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In Vitro Chemosensitivities of Human Tumor Stem Cells to the Phase II Drug 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide and Prospective *in Vivo* Correlations[1]

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ABSTRACT

A potential application of the human tumor stem cell colony assay is to guide Phase II clinical investigations by identifying classes of tumors (or individual patients) which are sensitive *in vitro* to a new antitumor compound. We have tested human tumor stem cells from 140 tumor biopsies representing 20 different tumor types for chemosensitivity to the Phase II drug 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. *In vitro* sensitivity was defined as a reduction in the number of tumor colony-forming cells to 30% of the control or less after a 1-hr exposure to one-tenth of the pharmacologically achievable plasma concentration of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. *In vitro* sensitivity was found in 29 cases: non-Hodgkin's lymphoma (2 of 2); cervical carcinoma (1 of 1); sarcoma (3 of 6); neuroblastoma (1 of 2); acute myelogenous leukemia (6 of 16); chronic myelogenous leukemia (1 of 3); melanoma (8 of 34); uterine carcinoma (1 of 5); lung carcinoma (1 of 9); ovarian carcinoma (4 of 36); and breast carcinoma (1 of 11). Prospective *in vitro-in vivo* correlations in eight patients with various tumor types showed that three of three patients sensitive *in vitro* to 4′-(9-acridinylamino)methanesulfon-*m*-anisidide responded *in vivo*, while five of five patients resistant *in vitro* had no clinical response. The results provide support for further evaluation of the utility of the human tumor stem cell colony assay for targeting Phase II clinical trials.

INTRODUCTION

In 1977, Hamburger and Salmon (15) reported an *in vitro* assay that permitted the growth of human tumors in soft agar. This assay has subsequently been modified and successfully utilized to obtain tumor colony formation in a wide variety of human tumor types (23). A variety of morphological, cytogenetic, and biomarker studies have shown that the colonies are composed of tumor cells. One of the major applications of this assay has been to quantify the effects of anticancer drugs. The combined experience in 316 *in vitro-in vivo* correlative trials using standard cytotoxic drugs at the University of Arizona and the University of Texas at San Antonio has demonstrated that if a drug reduces tumor colony formation less than 70%, clinical progression occurs over 95% of the time (2, 10, 18, 24, 31). In contrast, if the drug reduces colony formation by greater than 70%, an objective partial or complete clinical response occurs in 64% of patients. The assay also has promise for conducting "*in vitro* Phase II trials" and preclinical screening for entirely new compounds.

Each year, approximately 15,000 synthetic compounds and 400 purified natural products undergo anticancer screening with mouse tumor models at the Division of Cancer Treatment of the National Cancer Institute (9). In this study, we have focused on the Phase II drug m-AMSA,[2] an amino acridine derivative currently in clinical investigation. This compound demonstrated a wide spectrum of activity in mouse tumors, including L1210 and P388 murine leukemias, B16 melanoma, C3H and $CD8F_1$ mammary tumors, and Colon Tumors 26 and 38 (34). Over the last 6 years, extensive Phase I and II clinical trials have been ongoing.

The high frequency of successful prediction of *in vivo* responses for both resistance and sensitivity to standard cytotoxic drugs suggests that the human tumor stem cell colony assay may allow for targeting of Phase II clinical trials. Accordingly, we have analyzed our experience with the Phase II drug m-AMSA in the human tumor stem cell colony assay. We report here our total *in vitro* experience with m-AMSA and 8 prospective *in vitro-in vivo* correlations.

MATERIALS AND METHODS

Patient Material and Biopsy Specimens. All patients in this study were seen, evaluated, and treated at the University of Arizona Cancer Center or the Tucson Veterans Administration Hospital. All biopsy specimens were obtained by surgical excision after informed, written consent and prepared for culture as described previously (15, 25). Briefly, tumor biopsies were placed in a small amount of culture media in a sterile container and minced to approximately 1-mm square pieces using scalpels and then passed through sterile sieves. The resulting suspension was passed through needles of decreasing size and washed once with McCoy's Medium 5A containing 10% heat-inactivated fetal calf serum.

Drug Assay. A stock solution of m-AMSA (0.1 mg/ml) was prepared in sterile-buffered water and stored at −70°. Dilutions were made in medium for cell incubation. Single-cell tumor suspensions were transferred to tubes and adjusted to a concentration of 5×10^5 cells/ml with the appropriate m-AMSA dilution or control. Each tumor was tested, if possible, with a minimum of 3 concentrations of m-AMSA including at least one dose calculated as equal to or less than one-tenth of an achievable 1-hr concentration of a drug × time ($C \times t$) level *in vivo* (19). The final drug concentrations ranged from 0.0001 to 10 μg/ml. After a 1-hr incubation, the cells were centrifuged at 150 × *g* for 10 min, washed twice in medium, and plated in 35-mm Petri dishes in 1 ml of 0.3% agar layer over a 1 ml 0.5% agar underlayer containing an admixture of fetal bovine serum and growth factors (25). For assays of acute nonlymphocytic leukemia cells, the blast cell clonogenic assay designed by Buick *et al.* (4) was utilized, with methylcellulose as the semisolid support.

Plates were cultured in an incubator at 37° in a humidified 7.5% CO_2 environment. Plates were examined serially with an inverted-phase

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[2] The abbreviations used are: m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; TCFU, tumor colony-forming unit.

microscope for evidence of cluster or colony formation. This procedure included an examination of the final plate for clumps of cells that might be confused with colonies. In addition, a control plate was fixed and preserved for future comparative purposes if needed. Colonies were defined as aggregates of greater than 30 cells. At least 20 tumor colonies/plate were required in the control plates to assure an adequate number for the assessment of drug effect. Plates were counted when sufficient colonies were present (10 to 17 days). The average cloning efficiency was 0.0327% (range, 0.0039 to 0.1632%) (see Table 1).

Phase II Clinical Trial. Simultaneously, a broad phase II clinical trial using m-AMSA was conducted at the University of Arizona Cancer Center and the Tucson Veterans Administration Hospital. m-AMSA was administered as a single agent at a dose of 120 mg/sq m i.v. every 28 days. Patients were eligible to receive m-AMSA if they had measurable lesions, had failed standard therapy, and had a life expectancy of at least 8 weeks. Response to therapy was described as follows: complete response, the disappearance of all measurable lesions lasting at least 4 weeks; partial response, a decrease by at least 50% of all measurable lesions lasting at least 4 weeks; mixed response, a decrease in some measurable lesions with other lesions progressing or demonstrating a response. If a patient failed to achieve a tumor regression after 2 courses of m-AMSA, the drug was discontinued, and the patient was taken off study. In patients who achieved a partial response to m-AMSA, the drug was continued until disease progression.

Statistical Methods. All assay data were stored on a Wang 2200-C laboratory computer disc file. Cloning efficiencies were calculated from the total number of cells plated and were not corrected for the proportion of nontumor cells in the sample. This correction was not made, as normal cells do not form colonies in this system. All tumor specimens have all grossly normal tissue removed prior to the preparation of a single-cell suspension. Nevertheless, relative plating efficiencies are difficult to compare, because the percentage of normal cells varies from biopsy to biopsy. For the graphic presentation, the mean of triplicate observations of the survival of TCFU for each patient was plotted *versus* drug concentration.

Survival of TCFU at one-tenth the calculated pharmacologically achievable $C \times t$ (1 hr) ("cutoff" concentration) was determined. The cutoff concentration used was 0.1 μg/ml. On the basis of experiments with ovarian cancer and multiple myeloma, patients were classified as sensitive if survival of TCFU was less than 30% at the cutoff concentration and resistant if greater than 30% (2, 10). This simple approach to classifying *in vitro* response has developed from a careful analysis of the more complex "area under the curve" (19). Due to the fact that the critical concentration of a drug in the human tumor stem cell system which can be used to predict "sensitivity" can vary from drug to drug, it is essential in working with new drugs to utilize several different concentrations of drug and to plot colony formation at several concentrations. After adequate clinical correlation is available, the critical concentration can then be determined accurately.

RESULTS

Effect of m-AMSA on Tumor Colony Formation. Stem cells from 140 tumor biopsy specimens demonstrated greater than 20 colonies/plate and were tested against m-AMSA. Represented among these 140 specimens were 20 different tumor types (Table 1). Nine or more specimens were tested in breast carcinoma, lung carcinoma, acute myelogenous leukemia, ovarian carcinoma, and melanoma, and 4 or more specimens were tested in sarcomas, bladder carcinoma, uterine carcinoma, and colon carcinoma. The percentage of tumor specimens which had a 70% or greater inhibition of colony formation after exposure to m-AMSA at concentrations of 0.1 μg/ml or less are presented in Table 1. The *in vitro* response rate was higher (33% or more) in non-Hodgkin's lymphoma, acute myelogenous leukemia, chronic myelogenous leukemia, neuroblastoma, and sarcomas.

Prospective Correlation of *in Vitro-in Vivo* Effects of m-AMSA. Eight evaluable patients in the clinical trial also underwent biopsy and had successful human tumor stem cell colony formation prior to the initiation of therapy with m-AMSA. Chart 1 depicts the results and correlations as survival-concentration curves; the tumor specimen from the patient with papillary thyroid carcinoma had only enough cells to test one concentration of m-AMSA. The concentration of 1 μg/ml was selected prior to knowledge of the clinically achievable levels of m-AMSA. The tumor specimen from the patient with lymphoma

Table 1

In vitro sensitivity of tumor stem cells to m-AMSA at 0.1 μg/ml

Sensitivity was defined as a 70% or greater inhibition of tumor colony formation when compared to controls. Plating efficiency was calculated as the number of colonies formed on controls as a percentage of the total number of nucleated cells inoculated.

Tumor types	No. of specimens	Mean plating efficiency (%)	No. of sensitive *in vitro*	95% confidence limits
Non-Hodgkin's lymphoma	2	0.0184	2 (100)[a]	48, 100
Cervical carcinoma	1	0.0048	1 (100)	
Sarcoma	6	0.0088	3 (50)	15, 87
Neuroblastoma	2	0.0128	1 (50)	5, 95
Acute myelogenous leukemia	16	0.1256	6 (38)	16, 62
Chronic myelogenous leukemia	3	0.1632	1 (33)	2, 78
Melanoma	34	0.0184	8 (24)	12, 43
Uterine Carcinoma	5	0.0206	1 (20)	1, 63
Lung carcinoma	9	0.0222	1 (11)	0, 53
Ovarian carcinoma	36	0.0140	4 (11)	3, 27
Breast carcinoma	11	0.0125	1 (9)	0, 42
Bladder carcinoma	6	0.0333	0 (0)	0, 43
Colon carcinoma	4	0.0148	0 (0)	0, 50
Thyroid carcinoma	2	0.0142	0 (0)	0, 60
Pancreatic carcinoma	1	0.0041	0 (0)	
Prostatic carcinoma	1	0.0106	0 (0)	
Myeloma	1	0.0053	0 (0)	
Brain	1	0.0039	0 (0)	
Squamous cell carcinoma of the head and neck	1	0.0044	0 (0)	
Testicular carcinoma	1	0.0108	0 (0)	

[a] Numbers in parentheses, percentage.

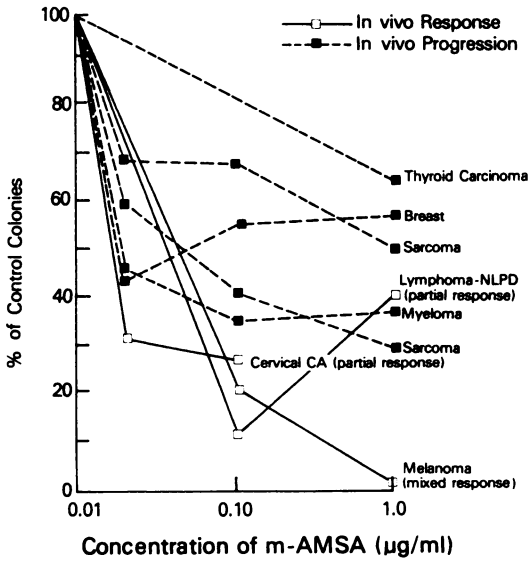

Chart 1. Survival-concentration curves for a 1-hr *in vitro* exposure of human tumor stem cells to m-AMSA with *in vivo* correlation. *CA*, carcinoma; *NLPD*, poorly differentiated modular lymphoma.

demonstrated a significant decrease in colony formation inhibition at the 1-μg/ml drug concentration compared to the 0.1-μg/ml drug concentration. This is a rare phenomenon which has unclear significance.

The 5 patients who demonstrated less than 70% colony-forming inhibition after a 1-hr exposure to 0.1 μg/ml of m-AMSA *in vitro* all failed to respond to m-AMSA in the clinical trial. All 3 patients who showed a reduction in survival of colony-forming cells to 30% or less after a 1-hr exposure to m-AMSA (0.1 μg/ml) had a mixed or partial clinical response to the drug *in vivo*. Two of the 3 patients who were sensitive to m-AMSA *in vitro* had partial responses to the drug. A patient with poorly differentiated nodular lymphoma had a greater than 50% shrinkage of his enlarged lymph nodes, which lasted 3 months, and a patient with a large pelvic mass secondary to cervical carcinoma achieved a greater than 50% reduction in tumor volume, which lasted 2 months. The other response was in a patient with metastatic melanoma who had a greater than 50% reduction in size of a grossly enlarged liver together with improved liver function tests, while a neck mass enlarged (mixed response). The tumor specimen from this patient was obtained from an excisional biopsy of a separate lymph node metastasis.

DISCUSSION

Correlative clinical trials have demonstrated that *in vitro* chemosensitivity to standard cytotoxic compounds in the tumor stem cell colony assay can predict clinical sensitivity or resistance with a high degree of accuracy (2, 10, 18, 24, 31). The *in vitro* experience with the assay reported here involved 20 different tumor types and suggested that m-AMSA may have clinical activity in leukemia. Our *in vitro* studies also suggest that there may not be significant antitumor activity for m-AMSA in lung carcinoma, breast carcinoma, and ovarian carcinoma. The data available on other tumor types tested *in vitro* are on a very small number of patients, and no firm conclusions can be drawn. m-AMSA has been the subject of extensive Phase I and II clinical trials since the late 1970's. Only a limited number of Phase II clinical trials using m-AMSA have been published in detail. m-AMSA appears to have minimal activity in colorectal carcinoma, lung cancer, hypernephroma, hepatoma, head and neck carcinoma, breast carcinoma, prostate cancer, and sarcoma (6–8, 11–14, 16, 20, 21, 26, 27, 29, 30, 35). The clinical utility of m-AMSA in non-Hodgkin's lymphoma is unclear, but the drug appears to be active in acute leukemia (3, 5, 28, 33).

The *in vitro* results in melanoma warrant separate comment. The chemosensitivity results as reported here in 34 human melanoma specimens would have predicted an *in vivo* sensitivity in 8 patients (24%). One of these 8 patients did receive m-AMSA and achieved a mixed response. In that instance, an axillary node biopsy was tested *in vitro*. After initiation of m-AMSA therapy, the patient had a striking reduction in massive hepatomegaly in 3 weeks, but simultaneously progressed with enlargement of a neck mass (mixed response). Only one detailed Phase II clinical trial with m-AMSA in melanoma has been reported, and in that trial plus our own experience little activity for the drug in this disease is suggested (1, 16). Therefore, we believe that the 24% *in vitro* response rate may include more "false positives" than are usually observed with this assay. Possible explanations for this false-positive finding include: (*a*) the critical *in vitro* $C \times t$ for m-AMSA in melanoma may be less than 0.1 μg·hr/ml; (*b*) binding of m-AMSA to melanin granules or some pharmacological quirk may distort the *in vitro* chemosensitivity results (22); (*c*) metastatic melanoma in any one patient may represent a particular heterogeneous tumor with several coexistent clones of malignant cells, each with differing chemosensitivity patterns. (The frequency of response *in vivo*, in fact, supports this latter possibility.) Additional *in vitro* studies, *in vivo-in vitro* correlations, and additional pharmacological studies in patients with melanoma treated with m-AMSA will be required to select among these alternatives.

With regard to the prediction of chemosensitivity to m-AMSA, the 8 prospective *in vitro/in vivo* trials reported in this study, while small in number, showed an excellent (100%) correlation with the human tumor stem cell colony assay results. These correlations support the reliability of the human tumor stem cell colony assay to predict *in vivo* responses to Phase II agents; however, since only partial or mixed responses were achieved, such responses were not of major clinical significance. It appears probable that more stringent criteria for *in vitro* chemosensitivity (*e.g.*, lower TCFU survival cutoff) may be required to predict complete responses successfully.

The major objective of Phase II clinical trial is to determine if a new agent has anticancer activity in humans. As currently conducted by the Division of Cancer Treatment of the National Cancer Institute, Phase II clinical trials have a number of problems (32). Evaluations are performed on extensively pretreated patients. A limited number of tumor types are tested. The process is expensive, it takes from 3 to 7 years to complete, and patients incur significant morbidity with a low probability of benefit. The human tumor stem cell colony assay provides a new methodology with which to evaluate potential Phase II agents and may allow identification of tumor types and specific patients who might benefit from a new drug. Our *in vitro* studies of m-AMSA together with 8 *in vivo* correlations support further investigations of this approach.

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