Free Rad. Res. Comms., Vol. 17, No. 6, pp. 387–398
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POSSIBLE REASONS FOR DIFFERENCES IN PHOTOTOXIC POTENTIAL OF 5 QUINOLONE ANTIBACTERIAL AGENTS: GENERATION OF TOXIC OXYGEN

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(*Received June 15, 1992; in revised form August 21, 1992*)

The reason for the differences in phototoxic potential between the 5 quinolone antibacterial agents lomefloxacin, enoxacin, ciprofloxacin, ofloxacin and DR-3355 (the s-isomer of ofloxacin) in mice was investigated. Superoxide anion, hydrogen peroxide (H_2O_2), and bleaching of p-nitrosodimethylaniline (B-NDMA) were detected in quinolone solutions during irradiation with ultraviolet-A (UVA). Apparent levels of H_2O_2 and the B-NDMA per mole of quinolone paralleled the phototoxic potentials in the mice. The N-NDMA induced by quinolones and UVA was inhibited partially by treatment with D-mannitol and dimethylsulfoxide, and also with diethylenetriamine-pentaaceticacid (DTPA), suggesting that Haber-Weiss and Fenton reactions occurred. UVA concentration-dependently increased the level of the B-NDMA in H_2O_2 solution and the swelling in the ear pretreated by intra-auricular injection of H_2O_2. Both augmentations were inhibited by DTPA or DMSO. The swelling induced by the 5 quinolones and UVA was completely inhibited by pretreatment with dimethylsulfoxide. Oxygen consumption was detectable during the photodegradation, and increased with time. These results showed that the phototoxic potentials of the 5 quinolones were probably related to the amounts of toxic oxygens generated in the target cells during irradiation.

KEY WORDS: Phototoxicity, ultraviolet-A, quinolone, toxic oxygens.

INTRODUCTION

Quinolone antibacterial agents, widely used as oral preparations, have been under development worldwide since the discovery of nalidixic acid in the 1960's. Great progress has been made in broadening their antibacterial spectrum against both gram-negative and gram-positive organisms, as well as in improving their pharmacokinetic profiles (absorption, distribution, metabolism and excretion). Occasionally, however, they produce side-effects in a very small percentage of patients.[1] One of these side effects is photosensitivity.

We recently demonstrated in a Balb/c mouse model that there were differences in phototoxic potential between the 5 quinolones lomefloxacin (LMFX), enoxacin (ENX), ciprofloxacin (CPFX), ofloxacin (OFLX) and DR-3355 (the s-isomer of OFLX).[2] The reasons for these differences and the related mechanisms require further investigation.

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Concerning the mechanism(s) of the phototoxicity induced by these 5 quinolones, we have already shown that the xanthine oxidase pathway participates in the inflammatory process,[3] and that both respiratory function and the lysosomal membrane are probably damaged by reactive oxygens derived from the quinolones during ultraviolet-A (UVA) irradiation.[4]

In this study, to examine the generation of reactive oxygens during photochemical reactions between these quinolones and UVA, we determined the apparent levels of superoxide anion radicals ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and of the bleaching of p-nitrosodimethylaniline (B-NDMA) by a modification of standard spectrophotometric methods. We demonstrated that the apparent levels of H_2O_2 generated and of the B-NDMA in the irradiated quinolone solutions reflect the cutaneous phototoxic potentials in mice described previously[2,5] and that the phototoxic potentials of the 5 quinolones were probably dependent on the amount of toxic oxygens generated in the target cells during irradiation.

MATERIALS AND METHODS

Chemicals

The quinolones LMFX, ENX, CPFX, OFLX, and DR-3355 used in this study (refer to Figure 1 for chemical structures) were synthesized or extracted from commercial tablets or powder in our laboratory. Superoxide dismutase (SOD; from bovine erythrocyte; 3200 units/mg protein), horse-heart ferricytochrome c (type VI), horseradish peroxidase (HRP; type VI; RZ = 3.1), guaiacol and diethylenetriaminepentaaceticacid (DTPA) were purchased from Sigma Chemical Co., St. Louis, MO, USA. p-Nitrosodimethylaniline was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, USA. All other chemicals employed were of reagent grade.

Light source and irradiation

Details of the light source and method of irradiation have been described previously.[6] Briefly, the light source consisted of a bank of 10 Toshiba FL20SBLB tubes, emitting

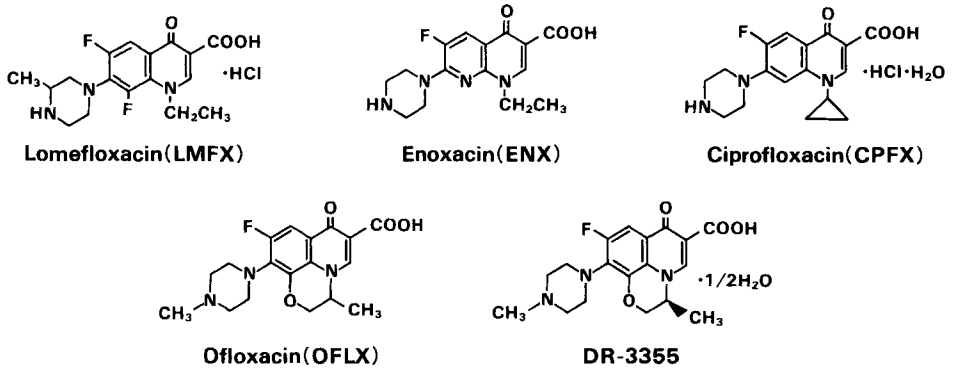

FIGURE 1 Chemical structures of the quinolone antibacterial agents used.

radiation in a wavelength range of 300–400 nm. A 3-mm-thick pane of glass (Floatglass, Asahi Glass, Tokyo, Japan) was used as a filter to eliminate wavelengths below 320 nm. The radiation dose at 15 cm from the tubes was 1.5 mW/sec·cm^2 in the irradiated region, as measured with a UVX Digital Radiometer fitted with a UVX-36 sensor responding at 365 nm (UVP Inc., San Gabriel, CA, USA). An electric fan prevented the temperature in the irradiated area from rising.

Determination of superoxide anion radicals, hydrogen peroxide and bleaching of p-nitrosodimethylaniline in quinolone solution under UVA irradiation

Superoxide anions, hydrogen peroxide and bleaching of p-nitrosodimethylaniline were determined spectrophotometrically with a Hitachi model 557 double-wavelength double-beam spectrophotometer. The baseline was corrected automatically. A quartz cuvette with a light-path of 1 cm length was used.

Superoxide anions ($\cdot O_2^-$) were determined by a modification of the cytochrome c reduction method used by Babior *et al.*[7] Sample and reference cuvettes were prepared. The sample cuvette contained a mixture of 100 nmole cytochrome c and 100 nmole of one of the quinolones other than ENX dissolved in 0.1 M phosphate buffer, pH 7.8. ENX was dissolved in dimethylsulfoxide (DMSO) and then diluted with the buffer (final DMSO concentration: 25 mM, about 0.2%). The final volume in each case was 3.0 ml. The reference cuvette contained the same mixture as was in the sample cuvette, mixed with 1000 units of SOD to give the same final volume as the sample cuvette. Both cuvettes were placed horizontally against a black background and exposed to UVA for 120 sec at an intensity of 1.5 mW/sec·cm^2 (total UVA energy was 0.18 joule/cm^2), and the difference spectrum between 500 to 600 nm was measured. Cytochrome c reduction was determined by measuring the absorbance at 550 nm. Preliminary studies showed no change in the cytochrome c spectrum under irradiation with UVA for 120 sec (data not shown). ΔEmM (ferrocytochrome c minus ferricytochrome c) at 550 nm was taken at 15.5.[8]

Hydrogen peroxide (H_2O_2) was determined by a modification of our previously described peroxidase assay method.[9] Assay of H_2O_2 (the first substrate of HRP) in each UVA-treated quinolone solution was performed with guaiacol as the hydrogen donor (the second substrate). Standard assay systems contained 33 mM guaiacol, 0.004 guaiacol units of HRP,[10] and 33 mM citrate-phosphate buffer, pH 4.5, in a total volume of 3.02 ml. Each quinolone (0.1 mM) except ENX was dissolved in Dulbecco's phosphate buffered saline (PBS, pH 7.2). ENX was dissolved in 25 mM DMSO-PBS. The solution was exposed to UVA for 120 sec (0.18 joule/cm^2). Five or more minutes after irradiation, the UVA-reacted quinolone solution was poured into the cuvette containing the second substrate, buffered. The reaction was started by adding 20 μl of 0.004 guaiacol units of HRP to the cuvette, and the absorbance at 470 nm was recorded. Initial velocity (ΔOD_{470}/sec) was then measured. The amount of H_2O_2 was calculated from the standard curve.

Bleaching of p-nitrosodimethylaniline was determined by a modification of the method of Bors *et al.*[11] The standard assay system contained 0.05 mM p-nitrosodimethylaniline, corresponding to an initial absorbance of 1.7, and 0.1 mM quinolone dissolved in PBS or 25 mM DMSO-PBS, total volume 3.0 ml (sample and reference cuvettes). The sample cuvette was exposed to UVA for 120 sec (0.18 joule/cm^2). The difference in absorbance between the two cuvettes at 440 nm was then measured. The specificity of the bleaching method to hydroxyl radical

(OH·) determination in irradiated quinolone solutions (0.1 or 0.3 mM) was confirmed by using two OH· scavengers, D-mannitol and DMSO.

Experimental animals and effect of DMSO on the H_2O_2- and quinolone-induced cutaneous phototoxicity (CP)

The participation of OH· in quinolone-induced CP was investigated. The method used was described previously.[3,4] Female Balb/c strain mice, 6-week old (Charles River Japan Inc., Hino, Tokyo, Japan) were used in the study. These mice were housed four per cage in plastic cages and maintained in air-conditioned room (temperature: 23 ± 2°C, relative humidity: 55 ± 15%) with free access to commercial laboratory chow (F-2, Funabashi Farm Ltd., Funabashi, Chiba, Japan) and tap water.

H_2O_2-induced CP: Mice (n = 4) were pretreated by subcutaneous injection of DMSO (0.1 mmole/kg) or saline. The dose level of DMSO was based on the statement of Athar *et al.*[12] Two hours later, 0.03 ml of H_2O_2 (1, 10 or 100 μM) was injected directly into the auricle of mice under ether anesthesia, immediately after which they were exposed to UVA for 2 hours in a partitioned chamber so that their ears were 15 cm from the lights. CP was determined by measuring the degree of ear swelling that developed 24 hours after irradiation in comparison with that before irradiation. Three measurements were made on each ear using a dial thickness gauge micrometer (Ozaki Co., Tokyo, Japan), and the mean values were calculated. Control mice were kept prone for 2 hours in the dark.

Quinolone-induced CP: Mice (n = 4) fasted overnight were pretreated with DMSO (0.1 mmole/kg) or saline. Two hours later, they were administered quinolone orally, and immediately exposed to UVA for 4 hours in the same conditions as above. The quinolones were suspended in 0.5% methylcellulose at a concentration of 5, 20 or 80 mg/kg, and a dose of 10 ml/kg was administered. Ear swelling was determined after irradiation.

The results were expressed as mean ± SD. For statistical comparison between groups, Student's or Aspin-Welch's t-test was used.

Measurement of O_2 consumption in irradiated quinolone solution

Oxygen consumption during photodegradation was measured with a Horiba model DO-8F dissolved oxygen meter (Horiba Ltd., Kyoto, Japan). Each quinolone (0.3 mM) was dissolved in PBS or in 25 mM DMSO-PBS. Air was bubbled through the solution for 30 min in the dark to saturate it with dissolved oxygen. The electrodes of the dissolved oxygen meter (a dual electrode unit containing a temperature sensor) was inserted into the bottom of a 20 ml Erlenmeyer flask (Iwaki Glass Co., Ltd., Tokyo, Japan). The flask was then filled with the solution to exclude air and sealed with a sheet of Parafilm® (American Can Company, Greenwich, CT, USA) to avoid contact with the outside air. The electrode equipped flask was then placed on a magnetic stirrer (about 1000 rpm) and exposed to UVA (2.0 mW/sec·cm^2, measured without a glass filter). O_2 concentration and temperature of the solution were monitored over 20 min.

RESULTS

Levels of $\cdot O_2^-$ and H_2O_2 generated and of the B-NDMA

Table I shows the relative concentrations of reactive oxygens generated and the level of the B-NDMA per mole of quinolone, and the phototoxic potentials in mice.[2,5] Since $\cdot O_2^-$ and H_2O_2 were detectable in each quinolone solution and a very small amount of Fe ions were present, all the values of reactive oxygens measured were considered as apparent amounts. OFLX generated $\cdot O_2^-$, 71 μmole/mole. This value was almost the same as that for $\cdot O_2^-$ generated by DR-3355. About twice the amount was generated by CPFX, and about 3 times by ENX. A relatively very small amount of $\cdot O_2^-$ was measured in the LMFX-treated solution. On the other hand, H_2O_2 levels showed marked differences, these seen with OFLX, DR-3355 and CPFX treatment being about 0.3, 0.3 and 0.7 mmole/mole respectively, and those with LMFX and ENX, about 7 mmole/mole, showing a difference of not less than 10 or 20 times. Furthermore, the B-NDMA level with LMFX was much higher than those yielded by the other four quinolones.

The specificity to OH· of the bleaching method of OH· determination claimed by Bors *et al.*[11] was investigated (Table II). Concentrations of quinolones were set at 0.1 mM in principle, but those of OFLX and DR-3355 were at 0.3 mM because of low level of the B-NDMA at their 0.1 mM (Table I). D-mannitol[13] or DMSO[14] was added to the B-NDMA assay system and the remaining ΔOD_{440} was determined. D-mannitol at 10 or 100 mM decreased the levels of the B-NDMA to about 60% or lower. DMSO showed a concentration-dependent, but gradual decrease of the B-NDMA level with all of quinolones used except for ENX, and 250 mM DMSO decreased the level with ENX. Neither reactive oxygens nor B-NDMA were detected

TABLE I

Apparent levels of reactive oxygens generated and the level of the B-NDMA during photochemical reactions between quinolones and UVA; comparison with phototoxic potential in mice

Quinolone	Apparent levels of $\cdot O_2^-$ μmole/mole	Apparent levels of H_2O_2 mmole/mole	Bleaching at ΔOD_{440}	Phototoxic potential in mice: $ETID_{50}$[a] (mg/kg)	Phototoxic potential in mice: Incidence of erythema[b]
LMFX	20 ± 2	8.37 ± 0.52	0.880 ± 0.002	24.8	N.T.
ENX	242 ± 6	5.99 ± 0.67	0.205 ± 0.014	81.9	N.T.
CPFX	156 ± 4	0.73 ± 0.20	0.077 ± 0.017	457.9	5/6
OFLX	71 ± 3	0.30 ± 0.10	0.009 ± 0.002	428.0	0/6
DR-3355	76 ± 3	0.30 ± 0.06	0.010 ± 0.003	526.6	0/6

Each quinolone (0.1 mM) was dissolved in PBS except for ENX. ENX was dissolved in PBS containing 25 mM DMSO. The solution was exposed to UVA for 120 sec (0.18 joule/cm^2).

[a]$ETID_{50}$ (50% ear thickness increment-inducing dose): Mice were orally administered quinolones and immediately exposed to UVA. The percent alteration in ear thickness was determined by measuring the ear swelling at 24 h after irradiation and before. $ETID_{50}$ was calculated from dose-response curve for each quinolone (reference 2).

[b]Incidence of erythema: Mice were injected with quinolone (100 mg/kg) and immediately exposed to UVA. The auricles were examined for the presence of erythema over 48 h after irradiation. Number of mice with erythema/number of mice used is indicated above (reference 5).

N.T.: Not tested.

TABLE II
Effect of D-Mannitol and DMSO on the B-NDMA induced by quinolone and UVA

	Percent of ΔOD_{440} remaining (Mean ± SD)				
		D-Mannitol		DMSO	
Quinolone (mM)	Control	10 mM	100 mM	25 mM	250 mM
LMFX (0.1)	100 ± 0.2	64 ± 5.0	44 ± 3.8	62 ± 0.5	52 ± 4.5
ENX* (0.1)	100 ± 6.8	39 ± 1.7	23 ± 2.1	N.T.	67 ± 7.2
CPFX (0.1)	100 ± 22	34 ± 4.7	32 ± 6.5	46 ± 5.0	28 ± 6.4
OFLX (0.3)	100 ± 16	44 ± 14	56 ± 20	49 ± 12	39 ± 4.7
DR-3355 (0.3)	100 ± 8.5	60 ± 15	46 ± 11	57 ± 5.1	46 ± 8.5

* Solvent of ENX contained 25 mM DMSO.
Sample and reference cuvettes contained 0.1 or 0.3 mM quinolone, 0.05 mM p-nitrosodimethylaniline and one of the two scavengers indicated above. After the baseline was corrected, a sample cuvette was exposed to UVA for 120 sec. The difference absorbance at 440 nm was then measured (n = 4). Cuvettes without scavenger served as control, and the difference absorbance from control was defined as 100%.
N.T.: Not tested.

in either the solution without quinolone after UVA irradiation or in the solution with quinolone before irradiation (data not shown). The amounts of H_2O_2 generated and the levels of B-NDMA paralleled to their previously described phototoxic potentials in mice.[2,5]

Effect of DTPA on UVA-irradiated H_2O_2 and quinolone solutions

Since $\cdot O_2^-$ and H_2O_2 were detected in the irradiated quinolone solution, some of the B-NDMA found may have been derived from them through the Haber-Weiss and Fenton reactions. In the solution of H_2O_2 alone, the B-NDMA was determined as a function of H_2O_2 concentration. On irradiation, differences in the levels of the B-NDMA with and without UVA were very small up to 10^{-3} M H_2O_2, but the differences became much greater at 10^{-2} and 10^{-1} M H_2O_2. DTPA, which chelates Fe ion,[15] decreased UVA-induced B-NDMA at high concentration range (Figure 2). DTPA also decreased the level of B-NDMA measured in all quinolone solutions to about 60%, or less (Table III).

Effect of DMSO on H_2O_2- and quinolone-induced CP

Figure 3 shows the effect of DMSO on H_2O_2-induced CP. H_2O_2 alone induced slight ear swelling, which, on irradiation, increased significantly depending on the H_2O_2 concentration. This augmented reaction was inhibited by pretreatment with DMSO (0.1 mmole/kg) at H_2O_2 concentrations of 10 and 100 μM. There was no significant difference between the swelling induced by H_2O_2 alone and that by a combination of H_2O_2, DMSO and UVA. UVA alone did not affect the swelling (data not shown).

DMSO was also effective in CP induced by the 5 quinolones. All of the swelling reactions were significantly inhibited by pretreatment with DMSO (Table IV).

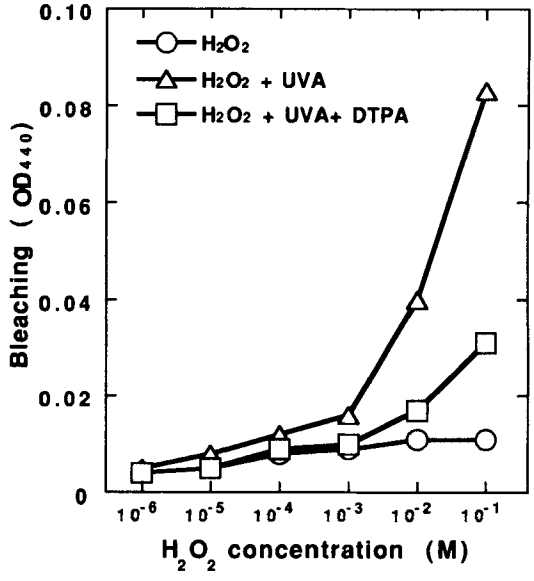

FIGURE 2 Effect of DTPA on UVA irradiated H_2O_2. H_2O_2 solutions with and without 1 mM DTPA were exposed to UVA for 120 sec. Non-irradiated H_2O_2 solution served as control. The B-NDMA was determined at 440 nm.

TABLE III
Effect of DTPA on the B-NDMA induced by quinolone and UVA

	Percent of ΔOD_{440} remaining (Mean ± SD)	
Quinolone (mM)	Control	DTPA (1 mM)
LMFX (0.1)	100 ± 0.9	65.2 ± 1.7
ENX (0.1)	100 ± 0.8	19.3 ± 3.7
CPFX (0.1)	100 ± 2.1	43.4 ± 8.2
OFLX (0.3)	100 ± 15.8	37.0 ± 7.6
DR-3355 (0.3)	100 ± 8.5	53.1 ± 10.8

Sample and reference cuvettes contained 0.1 or 0.3 mM quinolone, 0.05 mM p-nitrosodimethylaniline and 1 mM DTPA. After the baseline was corrected, a sample cuvette was exposed to UVA for 120 sec. The difference absorbance at 440 nm was then measured (n = 4). Cuvettes without DTPA served as control, and the difference absorbance from control was defined as 100%.

Oxygen consumption during photodegradation

Saturated O_2 concentrations at 23.5°C were about 8.0 to 8.8 ppm (about 0.3 mM). Although the temperature rose from 23.5 to 24.5°C during irradiation for 5 min, it remained constant at 24.5°C at each subsequent measurement. With the rise in temperature, saturated O_2 concentration in the solvent decreased slightly (about

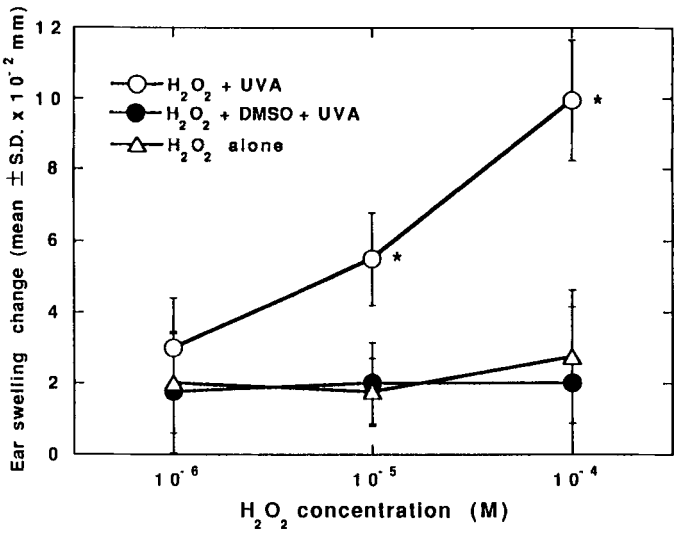

FIGURE 3 Effect of DMSO on H_2O_2-induced CP. The mice (n = 4) were pretreated with DMSO (0.1 mmole/kg) and 2 h later, were given injections of H_2O_2 in the auricle, followed immediately by exposure to UVA for 2 h. The control mice were pretreated with saline and exposed or kept for 2 h without irradiation. The increment in ear thickness was determined by subtracting ear thickness before irradiation from that 24 h after. * $p < 0.001$, significantly different from the non-irradiated group at the same H_2O_2 concentration.

TABLE IV
Effect of DMSO on quinolone-induced CP

Quinolone	Dose (mg/kg)	n	Ear thickness change (Mean ± SD; $\times 10^{-2}$ mm) Saline	DMSO
LMFX	50	4	9.5 ± 2.6	0.5 ± 3.1*
ENX	200	4	9.8 ± 1.7	2.0 ± 1.4*
CPFX	800	4	10.8 ± 2.2	−0.3 ± 1.0*
OFLX	800	4	8.5 ± 1.9	0.3 ± 2.2*
DR-3355	800	4	10.0 ± 3.4	1.0 ± 1.4*

* $p < 0.005$ vs saline control.

0.3 ppm) from the concentration before irradiation. When the quinolones (0.3 mM) were exposed to UVA, their O_2 consumption was much greater than that of the solvent, and increased with time. O_2 consumption by irradiated ENX and LMFX was much greater than that by irradiated OFLX, CPFX and DR-3355 for the same irradiation period (Figure 4).

DISCUSSION

Our previous reports showed differences in phototoxic potential between the 5 quinolones.[2,5] Oral and intravenous studies revealed that the phototoxic potency of

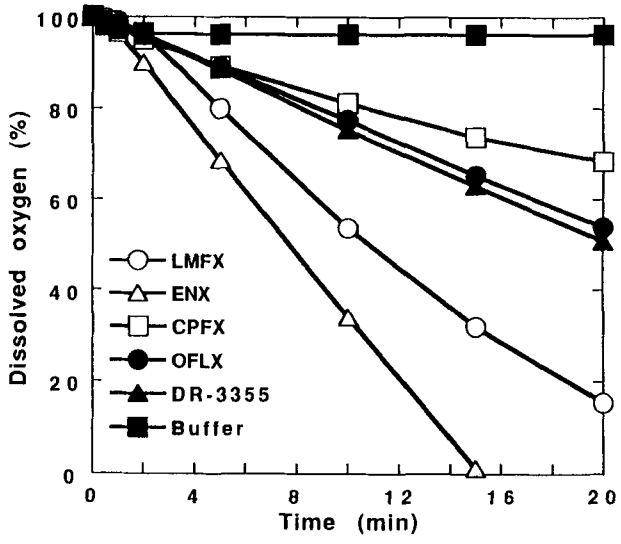

FIGURE 4 Oxygen consumption in quinolone solution during UVA irradiation. Air was bubbled through each quinolone solution (0.3 mM) for 30 min in dark to saturate it with dissolved oxygen. Then the solution was exposed to UVA for 20 min. O_2 concentration and temperature of the solution were monitored during irradiation. O_2 concentration after air bubbling was defined as 100%. The temperature change vs time was described in "Results".

the 5 quinolones decreased in the order, LMFX > ENX > CPFX > OFLX, DR-3355. The reason for these differences may have been the rate of generation of toxic reactive oxygens.[4] In the present study, we measured $\cdot O_2^-$, H_2O_2 and the level of the B-NDMA in order to try to account for the differences. The latter assay is alleged to measure $\cdot OH$, but it may have limited specificity.

The quinolone concentration for measuring the reactive oxygens and the B-NDMA was set at 0.1 mM principle, because these quinolones, at between 0.03 and 0.3 mM, induced concentration-dependent lysosomal membrane damage in Balb/c mouse 3T3 fibroblast cells when these were irradiated, and more than 50% of the cells were damaged when treated with 0.1 mM of quinolone and UVA exposure.[4] The measurement methods were devised in order to determine in a cuvette reactive oxygen radicals that, unlike H_2O_2 have very short half-lives, namely $\cdot O_2^-$ and $OH\cdot$.[16,17] Preliminary experiments demonstrated that UVA irradiation did not affect the assay system for $\cdot O_2^-$ or for B-NDMA and that no H_2O_2 was detected in the H_2O_2 assay system after the addition of catalase (data not shown). Specificity to $OH\cdot$ in measurements by the bleaching method was confirmed using $OH\cdot$ scavengers D-mannitol and DMSO. Addition of the scavenger, 10 mM D-mannitol or 250 mM DMSO in quinolone solution decreased the value derived from the bleaching method to about 60% or lower, but this inhibition was not complete and concentration-dependency was not so sharp in each quinolone (Table II). Therefore, the specificity to $OH\cdot$ measurement was regarded as comparatively low in the cases of these quinolones.

As Table I shows, apparent values for H_2O_2 and the level of the B-NDMA per mole of LMFX and ENX were very high compared with those for the other

quinolones, although the actual radical generation by ENX was estimated to be higher than that measured because of the presence of 25 mM DMSO as a solvent. These results reflected very well their phototoxic potential in mice. The level of $\cdot O_2^-$ generated with the 5 quinolones, however, was not reflected in *in vivo* results; LMFX treatment generated very low levels of $\cdot O_2^-$. The chemical reactivity of $\cdot O_2^-$ is modest in comparison with that of other reactive oxygen metabolites derived from it.[16] $\cdot O_2^-$ and H_2O_2 interact in the Haber-Weiss and Fenton reactions to yield OH·.[18,19] DTPA brought about decreases in the level of the B-NDMA in the quinolone solutions, suggesting that some amount of the OH· might be derived from these reactions.

In regard to the Fenton reaction, the generation of OH· was examined in H_2O_2 solution. As Figure 2 shows, UVA increased the level of the B-NDMA concentration-dependently and DTPA decreased UVA-induced the B-NDMA at 10^{-2} and 10^{-1} M H_2O_2. These findings suggest that the Fenton reaction was somewhat accelerated by UVA.

Damage to the auricle by H_2O_2 alone was then examined on the basis of ear swelling. The swelling was very slight, but irradiation augmented it significantly and concentration-dependently (10^{-5} and 10^{-4} M H_2O_2). This augmentation was inhibited by pretreatment with DMSO (Figure 3), demonstrating that toxic oxygen generation was probably accelerated *in vivo* by UVA irradiation. In addition, the quinolone-induced CP was completely inhibited by the DMSO pretreatment (Table III). We therefore suggest that the major cause of CP is toxic oxygen metabolites, which were probably generated during irradiation, both directly from the 5 quinolones and through the Haber-Weiss and Fenton reactions.[4]

Then we investigated the sources of the toxic oxygens. Mechanisms of phototoxic reactions have been divided into 4 types; those associated with reactive oxygens are categorized as Type I and Type II photodynamic reactions.[20] As Figure 4 indicates, measurement of O_2 consumption showed that all 5 quinolones consumed dissolved O_2 in the course of time. And in the poor oxygen conditions made by nitrogen gas bubbling, the level of the B-NDMA decreased for every quinolone used, to about 1/4 of that under O_2-saturated conditions (data not shown). The chemical structure of the major photodegradation product of OFLX was identified as that of the dialdehyde form of methylpiperazine,[21] and the 5 quinolones share the piperazine ring in common (Figure 1). These findings strongly suggest that dissolved O_2 was used mostly not only for oxidation of the parent compounds but also for the generation of toxic oxidants. The reason why ENX consumed O_2 faster than LMFX could be that ENX was more susceptible to oxidation in its chemical structure, not to generate toxic oxygens than LMFX was.

We have already reported the following in relation to the mechanisms of phototoxicity induced by the 5 quinolones: (1) the cause of phototoxicity did not depend on the photoproducts;[3] (2) xanthine oxidase participates in inflammatory reactions,[3] which were characterized by neutrophil infiltration;[5] (3) the enzyme substrates were derived from the damaged mitochondria;[4] and (4) SOD augmented and catalase inhibited the phototoxicity induced by these quinolones in *in vitro*.[4] In the present study, we confirmed both the generation of toxic oxygens from the 5 quinolones during irradiation, and their toxic effects in mice.

Other reports have cited cell damage caused by reactive oxygens.[22–25] Athar *et al.*[25] reported this as part of the mechanism of dihematoporphyrin ester-mediated photosensitization in mice. They proposed the following mechanism: after absorption

of light energy, dihematoporphyrin ester in an excited state transfers its energy to oxygen, thereby generating various reactive oxygen metabolites that damage the mitochondrial membranes. This damage leads to the generation of $\cdot O_2^-$ from xanthine oxidase. These anions are converted into various other strong oxidants that exacerbate tissue injury. In the case of ischemia, Grisham *et al.*[26] made a more precise proposal on the relationship between xanthine oxidase-generated $\cdot O_2^-$, neutrophil infiltration, and tissue injury. The generation of $\cdot O_2^-$ derived from xanthine oxidase not only results in the generation of other oxidants such as H_2O_2 and $OH\cdot$, but also causes the release of neutrophil activators and chemo-attractants. Stimulation of neutrophils increases the generation of strong oxidants, further aggravating tissue injury.

The 5 quinolones all show excellent pharmacokinetic profiles in both experimental animals[27,28,29] and humans.[30] These quinolones are taken up rapidly by cells, resulting in a high ratio of the intracellular to the extracellular level.[30,31] These findings show that large amounts of the quinolones are distributed rapidly into the various organs and into the cells themselves after oral administration. Once sufficient UVA energy reaches the target tissue or cells, therefore, tissue damage will start.

We therefore propose the following mechanism for the phototoxicity induced by the 5 quinolones and the reason for their differences in phototoxic potential. During UVA irradiation, quinolone in the target tissue generates toxic oxygen metabolites, which attack biological systems such as the mitochondria. The degree of cell damage is probably dependent on the balance between the amount of toxic oxygens generated from the quinolones and UVA, and the scavenging activity of the biological defense systems. If these defense systems are disrupted, cell destruction begins. Phototoxicity arises a result of the generation of toxic oxygens from quinolones during UVA irradiation and the phototoxic potentials of the 5 quinolones depend on the amounts of toxic oxygens. The spread of the tissue damage caused by this phototoxicity probably follows the same inflammatory process as occurs in ischemic disease.[26]

Acknowledgement

This work was supported by Daiichi Pharmaceutical Co. Ltd.

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Accepted by Professor B. Halliwell