Chem. Pharm. Bull. **40**(7) 1868—1870 (1992) Vol. 40, No. 7

Photodynamic Deoxyribonucleic Acid (DNA) Strand Breaking Activities of Enoxacin and Afloqualone

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Photodynamic deoxyribonucleic acid (DNA) strand breaking activities of 9 widely used drugs suspected of inducing photodermatological toxicity in humans, were examined. Enoxacin and afloqualone induced single strand breaks in supercoiled plasmid DNA following irradiation with common fluorescent lamps. The ED_{50} (effective dose which change 50% of supercoiled closed circular DNA to the open circular form) of enoxacin was 2 mM and that of afloqualone was approximately 860 μM. DNA strand breaking activity of photoirradiated enoxacin was markedly inhibited by NaN_3 and partly by superoxide dismutase and D-mannitol. That of afloqualone was inhibited by NaN_3 primarily and by catalase partly.

These results suggest that enoxacin and afloqualone are active photosensitizers and that these drugs induce phototoxicity in biological systems through type II photodynamic mechanisms. The experimental system used in assessing the photodynamic activity of drugs (*i.e.*, induction of single strand breaks in plasmid DNA) may also be useful for screening phototoxic compounds in our environment and for determining the active oxygen species involved.

Keywords photodynamic action; DNA strand break; plasmid DNA; drug; enoxacin; afloqualone; phototoxic; ED_{50}; type II; sodium azide

Many different kinds of compounds in our environment induce photobiological damages in various organisms.[1] Phototoxicity is an undesirable side effect of medicines intended for humans. Patients frequently experience photodermatological drug toxicity such as erythema, pigmentation, hardening or peeling of skin. However it is difficult to pinpoint which medicine causes these effects particularly in patients simultaneously medicated with several medicines. It is necessary to establish useful screening systems to detect drug phototoxicity.

In the previous work we demonstrated that some food additive dyes induced cell inactivation of yeast *Saccharomyces cerevisiae*[2] by photodynamic action and photoactivated acridine compounds induce not only nuclear mutation such as trp^- reversion but also mitochondrial mutation (petite mutation) in addition to cell inactivation.[3] Cell inactivation is thought to be related to membrane damage while petite induction is related to deoxyribonucleic acid (DNA) damages. Cell inactivation or petite mutation in yeast are good markers for detecting photodynamic activity of chemicals. However some chemicals which are active photosensitizers can not penetrate into the yeast cells. Recently several groups have demonstrated that DNA strand breaks are induced *in vitro* by photodynamic action of chemicals, such as thiopyronine,[4] proflavine,[5] riboflavin,[6] bilirubin,[7] promazines,[8] tetracyclines[9] and nonsteroidal antiinflammatory drugs.[10] We have also assessed the photodynamic activity of acridines[11] semiquantitatively in terms of the DNA single strand breaking (SSB) activity *in vitro*. Using this system we have also examined the phototoxic activity of 9 drugs which had been given to patients who subsequently developed photodermatological toxicity.

Materials and Methods

Plasmid DNA Plasmid YRp7 DNA was purified from *Escherichia coli* JA221/YRp7 by the method of Maniatis *et al.*[12] Crude plasmid DNA was purified by cesium chloride (CsCl)–ethidium bromide (EB) equilibrium density gradient ultra centrifugation by the method of Radloff *et al.*[13] Covalently closed circular DNA was collected and EB was removed by shaking with *n*-butanol. After dialysis with TE buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0), purified plasmid DNA was stored at 4 °C. Reagents, pipet tips and glassware were autoclaved to avoid digestion of supercoiled plasmid DNA by contaminant deoxyribonuclease.

Irradiation of Plasmid DNA in the Presence of Drugs Ten μl of plasmid DNA stock solution (415 ng) were mixed with 10 μl TE buffer including

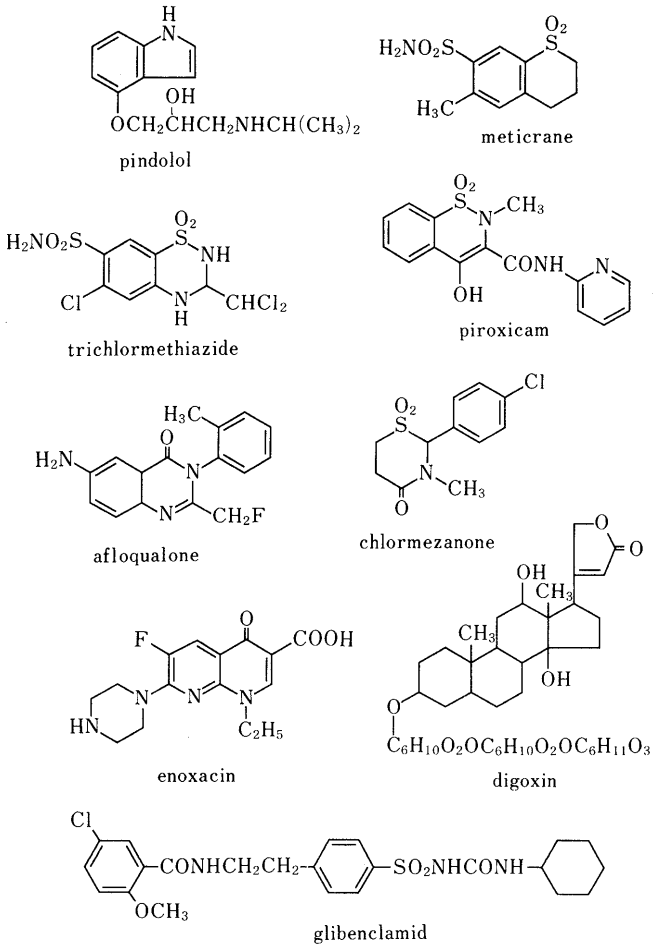

Fig. 1. Chemical Structures of Drugs Studied

one of the drugs (final concentration, 0.1—1 mM) in 1.5 ml Eppendorf tubes. Sample tubes were hung between two 15 W common fluorescent lamps each (Toshiba, mellow white FL 15N, 10 cm distance) which have broad emission spectra from 350—750 nm and were irradiated for 1 h at 9000 lux (measured with Topcon IM-3 illuminometer Tokyo Optical Co.).

Agarose Gel Electrophoresis Electrophoresis was performed with horizontal 0.7% agarose slab gels containing 0.5 μg/ml of EB in TBE buffer (0.089 M Tris–borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.3) as described in Molecular Cloning.[12] Twenty μl of photoirradiated DNA solution were mixed with 2 μl of gel loading buffer (0.25% bromphenol blue, 40% (w/v) sucrose in distilled water) and electrophoresed for 3 h at 60 mA (100 V). After migration, the gels were photographed using Polaroid type 667 positive film, through a red filter on a transilluminator (Funakoshi TM-20) or scanned with a Shimadzu chromato scanner (CS-910) at 600 nm (excitation wavelength at 330 nm). Relative portions of open circular (OC) DNA form and closed circular (CC) DNA form were calculated from resulting peak areas. Induction rates of OC formation or inhibition were calculated by the followed equations.

$$\text{induction rate } (\%) = \frac{d-b}{a} \times 100 \quad (1)$$

$$\text{inhibition rate } (\%) = \frac{e-c}{a-c} \times 100 \quad (2)$$

a: CC form (%) after photoirradiation in the absence of photosensitizer
b: OC form (%) after photoirradiation in the absence of photosensitizer
c: CC form (%) after photoirradiation in the presence of photosensitizer
d: OC form (%) after photoirradiation in the presence of photosensitizer
e: CC from (%) after photoirradiation in the presence of photosensitizer and inhibitor

Chemicals Pindolol, superoxide dismutase and catalase (C-100) were purchased from Sigma, D-mannitol and sodium azide from Nakarai Chemical Co. Methicrane was from Nihon Shin-yaku, trichlormethiazide from Sionogi Seiyaku, piroxicam from Pfeizer, afloqualone from Tanabe Seiyaku, chlormezanone from Daiichi Seiyaku, enoxacin from Dainihon Seiyaku, digoxin from Chugai Seiyaku Co. All other chemicals were of reagent grade. Chemical structures of drugs examined in this work are shown in Fig. 1.

Results

Photodynamic DNA SSB Activity of Enoxacin and Afloqualone Plasmid DNA was irradiated by fluorescent lamps in the presence of 100 μM (final concentration) drug. Samples were electrophoresed in 0.7% agarose slab gel. Enoxacin, an antimicrobial agent, and afloqualone which is used as a muscle relaxant, induced single strand breaks in plasmid YRp7 DNA and changed the CC DNA form to OC DNA. SSB activities of enoxacin and afloqualone were dose-dependent under our experimental conditions. As shown in Fig. 2a, most plasmid DNA in the photoirradiated sample without drug was CC DNA. Addition of 10 μM of enoxacin did not induce OC formation. But induction of the OC form was evident in the irradiated sample with 50 μM enoxacin, and OC formation (%) was increased with increasing enoxacin dose. No induction of OC formation was observed in the sample incubated with 1000 μM enoxacin without photoirradiation. Dose-dependent increases in OC (%) and decrease in CC (%) were obtained by photosensitization with afloqualone (Fig. 2b).

Quantitative Analysis of Photodynamic SSB by Enoxacin and Afloqualone Electrophoresed gels were scanned with a Shimadzu chromato scanner (CS-910) and fluorescent intensity of EB bound to DNA was measured at 600 nm using an excitation wavelength of 330 nm. Relative portions of OC form and CC form were calculated from the peak areas. As shown in Fig. 3, ED_{50} values (effective doses which induce 50% OC form) of enoxacin and afloqualone were assumed to be 2 mM and 860 μM, respectively.

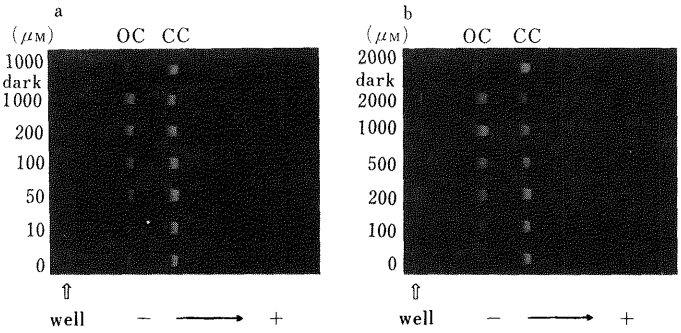

Fig. 2. Induction of OC Form by Photodynamic Action of Enoxacin and Afloqualone

Supercoiled plasmid YRp7 DNA photoirradiated with different concentration of enoxacin or afloqualone was electrophoresed in 0.7% agarose including 0.5 μg/ml EB. After migration, gels were photographed using Polaroid type 667 positive film, through a red filter on a transilluminator. a, enoxacin; b, afloqualone.

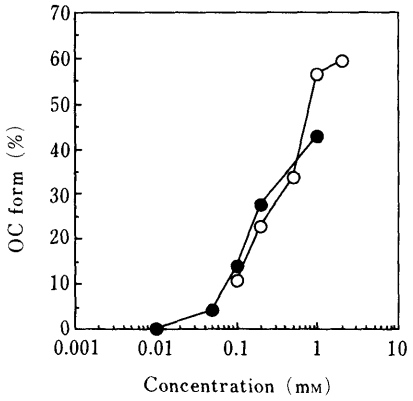

Fig. 3. Dose-Dependent Increase of OC Form (%) by Photodynamic Action of Enoxacin and Afloqualone

Electrophoresed agarose gels were scanned by a Shimadzu chromato scanner (SC-910) and fluorescence of EB bound to DNA was excited at 330 nm and measured at 600 nm. OC form (%) was calculated as described in the Methods. —●—, enoxacin; —○—, afloqualone.

TABLE I. Effects of Active Oxygen Scavengers on Photodynamic SSB Activity

Scavenger		Inhibition (%)[a]	
		Enoxacin	Afloqualone
NaN_3	100 mM	80.5	59.5
SOD	10000 U/ml	32.7	0
D-Mannitol	100 mM	27.6	0
Catalase	10000 U/ml	8.9	21.1

Plasmid DNA was irradiated with either drug in the presence of active oxygen scavenger. *a*) Inhibition rate was calculated as described in the Methods.

Effects of Active Oxygen Scavengers on the Photodynamic SSB Activities Inhibitory effects of different active oxygen scavenger on the photodynamic SSB activities of enoxacin and afloqualone were examined. As shown in Table I, SSB activities of photoirradiated enoxacin were markedly inhibited by NaN_3 and partly by superoxide dismutase (SOD) and D-mannitol. Activities of afloqualone were inhibited by NaN_3 primarily and were slightly inhibited by catalase.

Discussion

Enoxacin and afloqualone, induced SSB in supercoiled

plasmid DNA *in vitro* by photodynamic action. Enoxacin has been used widely as a new quinolone antimicrobial agent in chemotherapy and is reported to induce a combined phototoxic and photoallergic reaction in humans.[14] Afloqualone was explored as a new centrally acting muscle relaxant[15] and acts on vestibular nystagmus and the lateral vestibular nucleus.[16] Recently Tokura and Koide[17] reported that afloqualone induced photodermatological disorders in humans. Many phototoxic drugs induce photodynamic SSB activity of plasmid DNA[4-11] *in vitro*. Of course the reverse is not necessarily always true because some drugs are not uniformly distributed and are not incorporated into skin cells. However enoxacin and afloqualone were shown to induce phototoxic effects in this communication. Patients given these drugs should not be exposed to sun light or artificial lamps at close distances for long times.

The SSB activities of these drugs were inhibited by NaN_3, a singlet oxygen scavenger. That of enoxacin was partly inhibited by SOD or D-mannitol while that of afloqualone was only slightly inhibited by catalase. These suggest that the use of singlet oxygen scavengers may protect against phototoxic side effects of these agents. Hydroxyl radical ($HO^{\bullet}$) involvement in SSB induction has been shown in photosensitization by non-steroidal antiinflamatory drugs[10] or 2-thiouracil.[18] Therefore it is important to determine what kind of active oxygen species are involved in photosensitization by each agent to prevent the photodermatological disorders caused by various agents.

This experimental system may be useful for screening phototoxic substances in our environment and for analyzing the photosensitizing process by varius photosensitizers.

Acknowledgement This study was supported in part by a Grant-in-Aid for scientific research No. 62570994 from the Ministry of Education, Science and Culture of Japan and a Grant-in-Aid for scientific research from the Shizuoka Prefectural Foundation for the Advancement of Education, Japan.

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