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Chemosensitivities of Human Clonogenic Breast Tumor Cells

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Abstract—*We developed an* in vitro *system for the testing of the inherent chemosensitivity of clonogenic tumor cells, and we applied the system to the evaluation of* 104 *human breast tumors. We observed the following: clonogenic breast tumor cells were more sensitive to* 4-*hydroperoxycyclophosphamide (a metabolite of cyclophosphamide with* in vitro *activity) than to* 5-*flourouracil and to doxorubicin (the other two agents used in the frontline treatment of breast carcinoma). The sensitivity of these clonogenic breast tumor cells for mitoxantrone, bisantrene,* 4′-*epi-doxorubicin, and VP-*16 *was similar to that for doxorubicin and* 5-*fluorouracil, but it was less for* cis-*platinum.* In vivo *exposure to a combination of* 5-*fluorouracil, doxorubicin, and cyclophosphamide (FAC) did not change the sensitivity of cells towards* 5-*fluorouracil and doxorubicin, but lessened the sensitivity of some cells towards* 4-*hydroperoxycyclophosphamide. Furthermore,* in vivo *exposure to doxorubicin did not influence the sensitivity of cells towards the anthraquinone derivatives,* 4′-*epi-doxorubicin, mitoxantrone, and bisantrene. A comparison of the* in vitro *and* in vivo *chemosensitivity revealed that the assayed cell populations were biologically relevant: the concordance of sensitivity on* 41 *tumors was* 68%, *or* 95%, *if the* in vitro *sensitivity score was adjusted to the tumor bulk. We conclude that our system provides a valid tool to determine the inherent chemosensitivity pattern of the individual tumor types, and to compare the tumor cytotoxic potential of drugs.*

INTRODUCTION

INHERENT drug sensitivity of tumor stem cells is a prerequisite for treatment success and a major determinant of clinical response. Unfortunately, most antitumor agents have a limited spectrum of activity, and assays designed to distinguish sensitive from resistant tumors have not often been successful.

Technical difficulties, selection of irrelevant endpoints, and inappropriate use of these assays have caused many *in vitro* predictive assays of tumor response to fail. Although inhibition of self-renewal in the murine system correlates with the *in vivo* antitumor effect of chemotherapeutic agents [1], the success rate of clonogenic assays is low for most types of human tumors [2,3]. Furthermore, their application to the prediction of response in patients with far-advanced disease is limited, since, in this group of patients, other factors that also determine the disease course (such as tumor load and performance status) can obscure the treatment effect [4,5,6].

To eliminate some of these confounding variables, we devised a system of *in vitro* drug evaluation that measures the cytotoxic effects of drugs on tumor progenitor cells by using the chemosensitivity of myeloid progenitor cells of normal donors as the reference standard [7]. Myeloid progenitors are the dose-limiting host tissue cells for many anticancer drugs and hence, drugs are being evaluated for their tissue selective property, much in the sense of an *in vitro* therapeutic index. This system of *in vitro* drug evaluation permits, furthermore, the comparison of the antitumor potential of different agents. In this paper we report our observations from the application of this test system to the evaluation of the chemosensitivity of clonogenic breast tumor cells for agents used in the primary and secondary treatment of this tumor type. We further investigated the effects of *in vivo* exposure to chemotherapy on the chemosensitivity pattern of clonogenic breast tumors. To validate our *in vitro* system, we obtained 41 *in vitro/in vivo* correlations of tumor chemosensitivities.

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MATERIALS AND METHODS

Drugs

Anticancer agents were obtained from the following sources: doxorubicin, 4′-epi-doxorubicin and 5-fluorouracil from Farmitalia, Carlo Erba, SA, Italy; mitoxantrone, bisantrene from American Cyanamid, Pearl River, NY; *cis*-platinum, VP-16 from Bristol Laboratories, Syracuse, NY; and 4-hydroperoxycyclophosphamide from Dr. Michael Colvin, Johns Hopkins University School of Medicine, Baltimore, MD. (4-Hydroyperoxycyclophosphamide is a metabolite of cyclophosphamide active *in vitro*.) Drugs were reconstituted from the marketed form to the 100 X stock solution with calcium–magnesium-free Hank's balanced salt solution (CMF-HBSS). Subsequent dilutions were made in 2 X alpha-MEM, and 2 ml aliquots of the 2 X final concentrations were stored at −70°C until the time of experimentation.

Bone marrows

The initial 10 bone marrows were obtained from patients with breast carcinoma with no tumor involvement of that organ site. Their tumors were assayed simultaneously to compare the chemosensitivity of host and tumor target tissue cells. All subsequent bone marrows were obtained from normal donors, and were used to generate a reference standard for each of the eight drugs. An informed consent was obtained in each instance. Two to 3 ml bone marrow were aspirated from the posterior iliac crest and collected into 2 ml CMF-HBSS containing 100 units of preservative-free heparin (Fisher Scientific, Houston, Texas).

Tumors

Tumor samples from 158 patients with breast carcinoma were obtained in the Departments of Surgery and of Medical Oncology of the U.T. M.D. Anderson Hospital, after an informed consent had been given. Tumors were collected into 10 ml Ham's nutrient F12 (F12) (Gibco, Grand Island, NY), to which 15% fetal bovine serum (FBS)(KC Biological, Lenexa, KS) and 100 units of preservative-free heparin had been added. Ten units of preservative-free heparin were also added to each milliliter of aspirated fluid.

Cultures

Bone marrow cultures (GM-CFU). Mononuclear bone marrow cells were separated by Ficoll–Hypaque density centrifugation (density 1.077) to obtain light-density cells. The cell fraction was further depleted of cells adherent to plastic and of cells that formed rosettes with sheep red blood cells [8]. Cells were set in semisolid double-layer agar cultures as described by Bradley and Pike [9,10]. In brief, underlayers consisted of a mixture of α-Minimal Essential Medium (α-MEM) and 15% FBS in 0.5% agar. Placental conditioned medium was added as the source of colony-stimulating factor. The upper-layers consisted of a mixture of α-MEM and 15% FBS in 0.3% agar, in which 10^5 mononuclear cells were seeded. Drugs at graded concentrations were added to the upperlayer medium. Triplicate cultures were obtained for each drug concentration, and cultures were incubated in a fully humidified atmosphere of 5% CO_2, 12% O_2 in nitrogen at 37°C for 8 days. Aggregates of 40 or more cells were considered to represent the progenies of granulocyte-macrophage colony forming units and were scored for colonies using a dissecting microscope.

Breast tumor cell cultures (BT-CFU)

Solid tumor tissue samples were debrided and diced into 1 mm cubes with scalpels. Single cells were teased into suspension with 25-gauge needles. Cells were then suspended in an enzyme mixture of type III collagenase, elastase (Worthington Biochemical Corporation, Freehold, NJ), and deoxyribonuclease (Sigma Chemical Corporation, St. Louis, MO) at the final strengths of 0.7, 0.2, and 0.01%, respectively, for 16 hr at 37°C, under continuous agitation. No elastase was used for the treatment of cells obtained from malignant effusions. After completion of the enzymatic dissociation procedure, cells were washed in CMF-HBSS and set into semisolid cultures. The culture system described by Hamburger and Salmon [11,12] was used, but the conditions were modified for the growth of breast tumors as described previously [13]. Briefly, underlayers were prepared with 70% F12, 10% horse serum (KC Biological, Lenexa, KS), 10 μg/ml bovine crystalline insulin, 2.5 μg/ml hydrocortisone, 5×10^{-7}M 17-β-estradiol, and 50 ng/ml epidermal growth factor in 0.5% agar. Twenty per cent conditioned medium was also added. Conditioned medium was derived from supernatants of three established human breast tumor cell lines (MDA-468, MDA-435 and MDA-231) [13]. The upperlayers contained 5×10^5 cells in a mixture of 85% alpha-MEM and 15% FBS in 0.3% agar. Drugs, at graded concentrations, were contained in the medium of the upperlayers. One to three replicate cultures were obtained for each drug concentration and incubated in a fully humidified atmosphere of 5% CO_2, 12% O_2 in nitrogen at 37°C for 14 days. Since it is difficult to obtain single cell suspensions, one control plate was fixed with 0.5 ml 3% glutaraldehyde and stored at 4°C for a reference to measure culture contamination by tumor cell clumps. Aggregates of ≥ 40 cells with uniform morphology and a smallest diameter of ≥ 75 μm, were counted as colonies using a tissue culture microscope. The same criteria were used to score colonies of fixed plates, and the numbers of clumps were subtracted from the scores of culture plates.

RESULTS

The *in vitro* growth characteristics of tumor and bone marrow progenitors are summarized in Table 1. Only the 104 tumors that formed more than 50 colonies per 5×10^5 cells plated were included in the study (e.g., 66% of all tumors tested during this time period). The clonogenicity of tumors was lower, by a factor of 10, than that of bone marrow. Tumor growth was also more variable. Previously treated tumors formed over twice as many colonies as did untreated tumors (174 vs. 60, median number of colonies).

Table 1. *Comparison of bone marrow and breast tumor cultures*

Characteristics	GM-CFU	BT-CFU
	(%)	(%)
Plating efficiency,		
Median	0.30	0.03
Range	0.04–0.49	0.01–0.32
Coefficient of variation in colony formation of replicate cultures,		
Mean	8	23
Clumps		
Colonies		
Mean	0	7

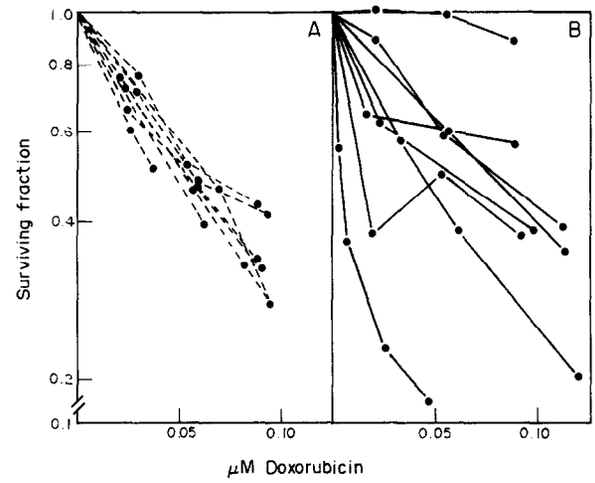

Fig. 1. *Cytotoxicity of doxorubicin for the clonogenic cells of the bone marrows* (A) *and tumors* (B) *from* 10 *patients with breast carcinoma. Points indicate the mean values of triplicate determinations for GM-CFU, and of one to three replicate determinations for BT-CFU, depending on sample size. The chemosensitivity of tumor cells is more heterogeneous than that of bone marrow cells.*

We first determined the dose-responses to doxorubicin of the progenitors of bone marrows and

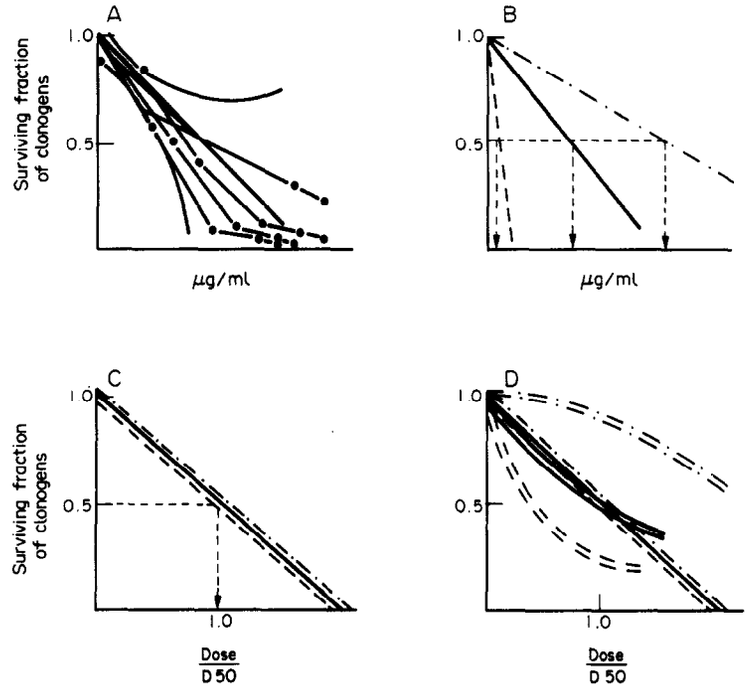

Fig. 2. *Normalization of the* in vitro *activity of drugs by their effects on bone marrow progenitor cells. Up to a cell-kill of approx.* 60–70%, *similar proportions of GM-CFU were killed by a given drug* (A). *Therefore, within the cell-kill range of* 0–60%, *the observed effects of a given drug on GM-CFU could be described by a log-linear regression line* (A,B). *We scaled the abscissa to doses in units of the D*50 *for drugs by calculating the ratios of exposure doses to the D*50 *of each drug. Thereby, the regression lines that described the sensitivities of the bone marrow to all drugs came to overlap* (C), *and a comparison of the inherent chemosensitivity of host and tumor cells became possible* (D). *In this graph, bone marrow responses are indicated by single lines, and tumor responses by double lines.*

tumors from 10 patients with breast carcinoma. As illustrated in Fig. 1, the bone marrows of these patients responded to doxorubicin in a more uniform manner than did their tumors. Similar cell-type specific responses were also observed for the three other drugs (4'-epi-doxorubicin, bisantrene, and mitoxantrone) that were tested on the bone marrows and tumors of these patients [14].

This relative uniformity in chemosensitivity of GM-CFU from different donors permitted us to standardize the *in vitro* activity of cytotoxic drugs by their effects on these cells, that is, on the progenitors of the dose-limiting host tissue for many antineoplastic drugs. We have described this *in vitro* system of evaluating drugs for their tissue-selective toxicity (in the sense of an *in vitro* therapeutic index) previously [7,15]. Figure 2 summarizes this test model: Graph A of Fig.2 illustrates the dose-responses of different bone marrows to a single drug. Up to a cell kill of approx. 60–70%, the effect of the drug was similar on all four bone marrows, and the sensitivity of the bone marrow progenitor cells towards this drug could therefore be expressed by a log-linear regression line. Responses of bone marrow progenitors beyond that dose range were unpredictable, as indicated by the width of the confidence band in the high exposure dose range. Hypothetical regression lines for the sensitivities of bone marrow progenitors towards three drugs are illustrated in graph B. In graph C, the abscissa was scaled to units of the D50s by dividing the exposure doses into the D50 of each drug. This normalization of the abscissa brought the regression lines that represent the bone marrow sensitivities of the different drugs to overlap. In graph D, tumor responses to these drugs were then superimposed on the bone marrow responses in order to display the differences in chemosensitivity of the two cell types.

We established the standard reference curve for each drug from the survival curves of six to ten donor bone marrows. The slopes of the standard reference curves for the eight drugs included in this study are listed in Table 2. The D50s for these bone marrow standards are also listed, and comparison is made with the pharmacologically-derived drug doses used for *in vitro* drug testing. A detailed report on the sensitivities of bone marrow cells to these and other drugs is in preparation.

In this study we confined the chemosensitivity evaluation of tumors to the linear part of the GM-CFU survival curves (e.g., to the 0–70%

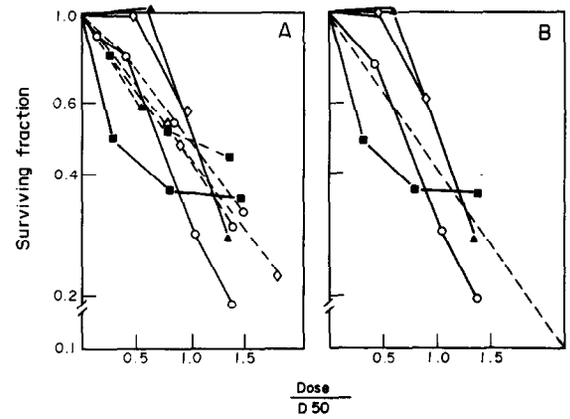

Fig. 3. Effects of four anthraquinone derivatives on tumor and bone marrow progenitors of a patient with breast carcinoma. The drug-sensitivities of tumors were scored against the patient's own bone marrow (A) *and against the reference standards obtained from bone marrows of normal donors* (B). *The bone marrow responses are indicated by broken lines, and the tumor responses are indicated by solid lines. Drugs are indicated by the following symbols:* ■, *doxorubicin;* ◊, *4'-epidoxorubicin;* ○, *mitoxantrone; and* ▲, *bisantrene. Symbols represent the means of triplicate determinations. The sensitivities of this tumor for all four drugs scored equal, whether evaluated against the patient's own bone marrow or against the reference standard.*

Table 2. Characteristics of bone marrow reference standards

Drug	D50* (μg/ml)	Survival slope† (μg/ml)	10% Peak plasma concentrations‡ (μg/ml)
Adriamycin	0.02450	−12.280	0.06[16]
HOOC	7.72000	− 0.039	3.50[16]
5-Fluorouracil	1.55000	− 0.194	6.00[16]
4'-Epi-doxorubicin	0.01960	−15.350	0.04[17]
Mitoxantrone	0.00019	−1551.230	0.01[18]
Bisantrene	0.00490	−61.250	0.30[19]
Cis-platinum	2.73000	− 0.170	0.25[16]
VP-16	0.04290	− 7.010	3.40[16]

$$*\text{Dose} = \frac{\log_{10}\text{ surviving fraction} - 2}{\text{Slope}}.$$

†The following form was used for the calculation of the survival slope:
$\log_{10}$ surviving fraction = 2 + slope × dose
‡Data obtained from the literature

range of cell kill). We tested two to three drug concentrations that corresponded roughly to the D30, D50, and D70 for GM-CFU (the exact doses are indexed by the points of determination for the individual survival curves). Only if the size of the tumor sample was large, did we obtain an additional determination at a higher exposure dose.

First, we compared the chemosensitivity scores of a patient's tumor for four different drugs in the testing (a) against her own bone marrow and (b) against the reference-standard, generated from normal donor bone marrows (Fig. 3). As can be seen, the sensitivities of all four drugs scored similarly in the two test systems. The testing of individual bone marrows could therefore be replaced by standard references.

We then applied this test system to define the sensitivity of clonogenic breast tumor cells; first, for the standard agents used in the treatment of this tumor; second, for alternative agents of less well established clinical efficacy. Third, we looked for the changes of chemosensitivity that may have occurred following *in vivo* exposure to chemotherapy. We used primarily log-linear survival curves as the measure of sensitivity. However, since fractional cell-kill may not occur throughout the entire exposure dose-range, we also used the weight areas under the survival curves up to the unit dose 1 for GM-CFU as measure for comparison.

We determined the effects of three drugs used for the primary treatment of breast carcinoma, [5-fluorouracil, doxorubicin, and cyclophosphamide (substituted by the *in vitro* active metabolite 4-hydroperoxycyclophosphamide)], at equitoxic bone marrow doses, on the tumors of 18 patients with breast carcinoma. Judged by the shapes of survival curves (Fig. 4) and the weights of areas under the survival curves (Table 3), 4-hydroperoxycyclophosphamide was the component of the FAC treatment regimen with the most consistent antitumor activity. The tumor tissue-selective cytotoxicity of 4-hydroperoxycyclophosphamide was of higher degree and was observed more consistently than for either of the other drugs. While the cytotoxicity of 5-fluorouracil was generally more for tumor cells than for bone marrow cells, the antitumor effect of adriamycin was variable: eight tumors were less, six more and four equally sensitive to adriamycin as were the bone marrow cells.

We then compared the antitumor effects of secondary treatment agents to that of doxorubicin. While the range of antitumor activity was wide, as expected, the sensitivities of tumors for VP-16 was similar to that for doxorubicin (Fig. 5, Table 3). Conversely, in each case, tumors were less sensitive to *cis*-platinum than to doxorubicin. Furthermore, all tumor progenitors were resistant to *cis*-

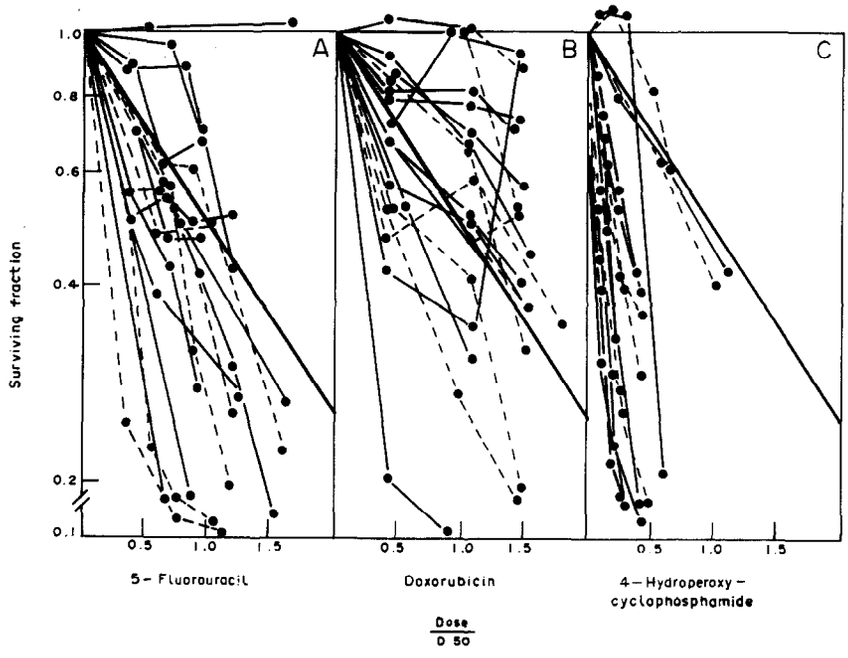

Fig. 4. Dose-responses to 5-fluorouracil (A), *doxorubicin* (B), *and 4'-hydroperoxycyclophosphamide* (C) *of tumors from* 18 *patients with breast carcinoma. Twelve tumors were obtained from patients who were not previously treated with chemotherapy, and six tumors were obtained from patients whose tumor recurred after exposure to adjuvant chemotherapy. The tumor responses from untreated patients are indicated by solid lines, and the tumor responses from patients previously treated with chemotherapy are indicated by broken lines. The bold line indicates the bone marrow reference standard, e.g. the sensitivity of progenitors from bone marrow towards these drugs. The effects of 5-flourouracil and doxorubicin on tumor cells were variable, while 4'-hydroperoxycyclophosphamide almost always exerted a selective cytotoxic effect on tumor cells.*

*Table 3. Comparative cytotoxicity on breast tumor cells of eight anticancer drugs**

Groups of drug comparisons	Area under curve (ng ± S.D.) All tumors	Untreated tumors	Treated tumors
2-*Drug comparisons*:			
	(6)†	(1)	(5)
Doxorubicin	120 ± 0.11	54	134 ± 0.11
VP-16	86 ± 0.10	12	100 ± 0.11
	(6)	(2)	(4)
Doxorubicin	159 ± 0.04	179 ± 0.06	149 ± 0.04
Cis-platinum	215 ± 0.04	202 ± 0.04	222 ± 0.05
3-*Drug comparisons*:			
	(12)	(7)	(5)
5-Fluorouracil	153 ± 0.08	174 ± 0.10	123 ± 0.02
Doxorubicin	168 ± 0.90	179 ± 0.08	155 ± 0.11
4'-Hydroperoxy-cyclophosphamide	105 ± 0.09	50 ± 0.05	132 ± 0.07
4-*Drug comparisons*:	(30)	(10)	(20)
Doxorubicin	143 ± 0.08	139 ± 0.08	145 ± 0.09
4'-Epi-doxorubicin	141 ± 0.09	156 ± 0.11	133 ± 0.07
Mitoxantrone	183 ± 0.08	180 ± 0.07	185 ± 0.08
Bisantrene	170 ± 0.07	178 ± 0.09	166 ± 0.07

*Weights of the areas under the curves (AUC) of surviving tumor progenitors within the unit dose-range of 0–1.0 for GM-CFU. The respective AUC for bone marrow progenitors is 160 ng.

†The number of observations is indicated in brackets.

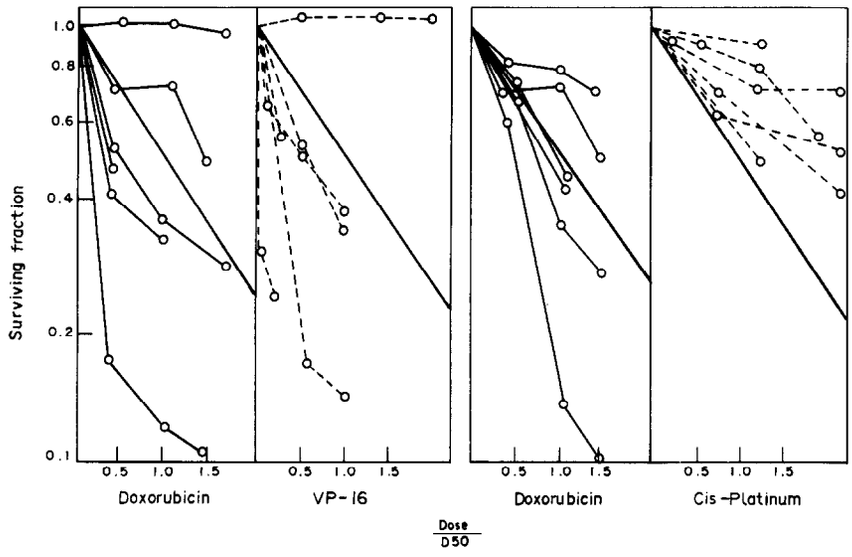

Fig. 5. Comparison of the cytotoxic effects of doxorubicin with those of VP-16 and cis-platinum (two agents used in the secondary treatment of patients with breast carcinoma). Tumor responses are in fine print, and the bone marrow responses in bold print. Points represent the mean values of up to three replicate determinations. The sensitivity of tumors for VP-16 was similar to that for doxorubicin. In contrast, the sensitivity for cis-platinum was less than that for doxorubicin for each of the six tumors tested. Furthermore, all tumor progenitors were more resistant to cis-platinum than bone marrow progenitors. Since myelotoxicity is not dose-limiting in vivo, our in vitro test dose was high compared to those of other drugs. Cis-platinum may therefore not exert meaningful cytotoxicity on clonogenic breast tumor cells.

platinum if comparison was made to bone marrow progenitors (Fig. 5, Table 3). Because myelotoxicity is not dose-limiting for *cis*-platinum, our test dose for this drug was high (10-fold higher than the pharmacologically-derived dose used in the conventional testing) (Table 2).

We next examined the effect of treatment on the chemosensitivity of clonogenic breast tumor cells

for (a) the drug of exposure, and (b) for analogues of the drug of exposure. There was no change in sensitivity for 5-fluorouracil and doxorubicin following *in vivo* exposure to FAC, but resistance had developed for 4′-hydroperoxycyclophosphamide in a proportion of tumors (Fig. 6). Conversely, the *in vivo* exposure to a doxorubicin-containing regimen did not affect the cellular sensitivity towards 3 anthraquinone derivatives (Fig. 7). However, doxorubicin and 4′-epi-doxorubicin, the two anthraquinones with a glycosamine moiety, more often exerted selective antitumor activity, both for untreated as well as for treated tumors (Fig. 7, Table 3).

The *in vitro* chemosensitivity of clonogenic tumor cells was compared retrospectively with the clinical response to one of the assayed drugs in 41 patients.

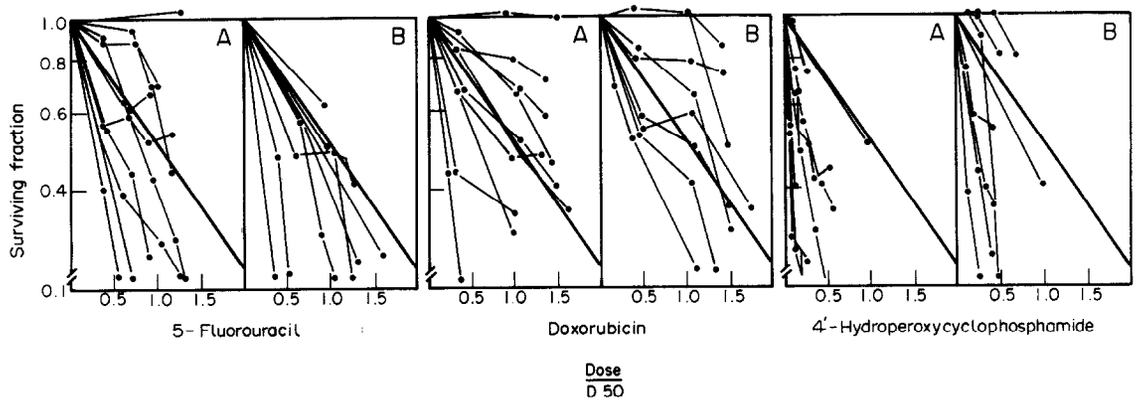

Fig. 6. Effect of in vivo *exposure to FAC on the chemosensitivity pattern of clonogenic breast tumor cells. The sensitivities to 5-fluorouracil, doxorubicin, and 4-hydroperoxycyclophosphamide of untreated tumors* (A), *and of tumors that have been exposed to FAC* (B) *were compared. Tumor responses are in fine print, and the reference standards of the bone marrow are in bold print. Points represent the mean values of one to three replicate determinations.* In vivo *drug exposure did not change the chemosensitivity of tumors toward 5-fluorouracil, but an increase in resistance toward 4-hydroperoxycyclophosphamide appeared to occur.*

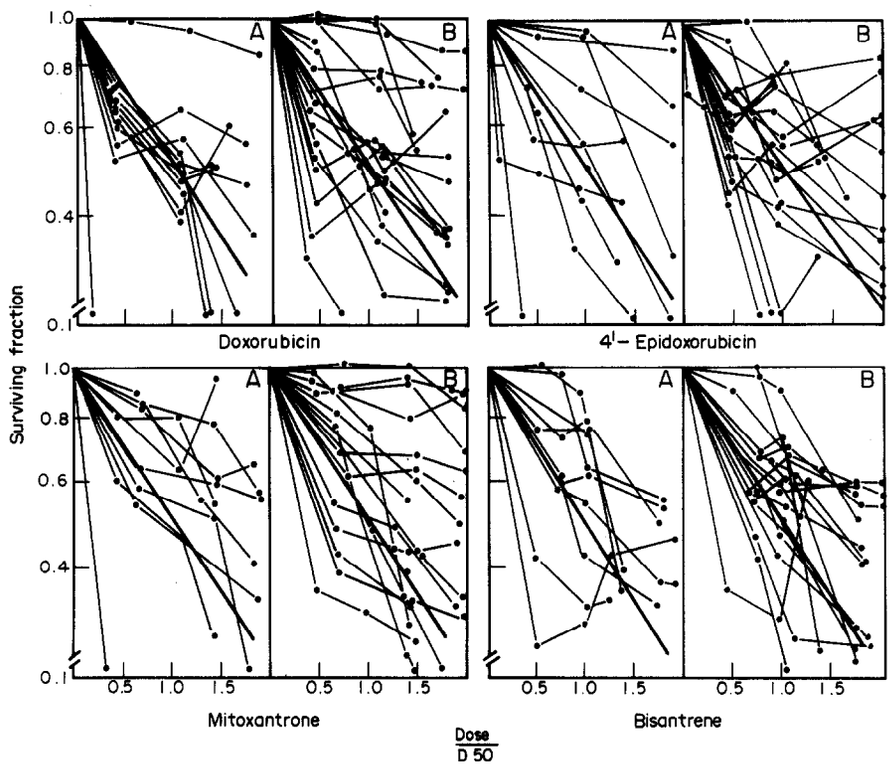

Fig. 7. Effect of in vivo *doxorubicin exposure on the sensitivity of clonogenic cells to doxorubicin analogues. The chemosensitivities of clonogenic breast tumor cells towards four anthraquinone derivatives from* 10 *untreated tumors* (A) *were compared to that from* 20 *tumors previously exposed to a doxorubicin-containing chemotherapy combination* (B). *Points represent the mean values of up to three replicate determinations. The tumor responses are in fine print, and the bone marrow responses in bold print.* In vivo *exposure to doxorubicin did not visibly modify the cellular sensitivity to its derivatives. However, of the four anthraquinone derivatives the two anthracyclines more often exerted selective tumor cytotoxicity (relative to bone marrow) than the two anthraquinones.*

The measures of sensitivity used for the comparisons are indicated in Fig. 8. The clinical criteria correspond to the UICC criteria of 'complete' or 'partial remission' (S), 'no change' (E), and 'progressive disease' (R) (20). The concordance of *in vitro* and *in vivo* findings was 68%. This concordance could be further increased to 95% after adjusting the *in vitro* sensitivity score to the tumor bulk, as described previously (Table 4), [7]. In no instance had an *in vitro* inactive drug clinical activity. The value of the test to predict for drug-resistance was 89% (expected 70%). The true positive rate of the test was 70%.

DISCUSSION

The use of equitoxic concentrations to determine drug sensitivities of tumor cells permits the comparison of the cytotoxic potentials of anticancer agents. By standardizing the *in vitro* drug activities by their effects on bone marrow cells, differences of inherent drug sensitivities of tumor and host progenitor cells can be determined, and an *in vitro* therapeutic index can be generated for each agent. Drug evaluation within such a defined *in vitro* system has the following advantages over the clinical evaluation: (1) the variables of drug delivery and elimination need not be considered, and (2) the treatment effect is not confounded by host factors. A test-system that can eliminate these variables may therefore provide a measure of the inherent chemosensitivity of tumor stem cells and may complement the clinical drug evaluation. Systems that evaluate drugs by comparing their tissue-selective toxicity for host and tumor targets have previously been described. Bruce *et al.* developed an *in vivo* murine system to define the modes of drug-action for leukemia cells [21], and Park *et al.* developed an *in vitro* human system to predict the clinical response of leukemia patients [22]. The role of such a model for the treatment of solid tumors may even be more important.

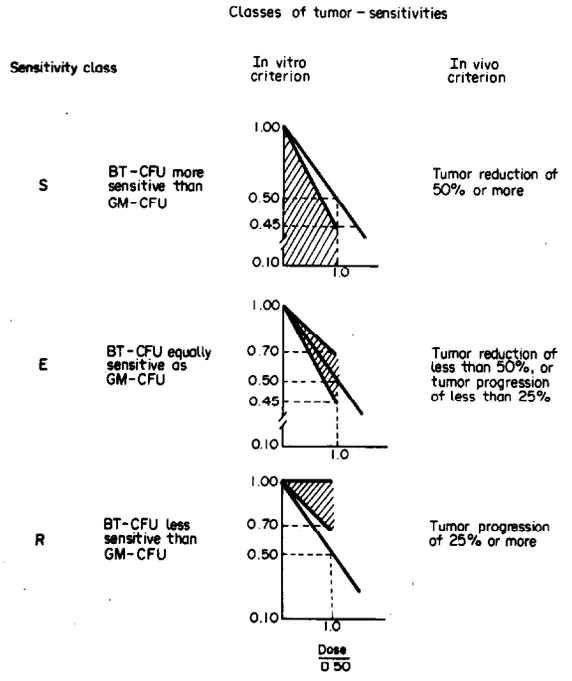

Fig. 8. *Criteria used for the classifications of* in vitro *and* in vivo *tumor chemosensitivities. The* in vitro *responses were evaluated over the* 0–1.0 *unit dose range. The unit dose* 1 (*D*50) *of each drug is listed in Table* 2. *Three classes of sensitivity were used* (*S, E, R*). *The criteria of the* in vitro *response classification are listed in Column* 1 *and illustrated in Column* 2. *The criteria of* in vivo *response classification correspond to the UICC categories of response, and are listed in Column* 3. *Class E,* in vitro, *was defined as response within the area encompassing the cell-kill range between* 40 and 55% *at the unit dose* 1, *and was equated with the clinical 'no change' category of response by the UICC criteria.*

We applied our *in vitro* model to test the inherent sensitivity of clonogenic breast tumor cells to agents used in the primary and secondary treatment of this disease, and to determine what effect the *in vivo* exposure to chemotherapy had on the chemosensitivity pattern of these cells. We made the following observations: (1) among the agents used for the frontline treatment, 4-hydroperoxycyclophosphamide most consistently had antitumor activity, (2) *cis*-platinum had no meaningful cytotoxic effects for clonogenic breast tumor cells, (3) anthracyclines were somewhat more often effective than anthraquinones, and (4) *in vivo* exposure to chemotherapy changed the sensitivity of the clonogenic cells of some tumors towards 4-hydroperoxycyclophosphamide, but not towards 5-fluorouracil or doxorubicin.

The combination of 5-fluorouracil, doxorubicin, and cyclophosphamide is widely used in the primary treatment of breast tumors. The antitumor activity of the three components, as evaluated in our system, was comparable to the reported clinical response rates of 20–30% for treatment with any of these agents [23–26]. However, tested at equitoxic doses on the same tumors, 4-hydroperoxycyclophosphamide most consistently exerted selective antitumor cytotoxicity. Since the equitoxic dose of 4-hydroperoxycyclophosphamide was high compared to plasma concentrations, it is possible that we were testing a metabolite with weak cytotoxicity and high tissue selectivity.

The *in vitro* antitumor effect of VP-16 was similar to that of doxorubicin, that is, better than its reported clinical efficacy [27]. However, as the effectiveness of front-line treatments improved, the clinical testing of second-line agents (comparing new to old drugs) has become more difficult, and underrating of their true efficacy can, in theory, be expected. Conversely, in no instance did *cis*-platinum selectively kill tumor progenitors, e.g. show *in vitro* antitumor activity. This in spite of the fact that our test dose was high in comparison to plasma levels, because myelotoxicity is not dose-limiting for this agent. Therefore, our observation

Table 4. Correlations of in vitro *and* in vivo *chemosensitivities of breast tumors*

A. Breakdown

Drug	Number of observations	% Response rate* *Expected* From literature†	*Predicted* By Assay‡	*Observed*	Concordance Predicted (before adjustment)§/observed	Predicted (after adjustment)§/observed
Adriamycin	7	40	29	43	2/3	2/3
4'-Epidoxorubicin	8	30	25	50	2/4	2/4
Bisantrene	9	20	11	0	1/0	0/0
5-Flourouracil	3	30	67	33	2/1	1/1
Vinblastine	14	30	71	43	10/6	7/6

B. Summary

Before adjustment:

in vivo \ *in vitro*	S	E	R
S	11	3	0
E	3	9	1
R	3	3	8

After adjustment:

in vivo \ *in vitro*	S	E	R
S	11	3	0
E	1	11	1
R	0	5	9

*S, for assay, Complete Remission or Partial Remission for clinical response.

†References [16–20].

‡Not adjusted for size of tumor load.

§Adjustment of the chemosensitivity score for the size of tumor: *in vitro* 'S' can be down-graded to 'E', and *in vitro* 'E' to 'R' for tumors that have metastasized to three or more sites (reference [7]).

may indicate true resistance of clonogenic breast tumor cells to this drug.

Doxorubicin is often considered a principle agent for the treatment of breast tumors, and analogues with an improved therapeutic index are searched. However, no test system is available for the comparison of the cytotoxic potentials of analogues. We evaluated the cytotoxic effects of four anthraquinone derivatives in our system, and observed, that anthracyclines tended to have, more often, a greater degree of antitumor activity than anthraquinones. It is possible that the sugar moiety mediates part of the cytotoxicities of the anthracyclines for breast tumor progenitor cells.

Adjuvant chemotherapy with FAC is added with increased frequency to the primary treatments of stages II and III breast tumors. In spite of this, a high rate of treatment failure is observed. Concern for harmful effects of adjuvant chemotherapy, to induce formation of, or to select for growth of resistant clones, therefore arises. We found that of the three drugs tested, only cyclophosphamide appeared to adversely effect the chemosensitivity of clonogenic cells that survived the *in vivo* treatment with these agents, while 5-fluorouracil did not change the cellular chemosensitivity of surviving cells to any degree. Our observation may indicate that either quiescent cells survive the exposure to antimetabolites unhurt, or that 5-fluorouracil is given ineffectively, while cells may repair the damage inflicted by alkylators by resistance development. Exposure to doxorubicin did not confer resistance development (nor the development of collateral sensitivity) to three anthraquinone derivatives. The clinical evaluation should therefore provide an accurate assessment of the efficacy of the analogues.

Our results indicate that while large differences in drug-sensitivities among progenitor cells of individual breast tumors occur, a sensitivity pattern distinctive of the tumor type as a whole may also exist. In the case of the breast tumor type, this pattern is characterized by a high sensitivity to the cyclophosphamide metabolite, 4-hydroperoxycyclophosphamide, and by a lack of sensitivity to *cis*-platinum.

Although we do not believe that the chemosensitivity of tumor clonogens as determined in our system has the capacity to predict for treatment response of advanced disease, the correlation of *in vivo* and *in vitro* of tumor *resistance* provides evidence that we are assaying a biologically relevant cell population. These correlations of *in vitro* sensitivities were not the primary objective of the study. We obtained these data merely to define the validity of our *in vitro* test system, and we collected them therefore retrospectively. Since size of tumor bulk interferes with the clinical measure of chemosensi-

tivity, we adjusted the *in vitro* score for this confounding variable. The enumeration of tumor sites may not be the best measure for the tumor load, but it is the most conventional, and we have selected it therefore for our preliminary analysis.

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