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CROSS-RESISTANCE STUDIES ON TWO K562 SUBLINES RESISTANT TO DIAZIRIDINYLBENZOQUINONES

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Abstract—Two resistant K562 sublines have been developed by treatment with AZQ (2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone) and BZQ (2,5-bis(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone). The ID_{50} values for AZQ on K562, the AZQ-resistant sublines (AZQR) and the BZQ-resistant sublines (BZQR) were 0.063, 1.47 and 0.244 μM, respectively. The relative ID_{50} values for BZQ on the same cell lines were 0.2, 0.67 and 0.83 μM, respectively. Although it is generally believed that these two quinones function by different mechanisms, the two sublines have similar decreased levels of cytochrome P-450 reductase and DT-diaphorase and increased levels of glutathione and superoxide dismutase, compared to the parent cell line. The sublines are also cross-resistant to adriamycin, mitozolamide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and mitomycin C. This work indicates the potential multifactorial mechanisms by which drug resistance can be induced in cell lines in the absence of conventional 'P'-glycoprotein multidrug resistance.

Key words: quinones; diaziridinylquinones; AZQ; BZQ; cytotoxicity; resistance; cross-resistance

Several aziridinylbenzoquinones have undergone clinical trials as potential antitumour drugs [1–3]. Recent work from our laboratory and others have shown that some aziridinylquinones can be activated towards alkylation as a result of bioreduction. This process can occur via NADPH:cytochrome P-450 reductase (EC 1.6.2.3), a one-electron reducing enzyme which produces a semiquinone radical or via the NAD(P)H: oxidoreductase (DT-diaphorase, EC 1.6.99.2) which is a two-electron reducing enzyme and results in the formation of an activated hydroquinone [4–6].

Further studies have shown that the cytotoxicity of many compounds which are capable of undergoing bioreduction can be related to the intracellular concentrations of reducing enzymes [7–9]. This work has highlighted the need to appreciate the different bioreductive pathways when designing antitumour drugs and in predicting their possible clinical response.

AZQ‖ and BZQ are two diaziridinylbenzoquinones which have similar structures (Fig. 1). However,

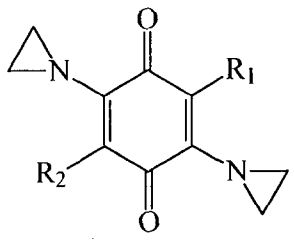

Fig. 1. Diaziridinylbenzoquinones. $R_1 = R_2 = -NHCH_2CH_2OH$ (BZQ). $R_1 = R_2 = -NHCOOC_2H_5$ (AZQ).

previous work from our laboratory has proposed that they are activated by different mechanisms [6]. AZQ does not readily alkylate DNA at physiologically relevant pH but has a relatively high reduction potential and is easily reduced by both cytochrome P-450 reductase and DT-diaphorase to form DNA alkylating species. In contrast, BZQ has a much more negative reduction potential and does not easily undergo reduction by either enzyme. However, BZQ is much less stable than AZQ and can readily alkylate in the absence of reduction [6, 10, 11].

The human erythroleukaemic cell line, K562, has been shown to be particularly sensitive towards these two compounds [6] and a previous study has demonstrated that AZQ is reduced within these cells to reactive forms [12]. In the present study, we report on the development of two K562 sublines which are resistant towards AZQ and BZQ. These two sublines were found to be cross-resistant, not

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‖ Abbreviations: AZQ, 2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone; BZQ, 2,5-bis(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone; DMSO, dimethyl sulfoxide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MITO, mitozolamide; ADR, adriamycin; MITO C, mitomycin C; SOD, superoxide dismutase; ATase, O^6-alkylguanine-DNA-alkyltransferase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ID_{50}, concentration required to inhibit cell growth by 50%.

Table 1a. ID_{50} values (μM) K562, AZQR and BZQR cell lines after continuous challenge with selected agents

	Drug					
Cell line	AZQ	BZQ	MITO	MNNG	ADR	MIT-C
K562	0.063	0.20	1.98	0.08	29.0	0.11
AZQR	1.470	0.67	5.8	0.13	78.0	0.92
BZQR	0.244	0.83	6.7	0.41	67.5	0.46

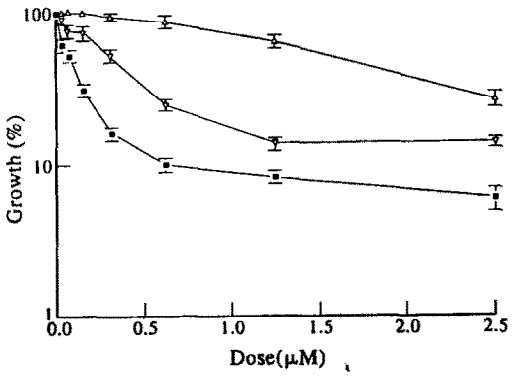

Fig. 2. Growth inhibition curves curves for the different cell lines after treatment with AZQ. ■, K562; △, AZQR; ▽, BZQR.

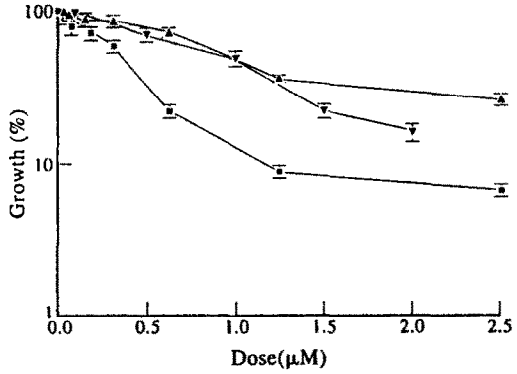

Fig. 3. Growth inhibition curves for the different cell lines after treatment with BZQ. ■, K562; ▲, AZQR; ▼, BZQR.

only towards AZQ and BZQ but also towards other antitumour compounds which do not undergo redox processes.

This work indicates the potential multifactorial mechanisms by which drug resistance can be induced in cell lines in the absence of conventional 'P'-glycoprotein multidrug resistance.

MATERIALS AND METHODS

Chemicals. AZQ was synthesized according to our published methods [13]. BZQ was synthesized according to our methods and the method of Chou *et al.* [6, 14].

Mitomycin C was obtained from Kyowa (Tokyo, Japan), Adriamycin was from Farmitalia Carlo, Erba (St Albans, U.K.). MNNG was obtained from Sigma (Poole, U.K.) and mitozolamide was a kind gift from Dr G.P. Margison (Dept. Carcinogenesis, Paterson Laboratories). All other reagents were of the highest purity commercially available. The protein concentrations were determined using the Biorad method (Biorad, Hemel Hempstead, U.K.).

Cell Culture. K562 cells were maintained in log phase growth in suspension in complete RPMI 1640 medium supplemented with 10% horse serum (GIBCO BRL) at 37°, 5% CO_2. Cell lines were sub-cultured weekly at 5×10^3, 5×10^2 and 50/mL.

Resistant cells were developed by continuous challenge with incremental concentrations of either AZQ (AZQR line) or BZQ (BZQR line), freshly prepared as concentrated solutions in DMSO. Cells were passaged as normal in the presence of the drug and when good cell growth was observed, the concentration was increased. Further increments in concentration was discontinued when the cells became tolerant to a 1 μM concentration of drug. The drug-resistant lines were then maintained routinely in medium containing this dose of drug.

Growth inhibition studies. Growth inhibition studies were carried out using the MTT method [15]. Briefly, cells were plated at 400 cells per well in 96-well plates (Bibby Sterilin), in a total volume of 200 μL of medium alone or medium containing drug. The plates were then incubated at 37°, 5% CO_2 for 5 days, prior to the addition of 50 μL of MTT (3 mg/mL). The plates were incubated for a further 3 hr and the medium was carefully aspirated. Formazan dye was then solubilized in 200 μL of DMSO and the plates read at 540 nm and 640 nm using a Multiscan plate reader (FLOW Ltd). Data were analysed and the percentage growth inhibition of the treated wells estimated. From these values, growth inhibition curves were constructed and ID_{50} values calculated. A minimum of two MTT tests were performed for each drug with triplicate measurements for each concentration.

DT-diaphorase. Cell lines in log phase growth were harvested by centrifugation, washed once with PBS, and then resuspended in a 1:1 mixture of Tris-

HCl buffer (25 mM, pH 7.4) and sucrose (250 mM). The cells were then sonicated on ice for 3 × 10 sec using a probe sonicator. DTD activity was estimated by the method of Ernster [16] as modified by Benson *et al.* [17]. Sonicates were assayed in 25 mM Tris-HCl, pH 7.4, containing 700 μg/mL bovine serum albumin, 0.2 mM NADH and 40 μM. Reduction of DCPIP was recorded spectrophotometrically at 600 nM. Because of the presence of other reducing enzymes, the assays were conducted both in the presence and absence of dicumarol (10 μM). The dicumarol sensitive activities were used as a measure of diaphorase activity [17].

P-450 reductase. The cytochrome P-450 reductase levels were measured from the above cell extracts using the reduction of cytochrome c according to the method of Vemillion and Coon [18].

Alkyltransferase. The cell extracts were prepared and assayed for ATase activity as previously described [19].

Glutathione. The glutathione levels were assayed using the GSH-400 method which relies on thioether and thione formation (Bioxytech SA, Bonneuil sur Marne, France).

MDR. DNA was extracted from each of the cell lines by the method of Miller [20]. DNA (10 μg) was digested overnight at 37° with a five-fold excess of restriction enzyme (EcoR1). A sample (3–5 μg) was then loaded onto 20 × 14 cm 0.7% agrose gels and electrophoresed for 22 hr at 50 V, using TBE (0.9 M, Tris; 25 mM, EDTA; 0.9 M, boric acid; pH 8.2) buffer. Molecular weight markers (Lambda DNA cut with Hind III, BRL) were run in outside lanes. Gels were then Southern blotted onto nylon membranes (Highbond nfp, Amersham), and UV fixed. Hybridization using a Chinese hamster derived MDR probe was carried out using the method of Bentzen [21]. This probe has been previously shown to cross-hybridize with homologous sequences within the human mdr1 gene [22, 23] and gives a positive response with a cell line which is known to be mdr amplified [24]. The membranes were wrapped in saran wrap and autoradiographed (Amersham Hyperfilm MP), at −70° using two intensifying screens for 24–72 hr.

In addition to the specific assays, total cellular proteins were observed using SDS polyacrylamide gels [25].

SOD. The SOD levels in the cells were determined from the inhibition of the reduction of cytochrome c by superoxide radicals, generated by xanthine oxidase/hypoxanthine [26]. One unit is defined as the amount of enzyme necessary to inhibit the reduction of cytochrome c by 50%.

RESULTS

The growth inhibition curves for AZQ on the K562, AZQR and BZQR cell lines are shown in Fig. 2. The inhibition curves after treatment with BZQ are shown in Fig. 3. The ID_{50} values are summarized in Table 1.

Hybridization experiments showed that there was no detectable amplification of mdr in the cells (data not shown). Similarly, the total protein gel electrophoresis did not show any measurable differences in protein bands between the three cell lines.

The levels of cytochrome P-450 reductase, DT-diaphorase, reduced glutathione, ATase and SOD are summarized in Table 2.

Several preliminary experiments showed that as the sublines were developing, there was an increase in cross-resistance in both sublines towards the other diaziridinylbenzoquinone. In order to further investigate cross resistance, several different anti-tumour compounds were similarly tested. The results are included in Table 1.

DISCUSSION

The curves in Figs 2 and 3 clearly show that it is possible to induce reasonable levels of resistance in K562 sublines by constant challenge of AZQ and BZQ. Although differential sensitivities in similar cell types have been observed due to differences in enzyme levels [7–9], we believe this is the first report of *in vitro* resistant sublines being developed specifically for these compounds. The southern blotting and the total protein electrophoresis show that there is no amplification of the mdr gene in the resistant cells and hence this mechanism of resistance is not present. This is to be expected for, as far as we are aware, there is no evidence for this type of resistance being induced by simple, synthetic alkylating agents.

Unfortunately, radiolabelled BZQ or AZQ were not available and hence reliable drug uptake studies could not be carried out. However, it is unlikely that differences in drug uptake could be responsible for the resistance as this would not explain why, in the absence of the MDR phenotype, the cell lines are cross-resistant towards the other drugs which have diverse structures, p*K* values and lipophilicities. Also since toxicities were carried under continuous challenge conditions, any differences in uptake should be essentially negated.

It is significant that the relative cross resistance in the AZQR cells is much higher with mitomycin than the other drugs (8.3-fold, see Table 1b). This is because both mitomycin C and AZQ are believed to have similar mechanism of action [27 and references therein].

The induced resistance in AZQ and mitomycin C in the AZQR cells can be partially explained by the differences in the DT-diaphorase and the P450 reductase levels (Tables 1b and 2). As both AZQ and mitomycin C can be activated to damaging forms by these enzymes [7, 9, 28], their decreased levels would prevent the activation processes.

The small, but significant increase in the GSH levels in these cells are also consistent with the general mechanisms wherein GSH and the GSH dependent enzymes (part of the phase II drug transforming proteins) can detoxify xenobiotics by direct reaction of the compound with GSH or by preventing free radical damage [27, 29, 30]. AZQ can react directly with GSH and form an adduct, presumably by reaction with the aziridine [31]. Similarly, it has recently been demonstrated that mitomycin C forms a glutathione–drug–DNA ternary complex [32] and glutathione is involved in pleiotropic

Table 1b. Relative increases in resistance of the different drugs compared to the K562 cell line

	Drug					
Cell line	AZQ	BZQ	MITO	MNNG	ADR	MITO-C
AZQR	23.3	3.3	2.9	1.6	2.7	8.3
BZQR	3.8	4.1	3.3	5.1	2.3	4.1

Table 2. Levels of enzymes and GSH in the cell lines

	Assay				
Cell line	P-450*	DT-Diaphorase†	ATase‡	GSH§	SOD¶
K562	4.9 ± 0.6	2.8 ± 0.3	6.6 ± 0.7	8.0 ± 0.6	18 ± 2
AZQR	1.7 ± 0.2	<0.3	5.4 ± 0.6	14.7 ± 1.2	44 ± 6
BZQR	2.3 ± 0.3	<0.3	5.4 ± 0.6	10.1 ± 0.9	37 ± 4

* nmole cytochrome c reduced/min/mg protein (average of three determinations).
† μmolar reduced DCPIP/min/mg protein (average of six determinations).
‡ fM/mg protein (average of four determinations).
§ mM/mg protein (average of two determinations).
¶ U/mg (average of four determinations).

mitomycin C resistance [33]. Bioreductive drugs can also form damaging superoxide radicals by the direct reaction of the one-electron reduced forms with oxygen [27 and references therein]. The increased levels of superoxide dismutase would therefore also inhibit DNA damage and lipid peroxidation.

The AZQR cells therefore appear to utilize several mechanisms to overcome cell damage and these mechanisms could also confer a cross-resistance towards other apparently unrelated compounds. The relatively small level of resistance to mitozolamide, MNNG, adriamycin and possibly BZQ (see below) could at least in part, be explained by the increase in GSH as all of these drugs can be detoxified directly by GSH or by the GSH detoxification pathways [34–36 and references therein]. Similarly, although Adriamycin is not a substrate for DT-diaphorase [28] it is readily reduced to potentially toxic free radicals by cytochrome P-450 reductase [37, 38] and hence the decrease in the level of this enzyme and the increase in SOD levels could explain this small cross resistance.

Therefore, if the results from the AZQR cells are taken in isolation then most of the mechanisms of resistance can be explained. However, the results from the BZQR cells would imply that there could be other underlying mechanisms.

Although BZQ is less toxic towards K562 cells than AZQ, it was very difficult to increase the relative levels of resistance in the BZQR cells to that of AZQR. (Table 1). This was despite the fact that both sublines were developed at the same time over a period of about 1 year and were treated with the respective drugs at the same time. It was apparent that relatively small increases in BZQ concentrations resulted in significant cell killing. A possible explanation for this effect may be due to the different mechanisms by which BZQ exerts its toxic effects.

It has been shown that BZQ is a much poorer substrate for the reducing enzymes than AZQ [6,11] and it has been proposed that BZQ merely acts as a simple proton-activated alkylating agent [11]. However, the BZQR cells also have reduced levels of DT-diaphorase and cytochrome P-450 reductase and increased levels of SOD, similar to AZQR (Table 2). This implies that redox processes may also be at least partially responsible for the cytotoxicity of BZQ. The changes in the enzyme levels may also explain the cross-resistance to the other compounds, but it is significant that although the DT diaphorase and P-450 reductase levels are reduced as with AZQR, the cross-resistance is not as large with AZQ or mitomycin C.

It can be postulated that there are at least two mechanisms involved in the cytotoxicity of BZQ. One mechanism involves bioreduction, although BZQ is a relatively poor substrate, while the other mechanism may involve simple proton activation. However, both mechanisms can result in DNA damage. In the developing BZQR subline, the bioreductive mechanism is readily suppressed by the changes in DT-diaphorase, P-450 reductase, SOD and GSH, similar to that found with AZQ. However, the other mechanism(s) is not so easily overcome.

This could partially explain the difficulty in inducing resistance in these sublines, for if one of the mechanisms of toxicity begins simply with the protonation of the aziridines, then the cells would have difficulty in overcoming this mechanism. It is

believed that the cytoxicity of AZQ, BZQ and mitomycin C is due to covalent binding with DNA bases [4, 39, 40]. In the present study we have shown that the levels of ATase are not dramatically altered in the resistant sublines. However, several other DNA repair systems could contribute towards the increased resistance in the BZQR and possibly, the AZQR sublines. We are currently investigating this possibility.

In conclusion, the higher levels of resistance in the AZQR line to some quinones shows that resistance to bioreductive activation is readily induced. However, this resistance is brought about not by simply reducing the expressed level of one enzyme, but by altering a variety of factors and this is also indicated for the BZQR line. It is this multifactorial ability, which comes about as a consequence of selective pressure to the growing cells, that confers cross resistance towards apparently unrelated compounds.

These results are very relevant not only to the design and use of bioreductive compounds but also in the treatment of cancers using combination chemotherapy. One of the implications from this study is that the compounds used in combination should be carefully chosen from the point of view of potential cross-reactivities (e.g. thiol reactions, reducibility, radical generation, sites of DNA reactions, etc.). The results would also suggest that the selection of drugs with differing cellular targets is likely to be more successful.

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