Synergistic Antitumor Activity of Taxol and Immunotoxin SS1P in Tumor-Bearing Mice

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Abstract

Purpose: To investigate the combined antitumor activity in mice of immunotoxin SS1P and Taxol.

Methods: Immunodeficient mice were implanted with A431/K5 tumors expressing mesothelin. Established tumors were treated i.v. with immunotoxin SS1P alone, i.p. with Taxol alone, or with the two agents together. SS1P was radiolabeled with ^{111}In and used to study the effect of Taxol on its uptake by A431/K5 tumors.

Results: Using doses at which either agent alone caused stabilization of tumor growth, the combination was synergistic causing long-lasting complete remissions in many animals. In contrast, synergy was not observed when the same cells were treated with these agents *in vitro*. Tumor uptake of ^{111}In-SS1P was not affected by treatment with Taxol.

Conclusion: The combination of Taxol and SS1P exerts a synergistic antitumor effect in animals but not in cell culture. This effect is not secondary to increased tumor uptake of the immunotoxin. Synergy could be due to improved immunotoxin distribution within the tumor or could involve factors released by other cell types in the tumors.

Recombinant immunotoxins are chimeric proteins in which the Fv portion of a tumor-specific monoclonal antibody is genetically fused to a bacterial toxin such as the 38 kDa portion of *Pseudomonas* exotoxin A (1). One of these immunotoxins, BL22 or anti-CD22(Fv)-PE38, which targets CD22 on the surface of certain leukemia cells, has produced many complete remissions in patients with drug-resistant Hairy Cell Leukemia (2). BL22 is a 63-kDa protein. After binding to CD22 on the cell surface, it is internalized by endocytosis, and after proteolytic processing, a 37-kDa fragment reaches the cytosol, inactivates elongation factor 2, and induces programmed cell death (3).

Immunotoxins like BL22 and LMB2, an immunotoxin that targets CD25, are very efficient in killing leukemia cells that are readily accessible in the circulation (4), but immunotoxins that target solid tumors are less active in part because the immunotoxin enters the tumor very slowly. One of these, SS1P or anti-mesothelin (Fv)-PE38, targets the mesothelin antigen that is expressed on mesothelioma, ovarian cancer, and pancreatic cancer. In cell culture, SS1P has the same cytotoxic activity against mesothelin-positive cells as BL22 does on CD22-positive leukemia cells, but its antitumor activity in patients is much less than that of BL22 (5, 6). This low activity is believed to be due, in large part, to the low entry of the immunotoxin into solid tumors.

It is well established that antibodies enter solid tumors slowly (7, 8). This has been attributed to several factors. One factor is high interstitial pressure within tumors, which slows the movement of proteins within the tumor. Second is the lack of functional lymphatics in the tumor. Lymphatics have an important role in the convective process by which proteins enter normal tissues. Antibodies often remain within the circulation for many days and, when given at high concentrations, eventually accumulate at effective levels within tumors (9). However, other types of antibody-based therapeutics such as immunotoxins, immunocytokines, and other immunoconjugates have much shorter lifetimes in the circulation and, consequently, their accumulation in tumors is impaired. Furthermore, these agents cannot be given in as high amounts as naked antibodies because they can be cytotoxic to normal cells. Therefore, it is necessary to devise new strategies to improve their activities.

One approach that has been used to enhance the uptake of protein-based therapeutics into tumors is to combine antibody-based therapy with cytotoxic agents that damage endothelial cells. This damage apparently allows the increased entry of the protein therapeutic into the tumor (10). Several different cytotoxic agents have been shown to damage endothelial cells. These include paclitaxel (Taxol), cyclophosphamide (Cytoxan), cisplatin [*cis*-diammine-dichloroplatinum (CDDP)], and Adriamycin (11–15).

The report that Taxol and Cytoxan increased the tumor uptake of an antibody-interleukin-2 fusion protein (10) suggested that these agents might also increase immunotoxin

uptake by tumors and, consequently, their antitumor activity. In this study, we employed a nude mouse model in which a human cancer cell line (A431/K5) expressing the mesothelin antigen forms solid tumors. The tumor-bearing mice were treated with SS1P alone, Taxol alone, or the two together. The results show that Taxol at a dose that has minimal antitumor effect by itself greatly increases the antitumor activity of SS1P. Unexpectedly, this effect is not observed in cell culture, and in mice, it is not associated with an increase in SS1P uptake, suggesting the synergy is due to a novel mechanism.

Materials and Methods

Chemicals. Taxol, CDDP, and Cytoxan were provided by the Division of Veterinary Resources (NIH). Immunotoxins SS1P and HA22 were prepared in Laboratory of Molecular Biology as previously described (16). Cremophor was from Sigma (St. Louis, MO). Taxol (6 mg/mL) dissolved in Cremophor (1.05 g/mL) was diluted 1:5 with 0.9% NaCl. Cremophor was diluted 1:1 with ethanol and further diluted 1:5 with 0.9% NaCl. The Cell Counting Kit-8 used for the cytotoxicity assay is from Dojindo (Kumamoto, Japan).

Cell culture. A431/K5 cells expressing the mesothelin protein were maintained as previously described in DMEM containing 10% fetal bovine serum and 750 μg/mL G418 (17).

In vitro *assays of SS1P and Taxol on A431/K5.* A431/K5 cells were seeded in 96-well plates at 5,000 per well and incubated at 37°C overnight. Taxol (vehicle described above) was added to a final concentration of 0, 0.8, 2.5, or 7.5 ng/mL. Serial dilutions of SS1P in 0.2% human serum albumin were added 24 hours after Taxol and cells were incubated at 37°C for another 48 hours. Inhibition of cell growth was determined using WST assays based on the reduction of tetrazolium salt to formazan by the mitochondrial dehydrogenases from viable cells. Ten microliters of WST-8 solution from Cell Counting Kit-8 were added to each well and incubated for 4 hours at 37°C. Absorbance at 450 nm was measured with a reference wavelength of 650 nm using a plate reader (SPECTRAmax, Molecular Devices, Sunnyvale, CA). Viability was expressed as percentage of the absorbance of untreated controls.

Tumor experiments. A431/K5 cells (1.5×10^6) were added to 30 mL of growth medium in T175 flasks. After 3 days, the cells were ~80% confluent and were trypsinized, harvested by centrifugation, washed thrice with serum-free DMEM, and then resuspended in the same medium with Matrigel (final concentration, 4.0 mg/mL). The cell suspension (2.0×10^6 cells/200 μL) was implanted s.c. into the thigh area of the right rear leg of athymic nude mice (5-6 weeks, 18-20 g). Tumor dimensions were determined every other day using calipers. Tumor size (mm^3) was calculated by the following formula: $(a) \times (b^2) \times 0.4$, where a is tumor length and b is tumor width in centimeters.

Treatment was started when tumors reached ~120 mm^3 in size. Taxol dissolved in Cremophor or Cremophor alone (diluted in 0.9% NaCl) was given i.p. CDDP or Cytoxan diluted with 0.9% NaCl was given i.p. as a single dose in 0.5 mL. SS1P or HA22 was diluted with PBS and 0.2 mL was given i.v. Due to institutional regulations, all animal experiments were stopped when the tumors reached 1,000 mm^3.

Labeling and the determination of immunoreactive fraction. SS1P was conjugated with the A stereoisomer of 2-(p-isothiocyanatobenzyl)-cyclohexyl-diethylenetriaminepentaacetic acid (CHX-A; Macrocyclics, Inc., Dallas, TX) and the conjugate was labeled with $^{111}InCl_3$ (Perkin-Elmer, Boston, MA) using a method similar to the method of Kobayashi et al. (18). In brief, SS1P (3 mg/mL) was reacted with a 100 times molar excess of CHX-A in 0.1 mol/L sodium bicarbonate (pH 8.4) for 3 hours at room temperature. The excess nonincorporated CHX-A was removed from the product by Microcon filtration using a 30K filter (Microcon YM-30, Millipore Corp., Bedford, MA). The conjugate SS1P-CHX-A (10 μg/13 μL) was then reacted with 18.5 MBq ^{111}In in 0.5 mol/L

sodium ascorbate (pH 6) at room temperature for 1 hour. To remove any nonincorporated ^{111}In, diethylenetriaminepetaacetic acid (Sigma-Aldrich, St. Louis, MO) was added to the labeling solution at a final concentration of 0.2 mol/L and the mixture was incubated for 15 minutes at room temperature. The product SS1P-CHX-A-^{111}In was purified by Microcon filtration as described above. The radiochemical purity of SS1P-CHX-A-^{111}In was confirmed by size-exclusion high-performance liquid chromatography equipped with a TSK 2000SWxl [0.067 mol/L sodium phosphate (pH 6.8)/0.10 mol/L KCl; 1 mL/min; Toso-Haas, Philadelphia, PA], a UV monitor (Gilson, Inc., Middleton, WI), and an online radioactivity detector (Bioscan, Inc., Washington, DC). The immunoreactivity of SS1P-CHX-A-^{111}In was estimated by the size-exclusion high-performance liquid chromatography after SS1P-CHX-A-^{111}In was incubated with mesothelin-Fc (2 μmol/L) at a 10 times molar excess to SS1P-CHX at room temperature for 30 minutes. The binding of SS1P-CHX-A-^{111}In to mesothelin-Fc shifted 73% of the initial SS1P-CHX-A-^{111}In to a higher molecular weight peak at a retention time of 6.9 minutes in addition to a peak (27%) at 8.1 minutes for SS1P-CHX-A-^{111}In.

Biodistribution of ^{111}In-labeled SS1P. In the initial experiment, 18 mice with tumors of ~120 mm^3 were divided into six groups. Three groups received 20 mg/kg Taxol i.p. and the other three groups received saline and, 24 hours later, 0.5 μg/2 μCi ^{111}In-SS1P in 0.2 mL PBS (pH 7.2) containing 1% bovine serum albumin was injected i.v. to all six groups. Groups of mice were sacrificed at 30 minutes, 2 hours, and 6 hours after injection. Tissues and organs were then harvested, weighed, and counted in a gamma counter.
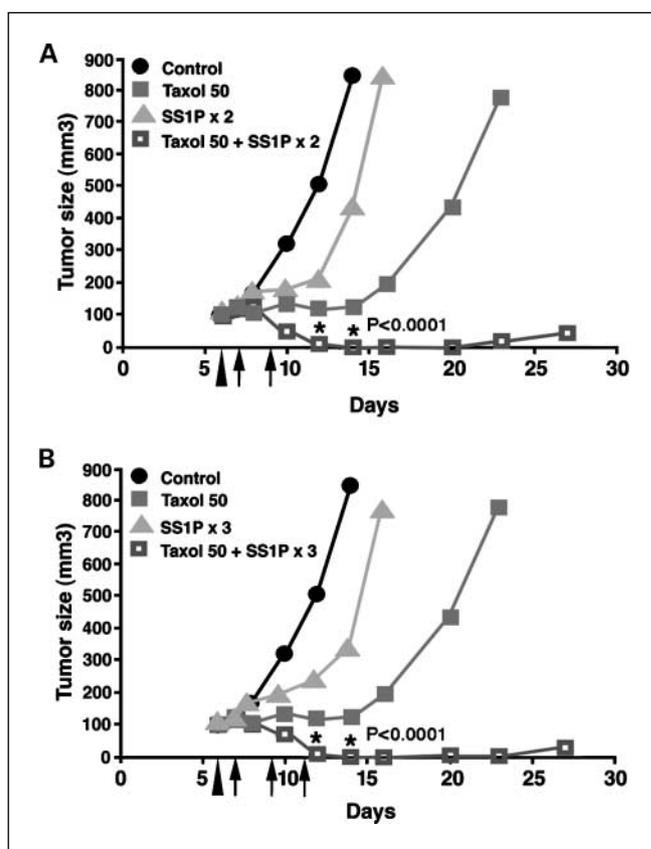

Fig. 1. Effect of Taxol and SS1P on A431/K5 tumors. Mice ($n = 5$) were implanted with 2.0×10^6 A431/K5 cells on day 0 in each treatment group. Taxol at 50 mg/kg was given i.p. on day 6 and SS1P was given i.v. at 0.2 mg/kg on days 7 and 9 (*A*) or on days 7, 9, and 11 (*B*). Synergy was analyzed by repeated measures analysis as described in Materials and Methods. Days on which synergy was statistically significant are marked with a star and *P* values are given.
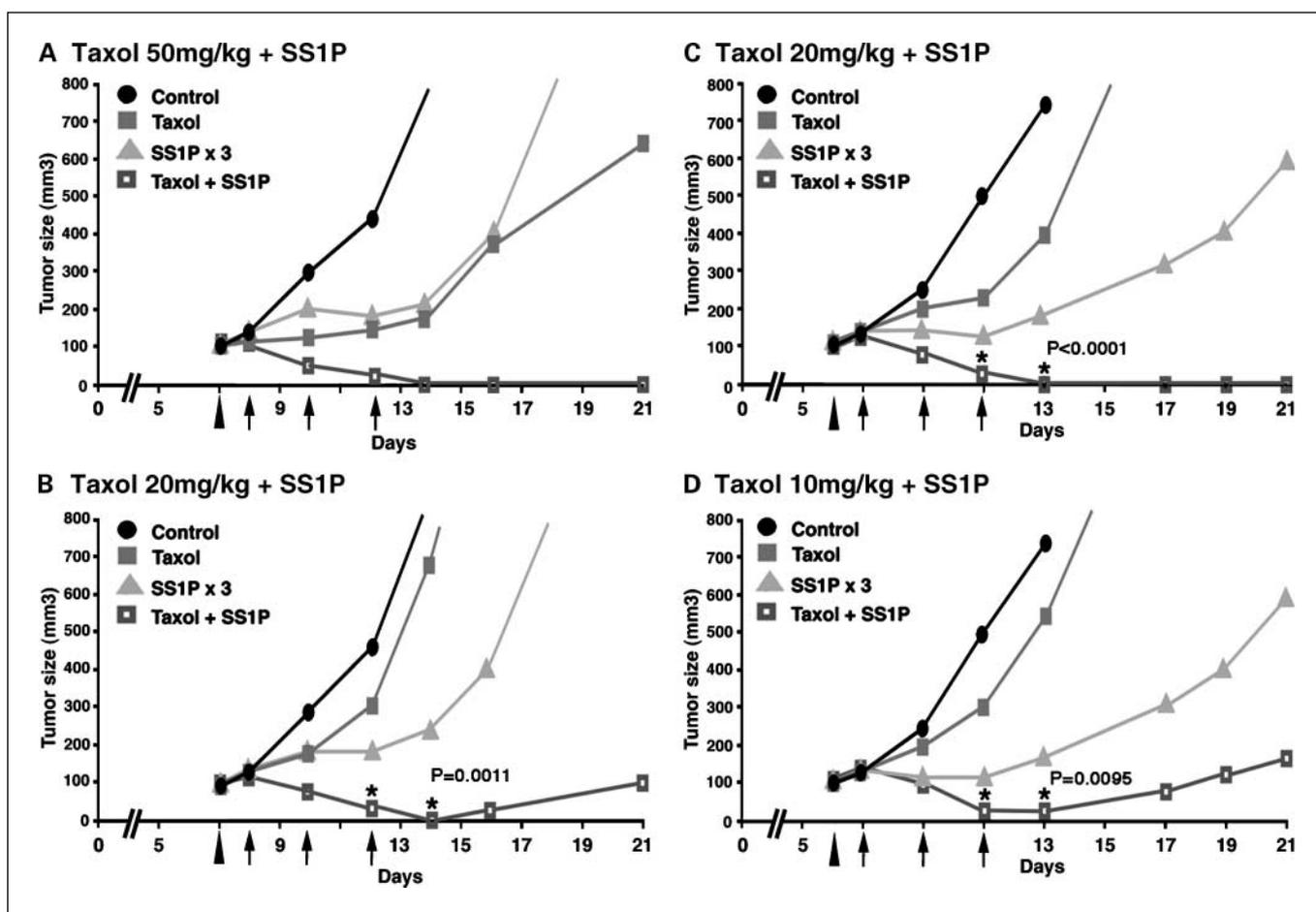
Fig. 2. Effect of different doses of Taxol and SS1P on A431/K5 tumors. The dose of SS1P was fixed at 0.2 mg/kg at each i.v. injection. *A,* tumor-bearing mice (*n* = 5) received Taxol at 50 mg/kg on day 7 and three doses of SS1P on days 8, 10, and 12. *B,* mice received 20 mg/kg Taxol and SS1P on days 8, 10, and 12. *C,* mice received 20 mg/kg Taxol on day 6 and SS1P on days 7, 9, and 11. *D,* mice received 10 mg/kg Taxol on day 6 and SS1P on days 7, 9, and 11. Synergy was analyzed by repeated measures analysis as mentioned in Materials and Methods. Days on which there was significant synergy are marked with a star and *P* values are shown.

In the next experiment, four groups, each containing six mice, were used for each experimental point. Two groups received 20 mg/kg Taxol i.p. when the tumors reached 120 mm^3. The other two groups received saline. Twenty-four hours later, 0.5 μg/2 μCi ^{111}In-SS1P or 4 μg/2 μCi obtained by the addition of unlabeled SS1P was injected i.v. The mice were sacrificed 6 hours after injection. Tissues and organs were harvested and weighed, and radioactive content was measured in a gamma counter. To determine whole-body retention, the remaining carcass was divided into three portions and also weighed and counted, and the sum of the activity in the carcasses, blood, and individual organs was determined. The data were expressed as percentage injected dose per gram of tissue and normalized to a 20-g mouse.

Statistics. Statistical analysis on synergy was done by David Venzon (Biostatistics and Data Management Section/Center for Cancer Research/National Cancer Institute, Bethesda, MD). Repeated measures ANOVA was applied to the changes in successive tumor spherical diameters. Synergy was defined as an interaction effect significantly greater than the sum of the Taxol and SS1P effect (19).

Results

Mice bearing mesothelin-expressing A431/K5 tumors were treated with Taxol alone, with SS1P alone, or with both agents, and the growth of the tumors was measured over several weeks.

SS1P was given at a dose of 0.2 mg/kg every other day ×2 or ×3; this dose was chosen because it prevents tumor growth during its period of administration but does not cause substantial tumor regressions that are seen with higher doses (Fig. 1). Therapy began 5 to 7 days after implantation of the cells when the tumors reached a size of ∼120 mm^3. Mice received a single dose of Taxol, followed 24 hours later by the immunotoxin SS1P. The 24-hour delay was chosen because there is evidence that there is a decrease in interstitial fluid pressure 24 hours after Taxol administration (20) and an increase in immunocytokine uptake 24 hours after Taxol (10), and because it seemed reasonable that it would take many hours for Taxol to damage endothelial cells and allow increased immunotoxin entry.

Figure 1A and B shows experiments in which mice received Taxol at 50 mg/kg on day 7 followed by two or three doses of SS1P at 0.2 mg/kg on days 8 and 10 or days 8, 10, and 12. Neither of the individual treatments caused any tumor shrinkage. A single dose of Taxol delayed tumor growth by 7 days. Two or three doses of SS1P delayed tumor growth until day 12. However, when Taxol and SS1P were combined, complete tumor regressions were observed in all the mice on day 10. Furthermore, the complete remissions were durable with two of five mice receiving two doses of SS1P still in

complete remission on day 109 and four of five mice receiving three doses still in complete remission on day 109.

Because Taxol at 50 mg/kg produced a potent antitumor effect by itself, we chose to test lower amounts that might not affect the tumor cells so dramatically. In these and all other experiments, we gave three doses of SS1P on a schedule of every other day × 3 because that is the schedule used to treat patients with immunotoxins (5, 6). Figure 2A to D shows a comparison of the effects of 50, 20, and 10 mg/kg of Taxol with and without SS1P. The antitumor effect of 20 mg/kg of Taxol alone was much less than that of 50 mg/kg but the combination of the two agents produced complete remissions in 6 of 10 mice and all six were durable and lasted for more than 40 days. The combination of 10 mg/kg Taxol and SS1P was also very effective although no complete regressions were observed.

It was clearly evident that the combination of Taxol and SS1P produced many durable complete remissions whereas neither SS1P alone nor Taxol alone produced tumor regressions but the two together were extremely active. To determine if the combination of SS1P and Taxol was synergistic or just additive, we analyzed the data from several different experiments with ANOVA as described in Materials and Methods. Statistical analysis shows that the combination of Taxol and SS1P was synergistic at 50 mg/kg Taxol (Fig. 1) and at 10 and 20 mg/kg Taxol tested (Fig. 2).

Several control experiments were carried out to establish the specificity of the antitumor responses. Because Taxol is poorly soluble in aqueous solutions, it is dissolved in Cremophor to
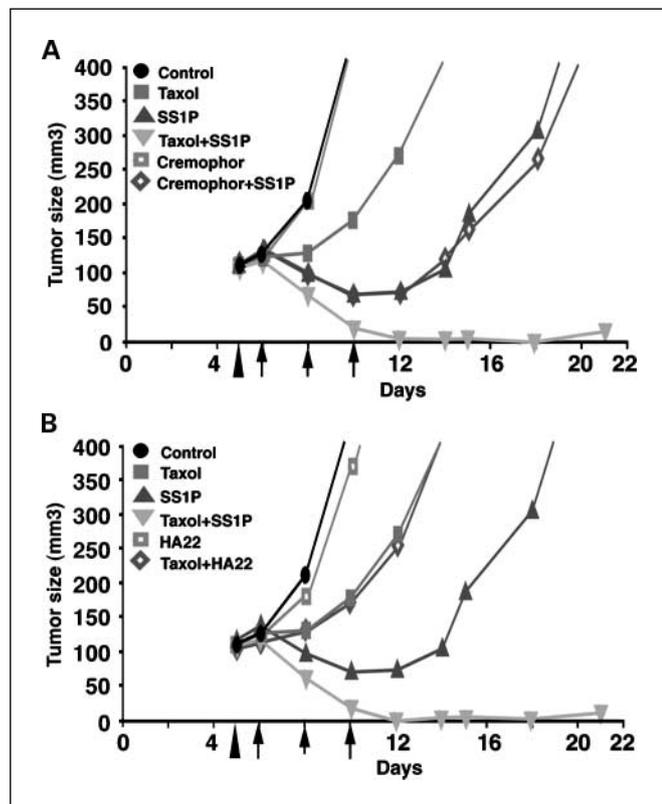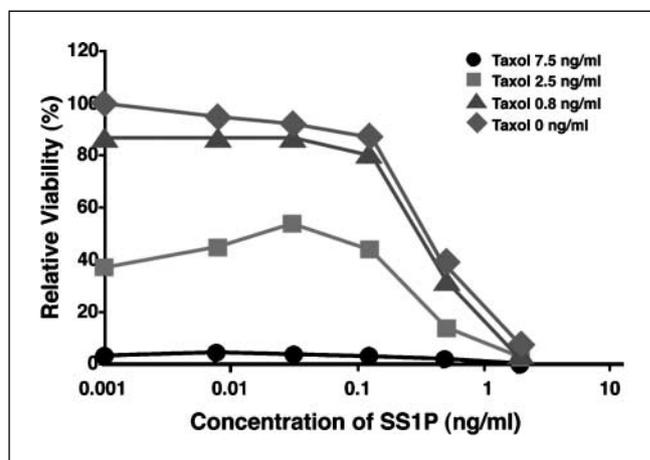

Fig. 4. Cultured A431/K5 cells were treated with various concentrations of SS1P and Taxol for 3 days and viability was measured with WST-8.

make it miscible with water. Cremophor has been shown to have a large effect on the pharmacokinetics of Taxol (21). It seemed possible that Cremophor might alter the permeability of capillaries and also affect immunotoxin pharmacokinetics or their entry into tumors. In the experiment shown in Fig. 3A, mice were treated with SS1P and the amount of Cremophor used to dissolve Taxol. No increase in the antitumor activity of SS1P was observed. In the experiment shown in Fig. 3B, mice were treated with an immunotoxin (HA22) that targets CD22, an antigen not expressed on the A431/K5 cells. As expected, this immunotoxin had no activity against A431/K5 tumors alone and the combination of 20 mg/kg Taxol and HA22 was no more effective than Taxol alone. These experiments show that the combination of Taxol and SS1P is specific.

To investigate if the synergistic effect of Taxol and SS1P could be due to a direct action on the target cells and not a response only observed in mice, we carried out experiments with cultured A431/K5 cells. To do this, we first determined the IC_{50}s for Taxol and SS1P on A431/K5 cells. The IC_{50} is 7 ng/mL for Taxol and 0.4 ng/mL for SS1P. We then treated cells with various concentrations of SS1P and Taxol using concentrations

Fig. 3. Lack of effect of HA22 and Cremophor on A431/K5 tumors. *A,* mice with tumors (*n* = 10) received 20 mg/kg of Taxol or Cremophor with equal concentration in Taxol formulation on day 5 either alone or in combination with SS1P at 0.2 mg/kg on days 6, 8, and 10. *B,* mice received 20 mg/kg of Taxol on day 5 and either SS1P or HA22 at 0.2 mg/kg on days 6, 8, and 10.
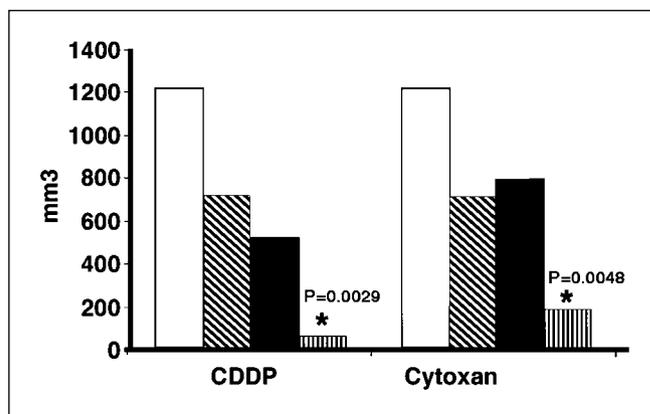
Fig. 5. Mice (*n* = 3) with A431/K5 tumors were treated on day 7 with CDDP at 5 mg/kg i.p. or Cytoxan at 15 mg/kg. SS1P at 0.2 mg/kg was given on days 9, 11, and 13. Tumor measures 8 days after treatment are shown. □, control; ◩, SS1P alone; ■, CDDP or Cytoxan alone; ▥, combination. Synergy was analyzed by repeated measures analysis as mentioned in Materials and Methods. Star with *P* value indicates statistical significance in synergy.

near the IC_{50} of each (Fig. 4) and calculated whether or not there was a synergistic effect. In contrast to the results *in vivo* where synergism was very evident, no synergism was observed *in vitro* (data not shown).

Other cytotoxic agents. Taxol is not the only cytotoxic agent shown to damage growing endothelial cells (13, 22). To determine if the enhanced tumor regressions observed with SS1P and Taxol could be shown with other commonly used cytotoxic drugs, we investigated both CDDP and Cytoxan. As shown in Fig. 5, both CDDP at 5 mg/kg and Cytoxan at 15 mg/kg greatly enhanced tumor regressions caused by SS1P. Statistical analysis showed that synergy was present with *P* values of 0.0029 and 0.0048, respectively. Thus, the action of Taxol on enhancing immunotoxin-induced tumor regressions is shared by other cytotoxic agents.

Radiolabeled SS1P uptake studies. To investigate if Taxol was increasing SS1P uptake, we prepared ^{111}In-SS1P as previously described for other recombinant immunotoxins and used it to measure tumor uptake (18). It has been previously shown that A431/K5 tumors specifically take up recombinant proteins targeted to the mesothelin present on the surface of these tumors by the Fv present in SS1P (23). When the A431/K5 tumors reached a size of ~120 mm³, Taxol was administered

and, 24 hours later, ^{111}In-SS1P was given i.v. In an initial experiment, we carried out a time-course experiment and found that by 6 hours there was a large difference in the tumor-to-blood ratio (Fig. 6A and B). In the next experiment, we examined SS1P uptake at the 6-hour time point with six animals in each group. The data in Fig. 6C were obtained with animals receiving a single injection of 4 μg of SS1P, a dose that is the same as the first dose used in the antitumor experiments. The data show that there is no difference in tumor uptake between the Taxol-treated and the untreated animals. As previously observed, significant amounts of ^{111}In were found in kidney and liver, organs in which immunotoxins are degraded (18), and in bone where ^{111}In released from the chelate accumulates. In another experiment (data not shown), the mice received a total dose of 0.5 μg SS1P, and again, no difference was observed between the Taxol-treated and untreated groups. We conclude that Taxol has no measurable effect on SS1P uptake by A431/K5 tumors.

Discussion

We have shown that the combination of Taxol and SS1P has a very potent and synergistic antitumor activity in mice, but no
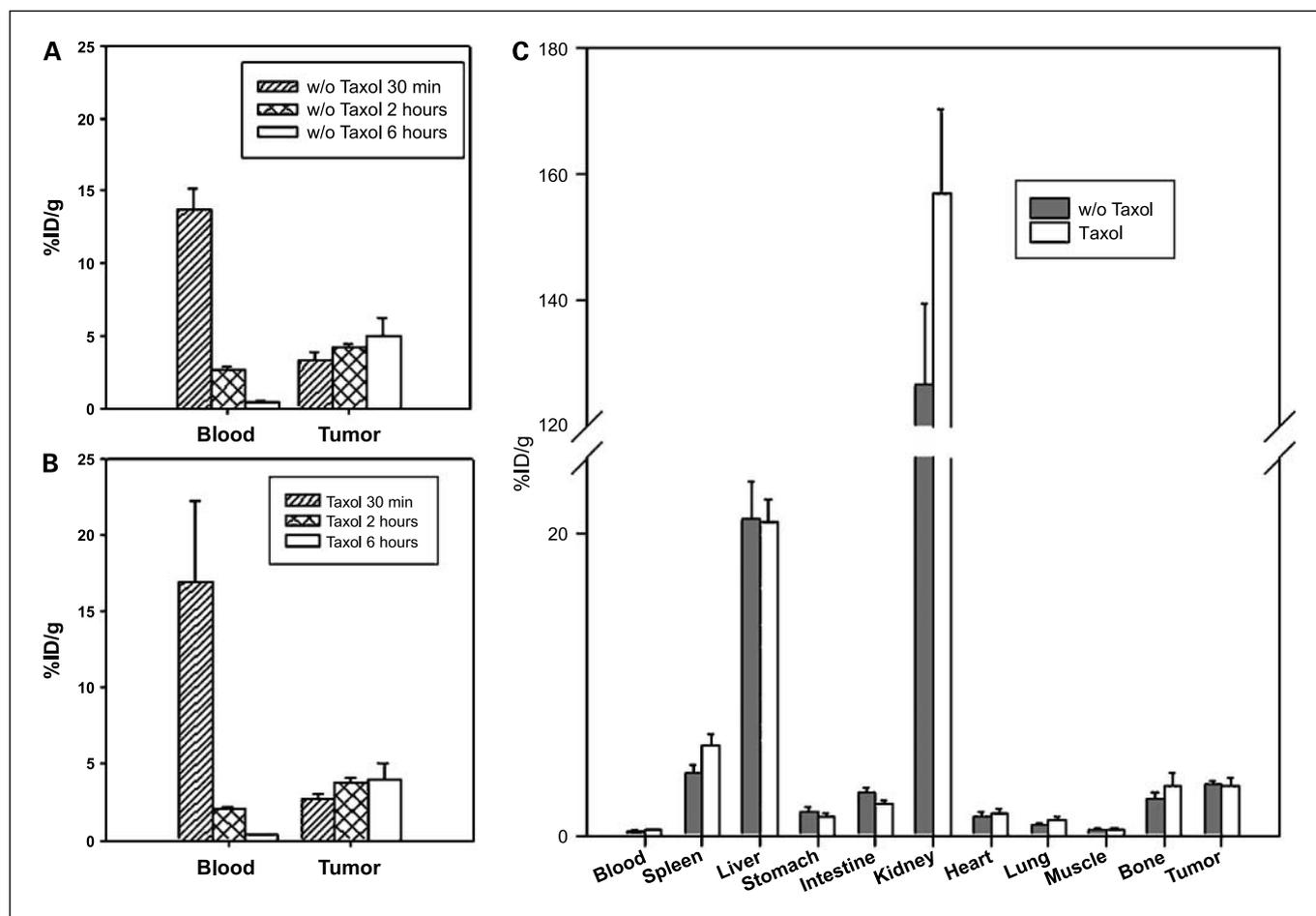

Fig. 6. Biodistribution of ^{111}In-labeled SS1P in nude mice (*n* = 3) with A431/K5 tumors. *A,* without Taxol; *B,* with Taxol biodistribution. Each mouse received a dose of 0.5 μg/2 μCi ^{111}In-labeled SS1P and uptake was measured at 30 minutes (▨), 2 hours (▧), and 6 hours (□). Data in blood and tumor are shown as percentage of injected dose per gram of tissue (ID%/g). At 6 hours after ^{111}In-labeled SSIP, most of immunotoxin had been cleared. *C,* ^{111}In-labeled SS1P with 4 μg/2 μCi/mouse was given 24 hours after Taxol (□) or saline (■). Mice were sacrificed 6 hours after SS1P (*n* = 6). Tissue uptake is expressed as percentage injected dose per gram of tissue (%ID/g). Columns, mean; bars, SD.

synergistic activity was evident when the agents were tested on the same tumor cells growing in tissue culture. Therefore, the synergy must involve an indirect effect on the tumor cells. Because the immunotoxin is specific for tumor cells and does not target and kill mouse cells, whereas Taxol can kill both the tumor cells and other cell types that are present in the tumor and not present in cell culture, we initially ascribed the synergy to an effect on mouse cells in the tumor and most likely to damage to endothelial cells that regulate the entry of macromolecules into tumors. Two studies, one in mice and another in humans, have shown increased uptake of radiolabeled antibody in tumors (24).

To evaluate this hypothesis, we measured the uptake of radiolabeled SS1P by the tumors and failed to observe a change in SS1P uptake. This was a surprising result because Holden et al. (10) have shown that Taxol treatment caused an increased uptake of an immunocytokine by tumors in mice and Miers et al. (24) have shown increased uptake of radiolabeled antibody by tumors in humans. Clearly, our synergistic effect implies a distinct mechanism. However, there are several differences in the two models. The model used in Gillies' study is a syngeneic one and the tumor types are different; we used A431/K5 tumors whereas Holden et al. (10) used murine mammary, colon, and lung tumors. The difference in tumor microenvironment could lead to distinct response to Taxol treatment. A second is the pharmacokinetics of the immuno-therapeutic: the blood clearance $t_{1/2}$ of 20 minutes for the immunotoxin SS1P and 4 to 6 hours for the immunocytokine (25). Therefore, if Taxol enhances the permeability of tumor capillaries, immunocytokine huKS-interleukin-2 will have a longer period to accumulate in the tumor. The third is that the interleukin-2 portion in huKS-interleukin-2 is a functional molecule with activity on capillaries, in addition to other possible activities. A fragment with permeability-enhancing effect has been derived from interleukin-2 and is called permeability-enhancing peptide (PEP; ref. 26). Its fusion protein with antibody NHS76 (NHS76/PEP) showed an increased uptake compared with NHS76 alone. Pretreatment of animals with NHS/PEP also augmented the therapeutic effect of several drugs (doxorubicin, Taxol, vinblastine, etoposide, and 5-fluorouracil) within a 2-hour interval (27).

Many publications have shown that cytotoxic drugs that are used to kill cancer cells are also very toxic to endothelial cells. Furthermore, some of these drugs have been shown to lower the interstitial fluid pressure within tumors (20). The tumor microenvironment is complex and consists of various types of stromal components that have an important role in tumor growth (28). Stromal components and tumor cells produce a variety of factors that support the formation of capillaries and the growth of tumor cells. It is possible that damage to tumor cells or to stromal components could decrease the levels of vascular endothelial growth factor or other factors that affect endothelial cell viability. It has been shown that vascular endothelial growth factor levels in the blood decrease following chemotherapy (14). We plan to measure the effect of Taxol on the levels of vascular endothelial growth factor and other cytokines in Taxol-treated tumors.

Immunologic process plays a very important role in the treatment of tumors. Taxol can also act as an immunomodulator (29) to change the behavior of macrophages. Taxol has been shown to activate macrophages in a similar manner to lipopolysaccharide, increasing the expression of interleukin-12, tumor necrosis factor α, and nitric oxide, and some tumor cell–derived cytokines can dysregulate the activation process (30). Thus, there are several mechanisms by which Taxol can affect SS1P action.

We have just completed phase I trials with the immunotoxin SS1P used in the current experiments (6) and are now planning phase II trials. The data shown here suggest that the combination of Taxol and SS1P would be a good choice for a phase II trial in ovarian cancer because Taxol is an effective drug in treating ovarian cancer and because we have already seen several minor responses in ovarian cancer in our phase I trial.[3]

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[3] In preparation.

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