MOLECULAR PHARMACOLOGY, 26:582-586

Free Radicals in Quinone-Containing Antitumor Agents

Electrochemical Reduction of Diaziquone (2,5-Diaziridine-3,6-bis(carboethoxyamino)-1,4-benzoquinone) and Two Analogues

PETER L. GUTIERREZ,[1] BONNIE M. FOX,[1] MAGDI M. MOSSOBA,[1] AND NICHOLAS R. BACHUR[2]

Division of Developmental Therapeutics, University of Maryland Cancer Center, University of Maryland Medical School, Baltimore, Maryland 21201 and Laboratory of Medicinal Chemistry and Pharmacology, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

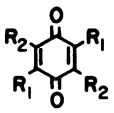
Received June 4, 1984; Accepted August 22, 1984

SUMMARY

The one-electron electrochemical reduction of diaziquone was analyzed using electron spin resonance and compared to its enzymatic reduction. Two analogues were used to help the analysis. The analogues contained chlorine atoms which substituted either the carboethoxyamino groups or the aziridine groups of the parent compound. The diaziquone free radical produced electrochemically in dimethyl sulfoxide exhibited an 11-line electron spin resonance spectrum. The hyperfine couplings responsible for this spectrum were due to the aziridine nitrogens (a_N^{Az}), and the imide hydrogens (a_H^{NHR}) and nitrogens (a_N^{NHR}) of the carboethoxyamino groups. The couplings had the following order of strength: $(a_N^{Az}) > (a_H^{NHR}) > (a_N^{NHR})$. The nuclei responsible for the (a_N^{Az}) and (a_H^{NHR}) coupling were found to be magnetically inequivalent. Adding water to the dimethyl sulfoxide solution of electrochemically reduced diaziquone changed its 11-line ESR spectrum to a 5-line spectrum identical to the one observed for enzymatically reduced diaziquone. Hence, the identity of this latter free radical was resolved. Electrochemically reduced free radicals of antitumor agents can be used to understand biologically reduced ones and thus explore drug activity-free radical structure relationships.

INTRODUCTION

Diaziquone (NSC 182986) belongs to a family of antitumor agents characterized by the presence of quinone groups. In general, quinone-containing antitumor agents can be reduced to their free radical anions by rat liver microsomes (1, 2), rat liver nuclei (3), purified NADPH-cytochrome *c* reductase (4–6), and Ehrlich ascites cells (1). We previously reported the activation of diaziquone (5) and analogues RQ2 and R14 (7) to free radicals by liver microsomes and NADPH-cytochrome *c* reductase. These two analogues contain chlorine atoms which substitute either the carboethoxyamino groups (RQ2) or the aziridine groups (RQ14) of the parent compound AZQ[3] (Fig. 1). The AZQ free radicals observed, at that time, exhibited electron spin resonance spectra, with broad unresolved lines. In this paper, we continue to investigate the diaziquone free radical and that of its two analogues using controlled potential electrolysis. This technique allows one to control the number of electrons involved in the reduction process as well as providing an environment free of metals, proteins, and other chemicals. ESR spectra obtained from free radicals reduced with controlled potential electrolysis have well resolved lines helpful in the indentification of the free radical. Diaziquone was shown to be active against intracerebral murine ependymoblastoma and L1210 tumors as well as intraperitoneal murine P388, B16, and L1210 (8). Currently,



COMPOUND	NSC NUMBER	R_1	R_2
AZQ	182986	N⊲	$NHCOOCH_2CH_3$
RQ2	30705	N⊲	Cl
RQ14	191295	Cl	$NHCOOCH_2CH_3$

FIG. 1. *Chemical structure of diaziquone, and its analogues RQ2 and RQ14*

This investigation was supported by United States Public Health Service Grant CA33681 awarded by the National Cancer Institute.

[1] University of Maryland Cancer Center.

[2] National Cancer Institute.

[3] The abbreviations used are: AZQ, diaziquone; DMSO, dimethyl sulfoxide.

0026-895X/84/060582-05$02.00/0

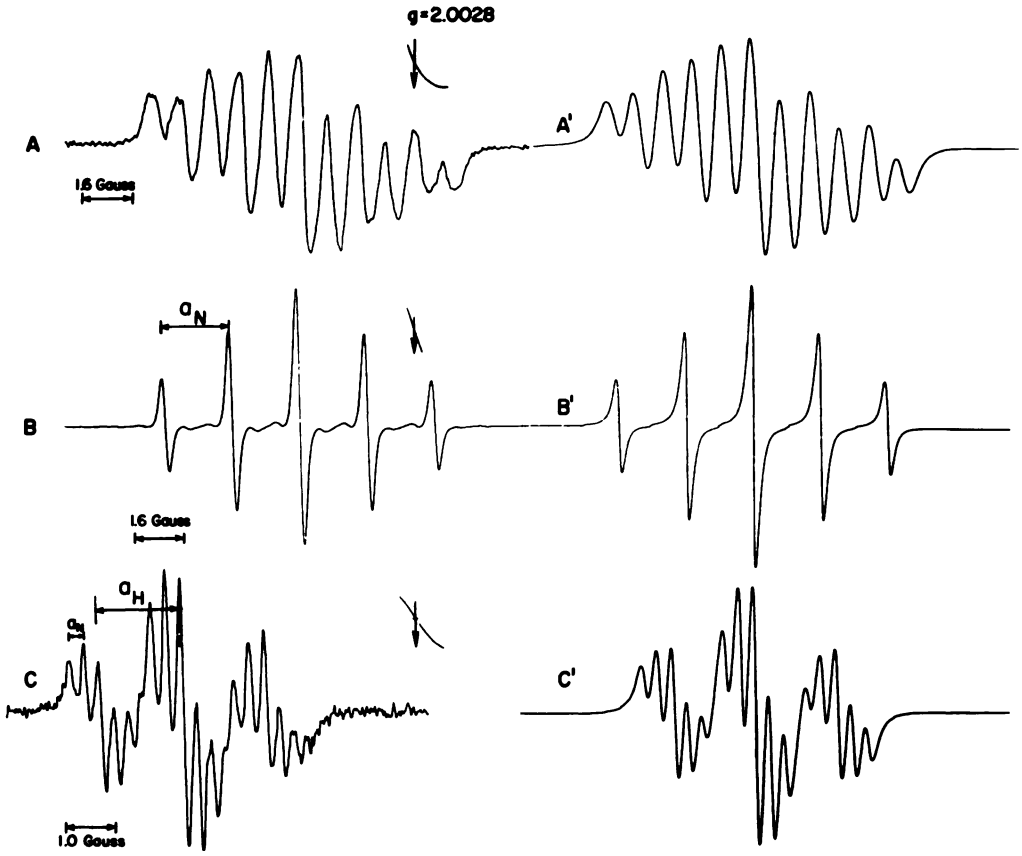


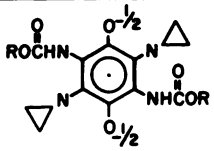
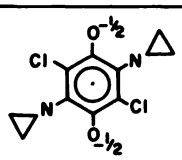
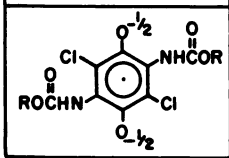
FIG. 2. *Electron spin resonance spectra of the one-electron electrochemical reduction of (A) diaziquone, (B) RQ2, and (C) RQ14*

Electrochemical reduction of 5×10^{-4} M compounds were carried out in anaerobic solutions of DMSO using 0.1 M tetraethyl ammonium perchlorate as electrolyte (see Materials and Methods). The ESR conditions at room temperature were 9.3-GHz microwave frequency, 10-mW incident microwave power, and 0.1 G modulation amplitude for A and B and 0.16 G for C. Spectral simulations for (A′) diaziquone, (B′) RQ2, and (C′) RQ14 free radicals were performed in a Nicolet 1180 computer using Lorentzian linewidths at 0.4 G for A′ and 0.2 G for B′ and C′.

TABLE 1

Electron spin resonance parameters for the electrochemically generated free radicals of diaziquone and its analogues RQ2 and RQ14

The hyperfine coupling constants of the electrochemically generated radicals were obtained by computer simulation.

FREE RADICAL	g-VALUE		HYPERFINE COUPLING (GAUSS)			
			AZIRIDINE		CARBOETHOXYAMINO	
	ELECTRO CHEM.	ENZYM.	ELECTRO CHEM.	ENZYM.	ELECTRO CHEM.	ENZYM
	2.0048	2.0046	1.88 (N) 1.78 (N)	1.8 (2N)	0.87 (H) 0.73 (H) 0.15 (2N)	—
	2.0049	2.0046	2.10 (2N)	1.9 (2N)		
	2.0055	2.0050			1.64 (2H) 0.30 (2N)	1.6 (2H)

diaziquone is undergoing Phase I (e.g., 9) and Phase II (e.g., 10) clinical trials and its mechanism of action is under investigation. It is possible that diaziquone free radicals play a role in the drug's mechanism of action either by affecting its alkylating activity or by rendering the drug more reactive. Mitomycin C, another quinone-containing antitumor agent, was shown to require the chemical reduction of the quinone moiety to its free radical species for drug activity (11, 12).

MATERIALS AND METHODS

Diaziquone was supplied by the Drug Synthesis and Chemistry Branch, Divisions of Cancer Treatment, National Cancer Institute and tested for purity by thin layer chromatography (13). No impurities were found, and so the drug was used without further purification. Analogues RQ2 and RQ14 were a gift of Dr. J. Driscoll, Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute.

ESR spectra were obtained with an X-band (9.3 GHz) Varian E-109 Century Series spectrometer (Varian Instruments Division, Palo Alto, CA) equipped with 100-kHz field modulation. A dual rectangular cavity (TE104) was used which contained strong pitch (g = 2.0028) in one section, and the sample in an ESR electrolytic flat cell (J. Scanlon, Solvang, CA) in the other. Strong pitch was used to evaluate g values. Diaziquone or analogues at 5×10^{-4} M were prepared in freshly opened dimethyl sulfoxide (spectrophotometric grade, Aldrich, Milwaukee, WI) containing 0.1 M (final concentration) tetraethylammonium perchlorate (Kodak Co., Rochester, NY).

All solutions were purged with nitrogen for the first 15 min of the electrolysis which was performed as follows: mercury at the bottom of the flat cell provided the cathode contact with the solution through contact with a platinum wire electrode. A second platinum wire electrode, inserted through the side port of the cell, was used as a counter-electrode. The reference electrode was a Ag/AgCl electrode (Bioanalytical Systems, West Lafayette, IN). The voltage across the solution was measured with a Fluke 800A digital multimeter and set with an AMEL model 550 potentiostat (ECO Instruments, Newton, MA). One-electron electrolyses were carried out in DMSO with the voltages set at −0.9 V for RQ2 and AZQ and −0.6 V for RQ14 to produce free radical anions. These voltages were determined by cyclic voltametry which will be discussed elsewhere. A second electron can be added at a more negative potential to produce diamagnetic dianions. Immediately after electrolysis, the flat cell containing the free radical solution was placed in the ESR cavity and spectra were recorded at room temperature.

Spectral simulation were carried out on a Nicolet 1180 computer (Nicolet Instrument Corp., Madison, WI) using the program ESRSIM provided by Nicolet.

RESULTS

The ESR spectrum of the one-electron reduced radical anion of diaziquone (Fig. 2A) can be best understood in terms of the spectra of RQ2 (Fig. 2B) and RQ14 (Fig. 2C). The spectrum of RQ2 free radical anion is a 1:2:3:2:1 quintet at g = 2.0049. This spectrum indicates the coupling of the unpaired electron to two equivalent nitrogens in the aziridine rings of RQ2 (Table 1). On the other hand, the spectrum of the RQ14 free radical indicates that the nitrogens on the carboethoxyamino group have a smaller coupling (0.30 G) than their adjacent hydrogens (1.64 G) two bond lengths away from the quinone ring (Table 1). This gave rise to a 1:2:1 triplet of 1:2:3:2:1 quintets (Fig. 2C) at g = 2.0055. The simulations shown on Fig. 2B′ and 2C′ verify our assignments. Hyperfine couplings for the two naturally occurring chlorine isotopes (^{35}Cl, ^{37}Cl; $I = 3/2$; natural abundance 76% and 24% respectively) are difficult to observe because of quadrupole relaxation effects (14). For RQ2, however, ^{13}C satellites ($I = 1/2$, natural abundance 1.11%) were observed.

The one-electron reduction of diaziquone produces a free radical at g = 2.0048 whose ESR spectrum exhibited 11 approximately equidistant lines with no standard intensity ratios. The analysis of the diaziquone free radical was based on the assumption that, as in the cases of RQ2 and RQ14, the largest couplings would be due to two aziridine nitrogens, while the protons and nitrogens in the carboethoxyamino groups would be responsible for the intermediate and smallest couplings, respectively. The simulation of the diaziquone radical revealed that the assumption mentioned above is true, but the aziridine nitrogens and imide protons were found to be slightly magnetically inequivalent (i.e., 1.88 and 1.78 G for the aziridine nitrogens and 0.87 and 0.73 G for the imide protons). The small nitrogen couplings from the carboethoxyamino groups were found to be equal with a hy-

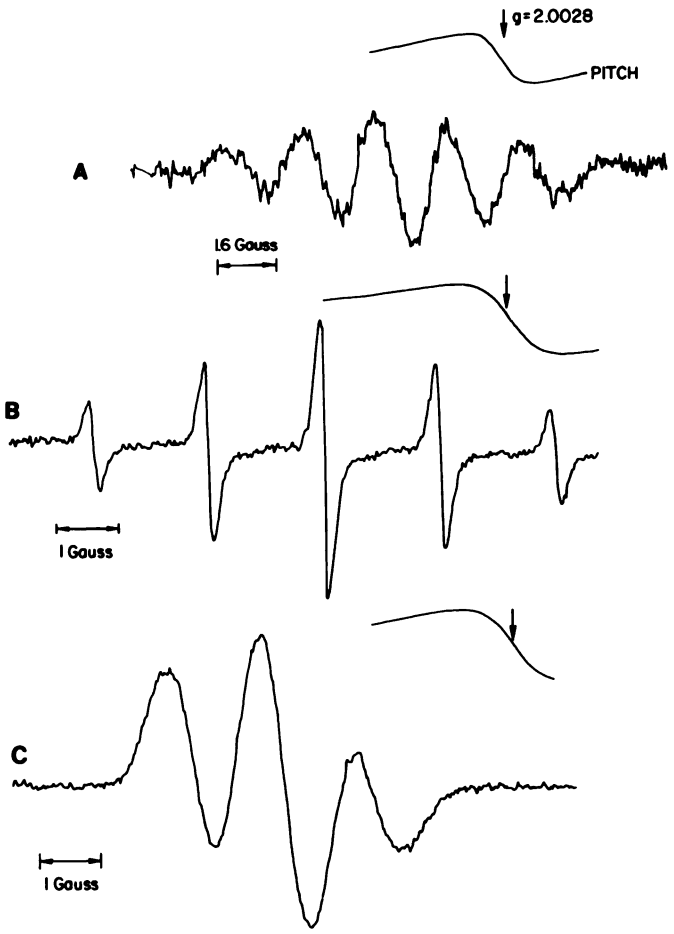


FIG. 3. *Electron spin resonance spectra of enzymatically reduced diaziquone (A), RQ2 (B), and RQ14 (C) taken from ref. 7 and presented here for comparison*

The aerated solutions contained 1 mM compounds, 3–4 mM NADPH, 200 mM phosphate buffer (pH 7.5), and NADPH-cytochrome c reductase (4 mg of protein). The ESR conditions at 9.3 GHz and room temperature were 10-mW incident microwave power and 0.1, 0.25, and 0.5 G modulation amplitude for A, B, and C respectively. Smaller modulation amplitudes did not further resolve the RQ14 spectrum (C).

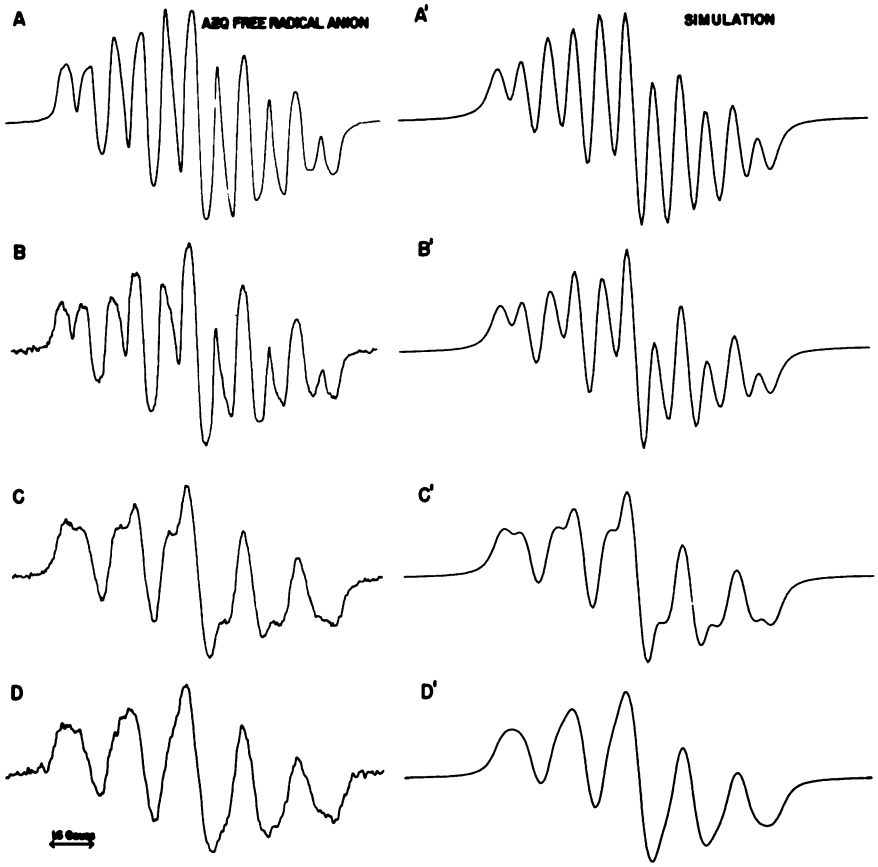


FIG. 4. *Electron spin resonance spectra of the one-electron electrochemical reduction of AZQ (A)*
Subsequent to the reduction in DMSO, water was added (20% by volume) and slow mixing of the solvents is observed in B and C. Final mixing is represented in D. The ESR conditions at room temperature were as in Fig. 2. Spectral simulations for the diaziquone free radical (A') and water-containing mixtures (B', C', and D') were performed in a Nicolet 1180 computer using Lorentzian linewidths at 0.4 G.

TABLE 2

Hyperfine coupling constants obtained by computer simulation for the electrochemically generated AZQ free radical in DMSO and DMSO/H_2O (80:20) by volume

Aziridine		Carboethoxyamino	
DMSO	DMSO/H_2O	DMSO	DMSO/H_2O
1.88 (N)	1.86 (2N)	0.87 (H)	0.63 (2H)
1.78 (N)		0.73 (H)	
		0.15 (2N)	0.21 (2N)

perfine coupling constant of 0.15 G (Table 1). In reality, these nitrogens may be slightly inequivalent, but not enough to alter or improve on the best simulation obtained.

The spectra of free radicals obtained by enzymatic reduction of diaziquone and analogues RQ2 and RQ14 have been previously described (5, 7) and are shown here on Fig. 3 for comparison. It can be seen that the ESR spectral linewidths are broad in the diaziquone and RQ14 free radicals spectra (0.80 and 0.96 G, respectively), when compared to the spectra of the electrochemically produced free radicals (0.48 G for AZQ and 0.15 G for RQ14). The narrow linewidth in the electrochemically obtained RQ14 free radical anion allowed for the detection of the smaller nitrogen coupling which was the important clue that led to the successful simulation of the AZQ free radical anion. The observed linewidths of the RQ2 free radical anion (0.25 G) remained the same in both electrochemical and enzymatic reductions.

When water (20% by volume) was added to a DMSO solution of the electrochemically reduced AZQ, the 11-line free radical spectrum gradually changed into the 5-line spectrum observed in enzymatic reductions (Figs. 4A–D and 3A). This experiment was carried out by adding a known volume of deaerated water to a DMSO solution containing AZQ free radical anion that was already present in the ESR flat cell. Consecutive spectra were recorded in the absence of electrochemical reduction, as the mixing progressed to completion. Spectral simulation of these water-induced spectral changes (Fig. 4A–D) indicated that each of the two pairs of aziridine nitrogens and imide protons involved in the coupling to the unpaired electron became magnetically equivalent (Table 2).

When the electrolysis of AZQ was carried out in an aqueous solution (Hanks' balanced salt solution, pH 7.5) containing 0.05 M KCl as supporting electrolyte, the 5-line (enzymatic) ESR spectrum was obtained. It is noted that this same spectrum was also obtained when AZQ was added to L1210 murine leukemic cells in Hanks' medium at the same pH.[4]

[4] P. L. Gutierrez, unpublished data

DISCUSSION

The spectra observed for the one-electron electrochemically reduced diaziquone, RQ2, and RQ14 have confirmed our previous statement that the free radicals obtained enzymatically (5, 7) from these compounds were indeed the corresponding one-electron free radical anions.

The broadening of the 1:2:1 triplet from enzymatically reduced RQ14 (Fig. 3C) did not allow the small nitrogen couplings to be detected (Fig. 2C). As a result, unambiguous coupling assignments could not be made (7). The spectrum of RQ2 free radical anion is basically the same in both generating systems (Figs. 2B and 3B). This implies that the difference observed in the diaziquone free radical (i.e., Figs. 2A and 3A) is due to the unresolved couplings of the carboethoxyamino groups. This line broadening arises in all probability from the protonation ($I = ½$) by water of the free radical anions generated in DMSO. Our data show that it is possible to obtain the spectrum of the enzymatically generated free radical (Fig. 3A) from that of the electrochemically reduced diaziquone in DMSO (Figs. 2A and 4A) by adding water (Fig. 4C).

The pertinent question of whether or not free radicals generated electrochemically are the same as those generated enzymatically has been answered in the affirmative. Hyperfine coupling constants are known to depend on the nature of the solvent used (15). Since the electrochemical measurements that lead to well resolved spectra were carried out in DMSO while the enzymatic reduction was done in aqueous medium, a difference in coupling constants is expected (15, 16) (Table 2).

It is reasonable to treat electrochemically reduced free radicals as having the same chemical structure as biologically generated ones. This means that one can study the basic structure of free radicals of quinone-containing agents generated electrochemically and draw biologially relevant conclusions with regards to the drug free radical structure and the agent's activity. For instance, because the spin density is proportional to the hyperfine coupling, one can deduce from our data that RQ2 has more spin density in the carbons adjacent to the aziridine rings than AZQ. One can speculate that a mechanism involving drug free radicals in the drug's antitumor activity is that these relative spin densities can favor the protonation of the aziridine ring leading the aziridinium ion, the required species for alkylation (17). With this hypothesis in mind, our results imply that RQ2 is more active that AZQ because of the former's greater spin density at the carbons attached to the aziridine rings.

REFERENCES

1. Sato, S., M. Iwaizumi, K. Handa, and Y. Tamura. Electron spin resonance study of the mode of generation of free radicals of daunomycin, Adriamycin and carboquone in NAD(P)H-microsome system. *Gann* **68**:603–608 (1977).
2. Bachur, N. R., S. Gordon, and M. Gee. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res.* **38**:1745–1750 (1978).
3. Bachur, N. R., M. V. Gee, and R. D. Friedman. Nuclear catalyzed antibiotic free radical formation. *Cancer Res.* **42**:1078–1081 (1982).
4. Bachur, N., S. Gordon, M. Gee, and H. Kon. NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. *Proc. Natl. Acad. Sci. USA* **76**:954–957 (1979).
5. Gutierrez, P. L., R. Friedman, and N. Bachur. Biochemical activation of AZQ (3,6,-diaziridinyl-2,5-bis(carboethoxyamino)-1,4-benzoquinone) to its free radical species. *Cancer Treat. Rep.* **66**:339–342 (1982).
6. Gutierrez, P. L., M. V. Gee, and N. R. Bachur. Kinetics of anthracycline antibiotic free radical formation and reductive glycosidase acivity. *Arch. Biochem. Biophys.* **223**:68–75 (1983).
7. Gutierrez, P. L., and N. R. Bachur. Free radicals in quinone containing antitumor agents: the nature of the diaziquone (2,5,-diaziridinyl-3,6,-bis(carboethoxyamiino), -1,4-benzoquinone) free radical. *Biochim. Biophys. Acta* **758**:37–41 (1983).
8. Driscoll, J. S., L. Dudeck, G. Congleton, and R. Geran. Potential CNS antitumor agents. VI. Aziridinylbenzoquinones III. *J. Pharm. Sci.* **68**:185–188 (1979).
9. Schilsky, R., J. Kelly, D. Ihde, D. Howser, R. Cordes, and R. Young. Phase I trial and pharmacokinetics of aziridinylbenzoquinone (NSC 182986) in humans. *Cancer Res.* **42**:1582–1586 (1982).
10. Curt, G., J. Kelly, C. Kufta, B. Smith, P. Kornblith, R. Young, and J. Collins. Phase II and pharmacokinetic study of aziridinylbenzoquinone 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone,diaziquone, NSC 182986 in high-grade gliomas. Phase II. *Cancer Res.* **43**:6102–6105 (1983).
11. Schwartz, H. S., J. E. Sodergren, and F. S. Phillips. Mitomycin C: chemical and biological studies on alkylation. *Science* **142**:1181–1183 (1963).
12. Moore, H. W. Bioactivation as a model for drug design bioreductive alkylation. *Science* **197**:527–532 (1977).
13. AZQ (NSC 182986) Clinical Brochure. *Cancer Therapy Evaluation Program, Division of Cancer Treatment.* National Cancer Institute, Bethesda, MD, August 1979.
14. Fairbowin, A., and E. A. Lucken. Spin density in subsituted 1,4-benzosemiquinones. *J. Chem. Soc.* 258–262 (1963).
15. Gendell, J., J. H. Freed, and G. K. Fraenkel. Solvent effects in electron spin resonance spectra. *J. Chem. Phys.* **37**:2832–2341 (1962).
16. Haak, R., and B. Benson. Effects of crystalline environment on pyrimidine free-radical formation: barbital crystal polymorphs and sodium barbital. *Int. J. Radiat. Biol.* **23**:157–166 (1973).
17. Dermer, O. C., and G. H. Ham. *Ethylenimine and Other Aziridines.* Academic Press, New York, 2-52 (1968).

Send reprint requests to: Peter L. Gutierrez, Ph.D., University of Maryland Cancer Center, University of Maryland Medical School, 655 W. Baltimore Street, Room 9-012, Baltimore, MD 21201.