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THE LACK OF CORRELATION BETWEEN TOXICITY AND FREE RADICAL FORMATION OF TWO DIAZIRIDINYL BENZOQUINONES

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Abstract—L1210 and K562 leukaemic cells have been used to study the relationship between cytotoxicity and free radical production by two aziridinyl benzoquinones, 2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone (AZQ) and 2,5-bis(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone (BZQ). BZQ showed a high level of toxicity in both cell lines, but no ESR signal was detectable, while AZQ readily produced an ESR signal but much lower cytotoxicity was observed, particularly in L1210 cells. The rate of superoxide formation was measured for each drug. The results demonstrate that cell killing and free radical production do not necessarily concur.

The pharmacological activity of quinone antitumour agents is believed to be brought about by their ability to undergo bioreductive activation to semiquinone moieties (e.g. [1–3]). The semiquinones are formed from one-electron reduction by various enzyme systems including xanthine oxidase, NADPH cytochrome P-450 reductase and NADH dehydrogenase (e.g. [3–5]). Semiquinones are believed to be responsible for cell death by undergoing intramolecular rearrangements to form more nucleophilic alkylating agents and cellular damage by the initiation of redox cycling, which leads to oxidative stress and DNA strand breaks due to the formation of superoxide radicals and other, more reactive, oxygen-derived species.

Various techniques have been used to demonstrate the production of semiquinone radicals in cell systems including electron spin resonance spectroscopy (ESR) and the formation of superoxide radicals (e.g. [5–8]. The implications from many of these studies have been that if an ESR signal can be detected for a semiquinone radical in a cellular system, then these species could be responsible for the toxicity of the quinone antitumour drug.

The diaziridinyl benzoquinones, 2,5-bis-(carboethoxyamino) - 3,6 - diaziridinyl - 1,4 - benzoquinone (AZQ) and 2,5-bis(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone (BZQ), are currently undergoing Phase II trials as potential antineoplastic agents. The structures of these drugs are shown in Fig. 1.

AZQ has been shown to produce DNA strand breaks, interstrand cross-links and DNA-protein cross-links in mammalian cell lines. The cytotoxicity of AZQ is believed to be mainly due to the formation of cross-links, which can be brought about via the production of reduced quinones [9, 10]. Well defined ESR signals have been detected from AZQ in certain cell lines grown in culture [8, 11, 12].

The main objective of this work is to compare the properties of AZQ and BZQ, and demonstrates that for two structurally similar quinones, the detection of free radicals in a cellular system is not a true reflection that semiquinone radicals are necessarily involved in the toxicity of a quinone drug.

MATERIALS AND METHODS

AZQ was a generous gift from the Division of Drug Treatment, NCI, Maryland. BZQ was synthesized according to the method of Chou *et al.* [13]. Both drugs were shown to be >98% pure by HPLC. The drugs were first solubilized in DMSO and then diluted with the appropriate aqueous buffers (final DMSO concentration <0.3%). All reagents were of the highest purity commercially available. L1210 murine leukaemia and K562 human leukaemia cells were maintained *in vitro* in RPMI 1640 medium, supplemented with L-glutamine (2 mM) and horse serum (10%) in 5% CO_2 atmosphere at 37°.

Electron spin resonance studies were conducted at room temperature (21–23°) using an incubation mixture consisting of drug (20 μM) and 10^5–10^7 cells/ml in Hanks buffer (pH 7.5) and were either air saturated or purged with nitrogen. The ESR spectra were scanned using a Varian E-9 X-band spectro-

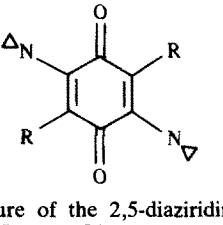

Fig. 1. The structure of the 2,5-diaziridinyl 1,4-benzoquinones, AZQ, R = $NHCOOC_2H_5$ and BZQ, R = $NHCH_2CH_2OH$.

meter with 100 kHz modulation. The data were collected on a Nicolet 1170 signal averager and further manipulations were carried out on a Hewlett-Packard HP85 computer.

The relative amount of superoxide formation was determined by monitoring the rate of reduction of acetylated cytochrome *c* at 550 nm. The extinction coefficient used was 19.6 mM^{-1} cm^{-1} which represents the difference in extinction coefficients between reduced and oxidized cytochrome *c*. The incubation mixture (3 ml, 37°) consisted of potassium phosphate buffer (pH 7.5, 50 mM), acetylated cytochrome *c* (70 μM), NADPH (1.0 mM), cellular protein (120 μg) in the absence or presence of quinone drug (50 μM). Cell preparations were obtained by washing the cells in phosphate buffer, followed by resuspension and sonication using an MSE ultrasonicator at 30 μ peak-to-peak setting for two periods of 5 sec each. The protease inhibitor, phenylmethylsulphonylfluoride, was added to give a final concentration of 0.5 mM. Cellular protein was measured using the Bio-rad assay. Superoxide dismutase (SOD) was added to the incubation mixture after 10 min to demonstrate the involvement of superoxide radicals. It was also observed that both drugs possessed the ability to react with acetylated cytochrome *c* in the presence of NADPH. This reaction was treated as a further blank and deducted from the measured rate in each case. It also precluded the direct measurement of NADPH consumption. All spectrophotometric determinations were carried out using a Hewlett-Packard 8451A diode array spectrophotometer with a temperature controlled cuvette.

Cell toxicity studies were carried out by treating the L1210 and K562 cells (10^5/ml) with AZQ (0–100 μM) or BZQ (0–5 μM) in the culture medium, as stated above. The cells were treated with the drug for one hour at 37° and then washed and resuspended at 10^3/ml in fresh medium. Cell counting was performed and percentage growth inhibition was calculated after 96 hr further incubation at 37°.

RESULTS

Direct evidence for the metabolism of AZQ into a semiquinone radical could be obtained by ESR spectroscopy in the intact L1210 and K562 cell lines (Fig. 2). The spectra are similar to those reported previously [12] in that the semiquinone signals observed in the K562 cells are stronger and much better resolved than those in the L1210 cells. It was possible to reduce the K562 cell concentration by two orders of magnitude from 10^7 to 10^5 cells/ml and still retain signal intensity comparable with the higher concentration L1210 cell samples. Additionally, with AZQ the signals from each cell line were found to be more intense in the presence of oxygen than in its absence. Despite numerous attempts to detect signals from BZQ, a signal could not be observed in the presence or absence of oxygen even when the cell concentration was increased to ($>10^7$) cells/ml and the BZQ concentration was increased to >150 μM.

Both BZQ and AZQ showed a slight increase on the basal rate of superoxide radical formation in each of the cell extracts when supplemented by NADPH

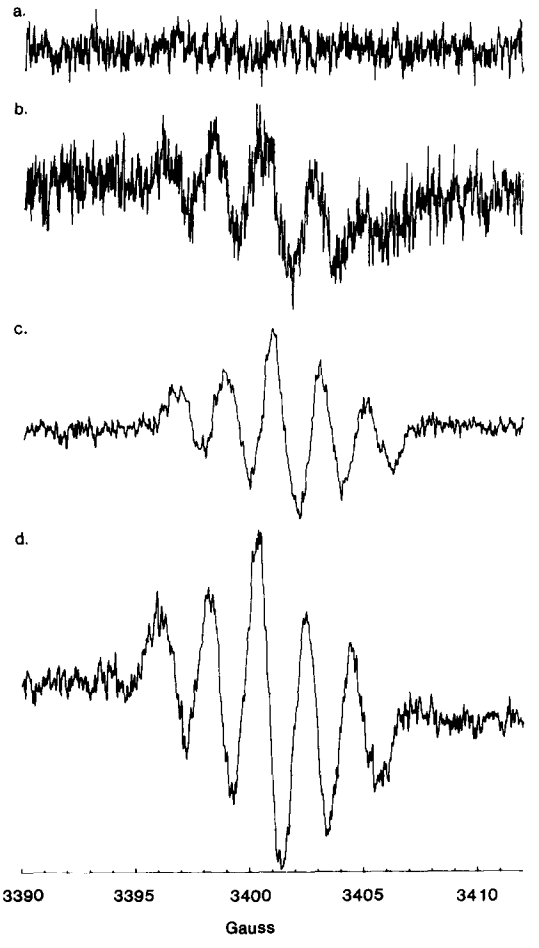

Fig. 2. X-band ESR spectra showing (a) BZQ in K562 cells, air saturated; (b) AZQ in L1210 cells, air saturated; (c) AZQ in K562 cells purged with nitrogen; (d) AZQ in K562 cells, air saturated. Spectra were taken under standard incubation conditions (see Materials and Methods) using instrument parameters: microwave power 10 mW: microwave frequency 9.53 GHz; scan range 20G; modulation amplitude 0.63G and gain 3.2×10^4. Each 4 min spectrum was averaged four times on a time constant of 0.3 sec. The spectra of AZQ were similar to those obtained from purified enzyme studies [16].

(Table 1). The rates observed in K562 cell extracts were significantly higher than those in the L1210 extracts and in both cases AZQ gave a higher rate than BZQ.

The concentration of BZQ required to inhibit cell growth by 50% (ID_{50}) was found to be very similar

Table 1. The effect of AZQ and BZQ on the rate of superoxide formation in L1210 and K562 cell extracts

	Rate of superoxide formation* (nmol min^{-1} mg^{-1} of protein)	
	L1210	K562
AZQ	8.0 ± 1.0	10.0 ± 1.0
BZQ	4.7 ± 1.0	6.5 ± 1.0

* The basal rate of superoxide formation has been subtracted from the data shown.

Table 2. Growth inhibition in L1210 and K562 cell lines (ID_{50} in μM)

	L1210	K562
AZQ	93.6	5.6
BZQ	2.2	2.3

for each of the cell lines (Table 2). However, the values for AZQ were strikingly different, i.e. ID_{50} = 5.6 μM and 93.6 μM for K562 and L1210s respectively.

DISCUSSION

The increased intensity of the ESR signals from the AZQ semiquinone radicals in the K562 cells compared to that from the L1210 cells is indicative of the various reducing enzyme systems in the K562 cells being more efficient than those in the L1210s. The large differences in the cytotoxicity of this drug in the two cell lines could partially be explained by this variation in the ability to metabolize the drug to semiquinone radicals.

The ESR signals from AZQ in both cell lines were more intense in the presence of air. This is in contrast to that observed for other antitumour drugs including mitozantrone, daunomycin and daunomycin analogues (e.g. [14, 15]) where oxygen prevents the production of ESR signals from the semiquinone. However, these results are consistent with our previous study which showed that the one-electron reductive potential of the $Q/Q^{\cdot-}$ couple for AZQ is -70 mV [16]. If the potential for the oxygen couple, $O_2/O_2^{\cdot-}$ is assumed to be -155 mV [17] then it can be shown that the equilibrium constant for the reaction:

$$O_2^{\cdot-} + Q \rightleftharpoons O_2 + Q^{\cdot-} \quad (1)$$

is 27.6. Hence in the cell systems the semiquinone radicals produced by the reducing enzyme systems will be stable with respect to oxygen and indeed could also be formed from superoxide radicals produced by other enzyme systems. However, this also implied that the rate of superoxide production in the acetylated cytochrome *c* assay represents the minimum value for AZQ. Nonetheless, the measured rate of superoxide formation is again higher in the K562 cell extracts than in the L1210 extracts.

The one-electron reduction potential of the $Q/Q^{\cdot-}$ couple for BZQ has been measured as -376 mV [16]. The equilibrium constant for reaction (1) can therefore be calculated to be 1.8×10^{-4}. Thus in the presence of oxygen the BZQ radicals will form superoxide. However, this large negative reduction potential would not favour the formation of semiquinone radicals and could explain why these radicals were not detected in the cell systems even in the absence of oxygen.

It is evident that the cytoxicity of the two drugs cannot simply be related to their ability to form semiquinone radicals and other mechanisms of cell killing must occur. In a previous study on AZQ, it was shown that there was a good correlation between the frequency of DNA cross-links and cytotoxicity [10]. Although the mechanism of cross-linking could involve the activation of AZQ to a free radical species [2, 9], the present results show that in the case of BZQ cross-linking must occur via another mechanism. BZQ is much more unstable than AZQ with respect to aziridine ring opening at acid or alkali pHs [18]. It is possible that BZQ could function as an alkylating agent solely by acid- or alkali-assisted ring opening within the cell. This possibility is currently being investigated.

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