Photodynamic DNA Strand Breaking Activities of Acridine Compounds

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Induction of single strand breaks in DNA was assessed by the conversion of supercoiled closed circular plasmid DNA into the open circular form. Euflavine produced single-strand breaks following irradiation but not in the control maintained in the dark. The single strand breaking activity of photoactivated euflavine was found to be dose-dependent. The effective dose conversion 50% (ED₅₀) of the closed circular DNA to the open circular form was 0.53 μ M. A comparison of 8 acridine compounds revealed that the ED₅₀ of diaminoacridines such as euflavine, proflavine and acridine yellow or the 3,6-dimethylamino-derivative (acridine orange) was less than 1 μ M while the ED₅₀ values of the other acridines were greater than 80 μ M. Euflavine was markedly inhibited by singlet oxygen scavengers such as NaN₃, histidine, α -tocopherol or β -carotene and partly inhibited by superoxide dismutase, mannitol or catalase. These results suggest that euflavine induces single strand breaks in DNA mainly by a type II photodynamic mechanism. Photodynamic single strand breaking activities appeared related to their mutagenic activities on yeast.

This experimental system described here is useful for the quantitative assessment of the single strand breaking activities of various photosensitizers *in vitro* and for the determination of active oxygen species involved in those processes.

Keywords photodynamic; DNA strand break; acridine compound; singlet oxygen production; active oxygen scavenger; ED₅₀

Various types of biological damage are induced by phototoxic drugs.¹⁻⁵ In previous reports we demonstrated that acridine (AC) compounds induce the cell inactivation and nuclear and mitochondrial mutations in yeast *via* mainly type II photodynamic mechanisms.⁶⁻¹² Several teams have demonstrated that DNA strand breaks are induced by the photodynamic action of thiopyronine,¹³ proflavine (PF),^{14,15} bilirubin,¹⁶ chlorpromazine,¹⁷⁻¹⁹ promazines,²⁰⁻²² tris(2,2'-bipyridyl)ruthenium(II),²³ tetracyclines²⁴ and non-steroidal anti-inflammatory drugs.²⁵

In order to clarify the differences in these photodynamic mutagenic activities among AC compounds were compared photodynamic strand breaking activity on plasmid DNA *in vitro*.

Materials and Methods

Chemicals Euflavine (EF) was purified by the method of Gupta *et al.*²⁶ from acriflavine hydrochloride purchased from Aldrich. PF hemisulfate was purchased from Tokyo Kasei, acridine orange (AO) from Merck, 6,9-diamino-2-ethoxyacridine (DAEA) lactate, ethidium bromide (EB) and ampicillin from Sigma, acridine yellow (AY) and AC hydrochloride from Wako, 9-aminoacridine (9-AA) hydrochloride and quinacrine (QA) hydrochloride from Nakarai. Superoxide dismutase (SOD) purified from bovine erythrocyte and catalase (C-100) were purchased from Sigma and D-mannitol from Kantoh Chemical Ltd. Chemical structures and registry numbers of the AC compounds used are shown in Fig. 1.

Preparation of Supercoiled Plasmid DNA Plasmid YRp7 DNA was extracted from *Escherichia coli* JA221/YRp7 by the method of Maniatis *et al.*²⁷ Crude plasmid DNA was purified by CsCl-EB equilibrium density gradient ultra-centrifugation by the method of Radloff *et al.*²⁸ The supercoiled closed circular (CC) DNA fraction was collected and EB was removed by shaking with *n*-butanol. After dialysis against TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), the CC DNA was stored at 4 °C. Reagents, tips of pipettes and glassware were autoclaved in order to avoid contamination with DNase which digests supercoiled plasmid DNA.

Irradiation of Plasmid YRp7 DNA in the Presence of Drugs Ten μ l of the stock solution of YRp7 (415 ng) was mixed with 10 μ l TE buffer containing one of the drugs (0.1 μ M—1 mM final concentration) in 1.5 ml Eppendorf tubes. Sample tubes were hung between two 15 W fluorescent lamps (Toshiba, mellow white FL 15N, 10 cm distance) which have a broad emission spectrum (350—750 nm) and irradiated for 1 h at 9000 lux

(measured by a Topcon IM-3 illuminometer [Tokyo Optical Co.]). The total dose was 425 mJ/cm² (measured by a Topcon UVR-1 UV meter).

Agarose Gel Electrophoresis Electrophoresis was performed using horizontal 0.7% agarose slab gels containing 0.5 μ g/ml EB in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.3) as described in elsewhere.²⁷ Twenty μ l samples of photoirradiated DNA solutions were mixed with 2 μ l gel loading buffer (0.25% bromophenol blue, 40% sucrose in distilled water) and electrophoresed for 3 h at 60 mA (100 V). The gels were then 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). The relative proportions of the open circular (OC) form and the CC form were then calculated using the peak areas. The

Fig. 1. Chemical Structure of the AC Compounds

degree of OC formation or inhibition rate was calculated using the following 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 % irradiated in the absence of AC

b: OC form % irradiated in the absence of AC

c: CC form % irradiated in the presence of AC

d: OC form % irradiated in the presence of AC

e: CC form % irradiated in the presence of AC and inhibitor

Results

Induction of Single Strand Breaks by Photoactivated EF Plasmid YRp7 DNA was irradiated for 1 h at 9000 lux (425 mJ/cm²) by fluorescent lamps in the presence of different concentrations of EF. As shown in Fig. 2, scarcely any of the OC form was observed in the control sample (plasmid DNA irradiated in the absence of EF) (bottom lane). Sixty minutes irradiation in the presence of 0.25 μM EF brought about single strand break(s) (SSB) in the plasmid DNA and resulted in a change from the CC form to the OC form. The proportion of the OC form increased with the applied concentration of EF under the same irradiation conditions. EF itself did not induce SSB when no irradiation was carried out (top lane).

The relative proportions of the OC and CC forms were determined as shown in Fig. 3. OC% increased with

Fig. 2. Induction of OC DNA by the Photodynamic Action of EF

Plasmid YRp7 DNA was electrophoresed in 0.7% agarose gel containing 0.5 μg/ml EB after photoirradiation, or kept in the dark in the presence of different concentrations of EF as described in the Materials and Methods.

Fig. 3. Induction of the OC Form by Photoactivated EF

Relative proportions of the OC and CC forms were calculated from the percentages of each peak area on the charts according to Eq. 1 in the Materials and Methods.

increasing concentration of EF. The ED₅₀ value of EF, expressed as the concentration of sensitizer which induces 50% of the OC form, was 0.53 μM.

Single Strand Breaking Activities of Photoactivated AC Compounds SSB activities, measured by the induction of the OC form, were examined using various AC compounds which are active or inactive photomutagens in yeast. As shown in Fig. 4, AO, PF, EF and AY exhibited potent OC inducing activity at less than 0.5 μM under photoirradiation while no OC induction was observed in the respective unirradiated controls. These ACs fully converted the CC form to the OC form, at less than 25 μM, under irradiation. OC induction by DAEA and QA started at 10 μM and increased in a dose-dependent fashion up to 250 μM, whereafter the breakage level decreased. The activity of 9-AA started at 40 μM and increased steeply with concentration, reaching 100% at 120 μM. AC breakage started at less than 50 μM and reached a plateau at 250 μM. The AC compounds studied clearly fell into two groups on the basis of their ED₅₀ values (Table I). The ED₅₀ values of AO, PF, EF and AY were all less than 1 μM while the ED₅₀ values of 9-AA, DAEA, AC and QA were all above 80 μM.

Inhibitory Effects of Different Active Oxygen Scavengers

The inhibitory effects of different active oxygen scavengers were examined. Photodynamic SSB was markedly inhibited by NaN₃, moderately by SOD as well as by mannitol, and poorly by catalase (Table II).

Fig. 4. Induction of the OC form by the Photodynamic Action of AC Compounds

—○—, AO; —●—, PF; —△—, EF; —▲—, AY; —◇—, QC; —■—, AA; —□—, DAEA; —◆—, AC.

TABLE I. Concentrations of AC Compounds Inducing 50% of the OC Form of a Plasmid DNA by Their Photodynamic Action

AC compound	ED ₅₀ (μM)
AO	0.24
PF	0.43
EF	0.53
AY	0.54
AA	84
DAEA	100 ^{a)}
AC	122 ^{b)}
QA	250

^{a)} Approximate value. ^{b)} Extrapolated value. The ED₅₀ values are derived from Fig. 4.

TABLE II. Inhibitory Effects of Active Oxygen Scavengers on the Activity of EF

Scavenger	Concentration	Inhibition (%)
NaN ₃	10 mM	89.9
Superoxide dismutase	1000 U/ml	37.4
D-Mannitol	100 mM	23.8
Catalase	1000 U/ml	13.0

Plasmid DNA was irradiated for 60 min in the presence of 1 μ M EF and different scavengers. Inhibition % was calculated as described in the Materials and Methods.

TABLE III. Inhibition of EF Activity by Singlet Oxygen Scavengers

Scavenger	Concentration	Inhibition (%)
NaN ₃	100 mM	100.0
Histidine	100 mM	75.0
DABCO	100 μ M	36.9
α -Tocopherol	100 μ M	56.6
β -Carotene	100 μ M	30.5

Inhibitory Effects of Different Singlet Oxygen Scavengers

The inhibitory effects of various singlet oxygen scavengers, other than NaN₃, on photodynamic SSB were examined. As shown in Table III, SSB activities were inhibited by all singlet oxygen scavengers, used here. This inhibition was 100% for 100 mM NaN₃, 75% for 100 mM histidine, about 60% for 100 μ M α -tocopherol, 30% for 100 μ M β -carotene and 37% for 100 μ M 1,4-diazabicyclo[2.2.2]octane (DABCO).

Discussion

We previously compared the photobiological effects of a number of AC compounds.^{6,7)} This showed that photoactivated AF, EF, DAEA, PF and AY were highly mutagenic in petite induction in yeast while AC, QC and AA were not. However, there were no specific differences in the singlet oxygen-producing activities among these ACs.¹⁰⁾ Mutation is a consequence of alterations in the base sequence of DNA followed by deletion, insertion and substitution of bases or frame shifts. Breaking of a phosphodiester bond,²⁹⁾ oxidative cleavage of a deoxyribose ring,³⁰⁾ liberation of a base by breaking a N-glycoside bond^{31,32)} or modification of a base such as production of 8-desoxyguanine^{33,34)} can occur. In order to clarify the differences in the photodynamic mutagenic activities of these AC compounds, DNA strand breaking activity was compared *in vitro*. All ACs used in this study exhibit some degree of SSB activity. Comparison of effective doses revealed that EF, PF, AY and AO were effective photosensitizers at concentrations < 1 μ M while DAEA, AA, AC and QC were less effective (ED₅₀ > 80 μ M). These results reflected their mutagenic activities in yeast.^{6,7)} AO which was a potent photosensitizer of cell inactivation and nuclear mutation, but not in petite induction in yeast^{8,10)} was found to be a potent photosensitizer for SSB in plasmid DNA. Many factors such as hydrophobicity, cellular uptake, intra-cellular distribution in organelles, the nature of the interaction with DNA, the fluence and wavelength of the photoirradiation and the photoactivation mechanism of the drugs all contribute to differences in photobiological activities. AO, for example, may be a potent photomutagen,

but may not be incorporated into yeast mitochondria because of its bulky two dimethylamino-groups. AA, which was neither potent in cell inactivation nor the mutation induction in yeast, effectively induced SSB in plasmid DNA. The profile of the dose-dependent curve of SSB induction by AA was unique compared with other AC compounds. OC induction started at 40 μ M and increased steeply with dose reaching 100% at 120 μ M. It is possible that there may be a special interaction between AA and plasmid DNA.

Rowe *et al.*³⁵⁾ and Nelson *et al.*³⁶⁾ have found that DNA damage by antitumor ACs was mediated by mammalian DNA topoisomerase II *in vivo*. However, AC compounds induce SSB in plasmid DNA *in vitro* by a photochemical process.

The SSB induction by photoactivated EF was markedly inhibited by the singlet oxygen scavengers such as NaN₃, histidine, α -tocopherol and β -carotene. These results are consistent with the observations that petite induction and cell inactivation were markedly inhibited by singlet oxygen scavengers.⁶⁻⁸⁾ Different active oxygen species are involved in the photosensitization processes of various drugs. DNA strand breakage by the reduction of sugar phosphates was inhibited by SOD, catalase, hydroxyl radical scavengers and singlet oxygen scavengers.³⁷⁾ Involvement of a hydroxyl radical in the relaxation of ϕ X174 DNA by photoactivated tetracyclines has been shown by Piette *et al.*²⁴⁾ Photodynamic DNA cleavage was quenched by NaN₃, but isopropanol and mannitol were much weaker³⁸⁾ while the DNA cleavage by non-steroidal anti-inflammatory drugs was inhibited by mannitol and SOD.²⁵⁾ In previous work we showed that photodynamic SSB by enoxacin was markedly inhibited by NaN₃ and partially by SOD or mannitol.³⁹⁾ These results suggest that AC compounds induce single strand breaks in DNA mainly by a ¹O₂ mediated type II photodynamic mechanism similar to rose bengal or enoxacin. Some AC compounds may prove useful as photo-chemotherapeutic drugs and their photobiological effects may be controlled by various singlet oxygen scavengers.

This assay system will be useful in evaluating semi-quantitatively the photodynamic mutagenic activities of drugs and in analyzing the photosensitizing mechanisms of various drugs.

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References

- 1) J. Amagasa, *Photochem. Photobiol.*, **33**, 947 (1981).
- 2) J. P. Pooler, *Med. Phys.*, **8**, 614 (1981).
- 3) M. A. Pathak, *J. Natl. Cancer Inst.*, **69**, 163 (1982).
- 4) T. Ito, *Photochem. Photobiol. Rev.*, **7**, 141 (1983).
- 5) J. H. Epstein, B. U. Wintroub, *Drugs*, **30**, 42 (1985).
- 6) Y. Iwamoto, I. Mifuchi, L. W. Yelding, W. J. Firth III, K. L. Yelding, *Mutat. Res.*, **125**, 213 (1984).
- 7) Y. Iwamoto, I. Mifuchi, *Chem. Pharm. Bull.*, **32**, 2759 (1984).
- 8) Y. Iwamoto, I. Mifuchi, K. L. Yelding, *Mutat. Res.*, **158**, 169 (1985).
- 9) Y. Iwamoto, H. Yoshioka, Y. Yanagihara, I. Mifuchi, *Chem. Pharm. Bull.*, **33**, 5529 (1985).
- 10) Y. Iwamoto, H. Yoshioka, Y. Yanagihara, *Chem. Pharm. Bull.*, **35**, 2478 (1987).

- 11) Y. Iwamoto, C. Tominaga, Y. Yanagihara, *Chem. Pharm. Bull.*, **37**, 1632 (1989).
- 12) M. Tsuchiya, Y. Iwamoto, T. Masuzawa, T. Shimizu, T. Morita, Y. Yanagihara, *Photochem. Photobiol.*, **48**, 545 (1988).
- 13) H. Triebel, H. Baer, H. E. Jacob, E. Sarfert, H. Berg, *Photochem. Photobiol.*, **28**, 331 (1978).
- 14) J. Piette, Calberg-Bacq, A. V. D. Vorst, *Photochem. Photobiol.*, **30**, 369 (1979).
- 15) J. Piette, M. Lopez, Calsberg-Bacq, A. V. D. Vorst, *Int. Radiat. Biol.*, **40**, 427 (1981).
- 16) B. S. Rosenstein, J. M. Ducore, S. W. Cummings, *Mutat. Res.*, **112**, 397 (1983).
- 17) A. A. Schothorst, D. Suurmond, R. Schouten, *Photochem. Photobiol.*, **38**, 659 (1983).
- 18) H. Fujita, *Mutat. Res.*, **158**, 135 (1985).
- 19) H. Fujita, I. Matsuo, *Chem.-Biol. Interact.*, **66**, 27 (1988).
- 20) J. Decuyper, J. Piette, M. Lopez, M. P. Merville, A. V. D. Vorst, *Biochem. Pharmacol.*, **33**, 4025 (1984).
- 21) J. Decuyper, J. Piette, M. P. Merville, A. V. D. Vorst, *Biochem. Pharmacol.*, **35**, 1345 (1986).
- 22) J. Decuyper, A. V. D. Vorst, "Oxygen Radicals in Chemistry and Biology," ed. by W. Bors, M. Saran, D. Tait, Walter de Gruyter & Co., Berlin-New York, 1984, pp. 555—558.
- 23) A. Aboul-Enein, P. Schuler-Frohlinde, *Photochem. Photobiol.*, **48**, 27 (1988).
- 24) J. Piette, J. Decuyper, A. V. D. Vorst, *J. Invest. Dermatol.*, **86**, 653 (1986).
- 25) T. Artuso, J. Bernadou, B. Meunier, J. Piette, N. Paillous, *Photochem. Photobiol.*, **54**, 205 (1991).
- 26) V. S. Gupta, S. C. Kraft, J. S. Samuelson, *J. Chromatogr.*, **26**, 158 (1967).
- 27) T. Maniatis, E. F. Fritsch, J. Sambrook, "Molecular Cloning, a Laboratory Manual," Cold Spring Harbor Laboratory, New York, 1982, pp. 86—96.
- 28) R. Radloff, W. Bauer, J. Vinograd, *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 1514 (1967).
- 29) M. Rosenberg-Arska, B. S. V. Asbeck, T. J. Martens, J. Verhoef, *J. Gen. Microbiol.*, **131**, 3325 (1985).
- 30) D. S. Frohlinde, C. V. Sonntag, "Oxidative Stress," ed. by H. Sies, Academic Press Inc., New York, 1985, pp. 11—36.
- 31) H. J. Rhaese, E. Freese, *Biochim. Biophys. Acta*, **155**, 476 (1968).
- 32) H. J. Rhaese, E. Freese, *Biochim. Biophys. Acta*, **155**, 491 (1968).
- 33) H. Kasai, S. Nishimura, *Nucleic Acids Res.*, **12**, 2137 (1984).
- 34) H. Kasai, S. Nishimura, "Oxidative Stress, Oxidants and Antioxidants," ed. by H. Sies, Academic Press Inc., New York, 1991, pp. 99—116.
- 35) T. C. Rowe, G. L. Chen, Y. H. Hsiang, L. F. Liu, *Cancer Res.*, **46**, 2021 (1986).
- 36) E. M. Nelson, K. M. Tewey, L. F. Liu, *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 1361 (1984).
- 37) J. Morita, T. Komano, *Agric. Biol. Chem.*, **47**, 11 (1983).
- 38) T. A. Ciulla, J. R. Van Camp, E. Rosenfeld, I. E. Kochevar, *Photochem. Photobiol.*, **49**, 293 (1989).
- 39) Y. Iwamoto, T. Itoyama, K. Yasuda, T. Uzuhashi, H. Tanizawa, Y. Takino, T. Oku, H. Hashizume, Y. Yanagihara, *Chem. Pharm. Bull.*, **40**, 1868 (1992).