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3-Substituted-5-aziridinyl-1-methylindole-4,7-diones as NQO1-directed antitumour agents: mechanism of activation and cytotoxicity *in vitro*

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Abstract

Indolequinone agents are a unique class of bioreductive cytotoxins that can function as dual substrates for both one- and two-electron reductases. This endows them with the potential to be either hypoxia-selective cytotoxins or NAD(P)H:quinone oxidoreductase 1 (NQO1)-directed prodrugs, respectively. We have studied the structure–activity relationships of four novel indolequinone analogues with regard to one- and/or two-electron activation. Single-electron metabolism was achieved by exposing the human carcinoma cell line T47D to each agent under hypoxic conditions, whilst concerted two-electron metabolism was assessed by stably expressing the cDNA for human NQO1 in a cloned cell line of T47D. The C-3 and C-5 positions of the indolequinone nucleus were modified to manipulate reactivity of the reduction products and the four prodrugs were identified as NQO1 substrates of varying specificity. Two of the four prodrugs, in which both C-3 and C-5 groups remained functional, proved to be NQO1-directed cytotoxins with selectivity ratios of 60- to 80-fold in the T47D (WT) versus the NQO1 overexpressing T47D cells. They also retained selectivity as hypoxic cytotoxins with oxic/hypoxic ratios of 20- to 22-fold. Replacement of the C-3 hydroxymethyl leaving group with an aldehyde group ablated all selectivity in air and hypoxia in both cell lines. Addition of a 2-methyl group on the C-5 aziridinyl group to introduce steric hinderance reduced but did not abolish NQO1-dependent metabolism. However, it enhanced single-electron metabolism-dependent DNA cross-linking in a manner that was independent of cytotoxicity. These data demonstrate that subtle structure–activity relationship exists for different cellular reductases and under certain circumstances distinct forms of DNA damage can arise, the cytotoxic consequences of which can vary. This study identifies a candidate indolequinone analogue for further development as a dual hypoxia and NQO1-directed prodrug.

Keywords: Indolequinone; NQO1; Enzyme-directed; Hypoxic cytotoxin; Bioreduction; Mitomycin C (MMC)

1. Introduction

Mitomycin C (MMC; **1**) is the prototypic quinone antineoplastic agent, requiring bioactivation to produce reactive alkylating species that can covalently bind DNA [1–3]. The biochemical mechanisms by which MMC and related indolequinones generate their active species have been elucidated and this has guided the development of structural analogues [4–10]. Of these, certain indolequinones (general structure **2**) and benzoquinones (e.g. 2-hydroxymethyl-3,6-diaziridinyl-5-methyl-1,4-benzoquinone, RH-1; **3**) can act as prodrug substrates for the reductive enzyme NQO1 (DT-diaphorase), a desirable feature since NQO1 is present in high levels in many tumours relative to normal

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Abbreviations: NQO1, NAD(P)H:quinone oxidoreductase 1; HQ, hydroquinone; SQ, semiquinone; SSB, single strand break; WT, wild type; x-links, cross-links.

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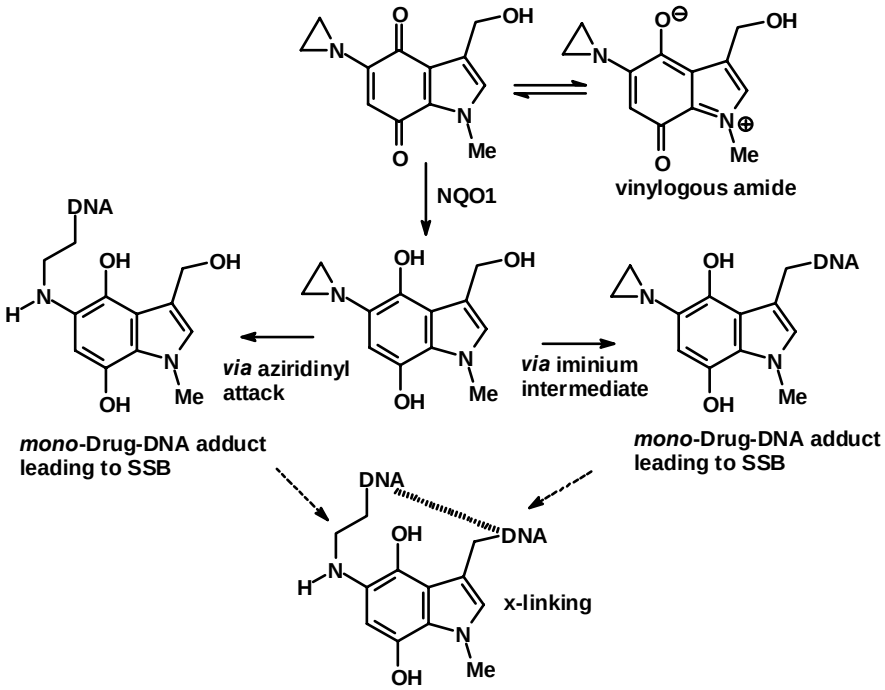


Scheme 1. Proposed mechanism of alkylation of compound **4**.

tissues [11,12]. This enzyme-directed approach to rational drug design is intended to focus chemotherapeutic activity to sites of neoplastic disease [13].

We have previously identified the indolequinone **4** (5-aziridinyl-3-hydroxymethyl-1-methylindole-4,7-dione) as an effective hypoxic cytotoxin *in vitro* by virtue of the efficient oxygen-inhibition of single-electron metabolism [14]. In this study, it was observed that two reactive groups at the C-3 (hydroxymethyl) and C-5 (aziridinyl) positions of structure **2** was sufficient for retention of both potency and hypoxia-selectivity, providing an analogue superior to compounds possessing a 2-hydroxypropenyl group (**5**, 5-aziridinyl-3-hydroxypropenyl-1-methylindole-4,7-dione) in place of the 3-hydroxymethyl (**4**), or retaining both C-2 and C-3 substituents on the indole ring (3-hydroxymethyl-5-aziridinyl-1-methyl-2-[1*H*-indole-4,7-dione]prop-2-en-1-ol, EO9; **6**).

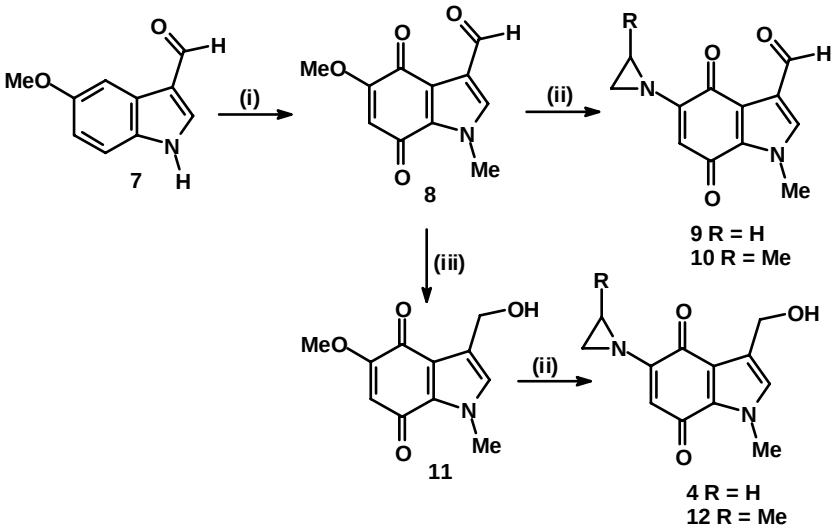
We postulated that analogue **4** may retain its substrate specificity for NQO1 and its selectivity and potency could be manipulated by modifying the nature of the functional groups at C-3 and C-5, which together are considered sufficient to provide a bis-alkylating species upon enzymatic reduction (Scheme 1). The cytotoxic HQ of **4** may be formed via NQO1-mediated two-electron reduction, leading to the formation of an iminium intermediate following a Michael-type elimination of the hydroxyl-leaving group. In conjunction with the 5-aziridinyl function a potential bis-alkylating species is generated. It was considered that (i) the absence of an efficient C-3 leaving group (LG) would prevent iminium intermediate formation, and (ii) the introduction of steric hindrance at the C-5 reactive centre would lower reactivity and thus modify prodrug selectivity [15–18]. To test these hypotheses the C-3 and C-5 groups of **4** were modified, both independently and simultaneously. The C-3 hydroxymethyl was replaced with an aldehyde group whilst the C-5 aziridinyl was substituted for a more bulky 2-methylaziridinyl moiety.

In order to determine the influence of these modifications upon NQO1-dependent metabolism, the indolequinone analogues were first evaluated in a cell-free system for their ability to act as substrates for NQO1. Next, each analogue was assessed for aerobic cytotoxicity (20% O_2, 5% CO_2) against the human breast tumour cell line T47D, using an isogenic cell line pair, WT and NQ-1, the latter of which was engineered to express high levels of functional NQO1. Then, analogue exposures were repeated under conditions of oxygen deprivation (95% N_2, 5% CO_2). MMC was included in the cytotoxicity experiments since it is a clinically relevant compound. Finally, analogues **4** and **12**, identified as dual NQO1 prodrugs and hypoxic cytotoxins, were evaluated by quantitative determination of DNA SSBs and DNA x-links in the T47D WT and NQO1 overexpressing cells under aerobic and hypoxic conditions.

2. Materials and methods

2.1. Chemical synthesis

As depicted in Scheme 2, the intermediate 3-formyl-5-methoxy-1-methylindole-4,7-dione (**8**) was synthesised in four steps starting from 5-methoxyindole-3-aldehyde (**7**) as by Naylor *et al.* [4]. Michael-type substitution by aziridine



Reagents and conditions : (i) 4 steps, References 4 and 5 ; (ii)aziridine or 2-methylaziridine, 1.5hr, rt; (iii) NaBH4, THF, 3 hr, rt.

Scheme 2. Chemical synthesis of 5-aziridinyl-3-substituted-1-methylindole-4,7-diones.

or 1-methylaziridine gave 5-aziridinyl- and 5-(1-methylaziridinyl)-3-formyl-1-methylindole-4,7-dione (**9** and **10**, respectively) in high yields. Reduction of the intermediate **7** with sodium borohydride gave the corresponding 3-alcohol (**11**). Similarly, treatment of either aziridine or methyl aziridine gave the corresponding 5-aziridinyl or 5-methylaziridinyl-3-hydroxymethyl-1-methylindole-4,7-dione (**4** and **12**) in reasonable yields.

NMR data were recorded on a Brucker JNMR-EX300 300 MHz spectrometer (270.05 MHz ^{1}H; 67.8 MHz ^{13}C). Tetramethylsilane (TMS) was used as an internal standard for ^{1}H NMR samples dissolved in $CDCl_3$ or DMSO-d_6. Multiplicities are indicated by s (singlet), brs (broad singlet), brd (broad doublet), d (doublet), dd (doublet of doublets), td (triplet of doublets), t (triplet), dt (doublet of triplets), q (quartet), m (multiplet), Ar (aromatic). High-resolution mass spectra (HRMS) were recorded on a Fisons VG Trio 2000 mass spectrometer. Melting points were determined using a Gallenkamp melting point apparatus. Thin layer chromatography (TLC) was performed on Merck 60F_{254} silica TLC aluminium plates. Flash column chromatography was performed using Prolabo silica gel 60 (35–75 μm). Chemicals were obtained either from Aldrich Chemical Co. or Lancaster Synthesis Ltd.

The intermediates 3-formyl-5-methoxy-1-methylindole-4,7-dione (**8**), 3-hydroxymethyl-5-methoxy-1-methylindole-4,7-dione (**11**), 5-aziridinyl-3-hydroxy-methyl-1-methylindole-4,7-dione (**4**) and 3-hydroxymethyl-5-methylaziridinyl-1-methylindole-4,7-dione (**12**) were synthesised as described previously [4,5].

2.1.1. 5-Aziridinyl-1-formyl-1-methylindole-4,7-dione (*9*)

To the aldehyde **8** (100 mg, 0.46 mmol) was added neat aziridine (2 mL, 46 mmol). The mixture was stirred for 2 hr at room temperature and then evaporated *in vacuo* to remove excess aziridine. The product was recrystallised from EtOAc to give the above named compound **9** as yellow crystals (72 mg, 68%): m.p. 242–244°. ^{1}H NMR ($CDCl_3$) δ: 6.82 (s, 1H, *H*-2), 5.67 (s, 1H, *H*-6), 5.26 (s, 2H, CH_2OH), 3.94 (s, 3H, OCH_3), 3.82 (s, 3H, N-CH_3), 2.08 (s, 3H, OCOCH_3), ^{13}C NMR ($CDCl_3$) δ: 187.0 (*C*HO), 179.7 (*C*-7), 179.4 (*C*-4), 158.1 (*C*-3), 131.8 (*C*-4a), 131.1 (*C*-7a), 123.7 (*C*-2), 123.6 (*C*-6), 117.0 (*C*-5), 37.4 (N-*C*H_3), 28.2 (2Az-*C*H_2), *m/z* 231 (M^+, 100%).

2.1.2. 1-Formuyl-5-[2-(methyl)laziridin-1-yl]-1-methylindole-4,7-dione (*12*)

2-Methylaziridine (100 mg, 0.46 mmol) was stirred with the aldehyde **8** (2 mL, 46 mmol) for 4 hr at room temperature. The mixture was condensed to dryness and the product recrystallised from EtOAc to give **12** as orange crystals (39 mg, 35%): m.p. 180–182°. ^{1}H NMR ($CDCl_3$) δ: 10.42 (s, 1H, C*H*O), 7.42 (s, 1H, *H*-2), 5.89 (s, 1H, *H*-6), 4.01 (s, 3H, N-CH_3), 2.12 (m, 2H, Az-*H*), 1.45 (d, 3H, $J = 5.5$ Hz, Az-CH_3), ^{13}C NMR ($CDCl_3$) δ: 186.6 (*C*HO), 179.4 (*C*-7), 179.1 (*C*-4), 157.8 (*C*-3), 131.3 (*C*-4a), 130.8 (*C*-7a), 123.2 (*C*-2), 123.2 (*C*-6), 115.8 (*C*-5), 37.0 (N-*C*H_3), 36.4 (Az-*C*H_3), 34.7 (Az-*C*H_2), 17.6 (Az-*C*H), *m/z* 274 (M^+, 92%).

2.1.3. Rates of reduction by NQO1

The ability of compounds to act as substrates for recombinant human NQO1 was determined by measuring the reduction of cytochrome *c* at 550 nm on a Beckman DU 650 spectrophotometer, details of which are described elsewhere [19,20]. The recombinant human NQO1 was purified by Chen *et al.* [21]. Briefly, all enzyme assays contained cytochrome *c* (70 μM), NADH (2 mM), purified

NQO1 (0.48 μg), and the test compound (25 μM) in Tris–HCl buffer (50 mM, pH 7.4) containing 0.14% bovine serum albumin in a final volume of 1 mL. The final concentration of DMSO in the cuvette was 0.5%. Reactions were carried out at room temperature and started by the addition of NADH. Rates of reduction were calculated from the initial linear part of the reaction curve (30 s) and results were expressed in terms of μmol cytochrome *c* reduced/min/mg protein using a molar extinction coefficient of 21.1 mM^{-1} cm^{-1} for cytochrome *c*. All reactions were carried out in triplicate and background rates of cytochrome *c* reduction (in the presence of 0.5% DMSO) were subtracted from test results.

2.1.4. Vector construction

The bistronic empty vector pEFIRES-P (F373) and derivative vector encoding NQO1 (pEFIRES-NQO1 [F397]), were a kind gift from Steve Hobbs and have been described elsewhere [22,23].

2.1.5. Transfection and clonal selection of T47D cells

5×10^6 cells in exponential growth were transfected with 10 μg of linearised pEFIRES-NQO1 DNA by electroporation (EasyjecT+, EquiBio Ltd.) in Optimix® buffer (EquiBio Ltd.) using manufacturer's recommended protocols. Cells were plated at low density and 48 hr later were exposed to 5 μg mL^{-1} puromycin. A number of puromycin resistant clones were isolated approximately 14 days later, expanded and analysed for the appropriate enzyme activity. NQO1 clones (NQ-1) were selected for use in these studies on the grounds of high expression of NQO1 activity, which was stably maintained in the absence of puromycin selection.

2.1.6. Determination of P450R and NQO1 enzyme activities

Cells were lysed in ice-cold nuclear buffer A (10 mM HEPES/KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 0.05 mM DTT, pH 7.4) by sonication. Lysate protein concentrations were determined by Bradford analysis using bovine serum albumen (BSA) as a protein standard. Both NQO1 and P450R assays have been described elsewhere [24,25]. P450R activity was determined as the lysate component able to catalyse the NADPH-dependent reduction of cytochrome *c* in a reaction mixture containing 100 μM NADPH, 1 mM KCN, 50 μM cytochrome *c* and 0.1–0.3 mg lysate protein in 100 mM phosphate buffer (pH 7.6). Control aliquots contained no NADPH. NQO1 activity was determined as the dicoumarol-inhibitable component of NADH-dependent reduction of cytochrome *c* in a reaction mixture containing 500 μM NADH, 70 μM cytochrome *c*, 0.14% (w/v) BSA, 0.1–0.3 mg lysate protein in 50 mM phosphate buffer (pH 6.8) using 10 μM menadione as an NQO1 substrate. One hundred micromolar dicoumarol was added to controls. Cytochrome *c* reduction was monitored at 550 nm by spectrophotometric assay and enzyme activity estimated from the difference between initial reduction rates in the sample and relevant control using a molar extinction coefficient of 21 mM^{-1} cm^{-1} for reduced cytochrome *c*.

2.1.7. Drug sensitivity

For anoxic exposures, cells were transferred as a centrifuged pellet into an anaerobic chamber (Sheldon Manufacturing Inc., Bactron Anaerobic/Environmental Chamber) then re-suspended to the appropriate volume using anoxic medium and seeded at 6000 cells/well in a 150 μL volume into replicate wells of plastic 96-well plates that had been preconditioned in the anoxic chamber for 72 hr. Cells were allowed to attach for approximately 2 hr. Drugs were made up at four times the required concentration in anoxic medium and 50 μL added per well in triplicate for each experiment. Drug exposure lasted 3 hr, then drug-containing medium was replaced with fresh medium plates removed from the hypoxic chamber and incubated in air for 4 days prior to cytotoxicity being determined by use of the MTT assay [26]. The procedures for aerobic exposures to drugs were identical to these detailed above except that all manipulations were carried out under ambient conditions and drugs exposures were carried out under 95% air plus 5% CO_2.

2.1.8. Comet assays

Drug treatments conducted under both aerobic and hypoxic conditions were carried out essentially as for cytotoxicity studies described above except that cells were seeded at 20,000 cells/well in 24-well plates containing glass inserts. Following drug treatment for 3 hr all of the cells were trypsinised, suspended in 0.5 mL of ice-cold fresh medium and then transferred into plastic 24-well dishes prior to embedding in agarose. For the cross-linking studies, the treated and control samples were chilled on ice to prevent any repair and then irradiated to 20 Gy in a Caesium-137 source (0.4 Gy min^{-1}). The control, unirradiated, non-drug treated cells were maintained on ice in the same manner as treated samples.

Glass microscope slides, frosted at one end, were pre-coated with 1% normal agarose in distilled water. These slides were allowed to air dry overnight prior to use. A 1% low melting point agarose (LMP) mixture in PBS was melted and held at 45°. One milliliter of LMP was then added to 0.5 mL of cell suspension on ice and the resultant mixture was pipetted onto a pre-coated glass microscope slide and allowed to gel for 1–2 min before being transferred to an ice tray. The cooling in ice was to inhibit repair. The slides were then immersed in ice-cold lysing solution (100 mM EDTA, 10 mM Tris–HCl, 1% Triton X-100, 1% DMSO, 2.5 M NaCl) for 1 hr. After lysis, the slides were washed by immersion in fresh double distilled water for 15 min. This process was repeated three times.

The slides were then placed onto a flat bed electrophoresis tank and covered (5–6 mm) with alkali unwinding solution (50 mM NaOH, 1 mM EDTA buffered to pH

12.5). The slides were left under subdued lighting for 45 min to allow the DNA to unwind before being subjected to electrophoresis at 0.6 V cm^{-1} for 25 min. Each slide was rinsed with 2 × 1 mL of 0.4 M Tris–HCl, pH 8.0 and allowed to dry in air. The dried slides were then rehydrated for 20 min with double distilled water, flooded with 2 × 1 mL of a 10,000-fold dilution of SYBR gold in water (Molecular Probes), and stained for 30 min in the dark. The slides were then immersed in 1 L of double distilled water for 30 min to reduce excess background staining. The slides were cover slipped and then examined at 250× magnification under an epifluorescent microscope (Zeiss-Jenamed) using green light from a 50 W mercury source with a 580 nm reflector and a 590 nm barrier filter set. Images were captured using an attached Sony HAD-1 interline CCD camera and Komet software analysis package (Kinetic Imaging). Twenty-five images from each of two duplicate slides from triplicate experiments were captured and analysed. The % tail DNA and % DNA cross-linked were calculated as previously described by Ward *et al.* [27].

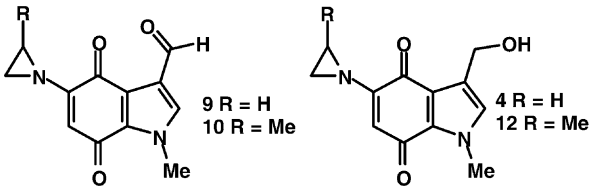
3. Results and discussion

All prodrugs were bioreduced by NQO1 (Table 1), ranking in the order **9** > **4** ≈ **10** > **12**. The addition of a methyl group (compounds **10** and **12**) to the 5-aziridine, reduces the rate of reduction by NQO1 [19]. In contrast, the replacement of 3-hydroxymethyl with a 3-aldehyde group significantly enhances reduction by NQO1 [28], suggesting an NQO1-mediated metabolic pathway. Thus modification of either the 3- or 5-substituents of the indole ring influenced reduction rates by NQO1, presumably reflecting changes in active site constraints. The structure–activity relationships between NQO1 and various quinone alkylating agents has proved complex and attempts to correlate rates of reduction to the two-electron reduction potentials have proved unsuccessful [29]. Active site constraints appear to contribute to the substrate preferences of NQO1, and the recent elucidation of the X-ray crystal structure of NQO1 has opened up the possibility of rationally predicting and designing quinone prodrugs as substrates for NQO1 [30,31].

Compounds **4** and **12** were 60-fold and 83-fold more potent, respectively, against the NQ-1 cell line when compared to MMC and analogues **9** and **10** (WT/NQ-1 data, Table 1) and hence are promising candidates for NQO1-directed bioreductive drug development. However, despite being adequate substrates for NQO1, neither **9** nor **10** displayed enhanced cytotoxicity as a consequence of NQO1 expression. The lack of NQO1-dependent sensitivity for **9** and **10** is consistent with the hypothesis that the absence of an LG at the C-3 position prevents formation of the iminium intermediate species, thus suppressing cross-link formation following bioreduction (see Scheme 1).

Compounds **4** and **12** were 20- and 22-fold more potent, respectively, under hypoxic compared to oxic conditions (HCR) in the parental cell line identifying both agents as hypoxic cytotoxins (Table 1). Notably, the NQ-1 cell line

Table 1
Toxicity data for the compounds in the WT and NQ-1 clone under both aerobic (air) and hypoxic (N_2) conditions



Drug	Cell line	IC_{50} (μM)		HCR[c] (air/N_2)	WT/NQ-1 (air)	Rates of reduction by NQO1[d]
		Air	N_2			
MMC	WT[a]	2.30 ± 2.00	0.75 ± 0.22	3.0	0.85	–
	NQ-1[b]	2.70 ± 0.13	1.01 ± 0.36	2.6		
9	WT[a]	1.33 ± 0.47	3.50 ± 2.50	0.38	0.82	75.5 ± 5.23
	NQ-1[b]	1.62 ± 0.37	2.54 ± 1.67	0.46		
10	WT[a]	1.89 ± 1.11	14.0 ± 7.50	0.14	0.51	39.2 ± 0.32
	NQ-1[b]	3.70 ± 1.80	27.8 ± 3.80	0.13		
4	WT[a]	11.20 ± 1.50	0.57 ± 0.34	19.6	62.2	42.1 ± 1.02
	NQ-1[b]	0.18 ± 0.13	0.025 ± 0.015	7.3		
12	WT[a]	54.8 ± 17.0	2.46 ± 2.40	22.2	83.0	12.8 ± 0.56
	NQ-1[b]	0.66 ± 0.30	0.418 ± 0.193	1.6		

[a] NQO1 activity: 31.6 mmol cytochrome *c* reduced min^{-1} mg^{-1} protein; P450R activity: 7.41 mmol cytochrome *c* reduced min^{-1} mg^{-1} protein.
[b] NQO1 activity: 9620.7 mmol cytochrome *c* reduced min^{-1} mg^{-1} protein; P450R activity: 8.37 mmol cytochrome *c* reduced min^{-1} mg^{-1} protein.
[c] HCR is the hypoxic cytotoxicity ratio.
[d] mmol cytochrome *c* reduced min^{-1} mg^{-1} protein.

Table 2
DNA cross-linking and % single strand breaks for compounds **4** and **12**

Drug	T47D cell line	Toxicity IC_{50} (μM)		X-linking (%)		SSB (%)	
		Air	N_2	Air	N_2	Air	N_2
4	WT	11.2 ± 1.5	0.52 ± 0.34	−4.41 ± 14.87	8.42 ± 1.78	22.59 ± 20.21	48.34 ± 17.91
	NQ-1	0.18 ± 0.13	0.025 ± 0.015	26.14 ± 0.80	50.71 ± 9.08	35.87 ± 14.31	18.09 ± 12.21
12	WT	54.8 ± 17.0	2.46 ± 2.4	−12.79 ± 20.80	41.25 ± −7.12	26.96 ± 21.88	18.02 ± 12.31
	NQ-1	0.66 ± 0.30	0.42 ± 0.19	9.93 ± 2.19	17.18 ± 5.90	20.24 ± 20.32	11.72 ± 11.41

SSB: single strand breaks.

was further sensitised to **4** under hypoxia, suggesting that mixed-reduction pathways were coordinately activating the prodrug. The apparently additive nature of one and two-electron reduction on cytotoxicity was not apparent for compound **12**. MMC showed modest selectivity for hypoxic conditions (HCR = 3), consistent with literature values [26,32–34].

Compounds **9** and **10** showed greater toxicity under aerobic as compared to hypoxic conditions, which may be due to inhibition of redox cycling. Moreover, the one-electron reduction of **9** and **10** under hypoxic conditions cannot provide a cytotoxic (cross-linking) species due to the absence of iminium intermediate formation (see Scheme 1), and hence the resultant toxicity from reactive oxygen species (ROS) production can thus be distinguished. The remaining toxicity is likely to be a result of the alkylating potential of the 5-aziridinyl substituents, although in the absence of reduction, the 5-methylaziridine may be moderately deactivated by delocalisation of the quinone via a vinylogous amide (Scheme 1) [35,36].

The lead compounds **4** and **12** were evaluated for their cross-linking abilities in the WT and NQ-1 cell lines under aerobic and hypoxic conditions. In air, neither compound produced detectable DNA x-links in the WT (parental) cell line, but a moderate level of SSBs were observed. Exposure of the NQO1 over-expressing line (NQ-1) to **4** (in air), resulted in extensive DNA x-link formation (26.1%) and a 1.6-fold increase in SSBs. For compound **12**, the NQ-1 cell line incurred fewer x-links (17.2%) and less SSBs (0.75-fold) than WT (Table 2).

Under conditions of hypoxia, surprisingly divergent patterns of DNA x-linking were observed for **4** and **12**. In WT cells, **4** gave rise to 8.4% x-links, whereas **12** generated 5-fold more x-links (41.3%) under identical conditions yet was observed to be 5-fold less cytotoxic. In the presence of NQO1 overexpressing cells, the % of x-links generated by **12** under hypoxic is reduced substantially (17.2%), or rather was restored to a level consistent with its cytotoxic effects. This incongruity is apparent when the correlation between % x-link formation and IC_{50} value is examined. Irrespective of oxygenation, the quantity of DNA x-links was consistent with IC_{50} values obtained for both compounds in either cell line ($R^2 = 0.91$; Fig. 2). The discrepancy between % DNA x-links and cytotoxicity is not apparent for NQO1 mediated activation of **12** under hypoxic conditions, being a manifestation of NQO1-independent hypoxic metabolism (Fig. 1). In the case of compound **4**, the presence of hypoxia and NQO1 raises the % of x-linking species beyond that seen for either alone (50.7%), suggesting both one and two-electron reduction activation pathways are operating in a parallel and complementary fashion [14].

Under conditions of oxygen deprivation, exposure of WT cells to **12** produces extensive x-links (41.3%). This is considered to be due to bioactivation by endogenous single-electron reductases, forming a SQ species. The

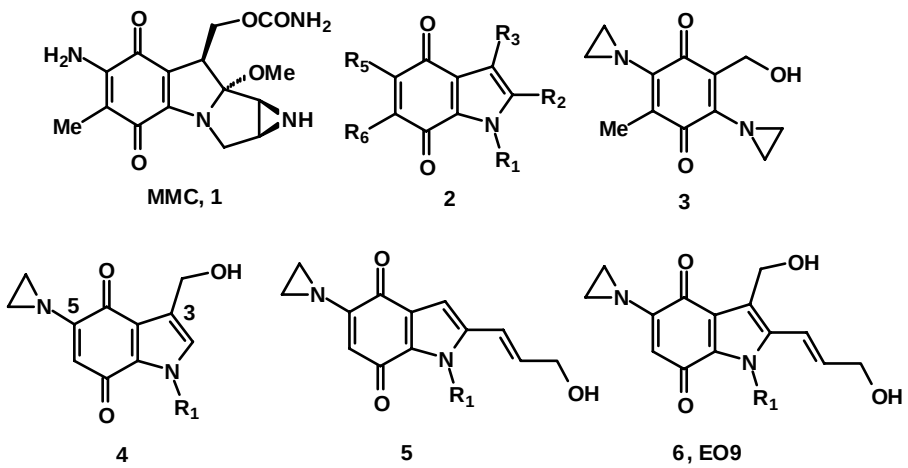


Fig. 1. Cytotoxic quinone compounds.

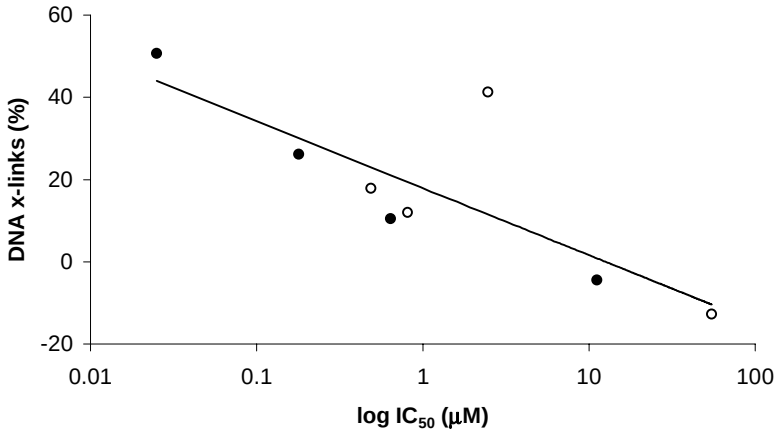


Fig. 2. Relationship between IC_{50} of **12** (○) and **4** (●) and the incident of DNA cross-linking in the T47D and NQ-1 cell lines under aerobic and hypoxic conditions.

SQ species generated by **12** is likely to be more stable than **4**, due to the presence of the electron donating effects of the methyl group of the aziridinyl moiety (inductive effect). The more stable SQ of **12** would result in extensive x-links via increased drug-DNA bond formation from the DNA strands as compared to **4**, whose cross-linking abilities are primarily due to the formation of bis-alkylating species (as depicted in Scheme 2). However, the % of x-links is in excess of that expected from the correlation between IC_{50} values and % x-links under all other conditions (Fig. 2). Consequently, **12** is approximately 50-fold less cytotoxic than predicted due to DNA x-linking, but only when single-electron reduction predominates (since direct (two-electron) formation of the HQ of **12** by NQO1 gives rise to the predicted level of DNA x-link damage). Thus, **12** appears to generate a large, but rapidly repaired burden of x-links from its SQ but not from its HQ species. No correlation was observed between SSBs and sensitivity to these agents, corroborating the conclusion that x-links are the relevant cytotoxic lesion.

4. Conclusion

This study suggests that minimal structural features within the indolequinone bioreductive class of compounds may result in substantial variation in activation and subsequent toxicity with respect to the bioreductive enzyme NQO1. However, as stated in our earlier work [19], NQO1 plays a major role in the activation and toxicity under aerobic conditions. The role of NQO1 in the bioactivation process of indolequinone (and other) bioreductive prodrugs is complex especially when comparing their activation processes under hypoxic conditions and with other reductive enzymes, which is the subject of ongoing studies in our laboratories. However, NQO1 does have a defining role in the activation of bioreductive prodrugs, especially under aerobic conditions, and this study has provided potential candidates for NQO1-directed prodrugs for cancer therapy.

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