Exisulind in Combination with Docetaxel Inhibits Growth and Metastasis of Human Lung Cancer and Prolongs Survival in Athymic Nude Rats with Orthotopic Lung Tumors[1]

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ABSTRACT

Docetaxel, a semisynthetic taxane, improves the survival of stage IIIB and IV non-small cell lung cancer patients. However, the 5-year survival remains poor, and few patients experience a complete remission. In this report, we evaluated the effects of exisulind, a novel proapoptotic agent that is a sulfone metabolite of sulindac, in combination with docetaxel on the growth of the human non-small cell lung cancer cell line A549 *in vitro* and *in vivo*. Exisulind is a novel sulindac metabolite in that it does not inhibit cyclooxygenase enzymes and has been shown to induce apoptosis in a variety of human cancers by inhibiting cyclic GMP-dependent phosphodiesterase. Exisulind alone increased the fraction of cells in the G_1 phase of the cell cycle from 46% to 65%, whereas it decreased the fraction of cells in the S phase from 38% to 14%. Docetaxel increased the fraction of cells in the S phase from 17% to 19%, and 10 nM docetaxel increased the G2-M phase by 23%. Docetaxel alone induced apoptosis from 11% to 64% at 12–24 h after incubation. The combination of exisulind with concentrations of docetaxel (in concentrations that alone did not alter cell cycle distribution) reduced the G_1 accumulation induced by exisulind, increased the fraction of cells in G_2-M (9–17%), and increased apoptosis (5–62%). The IC_{50} for *in vitro* growth inhibition by exisulind alone was ~200 μM and 2.5 nM for docetaxel. The *in vitro* combination of exisulind and docetaxel produced an additive to synergistic growth inhibition. In athymic nude rats with A549 orthotopic lung cancers, both exisulind and docetaxel alone moderately prolonged survival, inhibited tumor growth and metastases, and increased apoptosis compared with control animals treated with a carrier. However, the combination of exisulind with docetaxel significantly prolonged survival ($P = < 0.0004$), inhibited tumor growth and metastases ($P = < 0.0001$), and increased apoptosis ($P = < 0.001$) when compared with control animals. These results provide rationale for conducting clinical trials using the combination of exisulind and docetaxel in patients with advanced lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer death in both men and women in the United States causing 28% of all cancer deaths (1). The 5-year survival rate for all patients remains <15%, and a third of patients that are diagnosed with stage IV disease have a 2-year survival rate of <20% (1, 2). NSCLC[3] patients represent 80% of patients diagnosed with lung cancer. Despite recent advances in chemotherapy, response rates in NSCLC remain <50%, and complete remissions are rare (2). Docetaxel, one of the new chemotherapy agents that has improved survival of patients with advanced NSCLC in both the first and second line setting, inhibits depolymerization of microtubules inducing cell cycle arrest and apoptosis (2–7). Although taxene-based therapy improves survival of NSCLC patients, the effects are small, and new agents with novel mechanisms of action are needed to improve therapeutic outcomes.

Exisulind (Aptosyn) is a sulfone metabolite of the COX inhibitor, sulindac. Unlike, sulindac, exisulind is a poor COX1 and COX2 inhibitor and, therefore, lacks the gastrointestinal toxicity of the parent compound. Exisulind induced apoptosis in a variety of human cancer types (8). The mechanism of exisulind-induced apoptosis was independent of *bcl-2* and *p53* expression (8). Exisulind has been shown to inhibit cGMP-PDE isoforms of the PDE5 and PDE2 families (9). This exisulind cGMP-PDE inhibition produced a persistent increase in cellular cGMP levels and activation of protein kinase G in tumor cells containing high cGMP-PDE5 levels before therapy. This protein kinase G increase resulted in MEKK1 phosphorylation, subse-

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; SCLC, small cell lung cancer; MTT, modified tetrazolium salt assay; CI, combination index; COX, cyclooxygenase; cGMP, cyclic guanosine monophosphate; PDE, phosphodiesterase.

quent activation of Jun kinases, and inhibition of extracellular signal-regulated kinase 1/2 phosphorylation, which culminated in apoptosis (10–12). *In vivo* exisulind may have additional effects such as antiangiogenic properties (13). In mouse models, exisulind inhibited lung, breast, and prostate tumorigenesis (14–16). We showed previously that *in vitro* exisulind in combination with standard cytotoxic chemotherapy agents, including paclitaxel, produced synergistic growth inhibition of human lung cancer cell lines (17).

Because exisulind induced apoptosis through a mechanism unique from docetaxel and produced significant synergistic growth inhibition in combination with paclitaxel *in vitro*, we investigated the combination of exisulind and docetaxel on cell cycle, apoptosis, and growth of the human NSCLC cell line, A549, *in vitro* and *in vivo* in athymic nude rats bearing orthotopic A549 lung tumors. It has been documented previously that lung cancer cell lines grown orthotopically in nude rats metastasize in a manner similar to that seen in human lung cancer patients (18, 19). The studies were conducted with the NSCLC cell line A549, because 80% of lung cancer patients present with NSCLC at time of diagnosis and 50% of these patients have adenocarcinoma of the lung. A549 cells have been used extensively in the literature, and we have extensive experience with this orthotopic rat model.

MATERIALS AND METHODS

Cell Line Culture Conditions. The NSCLC cell line A549 was obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). The line was grown at 37°C in a 5% CO_2 incubator with 100% humidity.

Chemicals. Cell Pathways, Inc. (Horsham, PA) kindly provided the exisulind. Suspension exisulind was prepared in 0.5% carboxymethylcellulose (low viscosity; Aldrich Chemical Co., Milwaukee, WI) and administered p.o. to the rats by a single daily gastric gavage. The University of Colorado Institutional Animal Care and Use Committee approved this animal use protocol and drug delivery. Docetaxel was kindly provided by Aventis Pharmaceuticals (Bridgewater, NJ). A stock solution was prepared in DMSO, and working concentrations were diluted with sterile water.

Cell Cycle Distribution and Apoptosis. The effects of exisulind and docetaxel alone and in combination on cell cycle distribution and percentage of apoptotic cell induction *in vitro* was determined by flow cytometry. A549 cells (50,000) were plated in 25-cm^2 tissue culture flasks 24 h before drug addition. After 12–24 h of incubation with exisulind, docetaxel, or the combination, the A549 cells were harvested and stained with 2.5% propidium iodide solution/0.3%saponin/0.001% RNase A in 10 mM EDTA. Stained nuclei were analyzed using a Coulter EPICS XL-MCL (Hialeah, FL) for the proportion of cells in the G_1, S, and G_2-M phases of the cell cycle, and for the percentage of apoptotic cells. The resulting DNA distributions were analyzed using the Modfit LT Software (Verity House Software, Topsham, MA). The presence of apoptotic cells was confirmed by fluorescence microscopy after staining by *bis*-benzimide (Hoechst 33258; Sigma Chemical Co., St. Louis, MO) and counting 200 cells looking for the presence of apoptotic bodies.

Table 1 In vitro effects of exisulind and docetaxel alone and in combination on cell cycle distribution and % of apoptotic cells in A549 NSCLC cells

Treatment	% G_1[a]	% S	% G_2-M	% Apoptosis
Control 24 h	46	38	16	0
Exi 200 μM	60	22	18	0.1
Exi 300 μM	65	14	21	0
Doce 0.2 nM	42	39	19	0
Doce 0.5 nM	38	41	21	0.05
Doce 1 nM	46	40	14	0.58
Doce 3 nM	32	57	11	11
Control 12 h	51	33	16	0
Docetaxel 6 nM	30	50	20	34
Docetaxel 10 nM	10	51	39	64
Control 24 h	46	38	16	0
Exi 200 μM + Doce 0.2 nM	59	21	20	0.47
Exi 200 μM + Doce 0.5 nM	54	28	19	9.4
Exi 200 μM + Doce 1 nM	46	8	46	5
Exi 300 μM + Doce 0.2 nM	58	17	25	5
Exi 300 μM + Doce 0.5 nM	43	24	33	39
Exi 300 μM + Doce 1 nM	51	36	13	62

[a] Exi, exisulind; Doce, docetaxel; % Apoptosis, percentage of apoptotic cells.

MTT Growth Assay. Cell growth was assessed using an MTT assay (20). Briefly, 5000 viable cells were plated in 96-well microtiter plates (Corning, Corning, NY). After an overnight incubation, exisulind, docetaxel, or the combination were added at various concentrations and incubated for 6 days. The tetrazolium salt, MTT, was added at a concentration of 0.4 mg/well on day 6, and the plates were incubated at 37°C for 4 h. After 4 h, the medium was aspirated off leaving the dark blue formazan product at the bottom of the wells. The reduced MTT product was solubilized by adding 100 μl of 0.2 N HCl in 75% isopropanol to each well. Thorough mixing was done using a Titertek multichannel pipetman. The absorbency of each well was measured using an automated plate reader (Molecular Devices, Sunnyvale, CA). When combinations of exisulind and docetaxel were used, the combined growth inhibitory effects were assessed using the combination-index isobologram method of Chou and Talalay (21).

Orthotopic Rat Tumor Model. Female athymic nude rats (6–8 weeks old) obtained from the National Cancer Institute were maintained in pathogen-limited conditions at the Animal Resources Center, University of Colorado Health Sciences Center. One day before tumor cell implantation rats were treated with 400 cGY total body irradiation (Co^{60}) to increase immunosuppression and tumor take. Human adenocarcinoma A549 cells (1×10^7 in 100 μl of serum-free growth medium) were instilled intratracheally into the left lung of anesthetized rats by administration through a special 3-inch 22-guage catheter (Popper & Sons, Inc., New Hyde Park, NY). The procedure was <2–3 min, and the animals recovered in < 5 min without showing signs of stress or casualty. The animals were monitored for an additional 5 min after cell administration and returned to their cages. Seven days after tumor cell implantation, the nude rats were divided into groups of 8–15 rats and treated with saline (controls) or exisulind and/or docetaxel. Rats were treated with exisulind (25, 50, or 100 mg/kg) by a single

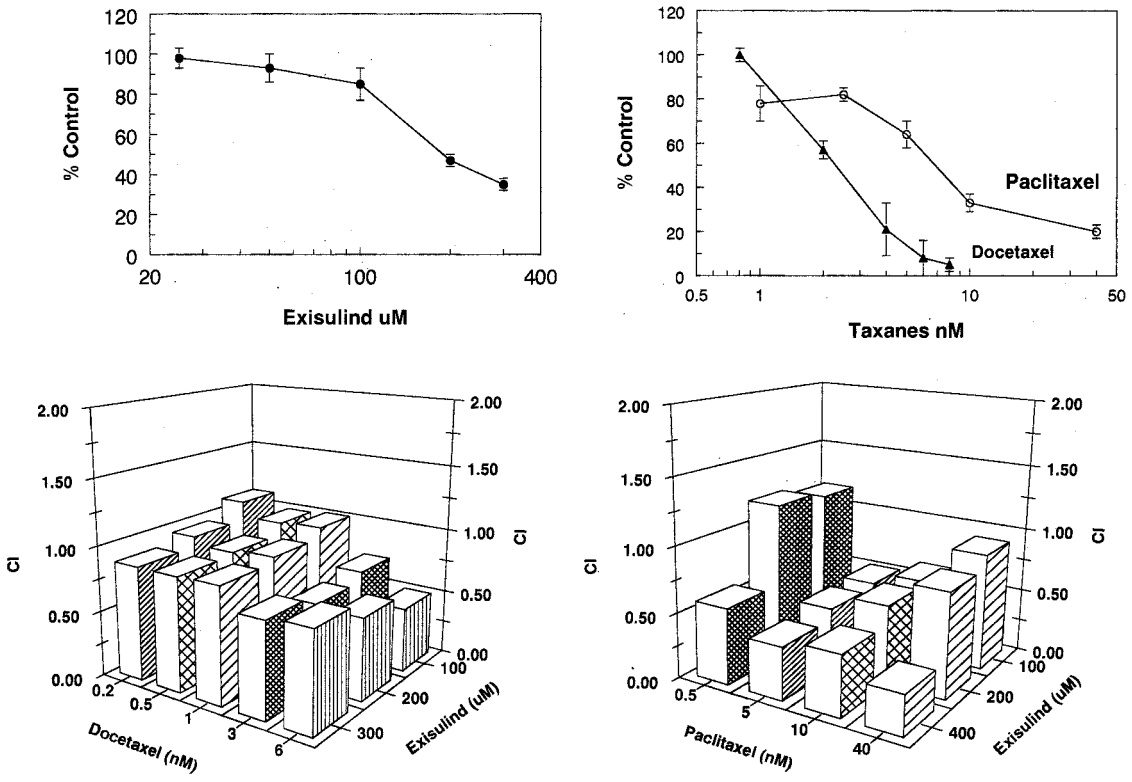

Fig. 1 *A*, the cytotoxicity of exisulind alone on the NSCLC line A549. *B*, the cytotoxicity of docetaxel alone and paclitaxel alone on the NSCLC line A549. *C*, the CI isobologram for the NSCLC line A549 adenocarcinoma treated *in vitro* with exisulind and paclitaxel. CI < 1 indicates synergism, CI $= 1$ indicates additive effects, and CI > 1 indicates antagonism. All data were obtained from 6-day MTT assays.

oral daily gavage with a special 18-guage feeding needle until day of sacrifice. The doses of exisulind were chosen from our previous nude mouse experiments where special diets blended with exisulind (250–1250 mg/kg of diet) reduced s.c. tumor growth (22). In our previous nude mice experiments, animal food consumption declined at the higher doses because of the taste of the exisulind, and there was a dose-related decrease in body weight for each 250 mg/kg of increasing exisulind in mice receiving dietary doses of 500-1250 mg/kg in their food. Therefore, exisulind was given by gavage to ensure reliable drug delivery, and the doses were decreased 10-fold to avoid toxicity (25–100 mg/kg/day). In the first study there were two docetaxel arms. In arm one, docetaxel was administered i.p. at 5 mg/kg twice during the second week after tumor cell implantation, followed by i.p. injections of 2.5 mg/kg weekly for 4-consecutive weeks. In arm two, 10 mg/kg of docetaxel was administered i.p. twice during the second week after tumor cell implantation and then at 5 mg/kg weekly for 4 weeks. The docetaxel schedule in the second study was as follows: 2.5 or 5 mg/kg docetaxel was injected i.p. once per week for 6 weeks or 5 mg/kg was administered i.p. once per week for 3 weeks followed by 2.5 mg/kg i.p. once per week for 3 weeks. Body weight was measured weekly, and the animals were monitored closely for clinical signs of stress on a daily basis for the duration of the study (80 days). On day 21 after tumor cell implantation, 3 rats from each group were sacrificed, and the lungs were fixed in 10% buffered formalin, sectioned, and analyzed for apoptotic cells as described below. Digital photos were taken of lungs isolated from all of the animals either at time of sacrifice or at the time they died from tumor burden using an Olympus 2500 L digital camera (Tokyo, Japan).

Tunel Analysis of Apoptotic Cells in Lung Tumor Tissue. Apoptotic cells containing oligonucleosomes were identified using the TdT-FragEL DNA fragmentation detection kit from Oncogene Research Products (San Diego, CA). Formalin-fixed, paraffin-embedded tissues were processed according to the manufacturer's protocol. Results of tumor tissues undergoing apoptosis because of drug treatment were normalized with the control group.

Statistical Analysis. The therapeutic effect of exisulind, docetaxel, and the combination on rat survival were analyzed using the Kaplan-Meier Survival Model (using a log-rank test). Hazard ratios for each treatment *versus* control were estimated with their 95% confidence intervals. Fisher's exact test was applied to examine differences in metastases associated with treatment *versus* control. All of the statistical analysis was carried out with SAS Software, Version 8.1 (SAS Institute, Cary, NC).

RESULTS

***In vitro* Effects of Exisulind, Docetaxel, and Combinations on Cell Cycle Distribution, Apoptosis, or Cell Growth.** The effects of exisulind, docetaxel, and the combination on cell cycle distribution and apoptosis in the NSCLC cell A549 are summarized in Table 1. A 24-h exisulind concentration of

Table 2 Effect of exisulind, docetaxel, and the combination on the number of athymic rats with metastases from orthotopic A549 tumors (first experiment)

Treatment	Dose and schedule	Mean survival days (*P*, log-rank)	# rats with mets/# treated (*P*, Fisher's exact test)
Control		43 days	9/10
Exisulind	50 mg/kg daily	52.5 days	7/8
		($P = 0.49$)	($P = 1.0$)
Exisulind	100 mg/kg daily	57.625 days	7/8
		($P = 0.26$)	($P = 1.0$)
Docetaxel[a]	5 mg/kg (2×/wk)	63.5 days	6/8
	2.5 mg/kg/wk (4×)	($P = 0.058$)	($P = 0.56$)
Docetaxel[a]	10 mg/kg (2×/1 wk)	63.25 days	5/8
	5 mg/kg/wk (4×)	($P = 0.038$)	($P = 0.27$)
Exisulind	50 mg/kg daily	>80 days	1/8
Docetaxel[a]	5 mg/kg (2×/wk)	($P < 0.001$)	($P = 0.003$)
	2.5 mg/kg/wk (4×)		
Exisulind	50 mg/kg daily	62.375 days	3/8
Docetaxel[a]	10 mg/kg (2×/wk)	($P = 0.12$)	($P = 0.043$)
	5 mg/kg/wk (4×)		
Exisulind	100 mg/kg daily	43.375 days	4/8
Docetaxel[a]	5 mg/kg (2×/wk)	($P = 0.99$)	($P = 0.12$)
	2.5 mg/kg/wk (4×)		
Exisulind	100 mg/kg daily	49 days	3/8
Docetaxel[a]	10 mg/kg (2×/wk)	($P = 0.40$)	($P = 0.043$)
	5 mg/kg/wk (4×)		

[a] Docetaxel was administered at the higher dose twice in the first week and once weekly at the lower dose for the next 4 weeks.

200–300 μM increased the proportion of cells in the G_0/G_1 of the cell cycle from 46% in the untreated control cells to 60%–65% in the treated cells with a corresponding decrease in the S phase fraction. Concentrations of exisulind <200 μM had no effect on cell cycle distribution in the A549 cell line (data not shown). Maximal concentration of exisulind induced apoptosis at 400 μM and 72–96 h, which is consistent with our data published previously for the SCLC cell line SHP77 (data not shown; Ref. 17). Interestingly, a 12-h exposure to docetaxel 6 nM or 10 nM and a 24-h of 3 nM docetaxel exposure increased the fraction of cells in the S phase from 33% to 38% in the controls to 50%, 51%, and 57%, respectively. Not surprisingly, a 12-h exposure of the above docetaxel concentrations increased the fraction of cells in the G_2-M phase from 16% in the controls to 20–39%. Docetaxel at 6–10 nM induced 34–64% apoptosis in the A549 cells after a 12-h exposure. No apoptosis was detected in the untreated control cells. Apoptosis did not occur with docetaxel concentrations below 3 nM.

As shown in Table 1, the combination of exisulind with concentrations of docetaxel that alone had no effect on the cell cycle increased the fraction of A549 cells in the G_2-M phase of the cell. For example, the combination of 200 μM exisulind with 1 nM docetaxel increased the proportion of cells in the G_2-M phase from 16% in the controls to 46%. An increase in the percentage of apoptotic cells was also observed with exisulind in combination with low doses of docetaxel. At 24 h, exisulind 300 μM in combination with 0.5 nM or 1 nM docetaxel induced apoptosis in 39–62% of the A549 cell population.

We studied the effects of exisulind, docetaxel, and paclitaxel alone and in combination on the growth of the A549 cell line in MTT assays. The growth inhibition by exisulind alone, docetaxel alone, and paclitaxel alone are shown in Fig. 1. All three of the compounds inhibited the growth of the A549 cell line in a dose-dependent manner. Docetaxel was the most potent with an IC_{50} of 2.5 nM. The IC_{50}s for paclitaxel and exisulind were 5 nM and 200 μM, respectively. Using the isobologram method of Chou and Talalay (21), we calculated the CI for each set of concentrations. A CI > 1 indicates antagonism, a CI = 1 indicates additive interactions, and a CI < 1 indicates synergy between the two drugs. As shown in Fig. 1 the interactions between exisulind and docetaxel, and exisulind and paclitaxel were additive to synergistic at all of the concentrations tested except at the lowest concentrations tested where the CI was > 1.

Effects of Exisulind, Docetaxel, and the Combination on the Growth of Orthotopic Tumors in Athymic Rats. Because of the synergy seen *in vitro* between exisulind and docetaxel, we compared the potential growth inhibitory effects of this combination on A549 orthotopic tumors in athymic rats to the growth inhibitory effects of each agent alone. The orthotopic model was chosen, because it closely mimics the human situation where the primary lung tumor metastasizes to other sites including the mediastinal lymph nodes and the contra-lateral lung lobes. We showed previously modest growth inhibition of s.c. SCLC-SHP77 and NSCLC-A549 xenografts in athymic mice fed a special chow with exisulind (22). The higher doses of exisulind decreased oral intake and reduced mouse body weights, presumably because of unfavorable taste of the exisulind feed. Therefore, gastric gavage with lower exisulind doses in the athymic rat model allowed us to control drug delivery and prevent weight loss.

Two experiments were done using this model bearing A549 orthotopic tumors. In the first study rats gavaged with either 50 or 100 mg/kg/day exisulind alone did not survive statistically longer than the controls animals (Table 2, Fig. 2A). In this experiment, the control rats had a mean survival of 43 days, and the mean survival was 52.5 days ($P = 0.49$) for the 50

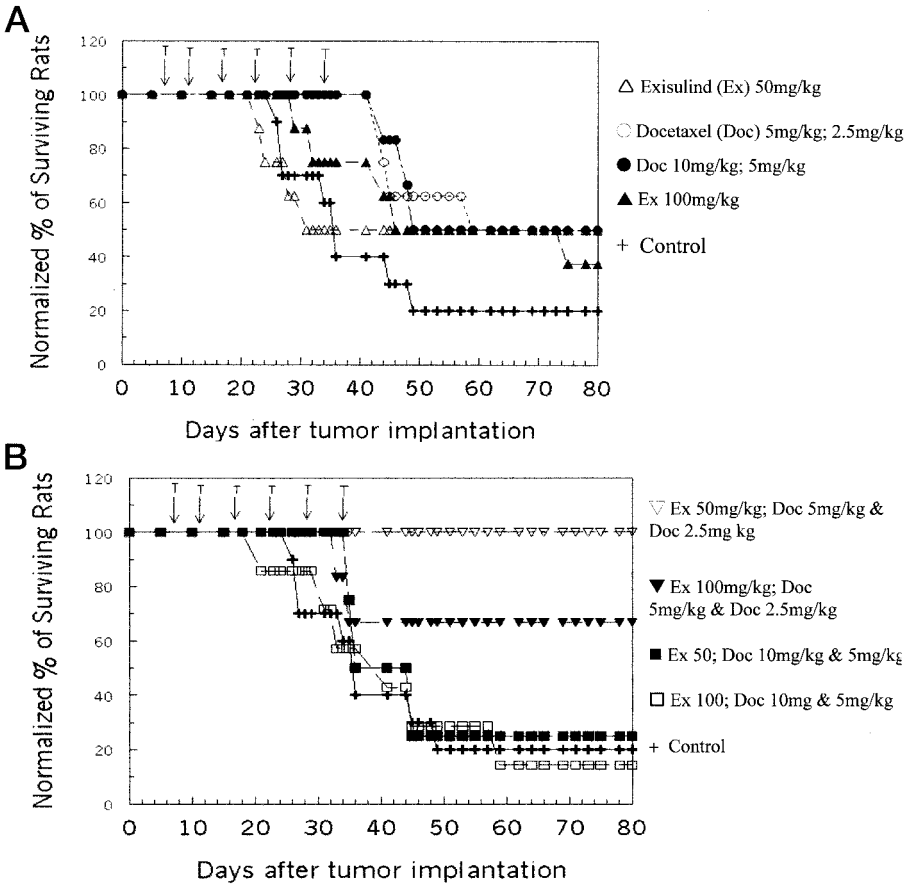

Fig. 2 First survival study of athymic nude rats with orthotopic A549 tumors. *A*, the survival curves for animals treated with exisulind and docetaxel alone compared with saline-treated controls (+); exisulind 50 mg/kg alone (△); exisulind 100 mg/kg alone (▲); docetaxel 5 mg/kg (2×) then 2.5 mg/kg (4×; ○); and docetaxel 10 mg/kg (2×) and 5 mg/kg (4×; ●). *B*, the survival curves for animals treated with the combination of exisulind + docetaxel compared with saline treated controls (+); exisulind 50 mg/kg plus docetaxel 5 mg/kg (2×) then 2.5 mg/kg (4×; ▽); exisulind 100 mg/kg plus docetaxel 5 mg/kg (2×) then 2.5 mg/kg (4×; ▼); exisulind 50 mg/kg plus docetaxel 10 mg/kg (2×) then 5 mg/kg (4×; □); and exisulind 100 mg/kg plus docetaxel 10 mg/kg (2×) then 5 mg/kg (4×; ■).

mg/kg/day group and 57.63 days ($P = 0.26$) for the 100 mg/kg/day group.

Docetaxel was administered i.p. in two arms. In arm 1, docetaxel was given at 5 mg/kg twice during the second week after tumor cell implantation followed by 2.5 mg/kg weekly for 4 consecutive weeks. For arm 2, docetaxel was administered at 10 mg/kg twice during the second week and 5 mg/kg weekly for 4 weeks (docetaxel concentrations were based on the mouse docetaxel literature). The initial 10 mg/kg docetaxel was acutely toxic, and 50% of the animals died. The mean survival in the rats treated with docetaxel alone was 63.5 days for the 5 mg/kg group and 63.25 days for the 10 mg/kg group alone, which was significantly better than the controls ($P = 0.058$ and $P = 0.038$, respectively; Table 2; Fig. 2*A*).

The combination of the 100 mg/kg dose of exisulind with either docetaxel arm was not well tolerated, and survival was not prolonged when compared with controls (Table 2; Fig. 2*B*). In the 100 mg/kg + 5 mg/kg docetaxel arm mean survival was 43.4 days ($P = 0.99$), and in the 100 mg/kg + 10 mg/kg docetaxel arm mean survival was 49 days ($P = 0.40$; Table 2; Fig. 2*B*). Again, the inferior survival was because of the combined toxicity from the high doses of both drugs, resulting in deaths in the majority of treated rats.

The combination of 50 mg/kg/day exisulind with the 5 mg/kg docetaxel arm had a superior survival rate compared with control animals or animals treated with either drug alone ($P < 0.001$; Table 2; Fig. 2*B*). The survival rate at the termination of the experiment at 80 days after tumor implantation was 100%. In the exisulind 50 mg/kg + 10 mg/kg docetaxel arm the 80-day survival rate was 67% with improved mean survival compared with exisulind alone and to controls, although the difference was not significant (62.4 mean survival days, $P = 0.12$; Table 2; Fig. 2*B*).

In the second orthotopic rat experiment, we decreased the exisulind to 25/mg/kg/day or 50 mg/kg/day and docetaxel doses to 2.5 mg/kg/wk or 5 mg/kg/wk for 6 weeks because of the toxicity seen in the first experiment. The results from this experiment are shown in Fig. 3, *A* and *B*, and Table 3. The control mice all died from tumor burden by day 62, and the mean survival was 35.7 days. Mean survival was not extended in the exisulind 25 mg/kg/day group (36 days, $P = 0.76$), and 100% of the rats had died by day 52 (Fig. 3*A*). In the 50 mg/kg/day exisulind-treated group 10% of the rats were still alive by day 60; however, survival was not significantly different from control animals ($P = 0.31$; Fig. 3*A*). Mean survival compared with control animals was also not significantly increased by docetaxel alone at 2.5 mg/kg (34.5 days, $P = 0.66$; Fig. 3*A*). In the docetaxel 5 mg/kg group, 20% of the animals were alive on day 60; however, 100% mortality occurring by day 80 and the mean survival of 43.6 days was not significantly different from controls ($P = 0.17$; Fig. 3*A*).

The combination of 2.5 mg/kg docetaxel with either exisu-

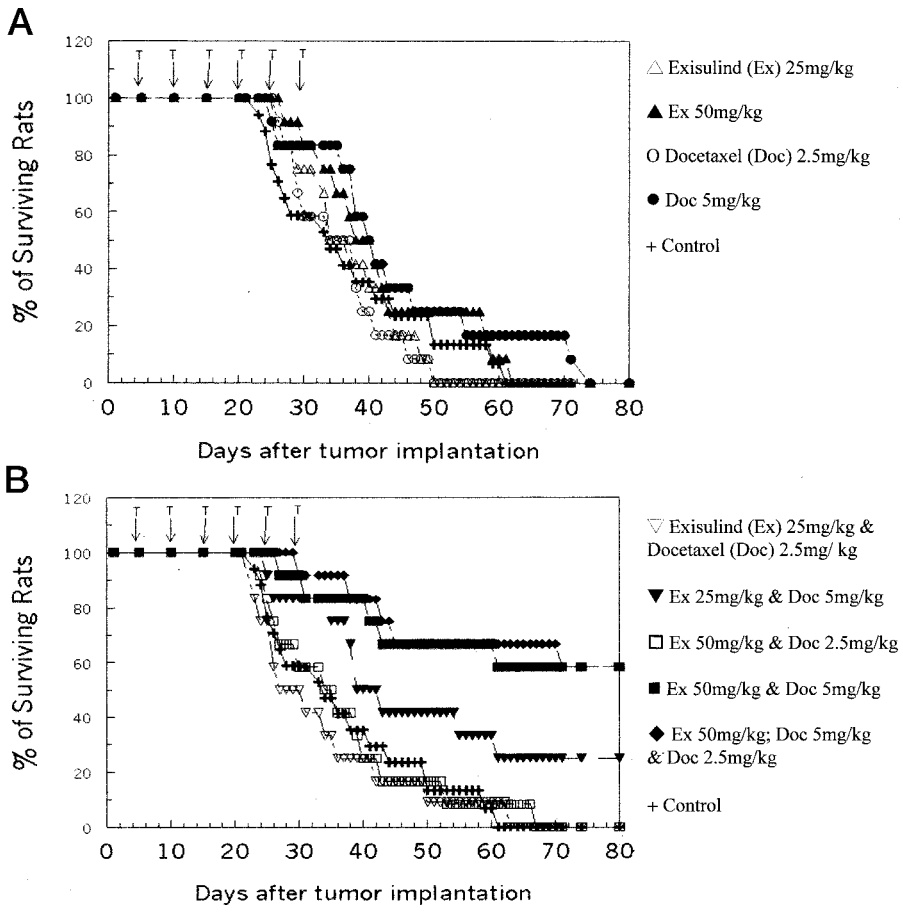

Fig. 3 Second survival study of athymic nude rats with orthotopic A549 tumors. *A*, the survival curves for animals treated with exisulind or docetaxel alone compared with saline treated controls (+); exisulind 25 mg/kg alone (△); exisulind 50 mg/kg alone (▲); docetaxel 2.5 mg/kg (6×; ○); and docetaxel 5 mg/kg (6×; ●). *B*, the survival curves for animals treated with exisulind + docetaxel compared with saline treated controls (+); exisulind 25 mg/kg plus docetaxel 2.5 mg/kg (6×; ▽); exisulind 25 mg/kg plus docetaxel 5 mg/kg (6×; ▼); exisulind 50 mg/kg plus docetaxel 2.5 mg/kg (6×; □); exisulind 50 mg/kg plus docetaxel 5 mg/kg (6×; ■); and exisulind 50 mg/kg plus docetaxel 5 mg/kg (2×) then 2.5 mg/kg (4×; ♦).

lind 25 mg/kg/day or 50 mg/kg/day also did not increase survival (35.5 days, $P = 0.68$ and 36 days, $P = 0.31$, respectively) and all of the animals died by day 70 from tumor burden (Fig. 3*B*). Superior survival rates were seen in the animals treated with exisulind (25 mg or 50 mg) with docetaxel 5 mg/kg/wk for 6 weeks or 5 mg/kg/wk for 3 weeks followed by docetaxel 2.5 mg/kg/wk for 3 weeks (Fig. 3*B*). The mean survival for the 25 mg/kg exisulind + docetaxel 5 mg/kg/wk (6 weeks), compared with controls, was 49.3 days, and 33% of the animals were alive on day 80 ($P = 0.04$). In 50 mg/kg exisulind-treated animals with 5 mg/kg/wk docetaxel or 5 mg/kg/wk for 3-weeks followed by 2.5 mg/kg/wk for 3 weeks, mean survival exceeded 60 days, and 60% of the animals were alive on day 80 in both groups. The survival in the above two groups was statistically significant when compared with controls (63.2 days, $P = <0.0004$ and 65.2 days, $P = <0.0001$, respectively). There was no significant difference in mean survival between these two 5 mg/kg docetaxel groups ($P = 0.92$).

Effects of Exisulind, Docetaxel, and the Combination on Metastases. Tumor burden and metastatic extent were evaluated by necropsy at the time of death or on day 80 when animals were sacrificed. Untreated control rats had visible primary tumors, and 90% (experiment 1) and 100% (experiment 2) had extensive metastases in the mediastinum and contra-lateral lungs as shown in Fig. 4*A* and summarized in Tables 2 and 3. Tumors in the majority of animals treated with exisulind or docetaxel alone were larger than control tumors and were localized to the left-lung lobes with metastases to the right lobes. In Fig. 4*B*, representative lungs and mediastinum from an animal treated with 50 mg/kg exisulind alone (died day 55) are shown. A total of 40 animals were treated with exisulind alone in the two experiments, and metastases were seen in 93%. The lungs and mediastinum from a representative animal treated with 5 mg/kg docetaxel alone (died day 50) are shown in Fig. 4*C*. In the ≥ 5 m/kg, 71% had metastatic disease. Metastases were seen in 100% of the animals treated with an initial dose 2.5 mg/kg docetaxel. The reduction in the number of metastases was most striking in the three groups of animals treated with 5 mg/kg docetaxel and exisulind 50 mg/kg (Table 2, $P = 0.003$ and Table 3, $P = < 0.0001$). The lungs of a surviving representative animal treated with 50 mg/kg exisulind + docetaxel 5 mg/kg and sacrificed on day 80 is shown in Fig. 4*D*. In these three groups, only 4 of 32 (12.5%) treated animals had evidence of metastases, and the majority of tumors in the left-lung lobes of the lung were smaller than control tumors and tumors in animals treated with exisulind or docetaxel alone.

Effects of Exisulind, Docetaxel, and the Combination on *in Vivo* Apoptosis. A hallmark of apoptotic cells is DNA cleavage into oligonucleosomal fragments with exposed 3′-OH ends, which can be labeled and identified microscop-

Table 3 Effect of exisulind, docetaxel, and the combination on the number of athymic rats with metastases from orthotopic A549 tumors (second experiment)

Treatment	Dose and schedule	Mean survival days (*P*, log-rank)	# rats with mets/# treated (*P*, Fisher's exact test)
Control		35.7 days	15/15
Exisulind	25 mg/kg daily	36 days	12/12
		($P = 0.76$)	($P = 1.0$)
Exisulind	50 mg/kg daily	41.08 days	11/12
		($P = 0.31$)	($P = 0.41$)
Docetaxel	2.5 mg/kg/wk (6×)	34.5 days	12/12
		($P = 0.66$)	($P = 1.0$)
Docetaxel	5 mg/kg/wk (6×)	43.58 days	9/12
		($P = 0.17$)	($P = 0.06$)
Exisulind	25 mg/kg daily	35.5 days	12/12
Docetaxel	2.5 mg/kg/wk (6×)	($P = 0.68$)	($P = 1.0$)
Exisulind	25 mg/kg daily	49.33 days	8/12
Docetaxel	5 mg/kg/wk (6×)	($P = 0.04$)	($P = 0.02$)
Exisulind	50 mg/kg daily	36 days	11/12
Docetaxel	2.5 mg/kg/wk (6×)	($P = 0.31$)	($P = 0.41$)
Exisulind	50 mg/kg daily	63.17 days	2/12
Docetaxel	5 mg/kg/wk (6×)	($P < 0.0004$)	($P < 0.0001$)
Exisulind	50 mg/kg daily	65.17 days	1/12
Docetaxel[a]	5 mg/kg/wk (3×)	($P < 0.0001$)	($P < 0.0001$)
	2.5 mg/kg/wk (3×)		

[a] Docetaxel was administered at 5 mg/kg/wk for the weeks 1–3 and at the lower dose of 2.5 mg/kg/wk for weeks 4–6.

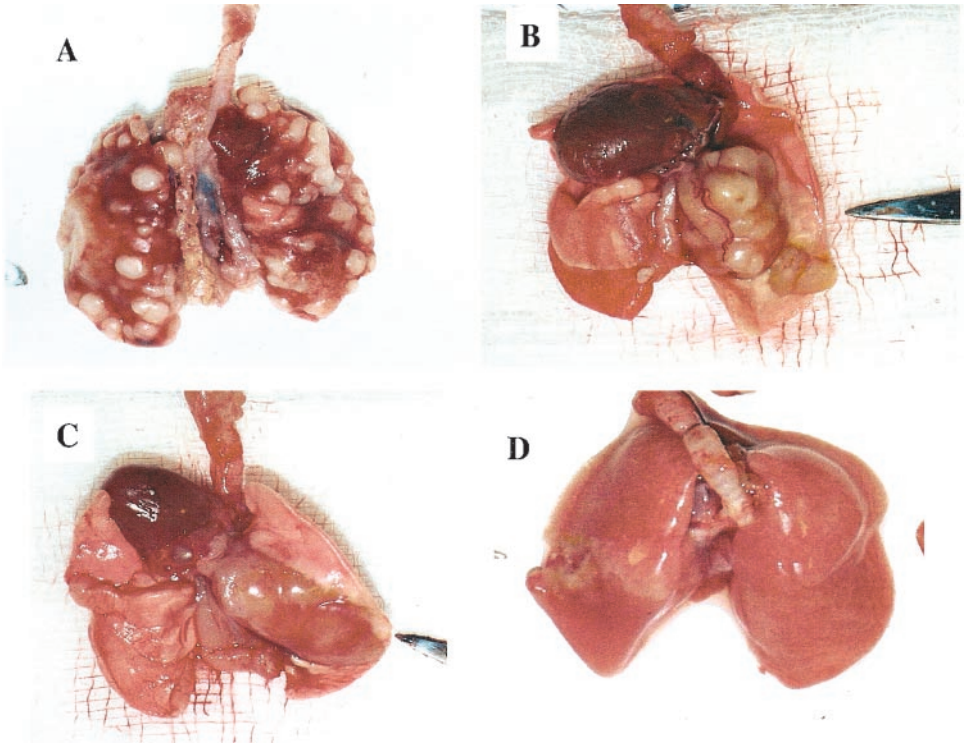

Fig. 4 Dissected lungs and mediastinum from athymic nude rats with A549 tumors treated with (*A*) saline control (day 34), (*B*) exisulind 50 mg/kg (day 55), (*C*) docetaxel 5 mg/kg (day 50), and (*D*) exisulind 50 mg/kg plus docetaxel 5 mg/kg (day 80).

ically (TUNEL). The number of apoptotic cells in the tumors from rats treated with exisulind, docetaxel, or the combination are shown in Fig. 5. Exisulind 25 mg/kg and 50 mg/kg alone increased the apoptotic index 3.8-fold and 4.4-fold, respectively, ($P < 0.01$) compared with control tumors. A 4.2-fold increase in the apoptotic index was seen in tumors from animals treated with 5 mg/kg docetaxel alone ($P = < 0.01$). The combination of exisulind 50 mg/kg + docetaxel 5 mg/kg dramatically augmented the apoptotic index level (7.2-fold) compared with control and to either agent alone ($P = < 0.001$). The increase in the rate of apoptosis seen in the tumors from animals treated with exisulind (50 mg/kg) +

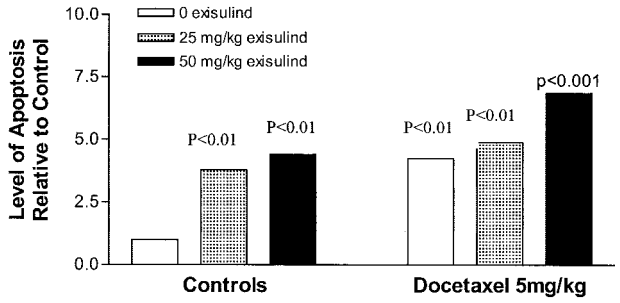

Fig. 5 Apoptotic rates determined by TUNEL analysis. The levels of apoptosis in the treatment groups were compared with the background apoptosis levels in the control group that received no treatment. Levels of apoptosis relative to controls are shown. The treatment group receiving the combination of 50 mg/kg/day of exisulind and 5 mg/kg/day docetaxel exhibited a significant increase in apoptosis.

docetaxel (5 mg/kg) correlates with their observed increase in survival.

DISCUSSION

The studies reported in this article show that exisulind inhibits the growth of human lung cancers of NSCLC histology *in vitro* and *in vivo* in the orthotopic nude rat model of lung cancer. The combination of exisulind with docetaxel produced additive or synergistic growth inhibition in the NSCLC cell line A549, which contain elevated levels of PDE5, a target of exisulind (23). Previous studies have shown that PDE5 is a primary target of exisulind and that sensitive tumor cell lines have a high expression of PDE5 (9). The combined antitumor effects of exisulind and docetaxel can be attributed primarily to increased apoptosis produced by exisulind alone or in combination with docetaxel. These study results have provided the basis for conducting clinical trials with a combination of exisulind and docetaxel in patients with advanced NSCLC.

We showed previously that exisulind increased the apoptotic rate in human lung SHP-77 SCLC cancer cells and created a G_1 arrest *in vitro* (17). The combination of this G_1 arrest and apoptosis was associated with marked growth inhibition in these *in vitro* studies (17). We additionally showed that combinations of exisulind with other chemoprevention agents (COX and lipoxygenase inhibitors, and retinoids) and standard cytotoxic chemotherapy agents (cisplatin and paclitaxel) increased the growth inhibition in an additive or synergistic manner (17). These data were consistent with data of other investigators that showed exisulind produced apoptosis, and growth inhibition in human breast and prostate cancer cell lines (15, 16). In this report we extend these observations to the combination of exisulind and docetaxel *in vitro* and *in vivo*. Docetaxel alone produced a dose-dependent G_2-M arrest, increased the apoptotic rate and growth arrest *in vitro* as predicted from other studies (7). A G_2-M block and apoptosis was not detected when docetaxel was given alone at concentrations below 3 nM. However, when docetaxel was combined with exisulind, increased apoptosis was noted even with docetaxel doses as low as 0.5 nM. In the current experiments exisulind alone produced a marked increase in apoptosis at concentrations of >200 μM and at 48 h or later (data not shown). This concentration is higher than the maximum steady state concentration C_{max} reported in phase I trials of exisulind in humans (24). We did not measure the serum exisulind concentrations in these rat experiments, but exisulind and the combination of exisulind with docetaxel clearly produced large increases in the apoptotic rates in the A549 cells *in vivo*. This could have been because of high concentrations (>200 μM) in the rats or because of other *in vivo* effects of exisulind including its reported antiangiogenic effects (13).

We observed previously that exisulind alone and in combination with cisplatin inhibited the growth of human SHP-77 lung cancers *in vivo* in athymic nude mice (22). Additive growth inhibition was produced by the combination of exisulind and cisplatin, but no additive growth inhibition was noted with the combination of exisulind and paclitaxel against SHP77, a SCLC cell line known to express high levels of multidrug-resistance phenotypes (17). However, the true impact of exisulind on tumor growth in this SCLC xenograft model was difficult to interpret, because higher doses of exisulind alone in the feed, especially in combination with cisplatin, increased weight loss (22). Because of toxicity the mice consumed less-formulated chow and, therefore, exisulind intake was less. To eliminate these problems and to study a more relevant human model, we conducted a series of studies using an orthotopic model in athymic nude rats. This allowed oral exisulind administration by gavage and allowed the assessment of survival, metastases, and tumor apoptosis analysis. In this model both exisulind and docetaxel alone prolonged survival in treated rats, but this was not statistically significant. Although exisulind and docetaxel alone prolonged survival, only docetaxel (≥5 mg/kg) reduced the number of rats with gross metastases.

The combination of exisulind and docetaxel produced a statistically significant increase in survival compared with either agent alone. Animals treated with optimal doses of exisulind (50 mg/kg) and docetaxel (5 mg/kg or 5 mg/kg followed by 2.5 mg/kg) had the best survival rates and significantly lower metastases. The *in vivo* effects of exisulind, docetaxel, and the combination on apoptosis were similar to the effects noted *in vitro*. There was a dose-dependent increase in the apoptotic rate observed with exisulind alone and docetaxel alone. However, the combination of high-dose exisulind and docetaxel produced a significant increase in the apoptotic rate compared with either drug alone. In companion experiments presented elsewhere, we showed that exisulind alone increased apoptosis and inhibited angiogenesis (23). Other groups have also shown that exisulind inhibits angiogenesis *in vivo* (13). Docetaxel alone is known to induce apoptosis, inhibit angiogenesis, and produce antiproliferative effects in tumor cells. Therefore, it can be inferred that the beneficial effects observed when exisulind and docetaxel are used in combination may be attributed to the increases in all biomarkers of drug activity.

In summary, our results show that the combination of exisulind with docetaxel prolongs survival in the orthotopic rat model of human NSCLC. This observation was corroborated with *in vitro* results, which demonstrated antitumor effects via induction of apoptosis and synergistic inhibition of cell growth when both drugs were combined. Apoptosis was also demonstrated in the tumors obtained from the treated animals. Significant reductions in tumor burden and metastases were observed

in the animals treated with both drugs. These findings provide a rationale for conducting clinical trials in patients with NSCLC.

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Exisulind in Combination with Docetaxel Inhibits Growth and Metastasis of Human Lung Cancer and Prolongs Survival in Athymic Nude Rats with Orthotopic Lung Tumors

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