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# Modifying rates of reductive elimination of leaving groups from indolequinone prodrugs: a key factor in controlling hypoxia-selective drug release

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**Abstract**

3-(4-Methylcoumarin-7-yloxy)methylindole-4,7-diones were synthesised as model prodrugs in order to investigate the correlation between rates of reductive elimination from the (indolyl-3-yl)methyl position with reductive metabolism by hypoxic tumor cells and NADPH: cytochrome P450. Rates of elimination of the chromophore/fluorophore (7-hydroxy-4-methylcoumarin) following one-electron reduction of indolequinones to their semiquinone radicals ($Q^{\bullet -}$) was measured by pulse radiolysis utilising spectrophotometric and fluorometric detection. Incorporation of a thienyl or methyl substituent at the (indol-3-yl)CHR-position (where R = thienyl or methyl adjacent to the phenolic ether linking bond) significantly shortened the half-life of reductive elimination from 87 to 6 and 2 ms, respectively. Elimination from the methyl substituted analogue can thus compete effectively with the reaction of the semiquinone radical with oxygen at levels typically present in tumours (half-life ~1.8 ms at 0.5% $O_2$). Chemical kinetic predictions were confirmed by metabolism in breast tumour MCF-7 cells between 0–2.1% $O_2$. Rates of reductive release of the fluorophore from the non-fluorescent parent indolequinones (R = H, Me, thienyl) were similar under anoxia (~1.7 nmol coumarin $min^{-1}$ mg $protein^{-1}$) reflecting the similarity in one-electron reduction potential. Whereas coumarin release from the indolequinone (R = H) was completely inhibited above 0.5% $O_2$, the enhanced rate of reductive elimination when R = thienyl or Me increased the metabolic rate of release to ~0.35 and 0.7 nmol coumarin $min^{-1}$ mg $protein^{-1}$, respectively at 0.5% $O_2$; complete inhibition occurring by 2.1% $O_2$. Similar 'oxygen profiles' of release were observed with NADPH: cytochrome P450 reductase. In conclusion, it is possible to modify rates of reductive elimination from indolequinones to control the release of drugs over a range of tumour hypoxia. 

*Keywords:* Indolequinone; 7-Hydroxy-4-methylcoumarin; Reduction; Chemical kinetics; Semiquinone radical; Pulse radiolysis; MCF-7 breast tumour cells; NADPH: cytochrome P450 reductase; Hypoxia

## 1. Introduction

There has been continued interest in the design of indolequinone-based bioreductively activated cytotoxins which target hypoxia and/or exploit the over-expression of reductase enzymes present in tumors [1–4]. A number of enzymes are known to be involved in the reduction of quinone compounds including those which catalyse one-electron reduction *via* the semiquinone radical ($Q^{\bullet -}$), such as NADPH: cytochrome P450 reductase and b5 reductase, and also NQO1 (DT-diaphorase) which performs two-electron reduction *via* hydride transfer to form the hydroquinone ($QH_2$) [5–10]. Drug activation by the one-electron reducing enzymes involves oxygen-sensitive reduction chemistry which protects normal tissues whereas reduction by NQO1 is oxygen independent. A particularly pertinent example of these two activating pathways is that of the bioreductive alkylating agent EO9 (Fig. 1, **1**) which has

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*Abbreviations:* EO9, 5-aziridinyl-1-yl-3-hydroxymethyl-2-(3-hydroxypropenyl)-1-methyl-1*H*-indole-4,7-dione; EORTC, European Organisation for Research and Treatment of Cancer; NQO1, DT-diaphorase, NAD(P)H: quinone acceptor oxidoreductase, EC 1.6.99.2.

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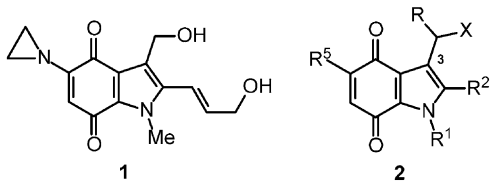
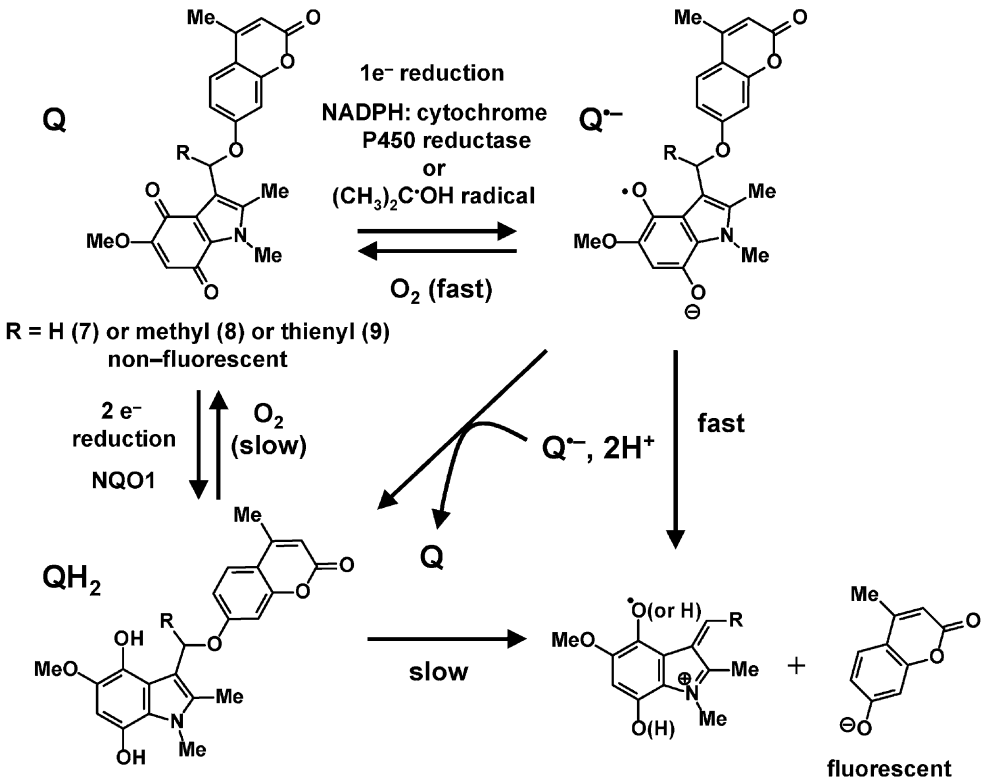


Fig. 1. Structures of bioreductive indolequinones: compound **1** =EO9; compound **2** =substituted indolequinone where X = leaving group.

been evaluated in phase clinical trials for the treatment of solid tumors by the EORTC [11–13]. The importance of NQO1 in the metabolism of EO9 to cytotoxic species has come from studies utilising either tumour cell lines which express high levels of NQO1 or ones which over-express human recombinant NQO1 [14–17]. NADPH: cytochrome P450 reductase will also reduce a range of bioreductive quinone compounds. Recent work by Saunders and co-workers involving transfection and over-expression of the reductase has implicated this enzyme in the cellular reduction of EO9, especially under hypoxia [18]. Work on indolequinones including derivatives of EO9 has begun to address the question of how to confer either substrate specificity for NQO1 or to achieve hypoxia-selective activation. Indolequinones bearing leaving groups at the (indol-3-yl)methyl position (e.g. Fig. 1, **2**) may well be good substrates for NQO1 but reductive fragmentation appears to rapidly inhibit enzyme activity [19–21]. Chemical kinetic studies both on EO9 and its derivatives have also demonstrated that the intermediate $Q^{\bullet -}$ radical is extremely reactive toward oxygen, which has important implications for the ability of these compounds to target all but extremely hypoxic tumours [22,23].

Indolequinones are capable of eliminating a range of leaving groups, coupled through various linkers, from the (indol-3-yl)methyl position following one- and two-electron reduction to the semiquinone radical and hydroquinone, respectively [22,24,25]. This work highlighted a shift in emphasis from 'direct' activation to cytotoxic species, to an alternative bioactivation route where reduction of an indolequinone initiates fragmentation of a linking bond and release of a bioactive agent, the biological activity of which is masked in the prodrug form [26]. From a chemical kinetic viewpoint it was predicted that a key factor in the design of prodrugs would be the ability to control rates of reductive fragmentation relative to the reactivities of the intermediate $Q^{\bullet -}/QH_2$ species with oxygen [22]. By incorporating stabilising substituents at the indolyl carbinyl position (e.g. Fig. 1, **2**; substituent R = methyl or thienyl) it was possible to significantly enhance the rate of reductive fragmentation of phenolic ether linking bonds directly from the $Q^{\bullet -}$ radical [27].

In this study we have synthesised 3-(4-methylcoumarin-7-yloxy)methylindole-4,7-diones in which fluorescence of the model leaving group (7-hydroxy-4-methylcoumarin) is




Scheme 1. Mechanisms for the reductive elimination of the fluorophore 7-hydroxy-4-methylcoumarin from the (indol-3-yl)methyl position of the non-fluorescent indolequinones (**7**, **8** and **9**), where the substituent R = H, Me or thienyl, respectively.

completely quenched when coupled to the indolequinone through a phenolic ether bond (Scheme 1). The indolequinones were reduced in a controlled and quantifiable manner using radiolytically-produced radicals which result in semiquinone formation and thus mimic reductive activation by NADPH: P450 reductase. Activation by NQO1 can also be modelled since semiquinone radicals disproportionate to form the hydroquinone. The effect of methyl or thienyl substitution at the (indol-3-yl)methyl position on the rate of reductive elimination of the chromophore/fluorophore was determined by pulse radiolysis. The impact of modifying rates of reductive elimination of the coumarin on the inhibition by oxygen of metabolic release by NADPH: cytochrome P450 reductase and in tumor cells was examined.

## 2. Materials and methods

### 2.1. Chemicals

Supersomes™ containing human NADPH: P450 reductase were obtained from Cambridge Bioscience *via* the Gentest Corporation. 7-Hydroxy-4-methylcoumarin, thallium chloride ($TlCl_3 \cdot 4H_2O$), cytochrome *c* (partially acetylated), β-NADPH and 2-propanol were obtained from the Sigma–Aldrich.

### 2.2. Synthesis

#### 2.2.1. General procedures

*2.2.1.1. NMR spectra.* *J* values are given in Hz. Elemental analyses were determined by MEDAC Ltd. and all compounds characterised by HRMS were chromatographically homogeneous. Solutions in organic solvents were dried by standard procedures, and dimethylformamide, toluene and tetrahydrofuran were anhydrous commercial grades. Silica gel for flash column chromatography was Merck Kieselgel 60 H grade (230–400 mesh) or Matrex silica 60.

#### 2.2.2. General method for Mitsunobu reaction

*2.2.2.1. The synthesis of the following indolequinone alcohols has been previously described in the literature.* 3-Hydroxymethyl-5-methoxy-1,2-dimethylindole-4,7-dione (**3**) [19,24], 3-(1-hydroxyethyl)-5-methoxy-1,2-dimethylindole-4,7-dione (**4**) and 3-(1-hydroxy-1-(2-thienyl)-methyl)-5-methoxy-1,2-dimethylindole-4,7-dione (**5**) [27]. The synthesis of the 3-isopropoxymethyl indolequinone (**6**) has also been described previously [24]. The 3-(4-methylcoumarin-7-yloxy)methylindole-4,7-diones (**7**, **8** and **9**) were prepared from the corresponding 3-hydroxymethylindolequinones (**3**, **4** and **5**), respectively with 7-hydroxy-4-methylcoumarin using the Mitsunobu reaction. Diethyl azodicarboxylate (3 eq.) was added to a solution of the 3-hydroxymethylindolequinone (**3**) or 3-(1-hydroxyethyl)-indolequinone (**4**) or 3-(1-hydroxy-1-(2-thienyl)indolequinone (**5**) (0.2 mmol, 1 eq.), triphenylphosphine (2.5 eq.) and 7-hydroxy-4-methylcoumarin (2.5 eq.) in THF (10 mL). The solution was stirred for 2 hr. The solvent was removed *in vacuo* and the residue dissolved in ethyl acetate and washed with sodium hydroxide (1 M), hydrochloric acid (1 M), water, dried ($MgSO_4$) and concentrated.

#### 2.2.3. 5-Methoxy-3-(4-methylcoumarin-7-yl)-oxymethyl-1,2-dimethylindole-4,7-dione (7)

The crude product was purified by column chromatography (5% ethyl acetate/dichloromethane elution) and recrystallised (ethyl acetate/hexane) to yield the title compound as an orange solid (28%), mp: 241–243°; (found: $M + NH_4$, 411.1559. $C_{22}H_{23}N_2O_6$ requires 411.1556); IR (KBr) 2955, 2848, 2919, 1727, 1716, 1681, 1642, and 1610 $cm^{-1}$; UV (DMF) 456 (ε 1763), 332 (12233), 316 (14144), 292 (15798) nm; $^1H$ (300 MHz; $CDCl_3$) δ: 7.48 (1H, d, $J = 8.7$ Hz, ArH), 6.94 (1H, dd, $J = 8.7$, 2.5 Hz, ArH), 6.90 (1H, d, $J = 2.4$ Hz, ArH), 6.12 (1H, s, ArH), 5.63 (1H, s, 6-H), 5.34 (2H, s, $CH_2$), 3.90 (3H, s, OMe), 3.81 (3H, s, NMe), 3.38 (3H, s, Me), 2.31 (3H, s, Me); $^{13}C$ (100 MHz; $CDCl_3$) δ: 178.7, 178.1, 161.6, 161.3, 159.6, 155.2, 152.4, 137.9, 129.0, 125.6 (CH), 121.3, 116.0, 113.8, 112.4 (CH), 112.0 (CH), 106.7 (CH), 102.5 (CH), 61.0 ($CH_2$), 56.4 (OMe), 32.4 (NMe), 18.6 (Me), 9.85 (Me); MS (CI) *m/z* 411 ($M + NH_4$, 100%), 394 (30).

#### 2.2.4. 5-Methoxy-3-(1-(4-methylcoumarin-7-yl)oxy)-ethyl-1,2-dimethylindole-4,7-dione (8)

The crude product was purified by column chromatography (3:1 light petroleum: ethyl acetate elution) and recrystallised from ethyl acetate/hexane to yield the title compound as an orange solid (23%), mp: 213–215°, (found: $M + NH_4$ 425.1720. $C_{23}H_{25}N_2O_6$ requires 425.1712); IR (KBr) 3063, 2919, 2853, 1726, 1665, 1629, 1603 $cm^{-1}$; UV (DMF) 456 (ε 1750), 320 (14092), 284 (16572) nm; $^1H$ (300 MHz; $CDCl_3$) δ: 7.42 (1H, d, $J = 8.8$ Hz, ArH), 6.85 (1H, dd, $J = 8.8$, 2.5 Hz, ArH), 6.73 (1H, d, $J = 2.5$ Hz, ArH), 6.28 (1H, q, $J = 6.5$ Hz, CHMe), 6.08 (1H, d, $J = 1.1$ Hz, ArH), 5.64 (1H, s, 6-H), 3.85 (3H, s, OMe), 3.80 (3H, s, NMe), 2.34 (3H, d, $J = 1.1$ Hz, Me), 2.27 (3H, s, Me); $^{13}C$ (100 MHz; $CDCl_3$) δ: 178.5, 178.4, 161.3, 160.7, 159.6, 155.1, 152.4, 135.0, 128.6, 125.5 (CH), 122.8, 120.1, 113.6, 112.5 (CH), 111.9 (CH), 106.9 (CH), 103.0 (CH), 69.4 (CH), 56.5 (OMe), 32.0 (NMe), 22.1 (Me), 18.5 (Me), 10.4 (Me); MS (CI) *m/z* 425 ($M + NH_4$, 100%), 266 (40).

#### 2.2.5. 5-Methoxy-3-(1-(4-methylcoumarin-7-yl)oxy-1-(thiophen-2-yl))methyl-1,2-dimethylindole-4,7-dione (9)

The crude product was purified by column chromatography (4:1 hexane:ethyl acetate elution) and recrystallised from ethyl acetate to yield the title compound as an orange solid (40%), mp: 183–185°, $^1H$ (400 MHz; $CDCl_3$) δ: 7.54 (1H, s, ArH), 7.46 (1H, d, $J = 8.8$ Hz, ArH), 7.27 (1H, dd,

$J = 6.3$, 1.2 Hz, ArH) 7.07 (1H, d, $J = 3.5$ Hz, ArH), 6.96 (dd, 1H, $J = 8.8$, 2.5 Hz, ArH), 6.95 (1H, d, $J = 2.5$ Hz), 6.87 (1H, d, $J = 2.5$ Hz, ArH), 6.11 (1H, s, CHAr), 5.65 (1H, s, 6-H), 3.85 (s, 3H, OMe), 3.83 (s, 3H, NMe), 2.36 (1H, s, Me), 2.32 (1H, s, Me); $^{13}$C (100 MHz; $CDCl_3$) $\delta$: 178.5, 178.4, 161.3, 160.1, 159.6, 155.0, 152.4, 143.1, 135.9, 128.7, 126.8 (CH), 125.7 (CH), 125.7, 120.7, 120.2, 114.2, 112.6 (CH), 112.3 (CH), 106.9 (CH), 103.4 (CH), 70.8 (CH), 56.6 (OMe), 32.2 (NMe), 18.7 (Me), 11.0 (Me); MS (EI) *m/z* 476 ($M^+$, 4%), 300 (100), 176 (75); Anal. C; 65.68, H; 4.48, N; 2.91% $C_{26}H_{21}NO_6S$ requires C; 65.67, H; 4.45, N; 2.94%.

### 2.3. Radiation chemistry

#### 2.3.1. Pulse radiolysis measurements of fragmentation rates

Pulse radiolysis and fast-kinetic spectrophotometry/fluorimetry was used to monitor the formation and decay of $Q^{\bullet -}$ radicals and the elimination of the 4-methyl-7-hydroxycoumarin anion. The semiquinone radical and hydroquinone were generated by reduction of the parent indolequinones (60 μM) by the 2-propanol radical ($(CH_3)_2C^{\bullet}OH$) generated radiolytically in an $N_2O$-saturated 2-propanol/water mixture (50%, v/v) with potassium phosphate buffer (4 mM) at pH 7.4–9 [22,24]. Experiments were performed using a 6 MeV linear accelerator to generate an electron pulse (typically ~ 500 ns) as described previously [28]. The absorbed radiation dose per electron pulse (typically 5–35 Gy equivalent to 3–23 μM reducing radicals) was determined by the thiocyanate dosimeter [29]. Changes in absorbance were measured using a tungsten lamp and photodiode detector preceded by a single-pass monochromator. The corresponding change in fluorescence was measured using a filtered xenon lamp for excitation and a photomultiplier detector. A 350 nm UV narrow band filter (excitation $\lambda_{max} \sim 350 \pm 10$ nm for 7-hydroxy-4-methylcoumarin) was placed between the lamp and the radiolysis cell and a 400 nm cut-off filter between the cell and the monochromator/detector. For indolequinones where the disproportionation of semiquinone radicals could compete with the release of 7-hydroxy-4-methylcoumarin data-fitting software (FACSIMILE for Windows version 3.0.30, 2000 AEA Technology) provided estimates of individual rate constants from experimental data as described previously [27].

#### 2.3.2. Steady-state γ-radiolysis

Product analysis was carried out using indolequinone solutions (40 μM) which were saturated with $N_2O$ gas in gas-tight syringes before irradiation in a $^{60}$Co source. An absorbed dose of 1 Gy = 0.67 μM $(CH_3)_2C^{\bullet}OH$ radicals in $N_2O$-saturated 2-propanol/water (50%, v/v) as determined by ferricyanide reduction. A dose rate of 5.9 Gy min$^{-1}$ was used, as determined by Fricke dosimetry [30].

### 2.4. Product analysis by HPLC

Product analysis following γ-radiolysis of indolequinone solutions was performed by gradient separation on a 100 mm × 3.2 mm base-deactivated reverse-phase column (Hichrom RPB) at a flow rate of 1 cm$^3$ min$^{-1}$. The eluents were A: 5 mM $KH_2PO_4$, 5 mM $H_3PO_4$ and B: 75% acetonitrile, 25% water with a gradient of 35–95% for 5–8 min. Detection was at 292 nm using a Waters 616 pump, 717 detector, 996 photodiode array detector and Millennium data acquisition. Alternatively, detection was performed with a Waters 474 fluorescence detector ($\lambda_{ex} = 380$ nm, $\lambda_{em} = 450$ nm). Tumour cell lysates and enzyme incubates were deproteinised with 50%, v/v acetonitrile and centrifuged prior to injection of the resultant supernatant. Hydroquinones autoxidise back to indolequinones following the unavoidable introduction of oxygen during HPLC sampling [22,27].

### 2.5. Cell culture

Human breast adenocarcinoma MCF-7 cells provided by the European Collection of Cell Cultures were propagated in Dulbecco's modified Eagle's medium supplemented by 10% bovine foetal calf serum, 1% non-essential amino acids, glutamine, 7.5% sodium hydrogen carbonate and 100 units of penicillin, and 100 μg of steptomycin per mL. Cultures were grown in flasks (75 cm$^2$) until confluent, the medium was removed and the cells washed twice with ice-cold phosphate buffered saline (10 mL). Immediately prior to experimentation the cells were scraped into PBS (~$3 \times 10^6$ cell mL$^{-1}$), sonicated using an MSE Soniprep 150 ($3 \times 5$ s at 23 kHz), then stored on ice. The total protein concentration was determined using the Bio-Rad protein assay kit that measures absorbance at 595 nm of an acidic coomassie blue-protein complex [31]. Standard concentrations of bovine albumin were used to construct a calibration curve. The NADPH: cytochrome P450 reductase activity was measured spectrophotometrically as the NADPH-dependent reduction of acetylated cytochrome *c* using a standard protocol [32]. NQO1 specific activity was measured as the dicoumarol-sensitive reduction of 2,6-dichlorophenol-indophenol [33].

### 2.6. The oxygen-dependence of 4-methyl-7-hydroxycoumarin release by NADPH: cytochrome P450 reductase and in tumour cells

Rates of reductive fragmentation of the 3-(4-methylcoumarin-7-yloxy)methylindole-4,7-diones (**7**, **8** and **9**) catalysed by NADPH: cytochrome P450 reductase and in MCF-7 cell lysates was determined by monitoring the increase in fluorescence intensity due to the formation of 7-hydroxy-4-methylcoumarin anion. Measurements were made in a 10 mm fluorescence sealable cell (Hellma) using a Perkin-Elmer Model LS-50B Luminescence Spec-

trometer (excitation wavelength 356 nm, emission wavelength 449 nm with excitation and emission slits set at 2.5 nm). Under the experimental conditions used one fluorescence intensity unit equalled 1.71 nM 4-methyl-7-hydroxycoumarin. The oxygen sensitivity of coumarin release was determined in solutions saturated with nitrous oxide (deoxygenated with an Oxisorb cartridge) or mixtures thereof with oxygen (0.5–2.1%). Tumour cell lysates or P450 reductase supersomes plus the cofactor NADPH buffered at pH 7.4 were gassed for 10 min in the fluorescence cell thermostated at 37° and the reaction initiated by the introduction of indolequinones (gassed separately in DMSO, <0.5% final concentration) *via* gas-tight syringes. Typical incubates contained 5 μM indolequinone, 200 μM NADPH in 25 mM buffer with either 0.25 mL lysate or 10 μL reductase supersomes (nominal activity ~500 nmol $min^{-1}$ mg $protein^{-1}$ with no detectable cytochrome P450 activity) in a final volume of 2 mL. The incubate was gassed gently over the surface to provide gentle agitation and to prevent depletion of oxygen by redox-cycling of the indolequinones particularly at intermediate oxygen concentrations.

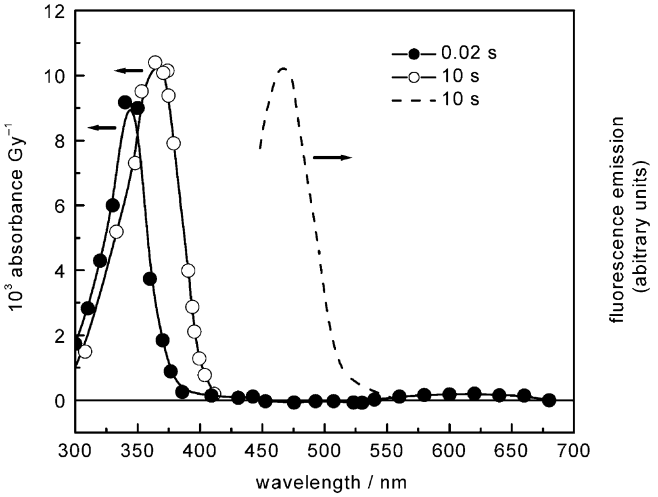



Fig. 2. Absorption and fluorescence spectra obtained on the reduction of indolequinone **7** (60 μM) by the $(CH_3)_2C^{\bullet}OH$ radical at pH 9.1: (●) absorbance after 200 μs due to semiquinone radical, (○) absorbance after 10 s due to 7-hydroxy-4-methylcoumarin anion, and (– – –) corresponding fluorescence after 10 s also ascribed to 7-hydroxy-4-methylcoumarin anion. All absorbances were normalised to a dose of 10 Gy corresponding to $[(CH_3)_2C^{\bullet}OH] \sim 7$ μM in a 2 cm path length optical cell.

## 3. Results

### *3.1. Effect of methyl or thienyl substitution on the rate of reductive fragmentation of the phenolic ether linking bond*

One-electron reduction of indolequinones (Q) (**7**, **8** and **9**) by the 2-propanol radical $(CH_3)_2C^{\bullet}OH$ generates the corresponding semiquinone radicals ($Q^{\bullet -}$), the spectra of which are characterised by two maxima at $\lambda_{max} \sim 350$ nm and $\lambda_{max} \sim 600$ nm, respectively. The indolequinones are reduced rapidly ($k_1 \sim 10^9$ $M^{-1}$ $s^{-1}$) and when the concentration of indolequinone (**6**) is 60 μM the absorbance due to the semiquinone radical is fully formed 200 μs after the electron pulse (see Fig. 2).

$$Q + (CH_3)_2C^{\bullet}OH \rightarrow Q^{\bullet -} + (CH_3)_2CO + H^+ \quad (1)$$

Similar semiquinone radical spectra have been observed following the reduction of 2-cyclopropyl indolequinones [28] and related (5-methoxy-1-methyl-4,7-dioxo-indol-3-yl)methyl derivatives [22,24] by the 2-propanol radical. The semiquinone radicals of the corresponding alcohols (**3**, **4** and **5**) do not exhibit leaving group chemistry and thus decay by pure second-order kinetics *via* the disproportionation reaction (2) to generate the hydroquinone [27].

$$Q^{\bullet -} + Q^{\bullet -} + 2H^+ \rightarrow QH_2 + Q \quad (2)$$

In 2-propanol/water (50%, v/v) the chromophore/fluorophore 7-hydroxy-4-methylcoumarin (LG = leaving group) has a $pK_a$ (LG-OH → LG-$O^-$ + $H^+$) = 9.43 ± 0.10 and when deprotonated has excitation $\lambda_{max} \sim 370$ nm and emission $\lambda_{max} \sim 485$ nm. At pH 9.4 the decay of the semiquinone radical of (**7**) results in an increase in absorbance $\lambda_{max} \sim 370$ nm due to release of the coumarin phenoxide anion reflecting reductive fragmentation of the ether 'linking' bond (see Fig. 2). The corresponding fluorescence emission spectrum $\lambda_{max} \sim 485$ nm of the coumarin phenoxide anion measured under the same experimental conditions is also shown in Fig. 2. As previously observed for the 4-nitrophenoxide anion [27] the reductive release of the coumarin phenoxide anion from (**7**) occurs in two distinct phases (see Fig. 3, panel a); the first phase complete within ~200 μs and a second slower phase which nears completion 10 s after reduction of (**7**) to the semiquinone radical. This biphasic release of the coumarin phenoxide anion reflects complex kinetics associated with reductive elimination initially from the $Q^{\bullet -}$ radical *via* reaction (3).

$$(Q\text{-}LG)^{\bullet -} \rightarrow Q^{\bullet +} + LG \quad (3)$$

In the case of indolequinone (**7**) the rate of release is sufficiently slow to allow a fraction of the $Q^{\bullet -}$ radicals generated to form the hydroquinone *via* reaction (2) which in turn releases the coumarin phenoxide anion on a timescale of seconds *via* reaction (4).

$$(QH_2\text{-}LG) \rightarrow QH_2^+ + LG^- \quad (4)$$

It should be noted that fragmentation can only be initiated by reduction of the indolequinone moiety since 7-hydroxy-4-methylcoumarin does not react with the $(CH_3)_2C^{\bullet}OH$ radical under the experimental condition employed. Furthermore, the equilibrium position of reaction (2) favours hydroquinone formation (even at low $[Q^{\bullet -}] < 2$ μM and at high pH ~ 9), the stability of which is reflected in slow rates of autoxidation back to the parent indolequinone by oxygen [22]. The rate constants for reductive elimination of the coumarin phenoxide anion

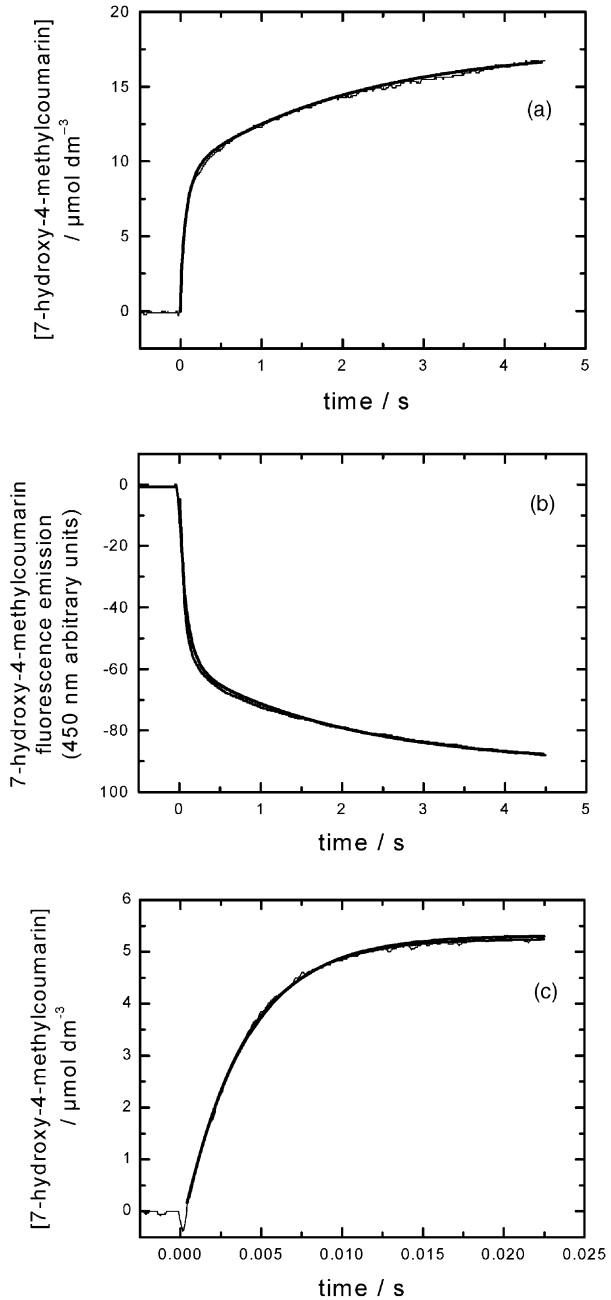



Fig. 3. Typical kinetic traces showing the fragmentation of the phenolic ether linking bond in indolequinones **7**, and **8** following reduction to the semiquinone radical and hydroquinone. Panel a: change in the concentration of the 7-hydroxy-4-methylcoumarin anion as measured by the change in absorbance at 385 nm after pulse radiolysis (31 Gy $\sim$ $[(CH_3)_2C^{\bullet}OH]$ $\sim$ 21 μM) of indolequinone **7** (60 μM) in an $N_2O$-saturated 2-propanol/water mixture (50%, v/v) at pH 9.4. Panel b: the corresponding increasing change in fluorescence intensity observed on the reduction of **7** recorded under identical experimental conditions as above. Panel c: change in the concentration of the 7-hydroxy-4-methylcoumarin anion as measured by the change in absorbance at 385 nm after pulse radiolysis (8 Gy $\sim$ $[(CH_3)_2C^{\bullet}OH]$ $\sim$ 5 μM) of indolequinone **8** (60 μM) in an $N_2O$-saturated 2-propanol/water mixture (50%, v/v) at pH 9.4.

from both the $Q^{\bullet-}$ radical and the hydroquinone were extracted using a data-fitting model in FACSIMILE. A model comprising reactions (2)–(4) was used to give best fits to the kinetic traces (see Fig. 2, upper panel for an example of a fitted kinetic trace) obtained by pulse radiolysis. Fig. 3, panel b shows the biphasic change in fluorescence intensity due to the release of the coumarin phenoxide anion which is identical to the corresponding change in absorbance in panel a. An average of the rate constants from both these absorbance and flourescence traces gave $k_3 = 7.9 \pm 0.1\ s^{-1}$ and $k_4 = 0.4 \pm 0.05\ s^{-1}$ for indolequinone (**7**). The rate constant for the reductive elimination of the coumarin phenoxide anion from the (indol-3-yl)methyl position of indolequinones (**8**) which contains a methyl substituent adjacent to the phenolic ether linking bond was determined to be $k_3 = 340 \pm 7\ s^{-1}$. A typical kinetic trace is shown in Fig. 3, panel c; with indolequinone (**8**) reaction (3) out-competes reaction (2) (even at high semiquinone radical concentrations $[Q^{\bullet-}] \sim 30$ μM), the formation of the hydroquinone is negligible and an accurate measurement of fragmentation rate ($k_4$) from the hydroquinone was not possible in this case. Indolequinone (**9**) which contains a thienyl substituent adjacent to the ether linker also fragments faster than the unsubstituted indolequinone (**7**) giving $k_3 = 120 \pm 8\ s^{-1}$.

Steady-state γ-radiolysis experiments confirmed that the reduction of the parent indolequinone (**7**) gave stoichiometric amounts of the corresponding 7-hydroxy-4-methylcoumarin. The radiation chemical yield for the loss of (**7**) $G(\text{-Q-LG}) = 1.02 \pm 0.1\ \mu mol\ J^{-1}$ equals the generation of the leaving group (LG = 7-hydroxy-4-methylcoumarin) $G(LG) = 1.0 \pm 0.1\ \mu mol\ J^{-1}$. The product profile and observed stoichiometry for the radiolytic reduction of indolequinone (**7**) in Fig. 4 is also typical not only for the indolequinones (**8** and **9**) in this study, but also for the reductive fragmentation of (5-methoxy-1-methyl-4,7-dioxo-indol-3-yl)methyl derivatives in general [22,24, 27]. The two remaining product peaks correspond to the alcohol (**3**) and the isopropyl ether (**6**) and are formed by reaction of the iminium ion with water and 2-propanol solvents, respectively. Their combined radiation chemical yields $G(\mathbf{3}) + G(\mathbf{6}) = 0.9 \pm 0.1\ \mu mol\ J^{-1}$ approximately equal the loss of the parent indolequinone (**7**) $G(\text{-Q-LG}) = 1.0 \pm 0.1\ \mu mol\ J^{-1}$.

### 3.2. 'Oxygen profiling' of reductive fragmentation

Fig. 5 shows the effect of substitution adjacent to the phenolic ether linker on the rate of release of the fluorophore coumarin phenoxide anion by NADPH: cytochrome P450 reductase and MCF-7 cells over a range of oxygen concentrations 0–2.1% $O_2$. Above 2.1% $O_2$ the release of the fluorophore was completely inhibited as the reaction of the semiquinone radical with oxygen out-competes the rate of reductive fragmentation. When comparing Fig. 5, panels a and b it is apparent that under anoxic conditions the rates of coumarin release from indolequinones (**7**, **8** and **9**) are comparable both in the enzyme and tumour cell incubates, respectively. These observations probably reflect similarities in the one-electron reduction

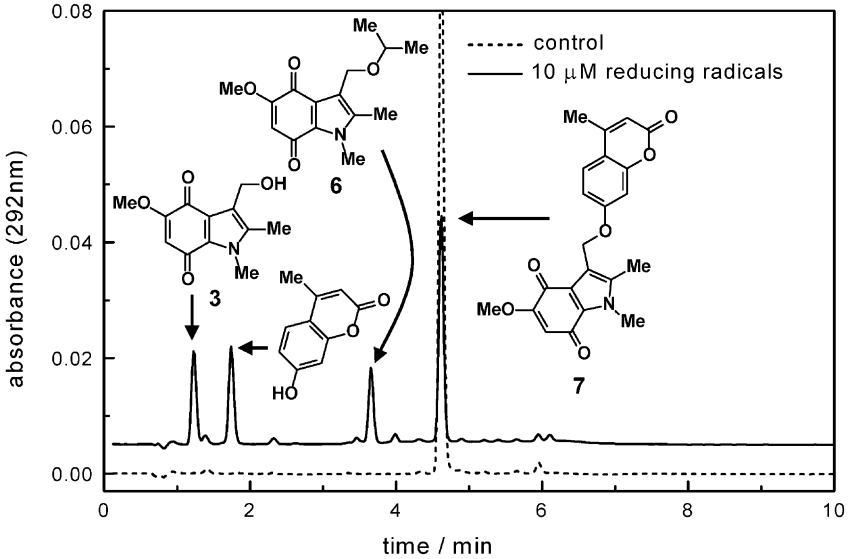



Fig. 4. An HPLC chromatogram showing typical product profile obtained after the steady-state γ-radiolysis (15 Gy) of indolequinone **7** (40 μM) in an $N_2O$-saturated 2-propanol/water mixture (50%, v/v) containing phosphate buffer (4 mM) at pH 7.4.

potentials of the individual indolequinones which are likely to fall in a narrow range based on previously determined measurements of reduction potential for their corresponding indolequinone alcohols (**3**, **4** and **5**) $E(Q/Q^{\bullet -}) \sim -376$ to $-350$ mV [27]. For quinones and nitroarenes in general with $E(Q/Q^{\bullet -}) \sim -400$ to $-165$ mV there is a good correlation between the ease of reduction and the corresponding rate of catalysis by NADPH: cytochrome P450 reductase [34,35]. In both systems anoxic metabolism was completely inhibited by the NADPH: cytochrome P450 reductase inhibitor $TlCl_3 \cdot 4H_2O$ [36,37], exclusion of NADPH and by denaturing the protein. Unfortunately, solubility limitations prevented determination of Michaelis–Menten parameters for each indolequinone although these are expected to be similar for indolequinones (**7**, **8** and **9**).

It is also well established that the reduction potential also governs the rate of electron transfer from the $Q^{\bullet -}$ radical to oxygen in reaction (5) [4,26].

$$Q^{\bullet -} + O_2 \rightarrow Q + O_2^{\bullet -} \quad (5)$$

For quinones with reduction potentials lower than $E(Q/Q^{\bullet -}) \sim -300$ mV manipulation of redox properties do not result in major variation in reaction (5). Thus, the semiquinone radicals of indolequinone alcohols (**3**, **4** and **5**) exhibit reactivity toward oxygen of the same order of

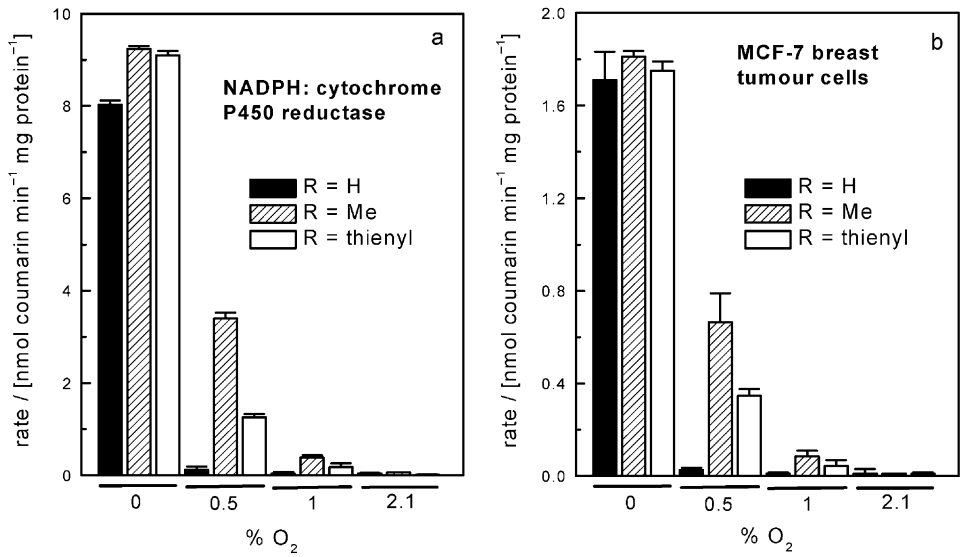



Fig. 5. The effect of substituent R (where R = H, Me or thienyl) adjacent to the phenolic ether linker on the rate of release of the coumarin phenoxide anion from indolequinones **7**, **8**, and **9** by NADPH: cytochrome P450 reductase (panel a) and MCF-7 tumour cells (panel b) over a range of oxygen concentrations 0–2.1% $O_2$.

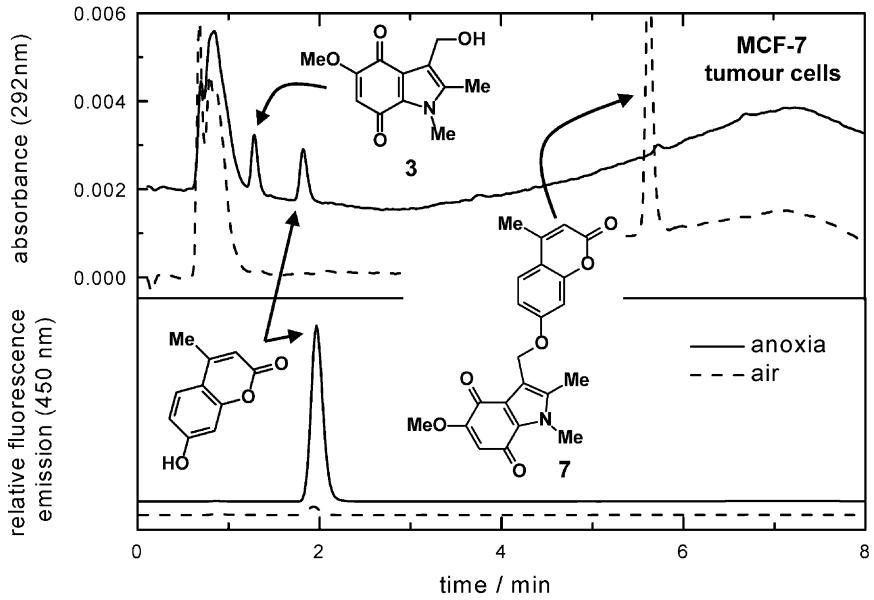



Fig. 6. An HPLC chromatogram showing typical product profile obtained on the metabolism of indolequinone **7** by MCF-7 tumour cell lysates in anoxia vs. air after 20 min incubation. The upper panel shows the absorbance detection and lower panel the corresponding fluorescence detection of reductive fragmentation products.

magnitude [27]. Nevertheless, the 'oxygen profiles' of release of 7-hydroxy-4-methylcoumarin from indolequinones (**7**, **8** and **9**) in both tumour cells and by NADPH: cytochrome P450 reductase are significantly different at intermediate oxygen concentrations (see Fig. 5. Panels a and b). In both systems indolequinone (**8**) (with R = Me) exhibits a greater efficiency of release between 0.5–2.1% $O_2$ than indolequinone (**7**) (with R = H) and indolequinone (**9**) (with R = thienyl). In MCF-7 tumour cells coumarin release from the indolequinone (**7**) was completely inhibited above 0.5% $O_2$, the rate of release from indolequinone (**8**) increased to ~0.7 nmol coumarin $min^{-1}$ mg $protein^{-1}$ at 0.5% $O_2$ and was still evident at 1% $O_2$. Fig. 5, panel a shows the effect of oxygen on the reductive release of coumarin from both indolequinones (**7**, **8** and **9**) with NADPH: cytochrome P450 reductase. As in MCF-7 cells release rates from these indolequinones were similar under anoxia but release from indolequinone (**8**) was again greater than for indolequinone (**9**) at 0.5% oxygen, a concentration of oxygen which completely inhibits reductive release from indolequinone (**7**).

Pulse radiolysis clearly demonstrated that the coumarin can be released from the hydroquinone *via* reaction (4). Although NQO1 is present at moderately high levels in MCF-7 cells [38], dicoumarol (a potent inhibitor of this 2e-reducing enzyme) did not affect the rate of coumarin release from the indolequinones under anoxia. This is consistent with previous observations showing that indolequinones bearing leaving groups at the (indol-3-yl)methyl position inhibit NQO1 following reductive activation [19,20]. Fig. 6 shows a typical product profile for the metabolism of indolequinone (**7**) in MCF-7 cells under anoxia compared to air. Whereas the reduction of indolequinone (**7**) under anoxia generated stoichiometric amounts of the coumarin, fragmentation was completely inhibited in air. The rate of reduction of indolequinone (**7**) under anoxia was similar to the rate of formation of the coumarin determined by fluorimetric methods described previously. The results are consistent with the one-electron reduction of indolequinone (**7**) to the $Q^{\bullet -}$ radical, fragmentation of the phenolic ether linker and release of the coumarin phenoxide anion. This process competes with reaction (5) which prevents fragmentation *via* reaction (3). The only other detectable product was the indolequinone alcohol (**3**) which accounts for ~ 20% of the loss of the parent indolequinone (**7**). This implies that ~80% of the reactive iminium derivative formed must be scavenged by cellular material which is then removed during the preparation of samples by HPLC. The reduction of indolequinones (**8** and **9**) by MCF-7 tumour cells also released the coumarin stoichiometrically under anoxic conditions and at a rate that was consistent with fluorimetric measurements.

## 4. Discussion

It is known that the fluorescence properties of coumarin derivatives depend on their molecular substitution; 7-hydroxycoumarins fluoresce strongly whereas the corresponding 7-alkoxy or 7-phenoxy analogues do not. For example, 4-methyl-7-hydroxycoumarin phosphate monoester has been used for studies of the kinetic behaviour of alkaline phosphatase and *O*-dealkylation of 7-alkoxycoumarin is commonly used to study metabolism by cytochrome P450 mono-oxygenases [39]. Recent work has

demonstrated that 7-phenoxycoumarin does not fluoresce [40]. Likewise, fluorescence of coumarin phenoxide anion is quenched when coupled to indolequinones (e.g. as in **7**, **8** and **9**) at the (indol-3-yl)methyl position *via* a phenolic ether linker. Indolequinones (**7**, **8** and **9**) therefore represent useful fluorescent probes to monitor bioreductively 'triggered' fragmentation of phenolic ether linkers with extremely high sensitivity in real time at known oxygen concentrations. The value of adopting this approach was immediately obvious as the extreme oxygen sensitivity of the semiquinone radical proved a major problem during repeat sampling of incubates which often resulted in the unavoidable introduction of oxygen. The latter approach is a less of a problem when studying the metabolism of nitroaromatics [41,42] since the corresponding nitro radical-anions react 2–3 orders of magnitude slower with oxygen [4].

In this study indolequinones (**7**, **8** and **9**) have been used to correlate fragmentation rates with the 'oxygen profile' of metabolic fragmentation by reductase enzymes and in tumour cells. It is possible to control rates of reductive fragmentation of indolequinones by substitution adjacent to the phenolic ether linking bond. Substitution of methyl for hydrogen significantly shortens the half-life of fragmentation from the semiquinone radical from $t_{1/2} = (0.7/k_3) =$ 87–2.4 ms. A similar change has been observed for the reductive release of the 4-nitrophenoxide anion where a methyl substituent shortens the half-life for fragmentation from 28 to 2 ms [27]. The observed differences in the rate of reductive elimination of these model leaving groups (where LG = 7-hydroxy-4-methylcoumarin or 4-nitrophenol) from the (indol-3-yl)methyl position of indolequinones probably reflect the corresponding difference in the p$K_a$ of the phenolic hydroxy group p$K_a$(LG-OH $\rightarrow$ LG-O$^-$ + H$^+$) = $9.3 \pm 0.1$ and $7.6 \pm 0.1$, respectively. Previous work on rates of reductive elimination of substitiuted nitrophenols from the (indol-3-yl)methyl position of indolequinones demonstrated that the half-life for reductive fragmentation from the semiquinone radical shortens (i.e. faster fragmentation of the phenolic ether linker) as the p$K_a$ of the phenolic hydroxyl group is lowered [43]. This is reflected in the slower rate of reductive release of coumarin from indolequinone (**7**) compared to that previously observed when 4-nitrophenol was the leaving group (half-lives $\sim$ 87 and 28 ms, respectively [27].

In most normal tissues, values of less than 1.3% $O_2$ are rarely observed, but such levels are common to many solid tumours [44]. In hypoxic tumour cells where $[O_2] \sim 5$ μM the semiquinone radical of (**4**) reacts with oxygen with a first-order rate constant of $k_5 = 5.2 \times 10 \sim 8.5 \times 10^{-6} \sim 2.6 \times 10^{-3}$ s$^{-1}$ (half-life, $t_{1/2} = (0.7/k_5[O_2]) \sim 0.3$ ms). At the same concentration of oxygen the half-life for the semiquinone radical of (**3**) is $t_{1/2} \sim 1.8$ ms. Reaction (5) represents the primary competing reaction to the reductive elimination reaction (3) and the balance between these reactions is likely to control release in hypoxic tumour cells. It is predicted that the half-life for the reductive elimination from indolequinone (**7**) of 87 ms would be too long to compete with the very short half-life of the semiquinone radical in 5 μM oxygen ($t_{1/2} \sim 0.3$ ms). Therefore, the coumarin would only be delivered to hypoxic tumour cells where $[O_2] \ll 5$ μM. The half-life for reductive fragmentation of the radical-anion of indolequinone (**7**) is $t_{1/2} \sim 2.4$ ms, which is on a timescale similar to the competing reaction of the $Q^{\bullet -}$ radical at 5 μM oxygen ($t_{1/2} \sim 1.8$ ms). Therefore, based on chemical kinetic arguments, it should be possible to modify rates of fragmentation from the semiquinone radical of prodrugs with a phenolic ether linkage to control the release of drugs to tumour cells with varying degrees of hypoxia.

Chemical kinetic predictions were confirmed by comparing the oxygen-sensitive reductive elimination of the fluorophore 7-hydroxy-4-methylcoumarin from indolequinones (**7**, **8** and **9**) in tumour cells and with NADPH: cytochrome P450 reductase. As predicted the oxygen sensitivity of the semiquinone radical of indolequinone (**7**) limited coumarin release to below <0.5% $O_2$. However, significantly increasing the rate of reductive fragmentation from the semiquinone radical through substitution of methyl or thienyl adjacent to the phenolic ether linking bond in indolequinones (**8** and **9**), respectively facilitated coumarin release over a broader range 0–2.1% $O_2$. It is noted that for indolequinone (**8**) 0.5% $O_2$ resulted in ~40% inhibition of coumarin release between anoxia and air. A similar oxygen concentration (0.48% $O_2$) was previously shown to inhibit the metabolism of the 2-nitroimidazole-based hypoxia probe (SR-4554) by 50% between anoxia and air [41]. This clearly demonstrates the ability to modify the rate of reductive fragmentation of these indolequinones to achieve hypoxic-selectivity despite the relative reactivity of the semiquinone and nitro radical-anions toward oxygen (which differ by orders of magnitude).

The mean diffusion distance $x$ for the semiquinone radical can be estimated from the equation,

$$x = (6Dt)^{1/2} \quad (6)$$

where $D$ is the diffusion coefficient in the cytoplasm, $\sim 2 \times 10^{-10}$ m$^2$ s$^{-1}$ [4,22]. If the half-life of the semiquinone radical at low $[O_2] = 1$ μM is taken as $t_{1/2} \sim 1$–10 ms, then $x \sim 4$ μm, it is predicted that almost all radicals would react in the cell where reduction occurred. Any so-called 'bystander effect' in neighbouring, better oxygenated tumor cells is therefore likely to depend on the ability of the released drug to diffuse and react with the target. This in turn will dictate whether it is better to closely match the rate of reductive fragmentation to the oxygen profile of tumours or to ensure release only in very hypoxic regions of tumors then rely on a bystander effect. Due to the much slower rates of both autoxidation and reductive fragmentation the hydroquinone is expected to diffuse a few cell diameters [22]. However, metabolic release of coumarin from indolequinone (**7**, **8** and **9**) by MCF-7 tumor

cells does not occur in air despite the fact that these cells contain moderate levels of NQO1 [38]. This result is consistent with previous studies with recombinant human NQO1 that the enzyme cannot tolerate leaving groups at the 3-position of indolequinones [19,20]. Although NQO1 is over-expressed in a number of tumors it is unlikely to be as important as NADPH: cytochrome P450 reductase in prodrug activation [45–48]. However, drug delivery to hypoxic tumour cells *in vivo* may be hampered by lack of prodrug metabolism in the aerobic sub-population of cells which in turn could prevent prodrug penetration to the avascular regions of the tumour.

In conclusion, by modifying the rate of reductive fragmentation of indolequinone prodrugs it is possible to control the release of drugs (at least those coupled *via* a phenolic ether linker) over a range of hypoxia expected in tumours.

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