

Sonodynamic Effects of Lomefloxacin Derivatives Conjugated with Methoxy Polyethylene Glycol on Sarcoma 180 Cells

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Abstract. *Background:* Sonodynamic therapy (SDT) is a promising methodology for cancer treatment. Lomefloxacin hydrochloride (LFLX) has been reported to have sonodynamic antitumor effects. *Materials and Methods:* We synthesized LFLX derivatives conjugated with methoxy polyethylene glycol (PEGylated LFLXs) and investigated their ultrasonically induced antitumor effects. *Results:* After ultrasound exposure at 2.0 MHz for 30 s, the survival rates of Sarcoma 180 cells in the presence of lower molecular weight PEGylated LFLXs (200 μ M) were significantly lower than those of the control and the LFLX at 1.5 and 2.0 W/cm². This enhancement was significantly inhibited by the addition of L-histidine, but not by D-mannitol or superoxide dismutase. There was no apparent cell damage in the presence of high molecular weight PEGylated LFLX even at 3.0 W/cm². *Conclusion:* These findings indicate that the sonodynamic antitumor effects of lower molecular weight PEGylated LFLXs are better than those of LFLX.

Sonodynamic therapy (SDT) is an enhancement of cytotoxic activities of chemicals (sonosensitizers) on tumor cells by exposure to ultrasound (US) (1-4). US can be focused in a small region and deeply penetrate tissue, thus SDT may be a useful tool for the clinical treatment of tumors located deep in the body (5-10). Umemura and colleagues first reported SDT, which involved the synergistic effect of US and hematoporphyrin (11, 12), and considerable research into sonosensitizers has been reported in recent years (13-16). Potent sonosensitizers for clinical use apparently have strong cytotoxicity on tumor cells following US exposure at low

intensity, few side-effects and high tumor-selectivity. Hematoporphyrin and its derivatives have sonodynamic antitumor effects and accumulate in tumor tissues, but these agents are liable to cause photodermatitis. There are few sonosensitizers that can satisfy all situations.

New quinolone (NQ) antibiotics have been widely used clinically for treating infectious diseases and have few side-effects. We have already reported that NQ antibiotics including lomefloxacin hydrochloride (LFLX) are promising sonosensitizer candidates (17, 18). LFLX has a prominent sonodynamic cytotoxic effect, but it may lack tumor-selectivity. If LFLX accumulation in tumors can be improved, this agent might have potential for successful clinical application.

Polyethylene glycol (PEG) is a nontoxic, non-immunogenic, biocompatible and water-soluble polymer used in biotechnology, biomaterials and pharmaceuticals (19, 20). It was well established that large macromolecules circulating for extended periods show substantial tumor accumulation (21). In addition, passive targeting of antitumor drugs to a tumor site can be achieved by increasing the apparent molecular size through conjugation with PEG (22, 23). In this study, we synthesized PEGylated LFLXs in order to improve the selectivity to tumor tissues; the ultrasonically induced antitumor effects of these agents on sarcoma 180 (S180) cells were investigated.

Materials and Methods

Chemicals. LFLX was provided by Shionogi & Co., Ltd. (Osaka, Japan). Monomethoxy PEG (mPEG) compounds with a terminal primary amino group were obtained from Nippon Oil & Fats Co., Ltd. (Tokyo, Japan). All other reagents were commercial products of analytical grade. PEGylated LFLXs (Figure 1) were synthesized from LFLX in three steps. Acetylation of the amino group in LFLX, followed by a reaction of the resulting acetylated LFLX (Ac-LFLX) with methyl chloroformate gave an unstable mixed anhydride which was condensed with each molecular weight (2,000, 5,000 and 20,000) of mPEG to provide the corresponding compounds (LFLX-PEG2000, LFLX-PEG5000 and LFLX-PEG20000), respectively

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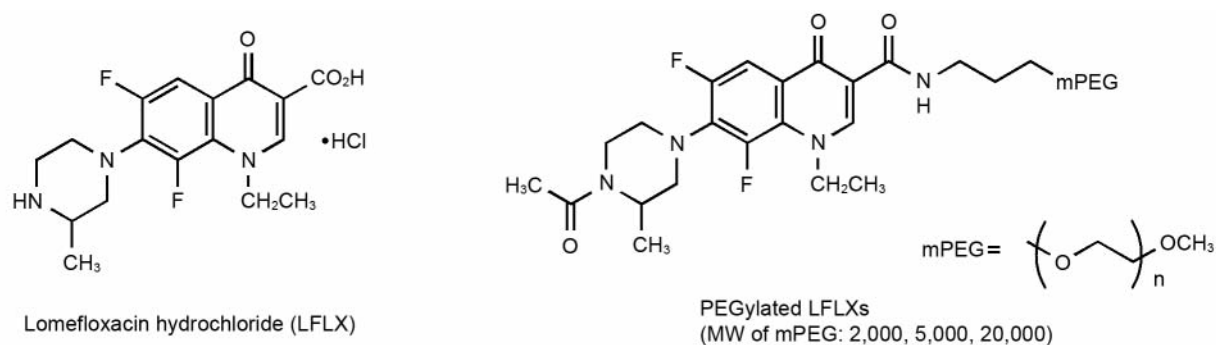


Figure 1. Chemical structures of lomefloxacin hydrochloride (LFLX) and PEGylated LFLXs.

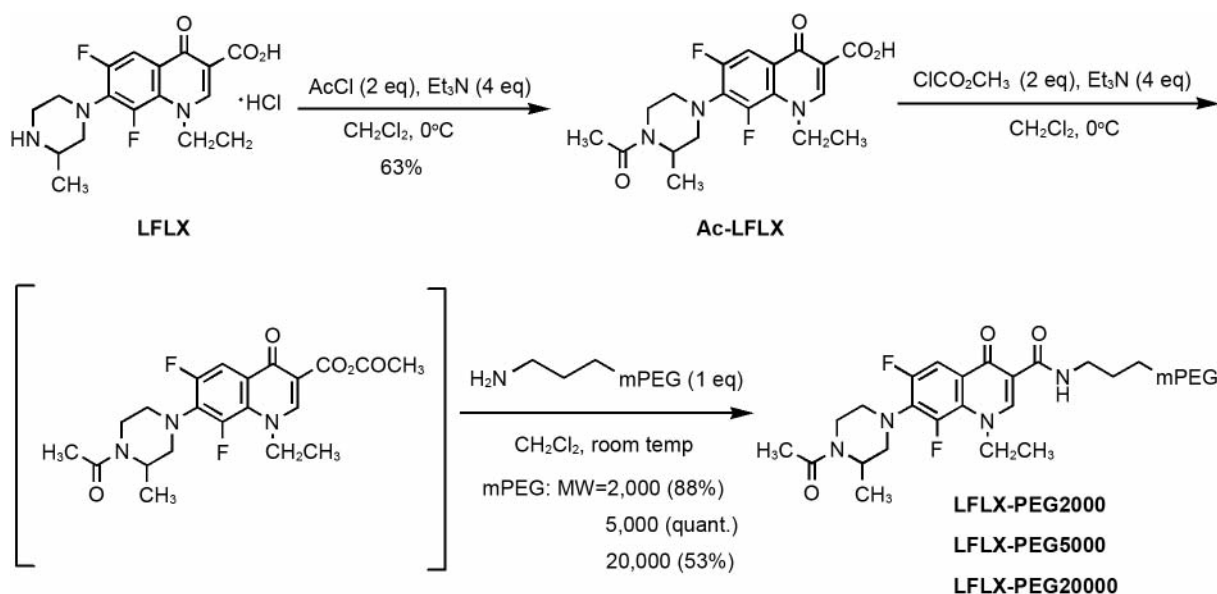


Figure 2. Synthetic pathway to PEGylated LFLXs.

(Figure 2). In the current study, we prepared 200 μM solutions of 4 drugs (LFLX, LFLX-PEG2000, LFLX-PEG5000 and LFLX-PEG20000) and then 5, 10, 50, 100 μM solutions of each of the 3 drugs (LFLX, LFLX-PEG2000 and LFLX-PEG5000).

Preparation of tumor cells. Ascitic S180 (Medical Cell Resource Center, Tohoku University Gerontology Research Institute, Sendai, Japan) was used as the experimental tumor. A suspension of S180 (about 1 ml) was injected intraperitoneally into 7-week-old ICR male mice (Japan SLC, Inc., Shizuoka, Japan). About 7-14 days later, 1.0 to 2.0 ml of ascitic fluid was collected and diluted in phosphate-buffered saline (PBS) to a cell density of $1.0 \times 10^6/\text{ml}$ ($1.4 \times 10^6/0.7 \text{ ml}$). The survival rate of tumor cells was evaluated by the trypan blue dye exclusion method using a hemocytometer (Kayagaki, Tokyo, Japan) under an optical microscope (Olympus BH-210, Tokyo; $\times 400$). Viability before treatment was always in excess of 98%.

Ultrasound exposure experiment. The experimental set-up for US exposure was basically the same as described in previous papers (24). The control solution was prepared by diluting the cell suspension (0.7 ml) with PBS (0.7 ml). The drug-treated groups were prepared by mixing the cell suspension (0.7 ml) and 200 μM solutions of 4 drugs (LFLX, LFLX-PEG2000, LFLX-PEG5000 and LFLX-PEG20000) (0.7 ml). They were introduced separately into a glass cell 20 mm diameter, 25 mm in height and with a base of 9- μm -thick Mylar film (glass cell, made at the Instrument Center, Akita University School of Medicine, Akita, Japan; Mylar film, Teijin DuPont Films Co., Tokyo, Japan).

First, we examined whether or not the survival rate of tumor cells in the drug-treated groups were changed without US exposure ($n=10$). The solution which did not contain LFLX and its derivatives was defined as the control group. The control and drug-containing solutions were then exposed to US at 1.5, 2.0 and 3.0 W/cm^2 (meter reading) at a frequency of 2 MHz for 30 s ($n=10$).

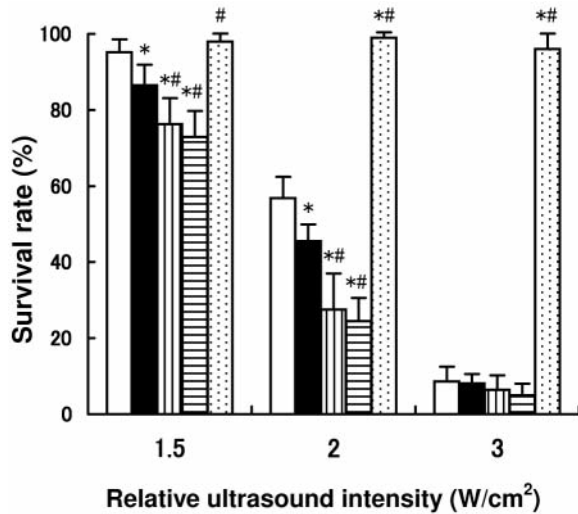


Figure 3. Effect of ultrasound exposure for 30 s on S180 cells. Open bar, the control group; solid bar, the LFLX group; vertically striped bar, the LFLX-PEG2000 group; horizontally striped bar, the LFLX-PEG5000 group; dotted bar, the LFLX-PEG20000 group (mean \pm SD, n=10). * p <0.01 Compared with the control group; # p <0.01 compared with the LFLX group.

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/cm² on the meter of the US device yielded effective output values of 0.18, 0.24, and 0.36 W/cm², respectively. However, in this paper, we use the meter reading of the device to refer to these measured values for convenience. Similarly, US-induced antitumor effects of 3 drugs (LFLX, LFLX-PEG2000 and LFLX-PEG5000) at different concentrations (5, 10, 50, 100 μ M) were compared with that of the control group under US exposure at 2.0 W/cm², 2 MHz for 30 s (n=10).

To ensure close adhesion of the piezo-electric element with the glass cell, transmission gel (US FINE GEL, Fukuda Denshi, Tokyo) was applied. All procedures in the US exposure experiment were performed within 1 h after aspiration of ascitic fluid from the mouse. The temperature of the solution in the glass was set at room temperature (22~26°C). During the sonication procedure, the temperature inside the glass cell did not rise by more than 2°C, as measured with a digital thermometer (TESTO 905-T1, Kanagawa, Japan). The cell survival rate in the experiment was calculated as (number of living cells after US exposure/number of living cells before US exposure) \times 100 (%); cells that were destroyed by US exposure were counted as dead cells.

Identification of active oxygen. L-histidine, D-mannitol and superoxide dismutase (SOD) were purchased from Wako Chemical Company (Tokyo, Japan). The survival rate of tumor cells was determined after US exposure at 2.0 W/cm², 2 MHz for 30 s (n=10) in the presence of 200 μ M LFLX-PEG2000 with SOD (100 μ g/ml), L-histidine hydrochloride monohydrate (50 mM) or D-mannitol (100 mM). The findings were compared with that in the presence of 200 μ M LFLX-PEG2000 alone.

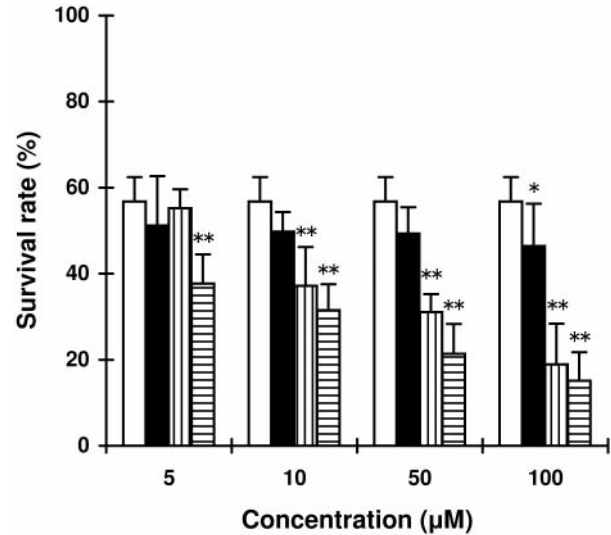


Figure 4. Effect of ultrasound exposure at an intensity of 2.0 W/cm² for 30 s on S180 cells (n=10) treated with different concentrations of LFLX, LFLX-PEG2000 and LFLX-PEG5000. Open bar, the control group; solid bar, the LFLX group; vertically striped bar, the LFLX-PEG2000 group; horizontally striped bar, the LFLX-PEG5000 group (mean \pm SD, n=10). * p <0.05; ** p <0.01 Compared with the control group.

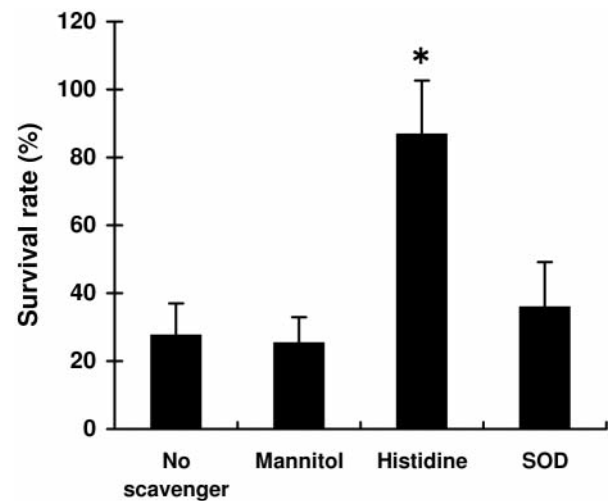


Figure 5. Effect of active oxygen scavengers on cell damage in the presence of LFLX-PEG2000 following ultrasound exposure at an intensity of 2.0 W/cm² for 30 s (mean \pm SD, n=10). * p <0.01 Compared with the no scavenger group.

Electron microscopy. Immediately after US exposure (2 W/cm², 2 MHz, 30 s) in the presence or absence 200 μ M LFLX-PEG2000, the cells were fixed in 3.0% glutaraldehyde solution buffered to pH 7.2 and postfixation was performed with 1% osmium tetroxide. The cells were then gradually dehydrated with increasing concentrations of ethanol, before being transferred to a freeze-drier, coated with gold and examined with a 1200 EX scanning electron microscope (Electron-microscope Optical Ltd. JOEL, Japan) at 10 kV.

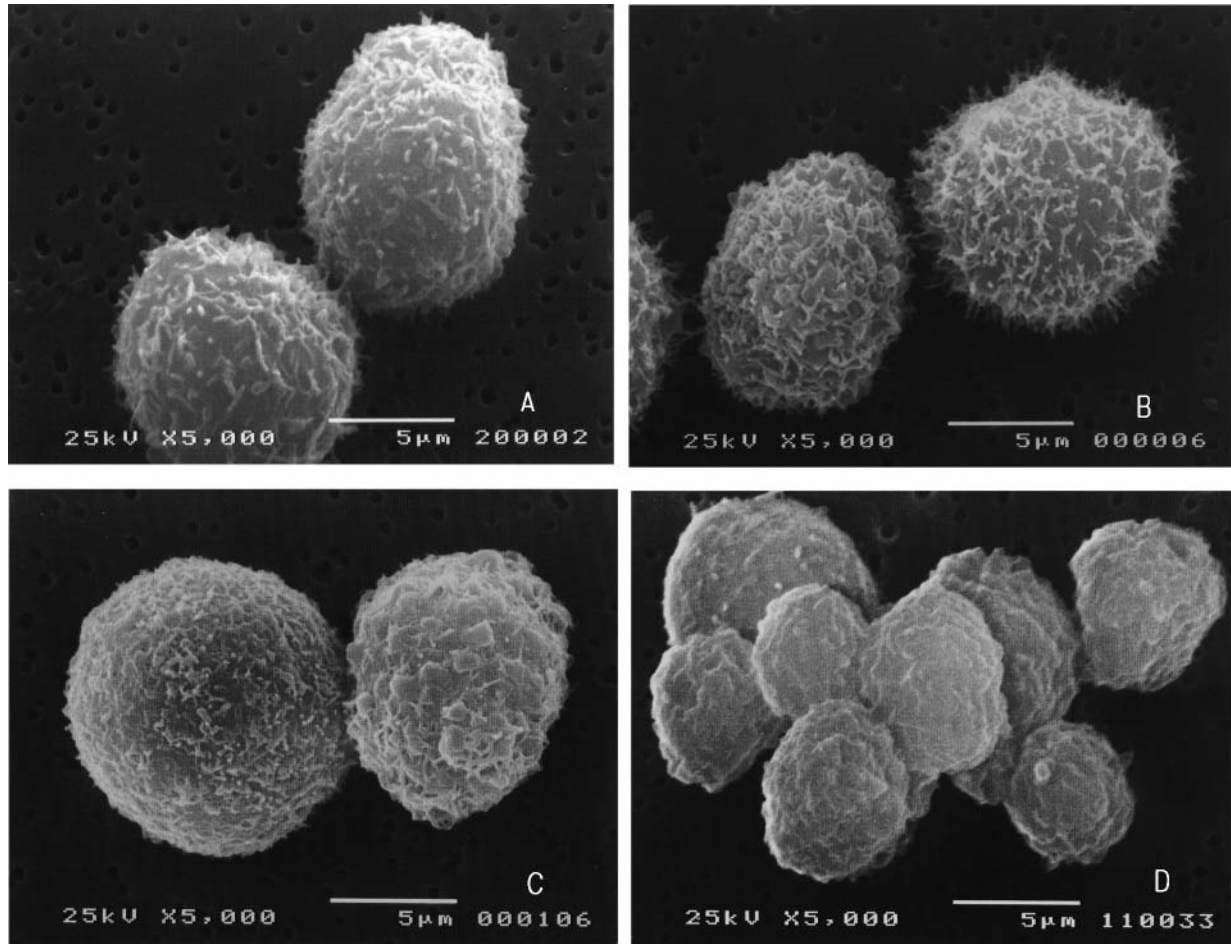


Figure 6. Scanning electron microscopic images of S180 cells: A, Cells in the control group without ultrasound exposure; B, cells in the LFLX-PEG2000 group (200 μM) without ultrasound exposure; C, cells in the control group exposed to ultrasound; D, cells in the LFLX-PEG2000 group (200 μM) exposed to ultrasound.

Statistical analysis. The mean and standard deviation of the survival rate of tumor cells was calculated for each group. Differences between the groups were considered significant when the *p*-value on Bonferroni-Dunnet's test was 0.05 or smaller.

Results

Cell damage. The survival rate of S180 cells in the presence of PEGylated LFLXs without US exposure was not significantly different from that of the control group within 1 h, nor was there any significant difference in survival rate between the PEGylated LFLX groups (data not shown). At an intensity of 1.5 W/cm^2 and 2.0 W/cm^2 , the survival rate in the presence of LFLX-PEG2000 and LFLX-PEG5000 was significantly lower than those of the control and LFLX groups. At 3.0 W/cm^2 , the survival rates were low in both the control group and the drug-treated groups except for that of LFLX-PEG2000. In the presence of LFLX-PEG2000, there was no cell damage at any intensity (1.5-3.0 W/cm^2) (Figure 3).

In the LFLX group, survival rates at concentrations greater than 100 μM were significantly lower than that of the control group. In the LFLX-PEG2000 group, survival rates at concentrations greater than 10 μM were significantly lower than that of the control group. In the LFLX-PEG5000 group, survival rates at concentrations greater than 5 μM were significantly lower than that of the control group (Figure 4).

Identification of active oxygen. Ultrasonically induced cell damage in the presence of LFLX-PEG2000 was significantly reduced in the presence of L-histidine, but not in the presence of D-mannitol or SOD (Figure 5).

Scanning electron microscope observations. In the control group without US exposure, the cells were round or oval with numerous microvilli over the surface of the cells (Figure 6A). LFLX-PEG2000 without US exposure did not cause any apparent cell damage (Figure 6B). Cells in the

control group exposed to US were mostly spherical in shape and the number of microvilli decreased (Figure 6C). Cells exposed to US in the presence of LFLX-PEG2000 had an irregular shape and disrupted cells adhered to each other. Most cells lost microvilli and numerous pores were observed in the cell surface (Figure 6D).

Discussion

The ultrasonically induced antitumor effects of PEGylated LFLXs depended on their molecular weight. PEGylated LFLXs with a low molecular weight (less than 5,000) enhanced ultrasonically induced cell damage, but that with a high molecular weight (more than 20,000) inhibited this effect. Ultrasonically induced cell damage was reported to be induced by acoustic cavitation (25). The occurrence of cavitation might be influenced by the viscosity of PEGylated LFLX. Since the viscosity of LFLX-PEG20000 is higher than that of LFLX-PEG2000 and LFLX-PEG5000, the occurrence of cavitation may decrease in the presence of LFLX-PEG20000. Thus, cytotoxicity might be suppressed in the presence of LFLX-PEG20000.

L-histidine is known to act as a scavenger of singlet oxygen and hydroxyl radicals, D-mannitol is a scavenger of hydroxyl radicals and SOD is a scavenger of H₂O₂ (18). Thus, significant reduction of ultrasonically induced cell damage by L-histidine in the presence of LFLX-PEG2000 and no obvious change with D-mannitol or SOD imply that singlet oxygen is an important mediator in inducing cell damage. Basically the same mechanism has been suggested for porphyrins and LFLX to explain enhancement of cell damage (12, 26). Under a scanning electron microscope, ultrasonically induced cell membrane damage was much more serious in the presence of 200 μM LFLX-PEG2000 than in the control group. Changes on the surface of the cell membrane affect membrane functions and eventually lead to cell death.

Many studies have been reported on SDT using porphyrins, since these agents were known to accumulate markedly in tumors (16). Among other sonosensitizers, alkylated rose bengals were also recognized for their high tumor accumulation (27). Conjugation with PEG has been known as one of the methods to enhance tumor accumulation, although this method was used only in fullerene among sonosensitizers (28). In addition, the influence of the molecular weight of mPEG on ultrasonically induced cell damage has not been previously reported.

Accordingly, it was found that adding mPEG to sonosensitizers promoted ultrasonically induced antitumor effects, and that the molecular weight of mPEG had an important influence on the effect. It should be stressed that when the molecular weight of mPEG was more than 20,000, the agent was not effective as a sonosensitizer. Tumor

selectivity and sonodynamic effects of PEGylated LFLXs in the treatment of tumor-bearing mice should also be investigated in the future.

In conclusion, PEGylated LFLXs are promising compounds as sonosensitizers for SDT because of their excellent antitumor effects under US exposure and their low toxicity. SDT using PEGylated LFLXs might be a useful tool for the treatment of malignant tumors located in deep tissues.

Acknowledgements

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