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Inhibitors of NQO1: Identification of compounds more potent than dicoumarol without associated off-target effects

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ABSTRACT

The enzyme NAD(P)H quinone oxidoreductase (NQO1) can function both as a detoxifying enzyme as well as chaperone protein. The latter property has been extensively characterized by the use of dicoumarol which inhibits the chaperone properties of NQO1 in cells. However, the use of this compound is compromised by its multiple "off-target" effects. Coumarin-based compounds that are more potent than dicoumarol as inhibitors of NQO1 in cells have been identified (Nolan et al., Biochem Pharmacol 2010;80:977–81). The purpose of the work reported here is to evaluate the off-target effects of these compounds when compared to dicoumarol. A range of these substituted coumarins are identified that are significantly less toxic than dicoumarol in a panel of nine cell lines. Further a number of the compounds generate much less intracellular superoxide, and many of them also show a reduced ability to induce apoptosis when compared to dicoumarol. None of these effects correlate with the ability of the compounds to inhibit the enzymatic activity of NQO1 in cells. In conclusion, potent inhibitors of NQO1 have been identified that will be more pharmacologically useful than dicoumarol for probing the function of NQO1 in cells and tissues.

1. Introduction

Human NAD(P)H quinone oxidoreductase (NQO1, DT-diaphorase, Quinone Reductase 1, Vitamin K Reductase, E.C.1.6.99.2.) is a flavoprotein with 274 residues per monomer and a molecular mass of 30,867 Da. NQO1 exists as a homodimer with one molecule of noncovalently bound flavin adenine dinucleotide (FAD) per monomer [1]. It has two active sites, which catalyse the obligate two-electron reduction of quinones, quinone epoxides and quinone-imines to hydroquinones [2]. The enzyme is unusual in that it has the capacity to utilise both the pyrimidine nucleotides NADH and NADPH, without preference, as reducing co-substrates [3]. The catalytic cycle of NQO1 functions via a "ping-pong" mechanism in two distinct steps: hydride transfer from the NAD(P)H to the FAD cofactor, followed by release of $NAD(P)^+$ and hydride transfer from the reduced cofactor to the quinone substrate [4]. This two electron reduction mechanism also means that NQO1 has antioxidant properties, as it will prevent reduction of quinones by one-electron reductases. The latter would cause the formation of semiquinones with concomitant formation of reactive oxygen species [2].

An additional function of NQO1 is its clear ability to act as a chaperone protein. It has been described as the "gate keeper" of the 20S proteosome [5] and regulates the degradation of certain oncoproteins such as p53, p73α and ornithine decarboxylase and proteins important in the regulation of mRNA translation [6,7]. The ability of NQO1 to stabilize proteins is dependent upon NADH [8] which suggests that the binding of the enzyme to its client protein(s) is most efficient when the protein FAD is in its reduced form. The anticoagulant dicoumarol (3,3′-methylenebis(4-hydroxycoumarin), has historically been regarded as the most potent inhibitor of NQO1 [9]; it acts through competitive binding with NAD(P)H and prevents the two-electron transfer to FAD from occurring [10]. Hence, addition of dicoumarol to cells has been shown to target p53 and other client proteins for degradation [6–8,11,12].

NQO1 is constitutively expressed in a variety of tissues throughout the body, but is overexpressed in many solid tumours [13,14]. These observations have made NQO1 a potential target for the activation of certain bioreductive anticancer agents [15] such as mitomycin C [16], EO9 (5-aziridinyl-3-hydroxymethyl-2-(3-hydroxyprop-1-enyl)-1-methylindole-4,7-dione) [17], streptonigrin [18], RH1 ((2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone) [19] and β-lapachone [20].

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Table 1
Substituents in the **S** and **AS** series (Fig. 1) together with the concentration of each compound that inhibits the activity of recombinant human NQO1 by 50% (IC_{50}) in the presence of 0.14% (w/v) BSA [22]. Also given are concentrations of each inhibitor that causes 50% toxicity in cells in the presence of 1 μM EO9. This concentration of EO9 alone will kill ≥90% of the HT29, A549 or MIA PaCa-2 cells; hence the values quoted give a measure of the efficiency of protection against EO9 toxicity [23]. This reflects the ability of the **S** and **AS** compounds to inhibit NQO1 activity in the cancer cells. Each experiment was repeated on at least three separate occasions and mean values are given ± standard deviation.

ID	R_5	R_6	R_7	R_8	X	IC_{50} (nM) with BSA	Concentration needed to protect from 50% of EO9 toxicity (μM)		
							HT29	A549	MIA PaCa-2
S1	H	H	H	H	H	404 ± 184	211 ± 83	118 ± 5.6	141 ± 48
S3	OCH_3	H	H	H	H	38 ± 2.1	18 ± 9.0	43 ± 25	54 ± 26
S4	H	OCH_3	H	H	H	3300 ± 600	>1000	> 250	> 250
S5	H	H	OCH_3	H	H	790 ± 355	875 ± 211	> 250	> 250
S10	H	CH_3	CH_3	H	H	233 ± 68	106 ± 29	165 ± 63	158 ± 72
S11	H	OCH_3	OCH_3	H	H	1497 ± 442	1954 ± 940	–	–
S13	H	H	CH_3	CH_3	H	149 ± 101	132 ± 9.8	68 ± 16	109 ± 21
AS1	H	CH_3	CH_3	H	1-Naphthyl	1095 ± 290	105 ± 77	251 ± 78	148 ± 42
AS2	H	CH_3	CH_3	H	2-Naphthyl	167 ± 83	50 ± 33	64 ± 38	89 ± 27
AS3	H	CH_3	CH_3	H	Phenyl	660 ± 108	128 ± 69	114 ± 7.2	312 ± 55
AS4	H	H	7,8-C_4H_4		1-Naphthyl	450 ± 325	58 ± 26	129 ± 29	359 ± 58
AS5	H	H	7,8-C_4H_4		2-Naphthyl	225 ± 151	97 ± 52	94 ± 27	132 ± 56
AS6	H	H	7,8-C_4H_4		Phenyl	880 ± 364	133 ± 41	> 250	205 ± 10
AS14	H	CH_3	CH_3	H	3,4 Dimethylbenzyl	192 ± 41	62 ± 19	61 ± 7.8	114 ± 2.3

Dicoumarol is often used as a pharmacological inhibitor to study the function of NQO1 in cells. This has not only been to establish the importance of the enzyme for the activation of bioreductive drugs [21,22], but also to identify the importance of NQO1 for determining protein stability [23]. However, the actions of dicoumarol are compromised by extensive protein binding [24] and confounding "off-target" effects such as mitochondrial uncoupling [25,26] and the increased production of intracellular superoxide [27,28]. These off-target effects can interfere with the interpretation of the function of NQO1 in cells. An example is where dicoumarol-induced production of superoxide was thought to be the basis for toxicity towards pancreatic cancer cells and this phenomenon was considered to be NQO1 dependent [29,30]. However, different research groups have subsequently presented data to show that these observations are not solely due to the inhibition of NQO1 but more importantly due to other properties of dicoumarol [28,31,32]. Thus, it is therefore of interest to develop novel inhibitors of NQO1 which retain the NQO1 inhibitory potency of dicoumarol but lack its unfavourable off-target effects.

The synthesis of a series of novel substituted coumarin-based compounds with improved inhibitory potency in both isolated enzyme systems and cells has recently been reported [28,33,34]. This series of compounds has now been evaluated for their "off-target" effects when compared with dicoumarol. This paper provides an evaluation of the toxicity of these compounds in vitro, effects on cell cycle, and their abilities to cause apoptosis and generate superoxide. The major aim of the study was to identify compounds that may be more pharmacologically acceptable than dicoumarol; these will be compounds that have a similar or greater NQO1 inhibitory potency to dicoumarol, but have a lower level of cellular toxicity and fewer off-target effects.

2. Materials and methods

2.1. Reagents, chemicals and NQO1 inhibitors

Unless otherwise stated, all reagents and chemicals were supplied by Sigma Aldrich (Poole, Dorset, UK). The NQO1 inhibitors have been previously described [28,33,34]. They comprise two series of substituted coumarins. The first includes a set of substituted dicoumarol analogues (the symmetric, **S** series, where dicoumarol is designated **S1**). The second group of compounds have one of the 4-hydroxycoumarol rings replaced by an aromatic ring system (the asymmetric, **AS** series). Structures are given in Fig. 1 and Table 1. The compounds were dissolved in either DMSO (Fisher Scientific, Leicestershire, UK) or 0.13 M NaOH (VWR, Leicestershire, UK) to give a final concentration of 25 mM.

2.2. Cell lines and cell culture

Human carcinoma cells with varying levels of NQO1 expression were selected for use. Cell lines were maintained at 37 °C in a humidified incubator with an atmosphere of air plus 5% CO_2. All cell lines were grown in RPMI-1640 medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) (Biosera, East Sussex, UK) and 2 mM L-glutamine (Invitrogen, Paisley, UK), except MIA PaCa-2 cells which were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% (v/v) FCS, 2.5% (v/v) horse

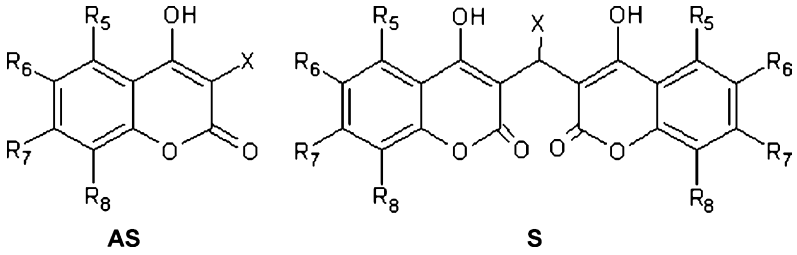

Fig. 1. Structures of substituted 4-hydroxycoumarins (**AS** series) and analogues of dicoumarol (**S** series). Substituents R_5–R_8 and X are given in Table 1.

serum (Invitrogen, Paisley, UK) and 2 mM L-glutamine. In the experiments using the MDA-MB-231, MDA-MB-468 and T47D cells which had been stably transfected with NQO1 (designated-DTD) [35], puromycin was added to the culture medium at a concentration of 5 μg/ml in order to maintain selection.

2.3. Determining the activity of NQO1 in cells

Cells at a concentration of 1.5×10^5 were seeded into 10 cm dishes and left to reach approximately 70% confluence. They were then washed in PBS (OXOID, Hampshire, UK) and scraped into phosphate buffer (pH 7.5) containing 5 μM FAD and 250 mM sucrose. The cells were sonicated twice for 5 s on ice, centrifuged at 13,000 rpm (16,200 × *g*) for 15 min at 4 °C, and the supernatants collected and stored at −80 °C. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay [36]. The assay for NQO1 enzyme activity has been described previously [28]. Briefly, 10 μl of cell lysate was mixed with 490 μl of 50 mM phosphate buffer at pH 7.5 containing 200 μM NADH, 70 μM cytochrome *c* and 20 μM menadione. Reactions were carried out at 25 °C and cytochrome *c* reduction was followed at 550 nm in a Beckman DU650 spectrophotometer. Dicoumarol (100 μM) was added to a parallel reaction mixture and the difference in the rates of reaction in the presence and absence of dicoumarol was then used to define the activity of NQO1 in the cellular lysates.

2.4. Assays of toxicity

The MTT proliferation assay [37] was used as a surrogate measure of the toxicity of the dicoumarol analogues. Cells were seeded at 7.5×10^3 cells/well into 96-well plates and were exposed to increasing concentrations of dicoumarol (**S1**) or each inhibitor from the **S** and **AS** series for 96 h. The number of surviving cells was then determined using the MTT assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved in sterile PBS and added to the wells at a final concentration of 1.5 mM. Cells were incubated with MTT for 4 h, at which time the resulting formazan crystals were solubilised with DMSO and absorbance read at 540 nm. Values of IC_{50} were calculated as the drug concentration required to reduce optical density by 50% relative to vehicle treated control cells.

In some experiments, A549 or MIA PaCa-2 cells were treated with EO9 (Apaziquone, synthesized in-house) with and without various non-toxic concentrations of the **S** and **AS** compounds for 3 h. EO9 and the compounds were then removed and cells left in growth medium for 96 h prior to addition of MTT for assessment of toxicity. As this has been described in detail previously [34], the ability of the putative enzyme inhibitors to protect against the toxicity of EO9 in cells is a surrogate measure for their ability to inhibit cellular NQO1.

2.5. Intracellular superoxide production

MIA PaCa-2 and MDA-MB-231 wild-type (WT) and DTD cells were grown to approximately 70% confluence, harvested using trypsin/EDTA, and 1×10^6 cells were resuspended in 1.5 ml culture media in 5 ml tubes. Dicoumarol or the other **S** and **AS** compounds were added to the cells (at varying concentrations up to 400 μM) and incubated for 4 h, with agitation every 20 min. Dihydroethidium was then added to each tube to give a final concentration of 10 μM. The cells were incubated for another 30 min, centrifuged and the cell pellets were resuspended in 1 ml ice-cold PBS. Samples were kept on ice and analysed immediately on a CyanADP flow cytometer using the Summit software package (Dako, Colorado, USA). Dihydroethidium is oxidised to 2-hydroxyethidium by superoxide, which then intercalates DNA and fluoresces red. This signal has been suggested to report the formation of intracellular superoxide [38]. The oxidised product, 2-hydroxyethidium, was excited by blue light (488 nm, argon laser) and fluorescence was measured using a 613/20 bandpass filter. The use of dihydroethidium is considered to be a relatively specific method for measuring superoxide [38]. However, it can be oxidised by other agents such as hydrogen peroxide in the presence of haem containing proteins [39]. These oxidation reactions can result in the production of different oxidation products with excitation and emission spectra overlapping with that of 2-hydroxyethidium [26]. Thus, to confirm that oxidation of dihydroethidium occurred via reaction with superoxide, experiments were carried out in the presence of ambroxol (2-amino-3,5-dibromo-N-[trans-4-hydroxycyclohexyl] benzylamine). Ambroxol is a, so-called, superoxide, dismutase mimetic and has been used previously [26,40] to confirm oxidation reactions occurring via superoxide. In all experiments, cells were exposed to 50 μM menadione as a positive control for generation of superoxide.

2.6. Cell cycle analysis

Cells were seeded into 10 cm dishes at a concentration of 1.5×10^5 cells per dish and left to adhere overnight. Cells were treated with 100, 200, 300 or 400 μM of dicoumarol (**S1**) or the **S** or **AS** compounds and incubated for 24 h. The cells were harvested using trypsin/EDTA, washed in PBS and then resuspended in 1 ml ice-cold 70% (v/v) ethanol added drop-wise while using a vortex mixer to avoid clumping. The cells were then left in ethanol at 4 °C for a minimum of 24 h. For cell cycle analysis, the cells were washed twice in PBS and finally resuspended in 500 μl of PBS containing propidium iodide (PI, 25 μg/ml) and RNase A (250 μg/ml), and incubated at 37 °C in the dark for 30 min. Cells were then washed and resuspended in 500 μl PBS and kept in the dark in preparation for analysis. Samples were analysed on a CyanADP flow cytometer using the Summit software package. Cell doublets were gated out using a plot of PI signal height against signal area and data were collected as histograms illustrating fluorescence intensity and hence DNA content of the cells. The percentage of cells in the different phases of the cell cycle were then calculated using gates positioned to encompass the respective phases of control cells in each individual experiment.

2.7. Western blot analysis of apoptotic markers

Exponentially growing cells were seeded into 6-well plates at a concentration of 1.5×10^5 cells per well and left overnight to adhere. Cells were treated with 200 μM of dicoumarol (**S1**) or the **S** or **AS** compounds, incubated for 24 h and then harvested by

Table 2
Intracellular NQO1 activity was measured as described in Section 2.3. Each measurement was made on lysates prepared on three separate occasions. Values given are means ± standard deviation.

Cell line	NQO1 activity (nmol cytochrome *c* reduced/min/mg protein)
HT29	1691 ± 715
A549	3011 ± 532
HCT116 WT	545 ± 115
HCT116 BVP $p53^{-/-}$	357 ± 42
MIA PaCa-2	1252 ± 142
MDA-MB-231 WT	40 ± 28
MDA-MB-231 DTD	4171 ± 816
MDA-MB-468 WT	20 ± 17
MDA-MB-468 DTD	4180 ± 1589
T47D WT	141 ± 33
T47D DTD	5284 ± 1598

Table 3
Toxicity of compounds in a panel of cell lines with varying NQO1 activity and p53 functionality. Values represent the means and standard deviations of at least three independent experiments.

Compound	96-h IC_{50} (μM)								
	MIA PaCa-2	HCT116 WT	HCT116 $p53^{-/-}$	MDA-MB-231		MDA-MB-468		T47D	
				WT	DTD	WT	DTD	WT	DTD
S1	52 ± 26	19 ± 13	22 ± 13	50 ± 37	67 ± 31	30 ± 12	47 ± 23	51 ± 6.2	41 ± 8.3
S3	350 ± 142	80 ± 27	58 ± 33	133 ±16	165 ± 37	174 ± 45	125 ± 5.2	98 ± 52	190 ± 77
S4	405 ± 22	48 ± 1.4	43 ± 13	77 ± 7.1	105 ± 60	58 ± 22	38 ± 4.2	283 ± 46	103 ± 84
S5	303 ± 32	187 ± 17	180 ± 7.1	202 ± 82	162 ± 78	148 ± 16	155 ± 35	109 ± 56	68 ± 31
S10	106 ± 33	35 ± 1.4	33 ± 5.6	66 ± 51	41 ± 2.6	51 ± 26	56 ± 30	66 ± 47	45 ± 16
S11	>400	>400	>400	>400	>400	>400	>400	>400	>400
S13	119 ± 42	35 ± 16	41 ± 13	114 ± 36	173 ± 15	123 ± 31	141 ± 22	111 ± 38	103 ± 32
AS1	188 ± 2.9	56 ± 4.9	76 ± 48	32 ± 9.0	51 ± 64	107 ± 17	45 ± 29	124 ± 67	116 ± 71
AS2	162 ± 28	180 ± 21	165 ± 44	119 ± 48	115 ± 59	142 ± 68	75 ± 51	143 ± 57	95 ± 74
AS3	267 ± 31	178 ± 2.8	122 ± 65	102 ± 70	185 ± 18	231 ± 70	99 ± 46	145 ± 42	117 ± 69
AS4	163 ± 20	102 ± 18	111 ± 21	41 ± 5.2	60 ± 58	100 ± 21	67 ± 41	94 ± 22	88 ± 29
AS5	152 ± 16	39 ± 2.8	31 ± 4.7	41 ± 15	58 ± 15	102 ± 22	58 ± 34	58 ± 36	51 ± 17
AS6	222 ± 60	40 ± 45	68 ± 48	124 ± 40	94 ± 21	173 ± 27	100 ± 36	13 ± 61	89 ± 16
AS14	407 ± 15	176 ± 6.4	172 ± 43	152 ± 36	164 ± 25	248 ± 37	154 ± 21	170 ± 7.0	138 ± 3.5

scraping into lysis buffer (50 mM Tris–HCl (pH 7.4) (Fisher Scientific, Leicestershire, UK); 120 mM NaCl; 5 mM EDTA; 0.5% (v/v) Nonidet P-40; 1 mM dithiothreitol; 1 mM phenylmethanesulphonyl fluoride; 2 mM sodium orthovanadate; 2 mM sodium fluoride; 20 mM B-glycerolphosphate; 5 mM sodium pyrophosphate; and 1 mg/ml Protease Inhibitor Cocktail tablet (Roche, Hertfordshire, UK). Samples were sonicated on ice and the protein concentration was estimated using the BCA protein assay. Protein (40 μg) was loaded for each sample, resolved on a 10% polyacrylamide gel and then transferred to a polyvinylidene (PVDF) membrane (Millipore, Hertfordshire, UK). Immunoblotting was carried out using a 1:1000 dilution of the cleaved PARP antibody (New England Biolabs, Hertfordshire, UK; anti-rabbit) or a 1:40,000 dilution of the actin antibody. Primary antibodies were detected using the correct horse radish peroxidise (HRP)-conjugated secondary antibody, and the ECL detection system (GE Healthcare, Buckinghamshire, UK) was used to visualise the respective bands.

3. Results

Novel analogues of dicoumarol (**S1**) have been synthesized (the **S** and **AS** series in Fig. 1 and Table 1) and shown to be potent inhibitors of recombinant NQO1 [33]. These molecules also show potent activity for inhibiting the activity of NQO1 in HT29 cells, with potency correlating well with that observed when measuring inhibition of recombinant enzyme in the presence of BSA [34]. These published data are given in Table 1. Inhibition of cellular NQO1 was characterized by the ability of the **S** and **AS** compounds to inhibit the cytotoxic activity of the indolequinone EO9, an agent known to be exquisitely dependent

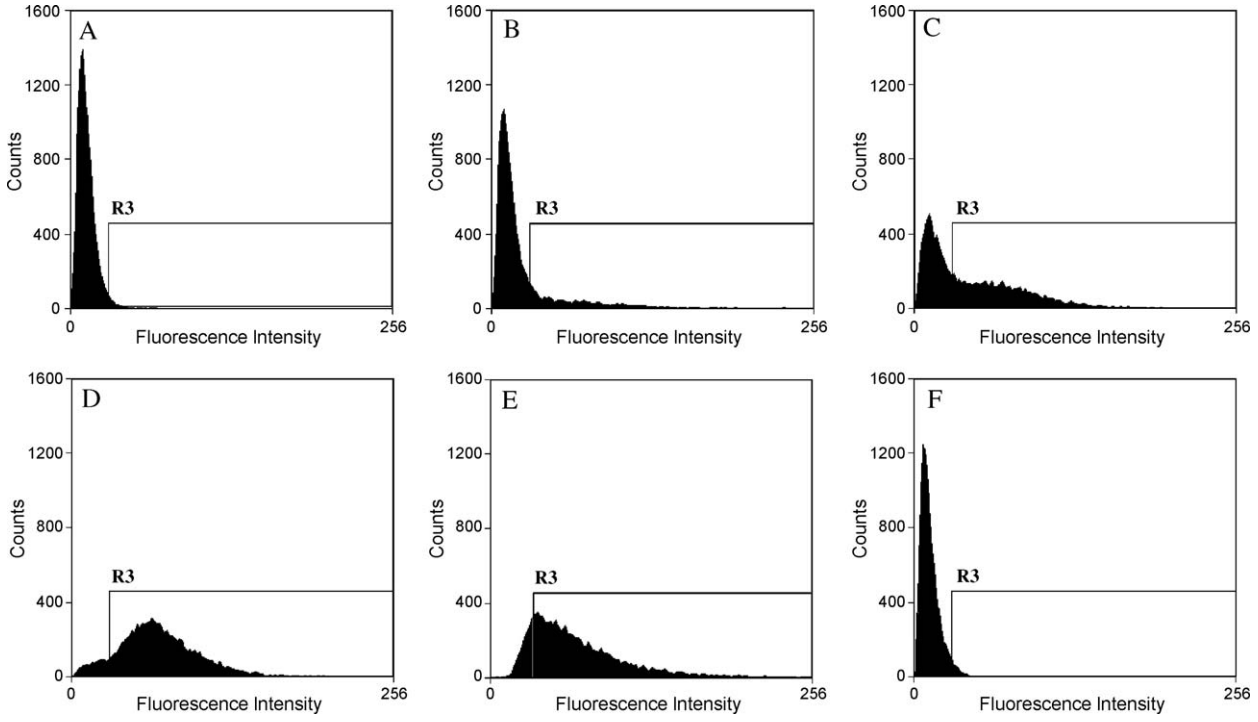

Fig. 2. Dose-dependent increase of intracellular superoxide following treatment of MIA PaCa-2 cells with dicoumarol for 4 h: (A) background fluorescence intensity in control cells. (B–D) Fluorescence intensity in cells treated with 100, 200 and 400 μM dicoumarol (**S1**) respectively. (E) Exposure of cells to 50 μM menadione. (F) Exposure of cells to 200 μM dicoumarol plus 500 μM ambroxol. Histograms are representative images of experiments performed on at least three independent occasions.

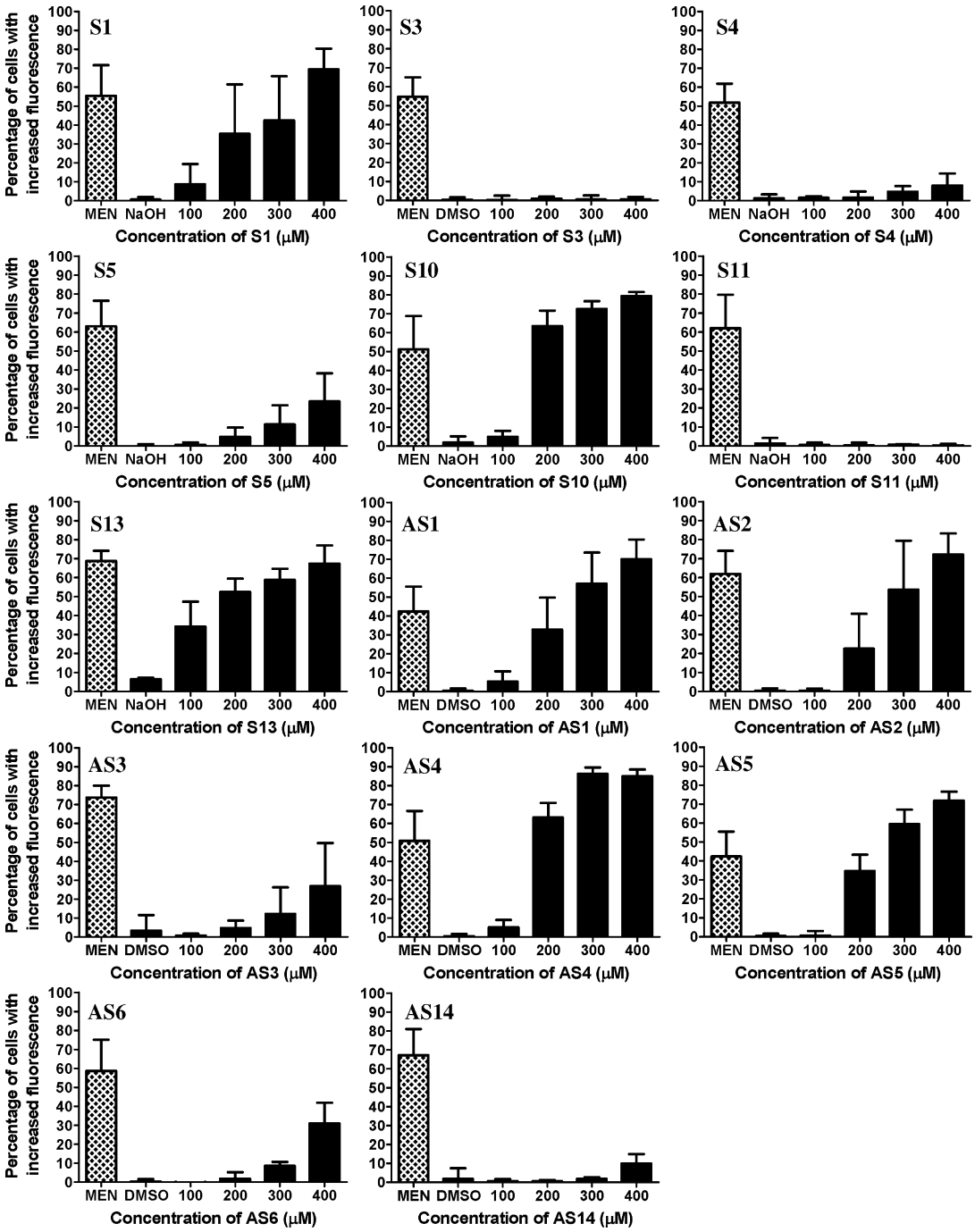

Fig. 3. Superoxide production in MIA PaCa-2 cells treated for 4 h with increasing concentrations of dicoumarol (**S1**) and the other **S** and **AS** compounds. Menadione (50 μM) and DMSO vehicle controls were carried out in each experiment. Bars represent mean and standard deviation of at least three independent experiments.

on NQO1 for toxicity under aerobic conditions [21,22]. Table 1 also shows the ability of the **S** and **AS** compounds to inhibit NQO1 in A549 and MIA PaCa-2 cells. Using the EO9 toxicity endpoint as a surrogate measure of NQO1 inhibition, it is apparent that **S3** is the most active compound in each cell line, being 2.5 to 10-fold more potent than dicoumarol for protecting against EO9 toxicity. In the **S** series, **S10** and **S13** are up to 2-fold more efficient than dicoumarol (**S1**), whereas the remaining compounds are less effective. On the contrary, in the **AS** series, all the compounds are at least as efficient as dicoumarol, with some (**AS2** and **AS14**) showing 2–4-fold greater potency in the different cell lines. With the knowledge that these compounds are functionally active as inhibitors of NQO1 in cells, it is now possible to assess any "off-target" activity and relate this to NQO1 inhibition.

3.1. The toxicity of the dicoumarol analogues in cell lines expressing varying levels of NQO1

The activity of NQO1 in the cell lines used in the present work is given in Table 2. Cell lines were chosen to have varying NQO1 activity. The MDA-MB-231, MDA-MB-468 and T47D WT cells are null for NQO1 due to a homozygous polymorphism (NQO1*2/*2) [41–43]. Each of these cell lines has been genetically engineered to stably overexpress functional NQO1 [35]. In order to evaluate any contribution of p53, the HCT116 p53 $^{+/+}$ and $^{-/-}$ pair of cell lines were

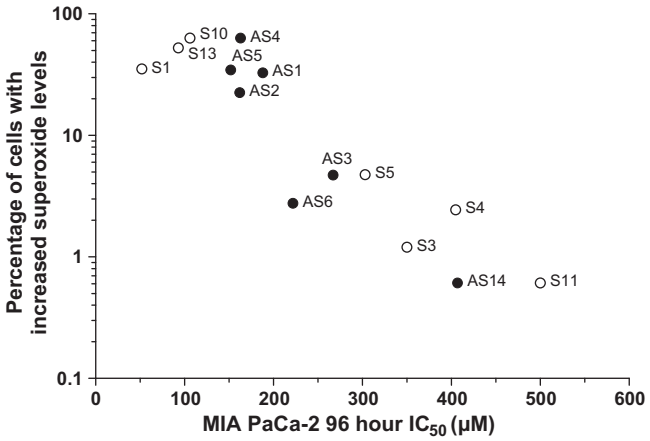

Fig. 4. The relationship between superoxide production and toxicity in the MIA PaCa-2 cell line, **AS** compounds (●); **S** compounds (○); and dicoumarol (**S1**).

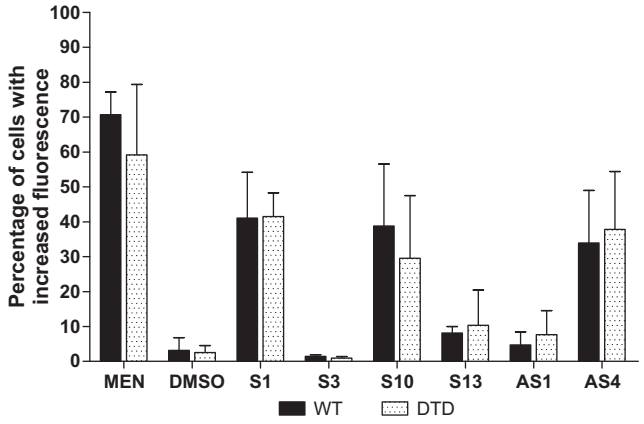

Fig. 5. Intracellular superoxide production is independent of the activity of NQO1 in MDA-MB-231 cells: cells were treated for 4 h with 100 μM of dicoumarol (**S1**) and the other **S** or **AS** compounds. Each measurement was made on three separate occasions and values are given ± standard deviation (black bars: WT cells; white bars: DTD cells).

used [44]. The values for enzyme activity given in Table 2 vary by a factor of 250. Endogenous values of enzyme activity range from 20 to 3011 (nmol of cytochrome *c* reduced/min/mg protein) in the MDA-MB-468 and A549 cells respectively. The maximum activity is 5284 in the overexpressing T47D DTD cells. Where measurements have been made previously, the values reported here compare extremely well [17]. It has been suggested [30] that the toxicity of dicoumarol is mediated by its ability to inhibit NQO1 thereby increasing cellular exposure to reactive oxygen species. Therefore, the toxicity of dicoumarol (**S1**) and analogues from the **S** and **AS** series were evaluated in all the cell lines. Values of IC_{50}, the concentration required to reduce proliferation by 50%, are given in Table 3. Dicoumarol is consistently one of the most toxic compounds in all of the cell lines and it is clear that neither NQO1 nor p53-status plays a role in determining toxicity. There also appears to be no relationship between the toxicity induced by the compounds and their ability to inhibit NQO1 enzymatic activity (Table 1).

3.2. Production of intracellular superoxide

Dicoumarol has been shown to dose-dependently increase the production of superoxide in MIA PaCa-2 pancreatic cancer cells [27]. This result was recapitulated here using flow cytometry to measure the superoxide-mediated oxidation of dihydroethidium to 2-hydroxyethidium. Fig. 2 shows fluorescence intensity histograms acquired following treatment of MIA PaCa-2 cells with increasing concentrations of dicoumarol (**S1**) for 4 h. In all experiments, 50 μM menadione was used as a positive control for superoxide production [45]. From the fluorescence intensity profile obtained for untreated control cells, the R3 region is defined as that containing cells with increased fluorescence. Clearly, treatment with dicoumarol results in a dose-dependent increase in the number of fluorescent cells, which is consistent with previous data [27] suggesting it was dicoumarol-induced superoxide formation that caused the increased intracellular fluorescence. To confirm this, the superoxide dismutase mimetic, ambroxol, was added to the cells exposed to dicoumarol. Fig. 2(panel F) shows the fluorescence profile obtained when cells were exposed to the combination of 200 μM dicoumarol plus 500 μM ambroxol. This non-toxic combination shows little, if any, formation of the fluorescent product 2-hydroxyethidium, which suggests that any superoxide formed by a dicoumarol-mediated process is scavenged by ambroxol, thereby preventing reduction of dihydroethidium.

The **S** and **AS** compounds were then evaluated for their ability to generate superoxide in MIA PaCa-2 cells. Fig. 3 shows the percentage of cells with increased fluorescence relative to untreated controls, following exposure of cells to varying concentrations of the compounds. There is substantial variation in the ability of the compounds to generate superoxide and there appears to be no relationship with the ability of the different compounds to inhibit NQO1. However, interestingly, the compounds in the **S** series that contain a methoxy group in the coumarin ring (**S3**, **S4**, **S5** and **S11**) and those in the **AS** series that contain the simpler X-substituent (**AS3**, **AS6** and **AS14**) show substantially lower induction of superoxide than dicoumarol (**S1**) and the other compounds. This is reflected in an apparent correlation between superoxide production and the ability of the compounds to inhibit the proliferation of MIA PaCa-2 cells as measured by the MTT assay (Fig. 4).

In order to confirm whether the phenomenon of intracellular superoxide production is not mediated by NQO1, measurements were made of changes in fluorescence intensity in the MDA-MB-231 WT and DTD cells, treated with a variety of compounds from the **S** and **AS** series. The cells were incubated for 4 h with 100 μM of each compound and the percentage of cells showing increased fluorescence relative to control is shown in Fig. 5. Clearly, there is no significant difference in the generation of intracellular superoxide between the two cell lines for any of the compounds. It is also apparent that the same lack of dependence on NQO1 for superoxide production is seen for menadione (also shown in Fig. 5).

3.3. Effects on cell cycle progression and induction of apoptosis

It has been shown previously that dicoumarol can promote the growth of HL-60 cancer cells via cell cycle perturbation [46]. In order to determine the effects of dicoumarol and the **S** and **AS** analogues on cell cycle progression, MIA PaCa-2 cells were exposed to 200 μM of each of the compounds for 24 h before staining with propidium iodide for cell cycle analysis. Fig. 6 shows typical DNA histograms of untreated cells and cells treated with dicoumarol (**S1**), **S3** and **AS2**. Inspection of these profiles suggests that there is no substantive change in the percentage of cells in each of the phases of the cell cycle. However, for both dicoumarol and **AS2** there is an indication of an increased number of cells in the sub-G1 region. The percentage of cells in each phase of the cell cycle following treatment with each compound is given in the Supplementary Data Table 1. The percentage of cells in the sub-

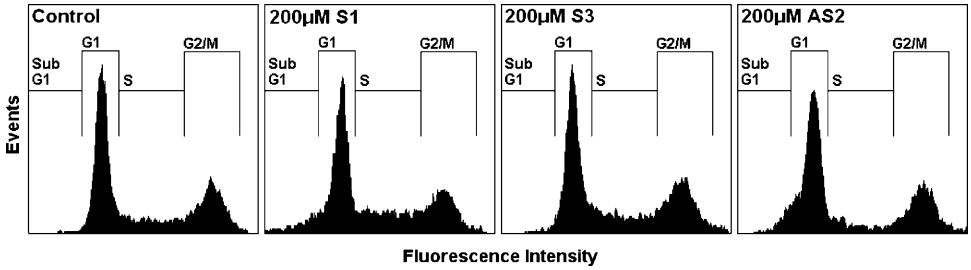

Fig. 6. Representative DNA histograms displaying cell distributions following 24-h treatment with 200 μM dicoumarol (**S1**), **S3** or **AS2**: The percentage of cells in the different phases of the cell cycle for all the compounds are given in the Supplementary data Table 1. Cell cycle profiles were generated on at least three independent occasions.

G1 region following exposure to a 200 μM concentration of each of the S and AS compounds is shown in the top panel in Fig. 7. Clearly, there are significant differences, with compounds such as **S3**, **S4**, **S5** and **S11** (those showing low superoxide production) providing little if any increase in the sub-G1 population compared to controls. In contrast, the remaining compounds, including dicoumarol (**S1**) show significantly higher numbers of cells in the sub-G1 region than in untreated populations. In order to determine whether the cells in the sub-G1 region are undergoing apoptosis [47], cells were collected after 24-h exposure to 200 μM of dicoumarol and each of the **S** and **AS** compounds, cell lysates prepared and Western blots probed for cleaved PARP. These are shown in the bottom panel of Fig. 7 and a clear concordance exists between expression of cleaved PARP and the percentage of cells in the sub-G1 area, which strongly suggests these cells are undergoing apoptosis. A comparison of the proportion of cells in the sub-G1 region and the generation of superoxide in MIA PaCa-2 cells treated with of each of the putative NQO1 inhibitors is given in the Supplementary Data Fig. 1. While there is no correlation between these end-points, it is quite clear that the compounds generating high levels of superoxide are those that also seem to cause the greatest amount of apoptosis. However, importantly, there is no clear relationship between induction of apoptosis or generation of superoxide and the ability of the compounds to inhibit the enzymatic activity of NQO1 (Table 1). Finally, to confirm that there was no link between induction of apoptosis and cellular levels of NQO1, cell cycle analysis of the MDA-MB-231 WT and DTD cells was made following treatment for 24 h with 200 μM of dicoumarol (**S1**). The proportion of cells in the sub-G1 region following treatment is given in the Supplementary data Fig. 2 and these results clearly show that the cellular NQO1 status has no influence on the number of cells in sub-G1.

4. Discussion

The purpose of this work has been to identify compounds that are potent inhibitors of NQO1 but with superior pharmacological acceptability than the conventional inhibitor, dicoumarol. A series of substituted coumarins has been synthesized (Fig. 1 and Table 1) and many of these have equivalent or greater potency for inhibiting NQO1 than dicoumarol [33,34]. Further, these agents can also inhibit the functional activity of NQO1 in intact HT29 colon cancer, A549 non small cell lung cancer and MIA PaCa-2 pancreatic cancer cell lines [34]. A number of these compounds (**S3**, **S10**, **S13**, **AS2** and **AS14**) are consistently more potent than dicoumarol, which suggests they may have general applicability as NQO1 inhibitors in cells. It is known that dicoumarol has a variety of "off-target" effects that can confound interpretation of its action as an inhibitor of NQO1 in cells. Therefore, to establish the potential pharmacological usefulness of the substituted coumarins, experiments have been carried out to determine their toxicity in a large panel of cell lines, their ability to generate superoxide and their ability to cause apoptosis.

In the toxicity studies reported here, there is no relationship between the ability of the compounds to inhibit NQO1 and the values of IC_{50} in any of the cell lines (irrespective of the endogenous level of NQO1). Furthermore, there appears to be no dependence on p53 (HCT116 cells). Interestingly, dicoumarol is the most toxic agent in each cell line, irrespective of NQO1 content. For the other compounds there is no systematic relationship between structure and toxicity. In contrast, there are clear differences between the compounds for their ability to generate superoxide. Experiments were initially done in MIA PaCa-2 cells as these cells were used by Du and co-workers [27,29,30,46] who initially proposed that dicoumarol generated superoxide via an NQO1-mediated mechanism. In the **S** series, compounds such as **S10** and **S13** are as efficient, if not more so, than dicoumarol for generating superoxide. These compounds are effective for inhibiting NQO1 in cells, but so is **S3**, and this compound generates little if any superoxide. A molecular feature of the compounds in the **S** series that do not generate superoxide is that they all contain a methoxy group in the coumarin ring. For the **AS** series, these compounds generally cause formation of superoxide. However, there are exceptions, with **AS3** and **AS6** generating substantially less superoxide than similar concentrations of dicoumarol; additionally, **AS14**, which is a more

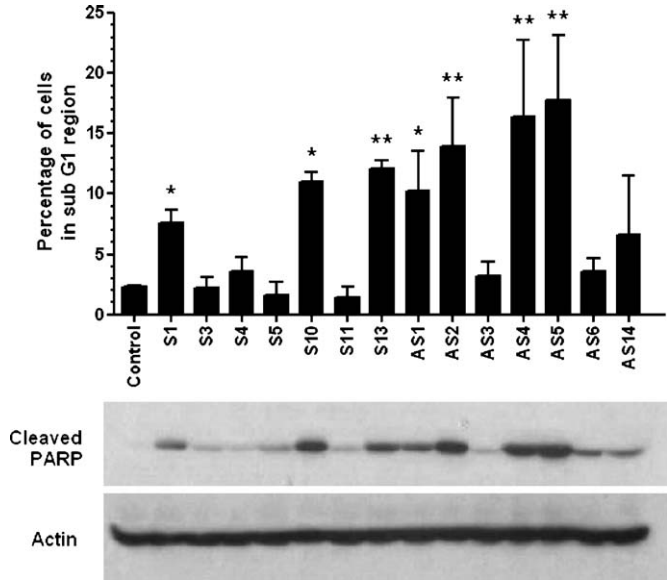

Fig. 7. The percentage of cells in the sub-G1 region of the cell cycle compared with measurement of cleaved PARP by Western blot: MIA PaCa-2 cells were treated for 24 h with vehicle or 200 μM of dicoumarol (**S1**) or the other **S** or **AS** compounds then either stained with PI for cell cycle analysis or cell lysates prepared for assessment of cleaved PARP. The values of the percentage of cells within the sub-G1 region were obtained from at least three independent experiments (±standard deviation), the asterisks give the significance from the controls in each experiment ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$). The Western blot is a representative from three separate experiments.

efficient inhibitor of NQO1 in cells than dicoumarol, generates little if any superoxide. Taken together with results showing similar superoxide production in isogenic cells expressing differing levels of NQO1 (MDA-MB-231 cells) and those in previous work reported by our group [28], and others [31,32], these data strongly suggest that cellular NQO1 plays little if any role in the generation of superoxide in cells following treatment with dicoumarol and its analogues. However, the generation of superoxide may be the basis for the toxicity of the coumarin analogues as is suggested by the correlation shown in Fig. 4. Interestingly, the relationship between superoxide production and apoptosis is less apparent, although it is quite clear that those compounds generating the greater amount of superoxide are those that produce most apoptosis.

Other off-target effects associated with dicoumarol include extensive protein binding and mitochondrial uncoupling. Protein binding has previously been shown [33] to be much reduced with some of the analogues, including **S3**. Further, preliminary experiments carried out to measure effects on oxygen respiration have shown that **S3** shows a much reduced ability to cause mitochondrial uncoupling when compared to dicoumarol (Scott KA, unpublished data).

It is not the purpose of this work to ascertain the underlying mechanism by which the coumarin analogues elicit their off-target effects. Instead, we are seeking to identify molecular features of compounds related to dicoumarol that would help facilitate their development as pharmacological inhibitors of NQO1. It is particularly obvious that the 5-methoxy substituted analogue of dicoumarol, **S3**, is the most potent inhibitor of NQO1 in cells, while being consistently less toxic in the nine cell lines used here. **S3** also shows much reduced generation of superoxide and does not induce apoptosis. From the **AS** series, **AS3** and **AS14** stand out, as they also show comparable NQO1-inhibitory potency to dicoumarol in cells with generally less generation of superoxide, less apoptosis and reduced toxicity. A potential advantage of the **AS** compounds compared to their **S** analogues is their improved water solubility [33]. Thus, we have provided a firm basis for development of compounds to evaluate the impact of NQO1 inhibition in cells.

Using gene knockout technology it has been shown that loss of NQO1 is associated with reduced p53 stability [48,49] and changes in NFκB signalling [50]. Treatment of cells with dicoumarol can also cause degradation of p53 and other onco-proteins in a process that is mediated by NQO1. There is good evidence to show that transient disruption of p53 during and after irradiation can protect against radiation damage [51,52]. Thus, there may well be substantial scope for developing NQO1 inhibitors for therapeutic use [34]. In summary, it has been shown that there are a number of compounds that act as potent inhibitors of NQO1 and, beneficially, they lack the off-target effects associated with dicoumarol.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.10.011.

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