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CELLULAR ACTIVATION OF DIAZIQUONE [2,5-DIAZIRIDINYL-3,6-BIS (CARBOETHOXYAMINO)-1,4-BENZOQUINONE] TO ITS FREE RADICAL SPECIES

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Abstract—Human leukemic cell lines K562 and HL60, and the murine leukemic cell line L1210, reduce Diaziquone (AZQ) (NCS182986) to its free radical anion. With all cell lines, the free radical was observed immediately in both aerobic and anaerobic cell suspensions. The steady-state concentration of AZQ free radicals was approximately 1% of the total AZQ concentration. L1210 cells treated with azide reduced AZQ, but cells treated with diamide and *N*-ethylmaleimide did not. NADPH and L-cysteine reduced AZQ. The latter did so under anaerobic conditions; the former did so under both anaerobic and aerobic conditions.

Previous work from this laboratory and others has shown that quinone-containing antitumor agents can be activated to their free radical species by rat liver microsomes [1–4], rat liver nuclei [5], purified NADPH-cytochrome P-450 reductase [6], xanthine oxidase [7, 8], and Ehrlich ascites cells [4]. Anthracycline antibiotics (i.e. Adriamycin and daunorubicin) have been the agents most widely studied in this manner (e.g. Refs. 4, 5 and 9). Recently, we described the biochemical activation of the aziridinyl quinone Diaziquone [2,5-diaziridinyl-3,6-bis (carboethoxyamino)-1,4-benzoquinone] (AZQ) to its free radical species by rat liver microsomes and NADPH-cytochrome *c* reductase [10]. In both cases, we detected a five line electron spin resonance (ESR) spectrum due to the AZQ free radical anion. AZQ is undergoing phase I and phase II clinical trials, so that it becomes increasingly important to define its metabolism and possible mechanism(s) of action. One probable mechanism of action is by alkylating biologically important molecules through the aziridine groups. A second possible mechanism is based on the hypothesis that drug free radical intermediates react readily with important macromolecules to render those macromolecules inactive (e.g. Ref. 11). A third possibility includes the effect that the drug free radical may have on generating oxygen free radicals which, in turn, can react in many ways deleterious to cells. In general, quinone-containing agents are a class of agents whose activity may be influenced by their ability to form free radicals. In this paper we investigate the possibility of whole cells activating AZQ to its free radical.

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MATERIALS AND METHODS

Diaziquone was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda MD, U.S.A., and tested for purity by high performance liquid chromatography (HPLC) [12]. No impurities were found and, therefore, the drug was used without further purification. For electron spin resonance (ESR) and oxygen measurement experiments, 0.1 M stock solutions were prepared by dissolving the agent in dimethyl sulfoxide (DMSO) (Gold Label, Aldrich Chemical Co., Milwaukee, WI). In all experiments, the final concentration of DMSO did not exceed 2%.

L1210 murine leukemia cells were maintained *in vitro* by serial culture in RPMI 1640 medium containing 50 units/ml of penicillin, 50 μg/ml of streptomycin, 2 μmoles/ml of L-glutamine (Flow Laboratories, McLean, VA), and 15% newborn calf serum (NBS, Flow Laboratories). Under these conditions, L1210 cells have a population doubling time of 14–18 hr, and achieve a maximum density of 1.5 to 2.0×10^6 cells/ml. Ten million cells were used per experiment. The HL60 cell line was derived from peripheral blood leukocytes of a patient with acute progranulocytic leukemia [13]. The particular HL60 cultures used in this study were started from cells cryopreserved after the seventy-first passage. The cells were used after several passages and maintained in culture using RPMI 1640 medium supplemented with 20% (v/v) heat-inactivated fetal calf serum, and L-glutamine, penicillin, and streptomycin as above. Under these conditions, HL60 cells had a population doubling time of approximately 24 hr and reached a saturation density of $3–4 \times 10^6$ cells/ml. Ten million cells were used per experiment. K562 cells were derived from the pleural fluid of a patient with

chronic myelogenous leukemia in blast crisis [14]. K562 cells are maintained in continuous liquid suspension culture in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, L-glutamine, penicillin and streptomycin as above. Under these conditions, K562 cells have a population doubling time of approximately 24 hr and achieve a saturation density of 2–4 $\times$ 10^6 cells/ml.

Oxygen uptake was determined with a Clark-type electrode in a Biological Oxygen Monitor (model 53, Yellow Springs Instrument Co.). The reaction mixtures contained 10 million cells in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) (pH 7.5) and 1 mM AZQ in a final volume of 1 ml. The cell suspension without AZQ was aerated in the chamber for 3 min. Endogenous oxygen consumption was monitored for 5 min. AZQ was then introduced into the system, and AZQ-induced oxygen consumption was monitored for 50–60 min at room temperature or 37°. Untreated cells survived up to 3 hr under these conditions. For kinetic data, the oxygen consumption evaluation was based on a 100% value of 1.58×10^{-7} moles/ml of dissolved oxygen [10]. Kinetic constants were determined by fitting data from at least three separate experiments to the Michaelis–Menten equation by the least-squares method assuming equal variances for the velocities. Calculations were performed on the Tymshare computer network using programs written by Cleland [15] which provide K_m, V_{max}, K_m/V_{max}, $1/V_{max}$ and the standard deviation of their estimates.

ESR measurements were made at room temperature with an X band (9.3 GHz) Varian E-109 Century series spectrometer equipped with 100 kHz field modulation. A dual cavity (TE104) was used which contained a strong pitch standard (g = 2.0028) in one section and the sample in a flat ESR cell in the other. The strong pitch marker was used to estimate g values. Reaction mixtures were the same as those described above for oxygen uptake experiments. Quantitative free radical analysis employed 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a standard. Solutions in benzene were freshly prepared for each determination, and concentrations were determined by using an extinction coefficient of 30.2 l g^{-1} cm^{-1} at 525 nm [16]. Double integrals were obtained with a Nicolet-1180 computer (Madison, WI, U.S.A.). Free radical kinetic experiments were attempted with the static field technique. The recorder pen is placed at the location of maximum displacement of the derivative signal. The instrument is locked to this signal with a Varian E-2723 Field/Frequency lock, and the field sweep is turned off. Under these conditions, the recorder sweeps time along the X-axis. At the end of the kinetic sweep or when the kinetic plot plateaus, the magnet sweep is turned on and a steady-state spectrum recorded. For calculations, one assumes that the deflection from the base line on a kinetic plot corresponds to half the total deflection of the derivative signal. The base line of the kinetic plot is obtained by extrapolating the plot to zero time. To obtain free radical concentrations as described by Hyde,* the integral of the sample's spectrum is compared to the integral of a standard DPPH solution of known concentration. These free radical concentrations represent steady-state concentrations used in calculating free radical production in terms of concentration per minute (see Fig. 4).

Oxygen consumption and free radical production were measured in aliquots of cells from the same cell harvest. The samples were treated identically, and the reactions were started at the same time by adding AZQ.

For certain experiments, anaerobic conditions were achieved by passing a stream of pure nitrogen through 1-ml solutions for at least 2 min.

The effects of azide and sulfhydryl inhibitors were investigated by adding them to the solution or cell suspension for 1 hr; the cells were washed and resuspended in 1 ml HBSS before adding AZQ. The final concentrations of inhibitors in a 1-ml suspension of 10^7 L1210 cells were: 10 mM azide and 1 mM for diamide and *N*-ethylmaleimide.

Protein concentrations were obtained by the method of Lowry *et al.* [17].

RESULTS

When Diaziquone was incubated with L1210, HL60 and K562 cells in HBSS, the AZQ free radical was formed (Figs. 1 and 2). AZQ alone did not give a signal in HBSS even after computer signal averaging for 30 min (Fig. 2C). For equal numbers of cells, the intensity of the free radical spectrum and therefore the number of free radicals produced varies with cell line. Thus, K562 cells yielded the most free radicals (Fig. 2F), while HL60 produced the least (Fig. 2D). L1210 cells yielded an intermediate amount (Fig. 2E). In all cases, the free radicals had the same number of lines and g value (g = 2.0046) as that obtained with NADPH-cytochrome *c* reductase in the presence of NADPH as a cofactor [10]. The free radical intensity, however, was approximately sixty to seventy times larger when induced with pure enzyme than with whole cells. The intensity of the free radical signal varied with the cell preparation, and, in the case of L1210, attempts to correlate this variation with cells in log or lag phase gave variable results.

Experiments where oxygen concentration and AZQ free radical production were measured under the same conditions show that free radicals were formed in the presence of relatively high oxygen content (50–60% of a fully areated solution) (Fig. 3). The radical was present within the 2 min it took to place the sample in the microwave cavity and begin recording. Hence, totally anaerobic solutions were not required for the accumulation of free radicals to detectable concentrations. As the solution became anaerobic, the free radical signal intensity increased by approximately 56%. This production of free radicals in aerobic mixtures is in contrast to our previous observations with anthracyclines, mitomycin, streptonigrin and other quinone antibiotics where the solutions had to be anaerobic before free radicals accumulated to detectable concentrations (e.g. Refs. 2, 4, 5, 6 and 18).

Diaziquone enhanced oxygen consumption in a

* J. Hyde, in *Proceedings of the Seventh Annual EPR-NMR Workshop*, p. 15, Palo Alto, CA (1963).

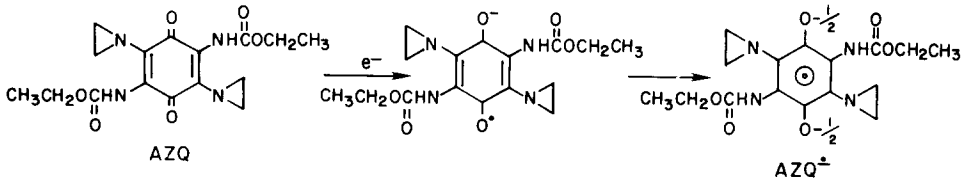

Fig. 1. One-electron reduction of AZQ.

reaction which is saturable by the drug. The data fit Michaelis–Menten kinetics and allowed for the calculation of the kinetic constants K_m and V_{max} with the drug as substrate. The values for these constants are $V_{max} = 2.0 \pm 0.05 \times 10^{-8}$ moles of O_2 utilized/min/mg protein (mean ± S.E.) and $K_m = 65 \pm 14\ \mu M$ (mean ± S.E.) The K_m values for liver nuclei microsomes are respectively, forty and eleven times lower than that for whole L1210 cells (Table 1) [10]. The K_m value for purified NADPH-cytochrome *c* reductase is thirty times lower than that of whole L1210 cells [10]. V_{max} values are considerably lower (*ca.* 500 times) for whole cells than for NADPH-cytochrome *c* reductase.

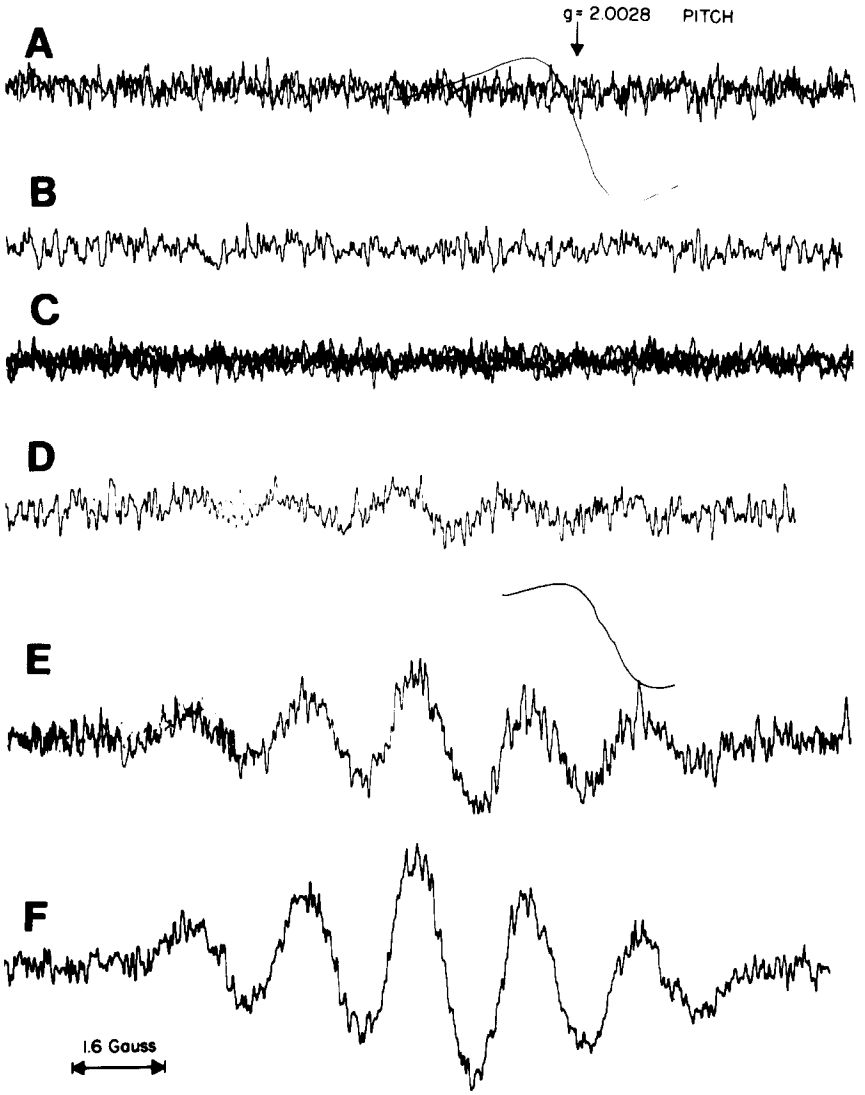

Fig. 2. ESR spectra of (A) L1210 cells in Hanks' balanced salt solution (HBSS) and 1% DMSO, (B) L1210 cells in HBSS, (C) Diaziquone in HBSS, (D) Diaziquone and HL60, (E) Diaziquone and L1210, and (F) Diaziquone and K562. The 1-ml solutions contained 10^7 cells and 1 mM Diaziquone. ESR conditions at room temperature were 9.3 GHz, 10 mW incident microwave power, 1 gauss modulation amplitude and 1.25×10^5 receiver gain.

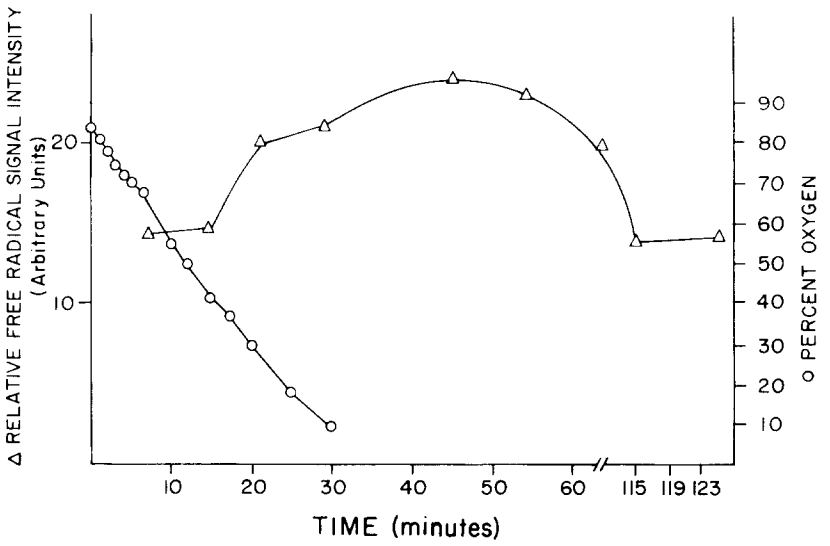

Fig. 3. Oxygen content and free radical signal intensity measured under the same conditions in L1210 cells (10^7) incubated with AZQ (1 mM); oxygen consumption (○) and Diaziquone free radical signal intensity (△) as a function of time. The measurements were performed in cells from the same cell harvest. The ESR conditions at room temperature were: 9.3 GHz, 1 gauss modulation amplitude, and 10 mW incident microwave power. Other conditions were as described in Materials and Methods.

Under steady-state conditions, a very small percentage (between 0.5 and 1%) of the total concentration of AZQ existed as a free radical (Table 2). Attempts at obtaining Michaelis–Menten kinetic parameters for free radical production were hampered by the low sensitivity of the method and substrate saturation. That is, at concentrations below 1 mM AZQ, the signals were too weak to obtain ESR static field kinetic plots and, at concentrations higher than 2 mM, the system had already been saturated (Table 2). The velocities reported in Table 2 are an attempt to calculate kinetic parameters (Fig. 4). The variability of cell preparations is reflected in the relatively large standard errors of free radical concentrations and velocities.

NADPH is a mild reducing agent and, at concentrations of 3–4 mM, it reduced AZQ to a detectable concentration of free radicals approximately equal to the free radical concentrations obtained with L1210 cells alone (Table 3). In a range of NADPH concentrations between 0.5 and 6 mM, there appeared to be a proportional increase in free

Table 1. Kinetics of AZQ-induced O_2 consumption in L1210 cells

System	Cofactor	K_m* (M)	V_{max}* (moles of O_2 utilized/min/mg protein)
Nuclei†	NADPH	$1.7 \pm 0.34 \times 10^{-6}$	$0.18 \pm 0.034 \times 10^{-7}$
Microsomes†	NADPH	$5.7 \pm 1.7 \times 10^{-6}$	$1.70 \pm 0.82 \times 10^{-7}$
NADPH-cytochrome *c* reductase†	NADPH	$2.1 \pm 1.0 \times 10^{-6}$	$100 \pm 5 \times 10^{-7}$
L1210 cells‡	None	$65 \pm 14 \times 10^{-6}$	$0.20 \pm 0.05 \times 10^{-7}$

* Values = means ± S.E.
† From Gutierrez *et al.* [10].
‡ This work.

Table 2. AZQ free radical activation of Diaziquone by L1210 cells

Number of experiments	AZQ (mM)	Steady state AZQ free radical* (μM)	AZQ free radical/AZQ (%)	Velocities* (μM/min)
5	1.0	10.64 ± 0.91	1.0	0.41 ± 0.03
5	2.0	15.67 ± 0.72	0.8	1.70 ± 0.13
8	3.0	15.41 ± 1.08	0.5	1.26 ± 0.18
3	4.0	27.18 ± 2.79	0.7	1.82 ± 0.15

* Values = means ± S.E.M.

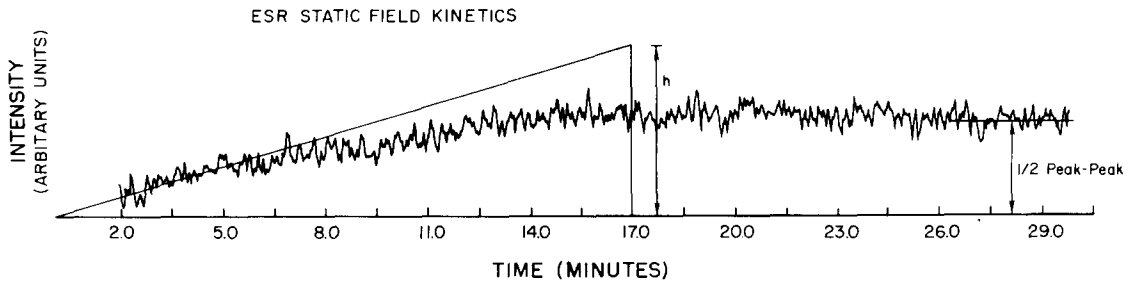

Fig. 4. ESR kinetics of Diaziquone free radicals in an aerobic solution of L1210 cell containing 3 mM Diaziquone final concentration in HBSS, pH 7.5. We chose the initial slope to calculate velocity parameters by $v = f_1 f_2$ μM/min (Table 2), where f_1 is the initial slope shown in the figure in units of mm per min; f_2 is the ratio of the free radical concentration in the plateau region to the peak to peak (mm) height of the center line of the Diaziquone free radical spectrum. This was the line followed in the kinetic trace above, and the line which the Varian E-2723 Field/Frequency Lock was locked onto. The free radical concentration was evaluated by double integration and compared to a DPPH standard of known concentration (see Materials and Methods). The ESR conditions at room temperature were 9.3 GHz, 1 gauss amplitude modulation, and 10 mW incident microwave power.

radical production. When NADPH was added to AZQ in the presence of L1210 cells, the free radical concentration increased by 40% from the concentration of NADPH generated radicals (i.e. 17.0 ± 0.67 μM for L1210 + NADPH versus 12.3 ± 0.42 for NADPH).

L-Cysteine and glutathione did not reduce AZQ in the presence of air. Under anaerobic conditions, only L-cysteine reduced AZQ to free radicals whose concentrations were similar to those generated by L1210 cells (Table 3).

To help elucidate the way in which cells reduce AZQ, we treated L1210 cells with azide, *N*-ethylmaleimide and diamide. Azide, which blocks the electron flow at the cytochrome ($a + a_3$) site in the respiratory chain [19] had no effect on free radical formation (Table 4). The number of free radicals generated by control cells in this case was less than the free radicals generated by control cells in Table 2 (1 mM). This reflects the effects of a 1-hr incubation followed by an additional wash (see Materials and Methods). *N*-Ethylmaleimide and diamide (azodicarboxylic acid-bis dimethylamide) are sulfhydryl blocking agents that inhibited the formation of AZQ free radicals by L1210 cells (Table 4). In the absence of cells, these agents had no effect on AZQ, but in the presence of NADPH generated AZQ free radicals, azide scavenged them [20], while diamide had little effect and *N*-ethylmaleimide appeared to aid their production.

Table 3. Chemical reduction of Diaziquone

	AZQ free radical concentration* (μM)
Control	0
NADPH (4 mM)	12.30 ± 0.42
L-Cysteine (4 mM)	0
Glutathione (GSH) (4 mM)	0
L-Cysteine (anaerobic) (4 mM)	10.21 ± 0.15
GSH (anaerobic) (4 mM)	0

* Values = averages ± S.E.M.

DISCUSSION

We have shown that the three cell lines studied mediate the formation of the AZQ free radical (Fig. 1). The intensity of the free radical generated varied with cell line, indicating that some cell lines possess a greater ability than others to reduce AZQ. If the hypothesis that drug free radicals are involved in cell toxicity is correct, then this result can be important in the sense that AZQ could potentially have preferential toxicity for some tumors and tissues. In this case, free radicals could also be involved in cytotoxic effects if free radicals were to enhance the alkylating activity of AZQ.

Diaziquone stimulated oxygen consumption in L1210 cells. The V_{max} value for the enzymatic reaction with purified NADPH-cytochrome *c* reductase was approximately 500 times greater than for L1210 cell suspensions. Our data show that AZQ free radicals were generated in the presence of oxygen in contrast to previous results with other quinone-containing antitumor agents [1, 2, 4, 21] and with other free radicals generated in biological systems [22, 23]. Of particular interest in the case of carboquone, a quinone-containing agent with two aziridine rings similar to AZQ, and Ehrlich ascites cells [4]. In all these cases, oxygen prevents the accumulation of free radicals to detectable concentrations by oxidizing them to the parent compound. One possible explanation for observing AZQ free radicals in aerobic solutions is the fact that AZQ may be easier to reduce than Adriamycin, daunorubicin or carboquone. For instance, one-electron reduction potentials for AZQ and Adriamycin quinone-semiquinone couples under identical conditions are −168 mV for AZQ and −289 mV for Adriamycin [24]. The reduction process for AZQ is probably more efficient than the oxidative process, allowing for the accumulation of detectable numbers of free radicals. Conversely, it is possible that AZQ is fully reduced by a two-electron transfer process and then it autoxidizes to the semiquinone free radical. Both these possibilities are under investigation in our laboratory.

Table 4. AZQ free radical submitted to various treatments

	AZQ free radical concentration* (μM)	Specific activity* (μM/mg protein)
L1210 control†	2.08 ± 0.59	4.72 ± 1.3
L1210 + azide† (10 mM)	2.39 ± 1.05	5.43 ± 2.39
L1210 + *N*-ethylmaleimide† (1 mM)	0	0
L1210 + diamide† (1 mM)	0	0
NADPH (4 mM)	12.30 ± 0.42	
NADPH + azide (10 mM)	5.00 ± 0.41	
NADPH + *N*-ethylmaleimide (1 mM)	14.38 ± 0.96	
NADPH + diamide (1 mM)	18.34 ± 0.67	

* Values = averages ± S.E.M.

† Mixtures of 10^{-7} cells in 1 ml HBSS, pH 7.5, were incubated with the agent for 1 hr at 37°, 5% CO_2 and 95% humidity, washed, and resuspended in 1 ml HBSS. AZQ (1 μM final concentration) was added to start the reaction.

The observed concentration of AZQ free radicals produced by L1210 cells does not exceed 1% of the initial concentration of AZQ. Despite the difficulties in obtaining kinetic data on free radical formation, the velocities of Table 2 suggest that the system is saturated at 2 mM concentrations of substrate.

AZQ has the ability to penetrate cells in culture [25], so that if only *N*-ethylmaleimide and diamide destroy the ability of L1210 cells to generate free radicals, the reducing electron may be enzymatic in nature. Of NADPH, GSH and L-cysteine, GSH is the only compound whose endogenous intracellular concentration (up to 5 mM) [26] is large enough to contribute significantly to the reduction of AZQ. However, we observed no AZQ free radical when GSH was incubated with AZQ (Table 3). GSH is a strong nucleophile so that an addition reaction (i.e. AZQ-GS) could very well interfere with free radical formation. The ability of NADPH and L-cysteine to reduce AZQ *in vitro* is significant in the sense that it points to reduction pathways other than enzymatic, and in the case of L-cysteine to anaerobicity as well. The possibility of chemical reduction of AZQ *in vivo* cannot be ruled out at this time, despite the fact that no AZQ free radicals were observed upon incubation with GSH, and that the concentrations required for reduction by NADPH and L-cysteine were relatively high. In reality, the reduction of AZQ in biological systems may involve a combination of both, enzymatic and chemical reductions.

As our data show, the cellular activation of AZQ to its free radical anion is a complicated process whose role in the activity of the drug cannot be ruled out. This is true especially since the opening of the aziridine rings of AZQ previous to cell treatment not only renders the drug inactive against L1210 cells, but the cells fail to generate a free radical [25]. One further contrast is that AZQ has a slow induced growth inhibition in L1210 cells (*ca.* 6 hr) [25] when compared to nor-nitrogen mustard and daunorubicin where growth inhibition effects can be observed within 1 hr of drug incubation [27]. It is possible that the AZQ free radical may be involved in this behaviour, either retarding the activity of the drug, or being part of a lengthy mechanism where the free radical is obligatory. We continue to investigate the relationship of drug activity to free radical activation using several analogs of AZQ.

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