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Comparison of Antitumor Activity of Standard and Investigational Drugs at Equivalent Granulocyte–Macrophage Colony-forming Cell Inhibitory Concentrations in the Adhesive Tumor Cell Culture System: An *in vitro* Method of Screening New Drugs

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Abstract—*We compared the* in vitro *growth inhibition of primary human tumor cells in the adhesive tumor cell culture system (ATCCS), exposed to the investigational agents caracemide, spirogermanium and taxol and to standard chemotherapy agents at equitoxic concentrations for granulocyte–macrophage colony-forming cells (GM-CFC)* in vitro. *Clinically active standard agents tested at up to GM-CFC* 90% *inhibitory concentrations* (IC_{90}) *resulted in* in vitro *activity* (≥ 50% *tumor growth inhibition) in at least* 30% *of tumors tested.* In vitro *responses for taxol, caracemide and spirogermanium were* 78%, 9% *and* 7%, *respectively. This paper proposes a model that incorporates two hypotheses*: (1) *myelotoxic drugs which inhibit tumor growth at concentrations equal to or less than equitoxic GM-CFC* IC_5 *will demonstrate clinical activity; and* (2) *both myelotoxic and particular nonmyelotoxic drugs inactive* in vitro *at these doses will not be active clinically. If this drug screening concept is valid, taxol may be clinically more active than caracemide and spirogermanium.*

INTRODUCTION

In vitro METHODS to screen new cytotoxic agents for potential clinical activity in human tumors could limit the number of costly and often ineffective phase I and II trials. Such methods could identify potentially useful drugs that are non-cross-resistant to clinically active agents [1–11].

Because of the number of problems associated with growing human tumors in soft agar [12, 13] and the reported superior growth of malignant cells on an extracellular matrix [14–16] we developed a drug sensitivity assay based on the adhesive tumor cell culture system (ATCCS) for the growth of fresh nonhematopoietic human tumors [17, 18]. This system has a high success rate of better than 70% in most solid human tumors and a cloning efficiency of approx. 0.2%. Preliminary clinical correlations with the ATCCS have been encouraging in that *in vitro* chemosensitivity at drug concentrations derived from GM-CFC dose range have correlated positively with clinical response [19].

The drug concentration at which investigational agents should be tested *in vitro* is an important factor. Inappropriately low or high concentrations could result in inaccurate predictions for use of drugs *in vivo*. We chose to establish an *in vitro* dose-range by testing drugs over equivalent *in vitro* toxic doses to human bone marrow GM-CFC [20–24]. This may establish a therapeutic criterion for *in vitro* concentrations for screening new drugs against tumors. This approach differs from previous

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methods described by others in the soft agar system [4–11]. Prior methods utilized either continuous exposure to drug concentrations ranging over two logs, usually at 0.1, 1 and 10 μg/ml or 1 h exposure at 1/10th of the peak plasma level of drugs.

This paper describes results using our *in vitro* GM-CFC equitoxicity approach with three investigational agents: (1) caracemide, (2) spirogermanium and (3) taxol.

MATERIALS AND METHODS

Drugs

All drugs were diluted with 0.9% NaCl or distilled water, stored in 0.1 ml aliquots at −70°C, and used within 1 month. Caracemide, spirogermanium and taxol were obtained from the National Cancer Institute, Bethesda, Maryland.

GM-CFC cultures

Human bone marrow cell cultures were utilized to normalize the inhibitory concentrations of the standard and experimental cytotoxic drugs. In brief, normal marrows were obtained from healthy volunteers or patients with no tumor involvement of marrow. Aspirations from the posterior iliac crest were collected in 1 ml of calcium-free Dulbecco's phosphate-buffered saline (PBS) solution containing 300 units (U) of preservative-free heparin (Fisher Scientific, Houston, TX). Mononuclear cells were separated by Ficoll–Hypaque density gradient (density, 1.077 g/ml). For colony formation of GM-CFC, a modification of a previously described [25, 26] bilayer soft-agar system was used. The underlayer was composed of 0.5% Bacto-agar, (Difco, detroit, MI) containing alpha minimal essential medium (MEM) (K.C. Biological, Kansas City, KS), 15% undialyzed fetal bovine serum (FBS) and 10% human placental conditioned medium as a source of colony-stimulating factor (CSF); 1 ml volumes of this under layer mixture were put in 35-mm plastic Petri dishes. After solidification of the under layer, the upper layer containing the desired drug concentrations (or drug diluent for the controls), 0.32% agar, alpha MEM, 15% FBS and 1.5×10^5 mononuclear cells were added. The cultures were incubated for 8–10 days in a humidified atmosphere containing 5% CO_2 and 12% O_2 at 37°C. Five different bone marrow specimens were tested at each concentration. The drugs were assayed for GM-CFC toxicity over a concentration range.

GM-CFC (aggregates of 40 or more cells) were scored using an Olympus 200-M stereo-microscope. Cultures yielding less than 30 colonies in the control plates were not analyzed for drug response. All experiments were performed in triplicate. The survival fractions of GM-CFC were calculated as the mean number of colonies in the experimental dishes divided by the mean number of the control and multiplied by 100%. Mean and standard deviations were calculated for each concentration from the group. Survival curves were generated for each assay and IC_{50} and IC_{90} were determined by extrapolation from a 'best-fit' curve drawn on a semi-log scale [27].

Primary human tumor cell culture

Primary human tumor cells were cultured in the ATCCS [17, 18]. In brief, this is a monolayer cell culture method optimized for growth by the use of culture surfaces composed of a cell adhesive matrix (CAM) (LifeTrac, Irvine, CA) and culture medium supplemented with hormones and epidermal growth factor [18, 28, 29]. Biopsies of human tumors or the fluid specimens containing malignant cells obtained during a therapeutic or diagnostic procedure were transported to our laboratory and placed in a tissue culture medium. Preservative-free heparin (10 U/ml) was added to effusions immediately after aspiration. Solid specimens were minced to 1 mm pieces. The solid-tissue and fluid-derived cells were disaggregated to single cells by incubating in 0.75% type III collagenase (Cooper Biomedical, Melvern, PA) and 0.005% DNase (Sigma Chemical, St. Louis, MO) in Ham's F12 medium K.C. Biological) with 10% FBS for 16 h with constant stirring. The yield of viable cells was determined by a hemocytometer count of trypan blue-negative nucleated cells larger than 10 μm (excluding lymphocytes, granulocytes and mesothelial cells). The cell suspension was diluted with a methylcellulose-based attachment medium to 25,000 cells/ml, and 24-well plates (six rows of four wells) were inoculated in duplicate with: (1) a cell inoculum titration of 25,000, 12,500, 6250 and 3125 cells/well in the first column; (2) 25,000 cells in each of the remaining wells. The inoculum titration enables the evaluation of colony-forming efficiency of cultures at lower cell density, and the extrapolation of the true control value in over-plated cultures. The second column contained a day-1 control culture fixed after 24 h of incubation to provide a record of the starting cell population. It also contained one well for estimating the nondividing fraction of the seeding cells by exposing the culture continuously to tritiated thymidine (10 μCi/ml; specific activity 56 Ci/mmole, ICN Radiochemicals, Irvine, CA). Another well in this column contained only culture medium to provide for a cell-free background. The remaining columns of this and additional plates were used for drug exposures.

After 24 h incubation, the medium was aspirated, and the adherent cells were washed with PBS and refed with culture medium consisting of Ham's F12, 2.7 μg/ml HEPES buffer (Sigma), 10% swine serum

(J.R. Scientific, Woodland, CA), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO, Grand Island, NY), 10 μg/ml transferrin, 0.5 ng/ml hydrocortisone, 5 ng/ml epidermal growth factor (EGF) and 5 μg/ml insulin (Collaborative Research Lexington, MA). Drugs, reconstituted in the same medium, were also added at this time. Each drug was tested at four concentrations over a six- to eight-fold range, covering an IC_{50}–IC_{90} and greater dose-range for GM-CFC *in vitro*. After a 5-day exposure period, the drugs were removed, the cultures replenished with drug-free medium, and then incubated for an additional 7 days. The plates were incubated in a humidified atmosphere of 5% CO_2 in air for a total duration of 13 days. At the end of the incubation period, the cultures were fixed in 70% ethanol for 20 min and stained with 0.05% crystal violet.

The surviving fraction for each concentration was determined quantitatively by image analysis, utilizing a Nikon/Joyce-Loeble Magiscan-2 analysis system [17, 18]. After subtraction of background (the integrated optical absorbance of the thymidine-suicide cultures), the survival fractions for each drug concentration were calculated, the survival curves plotted, and IC_{50} values determined from the survival curves.

Reproducibility

Intra-laboratory reproducibility of drug effects in the ATCCS was evaluated by dividing the cell yield following enzymatic digestion between two technicians, who then cultured the specimens with differentially prepared drug sets and independently derived the IC values. Inter-laboratory reproducibility was also tested similarly between our laboratory and the International Clinical Laboratories (Nashville, TN). Results were analyzed by the linear regression method. Adequate levels of reproducibility were indicated. The correlation coefficients were 0.99 for four intra-laboratory experiments (10 pairs of drug points), and 0.79 for six inter-laboratory determinations (six pairs of drugs points), respectively.

RESULTS

We determined the *in vitro* sensitivity of a large number of tumor specimens to clinically active chemotherapeutic agents. *In vitro* sensitivity was defined as 50% or greater inhibition of tumor cell growth at a concentration within the *in vitro* GM-CFC IC range. The *in vitro* response of 38–202 tumor specimens to each standard agent was analyzed. The results are summarized in Table 1. *In vitro* sensitivity to standard drugs was observed in at least 31% of the human tumors tested (median 75%, range 31–100%), at a drug concentration not exceeding GM-CFC IC_{90}. Drugs with minimal or no clinical myelosuppression (e.g. cisplatin and bleomycin) achieved the highest *in vitro* tumor response rates at lower GM-CFC concentrations (IC_{50}). This indicates that the concentrations determined in the bone marrow culture assays may be biologically higher for marrow-sparing agents than for marrow-suppressive agents.

All standard agents tested in this assay demonstrated a dose-dependent relationship against primary human tumors *in vitro*, i.e. tumor response rates increased with increasing drug concentrations (Table 1 and Fig. 1). To further illustrate this

Table 1. In vitro *activity of standard chemotherapy agents against human tumors at equivalent GM-CFC* IC_{50} *and* IC_{90} *concentrations*

Agents	No. specimens tested	*In vitro* responses (%) at GM-CFC IC_{50}	IC_{90}
Cisplatin	202	54	90
Adriamycin	171	7	31
Etoposide	157	31	46
5-Fluorouracil	137	67	81
Mitomycin-C	95	23	69
Actinomycin-D	81	35	74
Carmustine	62	30	75
Vinblastin	53	16	68
Bleomycin	38	89	100

GM-CFC: granulocyte–macrophage colony-forming cells.
IC: inhibitory concentration.
In vitro activity: ≥ 50% inhibition of tumor cell growth.

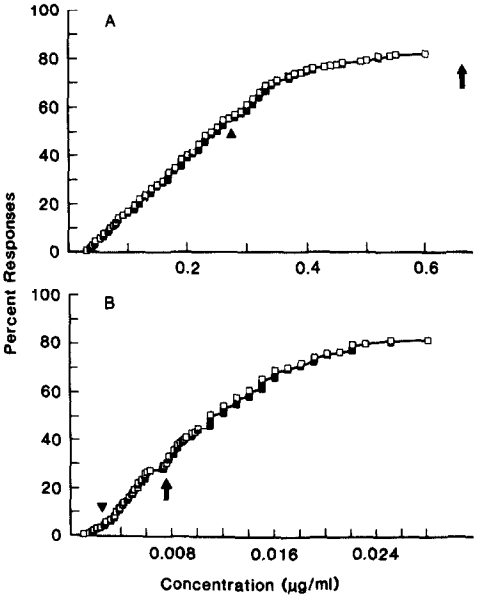

Fig. 1. *Cumulative frequency (percentage) of* in vitro *responses* (IC_{50} *values) at increasing drug concentrations* (μg/ml), *of tumors tested against standard chemotherapy agents. (A) Cisplatin* (n = 202); *(B) adriamycin* (n = 171). *The mean values of* IC_{50} *(arrowheads) and* IC_{90} *(arrows) for human bone marrow GM-CFC are also shown for each drug* (n = 5). *The GM-CFC* IC_{90} *value (arrow) for cisplatin is off-scale at* 0.92 μg/ml. IC *values of highly resistant human tumor cultures are not shown.*

Table 2. In vitro *activity of investigational chemotherapy agents against human tumors at equivalent GM-CFC* IC_{50} *and* IC_{90} *concentrations*

Agents	No. specimens tested	*In vitro* responses (%) at GM-CFC IC_{50}	IC_{90}
Taxol	36	58	78
Caracemide	33	3	9
Spirogermanium	15	0	7

GM-CFC: granulocyte–macrophage colony-forming cells.
IC: inhibitory concentration.
In vitro activity: ≥ 50% inhibition of tumor cell growth.

finding, the cumulative frequency of tumor responses to increasing drug concentrations of representative standard agents (cisplatin and adriamycin) is depicted in Figs 1A and 1B.

The disparity of cumulative tumor response within the GM-CFC toxic range between a representative clinically myelosuppressive agent (adriamycin) and clinically mildly myelosuppressive agent (cisplatin) is demonstrated here. Cisplatin achieved ≥ 50% growth inhibition in most human tumors tested at concentrations within its GM-CFC IC range (Fig. 1A). However, a plateau in response was reached in approx. 20% of tumors that were resistant to cisplatin in spite of exposure to a concentration (1 μg/ml) higher than the GM-CFC IC range (results not shown). The distribution of tumor types against which cisplatin was tested was 26% melanoma, 25% non-small cell carcinoma of the lung, 10% sarcoma, 9% genitourinary cancers, 9% gastrointestinal cancers, 7% breast carcinoma and 14% miscellaneous malignancies. Fifty-one per cent of the specimens were from patients who had not received chemotherapy. The cumulative *in vitro* response to adriamycin is shown in Fig. 1B; approximately 31% of the tumors achieved ≥ 50% inhibition within the GM-CFC IC_{50}–IC_{90} range (2–9.5 ng/ml). As the concentration of adriamycin was increased to three-fold (23 ng/ml) above the mean GM-CFC IC_{90} (7.8 ng/ml), approx. 80% of the tumors achieved ≥ 50% inhibition.The tumor types tested with adriamycin included: 26% non-small cell carcinoma of the lung, 20% melanoma, 12% sarcoma, 9% breast carcinoma, 9% gastrointestinal carcinoma, 9% genitourinary carcinoma and 15% miscellaneous tumors. Only 33% of the specimens were from patients who had not received prior chemotherapy.

In vitro antitumor activity of the investigational agents, defined as achievement of ≥ 50% inhibition of growth of human tumors at GM-CFC IC_{50} and IC_{90}, is depicted in Table 2 and Fig. 2. The *in vitro* response rate to both caracemide and spirogermanium was < 10% (Table 2). This is in contrast to standard agents that are relatively marrow-sparing, e.g. bleomycin and cisplatin (Table 1). Moreover, at equivalent GM-CFC ICs, the response rate of tumors to caracemide and spirogermanium was much lower than that achieved by most standard agents known to be clinically myelosuppressive.

The *in vitro* response rates to taxol (58% at GM-CFC IC_{50} and 78% at GM-CFC IC_{90}; Table 2), are similar to those derived from standard clinically myelosuppressive agents (Table 1), suggesting that taxol may be effective against human tumors. The cumulative tumor response to these investigational agents with increasing *in vitro* doses is also illustrated (Fig. 2). Caracemide had only a small increment in antitumor activity at concentrations above the GM-CFC IC range; concentrations four-fold (34 μg/ml) greater than the GM-CFC IC_{90} (8.6 μg/ml) resulted in response rate of only approx. 40% of human tumors tested. Spirogermanium was even less active *in vitro*, with a response rate of 0% at GM-CFC IC_{50} and 7% at GM-CFC IC_{90} (Table 2). However, at concentrations 1.5-fold (0.42 μg/ml) above the mean GM-CFC IC_{90} (0.28 μg/ml), a steep but limited *in vitro* response (62% of the tumors tested) which subsequently plateaued, was observed. With taxol, however, only 22% of tumors did not achieve 50% inhibition of growth at the maximum concentration tested (Fig. 2C).

Thirty-three tumors tested with caracemide, 31 with taxol and 15 with spirogermanium were also tested with cisplatin over a concentration range of 0.067–1.8 μg/ml. The cumulative responses of these tumors to cisplatin were compared with a reference cumulative response of 126 tumors tested previously with cisplatin, and there was no significant difference in their sensitivity profiles (Fig. 3A). Similarly, 31 tumors tested with caracemide, 33 with taxol and 14 with spirogermanium were also tested with adriamycin over a wide concentration range (1.5–27 ng/ml). The cumulative response of these tumors to adriamycin was compared with that of 104 reference tumors previously tested with adriamcyin and no significant difference was noted (Fig. 3B).

DISCUSSION

We have presented an alternative method of screening new agents using the ATCCS, a newly developed drug sensitivity assay that supports malignant cell growth as documented previously [17, 18] and recently by flow cytometric analysis of DNA content and by nude mouse xenograft experiments (unpublished data). Using the GM-CFC toxic range to define equitoxic doses, we observed that the clinically active chemotherapy agents resulted in ≥ 50% inhibition of tumor growth (*in vitro* responses) in approx. 40% of the tumors tested. Furthermore, the majority of a mis-

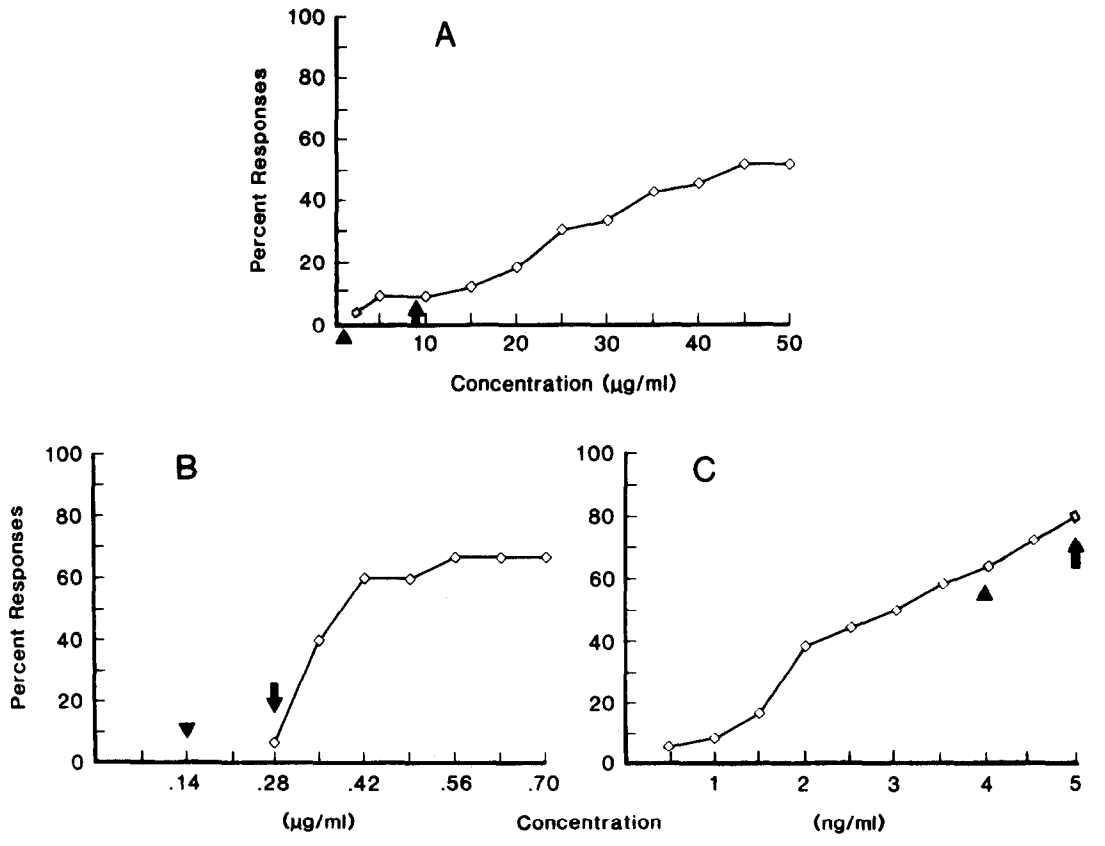

Fig. 2. Cumulative frequency (percentage) of in vitro *responses* (IC_{50} *values), at increasing concentrations* (μg/ml *and* ng/ml), *of tumors tested against investigational agents. (A) Caracemide* (n = 33); *(B) spirogermanium* (n = 15); *and (C) taxol* (n = 36). *The mean values of* IC_{50} *(arrowheads) and* IC_{90} *(arrows) for human bone marrow GM-CFC are also shown for each compound* (n = 5). IC *values of highly resistant human tumor cultures are not shown.*

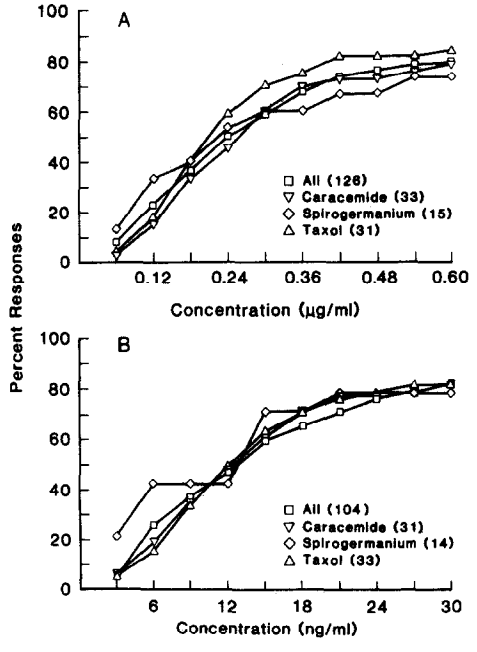

Fig. 3. Internal chemosensitivity profiles (cumulative percentage responses to IC_{50} *concentrations) of standard chemotherapy agents. (A) Cisplatin* (μg/ml), *(B) adriamycin* (ng/ml), *of the panel of tumors chosen to screen the investigational agents caracemide, spirogermanium and taxol. Shown also are the known sensitivity profiles (All) of a larger group of reference tumors initially tested with cisplatin and adriamycin. Figures in parentheses indicate the number of determinations.*

cellaneous group of tumors with various treatment backgrounds demonstrated a dose-dependent *in vitro* response to clinically active agents, e.g. adriamycin, at concentration up to approx. three-fold above the GM-CFC range. However, approx. 20–30% of these tumors were resistant regardless of the drug concentrations tested. Based on these observations, we would anticipate that myelotoxic drugs which are active *in vitro* in the ATCCS (30% response rate of greater) at concentrations equal to or less than the GM-CFC IC_{90} dose may have clinical activity against nonhematopoietic neoplasms, and both myelotoxic and particularly nonmyelotoxic drugs inactive *in vitro* at these doses may not be clinically active.

These relationships, derived from studies with standard agents, may be useful for interpretation of the *in vitro* data obtained from studies with the investigational agents. The phase I and II studies of caracemide and spirogermanium to date [30–37] have not reported myelosuppression as a dose-limiting toxicity; therefore, the *in vitro* concentrations derived from the GM-CFC assay were probably artificially high. This, and the fact that caracemide and spirogermanium had less than 10% response rates at concentrations corresponding to their IC_{90} of GM-CFC, would suggest that they may actually be relatively ineffective agents. This notion

should be viewed especially in the light of the *in vitro* data available on bleomycin and cisplatin; two drugs that share similar clinical marrow-sparing properties with caracemide and spirogermanium.

Caracemide was tested at concentrations between 5 and 50 μg/ml, higher than the *in vitro* concentrations of any clinically active agents listed in Table 1, and yet it was still found to have a very low antitumor activity. Taxol, on the contrary, appeared to be an active antitumor agent and achieved a 75% *in vitro* response rate at concentrations of only 0.005 μg/ml. However, if the *in vitro* equitoxic concentrations of taxol were not defined by GM-CFC assay prior to its screening against human tumors, an unduly optimistic projection of its efficacy would be made.

The panel of tumors tested with caracemide, spirogermanium and taxol included diverse histologic types. They were derived from both previously treated and untreated patients. Thus, it was thought that non-specific selection of specimens in the tumor panel might introduce erroneous conclusions. For example, a panel of resistant tumors selected for drug screening may determine that the drug under consideration is inactive (a false-negative result); on the contrary, a panel of sensitive tumors may produce artificially optimistic conclusions (a false-positive result). Such bias in our system was not obsrved when the *in vitro* response profiles of the tumor panel to standard agents, e.g. cisplatin and adriamycin, were compared to those of our general experience (i.e. reference profiles) with a large number of tumors to the same standard agents. As noted in Figs 3A and 3B, the *in vitro* chemosensitivity profiles to standard agents of tumor selected for screening new agents did not vary compared with that of large number of tumors tested in our laboratory. Therefore, tumor selection bias is either eliminated or insignificant in this assay.

The process of screening investigational agents described here requires firstly, a definition of inhibitory concentrations against GM-CFC; secondly, selection of a tumor panel that is well balanced in its sensitivity profile to clinically known agents; and thirdly, selection of at least three drug concentrations—the two lower concentrations should closely correspond to the GM-CFC IC_{50} and IC_{90}, and the highest concentration should be approx. three-fold higher than GM-CFC IC_{90}. The drug exposure to the tumor cells should be continuous to avoid possible false-negative results associated with cell-cycle specific agents. The prolonged exposure does not compromise activity of cell-cycle non-specific agents. This approach certainly needs further validation; however, it provides an attractive alternative or supplement to current approaches.

This concept of predicting clinical activity of an investigational agent is a complex process, particularly for marrow-sparing agents, because *in vitro* concentrations determined by the GM-CFC assay may be artificially (biologically) high. As evidenced by the outcome of our studies with standard agents, GM-CFC ICs for nonmyelosuppressive drugs could be one to two logs higher than the true predictive doses in nonhematopoietic tumor cells. A more appropriate method to establish accurate equitoxic concentrations for testing nonmyelosuppressive drugs would be to use malignant cell lines derived from sensitive tumors such as small cell lung carcinoma as target cells.

The GM-CFC assay-derived concentration range, however, may be accurate and appropriate for testing myelosuppressive investigational agents, such as taxol. Taxol demonstrated good activity at nanogram concentrations which is well within its GM-CFC toxic range.

Although seven clinical responses were reported with spirogermanium in 28 breast carcinoma patients [31] and two partial responses were noted in 18 previously treated ovarian carcinoma patients [32], no response was seen in 36 patients with renal cell carcinomas [33]. The lack of clinical activity with spirogermanium, as our assay would predict, has been more recently documented in patients with breast [34, 35], ovarian [36] lung [38], and colorectal [39] cancers.

Caracemide and taxol have completed phase I studies and should enter phase II studies shortly [40–44]. If this GM-CFC equitoxicity approach for screening drugs proves valid, our assay would predict that caracemide will be clinically relatively ineffective against nonhematopoietic neoplasms and that taxol has interesting prospects of being clinically active.

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