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The Tumoricidal Effect of Sonodynamic Therapy (SDT) on S-180 Sarcoma in Mice

Xiaohuai Wang, MD, Thomas J. Lewis, PhD, and Doug Mitchell, PhD

There are increasing data showing that sonodynamic therapy (SDT), which refers to a synergistic effect of drugs and ultrasound, is a promising new modality for cancer treatment. However, few clinical data on SDT have been published. One reason is the lack of suitable drugs for clinical SDT use. Recently a new sonosensitizing agent has been developed by SonneMed, LLC, referred to as SF1. In this study the effect of SDT with SF1 on S-180 sarcoma in mice was examined. The tumor bearing mice were allocated to the following groups: (1) sham-treatment (control, C); (2) ultrasound treatment (only ultrasound treatment, 1.2 mW/cm², without SF1, U); (3) SF1 treatment (SF1 20 mg/kg intraperitoneal [ip] without ultrasound treatment, S); and (4) SF1 + ultrasound treatment (SU). Following treatment, tumor volume was monitored. Tumor growth inhibition was seen only in

group SU, and with increasing ultrasound intensity, the inhibitory effect was enhanced. Tumor growth inhibition was also visible even when covered by a barrier of bone. Pathological slices showed coagulated necrosis or metamorphic tissue with inflammatory reaction in the tumor taken from 2 to 36 hours after SDT. These data revealed that SDT with SF1 did inhibit growth of mouse S-180 sarcoma and the inhibitory effect was sound intensity dependent. SDT also induced some inflammation while it destroyed the tumor, indicative of a "vaccine" effect. SF1 shows great promise for clinical use in the future.

Keywords: photodynamic therapy; sonodynamic therapy; cancer; cancer therapy; tumor necrosis; tumoricidal; porphyrins; SonneMed; mouse S-180 sarcoma; anticancer

There are increasing data showing that sonodynamic therapy (SDT), which refers to a synergistic effect of drugs and ultrasound, is a promising new modality for cancer treatment.¹ However, few clinical data on SDT have been published. One reason for this is the difficulty of finding a drug suitable for clinical SDT use. Recently SF1, a new sonosensitizing agent, has been developed by SonneMed, LLC (Boston, Massachusetts). This agent has very high sonodynamic activity, but almost no toxicity even for embryonic cells in the embryonic zebra fish assays of Phylonics Pharmaceuticals Inc, (Cambridge, Mass; results pending publication). In this study the effect of SDT with SF1 on S-180 sarcoma in mice was examined.

Materials and Methods

The sonosensitizer used in this study, SF1, was provided by SonneMed, LLC. Its average molecular weight is 942. The sensitizer has photosensitizer capabilities. Its absorption spectrum, which is shown in Figure 1, has been measured. SF1 has two absorption peaks. They occur at wavelengths 402 nm and 636 nm.

The agent was dissolved in 0.1 mol PSA under sterile conditions in a dark room. Its final concentration was adjusted to 2 mg/mL. The container of the suspension was constantly protected from any exposure to room and sunlight, placed into a thermos bottle to shield from light and sound, and stored in a refrigerator at 4°C to 16°C.

The animal tumor model used in this study was the KM mouse S-180 sarcoma, which is one of the tumor models appointed by the China Food and Drug Administration (CFDA) for new anticancer drug testing. The inbred strain of KM mice (female, about 18-20 g body weight) was bred and tested in a laboratory consistent with national standards for new drug tests (the Animal Experimental Centre of Guangzhou Chinese Traditional Medical University). The mouse S-180 sarcoma cell line was injected into and raised in the abdominal cavity of the KM mouse and regenerated

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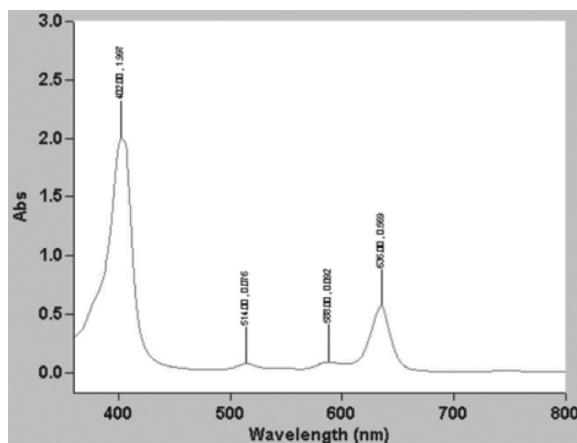


Figure 1. SF1 absorption scans: "Chem Lab" instrument. SF1 in 1:100 dilution.

3 times by passing malignant ascites from one mouse to another. Then the ascites with S-180 cell suspension was drawn out from the abdomen of the third passage mouse, and germ-free physiological saline solution was added. The final concentration of the cell suspension was 1×10^7 cells/mL. Next, 0.1 mL of the cell suspension was injected subcutaneously in the right axilla of the mouse to grow a solid tumor. Four days later, a small mass was seen and palpated on every implanted mouse axilla.

To prepare the mice for the experiment, the hair on the tumor-bearing area of the mice was removed with depilatory cream. Then 20 mg/kg of SF1 was injected into the mouse abdominal cavity in a dark room. Six hours after the injection, some of the tumor-bearing mice were partially submerged in water. A probe manufactured by Angel Ultrasound was then placed into the water to irradiate the tumor area at different intensities for 3 minutes.

Results

The Inhibitory Effect of SDT With SF1 on S-180 Sarcoma in Mice

Comparison of the tumor weight in each group. The tumor-bearing mice were allocated into 4 groups with 5 mice in each group: (1) sham-treatment (control, C); (2) ultrasound treatment (only ultrasound treatment, 1.2 W/cm², 1 MHz, without SF1, U); (3) SF1-treatment (SF1 20 mg/kg ip without ultrasound treatment, S); and (4) SF1 + ultrasound treatment (S 20 mg/kg ip + U 1.2 W/cm² and 1 MHz, SU). Fifteen days later, the mice in the 4 groups were all sacrificed. The tumors of the mice were separated and weighed (Table 1).

Comparing with group C (sham treatment), the tumor weight in group SU was significantly lower ($P < .01$). The

Table 1. Tumor Weight in Each Group 15 Days After Treatment

Group	Mean tumor weight (g)	P (compared with C)
C	0.361 ± 0.094	
U	0.440 ± 0.275	$>.05$
S	0.272 ± 0.328	$>.05$
SU	0.009 ± 0.003	$<.01$

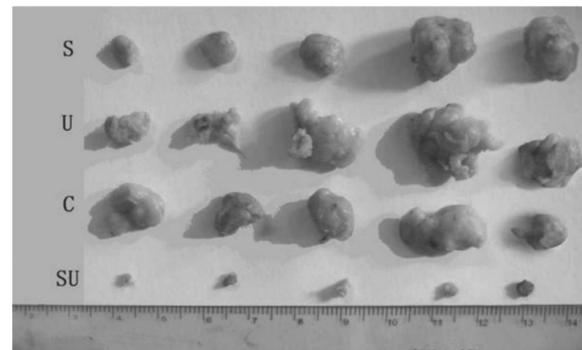


Figure 2. Image of tumors excised 15 days after treatment from each mice group.

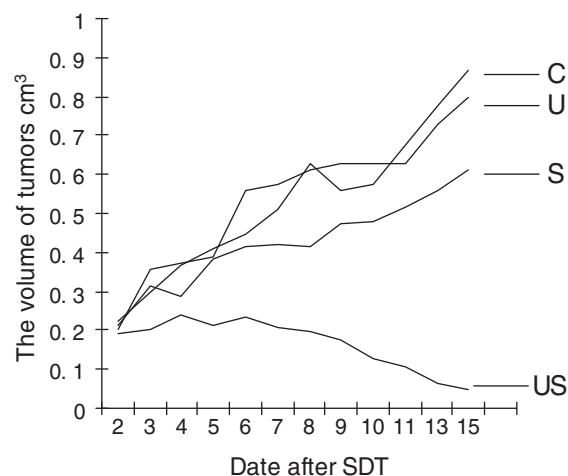


Figure 3. Growth curves of tumors in each group after sonodynamic therapy (SDT).

tumor weight in groups U and S showed no significant differences comparable to that of group C. This demonstrated that the SF1 plus sound treatment inhibited S-180 sarcoma in mice. This conclusion is demonstrated more clearly in Figure 2.

Comparison of the growth curves in each group. After treatment with SDT, primary tumor sizes were estimated by

Table 2. Tumor Size in Each Group 15 Days After Treatment

Group	Mean tumor size (cm^3)	P (compared with C)
C	0.865 \pm 0.124	
U	0.799 \pm 0.315	>.05
S	0.611 \pm 0.190	>.05
SU	0.047 \pm 0.019	<.01

measuring perpendicular minor dimension (W) and major dimension (L) using sliding calipers every 1 or 2 days. Approximate tumor volume was calculated by the formula: $W2L \times 1/2$. The results are shown in Figure 3 and Table 2.

As Figure 3 shows, the tumors in the control group continued to grow. The tumors in groups U and S grew slightly more slowly than those in group C. But as Table 2 shows, the end sizes in groups U and S were not significantly different from the control group. In group SU, some of the tumors enlarged after SDT, but 7 days later, the tumors in all 5 mice gradually shrank. At the end, their sizes were even smaller than before SDT therapy. These results demonstrated again that SDT with SF1 did inhibit the growth of S-180 sarcoma in mice. The data also suggest that SDT not only destroyed the tumors, but also caused an inflammatory reaction in the tumor area during the first 7 days.

The Influence of Sound Intensity on SDT Therapeutic Effect to S-180 Sarcoma in Mice

Comparison of tumor weights in each group. The tumor-bearing mice were allocated into 4 groups with 5 mice in each group: (1) sham-treatment (control, C); (2) S + 0.3 W/cm^2 , 1 MHz ultrasound treatment, SU1; (3) S + 0.6 W/cm^2 , 1 MHz ultrasound treatment, SU2; and (4) S + 1.2 W/cm^2 , 1 MHz ultrasound treatment, SU3. Here S signifies ip injection with SF1 20 mg/kg as described in "Materials and Methods." Fifteen days later, the mice in the 4 groups were all sacrificed and the tumors of the mice were excised and weighed.

As Table 3 and Figure 4 show, the tumor weight in the 3 SDT treated groups was much lower than in control group ($P < .05$). These results conform to the conclusion that SDT with SF1 did inhibit S-180 sarcoma in mice. The tumor weights within the SDT groups also had statistically significant differences between groups SU3 and SU1 ($P < .01$). It is clear that the higher the intensity of ultrasound used, the higher the inhibitory response produced in the range of ultrasound intensity from 0.3 W/cm^2 to 1.2 W/cm^2 .

Comparison of tumor growth curves in each group. After treatment with SDT, the tumor size was measured with sliding calipers every 1 or 2 days. The results are shown in Figure 5 and Table 4.

Table 3. Tumor Weight in Each Group 15 Days After Treatment

Group	Mean tumor weight (g)	P (compared with C)	P (compared with SU1)
Control	0.361 \pm 0.094		<.05
SU1	0.0425 \pm 0.025	<.05	
SU2	0.021 \pm 0.006	<.01	<.05
SU3	0.009 \pm 0.003	<.01	<.01

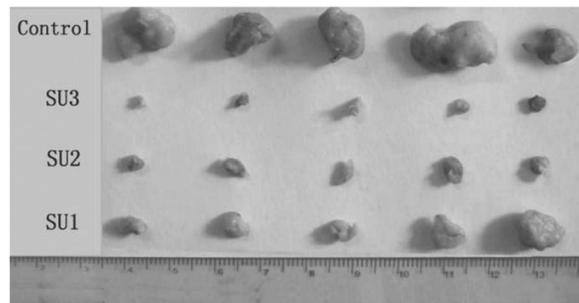


Figure 4. Image of tumors excised from each group of mice 15 days after treatment.

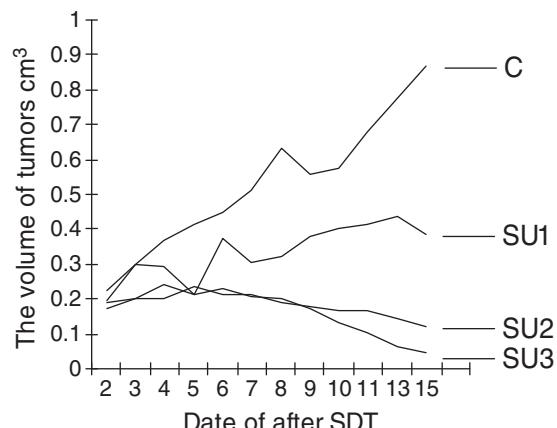


Figure 5. Growth curve of tumors in each group after sonodynamic therapy (SDT).

As Table 4 shows, the tumors in the 3 SDT treatment groups were much smaller than in the control group ($P < .05$). The tumor weights within the SDT groups also had statistically significant differences between group SU3 and SU1 ($P < .01$).

Figure 5 shows that in comparison with the control group, the tumors in group SU1 grew much more slowly, but 7 days later they still continued to enlarge. In groups SU2 and SU3, the tumors had stopped growing about 5

Table 4. Tumor Size in Each Group 15 Days After Treatment

Group	Mean tumor size (cm^3)	P (compared with SU1)
Control	0.865 ± 0.124	<.05
SU1	0.383 ± 0.113	
SU2	0.118 ± 0.020	<.05
SU3	0.047 ± 0.019	<.01

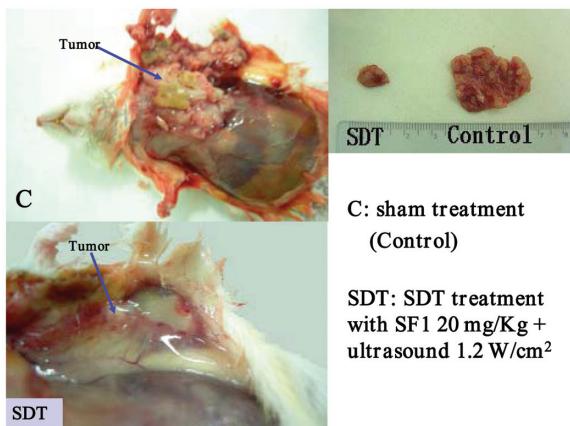


Figure 6. Gross tumor spread.

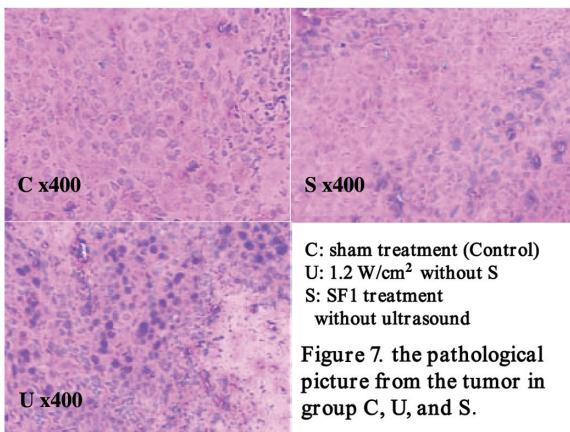


Figure 7. Micrographs of tissue from control (group C), ultrasound alone (group U), and SF1 alone (group S).

to 7 days after SDT treatment and then began to shrink. The tumors in group SU3 shrank faster than those in group SU2. Obviously, the inhibitory effect of SDT with SF1 was sound intensity dependent.

Pathology Study

Visual observations. After the tumor-bearing mice were sacrificed, the skin was cut and opened to observe the tumor. As Figure 6 shows, the tumor in the control group of mice was very large by the end of the study. It invaded into the muscle and sternum, and resulted in malignant ascites. Conversely, the tumors in the SDT-treated mice were very small and were confined to the originally injected site without ascites (see Figure 4 for precise measurements).

Observation with microscopy. Tumor-bearing mice were sacrificed on the 15th day of tumor induction. The tumors of mice were excised and processed for routine pathological study. Some of the tumors in group 1.2 W/cm² + SF1 were excised and examined 2 hours to 36 hours after SDT.

Figure 7C shows a typical pathological micrograph of an S-180 sarcoma mouse, which was taken from the control group. The pathological micrographs of the tumors taken from groups U and S were almost the same as the photos in group C. That means the treatment with ultrasound or SF1 alone had no significant effect on the S-180 sarcoma mouse.

Figure 8 shows the pathological study results in group SU (SF1 20 mg/kg and ultrasound of 1.2 W/cm²). Pathological slices were made from the mice sacrificed at 2 hours, 36 hours, and 15 days after SDT. Figure 8A was a slice taken 2 hours after SDT. There was coagulated necrosis or metamorphic tissue with inflammatory reaction in the tumor. Figure B shows that the processes of necrosis, degeneration, and inflammation further enhanced 36 hours after the SDT treatment. Fifteen days after SDT, as the images show in Figures C and D, only coagulated necrosis and vacuole degeneration were visible in the tumor; no living tumor cells could be identified. There was some inflammation and fibrosis around the necrotic or degenerative tumor. These data revealed that SDT with SF1 destroyed the S-180 sarcoma mouse tumor very rapidly. The degeneration of tumor induced by SDT occurred almost immediately or at least within 2 hours after SDT treatment. These data also revealed that along with the necrosis and degeneration of the tumor, SDT also induced inflammatory reaction in the tumor and that the reaction may last for 7 days.

Observation with confocal laser scanning microscope. The tumor bearing mice were divided into 2 groups: (1) sham-treatment (control, C); and (2) SF1 treatment (ip injection with SF1 40 mg/kg, T). Twelve hours later, the mice were sacrificed. The tumors of the mice were excised and processed for frozen slicing. As shown in Figure 9, the frozen slices were observed with confocal laser scanning microscope (TCS SP2 AOBS, Leica, Germany).

As Figure 9 shows, the SF1 specific fluorescence was seen only in the tumor of the mouse ip injected with SF1.

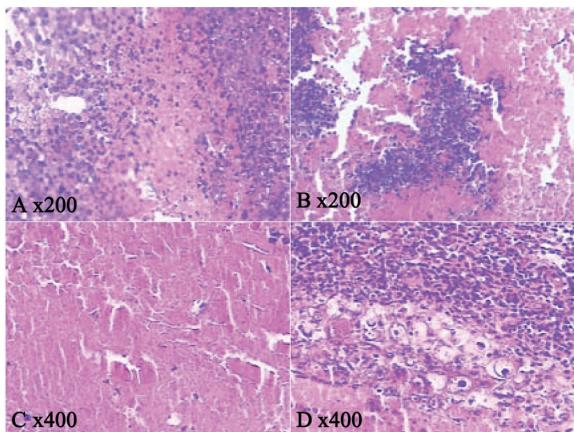


Figure 8. Slices of the tumor in group SU (SF1 + ultrasound treatment). A, Slice taken 2 hours after sonodynamic therapy (SDT). B, Slice taken 36 hours after SDT. C & D, Slices taken 15 days after SDT.

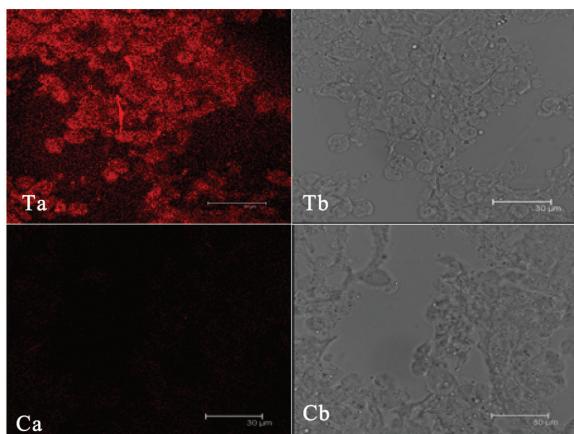


Figure 9. Confocal laser scanning microscopy. Ta and Tb, The same tumor slice in treatment mouse. Ta, Observation with activating laser. Tb, Observation without activating laser. Ca and Cb, The same tumor slice in control mouse. Ca, Observation with activating laser. Cb, Observation without activating laser.

This result suggested that the sensitizer specifically accumulated within tumor cells.

SDT With a Bone Barrier Between Tumor and Ultrasound

The tumor-bearing mice were allocated into 2 groups: (1) sham-treatment (control, C) group; and (2) SF1 and ultrasound treatment (SU). The mice in the SU group were injected intra-peritoneally with 20 mg/kg of SF1. Six hours later, the mice were put into the water, covered with a piece of dog thigh bone with an average thickness

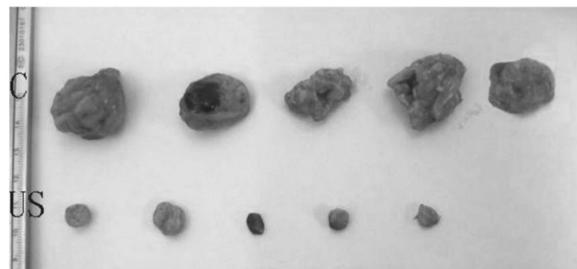


Figure 10. Tumors excised from each group of mice 15 days after treatment.

Table 5. Tumor Weight in Each Group 15 Days After Treatment

Group	Mean tumor weight (g)	P (compared with C)
C	0.73466 ± 0.0781	
SU	0.07416 ± 0.0158	>.01

Table 6. Tumor Weight in Each Group 8 Days After Treatment

Group	Mean tumor weight (g)	P (compared with C)	P (compared with SU2)
C	0.43208 ± 0.128413		
SU1	0.12515 ± 0.019856	<.01	>.05
SU2	0.111967 ± 0.031018	<.01	
SU3	0.121633 ± 0.020449	<.01	>.05

of 3 mm, and then irradiated with an ultrasound of 2 W/cm² and 1 MHz through the piece of bone to the tumor area for 6 minutes. Fifteen days later the tumors were excised from each group of mice and weighed.

The data (seen in Figure 10 and Table 5) showed that SDT with a bone barrier between tumor and ultrasound was still able to inhibit the tumor growth. This revealed that 1 MHz ultrasound can pass through bone, activate the sensitizer in the tumor, and lead to tumor destruction.

The Inhibitory Effect of SDT With Different Ultrasound Frequencies on S-180 Sarcoma in Mice

The tumor-bearing mice were allocated into 4 groups with more than 5 mice in each group: (1) sham-treatment (control, C); (2) S + 1 W/cm², 0.5 MHz ultrasound treatment, SU1); (3) S + 1 W/cm² and 1 MHz ultrasound treatment, SU2); and (4) S + 1 W/cm² and 2.5 MHz ultrasound treatment, SU3). Here S means ip injection

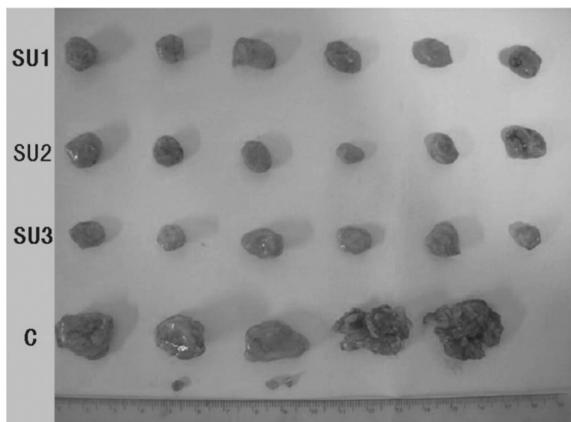


Figure 11. Tumors excised from each group of mice 8 days after treatment.

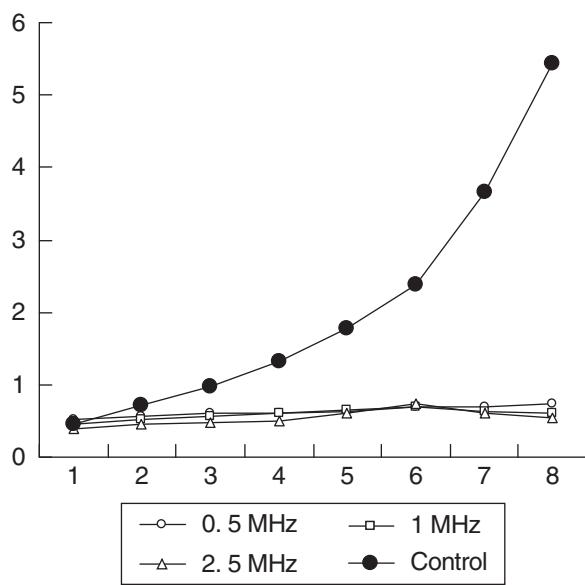


Figure 12. Growth curve of tumors in each group after SDT with bone barrier. SDT = sonodynamic therapy.

with SF1 20 mg/kg as described in "Materials and Methods." Following treatment, tumor volume was monitored. Eight days later, the mice in the 4 groups were all sacrificed and the tumors of the mice were excised and weighed (Table 6).

Comparing with group C (sham-treatment), the tumor weight in every SDT treated group was significantly lower ($P < .01$). This demonstrated again that the SF1 plus sound treatment did inhibit S-180 sarcoma in mice. But the tumor weight in groups SU1, SU2, and SU3 showed

no significant differences. This suggested that 0.5 to 2.5 MHz ultrasounds were all able to activate SF1 and destroy the tumor. This conclusion is demonstrated much more clearly in Figures 11 and 12.

Discussion

SDT is a treatment modality wherein sound energy-activated chemicals create a cascade of endogenous cytotoxic agents. SDT appears to fulfill all the promises of photodynamic therapy (PDT) while avoiding the inability of light to penetrate to deep tissue tumors. The basis for this belief was reviewed by Lane.² The clear advantage of SDT over PDT is the ability to address the limited depth of light energy penetration to sensitizer compounds and destroy deep cancerous tumors.² This fatal limitation of PDT is overcome when compounds can be sono-activated by deep penetrating sono-energy.

The exact mechanism by which sound activation causes a series of cytotoxic events is as yet unknown. However, the downstream mechanisms of action are postulated to be free radical formation,^{3,4} cavitation,⁵⁻⁷ and sonoporation.^{8,9} Ultrasonically induced cell damage via free radical generation is supported by various studies suggesting active oxygen radical generation⁴ and peroxy radical formation³ are responsible for cell damage during the ultrasound activation of the sensitizer compounds. The synergistic cell-killing effect of ultrasound and porphyrin-derivatives⁶ might be mediated through cavitation-related high temperatures within the microbubble itself,¹⁰ contributing to thermal destruction of the cancer cell, a unique feature of sonodynamic therapy. Sonoporation⁹ is a process by which chain peroxidation of membrane lipids destabilizes the cell membrane with the sonosensitizers and can therefore cause tumor cells to be more susceptible to drug transport antineoplastic compounds through the cell membrane.

Recently, a new sonosensitizing agent, SF1, was developed by SonneMed, LLC. In this study the effect of SDT with SF1 on S-180 sarcoma in mice was examined. Our data reveal that SDT with SF1 inhibits growth of mouse S-180 sarcoma. The inhibitory effect is sound intensity dependent.

Pathological study in this project shows that SF1 can be specifically accumulated within tumor cells, and that SDT with SF1 destroyed mouse S-180 sarcoma very rapidly. The degeneration of tumor occurred almost immediately or at least within 2 hours after SDT treatment. SDT also induced inflammation in the vicinity of the destroyed tumor indicating a rapid appearance of a "vaccine"-like effect.¹¹ Tumors treated with SDT enlarge in the first week and then shrink continuously thereafter.

It is very interesting that the tumor growth inhibition by SDT is also seen in tumors covered by bone. This indicates 1 MHz ultrasound is able to pass through bone, activate the sensitizer in the tumor, and lead to its destruction. SDT

with SF1 might be used for tumors in bone or in brain that are very difficult to treat by conventional anticancer therapy. Also, ultrasound treatments with frequencies of 0.5 to 2.5 MHz were all able to activate SF1 and destroy the tumor.

Taken together, our data indicate that SDT with SF1 does inhibit growth of mouse S-180 sarcoma and that SF1 has great promise for clinical SDT use in the future.

Acknowledgements

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