Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 68 (2004) 2107–2116
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Pharmacological and biological evaluation of a series of substituted 1,4-naphthoquinone bioreductive drugs

Roger M. Phillips[a,*], Mohammed Jaffar[b], Derek J. Maitland[c], Paul M. Loadman[a], Steven D. Shnyder[c], Gillian Steans[c], Patricia A. Cooper[c], Amanda Race[c], Adam V. Patterson[b], Ian J. Stratford[b]

[a]*Cancer Research Unit, Tom Connors Cancer Research Centre, University of Bradford, Bradford BD71DP, UK*
[b]*School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Oxford Road, Manchester, UK*
[c]*School of Pharmacy, University of Bradford, Bradford BD71DP, UK*

Received 14 April 2004; accepted 3 August 2004

Abstract

The indolequinone compound EO9 has good pharmacodynamic properties in terms of bioreductive activation and selectivity for either NAD(P)H:quinone oxidoreductase-1 (NQO1)-rich aerobic or NQO1-deficient hypoxic cells. However, its pharmacokinetic properties are poor and this fact is believed to be a major reason for EO9's lack of clinical efficacy. The purpose of this study was to develop quinone-based bioreductive drugs that retained EO9's good properties, in terms of bioreductive activation, but have improved pharmacokinetic properties. Out of 11 naphthoquinone compounds evaluated, 2-aziridinyl-5-hydroxy-1,4-naphthoquinone (compound **2**), 2,3-bis(aziridinyl)-5-hydroxy-1,4-naphthoquinone (compound **3**), and 2-aziridinyl-6-hydroxymethyl-1,4-naphthoquinone (compound **11**) were selected for further evaluation based on good substrate specificity for NQO1 and selectivity towards NQO1-rich cells in vitro. Compound **3** was of particular interest as it also demonstrated selectivity for NQO1-rich cells under hypoxic conditions. Compound **3** was not metabolised by murine whole blood in vitro (in contrast to compounds **2**, **11** and EO9) and pharmacokinetic studies in non-tumour-bearing mice in vivo (at the maximum soluble dose of 60 mg kg^{-1} administered intraperitoneally) demonstrated significant improvements in plasma half-life (16.2 min) and AUC values (22.5 μM h) compared to EO9 ($T_{1/2}$ = 1.8 min, AUC = 0.184 μM h). Compound **3** also demonstrated significant anti-tumour activity against H460 and HCT-116 human tumour xenografts in vivo, whereas EO9 was inactive against these tumours. In conclusion, compound **3** is a promising lead compound that may target both aerobic and hypoxic fractions of NQO1-rich tumours and further studies to elucidate its mechanism of action and improve solubility are warranted.

Keywords: NQO1; Hypoxia; Bioreductive drugs; Naphthoquinones

1. Introduction

The indolequinone compound EO9 (3-hydroxy-5-aziridinyl-1-methyl-2[indole-4,7-dione]-prop-β-en-α-ol) is a bioreductive drug that was selected for clinical evaluation in the early 1990s on the basis of a novel mechanism of action and promising preclinical activity [1,2]. The enzyme NAD(P)H:Quinone oxidoreductase-1 (NQO1, EC 1.6.99.2) plays a central role in bioreductively activating EO9 to DNA-damaging species [3,4] and good correlations between NQO1 activity and chemosensitivity in vitro under aerobic conditions have been reported [5–8]. EO9 is also selectively toxic to hypoxic cells, although good hypoxic cytotoxicity ratios (HCR) are only obtained in cells that have low NQO1 activity [5,9,10]. Despite evidence of activity (albeit modest) against a range of solid tumour models in vivo [1], EO9 failed to show activity in phase II clinical trials [11,12]. Several possible explanations for EO9's lack of clinical efficacy have been suggested [13], although the major causative factor is likely to be poor drug delivery to tumours as a result of rapid pharmacokinetic elimination and poor penetration through

Abbreviations: DMSO, dimethylsulphoxide; NQO1, NAD(P)H:Quinone oxidoreductase 1; P450R, NADPH cytochrome P450 reductase; NSCLC, non small cell lung cancer; MTD, maximum tolerated dose; MMC, mitomycin C

* Corresponding author. Tel.: +44 1274 233226; fax: +44 1274 233234.
E-mail address: r.m.phillips@bradford.ac.uk (R.M. Phillips).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.08.007

avascular tissue [14]. The targets for this class of compound (i.e. elevated NQO1 activity and hypoxia) have been extensively characterised in human tumours [15–17], and therefore further development of quinone-based bioreductive drugs is warranted. Based on experience with EO9, it is clear that future quinone-based bioreductive drugs must have improved properties in terms of drug delivery if therapeutic effects are to be obtained.

Numerous factors will determine how much drug is delivered to tumours following systemic administration with one of the most important factors being the drug's pharmacokinetic characteristics. In both rodents and humans, EO9 is rapidly cleared from the systemic circulation with plasma half-lives of 1.8 min in mice and between 0.8 and 19 min in humans [18,19]. The reasons for EO9's rapid pharmacokinetic elimination are not completely clear, although extra-hepatic metabolism by red blood cells is likely to be a significant contributing factor [18,19]. EO9 is rapidly metabolised by murine whole blood in vitro ($T_{1/2}$ = 15.6 ± 2.0 min), and recent studies have suggested that the identification of compounds that are metabolically stable in murine whole blood could be used as a filter to select compounds that are likely to have improved pharmacokinetic properties in vivo [19]. In this study a series of naphthoquinone compounds have been evaluated not only in terms of their selective toxicity towards NQO1-rich cells in vitro but also for metabolic stability in murine whole blood. Pharmacokinetic studies and anti-tumour activity against human tumour xenografts of selected compounds are also reported. Throughout this study, EO9 was used as a yardstick against which the relative merits of the naphthoquinones were measured.

2. Materials and methods

2.1. Compounds

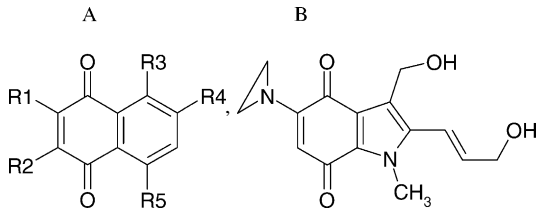
A series of 11 substituted naphthoquinones were synthesised by methods described elsewhere [20–22] and their chemical structures are presented in Table 1. EO9 was obtained from the Screening and Pharmacology Group of the European Organisation for Research and Treatment of Cancer. All compounds were initially dissolved in DMSO, aliquoted into Eppendorf tubes (100 μl per tube) and stock solutions at 5 mM were stored at −80 °C prior to biological and pharmacological evaluation.

2.2. Cell lines and chemosensitivity

A panel of human tumour cell lines was employed which had previously been characterised in terms of NQO1 enzyme activity. These included: A549 (NSCLC), H460 (NSCLC), HT-29 (colon adenocarcinoma), HCT-116 (colon adenocarcinoma) and BE (colon adenocarcinoma). A549, H460, HCT-116 and HT-29 cells were obtained from the American Tissue Type Collection (LGC Promochem) and BE cells were a gift from Dr. T. Ward (Paterson Institute for Cancer Research). All cell lines were routinely maintained at 37 °C in a humidified, CO_2-enriched (5%) environment and cultured in RPMI 1640 supplemented with 10% foetal calf serum, sodium pyruvate (2 mM), L-glutamine (2 mM), penicillin/streptomycin (50 IU ml^{-1}/50 μg ml^{-1}) and buffered with HEPES (25 mM). The NQO1 enzyme activities of A549, H460, HT-29, HCT-116 and BE cells are 1800 ± 122, 1652 ± 142, 688 ± 52, 565.6 ± 108.5 and <0.1 nmol DCPIP reduced/min/mg protein, respectively [23,24]. The BE cell line is devoid of functional NQO1 activity due to the presence of the C609T polymorphism [25]. In vitro chemosensitivity was determined using the MTT assay, details of which have been published elsewhere [26]. Briefly, cells in exponential growth were exposed to a range of drug concentrations for 1 h (at 37 °C) under aerobic conditions. Cells were washed twice in Hanks Balanced Salt Solution, re-suspended in RPMI 1640 growth medium and 1–2 × 10^3 cells plated into each well of a 96-well plate (8 wells per drug concentration with a final volume of 200 μl per well). Following incubation at 37 °C for 5 days, medium was removed and replaced with fresh RPMI 1640 medium prior to the addition of 20 μl MTT (5 mg ml^{-1}) per well. After a 4-h incubation at 37 °C, 200 μl of media plus MTT solution was removed and formazan crystals were dissolved in 150 μl of DMSO. The absorbance of the resulting solution was read at 550 nm using an ELISA spectrophotometer and percent cell survival determined as the absorbance of treated wells divided by the absorbance of the controls. The final DMSO concentration during drug exposure was 0.1% in all cases and each experiment was repeated in triplicate.

For analysis of hypoxia selectivity in vitro, T47D (human breast carcinoma) cell lines transfected with either NQO1 (DT-1) or P450R (R3) were employed [27,28]. Cells were routinely maintained as monolayer cultures in DMEM supplemented with 10% foetal calf serum, L-glutamine (2 mM), non-essential amino acids (1×), penicillin/streptomycin (50 IU ml^{-1}/50 μg ml^{-1}) and buffered with HEPES (25 mM). Puromycin (4 μg ml^{-1}) was also included in the media for culturing transfected cell lines. NQO1 enzyme activity in wild-type, DT-1 and R3 cells was 15.2, 1,234 and 12.1 nmol DCPIP reduced/min/mg protein, respectively. P450R activity in wild-type, DT-1 and R3 cells was 13, 11 and 441 nmol cytochrome *c* reduced/min/mg protein, respectively [27]. All compounds evaluated were exposed to cells under standard aerobic conditions and under nitrogen for 3 h, according to previously published protocols [28]. Following drug exposure, chemosensitivity was assessed using the MTT assay as described above and results were expressed in terms of IC_{50} values and hypoxic cytotoxicity ratios (HCR, defined as the ratio of IC_{50} values under air to IC_{50} values under nitrogen).

Table 1
Chemical structures and substrate specificity for purified human NQO1 of naphthoquinones (A) and EO9 (B)



Compound	R1	R2	R3	R4	R5	Substrate specificity (μmol/min/mg)
1	Az	CH_3	OH	H	H	41.87 ± 7.80
2	H	Az	OH	H	H	208.97 ± 30.48
3	Az	Az	OH	H	H	13.02 ± 1.10
4	Az	CH_3	H	H	H	61.44 ± 0.18
5	$NHCH_2CH_2Cl$	H	H	H	H	51.15 ± 5.95
6	Az	CH_3	OCH_3	H	H	2.49 ± 0.82
7	H	H	OCH_3	H	H	73.06 ± 7.48
8	H	CH_3	OCH_3	H	H	232.28 ± 13.86
9	H	H	OH	H	NH_2	7.81 ± 0.42
10	H	H	OH	H	I	137.95 ± 34.57
11	H	Az	H	CH_2OH	H	399.8 ± 26.4
EO9	–	–	–	–	–	15.39 ± 0.61

Values for substrate specificity are the mean ± standard deviation for three independent experiments.

2.3. Substrate specificity for NQO1

Purified human recombinant NQO1 was prepared as described elsewhere [29] and analysis of substrate specificity was conducted according to previously published methodology [29]. Briefly, each reaction consisted of NADH (2 mM), cytochrome *c* (75 μM), purified NQO1 (77.8 ng), test compounds (25 μM) in a final volume of 1 ml of tris–HCl buffer (50 mM, pH 7.4) containing bovine serum albumin (0.7% w/v). Reactions were started by the addition of NADH and the reduction of cytochrome *c* was monitored at 550 nm over the initial linear phase of the reaction curve (30 s). Results were expressed in terms of specific enzyme activity ± standard deviation for three independent experiments.

2.4. Animals

Two strains of mice aged 6–8 weeks were used: pure strain female NMRI mice (B and K Universal) and NCR/Nu (National Cancer Institute, USA). The latter were housed in isolated cabinets. Mice received CRM diet (SDS) and water ad libitum. Mice were kept in cages in an air-conditioned room with regular alternating cycles of light and darkness. All animal procedures were carried out under a project licence issued by the UK Home Office, and UKCCCR guidelines [30] were followed throughout.

2.5. Compound stability in murine whole blood

Blood from non-tumour-bearing NMRI mice was taken by cardiac puncture under terminal ether anaesthesia and collected in heparinised tubes. Whole blood (480 μl) was warmed to 37 °C on a heated reaction block for 30 min prior to the addition of 20 μl of test compounds (final drug concentration = 20 μM). Blood and drug solutions were vortexed and a 50 μl sample was taken (representing $t = 0$) to which 100 μl of acetonitrile was added. The mixture was vortexed and precipitated proteins were removed by centrifugation at 7000 × *g* for 3 min. The supernatant was collected and subjected to a further centrifugation step (7000 × *g* for 3 min) prior to drug analysis. Further samples were taken at various time intervals thereafter, and treated in the same manner as above. All samples were analysed by reverse phase HPLC using a Beckman system gold programmable solvent module 126, Beckman autosampler 50Y, diode array detector module 168 and version gold 711V software. Injection volumes were 50 μl and chromatographic separation was performed using a Lichrosorb RP8 column with a mobile phase of either 53% water (containing 1% phosphate buffer [0.5 mM, pH 7.4]):47% methanol (for analysis of compounds **2** and **3**) or 69% water (containing 1% phosphate buffer [0.5 mM, pH 7.4]):31% methanol (for analysis of compound **11** and EO9). Samples were run at a flow rate of 1.4 ml min^{-1} and compounds detected at a wavelength of 290 nm. The chemical stability in aqueous solutions of all compounds evaluated was determined by replacing murine whole blood with the same quantity of phosphate-buffered saline (pH 7.4). Sample preparation and analysis were identical to that described above.

2.6. Anti-tumour studies

H460 (human NSCLC) and HCT-116 (human colon carcinoma) human tumour xenografts were established

in NCR/Nu mice by subcutaneous inoculation of cell lines derived from cell cultures. The activity of NQO1 in these tumours in vivo have been determined elsewhere with specific activities of 1526 ± 42.6 (unpublished data) and 155 ± 3.9 nmol [31] DCPIP reduced/(min mg) protein for H460 and HCT-116 tumours, respectively. P450R activity in H460 and HCT-116 tumours in vivo were 8.2 ± 2.5 and 12.4 ± 4.8 nmol cytochrome *c* reduced/(min mg) protein, respectively (unpublished data). Tumours were excised from donor animals, placed in sterile physiological saline containing antibiotics and cut into small fragments of approximately 2 mm^3. Under brief general anaesthesia, a single fragment was implanted into the flank of each mouse using a trocar. Once the tumours could be accurately measured, the mice were allocated into groups of six by restricted randomisation to keep group mean tumour size variation to a minimum and treatment was commenced. To establish the MTD, compound **3** was dissolved in 10% DMSO/arachis oil and mice received escalating doses of compound (two mice per dose escalation) administered intraperitoneally. Toxicity was monitored by measuring body weight at various time intervals after drug administration and the percent maximum weight loss (relative to initial starting weight) was recorded with a weight loss of greater than 15% being considered toxic. The maximum amount of compound **3** that could be solubilised was 60 mg kg^{-1} (which was not toxic), and thus this dose was used for efficacy and pharmacokinetic studies. For efficacy studies, EO9 was administered in saline at its MTD of 6 mg kg^{-1} as a single intraperitoneal (i.p.) injection. Compound **3** was administered in 10% DMSO/arachis oil as either a single i.p. injection at 30 or 60 mg kg^{-1}, or as four consecutive daily i.p. injections of 12 or 15 mg kg^{-1} per day. The effects of therapy were assessed three to five times a week by two-dimensional calliper measurements of the tumours. Tumour volumes were then calculated using the formula $(a^2 \times b)/2$ where *a* is the smaller and *b* the larger diameter of the tumour. Tumour volume was then normalised relative to the respective volume on day 0 and semi-log plots of relative tumour volume (RTV) against time were made. Mann–Whitney *U*-tests were performed to determine the statistical significance of any differences in growth rate (based on tumour volume doubling time) between controlled and treated groups.

2.7. Pharmacokinetic analysis

Pharmacokinetic analysis was conducted in non-tumour-bearing NCR/Nu mice following the i.p. administration of compound **3** at 60 mg kg^{-1}. At 5, 15, 30, 60 and 120 min after administration, blood samples were taken by cardiac puncture under terminal ether anaesthesia and collected in heparinised collection tubes. Tubes were stored on ice during transport to the analytical laboratory. Three mice per time point were used and plasma was separated following centrifugation at 3000 × *g* for 15 min (at 4 °C). Compound **3** was extracted from plasma and analysed by HPLC as described above.

3. Results

3.1. Substrate specificity for purified human NQO1

A broad spectrum of substrate specificity for NQO1 existed within the panel of compounds evaluated ranging from 2.49 ± 0.82 to 399.8 ± 26.4 μmol/min/mg (Table 1). Compounds **2**, **11** and **8** were particularly good substrates for NQO1 with specific activities of 208.9 ± 30.5, 232.3 ± 13.9 and 399.8 ± 26.4 μmol/min/mg, respectively. In comparison with EO9, all compounds except **6** and **9** were better substrates for NQO1 with compound **3** being similar to EO9 in terms of reduction by NQO1.

3.2. Chemosensitivity studies in vitro under aerobic conditions

The response of a panel of cell lines with a broad spectrum of NQO1 activities to the various compounds tested is presented in Table 2. Whilst all compounds were less potent in vitro than EO9, preferential activity towards

Table 2
The response of cell lines in vitro following a 1-h exposure to test compounds under aerobic conditions

Compound	IC_{50} (μM) A549 cells	IC_{50} (μM) H460 cells	IC_{50} (μM) HT-29 cells	IC_{50} (μM) BE cells
1	7.2 ± 3.5 (24.2)	N/a	3.9 ± 0.8 (44.7)	174.2 ± 41.2
2	1.0 ± 0.1 (138.3)	0.6 ± 0.2 (230.5)	0.6 ± 0.1 (230.5)	138.3 ± 6.4
3	0.9 ± 0.2 (240.4)	1.5 ± 0.7 (144.3)	0.6 ± 0.3 (360.7)	216.4 ± 70.0
4	27.8 ± 4.4 (25.1)	690.4 ± 50.6 (1.0)	11.4 ± 2.0 (61.2)	679.2 ± 28.7
5	93.8 ± 1.8 (2.4)	206.5 ± 39.8 (1.1)	98.5 ± 4.0 (2.3)	223.1 ± 26.5
6	11.8 ± 5.6 (107.2)	29.5 ± 3.9 (42.9)	9.3 ± 3.5 (136.1)	1265.5 ± 85.0
7	10.8 ± 2.8 (0.4)	2.5 ± 1.9 (1.7)	12.9 ± 1.8 (0.3)	4.2 ± 0.5
8	95.3 ± 16.1 (0.6)	45.2 ± 1.8 (1.3)	36.4 ± 1.9 (1.6)	57.5 ± 13.5
9	45.5 ± 8.3 (0.7)	54.1 ± 4.1 (0.6)	15.3 ± 3.6 (2.0)	30.8 ± 5.5
10	6.8 ± 1.6 (0.3)	9.1 ± 1.2 (0.2)	5.2 ± 1.0 (0.4)	2.1 ± 0.1
11	2.5 ± 0.1 (61.9)	2.3 ± 0.1 (67.3)	5.3 ± 0.2 (29.2)	154.4 ± 6.4
EO9	0.1 ± 0.03 (475.0)	0.2 ± 0.1 (237.5)	0.3 ± 0.03 (158.2)	47.5 ± 9.5

Each value represents the mean ± standard deviation for three independent experiments. Values in parentheses represent the selectivity ratio defined as the ratio of IC_{50} values for the NQO1-deficient BE cell line to NQO1-rich cell lines. N/a represents data not available.

NQO1-rich cells was comparable in the case of compounds **2** and **3** (Table 2). Less-marked evidence of selectivity towards NQO1-rich cells was also observed in the case of compounds **6** and **11**. Similarly, modest selectivity for NQO1-rich cells was obtained for compounds **1** and **4**. In the case of compounds **5**, **7–10**, activity against NQO1-rich cells was less than the IC_{50} obtained in the NQO1-deficient BE cell line. Compound **3** was also evaluated against the HCT-116 cell line and an IC_{50} value of 0.72 ± 0.23 μM was obtained.

3.3. Chemosensitivity studies in vitro under hypoxic conditions

The response of wild-type (WT) and NQO1 (DT-1) plus P450R (R-3)-transfected cell lines to selected compounds under both aerobic and hypoxic conditions is presented in Table 3. Negligible hypoxia selectivity was demonstrated for compounds **2**, **4**, **6** and **11** in all three cell lines (Table 3). In the case of compound **3** however, significant hypoxia selectivity was observed with HCR values of 298, 82.5 and 365 in wild-type, NQO1 and P450R-transfected lines, respectively (Table 3). In addition, the potency of compound **3** (IC_{50} values ranging from 26.0 to 0.85 nM) under hypoxic conditions is similar to that of EO9 (IC_{50} values ranging from 33.0 to 1.1 nM) in all three cell lines. The HCR values are significantly greater than those obtained for EO9, particularly in the NQO1-transfected line where EO9 exhibited only modest potentiation (HCR = 6.0) of activity under hypoxic conditions (Table 3).

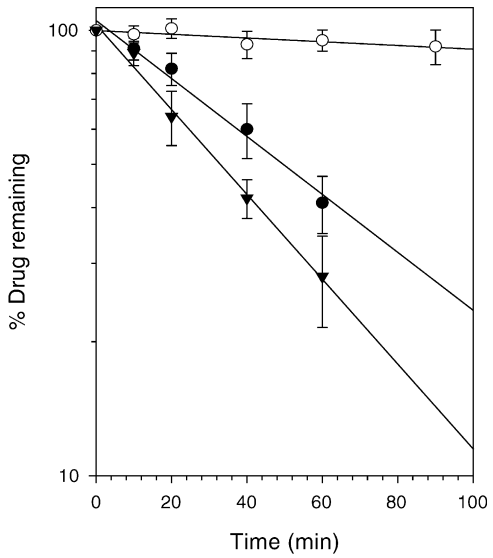


Fig. 1. Metabolism of compounds **2** (▼), **3** (○) and **11** (●) in murine whole blood in vitro at 37 °C. The half-lives of compounds **2**, **11** and **3** were 36 ± 7, 49 ± 12 and 543 ± 70 min, respectively. Each point represents the mean ± standard deviation of three independent experiments.

3.4. Metabolic stability of compounds in murine whole blood in vitro and pharmacokinetic analysis in vivo

The ability of murine whole blood to metabolise compounds **2**, **3** and **11** at 37 °C in vitro is presented in Fig. 1. Both compounds **2** and **11** were rapidly metabolised by murine whole blood with half-lives of 36 ± 7 and 49 ± 12 min, respectively. Half-lives are comparable with those

Table 3
Hypoxia selectivity of naphthoquinones and EO9 in vitro against T47D wild-type (WT) and T47D cells transfected with either NQO1 (DT-1) or P450R (R-3)

Compound	Exposure conditions	Cell line and IC_{50} values (μM except where indicated)			SR1	SR2
		Wt	DT-1	R-3		
2	Aerobic	1.59 ± 0.59	0.10 ± 0.003	0.071 ± 0.011	15.4	22.4
	Hypoxic	0.77 ± 0.40	0.31 ± 0.020	0.23 ± 0.10	2.5	3.3
	HCR	2.1	0.33	0.31		
3	Aerobic	7.75 ± 2.63	0.33 ± 0.05	0.31 ± 0.02	23.5	25.0
	Hypoxic (nM)	26.0 ± 1.0	4.0 ± 0.1	0.85 ± 0.15	6.5	30.6
	HCR	298	82.5	365		
4	Aerobic	59.7 ± 5.3	2.39 ± 0.08	1.60 ± 0.09	25.0	37.3
	Hypoxic	20.8 ± 3.8	3.24 ± 0.03	1.42 ± 0.02	6.4	14.7
	HCR	2.9	0.7	1.1		
6	Aerobic	90.7 ± 9.9	1.16 ± 0.13	5.19 ± 0.58	78.2	17.5
	Hypoxic	40.9 ± 2.6	1.04 ± 0.04	0.73 ± 0.08	39.3	56.0
	HCR	2.2	1.1	7.1		
11	Aerobic	0.45 ± 0.13	0.11 ± 0.01	0.14 ± 0.04	4.1	3.2
	Hypoxic	0.27 ± 0.18	0.27 ± 0.02	0.20 ± 0.02	1.0	1.35
	HCR	1.7	0.41	0.7		
EO9	Aerobic	3.80 ± 1.49	0.018 ± 0.01	0.153 ± 0.03	211	24.8
	Hypoxic (nM)	33.0 ± 5.0	3.0 ± 1.0	1.1 ± 0.1	11	30.0
	HCR	115	6.0	139		

Each value presented represents the mean ± standard deviation of three independent experiments. HCR denotes the hypoxic cytotoxicity ratio defined as the ration of IC_{50} values obtained in aerobic conditions divided by IC_{50} values obtained under hypoxic conditions; SR1 is the ratio of IC_{50} values in DT-1 to IC_{50} values in WT cells; SR2 is the ratio of IC_{50} values for R-3 cells to IC_{50} values in WT cells.

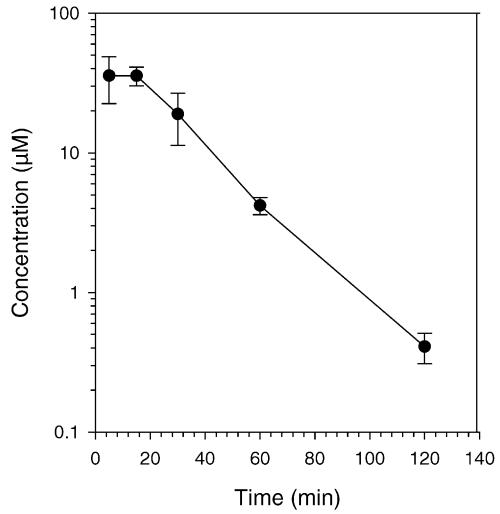


Fig. 2. Pharmacokinetic analysis of compound **3** in murine plasma (60 mg kg^{-1} intraperitoneally). All points represent the mean ± standard deviation using plasma from three mice per time point.

obtained for EO9 (15.6 ± 20 min) established previously [19]. In marked contrast, compound **3** was effectively metabolically stable in murine whole blood in vitro with an estimated half-life of 543 ± 70 min. Pharmacokinetic analysis following the i.p. administration of compound **3** (60 mg kg^{-1}) is presented in Fig. 2. Compound **3** could be detected in murine plasma up to 2 h after drug administration. Plasma pharmacokinetic parameters were as follows: C_{max} = 35.7 μM, T_{max} = 5 min, K_{el} = 2.56 h^{-1}, $T_{1/2}$ = 16.2 min (0.27 h) and area under the curve = 22.5 μM h.

3.5. Anti-tumour activity in vivo

The activity of compound **3** and EO9 against H460 and HCT-116 human tumour xenografts is presented in Table 4 and Fig. 3. Following a single i.p. administration of compound **3** at 60 mg kg^{-1} (maximum soluble dose) statistically significant growth delays of 3.6 and 3.1 days in HCT-116 and H460 xenografts, respectively, were

Table 4
Anti-tumour activity of compound **3** and EO9

Tumour	Compound (solvent)	Dose (mg kg^{-1}) /schedule	Median time to RTV2	Growth delay	Statistical significance
HCT–116	Solvent control	–	2.9	–	–
	Compound **3**	60/bolus	6.5	3.6	$P < 0.01$
	Compound **3**	12/daily for 4 days	4.6	1.7	$P < 0.01$
	Solvent control	–	1.8	–	
	Compound **3**	15/daily for 4 days	4.8	3.3	$P < 0.01$
H460	Solvent control	–	1.7	–	–
	Compound **3**	30/bolus	2.8	1.1	$P > 0.05$
	Compound **3**	60/bolus	4.8	3.1	$P < 0.01$
HCT–116	Solvent control	–	2.5		
	EO9	6/bolus	2.3	0	$P > 0.05$
H460	Solvent control	–	2.6	–	–
	EO9	6/bolus	2.4	0	$P > 0.05$

All compounds were administered intraperitoneally and statistical analysis was performed using the Mann–Whitney *U*-test. RTV2 denotes the time for relative tumour volume to double and growth delay is defined as the difference between the time for control and treated tumours to reach RTV2.

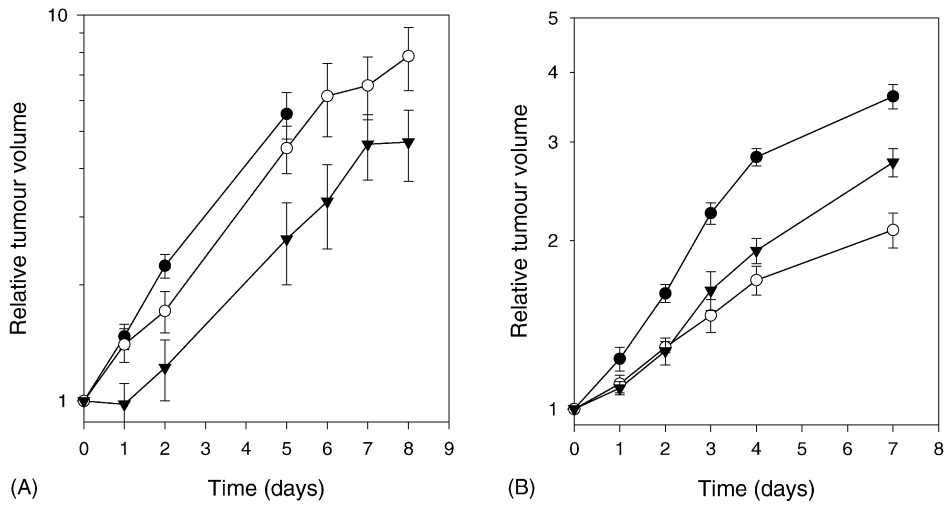


Fig. 3. Response of H460 (panel A) human tumour xenografts following an intraperitoneal administration of 30 mg kg^{-1} (○) and 60 mg kg^{-1} (▼) of compound **3**. Response of HCT-116 human tumour xenografts (panel B) following an intraperitoneal administration of compound **3** at 60 mg kg^{-1} (○) and a split schedule of 12 mg kg^{-1} compound **3** administered on days 1–4 (▼). Solvent controls (●) were 10% DMSO/arachis oil administered intraperitoneally. Each value presented represents the mean ± standard error with group sizes of six mice per treatment group.

obtained. Growth delays induced by compound **3** in the H460 model were dose-dependent with no significant growth delays seen at 30 mg kg^{-1}. Split-dose scheduling (15 mg kg^{-1} administered daily for 4 days) induced significant growth delays (3.3 days) in the HCT-116 xenograft. No anti-tumour effects were observed in both HCT-116 and H460 xenografts treated with EO9 at the MTD of 6 mg kg^{-1} administered i.p. (Table 4).

4. Discussion

Whilst the failure of EO9 to show efficacy in the clinic was a major disappointment for the field of quinone-based bioreductive drug development, the search for novel compounds that can exploit elevated tumour levels of NQO1 and/or tumour hypoxia continues. Medicinal chemistry approaches have predominantly focused on indolequinone derivatives of EO9 with comparatively little attention being paid to naphthoquinone compounds [23,28,32–34]. Early studies were conducted principally by Sartorelli's group and these demonstrated that 2,3-diaziridinyl-1,4-naphthoquinone sulphonate derivatives were active against a variety of tumour models in vivo [20]. It is interesting to note that good anti-tumour effects were observed only in compounds that possessed both the 5-*O*-sulphonyl and the 2,3-disubstituted aziridinyl groups [20]. Compounds such as 2-aziridinyl-5-hydroxy-1,4-naphthoquinone and 2,3-diaziridinyl-5-hydroxy-1,4-naphthoquinone (compounds **2** and **3** in this study) were classified as inactive against the experimental models employed (the L1210 murine leukaemia) by Lin et al. [20]. The results of this study clearly demonstrate, however, that aziridinyl substituted naphthoquinones such as compounds **1–3**, **6** and **11** are good substrates for human NQO1 and exhibit selective toxicity towards NQO1-rich cells in vitro under aerobic conditions (Table 2). The reasons for the discrepancy between this study and previous studies is likely to be the fact that haematological malignancies typically have low levels of NQO1 activity [6,7]. With the benefit of hindsight, the lack of anti-tumour activity of compounds **2** and **3**, for example, could be viewed as a positive result and the compounds activated by NQO1 would not be expected to demonstrate activity against a tumour model that lacks the appropriate enzymology.

In terms of structural features of naphthoquinones that determine substrate specificity, potency and selectivity for NQO1-rich cells, the presence or absence of an aziridinyl functional group appears to have a profound effect. In terms of substrate specificity for NQO1, the inclusion of an aziridine ring at position R1 generally reduces substrate specificity (compounds **1**, **4** and **6**, Table 1) that is reduced further if two aziridinyl groups are present (compare 1 and 3, Table 1). Whilst the inclusion of two aziridinyl groups reduces substrate specificity, it is important to note that compound **3** is comparable to EO9 which is widely recognised as a good substrate for NQO1 (Table 1). In marked contrast, the presence of an aziridinyl group at position R2 does not adversely affect substrate specificity and both compounds **2** and **11** are excellent substrates for NQO1 (Table 1). In terms of potency, the inclusion of an aziridinyl group is desirable but not absolutely essential. Compounds **7** and **10**, for example, lack aziridine groups but still show good potency in vitro (Table 2). Whilst substrate specificity and potency are important issues in the development of these compounds, the key parameter required is good selectivity for NQO1-rich cells in vitro. In this case, inclusion of the aziridinyl functional group is essential as all compounds that have this group (i.e. compounds **1–3**, **4**, **6** and **11**) are selectively toxic towards NQO1-rich cells in vitro to a greater or lesser extent (Table 2). These observations are essentially consistent with previous studies using indolequinone derivatives of EO9 [23,32]. Similarly, compounds that lack good leaving groups (compounds **7–10**) are likely to be detoxified by NQO1 under aerobic conditions (Table 2), as in the case with menadione [35,36]. These results suggest that reduction by NQO1 results in the generation of metabolites that can alkylate DNA and recent studies in our laboratory have shown that compound **3** induces interstrand cross-links in DNA (data not shown). It is of interest to note that whilst the aziridinyl analogues appear to be selectively toxic towards NQO1-rich cells compared to BE cells, IC_{50} are similar amongst NQO1-rich cells despite the fact that there is a broad range of NQO1 activities (Table 2). This suggests that there may be a threshold level of NQO1 activity, above which no further increase in toxicity occurs. Similar findings have been reported for other quinone-based compounds [37] and further studies are required to define this value.

One electron reductases such as P450R are known to reduce naphthoquinones effectively [38] and there is evidence that P450R is also involved in the activation of aziridinyl naphthoquinones used in this study (Table 3). In the case of compound **3**, T47D cells transfected with P450R are significantly more sensitive than T47D WT cells under aerobic conditions (selectivity ratio = 25.0). Furthermore, transfection of cells with P450R increases the potency of compound **3** under hypoxic conditions, although the role of P450R in determining hypoxia selectivity for compound **3** requires further investigation as HCR values for both T47D WT and P450R-transfected cells are comparable (298 and 365, respectively, Table 3). The mechanistic basis to explain these observations is not fully understood and further studies to characterise the type of DNA damage induced in cells following drug exposure are underway to address this question (i.e. single-strand DNA breaks indicate redox-mediated damage following one electron reduction or DNA alkylation cross-links formed following either one or two electron reduction). Whilst the mechanistic details are unclear, it is of particular interest to note that compound **3** exhibits the unusual

feature of selective toxicity towards NQO1-rich cells under hypoxic conditions (Table 3). In the case of EO9, good HCR values are generally seen only in cells that have low levels of NQO1 activity, whereas HCR values close to unity are obtained in NQO1-rich cells [5,9,10]. The mechanistic basis for selectivity towards NQO1-rich cells under hypoxic conditions is not fully understood, although, as stated above, the role of P450R is likely to be minimal in view of the fact that HCR values for T47D wild-type and P450R-transfected cells are comparable (Table 3). Whilst a mechanistic explanation for the observed biological effects of these compounds requires further investigation, compounds **2**, **3**, **6**, and **11** were selected for further evaluation with compound **3** showing particular promise in view of its selective toxicity towards NQO1-rich cells under hypoxic conditions.

A key requirement for the development of quinone-based bioreductive drugs in the aftermath of EO9 is the identification of compounds that retain the desirable features of EO9 but have better properties in terms of drug delivery. The pharmacokinetic profile of compounds is one factor that will have a significant bearing on drug delivery and a key objective of this study is to identify compounds that have significantly improved pharmacokinetic properties compared to EO9. Extra-hepatic metabolism of EO9 by red blood cells has been documented in both humans and rodents [18,19] and, whilst this alone cannot fully account for the rapid pharmacokinetic elimination of EO9, it is likely to be a significant contributing factor. As a means of selecting compounds that are likely to have improved pharmacokinetic properties in vivo, compounds that are poorly metabolised by blood cells in vitro would be attractive candidates. Analysis of the metabolic stability of compounds **2**, **3** and **11** in murine whole blood has demonstrated that compound **3** is metabolically stable whereas compounds **2** and **11** are metabolised by murine whole blood (Fig. 1) albeit at slightly slower rates than EO9 which has a $T_{1/2}$ of 15.6 min [19]. The metabolic basis for metabolism by the blood is not fully understood but it is clear that compounds that are rapidly metabolised by blood cells are unlikely to have good pharmacokinetic properties in vivo. On the basis of its chemosensitivity profile and stability in murine whole blood in vitro, compound **3** emerged as the lead compound for in vivo evaluation. An MTD was not obtained in this study due to solubility problems, but at 60 mg kg^{-1} administered intraperitoneally, compound **3** clearly had superior plasma pharmacokinetic properties compared to EO9. Plasma half-lives and AUC values for compound **3** were 16.2 min and 22.5 μM h, respectively (Fig. 2), which contrast sharply with the pharmacokinetic properties of EO9 ($T_{1/2}$ and AUC of 1.8 min and 0.184 μM h following an intravenous administration of 6 mg kg^{-1} [MTD] EO9, [19]). In view of the fact that compound **3** had only slightly reduced potency in vitro against NQO1-rich cells, the improved pharmacokinetic characteristics of compound **3** would potentially enhance delivery to tumour tissue. This is reflected in the fact that compound **3** did have significant activity against H460 and HCT-116 human tumour xenografts in vivo whereas EO9 was inactive against these models (Fig. 3, Table 4). No significant differences exist in the activity of compound **3** against HCT-116 xenografts when administered either as a bolus or via a split-dose schedule (Table 4). Furthermore, the activity of compound **3** against H460 and HCT-116 xenografts was similar, despite differences in NQO1 activity between the two models. This finding is consistent with in vitro chemosensitivity data and supports the concept of a threshold value for NQO1 activity above which no further enhancement of compound **3** activity occurs as discussed previously. In terms of accurately quantifying how much compound **3** is delivered to tumours, this is technically challenging as bioreductive activation to form a covalently bound product (DNA and/or protein) would be 'invisible' using the analytical techniques described here (covalently bound drug would be precipitated during extraction procedures employed). Further studies using pharmacodynamic endpoints (i.e. DNA damage using the comet assay) are currently underway to address this question.

In conclusion, the results of this study have identified a compound that has comparable properties to EO9 in terms of substrate specificity for NQO1 and selectivity for NQO1-rich cell lines in vitro. Of particular note is the fact that compound **3** has the ability to selectively target hypoxic cells that are NQO1 rich, which is not a common feature of the majority of quinone-based bioreductive drugs developed to date. The biochemical basis for this unique property of compound **3** is unknown and further studies to elucidate its mechanism of action under hypoxic and aerobic conditions are currently under investigation. The biological activity of compound **3** is potentially limited by the fact that its solubility is relatively poor (MTD in vivo could not be established), although further studies to determine whether or not the doses administered represent 'maximum target effects' are required. Promisingly though and in contrast to EO9, compound **3** had significantly improved pharmacokinetic properties and it was active in vivo against NQO1-rich tumour models.

Acknowledgements

The authors wish to acknowledge the financial support of The Association for International Cancer Research, Cancer Research UK (program grant number C459/A2579) and the Medical Research Council (program grant number G 9520193).

References

[1] Hendriks HR, Pizao PE, Berger DP, Kooistra KL, Bibby MC, Boven E, et al. EO9: a novel bioreductive alkylating indoloquinone with

preferential solid tumour activity and lack of bone marrow toxicity in preclinical models. Eur J Cancer 1993;29A:897–906.
[2] Workman P. Enzyme directed bioreductive drug development revisited: a commentary on recent progress and future prospects with emphasis on quinone anti-cancer agents and quinone metabolising enzymes, particularly DT-diaphorase. Oncol Res 1994;6: 461–75.
[3] Walton MI, Smith PJ, Workman P. The role of NAD(P)H quinone reductase (EC 1.6.99.2 DT-diaphorase) in the reductive bioactivation of the novel indoloquinone antitumour agent EO9. Cancer Commun 1991;3:199–206.
[4] Bailey SM, Wyatt MD, Friedlos F, Hartley JA, Knox RJ, Lewis AD, et al. Involvement of DT-diaphorase (EC 1.6.99.2) in the DNA cross-linking and sequence selectivity of the bioreductive anti-tumour agent EO9. Br J Cancer 1997;76:1596–603.
[5] Robertson N, Haigh A, Adams GE, Stratford IJ. Factors affecting sensitivity to EO9 in rodent and human tumour cells in vitro: DT-diaphorase activity and hypoxia. Eur J Cancer 1994;30A: 1013–9.
[6] Smitkamp-Wilms E, Peters GF, Pinedo HM, Van Arkotte J, Giaccone G. Chemosensitivity to the indolequinone EO9 is correlated with DT-diaphorase activity and gene expression. Biochem Pharmacol 1994;47:1325–32.
[7] Collard J, Matthew AM, Double JA, Bibby MC. EO9: relationship between DT-diaphorase levels and response in vitro and in vivo. Br J Cancer 1995;71:1199–203.
[8] Fitzsimmons SA, Workman P, Grever M, Paull K, Camalier R, Lewis AD. Reductase enzyme expression across the National Cancer Institute tumour cell line panel: correlation with sensitivity to mitomycin C and EO9. J Natl Cancer Inst 1996;88:259–69.
[9] Plumb JA, Workman P. Unusually marked hypoxic sensitisation to indoloquinone EO9 and Mitomycin C in a human colon cell line that lacks DT-diaphorase activity. Int J Cancer 1994;56: 134–9.
[10] Plumb JA, Gerritsen M, Workman P. DT-diaphorase protects cells from the hypoxic cytotoxicity of indoloquinone EO9. Br J Cancer 1994;70:1136–43.
[11] Dirix LY, Tonnesen F, Cassidy J, Epelbaum R, ten Bokkel Huinink WW, Pavlidis N, et al. EO9 phase II study in advanced breast, gastric, pancreatic and colorectal carcinoma by the EORTC Early Clinical Studies Group. Eur J Cancer 1996;32A:2019–22.
[12] Pavlidis N, Hanauske AR, Gamucci T, Smyth J, Lehnert M, te Velde A, et al. A randomized phase II study with two schedules of the novel indoloquinone EO9 in non-small-cell lung cancer: a study of the EORTC Early Clinical Studies Group (ECSG). Ann Oncol 1996;7:529–31.
[13] Connors TA. Bioreductive agents, hypoxic cells and therapy. Eur J Cancer 1996;32A:1833–4.
[14] Phillips RM, Loadman PM, Cronin BP. Evaluation of a novel in vitro assay for assessing drug penetration into avascular regions of tumours. Br J Cancer 1998;77:2112–9.
[15] Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply and metabolic microenvironment of human tumours: a review. Cancer Res 1989;49:6449–65.
[16] Malkinson AM, Siegel D, Forrest GL, Gazdar AF, Oie HK, Chan DC, et al. Elevated DT-diaphorase activity and messenger-RNA content in human non-small-cell lung-carcinoma–relationship to the response of lung tumor xenografts to mitomycin-C. Cancer Res 1992;52: 4752–7.
[17] Siegel D, Franklin WA, Ross D. Immunohistochemical detection of NAD(P)H:Quinone oxidoreductase in human lung and lung tumours. Clin Cancer Res 1999;4:2065–70.
[18] Schellens JHM, Planting AST, van Acker BAC, Loos WJ, de Boer-Dennert M, van der Burg MEL, et al. Phase I and pharmacological study of the novel indoloquinone bioreductive alkylating cytotoxic drug EO9. J Natl Cancer Inst 1994;86:906–12.
[19] Loadman PM, Bibby MC, Phillips RM. Pharmacological approach towards the development of indolequinone bioreductive drugs based on the clinically inactive agent EO9. Br J Pharmacol 2002;137:701–9.
[20] Lin TS, Xu SP, Zhu LY, Cosby LA, Sartorelli AC. Synthesis of 2,3-diaziridinyl-1,4-naphthoquinone sulfonate derivatives as potential antineoplastic agents. J Med Chem 1989;32:1467–71.
[21] Lin TS, Zhu LY, Xu SP, Divo AA, Sartorelli AC. Synthesis antimalarial activity of 2-aziridinyl- and 2,3-bis(aziridinyl)-1,4-naphthoquinonyl sulfonate and acylate derivatives. J Med Chem 1991;34: 1634–9.
[22] Sartorelli AC, Lin TS. 2,3-Bis(azridinyl)-1,4-naphthoquinone sulphonate derivatives having antineoplastic activity. United States Patent. Patent number 4,806,531 (1989).
[23] Phillips RM, Naylor MA, Jaffar M, Doughty SW, Everett SA, Breen AG, et al. Bioreductive activation of a series of indolequinones by human DT-diaphorase: structure activity relationships. J Med Chem 1999;42:4071–80.
[24] Choudry GA, Hamilton-Stewart PA, Double JA, Krul MRL, Naylor B, Flannigan GM, et al. A novel strategy for NQO1 (NAD(P)H:quinone oxidoreductase EC 1. 6. 99. 2) mediated therapy of bladder cancer based on the pharmacological properties of EO9. Br J Cancer 2001;85:1137–46.
[25] Traver RD, Horikoshi T, Dannenberg KD, Stadlbauer THW, Dannenberg PV, Ross D, et al. NAD(P)H:Quinone oxidoreductase gene expression in human colon carcinoma cells: characterisation of a mutation which modulates DT-diaphorase activity and mitomycin sensitivity. Cancer Res 1992;52:797–802.
[26] Phillips RM, Hulbert PB, Bibby MC, Sleigh NR, Double JA. In vitro activity of the novel indoloquinone EO9 and the influence of pH on cytotoxicity. Br J Cancer 1992;65:359–64.
[27] Cowen RL, Patterson AV, Telfer BA, Airley RE, Hobbs S, Phillips RM, et al. Viral delivery of P450 reductase recapitulates the ability of constitutive overexpression of reductase enzymes to potentiate the activity of mitomycin C in human breast cancer xenografts. Mol Cancer Ther 2003;2:901–9.
[28] Jaffar M, Phillips RM, Williams KJ, Mrema I, Cole C, Wind NS, et al. 3-Substituted-5-aziridinyl-1-methylindole-47-diones as NQO1-directed antitumour agents: mechanism of activation and cytotoxicity in vitro. Biochem Pharmacol 2003;66:1199–206.
[29] Phillips RM. Bioreductive activation of a series of analogues of 5-aziridinyl-3-hydroxymethyl-1-methyl-2-[1H-indole-47-dione] prop-β-en-α-ol (EO9) by human DT-diaphorase. Biochem Pharmacol 1996;52:1711–8.
[30] Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double JA, et al. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia. Br J Cancer 1998;77:1–10.
[31] Phillips RM, Burger AM, Loadman PM, Jarret CM, Swaine DJ, Fiebig HH. Predicting response to mitomycin C on the basis of DT-diaphorase activity or drug metabolism by tumour homogenates: implications for enzyme directed bioreductive drug development. Cancer Res 2000;60:6384–90.
[32] Naylor MA, Jaffar M, Nolan J, Stephens MA, Butler S, Patel KB, et al. 2-cyclopropylindoloquinones and their analogues as bioreductively activated antitumor agents: structure-activity in vitro and efficacy in vivo. J Med Chem 1997;40:2335–46.
[33] Beall HD, Winski S, Swann E, Hudnott AR, Cotterill AS, O'Sullivan N, et al. Indolequinone antitumor agents: correlation between quinone structure, rate of metabolism by recombinant human NAD(P)H:quinone oxidoreductase, and in vitro cytotoxicity. Med Chem 1998;41:4755–66.
[34] Swann E, Barraja P, Oberlander AM, Gardipee WT, Hudnott AR, Beall HD, et al. Indolequinone antitumor agents: correlation between quinone structure and rate of metabolism by recombinant human NAD(P)H:quinone oxidoreductase. Part 2. J Med Chem 2001;44: 3311–9.

[35] Cadenas E. Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism. Biochem Pharmacol 1995;49:127–40.
[36] Joseph P, Long II DJ, Klein-Szanto AJP, Jaiswal AK. Role of NAD(P)H:quinone oxidoreductase 1 (DT-diaphorase) in protection against quinone toxicity. Biochem Pharmacol 2000;60:207–14.
[37] Winski SL, Swann E, Hargreaves RHJ, Dehn DL, Butler J, Moody CJ, et al. Relationship between NAD(P)H:Quinone oxidoreductase 1 (NQO1) levels in a series of stably transfected cell lines and susceptibility to antitumour quinines. Biochem Pharmacol 2001; 61:1509–16.
[38] Giulivi C, Cadenas E. One and two electron reduction of 2-methyl-1,4-naphthoquinone bioreductive alkylating agents: kinetic studies, free radical production, thiol oxidation and DNA strand break formation. Biochem J 1994;301:21–30.