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Redox reactions and cytotoxicity mechanisms of anticancer aziridinyl-substituted-1,4-benzoquinones: a minireview

Jonas Šarlauskas[1],

Aušra Nemeikaitė-Čėnienė[2],

Henrikas Nivinskas[1],

Valė Miliukienė[1],

Rasa Jarašienė[1],

Milda Pečiukaitytė[1],

Daiva Baltriukienė[1],

Virginija Bukelskienė[1],

Kastis Krikštopaitis[1],

Narimantas Čėnas[1*]

[1] *Institute of Biochemistry of Vilnius University, Mokslininkų St. 12, LT-08662 Vilnius, Lithuania*

[2] *State Research Institute, Centre for Innovative Medicine, Molėtų Rd. 29, LT-08409 Vilnius, Lithuania*

In this paper, we summarize the data on the cytotoxicity mechanisms of aziridinyl-substituted-1,4-benzoquinones, which are potentially important antitumor agents known since 1980s. High antitumor activity of aziridinyl-1,4-benzoquinones arises from the combined effects of their two-electron enzymatic reduction into aziridinyl-substituted hydroquinones which alkylate DNA more readily than the parent quinones, the oxidative stress-type cytotoxicity due to the redox cycling of the corresponding quinone free radicals, and the direct alkylation of DNA by the low-potential aziridinyl-benzoquinones. However, it may also cause the undesirable side-effects, i. e. their cytotoxicity in primary mammalian cells. In this context, including our recent findings, we aim to review: i) the redox properties of aziridinyl-1,4-benzoquinones, ii) their reactivity and structure-activity relationships in single- and two-electron reduction by flavoenzymes and related redox systems, iii) the structure-activity relationships in their cytotoxicity, and iv) the mechanisms of the cell death and the expression of signaling proteins under their action.

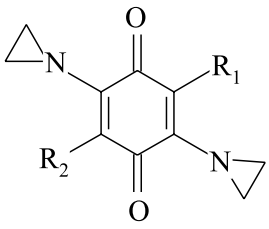
Key words: aziridinylbenzoquinones, cytotoxicity, NAD(P)H:quinone oxidoreductase, oxidative stress

INTRODUCTION

Aziridinyl-substituted 1,4-benzoquinones (Fig. 1) comprise an important group of potential antitumor agents, whose clinical application started in 1980's, but with a variable success later [1, 2]. Since that time, their numerous preclinical, cell culture cytotoxicity, enzymatic, and synthesis studies were carried out. It is accepted that their cytotoxic (antitumor) activity stems from the following mechanisms: a) their two-electron enzymatic reduction into aziridinyl-hydroquinones which alkylate DNA more readily than the parent quinones (Fig. 1); b) their oxidative stress-type cytotoxicity caused by the redox cycling of semiquinones formed during the single-electron enzymatic reduction of quinones (Fig. 2), and c) the direct alkylation of DNA by certain aziridinyl-benzoquinones with the strong electron-donating substituents, e. g. BZQ (2,5-bis(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone) or its analogues [1–4].

In general, the DNA-alkylating potency of aziridinyl-substitued quinones and their reduction products increases with an increase in the pK_a of their aziridinyl groups, i. e. the ease of their protonation which in turn accelerates the aziridine ring opening and DNA-alkylation [2]. Thus, due to the electron-donating character of hydroquinone -OH groups, the two-electron reduced forms of aziridinyl-substituted quinones should possess higher DNA-alkylating ability than the parent quinones, and their semiquinones

* Corresponding author. E-mail: narimantas.cenas@bchi.vu.lt



$R_1 = R_2 = -H$ (DZQ)
$R_1 = H$, $R_2 = -N(CH_2)_2$ (trenimon)
$R_1 = R_2 = -NHCOOC_2H_5$ (AZQ)
$R_1 = R_2 = -CH_3$ (MeDZQ)
$R_1 = -CH_3$, $R_2 = -CH_2OH$ (RH1)
$R_1 = -CH_3$, $R_2 = CH(OCH_3)CH_2OCONH_2$ (carboquone)
$R_1 = R_2 = -NHC_2H_4OH$ (BZQ)

Fig. 1. The formulae of the most commonly used diaziridinyl-1-4-benzoquinone antitumour agents

should occupy the intermediate position [2]. This is a basic explanation for an enhanced antitumor activity of aziridinyl-substituted quinones upon their bioreduction.

One may note that aziridinyl-benzoquinones also exert some side effects in vivo, e. g. lymphoid, bone marrow, and hematological toxicity [1, 5, 6]. However, the results of the preclinical and Phase-I clinical trials of a new representative of this group, RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone, Fig. 1), were quite encouraging [7–9], and may open the new perspectives in the application of this group of compounds. For this reason, here we summarize the recent achievements in the studies of aziridinyl-1,4-benzoquinones, including the characterization of their redox parameters, the activity in flavoenzyme-catalyzed reactions, and the quantitative relationships and mechanisms of their induced cell death, including the expression of cell death signaling proteins.

REDOX PROPERTIES OF AZIRIDINYL-1,4-BENZOQUINONES

Quinones (Q) are reduced in a two-electron way into hydroquinones (QH_2) with a transient formation of the radicals (semiquinones, $Q\cdot^-$) [10–16]:

$$Q \xrightarrow{e^-} Q^{\cdot -} \xrightarrow{e^- + 2H^+} OH_2\cdot \quad (1)$$

Because pK_a of semiquinones ($pK_a(QH\cdot)$) are low, 2.0–5.1, at pH 7.0 they exist in an anionic form. The energetics of the first electron transfer is described by the standard redox potential of the quinone/semiquinone couple, E^1_7 (Table 1). Because hydroquinones dissociate at much higher pH (pK_a (QH_2) = 7.0–13.0), two protons accompany the transfer of the second electron (Eq. (1)), whose energetics is characterized by E^2_7. The standard redox potential of Q/QH_2 couple, E^0_7, is equal to $(E^1_7 + E^2_7)/2$. Because at pH 7.0 E^1_7 is much more negative than E^2_7, the stability constant of $Q\cdot^-$ ($K_d = [Q\cdot^-]^2/([Q][QH_2])$), expressed according to the Nernst equation,

$$\log K_d = (E^1_7 - E^2_7)/0.059\ V, \quad (2)$$

is very low, and quinone radicals undergo rapid dismutation:

$$2Q\cdot^- + 2H^+ \rightleftarrows Q + QH_2\cdot \quad (3)$$

Typically, the rate constants of semiquinone dismutation ($2k_d$) vary between $5.0 \times 10^7 - 8.0 \times 10^8\ M^{-1}\ s^{-1}$ at pH 7.0 [12]. Therefore, only a single pair of peaks in cyclic voltammetry of

Table 1. **Redox properties of aziridinyl-1,4-benzoquinones and their aziridinyl-unsubstituted analogues [10–18]**

No.	Compound	E^1_7 (V)	$pK_a(QH\cdot)$	E^0_7 (V)	$pK_a(QH_2)$	Cyclic voltammetry data (v = 50 mV/s, vs Ag/AgCl) [20]	
						Ep(red)	Ep(ox)
1.	2-CH_3-1,4-BQ[a]	0.01	4.45	0.21	10.0		
2.	2,5-(Az)[b]$_2$-1,4-BQ (DZQ)	–0.054	5.0	0.12	11.0	–0.155	0.052
3.	2,5-(Az)$_2$-3,6-(NHCOO-C_2H_5)$_2$-1,4-BQ (AZQ)	–0.07	6.30	0.07	11.0	–0.205	0.084
4.	2,5-(CH_3)$_2$-1,4-BQ	–0.07	4.60	0.16	10.4		
5.	2,5-(Az)$_2$-3,6-(NHCOO-*i*-C_3H_7)$_2$-1,4-BQ	–0.079					
6.	2,5-(Az)$_2$-3,6-(NHCOO-*sec*-C_4H_9)$_2$-1,4-BQ	–0.125					
7.	2,5-(Az)$_2$-3,6-(NHCOO-*i*-C_4H_9)$_2$-1,4-BQ	–0.167					
8.	2,5-(Az)$_2$-3,6-(CH_3)$_2$-1,4-BQ (MeDZQ)	–0.23		0.01	11.4	–0.300	0.05
9.	2,5-(Az)$_2$-3-CH_3-6-CH_2OH-1,4-BQ (RH1)	–0.23				–0.290	0.072
10.	(CH_3)$_4$-1,4-BQ (DQ)	–0.26	5.10	0.04	11.2		
11.	2,5-(Az)$_2$-3,6-($NHCH_2CH_2OH$)$_2$-1,4-BQ (BZQ)	–0.38		–0.165	12.4	–0.527	–0.025

Abbreviations: [a] BQ, 1,4-benzoquinone; [b] Az, aziridine.

quinones is observed in an aqueous medium at pH 7.0, which correspond to their $2e^-$, $2H^+$ reduction, and to the electrochemical reoxidation of hydroquinone, respectively. It means that the conventional electrochemical methods cannot provide information on the single-electron transfer potentials of quinones, except in a strongly alkaline medium (pH > pK_a (QH_2)), where $Q\cdot^-$ is relatively stabilized [10, 11]. For this reason, the E^1_7 values of quinones are mainly obtained by the pulse-radiolysis technique, analyzing the equilibrium between the $Q/Q\cdot^-$ couple and the redox indicator [14].

Both E^1_7 and E^0_7 values of quinones decrease upon an increase in the number of their aromatic rings (benzoquinones > naphthoquinones > anthraquinones), which is caused by an increased efficiency of charge delocalization. The electron accepting substituents such as -Cl, $-SO_3^-$ etc. increase the redox potentials of quinones, whereas the electron donating ones such as $-CH_3$, $-OCH_3$, $-NH_2$, -NHR, and -OH decrease it. However, if the -OH group can form the intramolecular H-bond with the carbonyl group of quinone, it increases its redox potential and the stability of semiquinone [13]. It is important that the values of $pK_a(QH_2)$ and $pK_a(QH\cdot)$ for alkyl-substituted 1,4-benzoquinones decrease with an increase in their E^0_7 or E^1_7, respectively [16], however, this approach cannot be extended to a larger range of quinoidal compounds.

In this context, the redox properties of aziridinyl-1,4-benzoquinones appear as follows: a) aziridinyl group ($-N(CH_2)_2$) decreases the quinone reduction potentials, E^1_7 and E^0_7, by 50–80 mV, i. e. like the alkyl substituent (Table 1). It contrasts with the effect of $-NHC_2H_5$ group, which decreases the E^0_7 of benzoquinones by 200 mV [17, 18]. This is attributed to a weak interaction of an electron pair of aziridine N-atom with the π-system of quinone ring, because the aziridine group is not coplanar with the quinone ring due to steric hindrances. Thus, the E^1_7 and E^0_7 values of aziridinyl-substituted 1,4-benzoquinones are in line with those of alkyl-substituted ones (Table 1); b) 3,6- (NHCOO-alkyl) substituents of 2,5-diaziridinyl-1,4-benzoquinones, although possessing the electron-accepting character, modestly influence their E^1_7 (Table 1). This is also probably due to the steric hindrances; c) pK_a (QH·) values of aziridinyl-1,4-benzoquinones are scarce, besides, as in the case of AZQ (Table 1), having been determined indirectly.

One of the most important problems in the cytotoxicity of aziridinyl-1,4-benzoquinones is the pK_a of their aziridinyl group(s), which is related to their DNA-alkylating ability. The pK_a of aziridine groups in DZQ is between 3.8 and 4.0, whereas BZQ has the pK_a around 4.5 [17, 18]. These pK_a are significantly lower than that of a simple aziridine moiety, ~8.0, mainly because of the electron accepting character of carbonyl groups of the quinone ring. On the other hand, the reduction of quinone moiety into semiquinone may increase the pK_a of aziridine up to 6.3–6.6 (AZQ) and 5.0 (DZQ) [17, 18], or, as in the reduction into hydroquinone, to 7.5 (DZQ), 8.3 (AZQ), 8.7 (MeDZQ), and to 9.5 (BZQ) [17, 18]. It demonstrates the preferable thermodynamics of the DNA-alkylation by the reduced aziridinyl-benzoquinones as compared to the oxidized ones. However, because these data were obtained only by the electrochemical methods, they may be taken as approximate due to the problems with the electrochemical reversibility of quinones. For example, the couple AZQ/$AZQH_2$ acts as a reversible electrochemical system using a hanging Hg drop electrode [17, 18], but shows a significant irreversibility using a glassy carbon electrode [19]. The same is characteristic of other aziridinyl-substituted 1,4-benzoquinones using a glassy carbon electrode ([20], Table 1). Besides, the analysis of voltammograms is complicated by the electrochemical activity of Az substituents in the quinone moiety, which may be oxidized into the corresponding quinone imine groups.

SINGLE-AND TWO-ELECTRON REDUCTION OF AZIRIDINYL-1,4-BENZOQUINONES BY MAMMALIAN FLAVOENZYMES

In the cell, aziridinyl-1,4-benzoquinones are enzymatically reduced in a single-electron way into their free radicals which cause the oxidative stress-type cytotoxicity, or in a two-electron way causing the formation of aziridinyl-hydroquinones that alkylate DNA ([1–4], and references therein) (Fig. 2). First, let us analyze their single-electron reduction.

Typically, it is performed by NAD(P)H-oxidizing low-potential mammalian flavoenzymes electrontransferases, such as NADPH:cytochrome P-450 reductase (P-450R, EC 1.6.2.4), or NO-synthase (NOS, EC 1.14.13.39) [4, 21]. These enzymes transfer the redox equivalents in the sequence NADPH → FAD → FMN → heme (cytochrome P-450) (P-450R), or heme/tetrahydrobiopterin (NOS)). They form the

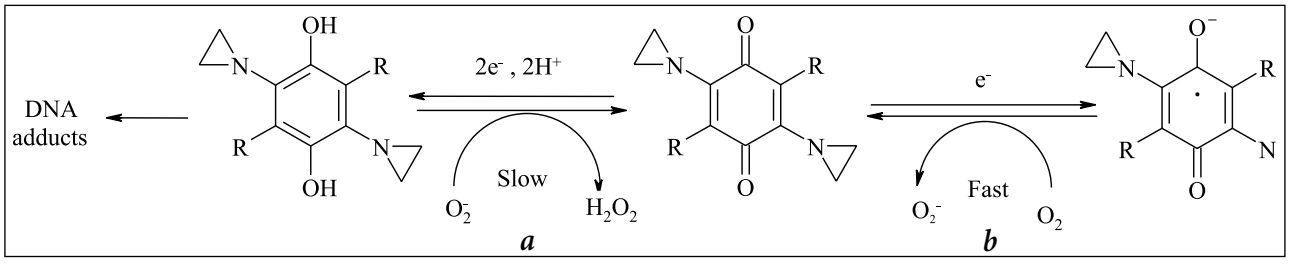


Fig. 2. Bioreduction of aziridinyl-1,4-hydroquinones: *a*) their two electron reduction into hydroquinones followed by their DNA alkylation and slow autooxidation, and *b*) single-electron reduction into their anion-radicals, followed by their fast reoxidation by oxygen (oxidative stress)

stable neutral (blue) flavin radicals ($FMNH^{\cdot}$ and $FADH^{\cdot}$) as the reaction intermediates, and, in both cases, reduce quinones via two-electron reduced FMN [22]. In both cases, quinone reduction rate constants ($\log k_{cat}/K_m$) do not depend on their structural features, e. g. benzo-, naphtho- or anthraquinone structure, but exhibit a linear or parabolic dependence on their E^1_7 [4, 21]. This agrees with an outer-sphere single-electron mechanism [23], where the rate constant of single-electron transfer at pH 7.0 between reagents (k_{12}) depends on the electron self-exchange constants of reagents (k_{11} and k_{22}), and the equilibrium constant of the reaction (K) ($\log K = \Delta E^1_7$ (V)/0.059), where ΔE^1_7 is the difference in the standard single-electron transfer potential of reactants:

$$k_{12} = (k_{11} \times k_{22} \times K \times f)^{1/2}, \quad (4)$$

and

$$\log f = (\log K)^2 / 4\log (k_{11} \times k_{22} / Z^2), \quad (5)$$

where Z is a frequency factor (10^{11} M^{-1} s^{-1}) [24]. Here quinones behave as a series of homologous compounds with $k_{22} \sim 10^8$ M^{-1} s^{-1} [12]. In these cases, the k_{cat}/K_m of reduction of aziridinyl-substituted quinones were similar to those of aziridinyl-unsubstituted ones with the similar values of E^1_7, being little sensitive to the Van der Waals volumes (VdWvol) of quinone ring or its substituents (Table 2). The other flavoenzymes of this group potentially reducing aziridinyl-substituted benzoquinones in a single-electron way are mitochondrial NADH:ubiquinone reductase (complex I, EC 1.6.5.3), microsomal NADH:cytochrome b_5 reductase (EC 1.6.2.2), and xanthine dehydrogenase (EC 1.17.1.4) [25–27], however, the kinetic analysis of these reactions has not been carried out. Besides, aziridinyl-benzoquinones are reduced in a single-electron way by FeS proteins. For example, the adrenal cortex mitochondria NADPH:adrenodoxin reductase (ADR) (EC 1.18.1.6) transfers redox equivalents in the sequence NADPH → FAD (ADR) → Fe_2S_2 (FeS protein adrenodoxin (ADX)) → heme (cytochrome P-450) [28]. ADR reduces quinones much slower than P-450R or NOS, besides, the reaction is competitively inhibited by NADPH (K_i = 5 μM) [29]. In this case, ADX prevents the inhibition of the reaction by NADPH, and stimulates it by increasing the k_{cat}/K_m of quinones by ≥ 10 times [29]. Because the k_{cat} of this reaction is equal to the maximal rate of ADR to ADX electron transfer, 10 s^{-1}, one may conclude that quinones are reduced through ADX. In this case, aziridinyl-substituted benzoquinones follow a similar $\log k_{cat}/K_m$ vs E^1_7 dependence as the aziridinyl-unsubstituted ones (Table 2).

One may also note that P-450R and NOS start to reduce MeDZQ and RH1 into their hydroquinones under incomplete anaerobiosis, $[O_2]$ = 40–50 μM at pH 7.0 [21]. It means that these reactions are incompletely inhibited by O_2, which reoxidizes quinone free radicals (Fig. 2), and that the hydroquinone formation efficiency is a result of the competition between the reoxidation of $Q^{\cdot -}$ by O_2 and the dismutation of $Q^{\cdot -}$ (Eq. (3), or even the repetitive reduction of $Q^{\cdot -}$. The hydroquinone formation efficiency increases with an increase in E^1_7 of quinones, because the formation of AZQ and DZQ hydroquinones starts even at completely aerobic conditions [21]. It means that the single-electron transferring flavoenzymes may also contribute to the bioreductively activated alkylation reactions of aziridinyl-benzoquinones under partly anaerobic conditions.

It is also important to analyze the two-electron reduction of aziridinyl-substituted benzoquinones by NAD(P)H: quinone oxidoreductase (NQO1, EC 1.6.99.2), which is supposed to be mainly responsible for their bioreductively activated DNA alkylation (Fig. 2). NQO1 contains the FAD with E^0_7 = –0.159 V in the active center. Its red (anionic) semiquinone ($FAD^{\cdot -}$) is unstable, with ca. 7% at the redox equilibrium [30]. The two-electron reduction of quinones by NQO1 is characterized by the very negative activation enthropies, which are in line with the efficient electronic coupling between quinone and FAD isoalloxazine due to the π-π interaction between their aromatic rings, as it is seen in the X-ray

Table 2. **Bimolecular rate constants of reduction (k_{cat}/K_m) of aziridinyl-benzoquinones and their aziridinyl-unsubstituted analogues by various flavoenzymes**

No.	Compound	E^1_7 (V)	k_{cat}/K_m ($M^{-1}s^{-1}$)				
			P-450R [4]	ADR/ADX[a]	NQO1 [4,32]	LipDH [38][b]	TrxR [40]
1.	2-CH_3-1,4-BQ	0.01	$1.5 \pm 0.1 \times 10^7$	$4.8 \pm 0.3 \times 10^6$	$1.3 \pm 0.2 \times 10^8$	$7.5 \pm 0.8 \times 10^{4a}$	$7.0 \pm 1.0 \times 10^4$
2.	DZQ	–0.054	$1.1 \pm 0.1 \times 10^7$	$2.0 \pm 0.2 \times 10^5$	$3.0 \pm 0.7 \times 10^8$	$9.3 \pm 0.3 \times 10^3$	$5.5 \pm 0.7 \times 10^3$
3.	AZQ	–0.07	$7.5 \pm 0.3 \times 10^6$	$2.8 \pm 0.2 \times 10^5$	$1.0 \pm 0.1 \times 10^5$	$1.0 \pm 0.1 \times 10^3$	$1.5 \pm 0.2 \times 10^{3a}$
4.	2,5-$(CH_3)_2$-1,4-BQ	–0.07	$8.8 \pm 0.6 \times 10^6$	$3.0 \pm 0.2 \times 10^6$	$1.4 \pm 0.4 \times 10^8$	$1.5 \pm 0.2 \times 10^{4a}$	$1.9 \pm 0.1 \times 10^5$
5.	$(CH_3)_3$-Az-1,4-BQ	–0.23	$4.2 \pm 0.2 \times 10^6$	$2.8 \pm 0.3 \times 10^5$	$6.5 \pm 0.1 \times 10^6$	300 ± 50	
6.	MeDZQ	–0.23	$2.6 \pm 0.2 \times 10^6$	$1.5 \pm 0.2 \times 10^5$	$1.9 \pm 0.4 \times 10^6$	$2.9 \pm 0.4 \times 10^3$	$1.0 \pm 0.1 \times 10^3$
7.	RH1	–0.23	$6.7 \pm 0.4 \times 10^6$	$3.2 \pm 0.3 \times 10^5$	$3.6 \pm 0.3 \times 10^7$	700 ± 100	$4.4 \pm 0.3 \times 10^3$
8.	$(CH_3)_4$-1,4-BQ	–0.26	$5.0 \pm 0.2 \times 10^6$	$5.0 \pm 0.4 \times 10^5$	$6.7 \pm 0.8 \times 10^7$	$1.2 \pm 0.2 \times 10^3$	600 ± 50
9.	BZQ	–0.38	$4.8 \pm 0.5 \times 10^5$	$1.5 \pm 0.1 \times 10^5$	$\leq 3.0 \times 10^3$	<100	<250
10.	2-OH-1,4-NQ[c]	–0.41	$4.4 \pm 0.3 \times 10^4$	$1.9 \pm 0.2 \times 10^4$	$5.9 \pm 0.5 \times 10^6$		700 ± 100

[a]H. Nivinskas, unpublished data; [b]at $[NAD^+]/[NADH] = 4.7$ (the reactions of 2e-, but not 4e-reduced LipDH); [c]NQ, naphthoquinone.

data of the complex of tetramethyl-1,4-benzoquinone with NQO1 [31]. A three-step (e^-, H^+. e^-) hydride transfer followed by a fast protonation is the most likely mechanism for the two-electron reduction of quinones by NQO1 [16, 32]. In contrast to the reactions of P-450R and NOS, the dependence of log k_{cat}/K_m of quinones on their E^1_7 in the case of NQO1 is almost absent. We found that the reactivity of both aziridinyl-substituted and unsubstituted quinones towards the rat NQO1 may be described by a two-parameter equation, with a positive role of their E^1_7, and the negative role of their VdWVol [32]. In accordance with this, BZQ (E^1_7 = –0.38 V) and its analogues with the large VdWVol, ≥330 Å^3 were much slower oxidants for NQO1 than 2-hydroxy-1,4-naphthoquinone (E^1_7 = –0.41 V, VdWVol = 180 Å^3) [32]. Later this model was refined using the molecular docking of homologous 1,4-benzoquinones in the active center of human NQO1 [33]. Although their reactivity in general increased upon a decrease in their VdWVol, the more appropriate correlation parameter appeared to be the distance between the N5 of dihydroalloxazine ring of FAD, and the C atom in an *o*-position to the carbonyl group of quinone [33].

Another group of flavoenzymes possibly participating in the reductive activation of aziridinyl-benzoquinones is C-S transhydrogenases-dehydrogenases, e. g. lipoamide dehydrogenase (LipDH, EC 1.8.1.4), glutathione reductase (GR, EC 1.8.1.7), and thioredoxin reductase (TrxR, EC 1.8.1.9) LipDH and GR contain FAD and redox active disulfide in their active center, and transfer the redox equivalents in the sequence NAD(P)H → FAD → -SS- → -SS- (lipoamide ($LipS_2$) or oxidized glutathione (GSSG)). LipDH is the component of mitochondrial pyruvate- and ketoglutarate dehydrogenase complexes and plays an important role in their metabolism, whereas GR and TrxR perform mainly the antioxidant functions in the cell. Mammalian TrxR contains the additional -SeS- cofactor at *C*-terminus, and transfers the redox equivalents in the sequence NADPH → FAD → -SS- → -SeS- → -SS- (thioredoxin (Trx)) [34]. These enzymes reduce quinones with 10–40% single-electron flux [35–40]. The kinetic analysis of *S. cerevisiae* and *Plasmodium falciparum* GR, and pig heart LipDH shows that the reactivity of quinones increased upon an increase in their E^1_7, including the aziridinyl-substituted quinones [35–38]. However, the reactivity (k_{cat}/K_m) of quinones was by 2–3 orders of magnitude lower than for P-450R. This is attributed to a low electron density on the isoalloxazine ring of FAD/reduced disulfide charge-transfer complexes of GR and LipDH, which reduces quinones via FAD [34]. Importantly, the two-electron reduced lipoamide ($Lip(SH)_2$), a product of LipDH-catalyzed reaction, also performs the two-electron reduction of fully-substituted quinones, including the aziridinyl-substituted ones [38, 39]. This is in contrast to reduced glutathione (GSH), and is attributed to an easier formation of intramolecular -SS- bond in $LipS_2$ [39]. In the case of TrxR, both aziridinyl-substituted and unsubstituted quinones are reduced in a single- and two-electron way through reduced FAD and/or reduced -SeS- [40]. Quinones react with mammalian TrxR at similar rates as with mammalian LipDH (Table 2), but do not display a well-expressed reactivity dependence on their E^1_7 [40]. In the case of C-S transhydrogenases-dehydrogenases, quinones act as their "subversive substrates", because their enzymatic redox cycling converts the antioxidant properties of the above flavoenzymes into the prooxidant ones. However, although being a subject of the considerable scientific interest, the role of these phenomena in the cytotoxicity of aziridinyl-benzoquinones may be minor because of the low reaction rates.

THE FACTORS RESPONSIBLE FOR THE ENHANCED CYTOTOXICITY OF 1,4-AZIRIDINYL-1,4-BENZOQUINONES

The quantitative structure-activity relationships (QSARs) in mammalian cell quinone cytotoxicity related to their electron-accepting properties were first obtained in 1990's. It was found that the log cL_{50} (the concentration of quinone causing the 50% cell death) decreases with an increase in its single-electron reduction potential (E^1_7) with a coefficient $\Delta \log cL_{50}/\Delta E^1_7 \sim -10\ V^{-1}$ [15, 41]. It follows from an increase of quinone reactivity towards the single-electron transferring flavoenzymes like P-450R with an increase in their E^1_7 (see "Single- and Two-Electron Reduction of Aziridinyl-1,4-Benzoquinones by Mammalian Flavoenzymes"). At sufficiently high values of E^1_7 of quinones, their cL_{50} may attain the limiting value [4, 15, 42]. It may be caused either by the decreased redox cycling rates of high-potential semiquinones, or by the metabolic instability of high-potential quinones, e. g. their rapid formation of the adducts with GSH [43]. The linear or parabolic log cL_{50} vs E^1_7 dependences were observed in the cytotoxicity of aziridinyl-unsubstituted quinones in FLK cells (bovine leukemia virus-transformed lamb embryo kidney fibroblasts) [4], MH22a murine hepatocytes [42], HL-60 human promyelocytic leukemia cells [44], primary mouse splenocytes [45], and primary rat hepatocytes [15]. Typically, the cytotoxicity of quinones is decreased by the antioxidant *N,N'*-diphenyl-*p*-phenylene diamine (DPPD) which prevents the lipid peroxidation, the Fe-ion chelator desferrioxamine (DESF) which prevents the Fenton reaction, and potentiated by the alkylating agent *bis*-chloroethylnitrosourea (BCNU), which inactivates the antioxidant flavoenzyme GR, and depletes the GSH pool, thus increasing the cell susceptibility to the oxidative stress [4, 46]. These data support the oxidative stress-type mechanism of quinone cytotoxicity.

Typically, the cytotoxicity of aziridinyl-substituted benzoquinones in several mammalian malignant cell lines was much higher than that of benzoquinones without aziridinyl moieties with the similar E^1_7 values (Table 3). However, their cL_{50} did not display a well-expressed dependence on E^1_7 [4, 42, 45, 47, 48]. Thus, their enhanced cytotoxicity may be considered as an additional factor to the prooxidant cytotoxicity of aziridinyl-unsubstituted quinones. This is because the reactivity of quinones in general does not depend on their redox

potential in NQO1-catalyzed two-electron reduction [32], and that the low-potential aziridinyl-quinones such as BZQ may alkylate DNA directly, without the bioreductive activation. This is evident from the comparison of the cytotoxicity of BZQ with that of 2-hydroxy-1,4-benzoquinone which possesses similar E^1_7 values in several cell lines (Table 3).

The cytotoxicity of bioreductively activated RH1 is also partly prevented by DPPD and DESF, and potentiated by BCNU [4, 42, 45], which points to an involvement of the oxidative stress. However, its cytotoxicity was also decreased by an inhibitor of NQO1, dicumarol, which shows an involvement of NQO1. In other cases, dicumarol also protected against the cytotoxicity of aziridinyl-substituted benzoquinones except BZQ and its analogues, which were the poor substrates for NQO1 [20, 32]. Dicumarol also did not affect or even increased the cytotoxicity of aziridinyl-unsubstituted quinones [4, 15, 42]. This was caused by the competition between the single-electron reduction of quinones leading to their redox cycling, and their two-electron reduction into hydroquinones. The subsequent glucuronylation or sulphonation of hydroquinones accelerates their removal from the cell, and decreases their cytotoxicity [15, 46].

In this context there was some uncertainty whether the enhanced cytotoxicity of aziridinyl-benzoquinones is not determined by their redox cycling or by the other modes of action of P-450R and/or the other single-electron transferring flavoenzymes [21, 49]. However, the parallel studies have shown that P-450R plays only a minor role in the activation of RH1 [50, 51]. Further, the numerous data point to a leading role of NQO1-catalyzed two-electron reduction in these cases: a) the cytotoxicity of RH1 in several sublines of human colon adenocarcinoma cells increases with an increasing amount of NQO1 [52]. The similar results were obtained in various sublines of human breast and non-small cell lung cancer cell lines [51], and in human breast adenocarcinoma xenographs [53]; b) the 16.6-fold resistance of murine hepatoma MH22a subline towards RH1 is related to a 24-fold decreased activity in NQO1, whereas only the 1.5-2-fold decreased activities in the prooxidant NAD(P)H: oxidase and NAD(P)H: cytochrome *c* reductase, and the antioxidant enzymes (catalase, superoxide dismutase, and glutathione reductase) were observed [42]. It points to an almost unchanged prooxidant/antioxidant balance in this cell subline, and is in line with an absence of cross-resistance towards the nonalkylating quinone DQ, and daunorubicin, which possesses both redox cycling and DNA-intercalating properties [42]; and c) similarly, the AZQ-resistant human erythroleukemic cell K562 sublines displayed a 10–20-fold decreased activity of NQO1 [54]. However, they may be regarded as more antioxidant ones as compared to the parent cells, because apart from a 2–2.5-fold decreased activity of P-450R, they possessed a 2–2.5-fold increased activity of superoxide dismutase. It may partly explain their increased resistance to the analogue of daunorubicin, adriamycin [54].

In the case of the derivatives of BZQ, the nature of alkylamino- or allylamino- substituents in 3,6-positions of 1,4-benzoquinone ring may change the cL_{50} of compound almost by one order of magnitude (Table 3). However, their cL_{50} do not depend on their reactivity towards NQO1, which

Table 3. **The single-electron reduction potentials (E^1_7) and compound concentrations causing 50% cell death (cL_{50}) of aziridinyl-substituted quinones and their aziridinyl-unsubstituted analogues in various cell lines**

No.	Compound	E^1_7 (V)	cL_{50} (μM)			
			FLK [4] (24 h)	MH22a [20, 42] (24 h)	K562 [47, 48]	Primary mouse splenocytes [45] (24 h)
1.	2-CH_3-1,4-BQ	0.01	5.4 ± 0.3		2.75 (2 h)	
2.	DZQ	–0.054	0.60 ± 0.03	0.25 ± 0.05	0.009 (2 h)	6.3 ± 0.7
3.	AZQ	–0.07			0.006 (120 h)	3.0 ± 0.4
4.	2,5-$(CH_3)_2$-1,4-BQ	–0.07	1.60 ± 0.15		0.75 (2 h)	5.0 ± 0.4
5.	2,5-$(Az)_2$-3-Phe[a]-1,4-BQ			1.2 ± 0.2	0.003 (120 h)	9.4 ± 1.0
6.	$(Az)_3$-1,4-BQ (trenimon)	–0.15[b]			0.003 (120 h)	
7.	$(CH_3)_3$-Az-1,4-BQ	–0.23	1.8 ± 0.2	1.3 ± 0.3		2.9 ± 0.4
8.	MeDZQ	–0.23	0.40 ± 0.03	0.31 ± 0.05	0.002 (120 h)	5.5 ± 0.4
9.	RH1	–0.23	0.11 ± 0.01	0.12 ± 0.02		4.2 ± 0.5
10.	$(CH_3)_4$-1,4-BQ	–0.26	16.0 ± 3.0	59.0 ± 5.0	>50 (2 h)	32.0 ± 4.0
11.	BZQ	–0.38	19.0 ± 1.5	28.0 ± 4.0	0.16 (120 h)	9.8 ± 0.8
12.	2,5-$(Az)_2$-3,6-$(NHC_3H_7)_2$-1,4-BQ	–0.38[c]		3.0 ± 0.4		9.4 ± 1.0
13.	2,5-$(Az)_2$-3,6-$(NHCH(CH_3)_2)_2$-1,4-BQ	–0.38[c]		25.0 ± 3.0		5.3 ± 0.5
14.	2,5-$(Az)^2$-3,6-$(NHCH_2CH{=}CH_2)_2$-1,4-BQ	–0.38[c]		11.0 ± 1.5		31.3 ± 5.0
15.	2,5-$(Az)_2$-3,6-(NH-cyclopropyl)$_2$-1.4-BQ	–0.38[c]		16.0 ± 1.5		10.9 ± 1.3
16.	2-OH-1,4-NQ	–0.41	700 ± 100	500 ± 80		1 000 ± 100

Abbreviations: [a] Phe, phenyl; [b] assumed to be the same as for trimethyl-1,4-benzoquinone; [c] assumed to be the same as for BZQ.

is low, and are probably related to the other currently poorly understood mechanisms, e. g. the DNA alkylation rates [20].

Another currently unclear problem is a strongly enhanced cytotoxicity of 2,5-diaziridinyl-1,4-benzoquinone-3-phenyl esters, which sometimes exceeded the cytotoxicity of MeDZQ by 10–50 times [55]. Their redox properties were not analyzed so far, but, in our opinion, their single-electron reduction potential should be similar to that of DZQ, ~–0.05 V. Using NQO1-rich and poor cell line pairs (H460 human lung cancer/H596 human lung, and HT29 human colon cancer/BE human colon), the ratios of cL_{50} for MeDZQ were equal to 63 (H596/H460), and to 7.6 (BE/HT29), which clearly points to the involvement of NQO1 in its cytotoxicity. In contrast, the ratios of cL_{50} for 2,5-diaziridinyl-1,4-benzoquinone-3-phenyl esters varied from 1.2 to 5.5 (H596/H460) and from 0.7 to 4.6 (BE/HT29), which points to a minor role of NQO1. Besides, there is no correlation between their cytotoxicity and activity towards NQO1 [55].

Finally, one may note that the cytotoxicity of aziridinyl-1,4-benzoquinones MeDZQ and RH1 in primary mouse splenocytes with a low amount of NQO1, 4 U/mg, is several times higher than that of their aziridinyl-unsubstituted analogue, DQ ([45], Table 3). Their cytotoxicity is insensitive to dicumarol. The simplest explanation is that it is caused by a net two-electron reduction of these quinones by single-electron transferring systems under partly hypoxic conditions [21]. BZQ and its analogues are also much more toxic than 2-hydroxy-1,4-naphthoquinone in mouse splenocytes ([20], Table 3), but this may be attributed to the direct alkylation of DNA. Taken together, these data show that even the novel representative of aziridinyl-1,4-benzoquinones, RH1, is not devoid of possible side effects, which may limit its clinical application.

THE MECHANISMS OF CELL DEATH AND THE EXPRESSION OF SIGNALING PROTEINS UNDER THE ACTION OF AZIRIDINYL-1,4-BENZOQUINONES

Like the other quinones, aziridinyl-substituted 1,4-benzoquinones cause the cell cycle arrest (prolonged S phase and G_2/M block) [56], the necrotic cell death (damage and permeabilisation of the cell membrane), and the apoptotic (programmed) cell death through the initial damage to DNA which further causes the nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation with the subsequent cell blebbing and shrinkage [57]. The aziridinyl-benzoquinone-induced damage to DNA in vitro is diverse [2, 3, 47, 48]: i) the reduced AZQ predominantly causes the interstrand crosslinks between dG residues separated by two intervening base pairs. The same is characteristic of nonreduced BZQ; ii) reduced DZQ, trenimon and several monosubstituted aziridinyl-benzoquinones alkylate DNA at 5'-TGC sequences; iii) reduced MeDZQ can efficiently crosslink DNA at 5'-GNC sequence.

The apoptotic and necrotic events take place in parallel, because the various types of damaged cells, i. e. the viable apoptotic, nonviable apoptotic, necrotic, and chromatin-free ones are observed during the action of quinones [58, 59]. The short time (1–3 h) incubation of primary rat hepatocytes with AZQ or trenimon or mouse epidermal cells with AZQ or DZQ leads to the typical oxidative stress-mediated necrotic cell death: i) the depletion of the intracellular GSH, ii) increased CN^- or N_3^--insensitive O_2 consumption, iii) an enhanced $OH^\cdot$ radical production, and iv) the Ca^{2+} release by mitochondria [60–62]. These phenomena are similar to those induced by aziridinyl-unsubstituted quinones. On the other hand, further studies of FLK and other cell lines gave more quantitative information: at concentrations close to cL_{50} at 24 h, MeDZQ and RH1 induced the formation of 51–66% viable apoptotic cells in the population of the viable FLK cells, which was higher than in the case of the equitoxic concentrations of nonalkylating DQ, 20.5 ± 3.9%, or H_2O_2, 32.0 ± 1.0% [59]. The multiparameter regression analysis of the apoptosis induction in FLK cells using the antioxidant and dicumarol protection data revealed that the main factor causing the apoptosis induction was the NQO1-catalyzed formation of alkylating products, whereas their prooxidant activities played a minor role [59]. Importantly, the close to cL_{50} concentrations of MeDZQ and RH1 in murine hepatoma MH-22a at 24 h also gave the 50–60% amount of apoptotic cells among the nonviable cells, which was higher than at the equitoxic concentration of DQ, 30%. Under these conditions, the data of the single cell electrophoresis (comet assay) were also in line with an increasing DNA damage in the order of RH1 > MeDZQ >> DQ, H_2O_2 (R. Jarašienė, M. Pečiukaitytė, D. Baltriukienė, V. Bukelskienė, unpublished data). It shows that MeDZQ and RH1 cause a more significant damage to DNA as compared to DQ. Overall, it is clear that the formation of two-electron reduction products of aziridinyl-benzoquinones which alkylate DNA mainly contributes to the apoptotic cell death, whereas the redox cycling of aziridinyl-benzoquinones especially in the short time experiments mainly contributes to the necrotic cell death. It is almost universally accepted that the apoptotic cell death mode is more desirable in the tumour chemotherapy.

Another important problem in the action of aziridinyl-substituted benzoquinones is the expression of cell signaling proteins during the cell death. This topic is insufficiently addressed so far, and is confined to the expression of the 'classical' signaling proteins like tumour suppressor p53, cell cycle inhibitor p21, and various caspases (cysteine-dependent aspartate-directed proteases, which are the executioners of the apoptotic cell death [63–67] (Table 4). The most important information in this case may be obtained from the action of RH1 in human breast cancer MDA-MB-231 sublines [67]: a) the proapoptotic action of RH1 is NQO1-dependent, and is accompanied by the disruption of mitochondrial membrane potential; b) although caspases are induced under the action of RH1 (Table 4), the caspase inhibitor z-VAD-fmk

does not affect the RH1-induced apoptosis, thus showing that caspases are not a decisive factor in this mode of the cell death; c) RH1 caused the translocation of mitochondrial apoptosis-inducing factor (AIF) from mitochondria to nucleus without changing its expression; d) the pro/antiapoptotic member of Bcl-2 family, Bax, is also involved in RH1-mediated signal transfer, including its cleavage into an 18 kD fragment; and e) the mitogen-activated kinases JNK, p38 and ERK are activated and phosphorylated during the action of RH1. In particular, the JNK inhibitor SP600125 inhibited the mitochondrial translocation of JNK and the generation of cleaved Bax. These data resulted in a tentative scheme of caspase-independent RH1-mediated apoptosis [67], which involves the activation of JNK and ERK by the reduced RH1, their subsequent activation of 18 kD cleaved Bax, and its mitochondrial translocation, which in turn activated JNK and decreased the mitochondrial membrane potential. It results in the translocation of AIF into the nucleuas, and the subsequent cell death. However, this scheme did not explain the chemical mechanisms of the activation of JNK and ERK by reduced RH1. Besides, the control experiments using the nonalkylating quinones like DQ and the other prooxidant compounds were not performed. On the other hand, our data (R. Jarašienė, M. Pečiukaitytė, D. Baltriukienė, V. Bukelskienė, unpublished data) add some information on the action of aziridinyl-substituted benzoquinones towards murine hepatoma MH22a cells: a) both RH1, MeDZQ, and DQ caused a transient (1–6 h) overexpression and phosphorylation of stress-activated kinases p38, JNK, and another protein kinase c-Jun, which responds to the various kinds of stress, and is supposed to be the protooncogene activated through its phosphorylation; b) the p38 kinase inhibitor SB 203580 protected the cells against the cytotoxicity of RH1 and MeDZQ, but potentiated the cytotoxicity of DQ and H_2O_2; and c) the inhibitor of JNK SP600125 potentiated the cytotoxicity of both RH1, MeDZQ, DQ, and H_2O_2. These data show that JNK in this case displays an antiapoptotic role protecting the cell from the oxidative stress, and that p38 may protect the cell from the oxidative stress, but may not protect DNA from the alkylating damage, or even enhance it. Naturally, the above described events may not be interpreted unequivocally, and warrant for a more thorough analysis and additional experiments.

CONCLUSIONS

In numerous malignant cell lines, the cytotoxicity of aziridinyl-substituted benzoquinones is much higher than that of their aziridinyl-unsubstituted analogues with the similar values of single-electron reduction potential, i. e. the ability to cause the oxidative stress-type cytotoxicity due to the single-electron enzymatic reduction. This phenomenon is related to the NQO1-activated DNA alkylation, and, in some cases, to the direct DNA akylation by low-potential aziridinyl-benzoquinones. The leading role of two-electron reduction of quinones by NQO1 in the enhanced cytotoxicity of aziridinyl-benzoquinones is evidenced by a significantly decreased NQO1 content in their resistant cell sublines. The enhanced DNA damage by aziridinyl-1,4-benzoquinones enhances an apoptotic mode of the cell death, which is desirable in tumour chemotherapy. However, there still exist several incompletely understood problems in their action: i) the enhancement of their cytotoxicity in primary cell cultures with a low NQO1 content, which may cause the side-effects in vivo, ii) the nature of NQO1–independent mechanisms, which may be responsible for a strongly enhanced cytotoxicity of some their groups, e. g. diaziridinyl-1,4-benzoquinone-3-phenyl esters; and iii) the mechanisms of expression and modification of cell death signaling proteins during their action. These problems warrant for a more thorough analysis and additional experiments.

Table 4. **The overexpression and other modes of action of cell signaling proteins during cell death induced by aziridinyl-1,4-benzoquinones**

Cell lines and tested compounds	Proteins	Quinone action mode
Human colon carcinoma HCT116, human chronic myelogenic leukemia K562, AZQ, DZQ, 2–15 h [63]	Overexpression of cell cycle inhibitor p21	Redox cycling
Human breast adenocarcinoma MCF-7, AZQ, DZQ, MeDZQ, 3–24 h [64]	Overexpression of tumour suppressor p53	Bioreductive alkylation (NQO1)
Oral cancer OEC-M1, AZQ, dimeric 2-aziridinyl-1,4-naphthoquinones, 24–48 h [66]	Overexpression of p53, protein cyclin B, antiapoptotic protein bcl-2, and caspase-3, cell cycle arrest	Unknown
Human breast cancer MDA-MB-231 sublines with different amount of NQO1, human colon cancer RKO, RH1, 2–48 h [67]	Overexpression of caspases 3, 8, 9, phosphorylation of mitogen-activated protein kinases JNK, p38, and ERK without their increased expression, generation and translocation of other proapoptotic proteins	Possibly related to the activation by NQO1
Murine hepatoma MH-22a, RH1, MeDZQ, 1–24 h[a]	Transient overexpression and increased phosphorylation of JNK, p38, and c-Jun	Redox cycling

[a] R. Jarašienė, M. Pečiukaitytė, D. Baltriukienė, V. Bukelskienė, unpublished data.

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Jonas Šarlauskas, Aušra Nemeikaitė-Čėnienė, Henrikas Nivinskas, Valė Miliukienė, Rasa Jarašienė, Milda Pečiukaitytė, Daiva Baltriukienė, Virginija Bukelskienė, Kastis Krikštopaitis, Narimantas Čėnas

PRIEŠNAVIKINIŲ AZIRIDINIL-PAVADUOTŲ 1,4-BENZOCHINONŲ REDOKSO REAKCIJOS IR CITOTOKSIŠKUMO MECHANIZMAI: TRUMPA APŽVALGA

S a n t r a u k a

Straipsnyje apibendriname duomenis apie aziridinil-pavaduotų-1,4-benzochinonų, kurie nuo 1980 m. yra žinomi kaip potencialūs priešnavikiniai agentai, citotoksiškumo mechanizmus. Aukštas priešnavikinis aziridinil-1,4-benzochinonų aktyvumas yra susietas su adityviu kelių mechanizmų poveikiu: 1) jų fermentine dvielektronine redukcija į aziridinil-pavaduotus hidrochinonus, kurie alkilina DNR efektyviau nei pradiniai chinonai; 2) oksidaciniu stresu, inicijuotu susidariusių laisvųjų radikalų ciklinių redoks virsmų, o atskirais atvejais ir 3) tiesioginiu DNR alkilinimu žemo redokso potencialo aziridinil-benzochinonais. Tačiau jų poveikis gali sukelti ir nepageidaujamų pašalinių efektų, t. y. citotoksiškumą pirminėse žinduolių ląstelėse. Taikydami mūsų pastaruosius pasiekimus, siekėme apžvelgti: 1) aziridinil-1,4-benzochinonų redokso savybes; 2) jų reaktingumą ir struktūros-aktyvumo ryšius vien- ir dvielektroninės redukcijos reakcijose, atliekamose flavininių fermentų ir su jais susietų redokso sistemų; 3) struktūros-aktyvumo ryšius jų citotoksiškume; 4) ląstelės žūties ir signalo perdavimo baltymų raiškos mechanizmus dėl jų poveikio.