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New Drugs in Ovarian Cancer and Malignant Melanoma: In Vitro Phase II Screening With the Human Tumor Stem Cell Assay[1,2]

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SUMMARY

The successful development of a soft agar clonogenic assay for human tumor stem cells provides an in vitro technique with a high degree of accuracy for predicting in vivo clinical response to standard anticancer drugs. We used this system to conduct an "in vitro phase II trial" in human ovarian cancer and melanoma. This approach can potentially identify active phase I–II drugs suitable for treatment of given tumor types for specific patients and eliminates the need to subject patients (who would be predicted not to respond) to toxic side effects. In vitro sensitivity for new agents was operationally defined as at least a 70% reduction of tumor colony-forming units (TCFU) at concentrations which are readily achievable pharmacologically. The new agents AMSA and vindesine (as well as vinblastine) appeared to have activity in melanoma, while PALA and thymidine were inactive. Pentamethylmelamine, mitomycin C, methyl-GAG, and AMSA were relatively ineffective in ovarian cancer. Vinblastine and vindesine had definite activity. The human tumor stem cell assay may thus provide the basis for a useful alternative to the current clinical phase II testing approach for identifying antitumor activity of new agents. Validation of this concept with correlative in vitro and in vivo phase II trials of new agents in patients with tumor types predicted to be sensitive is clearly warranted.

[Cancer Treat Rep 65:1–12, 1981]

Development of new drugs for ovarian cancer, malignant melanoma, or any other specific type of cancer has thus far been a very arduous and time-consuming procedure. The process, which initiates from rational design, serendipitous discovery, or random screening in a few signal mouse tumors has probably missed a number of compounds which were inactive in L1210 or P388 leukemia, but which would have had activity for other tumor types. Even using a broadened panel of five or six transplantable mouse tumors of different histopathologic types would be the conceptual equivalent of testing a new drug on five or six patients, each with a different type of cancer. After a new drug is found to be active in screening, it must pass preclinical and clinical toxicology trials before it can be brought to large-scale clinical phase II and III studies in various tumor types. Viewed in that context, it is perhaps remarkable that useful drugs have, in fact, been identified. In fact, many drugs have been identified through other mechanisms in various countries..

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We believe that the recent development of in vitro soft agar colony assays for human tumor stem cells (1–10) shows significant promise of shortening and simplifying the entire drug testing procedure including the preclinical drug screening for active compounds, clinical trials of new agents, and the final selection of treatment for individual patients. These comments are particularly germane for ovarian cancer and malignant melanoma because they are readily grown in the tumor stem cell assay, starting with either malignant effusions or solid tumors (3,5). Our initial approach to drug testing with the stem cell assay in ovarian cancer and melanoma was to use drugs with known activity in patients and correlate in vitro and in vivo sensitivity (7). These correlation studies continue to be quite encouraging (8,10). However, our central focus here will be the examination of new phase I–II agents to determine the frequency with which significant inhibition of ovarian and melanoma tumor colony-forming units (TCFU) is observed at pharmacologically achievable doses. In that sense, we are conducting an *"in vitro phase II trial"* of new anticancer drugs. Additionally, we have applied the assay to make some pharmacologic comparisons of 1-hour versus continuous contact of TCFU to a drug to evaluate potential schedule dependency of some of these agents.

METHODS

Tumor biopsies and malignant effusions were obtained from patients with adenocarcinoma of the ovary or melanoma. Techniques for preparing single-cell suspensions, incubating drugs, and plating the cells in agar culture were reported previously from our laboratory (3,5,7,8), except that conditioned medium was not used, since tumor colony growth was obtained without it. In our standard drug assay system, cells are exposed to various concentrations of drugs in tissue culture tubes for 1 hour at 37°C in McCoy's 5A medium with 10% heat-inactivated fetal calf serum, then washed and plated in the upper layer of the two-layer agar cultures. New agents studied included mitomycin C, vindesine, AMSA, dihydroxyanthracenedione, pentamethylmelamine (PMM), PALA, methyl-GAG, and thymidine. Dose-finding studies for new agents for which pharmacokinetic data were not available were carried out over a 3-log concentration range from 0.1 to 10 μg/ml. In the present study, some samples were also plated in the presence of standard concentrations of stable drugs incorporated into the agar. In most cases, 5×10^5 cells were plated in each 35-mm Petri dish. Freshly plated cultures were examined by inverted light microscopy to ascertain that aggregates were not present. Plates were cultured at 37°C in a humidified incubator with 5% CO_2 in air. Tumor clusters were apparent within 3–4 days. Ovarian tumor colonies usually were present in sufficient numbers and size to be counted 7–10 days after plating, while melanoma colonies could be counted after 10–14 days. Representative plates were prepared for morphologic analysis using our recently described dried-slide technique with Papanicolaou staining (9). Data from all experiments were entered into disc storage on a Wang 2300 laboratory computer which was used for data analysis and graphic output. Criteria for in vitro sensitivity for standard drugs were based on calculation of the area under linear survival-concentration curves and on ranking relative areas using results from an initial training set of patients for whom in vitro and in vivo studies were done (7,10). For new drugs with pharmacologic parameters that were less certain, we used an *operational* definition of sensitivity: ie, at least a 70% reduction in survival of TCFU at a relatively low dose of the drug. In all instances where pharmacokinetic data were available, the dose to achieve at least a 70% inhibition had to be < 10% of the maximal clinically achievable concentration × time (C × t) product to designate a drug as being sensitive in vitro.

RESULTS

Relation of Drug Schedule to Lethality on Clonogenic Tumor Cells

Studies comparing 1-hour exposure of cells to drugs before plating versus continuous contact, in the agar, to drugs thought to be stable in vitro have been only recently initiated in our program. Figure 1 summarizes comparative time exposures for ovarian cancer cells to vinblastine. For the two patients studied in this system, a somewhat greater in vitro lethality was observed with continuous contact to vinblastine for the entire culture period (approximately 10 days) in comparison to the 1-hour exposure. However, in view of the fact that the effective C × t product for continuous contact would be > 200 times that for the 1-hour exposure, it would appear that prolonged contact was relatively ineffective. Further studies will be required to clarify this phenomenon.

Individual Agents

A study of a number of anticancer drugs which have not been extensively utilized in ovarian cancer or melanoma or are currently investigational agents (IND status) is now under way in our laboratories.

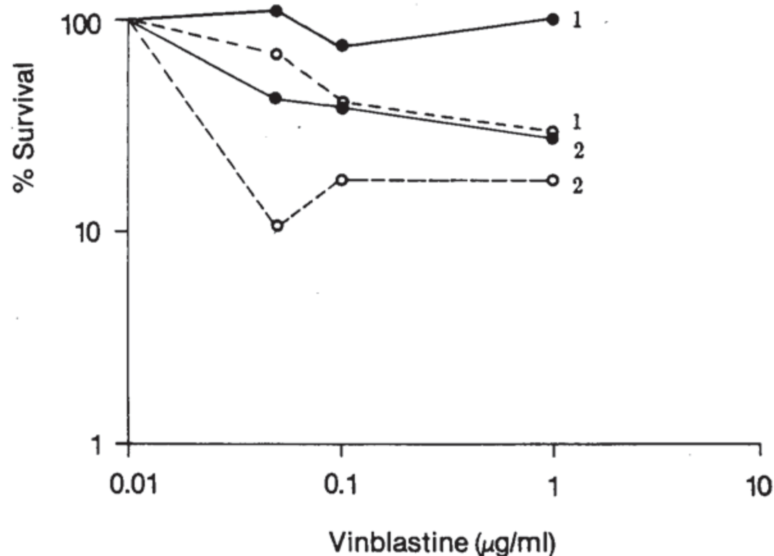


FIGURE 1.—Schedule-dependent TCFU inhibition by vinblastine in vitro: comparison of 1-hr exposure (—●—) and continuous contact (- - -○- - -) of ovarian tumor colony-forming cells from 2 patients (Nos. 1 and 2) to vinblastine.

Table 1 and figures 1–9 summarize the in vitro phase II investigations.

Mitomycin C

Mitomycin C is one of the standard anticancer agents that are only rarely used for early or advanced ovarian cancer. We investigated mitomycin C-induced lethality on tumor samples from 11 patients with ovarian cancer. The results of these studies (fig 2) indicate that for only one of these 11 patients, TCFU survival was reduced to < 30% of the control at pharmacologically achievable doses of mitomycin C. However, four of the 11 patients had steep curves to the 0.1-μg/ml dose level. Most of the patients who were studied were in relapse from prior alkylating agent therapy.

TABLE 1.—Sensitivity of TCFU to 1-hr exposure to single drugs*

	No. of sensitive cultures/ No. tested	
Drug	Ovarian cancer	Melanoma
Mitomycin C	1/11	NT†
PMM	2/5	NT
Vindesine	1/11	2/12
Methyl-GAG	2/11	0/5
PALA	0/8	0/5
AMSA	2/17	5/16
Dihydroxyanthracenedione	0/1	NT
Thymidine	NT	0/7
Vinblastine	1/7	NT

*For the purposes of this comparison, our operational definition of sensitivity is defined as a > 70% reduction in survival at 10% of the pharmacologically achievable 1-hr C × t.
†NT = not tested.

Pentamethylmelamine

Figure 3 summarizes our experience with the in vitro assay of the new agent PMM. This drug was formulated as an iv preparation because hexamethylmelamine (HMM) lacks sufficient solubility for iv use (11). PMM is under phase I–II study in several centers and is of interest in ovarian cancer because HMM is known to be active in this disease. Cells from two of the five patients studied thus far have shown a 70% reduction in survival of TCFU compared with the control. None of these patients had received HMM or PMM clinically. While one of these assays showed diminishing effect at increasing dose levels, this was not observed for the other patients studied. These preliminary data would suggest that PMM may prove to be an active drug for use in ovarian cancer treatment. Selection of this agent rather than HMM will, in part, depend on comparative toxicology and clinical pharmacology studies with both drugs. Since the activity of both HMM and PMM may be due to an intermediate product (12), the cells in the tumor suspensions appear to be capable of metabolizing PMM to the active form.

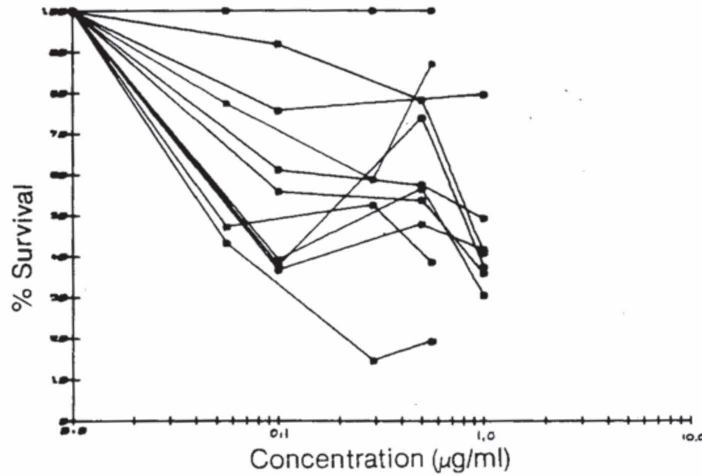


FIGURE 2.—Effect of 1-hr exposure of ovarian TCFU to mitomycin C.

Vindesine

The vinblastine analog vindesine (13) has been studied in vitro against TCFU from 11 patients with ovarian carcinoma (fig 4A) and from 12 with melanoma (fig 4B). Figure 4A shows comparative lethality of vindesine and vinblastine on ovarian tumor clonogenic cells from eight patients. When the two drugs were thus studied simultaneously in vitro, there appeared to be no clear advantage to vindesine over vinblastine. Reduction in survival to < 30% of the control TCFU at the 0.1-μg dose in the 1-hour exposure experiments was observed for one of the 11 patients with ovarian cancer (not all depicted in fig 4A) and for two of the 12 with melanoma (fig 4B). We have observed occasional excellent objective responses in patients with drug-refractory ovarian cancer treated with vinblastine (two partial remissions) and in patients with drug-refractory melanoma treated with vindesine (one partial remission) which were predicted by the stem cell assay.[5] Based on the comparative in vitro stud-

[5]Alberts DS, Salmon SE, Chen HSG, et al. Predictive chemotherapy of ovarian cancer using an in vitro clonogenic assay. Manuscript in press in *Lancet*.

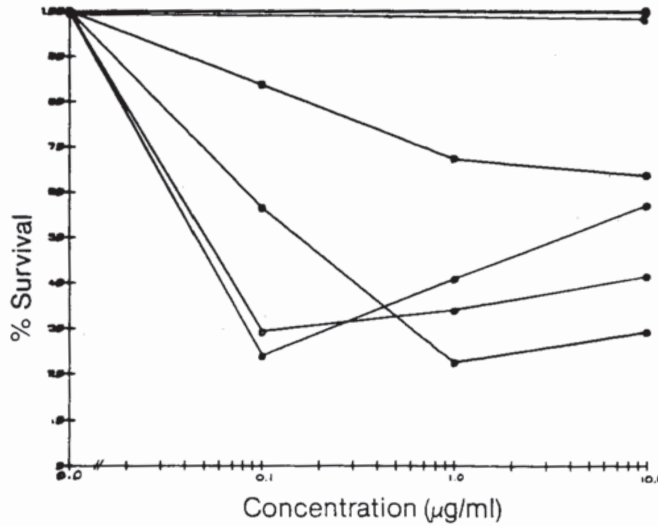


FIGURE 3.—Effect of 1-hr exposure of ovarian TCFU to PMM.

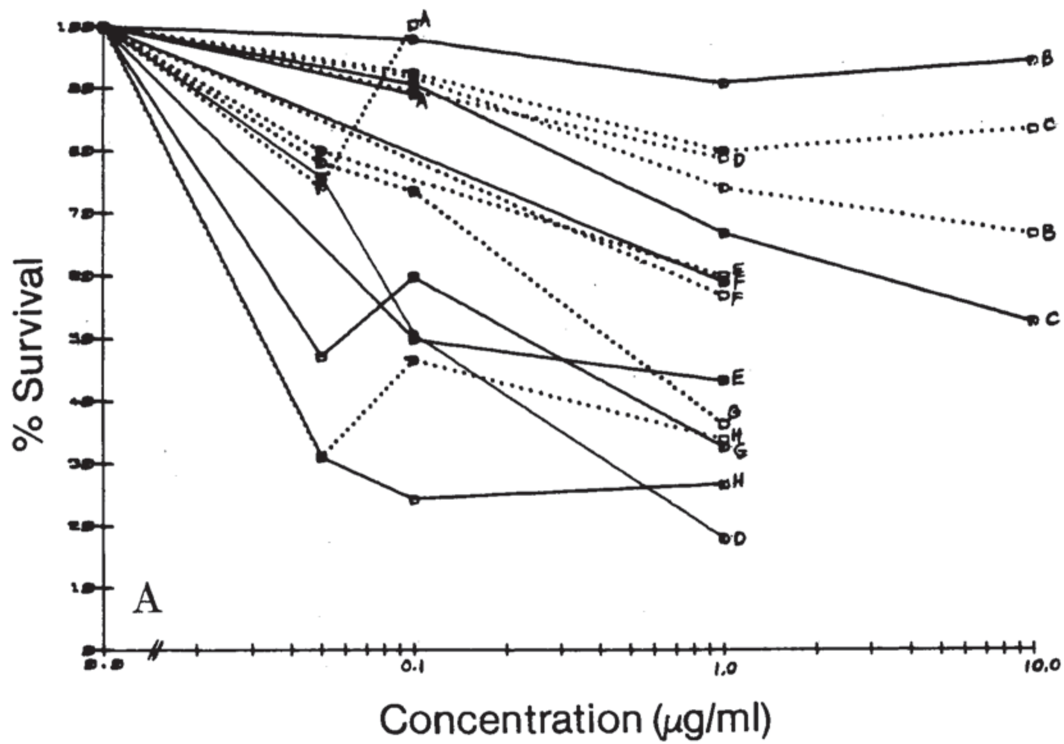
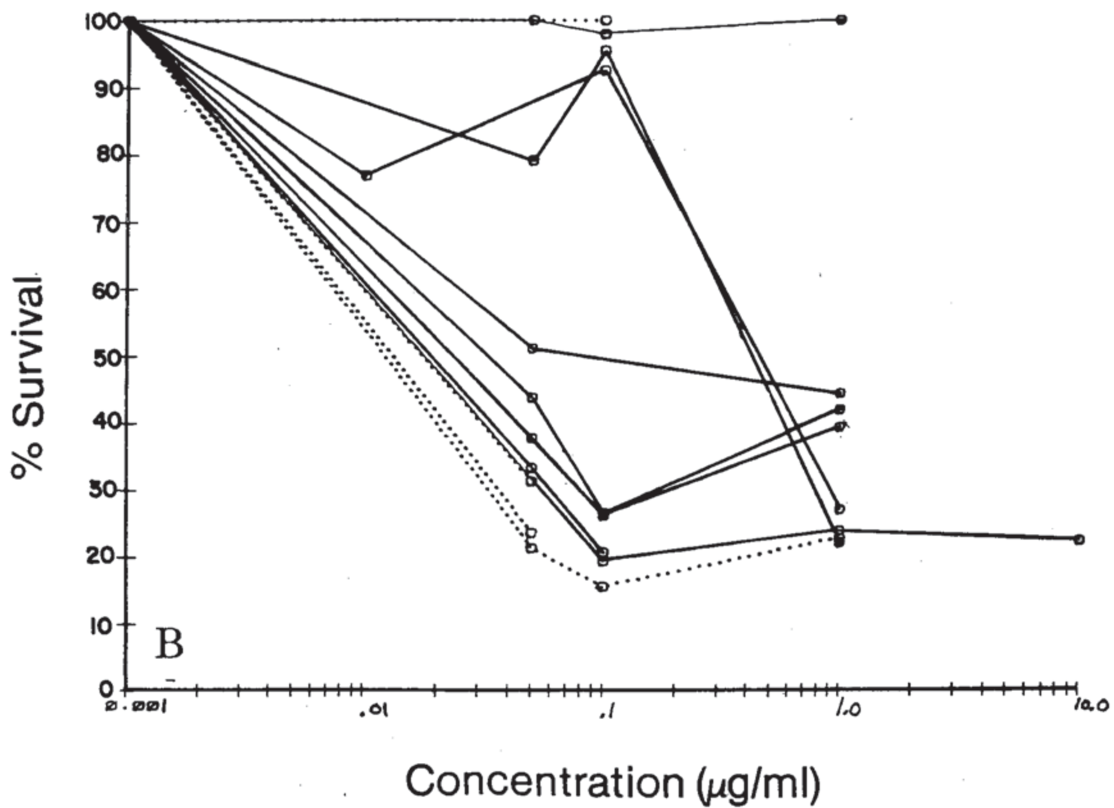


FIGURE 4.—(A) Comparative lethality of 1-hr exposure of ovarian TCFU to vindesine (————) or vinblastine (· · · · · ·). (B) Effect of vindesine on melanoma TCFU. ———— = 1-hr exposure; · · · · · · = continuous contact.



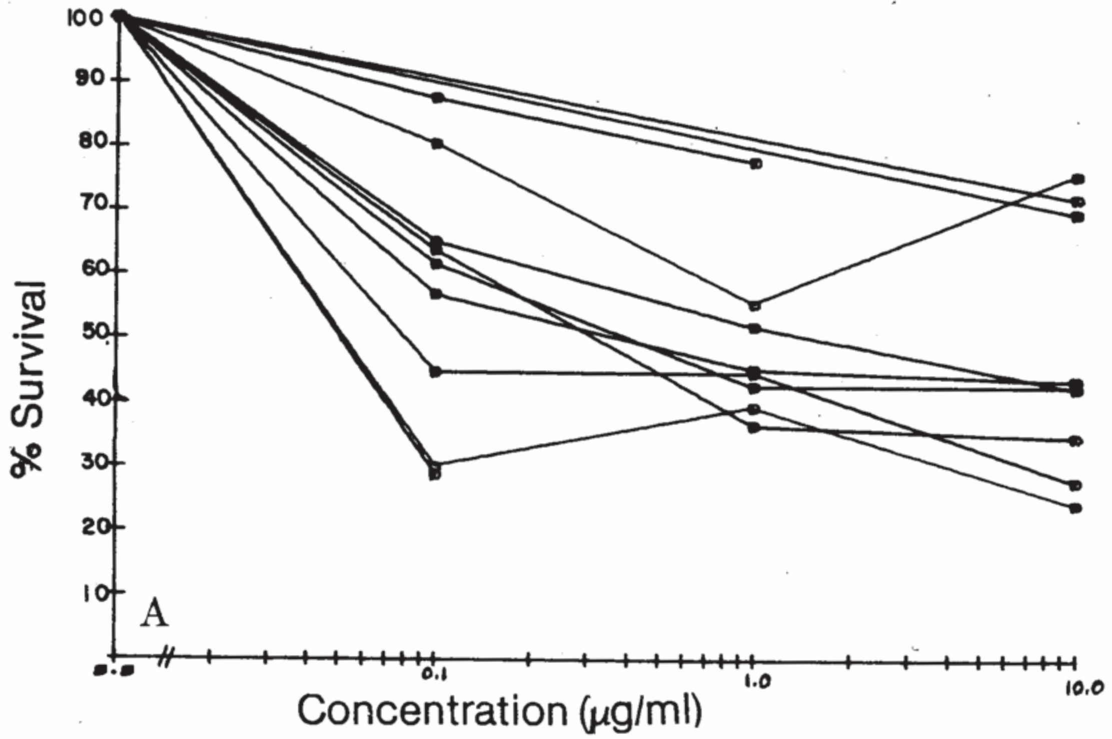
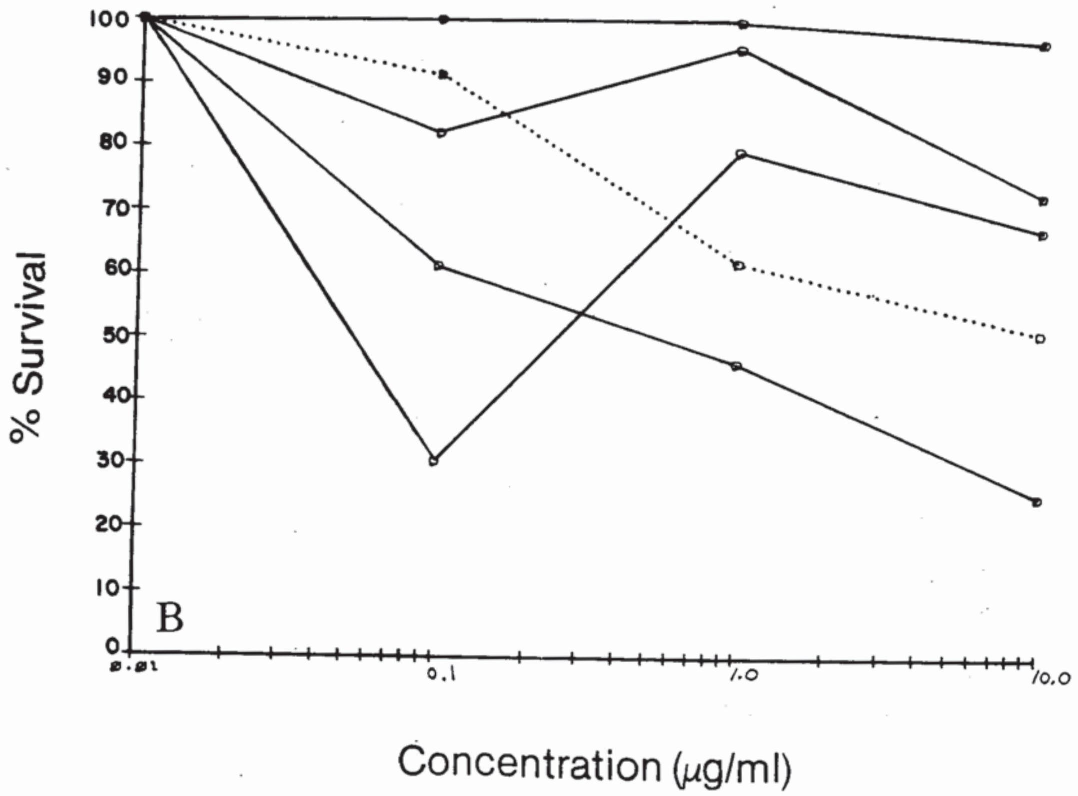


FIGURE 5.—Effect of exposure of TCFU to methyl-GAG (———— = 1-hr exposure; · · · · · · = continuous contact): (A) ovarian carcinoma; (B) melanoma.



ies in ovarian cancer we anticipate that vindesine and vinblastine may have comparable clinical effects in drug-resistant patients if the pharmacokinetics are similar.

Methyl-GAG

Methyl-GAG, an inhibitor of polyamine synthesis, was tested against tumor stem cells from 11 patients with ovarian carcinoma (fig 5A) and from five with melanoma (fig 5B). This long-standing investigational agent has recently been revived and found active in renal and blâdder cancer (14). In our in vitro assays, methyl-GAG had only intermediate lethality (or less) in cells from most of the patients studied, and survival of TCFU for only two of the 11 patients with ovarian cancer was reduced to 30% of control at the 0.1-μg/ml dose in vitro. While some increasing lethality was observed with increasing dose, it seems unlikely that such high doses of methyl-GAG would be achievable in vivo without excessive toxicity. Prior studies with high-dose methyl-GAG (in the 1960s) led to the premature abandonment of this drug. Our studies suggest that occasional patients with ovarian cancer (ie, 10%–20%)

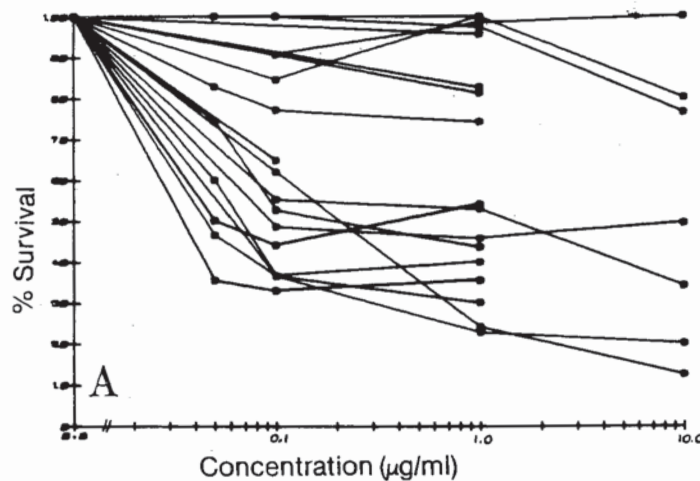
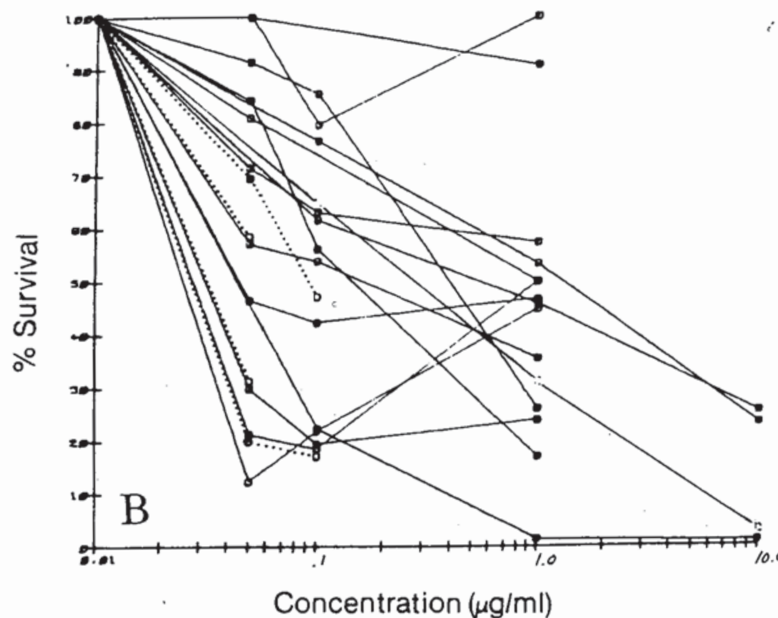


FIGURE 6.—Survival of TCFU after in vitro exposure to AMSA. ——— = 1-hr contact; · · · · · · = continuous exposure): (A) ovarian carcinoma; (B) melanoma.



may respond to methyl-GAG, but that patients with melanoma are less likely to respond.

AMSA

Promising preclinical results in a variety of tumors and acceptable phase I investigations of toxicity (15) have led to broad phase II trials with AMSA. In our clinical investigations we have seen significant activity of AMSA against a spectrum of tumors, including breast cancer, lymphoma, and melanoma (16). While animal studies suggested that this agent is cross-resistant with doxorubicin, some clinical observations in acute leukemia suggest that the drug may prove useful in patients who relapse after doxorubicin treatment (16). In our in vitro phase II study of AMSA, 17 patients with ovarian carcinoma (fig 6A) and 16 with melanoma (fig 6B) were studied at doses ranging from 0.05 to 10 μg/ml for 1 hour. We consider that 1 μg hour exposure should be pharmacologically achievable in vivo. Additionally, cells from four patients with melanoma were studied in vitro with continuous exposure to AMSA in the agar. Substantial heterogeneity in response to this agent was observed, and only two patients with ovarian carcinoma had a 30% reduction in cell survival compared to 30% of the control. However, five patients with melanoma had a > 70% reduction of TCFU with the 1 μg hour exposure to AMSA, which suggests that this agent would be active in melanoma. TCFU from an additional four patients with melanoma, studied with continuous contact to AMSA, showed no marked increase in lethality, suggesting that resistance may have more of a cellular uptake or biochemical basis than a cytokinetic one. Inasmuch as AMSA is known to bind to melanin granules, even a 1-hour exposure may provide "continuous contact" in melanoma. As with many of our other in vitro drug studies, AMSA was evaluated mainly in cells from patients who had received prior chemotherapy, including alkylating agents and anthracyclines. Additional studies on cells from patients who had not had previous drug exposure would be important.

PALA

The new agent PALA is a transition-state inhibitor of aspartate transcarbamoylase which blocks de novo pyrimidine biosynthesis (16–19). The drug has undergone phase I study (18,19) and recently entered phase II clinical trials in the US. We have completed in vitro studies for eight patients with ovarian cancer (fig 7A) and five with melanoma (fig 7B). A broad dose range was investigated (1–100 μg/ml), with 1 hour of in vitro exposure prior to culture. The highest in vitro concentration (100 μg/ml) approximates the plasma concentration attainable after a maximally tolerated clinical dose of 7.5 g/m^2. At exposures to relatively high in vitro doses, the TCFU of three of eight patients with ovarian cancer had survival reduced to < 30% of the control. Whether such high concentrations are achievable intratumorally remains to be established. Survival of TCFU for all of the five patients with melanoma was > 30% of the control even at these high doses, although one patient's clonogenic cells did manifest a sharp drop in survival at the highest dose tested (fig 7B). In our predictive studies with standard cytotoxic drugs, we found that, for in vivo activity, drugs had to exhibit substantial activity at 5%–10% of the clinically achievable C × t product of plasma concentration (7). If similar in vitro and in vivo dose-response relationships also apply to PALA, we would anticipate that this agent should be relatively inactive in ovarian cancer and in melanoma.

Dihydroxyanthracenedione

Certain anthraquinones have been studied as potential model compounds analogous to anthracyclines such as doxorubicin and daunorubicin (20,21). These new compounds have been designed with the thought that cardiac toxicity might be averted and therapeutic efficacy enhanced. Recently, a bis(hydroxyethylamino-ethylamino) anthraquinone was found to possess antitumor activity in several mouse tumors (21). The new agent dihydroxyanthracenedione has recently entered phase I clinical trial in San Antonio, TX, and Tucson, AZ. In conjunction with our phase I trial, we have carried out preliminary observations of the in vitro lethality of dihydroxyanthracenedione in the tumor stem cell assay for seven patients with breast or gynecologic cancers. Results of these in vitro survival curves are summarized in figure 8. Survival of TCFU was reduced to < 30% of the control for one patient with endometrial cancer (1-hour exposure) and for one with ovarian cancer (continuous exposure). Pharmacokinetic data as well as the results of phase I studies will be required to determine the achievable C × t products in vivo. However, in this pilot in vitro study, a log dose response covering doses of dihydroxyanthracenedione from 0.1 to 100 μg hour of exposure should more than bracket the pharmacokinetically achievable dose exposure in vivo.

Thymidine

The new agent thymidine is a naturally occurring nucleotide in the blood, which has been shown to inhibit DNA synthesis when given in excess amounts (22) and to inhibit human tumor growth in nude mice (23). At concentrations of ≥ 1 mM (242 μg/ml),

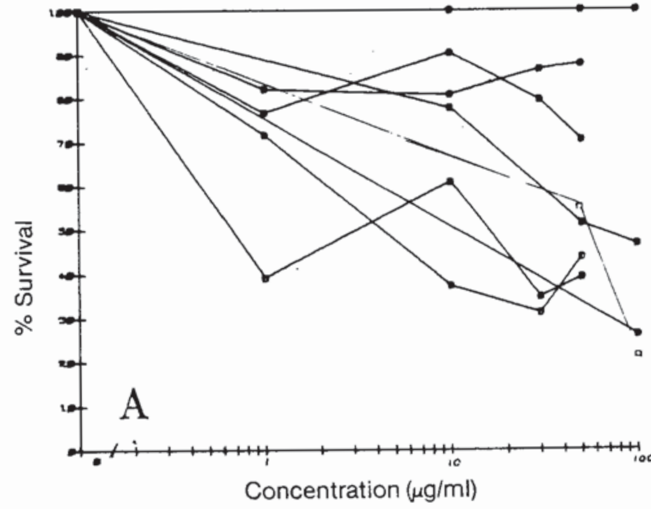
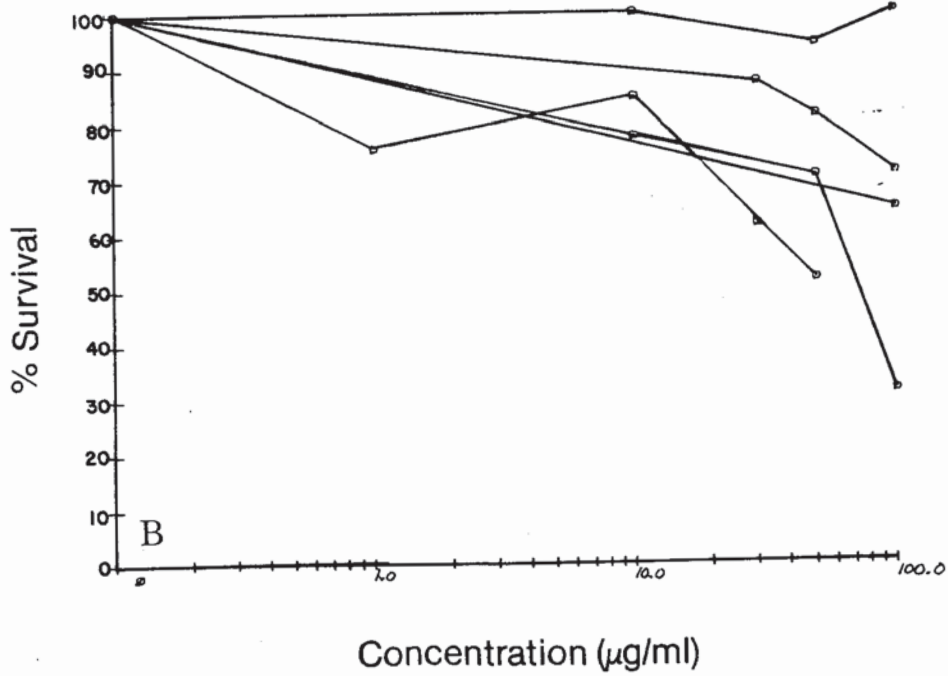


FIGURE 7.—Survival of TCFU after 1-hr exposure to PALA: (A) ovarian cancer; (B) melanoma.



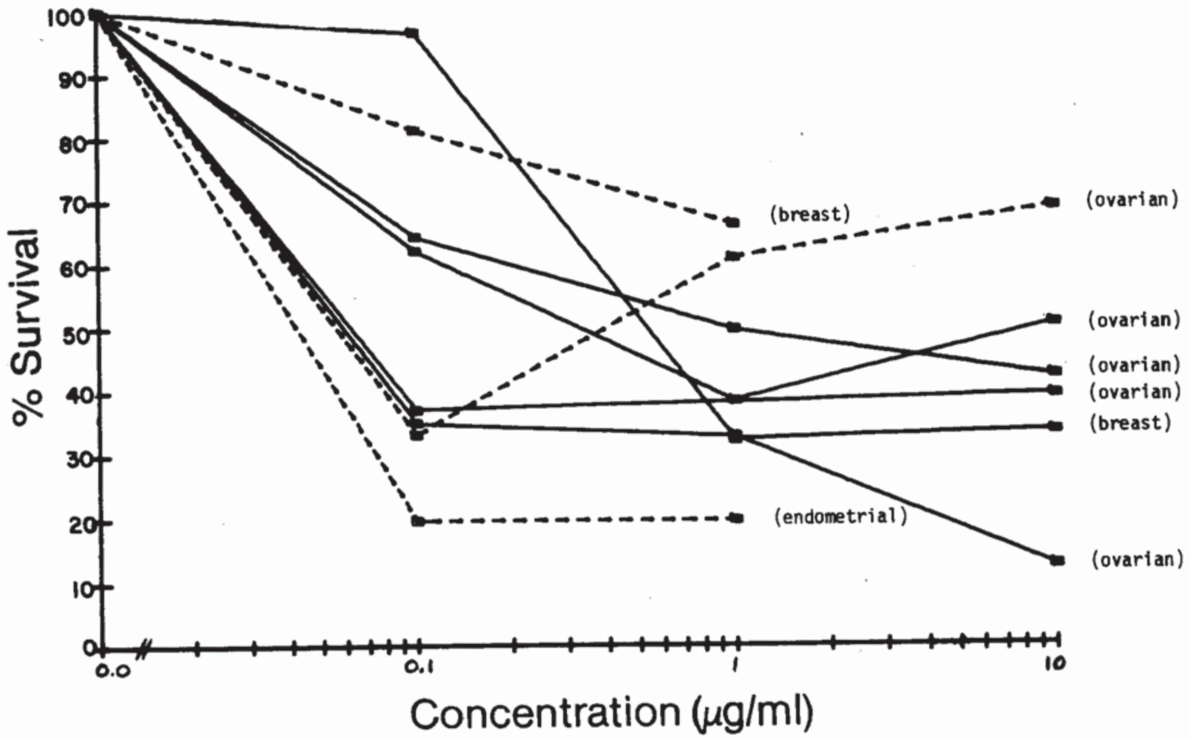


FIGURE 8.—Preliminary studies of 1-hr exposure (- - - - - -) and continuous contact (————) with dihydroxyanthracenedione on survival of gynecologic TCFU.

thymidine inhibits cellular proliferation and appears to have selective cytotoxic effects on neoplastic cells (22,23). This compound has undergone phase I study (24) and recently entered phase II trials. We have studied the effect of a broad dose range (50–1000 µg/ml) with 1-hour in vitro exposure to culture of cells from seven patients with melanoma (fig 9). One patient had reduction of TCFU to < 30% of the

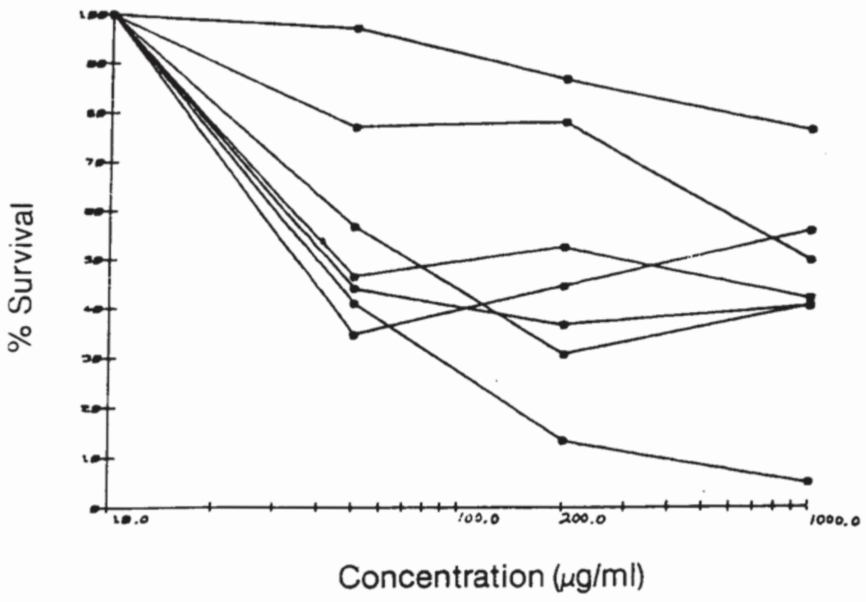


FIGURE 9.—Survival of melanoma TCFU after 1-hr exposure to thymidine.

control at a relatively high thymidine concentration (200 μg/ml). For the other patients, the plateau in survival seen with increasing drug concentration suggests that continuous exposure would not necessarily increase lethality.

DISCUSSION

In this investigation of in vitro phase II screening with the human tumor stem cell assay, we have focused on some of the new agents which are currently undergoing clinical trials in ovarian cancer and malignant melanoma. The feasibility of carrying out such in vitro studies rapidly on multiple agents is confirmed by our studies, inasmuch as we have been able to conduct simultaneous studies on these agents over the past year. It obviously has not yet been determined whether our in vitro results will correlate with the efficacy of these agents when given to heavily pretreated patients with ovarian cancer or melanoma. However, we have recently analyzed the in vitro effect of AMSA for ten patients with a variety of malignancies. Seven patients with a < 70% reduction in TCFU failed to respond while three patients with a > 70% reduction had a partial remission (16). Of greater interest in the future will be the application of this assay system to ovarian tumor and melanoma stem cells from patients who have received no prior treatment. When such data become available in assays carried out simultaneously with tests of standard agents known to be active (eg, melphalan and doxorubicin for ovarian cancer and actinomycin D and DTIC for melanoma), it might be possible to rank these drugs more realistically and to predict potential in vivo utility of new versus standard agents in such patients.

Our limited investigations of continuous contact versus 1-hour exposure for several drugs suggest that longer exposure may not offer an advantage. Clearly, this phenomenon will warrant further investigation in defining optimal drug exposures as well as in validating such observations through correlative clinical trials. The data which we have obtained thus far correlating the results of 1-hour exposure to standard anticancer drugs with results of clinical trials continue to be quite promising (10,25), and it is likely that studies from many other centers will also examine this question independently in ovarian cancer, melanoma, and other cancers over the next few years.

While many of the new phase I–II agents which we tested showed heterogeneity of response on TCFU from different patients with ovarian cancer and melanoma, it must be recognized that we have been carrying out "secondary" screening of these agents. That is, the drugs were developed on the basis of animal models employing leukemias and other neoplasms and not because they had any particular utility in ovarian cancer or melanoma. In at least one instance, we have advanced a drug to clinical trial on the basis of our in vitro sensitivity studies. We observed that 13-*cis*-retinoic acid is active in vitro in a variety of neoplasms including melanoma (5). Previously this agent had been considered as an "antipromoter" for cancer prevention rather than as a therapeutic agent. It will be of considerable interest to determine whether 13-*cis*-retinoic acid is therapeutically active and whether activity can be predicted in vitro for specific patients.

Recently, the National Cancer Institute, Bethesda, MD, has decided to make a major test of the human tumor clonogenic cell assay to determine whether it will be useful for primary drug screening of new compounds sent to the Institute for screening evaluation. The assay will thus be tested as an alternative to conventional screening in P388 leukemia and other in vivo mouse tumor systems. We believe that in primary screening, "positives" as defined with this assay should be identified when biopsy samples from the majority of a series (ie, > 50%) of untreated patients with a given tumor type manifest substantial lethality with the new agent tested. At that point, the new drug could be introduced into toxicology testing followed by clinical trial for the appropriate tumor type. We anticipate that ovarian cancer could be a signal neoplasm in the new drug screening effort (because it can be grown so readily). Melanoma, although slightly less readily grown, exhibits substantial heterogeneity (even among the clonogenic cells from each patient), and could be an excellent test system for this tumor type which is resistant to most drugs identified as active in murine systems. It seems reasonable to expect that the assay will detect new structures with more consistent efficacy in ovarian cancer and melanoma than those compounds which are currently available.

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