase I trial of taxol given as a 3-hour infusion every 21 days. Cancer Treat Rep 70: 605 607
6. National Cancer Institute (1990) Clinical brochure (revised): taxol IND 22850 NSC 125973. Division of Cancer Treatment, NCI, Bethesda, 10–17 August
7. Slichenmyer WJ, VonHoff DD (1991) Taxol, a new and effective anticancer drug. Anti-cancer drugs 2: 519–530
8. Sirotnak FM, Schmid FA, Samuels LL, DeGraw JI (1987) 10-Ethyl-10-deaza-aminopterin: structural design and biochemical, pharmacologic, and antitumor properties of folates and folic acid antagonists in cancer chemotherapy. NCI Monogr 5: 127–131
9. Sirotnak FM, DeGraw JI, Schmid FA, Goutas LJ, Moccio DM (1984) New folate analogs of the 10-deaza-aminopterin series. Further evidence for markedly increased antitumor efficacy compared with methotrexate in ascitic and solid murine tumor models. Cancer Chemother Pharmacol 12: 26–30
10. Schmid FA, Sirotnak FM, Otter GM, DeGraw JI (1985) New folate analogs of the 10-deazaaminopterin series: markedly increased antitumor activity of the 10-ethyl analog compared to the parent compound and methotrexate against some human tumor xenografts in nude mice. Cancer Treat Rep 69: 551–553
11. Grant SC, Kris MG, Young CW, Sirotnak FM (1993) Edatrexate, an antifolate with antitumor activity: a review. Cancer Invest 11: 36–45
12. Kris MG, Kinahan JJ, Gralla RJ, Fanucchi MP, Weitheim MS, O'Connell JP, Marks LD, William L, Farag F, Young CW, Sirotnak FM (1988) Phase I trial and clinical pharmacological evaluation of 10-ethyl-10-deazaminopterin in adult patients with advanced cancer. Cancer Res 48: 5573–5579
13. Shum KY, Kris MG, Gralla RJ, Burke MT, Marks LD, Heeland RT (1988) Phase II study of 10-ethyl-10-deaza-aminopterin in patients with stage II and IV non-small cell lung cancer. J Clin Oncol 6: 446–450
14. Lee JS, Libshitz MI, Murphy WK, et al (1990) Phase II study of 10-ethyl-10-deaza-aminopterin (10-EDAM: CGP 30694) for stage II B or IV non-small cell lung cancer. Invest New Drugs 8: 299–304
15. Schornagel JH, Vegt S van der, deGraeff A, Dullemond-Westlan A, vanDeijk WA, TenBokked Huinink WW (1992) Phase II study of edatrexate in chemotherapy-naive patients with metastatic breast cancer. Ann Oncol 3: 549–552
16. Vandenberg TA, Pritchard KI, Eisenhauer EA, Trudeau ME, Norris BD, Lopez P, Verma SS, Burckman RA, Muldal A (1993) Phase II study of weekly edatrexate as first-line chemotherapy for metastatic breast cancer: National Cancer Institute of Canada clinical trials group study. J. Clin Oncol 11: 1241–1244
17. Schmid FA, Sirotnak FM, Otter GM, DeGraw JI (1987) Combination chemotherapy with a new folate analog: activity of 10-ethyl-10-deazaminopterin compared to methotrexate with 5-fluorouracil and alkylating agents against advanced metastatic disease in murine tumor models. Cancer Treat Rep 71: 727–732
18. Sirotnak FM, Schmid FA, Degraw JI (1989) Intracavitary therapy of murine ovarian cancer with Cis-diaminodichloroplatinum (II) and 10-ethyl-10-deaza-aminopterin incorporating systemic leucovorin protection. Cancer Res 49: 2890–2893
19. Otter GM, Sirotnak FM (1994) Effective combination therapy of metastatic murine solid tumors with edatrexate and vinca alkaloids, vinblastine, navelbine and vindesine. Cancer Chemother Pharmacol 33: 286–290
20. Chou T-C, Talalay P (1981) Generalized equations for the analysis of inhibitors of Michaelis-Menten and higher order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. Eur J Biochem 115: 207–216
21. Chou T-C, Talalay P (1987) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors Adv Enzyme Regul 22: 27–55
22. Chou T-C, Talalay P (1987) Applications of the median-effect principle for the assessment of low-dose risk of carcinogens and for the quantitation of synergism and antagonism of chemotherapeutic agents. In: Harrap KR, Connors TA (eds) New avenues in developmental cancer chemotherapy. (Bristol-Myers Symposium series) Academic Press, New York, pp 37–64
23. Chou T -C (1991) The median-effect principle and the combination index for quantitation of synergism and antagonism. In: Chou T-C Rideout D (eds) Synergism and Antagonism in Chemotherapy. Academic Press, New York, pp 61–102
24. Chou T-C, Tan Q-H, Sirotnak FM (1993) Quantitation of the synergistic interaction of edatrexate and cisplatin in vitro. Cancer Chemother. Pharmacol 31: 259–264
25. Perez EZ, Hack FM, Webber LM, Chou T.-C (1993) Schedule dependent synergism of edatrexate and cisplatin combination in the A549 lung cancer cell line as assessed by median effect analysis. Cancer Chemother Pharmacol 33: 245–250
26. Lee JS, Libshity HL, Fassella FV, Murphy WK, Pang AC, Lippman SM, Dong MS, Dimery IW, Glisson BS, Hong KH (1992) Improved therapeutic index by leucovorin of edatrexate, cyclophosphamide and cisplatinum regimen for nonsmall cell lung cancer. J Natl Cancer Inst 84: 1039–1040
27. Chou J, Chou T-C (1987) Dose-effect analysis with microcomputers: quantitation of $ED_{50}$, $LD_{50}$, synergism, antagonism, low-dose risk, receptor-ligand binding and enzyme kinetics. Manual and software, Biosoft, Cambridge, UK
28. Chou J (1991) Quantitation of synergism and antagonism of two or more drugs by computerized analysis. In: Chou T-C, Rideout

DC (eds) Synergism and antagonism in chemotherapy. Academic Press, New York, pp 223–244
29. Skehan P, Storeny R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenny S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107–1112
30. Seidman AD (1994) Single-agent use of taxol (paclitaxel) in breast cancer. Ann Oncol 5: S17–S22
31. Reichman B S, Seidman A D, Crown J P A, Heelan R, et al (1993) Paclitaxel and recombinant human granulocyte colony-stimulating factor as initial chemotherapy for metastatic breast cancer. J Clin Oncol 11: 1943–1951
32. Seidman AD, Norton L, Reichman BS, Crown JPA, Yao TJ, et al (1994) Taxol (paclitaxel) plus recombinant human granulocyte colony-stimulating factor in the treatment of metastatic breast cancer. Oncology 51: S33–S39