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Irreversible binding of quinacrine to nucleic acids during horseradish peroxidase- and prostaglandin synthetase-catalyzed oxidation

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Acridine dyes possess antibacterial and antitumor properties. The biological activities of these compounds are believed to involve binding to DNA [1,2]. Two binding processes have been identified: (1) a weak binding resulting from electrostatic interactions between the positive charge of the dye and the negatively charged phosphates of DNA, and (2) intercalation of the planar dye molecule between adjacent base pairs [1–3]. Acridines induce frameshift mutations, and intercalation has been implicated in mutagenesis [4]. However, acridines with different mutagenic activities have similar DNA binding affinities [5]; hence, the biochemical events leading to frameshift mutation are not clear [6, 7].

A number of toxic xenobiotics form free radical intermediates, and the formation of reactive radicals may be involved in toxicity and carcinogenicity [8, 9]. Comprehensive review articles on the subject matter have been published recently [10, 11]. *N*-Hydroxy-*N*-acetylaminofluorene [8] and other aromatic amine carcinogens [9] form nitroxide free radicals during enzymatic activation. The mutagenic acridines, 9-aminoacridine and quinacrine, form free radicals during horseradish peroxidase(HRP) and prostaglandin synthetase (PES)-catalyzed activation [12]. This report shows that the intermediate(s) formed during HRP/H_2O_2 and PES/arachidonic acid (AA) oxidations of quinacrine binds irreversibly to nucleic acids.

Materials and methods

Quinacrine HCl was obtained from the Aldrich Chemical Co., Milwaukee, WI. [^{3}H-G]Quinacrine (sp. act. = 24 Ci/mmole; > 98% radiochemical purity) was purchased from Moravek Biochemicals, Inc., Bera, CA. Calf thymus DNA (highly polymerized, type I), yeast RNA (type XI), horseradish peroxidase (type VI, RZ = 3.0), and indomethacin were obtained from the Sigma Chemical Co., St. Louis, MO. Arachidonic acid was obtained from NuChek. Prep. Inc., Elysian, MN, and was stored in the dark at −70°.

Ram seminal vesicle (RSV; obtained from local slaughter houses) was freed of extraneous tissue and stored at −70°.

Ram seminal vesicle microsomes were prepared as described [13], and the protein concentration was determined according to the method of Sutherland *et al.* [14] with bovine serum albumin as the standard. The microsomal prostaglandin synthetase activity was determined by measuring AA-dependent oxygen uptake with a Clark-type electrode, and RSV microsomes with low enzymatic activity (less than 50% AA-dependent O_2 uptake in 15 sec/mg protein) were discarded.

Nucleic acids were dissolved in 50 mM phosphate-150 mM NaCl buffer (pH 7.4) at a concentration of 2 mg/ml, and an equal volume of [^{3}H-G]quinacrine (2 mM; 2 μCi/ml) solution was added. After mixing for 5 min at room temperature, protein (HRP or RSV microsomes, 0–2 mg/ml) was added, and the mixtures were incubated at 37° for 1 min with shaking. The reaction was initiated by adding 400 μM H_2O_2 or AA. The stable quinacrine–nucleic acid complexes were isolated by extraction with 15 ml of water-saturated phenol-chloroform (1:1) for 15 min, and centrifugation at 8000 *g* for 20 min as described [15]. The clear supernatant fraction was removed and precipitated in 4 vol. of absolute ethanol. The ethanol precipitates were cooled to −20° for 4 hr, collected by centrifugation, washed (3 × 20 ml) with ethanol, dried with nitrogen, and dissolved in the buffer. The nucleotide recovered under these conditions, is about 60–75% of the initial concentration. Under these conditions of isolation, all of the unbound quinacrine was removed from the controls, and no radioactivity could be detected. The irreversibly bound quinacrine intermediate(s) in the samples (detection limit ~ 1 drug/10^5 nucleotide) was measured with a Packard liquid scintillation counter (model A 300-C) using Aquasol (New England Nuclear, Boston, MA), and appropriate quenching corrections were made. The nucleotide concentration in the sample was determined spectrophotometrically as described [16]. The binding ratio is defined as the molar ratio of drug to mononucleotide unit.

Results and discussion

Incubation of quinacrine—DNA preformed complexes with HRP/H_2O_2 resulted in irreversible binding of quinacrine to DNA. The extent of this irreversible binding increased with time and reached maximum after 60 min of incubation (Fig. 1), yielding stable complexes containing 1 quinacrine molecule/650 nucleotides (Table 1). No binding was detected when either HRP or H_2O_2 was omitted from the incubation mixture. When quinacrine was activated with HRP/H_2O_2 and DNA was added after 5 min of activation, a decrease of 50% in binding of quinacrine to DNA was observed (Table 1). Longer incubation time in the absence of DNA further decreased the binding (data not shown). These observations indicate that the active intermediate that bound to DNA was unstable and decayed with time. Alternatively, the quinacrine intermediate(s) bound to HRP, which then inactivated the enzyme. When single-stranded nucleic acids (heat-denatured DNA or RNA) were used as substates, a significant increase in binding of quinacrine to these nucleic acids was observed (Table 1), suggesting that stable binding of quinacrine was facilitated by the loss of double-helical structures.

The HRP/H_2O_2 catalyzed binding to DNA was a function of protein concentration, and the highest binding was obtained between 0.2 and 0.4 mg/ml HRP (Fig. 2). The binding decreased with higher protein concentration (0.5 mg/ml), but further increase in HRP concentration was without effect. The decrease in DNA binding with

Table 1. Binding of quinacrine to nucleic acids in the presence of horseradish peroxidase (1 mg/ml) at 37°

Substrate	Conditions*	Binding ratio × 10^4
DNA	HRP	Not detected
DNA	H_2O_2	Not detected
DNA	HRP + H_2O_2	15.6 ± 1.2
DNA	HRP + H_2O_2†	8.5 ± 1.5
Denatured DNA	HRP + H_2O_2	150 ± 0.6
RNA	HRP + H_2O_2	44 ± 2.5

* Quinacrine–DNA complexes were incubated with HRP for 1 min at 37° before adding H_2O_2 (400 μM) and then incubated for another 60 min. Values for binding ratios are the mean ± standars error of four determinations.

† Quinacrine was incubated with HRP-H_2O_2 for 5 min before adding DNA.

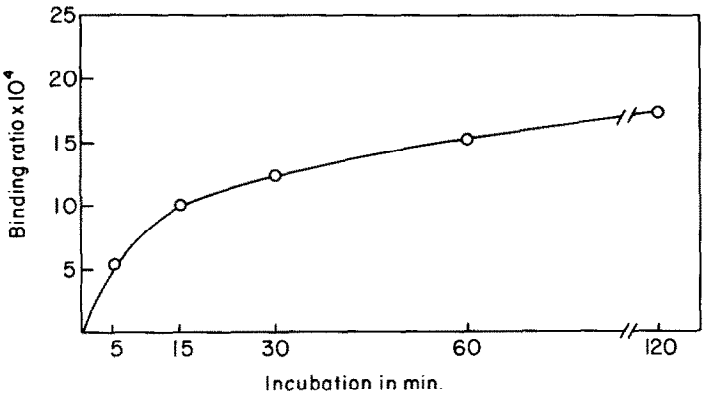


Fig. 1. Time course of binding of quinacrine (1 mM) to DNA (1 mg/ml) in the presence of horseradish peroxidase (1 mg/ml) and H_2O_2 (400 μM) at 37°.

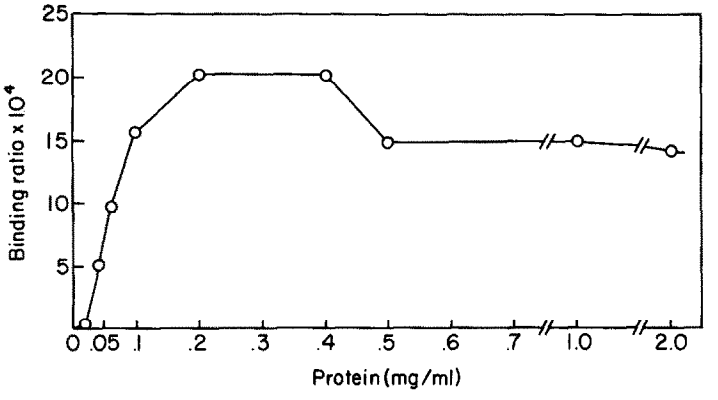


Fig. 2. Binding of quinacrine (1 mM) to DNA (1 mg/ml), at 60 min as a function of horseradish peroxidase concentration, in the presence of H_2O_2 (400 μM) at 37°

Table 2. Binding of quinacrine (1 mM) to DNA (1 mg/ml) in the presence of RSV microsomes (1 mg/ml) at 37°

Conditions*	Binding ratio × 10^5
RSV microsomes	Not detected
AA	Not detected
RSV microsomes + AA	4.2 ± 1.5
RSV microsomes† + AA	8.3 ± 1.0
RSV microsomes + AA + idomethacin (200 μM)	Not detected
RSV microsomes‡ + AA	2.7 ± 1.3

* Quinacrine–DNA complexes were incubated with RSV microsomes for 1 min at 37° before adding AA (400 μM) and then incubated for another 60 min. Stable complexes were isolated as described in Materials and Methods. Values for binding ratios are the mean ± standard error of four determinations.

† Quinacrine–DNA complexes were incubated with 0.2 mg/ml RSV microsomes, and the reaction was initiated by adding AA.

‡ Quinacrine–DNA complexes were incubated with RSV microsomes and AA at room temperature (~ 22°).

increasing protein concentations (0.5 mg/ml or higher) may indicate binding of the activated intermediate to the protein.

Activation of quinacrine with RSV microsomal protein, a source of prostaglandin synthetase, in the presence of arachidonic acid (AA) and DNA, also resulted in stable binding of quinacrine to DNA (Table 2). However, in this case the binding ratios (1 quinacrine molecule/12,000 nucleotides) obtained were significantly lower than those obtained with HRP/H_2O_2. The binding of quinacrine to DNA was a function of the RSV protein concetration, as with the HRP/H_2O_2 system, and the highest binding was obtained with 0.2 mg/ml protein. Increasing protein concentrations or incubations carried out at room temperature decreased binding (Table 2). Incubation of RSV microsomal protein with indomethacin (100–400 μM), an inhibitor of prostaglandin synthetase [17], abolished the binding (Table 2).

Acridine derivatives that contain a 9-amino group form free radical intermediates during HRP- and prostaglandin synthetase-catalyzed oxidation [12]. When quinacrine was incubated with HRP/H_2O_2, an ESR spectrum consisting of three lines was obtained (Fig. 3A), which with time was replaced by a single line spectrum (Fig. 3B). While saturation of the incubation mixtures with O_2 increased radical yield, anaerobic conditions inhibited radical formation. The G value of 2.0055 and the hyperfine splitting constant (a^N) of 7.5 G are characteristic of a nitrogen-centered radical, most probably a nitroxide radical. Neugebauer and Bamberger [18] have obtained a somewhat similar spectrum from the oxidation of 10-H-acridone with PbO_2 in benezene. The reported a^N of the resulting acridone-10-nitroxide is 6.89 G, with a G value of 2.0058, in benezene. These values compare favorably with a similar nitroxide radical from quinacrine. Thus, HRP/H_2O_2-catalyzed formation of quinacrine nitroxide may occur as shown in Scheme 1. In this proposal, HRP/H_2O_2 catalyzes the oxidation of II, a tautomeric form of quinacrine, to its 1- hydroxy derivative (III), which undergoes further oxidation to the quinacrine-10-nitroxide (IV). In one study, HRP has been shown to catalyze *N*-hydroxylation [19]. The question of whether formation and stabilization of II from I are enzymatic is not clear. The single line spectrum formed from quinacrine probably resulted from binding of the nitroxide (IV) to protein.

RSV microsomal protein, in the presence of AA, also catalyzed the formation of the nitroxide radical from quinacrine (Fig. 3C). In contrast to the HRP/H_2O_2 systems, the nitroxide spectrum consisted of a single line with a G value of 2.0055. The reason for the single line spectrum is not known but it may be that the nitroxide (IV) was bound to proteins present in the RSV microsomes. The formation of the radical was O_2-dependent and was inhibited by indomethacin. This would suggest that dioxygenation of AA to the cyclic hydroperoxy-endoperoxide prostaglandin (PGG_2) is required. PGG_2 would then be reduced to PGH_2, a hydroxy-endoperoxide, during co-oxidation of substrate [20]. Both of these reactions are catalyzed by prostaglandin synthetase (cyclooxygenase and peroxidase), an enzyme present in almost all mammalian cell types [21].

The prostaglandin synthetase-catalyzed quinacrine intermediate also was bound to DNA. The binding ratios obtained were considerably lower than those of HRP-catalyzed reactions. This may have been due to the binding of the active species to RSV microsomal proteins resulting in decreased DNA alkylation. Quinacrine binds to rat hepatic microsomal proteins [12].

The binding of quinacrine to DNA appears to be covalent, since quinacrine-DNA complexes were stable to heat, low pH, repeated precipitation from ethanol, and dialysis. Furthermore, all of the non-covalently bound quinacrine was removed in controls from which either the protein, H_2O_2, or AA was omitted.

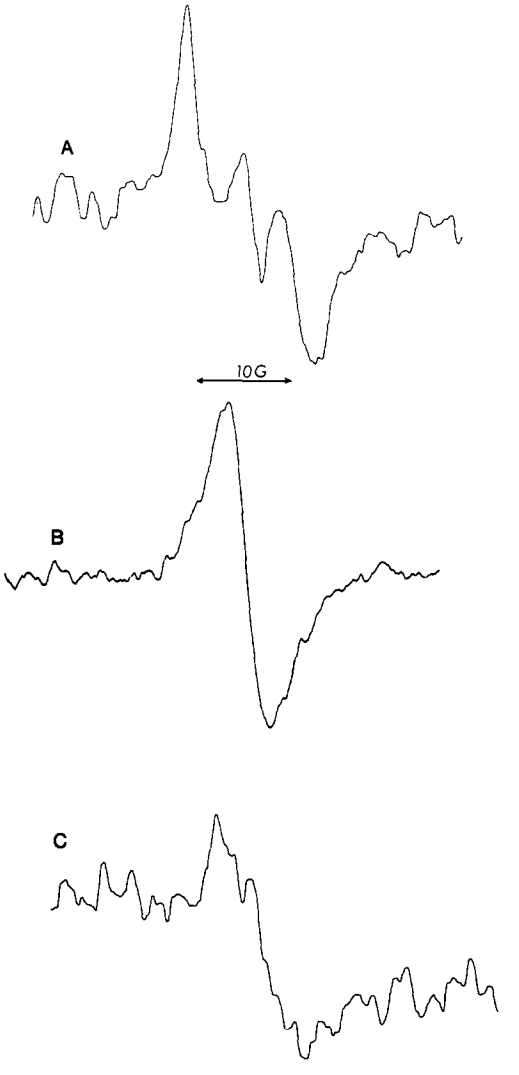
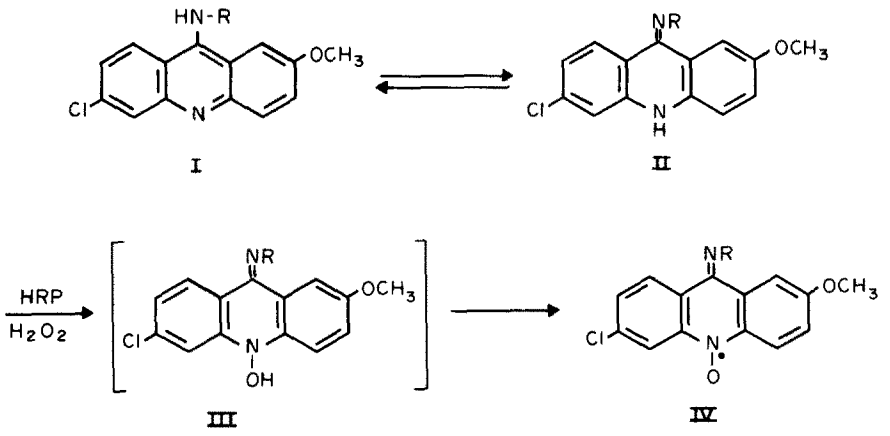


Fig. 3. Electron spin resonance (ESR) spectrum obtained from quinacrine (1 mM) during (A) incubation with horseradish peroxidase (1 mg/ml) and H_2O_2 (400 μM); (B) same as (A) except the spectrum was recorded after 60 min; and (C) incubation with RSV microsomal protein (1.5 mg/ml) and arachidonic acid (400 μM). The ESR settings were: field = 3500 G; field scan = 100 G; modulation frequency = 100 (k) Hz; nominal microwave power = 20 mW; and modulation amplitude = 2.0 G. Receiver gain was 4×10^5 for (A), 6.3×10^4 for (B), and 3.2×10^5 for (C).

The nature of the binding site(s) of quinacrine-derived species in nucleic acids, and the identity of the alkylating species, are not known. Whether the species II, III, or IV bind covalently to DNA is not clear.

In summary, quinacrine containing a -NH group in the C_9-position produced nitroxide radicals during HRP- and PES-catalyzed oxidation. The intermediate(s) formed during enzymatic oxidation bound irreversibly to nucleic acids. Significantly more drug was bound to denaturated DNA than to native DNA. Free radical metabolism and irreversible binding of the reactive intermediate(s) to cellular macromolecules may be important in the mutagenic properties of acridines.



Scheme 1. Proposed formation of quinacrine nitroxide radical by enzymatic activation.

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REFERENCES

1. A. R. Peacoke and J. N. Skerrett, *Trans. Faraday Soc.* **52**, 261 (1956).
2. L. S. Lerman, *J. molec. Biol.* **3**, 18 (1961).
3. L. S. Lerman, *Proc. natn. Acad. Sci. U.S.A.* **49**, 94 (1963).
4. A. Orgel and S. Brenner, *J. molec. biol.* **3**, 762 (1961).
5. S. A. Riva, *Biochem. biophys. Res. Commun.* **23**, 606 (1966).
6. J. R. Roth, *A. Rev. Gen.* **8**, 319 (1974).
7. J. W. Drake and R. H. Baltz, *A. Rev. Biochem.* **45**, 11 (1976).
8. R. A. Floyd, L. M. Soong, R. N. Walder and M. Stuart, *Cancer Res.* **36**, 2761 (1976).
9. A. Stier, R. Clause, A. Lucke and I. Reitz, *Xenobiotica* **10**, 661 (1980).
10. R. P. Mason, in *Free Radicals in Biology* (Ed. W. A. Pryor), Vol. 5, p. 161. Academic Press, New York (1982).
11. R. A. Floyd, *Radiat. Res.* **86**, 243 (1981).
12. B. K. Sinha, *Biochem. biophys. Res. Commun.* **103**, 1166 (1981).
13. K. Sivarajah, H. Mukthar and T. E. Eling, *Fedn. Eur. Biochem. Soc. Lett.* **106**, 17 (1979).
14. E. W. Sutherland, C. F. Cori, R. Hayes and N. S. Olson, *J. biol. Chem.* **180**, 825 (1949).
15. B. K. Sinha and J. L. Gregory, *Biochem. Pharmac.* **18**, 2626 (1981).
16. B. K. Sinha, *Chem. Biol. Interact.* **30**, 67 (1980).
17. S. Moncada, P. Neddleman, S. Bunting and J. R. Vane, *Prostaglandins* **12**, 323 (1976).
18. F. A. Neugebauer and S. Bamberger, *Chem. Ber.* **107**, 2362 (1974).
19. L. M. Bordeleau, J. D. Rosen and R. Bartha, *J. agric. Fd Chem.* **20**, 573 (1972).
20. S. Moncada and J. R. Vane, *Pharmac. Rev.* **30**, 293 (1979).
21. B. Samuelsson, M. Goldyne, E. Granstrom, H. Hamburg, S. Hammarstrom and C. Malmsten, *A. Rev. Biochem.* **47**, 997 (1978).

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Androgen dependence of glutathione metabolism in ventral prostate

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A relationship seems to exist between glutathione (GSH) metabolism and cellular proliferation [1]. Relatively high concentrations of glutathione and other sulfhydryl (SH) group containing compounds are required for mitosis and cell division in plants, animals and micro-organisms [2]. Studies on sulfhydryl metabolism during cell division suggest that glutathione might be subject to modulation during carcinogenesis [3]. Hosoda and Nakamura [4] showed that, in Ehrlich ascites tumor cells, the activity of the hexose monophosphate pathway, which supports the supply of materials for DNA and RNA synthesis, is largely dependent upon the availability of NADP, generated from the glu-