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Chemosensitivity Testing of Human Colorectal Carcinoma Cell Lines Using a Tetrazolium-based Colorimetric Assay[1]

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ABSTRACT

The *in vitro* chemosensitivity of 11 human colorectal cell lines to seven chemotherapeutic agents was determined using a semiautomated tetrazolium-based colorimetric assay (MTT assay). Four of the cell lines were from primary tumors and seven from metastases. Eight lines were from patients with no prior chemotherapy. From assay results, we predict 5-fluorouracil (5-FU) to be the sole active agent of the seven tested. This is based on two observations: the range of drug concentrations which produced 50% inhibition of cell growth was greatest with 5-FU (388-fold *versus* 5- to 30-fold with the other six agents); and the area under the curve (AUC) which produced 50% growth inhibition was within a clinically achievable range only for 5-FU. Since the assay AUC of 5-FU at 50% inhibition was in a clinically achievable range for only two of the 11 cell lines, we performed a multivariate analysis to explore parameters which predict 5-FU sensitivity. In the best fitting model, sensitivity was positively correlated with cloning efficiency in media and with cell surface TAG-72 (a tumor-associated glycoprotein found on epithelial tumors of ovary, lung, colon, and breast origin) expression. If validated with an *in vivo* test such as the nude mouse model, the MTT assay could be very useful in new drug screening for colorectal carcinoma, for examining combination chemotherapy for synergy, for exploring strategies for biochemical modulation, and perhaps in individualizing therapy when cell lines can be established from a patient.

INTRODUCTION

Colorectal cancer is one of the most common malignant tumors, with a reported incidence second only to lung cancer in the U. S. An estimated 140,000 new cases will be diagnosed in 1986, and 60,000 of them will ultimately die of their disease (1).

Chemotherapy for colorectal cancer is relatively ineffective (2). It is not clear that any systemic chemotherapeutic agent increases survival in the population of patients with advanced colorectal cancer. Some have suggested that patients with metastatic colorectal carcinoma be offered experimental chemotherapy as their initial treatment, in view of the disappointing results with currently available drugs (3, 4). Since most newly identified cytotoxic agents have been ineffective in the treatment of colorectal cancer, a predictive preclinical model would be very helpful.

Animal tumor models, such as murine P388 and L1210 leukemia models predict poorly for drug activity in the common human solid tumors. Hence, a major effort is under way to explore more disease-oriented assay systems in new drug development (5). One such model is the *in vitro* testing of human tumor cell lines. Human tumor stem cell assays have been used, but are inconvenient (6-8). They are also plagued by technical difficulties such as cell clumping (9). Such difficulties can translate into variability in results from different laboratories (10). Moreover, cloning efficiency of some cell lines is too low for practical application of this assay (8).

Recently, the semiautomated tetrazolium-based MTT[4] colorimetric assay has been used to measure cell survival and chemosensitivity (11-15). Our laboratory has reported that the assay yields results very similar to the clonogenic tumor stem cell assay using the CHO-AuxB1 cell line and the pleiotropic drug resistant CH^RC5 cell line (14).

We therefore used the MTT assay for 11 colorectal carcinoma cell lines established and characterized by us[5] to investigate the relationships between the characteristics of the cell lines and the *in vitro* response to seven chemotherapeutic agents.

MATERIALS AND METHODS

Cell Lines and Antigen Expression. We have previously published a detailed characterization of our human colorectal carcinoma cell lines.[5] They represent a wide range of morphologic phenotypes including well differentiated, moderately differentiated, poorly and undifferentiated, and mucinous morphology (see Table 1). As noted, four cell lines were from primary tumors and seven from metastases. Eight cell lines came from patients with no prior chemotherapy. Exponentially growing cultures of cell lines were grown in R10 and were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air. For determination of colony forming efficiency in liquid medium, single cell suspensions (one cell/two wells) in R10 medium were seeded into five replicate 96-well plates (0.2 ml/well). Growing colonies were counted 28-35 days later.

Expression of CEA (15), CA 19-9,[5] and TAG-72 (16, 17) surface antigens were measured as follows: Cells (1×10^6) were seeded into replicate 75-cm^2 flasks (15 ml total volume) and washed ×3 when semiconfluent. Three days later, cells from one flask were enumerated after trypsinization and then discarded. Cells from the other flask were harvested by using a rubber policeman, and by washing three times. They were resuspended in 1% Nonidet-P40 with 100 μM phenylmethylsulfonyl fluoride (approximately 5-10 ng of cellular protein/ml), sonicated twice on ice for 15 s and stored at −70°C until assayed. CEA was measured by a commercial enzyme-linked immunosorbent assay using a monoclonal antibody (Abbott Laboratories, North Chicago, IL). A sialylated Lewis A (CA 19-9) mucin antigen was determined by sandwich immunoassay using a CA 19-9 monoclonal antibody (17). TAG-72 was measured in a homologous sandwich radioimmunoassay using the B72.3 monoclonal antibody in a microtiter plate assay configuration (18, 19).

MTT Assay. Single cell suspensions were obtained by pipet disaggre-

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[4] The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (tetrazolyl blue); 5-FU, 5-fluorouracil; R10, RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum; CEA, carcinoembryonic antigen; AUC, area under the curve.

[5] Park, J-G., Oie, H. K., Sugarbaker, P. H., Henslee, J. G., Chen, T-R., Johnson, B. E., and Gazdar, A. F. Characteristics of cell lines established from human colorectal carcinoma. Cancer Res., in press, 1987.

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Table 1 *Characteristics of 11 human colorectal carcinoma cell lines*

Degree of differentiation	Primary tumor site	Cultured tumor site	Prior therapy	Cloning efficiency[a]	TAG-72 (ng/mg protein)
Well					
NCI-H548	Sigmoid	Primary	None	0	0
NCI-H630	Rectum	Liver	FAM[b] & R[c]	4	0
NCI-H684	Sigmoid	Liver	None	0	3833
Moderately					
NCI-H508	Cecum	Abdominal wall	5-FU	0	7889
NCI-H747	Cecum	Node	None	0	0
SNU-C1	Descending	Peritoneum	None	8	1200
Poorly/undifferentiated					
SNU-C2A	Cecum	Primary, xenograft	None	0	2400
SNU-C4	Transverse	Primary, xenograft	None	1	800
SNU-C5	Cecum	Primary, xenograft	None	12	0
NCI-H716	Cecum	Ascites	5-FU	11	0
Mucinous					
NCI-H498	Ileo-cecum	Peritoneum	None	0	250

[a] Cloning efficiency: percentage of cloning efficiency in liquid medium.
[b] FAM, 5-fluorouracil, doxorubicin (Adriamycin), and mitomycin-C.
[c] R, radiotherapy.

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gation of the floating cell lines or by trypsinization of monolayer cultures, and cell counts were performed using a hemocytometer. The MTT assay was performed as previously described (14). Briefly, the number of cells plated into 96 wells was determined after preliminary cell growth studies using the MTT assay so that untreated cells were in exponential growth phase at the time of initial harvest and at the end of the 4-day incubation. Equal numbers of cells were inoculated into each well in 0.18 ml of R10 medium, to which 0.02 ml of 10× concentration drug or phosphate buffered saline was added. For each drug, 5–10 concentrations were used, covering a 3-5-log concentration range chosen to span the 50% inhibitory concentration determined by preliminary assays. After 4 days of culture, with or without drug, 0.1 mg (50 μl of 2 mg/ml) of MTT (Sigma Chemical Co., St. Louis, MO) was added to each well and incubated at 37°C for a further 4 h. Plates were centrifuged at 450 × *g* for 5 min in a plate holder and then the media was aspirated from plates leaving about 30 μl of media in each well. Care was taken not to disturb the formazan crystals at the bottom of the wells. One hundred fifty μl of dimethyl sulfoxide (Sigma) was added to each well and the plates were placed on a shaker for 10 min to solubilize the formazan crystals. The plates were then read immediately at 540 nm on a scanning multiwell spectrophotometer (enzyme-linked immunosorbent assay reader; Biotek Instruments Inc., Burlington, VT). All data points represent the mean of a minimum of six wells. Percentage of control absorbance was considered to be the surviving fraction of the cells. This has been shown by others comparing cell numbers to absorbance (11, 15) and confirmed in our laboratory.[6] Additional controls consisted of media alone with no cells, with or without the various drugs. Absorbance levels from drug tested cells were corrected against untreated control absorbance values. The ID_{50} value was defined as the concentration of drug which produced 50% reduction of absorbance at 540 nm. Results of duplicate assays performed on line SNU-C4 for five drugs (5-FU, mitomycin-C, etoposide, doxorubicin, and cisplatin) were closely correlated ($r = 0.99$).

Calculation of Assay AUC. Drug stabilities in serum-containing media of a number of chemotherapeutic agents have been reported under incubation conditions at 37°C in the presence of 6% CO_2 (20). The half-life of 5-FU and mitomycin C are greater than 14 days. The half-lives of doxorubicin, cisplatin, melphalan, and carmustine are 29.0, 18.5, 1.8, and 1.0 h, respectively. Stability of etoposide (VP16) at 37°C in serum-containing media has been reported to be 60 h (21). For 5-FU and mitomycin C, the assay AUC was therefore calculated as the drug concentration × 96 h (the period of continuous exposure in the assay). For other drugs, the assay-AUC was calculated by the following formula:

$$\text{Assay-AUC} = \text{Initial concentration} \times t_{1/2} \times 1.44 \times [1 - e^{-(.693)(96)/t_{1/2}}] \quad \text{(A)}$$

where $t_{1/2}$ is the *in vitro* half-life of the drug at 37°C.

[6] Unpublished data.

Drugs. All drugs were obtained from commercial sources: 5-FU (Roche Lab., Nutley, NJ); doxorubicin (Adriamycin; Adria Lab., Columbus, OH); cisplatin (Ben Venue Lab., Bedford, OH); Melphalan (Burroughs Wellcome Co., Research Triangle Park, NC); etoposide (VP-16), mitomycin-C and carmustine (BCNU; Bristol Lab., Syracuse, NY). 100 mg powder of BCNU was dissolved in 3 ml ethanol and then diluted with PBS. 100 mg of melphalan was completely dissolved in 1 ml of acid alcohol; 9 ml of Wellcome diluent (containing 12 mg per ml of dipotassium phosphate, 0.6 ml per ml propylene glycol in water) was then added and then diluted with phosphate buffered saline for use. Other drugs were dissolved or diluted with phosphate buffered saline. All drugs were prepared immediately prior to use.

Analysis of Data. Data were key entered onto the NIH IBM mainframe computer, and sample statistics were computed for each of the seven drug concentrations used to generate a growth inhibitory curve and the descriptive variables. Tables were constructed which associated the concentrations of drugs with the other variables of interest in order to roughly characterize the association between the concentrations and the other variables. Those variables which were found to have some association with drug concentration were checked for correlation with one another (using the nonparametric Spearman rank correlation method). To avoid statistical difficulties, when two variables were found to be highly correlated with one another, one of the two (not both) was selected for use in the regression analysis. The variables that remained for further considerations were thus: (*a*) potentially associated with the drug concentration, and (*b*) relatively independent of one another. These were the variables to be used in the multiple regression modeling (22). We examined the distributional characteristics of these remaining variables and decided whether the variable itself should be included in the model, or whether a transformation was required. Several of the variables were in obvious need of transformation in order to be useful in a model—those with a range from 0 to 50,000, for example, were reasonable candidates for logarithmic transformation. Variables with values which extended from 0 to any very large number were transformed by adding 0.001 to their values before taking the logarithm in order to prevent infinitively small logarithms. Variables such as blood type were coded numerically to allow incorporation into the model (*i.e.*, if blood type = A+, then blood = 0; otherwise blood = 1). Finally, models using actual concentrations, as well as logarithm of concentration, were considered for analysis. The best model is detailed in the RESULTS section.

RESULTS

The ID_{50} means and standard deviation for each drug are catalogued in Table 2. The cell lines are categorized according to morphology (differentiation) in tissue culture. In all 11 lines degree of differentiation *in vitro* correlated well with the histo-

Table 2 *Mean values of ID_{50} (μg/ml) for the colorectal cell lines exposed to seven chemotherapeutic agents*

Differentiation	5-FU	Mit-C	VP-16	Platin	Adriamycin	Melphalan	BCNU
Well							
NCI-H548	233 (133)[a]	1.2 (0.1)	76 (51)	3.6 (1.3)	0.25 (0.02)	12 (0)	24 (10)
NCI-H630	43 (30)	1.0 (1.1)	30 (24)	3.5 (3.1)	0.30 (0.24)	20 (3.0)	30 (4.6)
NCI-H684	15 (11)	0.5 (0.47)	61 (26)	1.8 (0.8)	0.76 (0.38)	14 (0.6)	11 (5.5)
Moderately							
NCI-H508	39 (60)	0.29 (0.14)	15 (21)	2.0 (0.87)	0.21 (0.06)	13 (2.1)	17 (1.4)
NCI-H747	17 (4.6)	0.70 (0.14)	74 (30)	7.6 (0.51)	0.59 (0.22)	15 (4.0)	32 (4.6)
SNU-C1	0.60 (0.56)	0.17 (0.07)	3.0 (0.87)	0.93 (0.06)	0.10 (0.08)	9.7 (5.0)	19 (9.0)
Poorly/undifferentiated							
SNU-C2A	5.1 (3.3)	0.36 (0.09)	23 (15)	2.3 (0.32)	0.47 (0.24)	35 (4.0)	19 (5.0)
SNU-C4	0.93 (0.06)	0.11 (0.09)	28 (3.5)	0.80 (0.60)	0.41 (0.33)	10 (2.6)	18 (3.6)
SNU-C5	5.0 (5.3)	0.53 (0.04)	30 (42)	1.2 (0.45)	0.19 (0.05)	12 (7.5)	22 (11)
NCI-H716	6.6 (3.9)	0.04 (0.03)	4.0 (3.6)	0.77 (0.64)	0.11 (0.10)	6.3 (2.5)	7.0 (3.6)
Mucinous							
NCI-H498	1.4 (0.84)	0.10 (0.07)	58 (36)	1.5 (0.3)	0.16 (0.14)	10 (3.6)	19 (7.1)

[a] Numbers in parenthesis, standard deviations determined from three experiments.

Table 3 *ID_{50} Range of 11 tested cell lines to seven chemotherapeutic agents with comparison to clinically achievable area under the curve (CA-AUC) for standard doses of each drug*

	5-FU	Mit-C	VP-16	Cisplatin	Adriamycin	Melphalan	BCNU
ID_{50} range (μg/ml)	0.6–233	0.04–1.2	3–76	0.77–7.6	0.1–0.76	6.3–35	7.0–32
Variation (fold)	388	30	25	10	8	6	5
Assay AUC (μg-h/ml) at ID_{50}	58–22,368	3.8–115	174–2,384	20.5–202.5	3.8–28.5	16.3–90.7	10.1–46.1
Commonly used i.v. doses in patients	15 mg/kg (for up to 5 days)	20 mg	290 mg/m²	100 mg/m²	60 mg/m²	0.6 mg/kg	95 mg/m²
CA-AUC[a]	16–90	0.36	154	1.9	1.6–2.0	2.5	1.0
Peak serum concentrations clinically (μg/ml)[+]	60	1.5	34	2.5	0.36–0.6	3.4	2.0

[a] CA-AUC, Clinically achievable AUC (see "Results"). Values obtained from Ref. 22.

logical differentiation of the cell lines when grown as nude mouse xenografts by s.c. passage.

Table 3 shows the ID_{50} range of our 11 tested cell lines to the seven cytotoxic agents. For each ID_{50} value, we calculated an approximate assay AUC as described in "Materials and Methods." Clinically achievable AUC in humans are given for comparison in Table 3 for commonly used regimens of each drug (23).

As seen in Table 3, 5-FU had the largest range in ID_{50} from most to least sensitive cell line (388-fold) among the seven drugs tested. All other agents had a much narrower range of ID_{50} (from 5- to 30-fold). As an example, the two extremes are presented in Fig. 1, demonstrating the striking difference between 5-FU and BCNU.

Table 3 also demonstrates that the assay AUC at ID_{50} was well above a clinically achievable AUC for every agent except 5-FU. For 5-FU, two of the cell lines (SNU-C1 and SNU-C4) had an assay AUC within a clinically achievable range at ID_{50}.

To determine whether the results of the MTT assay are stable with *in vitro* cell passage, the SNU-C2A line was tested serially for sensitivity to BCNU, 5-FU, Adriamycin, and mitomycin at monthly intervals. As shown in Fig. 2 assay results were stable over a 3- to 4-month period (six passages).

A multiple regression analysis was performed in an attempt to identify parameters associated with sensitivity to 5-FU. Parameters evaluated in the models included: morphology, *in vitro* cell doubling time, plating efficiency, cloning efficiency in semisolid media, cloning efficiency in liquid media (see Methods), cell surface CEA, cell surface TAG-72, CA 19-9, media used to establish the line (R10 *versus* ACL-4) (24, 25), prior chemotherapy, patient blood type, and tumor site (primary *versus* metastasis). The list of all variables examined for the analysis is shown in Table 4. The best fitting model was:

$$\text{Log}_{10}(\text{5-FU concentration}) = 1.18 - 0.106(\text{\% cloning efficiency in media}) - 0.209(\text{TAG-72}) \quad (B)$$

where TAG-72 = $\log_{10}$(TAG-72 + 0.001). The F value was 15.1 with (2, 29) degrees of freedom ($P < 0.0001$), indicating an extremely good fit. In other words, concentration is inversely related to % cloning efficiency in media and TAG-72 antigen expression. No other models approached the above one in goodness of fit.

DISCUSSION

We have reason, on the basis of our experimental results, to predict that 5-FU would be the sole active agent of the seven tested in our cell lines and that it would be most likely active in the SNU-C1 and SNU-C4 lines. First, the range of drug concentrations which cause 50% inhibition of the cell lines (ID_{50}) is greatest for 5-FU. By contrast, the ranges of ID_{50} for all of the agents are relatively narrow. It is much more likely that all lines are resistant to these agents than that all are sensitive. In the case of 5-FU, it is certainly possible that all lines tested are resistant to 5-FU. However, it is encouraging that the calculated assay AUC for 5-FU is within the clinically achievable AUC of 5-FU in humans for two of the lines tested (Table 3). The assay predicts that a minority of the tested cell lines are sensitive to 5-FU, but this may simply reflect the true clinical situation, in which a minority of patients with colorectal carcinoma respond to 5-FU (2).

Our results are similar to those of Finlay *et al.* (15), who found a rather narrow range of inhibitory doses of a variety of cytotoxic agents against four human colon carcinoma lines. In contrast to our study, they found a narrow range using 5-fluorouracil. However, this was with fewer colon lines than in the present study.

The concentration over time concept has been reported by Alberts *et al.* as a means to calculate a sensitivity index for in vitro drug assays (26). With the use of continuous exposure, especially with "cycle active" drugs, it may be possible to decrease the frequency of false negative results reported in the

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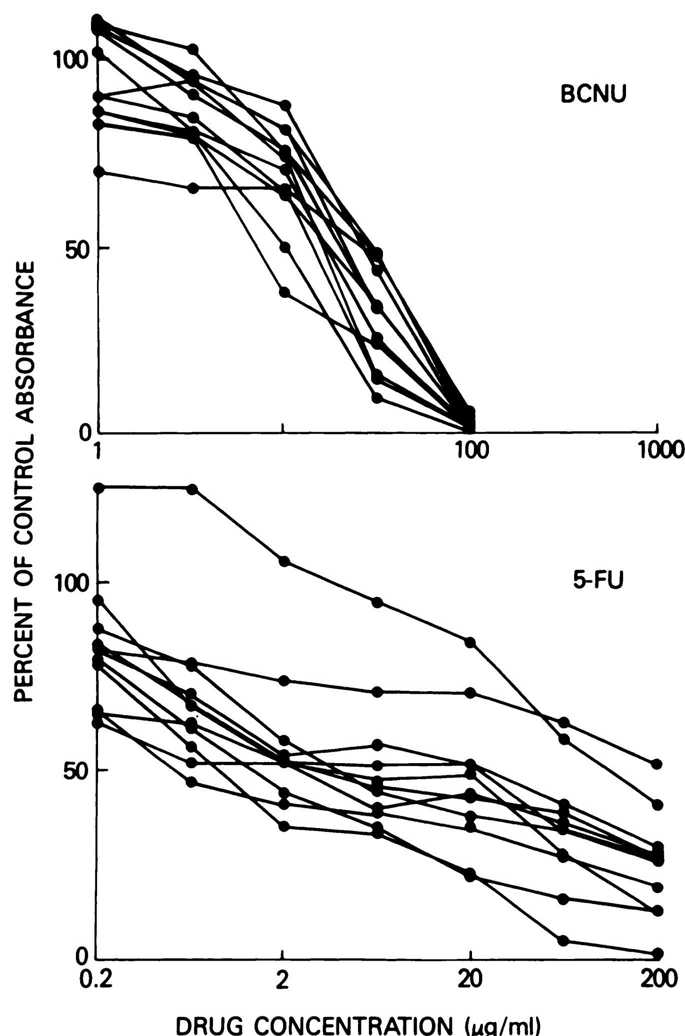

Fig. 1. Comparative effects of BCNU and 5-FU on colorectal carcinoma lines. Percentage of the control absorbance is plotted against the drug concentration in μg/ml. *Lines*, cell lines.

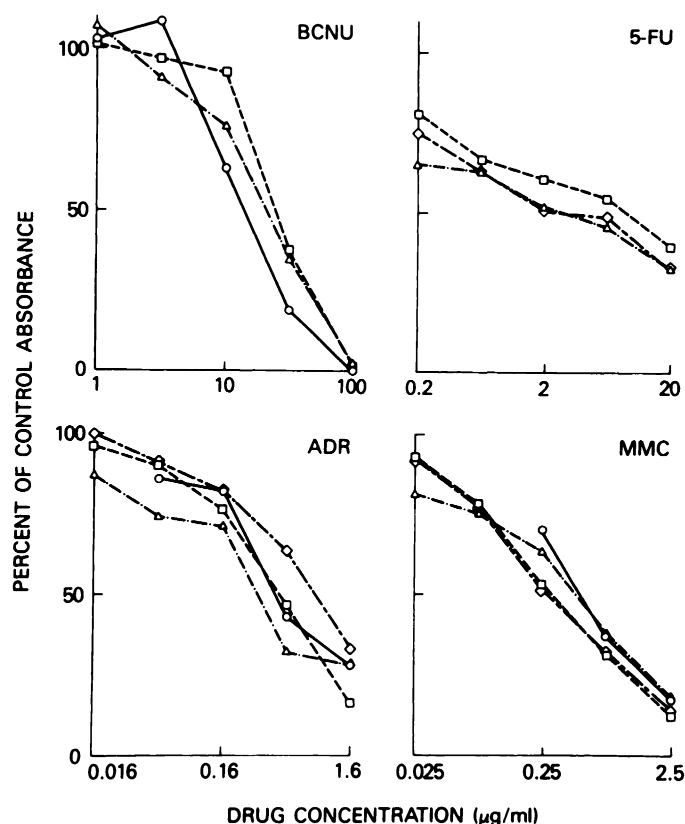

Fig. 2. Drug sensitivities of the cell line SNU-C2A were tested approximately monthly from July through October, 1986: (○– – –○) passage 24; (△–·–△) passage 27; (□– – –□) passage 28; (◇– –◇) passage 29.

Table 4 *Parameters examined for multiple regression analysis of correlation to 5-FU sensitivity*

Degree of morphological differentiation (in tissue culture and mouse xenografts)
Patient age
Patient sex
Patient race
Patient blood type
Prior chemotherapy
Primary tumor site
Source of culture (primary *versus* metastasis)
Initial growth medium (R10 *versus* ACL-4)
In vitro doubling time
Plating efficiency
Cloning efficiency in semisolid medium
Cloning efficiency in liquid medium
Dopa decarboxylase activity[a]
Chromosome number (pleudy)
CEA (carcinoembryonic antigen) expression
CA 19-9 expression
TAG-72 expression

[a] Dopa decarboxylase activity, a marker of neuroendocrine cells, has been found in 10 of these 11 lines, (Ref. 15).

human tumor stem cell assay using one hour exposure times (24).

No comment can be made on clinical response of the two patients from whom the two "sensitive" cell lines were derived. One never received chemotherapy, and the other received chemotherapy in the adjuvant setting, with no measurable tumor.

A multivariate analysis of parameters which predict *in vitro* 5-FU sensitivity produced one well-fitting model, which identified a positive correlation between chemosensitivity and cloning efficiency in media as well as cellular expression of TAG-72. The fact that cloning efficiency in media correlates well with sensitivity to 5-FU reasonably suggests that those lines with greatest stem cell proliferation capacity are most susceptible to attack on DNA synthesis. The TAG-72 is an antigen found on carcinoma cells of ovary, lung, colon, and breast. Its function is not known but it appears to reflect a more malignant phenotype of epithelium (27). Others, using the human tumor stem cell assay, have noted a similar relationship between sensitivity of human colorectal carcinoma cells to 5-FU and high proliferative potential (28). It is of interest that in a recent pilot study of the combination *N*-phosphonacetyl-L-aspartate, thymidine, and 5-FU in colorectal carcinoma, the response rate was felt to be particularly high in the subset of patients with anaplastic histology (29).

As more cell lines are accumulated the model derived from this multivariate analysis can be tested for generalizability. If it does apply to more cell lines and drugs, the model may help predict which lines are susceptible to systemic agents.

The MTT assay is well suited to the testing of characterized cell lines, and therefore could be useful in a disease-oriented screening program. There is not *a priori* reason to assume that clonogenic assays more accurately reflect *in vivo* drug activity than nonclonogenic assays such as the MTT test (9). In any case, results using the two assays are similar. A semiautomated assay such as the MTT may even become a useful tool in testing combinations of chemotherapeutic agents for synergy. Using human lung cancer lives we have demonstrated synergy between

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drugs commonly used in the therapy of small cell lung cancer.[7]

The utility of the above applications of the MTT assay, of course, hinges on the correlation of the assay with *in vivo* tumor sensitivity. It is of interest that the assay predicts 5-FU to be the most active agent overall in our cell lines, since 5-FU is the most widely used drug for colorectal carcinoma in the clinic. However, it would be important to validate the results with an *in vivo* assay, since it is not known how well the *in vitro* drug concentration and AUC correspond to clinical pharmacodynamics. For this reason, plans are underway to validate the *in vitro* results in nude mouse allografts of our human cell lines.

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[7] Unpublished data.

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