Approaches to Defining the Mechanism of Enhancement by Fluosol-DA 20% with carbogen of Melphalan Antitumor Activity

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ABSTRACT

Fluosol-DA with carbogen (95% oxygen and 5% carbon dioxide) breathing can increase the efficacy of melphalan. Addition of Fluosol-DA to treatment with melphalan leads to a greater increase in tumor growth delay under conditions of air breathing and carbogen breathing than does the fat emulsion Intralipid. The ability of melphalan to kill tumor cells increased with dose over the range of drug examined. At the lower doses of drug there is some increase in tumor cell killing seen with the addition of carbogen breathing or Fluosol-DA and air breathing; however, at the highest dose of the drug this difference disappeared. Throughout the melphalan dosage range examined there is approximately 1 log greater tumor cell kill observed with the addition of Fluosol-DA and carbogen breathing compared to the drug treatment alone. There was no significant difference in the survival of bone marrow cells under any of the treatment conditions. Fluosol-DA itself with air or carbogen breathing produced no detectable cross-links in DNA from tumors treated in vitro. The cross-linking factors for melphalan with air or carbogen breathing and for melphalan plus Fluosol-DA and air breathing were similar; when carbogen breathing was added to the treatment combination, the cross-linking factor increased almost 3-fold. When melphalan was dissolved in Fluosol-DA, the melphalan moved quickly into the lipophilic perfluorochemical particles so that after 1 h 60% of the drug was in the perfluorochemical layer. At 24 h, 85-90% of the melphalan was sequestered in the perfluorochemical particles. The pharmacokinetics of [14C]melphalan alone, [14C]melphalan plus Fluosol-DA, and [14C]melphalan prepared in Fluosol-DA were studied in several tissues of F344 male Sprague-Dawley rats. In general, the tissue absorption and distribution r,os for melphalan were shortened in the presence of Fluosol-DA to treatment with melphalan leads to a greater increase in tumor growth delay produced by the addition of Fluosol-DA and air breathing; however, at the highest dose of the drug this difference disappeared. Throughout the melphalan dosage range examined there is approximately 1 log greater tumor cell kill observed with the addition of Fluosol-DA and carbogen breathing compared to the drug treatment alone. There was no significant difference in the survival of bone marrow cells under any of the treatment conditions. Fluosol-DA itself with air or carbogen breathing produced no detectable cross-links in DNA from tumors treated in vitro. The cross-linking factors for melphalan with air or carbogen breathing and for melphalan plus Fluosol-DA and air breathing were similar; when carbogen breathing was added to the treatment combination, the cross-linking factor increased almost 3-fold. When melphalan was dissolved in Fluosol-DA, the melphalan moved quickly into the lipophilic perfluorochemical particles so that after 1 h 60% of the drug was in the perfluorochemical layer. At 24 h, 85-90% of the melphalan was sequestered in the perfluorochemical particles. The pharmacokinetics of [14C]melphalan alone, [14C]melphalan plus Fluosol-DA, and [14C]melphalan prepared in Fluosol-DA were studied in several tissues of F344 male Sprague-Dawley rats. In general, the tissue absorption and distribution r,os for melphalan were shortened in the presence of Fluosol-DA (except for kidneys). Shifting the r,os for absorption and distribution to shorter times produces a much sharper and earlier peak in the drug exposure of the tumor. Fluosol-DA provides a relatively nontoxic means of increasing oxygen delivery to tumors and a therapeutically meaningful way of improving melphalan antitumor activity.

INTRODUCTION

Hypoxia has long been known to protect cells from the cytotoxic effects of radiation. The level of cellular oxygenation is an important factor in the action of many antineoplastic agents. Reactive free radicals of oxygen may be responsible for the cleavage of DNA by several agents including bleomycin, procarbazine, streptonigrin, actinomycin D, and Adriamycin. The cellular state of oxygenation may also have a strong influence on the cytotoxicity of antimetabolites and Vinca alkaloids such as vincristine, which are most lethal to cells which are progressing through the cell cycle. Cells which are hypoxic may be either noncycling or slowly progressing through the cell cycle (1, 2). Several antineoplastic agents have been classified by their selective toxicities toward oxygenated or hypoxic tumor cells (3).

Compounds which mimic the effect of oxygen in cells such as the radiosensitizer misonidazole have been shown to enhance the cytotoxicity of several antitumor alkylating agents including melphalan, cyclophosphamide, 1,3-bis(2-chloroethyl)-1-nitrosourea, and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in vitro and in vivo. This phenomenon has been termed chemosensitization or chemopotentiation (1). The potentiation of the antitumor effect of these drugs by misonidazole in vitro was seen without a similar increase in normal tissue toxicity. The presence of hypoxic cells in solid tumors may account for the preferential effect, since chemosensitization in vitro occurs only when cells are exposed to misonidazole under hypoxic conditions, i.e., conditions in which reduction of misonidazole through formation of oxygen-mimicking free radicals can occur.

Like most bifunctional alkylating agents, melphalan exerts cytotoxic effects through interaction with DNA by forming either interstrand, intrastrand, or DNA-protein cross-links. For the most simple bifunctional alkylating agents, maximum levels of cross-linking are observed immediately following drug exposure (4). The formation of DNA cross-links in murine L1210 leukemia cells by melphalan increased most rapidly during the first 5 h following drug exposure (5, 6). The delay is most probably due to a slow conversion of monoadducts to cross-links. Although the molecular details of misonidazole enhancement of melphalan antitumor activity remain to be elucidated, it has been established that misonidazole pretreatment results in increased levels of melphalan-induced cross-links in DNA (7–10). Alterations in drug pharmacokinetics and in intracellular non-protein sulfhydryl levels may also play a role in the chemosensitization effects observed (9, 11–13).

Perfluorochemicals have excellent oxygen- and carbon dioxide-carrying capacity (14–16). 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 (14, 16). The uptake and release of oxygen from perfluorochemical emulsions are completely reversible. At least 90% of the emulsion particles in the preparation which we used are less than 0.2 μm in diameter, much smaller than RBCs (average diameter, 5–10 μm). The perfluorochemical emulsion, Fluosol-DA, in combination with breathing 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 (17–21) with little influence on normal tissues (22, 23). 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 (24).

Fluosol-DA with carbogen (95% oxygen and 5% carbon dioxide) breathing can also increase the efficacy of melphalan treatment of tumors (25). The perfluorochemical particles of Fluosol-DA form a highly lipophilic environment; thus, part of the increase in tumor growth delay produced by the addition of Fluosol-DA to melphalan with air breathing may be due to changes in the distribution and pharmacokinetics of the drug...
in the presence of the perfluorochemical emulsion particles. In this study, we attempt to approach the question of the relative contributions of oxygen and drug distribution into a lipophilic environment through studies using Intralipid, a fat emulsion, and we begin to explore the mechanism by which Fluosol-DA and carbogen breathing enhance the antitumor activity of melphalan.

MATERIALS AND METHODS

Drugs. Fluosol-DA 20% (Green Cross Corp., Osaka, Japan) was obtained from Alpha Therapeutics Corp. (Los Angeles, CA). The stem emulsion consists of 25% (w/v) perfluorochemicals: 7 parts perfluorodecalin; 3 parts perfluorotripropylamine; Pluronic F-68 (2.7%, w/v); yolk phospholipids (0.4%, w/v) as emulsifiers; and glycerol (0.8%, w/v) as a cryoprotecting agent. The annex solution (electrolyte/bicarbonate solution) furnishes the preparation with physiological osmolality. The stem emulsion particles provide a surface area of 1.82 × 10^6 cm²/liter available for oxygen diffusion (about 100 times the surface area of the RBC in whole blood). The half-life of Fluosol-DA in vivo is about 12 h. Intralipid 20% (KabiVitrum, Inc., Alameda, CA) was obtained from the Dana-Farber Cancer Institute pharmacy.

Melphalan (L-phenylalanine mustard) was a gift from Burroughs Wellcome Co. (Research Triangle Park, NC) and chlortetracyclol, 1-¿¿Cl-melphalan (specific activity, 20 mCi/mmol) was a gift from Dr. John A. Kepler, Research Triangle Institute (Research Triangle Park, NC).

Tumor. The FSaIIC fibrosarcoma (26) adapted for growth in culture (FSaIIC) (18) was carried in C3H/Be/FcI male mice (The Jackson Laboratory, Bar Harbor, ME). For the experiments, 2 × 10^5 tumor cells prepared from a brei of several stock tumors were implanted i.m. into the legs of C3H/Be/FcI male mice 8–10 weeks of age.

Tumor Growth Delay Experiments. When the tumors were approximately 50 mm³ in volume, the perfluorochemical emulsion Fluosol-DA (0.3 ml) was injected via the tail vein. Immediately afterward the drug was administered by i.p. injection. Melphalan was dissolved in 2% HCl/ethanol solution and then diluted in sterile 0.9% PBS³ or diluted into Fluosol-DA or Intralipid. The animals were then allowed to breathe air or were placed in a circulating atmosphere of 95% O₂ and 5% CO₂ (carbogen) for 1 or 2 h and then removed to air. The progress of each tumor was measured 3 times weekly until it reached a volume of 500 mm³. Tumor growth delay was calculated as the days taken by each individual tumor to reach 500 mm³ compared to the untreated controls. Each treatment group had 7 animals and the experiment was repeated 3 times. Days of tumor growth delay are the mean ± SE for the treatment group compared to the control.

Tumor Excision Assay. When the tumors were approximately 50 mm³ in volume (about 1 week after tumor cell implantation) the perfluorochemical emulsion Fluosol-DA (0.3 ml) was injected via the tail vein. Immediately afterward melphalan was administered by i.p. injection. The animals were then allowed to breathe air or were placed in a circulating atmosphere of 95% O₂ and 5% CO₂ (carbogen) for 1 h and then removed to air. Mice were sacrificed and soaked in 95% ethanol 24 h after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were washed twice, then resuspended in 450 units/ml collagenase (Sigma, St. Louis, MO) and 0.1 mg/ml 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 ml of 1 mg/ml DNase was added and incubation was continued for 5 min at 37°C. The samples were then filtered through 2 layers of sterile gauze. The samples were washed twice, then resuspended in minimal essential medium supplemented with 10% FBS (Sterile Systems, Logan, UT). These single-cell suspensions were counted and plated at 3 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 ± SE 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 2 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.) (27). The cells were washed and resuspended in supplemented medium. Granulocyte-macrophage colony-forming units were measured as follows. Bone marrow cells were suspended in supplemented McCoy's 5A medium containing 15% FBS, 0.3% agar (Difco, Detroit, MI) 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% 5A medium for 7 days in a humidified 5% CO₂ atmosphere at 37°C. The colony-stimulating activity-containing supernatant was obtained by centrifugation of the medium at 10,000 × g for 10 min at 4°C followed by filtration under sterile conditions (28). The bone marrow cell cultures were incubated for 7 days in a humidified 5% CO₂ 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 3 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.

Alkaline Elution. FSaIIC fibrosarcoma-bearing mice (7) as described above were given injections of 0.125 Ci/g [methyl-14C]thymidine (5 mCi/mol; New England Nuclear Research Products, Boston, MA) 24 h prior to drug treatment with Fluosol-DA (0.3 ml), melphalan (10 mg/kg), and carbogen or air breathing. Twenty-four h after treatment the tumors were excised, and a single-cell suspension was prepared as described above for the tumor excision assay. Alkaline elution was performed by standard procedures (29, 30). One-half of each group of cells was irradiated on ice with 600 rads using a Gamma cell 40 (Atomic Energy of Canada, Ltd.). Approximately 1.5 × 10⁶ cells/group were placed onto an alkaline elution filter (2.0-μm pore size) (Millipore, Bedford, MA). As an internal control, 1 × 10⁶ [3H]thymidine-labeled L1210 cells irradiated with 150 rads were also placed onto each filter. Cells were first washed with cold PBS, then lysed with 3 ml of 0.2% sodium dodecylsulfate/2 ml NaCl/0.04 M EDTA, pH 10, which was allowed to flow through by gravity. To study DNA-DNA interactions, 0.5 mg/ml proteinase K (Sigma) was added to the lysis solution and incubated on the filters for 60 min at room temperature. Alkaline elution was carried out in the dark using 2% tetrapropylammonium hydroxide (Fisher)/0.025 M EDTA at a rate of 2.4 ml/h. Fractions were collected at 90-min intervals and were assayed for radioactivity after adding 12 ml of Aquasol (New England Nuclear). The remaining DNA on the filters was removed by treatment with 0.4 ml of 1 N HCl for 60 min at 65°C, and the solution was neutralized with 2.5 ml of 0.4 N NaOH before Aquasol addition. Samples were counted on a LS 7000 Beckman scintillation counter. Each point was measured in 3 independent experiments. Cross-linking factor (CLF) was calculated as

\[
\text{CLF} = \frac{\log(\text{irradiated control})}{\log(\text{irradiated drug})} - 1
\]
Pharmacokinetics Study. FSaIIC fibrosarcoma-bearing mice, as described above, were given injections of 6 μCi of [ethylmethylen-2,4,8,12,16,20,24,32-14C] melphalan (10 mg/kg; 7.84 mCi/mmol) with Fluosol-DA (12 ml/kg, 0.3 ml) then with [14C]melphalan or with [14C]melphalan prepared in Fluosol-DA. All of the injections were i.v. Animals were sacrificed at 0.25, 0.5, 0.75, 1.00, 1.25, 3.00, 6.25, 24.00, and 24.25 h posttreatment. Known wet weights of tumor, liver, spleen, kidney, heart, lungs, skin, fat, gut, skeletal muscle, and blood were dissolved in a tissue solubilizer (Protosol; New England Nuclear), then counted by liquid scintillation in Aquasol (New England Nuclear). The data are expressed as μg 14C equivalents per g tissue wet weight. The data were fitted to a triexponential equation of the form

\[ C = A_0 e^{-\alpha t} + B e^{-\beta t} + C e^{-\gamma t} \]

which allowed derivation of the τ_{0.5} for absorption, distribution, and elimination of the drug as well as calculation of the area under the concentration versus time curve (31, 32).

Melphalan and Fluosol-DA Interaction Studies. A high-performance liquid chromatography–UV system was used to measure the levels of melphalan in the aqueous portion of Fluosol-DA. The mobile phase for high-performance liquid chromatography was 1 mM ammonium acetate, 0.7% acetic acid, and 40% ethanol delivered at a rate of 1.5 ml/min using a Spectra Physics pump (33, 34) and Brownlee C_{18} reverse phase column. Separation was effected isocratically at room temperature with detection at 254 nm. Quantitation was made by construction of a standard curve of peak height versus melphalan standards of known concentration. The calibration curve was linear on a log-log plot from 0.25–15 μg/ml melphalan.

In the first experiment, concentrations of melphalan of 5, 10, and 15 μg/ml were dissolved in Fluosol-DA and after allowing equilibration for 0.25, 0.50, 1.00, 4.00, and 24.00 h, aliquots were removed from each tube. The Fluosol-DA containing melphalan was centrifuged for 10 min in an Eppendorf microcentrifuge which separated the aqueous portion from the denser perfluorochemical portion of the Fluosol-DA. Ten μl of the supernatant were used to measure the amount of melphalan under each condition. The experiment was repeated 3 times.

In the second experiment, 15 μg/ml of melphalan were dissolved in Fluosol-DA and allowed to equilibrate for 1 h. Aliquots were removed and diluted 2-, 5-, or 10-fold with PBS and allowed to equilibrate once again for 0.25, 0.5, 1.0, 4.0, or 24.0 h. Then, as described above, aliquots were removed, centrifuged, and the amount of melphalan in the aqueous layer was measured. The experiment was repeated 3 times.

RESULTS

The influence of Fluosol-DA and Intralipid, a fat emulsion, with and without carbogen breathing, on the tumor growth delay of the FSaIIC fibrosarcoma caused by a single dose of 10 mg/kg melphalan was examined (Table 1). Under air breathing, Fluosol-DA produced a 2-fold increase in tumor growth delay compared to melphalan alone. Intralipid had no influence on the drug's effect. When carbogen breathing was added to the protocol, there was a 3-fold increase in tumor growth delay with Fluosol-DA and only a 1.6-fold increase in tumor growth delay with Intralipid. Preparing melphalan in Fluosol-DA produced a 4-fold increase in tumor growth delay with air breathing and a 10-fold increase in tumor growth delay with carbogen breathing. Preparing melphalan in Intralipid produced a 1.5-fold increase in tumor growth delay with air breathing and a 3.7-fold increase in tumor growth delay with carbogen breathing. Therefore, addition of Fluosol-DA to treatment with melphalan leads to a greater increase in tumor growth delay under both conditions of air and carbogen breathing than does Intralipid.

When FSaIIC tumors were excised 24 h after treatment with melphalan or melphalan plus Fluosol-DA with air or carbogen breathing and tumor cell survival was measured by colony formation, the results shown in Fig. 1 were obtained. Throughout the melphalan dosage range examined there is approximately 1 log greater tumor cell kill observed with the addition of Fluosol-DA and carbogen breathing compared to the drug treatment alone. In the mouse, 10 mg/kg of melphalan is a therapeutic dose, producing a FSaIIC tumor growth delay of 2.7 ± 0.3 (SE) days (25). The ability of melphalan to kill tumor cells increases with dose over the range of drug examined. At the lower doses of drug (5 and 10 mg/kg) there is some increase in tumor cell killing seen with the addition of carbogen breathing or Fluosol-DA and air breathing; however, at the highest dose of the drug, as shown by the near coincidence of the points, this difference disappears.

The survival of bone marrow cells from these same animals was measured by the colony-forming ability of granulocyte-macrophage colony-forming units in culture. The killing of bone marrow cells increases linearly over the dosage range examined at a level consistent with previous reports (Fig. 2; Refs. 35 and 36). There was no significant difference in the survival of bone marrow cells under any of the treatment conditions.

Following the methodology of Murray and Meyn (7) the alkaline elution of cells from FSaIIC tumors treated in vivo was used to examine the effect of these treatment conditions on the cross-linking or DNA in tumor cells (Table 2). Fluosol-DA itself with air of carbogen breathing produced no detectable cross-links in DNA. The cross-linking factors for melphalan (10 mg/kg) was 1.95 and 1.99 with air and carbogen breathing,
FLUOSOL-DA/CARBOGEN ENHANCEMENT OF MELPHALAN ACTIVITY

Fig. 2. Survival of bone marrow cells as measured by the granulocyte-macrophage colony-forming unit assay from mice treated with various doses of melphalan (M) with air breathing (●), melphalan followed by carbogen breathing for 1 h (○), melphalan and Fluosol-DA (F) with air breathing (☐), or melphalan and Fluosol-DA followed by carbogen breathing for 1 h (△). Bars, SE.

Table 2 DNA cross-linking factors from alkaline elution of FSaIC tumors treated in vivo

Fluosol-DA was administered i.v. at a dose of 12 ml/kg (0.3 ml). Melphalan was given as a single dose i.p. of 10 mg/kg.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cross-linking factor</th>
<th>Air</th>
<th>Carbogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluosol-DA</td>
<td>1.07*</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>Melphalan</td>
<td>1.95</td>
<td>1.99</td>
<td></td>
</tr>
<tr>
<td>Melphalan + Fluosol-DA</td>
<td>2.02</td>
<td>5.82</td>
<td></td>
</tr>
</tbody>
</table>

* Cross-linking factor = 1.00 indicates no cross-links.

respectively. Although the cross-linking factor for melphalan plus Fluosol-DA and air breathing was similar to melphalan alone, when carbogen breathing was added to the treatment combination, the cross-linking factor increased to 5.82. Therefore, there was more cross-linking of the DNA in the tumor as a whole with the combined treatment.

When melphalan was dissolved in Fluosol-DA and the amount of drug present in the aqueous portion of Fluosol-DA was followed over time, the results shown in Fig. 3 were obtained. At first, the melphalan moved quickly into the lipophilic perfluorochemical particles so that after 1 h 60% of the drug was in the perfluorochemical layer. At 24 h, 85–90% of the melphalan was sequestered in the perfluorochemical particles. To determine if the melphalan could be accessed from the perfluorochemical particles, 15 μg/ml of melphalan was allowed to equilibrate in Fluosol-DA for 1 h, and then the Fluosol-DA was diluted with PBS and allowed to reequilibrate for various periods of time (Fig. 4). Shortly after dilution, the melphalan was increased in the aqueous portion of the Fluosol-DA. However, after long periods of reequilibration, the melphalan moved back into the lipophilic perfluorochemical. The immediate effect of an increase of melphalan in the aqueous phase indicates that melphalan sequestered in the perfluorochemical can be accessed into a more hydrophilic environment.

The pharmacokinetics of [14C]melphalan alone, [14C]melphalan plus Fluosol-DA, and [14C]melphalan prepared in Fluosol-DA were studied in several tissues of FSaIC fibrosarcoma-bearing mice (Table 3). In general, the tissue absorption and distribution t1/2s for melphalan were shortened in the presence of Fluosol-DA (except for the kidneys). In the blood, the half-life for elimination for melphalan alone was only 20 min; however, with Fluosol-DA that time increased to 6.7 h, and when the drug was prepared in Fluosol-DA the half-life for elimination was 5.2 h. In the liver (and most other tissues) melphalan is absorbed and distributed much more rapidly in the presence of Fluosol-DA. The elimination time is also shortened and in general the area under the concentration versus time curve for exposure to the drug is smaller. In the kidneys, however, the half-time parameters for absorption, distribution, and elimination are all longer when Fluosol-DA is present, giving a 3.6-fold increase in the area under the curve for that tissue.

The melphalan concentration versus time curves for the 3 treatment conditions over the first 6 h are shown in Fig. 5. As can be seen, in the presence of Fluosol-DA there is a shifting of the t1/2s for absorption and distribution to shorter times which produces a much sharper and earlier peak in the drug exposure of the tumor. Since the drug in the presence of Fluosol-DA is eliminated from the tumor more slowly, the overall effect is to produce essentially no change in the area under the drug exposure curve for the tumor.

DISCUSSION

Solid tumors may be refractory to a cytotoxic agent for several reasons, including (a) the limited penetration of the
Table 3  Pharmacokinetic parameters for [14C]melphalan administered i.v. to mice bearing FSaIC tumors

These parameters were derived from curves based on 11 data points over 24 h. The data were analyzed via a computer program based on a 2-compartment model (31, 32). Data for blood, spleen, brain, heart, lungs, skin, fat, and gut are not shown. Melphalan was administered at a dose of 10 mg/kg. Fluosol-DA was administered at a dose of 12 ml/kg (0.3 ml). For melphalan in Fluosol-DA, melphalan (10 mg/kg) was prepared in Fluosol-DA (0.3 ml) and administered as a single injection.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment group</th>
<th>( t_{1/2} ) absorption (min)</th>
<th>( t_{1/2} ) distribution (min)</th>
<th>( t_{1/2} ) elimination (h)</th>
<th>Area under curve</th>
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<tr>
<td>Tumor</td>
<td>Melphalan</td>
<td>34</td>
<td>46</td>
<td>10.3</td>
<td>19.0</td>
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<tr>
<td></td>
<td>Melphalan + Fluosol-DA</td>
<td>20</td>
<td>25</td>
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<td>12</td>
<td>16</td>
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<tr>
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<td>56</td>
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<td></td>
<td>Melphalan + Fluosol-DA</td>
<td>8.5</td>
<td>14</td>
<td>28.9</td>
<td>16.6</td>
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<td></td>
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<td>13</td>
<td>24.0</td>
<td>14.9</td>
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<tr>
<td>Kidney</td>
<td>Melphalan</td>
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<td>3.4</td>
<td>2.3</td>
<td>44.1</td>
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<tr>
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<td>16</td>
<td>21</td>
<td>14.8</td>
<td>108.6</td>
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<tr>
<td></td>
<td>Melphalan in Fluosol-DA</td>
<td>40</td>
<td>47</td>
<td>39.7</td>
<td>158.0</td>
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</table>

Fig. 5. FSaIC tumor levels of [14C]melphalan (M) (10 mg/kg) alone (•), with Fluosol-DA (F) (12 ml/kg; 0.3 ml) (○), or prepared in Fluosol-DA (0.3 ml) (△).

Drug to poorly vascularized regions of the tumor, and (b) the physiological heterogeneity of the cellular populations of solid tumors with respect to oxygenation and proliferation (3, 37). Fluosol-DA and carbogen breathing may produce changes in vivo at several levels which could lead to enhancement of the antitumor activity of melphalan. In this study, we have shown that Fluosol-DA influences the pharmacokinetics of melphalan and probably the bioavailability of the drug. Moreover, from the tumor growth delay experiments, the tumor excision assay, and the alkaline elution assay of DNA cross-linking, it is clear that oxygen breathing combined with Fluosol-DA produces a marked enhancement in melphalan cytotoxicity. From the tumor growth delay results obtained with Intralipid compared with Fluosol-DA (Table 1), it is suggested that the lipophilic nature of these agents contributes to the enhancement in melphalan antitumor activity. However, it is the oxygen-carrying capacity of Fluosol-DA which leads to the greatest superiority over Intralipid. One possibility for the greater tumor growth delay observed with Fluosol-DA/air and melphalan versus Intralipid/air and melphalan is that Fluosol-DA provides a chemically inert lipophilic environment with no binding capacity, thus allowing molecules to move freely in and out. There may be some degree of hydrogen bonding involved in the lipophilic portion of Intralipid which may lead to a lessened release of melphalan from the Intralipid.

Unlike the case of enhancement of melphalan antitumor activity by high doses of misonidazole (38–41) we did not find a change in the slope of the tumor cell survival curves. The survival curves of melphalan and melphalan plus Fluosol-DA and carbogen breathing are parallel, which suggests that the addition of Fluosol-DA and carbogen breathing to treatment with melphalan is allowing the drug to be cytotoxic to an additional subpopulation of tumor cells. This notion is also supported by our previous finding using density gradient separation of FSaIC tumor cells where a band of denser cells became more sensitive to melphalan upon the addition of Fluosol-DA and carbogen breathing to the drug treatment (25). Consistent with these findings is the lack of enhancement in toxicity of the combination treatment to the bone marrow. The bone marrow is a well-oxygenated tissue; therefore, Fluosol-DA and carbogen breathing would not be expected to increase significantly the oxygenation of bone marrow cells as it does the hypoxic populations of tumors. The addition of misonidazole to treatment with melphalan also produces no increased toxicity to the bone marrow (38–41).

The increase in tumor growth delay and the decrease in tumor cell survival obtained with Fluosol-DA and air breathing represent the contribution to the therapeutic effect of melphalan treatment resulting from the alteration in the drug pharmacokinetics of the drug caused by Fluosol-DA. Melphalan (and probably many other lipophilic drugs) readily moves into the perfluorochemical particles of Fluosol-DA. This lipophilic environment may protect the drug from hydrolysis and nonspecific binding in circulation and thereby allow delivery of a greater proportion of active drug to the tumor. Similar results were obtained when melphalan was entrapped in small neutral liposomes (42). Although the area under the concentration versus time curve for melphalan in the presence of Fluosol-DA did not change, the shortened absorption and distribution half-life would tend to support the notion that more of the drug arriving at the tumor is the active agent, thus leading to enhanced antitumor activity. At the highest melphalan dose (15 mg/kg), no additional cell killing was seen upon addition of Fluosol-DA and air breathing, indicating that increasing the drug dose to a certain level can compensate for drug loss in the absence of Fluosol-DA.

The details of the involvement of oxygen in the antitumor activity of melphalan in combination with Fluosol-DA remain to be elucidated. The findings reported here demonstrate a relatively nontoxic means of increasing the delivery of oxygen to tumors, resulting in a therapeutically meaningful improvement in melphalan antitumor activity.

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Approaches to Defining the Mechanism of Enhancement by Fluosol-DA 20% with carbogen of Melphalan Antitumor Activity

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