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In Vitro Activity of Menogaril and *N*-Demethylmenogaril in a Human Tumor Cloning Assay*, †

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Abstract—*The activity of menogaril and its major metabolite in animals and humans,* N*-demethylmenogaril, has been investigated in the human stem cell assay as developed by Salmon* et al. *Among* 31 *evaluable samples, four were sensitive to menogaril, including one which responded to* N*-demethylmenogaril. Three samples resistant to menogaril responded to* N*-demethylmenogaril. None was sensitive to doxorubicin. Overall, one out of seven ovarian samples and one out of three breast samples responded to menogaril. Our data confirm the* in vitro *activity of menogaril in ovarian and breast cancer; in addition, they suggest incomplete cross-resistance between doxorubicin and menogaril and, considering the concentrations of* N*-demethylmenogaril in animals and humans, a minor role for this metabolite in the overall antitumor activity of the parent compound.*

INTRODUCTION

MENOGARIL (NSC 269148, 7-con-*O*-methylnogarol, menogarol, MEN) is a semi-synthetic anthracycline antibiotic derived from nogalamycin, an agent that has not been clinically investigated because of excessive toxicity in animals (Fig. 1) [1, 2]. MEN appeared as the most promising of several nogalamycin analogs [1, 3, 4]. Although they share the tetracyclic, anthraquinone-containing system, there are several structural differences between MEN and doxorubicin (DOX) (Fig. 1).

The exact mechanism of action of MEN is not yet defined. However, several lines of evidence point to a different mechanism of action for MEN and DOX: unlike DOX, MEN is a weak inhibitor of RNA and DNA syntheses [2] and causes no inhibition of the RNA polymerase even at highly lethal concentrations. MEN binds weakly to DNA compared to DOX. In cells treated with MEN, fluorescence is localized predominantly in the cytoplasm in contrast to DOX which is concentrated in the nucleus [5, 6]. MEN and DOX are also different in their cell cycle-phase-specific lethality [7].

MEN possesses broad spectrum activity against murine tumors [8, 9]. The spectra of antitumor activity of MEN and DOX differ by the absence of activity for MEN against the colon 38 carcinoma. The *in vitro* antitumor activity of MEN was investigated and compared to that of DOX in the human tumor stem cell assay [10]. Some antitumor activity was observed for MEN in breast, colon, lung and ovarian cancers. Incomplete cross resistance between MEN and DOX was also documented.

Pharmacokinetic studies have shown that *N*-demethylmenogaril (NDM) (Fig. 1) is a major plasma metabolite of MEN, both in animals and humans [11–16]. NDM is a cytotoxic compound although it has been shown to be less active than MEN *in vivo* [1].

This study was undertaken to determine the *in vitro* anti-tumor activity of NDM in comparison to that of MEN in the human tumor stem cell assay.

MATERIALS AND METHODS

Drug supply

MEN was supplied by the Upjohn Company (Kalamazoo, Michigan) in vials containing 50 mg

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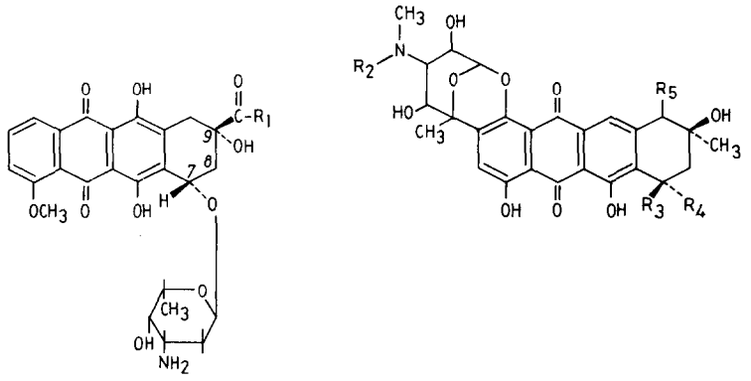

Compound	R_1
Daunorubicin	CH_3
Doxorubicin	CH_2OH

Compound	R_2	R_3	R_4	R_5
Nogalamycin	CH_3	H	Nogalose	$COOCH_3$
Menogaril	CH_3	OCH_3	H	H
N-Demethylmenogaril	H	OCH_3	H	H

Fig. 1. Comparative structures of daunorubicin, doxorubicin, nogalamycin, menogaril, and N-demethylmenogaril.

of MEN, 100 mg of mannitol and 16.6 mg of lactic acid. The drug was dissolved in sterile water. NDM was kindly supplied by the Upjohn Company (Kalamazoo, Michigan) and was dissolved in sterile water. DOX was a generous gift from Farmitalia Carlo Erba (Milan, Italy) and was dissolved in 0.9% sodium chloride.

Preparation of cells

Cells were obtained from ascitic fluids, pleural effusions and primary or metastatic solid tumors in 64 patients. Effusions were centrifuged at 2000 rpm for 5 min at room temperature. Red blood cells were eliminated over a Ficoll gradient. Solid tumors were first washed in Hank's balanced salt solution. Then, the samples were mechanically disrupted using scissors. Cells were passed 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 MEN and NDM*

Stock solutions of NDM and MEN 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 10, 1 and 0.1 μg/ml of MEN and NDM. The concentrations of MEN and NDM were based on the peak plasma concentrations observed in various animal species [1, 14–16]. Later studies indicated that peak plasma concentrations were in the same range in man, depending on the dose and schedule [11–13]. When the cell yield was sufficient (22 samples), cells were also exposed to DOX at concentrations of 1 and 0.1 μg/ml [17]. Cells were incubated with or without drugs for 1 hr at 37°C in Hank's balanced salt solution. The cells were then washed twice before culture. All experiments were conducted in triplicate.

Assay for tumor colony-forming units

Cultures were performed in 35 × 10 mm Petri dishes. The double agar layer was prepared as previously described [18]. Tumor colony formation was monitored using an inverted microscope. Immediately after plating, all dishes were checked for the presence of 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 mean of 20 colonies was required in the triplicate control plates. *In vitro* efficacy was defined as a ≥ 50 % decrease in tumor colony-forming units.

Patient's characteristics

Ninety-three tumor samples were freshly obtained. Six samples were contaminated. Out of the 87 cultured samples, the cell yield was insufficient for drug testing in 22, 23 did not grow at all and 11 yielded less than 20 colonies in the control plates, leaving 31 evaluable samples. Among these, 14 different tumor types were studied. The most common tumor types were ovarian cancer (seven cases) and carcinomas of unknown origin (four cases). There were three cases each of breast and lung (non-small cell types) cancer. Other tumor types accounted for the 14 remaining samples. There were 16 solid samples and 15 pleural effusions or ascites. Fourteen samples were obtained from patients previously treated with chemotherapy; among these, eight had received prior anthracycline antibiotics (four out of seven ovarian

Table 1. In vitro *activity of menogaril,* N-*demethylmenogaril, and doxorubicin*

Tumor	*In vitro* inhibition (%)							
	MEN (a,b)			NDM			DOX	
	10	1	0.1	10	1	0.1	1	0.1
Breast (c)	77	28	−19 (d)	32	4	−9	7	1
Ovary	67	67	37	73	63	10	33	47
Urothelial	ND	55	ND	ND	−15	ND	ND	ND
Unknown origin	ND	52	ND	ND	29	ND	ND	ND
Kidney	ND	−15	ND	ND	77	ND	37	−65
Lung (NSC)	ND	17	−2	ND	−7	55	0	ND
Colon	1	3	−2	51	16	3	−5	−11

(a) Abbreviations: ND: not tested because of insufficient cell yield; NSC: non small cell lung cancer; MEN: menogaril; NDM: *N*-demethylmenogaril; DOX: doxorubicin.

(b) MEN, NDM and DOX were tested at the indicated concentrations (μg/ml).

(c) This sample was obtained from a patient previously treated with cyclophosphamide, methotrexate, fluorouracil, doxorubicin, and vincristine. All other samples listed in this table were obtained from previously untreated patients.

(d) A negative value indicates an apparent stimulation.

cancers, two out of three breast cancers, two others).

RESULTS

Cell kill by 50 % or more with MEN was observed in four samples. Three of these were obtained from patients previously untreated with chemotherapy (Table 1). The last response was observed for a sample from a patient with breast cancer previously treated with cyclophosphamide, methotrexate, fluorouracil, doxorubicin and vincristine. No significant inhibition occurred at 0.1 μg/ml. Three of the four responses were observed at the concentration of 1 μg/ml, whereas the last one was obtained at the highest concentration (10 μg/ml).

One of the samples sensitive to MEN was also sensitive to NDM (Table 1). The three other samples sensitive to MEN did not respond to NDM. Three samples unresponsive to MEN were inhibited by 50 % or more by NDM at concentrations ranging from 0.1 to 10 μg/ml. Overall, MEN was active in one out of seven ovarian cancers, one out of four carcinomas of unknown origin, and one out of three breast cancers. NDM was active in one out of seven ovarian cancers. DOX did not induce any *in vitro* response in this study. The cell yield was sufficient to test DOX against five of the seven samples sensitive to MEN or NDM and none responded (Table 1).

The cell yield was sufficient to investigate the concentration–effect relationship for two sensitive samples; for both samples, this relationship was verified for MEN and NDM (Fig. 2).

DISCUSSION

The level of *in vitro* activity of MEN observed in this study is consistent with that reported by Weiss *et al.* [10]. With the exception of a breast cancer sample from a patient previously treated with DOX, all responses were observed at a concentra-

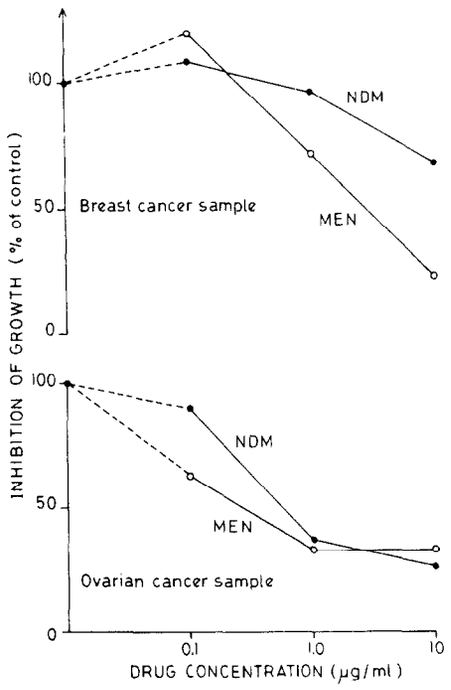

Fig. 2. *Dose–effect relationship of the antitumor activity of menogaril and* N-*demethylmenogaril in a case of ovarian cancer and a case of breast cancer.*

tion of 1 μg/ml, which is known to be achieved in patients treated with MEN [11–13]. The number of samples of each tumor type are too small to draw firm conclusions regarding the rate of *in vitro* activity of MEN; however, by pooling our results with those obtained by Weiss at a concentration of 0.2 μg/ml, MEN was active against 3 out of 20 breast cancer samples and 3 out of 17 ovarian cancer samples. The responses noted in this study were observed in samples resistant to DOX; this observation strengthens the statement by Weiss *et al.* of incomplete cross resistance between MEN and DOX.

Some *in vitro* antitumor activity was also observed with NDM. Although one sample was sensitive to both MEN and NDM, three samples sensitive to MEN were resistant to NDM. In contrast to MEN, for which no response was obtained at 0.1 μg/ml and all but one response was achieved at 1.0 μg/ml, responses to NDM were seen at concentrations ranging from 0.1 to 10 μg/ml. In plasma of patients treated with MEN, concentrations of NDM do not exceed 0.1 μg/ml [11–13]; at that concentration, only one sample obtained from a patient with lung cancer was sensitive to NDM in our study. At present, no data are available about the tissue concentrations of MEN and NDM in man; in animal tissues, very low concentrations of NDM have been noted [14]. In addition, although NDM was reported to be more potent than MEN [15], it was shown to be considerably less active than MEN at optimal doses. Therefore, our data suggest a minor role of NDM in the overall antitumor activity of MEN.

In conclusion, our data confirm the *in vitro* activity of MEN against breast and ovarian cancer. Whether this will be translated into an *in vivo* activity in patients remains to be proven by the ongoing phase II studies with MEN. In addition, our data suggest incomplete cross resistance between MEN and DOX and a minor role for NDM in the overall antitumor activity of MEN.

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