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Phase II Study of Ametantrone in a Human Tumor Cloning Assay*

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Abstract—*The anticancer activity of ametantrone was investigated in a human tumor cloning assay. Tumor samples were freshly obtained from* 105 *patients. Cells were exposed for* 1 hr *to drug concentrations of* 1 *and* 10 μg/ml. *A reduction in the number of tumor colony-forming units by* 50% *or more was seen in* 2/31 *breast cancers,* 2/25 *ovarian cancers,* 1/10 *primaries of unknown origin,* 1/10 *melanomas,* 2/8 *non-small cell lung cancers,* 1/5 *small cell lung cancers and* 1/3 *colon cancers. Only three of these* in vitro *responses were consistently obtained at the probably more relevant concentration of* 1 μg/ml. *These findings indicate that low efficacy should be expected in cancer patients with ametantrone. The predictive value of these* in vitro *phase II data remains to be demonstrated.*

AMETANTRONE, or 1,4-bis{2[(2-hydroxyethyl) amino]ethylamino}-9,10-anthracenedione diacetate (NSC-287513), is a new anticancer agent that was identified by random screening at the National Cancer Institute [1]. Its promising experimental antitumor properties stimulated extensive structure-activity relationship studies of bis-(substituted aminoalkylamino)-anthraquinones [1–3]. Phase I investigations revealed that the drug was very well tolerated and easy to handle. Leukopenia was dose-limiting, blue skin discoloration was seen at the highest doses and non-hematologic toxic effects were negligible [4–6]. Initial pharmacokinetic studies indicated that single drug administrations over 15–30 min at potentially therapeutic doses resulted in peak plasma levels varying between 4.1 and 15 μg/ml [6]. A variety of phase II trials are underway with interim reports of responses in breast, head and neck and non-small cell lung cancer [7].

The human tumor cloning assay developed by Hamburger and Salmon [8] appears to have great potential for *in vitro* drug studies despite a great number of limitations [9]. Prospective trials largely support its relevance as a chemosensitivity test [10, 11]. The assay could be helpful in the planning of clinical phase II trials [12] and in the screening of new drugs [13]. We have used this system to carry out an *in vitro* phase II evaluation of ametantrone following a procedure previously described by Von Hoff *et al.* [14, 15].

MATERIALS AND METHODS

Samples that yielded adequate growth in soft agar were freshly obtained from 105 cancer patients. Seventeen different tumor types were studied with breast and ovarian cancer accounting for more than 50% of the accrual (Table 1). One-half of the patients had not been previously treated with chemotherapy and none had received prior treatment with ametantrone, mitoxantrone or bisantrene. Sixty-seven had no prior anthracycline therapy.

Collection of cells

Cells were obtained from ascitic fluids, pleural effusions and primary or metastatic solid tumors. Effusions were centrifuged at 2000 rev/min for 5 min at room temperature. Red blood cells were eliminated over a Ficoll gradient. Solid tumors were first washed in Hanks' balanced salt solution. Then the samples were mechanically disrupted using scissors. Cells were passed

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Table 1. Patient selection

Tumor type	Total	No. of patients: No prior anthracyclines	No. of patients: No prior chemotherapy
Breast	31	11	7
Ovary	25	18	17
Unknown primary	10	7	6
Melanoma	10	8	6
Lung (non-small cell)	8	6	4
Lung (small cell)	5	2	2
Stomach	3	3	3
Colon	3	3	1
Cervix	2	2	1
Miscellaneous	8	7	7
Total	105	67	54

through raspers to disrupt small aggregates. Cell counting was done in a hemocytometer and cell viability was measured by the trypan blue dye exclusion method.

In vitro *exposure to ametantrone*

Stock solutions of the clinical formulation of ametantrone were stored at -20°C and sterile water was used for subsequent dilutions. Nucleated viable cells, at a concentration of 1.0×10^6 cells/ml, were exposed to 0.05 and 0.5 μg/ml of ametantrone in the first seven patients. The subsequent drug concentrations were 1 and 10 μg/ml. Most samples were also tested vs doxorubicin at a concentration of 1 μg/ml. Cells were incubated with or without drugs for 1 hr at 37°C in Hanks' balanced salt solution. The cells were then washed twice before culture. All experiments were conducted in triplicate.

Assay for tumor colony-forming units

Culture system. Cultures were performed in 35 × 10 mm Petri dishes. The double agar layer was prepared as previously described [14, 15]. The underlayer or feeder layer contained 40 ml of enriched McCoy's 5A, 10 ml of tryptic soy broth, 0.6 ml of asparagine (6.6 mg/ml) and 0.3 ml of DEAE dextran (50 mg/ml). The enriched McCoy's 5A was prepared as follows: 500 ml of McCoy's 5A, 75 ml of heat-inactivated fetal calf serum (FCS), 5 ml of Na-pyruvate (2.2%), 1 ml of L-serine (21 mg/ml), 5 ml of glutamine (200 nM) and 1 ml of penicillin (50,000 U)-streptomycin (50 mg). To prepare the 0.5% agar underlayer, 1 ml of a 3% agar solution kept liquid in a warm water bath (about 45-50°C) was added to 5 ml of prewarmed (37°C) enriched McCoy's. This material was sufficient for six plates. The upper layer was prepared with enriched CMRL 1066: 100 ml of CMRL 1066, 15 ml of FCS, 200 U of insulin in 5 ml, 1 ml of vitamin C (30 mM), 2 ml of glutamine and 0.2 ml penicillin-streptomycin. Immediately before plating, asparagine (0.6 ml/40 ml of medium), DEAE dextran (0.3 ml/40 ml of medium) and mercaptoethanol (1:100) were added. The 0.3% upper layer was prepared by adding 0.3 ml of 3% agar to 2.7 ml of the enriched CMRL containing 1.5×10^6 cells. This material was sufficient for three plates and layered over the 0.5% agar, which was solid at that point.

Tumor colony formation was monitored using an inverted microscope. Immediately after plating all dishes were checked for the presence of preformed cell aggregates. Thereafter, plates were incubated at 37°C in a 5% CO_2 humidified atmosphere and examined 2-3 times per week until final counting. Small clusters of 3-20 cells appeared within 10 days and colonies consisting of ⩾40 cells could be observed within 7-28 days. Final scoring of the number of colonies was generally possible during the third or fourth week.

Cell kill was measured by the percentage of the mean number of colonies after drug exposure relative to the mean number of control colonies. A minimum of 20 colonies were required in the control plates. *In vitro* efficacy was defined as a ⩾50% decrease in tumor colony-forming units. The standard deviation of the mean number of colonies per triplicate was below 10% in 66% of the cases.

RESULTS

Cell kill by 50% or more with ametantrone was found in samples obtained from 10 different patients, of whom five had prior chemotherapy that included doxorubicin in three (Table 2). The median number of control colonies in these samples was 109, with a range of 32-579. In five samples activity was detected at a concentration of 1 μg/ml, but in two of these efficacy was not confirmed at 10 μg/ml. In five other samples *in*

Table 2. In vitro *growth inhibition with ametantrone*

Tumor type	Prior chemotherapy (anthracyclines)		No. of control colonies* Mean	Range	% growth inhibition 1 μg/ml	10 μg/ml
Lung (small cell)	+	(+)	450	432-471	92	96
Ovary	0		85	78-95	82	94
Unknown	0		196	172-227	55	64
Lung (non-small cell)	+	(+)	386	376-392	-	84
Ovary	0		58	53-62	21	78
Breast	+	(+)	579	570-590	21	70
Lung (non-small cell)	+	(0)	41	39-44	0	59
Colon	+	(0)	133	130-138	10	58
Melanoma	0		33	29-37	55	27
Breast	0		32	30-33	53	16

*500,000 nucleated viable cells were seeded per plate.

vitro activity was noted at the higher concentration only. Overall, tumor sensitivity was found in 2/31 (7%) breast cancers, 2/25 (8%) ovarian cancers, 1/10 primaries of unknown origin, 1/10 melanomas, 2/8 non-small cell lung cancers, 1/5 small cell lung cancers and 1/3 colon cancers. An increase by 50% or more of the tumor colony-forming units was also observed, at concentrations of ametantrone ≥1 μg/ml, in two breast cancers, two melanomas and one gastric cancer.

Among 31 patients who had prior anthracycline therapy and whose tumor was tested vs doxorubicin, two showed *in vitro* sensitivity to this drug. One was a non-small cell lung cancer which was also sensitive to ametantrone in the assay. The effect of doxorubicin and ametantrone was tested concomitantly in samples from 57 anthracycline-naive patients. Of these, efficacy of doxorubicin was found in 4/16 ovarian cancers and 0/10 breast cancers. Overall, among the 57 patients with no prior anthracycline therapy, tumor sensitivity *in vitro* was found in four with ametantrone only, five with doxorubicin only and three with both (Table 3). Of the three samples that showed clear evidence of sensitivity to the lower concentration of ametantrone, two were also tested vs doxorubicin, and similar cell kill was found with both compounds.

Table 3. *Cross-resistance study of ametantrone and doxorubicin*

Activity of ametantrone (1 or 10 μg/ml)	No. of samples	Activity of doxorubicin (1 μg/ml) ++	+	0
++	2	1		1
+	5	1	1	3
0	50	1	4	45
Total	57	3	5	49

Growth inhibition: ≥70% = ++, 50-69% = +, <50% = 0.

DISCUSSION

Ametantrone exhibited *in vitro* efficacy in 10/105 tumors at clinically achievable concentrations. When all responses are considered this agent appears to elicit the same *in vitro* response rate as doxorubicin, with only partial cross-resistance. However, based on available clinical pharmacology data, growth inhibition would appear most significant at a drug concentration of 1 μg/ml. This was observed in only five samples, and of these, borderline cell kill in two was no longer seen at the higher drug concentration. Moreover, an increase by 50% or more in the number of tumor colony-forming units was found in five samples. This latter observation suggests either tumor stimulation, an unnoticed drug-induced clumping effect or other technical problems related to the assay.

In vitro responses were seen in malignancies from seven different origins. Due to small sample sizes, data regarding a specific drug sensitivity per tumour type are mostly inconclusive, with the possible exception of breast cancer. Using a decision theory proposed for the analysis and interpretation of phase II clinical trials [16], it would appear that ametantrone could be considered as an inactive agent for this disease since the true effectiveness rate with a β error of 5% would be below 20%. However, such conclusion should be tempered by an apparent selection of poor-risk breast cancer patients as evidenced by extensive prior chemotherapy in the majority of these and the concomitant lack of detectable *in vitro* efficacy with high concentrations of doxorubicin.

Markedly more favorable findings were found with mitoxantrone, the dihydro-derivative of ametantrone. Drug sensitivity to the former was noted in one-third of the samples tested in a large-scale *in vitro* phase II study of Von Hoff *et al.* [14]. The same culture system was used but the comparative effect of doxorubicin was not

reported. Published data are not sufficient to strictly compare our results, but it is worth noting that the proportion of patients previously treated with chemotherapy in general, and doxorubicin in particular, was superimposable in the two series. As predicted by the assay, mitoxantrone appears to be an active agent against breast cancer in humans. Other favorable *in vitro* findings with this compound remain to be confirmed in non-hematologic malignancies.

It is premature to determine the relevance of our data to the clinical situation. No attempts were made to correlate *in vitro* and *in vivo* observations in our study. In addition, information on the clinical efficacy of ametantrone is still lacking. Although the drug is, structurally, a very closely related analog of mitoxantrone, its *in vitro* antitumor efficacy is noticeably lower, especially when considering that only three samples exhibited undisputable drug sensitivity at the apparently more relevant concentration. Based on findings in the human tumor cloning assay, minimal activity could be expected in cancer patients with ametantrone.

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