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Pharmacologic Studies of Anticancer Drugs with the Human Tumor Stem Cell Assay

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Summary. *To optimize the human tumor stem cell assay (HTSCA) for clinical and research purposes we have carried out in vitro pharmacology studies. Useful observations were made in four areas. (1) Drug assay design: The predictive accuracy of the HTSCA depends on the in vitro testing of drug concentrations of less than 10% of those which are pharmacologically achievable with standard in vivo drug doses. The use of unrealistically high in vitro concentrations can accurately predict clinical drug resistance, but is likely to yield high false-positive rates of clinical response prediction. (2) Drug scheduling: For certain schedule-dependent drugs, as well as those with a prolonged plasma half-life and those used according to a repeated daily schedule, prolonged in vitro exposure (rather than 1 h) may be needed to provide an adequate in vitro design. For an accurate prediction of sensitivity of tumor colony-forming units (TCFUs) to continuous drug contact in the agar, concentrations should be in the range of 1/300 that used for the standard 1-h exposure prior to plating. (3) Drug combinations: In preliminary studies of combination chemotherapy in vitro we commonly observed at least additive effects with low doses of cis-platinum plus either vinblastine or adriamycin. (4) Drug bioactivation: Rat liver microsomes or S-9 fraction were used to activate cyclophosphamide for in vitro effect, and satisfactory dose-response curves were observed for the inhibition of TCFUs. Such pharmacologic studies will be required for a wide variety of standard and new agents and will probably become a regular aspect of investigation of new anticancer drugs.*

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Introduction

The in vitro human tumor stem cell assay (HTSCA) developed by Hamburger and Salmon [22–24, 43] has proven useful for testing new anticancer drugs for phase II activity [44, 45, 47] and for predicting clinical response to anticancer drug therapy in patients with multiple myeloma, melanoma, ovarian cancer, and other neoplasms [4, 43, 46, 48]. When applied in a research center, use of the HTSCA for the prediction of response to anticancer agents appears to have an accuracy rate at least comparable to that of the estrogen receptor assay for the prediction of response to hormonal manipulation in breast cancer [31]. However, additional pharmacologic studies are needed before the assay can be considered to be optimized for routine clinical and research applications. Some of these study areas include the following: (1) The use of pharmacokinetic principles to design clinically relevant drug dosing for the in vitro assay system [5, 34, 43]; (2) the evaluation of individual agents for possible schedule-dependent antitumor activity [5]; (3) the identification of two-drug combinations which are additive in their antitumor activity [7]; and (4) standardization of enzymatic systems for the in vitro bioactivation of drugs which require conversion to their active metabolites [29]. Preliminary results of studies in each of these research areas will be presented and discussed.

The Human Tumor Stem Cell Culture

Tumor samples for culture are obtained from solid tissues, the bone marrow, and malignant pleural and peritoneal effusions. Techniques for preparing single cell suspensions, drug incubations, and plating the cells in the agar cultures are as reported previously

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from our laboratory [22–24, 43], except that conditioned medium is not required for non-hematologic malignancies as sufficient tumor colony growth (e.g., 30–200 colonies per 35-mm petri dish) is usually obtained within 10–14 days without this addition. In addition to control specimens, triplicate samples are exposed to several drug concentrations, as discussed below under *Drug Scheduling*. Freshly plated cultures are examined by inverted light microscopy to ascertain that aggregates are not present. Plates are cultured under standard assay conditions. Clusters (15–30 cells) are apparent within 3–5 days and colonies (30-cell aggregates) are usually present in sufficient numbers and size to be counted by inverted microscopy or with the Bausch and Lomb FASII image analyzer 7–10 days after plating. Representative plates are prepared for morphologic analysis by a dried slide technique with Papanicolaou staining [42].

Pharmacokinetic Principles Used in Assay Design

Three principles have been used to design the concentrations and exposure times of a specific anticancer drug to be placed in the in vitro tumor stem cell assay system.

First, we have used several pharmacologically achievable drug concentrations for a minimum of 1 h incubation. The 1-h exposure time was established on practical grounds, including (1) the need to standardize time exposure for assays involving eight to ten different drugs, (2) considerations that short-term exposures (e.g., 5–10 min) might not be adequate with respect to time for uptake of all drugs, and (3) pharmacokinetic data suggesting that significant cellular exposure to most drugs is greatest during the first hour after administration. Two to three concentration points were used to ensure a representation of the dose-response curve for any given drug against a specific patient's tumor. Once a relatively large body of information is available from assays in which sensitivity is manifest, consideration can be given to using two drug doses plus a control.

Second, for new drugs a wide range of drug concentrations are used, usually covering three logs, and ranging from doses considerably below the clinically achievable CXT to doses far above those conventionably achievable. This is necessary to assure that the tumor colony-forming units (TCFUs) are given a maximum opportunity to manifest a dose response to the drug. This is particularly important in the area of new drug development, as pharmacologically achievable concentrations may well not have been defined. Unique to our approach has been the routine use of very low drug concentrations. Prior published studies of in vitro drug sensitivity of fresh human tumor cells to anticancer drugs have generally used unrealistically high dosages, often with long exposure times which are not clinically achievable. While TCFUs resistant to high drug concentrations will predict clinical drug resistance, high-dose studies will also yield high false-positive rates in the prediction of clinical response.

In our opinion, clonogenic assays employing only one drug dose level have very limited value. Both low and relatively high drug concentrations should be tested in vitro. For example, it is valuable to know whether a tumor is completely resistant to a given drug in vitro. Even if it does not show sensitivity to the drug at low concentrations and CXTs, inhibition of its growth at higher concentrations could lead to the design of a high-dose drug trial or suggest the use of the drug by local administration. For example, if a tumor stem cell assay predicted that a high melphalan CXT was needed to inhibit ovarian tumor colony formation to 10% of control, intraperitoneal (IP) administration of melphalan (e.g., 2–3 mg/kg) might prove useful for treatment of malignant ascites even though high concentrations are not achievable at that site after IV administration. In fact, we have had success with precisely this approach in relation to the drug assay for a patient with malignant ascites and intermediate sensitivity for melphalan. Investigators in the Division of Cancer Teatment, National Cancer Institutes, have explored this approach with methotrexate, adriamycin, and 5-fluorouracil 'belly baths' in ovarian cancer [16, 37, 38, 49]. Part of their rationale for these studies has been derived from use of the *ovarian* tumor colony assay for exploring effects of high doses of drugs. Disturbingly, some laboratories with clinical interests have used only unrealistically high drug concentrations in the stem cell assay, and often only a single drug concentration. In our opinion, reports of such assays will have little value for selection of clinically useful drugs.

Third, under normal conditions we select the lowest in vitro drug concentration to represent only 5%–10% of the achievable peak plasma concentration and plasma CXT in vivo for any given drug. This is done for three reasons. The drug concentration entering tumor cells in vivo may only be a small fraction of that measured in the plasma. Also, the CXT of a given anticancer drug may have marked variation from patient to patient. For example, drugs like 5-fluorouracil and melphalan, when taken orally, may have extremely variable absorption, leading to a wide range of plasma CXTs [2, 15]. Variation in the rate of elimination of various drugs (e.g., as a function of hepatic or renal function or pharmaco-

genetics) from patient to patient is also a well-known phenomenon which can influence the plasma CXT. Finally, correlations of in vitro sensitivity with clinical response have indicated that if inhibition of tumor colony growth is not achieved at low drug concentrations, then in vivo drug resistance to systemically administered drugs is almost a certainty [4, 43].

The Drug 'Sensitivity Index' Concept

Shown in Fig. 1 are linear survival-concentration curves of ovarian cancer TCFUs from series of patients whose cells were exposed to vinblastine for 1 h at 37° C prior to plating. Marked heterogeneity of response was notable, with some patients' tumor cells relatively sensitive and others much more resistant to vinblastine exposure. Several of the curves show a relatively steep initial decline in surviving TCFUs in response to low drug concentrations, followed by a later 'plateau' with no increase in lethality observed at higher drug doses. This biphasic-type dose-response curve may have two explanations. First, there may be at least two populations of tumor stem cells, one more sensitive than the other to drug exposure. Second, the steep portion of the dose-response curve may represent a cell population in the proliferative phase of the cell cycle, whereas the plateau portion of the curve may represent 'resting' clonogenic cells (or those in the wrong phase of the cell cycle), which would be kinetically resistant to a 1 h exposure to vinblastine. The latter consideration is clearly of importance for 'cycle-active' drugs such as methotrexate or cytosine arabinoside, the effects of which are largely limited to cycling TCFUs. In fact, for cycle-active drugs, it appears important routinely to relate the fractional lethality from the steep portion of the survival curve to the ^{3}H-thymidine suicide index. Preisler and Shaham [41] have recently reported on precisely such an approach in the response of normal myeloid progenitors (e.g., CFU-C) to cytosine arabinoside. They found an excellent correlation between cytosine arabinoside lethality and the ^{3}H-thymidine suicide index. However, with tumor cells such a study might also disclose a dichotonous relationship in which even though the ^{3}H-thymidine suicide index is high the TCFUs may still be inherently resistant to the anticancer drug at pharmacologically achievable concentrations.

We quantitate a sensitivity index for these in vitro drug assays by measuring the area under linear survival-drug concentration curves (1-h exposure) out to an upper concentration limit which is defined by clinically achievable dosage exposures [5, 34]. For consistency of interpretation, drug concentrations

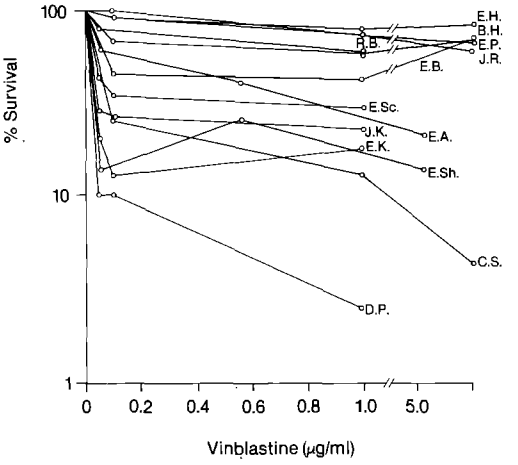

Fig. 1. In vitro survival of ovarian TCFUs following exposure to increasing concentrations of vinblastine for 1 h at 37° C. Vinblastine concentrations have been extrapolated between 1 and 5 μg/ml. Each *curve* corresponds to the results for an individual patient whose tumor biopsy was assayed. Reproduced from Alberts et al. 1980b [5], with permission of the publishers

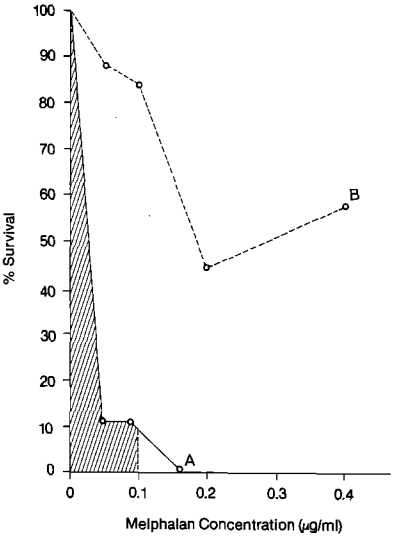

Fig. 2. In vitro survival of myeloma TCFUs following exposure to increasing concentrations of melphalan for 1 h at 37° C. The *shaded area* under the *melphalan dose-response curve* for patient A's TCFUs yields a sensitivity index of 3.1 area units. Patient A (●——●) went into a clinical complete remission following treatment with melphalan, whereas patient B's TCFUs (●– – –●) failed to respond to melphalan in vitro and his myeloma showed progressive growth in vivo. Reproduced from Alberts et al. 1980b [5], with permission of the publishers

used in calculating the sensitivity index should not be less than one half the upper concentration limit. The index can be expressed in area units and in relation to a cut-off or boundary concentration established from experimental observations for each drug. For example, Fig. 2 shows two examples of drug sensitivity in

ovarian cancer when the 0.1-μg dose is used as the upper boundary for measurement of area under the curve. One half of the upper concentration (i.e., 0.05 μg/ml) was also used to evaluate the dose-response curve. The shaded area under the melphalan dose-response curve for one patient represents the sensitivity index of myeloma TCFUs to the drug. This patient, whose sensitivity index was 3.1 units, went into a clinical partial remission following melphalan treatment, whereas a second patient, represented by the dotted line, failed to respond to melphalan and had a sensitivity index of 8.9 units.

Relation of Drug Sensitivity Index and in vivo Plasma Disappearance Kinetics

When we compared the in vitro drug concentrations and CXTs associated with the 'sensitive' range of sensitivity index areas to the in vivo peak plasma concentrations and CXTs for a series of drugs we discovered that the cut-off concentrations (an empirically established boundary for calculation of sensitivity by the area under the curve technique) were only 5%–10% of those which were clinically achievable. For example, for melphalan, the cut-off concentration for a sensitivity index of five would be a 1-h exposure to 0.1 μg of the drug/ml. In vivo this represents less than 10% of the achievable melphalan peak plasma concentration or CXT, which average 2.6 μg/ml and 2.5 μg · h/ml, respectively, after an IV bolus dose of 0.6 mg/kg [3].

As shown in Table 1 a similar relationship between the in vitro cut-off concentration and in vivo peak plasma concentration and CXT has been found for several other commonly used drugs. For example, our in vitro cut-off concentration for bleomycin is 0.1 mU/ml for 1 h. Its peak plasma concentration and CXT after a 15 U/m^2 IV bolus dose are 2.8 mU/ml and 5 mU · h/ml, respectively. Thus, the ratio of in vitro to in vivo peak concentration and CXT for bleomycin are 0.04 and 0.02, respectively. The observation that TCFUs show decreased survival for drug concentrations that are less than 10% of clinically achievable levels is analogous to reports for the in vitro assay for drugs effective against bacteria [27].

Relationship Between Sensitivity Index and Clinical Response

To relate the results of in vitro and in vivo tumor response to a specific drug, separate line plots have been constructed for each drug, correlating the sensitivity index with objective clinical response (Fig. 3) [34]. In vivo outcome was determined independently by the clinician, using standard response criteria, who did not know the results of the in vitro study. A distinct pattern was observed for all drugs tested. Patients with an in vitro sensitivity index to melphalan of less than 5 units frequently obtained an in vivo response, while patients with a sensitivity index greater than 5 units consistently did not achieve an in vivo response. The distinct pattern observed for these patients and similarly for patients receiving other anticancer drugs strongly suggested that a small value for the sensitivity index did correlate with

Table 1. In vitro-in vivo pharmacokinetic correlations

	Adriamycin IV	BCNU IV	Bleomycin IV	Melphalan		Methotrexate IV	*cis*-Platinum IV
				PO	IV		
In vivo							
Route and dosage	60 mg/m^2	95 mg/m^2	15 U/m^2	0.6 mg/kg	0.6 mg/kg	50 mg/m^2	100 mg/m^2
Peak plasma concentration (μg/ml)	1.0	1.97	2.81	2.64 (1.06–4.94)	0.28 (0.07–0.63)	4.58	2.49
CXT (μg · h/ml)	2.76	1.02	4.99	2.47 (0.87–5.50)	0.88 (0.15–2.50)	8.90	1.94
Reference number (Appendix 4)	12	28	1	3	2	13	39
In vitro							
Dose range studied (μg/ml)	0.01–1.0	0.1–3.0	0.01–1.0	0.01–1.0	0.01–1.0	0.05–5.0	0.01–1.0
'cut-off' concentration (μg/ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Peak concentration ratio (%)	10.0	5.0	3.6	3.8	35.7	2.2	4.0
CXT ratio (%)	3.7	9.0	2.0	4.0	11.4	1.1	5.2

Modified from Alberts et al. 1980b [5], with permission of the publisher

subsequent in vivo response and a large value correlated with in vivo resistance. Our first approach was to calculate a point of discrimination that could be used to classify each patient's sensitivity index into two disjoint prognostic regions [9]. Patients whose sensitivity index was less than this point of discrimination would be predicted to have an in vivo response, while patients whose sensitivity index or area under the curve was greater than the point would be predicted to have in vivo resistance.

To furnish the clinician with an explicit method of predicting patients' in vivo response to agents tested in vitro, a logistic regression model was used and the sensitivity index scale was separated into three disjoint regions [34]. The regions, called in vivo sensitive, intermediate, or resistant, enable the clinician to identify treatments that may have very different predicted probabilities of furnishing a patient with an in vivo response.

The sensitive region was defined to correspond with a predicted probability of in vivo response of at least 0.50. The resistant region was defined as the portion of the in vitro scale that corresponded with less than a 0.20 predicted probability of in vivo response. The region that corresponds with a predicted probability of in vivo response of less than 0.50 but greater than 0.20 was defined as the intermediate region. The sensitivity index regions for five standard cancer cytotoxic agents are shown in Table 2 [34]. The regions differ not only in boundary values, but also in width of the regions. While many other agents have been used in the in vitro assay system, the agents shown have both a number of retrospective correlations to estimate a logistic model and a number of prospective correlations to independently evaluate the model.

Moon et al. have provided boundaries for in vitro drug sensitivity for the combined data for 11 anticancer agents against a variety of solid and hematologic cancers [35, 36]. Their data suggest that a lower survival of TCFUs than 38% and a sensitivity index of less than 6.5 area units at low drug concentration (i.e., 0.1 μg/ml for a 1-h exposure, or approximately 10% of the CXT) represent average sensitivity boundaries which correspond to clinical responsiveness for most standard anticancer drugs. In vitro sensitivity boundaries did vary for different tumor types [35, 36].

Correlations of the percent surviving TCFUs and sensitivity indices with objective clinical response have shown that the predictive accuracy of the in vitro drug assays depends on the in vitro testing of drug concentrations of 10% or less than those which are pharmacologically achievable with standard in vivo drug doses [5, 34–36]. While the finding of in vitro drug resistance at drug concentrations in excess of 50% of those that are pharmacologically achievable will virtually always be associated with clinical drug resistance, a high false-positive in vitro sensitivity rate can be anticipated if these 'high' drug concentrations are used to correlate with the previously discussed percent survival and sensitivity index boundaries.

Table 2. In vitro sensitivity regions[a]

Drug		Sensitivity Index		
		Sensitive	Intermediate	Resistant
Adriamycin	(29)[b]	< 2.2	2.2– 7.3	> 7.3
Bleomycin	(15)	< 2.4	2.4– 3.8	> 3.8
Melphalan	(33)	< 3.0	3.0– 5.3	> 5.3
Platinum	(14)	< 8.7	8.7–11.4	> 11.4
Vinchristine	(8)	< 4.7	4.7– 6.4	> 6.4

[a] Area under the curve technique used in this evaluation
[b] The numbers in parentheses indicate the sample size of the training set used to estimate the logistic regression model
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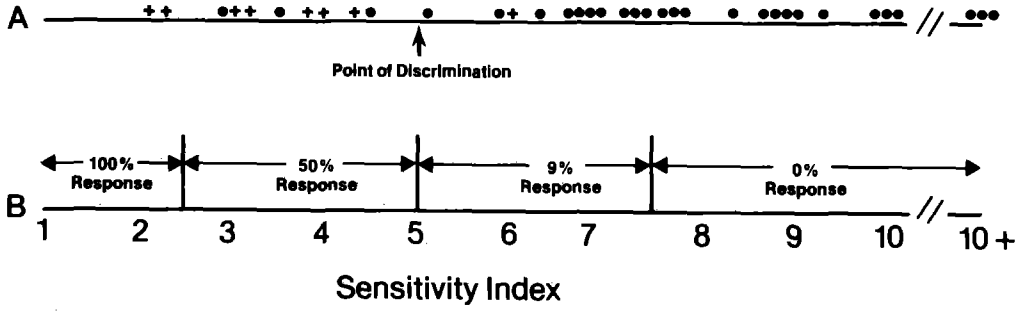

Fig. 3. Sensitivity index versus in vivo response (+, in vivo response; ●, lack of in vivo response). Reproduced from Moon et al. 1980 [34], with permission of the publishers (+) in vivo response; (●) no in vivo response

Studies of in vitro Drug Scheduling

There is clinical evidence that several anticancer drugs possess schedule dependency in their killing effects. A large body of literature has documented cytosine arabinoside's increased activity in acute myelogenous leukemia when administered by continuous infusion for 7–10 days [21]. More recently, Yap et al. [53, 54] have shown that vinblastine and vindesine can induce high rates of objective response in heavily pretreated breast cancer patients. Such clinical trials may require large numbers of patients to show improved clinical efficacy for an anticancer drug when used by continuous administration. The human tumor stem cell assay (HTSCA) provides a simple in vitro technique for evaluating the relative inhibitory effects on TCFUs of different time exposures to an anticancer drug. The results of such in vitro studies may provide important leads for clinical trials which test new schedules for drug treatment. However, while this approach is useful it does not distinguish between cytostatic and cytocidal effects of the agents tested. Brief exposures prior to plating (e.g., 1 h) measure cytocidal effects, and may therefore be more reliable.

We have carried out comparative studies of different time-course exposures for tumor cells to the same drug concentration. One-hour exposure in liquid culture prior to plating was compared to continuous contact of the TCFUs with the drug. We recognize that prolonged liquid culture could lead to selective alteration of cell populations attributable to factors other than drug sensitivity per se. Therefore, we carried out our studies of continuous exposure of cells to anticancer drugs by including stable drugs in the agar gel matrix. While it would be of definite interest to study varying time periods of continuous contact of drugs in agar, it is presently practical to compare drug incubation in liquid culture prior to plating with the incorporation of a drug into the agar for the entire period of culture. Our preliminary studies have focused on the cycle-active drugs methotrexate, vinblastine, and bleomycin, and the 'cycle-nonspecific' drugs adriamycin and *cis*-platinum. Methotrexate was evaluated at concentrations of 0.05, 0.5, and 5 μg/ml. Only two of seven ovarian cancers showed increased inhibition of TCFU growth resulting from continuous exposure for up to 14 days (Table 3). It is possible that various nutrients within the culture medium may have reversed methotrexate's antitumor activity during continuous in vitro exposure in agar.

Results of continuous contact in agar with vinblastine in five of ten cancers (one breast, two endometrial and seven ovarian cancer patients) showed a higher lethality than a 1-h exposure prior to plating, but a plateau of resistant TCFUs. These experiments provide clear evidence of inherent resistance to vinblastine, inasmuch as the in vitro CXT of continuous contact was often 300-fold that of the 1-h exposure. Hence resistance to vinblastine in these experiments cannot be explained on a kinetic basis, as colonies do indeed form in the plates. Recent interest in vinblastine infusions for breast cancer [53] might increase the relevance of continuous contact experiments such as those reported here.

Of the five drugs tested in these preliminary studies, bleomycin showed the greatest degree of schedule dependency for its inhibition of TCFU

Table 3. Continuous vs 1-h drug exposure effects on the inhibition of human tumor colony growth

Drug	No. of studies	Effect of 1-h[a] vs continous[b] drug exposure on colony growth[c]		
		Increased[d]	Unchanged	Decreased
Methotrexate	7	2 (29)	4	1
Vinblastine	10	5 (50)	5	0
Bleomycin	6	3 (50)	1	2
Adriamycin	7	1 (14)	6	0
cis-Platinum	2	0 (0)	2	0

[a] Drug and cells added together with culture medium in upper layer of agar

[b] Drug and cells incubated for 1 h at 37° C prior to cell washing and plating in agar upper layer

[c] In this analysis drugs were tested at the same concentration for both 1 h and continuous exposure. A more valuable comparison would use identical CXTs of the agent for 1 h and continuous testing. In that circumstance, continuous exposure would generally incorporate between $1/200$ and $1/300$ of the 1-h drug concentration $1/300$ for the concentration

[d] Figures in parentheses are percentages

growth. In three of six ovarian cancer patients bleomycin caused markedly greater cell kill when given by continuous in vitro exposure (Table 3). As shown in Fig. 4, bleomycin at the lowest in vitro dose (i.e., 0.01 mU/ml) resulted in a 22% survival of TCFU with continuous exposure, versus a 72% survival following a 1-h exposure. The fact that the highest (i.e., 1 mU/ml) 1-h incubation dose resulted in only a 50% reduction in survival (versus complete inhibition of TCFU growth with continuous exposure) suggests that bleomycin resistance in this tumor was on a cell kinetic rather than biochemical basis. Bleomycin is known to kill tumor cells by shearing DNA during the S phase of the cell cycle [26]. It follows that the longer the duration of cellular exposure to the drug the greater the chance for inhibition of tumor growth. For an accurate prediction of TCFU sensitivity to continuous drug contact (for bleomycin or any other stable anticancer drug), we would suggest that drug concentrations be in the range of 1/200 to 1/300 that of the 1-h exposure concentration. In that ratio a similar CXT would pertain in each circumstance. Correlating with these in vitro bleomycin data is evidence of increased inhibition of leukemic CFU-S by bleomycin administered by continuous IP infusion (versus daily IP bolus administration) in mice [40]. Individualized evaluation of drug schedule dependency based on the HTSCA may help select the patients in whom bleomycin is likely to have increased efficacy when administered by continuous infusion.

In a limited number of studies neither of the cell cycle-nonspecific agents, adriamycin or cis-platinum, showed important schedule dependency for their in vitro cytotoxic effects (Table 3). Several clinical studies of these two agents used by continuous administration for varying periods have confirmed that both lack schedule dependency in their inhibition of tumor growth.

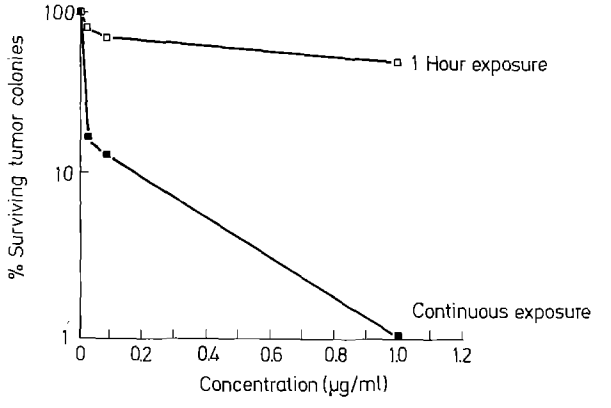

Fig. 4. Effect of in vitro bleomycin exposure duration on ovarian TCFU survival. *Upper curve* represents TCFU survival following a 1-h drug incubation. *Lower curve* represents TCFU survival with continuous contact with the drug in the agar gel matrix

Studies of the Additive Effects of Two-drug Combination

For many years medical oncologists have combined two or more anticancer drugs for the clinical management of most solid and hematologic cancers. These drug combinations are almost always designed on an empirical basis. The drugs are selected because they are known to be active as single agents and have different mechanisms of action and clinically compatible toxicities. A good example of such an empirically derived drug combination was the development of the MOPP regimen for the curative treatment of stage IV Hodgkin's disease [17]. The HTSCA could be used to develop a rational, rather than empirical, basis for combining two or more drugs for the treatment of specific cancers, and could help identify those drug combinations which might be additive or potentiating in clinical use.

In preliminary studies the HTSCA has been used to assess the additive effects of adriamycin-cis-platinum, cis-platinum-vinblastine, vinblastine-bleomycin, and actinomycin D-vindesine on the inhibition of TCFU growth from a variety of non-hematopoietic cancers. Each drug in the combination was tested for a 1-h incubation at two or three different drug concentrations (i.e., low, intermediate, and high). The two drugs were also combined simultaneously for 1-h incubations at one or two of the concentrations used for the single agents. Quantitation of in vitro drug effects was carried out according to the methods of Valeriote and Lin [51] and Momparler [33]. The surviving fraction of TCFUs resulting from each drug individualy (SF_A or SF_B) and the surviving fraction of the drug combination (SF_{A+B}) are determined experimentally. If the SF_{A+B} is equal to $(SF_A) \cdot (SF_B)$ the combined drug effects are additive. If the SF_{A+B} is less than $(SF_A) \cdot (SF_B)$, the combined effect is defined as greater than additive or synergistic. If the SF_{A+B} is greater than $(SF_A) \cdot (SF_B)$, the combination interaction is defined as less than additive. Statistical comparisons between the experimental and calculated data are carried out for each of the two or three concentrations tested for each drug.

The results of these preliminary drug combination studies are shown in Tables 4–6. There were 47 in vitro combination drug trials, performed in cancers from 27 patients. Fifteen patients had ovarian cancer,

Table 4. Evaluation of the additive effects of adriamycin[a] and *cis*-platinum[b] combination chemotherapy

Patient number	Percent of TCFUs				Statistical evaluation of two-drug effect[b]
	Single drug effects		Additive effects of two drugs		
	Adriamycin	*cis*-Platinum	Calculated	Experimental	
1	89.5	84.8	75.9	73.8	A
2	22.7	44.5	10.1	36.9	I
3	69.2	64.2	44.4	46.1	A
4	49.4	66.9	33.1	59.0	I
5	62.5	74.7	46.7	47.3	A
6	82.6	59.5	49.2	44.1	A
7	97.1	88.2	85.7	52.9	S
8	82.7	55.8	46.1	51.9	A
9	12.1	27.5	3.3	4.0	A
10	52.8	66.3	35.0	46.6	A
11	68.2	64.5	44.0	53.6	A

[a] All in vitro incubations for both agents were carried out for 1 h at 0.1 μg/ml
[b] A, additive; I, inhibitory; S, synergistic, according to statistical methods of Drewinko et al. [20]

Table 5. Quantitation of the additive effects of two-drug combination vs single-agent therapy on the inhibition of human TCFUs[a]

Number of studies[d]	Drug 1	Drug 2	SF_{1+2}[b] vs SF_1 Effect of adding drug 2 to drug 1			SF_{1+2}[b] vs SF_2 Effect of adding drug 1 to drug 2			SF_{1+2}[b] vs $SF_1 \cdot SF_2$[c] Experimental vs calculated effect of two drug Rx		
			Inhibitory	None	Additive	Inhibitory	None	Additive	Inhibitory	Additive	Synergistic
19	Adriamycin	*cis*-Platinum	2 ($P > 0.3$)	6	11	0 ($P = 0.08$)	6	13	4 ($P = 0.06$)	14	1
17	Bleomycin	Vinblastine	0 ($P > 0.3$)	7	10	2 ($P > 0.5$)	10	5	6 ($P = 0.17$)	8	3
10	*cis*-Platinum	Vinblastine	0 ($P = 0.001$)	0	10	0 ($P > 0.3$)	4	6	1 ($P = 0.01$)	8	1
10	Actinomycin D	Vindesine	2 ($P > 0.5$)	6	2	0 ($P > 0.5$)	5	5	3 ($P = 0.17$)	5	2

[a] Statistical analysis compares the inhibition of each patient's TCFU by one and two drug therapy according to the methods of Drewinko et al. [20]
[b] SF_{1+2} represents the 'surviving fraction' of TCFU resulting from the experimental addition of drug 1 and drug 2
[c] $SF_1 \cdot SF_2$ represents the calculated effect of multiplying the 'surviving fraction' of TCFUs resulting from drug 1 and drug 2, separately
[d] The results of some studies were inconclusive regarding the effect of one vs two drugs
The statistical P values represent a comparison of the data with respect to additive (+ synergistic) vs lack of additive effects

Table 6. Activity of vinblastine plus *cis*-Platinum against endometrial cancer TCFUs

Patient	Drug	Drug combination (μg/ml for 1 h)	% TCFU survival	Combination calculated % survival	Combination experimental % survival
1	Vinblastine	0.001	39	27.0	8.0_a
	cis-Platinum	0.001	69		
2	Vinblastine	0.001	1	0.4	0.6
	cis-Platinum	0.001	36		

[a] Synergistic result according to statistical criteria described in text

four had uterine cancer, seven had melanoma, and one, testicular cancer. As shown by comparing the percent surviving TCFUs in the 'calculated' and 'experimental' columns of Table 4, adriamycin plus *cis*-platinum at concentrations of 0.1 μg/ml were suggestive of being additive in their inhibition of TCFU growth in 8 of 11 trials, greater than additive in 1 of 11 trials, and less than additive in 2 of 11 trials. Thus, the adriamycin plus cis-platinum combination was additive or greater than additive in its inhibition of TCFU growth in 9 of 11 patients. These results correlate with the findings of broad phase II clinical trials of adriamycin plus cis-platinum in the treatment of solid cancers, which suggested at least additive effects from this drug combination [25].

Table 5 shows the overall results of the two-agent combination trials, with statistical comparisons of the inhibition of TCFU growth by the single agent and the two-agent combination. Statistical analysis was carried out to compare the inhibition of each patient's TCFU by one- and two-drug therapy according to the methods of Drewinko et al. [20]. A sign test was then used to determine whether most patients' TCFU growth was lower when two drugs were used as against one. In these studies adriamycin appeared to be the more important drug with respect to gaining an additive effect from the adriamycin-*cis*-platinum combination therapy. Again, this two-drug combination was at least additive in its effects in the majority of trials ($P < 0.06$). Vinblastine appeared to be the important drug in the vinblastine-bleomycin combination, but the two drugs were not statistically additive in this small trial. We do not know the reason for the antagonistic effects of vinblastine and bleomycin in five of the trials. Previous work in our laboratory showed that vinblastine did not inactivate bleomycin chemically [19]. Probably the most interesting data were those concerning the addition of vinblastine to *cis*-platinum. This drug combination was highly additive ($P < 0.01$), especially against endometrial cancer TCFU. Finally, there was no evidence of an additive effect between actinomycin D and vindesine on TCFUs from ten different melanomas tested ($P > 0.17$). Caution must be used in the interpretation of these data, because of the small number of trials; nevertheless, many of the findings correlate with the results of past clinical trials. On the basis of these preliminary data we conclude that the HTSCA can be used to assess the potential additive effects of two-drug combination chemotherapy for specific tumors, and can help identify those chemotherapy regimens that might be additive or potentiating in clinical use. We may have already identified one such new combination therapy for the treatment of advanced recurrent endometrial cancer. As shown in Table 6, the in vitro combination of extremely low concentrations (i.e., 0.001 μg/ml) of vinblastine and *cis*-platinum resulted in less than 10% TCFU survival.

There are some major problems inherent in these in vitro drug combination studies, which must also be addressed. Firstly, because of the low plating efficiencies and the multiple combinations to be tested, it requires in excess of 15 million fresh tumor cells to complete one of these two-agent combination studies, which uses at least three different drug concentrations (in triplicates) for the two single agents and the combination. It may be difficult to obtain enough cells for such studies if other single agents are being evaluated for cytotoxicity. Thus, such testing is limited to large tumor specimens. Secondly, it is preferable to evaluate the inhibition of TCFU growth by each agent and the two-drug combination at many different drug concentrations. Drug dose-TCFU survival curves can then be compared, rather than the inhibition of TCFU at only one or two drug concentrations. Such testing can best be carried out upon large samples of cryopreserved cells (i.e., preserved with 10% DMSO) which have first been found to grow after thawing. In our experience, TCFU proliferation occurs in 75% of tumors and allows the opportunity to repeat experiments. An isobologram analysis of these dose-survival curves can be attempted if enough drug concentrations are tested [50]. Thirdly, to finally validate this in vitro method of analyzing two-drug combination efficacy it will be necessary to carry out correlative clinical trials. Fourthly, when selecting two agents for these in vitro combination trials it is important to document that they do not activate or inactivate each other chemically. Chemical inactivation of one or both drugs would lead to a less than additive effect on TCFU growth inhibition.

Finally, it is essential for the evaluation of these studies either to use cancers from previously untreated patients or to take into account the prior therapy record when designing these in vitro combination chemotherapy studies. Prior therapy even with a single agent can cause cross resistance to a large number of anticancer drugs [6] and may invalidate or weaken the interpretation of the results of such studies. In our preliminary in vitro work in this area we have avoided using drugs and drug combinations to which the patients had been previously exposed, but these preliminary studies were not limited to samples from patients who had not received prior chemotherapy. The data derived from such studies may be of value in defining new approaches to combination chemotherapy of cancer.

Bioactivation of Anticaner Drugs for Use in HTSCA

Several standard anticancer drugs (e.g., cyclophosphamide [14], DTIC [11], mitomycin C [30], and hexamethylmelamine [52]) may require bioactivation in vitro by microsomal enzymes to express their cytotoxicity in the HTSCA. Additionally, in vitro screening for new anticancer drugs should include the capability for detecting agents which require metabolic activation. An analogous problem has been dealt with relatively effectively in Ames testing for mutagens and potential carcinogens. The technique uses a liver microsomal cocktail (S-9 mix) to activate agents for in vitro testing [8]. Several groups have applied similar metabolic activation techniques for in vitro screening of antineoplastic agents [10, 18, 32]. Lieber et al. [29] presented evidence that the same type S-9 mix could be used to activate compounds with anticancer activity, when the S-9 fraction of rat liver, $MgCl_2$, KCl, glucose-6-phosphate and NADP were used in phosphate buffer. They not only activated and showed in vitro activity of standard drugs such as cyclophosphamide in the HTSCA, but also showed that a pyrolizidine alkaloid (heliotrine) could be activated to express cytotoxicity in vitro against human tumor cell lines.

A number of important research questions must be answered before a final decision can be made concerning the bioactivation of agents to be tested for cytotoxicity in the HTSCA. Comparisons between rat liver slices, purified microsomes, and the S-9 mix should be carried out to the determine the optimal activating system for specific antineoplastic agents. Furthermore, isolated, active metabolites of these drugs (e.g., 4-hydroxycyclophosphamide, phosphoramide mustard, and acrolein from cyclophosphamide [14]) could prove more useful for in vitro cytotoxicity testing than any of the activating systems.

We have attempted to improve both the S-9 mix and purified microsomal systems to optimize cyclophosphamide activation for use in the HTSCA. Rat liver fractions were prepared by standard methods and an incubation mixture was put together containing cyclophosphamide, microsomes or S-9 mix, NADPH generating system, $MgCl_2$, and Tris-KCl buffer. Experiments have been carried out to determine the optimal amount of microsomal or S-9 protein and NADP to be included in the mix.

Cyclophosphamide can be activated in the presence of or prior to incubation with the tumor cells. We have preferred to pre-activate cyclophosphamide prior to use in the HTSCA. Aliquots of the resulting 'activated cyclophosphamide' are frozen at −80° C for later use. Shown in Fig. 5 is a dose-survival curve

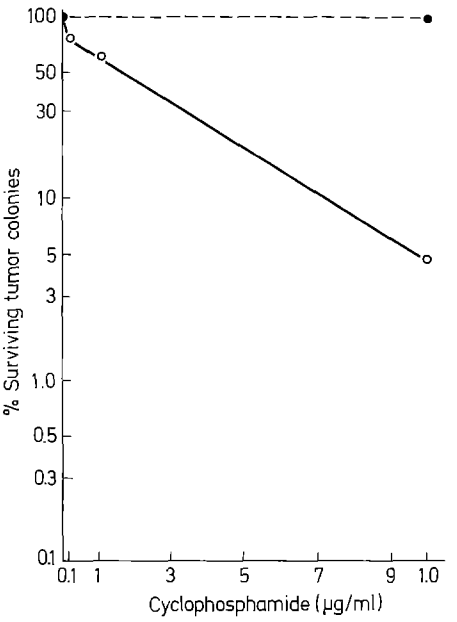

Fig. 5. Effect of cyclophosphamide following rat liver microsomal activation on the inhibition of TCFU growth from an advanced ovarian cancer. The *upper curve* represents TCFU growth inhibition by unactivated cyclophosphamide. The *lower curve* represents TCFU growth inhibition by the microsomally activated drug. Data courtesy of Gary Bignami. (●) non-activated drug; (○) activated drug

for microsomally activated cyclophosphamide against the growth of TCFUs from a patient with newly diagnosed stage III ovarian cancer. There was a greater than 90% inhibition of TCFUs at the pharmacologically achievable parent compound concentration of 10 μg/ml.

Conclusion

We have described our efforts at pharmacologic optimization of the HTSCA for use in the selection of anticancer drug therapy. It is clear that there is a need for continued evaluation of improved methods for drug sensitivity quantitation, scheduling, combination, and bioactivation. Such in vitro studies with the HTSCA are likely to become a regular component of the testing of new anticancer drugs.

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