ABSTRACT

Micronized droplets of oil loaded with docetaxel (1.0 mg ml$^{-1}$) and coated with fibrinogen were prepared and then characterized for physicochemical and cytotoxic properties in vitro and anticancer activity in vivo. The droplets remain readily dispersible and relatively stable in size for at least 24 h when stored at 4°C. During storage, the fibrinogen remains bound to the droplets and thrombin coagulable. Nucleoside incorporation assays, growth inhibition assays, and clonogenic assays involving several different tumor cell lines all indicate that the cytotoxicity in vitro of docetaxel applied in oil droplets is at least as great as that of docetaxel applied in olive oil droplets. When compared with Taxotere, an equivalent dose of docetaxel administered in fibrinogen-coated oil droplets improved the median survival time of B16F10 melanoma-bearing mice from 21 days to 69 days. Furthermore, whereas none of the Taxotere-treated mice survived longer than 34 days, 33% (three of nine) of the mice treated with docetaxel-loaded, fibrinogen-coated oil droplets were apparently free of disease after 139 days. Preliminary studies indicate fibrinogen adsorbed to docetaxel-loaded oil droplets facilitates the retention of the droplets within the fibrin-rich tumor microenvironment. We propose this new formulation may prove generally useful for the treatment of taxane-sensitive, fibrin-rich tumors.

INTRODUCTION

It was shown previously (1) that fibrinogen-coated droplets of oil administered i.v. to mice accumulate in the animals at an imposed fibrinogen-rich inflammatory site. That finding led to the proposal that such droplets could be used to deliver lipophilic drugs to any site of fibrin deposition. Inasmuch as fibrin accumulates about/within many, if not all, solid tumors (2–5), it occurred to us that fibrinogen-coated droplets of oil might be ideally suited as vehicles to deliver lipophilic chemotherapeutic agents to solid tumors. With this in mind, we set out to assess the feasibility of fibrinogen-coated oil droplets for the delivery of docetaxel, an important anticancer taxane adsorbed to fibrinogen-coated oil droplets facilitates the retention of the droplets within the fibrin-rich tumor microenvironment. We propose this new formulation may prove generally useful for the treatment of taxane-sensitive, fibrin-rich tumors.

Herein we report on the preparation and in vitro characterization of fibrinogen-coated olive oil droplets loaded with docetaxel and on preliminary studies in which those droplets were used successfully to treat B16F10 melanomas growing in the abdominal cavities of C57BL/6 mice.

MATERIALS AND METHODS

Reagents, Chemicals, and Mice. Docetaxel and Taxotere were from Avents Pharmaceuticals (Bridgewater, NJ). Human fibrinogen was from Enzyme Research Laboratories (Indianapolis, IN). The buffer composition of the commercial fibrinogen was changed by gel permeation chromatography using Sephadex G-25 as matrix material and 0.02 M Tris-HCI (pH 7.40) as eluent. The fibrinogen was then aliquoted and stored frozen until use. Fibrinogen concentration was determined using a HPLC method described below, such sonication did not result in any discernable deterioration of the docetaxel.

Docetaxel-free and docetaxel-loaded olive oil droplets coated with either F68 alone or with F68 and fibrinogen were prepared for use in vitro using the method of high-pressure extrusion as the first step in the process (1). To a clean, 12 × 75-mm glass tube were added 220 ml of either drug-loaded or drug-free olive oil, and 4.78 ml of an aqueous solution containing 0.01 M Na$_2$HPO$_4$/NaH$_2$PO$_4$ and 0.14 M NaCl (pH 7.40) and 1.0 mg ml$^{-1}$ F68 (PBS-F68). The oil/aqueous buffer mixture was first gently agitated and then extruded five times under high pressure (103.5 MPa) through the aperture of a homogenizer (EmulsiFlex-20,000-B3; Avestin, Ottawa, Canada). If the droplets were not to be coated with fibrinogen, then the resulting oil-in-water emulsion was used directly without further processing. If the droplets were to be coated with fibrinogen, however, then the resulting oil-in-water emulsion was added to 1.0 ml of PBS containing 4.0 mg ml$^{-1}$ fibrinogen. After sonicating them for 20 s using an ultrasonic water bath, oil droplets dispersed in fibrinogen-containing medium were floated using centrifugation at 1,500 × g for 15 min. Five ml of the resulting infranatant were removed, and the remaining 1.0 ml containing the fibrinogen-coated oil droplets was dispersed in 4.0 ml of PBS-F68. The droplets were then again floated using centrifugation.
tion at 1,500 \times g for 15 min. After discarding 4.0 ml of the infranatant, the remaining droplet-rich 1.0 ml was dispersed in 4.0 ml of PBS-F68 for use. As necessary, the resulting suspensions were further diluted with PBS-F68. If not applied in oil droplets, then the docetaxel that was used in tissue culture experiments was added to culture in 5.0 \mu l of DMSO.

Dosage requirement and a limitation in administered volume precluded the use in vivo of drug-loaded oil droplets formulated as described above. For use in vivo, more oil-rich formulations were needed, and they were prepared as follows. To a clean, 12 \times 75-mm glass tube were added 2.0 ml of either drug-loaded or drug-free olive oil, and 3.0 ml of PBS-F68. The oil/water mixture was gently agitated and then extruded five times through the aperture of the homogenizer. If the droplets were not to be coated with fibrinogen, then the resulting oil-in-water emulsion was used without further processing. If the droplets were to be coated with fibrinogen, however, then the resulting oil-in-water emulsion was added to 2.0 ml of PBS containing 4.0 mg/ml fibrinogen. After sonicating them for 20 s using an ultrasonic water bath, the fibrinogen-coated oil droplets were floated using centrifugation at 1,500 \times g for 15 min. Six ml of the resulting infranatant were removed, and the remaining 1.0 ml containing the cream layer was dispersed in 4.0 ml of PBS-F68. The droplets that had constituted the cream layer were then again floated using centrifugation at 1,500 \times g for 15 min. After discarding 4.0 ml of the resulting infranatant, the oil droplets in the remaining 1.0 ml were dispersed in 4.0 ml of PBS-F68 for use.

The concentrations of docetaxel in the various stock emulsions were determined using HPLC as described below. On the basis of those determinations, stock emulsions were diluted as necessary to the final desired concentrations of the drug.

Taxotere was prepared to a final concentration of 400 \mu g docetaxel/ml inoculum according to the manufacturer’s instructions. Unless specified otherwise, PBS was used as diluent for the commercial formulation.

**Size of Oil Droplets.** Oil droplets were minimized using a large droplet particle size analyzer (LS 230; Coulter, Miami, FL). Docetaxel-loaded oil droplets were added directly to the water-filled sizing chamber of the analyzer. Refractive indices of 1.47 (triolein) and 1.33 (water) were used when fitting droplet data to the analyzer’s preprogrammed sizing algorithm.

**Quantification and Coagulability of Fibrinogen on Oil Droplets.** The difference between the absorbance at 280 nm due to fibrinogen in the solution used to coat oil droplets and that due to fibrinogen in the post-coating, oil-free infranatant was used to determine the amount of fibrinogen associated with oil droplets. Thrombin-inducible agglutination of fibrinogen-coated olive oil droplets was monitored using a platelet aggregometer as described elsewhere (12). The treated sample as a percentage of the number of adherent cells from a control sample was determined as the result of a growth inhibition assay the number of adherent cells from a treated sample as a percentage of the number of adherent cells from an untreated control sample.

**Extraction and Quantification of Docetaxel from Cultured Tumor Cells.** B16F10 cells (5.0 \times 10^5) obtained from cultures in exponential growth phase were added in ~10 ml of medium to rectangular 75 cm^2 cell culture flasks (Corning Costar). After incubating the cells at 37^\circ C for 24 h, the culture medium and any nonadherent cells were decanted from the flasks and replaced with fresh medium to which docetaxel had been added, either in oil droplets or solubilized in 5.0 \mu l of DMSO. The final concentration of drug in the extracellular medium was 10.0 \mu M. Cells were then incubated in the presence of docetaxel at 37^\circ C for various lengths of time, after which the drug-containing medium was decanted, and the adherent cells were washed twice. After liberating the adherent cells from the surface of a flask by using a rubber cell scraper, the resulting cell suspension was collected, and the cells were sedimented using centrifugation at 1,500 \times g for 5 min. All but 1.0 ml of the supernatant overlying the cell pellet was aspirated from the sample. The cells were then resuspended in that residual volume, which was transferred to a tissue grinder tube. After homogenizing the cells, the docetaxel was first extracted using a solid-phase method (13) and then quantified using an established HPLC procedure (13) The amount of docetaxel in a homogenate was referenced against the homogenate’s protein content. Protein content was determined using the method of Lowry et al. (14).

**Tumor Model and Treatments.** Mice were each given i.p. 1.0 ml of PBS containing 1 \times 10^5 B16F10 melanoma cells. Tumor-bearing mice were then assigned randomly to experimental groups, the members of which were each treated i.p. 1 and 8 days later with 1.0 ml of a test or control formulation. All formulations were administered within 2 h of preparation. Whether administered as Taxotere or in oil droplets, the amount of docetaxel per test injection was 400 \mu g, i.e., ~25 mg/kg mouse body weight. Unless specified otherwise, there were nine mice in each group.

**Analysis of Antitumor Efficacy Data.** The number of mice surviving in an experimental group was monitored as a function of time after tumor cell implantation. Results of survival studies were plotted as percentage of original animals remaining alive versus day after tumor implantation. For the purposes of intergroup comparison, the %ILS attributable to a treatment was defined as: [median days of survival of treated group − median days of survival of PBS-F68-treated group]/median days of survival of PBS-F68-treated group] \times 100%.

Statistical significances of differences in the survivals of the various treatment groups were determined using Kaplan-Meier survival analysis and the log-rank test. Animals that were still alive 220 days after tumor cell implantation were censored from analysis.

**Demonstration of Fibrinogen in Tumor Tissue.** Mice that had each received 1.0 \times 10^5 B16F10 cells either i.p. or s.c. 7–10 days earlier were euthanized and sacrificed using cervical dislocation. Specimens of solid tumor were removed and then either embedded in Histo Prep (Fisher-Diagnostics, Fairlawn, NJ) and flash frozen in liquid nitrogen, or formalin-fixed and embedded in paraffin. Frozen specimens were cut into thin sections and immunostained for fibrinogen, and paraffin-embedded specimens were cut into thin sections and stained for fibrin using the traditional histochemical technique of Lendrum et al. (15).

In another experiment, two mice each received s.c. injection in the right flank with 0.3 ml of PBS containing 1 \times 10^5 B16F10 cells. Fourteen days later, when the resulting tumors in the mice had each grown to ~1 cm^3, the animals were each given via a lateral tail vein a 0.3-ml inoculum of PBS containing 1.7
mg of $^{125}$I-fibrinogen of specific activity $2.4 \times 10^6$ cpnmg$^{-1}$. Six h later, the animals were euthanized, and the tumors were excised from the carcasses. As control tissues, full thickness blocks of normal skin ($\sim 1 \mathrm{cm}^3$) were excised from the left flanks of the animals. After weighing the tumors and blocks of skin, the radioactivities associated with them were measured. Subsequently, the radioactivity in a tissue was normalized by dividing it by the weight of the corresponding tissue.

**Extraction and Quantification of Docetaxel from Tumor Tissue.** Three mice that had each received $1.0 \times 10^6 \mathrm{B16F10}$ melanoma cells 16 days earlier were each given an i.p. inoculum containing 400 $\mu$g of docetaxel. One animal received the drug as Taxotere, another received the drug within fibrinogen-free oil droplets, and the last received the drug within fibrinogen-coated oil droplets. Two h after having received the drug, the mice were sacrificed using cervical dislocation. Representative blocks of tumor from within the abdominal cavity of each animalwere harvested, rinsed extensively using deionized water, weighed, and then homogenized in 2.0 ml of water. To 1.0 ml of homogenate were added 4.0 ml of ethyl acetate. The resulting mixture was vortexed for 5.0 min, after which the organic phase was decanted and placed in a clean glass tube. That tube was placed in a heating block, where the solvent was evaporated to dryness under nitrogen. The nonvolatile material remaining in the tube was then dissolved in 200 $\mu$l of acetonitrile:water (50:50, v/v) for analysis using HPLC.

For analysis of docetaxel extracted from tissue homogenates, we used a HPLC procedure slightly different from that which was referenced above. One hundred $\mu$l of sample were applied to a $\mathrm{C}_{18}$ reverse-phase column (particle size, 10 $\mu$m) of dimensions $3.9 \times 300$ mm ($\mu$Bondapack; Waters Corp.). Acetonitrile:water (50:50, v/v) at a flow rate of 1.0 ml/min$^{-1}$ was used as eluent. Absorbance at 230 nm was used to detect docetaxel eluting from the column. Known amounts of docetaxel in 100-$\mu$l aliquots of acetonitrile:water (50:50, v/v) were used as external standards and to calibrate the assay. The area under the absorbance peak that was centered about a retention time of 13.23 min was linearly proportional to docetaxel content. Using this procedure, the interday coefficient of variation for the measurement of docetaxel over the range of 0.05–5.0 $\mu$g varied from 4% to 10%. After correcting for the total volumes of the various samples, data were recorded as the docetaxel content of a tissue, in picomoles, divided by the tissue’s wet weight, in milligrams.

**RESULTS**

**Preparation and Characterization in Vitro of Docetaxel-Loaded Olive Oil Droplets.** We prepared and characterized several different formulations of docetaxel-loaded olive oil droplets. Because we performed first the studies in vitro that required only relatively small doses of drug, control and test oil droplets for use in vitro were prepared from mixtures in which the oil:water ratio was $\sim 1:22$ (v/v). As we progressed to studies in vivo, however, we realized that much larger doses of drug in a relatively small volume of total inoculum would be needed. Thus, control and test oil droplets for use in vivo were prepared from mixtures in which the oil:water ratio was 2:3 (v/v).

Size distributions of fibrinogen-coated, docetaxel-loaded oil droplets are shown in Fig. 1. The figure shows droplets prepared 2 and 24 h earlier using oil:water ratios of either 1:22 or 2:3. Although the distributions overlap, they are not superimposable. Droplets made from the oil-poor mixture are smaller, on average, than those made from the oil-rich mixture. From absorbance measurements, we determined that $\sim 70\%$ of the fibrinogen binds to droplets of the oil-poor preparation, and $\sim 93\%$ of the fibrinogen binds to droplets of the oil-rich preparation. During storage at 4°C for 24 h, the droplets form cream layers that can be dispersed by simple agitation. The distributions shift somewhat to the left after 24 h due, perhaps, to the dissociation of aggregates of droplets formed during the fibrinogen-coating process. In support of that proposal, the distribution of a preparation of fibrinogen-free, docetaxel-loaded oil droplets made using the oil-rich mixture 2 h before sizing was virtually the same as that of fibrinogen-coated droplets after 24 h (data not shown).

For analysis of docetaxel extracted from tissue homogenates, we used a HPLC procedure slightly different from that which was referenced above. One hundred $\mu$l of sample were applied to a $\mathrm{C}_{18}$ reverse-phase column (particle size, 10 $\mu$m) of dimensions $3.9 \times 300$ mm ($\mu$Bondapack; Waters Corp.). Acetonitrile:water (50:50, v/v) at a flow rate of 1.0 ml/min$^{-1}$ was used as eluent. Absorbance at 230 nm was used to detect docetaxel eluting from the column. Known amounts of docetaxel in 100-$\mu$l aliquots of acetonitrile:water (50:50, v/v) were used as external standards and to calibrate the assay. The area under the absorbance peak that was centered about a retention time of 13.23 min was linearly proportional to docetaxel content. Using this procedure, the interday coefficient of variation for the measurement of docetaxel over the range of 0.05–5.0 $\mu$g varied from 4% to 10%. After correcting for the total volumes of the various samples, data were recorded as the docetaxel content of a tissue, in picomoles, divided by the tissue’s wet weight, in milligrams.

**Coagulability of Fibrinogen Bound to Docetaxel-Loaded Olive Oil Droplets.** When stirred in the presence of thrombin, hydrophobic microparticles coated with a layer of functional fibrinogen agglutinate, a process due to interparticle fibrin dimerization (11, 17). As shown in Fig. 2, fibrinogen-coated droplets of docetaxel-loaded olive oil agglutinate in thrombin-dependent fashion, confirming the functionality of the bound protein.

**Cytotoxicity in Vitro.** The antiproliferative effects of various formulations were assessed using Nb2-11 cells and $[^3H]$thymidine incorporation. For these and all other studies involving cultured tumor cells, we used DMSO instead of ethanolic polysorbate 80 as a control diluent for docetaxel because polysorbate 80, the surfactant used at high concentration in Taxotere, is itself cytotoxic (18). With respect to medium alone, neither DMSO alone nor fibrinogen-coated olive oil droplets alone had any effect on cellular proliferation. In contrast, docetaxel applied to a concentration of 5.0 nM in either fibrinogen-free or fibrinogen-coated olive oil droplets markedly reduced the proliferative response of Nb2-11 cells, i.e., $3.2 \pm 1.4\%$ and $2.8 \pm 0.5\%$,
respectively. The difference between the oil droplet formulations was not statistically significant. In comparison, docetaxel applied in DMSO to 5.0 nM reduced the proliferative response of Nb2-11 cells to only 44.2 ± 2.8% that of cells treated with medium alone.

Inhibition of the anchorage-dependent growth of cells was used as another indication of cytotoxicity in vitro. For this purpose, 1-day-old monolayer cultures of MCF-7 cells and B16F10 cells were treated with various materials for 48 h. We used only a single concentration of docetaxel, 5.0 nM, to assess the effect of the drug on the growth of MCF-7 cells. Whereas neither DMSO alone nor fibrinogen-coated oil droplets alone had any effect on cell growth, all docetaxel-containing formulations had a significant inhibitory effect. Once again, docetaxel administered in either fibrinogen-free or fibrinogen-coated oil droplets yielded significant, equivalent reductions in cell growth (30.4 ± 0.3% and 30.9 ± 0.5%, respectively). Those reductions, in turn, were significantly less than that yielded by DMSO-solubilized docetaxel (45.3 ± 0.7%). In the case of B16F10 cells, dose-response profiles relating to the various formulations were generated (Fig. 3). Whereas neither DMSO alone nor fibrinogen-coated oil droplets alone had any effect on tumor cell growth, docetaxel-containing formulations did inhibit cell growth in dose-dependent fashion. At all concentrations of docetaxel tested, the drug in olive oil droplets inhibited growth considerably. Once again, the presence of fibrinogen on the droplets made little or no difference to the observed cytotoxic effect. When fitting the data sets to various mathematical schemes, we found that each fit well an equation that relates, in double hyperbolic fashion, the observed adherent cells to docetaxel concentration, i.e., 

\[ y = \frac{\alpha(1 + x/\beta)}{(1 + x/\gamma)} + (100 - \alpha)/(1 + x/\gamma), \]

where \( y \) is the observed adherent cells, \( x \) is the docetaxel concentration, and \( \alpha, \beta, \) and \( \gamma \) are constants. Taken at face value, such fits suggest that two populations of adherent cells exist, one of which is more sensitive to docetaxel than the other.

To obtain yet another indication of cytotoxic activity in vitro, a clonogenic assay involving B16F10 cells was used (Fig. 4). Whereas neither DMSO alone nor fibrinogen-coated oil droplets alone limited colony formation, docetaxel in olive oil droplets, regardless of whether those droplets were coated with fibrinogen or not, was as effective at limiting colony formation as DMSO-solubilized docetaxel. Data sets corresponding to the dose dependencies fit well the simple hyperbolic relation 

\[ y = 100(1 + x/\delta), \]

where \( y \) is the observed clonogenic response, \( x \) is the concentration of docetaxel, and \( \delta \) is a constant equivalent to the concentration of drug that inhibits the clonogenic response by 50%. The \( \delta \)'s of these fits are relatively close to the \( \beta \)'s of the fits of the growth inhibition assay (see above and the legends for Figs. 3 and 4), suggesting B16F10 growth inhibition and clonogenicity are related mechanistically.

Taken together, the results of all of the cytotoxic assays indicate that the cytotoxicity in vitro of docetaxel delivered in olive oil droplets, regardless of whether the oil droplets are coated with fibrinogen or not, is at least as great as, if not greater than, that of docetaxel delivered in DMSO. We conclude that delivery in vitro of docetaxel within olive oil droplets does not preclude the pharmacological activity of the drug.

**Association of Docetaxel with Cultured Tumor Cells.** Fig. 5 shows the time dependencies of the association of docetaxel with cultured B16F10 cells after application of the drug in either DMSO or fibrinogen-coated oil droplets. After applying docetaxel in either of the two vehicles, there was an initial rapid rise in cell-associated drug, followed by a decay in drug level to an apparent equilibrium value. Although somewhat limited in number, the data points suggest that the approach to equilibrium may be slower for docetaxel applied in fibrinogen-coated oil droplets than for docetaxel applied in DMSO.

**Antitumor Efficacy of Docetaxel Formulations.** Before using docetaxel-loaded oil droplet formulations to treat melanoma-bearing
mice, we wanted to obtain at least some evidence that (a) fibrin(ogen) is enriched in the microenvironment of B16F10 melanomas and (b) delivery of docetaxel within fibrinogen-coated oil droplets would yield higher levels of the drug in tumor tissue than would delivery of docetaxel in control formulations. We found, first, that, at least histologically, fibrinogen does appear to be enriched within the melanoma microenvironment (Fig. 6). In support of the histological data, we next determined that the amount of $^{125}$I-fibrinogen in 14-day-old melanomas 6 h after having given tumor-bearing animals a dose of the radiolabeled protein was $14.7 \pm 0.5$ times that in samples of normal skin. We then assessed the influence of delivery vehicle on the association of docetaxel with melanomas grown in the abdominal cavities of mice. Three random mice with significant tumor burden were each given an i.p. injection of docetaxel, either as Taxotere or within fibrinogen-free or fibrinogen-coated droplets of olive oil. The amounts of docetaxel associated with the tumors 2 h later were as follows: from the Taxotere-treated mouse, 3.5 pmol/mg tissue; from the fibrinogen-free oil droplet-treated mouse, 4.8 pmol/mg tissue; and from the fibrinogen-coated oil droplet-treated mouse, 8.4 pmol/mg tissue.

Armed with the preceding results, we next treated groups of melanoma-bearing mice, hoping to prolong their survival. Survival plots related to various control and test formulations are given in Fig. 7. The survival plots of the mice treated with PBS-F68 or either of the docetaxel-free olive oil droplet formulations are virtually superimposable, and their differences are not statistically significant ($P = 0.5$). The median time to death of animals in the three control groups was 15 days; all animals died between 7 and 17 days after tumor cell implantation. Taxotere-treated mice lived longer than control animals ($P < 0.0001$), and their median time to death was 21 days; all Taxotere-treated mice died between 19 and 34 days after receiving tumor cells. Not unexpectedly (see “Discussion”), tumor-bearing mice treated with docetaxel in fibrinogen-free oil droplets lived significantly longer than the Taxotere-treated animals ($P < 0.0001$); their median time to death was 49 days, and all died between 17 and 69 days after tumor cell implantation. Mice treated with docetaxel-loaded, fibrinogen-coated oil droplets, however, lived even longer ($P < 0.03$); their median time to death was 69 days, with the first animal dying only after 32 days. Importantly, three mice from that last group were alive and well 139 days after having been given tumor cells and were apparently free of disease. With the exception of the mouse that died on day 140 (see Fig. 7 legend), the abdominal cavities of all mice that died during the course of the experiment were loaded with melanoma.

In a separate, control experiment, we tested whether the therapeutic efficacy of Taxotere would be improved by coadministration of that commercial preparation with fibrinogen-coated droplets of docetaxel-free olive oil. As shown in Fig. 8, such droplets had no significant effect ($P = 0.5$) on the survival of tumor-bearing mice treated with Taxotere. Such a result suggests the efficacy of docetaxel-loaded oil droplets is a synergistic (not additive) effect of drug and vehicular components.
DISCUSSION

Fibrinogen-coated olive oil droplets administered i.v. to mice accumulate at an imposed fibrinogen-rich inflammatory site (1). That finding led to the proposal that such droplets might be used advantageously to target lipophilic drugs to sites of fibrinogen deposition in vivo. Because fibrinogen accumulates within/around malignant tumors, it seemed worthwhile to determine whether fibrinogen-coated oil droplets could be used to deliver a lipophilic anticancer agent to a growing tumor. Not only might the droplets localize the agent to the tumor microenvironnent, but they also might provide a reservoir there for the agent’s slow release. As an anticancer agent, docetaxel seemed ideally suited because it is very lipophilic and because its existing formulation is associated with untoward side effects. As target, the B16F10 melanoma seemed a good choice because that tumor, which is uniformly fatal if propagated untreated in the abdominal cavity of a C57BL/6 mouse, elicits the local deposition of fibrinogen.

The results of our preliminary studies were most gratifying. We found, first, that incorporating docetaxel into micronized oil droplets did not limit in vitro the cytotoxicity of the drug. We showed, next, that melanoma-bearing mice treated with docetaxel-loaded, fibrinogen-coated oil droplets survived much longer (%ILS = 360) than melanoma-bearing mice treated similarly with fibrinogen-coated Taxotere (%ILS = 40). We conclude that fibrinogen-coated oil droplets are a more effective means by which to deliver docetaxel to melanoma growing in the abdominal cavity of a mouse than is Taxotere.

Because docetaxel administered in fibrinogen-free olive oil droplets also significantly prolonged survival (%ILS = 230), we cannot, at this time, attribute the survival benefit of the oil formulations solely to the presence of droplet-bound fibrinogen. It is entirely possible that the surfactant-stabilized oil phase acts synergistically with the drug to confer anticancer activity. As one of many plausible explanations for such a synergism, the benefit of the “naked” oil droplets may derive from an ability to interfere with the efflux of docetaxel from tumor cells (13, 19). It might also be the consequence of a simple depot effect. We favor and are focusing on another explanation, however; one that still invokes a role for adsorbed fibrinogen. Our experience with micronized particles of hydrophobic phases in contact with various fibrinogen-containing body fluids (11, 16, 20–24) has taught us that the protein invariably adsorbs from those fluids to the particles.

Thus, when introduced into a tumor-filled abdominal cavity, fibrinogen-free droplets of olive oil become coated with fibrinogen in situ. We are now using various means to test the hypothesis that such a coating is equivalent operationally to one that is applied to the droplets before they are administered parenterally. We are also modifying the formulations so as to make the droplets smaller and to optimize the fibrinogen packing density.

We are not the first to use fibrinogen as a component of a vehicle designed to deliver anticancer agents. Other investigators have covalently conjugated fibrinogen to methotrexate (25) or encapsulated various anticancer drugs within microspheres composed entirely of fibrinogen (26–28). The aim of the latter investigators was not to target the drugs to tumors but to create a biodegradable, sustained-release reservoir that could be loaded with an otherwise toxic amount of drug. In fact, fibrinogen microspheres worked well as drug reservoirs. Their ability to target tumors specifically, however, was never addressed.

We believe the docetaxel formulation described in this report will, in many circumstances, have greater therapeutic efficacy against fibrinogen-rich tumors than does the existing commercial formulation, i.e., Taxotere. Furthermore, because the new formulation favors sites of fibrinogen deposition, less total drug and/or less frequent drug dosing may be required for tumor treatment. This last possibility might limit drug-related side effects. We are now using other dosing regimens and various tumor models to address these issues.

Much remains to be determined regarding the general applicability of the new formulation to the treatment of solid tumors. The dependence of the preparation’s antitumor efficacy on its route of administration is not yet known. The cancer model described by us here was one in which the tumor was confined, at least macroscopically, to the abdominal cavity. Such confinement was conducive to i.p. administration of the droplets. Indeed, other fibrinogen-rich, docetaxel-sensitive tumors, when confined to the abdominal or other cavity, e.g., the pleural cavity, may also respond favorably to local application. When given i.v., however, fibrinogen-coated droplets of olive oil are retained not only at a site of fibrinogen deposition but also within organs of the reticuloendothelial system (1). Thus, delivery of drug-loaded droplets via an i.v. route to tumors in some anatomical locations may be less than optimal. On the other hand, i.v. delivery of droplets to tumors in the lungs, liver, spleen, and lymph nodes may be

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especially good. Finally, if a tumor is relatively large, intralesional application of the coated oil droplets may represent another route of administration, regardless of the tumor’s anatomical location.

We have not yet determined what, if any, side effects are associated with the new formulation. One potential side effect may be the development of humoral and/or cellular immune responses directed against fibrinogen, a protein critical to coagulation. In the experiments reported here, we used human fibrinogen to coat the droplets. The use of human fibrinogen was driven by a financial consideration: murine fibrinogen from commercial source was prohibitively expensive. Although we noted no overt manifestation of a change in the coagulation status of treated animals, the potential for such a change still exists. For that reason, it will be necessary to determine whether fibrinogen-coated olive oil droplets elicit antifibrinogen antibodies and/or cell-mediated immune responses in recipient animals and, if so, whether any such responses are associated with changes in the coagulation status of the recipients and/or contribute to therapeutic efficacy. We are also exploring the influences of solution-phase fibrinogen and both procoagulants and anticoagulants on the response to the drug-loaded droplets.

In closing, we have demonstrated the anticancer activity of docetaxel-loaded, fibrinogen-coated droplets of olive oil. We are confident other liquid hydrophobic phases and/or lipophilic agents can be used to formulate fibrinogen-coated microparticles that are equally, or even more, efficacious in the treatment of tumors and/or other lesions associated with fibrinogen deposition. We are particularly excited by the prospect that this formulation could be used to target a mixture of mechanistically dissimilar chemotherapeutic agents to fibrinogen-rich sites. We hope in the near future to develop several such formulations and assess their therapeutic utility using various model systems.

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Preparation, Characterization, and Preliminary Application of Fibrinogen-Coated Olive Oil Droplets for the Targeted Delivery of Docetaxel to Solid Malignancies

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