Vol. 132, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1985 Pages 445-452

DIAZIQUONE AS A POTENTIAL AGENT FOR PHOTOIRRADIATION THERAPY: FORMATION OF THE SEMIQUINONE AND HYDROXYL RADICALS BY VISIBLE LIGHT

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Received August 26, 1985

When diaziquone was irradiated with 500 nm visible light, hydroxyl free radicals as well as the diaziquone semiquinone were produced. The diaziquone semiquinone is a stable free radical that exhibits a characteristic 5-line electron spin resonance (ESR) spectrum. Since hydroxyl free radicals are short lived, and not observable by conventional ESR, the nitrone spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was used to convert hydroxyl radicals into longer lived ESR detectable spin adducts. The formation of hydroxyl radicals was further confirmed by investigating reactions in which hydroxyl radical scavangers, sodium formate and dimethylsulfoxide, compete with the spin traps DMPO or POBN (alpha-(4-Pyridyl-1-oxide)-N- tert-butylnitrone) for hydroxyl free radicals. The products of these scavenging reactions were also trapped with DMPO or POBN. If drug free radicals and hydroxyl free radicals are important in the activity of quinone-containing antitumor agents, AZQ may have a potential in photoirradiation therapy or photodynamic therapy.

INTRODUCTION: The aziridinyl quinone 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone (AZQ)** is a quinone containing alkylating agent with antitumor activity on malignancies of the central nervous system, intraperitoneal L1210 leukemia, P388 leukemia, B16 melanoma (1) and intracerebral L1210 leukemia (2,3). This drug has been undergoing phase I and phase II clinical trials (i.e. 4) and an increasing number of studies about its activity and metabolism have recently appeared (5-9). Electrochemical properties of AZQ and the possible role of its reductive alkylation have been recently investigated (10). AZQ can be reduced electrochemically in a single two-electron step to its dihydroquinone hydroquinone in aqueous medium and subsequently oxidized to the semiquinone AZQ̄H which exhibits a characteristic five line ESR spectrum (5,10). AZQ can also be reduced to its free radical anion by cells in culture (11) and by purified

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**Abbreviations: AZQ, Diaziquone; ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; POBN, alpha-(4-pyridyl-1-oxide)-N-ter-butylnitrone; DMSO, dimethylsulfoxide.

0006-291X/85 $1.50

enzymes (5). The activity of AZQ can also be enhanced by incubation with rat liver microsomes (7) which can in turn generate the drug's free radical (5).

In general, photochemical reactions of quinones are easily coupled to their electrode reactions (12-15). AZQ was found to exhibit an $n \rightarrow \pi^*$ band at 500 nm at neutral pH (11). It has been proposed that irradiation of quinones with light at wavelengths corresponding to this transition, causes the formation of their semiquinones as well as hydroxyl radicals (16-18). The present study provides conclusive evidence for the formation of AZQ semiquinone and hydroxyl radicals by the irradiation of AZQ with 500 nm visible light. Since hydroxyl radicals are short lived, and cannot be observed by conventional ESR, the spin trapping method was used (19,20). Spin traps are diamagnetic nitroso or nitrone compounds which react with transient radicals, such as $\dot{O}H$, to produce stable nitroxide radicals known as spin-adducts which can be conveniently detected by ESR.

MATERIALS AND METHODS: AZQ was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD. Stock solutions of AZQ (50 mM) were prepared in dimethylsulfoxide (DMSO). One millimolar solutions of AZQ were prepared in phosphate buffer pH 7.0 or phosphate buffered saline pH 7.4 such that the concentrations of DMSO were always less than 5%. Both DMPO and POBN spin traps were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were also purchased from Aldrich and were reagent grade. DMPO was purified before use according with the procedures of Buettner and Oberly (21). The concentrations of DMPO solutions were determined by UV spectroscopy (22) ($\epsilon_{277} = 8 \times 10^3\ M^{-1}\ cm^{-1}$). POBN was used without further purification.

ESR spectra were measured in an aqueous quartz flat cell (60 x 10 x 25 mm^3) with a Varian E-109 ESR spectrometer (X-band, 100 KHz field modulation). Photolysis was carried out in situ in the ESR cell, placed in the spectrometer cavity using a 500 $\pm$ 10 nm light from a 1000 watt Mercury-Xenon high pressure arc coupled to a grating monochromator.

RESULTS AND DISCUSSION: Spin traps have high affinity for reactive radicals and lead to the formation of persistant spin adducts as represented in equations 1 and 2. The ESR spectra of the spin-adducts exhibit a primary ^{14}N triplet which is split into a secondary doublet due to the beta proton. The magnitude of the hyperfine splitting constant (hpf) a_N and a_H^{β} are characteristic of the nature of the trapped radical $\dot{R}$.

When an aerated solution containing AZQ (1 mM), 5% dimethylsulfoxide and DMPO (10 mM) was irradiated with 500 $\pm$ 10 nm light for a short time, e.g. three minutes, an ESR spectrum appeared (Fig. 1A) consisting of four lines with 1:2:2:1 relative intensity.

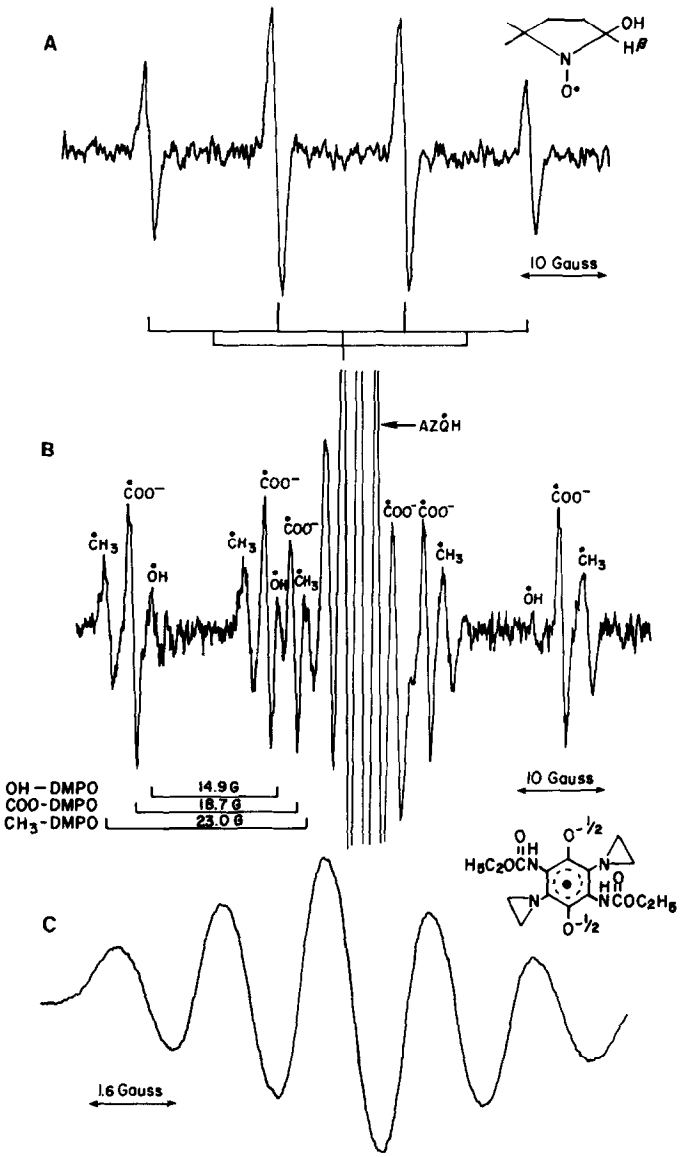
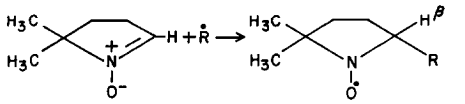


Figure 1A ESR spectrum of OH-DMPO obtained from an aqueous AZQ (1 mM) solution (5% DMSO) containing DMPO (10 mM) irradiated with 500 nm visible light for 3 min. Under identical experimental conditions, no spectrum was found in the absence of AZQ. The stick diagram shows the 14.9 G beta proton doublets. The ESR conditions at room temperature and 9.3 GHz were 10 mW incident microwave power and 1 Gauss modulation amplitude.

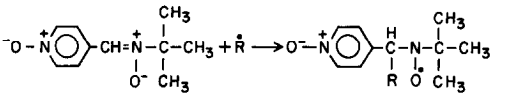
Figure 1B ESR spectra obtained from an aqueous AZQ (1 mM) solution (5% DMSO) containing DMPO (10 mM) and sodium formate (100 mM) irradiated with 500 nm visible light for 30 min. The COO^-, OH and CH_3 DMPO adducts gave rise to three sets of doublets each. The stick diagrams show the characteristic beta proton doublet for each adduct, for the low field $M_I = +1$ component. The semiquinone AZQH radical gave rise to a 5-line spectrum in the center of the figure which overlaps with the central $M_I = 0$ components of the DMPO adducts. The ESR conditions were as in Fig. 1A.

Figure 1C ESR spectrum of AZQH from Fig. 1B recorded 24 hr after irradiation at an expanded magnetic field range. The ESR conditions were as in Fig. 1A, except the receiver gain was six times less.



DMPO DMPO-R

1.



POBN POBN-R

2.

This spectrum is due to the coupling of the unpaired electron with a nitrogen (^{14}N) nucleus and the beta proton which yield an ESR spectrum with equal hyperfine constants of $a_N = a_H^{\beta} = 14.9$ Gauss. This spectrum is characteristic of the OH-DMPO adduct, and the value of the hyperfine splittings is in agreement with previously reported values (23,26,31,32). No characteristic spectrum due to the superoxide adduct (HOO-DMPO) was detected under our experimental conditions. Control experiments carried out in the absense of AZQ did not yield the OH-DMPO adduct.

Several reactions have been reported to produce the hydoxyl adduct of DMPO by pathways which do not involve the formation of $\dot{O}H$ (23). In order to verify that $\dot{O}H$ radicals are generated by the photoirradiation of aqueous solutions of AZQ, experiments where scavangers compete with DMPO for $\dot{O}H$ were undertaken. Sodium formate was used for this purpose.

Experiments in which the irradiation of AZQ (1 mM) was carried out for 30 minutes in the presence of DMPO (10 mM), sodium formate (10-100 mM) and 5% DMSO yielded detectable free radicals. There are four distinct sets of lines, corresponding to four different free radicals (Fig. 1B). Three free radicals are adduct products, and the fourth one is the AZQ semiquione ($AZ\dot{Q}H$). The three adduct products are: A relatively weak OH-DMPO adduct, due to the trapping of $\dot{O}H$ radicals as in Figure 1A; a relatively strong COO^- -DMPO adduct due to the trapping of the formyl free radical $\dot{C}OO^-$; and a CH_3-DMPO adduct of intermediate strength due to the trapping of methyl radicals ($\dot{C}H_3$) from the scavenging reaction of DMSO and $\dot{O}H$. Each spectrum is explained next.

The DMPO spin-adduct of the hydrogen abstraction radical from formate (COO^- - DMPO, eqs. 4a and 4b) gave rise to a primary triplet which was further split into a doublet due to the interaction of the unpaired electron with the nitrogen of the

nitroxide group and the beta proton, respectively. As the sodium formate concentration was increased at constant DMPO concentration, a decrease in the signal of the OH-DMPO adduct and a corresponding increase of the COO^--DMPO adduct was observed indicating competitive scavenging of $\dot{O}H$ by DMPO and $HCOO^-$. Since the rate constants for the reactions of DMPO with $\dot{O}H$ ($k = 4.3 \times 10^9\ M^{-1}\ s^{-1}$) (27) and $HCOO^-$ with $\dot{O}H$ ($k = 3 \times 10^9\ M^{-1}\ s^{-1}$) (28) are comparable, the observed increase in the COO^--DMPO ESR signal (labeled $\dot{C}OO^-$, Fig. 1B) relative to that of OH-DMPO (labeled $\dot{O}H$, Fig. 1B) occured because of the high sodium formate concentration used (100 mM). The signal due to the trapping of $\dot{C}H_3$, arising from the scavenging of $\dot{O}H$ by DMSO (29,30) gave rise to a triplet of doublets. The hyperfine constants a_N and a_H^β observed for these three adducts are given in Table I and agree with published values (25,26,31-33).

In the center of Fig. 1B is the distinct 5-line spectrum characteristic of the AZQ free radical $AZ\dot{Q}H$ (5) which gradually appeared and became detectable after about 20 min of irradiation. The spectrum is due to the coupling of the unpaired electron to the two equivalent nitrogens of the aziridine groups ($a_N^{Az} = 1.88$ gauss). Additional coupling from equivalent nitrogens ($a_N = 0.15$ gauss) and slightly inequivalent protons ($a_H = 0.87$, 0.73 gauss) in the carboethoxyamino groups help broaden the spectral lines (24).

As time progressed, the spectra of the DMPO adducts decayed and the AZQ free radical anion increased. Twenty-four hours later, the solution gave rise only to a strong $AZ\dot{Q}H$ signal shown in Fig. 1C at a smaller magnetic field range (16 gauss). As expected, this result indicates that the lifetime of $AZ\dot{Q}H$ is much longer than those of the DMPO adducts.

TABLE I

Hyperfine Coupling Constants of Spin Adducts Resulting
During the Photolysis of AZQ in Aqueous Solutions at 500 nm

Spin-Adduct	a_N (Gauss)	a_H^β (Gauss)
OH-DMPO	14.9	14.9
COO^--DMPO	15.6	18.7
CH_3-DMPO	16.1	23.0
COO^--POBN	15.5	3.0
CH_3-POBN	15.2	2.4

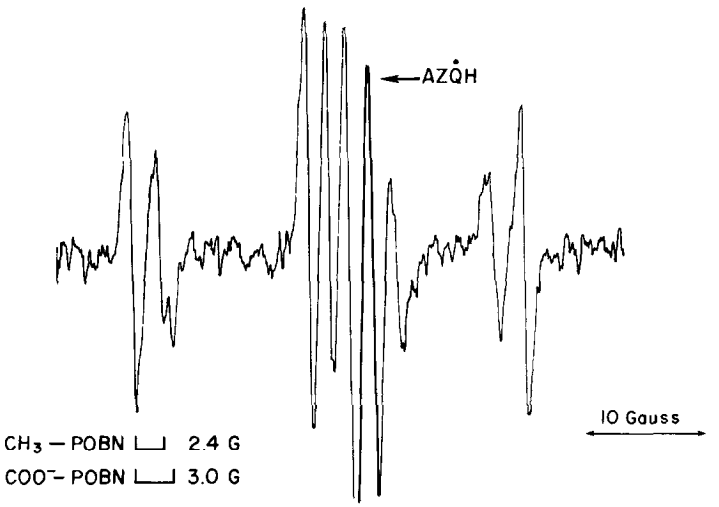


Figure 2 ESR spectra obtained from an aqueous AZQ (1 mM) solution (5% DMSO) containing POBN (25 mM) and sodium formate (100 mM) irradiated with 500 nm visible light for 30 min. The COO^- and CH_3 POBN adducts gave rise to three sets of doublets each. The stick diagram shows the characteristic beta proton doublet for each adduct, for the low M_I = +1 component. The $AZ\dot{Q}H$ spectrum is overlapping with the central M_I = 0 components of the POBN adducts. The ESR conditions are as in Fig. 1A.

In similar experiments in which POBN was used as the spin trap and sodium formate was used as a hydroxyl radical scavanger, COO^--POBN and CH_3-POBN were also obtained (Table I, Fig. 2). OH-POBN was not expected to be present because its life time is too short to be detected by conventional ESR (28). The observed hyperfine constants are in agreement with literature values (22). The generation of $AZ\dot{Q}H$ and $\dot{O}H$ from AZQ by visible light and the reactions used to detect $\dot{O}H$ in the present work are summarized in equations 1-8 below:

AZQ	$\xrightarrow[500\ nm]{h\nu}$	AZQ*	1
AZQ* + H_2O	$\longrightarrow$	$AZ\dot{Q}H$ + $\dot{O}H$	2
$\dot{O}H$ + DMPO	$\longrightarrow$	OH-DMPO	3
$\dot{O}H$ + $HCOO^-$	$\longrightarrow$	$\dot{C}OO^-$+H_2O	4a
$\dot{C}OO^-$ + DMPO	$\longrightarrow$	COO^--DMPO	4b
$\dot{O}H$ + $(CH_3)_2$ SO	$\xrightarrow[Steps]{Several}$	$\dot{C}H_3$ + Products	5a
$\dot{C}H_3$ + DMPO	$\longrightarrow$	CH_3-DMPO	5b

$$2AZ\dot{Q}H \rightleftharpoons AZQH_2 + AZQ \quad (6)$$

$$AZQH_2 \xrightarrow{\text{OXIDATION}} AZ\dot{Q}H \quad (7)$$

$$AZ\dot{Q}H \xrightarrow{\text{OXIDATION}} AZQ \quad (8)$$

Subsequent to the irradiation of AZQ which is the exclusive light absorption system in the reaction mixture at 500 nm, (eq. 1), the excited AZQ* reacts with water to form the stable radical $AZ\dot{Q}H$ and the short lived hydroxyl radical (eq. 2). The hydroxyl radical will follow competing pathways (eqs. 4a-5b) depending on the arithmetic product of the substrate concentration with which the hydroxyl radicals may react and the rate constant for the particular reaction. Thus, under the conditions employed in our experiments, $\dot{O}H$ can be trapped by DMPO (eq. 3) or scavanged by formate (eq. 4a) and/or dimethyl sulfoxide (eq. 5a) resulting in the $\dot{C}OO^-$ and $\dot{C}H_3$ radicals which are subsequently trapped by DMPO. The AZQ semiquinone free radical can also disproportionate to $AZQH_2$ and AZQ (eq. 6) while $AZQH_2$ can be oxidized in two one-electron steps (eqs. 7 & 8) to obtain the parent compound AZQ (11). At short irradiation times, the concentration of $AZ\dot{Q}H$ was below our detection level. At longer irradiation times, all oxygen was consumed by reactions 7 and 8 and eventually a steady state is reached (eq. 6) with detectable concentrations of $AZ\dot{Q}H$.

If drug free radicals are important in the activity of quinone-containing antitumor agents, as current data seems to indicate (e.g. 7, 9, 5, 11) these results indicate that AZQ may have a potential in photoirradiation therapy, or photodynamic therapy (e.g. 34, 35) a facet which has yet to be investigated. Photodynamic therapy, is a modality for the treatment of cancer, where photosensitizing porphyrins are administered systemically, and light is applied locally (35).

ACKNOWLEDGEMENTS

This investigation was supported by PHS grant CA33861 awarded by the National Cancer Institute, DHHS, USA.

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