Natural and synthetic quinones and their reduction by the quinone reductase enzyme NQO1: from synthetic organic chemistry to compounds with anticancer potential

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The quinone reductase enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1) is a ubiquitous flavoenzyme that catalyzes the two-electron reduction of quinones. This *Perspective* briefly reviews the structure and mechanism, physiological role, and upregulation and induction of the enzyme, but focuses on the synthesis of new heterocyclic quinones and their metabolism by recombinant human NQO1. Thus a range of indolequinones, some of which are novel analogues of mitomycin C, benzimidazolequinones, benzothiazolequinones and quinolinequinones have been prepared and evaluated, leading to detailed knowledge of the structural requirements for efficient metabolism by the enzyme. Potent mechanism-based inhibitors (suicide substrates) of NQO1 have also been developed. These indolequinones irreversibly alkylate the protein, preventing its function both in standard enzyme assays and also in cells. Some of these quinones are also potent inhibitors of growth of human pancreatic cancer cells, suggesting a potential role for such compounds as therapeutic agents.

Introduction

One group of natural products that exhibit wide-ranging properties are the quinones.[1–3] Even the simplest quinone, 1,4-benzoquinone itself—originally isolated by oxidation of quinic acid, a product of cinchona bark (hence the name quinone)—is bioactive; it is produced in the defensive spray of the bombardier beetle to ward off attackers. Not only do quinones constitute a large group of natural pigments, although surprisingly their contribution to natural colouring is relatively small, they participate in a range of important biological redox processes. The ubiquinones (coenzymes Q) **1** ($n = 1$–12) (Fig. 1) occur in virtually all aerobic organisms from bacteria to higher plants and animals, with nearly all vertebrates possessing ubiquinone-10 **1** ($n = 10$). The ubiquinones occur mainly in the mitochondria where they play a major role in electron transport in the respiratory chain. The closely related plastoquinones, for example plastoquinone-9 **2** ($n = 9$), occur in the chloroplast of green plants where they function in electron transport pathways in photosynthesis. Likewise the naphthoquinone, phylloquinone **3** (vitamin K_1) also occurs in green plants and participates in photosynthetic electron transport. The more recently discovered and structurally different heterocyclic pyrroloquinoline quinone (coenzyme PQQ) **4** functions as a cofactor for various dehydrogenases, and may also participate in electron transport processes.[4]

Our laboratory has been interested in the synthesis of quinone natural products for about 25 years, starting with an early synthesis of coenzyme PQQ **4**.[5] This was followed by work on other naturally occurring indolequinones murrayaquinones-A **5** and -B **6**,[6,7] and BE10988 **7**,[8] and more recently the benzoquinones primin **8**, pallasone-B **9**, verapliquinones-A and -B **10**, and panicein-A **11** (Fig. 2).[9]

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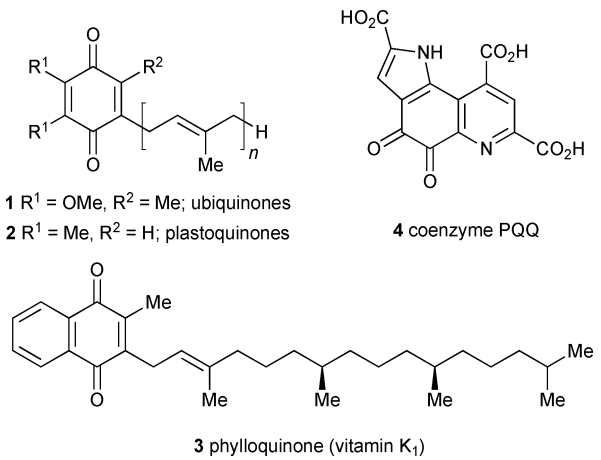


Fig. 1 Some naturally occurring quinones that participate in biological redox processes.

Some naturally occurring quinones also have anticancer properties (Fig. 3), and doxorubicin **12**, a member of the anthracycline family, is widely used as a frontline chemotherapeutic agent. However, it was another anticancer quinone, mitomycin C **13** (MMC), which captured our own attention in the mid-1980s. Initially our interest was in developing new synthetic routes to the pyrrolo[1,2-*a*]indole ring system of the mitomycins, but inspired by the contemporaneous work from the laboratories of Tomasz and Nakanishi,[10] Kohn[11] and Danishefsky,[12] it became clear to us that the mechanism of action of these anticancer quinones was just as interesting as their synthesis. Mitomycin C **13** is the archetypal bioreductive drug, a term coined to describe a range of anticancer compounds that are inactive in their own right, but upon metabolic reduction are transformed into a cytotoxic species that can interact with biomolecules.[13,14] In the case of MMC **13**, reactive electrophilic centres are generated at C-1 and C-10

Marie A. Colucci

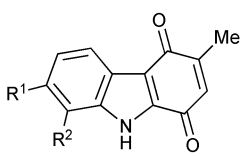
Marie Colucci was born in Swindon, and graduated from the University of Bath in 2000 with a BSc in Chemistry. After a period in industry, she joined the group of Professor Chris Moody firstly in Exeter and then in Nottingham as a PhD student. Her postgraduate research focused on the synthesis of inhibitors of NQO1.

Chris Moody

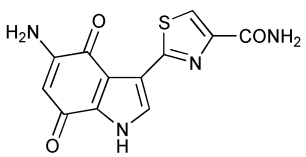
Chris Moody is the Sir Jesse Boot Professor in the University of Nottingham. He was educated at Manchester Grammar School and King's College, London, before carrying out PhD research at the University of Liverpool under the supervision of Charles Rees. He spent a postdoctoral year at the ETH in Zürich working with Albert Eschenmoser before taking up a post in industry at Roche. In 1979 he was appointed to a lectureship at Imperial College, London, and was promoted to a readership in 1989. In 1990 he moved to the chair of organic chemistry at Loughborough University, and in 1996 he was appointed Professor of Organic Chemistry at the University of Exeter. He moved to his current post in Nottingham in August 2005.

Gavin Couch

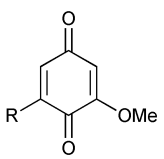
Gavin Couch is a native of Cornwall, and obtained a BSc in Medicinal and Biological Chemistry from the University of Exeter in 2003. He joined the group of Professor Chris Moody as a PhD student in Exeter later the same year, and migrated north to Nottingham in 2005. His postgraduate research was concerned with the synthesis of novel substrates for NQO1, NQO2 and nitroreductases.



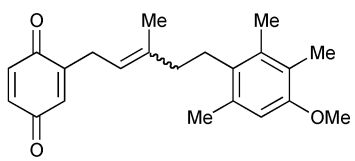
5 $R^1 = R^2 = H$; murrayaquinone-A
6 $R^1 = OMe$, $R^2 = CH_2CH{=}CMe_2$; murrayaquinone-B



7 BE10988



8 $R = C_5H_{11}$; primin
9 $R = C_{17}H_{35}$; pallasone-B
10 R = geranyl; verapliquinone-A/B



11 panicein-A

Fig. 2 Some naturally occurring quinones synthesized in our laboratory.

leading to cross-linking of DNA as shown later in Scheme 3. Hence bioreductive drugs such as MMC act as substrates for one or more of the reductases present in most cells, with the quinone suffering one-electron reduction to the semiquinone radical or two-electron reduction to the hydroquinone. Such one- or two-electron reductions would be catalyzed by, for example, NADPH: cytochrome *c* (P450) reductase or NAD(P)H: quinone oxidoreductase 1 (NQO1) respectively. It is this second enzyme, and its substrates and inhibitors, that forms the subject of this *Perspective*, written from a personal point of view, with emphasis on our work.

NAD(P)H: quinone oxidoreductase 1 (NQO1, QR1)

The enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1) (EC 1.6.99.2), sometimes simply referred to as quinone reductase 1 (QR1), was initially named DT-diaphorase for its unusual ability to use either NADH (originally designated **D**PNH) or NADPH (**T**PNH), and was first isolated in 1958 by Lars Ernster.[15] NQO1 is a ubiquitous flavoprotein and an obligate two-electron reductase that can catalyze, with varying degrees of efficiency,

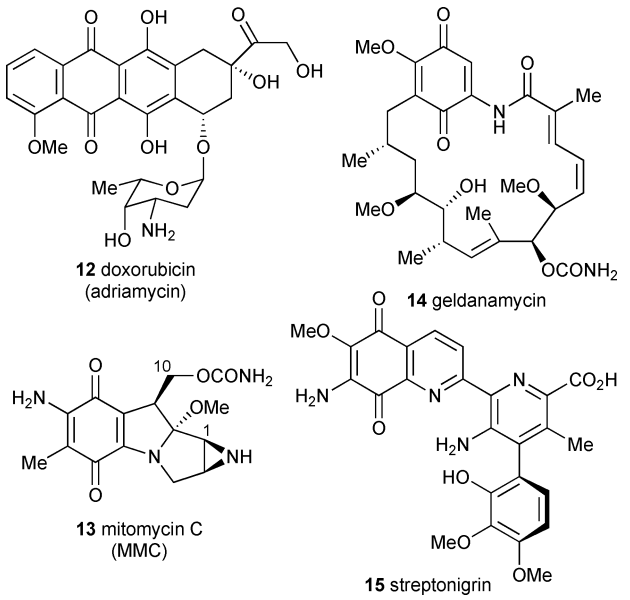


Fig. 3 Naturally occurring quinones with anticancer properties.

the reduction of quinones, quinone epoxides, aromatic nitro and nitroso compounds, azo dyes and Cr(VI) compounds. It is closely related to another flavoenzyme, NRH quinone oxidoreductase 2 (NQO2) that also catalyzes the reductive metabolism of quinones, although NQO2 is not discussed in detail here.[16]

NQO1 has long been regarded as a chemoprotective enzyme catalyzing the reduction and detoxification of exogenous quinones, although paradoxically, as we have already seen, it is also involved in the bioactivation of molecules such as MMC into toxic DNA-damaging agents. It also participates in the metabolism of endogenous quinones such as ubiquinone, and may be involved in scavenging superoxide in cells. The enzyme is upregulated in many tumours, and the recent discovery that it might be involved in the stabilization of tumour suppressor protein p53 has heightened interest. Hence the last decade has seen increased focus on this quinone reductase, discovered 50 years ago, and the enzyme has been the subject of a number of recent reviews,[17–20] including special issues of *Free Radical Biology and Medicine* (2000, **29**, 201–383) in commemoration of Ernster's original discovery, and of *Methods in Enzymology* (2004, **382**, 3–572). Notwithstanding these excellent review articles, the key features of the enzyme NQO1 are reiterated herein, although very much from an organic chemist's standpoint.

Structure and mechanism

NQO1 has a molecular weight of about 60 kDa, and is a homo-dimer of two interlocked monomers of 274 amino acids. Each subunit contains a non-covalently bound molecule of flavin adenine dinucleotide (FAD), and consists of two domains—a catalytic domain (residues 1–220) and a smaller C-terminal domain that forms part of the binding site for the hydrophilic regions of NAD(P)H.[21,22] The fold of the catalytic domain is similar to that found in other flavoproteins. The structure of the human protein has been solved by X-ray crystallography (Fig. 4).[23,24] The structures of the mouse (86% sequence identity to human) and rat

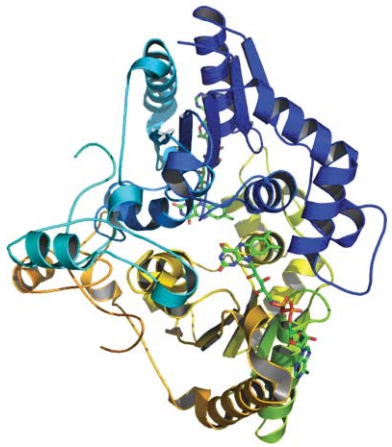


Fig. 4 X-Ray crystal structure of human NQO1 dimer (PDB accession code 1d4a). The non-covalently bound FAD is shown at both sites.[24]

(also 86% sequence identity to human; 94% sequence identity to mouse) enzymes have also been solved.[24,25]

The active site of human NQO1 (hNQO1) is located at the interface of the two monomer units, and is a flexible 360 Å^3 pocket lined with aromatic residues. The catalytic site has three regions: (i) the FAD binding site, (ii) a site near the C-terminus where the hydrophilic adenine–ribose portion of NAD(P)H binds, and (iii) the site occupied by the cofactor, the hydride donor (NADH or NAD(P)H), *or* the substrate, the hydride acceptor. The FAD prosthetic group is non-covalently, tightly bound and does not come off the enzyme readily under native conditions. The isoalloxazine moiety has extensive contact with the protein; the hydrophilic rings of the isoalloxazine make hydrogen bonding interactions with main chain NH groups and side chain hydroxyls of Thr and Tyr which anchor this side of the flavin in place. The hydrophobic dimethylbenzene ring of the FAD has the methyl groups in a hydrophobic pocket, composed of the aliphatic residues of the second monomer. The ribitol, phosphates and adenine ring of FAD interact non-covalently with several loops and helices, anchoring the FAD cofactor to the enzyme. The structure of the active site containing the bound FAD is shown in Fig. 5A.

Information on the binding of the cofactor (NADH or NADPH) comes from the structure of the rat enzyme with bound $NADP^+$.[25] $NADP^+$ has fewer specific interactions with the protein than does FAD. The nicotinamide ring is in van der Waals contact with the FAD and with the side chains of aromatic residues, and the carboxamide makes hydrogen bonds to two tyrosine residues. As already mentioned, the AMP part of the cofactor interacts mainly with the C-terminal domain. The binding of substrates to hNQO1 was initially studied with duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone).[24] The quinone is bound to the active site through a series of contacts involving the flavin and several hydrophobic and hydrophilic residues. The substrate is sandwiched between the five aromatic residues and the central portion of the isoalloxazine ring of FAD (Fig. 5B). The substrate binding site is highly flexible and can accommodate a wide range of quinone substrates (*q.v.*).

The fact that the cofactor and substrate occupy the same site is consistent with the "ping-pong" mechanism of the enzyme that is shared with other flavoproteins. In this mechanism, illustrated in Schemes 1 and 2, NAD(P)H occupies the binding site and transfers

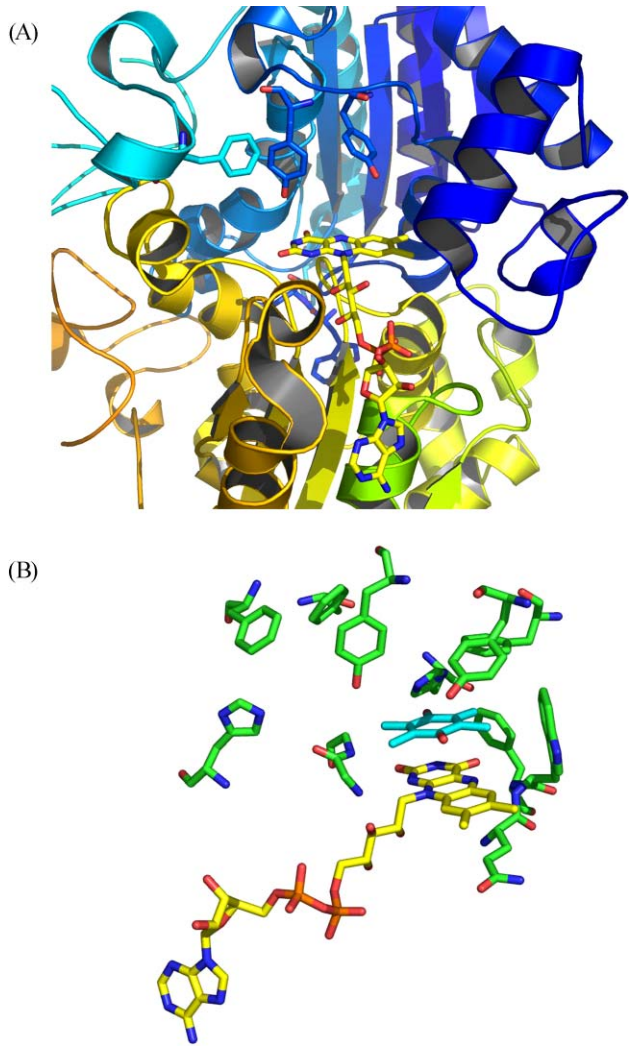
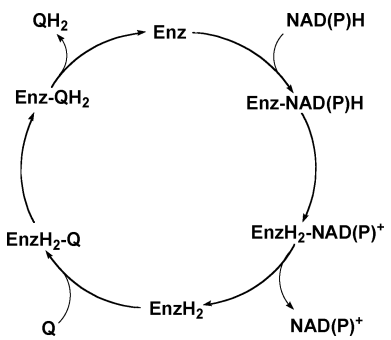


Fig. 5 Active site of hNQO1 showing (**A**) the apo enzyme with the bound FAD (PDB accession code 1d4a), and (**B**) the bound substrate duroquinone (shown in cyan) with the substrate quinone ring stacked above the isoalloxazine of the FAD (most of the surrounding residues omitted) (PDB accession code 1dxo).[24]

its hydride to FAD, the resulting NAD(P)+ leaves the site to be replaced by the quinone substrate. Hydride transfer from FADH results in reduction of the quinone, and finally the hydroquinone departs to restart the catalytic cycle (Scheme 1). The details of the hydride transfer steps are shown in Scheme 2.



Scheme 1 Ping-pong mechanism of NQO1.

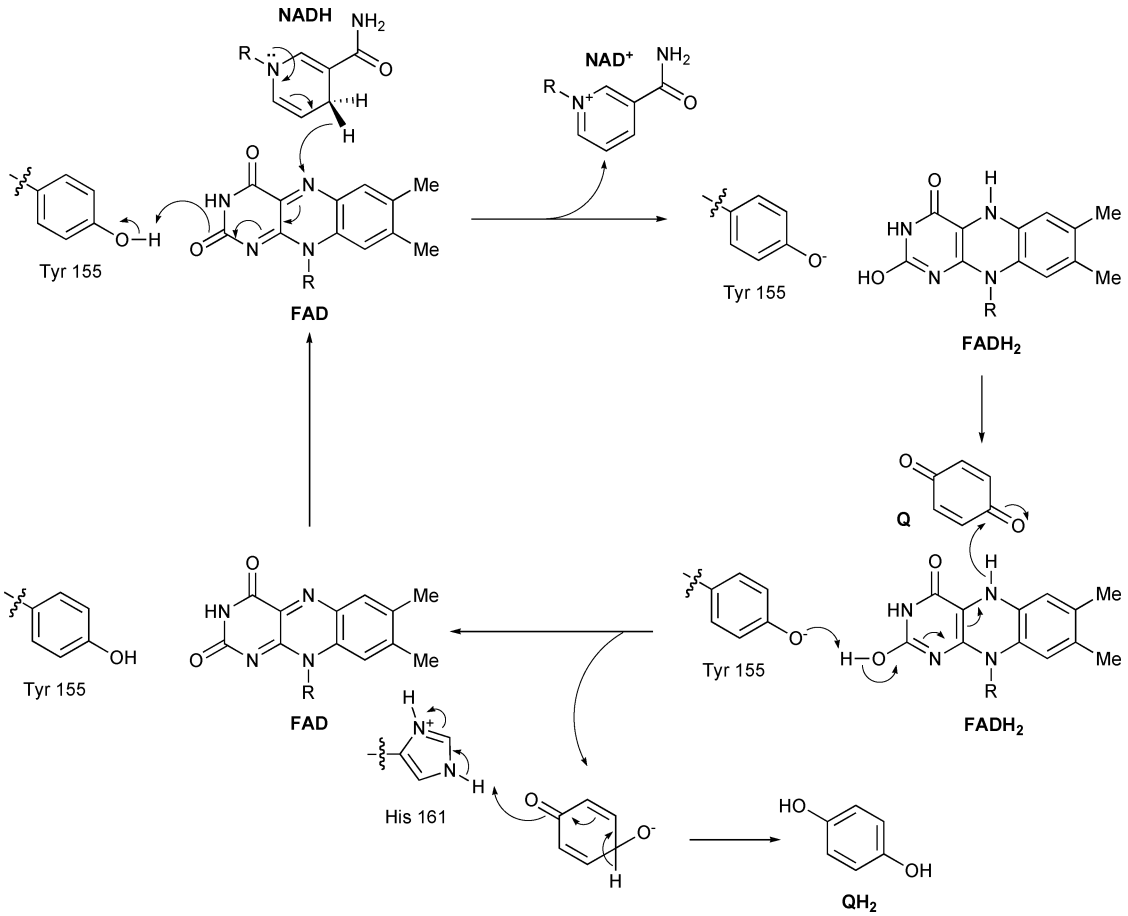
There is a high level of conservation between NQO1 and NQO2, but the NQO2 protein is 43 amino acids shorter. The overall structures of the enzymes are similar, although NQO2 lacks the C-terminal of hNQO1. Both enzymes bind FAD in similar ways but NQO2 fails in the binding of NAD(P)H due to the loss of the C-terminus. Hence NQO2 uniquely uses dihydronicotinamide riboside (NRH) as a hydride donor as opposed to NAD(P)H. Nevertheless, in the presence of NRH, NQO2 is able to catalyze the two-electron reduction of quinones. Unsurprisingly, due to differences in the protein structure—the active site of NQO2 is shorter and narrower than that for NQO1, and therefore suitable substrates have to be relatively short molecules with flat conformations—the two enzymes have a different substrate profile. NQO2 is of particular topical interest with respect to the bioactivation of prodrug CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] into a bifunctional alkylating agent. NQO2, in the presence of the non-biogenic cofactor NRH, can perform the four-electron reduction of the 4-nitro group in CB1954 some 3000 times more efficiently than hNQO1. The role of NQO2 in bioreduction processes has been covered in recent reviews,[26–28] and is not discussed further here.

Physiological role of NQO1

Although originally thought to be involved in mitochondrial electron transport,[15] NQO1 was subsequently shown not to be a component of the respiratory chain. Likewise, its purported role in vitamin K_1 metabolism was discounted when it was discovered that the naphthoquinone-based vitamin **3** was not a substrate for purified rat NQO1. However, the related quinones ubiquinones **1** (Fig. 1) and vitamin E quinone **16** (α-tocopherolquinone) (Fig. 8) are reduced by the rat enzyme, suggesting a role for NQO1 as an antioxidant enzyme.[20,29] NQO1 is also a phase II detoxifying enzyme, and protects cells from the damaging effects of reactive species formed upon metabolism of exogenous quinones.[30,31] On the other hand, NQO1 is also involved in the bioactivation of anti-tumour quinones such as MMC into cytotoxic species, *i.e.* it is also functioning as a toxification enzyme, prompting its classification as a double-edged sword.[29] More recent studies have suggested that NQO1 can stabilize the tumour suppressor protein p53 either by inhibiting its degradation, or by a direct protein–protein interaction.[29,32] Finally, it is noted that there are two well-characterized polymorphisms in NQO1—NQO1*2 and NQO1*3—both the result of single nucleotide changes. The more prevalent NQO1*2 polymorphism, the occurrence of which varies according to ethnic groups with frequencies of up to 22% in some Asian populations, results in lack of reductase activity. One possible consequence is that lack of NQO1 activity might increase the risk of certain types of toxicity. For example, individuals with the NQO1*2 allele are more susceptible to the toxic effects of benzene metabolites.[33,34]

Upregulation and induction of NQO1

NQO1 is expressed at high levels in many solid tumours, and its presence is readily detected by immunohistochemical staining.[35] An example illustrating NQO1 staining in human pancreatic cancer cells is shown in Fig. 6A. The upregulation or over expression of the enzyme in tumours compared to surrounding



Scheme 2 Mechanism of the two-electron reduction of quinones by NQO1.

healthy tissue, as illustrated for non-small cell lung cancer cells in Fig. 6B,[36] may be a result of several factors. Firstly it may give the tumour cell some sort of advantage to have NQO1 switched on and expressed. Secondly, NQO1 may be part of a stress response signalling system and tumour cells may be continually activated and in a state of "stress". A further explanation is that NQO1 is only elevated in solid tumours (not leukaemias), and solid tumours are most often derived from epithelial cells. Epithelial cells are one of the cell types that normally contain NQO1 and hence it may just reflect the cell of origin for the solid tumour.

NQO1 gene expression is regulated by two elements, the antioxidant response element and the xenobiotic response element (also known as AhRE since it involves the aromatic hydrocarbon receptor).[29,37] The expression of NQO1 can be induced by a number of dietary and synthetic agents with a wide range of structural diversity.[38,39] These include 1,2-dithiole-3-thiones such as D3T and oltipraz; extracts of vegetables such as the flavanol quercetin and the isothiocyanate sulforaphane; dietary antioxidants such as *tert*-butylhydroxy-anisole (BHA); xenobiotics such as aromatic hydrocarbons, azo dyes, diphenols, and dioxins (Fig. 7). D3T, isolated from cruciferous vegetables, is a very effective inducer of phase 2 enzymes such as NQO1 both *in vitro* and *in vivo*. The D3T analogue oltipraz also induces NQO1 activity. Isothiocyanates are widely distributed in higher plants, especially cruciferous vegetables, and sulforaphane, found in broccoli, is a very potent inducer of NQO1. Studies suggest that it might find use as a chemopreventative agent.[40,41]

Quinone substrates for NQO1

Although NQO1 can catalyze the two-electron reduction of a broad range of substrates, as its name implies, its major group of substrates are quinones. Its role in the metabolism of endogenous quinones such as the ubiquinones **1** and α-tocopherolquinone **16** has already been discussed, as has the bioactivation of MMC **13**. However, the flexible nature of the active site can accommodate a wide range of quinone substrates including the natural products geldanamycin **14** and streptonigrin **15** (Fig. 3), the benzoquinones AZQ, MeDZQ and RH1 **17**, the naphthoquinone β-lapachone and the indolequinone EO9 **18** (Fig. 8). The aziridinylbenzoquinones MeDZQ and RH1 **17** are both excellent substrates for hNQO1, and following reduction are activated into DNA cross-linking agents; the required ring opening of the aziridines is presumably easier in the hydroquinone than in the quinone.[42] RH1 **17** is in clinical trials. Similarly, the indolequinone diol EO9 **18** is also a potential tri-functional alkylating agent after loss of water from both the indole 3-carbinyl and vinylogous indole 2-carbinyl positions, and aziridine ring opening.[43] The compound has recently re-entered phase I clinical trials.[44]

The mechanism of action of MMC and related mitosenes has been widely studied, and the role of C-1 and C10 in the alkylation and cross-linking of DNA, following one- or two-electron reduction of the quinone, established.[10–13,45–47] An accepted mechanism for the activation of MMC by NQO1 is shown in Scheme 3. Thus two-electron reduction generates the hydroquinone followed by

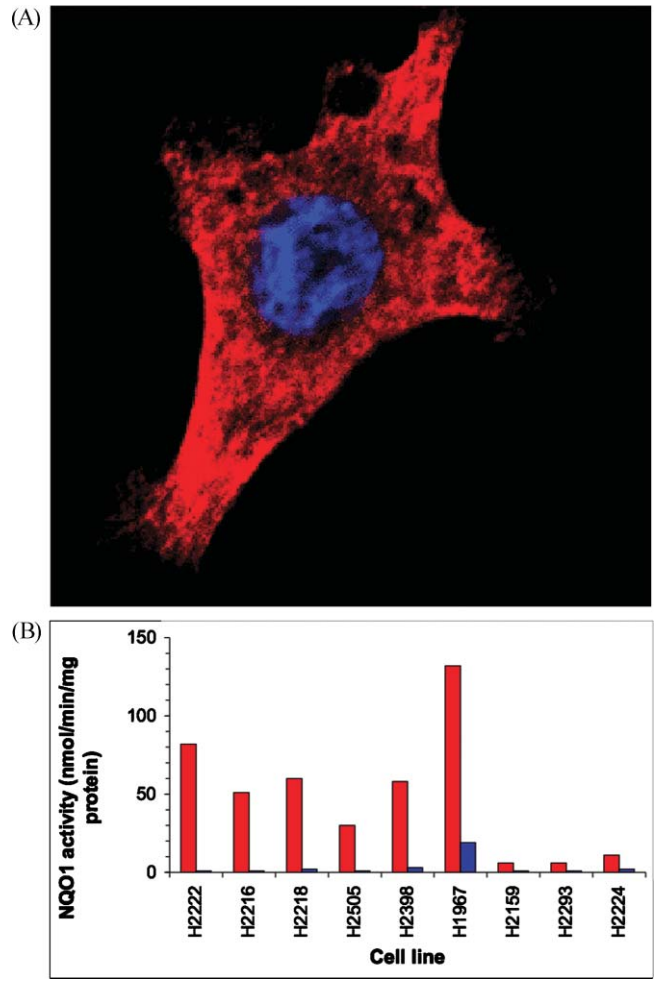


Fig. 6 (**A**) Confocal microscopy image of NQO1 immunostaining (red) in human pancreatic adenocarcinoma cells (BxPc-3). The cell nucleus is stained blue. Image is copyright David Siegel, and is used with permission. (**B**) Elevated levels of NQO1 in non-small cell lung cancer cells (red) compared with surrounding normal tissue (blue).[36]

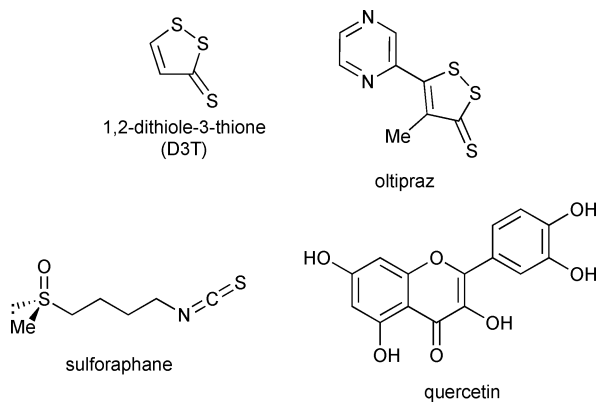


Fig. 7 Structures of some inducers of hNQO1 activity.

loss of the tertiary methoxy group, presumably assisted by the indole nitrogen, and opening of the aziridine to form an electrophile at C-1 capable of alkylating DNA, shown to be the N-2 of guanine. After elimination of the carbamate group, again initiated by the nitrogen lone pair of the indole ring, a second electrophile is

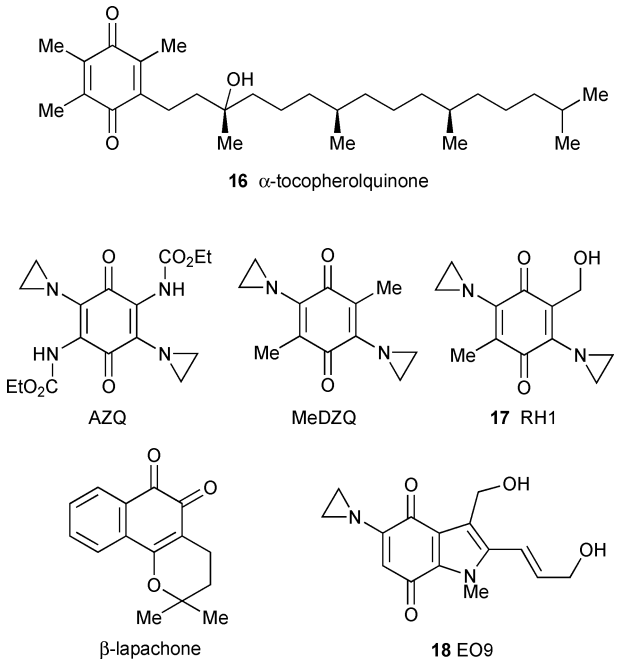


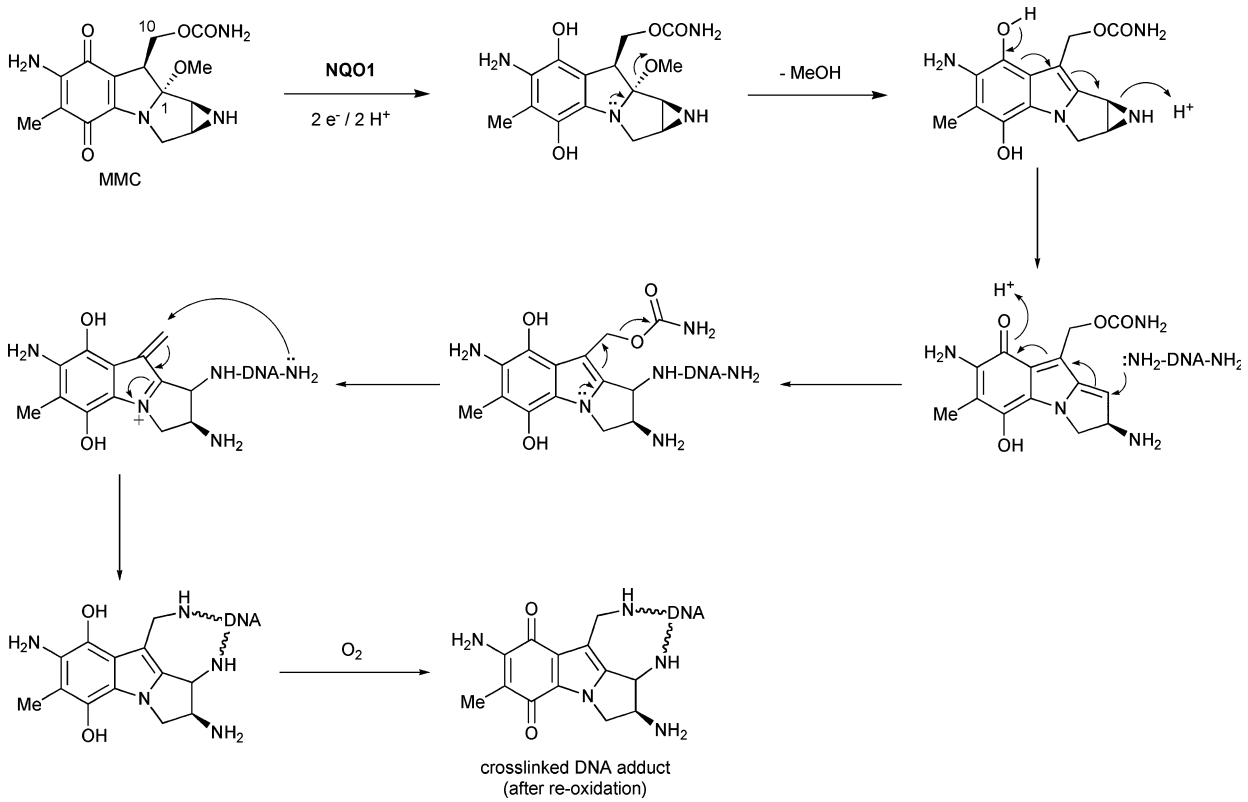
Fig. 8 Substrates for reduction by hNQO1. Ubiquinone **1**, MMC **13**, geldanamycin **14** and streptonigrin **15** are also substrates.

formed at C-10 that binds DNA once more, thereby completing the cross-linking. It is the intervention of 'normal' indole reactivity, which is suppressed in the quinone through delocalization into the quinonoid vinylogous amide system, which leads to the loss of the 3-indolyl carbinyl substituent and generation of the iminium electrophile, which is key to this process. In fact, the restoration of 'normal' indole reactivity following reduction of the quinone is the single most important feature of the indolequinone anticancer compounds discussed herein.

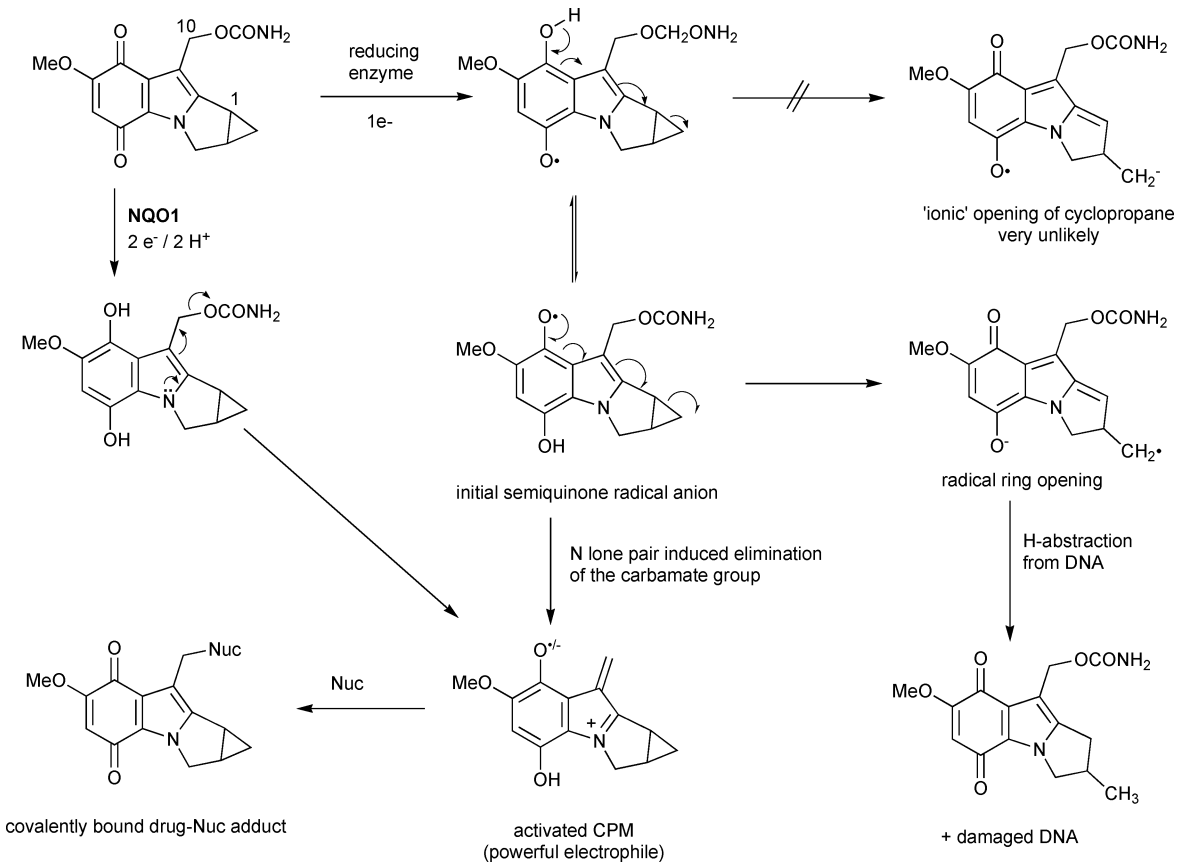
However, simply relating MMC cytotoxicity to NQO1 levels is complicated by the fact that the MMC quinone is a relatively poor substrate for hNQO1, is also bioactivated by one-electron reductases, and hence may be influenced by hypoxia, and the activation is also pH-dependent.

The above seminal studies on MMC formed the basis of our initial foray into the field of bioreductively-activated quinones some 20 years ago. Our first goal was to focus on the role of C-10 in the alkylation process by the design and synthesis of analogues in which the electrophilicity at C-1 is reduced by the presence of a cyclopropane in place of the aziridine. Such a cyclopropamitosene, it was reasoned, could upon reductive activation followed by loss of the carbamate, generate an electrophile at C-10 capable of alkylating DNA. However, ionic ring opening of the cyclopropane is very unlikely, although radical induced ring opening is an alternative pathway (Scheme 4).[48–51]

In order to test this hypothesis, a number of indolequinones, with and without the cyclopropane ring, were required. The novel cyclopropapyrrolo[1,2-*a*]indole ring system was prepared by an intramolecular cycloaddition as shown in Scheme 5. Allylation of the indole-2-carbaldehyde **19** was followed by conversion of the aldehyde into its tosyl hydrazone **20**. Heating the sodium salt of **20** resulted in cycloaddition of the derived diazo compound to give, after loss of nitrogen, the desired tetracycle **21**. Introduction of the C-10 carbon as an aldehyde group by Vilsmeier–Haack

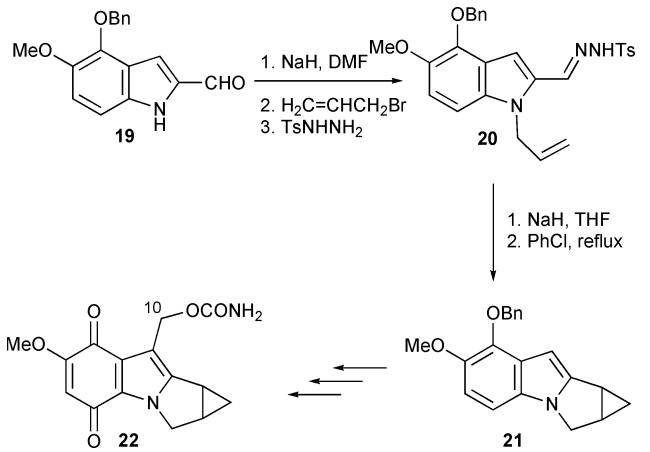


Scheme 3 DNA cross-linking by mitomycin C.



Scheme 4 Postulated activation cascade of unnatural cyclopropamitosenes.

formylation was followed by conversion into the cyclopropamitosene quinone **22** by conventional steps (Scheme 5).[49–51] The 7-methoxycyclopropamitosene **22** was converted into a range of 7-substituted derivatives by reaction with amine nucleophiles, and of these, the 7-aziridinyl compound proved the most interesting.

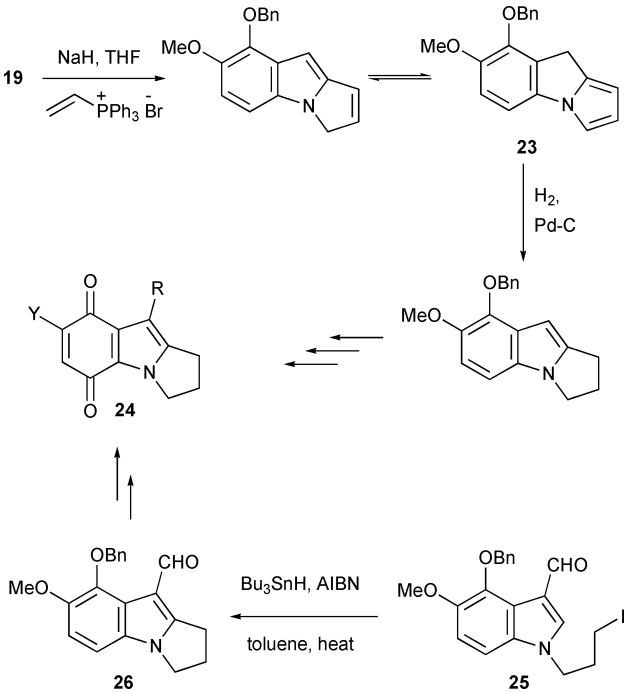


Scheme 5 Synthesis of cyclopropamitosenes.

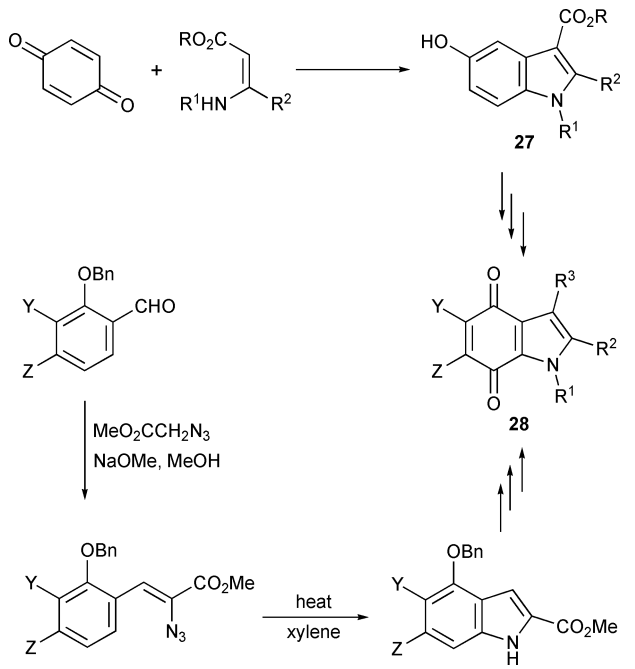
The biological properties of the cyclopropamitosenes were investigated in rodent cells. In air, the 7-aziridinyl compound was 1000 times more toxic than the 7-methoxy analogue **22**, with MMC and EO9 showing intermediate levels of toxicity. Under hypoxic conditions, there was no change in the level of toxicity of the aziridine whereas the potency of **22** increased 34 fold. This result indicates that one-electron reduction may contribute significantly to the toxicity of compound **22** under anaerobic conditions. In contrast, the 7-aziridinyl compound shows no additional toxicity under nitrogen and is far more toxic in air than **22**, which suggests O_2 independent two-electron reductive activation (*i.e.* NQO1) may contribute significantly to the toxicity of this compound. In fact, both cyclopropamitosenes are substrates for NQO1, with the 7-aziridinyl compound giving approximately three-fold faster rates. The compound was reduced four times more rapidly than MMC but 140 fold less efficiently than the related aziridinylindolequinone EO9 **18**.[52]

Having established that our novel cyclopropamitosenes possessed at least some of the relevant biological properties, simpler quinones lacking the cyclopropane were prepared for comparison. The corresponding mitosene **24** was prepared by two routes, firstly using an intramolecular Wittig reaction of an ylide formed by addition of the indole anion of **19** to vinylphosphonium bromide, followed by elaboration to the quinone as before.[51] Secondly, we developed a novel radical cyclization reaction starting from the 3-iodopropylindole-3-carbaldehyde **25** (Scheme 6).[53,54]

Simple indolequinones **28** were also prepared, and the Nenitzescu synthesis employing 1,4-benzoquinone and aminoacrylates proved a practical route to indoles **27**, versatile intermediates to a wide range of indolequinones **28** with the 3-carboxylate and 5-methoxy groups serving as precursors to a range of functionality (Scheme 7).[55–57] We have also used the Hemetsberger indole synthesis to good effect in the synthesis of indolequinones,[58,59] including our aforementioned synthesis of coenzyme PQQ.[5] As outlined in Scheme 7, condensation of a benzaldehyde with methyl



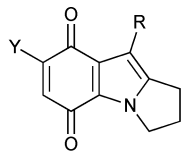
Scheme 6 Synthesis of mitosenes.



Scheme 7 Synthesis of indolequinones.

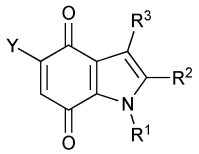
azidoacetate is followed by simple heating in xylene to deliver the indole nucleus, subsequently elaborated to the indolequinone **28**.

As with the cyclopropamitosenes, the cytotoxicity of the mitosenes and indolequinones towards aerobic and hypoxic tumour cells was determined, and in general, under aerobic conditions, the cyclopropamitosenes were more toxic than the corresponding mitosenes, which in turn were more toxic than simple 1,2-alkyl indolequinones, with many of the compounds in each series showing greater toxicity towards hypoxic cells.[58] However, it was



24

Y = OMe, cyclopropylamino, aziridinyl,
2-methylaziridinyl, pyrrolidinyl, piperidinyl
R = H, CHO, CH_2OH, CH_2OCONH_2



28

Y = OMe, NHMe, NMe_2, $NHCH_2CH_2OH$, $NHCH_2CH_2OMe$,
cyclopropylamino, aziridinyl, 2-methylaziridinyl, azetidinyl, pyrrolidinyl,
morpholinyl, N-methylpiperazinyl
R^1 = Me, n-Pr, c-Pr, Ph
R^2 = H, Me, Et, CO_2Me, CH_2OH, CHMeOH, CH_2OCONH_2,
$CH_2O(4\text{-}NO_2\text{-}C_6H_4)$, Ph, $4\text{-}C_6H_4Ph$, 2-naphthyl
R^3 = H, Me, CHO, CO_2Et, CH_2OH, CH_2OCONH_2, CH_2OAc,
CH_2OPh, $CH_2O(4\text{-}NO_2\text{-}C_6H_4)$, CHMeOH

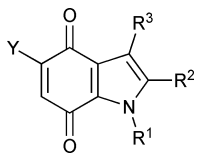
Fig. 9 Indolequinones studied for rate of metabolism by hNQO1.

the metabolism of the various quinones by hNQO1 that interested us most, and the large range of compounds studied is summarized in Fig. 9.

In total, our biology collaborators—David Ross and David Siegel (University of Colorado) and Howard Beall (University of Montana)—have evaluated over 100 of our novel indolequinones and mitosenes for their ability to act as a substrate for hNQO1, allowing us to build up a comprehensive picture of the structural requirements for a quinone to be an efficient substrate for hNQO1.[57,60–62] Some representative data are shown in Table 1; the rates of metabolism are measured by using an HPLC method capable of quantifying both NADH oxidation and quinone reduction. Quinone reduction is usually reversible due to redox cycling of the hydroquinone, so results (Table 1) are reported as μmol NADH oxidized min^{-1} mg^{-1} of NQO1 protein—the higher the number, the faster the reduction rate. This HPLC method gives average rates of reduction over a 30–40 minute period.

From the enzyme data, it was clear that substituents on the quinone ring (7-position in **24**, 5-position in **28**) had a marked effect on the rate of metabolism by hNQO1. In general those compounds bearing amine substituents (other than aziridine) were not substrates for the enzyme, either as a result of steric effects (*e.g.* pyrrolidine or piperidine) or because such substituents rendered the quinone more difficult to reduce by the donation of their nitrogen lone pair into the quinone. This was evidenced by electrochemical experiments: for example, for **28**, R = CH_2OH; E_{redox} −1.28 for Y = OMe, −1.48 for Y = pyrrolidinyl (data in V *vs.* ferrocene). Aziridines, on the other hand, because of their inability to donate electron density into the quinone that would require an unfavourable flattening of the nitrogen, have a similar electronic effect to a methoxy group (**28**, R = CH_2OH; E_{redox} −1.26 V for Y = aziridinyl). Conversely, the indolequinones that were among the easiest to reduce electrochemically were

Table 1 Metabolism of representative indolequinone and mitosenes by recombinant human NQO1



R^1	R^2	R^3	Y	Metabolism/μmol min^{-1} mg^{-1} (NADH oxidation)
Me	H	CH_2OH	OMe	13.6 ± 3.5
Me	Me	H	OMe	5.31 ± 0.93
Me	Me	CH_2OH	OMe	1.25 ± 0.03
Me	H	CHO	OMe	8.78 ± 1.91
Me	Me	CO_2Et	OMe	14.3 ± 34.9
Me	Me	CH_2OH	NMe_2	0.46 ± 0.04
Me	Me	CH_2OH	Piperidinyl	0.22 ± 0.06
Me	Me	CH_2OH	Aziridinyl	3.35 ± 0.65
Me	Me	CH_2OAc	OMe	nd[a]
Me	Me	CH_2OCONH_2	OMe	nd[a]
n-Pr	Me	CH_2OH	OMe	1.30 ± 0.10
Ph	Me	CH_2OH	OMe	6.0 ± 0.8
Me	Ph	CH_2OH	OMe	11.0 ± 0.9
Me	$4\text{-Ph-}C_6H_4$	CH_2OH	OMe	5.40 ± 0.83
Me	2-Naphthyl	CH_2OH	OMe	4.0 ± 0.7
Me	CH_2OH	Me	OMe	2.49 ± 1.27
$-(CH_2)_3-$		CH_2OH	OMe	4.80 ± 0.38
$-(CH_2)_3-$		CH_2OH	Me-aziridinyl	2.22 ± 0.41
$-(CH_2)_3-$		CH_2OH	Pyrrolidinyl	0.06 ± 0.02
$-(CH_2)_3-$		CH_2OCONH_2	OMe	nd[a]

[a] No quinone metabolism detected; enzyme is deactivated.

among the best substrates for hNQO1. However, although the ease of reduction is a factor, there was no overall correlation between reduction potential and rate of metabolism by hNQO1. The substituent at the indole 3-position (9-position in mitosene **24**) also had a considerable effect on metabolism by hNQO1. Compounds with an electron-withdrawing group at this position were the best substrates for hNQO1 in both series, although the 3 (or 9)-unsubstituted compounds were also efficiently metabolized. Within the 5-methoxyindolequinones, the rate of metabolism by the enzyme decreased as the 3-substituent was altered in the order: $CO_2Et > CHO > H > CH_2OH > CH_3 > CH_2OCONH_2$. The indolequinones and mitosenes bearing a leaving group at the C-3 (or 9) position (*e.g.* carbamate) were generally much poorer substrates for the enzyme, and in some cases caused inactivation (*q.v.*). The enzyme can also accommodate some larger substituents around the indolequinone nucleus. For example, the 2-phenylindolequinones **28** ($R^2 = Ph$) were excellent substrates for hNQO1, better than their corresponding 2-methyl derivatives. Overall the structure–activity relationships for indolequinones **28** can be summarized as: large groups tolerated at R^1 and R^2; electron-withdrawing groups at R^3 cause faster reduction ($CO_2Et > Me$) but CH_2Y groups at R^3, where Y is a good leaving group, tend to deactivate the enzyme; amines such as piperidine and pyrrolidine at R^5 (but not aziridines) are poor substrates.[57,60–62]

The cytotoxicity of representative indolequinones and mitosenes toward non-small cell lung cancer (NSCLC) cell lines was also tested. Overall the toxicity in these cell lines was greatest for the indolequinones that can potentially act as bifunctional alkylating agents, namely those with an aziridine at C-5 (C-7) and a CH_2OH or CH_2OCONH_2 at C-3 (C-9). Compounds that could only function as monoalkylating agents were less cytotoxic, whereas compounds that have no possible alkylating centres were not toxic. In agreement with their ability to act as efficient substrates for hNQO1, the aziridinyl quinones were usually the most selective agents in each series, exhibiting much greater toxicity toward the high hNQO1 cells than to the hNQO1 deficient cells.[57,61]

In order to obtain more information about the interaction of our novel indolequinones with the enzyme, one of the indolequinones **28** ($R^1 = Me$, $R^2 = Ph$, $R^3 = CH_2OH$, Y = 2-methylaziridinyl), a compound known as ARH019, was co-crystallized with the protein, and the structure of its enzyme complex compared with that of the benzoquinone RH1 **17** and the indolequinone EO9 **18**. RH1 and ARH019 bind to hNQO1 with a similar spatial arrangement such that the quinone atoms almost overlap. Surprisingly ARH019 and EO9, which are chemically very similar indolequinones, bind to the enzyme in different orientations. In ARH019, both the N-1 and C-2 positions are oriented toward the active site entrance with the large 2-phenyl group pointing towards the outside of the active site pocket. The bound structures of the three quinones are shown in Fig. 10.[63]

Although we now had a good understanding of the enzymatic reduction of indolequinones, the range of heterocyclic quinones remained rather small. Therefore in an attempt to widen the group of NQO1 substrates, and to probe further the active site of the enzyme, we explored a new series of heterocyclic quinones based on benzimidazole and benzothiazole. Both benzimidazole-4,7-diones and benzothiazole-4,7-diones have been described previously, and reported to have a range of biological properties.

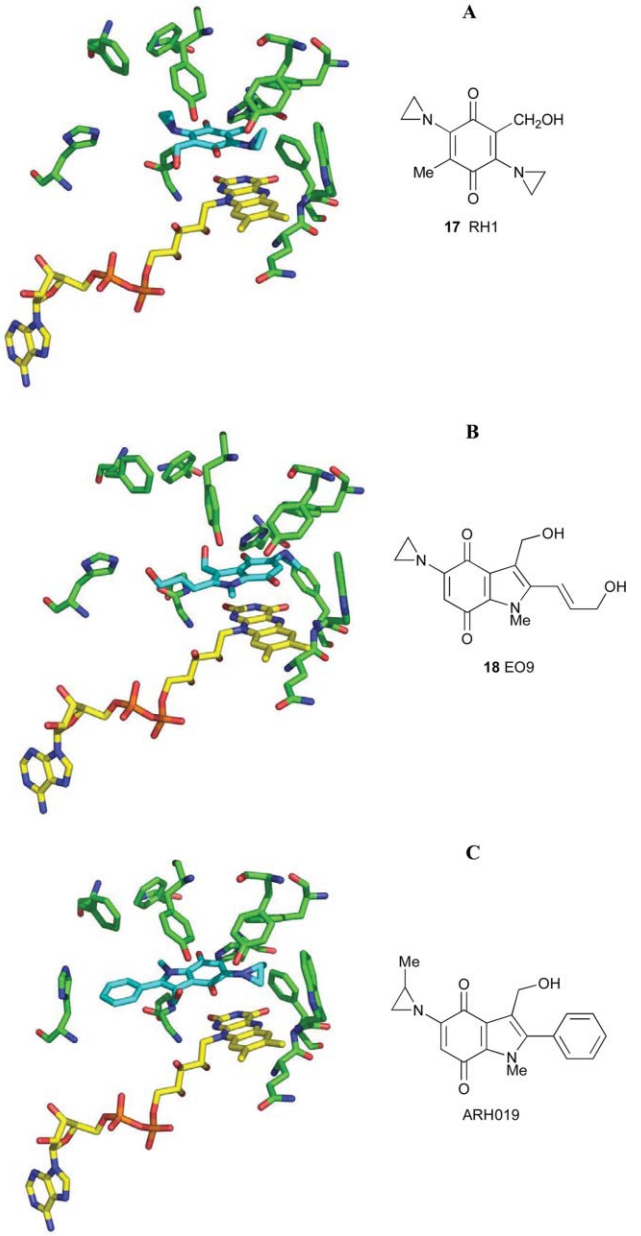


Fig. 10 X-Ray crystal structure of active site of hNQO1 with bound quinones (shown in cyan): (**A**) RH1 (PDB accession code 1h66); (**B**) EO9 (PDB accession code 1gg5); (**C**) ARH019 (PDB accession code 1h69).[22,63] Protein side chain residues are shown in green; the carbon framework of FAD is shown in yellow.

The benzimidazole quinones are better known, and pyrrolo[1,2-*a*]benzimidazoles such as **29** have been extensively investigated by Skibo and co-workers as analogues of MMC (Fig. 11).[64,65] Benzimidazolequinone phosphorodiamidates **30** have also been studied as potential prodrugs for bioreductive activation (*q.v.*).[66] The most widely studied benzothiazolequinone is 5-undecyl-6-hydroxybenzothiazole-4,7-dione (UHDBT) **31**, an analogue of ubiquinone that inhibits electron transport by binding to cytochrome bc_1.[67,68]

In order to make meaningful comparisons with the more widely studied indolequinones, we elected to investigate relatively simple

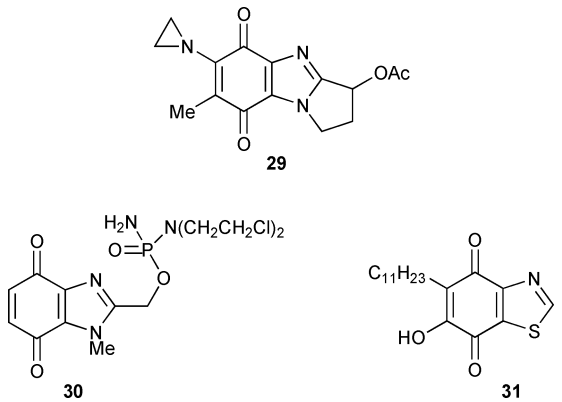
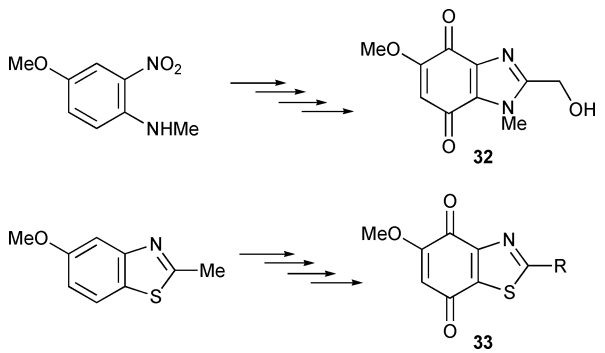


Fig. 11 Benzimidazole- and benzothiazole-quinones.

5-methoxybenzimidazolequinones. Thus the benzimidazole-quinone **32** was prepared by a standard benzimidazole synthesis starting from the corresponding *o*-phenylenediamine (Scheme 8). The synthesis of the benzothiazolequinones **33** started with commercially available 5-methoxy-2-methylbenzothiazole, and proceeded by conventional elaboration of the quinone ring.[69]



Scheme 8 Synthesis of benzimidazole- and benzothiazole-quinones.

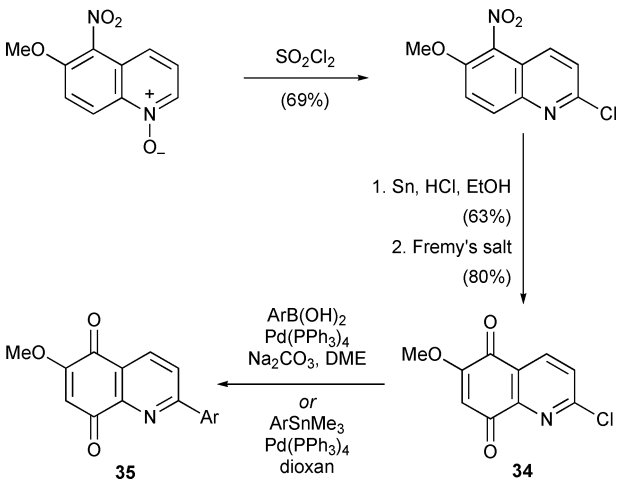
Table 2 Metabolism of benzimidazole and benzothiazole quinones by recombinant human NQO1

Compound	R	Metabolism/μmol min^{-1} mg^{-1} (NADH oxidation)
32	—	43.5 ± 6.3
33	CH_2OH	38.3 ± 8.0
33	Me	49.7 ± 4.0

We next examined the ability of the new quinones to act as substrates for hNQO1. The enzyme data show that the new quinones are excellent substrates for hNQO1.[69] Reduction rates for the benzimidazole- and benzothiazole-quinones were similar (Table 2), but all of these new quinones were much better substrates for hNQO1 than the corresponding indolequinones described above.

One of the best substrates for NQO1 is the naturally occurring antitumour antibiotic streptonigrin **15** (Fig. 3). Isolated from *Streptomyces flocculus* almost 50 years ago, streptonigrin was studied clinically in the 1960s and 1970s as an antitumour agent, but its use was limited by reports of delayed myelotoxicity. The mechanism of action is thought to involve hydroxyl radical (HO·) production following reduction of the quinolinequinone moiety and metal complexation, leading to DNA degradation.[70] Streptonigrin is an excellent substrate for NQO1,[71] and has been shown to be selectively toxic to cancer cell lines with elevated NQO1,[72] although its facile two-electron reduction by NQO1 may be independent from the hydroxyl radical production referred to above. We therefore decided to extend our studies on heterocyclic quinones, and examine the effects of functional group substitutions on the metabolism of a range of novel quinolinequinones **35**.

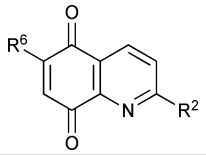
The main synthetic route involved the preparation of 2-chloro-6-methoxyquinolinequinone **34** from 6-methoxy-5-nitroquinoline-*N*-oxide as shown in Scheme 9, followed by palladium catalyzed coupling with areneboronic acids or arylstannanes under typical Suzuki or Stille reaction conditions to introduce a range of aryl groups into the C-2 position, although we also employed strategies based on the classical Friedländer quinoline synthesis, and the "double-Vilsmeier" reaction of acetanilides.[73,74]



Scheme 9 Synthesis of quinolinequinones.

Although the metabolism of streptonigrin itself by hNQO1 has been investigated, there has been no similar study of quinolinequinones in general. Therefore our work represented the first detailed study of the metabolism of such quinones by hNQO1. The rates of metabolism of the new quinolinequinones by purified recombinant hNQO1 are shown in Table 3. In general, the quinolinequinones were much better substrates for hNQO1 than related indolequinones, this greater ease of reduction being borne out by the electrochemical data. Within the series of 6-methoxy substituted quinones several trends are apparent from the metabolism data (Table 3). For simple substituents at the 2-position, the rates of metabolism by hNQO1 are R^2 = H ~ Me > Ph. For aromatic substituents at C-2, the compounds possessing the smaller substituents are metabolized faster. Thus the rate of reduction decreases dramatically for R^2 = Ph > 1-naphthyl > 2-naphthyl > 4-biphenyl, with the last compound being a very poor substrate. Replacement of the C-6 methoxy group by a more electron-releasing secondary amine deactivated the quinolinequinone towards reduction, as observed throughout our studies on indolequinones. In the 6-methoxyquinolinequinones containing heteroaromatic groups, the 2-thienyl derivative is close to the 2-phenyl compound in rate of metabolism, a result that is perhaps not surprising given the similarity in electronic properties of benzene and thiophene rings. However it is the pyridine

Table 3 Metabolism of representative quinolinequinones by recombinant human NQO1



R^2	R^6	Metabolism/μmol min^{-1} mg^{-1} (NADH oxidation)
Streptonigrin	—	47.4 ± 11.5
H	OMe	58.7 ± 18.5
Me	OMe	20.7 ± 3.7
Ph	OMe	28.8 ± 7.9
4-Biphenyl	OMe	4.75 ± 1.01
1-Naphthyl	OMe	25.1 ± 5.1
2-Naphthyl	OMe	14.0 ± 5.2
2-Pyridyl	OMe	50.5 ± 10.6
5-Me-2-pyridyl	OMe	45.7 ± 13.4
3-Pyridyl	OMe	61.9 ± 8.6
4-Pyridyl	OMe	66.1 ± 12.0
2-Thienyl	OMe	32.1 ± 7.8
Ph	Pyrrolidinyl	2.02 ± 0.21
Ph	Morpholinyl	1.40 ± 0.20

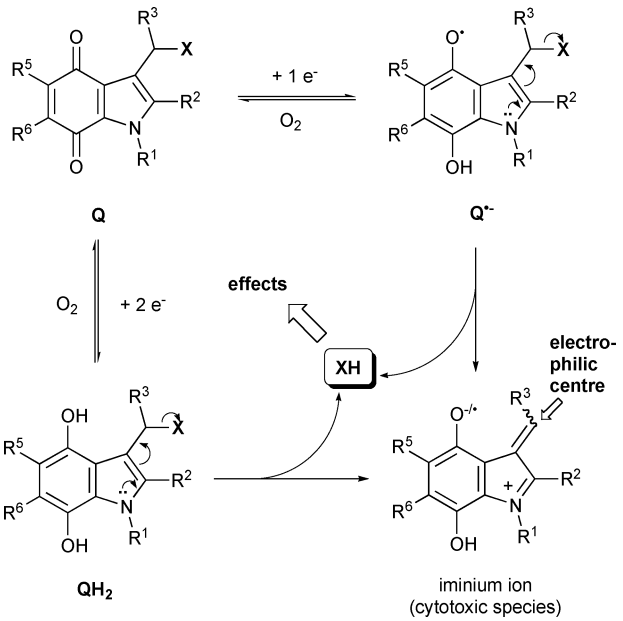
derivatives that are the best substrates, with the 2-, 3- and 4-pyridyl compounds being better substrates than streptonigrin itself. The reason for investigating such compounds was, of course, the presence of a 2-pyridyl group in streptonigrin.

Cytotoxicity data were obtained for selected quinolinequinones. In comparison to streptonigrin, all the synthetic analogues are much less cytotoxic, since presumably they do not possess the metal-binding features that lead, after reduction, to hydroxyl radical production, and toxicity. As expected, quinones that are good substrates for hNQO1 are more toxic to the NQO1 containing or expressing tumour cell lines than the NQO1 deficient cell lines. Quinones such as the biphenyl and naphthyl derivatives that are poor substrates show no selectivity or have no measurable cytotoxicity.[73,74]

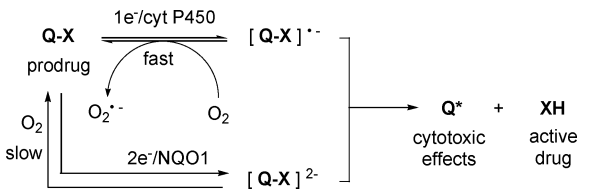
Indolequinones as prodrugs: bioreductive drug delivery

As a result of their structural relationship to MMC, the indolequinones have been widely studied, particularly because of the ability of 3-indolyl carbinyl substituents in such compounds to undergo an elimination process upon either one-or two-electron reductive activation. The resulting iminium species is then a potential electrophile capable of DNA-alkylation or other cellular-damaging events (Scheme 10). However, bioreductive drugs such as indolequinones are also of interest since they could act as reductively activated drug delivery vehicles by releasing a variety of leaving groups X^- in a reductive environment. Thus they may have secondary biological effects due to the eliminated molecule XH (after protonation), in addition to the cytotoxic iminium derivative formed on reduction and elimination (Scheme 10).

Hence a simple bioreductive drug delivery system can be envisaged as outlined schematically in Scheme 11 for a quinone prodrug molecule **Q–X**, where XH represents the free drug. Although the concept is illustrated for a quinone, other reductively activated prodrug molecules, notably aromatic nitro compounds



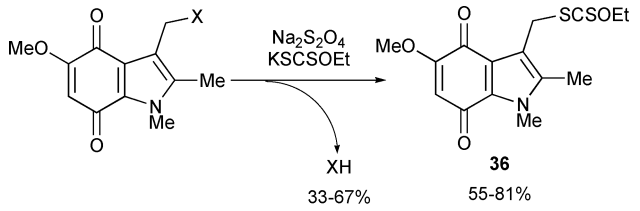
Scheme 10 Elimination of leaving groups upon reductive activation of indolequinones.



Scheme 11 Reduction induced fragmentation of potential prodrugs **Q–X**.

have also been investigated.[75–77] As discussed, a quinone **Q–X** could be activated by one- or two-electron processes according to which enzyme is involved. A one-electron reduction results in the formation of a semiquinone radical anion in which rapid re-oxidation (with simultaneous formation of superoxide) will compete with fragmentation and loss of the X^- leaving group. Hence such drug delivery mechanisms are only likely to operate in regions of very low (or zero) oxygen concentration such as the hypoxic (or anoxic) region of a solid tumour. Two-electron reduction by NQO1, however, leads to a hydroquinone that would react more slowly with oxygen, and hence the desired fragmentation to release the drug molecule XH (after protonation) may be favoured. Hence, in principle, the reduction properties of the quinone allows the targeting of two environments inherent in tumours: the hypoxic regions of solid tumours, and tumours where NQO1 is upregulated.

However, it was important to understand the relative rates of the processes outlined in Schemes 10 and 11, and therefore we initiated a study of the elimination of various groups X from a range of indolequinones. Initially the reductive activation was carried out under chemical conditions using sodium dithionite as reducing agent, and potassium ethyl xanthate as a thiol to trap the intermediate iminium ion (Scheme 12). Elimination of a range of leaving groups X (phenolates, carboxylates, thiophenolates) was observed. Concurrent with the elimination of X^-, the formation of the thiol trapped product **36** was also observed and was isolated following oxidative work up in air. Blank experiments established



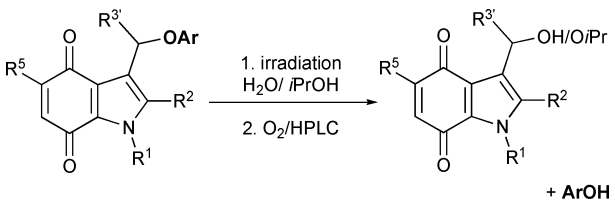
X = OPh, OC_6H_4-4-F, OC_6H_4-3-NHAc, $OCOC_6H_4$-4-F, S-2-pyrimidyl

Scheme 12 Elimination of various leaving groups upon reductive activation of indolequinones with trapping of the iminium ion with a thiol.

that **36** was not formed in the absence of the reducing agent, *i.e.* no direct nucleophilic substitution occurs.[55,56]

In a productive collaboration with Peter Wardman and Steven Everett and their colleagues of the Gray Cancer Institute (Mount Vernon Hospital, Middlesex), detailed physical chemical studies using radiolytic reduction (pulse radiolysis) enabled the measurement of various kinetic parameters pertaining to the processes outlined in Scheme 11, and established that for indolequinones with good leaving groups X, the elimination occurred from the hydroquinone. Fragmentation of the semiquinone radical anion is slow, and in the absence of oxygen, it most likely decays by disproportionation to the hydroquinone plus quinone. In terms of toxicity towards tumour cells, compounds with good leaving groups were generally more cytotoxic, although the nature of the R^2 substituent has a marked effect on potency. For compounds with the same leaving group (X = OCOPh), potency decreases as R^2 is changed from H to Me to cyclopropyl. In general, 2-unsubstituted indolequinones were the most toxic.

In order to investigate the effects of substituents on the indolequinone on the rate of elimination of potential drug molecules, a series of indolequinones with the same leaving group (4-nitrophenoxide) was prepared, and studied by pulse radiolysis to provide detailed kinetic data. Product analysis was carried out by HPLC following oxidative work up of the radiolysis mixture, with products of reductive fragmentation of the phenolic linker—4-nitrophenol and the hydroxyl- or isopropoxymethyl indolequinone (formed by addition of the solvents to the intermediate iminium ion)—being readily identified (Scheme 13).



R^1, R^2 as Figure 9; $R^{3'}$ = H or Me

Ar = Ph, 4-NO_2-C_6H_4, 2,4-$(NO_2)_2$-C_6H_3, 2-MeO-5-NO_2-C_6H_3, 2-MeO-4-NO_2-C_6H_3, 2-CHO-4-NO_2-C_6H_3, 3-CHO-4-NO_2-C_6H_3, 2-F-4-NO_2-C_6H_3

Scheme 13 Fragmentation of indolequinones on pulse radiolysis.

After reduction of the indolequinones to either the semiquinone ($Q^{\bullet -}$) or hydroquinone (QH_2), elimination of 4-nitrophenol occurred. The half-lives of semiquinone ($Q^{\bullet -}$) radicals at $[O_2] = 5$ μmol dm^{-3}, typical of tumour hypoxia, were $t_{1/2} = 0.3$–1.8 ms, the higher values associated with higher reduction potentials. The half-lives of the hydroquinone (QH_2) were markedly longer ($t_{1/2} =$ 8–102 min). Although the indolequinones were able to eliminate 4-nitrophenol with high efficiency, only the semiquinone ($Q^{\bullet -}$) radicals of the 3-indolylmethyl carbinyl substituted derivatives ($R^{3'}$ = Me) did so with sufficiently short half-lives to compete with electron transfer to oxygen. In contrast, the elimination of 4-nitrophenol from the hydroquinone ($t_{1/2} \approx 1.5$–3.5 s) was not inhibited even at normal tissue oxygen concentrations. The semiquinone radical derived from the $R^{3'}$ substituted analogue (R^1 = Me, R^2 = Me, $R^{3'}$ = Me, Y = OMe) exhibited the fastest rate of elimination of 4-nitrophenol ($t_{1/2} \approx 2$ ms) and was therefore capable of competing against electron transfer to oxygen ($t_{1/2} \approx$ 1.6 ms) at $[O_2] \approx 5$ μmol dm^{-3}. This study demonstrated that by incorporating radical-stabilizing substituents ($R^{3'}$) such as a simple alkyl group at the indolyl carbinyl position, it was possible to control the rate of reductive fragmentation and target the leaving group to hypoxic tissues.[78,79]

In a related study we also looked at the effect of pK_a of the phenolic leaving group on the rates of reductive elimination from the (indol-3-yl)methyl position of indolequinones by pulse radiolysis. The rate of reductive elimination of phenoxide anions from the (indol-3-yl)methyl position of semiquinone radicals was dependent upon this pK_a, with a decrease in 3.8 pK units shortening the half-life from 28 to 1.5 ms. Only 2,4-dinitrophenol ($pK_a = 3.9$) was eliminated from an unsubstituted (indol-3-yl)methyl position at a rate that would compete with reoxidation of the radical by oxygen. Substitution by methyl on the linker increased the rate of elimination of the leaving group by a factor of ~12. Clearly both lowering the pK_a of the leaving group and incorporation of a suitable radical stabilizing substituent at the indolyl carbinyl position can have a dramatic effect on rates of elimination.[80]

The above studies on model drug molecules as leaving groups have defined the physico-chemical parameters for a successful indolequinone based bioreductive drug delivery strategy, although we have yet to put this into practice with "real" anticancer drug molecules. Nevertheless, some further progress has been made recently. Thus Borch and co-workers have reported the elimination of a cytotoxic phosphoramidate from the indolequinone **37** upon reductive activation (Fig. 12),[81,82] Tanabe *et al.* have shown that 5-fluorodeoxyuridine is released from the indolequinone

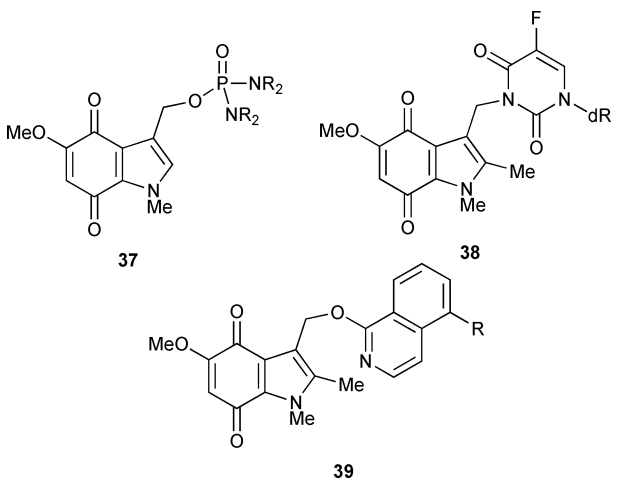


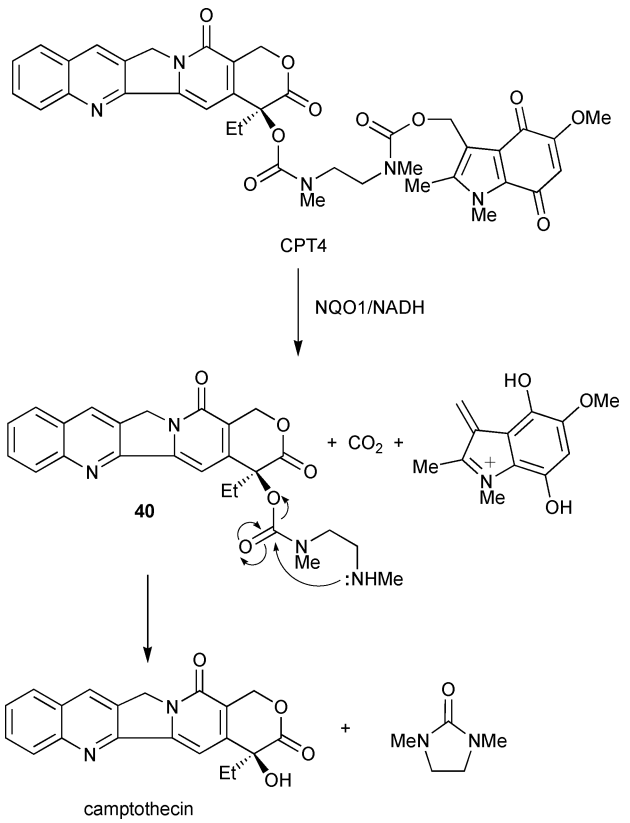
Fig. 12 Indolequinone based prodrug systems.

conjugate **38** upon radiolytic reduction,[83] and Threadgill and co-workers have described an indolequinone **39** conjugated with an isoquinoline—the 1-hydroxyisoquinolines (as the 1-isoquinolone tautomers) released upon reductive activation are potent inhibitors of poly-ADP-ribose polymerase (PARP) with potential therapeutic applications.[84,85]

One particularly elegant application of indolequinones as bioreductively activated prodrugs involves camptothecin, a naturally occurring, potent inhibitor of DNA topoisomerase 1 (Topo 1) that can stabilize covalent binding of Topo 1 to DNA, thereby resulting in irreversible and lethal strand breaks of DNA during replication. However, the clinical application of camptothecin itself in cancer treatment was suspended due to its non-specific toxicity and negligible water solubility, although new synthetic derivatives such as irinotecan (Camptosar®) and topotecan (Hycamtin®) are now in clinical use. Zhang *et al.* have developed a new class of water-soluble, bioreductively activated indolequinone prodrugs of camptothecin such as CPT4 that are subject to metabolism by cellular reductases, thereby releasing a potent Topo 1 inhibitor. In the presence of NQO1 and NADH, CPT4 was quickly reduced, thereby producing both the indolequinone iminium ion moiety, and compound **40**, which fragments chemically to release camptothecin as shown in Scheme 14.[86]

To date the focus had been the fragmentation of groups from the (indol-3-yl)methyl position of indolequinones (Scheme 10), notwithstanding the fact that in both MMC **13** and EO9 **18** elimination from the (indol-2-yl)methyl position must also occur. The role of 2-indolyl substituents in other indolequinones is less clear, since relatively few studies have been reported. For example, no elimination of a carboxylate leaving group—2-acetoxybenzoate (aspirin)—occurred upon (one-electron) radiolytic reduction of the 2-substituted indolequinone **28** ($R^1 = R^3 =$ H, $R^2 = CH_2OCOAr$, Y = OMe). On the other hand, the 3-substituted analogue **28** ($R^1 = R^2 =$ H, $R^3 = CH_2OCOAr$, Y = OMe) underwent efficient fragmentation upon reduction.[87] Similar results were observed with indolequinone phosphoramidate prodrugs: upon one-electron reduction, elimination from the 2-substituted analogue of quinone **37** was slow in comparison to **37** itself (Fig. 12). In contrast, the phosphoramidate was rapidly released from both the 2-and 3-substituted indolequinones upon two-electron reduction suggesting that potential drug delivery mechanisms from the indolequinone 2-position proceed better from the hydroquinone QH_2.[81,82] In order to investigate this suggestion more fully, we investigated a series of indolequinones bearing a range of substituents at the 2-position, and evaluated both their fragmentation upon reduction and their metabolism by NQO1.

Firstly, using pulse radiolysis physical chemical kinetic studies, we demonstrated that reductive elimination of a good leaving group such as 4-nitrophenoxide from the (indol-2-yl)methyl position occurs most readily *via* the hydroquinone rather than the semiquinone, but it is very significantly slower than elimination from the (indol-3-yl)methyl position in an analogous compound (unpublished data). Secondly, the enzyme data showed that such indolequinones were generally quite poor substrates for hNQO1. Interestingly, whilst in the 3-substituted series, compounds with potential leaving groups, for example **28** ($R^3 = CH_2OAc$) appeared to inactivate the enzyme, in the 2-substituted series, such compounds with leaving groups are substrates.[62]



Scheme 14 Bioreductive activation of the prodrug CPT4 by NQO1 resulting in release of camptothecin.

Inhibitors of NQO1

A number of compounds are known to inhibit the activity of NQO1 by competing with NAD(P)H for binding to the enzyme thereby preventing reduction of the FAD. These include a number of flavones, coumarins, and the turmeric spice-derived curcumin, but the most potent competitive inhibitor (K_i = 1–10 nM) is dicumarol **41** (Fig. 13).[88,89] Dicumarol, an anticoagulant that functions in a similar manner to warfarin, has used been used as a competitive inhibitor in NQO1 assays for many years, and has recently been co-crystallized with hNQO1 and the bound complex studied by X-ray crystallography.[89] The inhibitor stacks

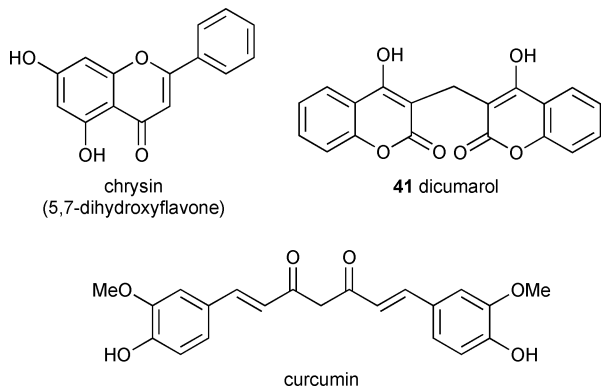


Fig. 13 Competitive inhibitors of NQO1.

parallel to the isoalloxazine ring of the FAD (Fig. 14A), and is bound by a series of hydrophobic interactions and hydrogen bonds to both monomer units of the protein. However, there are potential disadvantages with dicumarol in that it is not selective and can inhibit other enzymes in addition to NQO1. It can also be extensively protein bound complicating its use in cellular assays. Hence there was a need for a more efficient inhibitor of NQO1, and in collaboration with David Ross (University of Colorado) this is what we set out to look for.

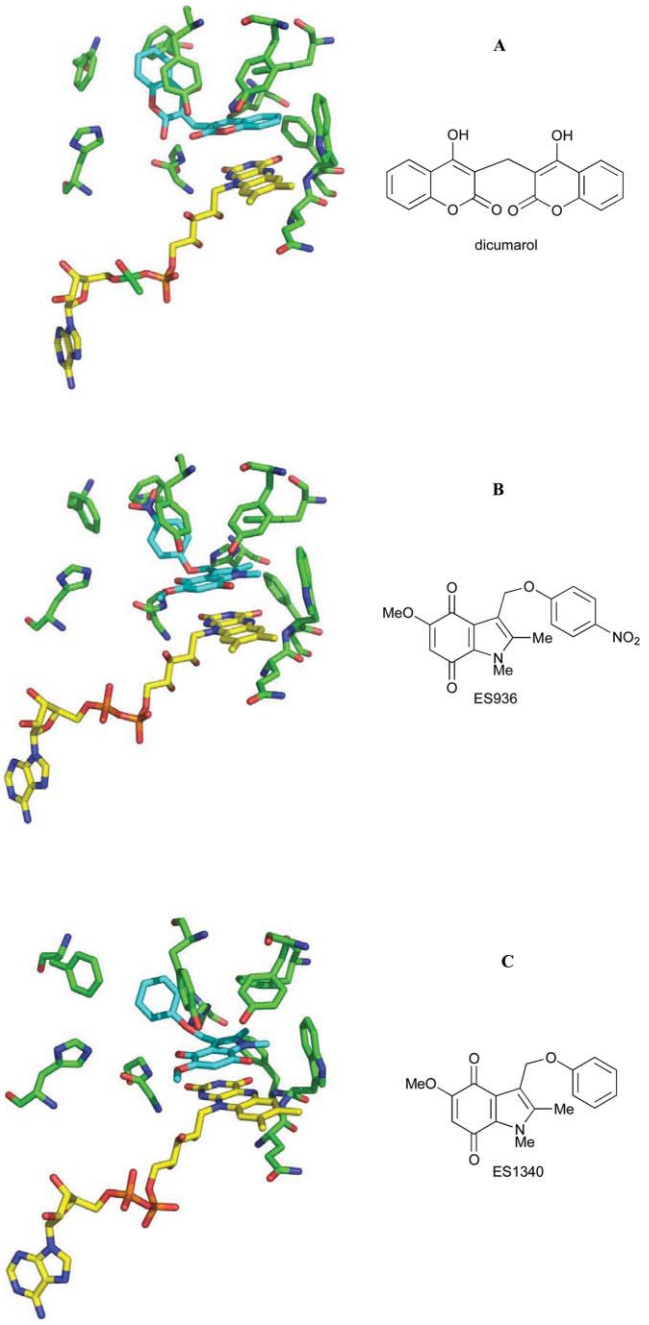
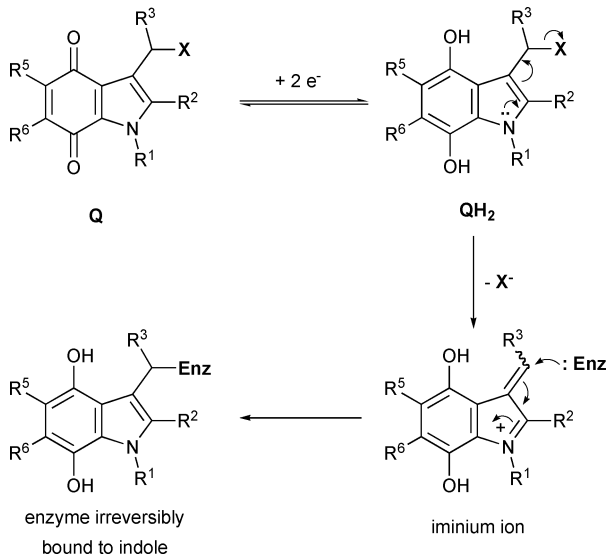


Fig. 14 X-Ray crystal structure of active site of hNQO1 with bound inhibitors (shown in cyan): (**A**) dicumarol (PDB accession code 2f1o);[89] (**B**) ES936 (PDB accession code 1kbq);[22,90] (**C**) ES1340 (PDB accession code 1kbo).[22,90] Protein side chain residues are shown in green; the carbon framework of FAD is shown in yellow.

Our efforts to develop efficient inhibitors of NQO1 started with the observation, noted above, that indolequinones with a good leaving group at the (indol-3-yl)methyl position tended to inactivate the enzyme during the assays. We rationalized this on the basis of NQO1 activated reduction of the indolequinone (*cf.* Scheme 10) that leads to an electrophilic iminium ion following elimination of the leaving group in the hydroquinone. Since the hydroquinone must be generated in the enzyme active site, if it fragments quickly, then the iminium ion is also formed in the active site and can alkylate nearby nucleophilic residues leading to irreversible binding of the enzyme. This mechanism-based (or suicide substrate) inhibition is outlined in Scheme 15. Furthermore, the rates of elimination of various leaving groups were available from our detailed kinetic studies described above.



Scheme 15 Mechanism-based inhibition of NQO1 by indolequinones bearing good leaving groups X at the (indol-3-yl)methyl position.

One compound that proved a particularly effective mechanism-based inhibitor of hNQO1 was the indolequinone with a 4-nitrophenoxide leaving group, a compound known as ES936 (**28**, $R^1 = R^2 = Me$, $R^3 = CH_2OC_6H_4NO_2$, Y = OMe).[90,91] ES936 inhibited hNQO1 in a time- and concentration-dependent manner, and in agreement with its postulated role as mechanism-based (suicide substrate) inhibitor, required the presence of cofactor NADH, and therefore a catalytic turnover, for effective enzyme inhibition. An important measure of mechanism based-enzyme inhibitors is the partition ratio–the number of molecules released from the active site in proportion to the number that remain to inactivate the enzyme. The partition ratio for ES936 was initially measured as 1.40 indicating that it is an extremely efficient inactivator of the enzyme. Detailed information on the binding of ES936 to the enzyme came from the co-crystal structure of the enzyme complex (Fig. 14B). Interestingly, the orientation of the ES936 in the active site is reversed relative to that of the indolequinone substrates EO9 **18** and ARH019 (Fig. 10B and 10C). The nitro group is not an important factor in determining the binding mode of ES936 since the phenoxy analogue lacking the 4-nitro substituent (ES1340) binds in an identical manner (Fig. 14C), although the compound is a very poor inhibitor of

the enzyme (*q.v.*).[90] The comparison of the binding of ES936 with that of dicumarol (Fig. 14A) has been discussed.[89]

The orientation of ES936 in the active site (Fig. 14B) shows that the neighbouring nucleophilic residues are two tyrosines (Tyr126′ and Tyr128′) and one histidine (His161), and hence one would expect that if a reactive iminium species were generated from ES936 in the active site, it would be one of these residues that would be alkylated. Hence our next task was to ascertain the site of covalent binding (if any) to the enzyme and prove the original mechanism-based inhibition theory. The molecular mass of the monomeric protein was measured by ESI mass spectrometry as 30864 ± 6 Da compared to a calculated value of 30868.6 Da. After incubation with NADH and ES936, the measured molecular mass increased to 31081 ± 7. The difference of 217 mass units is consistent with the proposed adduct (*cf.* Scheme 15); alkylation with an ES936-derived iminium ion would result in a theoretical increase of mass of 218 after reoxidation to the quinone. Hence there is excellent evidence for the mechanism outlined in Scheme 15. In order to determine the actual site of binding, NQO1 and the ES936-treated protein were both digested using chymotrypsin, and the resulting fragment peptides analyzed by LC-MS-MS (Fig. 15). Chromatography identified a peptide (m/z = 734.2 Da) that was not present in the control protein, and subsequent MS-MS analysis established that this was a tetrapeptide of sequence AlaTyrThrTyr corresponding to residues 125–128 of hNQO1. Hence the likely site of covalent bond formation to the protein is one of the two tyrosine residues (Tyr126′ or Tyr128′).[90]

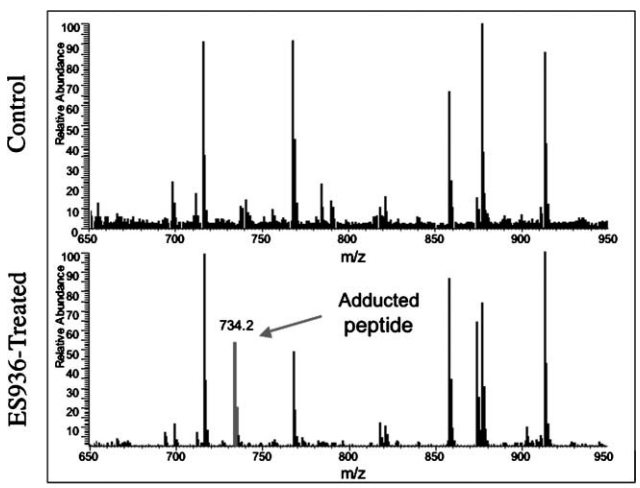


Fig. 15 ES-LC-MS analysis of chymotrypsin digests of control hNQO1 (top) and after incubation with ES936 (bottom). The adducted peptide fragment is seen at m/z = 734.2.[90]

Hence we had achieved our goal and developed an efficient mechanism-based inhibitor of hNQO1. At 100 nM concentration, ES936 inhibits >95% of NQO1 activity in cells within 30 min,[92] and since it is specific, appears to be a more useful biochemical tool than dicumarol for use in routine NQO1 assays. At this point, our work on inhibitors of NQO1 was put on hold, only to be reactivated in late 2003 following the report by Cullen *et al.* that dicumarol inhibition of NQO1 led to inhibition of growth in pancreatic cancer cells.[93] The authors also showed that the dicumarol inhibition of NQO1 caused increased intracellular levels of superoxide, and proposed a novel mechanism whereby NQO1 inhibition leads to increased superoxide levels and inhibition of growth of the malignant phenotype. Our biology collaborators had already established that NQO1 can directly scavenge superoxide,[94] and in cells containing high levels of NQO1, such as pancreatic tumour cells, scavenging of superoxide by NQO1 may compete with superoxide dismutase as an alternative pathway for detoxification. Hence there was a mechanistic basis for the possible beneficial effects of NQO1 inhibitors in pancreatic cancer, a disease that has one of the worst prognoses of all cancers.

Our first task, therefore, was to show that our potent NQO1 inhibitor ES936 had an effect in pancreatic cancer cell lines. Firstly it was established that ES936 inhibited NQO1 activity in human pancreatic cancer cells (MIA PaCa-2 and BxPc-3 lines) and in a time-dependent manner. Secondly, ES936 was also effective in inhibiting the growth of the same two cancer cell lines with IC_{50} values of 100–365 nM, and finally it was shown that pancreatic tumour xenografts in mice grew significantly slower following treatment with ES936.[95] In separate experiments, it was also shown that the cytotoxicity was independent of superoxide generation, suggesting that NQO1 inhibition and increased superoxide levels may not be the only factors contributing to the mechanism of action of ES936 in pancreatic cancer cells.

The initial biological data on ES936 encouraged us to undertake a much wider study of indolequinones, and hence establish whether NQO1 inhibition is a potential therapeutic approach to pancreatic cancer. Hence we prepared a number of analogues of ES936 in which the 4-nitrophenoxy leaving group was replaced by a range of other phenolic leaving groups. These were chosen to explore both the effect of leaving group ability, as evidenced by the pK_a of the corresponding phenol, and the steric effects of the 3-aryloxymethyl group. We were also conscious of the need to identify possible alternative electron-withdrawing groups on the phenolic moiety, since nitro groups are not without problems in potential drug molecules due to their metabolism. Hence, for example, the choice of the 2,4,6-trifluorophenoxy group, since 2,4,6-trifluorophenol has a similar pK_a to 4-nitrophenol (7.5 and 7.2 respectively). We also investigated an isomeric series of NQO1 inhibitors based on the 6-methoxyindolequinone ring system. Although this may appear a trivial change, the switch of the electron-releasing methoxy group from the 5- to the 6-position has an effect on the electronic properties of the quinone ring system, and hence on the reductive elimination of the phenoxide as required for NQO1 inhibition (*cf.* Scheme 15). Hence it was by no means obvious that such analogues would be potent inhibitors.

David Ross and colleagues at the University of Colorado evaluated our new quinones as mechanism-based inhibitors of NQO1 (dependence on NADH and partition ratio), as inhibitors of the enzyme in cells, and for their ability to inhibit the growth of the human pancreatic MIA PaCa-2 tumour cell line.[59,96] In order to illustrate these biological tests, we show the comparisons between ES936 and its 6-methoxy analogue **43**. As already mentioned, a key feature of a mechanism-based enzyme inhibitor is the requirement for a catalytic turnover, and hence the presence of the cofactor, for effective inhibition. Therefore an essential experiment is to measure NQO1 activity after incubation with the indolequinone in the presence and absence of the cofactor NADH. The results are shown in Fig. 16A, and for both ES936 and **43** there is a clear dependence on the cofactor. The data for

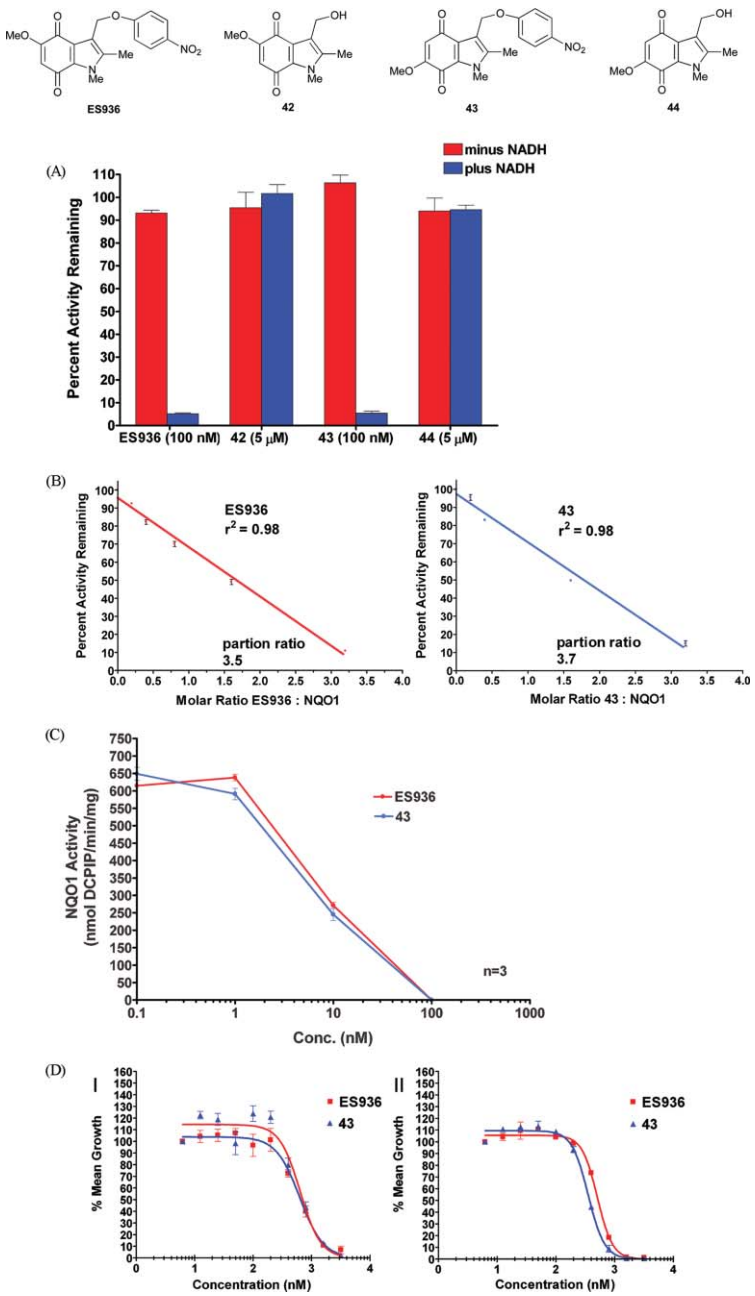


Fig. 16 Mechanism-based inhibition of hNQO1 by the indolequinones ES936 and **43**, and effects on growth of human pancreatic cancer cells.[59,96] (**A**) Dependence on NADH. NQO1 activity was assayed following the incubation of indolequinone (100 nM or 5 μM) with recombinant human NQO1 in the absence (red bars) and presence (blue bars) of 0.2 mM NADH. Incubations were performed for 5 min at 32 °C. Results are the mean ± standard deviations of three separate determinations. (**B**) Partition ratios for the inactivation of NQO1. Indolequinones and recombinant human NQO1 were incubated in the presence of 0.2 mM NADH for 15 min, with defined molar ratios of indolequinone to enzyme (range 0.2 : 1 to 1250 : 1). (**C**) Inhibition of NQO1 catalytic activity in human pancreatic MIA PaCa-2 cells. Cells were treated with the indolequinones (ES936 and **43**) at the indicated concentrations in complete growth media for 1 h, after which the cells were harvested and NQO1 catalytic activity was measured using the reduction of DCPIP. Results are the mean (± standard deviation) of three independent determinations. (**D**) Growth inhibition in the human pancreatic MIA PaCa-2 cancer cell line measured using the MTT colourimetric assay. MIA PaCa-2 cells were treated with the appropriate indolequinone (6.25–3200 nM) in complete medium for 4 h (**Graph I**) and 72 h (**Graph II**) time periods. The IC_{50} values were defined as the concentration of indolequinone that resulted in 50% reduction in cell number compared to the DMSO treated control, determined from semi-log plots of percentage of control *versus* indolequinone concentration.

the corresponding hydroxymethyl compounds **42** and **44** are also shown; no enzyme inhibition is observed in the absence or presence of NADH indicating that these compounds are not mechanism-based inhibitors of NQO1. Presumably the poor leaving group (hydroxide) prevents the formation of the electrophilic iminium ion (Scheme 15).

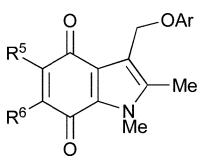
The dependence of indolequinones ES936 and **43** on NADH for inhibition was taken as preliminary evidence for mechanism-based

inactivation. To compare the efficiency with which the indolequinones inactivated NQO1, their partition ratios were calculated from plots of enzyme activity against molar ratio of inhibitor to enzyme (Fig. 16B). As described previously, the partition ratio for a mechanism-based inhibitor is the number of catalytic cycles required to inactivate one molecule of enzyme, therefore the lower the partition ratio, the more efficient the inhibitor. The partition ratios for ES936 and **43** were 3.5 (remeasured) and 3.7, respectively, indicating that these indolequinones were both efficient mechanism-based inhibitors with similar potency.

The next biological assay was to determine whether the partition ratios for NQO1 inhibition using purified recombinant human NQO1 reflected NQO1 inhibition in cellular systems. The concentration dependences of NQO1 inhibition following treatment of human pancreatic MIA PaCa-2 cancer cells with compounds ES936 and **43** were determined after 1 h. For both indolequinones, greater than 95% of NQO1 activity in the cells could be inhibited at concentrations between 10–100 nM (Fig. 16C). Finally the ability of ES936 and **43** to induce cytotoxicity was measured in MIA PaCa-2 cells using the MTT growth inhibition assay. Although it has been shown that greater than 95% inhibition of NQO1 occurs after a 1 hour exposure to indolequinones, MIA PaCa-2 cells were treated with indolequinones for 4 hours in order to maintain a prolonged period of NQO1 inhibition, since once these mechanism-based inhibitors are removed, NQO1 activity slowly returns due to the synthesis of new NQO1 protein. A longer exposure period was also used (72 h) for comparative purposes. The IC_{50} values are shown for both the 4 h and 72 h treatments with the appropriate indolequinone. The growth inhibitory potency of these compounds after 4 h and 72 h indicated that both indolequinones were effective growth inhibitors in MIA PaCa-2 cells ($IC_{50} < 640$ nM) (Fig. 16D).

The above data show that notwithstanding the electronic effect of the 5- or 6-methoxy group, the indolequinones ES936 and **43** are very similar biologically. Data for a larger range of quinones are shown in Table 4. Within the series of analogues, there was some correlation of the partition ratio with the leaving group ability of the aryloxy group at the (indol-3-yl)methyl position, as evidenced by the pK_a of the corresponding phenol. This is nicely illustrated by the series of fluorophenoxy compounds (entries 6–8), where the compounds become more potent inhibitors as the pK_a of the leaving group decreases as the degree of fluorine substitution increases (4-fluorophenol $pK_a = 9.9$; 2,4-difluorophenol $pK_a = 8.7$; 2,4,6-trifluorophenol $pK_a = 7.5$). It is noteworthy that a nitrophenolate leaving group is not essential since the indolequinones bearing a 2,4,6-trifluorophenoxy group (entries 8 and 9) are highly efficient inhibitors. Likewise, the compounds with 4-hydroxypyridine (4-pyridone) leaving groups were also efficient inhibitors of NQO1 (entries 10 and 11). Importantly, there was also a general relationship between the partition ratio measured using purified NQO1 and the ability of the indolequinones to inhibit NQO1 in cells. Finally, it was shown that the indolequinones inhibited the growth of human pancreatic MIA PaCa-2 cancer cells. The complete range of indolequinones tested exhibited IC_{50} values in the range *ca.* 0.1–9.5 μM (100–9500 nM) with the 2-nitrophenyl, and the di- and tri-fluorophenyl derivatives (entries 3, 4, 7–9) being among the most potent growth inhibitors, and the pyridyloxy derivatives (entries 10 and 11) the least effective. Hence it is clear that potent NQO1 inhibitors such as the pyridyloxy derivatives do not exhibit the highest levels of cytotoxicity. Conversely, relatively poor inhibitors such as the 4-trifluoromethylphenoxy compound (entry 5) demonstrated more potent cell growth inhibition. Recently we have identified indolequinones, which are even more potent inhibitors of cell growth (IC_{50} 26–78 nM) but are not mechanism-based inhibitors of NQO1 at all. These data on a large set of indolequinones demonstrate that NQO1 inhibition does not correlate with growth inhibitory activity, at least in the MIA PaCa-2 tumour cell line,

Table 4 Inhibition of recombinant human NQO1 by indolequinones, and inhibition of cell growth in the MIA PaCa-2 cell line



Entry	R^5	R^6	Ar	Partition ratio	>90% Inhibition of NQO1 in MIA PaCa-2 cells[a]/nM	IC_{50} MIA PaCa-2 at 4 h/nM
ES936	OMe	H	4-NO_2-C_6H_4	3.5	10–100	629 ± 17
43	H	OMe	4-NO_2-C_6H_4	3.7	10–100	638 ± 15
1	OMe	H	C_6H_5	4000	nd	1385 ± 24
2	H	OMe	C_6H_5	3800	nd	4563 ± 26
3	OMe	H	2-NO_2-C_6H_4	1.0	nd	345 ± 20
4	H	OMe	2-NO_2-C_6H_4	1.0	10–100	363 ± 9
5	H	OMe	4-CF_3-C_6H_4	652	5000–10 000	496 ± 3
6	H	OMe	4-F-C_6H_4	>100 000	5000–10 000	905 ± 25
7	H	OMe	2,4-F_2-C_6H_4	21.3	10–100	255 ± 5
8	H	OMe	2,4,6-F_3-C_6H_4	1.7	10–100	452 ± 4
9	OMe	H	2,4,6-F_3-C_6H_4	1.9	nd	427 ± 5
10	OMe	H	4-Pyridyl	1.3	nd	2007 ± 16
11	H	OMe	4-Pyridyl	0.9	10–100	2560 ± 7

[a] nd not determined.

suggesting that targets in addition to NQO1 need to be considered to explain the potent activity of this series of indolequinones in human pancreatic cancer cells.[59,96] The search for these target(s) is ongoing.

Future directions

In the mid 1980s we started a project in mainstream synthetic organic chemistry aimed at developing new routes towards a natural product, mitomycin C. Over the intervening 20 years, the project has evolved considerably, and moved in directions we could not have imagined. In this period, as well as pursuing organic synthesis, we have collaborated with electrochemists, physical organic chemists, biochemists, cell biologists and protein crystallographers. Through these collaborations, we have been able to increase significantly our knowledge of the quinone reductase NQO1. Hence through this combination of synthetic chemistry and biology, we have developed efficient substrates for, and effective mechanism-based inhibitors of this fascinating enzyme.

What next? Clearly our original hypothesis that NQO1 inhibitors would be useful against pancreatic cancer is not the whole story—our most potent inhibitors of cell growth of human pancreatic cancer cell lines are not mechanism-based inhibitors of the enzyme, so another biological target(s) must be involved. A priority is to find this biological target, and establish how it links to NQO1, if at all. Even if it turns out that the inhibition of NQO1 plays little role in pancreatic cancer, following this hypothesis has led us, maybe fortuitously, to potent compounds that may have potential as therapeutic agents. Hence we will continue to work on NQO1, since new quinones and new roles for the enzyme are still being discovered. For example, it has been discovered that the naturally occurring quinone geldanamycin **14** (Fig. 3), a derivative of which is in clinical trials as the first in class inhibitor of Hsp90 against cancer, is a substrate for NQO1.[97] Indeed, it appears that the hydroquinone may be more biologically active.[98,99] Given the potential of Hsp90 inhibitors such as geldanamycin derivatives, not only in cancer but also in neuro-degenerative diseases, it seems likely that attention will continue to focus on the bioreduction of quinones.

Quinones are ubiquitous in Nature, and their properties are wide-ranging. We hope that we have demonstrated herein that not only do they provide challenges for synthetic organic chemists, they also provide opportunities for biological investigations, particularly their reduction by the intriguing enzyme NQO1.

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