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Intracavitary Therapy of Murine Ovarian Cancer with *cis*-Diamminedichloroplatinum(II) and 10-Ethyl-10-deazaaminopterin Incorporating Systemic Leucovorin Protection[1]

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ABSTRACT

Administration i.p. of 10-ethyl-10-deazaaminopterin (10EDAM) with *cis*-diamminedichloroplatinum(II) (*cis*-Pt) had significant antitumor activity against the murine ovarian tumor. This tumor is a teratoma originating in the ovary with pathogenesis and metastatic properties similar to those of human ovarian cancer. Drug was given on a schedule of once every 3 days for 3 doses 1 or 2 days after i.p. implant of 10^7 tumor cells. Despite the 2-fold attenuation of dosage required, antitumor activity of the combination (increased life span, 161%) was approximately twice that obtained with maximum tolerated doses of either agent alone and tumor-free, long-term survivors were obtained. Incorporation of s.c. calcium leucovorin administration 16 h after each dose of 10EDAM and *cis*-Pt allowed a 4-fold increase in dosage of 10-EDAM without an increase in toxicity, increased median survival by an additional 120%, and quadrupled the number of tumor-free, long-term survivors to 40% of treated animals. By comparison, methotrexate was only modestly active against this tumor model either as a single agent, with *cis*-Pt, or with delayed s.c. calcium leucovorin administration. These results appear to suggest that 10EDAM with *cis*-Pt may have considerable potential for intracavitary therapy of human cancer, including ovarian carcinoma, particularly when incorporating delayed systemic calcium leucovorin administration.

INTRODUCTION

As an approach to improved clinical management of patients with ovarian cancer, intracavitary chemotherapy (1–5) represents an attractive option. However, further progress in the development of this modality will require the identification of additional agents that can be safely administered in this fashion with significant antitumor effects. With this objective in mind, we have been examining the new folate analogue, 10EDAM,[3] in a murine model of ovarian cancer. This analogue was recently developed in the laboratories of the Memorial Sloan-Kettering Cancer Center and SRI International and is currently undergoing multicenter clinical trials. It represents an interesting candidate for possible intracavitary use because of its favorable toxicity profile and greater antitumor activity in many model systems (6–8) and in patients with advanced non-small cell lung cancer (9) compared to MTX and its marked therapeutic potentiation (8) with alkylating agents and *cis*-Pt against metastatic murine solid tumors. A number of studies have documented (1–3, 10) the usefulness of *cis*-Pt for intracavitary therapy of human ovarian cancer. We now report on studies in a murine model of ovarian cancer which appears to demonstrate that 10EDAM may have substantial potential in this neoplastic disorder during intracavitary administration with *cis*-Pt. These studies also demonstrated that antitumor effects of this combination of agents may be markedly enhanced by delayed systemic administration of calcium leucovorin which allowed dose escalation of 10EDAM to levels far beyond that which was toxic with *cis*-Pt.

MATERIALS AND METHODS

The results described here were obtained in experiments in which a murine ovarian tumor (11) was transplanted as an ascites to C3HeB/Fe mice. This murine teratoma exhibits (11–14) biological and metastatic behavior similar to those of human ovarian carcinoma. Within 1 day after implantation of 10^6 cells i.p., diaphragmatic lymphatic obstruction could be histologically demonstrated followed by rapid appearance of ascites, subdiaphragmatic tumor deposits, and intraabdominal carcinomatosis. Microscopically the tumor is composed (11–14) of both undifferentiated "embryonal" cells and differentiated cells. Since this tumor will grow well (11) in either male or female mice, we carried out our experiments with the former because of similar toxicity profiles and their greater availability from our supplier (The Jackson Laboratory, Bar Harbor, ME). Tumor-bearing mice were treated either i.p. (0.25–0.5 ml) or s.c. (0.1–0.2 ml) after varying delay with either *cis*-Pt or 10EDAM on a schedule of once every 3 days for 3 doses. This schedule of administration was used in our earlier (8) studies that demonstrated therapeutic potentiation between 10EDAM and *cis*-Pt against metastatic murine tumors. All of the experimental procedures used during these studies have already been described in detail (6–8) and were carried out in compliance with the *Guide for Care and Use of Laboratory Animals*.

The folate compounds in these studies were used as the sodium salt and along with *cis*-Pt given at a neutral pH in isotonic saline. Samples of MTX and *cis*-Pt were provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 10EDAM was made available by the Ciba-Geigy Corporation, Summit, NJ. Tolerances to the various agents used on this schedule and these routes of administration were first established in preliminary experiments and then redetermined again with tumor-bearing mice. Dosing during therapeutic experiments was at or near the LD_{10} dosage (10EDAM, MTX, and *cis*-Pt, 60, 30, and 4 mg/kg, respectively) or at a defined fraction thereof. LD_{10} dosages were the same when drug was given either i.p. or s.c. to tumor-bearing mice. Antitumor effects were expressed as percentage ILS based upon median survival time of treated *versus* control (untreated) groups. Animals implanted with tumor were selected randomly for each group. Toxicity was monitored by weight loss and drug-induced deaths in animals with no evidence of disease. Analysis for statistical significance between values for increased life span for different experimental groups was carried out using the χ^2 test as described by Zar (15).

RESULTS

In our initial experiments, we compared (Table 1) the effectiveness of i.p. or s.c. administered 10EDAM and *cis*-Pt against the murine ovarian tumor. Therapy was given at the LD_{10} dosage and was initiated 1 day after transplantation of 10^7 ascites cells on a schedule of once every 3 days for a total of three doses. When given s.c., MTX and *cis*-Pt were essentially inactive against this tumor, while 10EDAM exhibited modest antitumor activity (ILS 52%). When given i.p., 10EDAM and

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[3] The abbreviations used are: 10EDAM, 10-ethyl-10-deazaaminopterin; MTX, methotrexate; *cis*-Pt, *cis*-diamminedichloroplatinum(II); LD_{10}, dose lethal to 10% of subjects; ILS, increase in life span.

Table 1 *Therapeutic effects of MTX, 10EDAM, and cis-Pt against murine ovarian cancer*

Animals were implanted i.p. with 10^7 ascites cells and selected randomly in either treated or control groups. Therapy was initiated at the LD_{10} dosage either i.p. or s.c. 1 day after tumor implant. LD_{10} dosages were determined separately for i.p. and s.c. administration of drug in tumor bearing mice in a standard dose-finding assay determining toxic deaths at each dose level administered.

	Route	LD_{10}[a] (mg/kg)	Median survival time (days ± SE)	ILS	No. of mice
			14.2 ± 2		15
MTX	s.c.	30	16.5 ± 2	16	10
	i.p.	30	19.0 ± 3	25	15
10EDAM	s.c.	60	21.8 ± 3	52	10
	i.p.	60	26.5 ± 3	88	14[b]
cis-PT	s.c.	4	14.8 ± 2	5	10
	i.p.	4	28.5 ± 3	100	15

[a] Determined from separate toxicity assays using either s.c. or i.p. administration.
[b] Toxic death not included in calculation of median survival time.

Table 2 *Therapeutic effects of i.p. administered MTX or 10EDAM with and without cis-Pt against murine ovarian cancer*

Most of the experimental details are provided in the text and legend of Table 1. In addition, animals receiving both an antifolate and *cis*-Pt were given injections of these drugs essentially simultaneously. Dosing was at the LD_{10} dose or one-half the LD_{10} dosage.

	LD_{10} dosage[a] (fraction)	Median survival time[b] (days ± SE)	ILS (%)	60-day survivors[c] (no./total)
		13.2 ± 2		0/25
MTX	1	16.7 ± 3	27	0/24[d]
	0.5	15.3 ± 3	16	0/15
10EDAM	1	23.6 ± 3	78	0/24[d]
	0.5	19.5 ± 3	48	0/15
cis-Pt	1	25.5 ± 3	94	0/25
	0.5	15.8 ± 2	20	0/15
MTX + *cis*-Pt	1	Toxic		
	0.5	21.3 ± 3	62	0/24[d]
10EDAM + *cis*-Pt	1	Toxic		
	0.5	34.4 ± 4	>161	3/24[d]

[a] MTX, 30 mg/kg; 10EDAM, 60 mg/kg; *cis*-Pt, 4 mg/kg given on a schedule of once per day every 3 days for 3 doses.
[b] Average of 3 to 5 individual experiments of 5 mice each.
[c] Tumor free at the time of evaluation.
[d] One toxic death in each group not included in calculation of median survival time.

cis-Pt, but not MTX, were more effective against this tumor. While MTX was minimally effective by this route, both 10EDAM and *cis*-Pt were highly effective ($P < 0.001$), yielding values of ILS approaching 90 to 100%. Either MTX or 10EDAM were then given with *cis*-Pt i.p. on the same schedule against this ascites tumor. However, because of toxicity, it was necessary to attenuate the dosage of each agent to one-half the LD_{10} dosage. These relative tolerances for the combination of agents *versus* single agent therapy were consistent with our prior (8) results obtained with these same combinations in tumor-bearing C57BL × DBA/2 F_1 (hereafter called $BD2F_1$) mice. However, tolerances to 10EDAM and MTX in C3HeB/Fe mice were more than 2-fold lower than tolerances for these same drugs in $BD2F_1$ mice. The results (Table 2) show that MTX with *cis*-Pt at these doses and schedule was relatively ineffective (ILS 60%) against this tumor, while 10EDAM with *cis*-Pt was highly effective (ILS >160%; $P < 0.001$). In mice receiving this latter combination, there was also a significant increase ($P < 0.05$) in ILS above that obtained with either agent alone at their LD_{10} dosages. In addition, some tumor-free, long-term survivors were obtained with 10EDAM plus *cis*-Pt.

The increased efficacy of 10EDAM when given (Table 1) i.p. rather than s.c. most likely reflected the overall higher potency of this analogue (6–8) compared to MTX and the higher initial accumulation and persistence of this analogue achievable at the site of the tumor by this route. This is entirely consistent with earlier pharmacokinetic studies from our laboratory (6, 16, 17) and elsewhere (13) of this agent and MTX in murine tumor models. Since the same pharmacokinetic considerations would be expected to apply to calcium leucovorin and all three folate compounds as well share (18, 19) the same transport route for entry into tumor cells, it seemed likely that we would be able to increase the dosage of the folate analogues given i.p. and at the same time preserve the antitumor effect and prevent toxicity to the animal by s.c. administration of an appropriate dose of calcium leucovorin; *i.e.*, administration of calcium leucovorin by this route after i.p. 10EDAM should favor the accumulation of each at a higher 10EDAM/calcium leucovorin ratio in the peritoneal cavity than following i.p. administration of both.

The results of these experiments are given in Table 3 and Fig. 1. Tumor-bearing mice were given 10EDAM and *cis*-Pt i.p. on a schedule of every 3 days for 3 doses at a dosage 4 times greater (120 mg/kg) than that normally tolerated as a

Table 3 *Therapeutic effect of i.p. administered 10EDAM or 10EDAM with cis-Pt and delayed s.c. calcium leucovorin against murine ovarian cancer*

Most of the experimental details were provided in the text or the legend of Table 1. In addition, animals receiving both 10EDAM and *cis*-Pt were given injections of these drugs essentially simultaneously. Calcium leucovorin (CF) was administered in a single dose s.c. 16 h after the administration of drug.

10EDAM (mg/kg)	*cis*-Pt (mg/kg)	CF (mg/kg)	Median survival time[a] (days ± SE)	ILS (%)	60-day survivors[b] (no./total)
			14.2 ± 2		
120			Toxic		0/10
120		4	27.1 ± 3	90	0/10
		12	22.4 ± 3	55	0/10
		36	21.0 ± 2	49	0/10
120	2		Toxic		0/10
120	2	4	Toxic		
		12	48	>235	7/21[c]
		36	>55	>278	9/21[c]
240	2		Toxic		0/10
240	2	12	Toxic		0/10
		36	Toxic		0/10

[a] Average of 2 to 4 individual experiments of 5 or 6 mice each.
[b] Tumor free at the time of evaluation.
[c] Toxic death not included in calculation of survival.

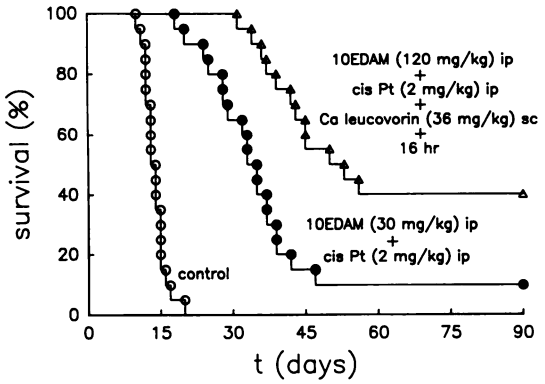

Fig. 1. Survival of mice bearing i.p. murine ovarian cancer following treatment with 10EDAM and *cis*-Pt i.p. with and without systemic calcium leucovorin protection. Mice were treated 1 day following implantation of 10^7 tumor cells on a schedule of once per day every 3 days for 3 doses. Calcium leucovorin was administered s.c. 16 h after each dose of 10EDAM and *cis*-Pt.

combination. This was followed 16 h later, after each dose of 10EDAM plus *cis*-Pt, by a single dose of calcium leucovorin given s.c. This interval between folate analogue and calcium leucovorin administration was found in our prior studies (20) to be optimum for mice receiving high-dose MTX with low-dose calcium leucovorin "rescue." On this schedule, a dose of calcium leucovorin of at least 12 mg/kg was necessary to prevent toxicity. However, at doses of either 12 mg/kg ($P < 0.025$) or 36 mg/kg ($P < 0.001$) calcium leucovorin with 10EDAM (120 mg/kg) and *cis*-Pt, there was a marked increase in antitumor effect (Table 3; Fig. 1) when compared to that obtained with tolerated doses of 10EDAM with *cis*-Pt alone. In addition, the number of tumor-free, long-term survivors was increased to 40% of the animals treated in this way. Similar antitumor effects and total number of tumor-free long-term survivors were obtained (Table 4) with 10EDAM, *cis*-Pt and calcium leucovorin (12 mg/kg) when therapy was delayed 2 days after 10^7 cells were implanted i.p. Significant but reduced antitumor activity was obtained even after a 4-day delay, but not after a 6-day delay following tumor implant. The same regimen incorporating MTX and *cis*-Pt with calcium leucovorin was somewhat more effective (data not shown) than MTX and *cis*-Pt without calcium leucovorin, but markedly less effective ($P < 0.001$, >2-fold lower median survival time, and no tumor-free survivors) than in the case of 10EDAM, *cis*-Pt, and calcium leucovorin. Interestingly, when 10EDAM alone was given at a dose (120 or 240 mg/kg) ordinarily toxic by itself, but with calcium leucovorin, there was no improvement (Table 3) in the therapeutic effect over that obtained with this antifolate alone.

DISCUSSION

In earlier studies by others (12) the responsiveness of this murine ovarian tumor was determined for MTX, *cis*-Pt, Adriamycin, hexamethylamine, cyclophosphamide, and 5-fluorouracil. This tumor, implanted at 10^6 cells/mouse, was relatively insensitive to all of these agents except i.p. administered Adriamycin, MTX, and *cis*-Pt. These workers also incorporated i.p. administration of calcium leucovorin 24 h after a toxic (4-fold higher than the maximum tolerated dose) dose i.p. of MTX with some enhancement of antitumor activity over MTX alone but no tumor-free, long-term survivors were obtained. This study also documented (13) a slower reduction with time in levels of MTX in ascitic fluid following i.p. *versus* i.v. administration of drug. The difference in the results on antitumor activity obtained with MTX in each study most likely relates to the large difference in tumor burden at the time of therapy and the dissimilarity in dosage and scheduling of drug administration used in both studies.

We also note that in earlier pharmacokinetic studies in patients (21, 22), intracavitary infusion of MTX maintained concentrations of this drug in these cavities (peritoneal, pleural, and pericardial) that were 20–40-fold higher than in plasma. A similar differential would be expected in the case of 10EDAM following intracavitary administration in patients since the pharmacokinetic behavior of both agents is essentially the same (22). In one of these studies (21), the simultaneous systemic administration of calcium leucovorin with intracavitary MTX was also explored in patients bearing various malignant effusions including those that were ovarian derived. In this study, calcium leucovorin administration allowed a 4-fold increase in the infusion time from 24 to 120 h with acceptable systemic toxicity. Of interest as well was the finding that all 8 evaluable patients entered into this study exhibited some sort of clinical response.

The lack of improved therapeutic selectivity in this model system of 10EDAM alone with calcium leucovorin when compared to 10EDAM and *cis*-Pt with calcium leucovorin was curious but not entirely unexpected. There are numerous studies in the literature (23) that were unable to document the efficacy of this modality in the case of MTX and calcium leucovorin for various cancers in patients. Although we have provided evidence of therapeutic efficacy (20) for MTX with delayed calcium leucovorin in the case of two murine tumor models, there are most likely many animal tumors in which this modality of therapy would show little improvement over MTX alone. The results derived here might suggest that the marked therapeutic effect observed (Tables 3 and 4; Fig. 1) with 10EDAM plus *cis*-Pt and calcium leucovorin is more likely related to an antifolate-mediated potentiation in effects of *cis*-Pt on tumor (Table 1) compared to normal proliferative tissue in small intestine as well as the higher ratio of 10EDAM/calcium leucovorin probably achieved in the peritoneal cavity compared to small intestine.

Our results also suggest that 10EDAM with *cis*-Pt may have utility for intracavitary therapy of human cancer, including that which is ovarian derived, particularly when incorporating delayed systemic calcium leucovorin protection. We point out that both 10EDAM (9) and *cis*-Pt (1, 2, 5) are clinically active agents and the therapeutic effect observed with this combination of agents in these tumor-bearing animals was obtained at a tumor burden at least 10-fold larger than that used in conventional model systems. In addition, the pharmacokinetic advantage demonstrated (6, 21) for MTX in patients following intracavitary therapy would also apply to 10EDAM, since both agents exhibit similar (22) pharmacokinetic behavior in patients. With regard to human ovarian cancer, the model system used, although differing in its histological origin from this human carcinoma, exhibited biological behavior (11–13) and relative sensitivities to MTX and *cis*-Pt and other therapeutic agents [here and in Ref. 13] that were similar to that seen for the human neoplasm. In addition, the greater antitumor potency for 10EDAM compared to MTX observed in the murine model might also be anticipated for this human neoplasm on the basis of our prior published data (24) on their membrane transport and cytotoxicity for human ovarian carcinoma cells derived directly from patients.

The effective clinical utility of this regimen of therapy will, of course, depend upon tolerances of this combination of agents in patients. For *cis*-Pt, dose-limiting toxicities in both animals and humans appear (25) to occur in the kidney. Myelosuppres-

Table 4 *Treatment of murine ovarian tumor with cis-Pt and 10EDAM with delayed calcium leucovorin: effect of delay in therapy following tumor implant*

10EDAM and *cis*-Pt were administered i.p. to mice at one-half their respective LD_{10} dosage at intervals between 1 and 6 days after implant of 10^7 ascites cells. Calcium leucovorin was given s.c. 16 h after administration of drug.

Delay in therapy[a] (days)	Median survival time[b] (days)	ILS (%)	60-day survivors[c] (no./total)
1	43.5	>229	5/13[d]
2	44.0	>230	4/13[d]
4	25.0 ± 4	85	2/14
6	18.5 ± 3	44	0/14
Control	13.5 ± 2		0/20

[a] Therapy was given on a schedule of every 3 days for 3 doses. Dosages were 2 mg/kg *cis*-Pt, 240 mg/kg 10EDAM, and 12 mg/kg calcium leucovorin.
[b] Average of 2 experiments of 7 to 10 mice each.
[c] Tumor free at the time of death.
[d] One toxic death in each group not included in calculation of median survival time.

sion is also a common occurrence in both species. Some differences do occur between animals and humans in the case of 10EDAM. This agent, when administered to animals (6–8, 26), has dose-limiting effects in the small intestine with no or only transient myelosuppression observed. In patients (22), 10EDAM is also only minimally myelosuppressive with dose-limiting toxicity (mucositis) occurring in the buccal cavity. Because of these nonoverlapping toxicities, the use of this combination of agents in patients may be possible without pronounced dosage attenuation.

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