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FREE RADICALS IN QUINONE CONTAINING ANTITUMOR AGENTS

THE NATURE OF THE DIAZIQUONE (3,6,-DIAZIRIDINYL-2,5-BIS(CARBOETHOXYAMINO)-1,4-BENZOQUINONE) FREE RADICAL

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The enzymatically generated free radical of the antitumor agent diaziquone is analyzed with the help of two analogs where either the aziridine rings (RQ14) or the carboethoxyamino groups (RQ2) were substituted by chlorine atoms. The hyperfine couplings observed in the diaziquone free radical are due to the nitrogens in the aziridine group. Unresolved coupling and hindered rotation contribute to line broadening. We find that diaziquone free radicals are more stable than RQ14 but less stable than RQ2 free radicals. The reason for this is that the carboethoxyamino groups make the aromatic ring unstable, while the aziridines contribute to its stability. The free radical observed in diaziquone is in all probability that of the parent compound and not that of an intermediate metabolite.

Introduction

Quinone-containing antitumor agents can be biologically reduced to their free radical anions by rat liver microsomes [1], rat liver nuclei [2] and purified NADPH cytochrome *c* reductase [3]. Anthracycline antibiotics (e.g. Adriamycin) have been the agents most widely studied in this manner (e.g. Refs. 1–4). Recently we reported the activation of diaziquone (3,6-diaziridinyl-2,5-bis(carboethoxyamino)-1,4 benzoquinone) to a free radical by liver microsomes and NADPH-cytochrome *c* reductase [5]. This free radical produced a 5-line ESR spectrum of 1 : 2 : 3 : 2 : 1 relative peak intensities and 1.8 G hyperfine coupling. We concluded that we had generated a semi-quinone free radical anion. The 5-line spectrum indicated hyperfine coupling of the unpaired electron to two magnetically equivalent nitrogens, but it was not determined which two of the four nitrogens in the diaziquone molecule was responsible for this interaction. In addition, the origin of the molecule that gives rise to the free radical was not certain, that is, the ESR spectrum could originate from the parent compound, a metabolite or a mixture of both. In this paper we continue to investigate the diaziquone free radical by using two diaziquone analogs (Fig. 1). These analogs contain chlorine atoms substituting either the carboethoxyamino groups (RQ2) or the aziridine groups (RQ14) of the parent compound diaziquone. These analogs will enable us to evaluate separately the influence of the aziridine and carboethoxyamino groups on the free radical stability, and the observed hyperfine coupling. Analogs RQ2 and RQ14 are interesting not only because they can help understand the enzymatically induced free radical anion of diaziquone, but because they are moderately active

as well. Diaziquone and RQ2 were tested in vivo for biological activity against intracerebral murine ependymoblastoma and L1210 tumors and against intraperitoneal murine P388, B16 and L1210 [6]. Diaziquone was shown to be active against all the above tumors and is currently undergoing phase II trials on patients with refractory small cell carcinoma of the lung [7], and brain tumors [8]. Analog RQ2 was active only against intraperitoneal L1210 and P388 [6]. Analog RQ14 was not tested in vivo. However, in vitro, it shows activity against L1210 cells in culture (Gutierrez, Fox, Nakasawa and Bachur, unpublished data).

Materials and Methods

Diaziquone was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health and tested for purity by TLC. No impurities were found, and so the drug was used without further purification. Analogs RQ2 and RQ14 were a gift of Dr. J. Driscoll, Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, National Institute of Health.

Electron spin resonance (ESR) spectra were obtained in a Varian E-109 century series spectrometer using a rectangular TE-104 dual cavity with strong pitch ($g = 2.0028$) in the reference cavity. Strong pitch was used to evaluate g values.

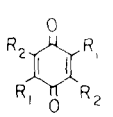
Biochemical reductions were performed in aerobic solutions containing 1 mM diaziquone, RQ2 or RQ14, 1–4 mM NADPH, 200 mM phosphate buffer (pH 7.5) and NADPH-cytochrome c reductase. NADPH-cytochrome c reductase was prepared by the method of Omura and Takesue [9]

Diaziquone was extracted from enzymatic incubations with equal volumes of tetrahydrofuran. The extraction was repeated twice, the extracts were then combined and dried under nitrogen. The residue was taken up in 40 microliters of methanol and spotted (10 microliters) on silica TLC plates containing fluorescence indicators. The TLC plates were then developed in a solvent system containing 20 : 5 : 1 chloroform, methanol and amonium hydroxide and read for quenched fluorescence.

Results

The enzymatically aerobic reduction of diaziquone (Fig. 1) to its free radical anion species, gives a 5-line ESR spectrum at $g = 2.0046$ (5) (Fig. 2A). The width of the lines is approx. 0.96 G with a nitrogen coupling of 1.8 G. The free radical anion of RQ2 produced under identical conditions, consists of a quintet with relative line intensities 1 : 2 : 3 : 2 : 1 at $g = 2.0046$ (Fig. 2B). The line width in this case is 0.25 G. This spectrum is typical of the coupling of an unpaired electron to two equivalent nitrogens. The hyperfine coupling is $a_N = 1.9$ G. The free radical anion of RQ14 produced under the same conditions as above, consists of a triplet at $g = 2.0050$ (Fig. 2C). The line width in this case is 0.80 G. No additional hyperfine was observed in this spectrum, even at modulations of 0.1 G.

The biochemical reduction of RQ14 is much faster than that of RQ2 in aereated solutions (Fig. 3). The RQ14 free radicals are originally very intense and decay very quickly. Using peak to peak measurements of the center line, these free radicals decay within 20 min after the onset of the reaction. On the other hand, RQ2 free radicals are initially very weak, increase gradually and remain in detectable concentrations for up to 6 h. Diaziquone free radicals are observed immediately after the onset of the reaction, but they do not increase considerably over a period of time, decaying before RQ2 radicals but after those of RQ14. The data on Fig. 3 are intended to show the relative trend of free radical production, not absolute values.



COMPOUND	NSC NUMBER	R_1	R_2
DZQ	182986	N◁	$NHCOOCH_2CH_3$
RQ2	30705	N◁	Cl
RQ14	191295	Cl	$NHCOOCH_2CH_3$

Fig. 1. Chemical structures of diaziquone (DZQ) and its RQ2 and RQ14 analogs.

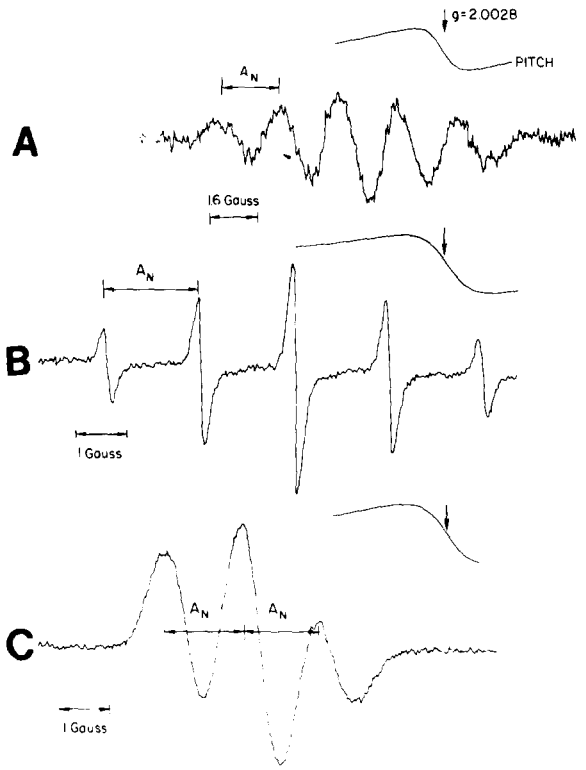


Fig. 2. Electron spin resonance (ESR) spectrum of: (A) diaziquone free radical anion; (B) RQ2 free radical anion and (C) RQ14 free radical anion in aereated mixtures containing 1 mM compounds, 4 mM NADPH, 200 mM phosphate buffer (pH 7.5) and NADPH-cytochrome *c* reductase (4 μg protein). The ESR conditions at 9.3 GHz and room temperature were 10 mW recident microwave power and 0.1, 0.25 and 0.5 G modulation amplitude for A, B and C, respectively.

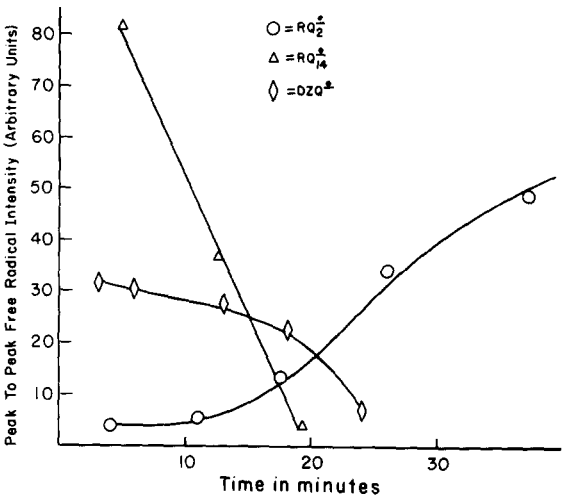
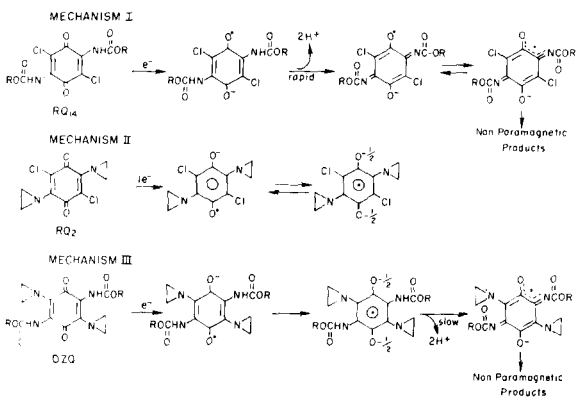


Fig. 3. Typical kinetics of the enzymatically generated free radicals of diaziquone, RQ2 and RQ14 under aerobic conditions. The mixtures and ESR conditions are as described in Fig. 2.

It is of interest to know whether the parent compound, a metabolite or a combination of both is responsible for the free radical observed in the biologically reduced diaziquone. To investigate this, diaziquone (1 mM) was incubated in identical mixtures as those used in the ESR experiments and described in Materials and Methods. Mixtures with and without NADPH-cytochrome *c* reductase were incubated at 37°C for 16 h. NADPH-cytochrome *c* reductase without NADPH and drug was also incubated for 16 h and later assayed for activity. The reaction mixtures were extracted with tetrahydrofuran, spotted on thin layer chromatographic plates with fluorescence indicator and developed in the solvent mixture described in Methods. Results indicate that there is no difference between the controls (no enzyme) and enzyme-treated samples. The spots in both extracts co-chromatograph with diaziquone at $R_F = 0.80$. The R_F values of RQ_2 and RQ_{14} do not co-chromatograph with any of the spots observed in the enzyme-treated diaziquone. The enzyme was found to be active after 16 h, as judged by the stimulation of oxygen consumption by adriamycin [3].

Discussion and Conclusions

The spectrum of RQ14 free radical anion is not what one would expect from the coupling of two nitrogens to an unpaired electron in the quinone pi-system. Rather it is more like the coupling of an unpaired electron with one nitrogen atom where the lines have been broadened, or the coupling of a pi-electron to two equivalent protons. The broad lines could be the result of unresolved coupling and/or hindered rotation. If they were a result of unresolved coupling, one would expect this coupling to be in the order of 0.15 to 0.2 G since 0.1 was the smallest modulation amplitude used. We did not observe such couplings. This result points to the fact that one may not have a pi-electron delocalized in the quinone ring. The RQ14 free radical spectrum is better understood in terms of mechanism I (Scheme 1) where the amide protons



Scheme 1. Mechanisms.

are lost leading to the break down of the aromatic system, and the loss of stability. This mechanism successfully explains coupling to only one nitrogen instead of two. On the other hand, pi-electron coupling to two equivalent protons cannot be ruled out. Electrochemical experiments are underway to investigate this. The carboethoxyamino groups outweigh the quinone ring by 1.9-times. This could be significant, forcing the molecule to rotate preferentially along the carboethoxyamino axis. The restricted motion of the molecule is reflected on the shape of the spectrum which is typically found in spin labels (nitroxyl radicals) undergoing restricted rotation. The unpaired electron in RQ14 free radicals then may not be a pi-system but mainly in the oxygens and delocalizes to a nitrogen in the carboethoxyamino group to generate the small couplings observed.

The RQ2 free radical anion shows the typical 5-line ESR spectrum that one would expect from the coupling of two equivalent nitrogens to an unpaired electron. The hyperfine coupling in this case (1.9 G) compares with that of the diaziquone free radical (1.8 G). The spectrum indicates no motional restriction. In RQ2, (mechanism II) and diaziquone (mechanism III) free radicals, the steric requirements of the system are such that the plane of the aziridine ring is perpendicular to the semiquinone ring. This configuration, confirmed by space filling models, makes the orbital containing the lone pair electrons of the nitrogen to be perpendicular to the pi-orbital of the six membered ring. This arrangement explains the small nitrogen couplings because with this geometry, the coupling is through a sigma bond. This geometry also explains the stability of the RQ2 free radical because there is no additional electrons which could interfere with the stable pi-system in the six membered ring.

The diaziquone free radical anion is consistent with the results described above if we assign the 5 lines observed in the spectra to the hyperfine coupling of the unpaired electron to the nitrogens in the two aziridine groups, and if we attribute the broad lines to restricted motion from the bulky carboethoxyamino groups and unresolved coupling from these groups. More importantly, the breakdown of the aromatic system as in RQ14 explains the intermediate stability of diaziquone as shown in mechanism III. Similarly, the carboethoxyamino groups are probably responsible for the fact that diaziquone free radicals can be generated rather quickly as previously described [5] and as shown in Fig. 3. This is consistent with the fact that RQ14 has a lower half wave reduction potential than RQ2 (Gutierrez et al., unpublished data).

Our diaziquone incubation and TLC analysis revealed that the parent compound is present after long periods of incubation in mixtures identical to those used in the ESR experiments. No other major spots were found.

From the data presented here, it is reasonable to conclude that the ESR spectrum of the enzymatically induced diaziquone free radical anion represents the coupling of an unpaired electron in the pi-system of the quinone ring with the two magnetically equivalent nitrogens of the aziridine groups. Secondly, that the combination of the aziridine and carboethoxy amino groups give diaziquone free radicals the ability to form rapidly in aerobic systems, (carboethoxyamino groups) and its relative stability (aziridines). Finally, that the free radical observed is due to the parent compound. If there are any free radicals from metabolites, they are present at concentrations too low to detect, or are diamagnetic in nature.

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