ACTA ABP BIOCHIMICA POLONICA

Vol. 61, No 4/2014
833–836
on-line at: www.actabp.pl

Communication

Cytotoxicity of anticancer aziridinyl-substituted benzoquinones in primary mice splenocytes

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The anticancer activity of aziridinyl-quinones is mainly attributed to their NAD(P)H:quinone oxidoreductase 1 (NQO1)-catalyzed two-electron reduction into DNA-alkylating products. However, little is known about their cytotoxicity in primary cells, which may be important in understanding their side effects. We found that the cytotoxicity of aziridinyl-unsubstituted quinones (n = 12) in mice splenocytes with a low amount of NQO1, 4 nmol × mg^{-1} × min^{-1}, was caused mainly by the oxidative stress. Aziridinyl-benzoquinones (n = 6) including a novel anticancer agent RH1 were more cytotoxic than aziridinyl-unsubstituted ones with the similar redox properties, and their cytotoxicity was not decreased by an inhibitor of NQO1, dicumarol. The possible reasons for their enhanced cytotoxicity are discussed.

Key words: aziridinyl-substituted quinones, cytotoxicity, oxidative stress

Received: 13 July, 2014; revised: 07 October, 2014; accepted: 12 November, 2014; available on-line: 18 December, 2014

INTRODUCTION

Aziridinyl-substituted benzoquinones (Fig. 1) comprise an important group of potential antitumour agents (DiFrancesco *et al*., 2004; Alcain & Villalba, 2007; Parkinson *et al.,* 2013, and references therein). Their antitumour activity stems mainly from the net two-electron reduction by flavoenzyme NAD(P)H:quinone oxidoreductase (NQO1, EC 1.6.99.2) into corresponding hydroquinones, which alkylate DNA more rapidly than the parent quinones (Lee *et al*., 1992; DiFrancesco *et al*., 2004). It determines their enhanced cytotoxicity towards the cancer cell lines with high levels of NQO1 (Winski *et al*., 2001, and references therein). In parallel, they may exert the 'oxidative stress-type' cytotoxicity due to their reduction by NADPH: cytochrome P-450 reductase (P-450R, EC 1.6.2.4) or by other single-electron transferring flavoenzymes into their free radicals, which further undergo redox cycling (Hargreaves *et al*., 2000; Nemeikaitė-Čėnienė *et al*., 2003). Besides, the aziridinyl-substituted benzoquinones with strong electron-donating substituents, e.g., 2,5-bis(2'-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone (BZQ, Fig. 1) may alkylate DNA directly, without bioreductive activation (Butler *et al*., 1990; Lee *et al*., 1992; Hargreaves *et al*., 2000).

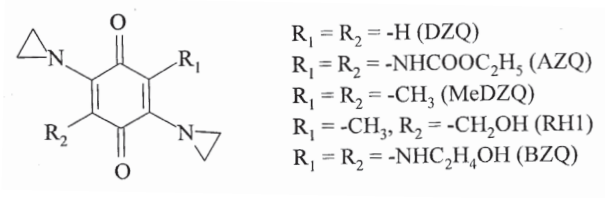

Figure 1. The formulae of diaziridinyl-benzoquinones used in this study.

On the other hand, certain aziridinyl-benzoquinones exert substantial side effects *in vivo*, e.g., lymphoid, bone marrow, and hematological toxicity (Hacker *et al*., 1982). It caused the withdrawal of trenimon (triaziridinyl-1,4-benzoquinone) from the clinical use, and AZQ and BZQ (Fig. 1) from further clinical studies (Begleiter, 2000; Hargreaves *et al*., 2000). In our opinion, the cytotoxicity studies of aziridinyl-benzoquinones in primary (non-transformed) cell cultures may be helpful in understanding of the mechanisms of their side effects. However, their studies in primary cells are scarce, being mainly concentrated on the action of AZQ (O'Brien, 1991; Begleiter, 2000). In order to expand the knowledge in this field, we examined the cytotoxicity of two series of quinones, aziridinyl-substituted and -unsubstituted ones, in primary mice splenocytes. Our studies involved the new representative of aziridinyl-benzoquinones, RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone; Fig. 1), which recently underwent preclinical and Phase-I clinical trials (Ward *et al*., 2005; Hussein *et al*., 2009; Danson *et al*., 2011).

MATERIALS AND METHODS

Enzymes and chemicals. Xanthine oxidase, xanthine, H_2O_2, NADH, NADPH, cytochrome *c,* dicumarol, desferrioxamine, *N,N'*-diphenyl-*p*-phenylene diamine (DPPD), 1,3-bis(2-chloromethyl)-1-nitrosourea (BCNU), model quinones, glutathione (GSSG) were obtained from Sigma-Aldrich, and were used as received. Aziridinyl-substituted quinones (Fig. 1) were a generous gift from Dr. Jonas Šarlauskas (Institute of Biochemistry of Vilnius University). Their synthesis was performed according to the established methods (Cameron *et* al., 1968; Chou *et al*., 1976; Petersen *et al*., 1955; Winski *et al.,* 1998). All the synthesized compounds

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Abbreviations: AZQ, 2,5-bis(carboethoxy-amino)-3,6-diaziridinyl-1,4-benzoquinone; BCNU, 1,3-bis(2-chloromethyl)-1-nitrosourea; BZQ, 2,5-bis(2'-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone; cL_{50}, the concentration of compound for 50% cell survival; DPPD, *N,N*-diphenyl-*p*-phenylene diamine; DZQ, 2,5-diaziridinyl-1,4-benzoquinone; E^1_7, redox potential of quinone/semiquinone couple at pH 7.0; MeDZQ, 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone; NQO1, NAD(P)H:quinone oxidoreductase; P-450R, NADPH:cytochrome P-450 reductase; RH1, 2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone

were characterized by a melting point and ^{1}H-NMR, UV, and IR spectroscopy.

Cell culture growth and cytotoxicity studies. In the studies of primary mice splenocytes, 4- to 8-week old male and female BALB/c mice (24±2.0 g) were kept under standard conditions, and were given food and water *ad libitum*. The mice were sacrificed by decapitation, their spleen was removed according to an established procedure (Stack *et al*., 1999). These experiments were approved by the Lithuanian Veterinary and Food Service (License No. 0215, 2011). For each experiment, spleens of 3-5 mice were used as a source of splenocytes. Erythrocytes were lysed by 5 min exposure in lysis solution (0.155 M NH_4Cl, 10 mM KCl, 0.1 mM EDTA). After washing the cells twice with RPMI 1640 medium, they were resuspended at the concentration of 10^6 cells /ml in RPMI 1640 medium with 5% fetal bovine serum and antibiotics, and were used for the further experiments. Cell viability was determined after 24 h of incubation of splenocytes with the examined compounds in 96-well cell culture plates (200 µl suspension per well), according to a Trypan blue exclusion test. The stock solutions of examined compounds were made in DMSO. The total concentration of DMSO in all the experiments was equal to 0.2%, and did not affect the viability of the cells, 98-96%, after their 24 h incubation.

Enzyme activity determination. Splenocytes were washed with PBS twice, and sonicated on ice in four cycles of 20 s. The homogenate was centrifuged at 14000 × *g* for 45 min and the resulting supernatant with added 1.0 mM PMSF was used for the further analysis. Protein amount was determined according to the method of Bradford. All the spectrometric measurements were performed using a Hitachi-557 spectrophotometer at 25°C in 0.1 M K-phosphate (pH 7.0) containing 1mM EDTA. The activity of catalase was determined following the decomposition of 10 mM H_2O_2 (Schallreuter *et al*., 1991). The activity of superoxide dismutase was determined from the inhibition of reduction of nitroblue tetrazolium by xanthine oxidase/xanthine system (Oberley & Buettner, 1979). One unit of superoxide dismutase activity corresponds to an amount of protein needed to inhibit the reduction of nitroblue tetrazolium by 50%. The activity of NAD(P)H:oxidase was determined according to the rate of oxidation of 100 µM NAD(P)H ($\Delta\varepsilon_{340}$= 6.2 mM^{-1} cm^{-1}). The activity of NAD(P)H:cytochrome *c* reductase was determined according to the rate of reduction of 50 µM cytochrome *c* ($\Delta\varepsilon_{550}$ = 20 $mM^{-1}cm^{-1}$) in the presence of 100 µM NAD(P)H. The activity of glutathione reductase was determined according to the rate of oxidation of 100 µM NADPH in the presence of 1.0 mM GSSG. The activity of NAD(P)H:quinone oxidoreductase 1 (NQO1) was determined following the rate of reduction of 50 µM cytochrome *c* in the presence of 10 µM menadione (2-methyl-1,4-naphthoquinone) and 100 µM NADPH, as the difference between the reduction rate in the absence of dicumarol, and in its presence (20 µM) (Lind *et al*., 1990). In this assay, Tween 20 (0.01%) and bovine serum albumin (0.25 mg × ml^{-1}) were used as the activators of NQO1.

Statistical analysis. Statistical data analysis was performed using Statistica (version 4.3, Statsoft Inc., 1993). Values are shown as standard error of mean. Where appropriate, the data were analyzed using a Student's *t*-test.

RESULTS

First, we determined the activities of redox enzymes which may be important in quinone cytotoxicity in splenocytes. The activity of NQO1 was relatively low, being equal to 4.0±0.3 nmol × mg^{-1} × min^{-1}. The activities of the prooxidant enzymes NADH:oxidase, NADPH:oxidase, NADH: cytochrome *c* reductase, and NADPH:-cytochrome *c* reductase (P-450R), were equal to 2.9±0.3 nmol × mg^{-1} × min^{-1}, 4.2±0.3 nmol × mg^{-1} × min^{-1}, 7.2±0.5 nmol × mg^{-1} × min^{-1}, and 11.1±1.0 nmol × mg^{-1} x min^{-1}, respectively. The activities of the antioxidant enzymes glutathione reductase, catalase, and superoxide dismutase were equal to 4.3±0.5 nmol × mg^{-1} × min^{-1}, 43.7±3.0 µmol × mg^{-1} × min^{-1}, and 1.0±0.2 units/mg, respectively.

Next, we examined the 24-h cytotoxicity of model aziridinyl-unsubstituted quinones (n = 12) in splenocytes. The data of Table 1 show that their concentrations for 50% cell survival (cL_{50}) decreased with an increase in their single-electron reduction potential (E^1_7), and ranged from 4.0±0.4 µM (5-hydroxy-1,4-naphthoquinone, E^1_7 = –0.09 V) to 1000±100 µM (2-hydroxy-1,4-naphthoquinone, E^1_7 = –0.41 V). The dependence of log cL_{50} on E^1_7 of quinones is parabolic (Fig. 2), being in line with the previously observed parabolic or linear log cL_{50} *vs.* E^1_7 relationships with $\Delta\log cL_{50}/\Delta E^1_7 \sim -10$ V^{-1} in their aerobic cytotoxicity in several mammalian cell lines (Nemeikaitė-Čėnienė *et al*., 2003, and references therein). It points to the dominating role of their oxidative stress-type cytotoxicity. It reflects the ease of the single-electron reduction of quinones by P-450R or by similar single-electron transferring flavoenzymes, which initiate their redox cycling. The reaching of the limiting value of cL_{50} of quinones at high redox potentials may be explained by the instability of high potential partly-substituted quinones, i.e., their rapid trapping by reduced

Table 1. Redox potentials of quinone/semiquinone couples of quinones at pH 7.0 (E^1_7) (Wardman, 1989; Čėnas *et al*., 2004, and references therein), and their concentrations for 50% survival of primary mice splenocytes during 24 h incubation (cL_{50}).

No. Compound	E^1_7 (V)	cL_{50} (µM)
1. 2-Methyl-1,4-benzoquinone	0.01	6.3±0.4
2. 2,3-Dichloro-1,4-naphthoquinone	–0.035	13.0±1.9
3. DZQ	–0.054	6.3±0.7
4. AZQ	–0.07	3.0±0.4
5. 5,6-Dimethyl-1,4-benzoquinone	–0.08	5.0±0.4
6. 5-Hydroxy-1,4-naphthoquinone	–0.09	4.0±0.4
7. 5,8-Dihydroxy-1,4-naphthoquinone	–0.11	6.0±0.5
8. 9,10-Phenanthrene quinone	–0.12	6.3±0.6
9. 1,4-Naphthoquinone	–0.15	11.5±1.9
10. 2-Methyl-1,4-naphthoquinone	–0.20	14.0±1.0
11. MeDZQ	–0.23	5.5±0.4
12. RH1	–0.23	4.2±0.5
13. Trimethyl-aziridinyl-1,4-benzoquinone	–0.23	2.9±0.4
14. Tetramethyl-1,4-benzoquinone	–0.26	32.0±4.0
15. 1,4-Dihydroxy-9,10-anthraquinone	–0.30	106±12
16. 1,8-Dihydroxy-9,10-anthraquinone	–0.33	≥250
17. BZQ	–0.38	9.8±0.8
18. 2-Hydroxy-1,4-naphthoquinone	–0.41	1000±100

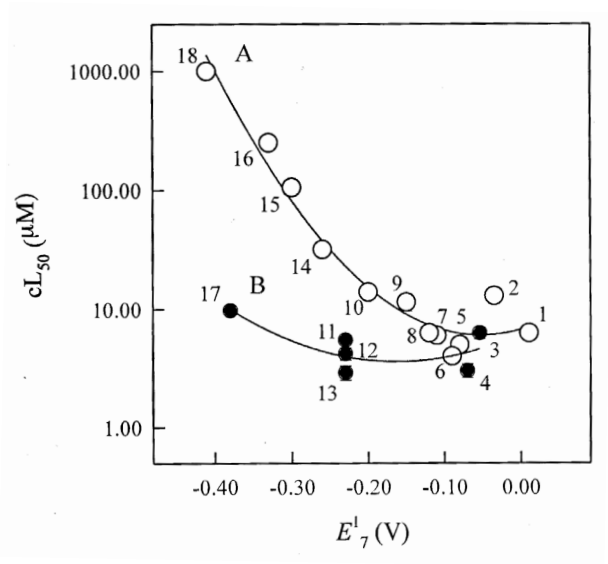

Figure 2. The dependence of the cytotoxicity of aziridinyl-unsubstituted and -substituted quinones in primary splenocytes on their single-electron reduction potential (E^1_7).
The dependences of the concentrations of aziridinyl-unsubstituted (**A**) and -substituted (**B**) quinones causing the 50% survival of the cells (cL_{50}) are presented as the second order approximations, the numbers of quinones are taken from Table 1.

glutathione (GSH) and other cellular nucleophiles, and/or their impaired redox cycling ability (Wardman *et al.*, 1995).

Importantly, aziridinyl-substituted quinones RH1, MeDZQ, trimethyl-2-aziridinyl-1,4-benzoquinone, and, especially, BZQ (Fig. 1) exhibited higher cytotoxicity than it may be expected from their E^1_7 value (Fig. 2). It points to the additional mechanisms of their action.

Further, we found that the cytotoxicity of RH1 at close to cL_{50} concentration was partly protected by the antioxidant *N,N´*-diphenyl-*p*-phenylene diamine (DPPD), the iron ion chelator desferrioxamine, and potentiated by the prooxidant BCNU, the latter inactivating the antioxidant enzyme glutathione reductase, and depleting GSH

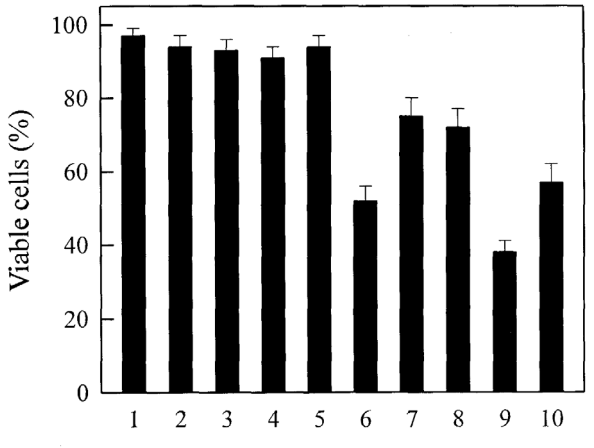

Figure 3. The modulation of the cytotoxicity of RH1.
Viability of control splenocytes (1–5), additions — none (1), 3.0 μM DPPD (2), 300 μM desferrioxamine (3), 20 μM BCNU (4), 20 μM dicumarol (5), 4.0 μM RH1 (6), 4.0 μM RH1 + 3.0 μM DPPD (7), 4.0 μM RH1 + 300 μM desferrioxamine (8), 4.0 μM RH1 + 20 μM BCNU (9), and 4.0 μM RH1 + 20 μM dicumarol (10), $n = 3$, $P>0.2$ for 2–5 against 1, $P<0.002$ for 7,8 against 6, $P<0.02$ for 9 against 6, and $P>0.05$ for 10 against 6.

(Öllinger & Brunmark, 1991) (Fig. 3). Importantly, the cytotoxicity of RH1 was not protected by an inhibitor of NQO1, dicumarol (Fig. 3). Analogous protection/potentiation effects by DPPD, desferrioxamine, and BCNU were observed in the cytotoxicity of tetramethyl-1,4-benzoquinone (duroquinone) and BZQ (data not shown). Duroquinone is a nonalkylating analogue of RH1 with similar reactivity towards the single-electron transferring P-450R and two-electron transferring NQO1 (Nemeikaitė-Čėnienė *et al.*, 2003). On the other hand, BZQ undergoes redox cycling and directly alkylates DNA, but is not a substrate for NQO1 (DiFrancesco *et al.*, 2004). Analogously, dicumarol also did not protect against the cytotoxicity of duroquinone and BZQ (data not shown).

DISCUSSION

According to our best knowledge, the previous primary cell cytotoxicity studies of aziridinyl-benzoquinones were scarce, and did not disclose their specific mode(s) of action. They involved the short-time testing of AZQ in rat hepatocytes (O'Brien, 1991), a study of trenimon derivatives in primary human skin fibroblasts (Huang *et al.*, 2009), and the related genotoxic studies of AZQ in lymphocytes (Klingerman *et al.*, 1988), as well as the cytotoxicity of AZQ and DZQ in mice epidermal cells (Li *et al.*, 1999). In contrast, our data provide a more quantitative insight into the cytotoxicity of aziridinyl-substituted quinones in primary cells with a low amount of NQO1. In our opinion, the primary splenocytes used in this study, are a more advantageous model as compared to primary hepatocytes, because of the possibility to perform longer 24-h cytotoxicity tests.

Previously, we found that the cytotoxicity of aziridinyl-benzoquinones in transformed cells with high activity of NQO1, 250 nmol × mg^{-1} × min^{-1}, was 10-100 times higher than that of aziridinyl-unsubstituted quinones possessing the same E^1_7 values (Nemeikaitė-Čėnienė *et al.*, 2003). The current data show that in spite of the low activity of NQO1 in splenocytes, 4.0 nmol × mg^{-1} × min^{-1}, the cytotoxicity of aziridinyl-benzoquinones is still enhanced (Fig. 2). The much enhanced cytotoxicity of BZQ (Fig. 2) may be explained by its direct alkylation of DNA without the involvement of enzymatic activation (Butler *et al.*, 1990). In contrast, MeDZQ and RH1 do not alkylate DNA directly (Lee *et al.*, 1992; DiFrancesco *et al.*, 2004), but they still possess increased cytotoxicity, which, in the case of RH1, is not related to the action of NQO1, because it is not inhibited by dicumarol (Fig. 3). The protective effects of DPPD and desferrioxamine against the action of RH1 (Fig. 3) indicate that its cytotoxicity is partly attributed to the oxidative stress. However, in our opinion, the higher cytotoxicity of RH1, MeDZQ and aziridinyl-benzoquinones with the higher values of E^1_7 (Table 1, Fig. 2) may not be attributed to their enhanced redox cycling, because both aziridinyl-substituted and –unsubstituted quinones follow the same log (rate constant) *vs.* E^1_7 relationship in their P-450R-catalyzed single-electron reduction (Nemeikaitė-Čėnienė *et al.*, 2003). On the other hand, it may be caused by other currently insufficiently addressed NQO1-independent cytotoxicity modes (Tudor *et al.*, 2005), e.g., a net two-electron reduction of aziridinyl-benzoquinones by single-electron transferring enzymes into their DNA-alkylating hydroquinones under partly anaerobic conditions (Anusevičius *et al.*, 2013), which may take place in relatively anaerobic cell compartments, or, possibly, the action of dihydronicotinamide riboside:qui-

none oxidoreductase 2 (NQO2) (Yan *et al.,* 2008). These problems warrant a more thorough examination. However, the enhanced cytotoxicity of RH1 in splenocytes (Fig. 2) is in line with its significant suppressive effects on the cells of the immune system, which were observed during its Phase-I clinical trials (Danson *et al.*, 2011). It shows that this novel compound may be not devoid of significant side effects even in the cells with low content of NQO1, which may limit its further clinical application.

Acknowledgements

This research was funded by the European Social fund under the Global Grant Measure No. VP1-3.1-ŠMM-07-K01-103 (Scientific Council of Lithuania).

REFERENCES

Alcain FJ, Villalba JM (2007) NQO1-directed antitumour quinones. *Expert Opin Ther Pat* **17**: 649–665.

Anusevičius Ž, Nivinskas H, Šarlauskas J, Sari MA, Boucher J-L, Čėnas N (2013) Single-electron reduction of quinone- and nitroaromatic xenobiotics by recombinant rat neuronal nitric oxide synthase. *Acta Biochim Pol* **60**: 217–222.

Begleiter A (2000) Clinical applications of quinone-containing alkylating agents. *Frontiers Biosci* **5**: e153–e171.

Butler J, Dzieliendziak A, Lea JS, Ward TH, Hoey BM (1990) Contrasting cytotoxic mechanisms of similar antitumour diaziridinyl-benzoquinones. *Free Rad Res Commun* **8**: 231–239.

Cameron DW, Gilles RGF, (1968) Photochemical formation of benzoxalizone derivatives from aminated quinones. *J Chem Soc C* 1461–1464.

Čėnas N, Anusevičius Ž, Nivinskas H, Misevičienė L, Šarlauskas J (2004) Structure-activity relationships in two-electron reduction of quinones. *Methods Enzymol* **382B**: 258–277.

Chou F, Chan AH, Driscoll JS (1976) Potential central nervous system antitumour agents. Aziridinylbenzoquinones. *J Med Chem* **28**: 1302–1308.

Danson SJ, Johnson P, Ward TH, Dawson M, Denneny O, Dickinson G, Aarons L, Watson A, Jowle D, Cummings J, Robson L, Halbert G, Dive C, Ranson M (2011) Phase I pharmacokinetic and pharmacodynamic study of the bioreductive drug RH1. *Ann Oncol* **22**: 1853–1860.

DiFrancesco A, Ward TH, Butler J (2004) Diaziridinylbenzoquinones. *Methods Enzymol* **382B**: 174–193.

Hacker MP, Hong CB, McKee MJ, Unwin SE, Urbanek SA (1982) Toxicity of aziridinyl benzo quinone administrated intra venously to beagle dogs. *Cancer Treat Rep* **66**: 1845–1852.

Hargreaves RHJ, Hartley JA, Butler J (2000) Mechanisms of action of quinone-containing alkylating agents: DNA alkylation by aziridinylquinones. *Frontiers Biosci* **5**: e172–e180.

Huang CH, Kuo H-S, Liu J-W, Lin Y-L (2009) Synthesis and antitumour evaluation of novel bis-triaziquone derivatives. *Molecules* **14**: 2306–2316.

Hussein D, Holt SV, Brookes KE, Klymenko T, Adamski JK, Hogg A, Estlin EJ, Ward T, Dive C (2009) Preclinical efficacy of the bioreductive alkylating agent RH1 against paediatric tumours. *Br J Cancer* **101**: 55–63.

Klingerman AD, Erexson GL, Bryant MF (1988) Sister chromatid exchange induction by diaziquone in human and mouse lymphocytes following both *in vivo* and *in vitro* exposures. *Cancer Res* **48**: 27–31.

Lee C-S, Hartley JA, Berardini MD, Butler J, Siegel D, Ross D, Gibson NW (1992) Alteration in DNA cross-linking and sequence selectivity of a series of aziridinylbenzoquinones after enzymatic reduction by DT-diaphorase. *Biochemistry* **31**: 3019–3025.

Li B, Guttierrez PL, Amstad P, Blough NV (1999) Hydroxyl radical production by mouse epidermal cell lines in the presence of quinone anti-cancer compounds. *Chem Res Toxicol* **12**: 1042–1049.

Lind C, Cadenas E, Hochstein P, Ernster L (1990) DT-diaphorase: purification, properties, and function. *Methods Enzymol* **186**: 287–301.

Nemeikaitė-Čėnienė A, Šarlauskas J, Anusevičius Ž, Nivinskas H, Čėnas N (2003) Cytotoxicity of RH1 and related aziridinylbenzoquinones: involvement of activation by NAD(P)H:quinone oxidoreductase (NQO1) and oxidative stress. *Arch Biochem Biophys* **416**: 110–118.

Oberley LW, Buettner GR (1979) Role of superoxide dismutase in cancer: a review. *Cancer Res* **39**: 1141–1149.

O'Brien PJ (1991) Molecular mechanisms of quinone cytotoxicity. *Chem-Biol Interact* **80**: 1–41.

Öllinger K, Brunmark A (1991) Effect of hydroxyl substituent position on 1,4-naphthoquinone toxicity to rat hepatocytes. *J Biol Chem* **266**: 21496–21503.

Parkinson EI, Bair JS, Cismesia M, Hergenrother PJ (2013) Efficient NQO1 substrates are potent and selective anticancer agents. *ACS Chem Biol* **8**: 2173–2183.

Petersen S, Gauss W, Urbschat E (1955) Synthese einfacher chinonderivation mit fungiziden, bakteriostatischen oder cytostatischen eigenschaften. *Angew Chem* **67**: 1461–1464.

Schallreuter KU, Wood JK, Berger J (1991) Low catalase levels in the epidermis of patients with vitiligo. *J Invest Dermacol* **97**: 1081–1085.

Stack AS, Altman-Hamamdzic S, Morris PJ, London SD, London L (1999) Polychlorinated biphenyl mixtures (Aroclors) inhibit LPS-induced murine splenocyte proliferation *in vitro. Toxicology* **139**: 137–154.

Tudor G, Alley M, Nelson CM, Huang R, Covell D, Gutierrez P, Sausville EA (2005) Cytotoxicity of RH1: NAD(P)H:quinone acceptor oxidoreductase (NQO1)-independent oxidative stress and apoptosis induction. *Anti-Cancer Drugs* **16**: 381–391.

Ward TH, Danson S, McGown AT, Ranson M, Coe NA, Jayson GC, Hargreaves RHJ, Butler J (2005) Preclinical evaluation of the pharmacodynamic properties of 2,3-diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone. *Clin Canc Res* **11**: 2695–2701.

Wardman P (1989) Reduction potentials of one-electron couples involving their radicals in aqueous solutions. *J Phys Chem Ref Data* **18**: 1637–1755.

Wardman P, Dennis MF, Everet SA, Patel KB, Stratford MR, Tracy M (1995) Radicals from one-electron reduction of nitro compounds, aromatic *N*-oxides and quinones: the kinetic basisfor hypoxia-selective bioreductive drugs. *Biochem Soc Symp* **61**: 171–194.

Winski SL, Hargreaves RHJ, Butler J, Ross D (1998) A new screening system for NAD(P)H:quinone oxidoreductase (NQO1)-directed antitumour quinones: Identification of a new aziridinylbenzoquinone RH1, an NQO1-directed antitumour agent. *Clin Cancer Res* **4**: 3083–3088.

Winski SL, Swann E, Hargreaves RHJ, Dehn DL, Butler J, Moody CJ, Ross D (2001) Relationship between NAD(P)H:quinone oxidoreductase 1 (NQO1) levels in a series of stably transfected cell lines and susceptibility to antitumor quinones. *Biochem Pharmacol* **61**: 1509–1516.

Yan C, Kepa JK, Siegel D, Stratford IJ, Ross D (2008) Dissecting the role of multiple reductases in bioactivation and cytotoxicity of the antitumour agent 2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone (RH1). *Mol Pharmacol* **74**: 1657–1665.