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Original Contribution

REDOX CYCLING OF POTENTIAL ANTITUMOR AZIRIDINYL QUINONES

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Abstract—The formation of reactive oxygen intermediates (ROI) during redox cycling of newly synthesized potential antitumor 2,5-*bis* (1-aziridinyl)-1,4-benzoquinone (BABQ) derivatives has been studied by assaying the production of ROI (superoxide, hydroxyl radical, and hydrogen peroxide) by xanthine oxidase in the presence of BABQ derivatives. At low concentrations (< 10 μM) some BABQ derivatives turned out to inhibit the production of superoxide and hydroxyl radicals by xanthine oxidase, while the effect on the xanthine-oxidase-induced production of hydrogen peroxide was much less pronounced. Induction of DNA strand breaks by reactive oxygen species generated by xanthine oxidase was also inhibited by BABQ derivatives. The DNA damage was comparable to the amount of hydroxyl radicals produced. The inhibiting effect on hydroxyl radical production can be explained as a consequence of the lowered level of superoxide, which disrupts the Haber-Weiss reaction sequence. The inhibitory effect of BABQ derivatives on superoxide formation correlated with their one-electron reduction potentials: BABQ derivatives with a high reduction potential scavenge superoxide anion radicals produced by xanthine oxidase, leading to reduced BABQ species and production of hydrogen peroxide from reoxidation of reduced BABQ. This study, using a unique series of BABQ derivatives with an extended range of reduction potentials, demonstrates that the formation of superoxide and hydroxyl radicals by bioreductively activated antitumor quinones can in principle be uncoupled from alkylating activity.

Keywords—Aziridinyl quinones, Reactive oxygen species, Redox cycling, DNA single-strand breaks, Superoxide anion radical, Hydroxyl radical, tumor therapy, Free radicals

INTRODUCTION

Among the drugs used in antitumor chemotherapy today are several drugs containing a quinone moiety —for example, anthracyclines (e.g., adriamycin), mitomycin, aziridinylbenzoquinones (e.g., diaziquone [AZQ]), and others (for reviews, see Refs. 1 and 2). The antitumor activity of compounds containing a benzoquinone moiety and an alkylating function has been claimed to be due to alkylating properties as well as to the formation of reactive oxygen intermediates (ROI) during redox cycling of these compounds.[3] These two mechanisms are related: Reduction of the quinone moiety may activate the alkylating properties of the compound, as demonstrated for adriamycin, mitomycin C (Ref. 4), and aziridinylbenzoquinone derivatives,[5,6] while oxidation of the reduced quinone moiety by molecular oxygen generates ROI.

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Continuous reduction and reoxidation of the quinone-containing compound (redox cycling) is thought to produce high amounts of ROI, ultimately leading to toxicity (e.g., cardiotoxicity).[7] In this model of redox cycling, one-electron reduced quinone (semiquinone) produces superoxide anion radicals (O_2^-), which dismutate to yield H_2O_2. Reaction between O_2^- and H_2O_2 can lead to the formation of $^{\cdot}OH$ in an iron-mediated Haber-Weiss sequence represented by Eqs. 1–3 (Ref. 8).

$$Fe^{3+} + O_2^- \rightleftharpoons Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad (2)$$

$$O_2^- + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^- \quad (3)$$

$^{\cdot}OH$ is considered to be the most reactive oxygen intermediate and can give rise to lipid peroxidation and degradation of proteins and DNA.[9–11]

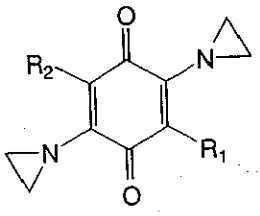


Fig. 1. General structure of BABQ derivatives.

Bis(aziridinyl)benzoquinone (BABQ) derivatives have been synthesized as potential antitumor compounds. They contain a benzoquinone moiety and two alkylating aziridine groups (Fig. 1; Table 1). Several derivatives have reached clinical application (e.g., AZQ, trenimon, and carboquone), although their importance for cancer chemotherapy has yet to be established.[12] BABQ derivatives alkylate DNA after reductive and/or acidic activation.[6] Therefore they are of potential use against hypoxic tumors and tumor cells with decreased intracellular pH.[13]

Previously, we studied xanthine oxidase (xanthine: oxygen oxidoreductase; EC 1.1.3.22) as a reducing enzyme for a series of potential antitumor BABQ derivatives.[14] In vivo, xanthine oxidase appears to function as a reduced nicotinamide adenine dinucleotide (NADH)-dependent dehydrogenase, but in hypoxic cells and during ischemia it becomes an O_2-utilizing oxidase capable of reducing a wide range of substrates.[15] We showed that under air, xanthine oxidase reduces both BABQ derivatives and molecular oxygen.[14] Reduction of BABQ derivatives by xanthine oxidase produces the one-electron reduced BABQ semiquinone ($BABQ^-$) and possibly some BABQ hydroquinone ($BABQH_2$). $BABQH_2$ can also be formed by dismutation of $BABQ^-$ to quinone (BABQ) and two-electron reduced quinone ($BABQ^{2-}$) (Eq. 4).

$$2\ BABQ^- \rightleftharpoons BABQ + BABQ^{2-} \qquad (4)$$

Two-electron reduced quinone species are assumed to be readily protonated at physiological pH.[16]

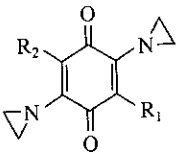
The present investigation was aimed at determining the effect of a series of newly synthesized BABQ derivatives, with various one-electron reduction potentials, on ROI production during reduction. Xanthine oxidase was used, which also enables us to study effects of BABQ derivatives on already formed ROI. Furthermore, we studied DNA damage, especially single-strand breaks in the system containing xanthine oxidase and BABQ derivatives.

MATERIALS AND METHODS

BABQ, TW14 (Ref. 17), TW22, TW25, TW39 (Refs. 18 and 19), and TW19 (Ref. 20) were prepared as described in the literature. Diaziquone was a gift from the Drug Synthesis and Chemistry Branch, National Cancer Institution (Bethesda, MD). Adriamycin was kindly provided by Dr. S. Penco, Farmitalia, Milan, Italy. 2-Methylnaphthoquinone (menadione) was from Aldrich Europe (Bornum, Belgium). All compounds appeared to be pure by high-pressure liquid chromatography (HPLC) analysis. Stock solutions of the compounds contained 5 mM quinone in *N,N*-dimethylformamide and were kept at −20°C in the dark. Catalase (H_2O_2: H_2O_2 oxidoreductase; EC 1.11.1.6 from beef liver; 20 $mg \cdot mL^{-1}$, 65,000 $U \cdot mg^{-1}$) and superoxide dismutase (Superoxide: superoxide oxidoreductase; EC 1.15.1.1 from bovine erythrocytes; 5000 $U \cdot mg^{-1}$) were from Boehringer (Mannheim, Germany). Horseradish peroxidase (Donor: H_2O_2 oxidoreductase; EC 1.11.1.7), desferrioxamine mesylate, lucigenin, xanthine (sodium salt), and xanthine oxidase (Grade III, from buttermilk, EC 1.2.3.2; 15.4 $U \cdot mL^{-1}$; 1.2 U/mg protein) were from Sigma Chemical Co. (St. Louis, MO). Perhydrol (30% H_2O_2 pro analysi [p.A.]) was from Merck (Darmstadt, Germany). Agarose was from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of analytical grade. Double distilled or Milli-Q purified water was used. TE buffer contained 10 mM Tris buffer and 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0. *E. coli* JM101 and pBr322 plasmid were a gift from Dr. P. Baas, Department of Molecular Biology, Utrecht University, The Netherlands. Plasmids were transfected and grown in *E. coli* JM101 and isolated by standard methods.[21]

Luminescence measurements were performed on a Hewlett-Packard (Downers Grove, IL) Pico-Lite luminometer. Absorbances were measured on a Perkin Elmer (Norwalk, CT) Lambda 5 spectrophotometer.

Table 1. Structures of 2,5-*bis*(1-aziridinyl)-1,4-benzoquinone (BABQ) and Derivatives



Compound	R_1	R_2
BABQ	H	H
TW14	Cl	Cl
TW19	F	F
TW22	Br	CH_3
TW25	Br	C_2H_5
TW39	CH_3	$C_2H_4OCONH_2$
AZQ	$NHCOOC_2H_5$	$NHCOOC_2H_5$
Carboquone	CH_3	$CH(OCH_3)CH_2OCONH_2$
Trenimon	H	Aziridinyl

Electron spin resonance (ESR) spectra were recorded on a Bruker (Karlsruhe, Germany) ESP-300, at room temperature and 100 kHz frequency, with 20 mW incident power and 0.4 G modulation amplitude.

Superoxide assay

The amount of superoxide anion radical was determined by measuring the chemiluminescence in the presence of lucigenin. Reaction mixtures contained 0.1 mM xanthine, 0.1 mM EDTA, 5 μM $FeNH_4(SO_4)_2$, 0.05 mM lucigenin and quinone compound at the indicated concentrations, in 0.1 mM phosphate buffer, pH 7.5. The reactions were started by adding xanthine oxidase to a final concentration of 2 mU/mL. The reaction mixture was kept at 30°C and the luminescence (counts per min) was measured by monitoring the reaction for 15 min. After correction for quenching by *N,N*-dimethylformamide, the intensity of the peak levels was used to quantify the chemiluminescence, as has been described.[22]

Assay of oxygen consumption

Oxygen consumption assays were performed under the same reaction conditions as for the superoxide assay using a YSI model 53 oxygen monitor (Yellow Springs Instruments Inc., Yellow Springs, OH), equipped with a Clark electrode.

Hydrogen peroxide assay

The hydrogen peroxide assay is based on a horseradish peroxidase (HRP)-coupled color reaction.[23,24] The reaction mixture contained 0.1 mM xanthine, 0.1 mM EDTA, 2 μM $FeNH_4(SO_4)_2$, and quinone at the indicated concentrations, in 5 mM phosphate buffer, pH 6.8. The measuring solution consisted of 0.1% (w/v) sulphonated 2,4-dichlorophenol, 0.1% (w/v) 4-aminoantipyrine, and 0.0088% (w/v) horseradish peroxidase in 5 mM phosphate buffer, pH 6.8. After addition of 1 mL of measuring solution to 3 mL reaction mixture, the reaction was started by adding xanthine oxidase to a final concentration of 3 mU/mL. The mixture was kept at 25°C and the absorbance at 505 nm was read at timed intervals. The rate of H_2O_2 production (slope of the linear increase in absorbance vs. time) was corrected for *N,N*-dimethylformamide effects and plotted against concentration of quinone. As a control, the effect of BABQ on HRP activity was tested by following spectrophotometrically the oxidation of chlorpromazine. BABQ (20 μM) did not significantly affect the HRP activity.

Hydroxyl radical assay

$^{\cdot}OH$ production was assayed by measuring the formation of degradation products from deoxyribose, which upon heating with thiobarbituric acid at low pH yield a pink chromogen.[25] The reaction mixture contained 0.1 mM xanthine, 0.1 mM EDTA, 5 μM $FeNH_4(SO_4)_2$, 3 mM deoxyribose, and quinone at the indicated concentrations, in 50 mM phosphate buffer, pH 7.5. The reaction was started by adding xanthine oxidase to a final concentration of 5 mU/mL. The reaction mixture was kept at 25°C. At timed intervals, samples were taken and mixed with equal volumes of thiobarbituric acid solution (1% w/v in 50 mM NaOH) and trichloroacetic acid (2.8% w/v in water). After heating for 15 min at 100°C and subsequent cooling, absorbance was read at 532 nm vs. blank. The rate of $^{\cdot}OH$ production (slope of the linear increase in absorbance vs. time) was corrected for *N,N*-dimethylformamide-induced effects and plotted against concentration of added compound.

Assay of one-electron reduction potential of BABQ derivatives

The one-electron reduction potentials of the BABQ derivatives were measured using the pulse-radiolysis facility at the Paterson Institute. The system consisted of a tungsten analyzing lamp and a Kratos (Manchester, England) monochromator. Microcells with an absorbance path length of 2.5 cm were used throughout. The method for measuring the potentials is similar to that previously reported.[26] The standards used were oxygen ($E_7^1(O_2/O_2^-)$ = −155 mV) and 2,5-dimethylbenzoquinone ($E_7^1(BQ/BQ^-)$ = −66 mV).

Essentially the quinone radicals, which are rapidly generated by pulse radiolysis, are allowed to come into equilibrium with compounds of known one-electron reduction potentials. The equilibrium constant was measured before the decay of the radicals, and from these values the reduction potentials could be determined.

Assay of strand breaks in plasmid DNA

The reaction mixture was the same as used in the hydroxyl radical assay, except that instead of 3 mM deoxyribose, 0.1 mg/mL pBr322 plasmid DNA was present. The reaction was started by adding xanthine oxidase to a final concentration of 5 mU/mL. The reaction mixture was incubated at 25°C for 30 min and was then stopped by putting the samples on ice. The samples were loaded on an agarose gel (1% in TE buffer) and separated by electrophoresis in TE buffer

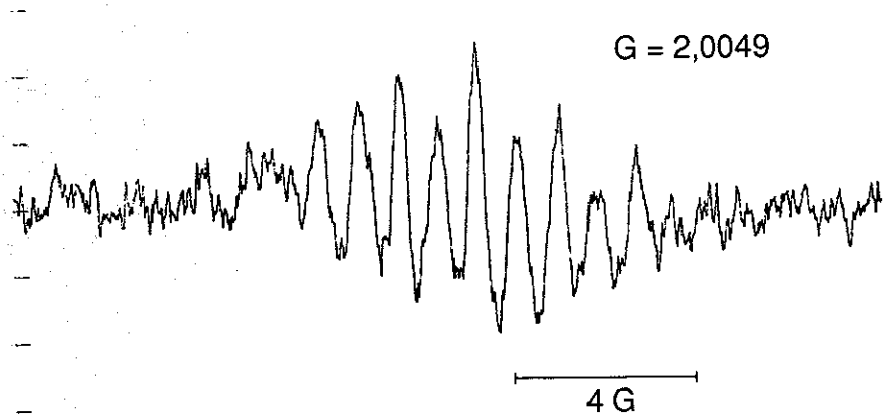


Fig. 2. ESR spectrum of BABQ semiquinone radical in an aerobic mixture containing BABQ (0.1 mM), xanthine (500 μM), xanthine oxidase (100 mU/mL), Fe^{3+} (5 μM), and EDTA (0.1 mM) in 50 mM phosphate buffer pH 7.5 (g = 2.0049).

during 90 min at 50 V. DNA bands were made visible by ethidium bromide fluorescence and photographed as described.[21] Photograph negatives were densitometrically scanned on a Zeiss (Württemberg, Germany) KM3 chromatogram spectrometer, in transmission mode.

Autooxidation of electrochemically reduced BABQ derivatives

A solution of 0.05 mM BABQ derivative in 50 mM phosphate buffer, pH 7.5, was electrochemically reduced under nitrogen at a potential 200 mV more negative than the halfwave potential ($E_{1/2}$) of the BABQ derivative, as previously described.[6] After reoxidation under exposure to air, H_2O_2 concentration was assayed.

RESULTS AND DISCUSSION

Generation of superoxide anion radical

When BABQ derivatives are reduced aerobically by xanthine oxidase, formation of $BABQ^-$ can be demonstrated using ESR spectrometry (Fig. 2). The spectra of the $BABQ^-$ radicals are similar to the spectra obtained by Gutierrez et al.[27] for diaziquone and other BABQ derivatives. A high concentration of xanthine oxidase was required to obtain an ESR signal. The intensity of the ESR signal was generally higher for compounds that have a higher rate of reduction by xanthine oxidase as assayed by cytochrome *c* reduction.[14] The low intensity of the ESR signal indicates that the steady-state concentration of $BABQ^-$ in the presence of oxygen is low, as has also been reported for AZQ semiquinone radicals.[5]

To detect O_2^-, chemiluminescence in the presence of lucigenin was used. In the xanthine/xanthine oxidase system, lucigenin is a specific indicator for O_2^- (Ref. 28). Assay of O_2^- with the frequently used succinylated cytochrome *c* reduction method was not successful, as under anaerobic conditions succinylated cytochrome *c* was also reduced by reduced aziridinyl quinone species.

Figure 3 shows the effects of addition of BABQ on the production rate of O_2^- and H_2O_2. The inhibiting effect of a series of BABQ derivatives on O_2^- production is presented in Table 2. In the presence of BABQ derivatives under aerobic conditions, cytochrome *c* reduction increases[14] while the xanthine oxidation rate also increases, as has been shown for TW19 (Ref. 14). This indicates that BABQ derivatives are efficiently reduced by xanthine oxidase also in the presence of oxygen: The concentrations of BABQ derivatives that inhibit 50% of O_2^- production are much lower than the K_M values for anaerobic BABQ reduction by xanthine oxidase (Table 2). For example, TW19 inhibits 50% of the O_2^- production at a concentration of 0.062 μM, but its anaerobic reduction rate by xanthine oxidase is half maximal at a concentration of 16 μM (Ref. 14). Efficient reduction of BABQs compared to oxygen also appears from the K_M value of 800 μM for oxygen reduction by xanthine oxidase as assayed with cytochrome *c* reduction,[29] which is larger than corresponding BABQ K_M values (Table 2). Comparing the aerobic and anaerobic xanthine oxidation rate in the presence of TW19 (Ref. 14), it can be concluded that at TW19 concentrations higher than 10 μM no substantial direct oxygen reduction by xanthine oxidase occurs and that the electron flow is directed toward quinone reduction. Therefore, competition between molecular oxygen and BABQ derivative for reduction by xanthine oxidase is insufficient to explain the observed results. The decreasing effect of the quinones on the lucigenine chemiluminescence can in principle also be caused by quenching of the chemiluminescence by the quinones. This possibility

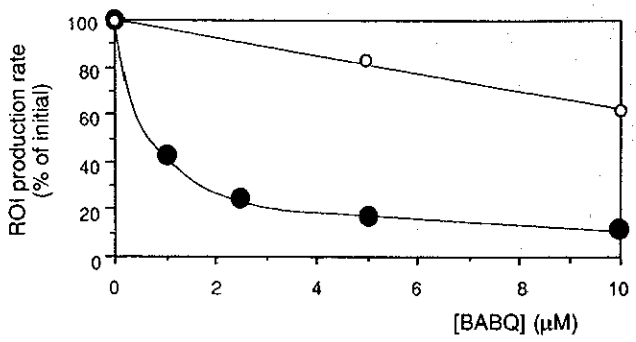


Fig. 3. Effects of various concentrations of BABQ on the production of superoxide anion radicals (●) and hydrogen peroxide (○) in a mixture containing 2 mU/mL xanthine oxidase, 0.1 mM xanthine, 5 μM Fe^{3+}, and 0.1 mM EDTA in phosphate buffer pH 7.5 at 30°C.

is difficult to test. However, the fact that the inhibiting effect on the chemiluminescence correlates with that on the other reactive oxygen species ($^{\cdot}OH$, H_2O_2) strongly favors the interpretation that an effect on O_2^- is observed. The data from the O_2^- assay in Table 2 are obtained in the presence of Fe^{3+}/EDTA in order to have similar conditions in all assays of the reactive oxygen species. When Fe^{3+}/EDTA was omitted higher O_2^- yields were observed, likely caused by a decrease of reaction 1. The effects of the BABQ derivatives indicate that O_2^- is efficiently removed by some BABQ species. BABQ has no significant effect on O_2 consumption in the concentration range that decreases O_2^- levels (0–10 μM). Only at concentrations > 100 μM BABQ, less O_2^- is consumed (e.g., with 150 μM BABQ, oxygen consumption is 60% of that without BABQ). These results show that BABQ redox cycling consumes oxygen.

In principle, the amount of O_2^- can be affected by BABQ by the following reactions:

$$BABQ + O_2^- \underset{k_b}{\overset{k_f}{\rightleftharpoons}} BABQ^- + O_2 \qquad (5)$$

$$BABQ^- + O_2^- + 2H^+ \rightarrow BABQ + H_2O_2 \qquad (6)$$

The position of equilibrium 5 is defined by

$$E(O_2/O_2^-) - E_7^1 = (RT/F)\ln K_5 \qquad (7)$$

E_7^1 is the one-electron reduction potential (E(BABQ/$BABQ^-$)) at pH 7.0 under standard conditions. The

Table 2. Inhibitory Effect of BABQ Derivatives on the Production of Reactive Oxygen Intermediates by Xanthine Oxidase

Compound	Superoxide Assay IC_{50} (μM)	Hydrogen Peroxyde Assay IC_{50} (μM)	Hydroxyl Radical Assay IC_{50} (μM)	E_7^1 (mV)	K_M^1 (μM)
TW39	9.8 ± 2.6	242 ± 151	15.4 ± 2.2	−163 ± 5	n.d.
AZQ	3.6 ± 0.8	62.6 ± 9.3	7.3 ± 1.8	−65 ± 8	257
TW25	3.0 ± 0.3	21.7 ± 2.2	2.0 ± 0.2	−88 ± 10	21
TW22	1.5 ± 0.6	16.5 ± 1.0	4.4 ± 0.9	−78 ± 8	11
BABQ	0.95 ± 0.36	11.9 ± 0.3	1.99 ± 0.28	−54 ± 8	n.d.[3]
TW14	0.35 ± 0.08	1.37 ± 0.05	1.30 ± 0.26	−26 ± 3	n.d.[3]
TW19	0.06 ± 0.04	0.50 ± 0.04	0.58 ± 0.23	3 ± 8	16
Adriamycin	0.46 ± 0.26	362 ± 208	Stim.[2]	−341 ± 15[5]	n.d.[3]
Menadion	Stim.[2]	2.76 ± 0.34	48.8 ± 9.7	−203 ± 5[4]	n.d.[3]

Note: IC_{50} values are concentrations that inhibit the reactive oxygen intermediate production by 50%. E_7^1 is the one-electron reduction potential at pH 7.0 (see Materials and Methods). K_M is the Michaelis constant for reduction by xanthine oxidase. IC values are ± SE obtained from an exponential fit (O_2^-, $^{\cdot}OH$) or linear fit (H_2O_2). E_7^1 values are ± SE from three experiments. In those cases where it could be experimentally tested, maximum inhibition was full for all assays.

[1] Ref 14.
[2] Stim. = (small) stimulatory effect.
[3] n.d. = not determined.
[4] Ref. 33.
[5] Ref. 34.

Table 3. Effects of Iron Complexing Agents and BABQ on the Rate of H_2O_2 Formation

Chelator	Rate of H_2O_2 Formation (μM/min)
0.1 mM Desferrioxamine	11.3
0.1 mM EDTA	8.0
0.01 mM BABQ	5.3

Reaction mixture contained xanthine (0.1 mM) xanthine oxidase (3 mU/mL), Fe^{3+} (5 μH), H_2O_2 measuring solution (see Materials and Methods) in 50 mM phosphate buffer pH 7.5.

standard reduction potential at pH 7.0 of the $O_{2(aq.)}/O_2^-$ (1 M O_2) couple is −155 mV (Ref. 16). Equilibrium 5 has been studied in more detail for AZQ.[26] The reaction rates k_f and k_b were found to be 2.7×10^8 and $1.1 \times 10^7\ M^{-1} \cdot s^{-1}$, respectively. Continuous decay of $BABQ^-$ will shift the equilibrium to the right-hand side, which leads to a decrease in the O_2^- concentration. This shift to the right-hand side will be favored for BABQ derivatives with a high reduction potential. O_2^- is not stable: It undergoes spontaneous dismutation (Eq. 8). It can be reasonably assumed that the forward reaction in Eq. 5 is much faster than the spontaneous dismutation rate. This means that equilibrium 5 can prevail over O_2^- decay under our conditions.

E_7^1 values have been obtained from pulse radiolysis experiments and are included in Table 2. Indeed, a correlation between E_7^1 and IC_{50} values for the O_2^- production is observed (Table 3).

The higher the reduction potential of a BABQ derivative, the more the equilibrium of Eq. 5 is shifted to the right, and the less BABQ derivative is required to decrease O_2^- concentration. A low quinone reduction potential will in principle thermodynamically favor reaction 6, leading to diminished O_2^- levels for BABQ derivatives with low redox potentials. The opposite effect on O_2^- level is observed, however, for quinones with a lower reduction potential $BABQ^-$ will be formed slower, and this may cause these compounds to be less effective in trapping O_2^-. Therefore, the contribution of reaction 6 is not to be excluded.

Based on these results, it can be concluded that E_7^1 values can be used to predict the reactivity of $BABQ^-$ with O_2. This is not self-evident because the relation between K_5 and E_7^1 (Eq. 7) applies to equilibrium conditions. However, in our system competing radical decay processes also exist, like dismutation of quinone (Eq. 4), O_2^- dismutation (Eq. 8), and reduction of Fe^{3+} (Eq. 1).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (8)$$

Therefore, reaction rates are expected to be more important for the obtained steady-state concentrations of radical intermediates than equilibrium constants, and the amount of O_2^- is expected to be kinetically controlled rather than thermodynamically. Marcus theory reconciles the thermodynamic and kinetic aspects: A relation between the equilibrium constant of, for example, equilibrium 5 (K_5) and the rate constant for electron transfer reactions (e.g., k_f from Eq. 5) is predicted. For a series of quinones, a correlation between the rate constants with O_2^- (i.e., k_f from Eq. 5) and the quinone one-electron reduction potential has been reported, which is in accordance with Marcus theory for electron-transfer reactions.[30] The BABQ derivative AZQ fitted well into the observed relation.[31] Our finding that E_7^1 predicts the reactivity of BABQ with O_2^- suggests that also for the other BABQ derivatives k_f from Eq. 5 correlates with E_7^1 and that reaction 5 is faster than competing reactions. E.g. k_8 at pH 7 is lower than $10^5\ M^{-1} \cdot s^{-1}$ (Ref. 31), compared to k_f (Eq. 5) for AZQ is $2.7 \times 10^8\ M^{-1} \cdot s^{-1}$ (Ref. 26). It has also been reported for a series of simple quinones that equilibrium 5 is maintained much faster than the time scale of quinone disproportionation (reaction 4).[32]

In the presence of adriamycin, which is efficiently reduced by xanthine oxidase,[35] decreased O_2^- production is observed with increased $^{\cdot}OH$ production (Table 2). Adriamycin forms complexes with Fe^{3+}, and its semiquinone radical reduces Fe^{3+}/EDTA efficiently.[36] This leads to less availability of adriamycin semiquinone for production of O_2^- (Eq. 5) and to increased $^{\cdot}OH$ production (reaction 2). Menadione, a well-known redox cycling drug, slightly increased the production of superoxide.

Generation of hydrogen peroxide

The production of H_2O_2 also decreases upon addition of BABQ derivatives to the xanthine/xanthine oxidase mixture. The effect tends to be larger for compounds with a high reduction potential. However, the IC_{50} values for inhibition of H_2O_2 production by BABQ derivatives are much higher than for inhibition of O_2^- production (Table 2, Fig. 3).

Adriamycin hardly influenced the production of H_2O_2, but surprisingly menadione strongly decreased H_2O_2 production.

H_2O_2 can be produced by xanthine oxidase directly via a two-electron outlet and via a one-electron outlet after dismutation of superoxide (reaction 8).[37] As discussed earlier, at concentrations of BABQ derivative higher than 10 μM, mainly quinone reduction occurs at the cost of one-electron oxygen reduction.[14] Concerning the origin of H_2O_2 production in the presence of BABQ derivatives, three explanations may be given: (1) the aforementioned direct two-electron reduction of oxygen to H_2O_2 by xanthine oxidase,

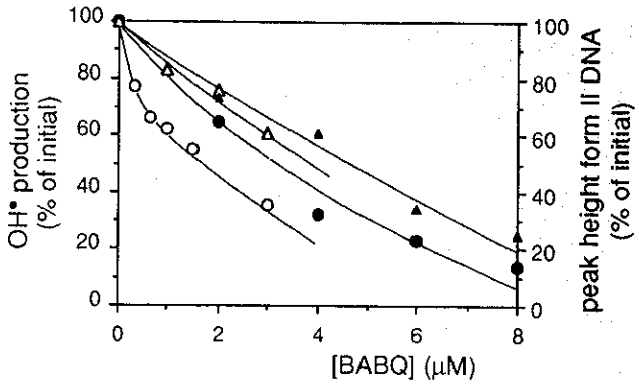


Fig. 4. Effects of various concentrations of BABQ and TW39 on the production of hydroxyl radicals and DNA single-strand breaks. The reaction mixture contained 0.1 mM xanthine, 5 μM Fe^{3+}, 0.1 mM EDTA, and 5 mU/mL xanthine oxidase in 50 mM phosphate buffer pH 7.5. Formation of hydroxyl radicals, O: BABQ, Δ:TW39. Formation of DNA breaks, ●: BABQ, ▲:TW39.

which is then not (much) affected by quinone reduction; (2) redox cycling of BABQ hydroquinone and semiquinone yielding H_2O_2, without a detectable level of O_2^- production (Reoxidation of electrochemically reduced BABQ derivatives indeed yields H_2O_2; results not shown.); or (3) production of H_2O_2 in reaction 6.

As mentioned earlier, in a concentration range from 0 to 10 μM BABQ, oxygen consumption is not affected, while the rate of H_2O_2 formation is decreased by approximately 60% (Fig. 3). Therefore, the decreased H_2O_2 production is not caused by inhibition of xanthine oxidase. It is likely that produced H_2O_2 undergoes decomposition, resulting in a decreased apparent rate of formation. In this H_2O_2 decomposition, Fe^{3+} may play a role: Two-electron reduction of H_2O_2 with formation of higher oxidation states of iron is plausible.[38] Therefore, we studied the effect of desferrioxamine, a chelating agent which effectively protects against iron-mediated effects on reactive oxygen species.[39] Complexing of iron with desferrioxamine increases the apparent rate of H_2O_2 formation (Table 3). In the presence of 10 μM BABQ, without EDTA the apparent rate of H_2O_2 formation is decreased compared to EDTA. These results suggest that BABQ (semiquinone) and iron are indeed responsible for H_2O_2 decomposition.

Generation of hydroxyl radicals

The production of $^{\bullet}OH$ was investigated using degradation of deoxyribose by $^{\bullet}OH$, which is regarded as a reliable test to study generation of $^{\bullet}OH$.[40] Addition of BABQ derivatives results in a strong decrease of $^{\bullet}OH$ production, as shown in Fig. 4 for BABQ and TW39. The IC_{50} values for inhibition of $^{\bullet}OH$ production (Table 2) are slightly higher than for inhibition of O_2^-, but much lower than that for inhibition of H_2O_2 production. From the correlation matrix (Table 4) it can be seen that the effects of the BABQ derivatives on the production of O_2^-, H_2O_2, and $^{\bullet}OH$ are correlated with each other. The correlation between O_2^- and $^{\bullet}OH$ production is in agreement with formation of $^{\bullet}OH$ in a Fenton reaction in a Haber-Weiss sequence (Eqs. 1–3), which requires the presence of both O_2^- and H_2O_2 to produce $^{\bullet}OH$. Another mechanism with which $^{\bullet}OH$ may be formed is via reduction of Fe^{3+} by $BABQ^-$ (Eq. 9), which is a very efficient reaction for adriamycin.[36]

$$BABQ^- + Fe^{3+} \rightarrow BABQ + Fe^{2+} \qquad (9)$$

In this mechanism a correlation of Fe^{2+} and $^{\bullet}OH$ formation with the BABQ reduction potential is not straightforward: BABQ derivates with a low reduction potential will produce lower levels of $BABQ^-$. On the other hand, reaction 9 will proceed more efficiently for quinones with a low reduction potential. As a fair correlation between $^{\bullet}OH$ production and reduction potential is observed, we conclude that Fe^{3+} reduction by O_2^- rather than by $BABQ^-$ determines the rate of $^{\bullet}OH$ production.

The high $^{\bullet}OH$ production by adriamycin was discussed earlier. The effect of menadione on $^{\bullet}OH$ production is in agreement with its relatively low reduc-

Table 4. Correlation Matrix of IC_{50} Values for O_2^-, H_2O_2, and $^{\bullet}OH$ Production Inhibition, and E_7^1 for BABQ Derivatives (Data from Table 2)

	$IC_{50}(O_2^-)$	$IC_{50}(H_2O_2)$	$IC_{50}(^{\bullet}OH)$	E_7^1
$IC_{50}(O_2^-)$	1.00	0.98	0.91	0.92
$IC_{50}(H_2O_2)$		1.00	0.95	0.86
$IC_{50}(^{\bullet}OH)$			1.00	0.86

Using ANOVA statistics, all $p \leq .01$.

tion potential (Table 2): For quinones with a low reduction potential, equilibrium 5 will be shifted to the left, leading to higher levels of O_2^- and $^{\cdot}OH$.

$^{\cdot}OH$ is considered to be the ultimate damaging species formed during redox cycling,[25] although higher oxidized iron species are hold responsible for biological damage, formerly ascribed to $^{\cdot}OH$.[41]

Effects of BABQ derivatives on the formation of DNA strand breaks

The inhibition by BABQ derivatives of $^{\cdot}OH$ production by xanthine oxidase may protect against deleterious effects produced by $^{\cdot}OH$, such as lipid peroxidation and DNA damage. The effects of BABQ derivatives on DNA damage induced by xanthine oxidase were studied using supercoiled plasmid DNA. Strand breaks in plasmid DNA are visible as a decrease in the supercoiled form I DNA and an increase in the open circular form II DNA, arising from single-strand breaks, and the linear form III DNA arising from double-strand breaks. Figure 5 shows the occurrence of DNA strand breaks upon reduction with xanthine oxidase in the presence of increasing amounts of BABQ, TW39, and adriamycin. Only single-strand breaks are observed, as is common with $^{\cdot}OH$-mediated reactions.[42] The addition of BABQ derivatives inhibits the formation of DNA single-strand breaks by ROI from xanthine oxidase; BABQ inhibits DNA strand break formation more strongly than TW39, and IC_{50} values are 3.0 and 4.6 μM, respectively. This corresponds well with the inhibition of $^{\cdot}OH$ generation by BABQ and TW39 as measured with deoxyribose degradation (Fig. 4). Reduction of adriamycin by xanthine oxidase produces DNA single-strand breaks, as can be seen by an increase in form II DNA. This is in accordance with the stimulated production of $^{\cdot}OH$ (Table 2). The electrophoretic mobility of form I DNA is altered by addition of adriamycin, even without reduction, probably because adriamycin intercalates into DNA.[43]

This study shows that BABQ derivatives strongly decrease the production of O_2^- and $^{\cdot}OH$ in the presence of xanthine oxidase. The results can be explained by an efficient reaction of BABQ with O_2^- (Eq. 5). This deviant behavior from classical antitumor drugs like adriamycin is caused by the relatively high reduction potential of BABQ derivatives. BABQ derivatives with a relatively low reduction potential (e.g., TW39) show $^{\cdot}OH$ levels comparable with adriamycin. Production of O_2^- and $^{\cdot}OH$ by redox cycling apparently does not seem to be relevant in the biological activity of BABQ derivatives, but H_2O_2 production may be as appears from the recent study of Fisher and Gutierrez.[44] These results seem to contradict reports on the formation of reactive oxygen intermediates and DNA damage from redox cycling of BABQ derivatives, especially AZQ.[45–48]

Szmigiero and Kohn[46] ascribe DNA strand breaks in cells and isolated nuclei by AZQ to ROI. This is based on the protective effect of superoxide dismutase. However, superoxide dismutase also decreases the amount of AZQ semiquinones.[14] The effect of AZQ on strand breaks in plasmid DNA reported by Gutierrez et al.[47] is only observed at AZQ concentrations higher than 50 μM up to 400 μM, which is far from biologically relevant. Also, under anaerobic conditions an effect of AZQ on the plasmid DNA was observed.[47] Furthermore, in this study reduction was achieved with sodium borohydride, which likely gives rise to artifacts due to, for example, pH changes.

Doroshow[48] investigated the cytotoxic effect of AZQ on Ehrlich tumour cells. The protective effect of as series of $^{\cdot}OH$ scavengers is taken as an indication that the cytotoxicity is an $^{\cdot}OH$-mediated effect. However, scavenging of semiquinone radicals by these $^{\cdot}OH$ scavengers is not to be excluded.

Fisher and Gutierrez[44] recently reported AZQ redox cycling with production of H_2O_2. An ESR signal of a short-lived 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO)-OOH adduct was observed which was sensitive to SOD. In these experiments very high AZQ concentrations (200 μM) have been used. It is possible that under these conditions some O_2^- can be formed in equilibrium 5, which is efficiently trapped by the ESR spin trap. Remarkably, very high amounts of SOD (400 U/mL) are needed to quench this DMPO-OOH signal, compared to approximately 0.2 U/mL SOD to stop O_2^--induced cytochrome *c* reduction.[28,49]

In conclusion, we can state that the one-electron reduction potential of the BABQ derivatives is an important predictive parameter in the formation of reactive oxygen intermediates as well as for their reactivity with O_2^-. The reactions between BABQ and reduced oxygen species studied here may also be relevant in in vivo systems. The results indicate that H_2O_2 may be more relevant in the oxygen-derived toxicity of the BABQ derivatives than O_2^- and $^{\cdot}OH$. H_2O_2 has indeed been shown to be important in the AZQ-induced cytotoxicity in isolated rat hepatocytes.[50] Reactive oxygen intermediates are considered to contribute to the toxicity of antitumor drugs.[48] On the other hand redox cycling may also play an important role in the neoplastic activity of anticancer quinones.[51] The series of BABQ derivatives possessing a wide range of reduction potentials offers the possibility to separate alkylation from the formation of deleterious oxygen radicals like $^{\cdot}OH$ and O_2^-.

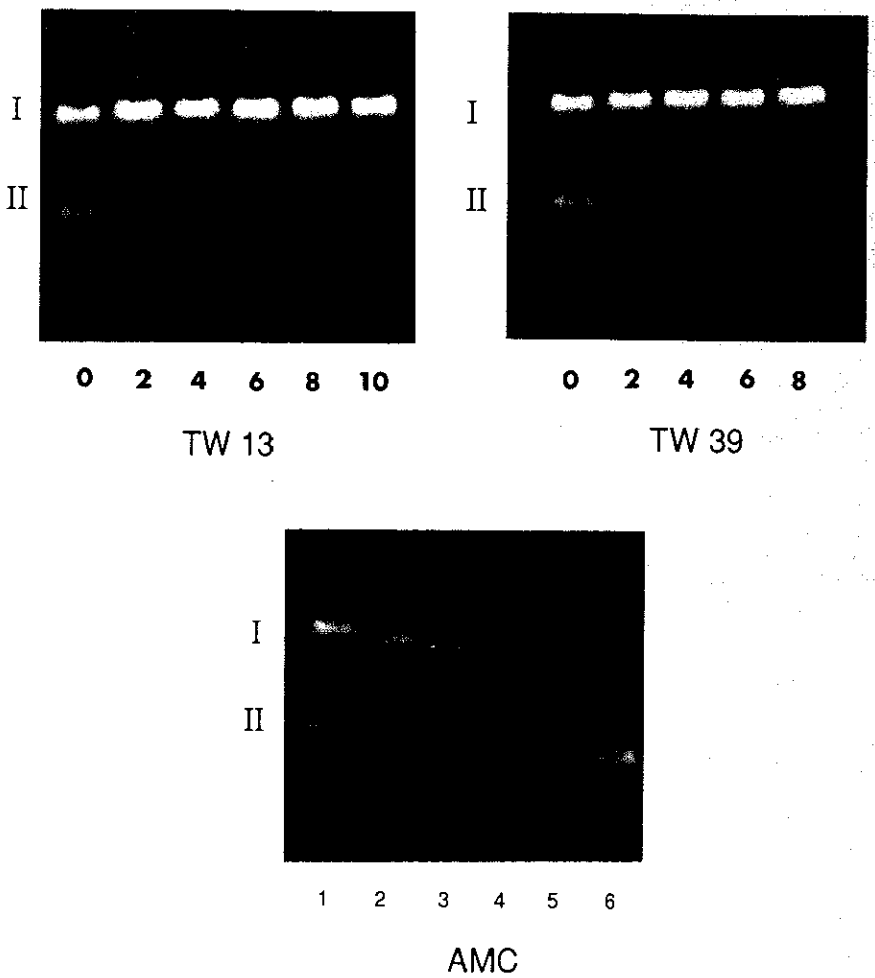


Fig. 5. Agarose gel of pBr322 plasmid DNA, incubated for 30 min at 25°C under air in a mixture containing 0.1 mM xanthine, 5 μM Fe^{3+}, 0.1 mM EDTA, and 5 mU/mL xanthine oxidase in 50 mM phosphate buffer pH 7.5 and various concentrations of BABQ (0–10 μM), TW39 (0–8 μM), and adriamycin (0–10 μM). DNA strand break induction is visible as a decrease in form I DNA and an increase in form II DNA. Increasing concentrations of BABQ and TW39 protect against oxygen-radical-mediated DNA damage. In the presence of adriamycin, DNA damage increases.

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ABBREVIATIONS

AZQ—diaziquone, 2,5-*bis*(1-aziridinyl)-3,6-*bis*(2-hydroxyethylamino)-1,4-benzoquinone
BABQ—2,5-*bis*(1-aziridinyl)-1,4-benzoquinone
$BABQ^{\cdot}$, $BABQH_2$, and $BABQ^{2-}$—BABQ semiquinone, hydroquinone, and deprotonated hydroquinone, respectively
E_7^1—one-electron reduction potential (E(BABQ/$BABQ^{\cdot}$)) at pH 7.0
$O_2^{\cdot}$—superoxide anion radical
$^{\cdot}OH$—hydroxyl radical
ROI—reactive oxygen intermediates
TE-buffer—*tris*-EDTA buffer