Cancer Chemother Pharmacol (1988) 21: 281–285

Cancer Chemotherapy and Pharmacology

Effect of fluosol-DA/carbogen on etoposide/alkylating agent antitumor activity*

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Summary. The tumor growth delay produced by the combination of etoposide with the alkylating agent CDDP or BCNU and Fluosol-DA with carbogen breathing in three model tumor systems was examined. The addition of Fluosol-DA to etoposide treatment increased tumor growth delay 2.8-fold, 3.3-fold and 2.2-fold in the FSaIIC fibrosarcoma, the Lewis lung carcinoma and the SW2 small-cell xenograft, respectively. In both the FSaIIC fibrosarcoma and the Lewis lung carcinoma the combination of etoposide treatment with CDDP produced an additive effect. When Fluosol-DA was added to this combination the tumor growth delay increased 1.9-fold and 1.4-fold in the FSaIIC fibrosarcoma and the Lewis lung carcinoma, respectively. Adding Fluosol-DA to a treatment regimen with etoposide and BCNU produced a 2.2-fold, 2.0-fold and 1.6-fold increase in the tumor growth delay of the FSaIIC fibrosarcoma, the Lewis lung carcinoma and the SW2 small-cell xenograft, respectively. The effect of these various treatment combinations on tumor cell survival was assessed in the FSaIIC fibrosarcoma. When the alkylating agents CDDP or BCNU were prepared in Fluosol-DA, there was an additional increase in tumor cell kill, so that with CDDP there was 2.1-fold and 4.7-fold increase in tumor cell kill and with BCNU there was 1.5-fold and 1.2-fold increase in tumor cell kill compared to the drug plus Fluosol-DA and the drug plus Fluosol-DA/carbogen breathing, respectively. The combination of etoposide and CDDP led to less than additive cell killing, and the combination of etoposide and BCNU appeared to be additive, as predicted by simple product summation, in all of the treatment conditions examined. Both etoposide + CDDP and etoposide + BCNU produced additive or less than additive toxicity to bone marrow as measured by CFU-GM.

Introduction

Perfluorochemicals have excellent oxygen-carrying capacity [6 – 8]. To fully utilize the oxygen-carrying capacity of these materials, high partial pressures of oxygen are used. Unlike hemoglobin, where oxygen is bound to the molecule, the solvent action of the perfluorochemicals for oxygen does not involve any kind of chemical or chelating process. The gas molecules situate themselves in the spaces between the molecules [6, 8]. The uptake and release of oxygen from perfluorchemical emulsions are completely reversible. The perfluorchemical emulsion, Fluosol-DA, in combination with the breathing of a 100% or 95% oxygen atmosphere has been shown to enhance the response of several solid rodent tumors to single-dose and fractionated radiation treatment [24, 32–34, 41] with little influence on normal tissues [18, 25]. As a result of these studies, Fluosol-DA plus oxygen breathing has recently entered clinical trials in combination with radiotherapy in advanced head and neck cancer [26].

Etoposide, a podophyllotoxin derivative, has been shown to cause both single-strand and double-strand DNA breaks and DNA protein cross-links in mammalian cells in a process which requires a nuclear protein. Etoposide does not bind directly to DNA, and evidence indicates that the protein-associated DNA breaks resulting from etoposide treatment are mediated through an interaction between the drug and topoisomerase II and perhaps oxygen [11, 15, 16, 22, 27]. Flow cytometry in etoposide-treated cells shows a delay in S-phase transit before arrest of cells in G_2. Correlating with the S-phase delay is a selective inhibition of thymidine incorporation into DNA as well as a concentration-dependent scission of DNA strands [12, 14]. Inhibition of DNA repair enhances cytotoxicity [2]. However, etoposide when incubated with purified DNA in vitro does not induce such damage [14]. Metabolic activation of etoposide to reactive intermediates by rat liver and HeLa microsomal fractions has been reported [37], and free radical formation at the pendant phenolic group has been implicated [38, 39]. A free hydroxyl group at the 4′position is essential for DNA breakage activity [16]. Free radical scavengers, dehydrogenase inhibitors, and dehydrogenase substrates prevent the formation of single-strand DNA breaks and inhibit the cytotoxicity of etoposide [38, 39]. Verapamil and a number of other calcium channel antagonists potentiate DNA damage by etoposide, perhaps by overcoming resistance to transport or drug action [40]. Etoposide shows a selective cytotoxicity to normally oxygenated cells in vitro, and when combined with an oxygen-carrying perfluorchemical emulsion, the antitumor activity and therapeutic efficacy of etoposide are enhanced [35].

Lung cancer is the most common cause of death due to cancer among men and women in the USA. Several chem-

* This work was supported by NCI grant # 2PO1-CA19589-10 (BAT), RO1-CA36498-03 (SDB), grants from the American Cancer Society of New York (CH 340) and Alpha Therapeutics Co., Los Angeles, CA 90032 (BAT) and NIH fellowship # 5F32-CA07821-02 (SAH)

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otherapeutic regimens have been developed which can achieve high response rates in small cell lung carcinoma. Etoposide has been used in the treatment of small cell lung cancer for some years [3, 9, 20]. In non-small cell lung cancer the combination of *cis*-diamminedichloroplatinum (II) (CDDP) with etoposide has been used in a number of studies with response rates varying from 20% to 50% [1, 5, 10, 13, 19]. The development of chemotherapy-resistant disease remains a major problem in both small and non-small cell lung cancers [3, 20].

The antitumor alkylating agents are a heterogenous class of drugs having in common the capacity to bifunctionally alkylate DNA. In this report, we examine the effect of combination of etoposide and the alkylating agents CDDP or carmustine (BCNU) with Fluosol-DA and carbogen breathing on tumor growth delay in three solid tumor systems, including the SW2 small cell lung carcinoma xenograft, and on tumor cell and bone marrow survival in mice bearing the FSaIIC fibrosarcoma.

Material and methods

Drugs. Etoposide was provided by Bristol Laboratories Inc. (Syracuse, NY). CDDP was a gift from Dr. Michael Abrams, Johnson Matthey Inc. (West Chester, Pa.). BCNU was obtained from the Dana-Farber Cancer Institute pharmacy. Fluosol-DA, 20%, (manufacturer: Green Cross Corp.) was provided by Alpha Therapeutics Corp. (Los Angeles, Calif.). The stem emulsion was stored frozen and the complete emulsion was prepared immediately prior to use.

Tumors. FSaIIC fibrosarcoma [23, 34] was carried in male C3HeB/FeJ mice (Jackson Laboratory, Bar Harbor, Me.), and the Lewis lung tumor [28, 30, 31] was carried in male C57BL/6J mice (Jackson Laboratory). For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted s.c. in the flanks of mice 8–10 weeks of age. When the tumors were approximately 50 mm^3 in volume ($\approx$ 1 week after tumor cell implantation), treatment was initiated. The human small cell carcinoma SW2 was grown in culture in RPMI medium with 10% fetal bovine serum (FBS) and 1 m*M* glutamine [29]. For implantation in outbred Swiss nu/nu male mice (Taconic Laboratories), 8×10^6 cells in 0.5 ml serum-free medium were injected s.c. in the flanks and the tumors were allowed to grow to approximately 50 mm^3 before initiation of treatment.

Tumor growth delay. The animals were given six daily i.v. injections of etoposide of 15 mg/kg. CDDP (4.5 mg/kg) or BCNU (10 mg/kg) was administered i.p. on days 1, 3 and 5 of treatment. Fluosol-DA (12 ml/kg, 0.3 ml) was administered i.v. immediately following the drugs and the animals were placed in a carbogen (95% O_2/5% CO_2) atmosphere for 2 h and then removed to air. In some groups, etoposide was prepared in Fluosol-DA and administered i.v. as a single injection. The progress of each tumor was measured thrice weekly until it reached a volume of 500 mm^3. Tumor growth delay was calculated as the days taken by each individual tumor to reach 500 mm^3 compared to the untreated controls. Each treatment group had seven animals, except for the SW2 xenograft model where there were nine animals per treatment group, and the experiment was repeated three times. Tumor growth delay was expressed as the mean ± standard error (SEM) in days for the treatment group compared to the control.

Data analysis. Statistical comparisons were made using Student's *t*-test. Data from the tumor growth delay experiments were analyzed using a computer programm written in BASIC for the Apple II+ microcomputer. The program first derives the best-fit curve for each individual set of tumor volume data and then calculates the median, mean and SEM for each experimental group. The day on which each tumor reaches 500 mm^3 and the median, mean and SEM are then derived. A second program provides statistical comparisons between any number of groups using the *t*-test and deriving degrees of freedom (df) and *P* values.

Tumor excision assay. When FSaIIC tumors were approximately 50 mm^3 in volume (about 1 week after tumor cell implantation), etoposide (20 mg/kg) and/or the perfluorochemical emulsion Fluosol-DA (0.3 ml) were injected via the tail vein. In some groups, immediately afterward CDDP (4.5 mg/kg) or BCNU (10 mg/kg) was administered by i.p. injection. In some groups, etoposide was prepared in Fluosol-DA and was administered i.v. as a single injection. The animals were then allowed to breathe air or were placed in a circulating atmosphere of carbogen for 1 h and then removed to air. Mice were killed and soaked in 95% ethanol 24 h after treatment. The tumors were excised under sterile conditions in a laminar flow hood and minced to a fine brei with two scalpels. Four tumors were pooled to make each treatment group. Approximately 0.2–0.3 g tumor brei was used to make each single-cell suspension. All reagents were sterilized with 0.22-μm Millipore membranes and were added aseptically to the tumor cells. Each sample was washed in 20 ml alpha-minimal essential medium (α-MEM) (Grand Island Biological Co., Grand Island, NY) in a 50-ml centrifuge tube, after which the liquid was gently decanted and discarded. The samples were resuspended in 450 units/ml collagenase (Sigma, St. Louis, Mo.) and 0.1 mg/ml deoxyribonuclease (DNase, Sigma) and incubated for 10 min at 37°C in a shaking water bath. The samples were centrifuged at 200 *g* and the supernatant was discarded. The samples were resuspended as above and incubated for another 15 min at 37°C. One milliliter of 1 mg/ml DNase was added and incubation was continued for 5 min at 37°C. The samples were then filtered through two layers of sterile gauze. The samples were washed twice, then resuspended in α-MEM supplemented with 10% fetal bovine serum (FBS) (Sterile Systems, Logan, Utah). These single-cell suspensions were counted and plated at three different cell concentrations in duplicate for the colony-forming assay. One week later the plates were stained with crystal violet and colonies of more than 50 cells were counted. The untreated tumor cell suspensions had a plating efficiency of 10% –16%. The results are expressed as the surviving fraction ± SEM of cells from treated groups compared to 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 using ice-cold McCoy's 5A medium (Grand Island Biological Co.), supplemented with 2% FBS and nonessential

amino acids (100 × ; 2 ml/liter) (Grand Island Biological Co.), sodium pyruvate (50 μg/ml), L-glutamine (146 μg/ml), L-asparagine (8 μg/ml), L-serine (4.2 μg/ml), vitamins (100 × ; 2 ml/liter), penicillin (50 units/ml) and streptomycin (50 μg/ml) (Grand Island Biological Co.) [21]. The cells were washed and resuspended in supplemented medium. CFU-GM were measured as follows: bone marrow cells were suspended in supplemented McCoy's 5A medium containing 15% FBS, 0.3% agar (Difco, Detroit, Mich.) and 10% L-cell conditioned medium as a source of colony-stimulating activity. The colony-stimulating activity supplement was prepared by incubating-L-929 mouse fibroblasts (2500 cells/ml; Microbiological Associates, Bethesda, Md.) with 30% FBS in McCoy's 5A medium for 7 days in humidified 5% CO_2 atmosphere at 37°C. The supernatant containing colony-stimulating activity was obtained by centrifugation of the medium at 10000 g for 10 min at 4°C then filtration under sterile conditions [4]. The bone marrow cell cultures were incubated for 7 days in a humidified 5% CO_2 atmosphere at 37°C and then fixed with 10% glutaraldehyde. Colonies of at least 50 cells were scored on an Acculite Colony counter (Fisher, Springfield, NJ). The results from three experiments in which each group was measured in triplicate were averaged. The results are expressed as the surviving fraction of treated groups compared to untreated controls.

Results

The tumor growth delay produced by combination of etoposide with the alkylating agent CDDP or BCNU and Fluosol-DA with carbogen breathing in three model tumor systems is shown in Table 1. All of the animals in these experiments breathed carbogen for 2 h after drug treatment. The addition of Fluosol-DA to treatment with etoposide increased tumor growth delay 2.8-fold, 3.3-fold and 2.2-fold in the FSaIIC fibrosarcoma, the Lewis lung carcinoma and the SW2 small cell xenograft respectively. Fluosol-DA and carbogen breathing alone produced no effect on tumor growth. In all three tumor systems the combination of etoposide treatment with CDDP appeared to produce an additive effect. When Fluosol-DA was added to this combination the tumor growth delay increased 1.9-fold, 1.4-fold and >1.3-fold in the FSaIIC fibrosarcoma, the Lewis lung carcinoma and the SW2 small cell xenograft, respectively.

Table 1. Tumor growth delay caused by etoposide ± CDDP or BCNU ± Fluosol-DA/carbogen in three solid model systems

Treatment group[a]	Tumor growth delay (days)		
	FSaIIC fibrosarcoma	Lewis lung carcinoma	SW2 small cell xenograft
Etoposide	3.9 ± 1.0	4.1 ± 1.0	7.9 ± 2.5
Etoposide + FDA	11.0 ± 3.6	13.5 ± 2.4	17.6 ± 3.5
CDDP	6.0 ± 1.8	3.7 ± 0.9	13.1 ± 4.3
Etoposide + CDDP	9.9 ± 1.2	5.7 ± 1.4	44.7 ± 4.5
Etoposide + CDDP + FDA	18.7 ± 3.0	7.9 ± 2.0	>60
BCNU	8.3 ± 2.0	3.2 ± 0.7	11.1 ± 3.6
Etoposide + BCNU	8.4 ± 2.0	9.3 ± 1.2	32.8 ± 3.7
Etoposide + BCNU + FDA	18.1 ± 2.4	18.6 ± 2.5	52.7 ± 6.5

FDA, Fluosol-DA

* In all treatment groups the animals breathed carbogen for 2 h after drug administration. Etoposide (15 mg/kg) and Fluosol-DA (12 ml/kg; 0.3 ml) were administered i.v. for 6 days. CDDP (4.5 mg/kg) or BCNU (10 mg/kg) was administered i.p. on days 1, 3 and 5 of treatment

BCNU was a very effective single agent in the FSaIIC fibrosarcoma [36] and SW2 small cell xenograft and somewhat less effective in the Lewis lung carcinoma. The combination of etoposide and BCNU produced no increase in tumor growth delay in the FSaIIC fibrosarcoma, increased tumor growth delay in the Lewis lung carcinoma to a level which appeared to be slightly greater than additive and increased tumor growth delay in the SW2 small cell xenograft to approximately twofold greater than the single agent effects. When Fluosol-DA was included in the treatment regimen with etoposide and BCNU there was a 2.2-fold, 2.0-fold and 1.6-fold increase in the tumor growth delay of the FSaIIC fibrosarcoma, the Lewis lung carcinoma and the SW2 small-cell xenograft, respectively.

The effect of these various treatment combinations on tumor cell survival was assessed in the FSaIIC fibrosarcoma (Table 2). In these experiments, the treatment agents were administered in single doses followed by 2 h carbogen breathing. The tumors were excised 24 h after treatment and cell survival was measured as colony formation in vitro. The addition of a period of carbogen breathing after drug injection slightly increased the tumor cell killing observed with each of the individual agents. Similarly, the

Table 2. Survival of FSaIIC tumor cells from mice treated with etoposide ± CDDP or BCNU ± Fluosol-DA/carbogen

Treatment	Surviving fraction[a]				
	Etoposide[2]	CDDP	Etoposide ± CDDP	BCNU	Etoposide ± BCNU
Air	0.56 (± 0.12)	0.24 (± 0.07)	0.49 (± 0.10)	0.38 (± 0.09)	0.12 (± 0.01)
Carbogen[b]	0.36 (± 0.08)	0.14 (± 0.03)	0.48 (± 0.09)	0.28 (± 0.10)	0.11 (± 0.01)
Fluosol-DA[c]	0.23 (± 0.07)	0.15 (± 0.03)	0.22 (± 0.08)	0.24 (± 0.07)	0.06 (± 0.005)
FDA/carbogen	0.17 (± 0.05)	0.14 (± 0.06)	0.13 (± 0.06)	0.11 (± 0.06)	0.02 (± 0.007)
In FDA[d]	0.22 (± 0.08)	0.07 (± 0.01)	0.04 (± 0.01)	0.16 (± 0.03)	0.05 (± 0.02)
In FDA/carbogen	0.11 (± 0.03)	0.03 (± 0.01)	0.03 (± 0.01)	0.09 (± 0.01)	0.007 (± 0.004)

Drug doses: etoposide, 20 mg/kg; CDDP, 4.5 mg/kg; BCNU, 15 mg/kg

[a] Tumors were excised 24 h after treatment. Data are the means of three independent measurements. Values in parentheses are the SEM

[b] Carbogen breathing was maintained for 2 h immediately following drug administration

[c] The Fluosol-DA dose was 12 ml/kg (0.3 ml) i.v.

[d] The drug(s) was prepared in Fluosol-DA

Table 3. Survival of bone marrow cells (CFU-GM) from mice treated with etoposide ± CDDP or BCNU ± Fluosol-DA/carbogen*

Treatment	Surviving fraction[a]				
	Etoposide	CDDP	Etoposide ± CDDP	BCNU	Etoposide ± BCNU
Air	0.61 (± 0.08)	0.80 (± 0.27)	0.54 (± 0.09)	0.38 (± 0.12)	0.55 (± 0.15)
Carbogen	0.69 (± 0.22)	0.85 (± 0.24)	0.57 (± 0.17)	0.44 (± 0.15)	0.44 (± 0.09)
Fluosol-DA	0.68 (± 0.25)	0.84 (± 0.24)	0.52 (± 0.20)	0.55 (± 0.09)	0.51 (± 0.13)
FDA/carbogen	0.67 (± 0.20)	0.63 (± 0.08)	0.60 (± 0.18)	0.59 (± 0.20)	0.46 (± 0.14)
In FDA[a]	0.56 (± 0.17)	0.36 (± 0.08)	0.43 (± 0.12)	0.50 (± 0.08)	0.35 (± 0.08)
In FDA[a]/carbogen	0.45 (± 0.09)	0.36 (± 0.07)	0.27 (± 0.09)	0.55 (± 0.17)	0.31 (± 0.06)

* Bone marrow was harvested from the femurs of the same animals described in Table 2
Drug doses: see note to Table 2
[a] Drug preparation in Fluosol-DA

combination of each drug with Fluosol-DA and air breathing produced a small increase in tumor cell killing compared to the drug and air breathing. When carbogen breathing was added to each drug plus Fluosol-DA there was an additional increase in tumor cell killing with etoposide and BCNU; however, with CDDP there was no increase in tumor cell killing compared to CDDP with Fluosol-DA and air breathing.

Preparing etoposide in Fluosol-DA followed by air breathing or carbogen breathing produced tumor cell kill comparable to that obtained when the two agents were administered individually. However, when the alkylating agents CDDP or BCNU were prepared in Fluosol-DA, there was an additional increase in tumor cell kill, so that with CDDP there was 2.1-fold and 4.7-fold increase in tumor cell kill and with BCNU there was 1.5-fold and 1.2-fold increase in tumor cell kill compared to the drug plus Fluosol-DA and the drug plus Fluosol-DA/carbogen breathing.

The combination of etoposide and CDDP led to less than additive cell killing, as predicted by simple product summation, in all of the treatment conditions examined. The combination of etoposide and BCNU, by simple product summation, appears to be additive under all of the treatment conditions.

Bone marrow was harvested from the femurs of the same animals used for the tumor cell survival experiments (Table 3). Toxicity to the bone marrow was measured by colony formation in vitro of granulocyte-macrophage progenitors (CFU-GM). The addition of Fluosol-DA and/or carbogen breathing did not increase the bone marrow toxicity of any of the individual drugs or the combination treatments. Preparing the drugs in Fluosol-DA with or without carbogen breathing also did not significantly increase the toxicity to the bone marrow. Using simple product summation, both etoposide plus CDDP and etoposide plus BCNU produced additive or less than additive toxicity under all of the treatment conditions.

Discussion

The addition of Fluosol-DA and carbogen breathing to treatment regimens has been shown to improve the antitumor activity of several individual chemotherapeutic agents, including etoposide [35] and the nitrosoureas [36]. In both of these cases, the increase in tumor growth delay or tumor cell kill was not accompanied by an increase in bone marrow killing, indicating an increase in therapeutic efficacy with Fluosol-DA and carbogen breathing.

In addition to the therapeutic enhancement afforded by Fluosol-DA and carbogen breathing, the combination of etoposide and alkylating agents may take advantage of the binding of etoposide to topoisomerase II, leading to a DNA complex which allows better access of the alkylating drug to the DNA. Although some evidence for therapeutic synergism of etoposide and CDDP in P388 leukemia and B16 melanoma has been reported previously [17], tumor cell killing by etoposide and either CDDP or BCNU in the FSaIIC fibrosarcoma survival assay appeared to be additive. Tumor growth delay studies in the FSaIIC fibrosarcoma, the Lewis lung carcinoma and the SW2 human small cell xenograft with etoposide and CDDP also appeared to produce additive or subadditive results. The combination of etoposide and BCNU appeared to produce greater than additive tumor growth delay in the SW2 small cell xenograft.

Drug combinations including etoposide and frequently CDDP, alone or with other agents, have shown good levels of activity in small cell and non-small cell lung cancer [1, 3, 5, 9, 10, 13, 19, 20]. These therapies fall short of achieving durable responses in small cell lung cancer and complete responses in non-small cell lung cancer. One possible strategy for improving the efficacy of current drug combinations would be to increase the degree of activity of active agents on the initial course(s) of treatment when the tumor is most sensitive to chemotherapy. The addition of Fluosol-DA and carbogen breathing to treatment regimens such as etoposide + CDDP or etoposide + BCNU may provide a means of increasing tumor cell kill without increasing normal tissue toxicity.

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Received August 26, 1987/Accepted January 15, 1988