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REDUCTIVE METABOLISM OF DIAZIQUONE (AZQ) IN THE S9 FRACTION OF MCF-7 CELLS

II. ENHANCEMENT OF THE ALKYLATING ACTIVITY OF AZQ BY NAD(P)H: QUINONE-ACCEPTOR OXIDOREDUCTASE (DT-DIAPHORASE)

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Abstract—The alkylating activity of reduced diaziquone was studied by the nitrobenzylpyridine (NBP) assay and was compared to those of the parent compound and aziridine-containing *N,N′,N″*-triethylenethiophosphoramide (Thio-TEPA). Diaziquone (AZQ) was reduced enzymatically by $2e^-$ using S9 cell fraction from MCF-7 cells which is rich in NAD(P)H:quinone-acceptor oxidoreductase (DT-diaphorase) (QAO) activity. One electron enzymatic reduction was performed with NADPH-cytochrome *c* reductase. The alkylating activity of AZQ increased 3-fold when reduced by $2e^-$. This increase was inhibited by dicumarol, an inhibitor of QAO. In contrast, the alkylating activity of AZQ did not increase beyond that of the parent compound when reduced by $1e^-$ using purified NADPH-cytochrome *c* reductase. Similar results were obtained when AZQ was reduced chemically with borohydride ($2e^-$) and with NADPH ($1e^-$). Anaerobic incubations of AZQ with the S9 fraction of MCF-7 cells ($2e^-$ reduction) resulted in an increase in NBP alkylation over its aerobic counterpart (1.8-fold) while maintaining the near 3-fold increase in alkylation over untreated AZQ. In contrast, AZQ incubations with NADPH-cytochrome *c* reductase ($1e^-$ reduction) under the same conditions did not result in an NBP alkylation increase over untreated AZQ. These results indicate that AZQ hydroquinone is most likely the responsible species for the observed alkylation of this antitumor agent to DNA and other nucleophiles. The results also suggest that NAD(P)H:quinone-acceptor oxidoreductase is a very important enzyme in the bioactivation of AZQ.

Diaziquone (AZQ†, 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone) (Fig. 1) is a diaziridinyl quinone compound that has activity against brain tumors [1] and acute lymphocytic leukemia [2, 3]. AZQ is a bioreductive alkylating agent whose carboethoxyamino groups make it lipophilic. The aziridine groups provide bifunctional alkylation [4–7]. The quinone chromophore undergoes enzymatic reduction in both purified enzyme systems and whole cells. In the presence of molecular oxygen, the semiquinone leads to the production of reactive oxygen species including $O_2^{\cdot}$, H_2O_2, $\cdot OH$ in a process known as redox cycling [8–11]. Bifunctional alkylation to calf thymus DNA was shown to be dependent on the reduction of AZQ with borohydride, a two electron reducing agent [6]. In whole cells, this bifunctional alkylation is enhanced by borohydride [5, 7]. DNA single-strand breaks also have been found in AZQ-treated whole cells, and these strand breaks are also enhanced under reducing conditions [5, 7]. In cell nuclei, superoxide dismutase (SOD) inhibits AZQ-dependent DNA strand break formation but not DNA interstrand cross-linking [4]. These results as well as detailed studies of electrochemical and chemical reductions of AZQ [10] suggested that the mechanism of AZQ cytotoxicity is due to: (a) redox cycling leading to the generation of reactive oxygen species; and (b) aziridine alkylation enhanced by the reduction of the quinone.

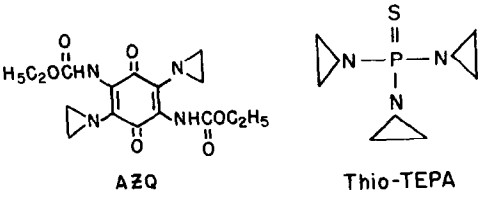

Fig. 1. Chemical structures of AZQ and Thio-TEPA.

Recently, AZQ was shown to be more cytotoxic against Chinese hamster ovary cells under anaerobic conditions [12]. Anaerobic conditions favor the stability of reduced AZQ species and the absence of reactive oxygen species. Thus, these conditions

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† Abbreviations: AZQ, diaziquone; $AZQH_2$, AZQ hydroquinone; NBP, nitrobenzylpyridine; Thio-TEPA, *N,N′,N″*-triethylenethiophosphoramide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; PBS, phosphate-buffered saline; QAO, NAD(P)H:quinone-acceptor oxidoreductase (DT-diaphorase); SOD, superoxide dismutase; and FBS, fetal bovine serum.

should result in enhanced alkylation by AZQ and minimum reactive oxygen-mediated DNA strand breaks. This result is consistent with the importance of AZQ alkylation in cytotoxicity as mediated by the reduced quinone. Consistent with the importance of oxidative stress in the toxicity of AZQ is the ability of catalase to abrogate this activity [12, 13]. Catalase did not abrogate the cytotoxicity of AZQ under anaerobiosis [12].

Diaziquone can be reduced by one or two electrons to produce the semiquinone (AZQH) or the hydroquinone ($AZQH_2$), respectively. Both of these reduced species can auto-oxidize with the production of oxygen radicals [14–16]. It is likely that the results of previous DNA alkylation experiments with borohydride were the consequence of the reaction of DNA with the colorless hydroquinone [16] and that strand breaks [4, 5, 7] were the result of the auto-oxidation of this hydroquinone. This latter point has been substantiated recently with purified NAD(P)H (quinone-acceptor) oxidoreductase (see below) in experiments that showed AZQ-dependent strand breaks of PM2 plasmid DNA [17].

We are interested in the metabolism of AZQ by MCF-7 human breast cancer cells. Recently, we demonstrated that the S9 fraction of these cells is rich in NAD(P)H:quinone-acceptor oxidoreductase (DT-diaphorase) (QAO) activity [18]. NAD(P)H:quinone-acceptor oxidoreductase (EC 1.6.99.2) is an enzyme that reduces quinone-containing xenobiotics by two electrons [19]. These include menadione [20], naphthoquinones [21] and benzopyrene quinone [22]. NADPH-cytochrome *c* reductase, on the other hand, reduces quinones by one electron [23]. The direct two electron reduction of quinones by QAO has been proposed to represent a protective mechanism against redox cycling because hydroquinones are substrates for cellular detoxification [24]. This hypothesis is supported by evidence indicating that dicumarol, an inhibitor of QAO, increases the cytotoxicity of some quinones including menadione [25, 26]. AZQ is a substrate for QAO [18, 27]. The metabolism of AZQ by QAO is associated with the dicumarol-inhibitable production of oxyradicals and AZQ semiquinone in a mechanism that involves a two electron oxidation pathway, another form of redox cycling [17, 18]. Therefore, this enzyme quite possibly plays a very important role in the bioactivation of AZQ with respect to both alkylation and oxidative stress.

To continue to study the metabolism of AZQ in MCF-7 cells in particular, and to explore the dual nature of the proposed mechanism of AZQ in general, we focused on the AZQ reduced species ($1e^-$ or $2e^-$) responsible for its alkylation. We quantified the alkylating power by the nitrobenzylpyridine (NBP) assay (described below). This is a relatively simple assay which allows for quantification of alkylating activity in a way that experiments with relevant nucleophiles, such as DNA, do not make possible. Two electron reduction was accomplished chemically with borhydride [16] or enzymatically with the S9 cell fraction of MCF-7 cells. We chose this preparation because we have shown that it is 29-fold more active in QAO than in NADPH-cytochrome *c* reductase [17], and because it will reflect the metabolism of AZQ in MCF-7 cells. For comparison, one electron reductions were performed with NADPH and purified NADPH-cytochrome *c* reductase.

MATERIALS AND METHODS

Materials. Diaziquone was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, and used without further purification. *N,N',N''*-Triethylenethiophosphoramide (Thio-TEPA) was provided by Lederle Laboratories (Pearl River, NY). NBP, dicumarol [3,3'-methelenebis(4-hydroxycoumarin)], NADH, NADPH, EDTA, Tween 20 and SOD were purchased from the Sigma Chemical Co. (St. Louis, MO). The spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Bovine serum albumin (BSA) was obtained from Miles Laboratories (Elkhart, IN).

Colorimetric determination of alkylating activity. Alkylating activity was assayed by the method of Friedman and Boger [28]. This assay is based on the fact that 4-(*p*-nitrobenzylpyridine) is a nucleophile that reacts with an alkylating agent to give a pyridinium salt. This salt produces a blue color in alkaline solutions detectable at 540 nm. NBP (5%, by wt 233 mM in acetone) was added to a solution of 45 mM sodium acetate buffer (pH 4.6) to achieve a final concentration of 21 mM NBP. Appropriate concentrations of alkylating agent were added to this mixture which was boiled for 20 min. After cooling on ice for 10 min, the alkylated NBP was extracted with 2 mL of acetone and 5 mL of ethyl acetate. To develop the color, 1.5 mL of 0.25 N sodium hydroxide was added, the sample was shaken and centrifuged, and absorbance was read at 540 nm immediately. Because the color fades with time, a maximum of four samples was developed at a time. Chemical or enzymatic reactions were carried out in a separate test tube and incubated for 10–15 min at 25°. The incubate was then added quantitatively to NBP. In the absence of drug, the background absorbance at 540 nm was consistently 0.0029 to 0.0035.

Reactions with S9 fraction were carried out in Tris or phosphate buffer (pH 7.4) in the presence of QAO activators Tween 20 (0.01%) and BSA (0.23 mg/mL). The concentrations of AZQ and NADPH were 400 μM. Reactions with NADPH-cytochrome *c* reductase were carried out in 25 mM phosphate buffer (pH 7.4), NADPH (1000 μM) and AZQ (500 μM). Dicumarol (100 μM) was used as an inhibitor in both enzymatic reactions. The final concentrations of AZQ achieved after aliquots of the reaction were added to NBP are shown in the figures. NBP concentrations were estimated using the extinction coefficient of 12 $mM^{-1}\,cm^{-1}$ in ethanol at 264 nm. Standard curves using nitrogen mustard or Thio-TEPA were used to validate the assay. Results are reported here in terms of the optical absorption at 540 nm.

Anaerobic experiments were carried out by preparing the NBP solution anaerobically (i.e. water, buffers and organic solvents were deaerated by

passing nitrogen gass through them for 30 min). Anaerobic incubations of AZQ and enzyme were carried out in deaerated buffers containing AZQ and required cofactors and activators. The 1-mL solutions were further deaerated by passing nitrogen gas through them for 2 min. The enzyme was added to start the reaction followed by a 30-sec deaeration. Aliquots of these reactions were added anaerobically to NBP.

The measurement of alkylating activity in whole cells was performed with 10^6 cells harvested as described below and suspended in 1 mL of phosphate-buffered saline (PBS). Alkylating activity was evaluated immediately after adding 400–600 μM AZQ as above and 15, 30, or 60 min thereafter. These are highly toxic AZQ concentrations. In some experiments, the cells were disrupted by sonication prior to adding AZQ and assaying for alkylating activity to ensure NBP cell penetration.

Electron spin resonance measurements. Electron spin resonance (ESR) spectra of AZQ incubates with 2×10^6 cells/mL were obtained with a Varian E109 Century Series X-band (9.3 GHx) spectrometer. The cells were suspended in growth medium supplemented with 10% fetal bovine serum (FBS) and treated with AZQ (200 μM) in the presence of DMPO (100 mM). For other treatments, cells were preincubated on ice for 40 min with SOD, catalase, dicumarol or ethanol.

Cell culture, S9 cell fraction preparation and cytotoxicity assay. The human breast carcinoma cell line MCF-7 was purchased from the American Type Culture Collections (Rockville, MD). Iscove's modified Dulbecco's medium (IMDM) and FBS were obtained from Gibco Laboratories (Grand Island, NY). Cells were grown in monolayer cultures in IMDM medium, supplemented with 10% FBS and maintained in an atmosphere of 5% CO_2, 95% relative humidity at 37°. Cells were plated at a density of 10^5 in 75 cm^2 flasks. Cell medium was changed every 3 days and the cells were passed once weekly.

To prepare S9 cell fraction from MCF-7 cells, 10^7 to 10^9 cells were harvested in exponential growth using 0.1% EDTA to dislodge them. The cells were then suspended in PBS and centrifuged at 100 *g* for 10 min. The precipitate was resuspended in 100–500 μL of ice-cold saline and sonicated for 2 min with a microtip using a Branson Sonifier 250 set at 7. The resulting sonicate was centrifuged (10,000 *g*, 30 min), and the supernatant (S9 fraction) was collected and stored under liquid nitrogen until required. Protein content was determined by using the Pierce BSA assay [29].

NADPH-cytochrome *c* reductase was prepared by the method of Omura and Takesue [30].

RESULTS

Aerobic NBP alkylating activity. To study the alkylating activity mediated enzymatically by the S9 cell fraction of MCF-7 cells, AZQ was incubated with this cell fraction in the presence of NADPH and the QAO enhancers Tween 20 and BSA. After 10–15 min, appropriate amounts of this mixture were added to NBP. During this time, the solution became

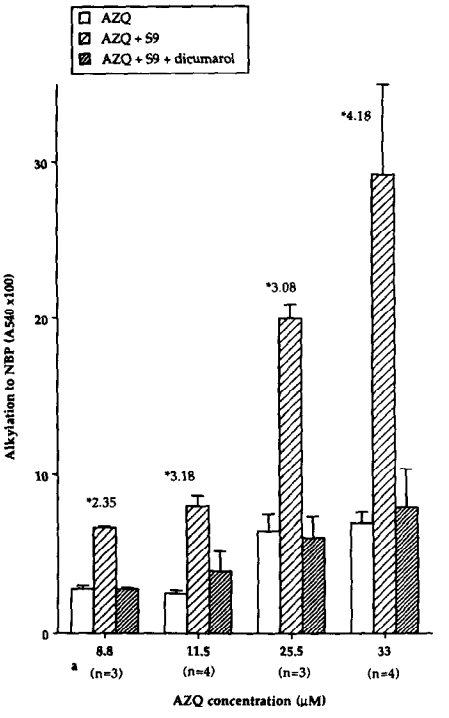


Fig. 2. Alkylating activity of AZQ and AZQ incubated with MCF-7 S9 cell fraction. *Alkylating activity ratio of S9 activated AZQ to parent compound. The mean ratio ± SD of these four experiments is 3.19 ± 0.75. [a]Number of experiments. Each experiment consisted of triplicate measurements; values are given as means ± SD. Experiments were performed in 25 mM phosphate or Tris buffer (pH 7.4) with an S9 protein concentration of 0.6 to 0.8 mg/mL. QAO activators Tween 20 (0.01%) and BSA (0.23 mg/mL) were used. Incubation times were 10–15 min, before adding aliquots quantitatively to NBP. The QAO inhibitor dicumarol was added at 100 μM.

clear as one would expect for $AZQH_2$ [16–18]. This incubation resulted in a mean alkylation activity increase of 3.19 ± 0.75 (mean ± SD) over untreated AZQ (Fig. 2). This increase was proportional to AZQ concentration and was abrogated by dicumarol, an inhibitor of QAO (Fig. 2). The NAD(P)H-dependent enzymatic activity has been described recently in terms of the free radical metabolism of AZQ [18]. It was shown that a $2e^-$ reduction by QAO predominates over the $1e^-$ reduction by cytochrome *c* reductase [18].

The free radical metabolism of AZQ in the S9 fraction used in the alkylating assay was the same as previously described [18]. Briefly, S9 catalyzes the formation of AZQ-dependent hydroxyl radicals which are trapped by DMPO [18]. This signal is totally abolished by catalase and inhibited by dicumarol (100 μM). Under anaerobic conditions, DMPO adducts are not observed but the AZO semiquinone is detected [18]. AZQ is reduced with QAO by two electrons to produce the hydroquinone, and thus, no semiquinone of oxyradicals should be present. However, a transient semiquinone due to

comproportionation ($AZQ + AZQH_2 \rightarrow 2AZQ\dot{H}$) exists, especially in the presence of excess reducing agent [16]. In the case of the MCF-7 S9 cell fraction, there is also a contribution from one-electron enzymatic reduction that produces semiquinone to maintain the comproportionation/disproportionation equilibrium ($AZQ + AZQH_2 \rightleftarrows 2AZQ\dot{H}$) for as long as 14 hr in anaerobic preparations [18]. In general, the maximum production of hydroxyl radicals is reached 15 min after the onset of the reaction. By 10 min the hydroxyl radicals are 73–75% of the maximum concentration. Similarly, the AZQ absorbance at 340 nm decreases reaching minimum absorption by 20 min. By 10 min the solution appears colorless and the absorbance is decreased by 62%. It is for this reason that an incubation time of 10–15 min was adopted as the standard protocol to react AZQ with S9 and its QAO enhancers before adding appropriate aliquots to NBP for alkylating measurements.

To demonstrate that the results observed in the experiment with S9 fraction were due to $2e^-$ reduction, AZQ was reduced with 2- to 3-fold excess borohydride and assayed for alkylating activity. When an orange solution of AZQ is reduced with excess borohydride, the solution becomes temporarily yellow and then goes on to become colorless as the hydroquinone $AZQH_2$ is formed [16]. The yellow color indicates the presence of the AZQ free radical anion formed by the equilibrium $AZQ + AZQH_2 \rightleftarrows 2AZQ\dot{H}$. The solution remains colorless for at least 1 hr, but aeration of this solution by shaking the test tube can temporarily turn the solution yellow by the oxidation of the hydroquinone [16]. Leaving this solution exposed to air overnight results in the colorless solution returning to orange, the color of the parent compound.

When the colorless $AZQH_2$, was added to NBP, the alkylation of AZQ to NBP increased 3-fold over the parent oxidized compound (Fig. 3). This alkylation activity increased proportionally with AZQ concentration in both reduced and oxidized species. The relative alkylating activity of reduced AZQ to parent compound did not change significantly as the drug concentration increased. The mean alkylating activity increase in the four AZQ concentrations used was 3.12 ± 0.06-fold (mean ± SD) (Fig. 3).

To ascertain the influence that the quinone moiety might have on the observed increase in alkylation activity of AZQ with reduction, we performed similar experiments with Thio-TEPA (Fig. 1). This clinically used triaziridine compound lacks the quinone moiety. As can be seen in Fig. 4, the alkylating activity of Thio-TEPA increased linearly with the drug's concentration. However, no increase in alkylating activity was observed when Thio-TEPA was treated with borohydride. It appears that Thio-TEPA has more alkylating power than AZQ before reduction, but after reduction the alkylating power is comparable. That is, when the alkylating activity per aziridine at a 30 μM concentration is normalized, the result is 0.0976 O.D. units for Thio-TEPA and 0.0376 for AZQ. Upon reduction, this same measurement becomes 0.096 O.D. units for AZQ and 0.088 for Thio-TEPA. It must be mentioned at this point that the aziridine rings are difficult to reduce. They can be reduced electrochemically at a very high negative potential [15]. Borohydride does not have the power to reduce these rings chemically; thus, the quinone is required in the alkylating effects observed.

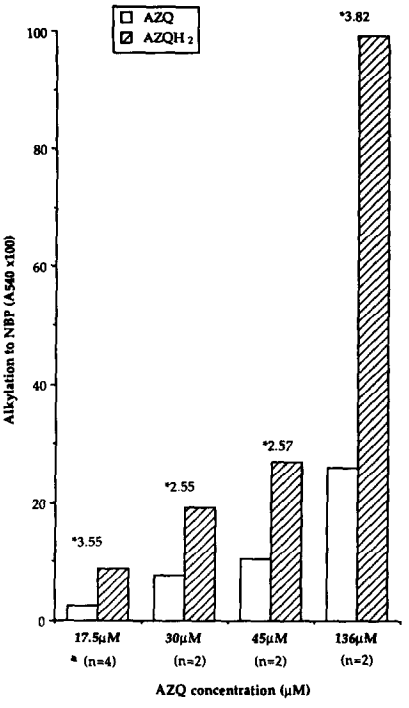


Fig. 3. Alkylating activity of AZQ and its borohydride reduced species $AZQH_2$. Borohydride was added in 2- or 3-fold excess. *Ratio of the alkylating activity of $AZQH_2$ to AZQ. The mean ratio ± SD of these four measurements is 3.12 ± 0.06. [a]Number of experiments. Each measurement in an experiment was performed in triplicate. Reactions were carried out in Tris or phosphate buffer (pH 7.4) for 10–15 min and then added quantitatively to NBP. Adding colorless $AZQH_2$ before the 10-min incubation time did not change the outcome.

The alkylating activity of AZQ reduced by $1e^-$ was investigated enzymatically with NADPH-cytochrome *c* reductase and chemically with excess NADPH. The alkylating activity increased with AZQ concentration, but it did not increase when AZQ was incubated with NADPH-cytochrome *c* reductase (Fig. 5). Dicumarol had no effect on the outcome of this reaction. The alkylating activity ratio of reduced AZQ to parent compound remained at 1 (Fig. 5).

NADPH, a mild $1e^-$ reducing agent, also did not increase the alkylating activity of AZQ at NADPH concentrations that were 2-, 3-, 5- and 10-fold excess over AZQ. The alkylating activity ratios of NADPH-reduced AZQ to parent compound for 14.7 μM AZQ were in the range of 1.01 to 0.89 for the NADPH concentrations used (minimum N = 6). Similar values were obtained for 28.7 μM AZQ. These concentrations of NADPH reduce AZQ to its semiquinone [11].

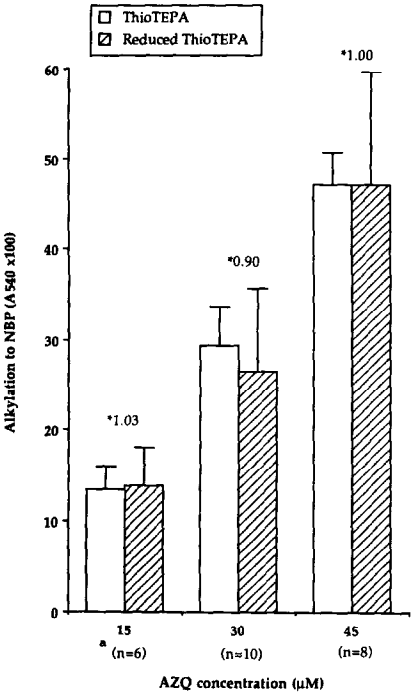


Fig. 4. Alkylating activity of Thio-TEPA and Thio-TEPA treated with borohydride. Borohydride was added in 2- or 3-fold excess. *Ratio of the alkylating activity of reduced Thio-TEPA to parent compound. The mean ratio ± SD of these three measurements is 0.98 ± 0.07. [a]Number of experiments. Each measurement in an experiment was performed in triplicate; values are given as means ± SD. Reactions were carried out in Tris or phosphate buffer (pH 7.4) for 10 min before adding quantitative aliquots to NBP.

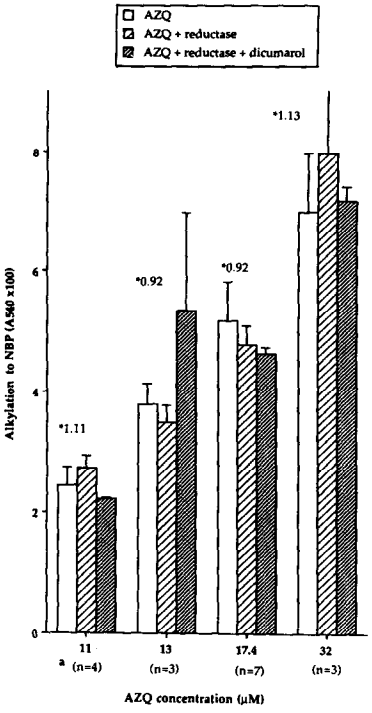


Fig. 5. Alkylating activity of AZQ incubated with NADPH-cytochrome *c* reductase. *Alkylating activity ratio of AZQ activated with NADPH-cytochrome *c* reductase to parent compound. The mean ratio ± SD of these four experiments is 1.02 ± 0.11. [a]Number of experiments. Each experiment consisted of triplicate measurements; values are given as means ± SD. Experiments were performed in 25 mM phosphate buffer (pH 7.4) with a protein concentration of 0.68 mg/mL. Dicumarol was added at 100 μM. No difference in alkylating activity was observed with NADPH alone at 16.2 and 48.5 μM.

Electron spin resonance experiments were performed with the same NADPH-cytochrome *c* reductase used in the alkylating assay to verify its reductive power. This enzyme was capable of inducing AZQ-dependent oxyradicals and AZQ semiquinone as previously described [10, 11, 17].

The growth of the semiquinone ESR signal lagged behind the DMPO-hydroxyl radical signal in a typical redox-cycling manner [11, 17]. In this case, 10–15 min after the start of the reaction, the hydroxyl radical reached 68–75% of its maximum intensity, while the semiquinone was nearly 44% of its maximum intensity. Spectral data for the alkylating assay were obtained 10–15 min after the onset of the reaction.

Alkylating activity experiments with whole cells were performed with 10^6 cells incubated with 400–600 μM AZQ. Aliquots of these incubates added to NBP resulted in a range of AZQ concentrations from 44 to 157 μM. No whole cell mediated increase in alkylating activity was observed. The same result was obtained when MCF-7 cells were quickly sonicated prior to adding AZQ. Electron spin resonance data indicate that 2×10^6 MCF-7 cells/mL incubated with 200 μM AZQ and 100 mM DMPO in cell growth medium containing 10% FBS were able to induce AZQ redox cycling as judged by the typical DMPO-OH quartet of ESR lines (Fig. 6). The intensity of the DMPO-OH ESR spectrum increased with time for up to 40 min. Dicumarol (100 μM) reduced hydroxyl radical formation to background levels (Fig. 6) implying inhibition of AZQ redox cycling. No AZQ semiquinone was detected at the AZQ concentration used (200 μM) except in trace amounts. At higher AZQ concentrations the semiquinone was evident (data not shown). Catalase diminished the DMPO-OH signal while SOD abrogated it (Fig. 6). Addition of ethanol resulted in very weak DMPO-ethyl adduct signals. Furthermore, ethanol did not decrease significantly the intensity of AZQ-dependent DMPO-OH signals. Sonicated cells gave no ESR signals in the presence of AZQ. AZQ alone in complete medium gave weak background DMPO-OH signals. These ESR studies were performed in the presence of 10% FBS which no doubt scavanges some free radicals, hence the low levels of free radicals detected. The cell viability throughout these experiments by trypan blue was consistently high. At the beginning of the experiments, the cells were 94.2 ± 2.8% viable.

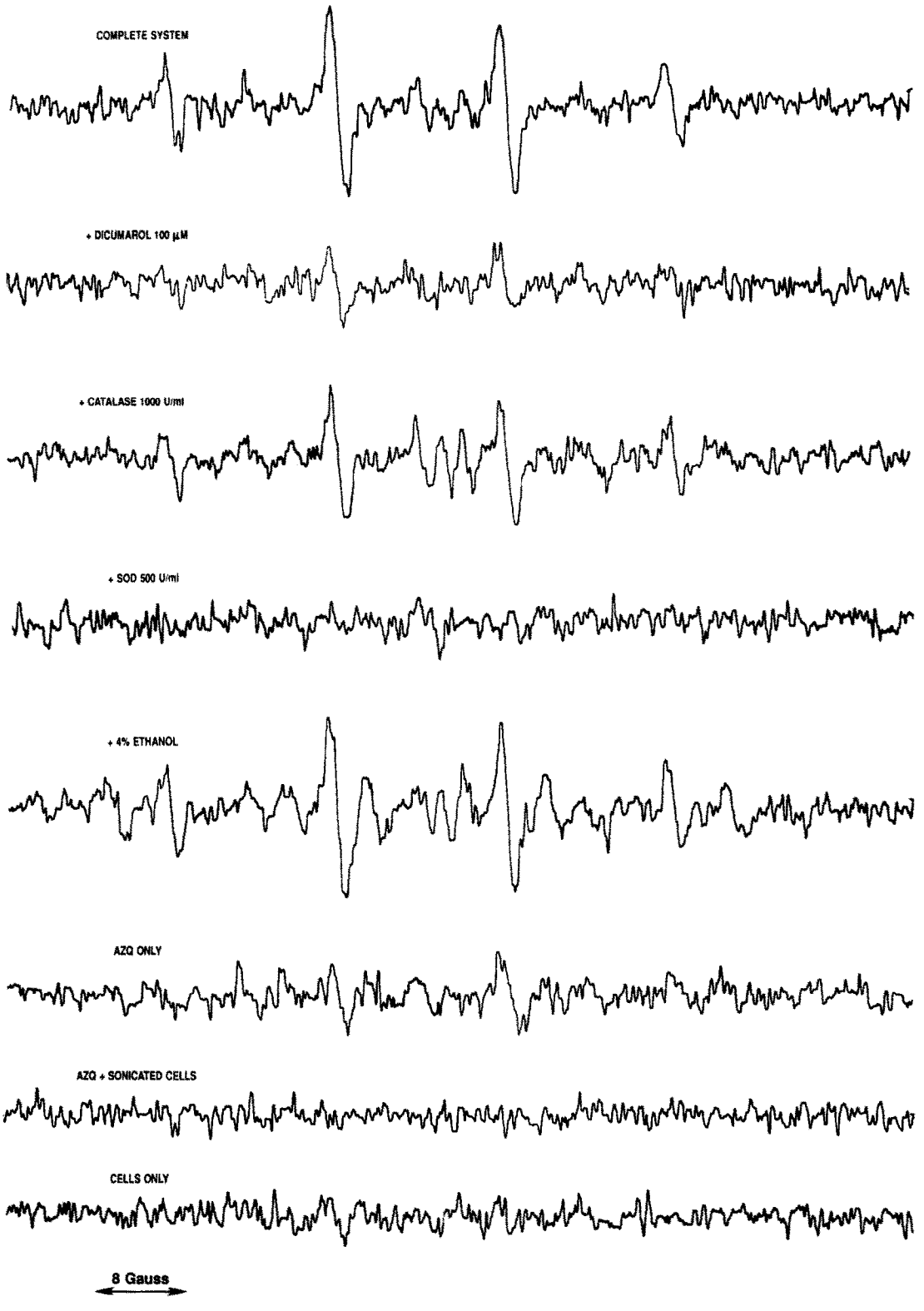


Fig. 6. ESR spectra of DMPO-OH in a system containing 2×10^6 MCF-7 cells/mL, 200 μM AZQ, 100 mM DMPO and 10% FBS supplemented Iscove's modified Dulbecco's medium. The spectra were recorded 40 min after incubation. Cells were preincubated with catalase, SOD, dicumarol and ethanol for 40 min on ice before adding AZQ and DMPO. ESR conditions were: 10 mW incident microwave power, 1.25 G modulation amplitude and 9.51 GHz.

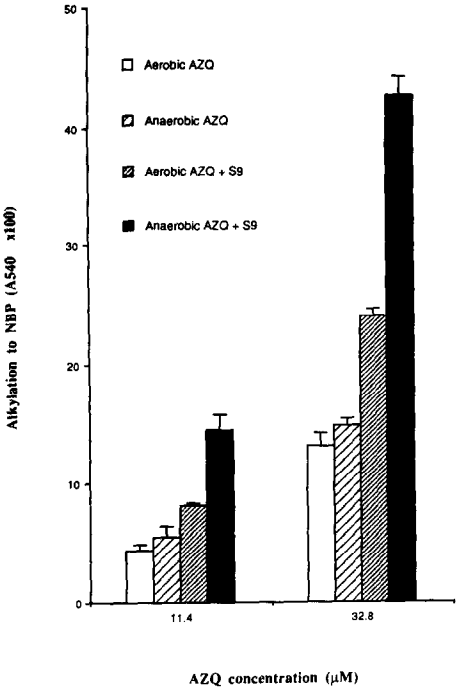


Fig. 7. Anaerobic and aerobic alkylating activity of AZQ and AZQ incubated with MCF-7 S9 cell fraction. Values are means ± SD. The average increase of alkylating activity with anaerobic conditions was 1.78. The number of independent measurements ranged from 3 for aerobic AZQ + S9 to 6 for anaerobic AZQ + S9. Experimental conditions were as described in the legend of Fig. 2.

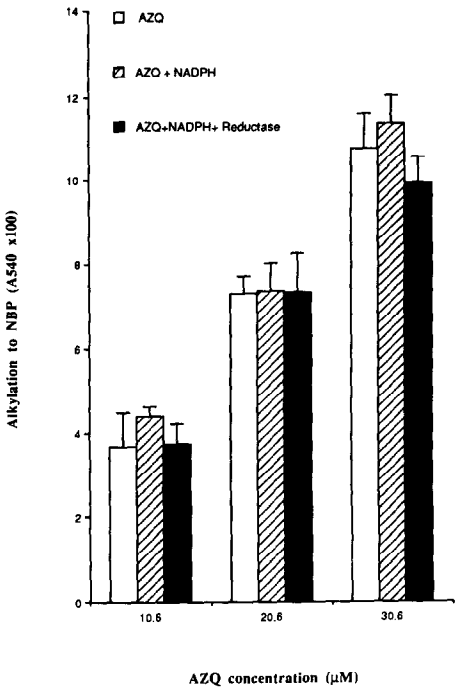


Fig. 8. Anaerobic alkylating activity of AZQ and AZQ incubated with NADPH-cytochrome *c* reductase. Values are means ± SD. The number of independent measurements ranged from 3 to 6. Experimental conditions were the same as described in the legend of Fig. 5.

After 2 or 4 hr, the cells were 91.7 ± 2.9 or 89.8 ± 2.2% viable, respectively. These values represent the means ± SD of three independent measurements.

Anaerobic NBP alkylating activity. The alkylating activity of AZQ metabolized by the S9 cell fraction of MCF-7 cells under anaerobic conditions and measured by the NBP assay under the same conditions showed a 1.8-fold increase over its aerobic counterpart. This result was obtained at AZQ concentrations of 11.4 and 32.8 μM (N = 3 to N = 6) (Fig. 7). The alkylating activity of AZQ alone tended to be higher in anaerobic incubations, but this difference was not statistically significant. In anaerobic incubations, the increase in the alkylating activity ratio between AZQ treated with S9 cell fraction and nontreated AZQ was 2.6 for 11.4 μM AZQ and 2.87 for 32.8 μM AZQ. This increase was smaller than the 3.1 average obtained in the experiments depicted in Fig. 2. In general, the alkylating activity of AZQ alone was slightly larger in the anaerobic experiments depicted in Fig. 7 than in the aerobic experiments of Fig. 2.

Anaerobic experiments with NADPH-cytochrome *c* reductase at three different concentrations of AZQ indicated that the alkylating activity increased with AZQ concentration (Fig. 8). However, AZQ alkylation did not increase when reduced enzymatically or when treated with NADPH alone (Fig. 8). As with S9 incubations, these experiments (i.e. Fig. 8) showed a slight increase in the alkylating activity of AZQ alone when compared to aerobic experiments (Fig. 5).

DISCUSSION

The relationship between chemical and enzymatic reduction of AZQ and its alkylating activity has been investigated. Our data show that only two electron reductions enhanced the alkylating activity of AZQ and that this activity was further enhanced by anaerobic conditions. The alkylating activity per aziridine was less in AZQ than in the phosphorus containing alkylator Thio-TEPA, reflecting the difference in chemical structure. The quinone moiety, however, upon reduction by two electrons rendered the two drugs equal in alkylating power.

These data support the proposed involvement of the hydroquinone in the bioreductive alkylation of AZQ. Our earlier work has supported the idea that the most probable mechanism for AZQ alkylation involved quinone reduction followed by protonation on the aziridine rings [14, 15]. This reduction was suggested to be a $2e^-$ reduction. In early experiments with calf thymus DNA, the semiquinone was suggested as the primary alkylating species [6]. This arose from the observation that under anaerobic conditions AZQ reduced with 10-fold excess borohydride showed substantially lower cross-linking

than under aerobic conditions. Thus, because aerobic conditions favor AZQH the importance of this species in alkylation was postulated. However, Szmigiero and Kohn [4] showed in a concurrent publication that an 8-fold excess borohydride results in a marked decrease in AZQ alkylating activity. Furthermore, at borohydride concentrations that are 3-fold higher than AZQ, an aziridine can be replaced to produce a monoaziridine quinone product [16]. Anaerobic incubations of AZQ with 10-fold excess borohydride may have resulted in AZQ breakdown (since $AZQH_2$ is favored) leading to lower DNA alklyation products. Under aerobic conditions auto-oxidation may have slightly prevented AZQ breakdown leading to more DNA alkylation products. In other work, Lusthof in his Ph.D. thesis recently suggested that in electrochemically reduced bis-aziridine benzoquinones, the $2e^-$ reduction does not lead to higher alkylating activity; only the $1e^-$ reduction does [31]. In another publication, similar data with electrochemically reduced compounds were presented by this same author and coworkers [32]. The suggestion was made that reduced aziridine quinones including AZQ are the reactive alkylating species, but no distinction was made between $1e^-$ and $2e^-$ reductions [32]. It has been our experience and that of others that AZQ, aziridine quinones and other quinones can only be reduced by $2e^-$ in aqueous solutions when using cyclic voltammetry and polyography [14, 33]. The $1e^-$ reduced species may exist in equilibrium with the $2e^-$ reduced species but the aziridine ring is quite unstable in a $2e^-$ reduction ($QH_2N \lhd\rightarrow QH_2NHCH_2CH_3$, [34]). Thus, when speaking of protonated reduced species of AZQ and other aziridine quinones, it is important to describe the nature of the reduced species. Unfortunately, for these compounds, ESR data can also lead to erroneous conclusions because the semiquinone, although transient, can be readily observed in two electron reductions.

Despite this apparent discrepancy, the data presented here support increased alkylation by the AZQ hydroquinone over the parent compound and can explain previous and recent data on the variability of AZQ toxicity. In early work, Szmigiero *et al.* [5] observed a marked variability between AZQ-stimulated DNA strand scission and AZQ-dependent DNA cross-linking in three human cell lines (human embryo cell lines IMR-90 and VA-13, and the human colon carcinoma line HT-29) and one mouse line (L1210 leukemia). The colon cancer line HT-29 had high interstrand cross-linking and no detectable strand breaks, while the opposite was true for the embryo lines IMR-90 and VA-13. Recent work [27] re-investigated the HT-29 cancer line in the light of its high QAO content. For comparison a cell line deficient in QAO was used. The results were the same as previously observed for HT-29. That is, the AZQ-induced damage to DNA is almost exclusively interstrand cross-linking with no detectable strand breaks. The QAO-deficient cell line exhibits very low AZQ-dependent cross-linking [27].

Early reports on AZQ-mediated DNA strand breaks and DNA cross-linking in isolated nuclei from human cells used borohydride and low pH to activate AZQ (e.g. Ref. 4). The effect of low pH can be understood in terms of aziridine protonation. The effect of borohydride can be understood in terms of the $AZQH_2$ species and its enhanced ability to alkylate over AZQ. These early experiments [4] also reported that AZQ-induced DNA strand breaks required the addition of NADPH. This result can be understood in terms of reactive oxygen species formation. NADPH reduces AZQ by one electron because the concentration of the semiquinone that results is proportional to the NADPH concentration [11], and because the yellow color of the free radical is observed and persists under anaerobic conditions [11]. Another result is that NADPH also enhanced interstrand cross-linking as borohydride did in a reaction that was not inhibited by superoxide dismutase [4]. This result would tend to give a role to the semiquinone as an alkylator. In contrast, another early work reported the lack of AZQ alkylation to DNA upon the reduction of AZQ with NADPH [6]. This latter result is in agreement with the data presented here. We explain the NADPH-dependent DNA cross-links in isolated nuclei in terms of enzymatic activity. We theorize that NADPH could serve as an electron source to provide QAO-like two electron reductions in isolated nuclei. Nuclei are not likely to have QAO activity, but there are other enzymes with such properties (e.g. Ref. 35).

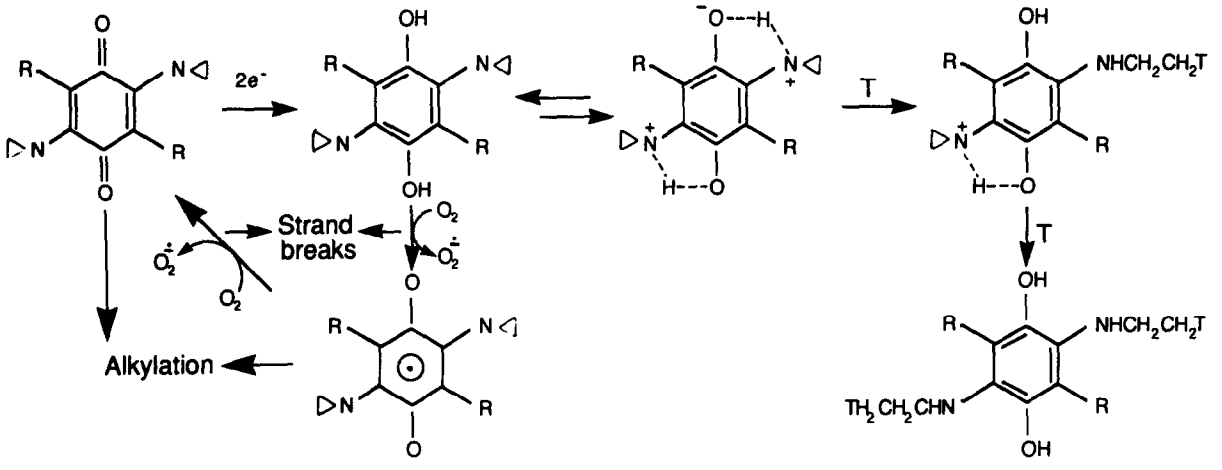
The influence of $AZQH_2$ in aklylation may also explain the sequence selectivity recently observed when analogs of AZQ were studied for their ability to alkylate DNA [36]. Two AZQ analogs, diaziridinyl benzoquinone (DZQ) and a dimethylaziridinyl quinone analog, show alkylation to guanine at pH 7.2. Upon reduction by QAO, DZQ appears to preferentially alkylate guanine, while the dimethyl derivative preferentially alkylates adenines [36]. We and others observed similar adenine alkylation preference with untreated AZQ [37, 38] and with borohydride-reduced AZQ* in mononucleotides [38,*] and in polynucleotides [37].

The proposed mechanism of action that emerges is depicted in Scheme 1 [14, 15]. Although we have no evidence for the intermediate hydroquinone with intramolecular hydrogen bonds that result in destabilizing the aziridine ring in an identical way as protonation would, there is no precursor that can lead to alkylation at neutral pH. The proposed five-membered hydrogen bounded structure is not ideal but it can lead to aziridine ring opening and alkylation. Perhaps a similar structure involving the carboethoxyamino groups is possible. Nevertheless, the evidence for alkylation with the hydroquinone as an intermediate is compelling.

Scheme 1 also shows the role of the semiquinone and the hydroquinone in producing reactive oxygen species that result in DNA strand breaks among other toxic consequences. It also indicates that the alkylating potential of the AZQ semiquinone is similar to that of the parent compound.

The fate of the hydroquinone in Scheme 1 is shown in the presence of nucleophile target T. In the absence of a nucleophile we have shown that the main product of the borohydride reduction is the

* Gutierrez P, unpublished data.



Scheme 1.

monoaziridine AZQ where one aziridine is still intact and ready for alkylation [16]. This can easily lead to monoalkylated DNA adducts. Another product found in smaller concentrations was the monoamino alcohol where one aziridine was opened with the addition of a hydroxyl group while the other aziridine remained unchanged. This last product as well as the dihydroxylethylamino compound (diaminoalcohol) were the major degradation products in acidic media [39]. The reduction rate of AZQ by borohydride depends on the concentration of the reducing agent. At the excess concentrations of 2:1 and 3:1 used here, the rate constants of reduction were of the order of 0.020 to 0.065 O.D. sec^{-1} at 342 nm [16]. The colorless $AZQH_2$ formed remains stable for 1–2 hr at the concentrations of borohydride used here, and in the absence of nucleophiles, it yields the products described above.

The question as to whether or not NBP is an adequate nucleophile to study the alkylating activity of AZQ and other aziridine quinones should be addressed. Our data show that for AZQ (this work) and for other AZQ analogs,* alkylating activity can be quantified and it depends on alkylator concentration. NBP is also able to reflect stearic hindrances that can affect alkylation in aziridine quinones that have bulky groups.* In this respect NBP is faster and less labor intensive in evaluating alkylation than nucleophiles such as DNA and thiols. There is a question about the background alkylation observed in the absence of reduction. The NBP assay requires that NBP (dissolved in acetone) be mixed with acetate buffer at pH 4.6. This short incubation at low pH could lead to the alkylating activity observed in untreated AZQ. However, at pH 7.4 there is also a background alkylation by untreated AZQ [4,*].

No alkylation to DNA was reported in the absence of electrochemical reduction for a series of aziridine quinones [32] that include DZQ. However, using other analogs including DZQ, Lee *et al.* [36] have shown recently that this compound (untreated) alkylates to DNA at pH 7.2. Detecting AZQ–DNA adducts by light spectroscopy [32] or by isolating DNA–AZQ adducts from whole DNA may not be as sensitive as gel electrophoresis [36] to determine if DNA–AZQ adducts are formed by untreated AZQ.

The increase in alkylating activity observed with anaerobic conditions was relatively low (near 1.8-fold) (Fig. 7). The ratio of S9-treated to nontreated AZQ remained in the same range (2.6 to 2.9) as aerobic experiments (3.1, Fig. 2). The relatively low 1.8-fold increase with anaerobicity in S9-treated AZQ can be explained in terms of the relative stability of $AZQH_2$ to auto-oxidation. The lack of oxygen prolongs the life of $AZQH_2$ for long periods of time (14–28 hr) [16]. Under aerobic conditions $AZQH_2$ can be stable for up to 1 hr [16]. In the time frame of the experiments performed here (10–15 min after reduction), $AZQH_2$ may not have had time to completely auto-oxidize as it is added to the NBP solution. Thus, under both anaerobic and aerobic conditions, NBP was probably exposed to $AZQH_2$ concentrations that were not very different.

Anaerobic conditions are essential to extend the life of the semiquinone. The AZQ semiquinone is very unstable to oxygen and it auto-oxidizes rather quickly (e.g. Ref. 15). Anaerobic conditions, do not help increase the alkylating activity of the $1e^-$ reduction of AZQ. We observed a tendency of untreated AZQ to alkylate more under anaerobic conditions than in the presence of air (e.g. Fig. 7). This increase was in the range of 1.1- to 1.45-fold (Figs. 7 and 8 vs Figs. 2 and 5) and persisted even after enzymatic reductions (Fig. 7, not shown in Fig. 8). This is probably due to slightly higher concentrations of NBP that result from acetone evaporation during gentle nitrogen gas purging while adding drug to the NBP solutions. The NBP solutions were prepared with pre-deaerated acetone.

We used dicumarol to emphasize the importance of QAO on the activation of AZQ. Reservations about using this inhibitor have been made especially

* Gutierrez P, unpublished data.

when dealing with mitomycin C [40]. Criticism has been directed at studies which modulate quinone cytotoxicity with dicumarol without defining first if the quinone in question is a substrate for QAO or not. We have shown that AZQ is a substrate for QAO activity in the S-9 fraction of MCF-7 cells [18]. In addition, dicumarol was added to NADPH-cytochrome *c* reductase to observe its effect on this enzyme and for the sake of completion.

The difficulty in enhancing alkylating activity with whole cells is not clear. One explanation is that, due to the hydrophobic nature of AZQ, it may remain in the lipid environment of the cell membranes and thus it is not available to akylate NBP. Another explanation is that even though AZQ redox cycling was observed by ESR with 2×10^6 cells/mL, the actual number of cells used in the NBP assay (10^6 cells/mL) may not have been adequate to reduce AZQ and observe NBP alkylation. Most likely, however, is the possibility that AZQ preferentially alkylates cellular macromolecules/membranes rather than NBP.

Taken together these data point to QAO in MCF-7 cells as a particularly important enzyme in the bioactivation of AZQ.

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