ESR Detection of Free Radical and Active Oxygen Species Generated during Photolysis of Fluoroquinolones

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The photolysis of six fluoroquinolone agents was investigated in neutral aqueous solution by means of ESR spectroscopy. Lomefloxacin (LFLX), sparfloxacin (SPFX), ciprofloxacin (CPFX), enoxacin (ENX) and sitafloxacin (STFX) generated free radicals in the process of photochemical degradation, while levofloxacin (LVFX) did not. The free radicals and active oxygen species observed were presumed to be carbon-centered radical (·C), hydroxyl radical (·OH) and singlet oxygen ($^1O_2$). No superoxide anion radical ($O_2^{\cdot -}$) was detected by the 5,5-dimethylpyrroline*N*-oxide (DMPO) spin trapping method in our system. 8-Fluorine-substituted fluoroquinolones (LFLX, SPFX) produced ·C, and generated a high degree of $^1O_2$. On the other hand, the fluoroquinolones which have hydrogen, oxygen or chlorine at the 8-position (CPFX, LVFX, STFX) did not produce ·C, and generated a low degree of $^1O_2$. ENX produced ·C, and generated a low degree of $^1O_2$. These results suggest that the nature of the substituent at the 8-position of the fluoroquinolone ring plays an important role in the formation of free radicals and active oxygen species during photoirradiation.

Key words free radical; active oxygen; ESR; photolysis; spin trapping; fluoroquinolone

Fluoroquinolone antibacterial agents (the generic structure is shown in Figure 1) have been widely used as therapeutic agents for general bacterial infections. However, fluoroquinolones are known to induce phototoxicity as a side effect.[1] Photolysis of a first generation fluoroquinolone, nalidixic acid, leads to the generation of free radicals and active oxygen species.[2] Furthermore, the phototoxicity of fluoroquinolones is inhibited by the addition of catalase, and is augmented by the addition of superoxide dismutase.[3] This suggests that the active oxygen species, especially the hydroxyl radical, and more potent oxygen toxic species, might be the cause of this phototoxicity. There are a few reports providing direct proof of the generation of the superoxide and the singlet oxygen,[4] however, there has been no report for the other active species, such as the hydroxyl radical and free radicals. Hence, ESR spectroscopy along with a trapping agent provides a method to directly observe free radicals. One method which has become a valuable means for studying reaction mechanisms is the spin trapping of short-lived radical intermediates using 5,5-dimethylpyrroline *N*-oxide (DMPO).[5,6] The resultant spin adducts are usually relatively stable nitroxide radicals which can be characterized by ESR. In favorable cases, it is possible to identify the radical structure from the *g*-value and hyperfine coupling constant. Another method employs 2,2,6,6-tetramethyl-4-piperidone (TEMP) which results in the *N*-oxyl radical (TEMPO) reacting with singlet oxygen ($^1O_2$).[7,8]

The purpose of this study was to show by means of ESR spectroscopy that the fluoroquinolones generate these active oxygen species during photolysis in aqueous neutral solutions. In addition, a mechanism of species generation was proposed based on the nature of the substituent at the 8-position of the fluoroquinolones.

Experimental

Materials Levofloxacin (LVFX) and sitafloxacin (STFX) were synthesized at Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan. Sparfloxacin (SPFX; Dai-Nippon Pharmaceutical Co., Ltd., Osaka, Japan), enoxacin (ENX; Dai-Nippon Pharmaceutical Co., Ltd., Osaka, Japan), lomefloxacin (LFLX; Hokuriku Pharmaceutical Co., Ltd., Fukui, Japan) and ciprofloxacin (CPFX; Bayer A.G., Leverkusen-Bayerwerk., Germany) were commercial products. DMPO, 2-methyl-2-nitrosopropane dimer (MNP dimer) and catalase were purchased from Sigma Co., Ltd., (St. Louis, MO, U.S.A). TEMP monohydrate and 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene ; BHT) were purchased from Aldrich Chemical Co., Ltd., (Milwaukee, WI, U.S.A) and Tokyo Kasei Co., Ltd., (Tokyo, Japan), respectively.

Detection of Free Radicals and Active Oxygen during Photolysis of Fluoroquinolone Solution by Spin-Trapping ESR measurements were recorded on a JEOL JES-FE2XG spectrometer (JEOL, Tokyo, Japan) with 100 KHz field modulation operating at 9.42 GHz and at room temperature. The following instrumental parameters were employed: modulation amplitude, 0.063 mT; microwave power, 8.0 mW; scan time, 2 min.

The fluoroquinolones (200 μM/100 μl pH 7 Sörensen buffer solution) containing DMPO (*ca.* 180 mM) or MNP (230 mM) (20 μl and 25 μl, respectively) were irradiated with fluorescent lamps (Biophotochamber LX-2100 (TAITEC, Tokyo)) at approximately 10000 lux for 20 min. The resultant solution was transferred to capillary tubes (Drummond Microcaps: 50 μl) sealed with Terumoseal (Terumo, Tokyo) and measured by ESR.

Effect of Various Scavengers on Generation of Free Radical and Active Oxygen during Photolysis of Fluoroquinolone Solution by Spin-Trapping SPFX (2 mM/100 μl pH 7 Sörensen buffer solution) containing DMPO (180 mM/20 μl) in the presence of BHT (0.1 M) or catalase (350 units) was irradiated with fluorescent lamps at approximately 10000 lux. At specified time points, aliquots were removed, placed into a capillary cell and analyzed by ESR. ESR conditions were as described above.

Detection of $^1O_2$ during Photolysis of Fluoroquinolone Solution by TEMP Fluoroquinolones (200 mM/1 μl pH 7 Sörensen buffer solution) in the presence of TEMP (20 mM) were irradiated with fluorescent lamps of approximately 10000 lux for several hours. At specified time points, aliquots were removed, placed into a capillary cell and analyzed by ESR.

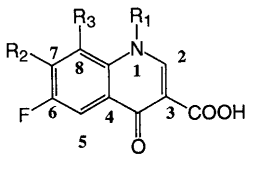

Fig. 1. Basic Structure of New Quinolones

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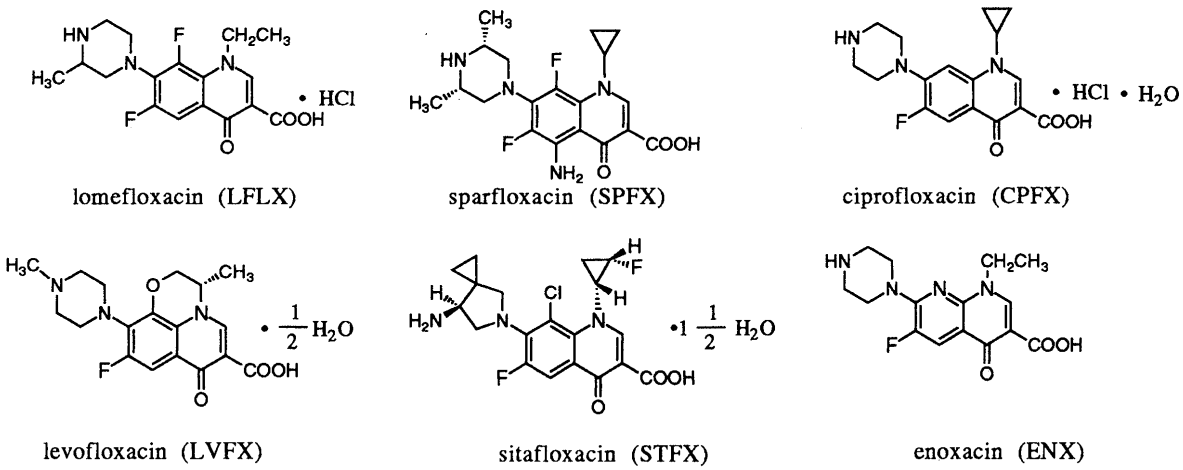

Fig. 2. Chemical Structure of Fluoroquinolones

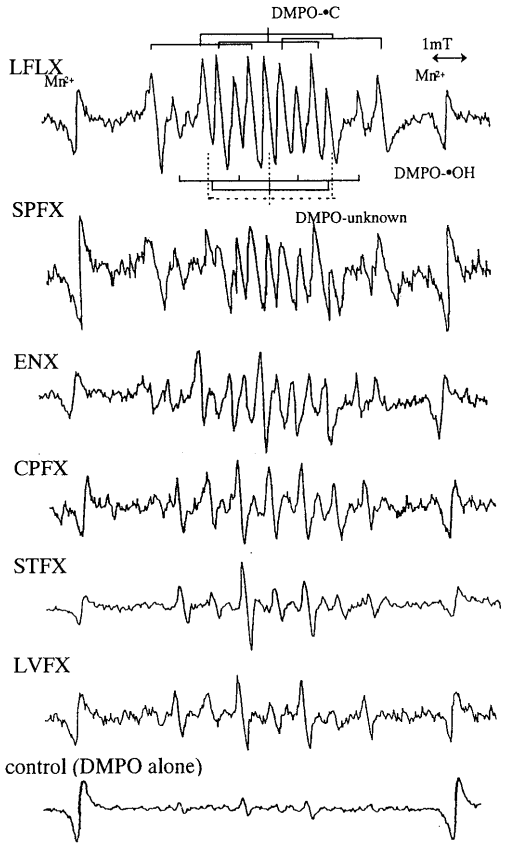

Fig. 3. ESR Spectra of Photoirradiated Aqueous Fluoroquinolone Solution in the Presence of DMPO

Samples containing 200 μM fluoroquinolone solution and DMPO 20 μl in Sörensen buffer (pH 7.0) were irradiated with fluorescent lamps (approximately 10000 lux) for 20 min.

The following instrumental parameters were employed: modulation amplitude, 0.08 mT; microwave power, 8.0 mW; scan time, 2 min.

After recording, the intensity of the lowest field peak of the TEMPO signal was normalized as a relative value when compared to the standard signal intensity of manganese oxide.

Results and Discussion

Detection of Free Radicals and Active Oxygen Species during Photolysis of Fluoroquinolone Solutions by Spin-Trapping Various aqueous fluoroquinolone solutions containing DMPO were irradiated with fluorescent lamps at approximately 10000 lux and were then measured by ESR spectroscopy. Figure 3 shows the ESR spectra of the fluoroquinolone solutions in the presence of DMPO after photoirradiation for 20 min. The fluoroquinolones examined either displayed spectra with many signals (LFLX, SPFX, ENX) or spectra with relatively few signals (CPFX, LVFX, STFX). These results are independent of irradiation time.

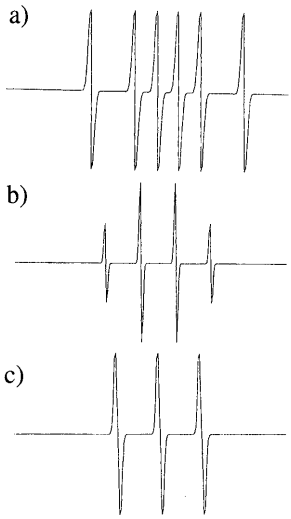

Fig. 4. Computer-Simulated Spectrum Using the Coupling Constant Given in the Text and a Line Width of 0.06 mT (Gaussian Line Shape)

a) DMPO-·C adduct, b) DMPO-·OH adduct, c) Unknown adduct

The ESR spectrum of LFLX was found to consist of overlapping sextet ($a_N = 1.47$ mT, $a_H = 2.21$ mT, $g = 2.0062$, Figure 4a), quartet ($a_N = 1.42$ mT and hyperfine coupling constant of hydrogen, $a_H = 1.42$ mT, Figure 4b), as well as a triplet (hyperfine coupling constant of nitrogen, $a_N = 1.42$ mT, Figure 4c). The sextet signal was assigned to an aryl carbon-centered radical (·C), because the values found here are close to those reported for the 2,6-$Cl_2C_6H_4$ radical ($a_N = 1.50$ mT, $a_H = 2.12$ mT, $g = 2.00616$).[9]

In order to confirm the generation of this aryl carbon centered radical, the MNP spin trapping agent was employed because it only reacts with carbon-centered radicals. When LFLX in aqueous solution was irradiated in the presence of MNP, a triplet signal without additional

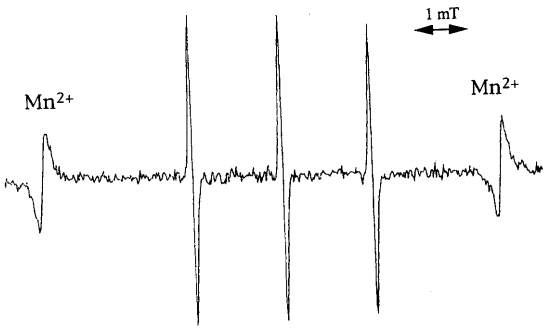

Fig. 5. ESR Spectrum of a Photoirradiated Aqueous LFLX Solution in the Presence of MNP

Samples containing 200 μM LFLX and 230 mM MNP in Sörensen buffer (pH 7.0) were irradiated with fluorescent lamps (approximately 10000 lux) for 10 min.

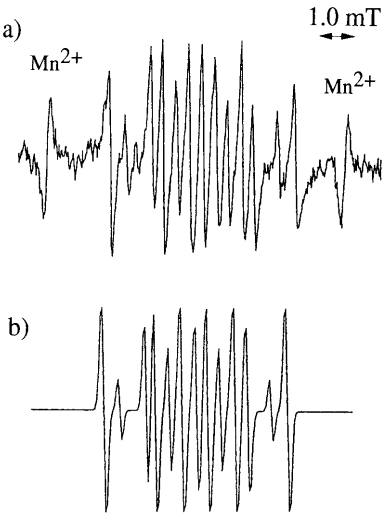

Fig. 6. ESR Spectrum of a Photoirradiated Aqueous LFLX Solution in the Presence of DMPO (a), and Computer-simulated Spectrum using Coupling Constant Given in the Text and a Line Width of 0.06 mT (Gaussian Line Shape) (b)

hyperfine splitting was observed in the ESR spectrum seen in Figure 5. This triplet signal must be due to trapping of the carbon centered radical formed during photoirradiation. Furthermore, the triplet signal suggested that the structure of the radical did not contain hydrogen coupled to an unpaired electron. Morimura *et al.* reported that orbifloxacin (ORFX), which is a fluoroquinolone derivative having a fluorine at the 8-position, was easily photodecomposed,[10] and the structure of the main products are a defluorinated analogue of ORFX. That is, the carbon-centered radical must be formed by the loss of fluorine at the 8 position of a fluoroquinolone skeleton.

The quartet-signal had a typical 1:2:2:1 pattern and was readily attributed to the hydroxyl radical (·OH) adduct. From this pattern, the hyperfine coupling constants of nitrogen and hydrogen are calculated to be the same, 1.42 mT (Figure 4b). These constants are consistent with those values in the literature.

The last triplet signal was due to an unknown adduct ($a_N = 1.42$ mT: Figure 4c). This may represent a dialkyl adduct formed after the loss of a hydrogen atom at the α-position of DMPO.

The ESR spectrum simulated using these hyperfine coupling constants corresponded well with the observed

Table 1. ESR Parameters of Radicals Trapped during the Photoirradiation of Fluoroquinolones

Fluoroquinolone	Trapping agent	Trapped radical	Hyperfine coupling constant
LFLX	DMPO	·OH	$a_N = 1.42$ mT, $a_H = 1.42$ mT
		·C	$a_N = 1.47$ mT, $a_H = 2.21$ mT
		Unknown	$a_N = 1.42$ mT
	MNP	·C	$a_N = 1.42$ mT
SPFX	DMPO	·OH	$a_N = 1.42$ mT, $a_H = 1.42$ mT
		·C	$a_N = 1.47$ mT, $a_H = 2.21$ mT
		Unknown	$a_N = 1.42$ mT
ENX	DMPO	·OH	$a_N = 1.42$ mT, $a_H = 1.42$ mT
		·C	$a_N = 1.47$ mT, $a_H = 2.21$ mT
		Unknown	$a_N = 1.42$ mT
CPFX	DMPO	·OH	$a_N = 1.42$ mT, $a_H = 1.42$ mT
		Unknown	$a_N = 1.42$ mT
STFX	DMPO	·OH	$a_N = 1.42$ mT, $a_H = 1.42$ mT
		Unknown	$a_N = 1.42$ mT
LVFX	DMPO	·OH	$a_N = 1.42$ mT, $a_H = 1.42$ mT
		Unknown	$a_N = 1.42$ mT

one, suggesting that no other radicals were presented (Figure 6). Although we could not detect the superoxide anion radical ($O_2^{\cdot -}$) adduct,[11] we could detect the hydroxyl radical adduct. It is well known that the superoxide anion radical is unstable in an aqueous solution and rapidly disproportionates into hydrogen peroxide and then degrades into a hydroxyl radical by exposure to light. To ascertain the generation of H_2O_2, we performed the effect of scavengers on the generation of free radical and active oxygen species during photoirradiation, which is described in the next section. The generation of the superoxide anion radical was also supported by the report of Nagano and his co-workers that LFLX, ENX, CPFX and ofloxacin produce a superoxide anion radical exposed to sunlight for 1.5 h by means of optical spectrometry using a nitroblue tetrazolium reduction method in the aqueous solution.[4] In general, the DMPO spin trapping method is not effective for detecting the superoxide anion radical because of its low reactivity with DMPO, $k = 1.6 \times 10^3$ mol^{-1} s^{-1}, as well as the low stability of the DMPO–$O_2^{\cdot -}$ adduct.

The spectra of SPFX and ENX were analyzed in the same manner as LFLX. As seen in the LFLX spectrum, the spectra of SPFX and ENX were considered to contain signals from the same three radicals, an aryl carbon centered radical, a hydroxyl radical and an unknown radical.

In contrast, the ESR spectra of CPFX, STFX and LVFX did not show the same pattern of signals. The only signals observed in the spectra were the 1:2:2:1 quartet signal due to a hydroxyl radical and the triplet signal due to an unknown radical. The ESR parameters of radicals trapped during photolysis of the fluoroquinolones are listed in Table 1.

Effect of Scavenger The scavengers used were BHT and catalase. BHT is known to scavenge any free radicals, whereas catalase scavenges only hydrogen peroxide. Among fluoroquinolones, SPFX was chosen for this further study because it is the most soluble of the fluoroquinolones.

The scavengers were added to aqueous solutions of

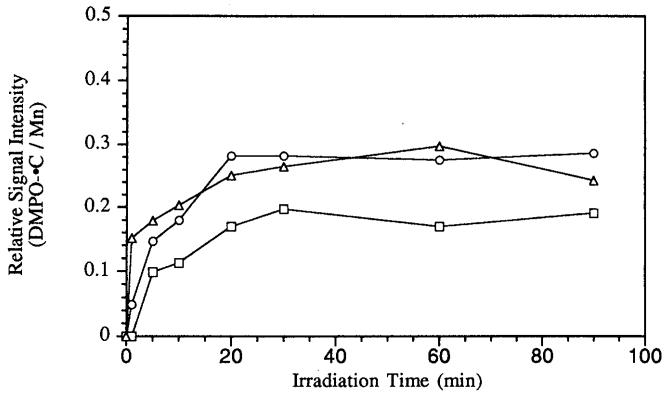

Fig. 7. Time Course of DMPO–·C Production of Photoirradiated Aqueous SPFX Solution in the Presence or Absence of Scavengers

Samples containing 2 mM SPFX, DMPO 20 μl and 0.1 M BHT or 350 units of catalase in Sörensen buffer (pH 7.0) were irradiated with fluorescent lamps (approximately 10000 lux). —○— control, —□— BHT, —△— catalase

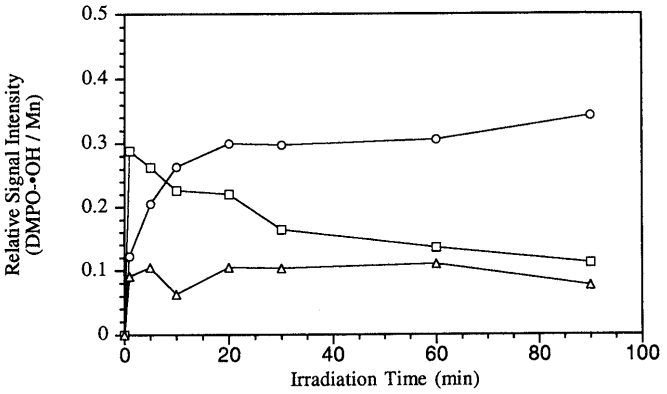

Fig. 8. Time Course of DMPO–·OH Production of Photoirradiated Aqueous SPFX Solution in the Presence or Absence of Scavengers

Samples containing 2 mM SPFX, DMPO 20 μl and 0.1 M BHT or 350 units of catalase in Sörensen buffer (pH 7.0) were irradiated with fluorescent lamps (approximately 10000 lux). —○— control, —□— BHT, —△— catalase

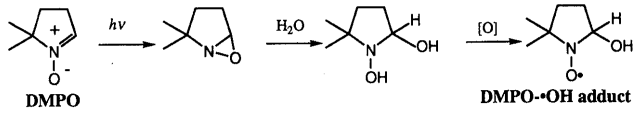

Chart 1

SPFX and irradiated under similar conditions. Figures 7 and 8 show the effect of the scavengers on the generation of free radicals. Since DMPO and the DMPO adducts are unstable, this method does not allow for accurate quantification. Also, the DMPO–OH adduct can be produced not only by reaction with a hydroxyl radical but also by the self-decomposition of DMPO by light (Chart 1). Here, the addition of BHT to the SPFX solution decreased the signal intensity of both ·C and ·OH adducts in the ESR spectrum. The addition of catalase to the SPFX solution also decreased the signal intensity of the ·OH adduct. These effects of scavengers supported the proposed generation of carbon-centered and hydroxyl radicals. Moreover, the source of the hydroxyl radical is assumed to be the superoxide anion radical.

Detection of $^1O_2$ during Photolysis of the Fluoroquinolone Solution in the Presence of TEMP TEMP is oxidized by $^1O_2$ to TEMPO, a stable nitroxide radical

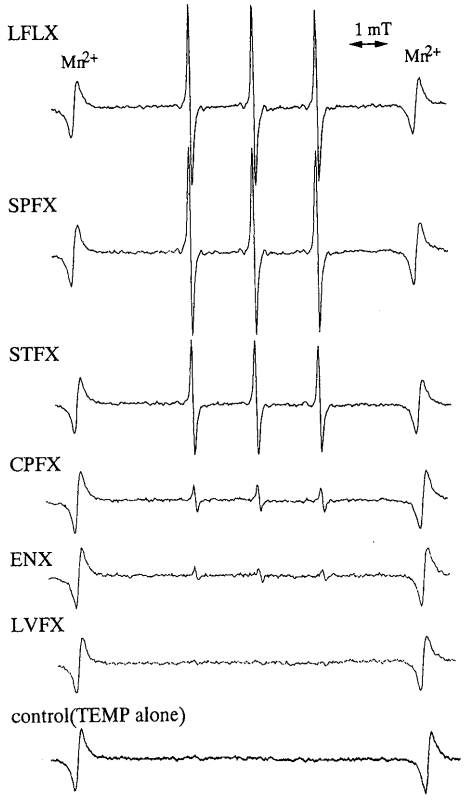

Fig. 9. ESR Spectra of Photoirradiated Aqueous Fluoroquinolone Solution in the Presence of TEMP

Samples containing 200 μM fluoroquinolone solution and TEMP 20 mM in Sörensen buffer (pH 7.0) were irradiated with fluorescent lamps (approximately 10000 lux) for 48 h.

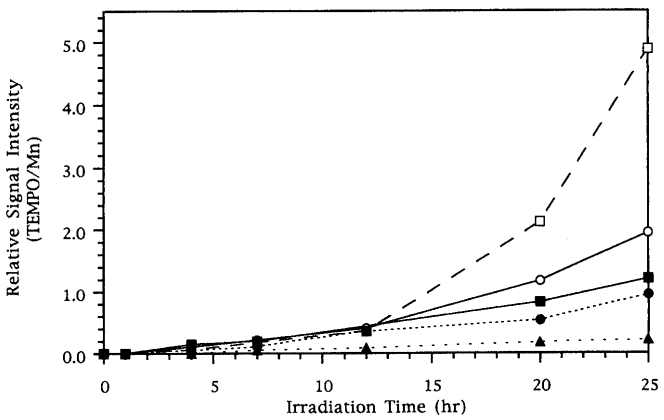

Fig. 10. Time Course of TEMPO Production of Photoirradiated Aqueous SPFX Solution in the Presence or Absence of Various Scavengers

Samples containing 200 μM SPFX solution and 20 mM TEMP in Sörensen buffer (pH 7.0) were irradiated with fluorescent lamps (approximately 10000 lux). —○— SPFX alone, —□— in D_2O, —▲— with NaN_3 (0.1 M), –●– with β-carotene (0.1 M), —■— with BHT (0.1 M)

that shows up as a triplet signal when analyzed by ESR spectrometry. Using this method, we demonstrated that the fluoroquinolone solutions generate $^1O_2$ during photoirradiation. As shown in Figure 9, LFLX, SPFX and STFX gave triplet signals, while LVFX did not. In order to confirm the generation of $^1O_2$, we performed further experiments. The lifetime of $^1O_2$ in D_2O is longer than in H_2O, illustrating the deuterium effect. When SPFX D_2O solution was exposed to light, TEMPO production

was increased (Figure 10). It is well known that sodium azide quenches $^1O_2$ efficiently. When sodium azide was added to SPFX aqueous solution, TEMPO production was extremely decreased. The result confirmed that TEMPO was produced mainly by the reaction with $^1O_2$ in aqueous fluoroquinolone solution irradiated with light. TEMPO itself was very stable, even upon UV irradiation.[12] Figure 11 shows the change in TEMPO production with time for various fluoroquinolones during photoirradiation. SPFX and LFLX produced the greatest amounts of $^1O_2$, while LVFX generated very little. The fluoroquinolones having a fluorine substituent at the 8-position were the most efficient generators of $^1O_2$.

Wagai *et al.* studied the phototoxicity of fluoroquinolones and showed that the potency of LFLX was greater than that of LVFX.[13] Also, Matsumoto *et al.* reported that the fluoroquinolones which are substituted with fluorine at the 8-position had increased levels of cytotoxicities when they were degraded using long-wavelength UV light (UVA) irradiation.[14] $^1O_2$ is a

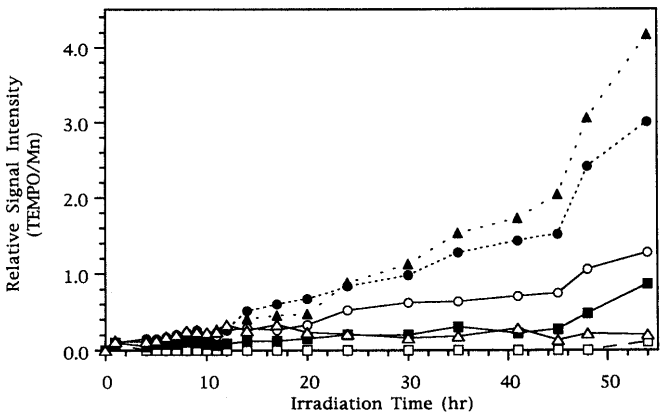

Fig. 11. Time Course of TEMPO Production of Photoirradiated Aqueous Fluoroquinolone Solution

Samples containing 200 μM fluoroquinolone solution and 20 mM TEMP in Sörensen buffer (pH 7.0) were irradiated with fluorescent lamps (approximately 10000 lux). --●-- LFLX, —▲— SPFX, —■— CPFX, —△— ENX, —○— STFX, —□— LVFX

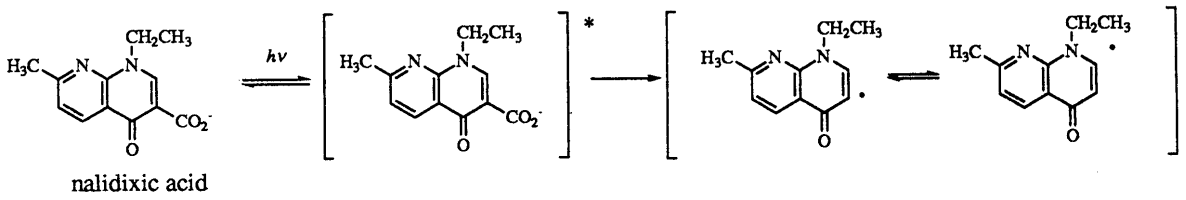

Chart 2

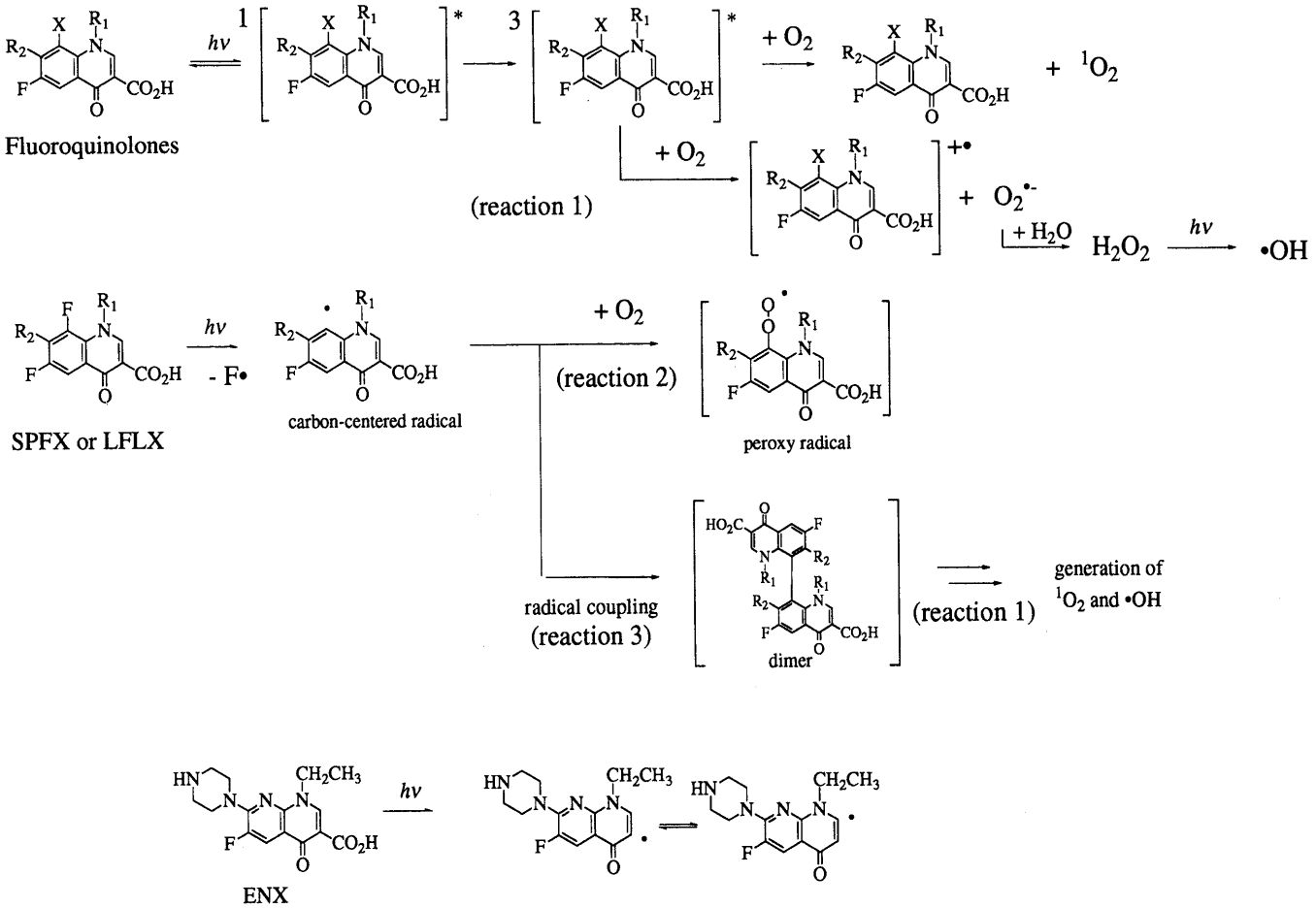

Chart 3. Proposed Mechanisms of the Generation of Active Species by the Photoirradiation of Fluoroquinolones

strong oxidizing agent, so these relative cytotoxicity might be related to the amount of $^1O_2$ present in the aqueous solution system.

Mechanism of Generation of Free Radical and Active Oxygen Species As described above, the different fluoroquinolones produced qualitatively and quantitatively different active oxygen species in our ESR experiments. These findings allowed the fluoroquinolones to be classified into three types based on the radical production and structure: 1) fluoroquinolones which generate large amounts of $^1O_2$ and have fluorine at the 8-position (LFLX, SPFX); 2) fluoroquinolones which generate intermediate amounts of $^1O_2$ and have no fluorine at the 8-position (CPFX, STFX, ENX); 3) a fluoroquinolone which generates very little $^1O_2$ and has an oxazine ring (LVFX).

The mechanism for active oxygen generation that all of the compounds have available to them is through a photosensitizing reaction, which may formed by triplet excited fluoroquinolones reacting with molecular oxygen (reaction 1). There might be two reaction pathways of triplet excited fluoroquinolones with oxygen molecules. One generates $^1O_2$ due to energy transfer. The other generates $O_2^{\cdot -}$ and fluoroquinolone cation radicals due to electron transfer. $O_2^{\cdot -}$ immediately disproportionates into hydrogen peroxide. Hydrogen peroxide cleaves into ·OH by light. We propose that the observed differences in $^1O_2$ may be explained by the availability of two additional reaction pathways specific for the photoirradiation of the 8-fluoro substituted compounds in aqueous solution. The first pathway begins with the homolytic elimination of fluorine to produce ·C. Molecular oxygen then joins to the ·C, resulting in the formation of a peroxy radical at the 8-position (reaction 2). The second, additional pathway involves of the ·C (reaction 3). This is feasible as this radical is not sterically hindered. The photosensitizer could then generate large amounts of $^1O_2$.

To explain the differences in $^1O_2$ generation, the fluoroquinolones without an 8-fluoro substituent should not be able to generate a radical at that position. However, ENX, STFX and CPFX produced low amounts of $^1O_2$. The ENX radical may be formed concomitantly with decarboxylation, thus forming a ·C at positions 2 or 3, a known reaction of nalidixic acid (Chart 2).[15] The ·C at the 2 or 3 position may not react with molecular oxygen and may not generate a dimer.

Alternatively, ENX as well as STFX and CPFX may only proceed through a photosensitizing reaction (reaction 1) which generates $^1O_2$ or ·OH.

As LVFX did not produce any detectable $^1O_2$, LVFX or its degradation products are not active photosensitizers. Possible structures for these degradation products are summarized in Chart 3. Further studies are underway to clarify the structure of the degradation products and to fully explain the results found here.

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