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Communication

Role of NAD(P)H:quinone oxidoreductase (NQO1) in apoptosis induction by aziridinylbenzoquinones RH1 and MeDZQ

Aušra Nemeikaitė-Čėnienė[1], Aldona Dringelienė[1], Jonas Šarlauskas[2] and Narimantas Čėnas[2]✉

[1]*Institute of Immunology of Vilnius University, Vilnius, Lithuania,* [2]*Institute of Biochemistry, Vilnius, Lithuania;* ✉*e-mail: ncenas@bchi.lt*

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We aimed to characterize the role of NAD(P)H:quinone oxidoreductase (NQO1) in apoptosis induction by antitumour quinones RH1 (2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone) and MeDZQ (2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone). Digitonin-permeabilized FLK cells catalyzed NADPH-dependent single- and two-electron reduction of RH1 and MeDZQ. At equitoxic concentrations, RH1 and MeDZQ induced apoptosis more efficiently than the non-alkylating duroquinone or H_2O_2. The antioxidant *N,N'*-diphenyl-*p*-phenylene diamine, desferrioxamine, and the inhibitor of NQO1 dicumarol, protected against apoptosis induction by all compounds investigated, but to a different extent. The results of multiparameter regression analysis indicate that RH1 and MeDZQ most likely induce apoptosis *via* NQO1-linked formation of alkylating species but not *via* NQO1-linked redox cycling.

Keywords: aziridinylbenzoquinones, NAD(P)H:quinone oxidoreductase, apoptosis, cytotoxicity, oxidative stress

The antitumour properties of aziridinylbenzoquinones have been known for several decades (DiFrancesco *et al.*, 2004, and references therein). Recently, a new representative of this group, RH1 (2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone) (Fig. 1), entered phase I clinical trials (Danson *et al.*, 2004). The high activity of RH1 and its analogue MeDZQ (2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone) is commonly attributed to their two-electron reduction by NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase, EC 1.6.99.2), whose levels are markedly increased in a number of cancer cell lines (Ross *et al.*, 2000; Danson *et al.*, 2004). The reaction products, aziridinylhydroquinones, alkylate DNA much more efficiently than the parent quinones (Fig. 1, route (a)) (Lee *et al.*, 1992).

Like other quinones, RH1 and MeDZQ exhibit oxidative stress-type cytotoxicity, due to their single-electron reduction by flavoenzymes electron-transferases, e.g., NADPH:cytochrome P-450 reductase (P-450R, EC 1.6.2.4), and subsequent redox cycling of free radicals (O'Brien, 1991) (Fig. 1, route (b)). However, the latter is a factor of minor importance in the induction of necrotic cell death (Nemeikaitė-Čėnienė *et al.*, 2003, and references therein). On the other hand, the proposed mechanisms of induction of apoptosis, the morphologically distinct programmed cell death, by MeDZQ and other aziridinylbenzoquinones are controversial (Sun & Ross, 1996; Qiu *et al.*, 1996; 1998; Ngo *et al.*, 1998; Tudor *et al.*, 2003), or almost uncharacterized (RH1) (Kim *et al.*, 2004). Interestingly, in several cases induction of apoptosis has been attributed not to DNA alkylation, but to the prooxidant action, partly arising from autoxidation of aziridinylquinones, the products of NQO1-catalyzed reduction (Fig. 1, route (c)) (Qiu *et al.*, 1996; 1998).

In order to assess the role of NQO1 in apoptosis induction, we examined the effects of antioxidants and an NQO1 inhibitor, dicumarol, on the induction of apoptosis by RH1, MeDZQ, the nonalkylating tetramethyl-1,4-benzoquinone (duroquinone), and H_2O_2, whose prooxidant action is unrelated to NQO1. Multiparameter regression analysis of data obtained indicates that RH1 and MeDZQ most likely induce apoptosis *via* NQO1-linked formation of alkylating species but not *via* NQO1-linked redox cycling.

Abbreviations: NQO1, NAD(P)H:quinone oxidoreductase; P-450R, NADPH:cytochrome P-450 reductase; PBS, phosphate-buffered saline; MeDZQ, 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone; RH1, 2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone; DPPD, *N,N'*-diphenyl-*p*-phenylene diamine.

MATERIALS AND METHODS

RH1 and MeDZQ were synthesized according to the described procedures (Cameron & Giles, 1968; Winski *et al.,* 1998). Other compounds were obtained from Sigma and used as received. A culture of bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) was maintained in Eagle's medium supplemented with 10% fetal bovine serum at 37°C as described previously (Nemeikaitė-Čėnienė *et al.,* 2003). In cytotoxicity experiments, cells (2.5×10^4/ml) were seeded on 18 × 18 mm glass slides in 5 ml flasks in the presence or in the absence of compounds, and were grown for 24 h. Further, the slides were rinsed 3–4 times with phosphate-buffered saline (PBS) and stained with Trypan blue. The cells on the slides were calculated under a light microscope. Cell viability is expressed as the percentage of viable (Trypan blue excluding) cells remaining adherent after the compound treatment with respect to their amount after 24 h growth in the absence of the compound. The adherent FLK cells showed 98–99% viability, while the detached ones were found to be 98–99% nonviable. For the evaluation of apoptosis, cells were grown for 24 h at a concentration of each compound causing 50% cell survival. The slides were rinsed 3–4 times with PBS, the number of normal (N) and apoptotic cells (A) was determined under a fluorescence microscope Leica DMBL by staining with ethidium bromide and acridine orange (Mercille & Massie, 1994). A minimum of 200 cells were counted on each slide. The apoptotic index is expressed as the percentage of apoptotic cells (A/(N + A) × 100%).

For studies of RH1 and MeDZQ reduction, FLK cells were detached by trypsinization and suspended in 0.1 M K-phosphate buffer, pH 7.0 (final concentration, $1.0–3.0 \times 10^5$ cells/ml). Cells were permeabilized by 0.16 mg/ml digitonin. Next, RH1 or MeDZQ, and NADPH regeneration system (20 μM NADPH, 10 mM glucose-6-phosphate, and 10 U/ml glucose-6-phosphate dehydrogenase) were added. The reactions were followed spectrophotometrically, using a Hitachi-557 UV-VIS spectrophotometer, at 37°C. In separate experiments, 0.5 mM nitrotetrazolium blue was added into the reaction mixture, its reduction monitored at 540 nm.

Multiparameter regression and statistical analysis was performed using Statistica (version 4.3) software (Statsoft Inc., 1993).

RESULTS AND DISCUSSION

The FLK cell line used in this work and in our previous studies (Nemeikaitė & Čėnas, 1993; Nemeikaitė-Čėnienė *et al.,* 2003), is characterized by an activity of NQO1 of 260 nmol NADPH oxidized/mg protein × min, and an activity of NADPH: cytochrome P-450 (*c*) reductase of 43 nmol cytochrome *c* reduced/mg protein × min. With respect to an involvement of bioreductive activation in the cytotoxicity of RH1 and MeDZQ, we examined their reduction by permeabilized cells. In the presence of NADPH regeneration system, RH1 is rapidly reduced to hydroquinone (Fig. 2A). Dicumarol (20 μM) decreases the reduction rate by 70%, thus pointing to an involvement of NQO1 in the reaction. Next, we examined the possibility of parallel single-electron reduction. RH1 (10–50 μM) increased the rate of NADPH-dependent reduction of nitrotetrazolium by FLK cells 1.5-fold (Fig. 2B). The reaction was markedly inhibited by superoxide dismutase (Fig. 2B), but insensitive to dicumarol. Analogous results were obtained using 20–50 μM MeDZQ as an electron acceptor (not shown). This shows that FLK cells may perform single-electron reduction of RH1 and MeDZQ to their radicals being in equilibrium with the $O_2/O_2^{-\bullet}$ couple (Nemeikaitė-Čėnienė *et al.,* 2003; DiFrancesco *et al.,* 2004).

Next, we examined the effects of the antioxidant *N,N′*-diphenyl-*p*-phenylene diamine (DPPD) (Ollinger & Brunmark, 1991), the iron chelator desferrioxamine, and an NQO1 inhibitor, dicumarol, on cytotoxicity and apoptosis induction. Our findings are as follows: i) DPPD and desferrioxamine decreased the cytotoxicity of MeDZQ and RH1 (not shown), duroquinone, and H_2O_2 (Fig. 3A–C) in terms of Trypan blue uptake and apoptosis induction (Table 1). This shows that for all compounds investigated, there exists a common apoptosis and cytotoxicity mechanism, oxidative stress; ii) dicumarol decreased the cytotoxicity of MeDZQ (Fig. 3A) and RH1 (not shown), and did not decrease the cytotoxicity of duroquinone or H_2O_2 (Fig. 3B, C). It is evident that NQO1 selectively contributes to the cytotoxicity of MeDZQ and RH1. DNA alkylation by aziridinylhydroquinones (Fig. 1, route (a)), but not their autoxidation (Fig. 1, route (c)), seems to be responsible for cytotoxicity. Hydroquinones of RH1, MeDZQ, and duroquinone autoxidize at similar rates, $t_{1/2}$ = 40–60 min at pH 7.0 (Nemeikaitė-Čėnienė *et al.,* 2003). If their autoxidation were important in cytotoxicity, the action of duroquinone ought to be inhibited by dicumarol as well. This also indicates that the prooxidant cytotoxicity of quinones is caused by their single-electron reduction (Fig. 2B); iii) dicumarol protected against the apoptosis induction by MeDZQ and RH1 (Table 1). However, in contrast to the data of Figs. 3B and C, it also protected against the apoptosis induction by duroquinone (Table 1). Thus, it is possible that NQO1-catalyzed redox cycling of duroquinone, RH1 and MeDZQ (Fig. 1, route (c)), is partly responsible for the apoptosis induction. However, this possibility is challenged by the dicumarol protection against apoptosis induction by H_2O_2 (Table 1). This shows that, in contrast to cytotoxicity,

Table 1. Apoptosis induction in FLK cells.

Viable normal (N) and apoptotic (A) cells and apoptotic index after 24 h growth in the presence of 0.08 μM RH1, 0.4 μM MeDZQ, 40 μM duroquinone, or 50 μM H_2O_2. Cell number is expressed as the percentage with respect to the number of viable cells after 24 h growth in the absence of the compound, n = 3–4. For the number of apoptotic cells, $P < 0.02$ for 2–4 against 1, for 6, 8 against 5, and for 14–16 against 13, $P < 0.05$ for 12 against 9. For apoptotic index, $P < 0.005$ for 2–4 against 1, and for 14–16 against 13, $P < 0.02$ for 6–8 against 5, and $P < 0.05$ for 10–12 against 9. In control experiments, the apoptotic index was equal to 3–5%, being unaffected by DPPD or desferrioxamine or dicumarol. The presence of DPPD + desferrioxamine or dicumarol, or desferrioxamine + dicumarol, increased the apoptotic index to 7–10%.

No. Additions	Cell fraction (%)		Apoptotic index (%)
	N	A	(A/(N + A))
1. MeDZQ	23 ± 1.9	23.8 ± 1.5	50.9 ± 1.0
2. MeDZQ + DPPD	63.9 ± 5.4	7.9 ± 2.2	11.5 ± 3.5
3. MeDZQ + desferrioxamine	70.2 ± 2.9	11.5 ± 0.3	13.2 ± 0.6
4. MeDZQ + dicumarol	54.0 ± 8.4	14.2 ± 2.1	22.4 ± 4.7
5. RH1	16.7 ± 1.5	32.0 ± 2.0	65.8 ± 2.8
6. RH1 + DPPD	51.1 ± 2.3	8.1 ± 1.2	13.6 ± 1.0
7. RH1 + desferrioxamine	33.9 ± 1.8	27.2 ± 2.5	44.4 ± 4.5
8. RH1 + dicumarol	50.6 ± 1.7	12.0 ± 1.8	19.1 ± 2.7
9. Duroquinone	39.8 ± 3.6	10.3 ± 2.4	20.5 ± 3.9
10. Duroquinone + DPPD	65.0 ± 2.8	4.9 ± 1.8	7.0 ± 2.0
11. Duroquinone + desferrioxamine	59.2 ± 3.0	6.6 ± 2.0	10.0 ± 2.5
12. Duroquinone + dicumarol	51.0 ± 3.8	3.5 ± 0.5	6.4 ± 1.4
13. H_2O_2	33.2 ± 2.0	15.7 ± 1.0	32.0 ± 1.0
14. H_2O_2 + DPPD	69.1 ± 2.0	3.0 ± 1.0	4.2 ± 1.0
15. H_2O_2 + desferrioxamine	81.3 ± 2.0	3.0 ± 1.0	3.6 ± 1.0
16. H_2O_2 + dicumarol	42.8 ± 2.0	4.0 ± 1.0	8.5 ± 1.1

the effects of dicumarol in apoptosis induction may be not entirely related to the inhibition of quinone reduction by NQO1. Mechanistic studies of this phenomenon are beyond the scope of this paper. Possibly, dicumarol inhibits c-Jun N-terminal kinase, which is an important mediator in H_2O_2-induced apoptosis (McGee *et al.*, 2002; Pontano *et al.*, 2003). Because FLK cells express another important mediator of oxidative stress-induced apoptosis, the tumor suppressor p53 (Dees *et al.*, 1994), it is also possible that dicumarol inhibits p53 stabilization by NQO1 (Asher *et al.*, 2004, and references therein) and, subsequently, the p53-dependent apoptosis. Studies in these directions are currently underway; and iv) at equitoxic concentrations, RH1 and MeDZQ induced a larger number of apoptotic cells than duroquinone or H_2O_2 (Table 1). This shows that irrespective of other factors, the presence of aziridine groups contributes to enhanced apoptosis induction, which may be attributed to NQO1-mediated alkylation (Fig. 1, route (a)).

We attempted to distinguish between the mechanisms (a), (b), and (c) (Fig. 1) in apoptosis induction, using multiparameter regression analysis. We suggest that at equitoxic concentrations of the compounds, the apoptotic index or the total number of viable apoptotic cells (Table 1) may be expressed as a linear function of variables *A*, *B*, *C* and *D*, where *A* reflects the presence of aziridine groups (formation of DNA-alkylating species by NQO1), *B* reflects the prooxidant action unrelated to the reactions of NQO1 and not inhibited by dicumarol, *C* reflects the prooxidant action of hydroquinones formed in NQO1-catalyzed reactions, and *D* reflects other prooxidant ways of apoptosis induction inhibited by dicumarol, e.g. those mediated by JNK or by p53, unrelated to quinone reduction by NQO1. We assume that: a) $A = 1$ for RH1 and MeDZQ in the absence of dicumarol, and $B = 0$ for RH1 and MeDZQ in its presence, and for duroquinone and H_2O_2 in all cases; b) $B = 1$ for all the compounds in the absence of DPPD or desferrioxamine, and $B = 0$ in their pre-

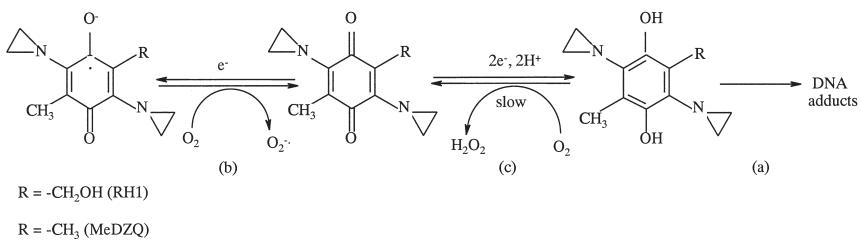

Figure 1. Structural formulae of RH1 and MeDZQ and pathways of their bioreductive activation: DNA alkylation after two-electron reduction (a), redox cycling of free radicals (b) or hydroquinones (c).

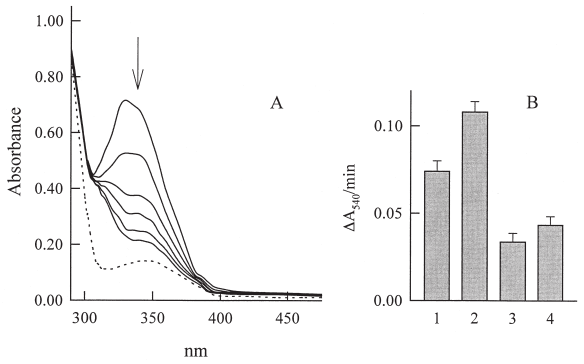

Figure 2. A. Reduction of 50 μM RH1 in the presence of 3.0 × 10^5/ml digitonin-permeabilized FLK cells and NADPH regeneration system.
The spectra were recorded in 4 min intervals. The dashed line shows the absorbance of the reaction mixture in the absence of RH1. The arrow shows the direction of absorbance changes.
B. Stimulation by 50 μM RH1 of nitrotetrazolium blue reduction by 2.0 × 10^5/ml permeabilized FLK cells and NADPH regeneration system.
RH1 was absent (1, 3) or present (2, 4), superoxide dismutase (5 units/ml) was added into the reaction mixture (3, 4), $P < 0.05$ for 1 against 2 (n = 3). One unit of superoxide dismutase activity is defined as the amount of protein needed to inhibit xanthine oxidase-catalyzed reduction of cytochrome *c* by 50% (McCord & Fridovich, 1969).

sence; c) $C = 1$ for RH1, MeDZQ and duroquinone in the absence of DPPD, desferrioxamine, and dicumarol, and $C = 0$ in all other cases; and d) $D = 1$ for all the compounds in the absence of DPPD, desferrioxamine, and dicumarol, and $D = 0$ in all other cases. Using this approach, the data of Table 1 may be expressed by Eqns. 1, 2:

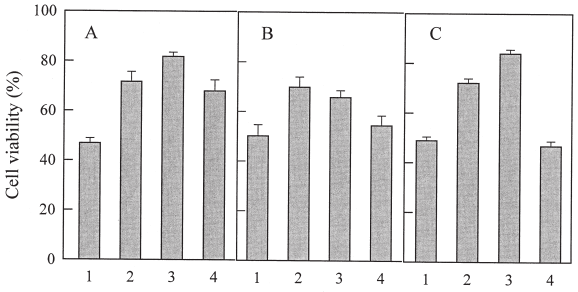

Figure 3. The effects of DPPD (2 μM), desferrioxamine (300 μM), and dicumarol (20 μM) on the toxicity of 0.4 μM MeDZQ (A), 40 μM duroquinone (B), and 50 μM H_2O_2 (C) to FLK cells.
Additions: compound (1), compound + DPPD (2), compound + desferrioxamine (3), and compound + dicumarol (4), n = 3–4; $P < 0.02$ for 2–4 against 1 (A), $P < 0.05$ for 2, 3 against 1 (B), and $P < 0.01$ for 2, 3 against 1 (C). Desferrioxamine or DPPD or dicumarol did not affect cell viability by more than ±3%, DPPD + desferrioxamine or dicumarol, and desferrioxamine + dicumarol, decreased the viability by >5%.

$$100A/(A + N)\ (\%) = (3.278 \pm 5.177) + (20.319 \pm 6.778)\ A + (10.822 \pm 7.578)\ B + (0.188 \pm 13.556)\ C + (17.900 \pm 12.375)\ D\ (r^2 = 0.7333,\ F(4.11) = 7.560), \quad (1)$$

and

$$A\ (\%) = (3.338 \pm 2.887) + (11.357 \pm 3.780)\ A + (5.088 \pm 4.226)\ B - (1.250 \pm 7.560)\ C + (7.275 \pm 6.901)\ D\ (r^2 = 0.6509,\ F(4.11) = 5.128). \quad (2)$$

These equations show that the role of NQO1-catalyzed redox cycling (Fig. 1, route (c)) in apoptosis induction is uncertain. Omission of variable *C* does not increase the r^2 values, but it improves the correlations by increasing the Fisher coefficient *F*, e.g., $F(3.12) = 10.966$ for apoptotic index, and $F(3.12) = 7.430$ for the number of apoptotic cells. Although the arbitrarily assigned values for *A*, *B*, *C* and *D* may be responsible for some uncertainty in Eqns. (1, 2), they show that under our conditions, the NQO1-linked formation of alkylating products of RH1 and MeDZQ is the most important factor in apoptosis induction in FLK cells. In our opinion, this approach may resolve the present controversy between the proposed mechanisms of apoptosis induction by aziridinylbenzoquinones, and may be useful in studies of other cell lines.

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