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Preclinical evaluation of aclacinomycin A for the intraperitoneal treatment of human ovarian carcinoma

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Summary. Combination chemotherapy regimens have produced a pathological complete response rate of only 1%–25% in patients with advanced ovarian cancer. Patients with small-volume residual disease after treatment are refractory to further systemic therapy, and most eventually die of their disease. Intraperitoneal (i.p.) chemotherapy, particularly with adriamycin or cisplatin has shown promise in these patients. However, the dose-limiting painful peritonitis associated with i.p. adriamycin makes this regimen potentially too toxic for many patients. Aclacinomycin A, another anthracycline antibiotic, has been found to have activity against a wide variety of murine tumors and human xenografts. It has also demonstrated clinical efficacy in phase I and II trials against refractory ovarian cancer and has less pronounced vesicant properties than adriamycin, making it an ideal candidate for i.p. use in ovarian cancer patients. In vitro clonogenic assays utilizing a battery of adriamycin-sensitive and -resistant human ovarian carcinoma cell lines have shown that aclacinomycin A is more cytotoxic than adriamycin in all cell lines tested. In addition, aclacinomycin A was found to prolong survival in a nude mouse xenograft of i.p. human ovarian cancer. These results have provided the experimental rationale for an ongoing clinical trial of i.p. aclacinomycin in refractory ovarian cancer patients at the Medicine Branch, NCI.

Introduction

The majority of patients with ovarian cancer have advanced (FIGO stage III–IV) diesease at the time of diagnosis [2]. Combination chemotherapy regimens have achieved clinical complete responses in approximately 50% of patients with bulky disease [1, 4, 23, 25, 26]; however, the pathological complete response rate is less than 25% [26]. The vast majority of patients who are left with small-volume (<2 cm) residual disease after induction chemotherapy are refractory to further systemic therapy and will eventually die of their disease. Phase I and II trials utilizing intraperitoneal (i.p.) dialysis with various antineoplastic agents have been performed in such patients [9, 10, 21, 22] in an effort to convert their partial responses into complete remissions. The rationale and preliminary results of i.p. chemotherapy have recently been reviewed [13]. Most anticancer drugs used in the treatment of ovarian cancer have been shown to have a pharmacologic advantage (peak i.p. drug level/peak plasma level) when given i.p.; however, the optimum drug remains to be determined.

Adriamycin was initially evaluated in patients with ovarian cancer for the following reasons: (a) It is an active agent in ovarian cancer with a 40% response rate in untreated patients [3, 27]; (b) in vitro studies with the human tumor stem cell assay showed a response at high drug doses which could potentially be achieved by i.p. drug delivery [20]; (c) i.p. adriamycin was shown to be curative in 70% of mice with murine ovarian cancer [17]; and (d) i.p. adriamycin penetrated into small tumor masses in the murine model [18]. The phase I study of i.p. adriamycin in patients with advanced ovarian cancer demonstrated a pharmacologic advantage for adriamycin of 474 [21]. In addition, there were three objective responses among the ten patients treated, and two other patients had a marked decrease in ascites production. Despite the encouraging therapeutic results, the major dose-limiting toxicity was a painful, sterile peritonitis, making this treatment potentially too toxic for many patients.

Aclacinomycin A is an anthracycline antibiotic isolated from a culture broth of *Streptomyces galilaeus* in 1974, which may be a useful alternative to adriamycin for i.p. use in ovarian cancer [14]. It has been studied extensively in Japan and has been found to have activity against a wide spectrum of murine tumors as well as some human xenografts [8, 15, 16]. Phase I and II trials have demonstrated responses in breast cancer (21%), ovarian cancer (27%), and especially in acute leukemia (38% complete responses in previously untreated patients) [16, 24]. The maximum tolerable dose in man was 4 mg/kg, the safe dose for weekly administration being 2 mg/kg. The peak blood level achieved after an i.v. injection of 2 mg/kg in patients was 0.2 μg/ml (0.23 μ*M*) [16]. Of particular interest in these studies is the very low incidence of phlebitis and the absence of tissue necrosis after extravasation [15]. The clinical efficacy of the drug coupled with its decreased vesicant properties prompted its evaluation as an alterative to adriamycin i.p.

In this study the activity of aclacinomycin A has been compared with that of adriamycin in clonogenic assays using several human ovarian cancer cell lines. Cell lines

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studied included those derived from previously untreated patients and those from patients currently refractory to adriamycin-containing combination chemotherapy regimens when cells were taken. Additional adriamycin-resistant cell lines were developed in vitro from the sensitive parental cell line [7, 11]. Finally, a nude mouse xenograft model of human ovarian cancer was also used to examine the efficacy of i.p. aclacinomycin A [6].

Materials and methods

Chemicals and reagents. Roswell Park Memorial Institute (RPMI) 1640, Fetal Bovine Serum (FBS), penicillin/streptomycin, and glutamine were obtained from Grand Island Biological Company, Chagrin Falls, Ohio. Type VII agarose was from Sigma Chemical Company, St. Louis, Mo. Insulin (Iletin U-100) was obtained from Eli Lilly and Co., Indianapolis, Ind. Adriamycin and aclacinomycin A were from the Pharmaceutical Resources Branch, National Cancer Institute, Bethesda, Md.

Ovarian cancer cell lines. The human ovarian cancer cell lines used in this study have previously been characterized. Cell line A2780 was established from a previously untreated patient with ovarian cancer [5], and the adriamycin-resistant variant $A2780^{AD}$ was developed by stepwise incubation of A2780 with increasing concentrations of adriamycin [11]. Cell lines OVCAR-$3^{Nu(Ag+)}$ and OVCAR-$4^{(Ag+)}$ were established from ovarian cancer patients while they were refractory to adriamycin-containing combination chemotherapy regimens [7, 11].

Tissue culture media. Cells were passaged and maintained in RPMI 1640 supplemented with 10% (v/v) FBS, insulin 0.25 units/ml, streptomycin 100 μg/ml, penicillin 100 units/ml, and glutamine 0.3 mg/ml, as previously described [19]. Cells were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2.

Clonogenic assay. Drug-sensitivity curves were determined using clonogenicity in soft agarose as previously described [19]. Briefly, cells were harvested with a trypsin (0.05%, w/v)/EDTA (0.02% w/v) solution and counted on a Coulter Counter (Coulter Electronics Model ZBI). Cells in single-cell suspension were plated in a mixture of 0.3% (w/v) agarose, RPMI 1640 (including the ingredients listed above) and drug at various concentrations, over a layer of solidified 0.6% (w/v) agarose in 10-cm^2 dishes. Colonies measuring greater than 60 μm and containing more than 50 cells were scored on a Bausch and Lomb Omnicon Fas II System. A2780 and 2780^{AD} were counted on day 7, and OVCAR-$3^{nu(Ag+)}$ and OVCAR-$4^{(AG+)}$ were counted on day 21 and day 14, respectively.

Nude mouse survival study. An athymic nude mouse model (Balb/c) with an i.p. xenograft of the OVCAR-$3^{nu(Ag+)}$ cell line has been previously described in detail [6]. LD_{10} doses for i.p. adriamycin and i.p. aclacinomycin A in normal nude mice were previously determined to be 2 mg/kg for adriamycin and approximately 16 mg/kg for aclacinomycin. The survival of mice is dependent upon the dose of tumor inoculum. The animals died of intraperitoneal carcinomatosis and massive ascites. Ten nude mice received injections i.p. of approximately 30×10^6 nucleated cells harvested from the ascites of animals bearing the xenograft [6]. Four days later, five of the animals received aclacinomycin A 16 mg/kg by i.p. injection, with five animals serving as untreated controls. Animals were observed and deaths charted through 70 days from the time of tumor injection.

Results

Clonogenic assay

Figure 1A illustrates the dose-response curves for adriamycin and aclacinomycin A against the untreated ovarian cancer cell line A2780 [5]. The IC_{50} (dose which reduces colony formation by 50%) for adriamycin is 0.017 μ*M*, as against 0.0085 μ*M* for aclacinomycin A. This indicates that A2780 is twice as sensitive to aclacinomycin A as to adriamycin on a molar basis.

Figure 1B compares the cytotoxicity of adriamycin and aclacinomycin A in the adriamycin-resistant cell line 2780^{AD}, which is 100-fold more resistant to adriamycin than the parent line, A2780 [11]. The IC_{50} for adriamycin vs 2780^{AD} is 1.5 μ*M*, as against 0.03 μ*M* for aclacinomycin A, demonstrating that 2780^{AD} is 50 times more sensitive to aclacinomycin A than to adriamycin. However, 2780^{AD} is still 3.5-fold cross-resistant to aclacinomycin A.

Ovarian cancer cell lines OVCAR-$3^{nu(Ag+)}$ and OVCAR-$4^{(Ag+)}$ were established from patients clinically refractory to cisplatin, adriamycin, and cyclophosphamide [7, 11]. Figures 1C and 1D show the dose-response relationships for adriamycin and aclacinomycin A against OVCAR-$3^{nu(Ag+)}$ and OVCAR-$4^{(Ag+)}$, respectively. The IC_{50} for adriamycin against OVCAR-$3^{nu(Ag+)}$ is 0.047 μ*M*, as apposed to 0.019 μ*M* for aclacinomycin A. Thus OVCAR-$3^{nu(Ag+)}$ is 3 times as resistant to adriamycin as the untreated cell line A2780. It is also 2.5 times more sensitive to aclacinomycin A than to adriamycin. The results with OVCAR-$4^{(Ag+)}$ (Fig. 1D) are similar: the IC_{50} for adriamycin is 0.07 μ*M* and for aclacinomycin A is 0.026 μ*M*. Therefore OVCAR-$4^{(Ag+)}$ is approximately 4 times as resistant to adriamycin as A2780, and 2.7 times more sensitive to aclacinomycin A than adriamycin.

Nude mouse survival study

In a survival study designed to evaluate the in vivo efficacy in of i.p. aclacinomycin A in our i.p. xenograft model of human ovarian cancer, we found that i.p. aclacinomycin A at 16 mg/kg (less than the LD_{10}) increased median survival to 54 days, as against 37 days in untreated controls, leading to a T/C of 146%. We observed no early deaths in aclacinomycin-treated animals which could be attributed to drug toxicity, and deaths of treated animals appeared grossly to be the result of complications associated with i.p. carcinomatosis, as with untreated controls. In addition, at this aclacinomycin dose animals showed no sign of acute toxicity. This is in contrast to the acute distress observed in animals treated with i.p. adriamycin at doses as low as 5 mg/kg in separate studies.

Discussion

Despite promising therapeutic results in the phase I study, i.p. adriamycin has been found to be too toxic for most patients with ovarian cancer, owing to a painful chemical

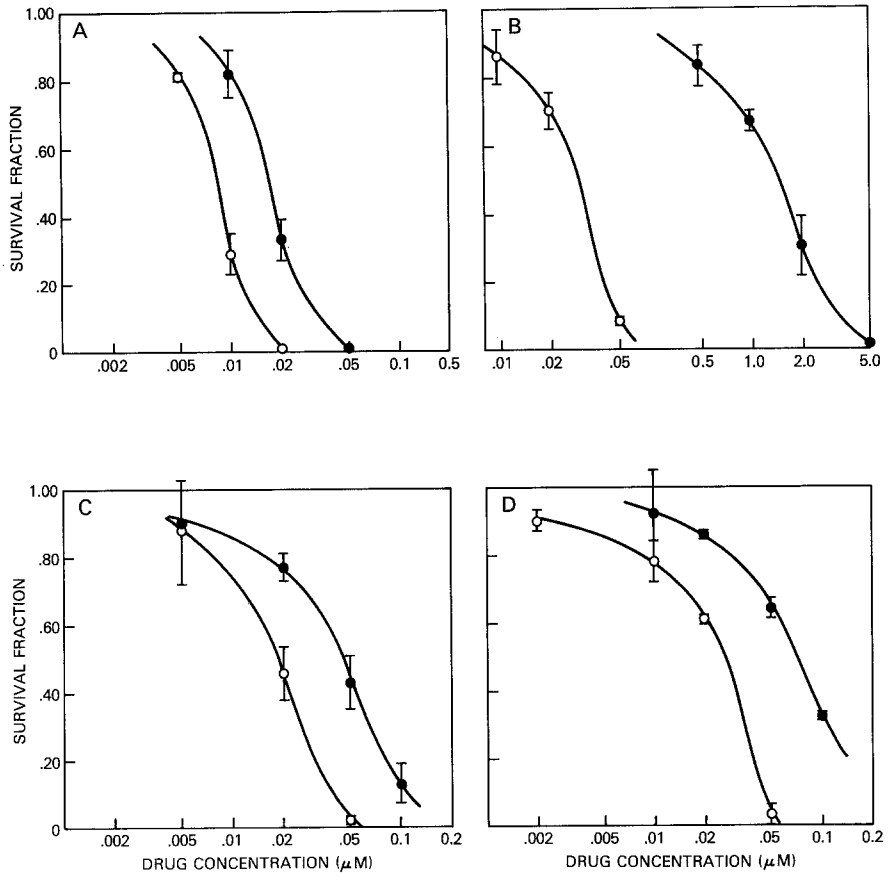

Fig. 1A–D. Continuous-exposure clonogenic assays of adriamycin (●) and aclacinomycin A (○) versus A2780 **(A)**, 270^{AD} **(B)**, OVCAR-$3^{Nu(Ag+)}$ **(C)**, and OVCAR-$4^{(AG+)}$ **(D)**. Each *point* represents the mean (±1 SD) of two experiments, each with triplicate dishes

peritonitis [21]. Aclacinomycin A, another anthracycline antibiotic, has been shown to have activity against a wide range of murine and human tumors, as well as clinical efficacy in phase I/II trials with ovarian cancer.[8, 15, 16, 24]. In addition, it has a greatly reduced incidence of phlebitis, vascular pain, and tissue necrosis after extravasation, suggesting that it might be a more suitable anthracycline for i.p. use [15].

A battery of human ovarian cancer cell lines, which included lines established from an untreated patient, from two patients who had relapsed after combination chemotherapy, and a subline with in vitro-induced resistance to single-agent adriamycin, was used to test the dose-response relationship of aclacinomycin A in comparison with that of adriamycin. Figures 1A–D demonstrates that aclacinomycin A was more effective than adriamycin in all the cell lines tested. It is not clear whether the 2- to 3-fold increase in cytotoxicity with aclacinomycin A compared with adriamycin may result in a clinically significant advantage. 2780^{AD}, a cell line with a high degree of resistance to adriamycin, was 50-fold more sensitive to aclacinomycin A than adriamycin, suggesting that the mechanism of in vitro-induced resistance to adriamycin is different from the mechanism of resistance to aclacinomycin A in at least some particulars.

Phase I/II trials with aclacinomycin A have revealed the maximum tolerated dose in humans to be 4 mg/kg per injection and shown that the safe i.v. dose for weekyl injections is 2 mg/kg. A 2-mg/kg i.v. dose of aclacinomycin A produces a blood level of 0.23 μ*M* with an initial half-life of 2–5 min. From Fig. 1A–D it can be seen that such a dose is higher than the IC_{50} for all cell lines tested. While it is difficult to compare pharmacokinetic data in humans with the exact doses required for in vitro cytotoxicity, these results still suggest that cytotoxic drug levels can be achieved in ovarian cancer patients. However, there are no available data regarding the penetration of aclacinomycin A into the peritoneal fluid after an i.v. dose. One would expect peritoneal fluid levels to be substantially lower. Whether the i.p. level of aclacinomycin A would be below the IC_{50} of resistant cells and whether this would translate into a noticeable effect on clinical efficacy remains to be established. The level of aclacinomycin A that may be achieved via peritoneal dialysis (assuming the starting dose of 100 mg/2 l dialysate) would be 56.5 μ*M* or approximately 245 times the level clinically achievable in blood after an i.v. dose.

Aclacinomycin A is effective in the nude mouse xenograft model of i.p. human ovarian cancer, as monitored by prolongation of survival. These results, when taken together with the clonogenic cytotoxicity data, strongly, suggest that i.p. aclacinomycin A may be effective in ovarian cancer patients with minimal (<2 cm) residual disease.

On the basis of these studies, The National Cancer Institute has an ongoing phase I–II trial of i.p. aclacinomycin A in relapsed ovarian cancer patients with <2 cm residual disease. To date, one stage III patient with minimal residual disease after cyclophosphamide, hexamethylmelamine, cisplatin, and abdominopelvic irradiation has been treated with minimal side effects, demonstrating the clini-

cal feasibility of administering the drug i.p. In addition, after six cycles of treatment (100 mg drug in 2 l saline given every 2–3 weeks via a Tenckhoff catheter), repeat peritineoscopy and "second-look" laparotomy with multiple biopsies demonstrated a complete remission. A second patient is now undergoing therapy, which is being well tolerated; disease evaluation awaits the completion of therapy. It is of note that pathological complete remissions are very difficult to achieve in previously treated patients with minimal residual disease. In the phase I trial of i.p. adriamycin there were no pathological complete remissions [21]. In addition, only about 30% of patients with small-volume disease will attain a pathological complete remission with i.p. cisplatin [12]. Thus, alternative drugs are needed and aclacinomycin A appears to be a very promising candidate.

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