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Fluosol-DA/carbogen with lonidamine or pentoxifylline as modulators of alkylating agents in the FSaIIC fibrosarcoma*

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Summary. In an effort to increase the efficacy of several antineoplastic alkylating agents (CDDP, L-PAM, CTX, or BCNU), we examined the effect of the modulator Fluosol-DA/carbogen in combination with a second modulator, either lonidamine or pentoxifylline, on the survival of FSaIIC tumor cells and of bone marrow CFU-GM from tumor-bearing C3H mice. Fluosol-DA/carbogen increased the tumor-cell killing activity of each alkylating agent by about 10 times. In contrast, lonidamine alone did not significantly increase the cytocidal activity of any of the alkylating agents tested. However, in combination with Fluosol-DA/carbogen, the use of lonidamine produced about a 100-fold increase in the tumor cell kill achieved with CDDP as compared with CDDP alone. No increase in tumor cell kill over that produced with the single modulator Fluosol-DA/carbogen was seen following the addition of lonidamine to the combination treatment with L-PAM, CTX, or BCNU. Unfortunately, although neither lonidamine nor Fluosol-DA/carbogen alone significantly increased alkylator toxicity to bone marrow CFU-GM, the combination of modulators increased the toxicity of each alkylating agent to bone marrow by about 10 times. Pentoxifylline caused an increase in alkylator activity against the FSaIIC fibrosarcoma only when used with BCNU; this effect was further augmented by the addition of Fluosol-DA/carbogen. The combination of modulators pentoxifylline plus Fluosol-DA/carbogen was more effective than Fluosol-DA/carbogen alone only when the former was used with BCNU, whereas only minimal increases in tumor-cell killing activity were obtained with this modulator combination and CDDP, L-PAM, or CTX. Pentoxifylline increased the bone marrow CFU-GM toxicity of L-PAM by about 10 times. The bone marrow CFU-GM toxicity was further increased by Fluosol-DA/carbogen, as was the toxicity of each of the other alkylating agents. Lonidamine plus Fluosol-DA/carbogen may be useful in increasing the therapeutic efficacy of CDDP, and the combination of pentoxifylline plus Fluosol-DA/carbogen might improve the antitumor activity of BCNU.

Introduction

In developing strategies for the improved treatment of solid tumors, one possible avenue is to take advantage of physiological differences between solid tumors and normal tissues. Hypoxic cells in solid tumors are presumed to be an obstacle to successful cancer treatment because these cells are relatively protected from the cytotoxic effects of radiotherapy and certain anticancer drugs [28, 29, 46, 50]. The importance of hypoxic cells in limiting the curability of human tumors remains a controversial issue, although some clinical [5, 10] and laboratory data [10, 20, 40, 47] strongly suggest that hypoxic cells are a cause of in vivo treatment failure.

The perfluorochemical emulsion Fluosol-DA in combination with inspiration of atmospheres comprising 100% or 95% oxygen has been shown to enhance the response of several solid rodent tumors to single-dose and fractionated radiation treatment [26, 38, 44, 45, 57]. The level of cellular oxygenation is also an important factor in the action of many antineoplastic agents, many of which have been classified in vitro [47] and in vivo [58] by their relative cytotoxicity to oxygenated vs hypoxic tumor cells. Fluosol-DA and carbogen breathing have also been shown to enhance the antitumor and cytocidal activity of many of these chemotherapeutic agents in vivo [43, 47–49, 51, 54–56, 58].

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Abbreviations: CDDP, *cis*-diamminedichloroplatinum(II); L-PAM, L-phenylalanine mustard, melphalan; thioTEPA, *N,N',N''*-triethylenethiophosphoramide; CTX, cyclophosphamide; BCNU, *N,N'-bis*(2-chloroethyl)-*N*-nitrosourea; CFU-GM, granulocyte-macrophage colony-forming unit; αMEM, alpha minimal essential medium; PBS, phosphate-buffered saline

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In the present study, we combined two other modulators, lonidamine and pentoxifylline, which work through mechanisms other than those of perfluorochemicals, with Fluosol-DA/carbogen in an attempt to improve the antineoplastic efficacy of several different alkylating agents. Londiamine, 1-[(2,4-dichlorophenyl)methyl]-1H-indazol-3-carboxylic acid, affects the energy metabolism of cells [7, 8, 16–19] by inhibiting oxygen consumption and that of tumor cells by blocking anaerobic glycolysis. Based on these data, mitochondria have been considered the primary intracellular targets of this drug. Lonidamine has been shown to enhance the cytotoxicity of radiation [24], hyperthermia [8, 11], and several alkylating agents [39].

Pentoxifylline is a methylxanthine that is used to treat vascular occlusive diseases in human beings [2, 3, 11, 12, 32, 33, 35, 36, 42]. Like other methylxanthines [5, 6, 14, 21, 22, 25, 30, 31], at relatively high concentrations pentoxifylline has been shown to enhance the cytotoxicity of radiation, alkylating agents, and other anticancer drugs in cell culture [9, 14, 15]. At doses readily achievable in man, pentoxifylline exerts significant hemorheologic effects on red blood cells (*RBCs*) and platelets. Pentoxifylline increases RBC deformability and inhibits platelet aggregation such that RBCs can better traverse narrowed arterioles and capillaries [2, 3, 9, 11, 12, 32, 33, 35, 36, 42]. These properties of pentoxifylline would be expected to lead to improved tumor blood flow and, hence, to improved tumor oxygenation and increased drug delivery.

Since Fluosol-DA plus oxygen breathing is most likely not 100% effective in reoxygenating hypoxic tumors, we combined this modulator with lonidamine, which should be effective in reducing cellular energy stores, or with pentoxifylline, which may further improve tumor oxygenation as well as drug delivery. We examined these interactions by measuring the effect of these modulator combinations on the tumor-cell killing activity and bone marrow CFU-GM cytotoxicity of CDDP, L-PAM, CTX, and BCNU in animals bearing the FSaIIC fibrosarcoma.

Materials and methods

Drugs. Fluosol-DA 20%, a product of Green Cross Corp. (Osaka, Japan), was obtained from Alpha Therapeutics Corp. (Los Angeles, Calif.). The stem emulsion consists of 25% (wt/vol) perfluorochemicals: 7 parts perfluorodecalin; 3 parts perfluorotripropylamine Pluronic F-68 (2.7%, wt/vol); yolk phospholipids (0.4%, wt/vol) as emulsifiers; and glycerol (0.8%, wt/vol) as a cryoprotecting agent. The annex solution (electrolyte/bicarbonnate solution) furnishes the preparation with physiologic osmolarity. The stem-emulsion particles provide a surface area of 1.82×10^8 cm^2/l that is available for oxygen diffusion (about 100 times the surface area of the RBC in whole blood). The half-life of Fluosol-DA in circulation in vivo is about 12 h [23]. Carbogen comprises 95% oxygen and 5% carbon dioxide.

Lonidamine was obtained as a gift from DeSanctis Consultants (Montreal, Canada), prepared in phosphate-buffered 0.9% saline (PBS), and stored at –20° C. Pentoxifylline (Trental), CTX, and melphalan were purchased from Sigma Chemical Company (St. Louis, Mo.). CDDP was a gift from Drs. D. H. Picker and M. J. Abrams, Johnson Matthey, Inc. (West Chester, Pa.) and was prepared in PBS and stored at –20° C. Melphalan was dissolved in HCl-acidified ethanol and diluted with PBS just prior to use. BCNU (carmustine) was obtained from the Dana-Farber Cancer Institute's pharmacy.

Tumor. The FSaII fibrosarcoma [37] adapted for growth in culture (FSaIIC) [57], was carried in C3H/He male mice (Jackson Laboratories, Bar Harbor, Me.) For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted i.m. into the legs of C3H/He male mice and 8–10 weeks.

Tumor excision assay. When the tumors had reached a volume of approximately 100 mm^3 (about 1 week after tumor cell implantation), the animals were injected i.p. with: (1) each of the alkylating agents alone; (2) each of the alkylating agents preceded by lonidamine (100 mg/kg) or pentoxifylline (100 mg/kg); (3) each of the alkylating agents preceded by Fluosol-DA (0.3 ml, 12 ml/kg) given by tail-vein injection and followed by 6 h carbogen breathing; or (4) each of the alkylating agents preceded by Fluosol-DA (0.3 ml, 12 ml/kg) given i.v. just prior to the i.p. administration of lonidamine (100 mg/kg) or pentoxifylline (100 mg/kg) and followed by 6 h carbogen breathing. Mice were killed at 24 h after treatment to enable full expression of drug cytotoxicity and repair of potentially lethal damage.

The tumors were excised under sterile conditions in a laminar flow hood and minced to a fine brei using two scalpels. Four tumors were pooled for each treatment group. Approximately 500 mg tumor brei was used to make each single-cell suspension. All reagents were sterilized with 0.22-μm Millipore filters and were added aseptically to the tumor cells. Each sample was washed in 20 ml α-MEM, after which the liquid was gently decanted and discarded. The samples wee resuspended in 450 units collagenase/ml (Sigma, St. Louis, Mo.) and 0.1 mg DNase/ml (Sigma) and were incubated for 10 min at 37° C in a shaking water bath. The samples were resuspended as above and incubated for another 15 min at 37° C. Then, 1 ml DNase at 1 mg/ml was added and incubation was continued for 5 min at 37° C, following which the samples were filtered through two layers of sterile gauze. The samples were washed twice, then resuspended in α-MEM supplemented with 10% fetal bovine serum (FBS).

These single-cell suspensions were counted and plated in duplicate at three different cell concentrations for the colony-forming assay. No significant difference was observed in total cell yield from the pooled tumors in any treatment group. After 1 week, the plates were stained with crystal violet and colonies of >50 cells were counted. The untreated tumor cell suspensions had a plating efficiency of 10%–16%. The results are expressed as the surviving fraction (± SE) of cells from treated groups as compared with untreated controls [51, 52].

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 [51, 52]. CFU-GMS 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% conditioned medium as a source of colony-stimulating activity. The colony-stimulating activity supplement was prepared by incubating L-929 mouse fibroblasts (2,500 cells/ml; Microbiological Associates, Bethesda, Md.) with 30% FBS in McCoy's 5A medium for 7 days at 37° C in a humidified atmosphere containing 5% CO_2. The supernatant containing colony-stimulating activity was obtained by centrifugation of the medium at 10,000 *g* for 10 min at 4° C followed by filtration under sterile conditions. The bone-marrow cell cultures were incubated for 7 days at 37° C in a humidified atmosphere containing 5% CO_2 37° C and were fixed with 10% glutaraldehyde. Colonies of at least 50 cells were scored on an Acculite colony counter (Fisher Scientific, Springfield, N. J.). The results of three experiments, in which determinations for each group were made in duplicate at three cell concentrations, were averaged. The results are expressed as the surviving fraction from treated groups as compared with untreated controls.

Results

When lonidamine (100 mg/kg) was given i.p. to animals bearing the FSaIIC fibrosarcoma just prior to a range of

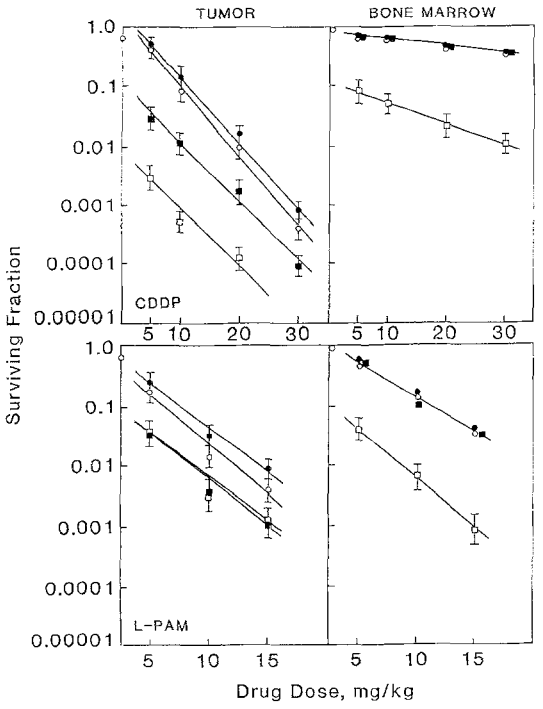

Fig. 1. Survival of FSaIIC tumor cells and bone marrow CFU-GM from animals treated in vivo with single doses of CDDP or L-PAM alone (●), preceded by a single dose of lonidamine (100 mg/kg; ○), preceded by a single dose of Fluosol-DA (0.3 ml, 12 ml/kg) and followed by carbogen breathing (6 h; ■) or with the combination of the two modulators (□). *Points*, Means of three independent experiments; *bars*, SE

i.p. doses of CDDP, there was no significant increase in the killing of tumors as compared with CDDP alone (Fig. 1, top). Fluosol-DA (0.3 ml, 12 ml/kg) given i.v. just prior to i.p. injection of CDDP and followed by 6 h carbogen breathing resulted in about a 10-fold increase in tumor cell kill as compared with CDDP alone. The combination of modulators lonidamine and Fluosol-DA/carbogen (6 h), resulted either in an additional 10-fold increase in tumor-cell killing activity as compared with Fluosol-DA/carbogen/CDDP or in a 100-fold increase in tumor cell kill as compared with CDDP alone. The cytotoxicity of CDDP to bone marrow CFU-GM was unaffected by the addition of lonidamine (100 mg/kg) or Fluosol-DA (0.3 ml, 12 ml/kg) carbogen (6 h). The combination of modulators produced about a 10-fold increase in the cytotoxicity of CDDP to bone marrow CFU-GM, which nevertheless resulted in an overall therapeutic gain.

Administration of lonidamine (100 mg/kg) just prior to treatment with L-PAM resulted in about a 2-fold increase in the killing of FSaIIC tumors as compared with L-PAM alone, but this increase was not significant (Fig. 1, bottom). Fluosol-DA (0.3 ml, 12 ml/kg) given i.v. immediately prior to L-PAM and followed by 6 h carbogen breathing resulted in about a 10-fold increase in the killing of FSaIIC tumor cells as compared with L-PAM alone. Use of the combination of modulators lonidamine and Fluosol-DA/carbogen did not further increase the tumor cell kill as compared with that of Fluosol-DA/carbogen and L-PAM. Neither lonidamine nor Fluosol-DA/carbogren increased the toxicity of L-PAM to bone marrow as compared with the drug alone. However, as was the case with CDDP, the combination of modulators markedly increased the cytotoxicity of L-PAM to bone marrow CFU-GM by 10 to 50-fold over the L-PAM dose range as compared with L-PAM alone. Only with the use of Fluosol-DA/carbogen was the therapeutic index of L-PAM improved (Table 1).

The addition of lonidamine (100 mg/kg) to treatment with CTX did not significantly alter the tumor cell kill as compared with CTX alone (Fig. 2, top). Fluosol-DA (0.3 ml, 12 ml/kg) given i.v. just prior to CTX and followed by 6 h carbogen breathing had a dose-modifying effect on tumor cell killing by CTX (greater at a high dose). Fluosol-DA/carbogen increased tumor cell killing by CTX by about 9 times at 100 mg/kg CTX and about 90 times at 300 mg/kg CTX. The addition of lonidamine (100 mg/kg) to treatment with Fluosol-DA/carbogen and CTX did not alter the tumor cell kill produced by the latter. There was no significant difference in the toxicity of CTX to bone marrow CFU-GM when the drug was given in combination with lonidamine or with Fluosol-DA/carbogen. The combination of modulators with CTX, however, resulted in about a 10-fold increase in killing of bone marrow CFU-GM as compared with CTX alone. As with L-PAM, the greatest therapeutic gain was achieved using Fluosol-DA/carbogen and CTX (Table 1).

The addition of lonidamine (100 mg/kg) to treatment with BCNU resulted in about a 2-fold increase in tumor cell kill, but this increase was not significant (Fig. 2, bottom). The addition of Fluosol-DA (0.3 ml, 12 ml/kg) just prior to BCNU administration followed by 6 h carbogen breathing resulted in about a 10-fold increase in tumor cell killing as compared with BCNU alone. The combination of the two modulators lonidamine and Fluosol-DA/carbogen did not further increase tumor cell killing by BCNU as

Table 1. Ratios of FSaIIC tumor cell survival to bone marrow CFU-GM survival for various alkylating agents and modulator combinations[a]

Alkylating agent	Dose (mg/kg)	*Bone marrow CFU-GM survival* Tumor cell survival					
		AA alone	+ Fluosol-DA/ carbogen	+ Lonidamine	+ Pentoxifylline	+ Fluosol-DA/ carbogen + Lonidamine	+ Fluosol-DA/ carbogen + pentoxifylline
CDDP	10	4	50	8.5	2.3	93	31
L-PAM	10	5.3	28	1	1	2	1
CTX	300	2.4	363	4	3.4	125	192
BCNU	50	5.4	43	4.3	11	8	12.5

[a] Ratios are derived from the experiments shown in Fig. 1–4

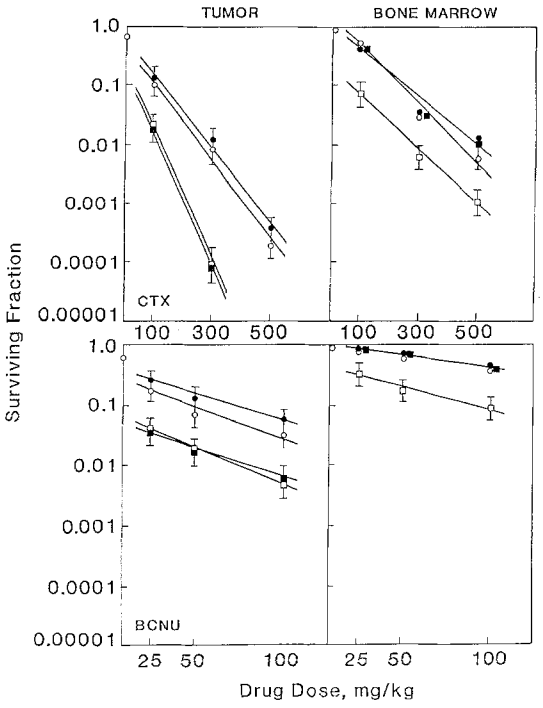

Fig. 2. Survival of FSaIIC tumor cells and bone marrow CFU-GM from animals treated in vivo with single doses of CTX or BCNU alone (●), preceded by a single dose of lonidamine (100 mg/kg), preceded by a single dose of Fluosol-DA (0.3 ml, 12 ml/kg) and followed by carbogen breathing (6 h; ■) or with the combination of the two modulators (□). *Points*, Means of three independent experiments; *bars*, SE

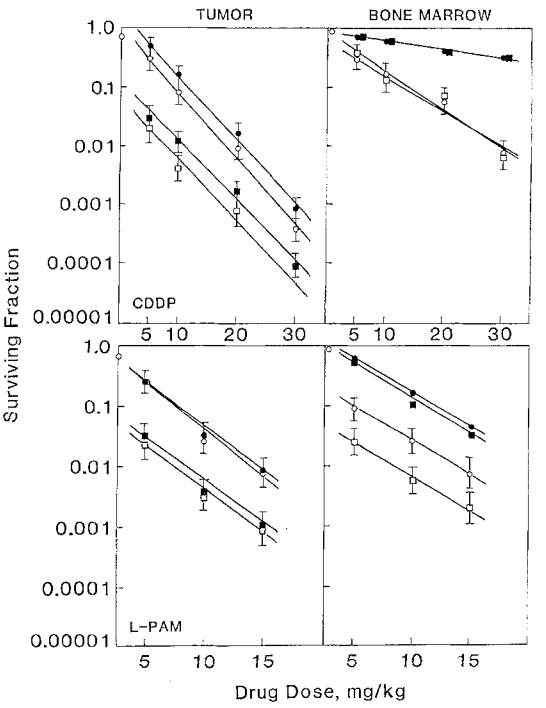

Fig. 3. Survival of FSaIIC tumor cells and bone marrow CFU-GM from animals treated in vivo with single doses of CDDP or L-PAM alone (●), preceded by a single dose of pentoxifylline (100 mg/kg), preceded by a single dose of Fluosol-DA (0.3 ml, 12 ml/kg) and followed by carbogen breathing (6 h; ■) or with the combination of the two modulators (□). *Points*, Means of three independent experiments; *bars*, SE

compared with Fluosol-DA/carbogen plus BCNU. As with the other three drugs, neither lonidamine nor Fluosol-DA/carbogen alone increased the cytotoxicity of BCNU to bone marrow CFU-GM; however, the combination of modulators increased the killing of bone marrow CFU-GM by BCNU by about 5 times.

When pentoxifylline (100 mg/kg) was added to treatment with CDDP, there was about a 2 to 2.5-fold increase in the killing of tumor cells (Fig. 3, top). In combination with Fluosol-DA/carbogen and CDDP, pentoxifylline also increased the tumor cell kill by about 2 times. Pentoxifylline (100 mg/kg) alone and in combination with Fluosol-DA/carbogen had a dose-modifying effect on the cytotoxicity of CDDP to bone marrow CFU-GM. The cytotoxicity of CDDP to bone marrow CFU-GM was increased about 2 times at 5 mg/kg CDDP (with or without Fluosol-DA/carbogen) and about 50 times at 30 mg/kg CDDP (with or without Fluosol-DA/carbogen). However, at 10 mg/kg CDDP, there was a 31-fold increase in tumor cell kill over bone marrow CFU-GM kill (Table 1).

The addition of pentoxifylline (100 mg/kg) to treatment with L-PAM had no effect on the tumor cell killing by L-PAM in either the presence or the absence of Fluosol-DA/carbogen (Fig. 3, bottom). Although pentoxifylline did not increase the toxicity of L-PAM to the tumor, it did increase the cytotoxicity of the latter to bone marrow CFU-GM by about 6 times. The combination of pentoxifylline and Fluosol-DA/carbogen further increased the killing of bone marrow CFU-GM by L-PAM such that the level of bone marrow CFU-GM kill achieved by the combination

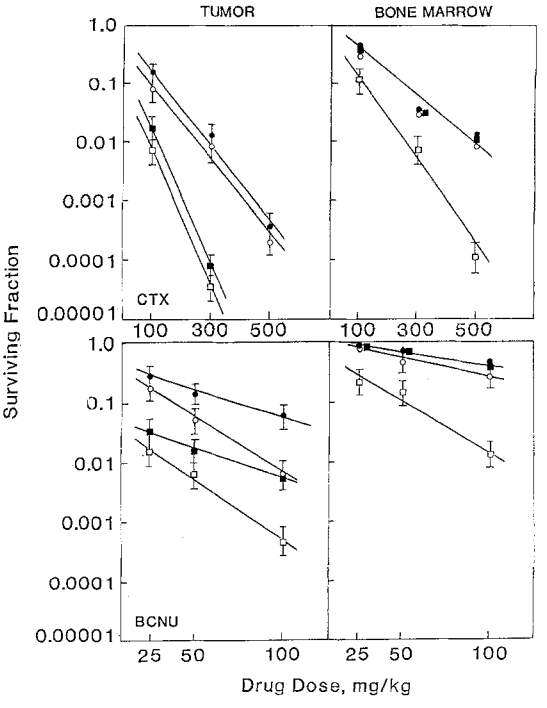

Fig. 4. Survival of FSaIIC tumor cells and bone marrow CFU-GM from animals treated in vivo with single doses of CTX or BCNU alone (●), preceded by a single dose of pentoxifylline (100 mg/kg), preceded by a single dose of Fluosol-DA (0.3 ml, 12 ml/kg) and followed by carbogen breathing (6 h; ■) or with the combination of the two modulators (□). *Points*, Means of three independent experiments; *bars*, SE

was about 20-fold that of L-PAM alone. At 10 mg/kg L-PAM, there was no therapeutic index with these combination treatments (Table 1).

When used with CTX, pentoxifylline (100 mg/kg) with or without Fluosol-DA/carbogen resulted in a 1.5 to 2-fold increase in the killing of tumor cells as compared with CTX or Fluosol-DA/carbogen plus CTX (Fig. 4, top). Although neither pentoxifylline or Fluosol-DA/carbogen increased the cytotoxicity of CTX bone marrow CFU-GM, the combination of modulators produced a dose-modifying effect on the cytotoxicity of CTX bone marrow CFU-GM. The enhancement in bone marrow CFU-GM cytotoxicity increased from about a 5-fold increase at 100 mg/kg CTX to about a 100-fold increase at 500 mg/kg CTX. There was a marked increase in therapeutic index with the combination of modulators and CTX (Table 1).

In combination with BCNU, pentoxifylline (100 mg/kg) had a dose-modifying effect. The enhancement in the killing of tumor cells by BCNU in combination with pentoxifylline increased from about 1.5-fold at 25 mg/kg BCNU to about 10-fold at 100 mg/kg BCNU. This type of enhancement in the killing of tumor cells persisted when pentoxifylline was used in combination with Fluosol-DA/carbogen and BCNU. The combination of modulators with BCNU resulted in about a 20-fold enhancement in the tumor cell kill achieved by 25 mg/kg BCNU, which increased to about a 200-fold enhancement at 100 mg/kg BCNU. Neither pentoxifylline nor Fluosol-DA/carbogen increased the cytotoxicity of BCNU to bone marrow CFU-GM; however, the combination of the two modulators had a dose-modifying effect on the cytotoxicity of BCNU to bone marrow CFU-GM such that there was about a 4-fold increase in cytotoxicity at 25 mg/kg BCNU and about a 25-fold increase at 100 mg/kg BCNU.

Discussion

Fluosol-DA/carbogen breathing has previously been shown to increase the tumor growth delay [43, 48–56, 58] and tumor-cell killing activity [49–53, 58] of a wide variety of antitumor agents. In most cases, no increase in the toxicity of the drugs to critical normal tissues was observed [50, 52, 55, 56]. An exception to this general finding was the enhancement in pulmonary toxicity by the combination of Fluosol-DA/carbogen and bleomycin as compared with bleomycin alone [50].

We used the tumor-cell survival assay to evaluate the ability of the mitochondrial toxin londiamine or the rheological agent pentoxifylline, used alone or with Fluosol-DA/carbogen, to modulate the antitumor activity of alkylating agents. The advantage of this assay method is that it enables quantitative determination of the in vivo tumor cell kill achieved by the treatment combination being examined. The main limitation of the tumor-cell survival assay is that primarily involves a single-dose experiment.

Lonidamine significantly increased the tumor-cell killing activity only of CDDP when used with Fluosol-DA/carbogen. However, when used with each of the four alkylating agents, the combination of lonidamine and Fluosol-DA/carbogen led to significantly increased toxicity to bone marrow CFU-GM. Pentoxifylline produced a dose-modifying effect on the tumor-cell killing ability of BCNU in both the presence and the absence of Fluosol-DA/carbogen, but it did not significantly influence the cytotoxicity of any of the other three alkylating agents. However, pentoxifylline administration prior to treatment with CDDP or L-PAM significantly increased the bone marrow toxicity of these drugs. In addition, the combination of modulator spentoxifylline and Fluosol-DA/carbogen led to significant increase in the toxicity of each of the four alkylating agents to bone marrow CFU-GM.

In contrast, Fluosol-DA/carbogen produced significant increases in the cytotoxicity of each of the alkylating agents tested without significantly increasing the bone marrow toxicity of any drug. The greatest differences between tumor cell killing and the killing of bone marrow CFU-GM were obtained using Fluosol-DA/carbogen. The addition of either lonidamine or pentoxifylline decreased the therapeutic index (Table 1). Thus, Fluosol-DA/carbogen appears to be a far better modulator of these alkylating agents than is either lonidamine or pentoxifylline.

These studies point out the importance of proper preclinical modeling prior to clinical testing of single or combination modulators. Although combinations of lonidamine or pentoxifylline with Fluosol-DA/carbogen were based on rational considerations, testing in this model system indicated that these combinations could result in widely varying therapeutic indices, depending on the alkylating agent involved.

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