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Research Paper

Nontoxic Suramin Treatments Enhance Docetaxel Activity in Chemotherapy-Pretreated Non-Small Cell Lung Xenograft Tumors

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Purpose. We reported that nontoxic suramin treatments enhance the activity of chemotherapy in preclinical models, a finding supported by the results of subsequent phase I/II trials in chemotherapy-naive non-small cell lung cancer (NSCLC) patients who received/carboplatin (P/C) combination therapy. The present study evaluated whether suramin enhances the activity of docetaxel in human NSCLC xenografts.

Methods. The *in vitro* effect of suramin on docetaxel activity was evaluated using 3-D histocultures of chemotherapy-naive A549 tumors. For *in vivo* activity evaluation, we first established the P/C pretreatment schedule that produced tumor growth inhibition, but not tumor eradication, and established the maximally tolerated docetaxel/suramin regimens. In the second study, P/C-treated animals received physiological saline, single-agent suramin (10 mg/kg), docetaxel (10 mg/kg), or the combination twice weekly for 3 weeks.

Results. The *in vitro* results showed that 20 μM suramin, which had no activity as single agent, enhanced the docetaxel activity (measured as 50% inhibition of DNA synthesis) by more than 10-fold. The *in vivo* studies showed reduced tumor growth by P/C (30% growth in 14 days *vs.* 75% in control). In contrast, docetaxel produced tumor regression (15% reduction) in P/C-treated animals, significantly reduced, on a cellular level, the viable cell density and the proliferating fraction (40% reduction for both measurements), and enhanced the apoptotic fraction 4-fold ($p < 0.05$ for all effects). Suramin had no activity or toxicity (measured as body weight loss) but significantly enhanced the docetaxel activity. Compared to docetaxel alone, the combination showed earlier onset of tumor size reduction, greater extent of tumor regression (31 *vs.* 15%), greater reduction of viable cell density and proliferating fraction (additional 15–25% reduction), and greater apoptotic fraction (additional 2.5-fold increase) ($p < 0.05$ for all parameters).

Conclusions. Results of the present study indicate that nontoxic suramin treatments enhanced the activity of docetaxel in P/C-pretreated A549 xenograft tumors in mice without enhancing host toxicity. These encouraging results provided the basis for phase I/II trials of docetaxel plus low-dose suramin in patients with NSCLC in second-/third-line settings.

KEY WORDS: chemosensitization; docetaxel; *in vivo* chemoresistance model; low-dose suramin; lung cancer.

INTRODUCTION

We recently reported an epigenetic, broad-spectrum mechanism of anticancer drug resistance caused by acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) that are expressed in solid tumors. These two proteins, at clinically relevant concentrations, induce an up to 10-fold resistance to drugs with diverse structures and action mechanisms. The resistance was not due to alteration in drug accumulation (1).

Suramin, a polysulfonyl-naphthylurea, inhibits the binding of several polypeptide growth factors such as platelet-derived growth factor, aFGF, bFGF, vascular endothelial growth factor, transforming growth factor β, and insulin-like growth factor-1 to their receptors (2–8). Our earlier studies showed that nontoxic concentrations of suramin (15 μM) were sufficient to completely reverse the FGF-induced resistance to paclitaxel, doxorubicin, and 5-fluorouracil in cultured prostate tumor cells. We also showed that suramin, at nontoxic and subtherapeutic doses, significantly enhanced the therapeutic efficacy of chemotherapy without enhancing the host toxicity in multiple xenograft tumors in mice, including (a) paclitaxel and doxorubicin in subcutaneous or lung metastases of human prostate PC3 xenografts, (b) irinotecan in human colon HT29 xenografts, (c) gemcitabine and paclitaxel in human pancreatic Hs766T xenografts, and

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ABBREVIATIONS: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; IC_{50}, concentration producing 50% inhibition of DNA precursor; NSCLC, non-small cell lung cancer; P/C, paclitaxel/carboplatin.

(d) mitomycin C in human bladder RT4 xenografts (9–13). Furthermore, suramin at concentrations less than 50 μM also enhanced the activity of 5-fluorouracil in histocultures of human renal cell cancer (14). These encouraging preclinical results have led to several phase I/II trials using low-dose suramin to enhance the antitumor activity of chemotherapy in patients with lung, breast, and kidney cancer. The first-phase phase I/II trial in patients with advanced non-small cell lung cancer (NSCLC) has been completed. The total response rate was 40.8% including a 6.1% complete response rate, time to progression was 7.2 months, and median survival time was 11.4 months (15,16). These clinical outcomes are more favorable compared to historical controls (<20% total response and <1% complete response, 3–4 months for time to progression, and median survival time of 7–8 months) (17,18).

There are few treatment options for chemotherapy-pretreated patients with NSCLC (19). Docetaxel is the first agent approved by the US Food and Drug Administration in this setting. Compared to best supportive care, docetaxel significantly delayed disease progression and prolonged survival in patients with NSCLC previously treated with chemotherapy. In patients who failed platinum-containing chemotherapy, docetaxel was superior to vinorelbine or ifosfamide, producing a higher overall response rate, a higher progression-free rate at 26 weeks, and a longer 1-year survival rate (6.7 *vs.* 0.8, 17 *vs.* 8, and 32 *vs.* 19%, respectively) (20).

Several other new treatments for chemotherapy-pretreated patients with NSCLC have been evaluated. These include gefitinib, an inhibitor of epidermal growth factor receptor tyrosine kinase. Gefitinib shows activity as single agent as third-line treatment in patients with advanced NSCLC who have failed platinum-based and docetaxel treatments (21). However, the fraction of patients responding to gefitinib is relatively small and appears to be related to mutations in the receptor, i.e., 26% in Japanese patients and 2% in North American patients (22). More recently, pemetrexed, a multi-target antifolate, was approved for use in chemotherapy-pretreated patients on the basis of a more favorable toxicity profile compared to docetaxel although pemetrexed does not produce superior antitumor activity (23). These recent developments of molecular-targeted therapies for advanced NSCLC are encouraging. However, the limited extent of therapeutic improvements indicates a continuing need for further development of effective treatments for patients with advanced NSCLC in the second- or third-line settings.

The present study evaluated whether low and nontoxic suramin regimens can enhance the activity of docetaxel, under *in vitro* and *in vivo* conditions, with the use of a human NSCLC A549 xenograft tumor model. The *in vitro* studies used A549 tumors maintained in 3-D histocultures consisting of tumor and stromal cells. The *in vivo* studies used A549-bearing animals pretreated with maximally tolerated doses of a paclitaxel and carboplatin (P/C) combination, which is a standard treatment of chemotherapy-naive NSCLC patients.

MATERIALS AND METHODS

Chemicals and Reagents

Paclitaxel was purchased from Hande Tech (Houston, TX, USA), suramin from Sigma (St. Louis, MO, USA), cefotaxime sodium from Hoechst-Roussel (Somerville, NJ), and other cell culture supplies from GIBCO Laboratories (Grand Island, NY, USA). The chemical form and clinical formulation of docetaxel were provided by Aventis, Inc. (Bridgewater, NJ, USA).

Cell Cultures

Human non-small cell lung A549 tumor cells were purchased from ATCC (Manassas, VA, USA). Tumor cells were maintained as monolayer cultures at 37°C in a humidified atmosphere containing 5% CO_2, in RPMI 1640 culture medium supplemented with 9% fetal bovine serum, 2 mM L-glutamine, 90 μg/ml gentamicin, and 90 μg/ml cefotaxime.

Establishment of A549 Xenograft Tumors

Male athymic nude mice (5–6 weeks old) were purchased from the National Cancer Institute (Bethesda, MD, USA). Animals had free access to food and water and were cared for in accordance with institutional guidelines. Mice were housed in air-filtered laminar flow cabinets.

A549 cells were harvested from subconfluent cultures using EDTA/trypsin and injected subcutaneously into the flanks on both sides of a mouse (2×10^6 cells in 200 μl 1:1 mixture of RPMI 1640 and Matrigel per site). After 2 weeks, animals bearing tumors that reached a diameter of more than 5 mm were used for experiments. One group of animals was used as donors of tumors for the *in vitro* chemosensitivity study. Another group was used for *in vivo* antitumor activity evaluation.

In Vitro Chemosensitivity Evaluation

Evaluation of the *in vitro* effect of suramin on docetaxel activity was conducted using histocultures of A549 tumors. Stock solutions were prepared by dissolving the drug in water (suramin) or ethanol (docetaxel). The final concentration of ethanol was less than 0.1%. Drug effect was measured as inhibition of DNA precursor (bromodeoxyuridine or BrdU) incorporation in tumor cells, as described previously [e.g., (24,25)]. Briefly, tumor histocultures were incubated with vehicle (control) or docetaxel and/or suramin for 48 h, at which time BrdU (40 μM) was added and incubated for an additional 48 h. Afterward, tumors were washed three times with 4 ml phosphate-buffered saline (pH 7.4), fixed in 10% formalin, embedded in paraffin, and then cut into 8-μm-thick sections with a microtome. The sections were deparaffinized and processed for immunostaining for BrdU using the LSAB kit (Dako, Carpinteria, CA, USA), and counterstained with hematoxylin. 3,3′-Diaminobenzidine was the chromogen. Controls were processed in the same manner, with the exclusion of drug treatment. The fraction of BrdU-labeled cells in tissue sections was determined by microscope examination. Typically, five to six tumor pieces were used per drug concentration. A minimum of 150 cells per tumor piece or more than 600 cells per concentration were counted.

The drug concentration–effect data were analyzed using the following equation and nonlinear regression with the PROC NLIN routine of SAS (Cary, NC, USA):

$$E = (E_o - R_e)\left(1 - \frac{C^n}{K^n + C^n}\right) + R_e$$

where E is the labeling index of drug-treated tissues, E_o is the labeling index of untreated controls, R_e is the residual fraction, C is the drug concentration, K is the drug concentration at one-half E_o, and n is a curve shape parameter. Values of drug concentration needed to inhibit DNA precursor incorporation by 50% (IC_{50}) were determined.

Animal Treatment Protocols

For *in vivo* studies, stock solutions of suramin or carboplatin were diluted with physiological saline to the desired concentrations. Paclitaxel was dissolved in ethanol–Cremophor (50:50 v/v) at a concentration of 18 mg/ml and diluted with physiological saline before administration. The clinical formulation of docetaxel was used as received in accordance with manufacturer recommendations.

We performed two *in vivo* studies to evaluate the activity of docetaxel plus suramin in P/C-pretreated A549-bearing animals. As there are no data in the literature on the *in vivo* antitumor activity of P/C in A549 tumors, we first established the P/C pretreatment that produced tumor growth inhibition but not tumor eradication and identified the maximally tolerated docetaxel/suramin regimens. These data were used to design the second study that evaluated whether low-dose suramin enhanced the activity of docetaxel in P/C-treated animals. For all animal studies, mice were implanted with A549 cells in the right and left flank regions, and drug treatments were initiated when the tumor size reached 5 mm in diameter. Initial treatments included paclitaxel (30 mg/kg) with or without carboplatin (50 or 100 mg/kg). For treatment with suramin and docetaxel, P/C-pretreated animals were given physiological saline or docetaxel (10 mg/kg) with or without suramin (10 mg/kg), twice weekly for 3 weeks. With the exception of carboplatin, all drugs were injected intravenously (200 μl) for 1 min into a tail vein. Carboplatin could not be dissolved in the Cremophor–ethanol solution of paclitaxel and was separately administered by intraperitoneal injection. Carboplatin is completely and rapidly absorbed from the peritoneum (26,27). We previously showed that the selected suramin dose yielded between 10 and 50 μM plasma concentrations in mice, which were sufficient to reverse the FGF-induced chemoresistance, but had no *in vivo* antitumor activity against other xenograft tumors (1,10).

In study 1, which established the P/C pretreatment schedule, animals were randomized to control and treatment groups according to their initial tumor weight, such that all groups within each study had comparable initial tumor sizes. In study 2, which evaluated the activity of docetaxel with or without suramin, the tumor size distribution was such that randomization could not be accomplished. In this case, we placed the animals with smaller initial tumors in the single-agent docetaxel group in order to eliminate potential bias in favor of the docetaxel/suramin combination due to a better treatment outcome associated with smaller tumor size. The second randomization criterion was the initial body weight.

In Vivo Chemosensitivity Evaluation

The effects of drug treatments were examined using two pharmacodynamic endpoints. The first is the conventional tumor size measurement. Tumor width and length were measured using calipers, and the tumor volume was calculated as the product of $0.5 \times (\text{width})^2 \times (\text{length})$. The relative change in tumor size was calculated as the ratio of tumor volume at time t to the tumor volume at the start of docetaxel treatment.

The second set of pharmacodynamic endpoints was tumor morphological changes induced by drug treatments. These included the extent of apoptosis induction, as described previously (1). Because apoptotic cells disappear over time, a second measure of the extent of apoptosis was the density of nonapoptotic cells in the residual tumors. This was determined by counting the number of nonapoptotic tumor cells in three to five randomly selected microscopic fields per tumor at 100× magnification. Briefly, 3 days after the last drug treatment, a mouse was anesthetized and its tumors excised, fixed in 10% formalin, and embedded in paraffin. Five-micrometer histologic sections were obtained, stained with hematoxylin and eosin, and examined microscopically. Apoptotic cells were identified by their condensed and fragmented nuclei. Apoptotic cells identified by these morphological changes are identical to the apoptotic cells identified by the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) method (25,28).

Proliferating cells were identified by staining for Ki67 nuclear antigen (Ki67 Antigen Kit, Novocastra Lab Ltd, Newcastle, UK), which is expressed throughout the cell cycle except in the resting G_0 cells. Briefly, sections of paraffin-embedded tumors were deparaffinized, rehydrated, and boiled in 10 mM sodium citrate buffer (pH 6) for 15 min for antigen retrieval. After washing, quenching of endogenous peroxidase activity with 1% hydrogen peroxide for 10 min, and blocking, tissue sections were incubated with anti-Ki67 monoclonal antibody at room temperature for 2 h, followed by incubation with biotinylated secondary antibody

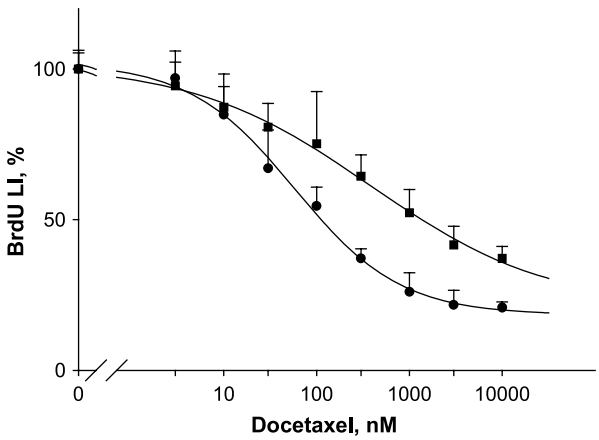

Fig. 1. Suramin enhanced the activity of docetaxel in A549 tumor histocultures. Tumors were harvested from hosts and maintained as 3-D histocultures. Histocultures were treated with docetaxel in the absence (squares) or presence (circles) of 20 μM suramin (Mean ± SD, n = 4–5 replicates).

(diluted 1:500) for 30 min and then streptavidin–peroxidase complex for 30 min. 3,3′-Diaminobenzidine was the chromogen. Sections were counterstained with hematoxylin, dehydrated, and mounted using Permount (Fisher). Human tonsil sections were used as positive controls. For negative controls, we replaced the primary antibody with the blocking reagent.

We determined the number of tumor cells, apoptotic cells and nonapoptotic cells in randomly selected microscopic fields at 100× magnification and counted, on average per animal, >600, >500, >350, and >250 cells in control, suramin, docetaxel, and docetaxel/suramin combination groups. For Ki-67 staining, we selected three to five most intensely labeled fields also at 100× magnification, and counted, on average per animal, >500, >550, >400, and >300 cells in control, suramin, docetaxel, and combination groups.

Statistical Analysis

Statistical analyses of the differences in initial animal body weight and initial tumor size were assessed by analysis

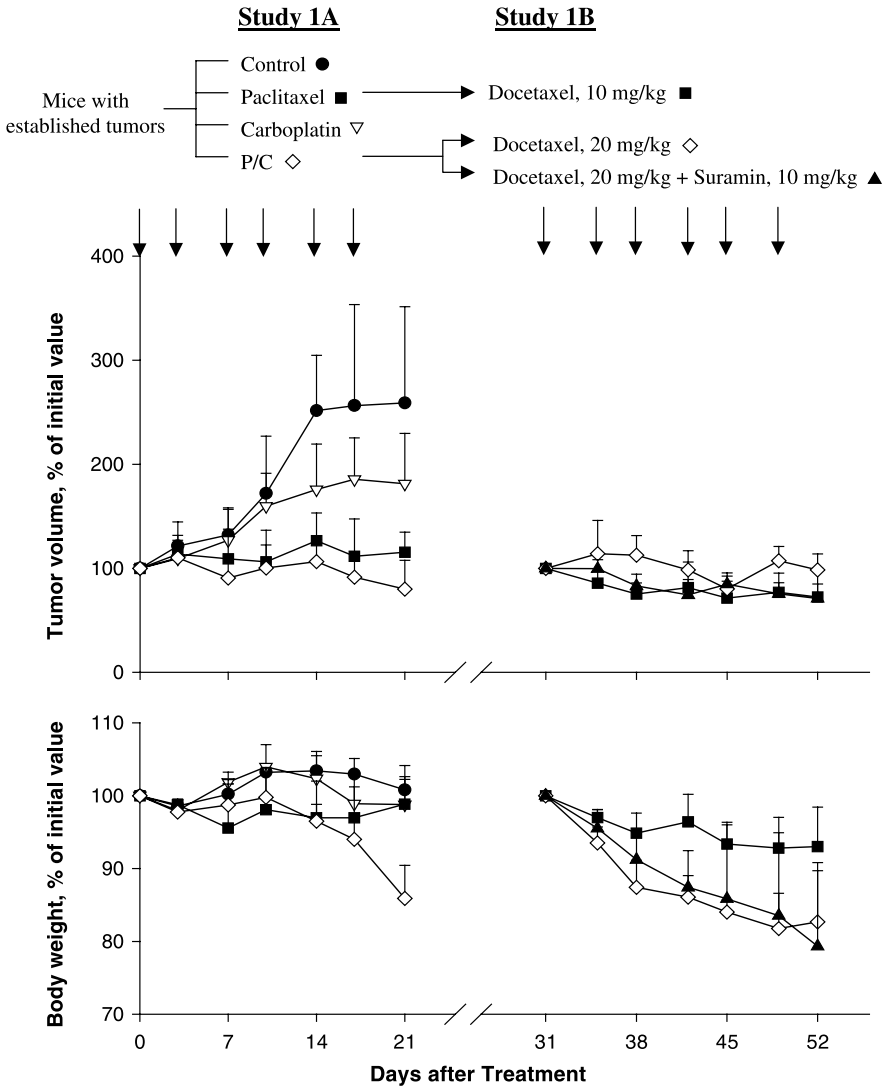

Fig. 2. Effect of paclitaxel and carboplatin in mice bearing A549 xenograft tumors. For study 1A, animals with well-established, subcutaneously implanted A549 tumors were treated with Cremophor vehicle (control, $n = 6$), 30 mg/kg paclitaxel ($n = 5$), 50–100 mg/kg carboplatin ($n = 6$), or P/C combination ($n = 10$) for a total of six treatments. For study 1B, mice in the paclitaxel-pretreated group were treated with 10 mg/kg docetaxel ($n = 5$), and mice in the P/C-pretreated group were randomized into two groups and treated with 20 mg/kg docetaxel or 20 mg/kg docetaxel plus 10 mg/kg suramin ($n = 5$ for each group) for a total of six treatments. Changes in tumor volume and body weight for both treatments were monitored. Mean and one SD. Arrows indicate times of treatments. Data are expressed as percent of initial tumor size. The initial tumor size was 120 ± 40, 102 ± 23, 101 ± 30, and 101 ± 19 mm^3 in the control, carboplatin, paclitaxel, and P/C groups, respectively, in study 1A and 114 ± 38, 96 ± 26, and 82 ± 19 mm^3 in the docetaxel (10 mg/kg), docetaxel (20 mg/kg), and docetaxel (20 mg/kg) plus suramin (10 mg/kg) groups, respectively, in study 1B. The differences in the initial tumor sizes among the different groups within each study were not significant ($p > 0.05$, ANOVA).

of variance (ANOVA). Differences in nonapoptotic cell density, apoptotic fraction, and body weight changes were assessed by unpaired Student's *t* test. Differences in tumor growth rate were determined by ANOVA for repeated measurements and the nonparametric Mann–Whitney *U* test. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Suramin Enhanced Chemosensitivity of A549 Tumors to Docetaxel: *In Vitro* Results

Figure 1 shows the results of drug-induced inhibition of BrdU incorporation in tumor cells grown in histocultures.

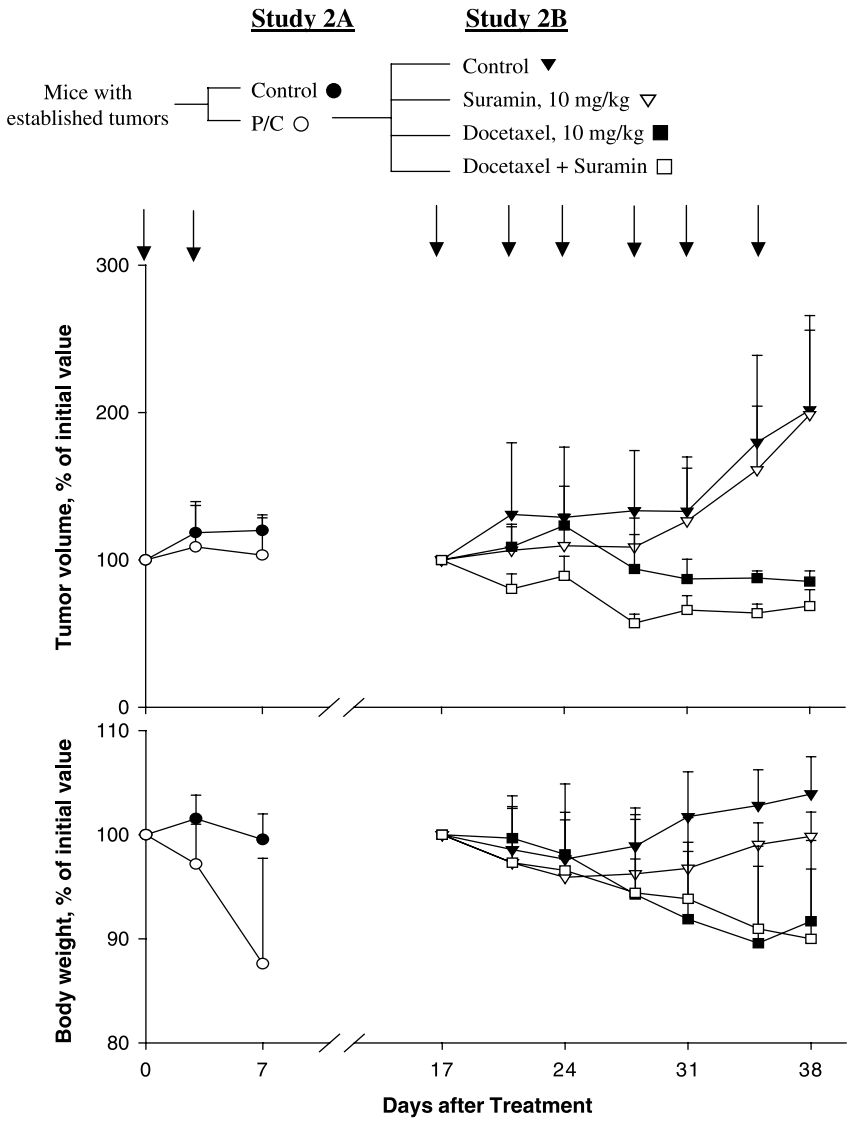

Fig. 3. Low and nontoxic suramin regimens enhanced activity of docetaxel in P/C-pretreated mice bearing A549 xenograft tumors. For study 2A, mice bearing well-established, subcutaneously implanted A549 tumors were treated with Cremophor vehicle (control, $n = 6$) or 30 mg/kg paclitaxel intravenously plus 100 mg/kg carboplatin intraperitoneally ($n = 20$) twice weekly for two treatments. For study 2B, the P/C-pretreated mice were retreated with physiological saline (control, $n = 5$), 10 mg/kg suramin ($n = 5$), 10 mg/kg docetaxel ($n = 5$), or docetaxel plus 10 mg/kg suramin ($n = 5$) twice weekly for 3 weeks. Changes in tumor volume and body weight for both treatments were monitored. Mean and one SD. Arrows indicate times of treatments. Data are expressed as percent of initial tumor size. The initial tumor size was 78 ± 24 and 80 ± 19 mm^3 in control and P/C groups, respectively, in study 2A and 131 ± 28, 130 ± 28, 103 ± 30, and 141 ± 27 mm^3 in control, suramin, docetaxel, and docetaxel plus suramin groups, respectively, in study 2B. The differences in the initial tumor sizes among the different groups within each study were not significant ($p > 0.05$, ANOVA).

Table I. Effect of Low-Dose Suramin on *in Vivo* Docetaxel Activity

	Initial body weight (g)			Final body weight (% of initial value)			Initial tumor size (mm^3)			Final tumor size (% of initial size)		
Group (n)	Mean ± SD	Range	Median	Mean ± SD	Range	Median	Mean ± SD	Range	Median	Mean ± SD	Range	Median
Control ($n = 5$)	30.7 ± 2.2	27.0–33.0	31.2	103 ± 3	99–106	102	131 ± 28	94–171	129	202 ± 54	141–259	196
Suramin ($n = 5$)	29.4 ± 2.1	27.0–31.7	29.2	100 ± 2	98–104	99	130 ± 28	96–166	126	199 ± 67	110–264	226
Docetaxel ($n = 5$)	28.9 ± 1.7	27.0–31.5	29.0	92 ± 8	80–100	92	103 ± 30	72–152	97	85 ± 7	73–92	88
Combination ($n = 5$)	31.3 ± 1.4	29.3–32.8	31.3	90 ± 7	85–101	89	141 ± 27	95–162	153	69 ± 11	54–82	66
P value		0.23[a]			0.72[b]			0.38[a]			0.03[c]	

Human non-small cell lung A549 tumor cells were injected subcutaneously into the right and left flank regions of immunodeficient mice. When tumors reached a diameter of more than 5 mm (day 0), animals received two treatments of intravenous 30 mg/kg paclitaxel plus intraperitoneal 100 mg/kg of carboplatin given 4 days apart. On day 17, P/C-treated mice were given intravenous injection of physiological saline, 10 mg/kg suramin, 10 mg/kg docetaxel, or docetaxel/suramin combination twice a week for a total of six treatments. Mean ± SD.
[a] p value among all treatment groups (ANOVA).
[b] p value between docetaxel and combination groups (unpaired Student's t test).
[c] p value between docetaxel and combination groups (nonparametric Mann–Whitney U test).

Single-agent docetaxel produced a concentration-dependent inhibition of BrdU labeling, with an IC_{50} of 1.15 ± 0.20 μM. Single-agent suramin at 20 μM had no activity, as indicated by the identical BrdU labeling indices in untreated controls and suramin-treated tumors (66.3 ± 4.6 *vs.* 66.5 ± 5.0%, $p > 0.05$). Suramin did significantly enhance the activity of docetaxel, reducing the IC_{50} by more than 10-fold to 0.10 ± 0.01 μM ($p < 0.01$).

Identification of Maximally Effective and Maximally Tolerated Doses of Paclitaxel and Carboplatin in Tumor-Bearing Animals

We performed two *in vivo* studies. The first study consisted of two parts; the first part established the maximally tolerated P/C pretreatment schedule that also produced minor antitumor effect (study 1A) and the second part identified the maximally tolerated and maximally effective docetaxel/suramin regimens in the P/C-pretreated animals (study 1B). These studies were designed to enable the identification of the appropriate treatment schedules with a minimal number of animals.

Figure 2A shows the results of study 1A. Tumor-bearing animals were initially treated with vehicle, 50 mg/kg carboplatin, 30 mg/kg paclitaxel, or P/C combination twice weekly. After four treatments, the respective tumor sizes on day 14 in these four groups were 252, 176, 126, and 106% of the initial values (Fig. 2A), indicating that the treatments were not sufficient to produce tumor regression. In addition, all drug-treated groups showed minimal body weight loss of 4% on day 14, suggesting that maximally tolerated doses had not been reached. Hence, we escalated the carboplatin dose to 100 mg/kg and animals received two additional treatments given for 1 week. A comparison of the tumor size changes from day 14 to day 21 indicates a higher antitumor activity for carboplatin at this higher dose; that is, single-agent carboplatin completely inhibited tumor growth and the P/C combination produced 20% tumor regression. The toxicity was also higher with a 14% total body weight loss for the P/C combination group at day 21, suggesting 100 mg/kg as the maximally tolerated dose of carboplatin in combination with paclitaxel.

The P/C-pretreated animals were then used for study 1B. When the body weights of all groups returned to at least ~100% of the pretreatment levels, the animals were treated with docetaxel, with or without suramin. The P/C-treated group consisted of ten animals, which were randomized to receive either docetaxel (20 mg/kg) or docetaxel plus suramin (10 mg/kg). The 20-mg/kg docetaxel dose was selected based on the previous observation that single 40-mg/kg and multiple 20-mg/kg doses were well tolerated in rodents (29,30). Under these conditions, single-agent docetaxel retarded tumor growth in P/C-treated animals but did not produce tumor regression, whereas the docetaxel/suramin combination produced 29% tumor regression. The differences in tumor sizes between the doxotaxel and combination groups are significant ($p < 0.05$, repeated measurement test). The two groups showed similar body weight losses of 19–21% ($p > 0.05$). These data indicate that suramin enhanced docetaxel activity without enhancing toxicity.

As dose-limiting grade 3 or 4 myelosuppression is common in chemotherapy-pretreated NSCLC patients and results in dose reduction (31), we also evaluated a lower docetaxel dose, i.e., 10 mg/kg. This was performed using the group pretreated with single-agent paclitaxel, and we limited the study to single-agent docetaxel due to the smaller group size (i.e., five animals). The results, shown in Fig. 2B, indicate antitumor activity of docetaxel in paclitaxel-treated animals. Of note, the effect of docetaxel (10 mg/kg) in paclitaxel-pretreated animals exceeded the paclitaxel effect in chemotherapy-naive animals (17% tumor regression *vs.* 18% growth, $p < 0.05$), whereas both treatments resulted in similar body weight loss (4–5%, $p > 0.05$).

A comparison of the two docetaxel doses indicated that body weight loss associated with the 20-mg/kg dose (17%) exceeded the desired upper limit of 10%, whereas the 10 mg/

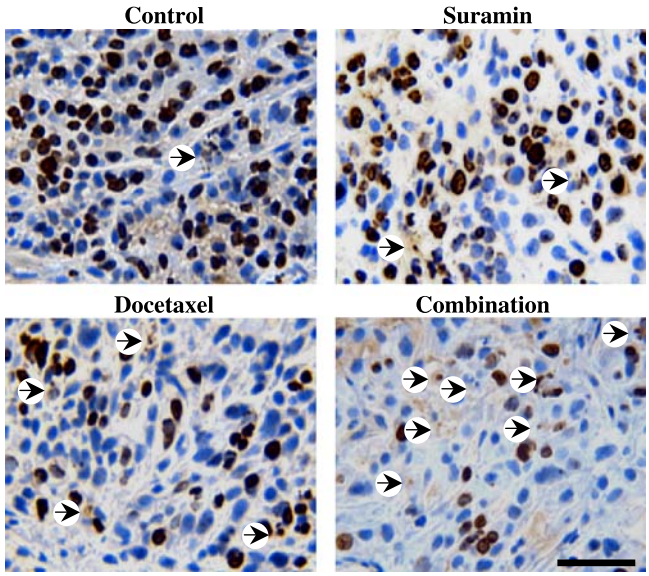

Fig. 4. Tumor morphology. A549 tumor-bearing animals were treated with vehicle, single-agent suramin (10 mg/kg) or docetaxel (10 mg/kg), or docetaxel/suramin combination. Tumors were harvested 3 days after the last treatment. Paraffin-embedded tumor specimens were processed for immunohistochemical staining. Brown color indicates positive Ki-67 staining. Arrow indicates apoptotic cells. Note the decrease in cell density in the combination group. Bar represents 50 μm.

kg produced significant less toxicity (7% body weight loss, $p < 0.05$ compared to 20 mg/kg).

Suramin Enhanced Chemosensitivity of Chemotherapy-Treated A549 Tumors to Docetaxel: *In Vivo* Results

Based on the above antitumor activity and toxicity data, we concluded that the maximally tolerated regimens were (a) paclitaxel 30 mg/kg plus 100 mg/kg carboplatin in chemotherapy-naive animals, twice weekly for 1 week, and (b) docetaxel 10 mg/kg, with or without suramin, in chemotherapy-treated animals, twice weekly for 3 weeks. These regimens were used to design the second study that evaluated whether low-dose suramin enhanced the activity of docetaxel in P/C-treated animals. The results are shown in Fig. 3 and summarized in Table I.

For the pretreatment, the tumor size increased 74% in the control group and 30% in the P/C group by day 14. This finding indicates that P/C pretreatment reduced tumor growth but did not produce tumor regression. On day 17, the P/C-pretreated animals were randomized into four groups, i.e., control, single-agent suramin, docetaxel, and docetaxel/suramin combination. The results show that tumor volumes in control and single suramin groups doubled in 21 days. The single-agent docetaxel group showed increases in tumor sizes initially, followed by minor tumor regression after three treatments (final tumor size reduction of 15%); decreases in tumor sizes became statistically significant on day 35. In comparison, the combination group showed tumor regression after the first treatment; reduction in tumor sizes became statistically significant on day 21. Tumor size reduction was also greater in the combination group compared to single-agent docetaxel group (final tumor size reduction of 31 *vs.* 15%, $p < 0.05$).

Figure 4 shows the tumor morphology, and Table II summarizes the drug effects on a cellular level. The vehicle-treated controls showed about 200 viable cells per field (0.03 mm^2), a Ki67 labeling index of 34%, and an apoptotic index of 2%. Single-agent suramin did not significantly reduce the density of nonapoptotic cells or increase the apoptotic fraction and did not reduce the Ki67 labeling index. Single-agent docetaxel reduced the nonapoptotic cell density by 40%, reduced the Ki67 labeling index by 38%, and increased the fraction of apoptotic cells by 4-fold ($p < 0.01$ compared to control and single-agent suramin groups). Addition of suramin to docetaxel further decreased the nonapoptotic cell density by an additional 15%, reduced the Ki67 labeling index by an additional 26%, and increased the apoptotic cell fraction by an additional 2.5-fold. The differences between single-agent docetaxel and combination groups are statistically significant ($p < 0.01$).

DISCUSSION

The goal of the present study was to determine whether nontoxic suramin treatments enhance the activity of docetaxel in NSCLC. This was evaluated in two ways, using the human A549 xenograft model. We first evaluated whether suramin can reduce the IC_{50} of docetaxel in 3-D histocultures of chemotherapy-naive A549 tumor. Histocultures are tumor fragments cultured on a collagen gel matrix and retain many features of solid tumors, i.e., 3-D multicellular structure, cell-to-cell communication, coexistence of epithelial tumor cells and normal stromal tissue and, accordingly, the organ

Table II. Enhancement of *in Vivo* Antitumor Effect of Docetaxel by Suramin (Histological Evaluation)

Group	Viable cell/field[a]	Apoptotic index (%)	Ki67 labeling index (%)
Control	197 ± 31	1.9 ± 0.8	33.7 ± 2.1
Suramin	168 ± 19	2.7 ± 1.0	31.9 ± 5.3
Docetaxel	117 ± 24	7.7 ± 1.7	20.9 ± 3.3
Combination	87 ± 8[b]	12.9 ± 3.6[b]	12.0 ± 5.2[b]

Animals were treated as described in Table I. Tumors were removed and processed for histological evaluation. Cell density and apoptotic cell fractions were determined in three to five randomly selected microscopic fields per animal. Ki67-positive cells were determined in three to five most intensely stained fields per animal. Ki67 labeling index was calculated as number of positively stained cells divided by total cell number. All evaluations were performed at 100× magnification. Mean ± SD of at least three animals per group.

[a] Area per field is 0.03 mm^2.

[b] $p < 0.01$ compared with all other groups.

microenvironment including the high expression of fibroblast growth factors. The clinical relevance of the histoculture model in predicting clinical response has been demonstrated in retrospective and semiprospective studies (32). Our results show a 10-fold enhancement of docetaxel activity by noncytotoxic concentrations of suramin.

We also evaluated the effects of suramin in mice bearing chemotherapy-pretreated A549 tumors. The three key findings are as follows. First, this tumor model was resistant to P/C, as indicated by the continuing tumor growth during treatment with the maximally tolerated doses of P/C. Second, equitoxic docetaxel treatments (as indicated by the similar body weight losses) produced greater antitumor activity compared to P/C (i.e., tumor regression instead of incomplete tumor growth). Third, a low and nontoxic suramin regimen enhanced the docetaxel activity without enhancing the host toxicity in P/C-pretreated A549 xenograft tumors.

Paclitaxel and docetaxel appear to have different activities in NSCLC. In chemotherapy-naive patients, docetaxel/cisplatin combination but not paclitaxel/platinum combination has superior antitumor activity compared to other platinum-containing combinations (33,34). Compared to docetaxel, paclitaxel is less active in patients who failed platinum-based therapy (35,36). Our present results show antitumor activity of docetaxel in animals pretreated with single-agent paclitaxel or P/C. This is consistent with the clinical observation in NSCLC patients that prior exposure to paclitaxel does not alter the likelihood of response to docetaxel (20). Similarly, patients with breast cancer who progress on paclitaxel can respond to docetaxel. This may be explained by the finding that docetaxel shows incomplete cross-resistance to paclitaxel in cultured ovarian and breast tumor cells (37,38). The finding that the A549 xenograft tumor shows similar response profiles to paclitaxel and docetaxel as in NSCLC patients suggests A549 as a good preclinical model for evaluating the activity of these taxanes.

Our earlier data on cultured prostate tumor cells, showing that addition of bFGF antibody did not enhance the synergy between doxorubicin and suramin, suggest a bFGF-mediated mechanism under *in vitro* conditions (1,10,13). However, suramin has multiple additional pharmacological effects, including inhibition of other growth factors and other targets such as reverse transcriptase, DNA polymerase, interleukin-2, tumor necrosis factor α, topoisomerase II, protein kinase C, RNA polymerase, and transforming growth factor β (39). Further studies are needed to elucidate the mechanisms of the *in vivo* chemosensitization effect of suramin.

Suramin has shown some activity in prostate cancer and has been tested in a variety of solid tumors, either as single agent or in combination with other chemotherapeutics. At least 33 trials have been published since clinical evaluation began in the early 1980s [e.g., (40,41)]. In all these trials, suramin was used as a cytotoxic agent, at plasma concentrations of between 100 and 200 μM. Suramin has also been tested in patients with breast cancer as an antiangiogenic, again requiring the maintenance of concentrations above 140 μM (42). At these concentrations, suramin shows significant toxicities and only modest activity in patients. Furthermore, suramin-containing combination therapy did not show a benefit over monotherapy. This has led to recommendations by multiple investigators against its future use (40–48). The major difference between the previous clinical studies with suramin and our ongoing studies is the intended use of suramin and, accordingly, the selection of the dose/concentration. In the current study, suramin is used to reverse bFGF-induced resistance, an effect that requires only 10–20 μM suramin, concentrations that are not associated with cytotoxicity in cultured tumor cells nor host toxicity in animals or patients. We further showed in *in vitro* and *in vivo* studies that the chemosensitization effect of suramin is lost at concentrations exceeding 50 μM, possibly related to its effects on cell cycle kinetics. Suramin at concentrations above 50 μM arrests cells in the G_1 phase (49–51). A blockage in the G_1 phase may prohibit cells from progressing to the later phases such as the S and M phases where other agents exert their action.

In summary, results of the present study indicate that low-dose suramin enhanced the activity of docetaxel in P/C-pretreated A549 xenograft tumors in mice without enhancing host toxicity. These encouraging results provided the basis for phase I/II trials of docetaxel plus low-dose suramin as second and third line treatments in patients with NSCLC.

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