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# Combination of etanidazole with cyclophosphamide and platinum complexes*

**Beverly A. Teicher[1,2], Terence S. Herman[1,2], Lawrence Shulman[4], Glenn Bubley[6], C. Norman Coleman[2,3], and Emil Frei III[2]**

[1] Dana-Farber Cancer Institute and [2] Joint Center for Radiation Therapy, 44 Binney Street, Boston, MA 02115, USA
[3] Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA
[4] Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215, USA

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**Summary.** In an effort to improve the therapeutic efficacy and selectivity of cyclophosphamide (CTX), *cis*-diamminedichloroplatinum(II) (CDDP), and carboplatin (Carbo), these antitumor alkylating agents were combined with the 2-nitroimidazole drug etanidazole (ETA). As revealed by tumor-cell survival assay in the FSaIIC murine fibrosarcoma, the addition of ETA (1 g/kg, i.p.) just prior to the i.p. injection of various doses of the alkylating agents resulted in increases in the tumor-cell kill produced by each drug (CTX, 10-fold; CDDP, 20-fold; and Carbo, 5- to 15-fold), whereas toxicity to bone marrow granulocyte-macrophage colony-forming units (CFU-GM) increased only about 0- to 3-fold. When CTX was combined with either CDDP or Carbo, striking increases in tumor-cell killing were observed (20- to 100-fold across the CDDP dose range and 5- to 20-fold across the dose range of Carbo), which were supra-additive for CDDP and additive for Carbo as revealed by isobologram analysis. The addition of ETA to these alkylating-agent combinations produced a further approx. 20-fold increase in tumor-cell kill for both CTX/CDDP and CTX/Carbo. This effect was greatest at the lowest dose of the platinum drug tested and was supra-additive in the case of CDDP and additive for Carbo. Following treatment with ETA/CTX/CDDP, bone marrow CFU-GM toxicity increased only about 5-fold over that of CTX/CDDP alone, but the injection of ETA/CTX/Carbo resulted in a 10- to 20-fold increase in bone marrow toxicity as compared with that obtained using CTX/Carbo alone. Tumor growth-delay studies revealed significant increases in the antitumor effect of the alkylating agents when these were given in combination with ETA. Both the ETA/CTX/CDDP and the ETA/CTX/Carbo combinations produced tumor growth delays of 23 days, which represented approx. 1.6-fold increases over those obtained using the alkylating-agent combinations alone. These results suggest that ETA could significantly improve the therapeutic efficacy of these alkylating agents, whether they are given individually or in combination.

## Introduction

The combination of *cis*-diamminedichloroplatinum(II) (CDDP) and cyclophosphamide (CTX) has been well established in the treatment of ovarian carcinoma [1, 4]. In recent years, several clinical trials have been conducted comparing the efficacy of carboplatin (Carbo) [8, 20] with that of CDDP given alone [3, 12, 19, 22] and in combination with CTX [1, 4] to patients with ovarian carcinoma. The overall conclusion drawn from many of the studies has been that at doses that produce response rates equal to those achieved with CDDP, the spectrum of normal tissue toxicities resulting from Carbo treatment is much more tolerable than that seen following therapy with CDDP [19]. However, complete response rates in these clinical trials remain in the range of 20%–35%, indicating that there is much room for improvement.

The 2-nitroimidazole radiosensitizer etanidazole (ETA) is also a hypoxic-cell-selective cytotoxic agent [5, 31] and is a chemosensitizer or modulator of some antitumor drugs [11, 21, 28]. Although the precise mechanism by which ETA acts as a chemosensitizer is not known, the most important mechanisms appear to occur at the cellular level and may involve the interaction of a metabolite of the 2-nitroimidazole with the alkylating agent in the vicinity of the DNA [9, 13, 15, 18, 21, 33]. In the present study, we used isobologram analysis [30] to examine the ability of

*Abbreviations:* ETA, etanidazole; CTX, cyclophophamide; CDDP, *cis*-diamminedichloroplatinum(II); Carbo, carboplatin; PBS, phosphate-buffered saline; FBS, fetal bovine serum; DMEM, Dulbecco's minimal essential medium; CFU-GM, granulocyte-macrophage colony-forming units

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*Offprint requests to:* Beverly A. Teicher, Dana-Farber Cancer Insitute, 44 Binney Street, Boston, MA 02115, USA

ETA to potentiate the tumor-cell kill and tumor growth delay produced by CTX, CDDP, and Carbo used alone and in combination in the FSaIIC fibrosarcoma. Survival of bone marrow granulocyte-macrophage colony-forming units (CFU-GM) was used to estimate the effects of the combinations on representative normal tissue. Our objective in conducting these studies was to attempt to achieve a marked improvement in the antitumor efficacy of these alkylating agents against this tumor model using the relatively nontoxic drug ETA.

## Materials and methods

*Drugs.* (ETA was obtained as a gift from the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, Md.). CTX was purchased from Sigma Chemical Co. (St. Louis, Mo.). CDDP and Carbo were gifts from Drs. D. H. Picker and M. J. Abrams, Johnson Matthey, Inc. (West Chester, Pa.).

*Tumor.* The FSaII fibrosarcoma [17], adapted for growth in culture (FSaIIC) [25], was maintained in male C3H/FeJ mice (Jackson laboratories, Bar Harbor, Me.). For the experiments, $2 \times 10^6$ tumor cells prepared from a brei of several stock tumors were implanted s.c. into the legs of male C3H/FeJ mice 8–10 weeks of age.

*Tumor excision assay.* When the volume of the tumors had reached approx. 100 $mm^3$ (about 1 week after tumor-cell implantation), animals were treated either with single doses of alkylating agents (CTX, CDDP, or Carbo) prepared freshly in phosphate-buffered normal saline (PBS) and given i.p. in a volume of about 0.2 ml either alone or preceded by an i.p. injection of 1 g/kg ETA or with i.p. injections of 1 g/kg ETA plus 150 mg/kg CTX immediately prior to the i.p. administration of various doses of CDDP or Carbo. Mice were killed at 24 h after treatment to ensure full expression of drug cytotoxicity and repair of potentially lethal damage and were then immersed in 95% ethanol. The tumors were excised under sterile conditions, and single-cell suspensions were prepared for the colony-forming assay [25, 27]. At 1 week thereafter, the plates were stained with crystal violet, and colonies of $\geq 50$ cells were counted. The untreated tumor-cell suspensions showed a plating efficiency of 8%–12%. The results were expressed as the surviving fraction ± SE of cells from treated groups as compared with untreated controls.

*Bone marrow toxicity.* Bone marrow was taken from the same animals used for the tumor excision assay. A pool of marrow from the femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle and the CFU-GM assay was carried out as previously described [27]. Colonies of $\geq 50$ cells were scored on an Acculite colony counter (Fisher Scientific, Springfield, N. J.). The results of three experiments, in which determinations were made in each group at three cell concentrations in duplicate, were averaged. The results were expressed as the surviving fraction ± SE of treated groups as compared with untreated controls.

*Tumor growth-delay experiments.* All drugs were given by i.p. injection. Animals were either treated with ETA immediately prior to each administration of the various allkylating agents or were given 1 g/kg ETA alone on days 7, 9, and 11 following tumor-cell implantation. CTX (100 mg/kg) was given on days 7, 9, and 11; 10 mg/kg CDDP was injected as a single dose on day 7, as was 50 mg/kg Carbo. In the combination studies, ETA was injected as a single dose of 1 g/kg immediately prior to the administration of the alkylating agent(s) or at a dose of 350 mg/kg at 1 h before treatment with the alkylating agent(s) followed by a dose of 650 mg/kg given immediately prior to their administration. The progress of each tumor was measured three times weekly until it reached a volume of 500 $mm^3$; tumor growth delay was calculated as the number of days (± SE) required for each tumor to reach this volume as compared with untreated controls. Each treatment group comprised seven animals and each experiment was repeated three times.

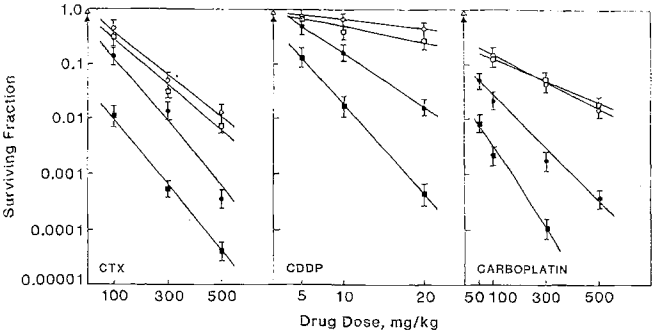


**Fig. 1.** Survival of FSaIIC tumor cells (●, ■, ▲) and bone marrow CFU-GM (○, □, △) from animals treated in vivo with 1 g/kg ETA given immediately prior to a single dose of each alkylating agent (■ □) or with a single dose of the alkylating agent alone (●, ○). Shown on the axis is 1 g/kg ETA (▲, △). *Points* represent the means of 3 independent determinations; *bars* represent the SE

*Data analysis.* Quantitative analysis of survival curves was carried out using the log-probit iterative least-squares method of Litchfield and Wilcoxon [14] as revised by Tallarida and Murray [24]. Calculations were done on an Apple IIC microcomputer. The method of Deen and Williams [6] was used to generate isobolograms for the special case in which the dose of one agent is held constant. This method produces envelopes of additive effect for different levels of the variable agent; it is conceptually identical to our generating a series of isobolograms and replotting the results at a constant dose of one agent on a log effect by dose of the second agent in a coordinate system. Dose-response curves were first generated for each individual agent using dose or log dose and effect, log effect, probit percentage of effect, or logit percentage of effect relations; for cell-survival dose-response curves, correlations of $\geq 0.96$ were obtained. The envelopes of additivity shown in Figs. 2 and 3 were generated from a series of iso-effect curves derived from the complete dose-response curves for each agent.

Overall, combinations producing the desired effect that lie within the envelope boundaries of modes I and II are considered to be additive; those displaced to the left are supra-additive and those displaced to the right are subadditive [2, 23]. This general approach can be extrapolated to the special case in which the level of an agent is held constant. Under these conditions, an isobologram can be derived that plots the expected effect (modes I and II) for any level of the variable agent plus the constant-agent combinations [7]. Experimentally, this approach is quite simple and readily facilitates the determination of additive and nonadditive combinations.

## Results

FSaIIC tumor-cell survival curves and bone marrow CFU-GM survival curves for CTX, CDDP, and Carbo given as single bolus injections with or without ETA are shown in Fig. 1 [23, 29]. Treatment with 1 g/kg ETA produced very little killing of FSaIIC tumor cells (S.F. = 0.65) and no measurable killing of bone marrow CFU-GM from the same animals. However, when the administration of ETA just preceded treatment with CTX, there was a 10-fold increase in tumor-cell kill across the dose range tested, whereas only a 1.5- to 2-fold increase was observed in the killing of bone marrow CFU-GM from the same animals.

ETA had a dose-modifying effect on tumor-cell killing by CDDP; that is, its enhancement of the tumor-cell kill

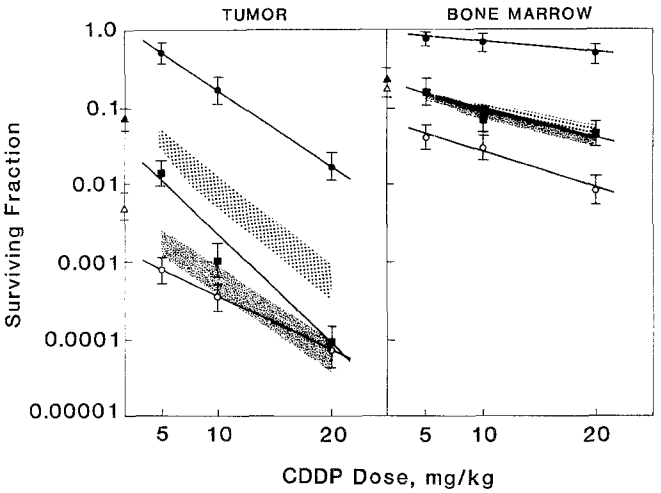


**Fig. 2.** Survival of FSaIIC tumor cells and bone marrow CFU-GM from animals treated in vivo with single doses of CDDP alone (●) preceded by 150 mg/kg CTX (■) or by 1 g/kg ETA together with 150 mg/kg CTX (○). Shown on the axis are 150 mg/kg CTX (▲) and 1 g/kg ETA together with 150 mg/kg CTX (△). *Shaded areas* indicate the envelopes of additivity determine by isobologram analysis of the survival curves for each drug combination. *Points* represent the means of 3 independent determinations; *bars* represent the SE

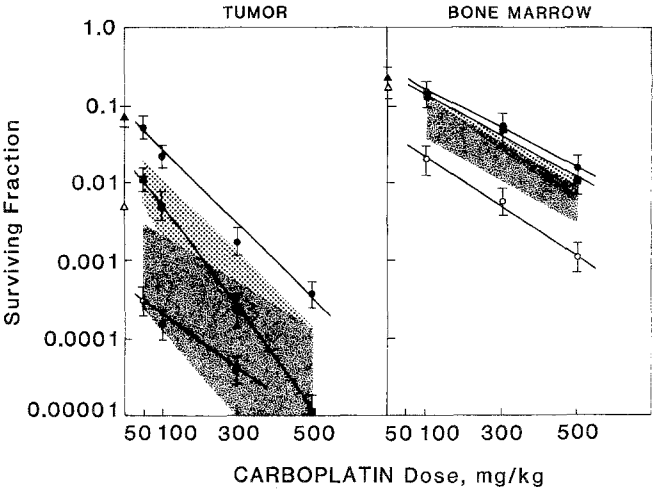


**Fig. 3.** Survival of FSaIIC tumor cells and bone marrow CFU-GM from animals treated in vivo with single doses of carboplatin alone (●) preceded by 150 mg/kg CTX (■) or by 1 g/kg ETA together with 150 mg/kg CTX (○). Shown on the axis are 150 mg/kg CTX (▲) and 1 g/kg ETA together with 150 mg/kg CTX (△). *Shaded areas* indicate the envelopes of additivity determined by isobologram analysis of the survival curves for each drug combination. *Points* represent the means of 3 independent determinations; *bars* represent the SE

produced by CDDP increased from about 4-fold to about 30-fold over the CDDP dose range examined. The cytotoxicity of CDDP toward bone marrow CFU-GM was limited, and the addition of ETA resulted in an increase of about 1.2- to 2-fold in tumor-cell kill over the CDDP dose range examined.

ETA also had a dose-modifying effect on the cytotoxicity of Carbo toward FSaIIC tumor cells. The enhancement by ETA of the tumor-cell kill achieved using Carbo increased from about 6-fold to >37-fold over the Carbo dose range tested. Treatment with ETA did not increase the cytotoxicity of Carbo toward bone marrow CFU-GM. Overall, assuming that, if bone marrow CFU-GM is a representative sensitive normal tissue, a marked increase in the therapeutic index of each of these three drugs was obtained when they were used in combination with ETA.

For examination of the combination of CTX and CDDP, a dose of 150 mg/kg CTX was selected (Fig. 2). When CTX was given just prior to treatment with CDDP, the resulting tumor-cell kill was supra-additive as determined by isobologram analysis. When administration of the combination of ETA and CTX preceded treatment with CDDP, the complete treatment regimen produced supra-additive tumor-cell killing at lower doses of CDDP and additive tumor-cell killing at high CDDP doses. The bone marrow CFU-GM cytotoxicity of the combination of CTX (150 mg/kg) and CDDP appeared to be primarily additive. However, there was an additional approx. 3-fold enhancement of bone marrow CFU-GM cytotoxicity when ETA was added to the CTX-CDDP treatment.

Similar experiments were conducted to examine the effect of the combination of CTX (150 mg/kg) and a range of Carbo doses on tumor-cell killing in the FSaIIC fibrosarcoma (Fig. 3). The tumor-cell kill achieved using CTX with Carbo was additive over the range of Carbo doses examined. When ETA + CTX was added to treatment with Carbo, the combination was most effective at lower Carbo doses, as described above for CDDP. At these lower doses, Carbo lay at the limits of the envelope of additivity, almost reaching supra-additivity, but the combination using higher doses of Carbo lay well within the envelope of additivity. The combination of CTX and Carbo produced killing of bone marrow CFU-GM that was only equivalent to that of Carbo alone. However, the addition of ETA to the CTX-Carbo treatment increased the killing of bone marrow CFU-GM about 8- to 10-fold over the Carbo dose range tested.

Ratios of bone marrow to tumor-cell survival for standard and high doses of the alkylating agents are shown in Table 1. Under both standard- and high-dose conditions, the addition of ETA to treatment with the alkylating agents resulted in an improvement in the ratio of bone marrow to tumor-cell killing. At standard doses of alkylating agents, the addition of ETA to treatment with CTX and CDDP or Carbo also improved the ratio of bone marrow to tumor-cell killing. However, caution is warranted: although higher ratios of bone marrow to tumor-cell killing may occur following some of these treatments, the quantitative level of bone marrow killing may be dose-limiting.

For tumor growth-delay studies 100 mg/kg CTX was given on an alternate-day schedule for three injections (days 7, 9, and 11), whereas 10 mg/kg CDDP and 50 mg/kg Carbo were injected as single doses on day 7 (Table 2). When ETA was added to these treatments, it was given as a single dose of 1 g/kg immediately prior to the injection of each alkylating agent or as a priming dose of 350 mg/kg followed 1 h later by a second dose of 650 mg/kg given immediately prior to the administration of the alkylating agent. ETA ($3 \times 1$ g/kg) essentially produced no tumor growth delay in the FSaIIC fibrosarcoma.

**Table 1.** Ratios of bone marrow CFU-GM survival to FSaIIC tumor-cell survival in mice treated with CDDP or carboplatin and/or CTX with or without ETA at a standard or high dose

| Treatment group[a] | Bone marrow survival ratio, standard dose | Tumor-cell survival ratio high dose |
|---|---|---|
| CDDP | 1.5 | 28.1 |
| Carbo | 2.7 | 40.5 |
| CTX | 3 | 28.9 |
| ETA/CDDP | 5.4 | 583 |
| ETA/Carbo | 14.1 | >1,700 |
| ETA/CTX | 30 | 160 |
| CTX/CDDP | 11.5 | 500 |
| CTX/Carbo | 11.8 | 1,000 |
| ETA/CTX/CDDP | 53.3 | 1,000 |
| ETA/CTX/Carbo | 62.5 | >110 |

[a] Treatment groups correspond to those shown in Fig. 1–3

**Table 2.** Delay in growth of the FSaIIC fibrosarcoma produced by combinations of ETA and CTX with CDDP or carbo

| Treatment group[a] | Tumor growth delay[b] (days) |
|---|---|
| ETA (3 × 1 g/kg) | 0.3 ± 0.5 |
| CTX (3 × 100 mg/kg) | 10.4 ± 1.8 |
| CDDP (10 mg/kg) | 7.8 ± 1.4 |
| Carbo (50 mg/kg) | 6.2 ± 1.5 |
| CTX (3 × 100 mg/kg)/CDDP (10 mg/kg) | 14.4 ± 1.7 |
| CTX (3 × 100 mg/kg)/Carbo (50 mg/kg) | 12.8 ± 1.6 |
| ETA (3 × 1 g/kg)/CTX (3 × 100 mg/kg) | 13.1 ± 1.8 |
| ETA (3 × 350/650 mg/kg)/CTX (3 × 100 mg/kg) | 13.4 ± 1.8 |
| ETA (1 g/kg)/CDDP (10 mg/kg) | 12.3 ± 1.6 |
| ETA (350/650 mg/kg)/CDDP (10 mg/kg) | 12.7 ± 1.4 |
| ETA (1 g/kg/Carbo (50 mg/kg) | 7.8 ± 1.3 |
| ETA (350/650 mg/kg)/Carbo (50 mg/kg) | 10.5 ± 1.4 |
| ETA (3 × 1 g/kg)/CTX (3 × 100 mg/kg)/CDDP (10 mg/kg) | 22 ± 1.9 |
| ETA (3 × 350/650 mg/kg)/CTX (3 × 100 mg/kg)/CDDP (10 mg/kg) | 25.4 ± 2.8 |
| ETA (3 × 1 g/kg)/CTX (3 × 100 mg/kg)/Carbo (50 mg/kg) | 20.4 ± 2.3 |
| ETA (3 × 350/650 mg/kg)/CTX (3 × 100 mg/kg/Carbo (50 mg/kg) | 23.8 ± 2.5 |

[a] ETA (1 g/kg or 350/650 mg/kg) was injected i. p. prior to each dose of alkylating agent; CDDP (10 mg/kg) and Carbo (50 mg/kg) were given i. p. on day 7; CTX (3 × 100 mg/kg) was injected i. p. on days 7, 9, and 11
[b] Tumor growth delay is the difference in days required for treated tumors to reach 500 $mm^3$ as compared with untreated control tumors. The data represent the mean value for 14 animals ± SE. Untreated control tumors reach 500 $mm^3$ in 12.2 ± 0.4 days

The addition of ETA on either the single- or split-dose schedule to treatment with CTX increased the tumor growth delay prduced by the latter from about 10 days to about 13 days. The addition of ETA given as a single or a split dose to treatment with CDDP resulted in an increase in tumor growth delay from about 8 days to about 12.5 days. A single, full dose of ETA added to treatment with Carbo increased the tumor growth delay from about 6 days for Carbo alone to about 8 days; however, when ETA was given on the split-dose schedule, there was a greater increase in the tumor growth delay form about 6 days for Carbo alone to about 10.5 days for the combination.

The combination of CTX (3 × 100 mg/kg; days 7, 9, 11) and CDDP (10 mg/kg, day 7) produced about 14 days of tumor growth delay; the addition of ETA (3 × 1 g/kg) to this treatment resulted in about 22 days of tumor growth delay, whereas that of ETA given on the split-dose schedule increased the delay in tumor growth to about 25 days. These results indicate that the additon of ETA resulted in a 1.6- to 1.8-fold increase in the effectiveness of the CTX-CDDP combination. Similarly, CTX (3 × 100 mg/kg; days 7, 9, 11) and Carbo (50 mg/kg, day 7) produced a tumor growth delay of about 13 days. When ETA (3 × 1 g/kg) was added to CTX + Carbo, the delay in tumor growth increased to about 20 days, whereas treatment with ETA given on the split-dose schedule in combination with CTX and Carbo produced a tumor growth delay of about 24 days. Therefore, the addition of ETA to CTX + Carbo resulted in a 1.5- to 1.8-fold increase in tumor growth delay.

## Discussion

To improve the efficacy of chemotherapy in the treatment of solid tumors, regimens must be developed that result in increased tumor-cell killing without increasing the damage to normal tissue. ETA is a promising "modulator" or "chemosensitizer" of chemotherapy for the treatment of solid tumors because it is selectively cytotoxic toward the hypoxic tumor subpopulation [32] and because this 2-nitroimidazole appears to cause a relatively selective enhancement of the cytotoxicity of several antitumor alkylating agents toward tumor cells as compared with bone marrow CFU-GM as a representative normal tissue. Although the mechanism by which ETA acts as a chemosensitizer is not known, greater efficiency of DNA cross-link formation by the alkylating agents appears to occur in tumors treated with a combination of ETA and these agents [9, 13, 15, 18, 21, 33].

The current study explored the effects of ETA on two alkylating-agent combinations, CTX-CDDP and CTX-Carbo. Carbo is a less potent platinum-containing antitumor agent than is CDDP; the production of a tumor-cell kill equivalent to that caused by CDDP requires approx. A 5-fold dose of Carbo. This differential was maintained when ETA was used in combination with these drugs (Fig. 1). However, when 150 mg/kg CTX was combined with each platinum drug, tumor-cell killing was supra-additive for CTX-CDDP, leading to 3 logs of tumor-cell kill for CTX +10 mg/kg CDDP (Fig. 2), whereas only additivity was found for the combination of CTX and Carbo, leading to 2 logs of tumor-cell kill for CTX + 50 mg/kg Carbo (Fig. 3). When ETA was added to the combination of CTX and CDDP, the tumor-cell kill produced continued to be slightly supra-additive at the low CDDP dose, which

is similar to results that are clinically achievable using standard CDDP doses. The combination of ETA with CTX and Carbo was additive over the Carbo dose range examined, although at the lower dose of Carbo, this combination was also almost supra-additive. Using the complete treatment combinations, the 5-fold difference between CDDP and Carbo doses was again observed: ETA/CTX/CDDP (10 mg/kg) killed about 3.5 logs of tumor cells, as did ETA/CTX/Carbo (50 mg/kg).

Although ETA did not significantly increase the single-agent toxicity of any of the drugs to bone marrow CFU-GM a significant increase in the killing of bone marrow CFU-GM was observed following treatment with the complete combinations of ETA/CTX/CDDP and ETA/CTX/Carbo. However, in either case, a substantial selectivity remained in the enhancement of tumor-cell killing as compared with bone marrow lethality. Although Carbo (50 mg/kg) was somewhat less effective than CDDP (10 mg/kg) in the tumor growth-delay studies, there was no significant difference in the treatment results obtained using ETA/CTX/CDDP and those achieved using ETA/CTX/Carbo. The tumor growth delays produced by both the CTX/CDDP and the CTX/Carbo combinations were improved by factors of 1.5–1.8 by the addition of ETA.

Clinically, the spectra of normal tissue toxicities produced by Carbo are sometimes more manageable than those produced by CDDP [19]. If Carbo can be given at a dose approximately 5-fold that of CDDP, approximately equal tumor-cell kill should be achieved. The available evidence indicates cross-sensitivity and -resistance between these drugs [26, 34]; therefore, tumors responsive to CDDP will most likely respond to Carbo. ETA was equally effective when used with Carbo (± CTX) and with CDDP (± CTX) in these studies. Based on these data, the addition of ETA to clinical protcols based on CTX/CDDP or CTX/Carbo may be warranted.

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