# Enhanced antitumor activity of ultrasonic irradiation in the presence of new quinolone antibiotics *in vitro*

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**To determine if there is any synergistic antitumor effect of ultrasound (US) in the presence of new quinolone (NQ) antibiotics, 0.2 m*M* solutions of lomefloxacin hydrochloride (LFLX), sparfloxacin (SPFX), ciprofloxacin hydrochloride (CPFX), and gatifloxacin hydrate (GFLX) were tested as sonodynamic agents against sarcoma 180 cells *in vitro*. After US irradiation at 2 W/cm$^2$ for 30 and 60 s, the survival rates of tumor cells in the presence of NQ antibiotics were significantly lower than those in their absence ($P$<0.001). In May-Giemsa smears, most of the tumor cells remained intact in the control group. However, in the 0.2 m*M* SPFX group, the tumor cells were mostly fragmented. The synergistic antitumor effect of SPFX was dose-dependent. Furthermore, when D-mannitol was used with SPFX, the survival rate of tumor cells after irradiation was comparable with that when SPFX alone was applied, but when L-histidine was used concurrently, the survival rate of tumor cells was significantly higher than that when SPFX alone was applied. These findings suggest that NQ antibiotics would exhibit useful antitumor activity under US irradiation, and that generation of singlet oxygen is involved in the process of cell damage. (Cancer Sci 2004; 95: 845–849)**

Sonodynamic therapy, which is based on a synergistic effect of drugs and ultrasound (US), is a promising new methodology for cancer treatment.[1–3] Hematoporphyrin (Hp), a photodynamic compound, and its derivatives were found to enhance the cell-damaging effect of US irradiation at a concentration at which the chemical alone caused no cell damage. The combination of Hp and US provided increased effectiveness, as well as reducing the required ultrasonic irradiation intensity.[4,5] However, Hp has several clinical side effects, such as severe photodermatitis. Some clinical reports indicate that patients have to be shielded from sunlight for 4 weeks or longer.[6,7] Therefore, Hp has not so far been widely used clinically. Recently, some non-steroidal anti-inflammatory drugs, such as piroxicam, were also found to have a synergistic antitumor effect with US, but they are still far from ideal for clinical use.[2,8] New quinolone (NQ) antibiotics have been widely used clinically for treating infectious diseases, because they are highly potent, broad-spectrum antibacterial agents and have few side effects.[9–11] However, there has been no previous report investigating the feasibility of using NQ antibiotics as sonodynamic compounds.

This study was designed to examine whether or not the sensitivity of tumor cells to US is increased in the presence of NQ antibiotics, and to investigate the feasibility of using combinations of US and NQ antibiotics in the treatment of tumors.

## Materials and Methods

**Preparation of tumor cells.** Ascitic sarcoma 180 (Medical Cell Resource Center, Tohoku University Gerontology Research Institute, Sendai, Japan) was used as the experimental tumor. A suspension of sarcoma 180 (about 1 ml) was injected intraperitoneally into 7-week-old ICR male mice (Shizuoka Laboratory Co., Shizuoka, Japan), and 1.0 to 2.0 ml of ascitic fluid, collected about 7–10 days later, was diluted in phosphate-buffered saline (PBS) so that the number of cells was $7.5\times10^5$/ml ($5.3\times10^5$/0.7 ml) (hereinafter referred to as the stock solution). The control solution was prepared by diluting the stock solution 2-fold in 100 ml of PBS with 0.3 ml of dimethylformamide (DMF) added. The survival rate of tumor cells was evaluated by means of the trypan blue dye exclusion method using a hemocytometer (Kayagaki, Tokyo) under an optical microscope (Olympus BH-210, Tokyo, ×400). Viability before treatment was always over 98%.

**Chemicals.** Antitumor activities of the following 5 drugs as sonodynamic compounds against sarcoma 180 were studied: 1) piroxicam (supplied by Taito Pfizer Co., Ltd., Tokyo) $C_{15}H_{13}N_3O_4S$, molecular weight 331.35; 2) lomefloxacin hydrochloride (LFLX, supplied by Shionogi & Co., Ltd, Osaka, Japan) $C_{17}H_{19}F_2N_3O_3\cdot HCl$, molecular weight 387.81; 3) sparfloxacin (SPFX, supplied by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) $C_{19}H_{22}F_2N_4O_3\cdot HCl$, molecular weight 392.41; 4) ciprofloxacin hydrochloride (CPFX, supplied by Bayer Yakuhin, Ltd., Osaka, Japan) $C_{17}H_{18}FN_3O_3\cdot HCl\cdot H_2O$, molecular weight 385.82; 5) gatifloxacin hydrate (GFLX, supplied by Kyorin Pharmaceutical Co., Ltd., Tokyo) $C_{19}H_{22}F_2N_3O_4\cdot HCl$ 1/2 $H_2O$, molecular weight 402.42 (Fig. 1). To prepare a 0.2 m*M* solution of each drug, 6.63 mg of piroxicam, 7.76 mg of LFLX, 7.85 mg of SPFX, 7.72 mg of CPFX, or 8.04 mg of GFLX was dissolved in 0.3 ml of DMF and diluted with 100 ml of PBS. The concentration of 0.2 m*M* was based on the amount of drug that could be dissolved in 0.3 ml of DMF, which was expected to have no influence on the experimental system.[2,8]

**Ultrasonic generator.** The ultrasonic generator used in this study was made at the Department of Electronic Engineering, Akita University Mining College, Akita, Japan. Basically, the generator consists of a ceramic cylindrical transducer combined with a function generator. It can be used at a resonance frequency of 2 MHz on a piezo-electric element. A round ceramic plate of 20 mm in diameter and 1 mm in thickness (2Z 20D SYIC, Fuji Ceramics, Shizuoka, Japan) was used as the piezoelectric element. The resonance frequency after adhesion to aluminum is 2.256 MHz. A function generator (FG-350, Iwatsu Denshi, Tokyo) that can be operated over a frequency bandwidth of 0.1 Hz to 10 MHz was used. The sine wave mode was used in this experiment. The system also included a power amplifier (PA 40-2801, Someway, Shizuoka, Japan) with a frequency bandwidth of 100 kHz to 350 MHz and an output of 0 to 10 W, and a power meter (SX-200, Daiichi Denpa Kogyo, Tokyo) with a frequency range of 1.8 to 200 MHz and a power measurement range of 0 to 200 W (Fig. 2).

**Ultrasonic irradiation experiment.** The control solution (1.4 ml), and 0.7 ml of the stock solution mixed with 0.7 ml of 0.2 m*M* solution of each drug (6 groups) were introduced separately into a glass cell 20 mm in diameter, 40 mm in height and with a base 1 mm in thickness (made at the Instrument Center, Akita University School of Medicine, Akita, Japan). The number of tumor cells contained in each glass cell was set at about

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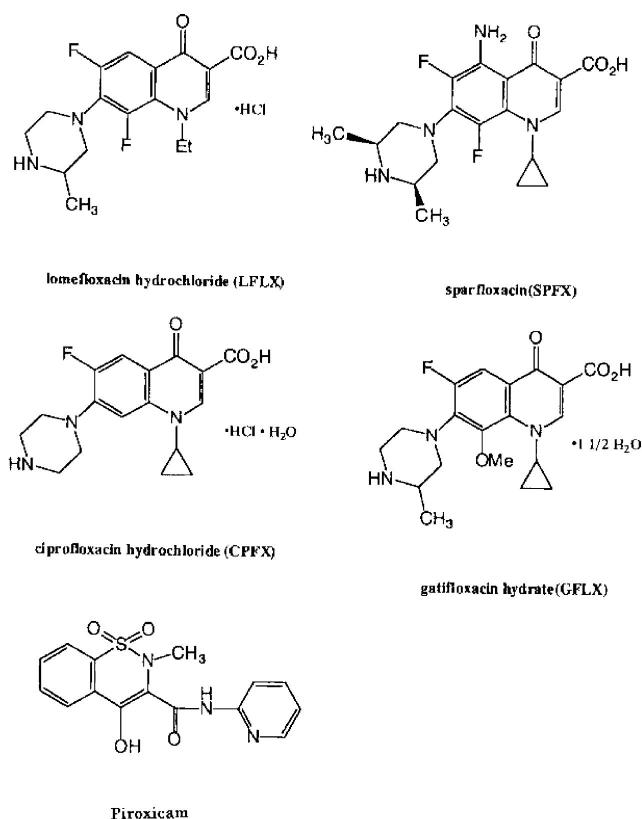


**Fig. 1.** Structural formulae of piroxicam, LPLX, SPFX, CPFX, and GFLX.

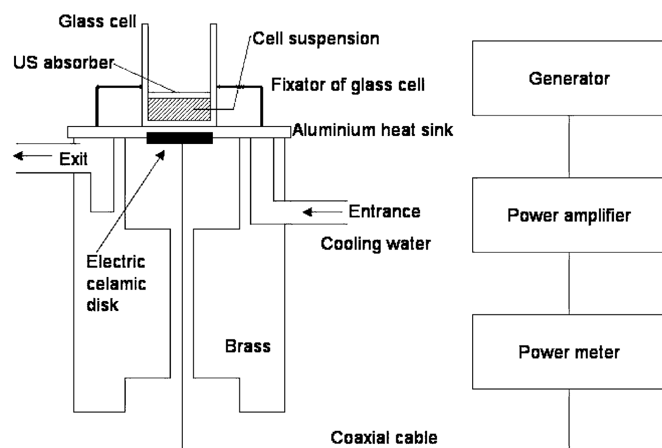


**Fig. 2.** Overview of the US apparatus.

$5.3\times10^5$ as mentioned earlier. First, we examined whether the survival rate of tumor cells in the stock solution was changed by adding the drug alone ($n$=10). Next, the control and the drug-containing solutions were exposed to ultrasonic radiation at 1.5, 2.0, and 3.0 W/cm$^2$ (meter reading) at a frequency of 2 MHz for 30 s and 60 s, respectively ($n$=10). Using a US power meter (UPM-DT-10E, Ohmic Instrument Co., Easton, USA), the effective output was determined and it was found that intensities of 1.5. 2.0, and 3.0 W/cm$^2$ on the meter of the US device gave effective output values of 0.07, 0.11, and 0.18 W/cm$^2$, respectively. However, in this paper, we use the meter reading of the device to refer to these measured values for convenience. To ensure close adhesion of the piezo-electric element with the glass cell, US transmission gel (Parker, Aquasonic 100, Fairfield, NJ, USA) was applied. All procedures in the US irradiation experiment were performed within 1 h after the aspiration of ascitic fluid of the mouse. The temperature of the solution in the glass cell was set at room temperature (23–25°C). During the sonication procedure the temperature inside the glass cell did not rise by more than 0.1°C, as measured with a digital thermometer (TESTO 905-T1). The cell survival rate in the experiment was calculated as (number of living cells after irradiation/number of living cells before irradiation)×100 (%); cells that were destroyed by ultrasonic irradiation were counted as dead cells. A May-Giemsa smear was prepared from the cell suspension before and after the US irradiation to visualize the extent of degeneration and necrosis of cells.

**Identification of active oxygen.** The survival rate of tumor cells was determined under US irradiation at 1.5, 2.0, and 3.0 W/cm$^2$ (meter reading) at a frequency of 2 MHz for 30 s ($n$=10) in the presence of superoxide dismutase (Wako, Osaka, Japan) and catalase (Wako), scavengers of $H_2O_2$,[12] L-histidine hydrochloride monohydrate (Wako), a scavenger of singlet oxygen and hydroxyl radical,[13] and D-mannitol (Nacalai Chemicals, Ltd., Kyoto, Japan), a scavenger of hydroxyl radical.[14] The results were compared with that in the presence of 0.2 m$M$ SPFX. The concentrations of superoxide dismutase and catalase were each set at 50 ng/ml, and those of L-histidine and D-mannitol were each set at 0.2 $M$.

**Statistical analysis.** The mean and standard deviation of survival rate of tumor cells were calculated for each group. Differences between the groups were considered significant when the $P$ value in the Mann-Whitney test was 0.05 or smaller.

## Results

**Influence of drugs on tumor cell viability over time.** The survival rate of tumor cells in the control group was not significantly different from those of the 0.2 m$M$ drug-added groups within 3 h. No significant difference was observed in survival rate between the drug-added groups within 3 h.

**Influence of ultrasonic irradiation on survival rate of tumor cells.** The survival rate of tumor cells in both the control and the drug-added groups at irradiation times of 30 s and 60 s declined as the intensity of US increased. When the intensity of US was 1.5 W/cm$^2$, no significant difference was found between the control group and drug-added groups at an irradiation time of 30 s or 60 s. When the intensity of US was increased to 2 W/cm$^2$, the survival rate of tumor cells was significantly lower in the drug-added groups compared with the control group at 30 s (control, 78.60±12.98%; LFLX, 49.46±14.62%; SPFX, 30.85±15.41%; CPFX, 66.47±11.27%; GFLX, 38.84±8.35%; piroxicam, 40.43±12.33%), or 60 s (control, 52.14±22.41%; LFLX, 28.38±9.20%; SPFX, 23.41±8.74%; CPFX, 38.31±8.99%; GFLX, 26.08±9.52%; piroxicam, 28.19±9.40%). The survival rate of tumor cells was significantly lower in the LFLX, SPFX, GFLX, and piroxicam groups compared with the CPFX group at 30 s and 60 s (Fig. 3, A and B, $P$<0.001). At 3 W/cm$^2$ for 30 s or 60 s, the survival rate was low in both the control and drug-added groups, and no significant difference was seen. After this experiment, different concentrations (0.2 m$M$, 0.1 m$M$, 0.05 m$M$, and 0.01 m$M$) of SPFX, which gave the most effective results, were chosen for further experiments under the same conditions, as mentioned earlier.

At 2 W/cm$^2$, the survival rate of tumor cells was significantly lower in the 0.2 m$M$ (30.85±15.41%), 0.1 m$M$ (34.58±15.90%), and 0.05 m$M$ (44.15±8.35%) SPFX groups compared with the control group for 30 s ($P$<0.001) and for 60 s ($P$<0.001) (0.2 m$M$, 23.41±8.74%; 0.1 m$M$, 28.72±5.70%; 0.05 m$M$, 34.05±8.77%), and no significant difference was

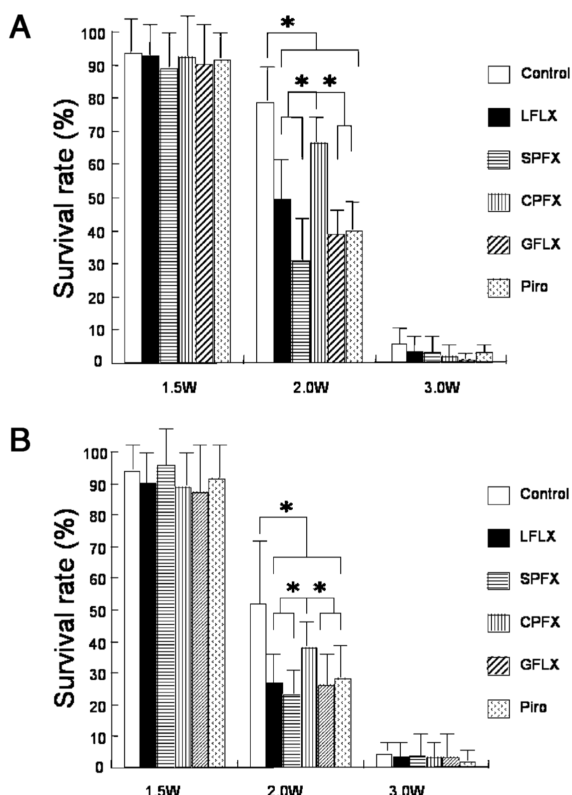


**Fig. 3.** Survival rate of tumor cells after US irradiation. A significant difference was seen between the control and 0.2 m*M* drug-added groups, and between the CPFX group and other drug-added groups at 2 W/cm² for 30 s (A) and 60 s (B).

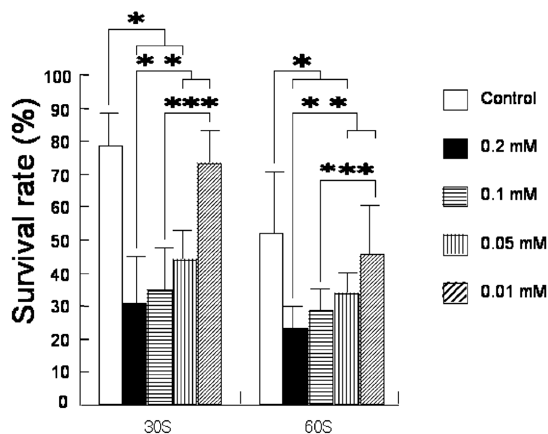


**Fig. 4.** Survival rate of tumor cells after US irradiation in the presence of various concentrations of SPFX. At 2 W/cm², a significant difference was seen between the control group and SPFX-added groups at a concentration of 0.05 m*M* or higher for 30 s and 60 s.

found between the control and 0.01 m*M* group for 30 s or 60 s. The survival rate of tumor cells in 0.2 m*M* group was significantly lower than that in 0.05 m*M* group at 30 s ($P$=0.0281) (Fig. 4).

**May-Giemsa smear.** After US irradiation at 2 W/cm² for 30 s, sarcoma 180 cells stained purplish blue were seen diffusely, and most of the nucleus and cytoplasm were well maintained in the control group (Fig. 5A). In the 0.2 m*M* SPFX group, most of the tumor cells were lost or broken into small pieces, the number of intact tumor cells sharply decreased, and the cells showed a concentrated nucleus and bright cytoplasm (Fig. 5B).

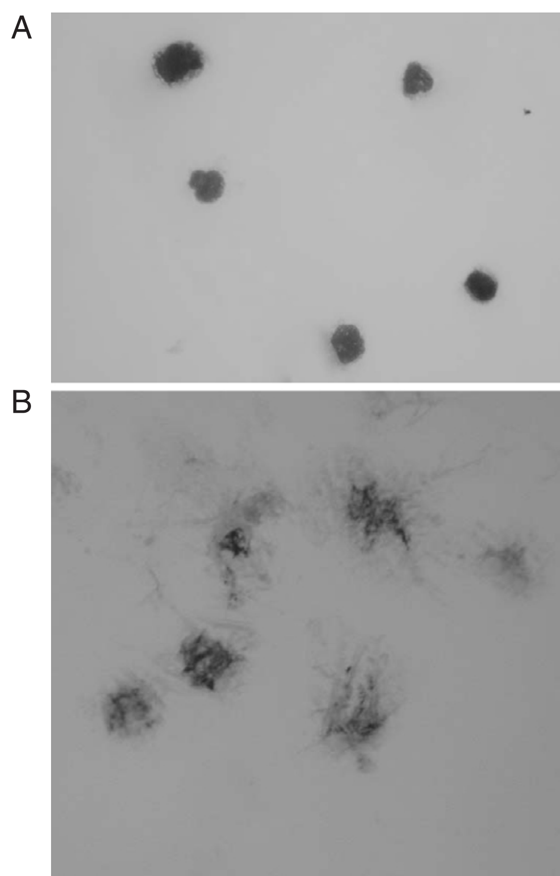


**Fig. 5.** May-Giemsa smear. At 2 W/cm² for 30 s. (A) Control group. The tumor cells were stained purplish blue. Both the nucleus and cytoplasm are maintained intact (×400). (B) 0.2 m*M* SPFX group. The tumor cells were mostly lost or fragmented (×400).

**Effect of scavengers of active oxygen.** The survival rate of tumor cells was significantly higher in the presence of 0.2 m*M* SPFX with 0.2 *M* L-histidine than that in the presence of SPFX alone at 2 W/cm² for 30 s ($P$<0.001). In the superoxide dismutase-added, catalase-added, and D-mannitol-added groups, however, suppression of the death of tumor cells was not seen (Fig. 6).

## Discussion

Recently, various drugs, such as Hp and its derivatives, and some non-steroidal anti-inflammatory drugs (NSAIDs), have been found to have the ability to enhance the cell-damaging effect of US irradiation at a concentration at which the chemical alone causes no cell damage.[2, 8, 15] However, Hp has several clinical side effects, such as severe photodermatitis, and has not so far been widely used clinically.[6, 7] Piroxicam has a similar synergistic antitumor effect to Hp and fewer side effects, but a high concentration is required for clinical use as a sonodynamic compound.[2, 8] Therefore, new kinds of sonodynamic compounds would be of potential value.

NQs are a highly effective class of antibiotics used clinically as a first choice for treating general bacterial infectious diseases. Some of them are weak inducers of photodermatitis, depending on their structures. Our original idea was based on the following hypothesis. NQ antibiotics with functional groups such as fluorine and methoxyl groups at the $C_8$ position would be promising candidates for sonodynamic compounds because

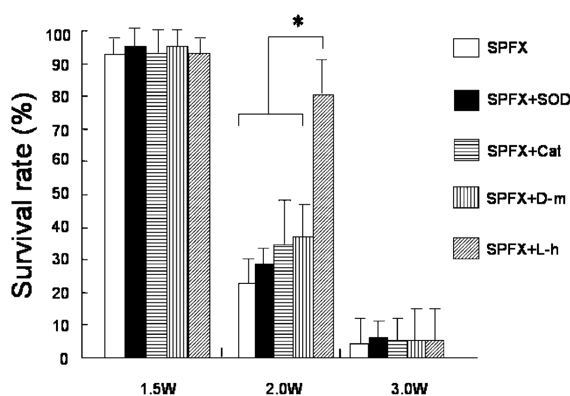


Fig. 6. Tumor cell death-suppressing effects of superoxide dismutase (SOD), catalase (Cat), L-histidine (L-h), and D-mannitol (D-m). The survival rate of tumor cells was significantly higher in the SPFX-L-h-added group than in the SPFX group, whereas no significant difference was found among SPFX-D-m-added, SPFX-SOD-added, SPFX-Cat-added, and SPFX groups at 2 W/cm² for 30 s.

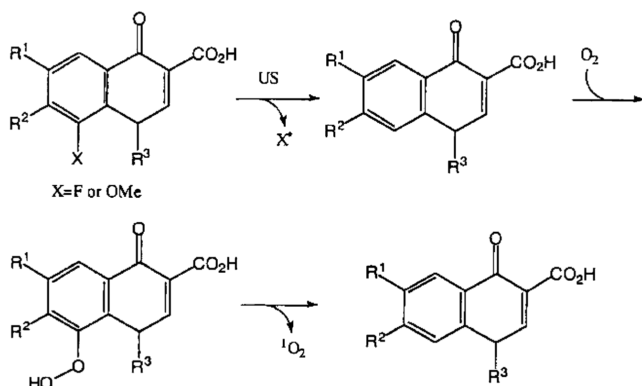


Fig. 7. Possible mechanism of generation of singlet oxygen from NQ antibiotics.

of radical formation at the $C_8$ position under ultrasonic irradiation, followed by reaction with oxygen to generate unstable perhydroxyl compounds, leading to formation of singlet oxygen[9, 11] (Fig. 7).

In the present experiment, the survival rate of tumor cells under ultrasonic irradiation was significantly lower in the drug-added groups. Significantly lower survival rates of tumor cells were also observed in the LFLX, SPFX, GFLX, and piroxicam groups compared with the CPFX group. No significant difference was found in the LFLX, SPFX, or GFLX group versus the piroxicam group. At 1.5 W/cm², no difference in the survival rate was found among the groups. At 3 W/cm², both the control and the drug-added groups showed a tumor cell survival rate close to 0%. From this result, it seems that US irradiation at high power alone can destroy sarcoma 180 cells, and a considerable antitumor effect could be achieved at a lower power in a shorter period of time in the presence of the NQ antibiotics and piroxicam. All four NQ antibiotics are effective as sonodynamic compounds, but the effectiveness varied. At 2 W/cm², the survival rate of tumor cells was significantly lower in the 0.2 m*M*, 0.1 m*M*, and 0.05 m*M* SPFX groups compared with the control group for 30 s or 60 s. No significant difference was found between the 0.01 m*M* SPFX group and the control group. The survival rate of tumor cells in the 0.2 m*M* group was also significantly lower than that in the 0.05 m*M* group at 30 s or 60 s. These results indicated that the effect of US irradiation in the presence of SPFX was dose-dependent. US over 2 W/cm² in the presence of SPFX at a dose greater than 0.05 m*M* is required for eradicating sarcoma 180 cells in the glass cell.

In the present experiment, DMF, a solubilizer, was used concurrently with NQ and piroxicam to aid dissolution of the drugs in PBS.[2, 8] There has been no report of an antitumor effect of DMF. In regard to NQs, some of them may induce apoptosis in some cancer cell lines.[16, 17] Therefore, we compared the survival rate of tumor cells in the control and drug-added groups without US. As shown in the present study, no change in the survival rate of tumor cells was found, even after 3 h, in the presence of 0.3% DMF or DMF and the drugs. These findings indicated that the enhanced antitumor effect is attributable to the sonodynamic effect of US in combination with the drugs.

In the case of sonodynamic reaction of Hp, several lines of evidence suggest that singlet oxygen generated by sonochemically activated Hp is likely to be the most important cell-damaging mediator.[18, 19] It seems natural to assume that the mechanism of the sonodynamic reaction of NQs is similar to that of Hp,[20] but this should be confirmed. Superoxide dismutase and catalase are known to act as scavengers of $H_2O_2$, and L-histidine is a scavenger of singlet oxygen and hydroxyl radical. A D-mannitol concentration of 0.2 *M* is more than enough to scavenge ultrasonically induced hydroxyl radical. Thus, the significant reduction by L-histidine of ultrasonically induced cell damage in the presence of SPFX and the lack of a significant effect of superoxide dismutase, catalase, and D-mannitol imply that singlet oxygen is an important mediator of the cell damage. At 3 W/cm², no reduction of cell damage by any scavenger was observed, since high-power ultrasound alone can destroy sarcoma 180 cells. This putative mechanism of the synergistic effect of US with SPFX is basically the same as that of US with Hp.

As mentioned earlier, Hp results in high tumor mortality because it accumulates in tumors.[4, 5, 10, 21] In the case of NQ, the side-effects are mild,[9, 11] but it is unclear whether or not they accumulate in tumors. The minimum effective concentration of SPFX used in this experiment was 0.05 m*M*. SPFX is a newly developed quinolone for oral use that has broad and potent antibacterial activity. It has the following pharmacological advantages: 1) great penetration of the dense, largely lipid outer capsule and cell wall; 2) superior tissue penetration, resulting in levels 2 to 11 times higher than those obtained in plasma; 3) superior accumulation within macrophages; and 4) long half-life in plasma in mice, as well as in humans. With these excellent pharmocokinetic properties, SPFX or more powerful NQs should prove to be not only a useful antimicrobial agent, but also a potentially valuable sonodynamic compound.

In conclusion, the sensitization of sarcoma 180 to US by four NQ antibiotics *in vitro* has been demonstrated. Although the results reported in this paper are preliminary, clinical application of the combination treatment of US and some of the NQ antibiotics seems likely to be useful. Work on the the synergistic effects of US and NQ antibiotics in the treatment of tumor-bearing mice is in progress. US after administration of NQ antibiotics may be useful as a tool in the clinical treatment of tumors located deep in the body.


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