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

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1  
2 **Abstract**  
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6 The enzyme NAD(P)H quinone oxidoreductase (NQO1) can function both as a detoxifying  
7 enzyme as well as chaperone protein. The latter property has been extensively characterized  
8 by the use of dicoumarol which inhibits the chaperone properties of NQO1 in cells. However,  
9 the use of this compound is compromised by its multiple “off-target” effects. Coumarin-based  
10 compounds that are more potent than dicoumarol as inhibitors of NQO1 in cells have been  
11 identified (Nolan et al., *Biochem Pharmacol* 2010;80:977-81). The purpose of the work  
12 reported here is to evaluate the off-target effects of these compounds when compared to  
13 dicoumarol. A range of these substituted coumarins are identified that are significantly less  
14 toxic than dicoumarol in a panel of nine cell lines. Further a number of the compounds  
15 generate much less intracellular superoxide, and many of them also show a reduced ability to  
16 induce apoptosis when compared to dicoumarol. None of these effects correlate with the  
17 ability of the compounds to inhibit the enzymatic activity of NQO1 in cells. In conclusion,  
18 potent inhibitors of NQO1 have been identified that will be more pharmacologically useful  
19 than dicoumarol for probing the function of NQO1 in cells and tissues.  
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44 **Keywords** : NQO1, Dicoumarol, Coumarins, Superoxide, Apoptosis  
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## 1. Introduction

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5 Human NAD(P)H quinone oxidoreductase (NQO1, DT-diaphorase, Quinone Reductase 1,  
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7 Vitamin K Reductase, E.C.1.6.99.2.) is a flavoprotein with 274 residues per monomer and a  
8  
9 molecular mass of 30,867 Da. NQO1 exists as a homodimer with one molecule of  
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11 noncovalently bound flavin adenine dinucleotide (FAD) per monomer [1]. It has two active  
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13 sites, which catalyse the obligate two-electron reduction of quinones, quinone epoxides and  
14  
15 quinone-imines to hydroquinones [2]. The enzyme is unusual in that it has the capacity to  
16  
17 utilise both the pyrimidine nucleotides NADH and NADPH, without preference, as reducing  
18  
19 co-substrates [3]. The catalytic cycle of NQO1 functions via a “ping-pong” mechanism in two  
20  
21 distinct steps: hydride transfer from the NAD(P)H to the FAD cofactor, followed by release  
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23 of NAD(P)<sup>+</sup> and hydride transfer from the reduced cofactor to the quinone substrate [4]. This  
24  
25 two electron reduction mechanism also means that NQO1 has antioxidant properties, as it will  
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27 prevent reduction of quinones by one-electron reductases. The latter would cause the  
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29 formation of semiquinones with concomitant formation of reactive oxygen species [2].  
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39 An additional function of NQO1 is its clear ability to act as a chaperone protein. It has been  
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41 described as the “gate keeper” of the 20S proteasome [5] and regulates the degradation of  
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43 certain onco-proteins such as p53, p73 $\alpha$  and ornithine decarboxylase and proteins important in  
44  
45 the regulation of mRNA translation [6,7]. The ability of NQO1 to stabilize proteins is  
46  
47 dependent upon NADH [8] which suggests that the binding of the enzyme to its client  
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49 protein(s) is most efficient when the protein FAD is in its reduced form. The anticoagulant  
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51 dicoumarol (3,3'-methylenebis(4-hydroxycoumarin), has historically been regarded as the  
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53 most potent inhibitor of NQO1 [9]; it acts through competitive binding with NAD(P)H and  
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1 prevents the two-electron transfer to FAD from occurring [10]. Hence, addition of dicoumarol  
2 to cells has been shown to target p53 and other client proteins for degradation [6-8, 11, 12].  
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7 NQO1 is constitutively expressed in a variety of tissues throughout the body, but is  
8  
9 overexpressed in many solid tumours [13, 14]. These observations have made NQO1 a  
10  
11 potential target for the activation of certain bioreductive anticancer agents [15] such as  
12  
13 mitomycin C [16], EO9 (5-aziridinyl-3-hydroxymethyl-2-(3-hydroxyprop-1-enyl)-1-  
14  
15 methylindole-4,7-dione) [17], streptonigrin [18], RH1 ((2,5-diaziridinyl-3-(hydroxymethyl)-  
16  
17 methylindole-4,7-dione) [17], streptonigrin [18], RH1 ((2,5-diaziridinyl-3-(hydroxymethyl)-  
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19 6-methyl-1,4-benzoquinone) [19] and  $\beta$ -lapachone [20].  
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24 Dicoumarol is often used as a pharmacological inhibitor to study the function of NQO1 in  
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26 cells. This has not only been to establish the importance of the enzyme for the activation of  
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28 bioreductive drugs [21, 22], but also to identify the importance of NQO1 for determining  
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30 protein stability [23]. However, the actions of dicoumarol are compromised by extensive  
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32 protein binding [24] and confounding “off-target” effects such as mitochondrial uncoupling  
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34 [25, 26] and the increased production of intracellular superoxide [27, 28]. These off-target  
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36 effects can interfere with the interpretation of the function of NQO1 in cells. An example is  
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38 where dicoumarol-induced production of superoxide was thought to be the basis for toxicity  
39  
40 towards pancreatic cancer cells and this phenomena was considered to be NQO1 dependent  
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42 [29, 30]. However, different research group have subsequently presented data to show that  
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44 these observations are not solely due to the inhibition of NQO1 but more importantly due to  
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46 other properties of dicoumarol [28, 31, 32]. Thus, it is therefore of interest to develop novel  
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48 inhibitors of NQO1 which retain the NQO1 inhibitory potency of dicoumarol but lack its  
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50 unfavourable off-target effects.  
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1 The synthesis of a series of novel substituted coumarin-based compounds with improved  
2 inhibitory potency in both isolated enzyme systems and cells has recently been reported [28,  
3 33, 34]. This series of compounds have now been evaluated for their “off-target” effects when  
4 compared with dicoumarol. This paper provides an evaluation of the toxicity of these  
5 compounds in vitro, effects on cell cycle, and their abilities to cause apoptosis and generate  
6 superoxide. The major aim of the study was to identify compounds that may be more  
7 pharmacologically acceptable than dicoumarol; these will be compounds that have a similar  
8 or greater NQO1 inhibitory potency to dicoumarol, but have a lower level of cellular toxicity  
9 and fewer off-target effects.  
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## 24 **2. Materials and Methods**

### 25 *2.1. Reagents, chemicals and NQO1 inhibitors*

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

## 2.2. Cell lines and cell culture

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5 Human carcinoma cells with varying levels of NQO1 expression were selected for use. Cell  
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7 lines were maintained at 37°C in a humidified incubator with an atmosphere of air plus 5%  
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9 CO<sub>2</sub>. All cell lines were grown in RPMI-1640 medium (Invitrogen, Paisley, UK)  
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11 supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) (Biosera, East Sussex,  
12  
13 UK) and 2mM L-glutamine (Invitrogen, Paisley, UK), except MIA PaCa-2 cells which were  
14  
15 grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Paisley, UK)  
16  
17 supplemented with 10% (v/v) FCS, 2.5% (v/v) horse serum (Invitrogen, Paisley, UK) and  
18  
19 2mM L-glutamine. In the experiments using the MDA-MB-231, MDA-MB-468 and T47D  
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21 cells which had been stably transfected with NQO1 (designated-DTD) [35], puromycin was  
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23 added to the culture medium at a concentration of 5µg/ml in order to maintain selection.  
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## 2.3. Determining the activity of NQO1 in cells

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36 Cells at a concentration of  $1.5 \times 10^5$  were seeded into 10cm dishes and left to reach  
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38 approximately 70% confluence. They were then washed in PBS (OXOID, Hampshire, UK)  
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40 and scraped into phosphate buffer (pH 7.5) containing 5µM FAD and 250mM sucrose. The  
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42 cells were sonicated twice for 5 seconds on ice, centrifuged at 13,000rpm (16,200g) for 15  
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44 minutes at 4°C, and the supernatants collected and stored at -80°C. Protein concentrations  
45  
46 were determined using the Bicinchoninic acid (BCA) protein assay [36]. The assay for NQO1  
47  
48 enzyme activity has been described previously [28]. Briefly, 10µl of cell lysate was mixed  
49  
50 with 490µl of 50mM phosphate buffer at pH 7.5 containing 200µM NADH, 70µM  
51  
52 cytochrome c and 20µM menadione. Reactions were carried out at 25°C and cytochrome c  
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54 reduction was followed at 550nm in a Beckman DU650 spectrophotometer. Dicoumarol  
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1 (100 $\mu$ M) was added to a parallel reaction mixture and the difference in the rates of reaction in  
2 the presence and absence of dicoumarol was then used to define the activity of NQO1 in the  
3 cellular lysates.  
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#### 9 *2.4. Assays of toxicity*

10 The MTT proliferation assay [37] was used as a surrogate measure of the toxicity of the  
11 dicoumarol analogues. Cells were seeded at  $7.5 \times 10^3$  cells/well into 96-well plates and were  
12 exposed to increasing concentrations of dicoumarol (**S1**) or each inhibitor from the **S** and **AS**  
13 series for 96 hours. The number of surviving cells was then determined using the MTT assay.  
14 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved in sterile  
15 PBS and added to the wells at a final concentration of 1.5mM. Cells were incubated with  
16 MTT for 4 hours, at which time the resulting formazan crystals were solubilised with DMSO  
17 and absorbance read at 540nm. Values of IC<sub>50</sub> were calculated as the drug concentration  
18 required to reduce optical density by 50% relative to vehicle treated control cells.  
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39 In some experiments, A549 or MIA PaCa-2 cells were treated with EO9 (Apaziquone,  
40 synthesized in-house) with and without various non-toxic concentrations of the **S** and **AS**  
41 compounds for 3 hours. EO9 and the compounds were then removed and cells left in growth  
42 medium for 96 hours prior to addition of MTT for assessment of toxicity. As this has been  
43 described in detail previously [34], the ability of the putative enzyme inhibitors to protect  
44 against the toxicity of EO9 in cells is a surrogate measure for their ability to inhibit cellular  
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### 2.5. Intracellular superoxide production

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5 MIA PaCa-2 and MDA-MB-231 wild-type (WT) and DTD cells were grown to  
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7 approximately 70% confluence, harvested using trypsin/EDTA, and  $1 \times 10^6$  cells were  
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9 resuspended in 1.5ml culture media in 5ml tubes. Dicoumarol or the other **S** and **AS**  
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11 compounds were added to the cells (at varying concentrations up to  $400\mu\text{M}$ ) and incubated for  
12  
13 4 hours, with agitation every 20 minutes. Dihydroethidium was then added to each tube to  
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15 give a final concentration of  $10\mu\text{M}$ . The cells were incubated for another 30 minutes,  
16  
17 centrifuged and the cell pellets were resuspended in 1ml ice-cold PBS. Samples were kept on  
18  
19 ice and analysed immediately on a CyanADP flow cytometer using the Summit software  
20  
21 package (Dako, Colorado, USA). Dihydroethidium is oxidised to 2-hydroxyethidium by  
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23 superoxide, which then intercalates DNA and fluoresces red. This signal has been suggested  
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25 to report the formation of intracellular superoxide [38]. The oxidized product, 2-  
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27 hydroxyethidium, was excited by blue light (488nm, argon laser) and fluorescence was  
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29 measured using a 613/20 bandpass filter. The use of dihydroethidium is considered to be a  
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31 relatively specific method for measuring superoxide [38]. However, it can be oxidized by  
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33 other agents such as hydrogen peroxide in the presence of haem containing proteins [39].  
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35 These oxidation reactions can result in the production of different oxidation products with  
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37 excitation and emission spectra overlapping with that of 2-hydroxyethidium [26]. Thus, to  
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39 confirm that oxidation of dihydroethidium occurred via reaction with superoxide, experiments  
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41 were carried out in the presence of ambroxol (2-amino-3,5-dibromo-N-[trans-4-  
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43 hydroxycyclohexyl] benzylamine). Ambroxol is a, so-called, superoxide, dismutase mimetic  
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45 and has been used previously [26,40] to confirm oxidation reactions occurring via superoxide.  
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47 In all experiments, cells were exposed to  $50\mu\text{M}$  menadione as a positive control for  
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49 generation of superoxide.  
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## 2.6. Cell cycle analysis

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5 Cells were seeded into 10cm dishes at a concentration of  $1.5 \times 10^5$  cells per dish and left to  
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7 adhere overnight. Cells were treated with 100, 200, 300 or 400 $\mu$ M of dicoumarol (**S1**) or the **S**  
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9 or **AS** compounds and incubated for 24 hours. The cells were harvested using trypsin/EDTA,  
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11 washed in PBS and then resuspended in 1ml ice-cold 70% (v/v) ethanol added drop-wise  
12  
13 while using a vortex mixer to avoid clumping. The cells were then left in ethanol at 4°C for a  
14  
15 minimum of 24 hours. For cell cycle analysis, the cells were washed twice in PBS and finally  
16  
17 resuspended in 500 $\mu$ l of PBS containing propidium iodide (PI, 25 $\mu$ g/ml) and RNase A  
18  
19 (250 $\mu$ g/ml), and incubated at 37°C in the dark for 30 minutes. Cells were then washed and  
20  
21 resuspended in 500 $\mu$ l PBS and kept in the dark in preparation for analysis. Samples were  
22  
23 analysed on a CyanADP flow cytometer using the Summit software package. Cell doublets  
24  
25 were gated out using a plot of PI signal height against signal area and data was collected as  
26  
27 histograms illustrating fluorescence intensity and hence DNA content of the cells. The  
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29 percentage of cells in the different phases of the cell cycle were then calculated using gates  
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31 positioned to encompass the respective phases of control cells in each individual experiment.  
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## 2.7. Western blot analysis of apoptotic markers

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46 Exponentially growing cells were seeded into 6-well plates at a concentration of  $1.5 \times 10^5$   
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48 cells per well and left overnight to adhere. Cells were treated with 200 $\mu$ M of dicoumarol (**S1**)  
49  
50 or the **S** or **AS** compounds, incubated for 24 hours and then harvested by scraping into lysis  
51  
52 buffer (50mM Tris-HCl (pH 7.4) (Fisher Scientific, Leicestershire, UK); 120mM NaCl; 5mM  
53  
54 EDTA; 0.5% (v/v) Nonidet P-40; 1mM dithiothreitol; 1mM phenylmethanesulphonyl fluoride;  
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56 2mM sodium orthovanadate; 2mM sodium fluoride; 20mM B-glycerolphosphate; 5mM  
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1 sodium pyrophosphate; and 1mg/ml Protease Inhibitor Cocktail tablet (Roche, Hertfordshire,  
2 UK). Samples were sonicated on ice and the protein concentration was estimated using the  
3  
4 BCA protein assay. Protein (40µg) was loaded for each sample, resolved on a 10%  
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6 polyacrylamide gel and then transferred to a polyvinylidene (PVDF) membrane (Millipore,  
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8 Hertfordshire, UK). Immunoblotting was carried out using a 1:1000 dilution of the cleaved  
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10 PARP antibody (New England Biolabs, HertfordshireUK; anti-rabbit) or a 1:40000 dilution of  
11  
12 the actin antibody. Primary antibodies were detected using the correct horse radish peroxidase  
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14 (HRP)-conjugated secondary antibody, and the ECL detection system (GE Healthcare,  
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16 Buckinghamshire, UK) was used to visualise the respective bands.  
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### 24 3. Results

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29 Novel analogues of dicoumarol (**S1**) have been synthesized (the **S** and **AS** series in Figure 1  
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31 and Table 1) and shown to be potent inhibitors of recombinant NQO1 [33]. These molecules  
32  
33 also show potent activity for inhibiting the activity of NQO1 in HT29 cells, with potency  
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35 correlating well with that observed when measuring inhibition of recombinant enzyme in the  
36  
37 presence of BSA [34]. These published data are given in Table 1. Inhibition of cellular NQO1  
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39 was characterized by the ability of the **S** and **AS** compounds to inhibit the cytotoxic activity of  
40  
41 the indolequinone EO9, an agent known to be exquisitely dependent on NQO1 for toxicity  
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43 under aerobic conditions [21, 22]. Table 1 also shows the ability of the **S** and **AS** compounds  
44  
45 to inhibit NQO1 in A549 and MIA PaCa-2 cells. Using the EO9 toxicity end-point as a  
46  
47 surrogate measure of NQO1 inhibition, it is apparent that **S3** is the most active compound in  
48  
49 each cell line, being 2.5 to 10-fold more potent than dicoumarol for protecting against EO9  
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51 toxicity. In the **S** series, **S10** and **S13** are up to 2-fold more efficient than dicoumarol (**S1**),  
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65 whereas the remaining compounds are less effective. On the contrary, in the **AS** series, all the

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compounds are at least as efficient as dicoumarol, with some (**AS2** and **AS14**) showing 2- to 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, 42, 43]. Each of these cell lines have been genetically engineered to stably overexpress functional NQO1 [35]. In order to evaluate any contribution of p53, the HCT116 p53<sup>+/+</sup> and <sup>-/-</sup> pair of cell lines were 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 (nmoles 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<sub>50</sub>, 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

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5 Dicoumarol has been shown to dose-dependently increase the production of superoxide in  
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7 MIA PaCa-2 pancreatic cancer cells [27]. This result was recapitulated here using flow  
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9 cytometry to measure the superoxide-mediated oxidation of dihydroethidium to 2-  
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11 hydroxyethidium. Figure 2 shows fluorescence intensity histograms acquired following  
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13 treatment of MIA PaCa-2 cells with increasing concentrations of dicoumarol (**S1**) for 4 hours.  
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15 In all experiments, 50 $\mu$ M menadione was used as a positive control for superoxide production  
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17 [45]. From the fluorescence intensity profile obtained for untreated control cells, the R3  
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19 region is defined as that containing cells with increased fluorescence. Clearly, treatment with  
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21 dicoumarol results in a dose-dependent increase in the number of fluorescent cells, which is  
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23 consistent with previous data [27] suggesting it was dicoumarol-induced superoxide  
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25 formation that caused the increased intracellular fluorescence. To confirm this, the superoxide  
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27 dismutase mimetic, ambroxol, was added to the cells exposed to dicoumarol. Figure 2, panel  
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29 F, shows the fluorescence profile obtained when cells were exposed to the combination of  
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31 200 $\mu$ M dicoumarol plus 500 $\mu$ M ambroxol. This non-toxic combination shows little, if any,  
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33 formation of the fluorescent product 2-hydroxyethidium, which suggests that any superoxide  
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35 formed by a dicoumarol-mediated process is scavenged by ambroxol, thereby preventing  
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37 reduction of dihydroethidium.  
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49 The **S** and **AS** compounds were then evaluated for their ability to generate superoxide in MIA  
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51 PaCa-2 cells. Figure 3 shows the percentage of cells with increased fluorescence relative to  
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53 untreated controls, following exposure of cells to varying concentrations of the compounds.  
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55 There is substantial variation in the ability of the compounds to generate superoxide and there  
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57 appears to be no relationship with the ability of the different compounds to inhibit NQO1.  
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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 (Figure 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 hours with 100 $\mu$ M of each compound and the percentage of cells showing increased fluorescence relative to control is shown in Figure 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 Figure 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 $\mu$ M of each of the compounds for 24 hours before staining with propidium iodide for cell cycle analysis. Figure 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

1 the percentage of cells in each of the phases of the cell cycle. However, for both dicoumarol  
2 and **AS2** there is an indication of an increased number of cells in the sub-G1 region. The  
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4 percentage of cells in each phase of the cell cycle following treatment with each compound is  
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6 given in the Supplementary Data Table 1. The percentage of cells in the sub-G1 region  
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8 following exposure to a 200 $\mu$ M concentration of each of the **S** and **AS** compounds is shown  
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10 in the top panel in Figure 7. Clearly, there are significant differences, with compounds such as  
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12 **S3, S4, S5** and **S11** (those showing low superoxide production) providing little if any increase  
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14 in the sub-G1 population compared to controls. In contrast, the remaining compounds,  
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16 including dicoumarol (**S1**) show significantly higher numbers of cells in the sub-G1 region  
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18 than in untreated populations. In order to determine whether the cells in the sub-G1 region are  
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20 undergoing apoptosis [47], cells were collected after 24 hour exposure to 200 $\mu$ M of  
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22 dicoumarol and each of the **S** and **AS** compounds, cell lysates prepared and Western blots  
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24 probed for cleaved PARP. These are shown in the bottom panel of Figure 7 and a clear  
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26 concordance exists between expression of cleaved PARP and the percentage of cells in the  
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28 sub-G1 area, which strongly suggests these cells are undergoing apoptosis. A comparison of  
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30 the proportion of cells in the sub-G1 region and the generation of superoxide in MIA PaCa-2  
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32 cells treated with of each of the putative NQO1 inhibitors is given in the Supplementary Data  
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34 Figure 1. While there is no correlation between these end-points, it is quite clear that the  
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36 compounds generating high levels of superoxide are those that also seem to cause the greatest  
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38 amount of apoptosis. However, importantly, there is no clear relationship between induction  
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40 of apoptosis or generation of superoxide and the ability of the compounds to inhibit the  
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42 enzymatic activity of NQO1 (Table 1). Finally, to confirm that there was no link between  
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44 induction of apoptosis and cellular levels of NQO1, cell cycle analysis of the MDA-MB-231  
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46 WT and DTD cells was made following treatment for 24 hours with 200 $\mu$ M of dicoumarol  
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48 (**S1**). The proportion of cells in the sub-G1 region following treatment is given in the  
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Supplementary data Figure 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 (Figure 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

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for their ability to generate superoxide. Experiments were initially done in MIA PaCa-2 cells as these cells were used by Du, Cullen and colleagues [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 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 Figure 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.

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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

1 ability to cause mitochondrial uncoupling when compared to dicoumarol (Scott KA  
2 unpublished data).  
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7 It is not the purpose of this work to ascertain the underlying mechanism by which the  
8 coumarin analogues elicit their off-target effects. Instead, we are seeking to identify molecular  
9 features of compounds related to dicoumarol that would help facilitate their development as  
10 pharmacological inhibitors of NQO1. It is particularly obvious that the 5-methoxy substituted  
11 analogue of dicoumarol, **S3**, is the most potent inhibitor of NQO1 in cells, while being  
12 consistently less toxic in the nine cell lines used here. **S3** also shows much reduced generation  
13 of superoxide and does not induce apoptosis. From the **AS** series, **AS3** and **AS14** stand out, as  
14 they also show comparable NQO1-inhibitory potency to dicoumarol in cells with generally  
15 less generation of superoxide, less apoptosis and reduced toxicity. A potential advantage of  
16 the **AS** compounds compared to their **S** analogues is their improved water solubility [33].  
17 Thus, we have provided a firm basis for development of compounds to evaluate the impact of  
18 NQO1 inhibition in cells.  
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39 Using gene knockout technology it has been shown that loss of NQO1 is associated with  
40 reduced p53 stability [48, 49] and changes in NFκB signalling [50]. Treatment of cells with  
41 dicoumarol can also cause degradation of p53 and other onco-proteins in a process that is  
42 mediated by NQO1. There is good evidence to show that transient disruption of p53 during  
43 and after irradiation can protect against radiation damage [51, 52]. Thus, there may well be  
44 substantial scope for developing NQO1 inhibitors for therapeutic use [34]. In summary, it has  
45 been shown that there are a number of compounds that act as potent inhibitors of NQO1 and,  
46 beneficially, they lack the off-target effects associated with dicoumarol.  
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## 5. References

- [1] Li R, Bianchet MA, Talalay P, Amzel LM. The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. *Proc Natl Acad Sci USA* 1995;92:8846-50.
- [2] Siegel D, Gustafson DL, Dehn DL, Han JY, Boonchoong P, Berliner LJ, et al. NAD(P)H:Quinone Oxidoreductase 1: Role as a Superoxide Scavenger. *Molecular Pharmacology* 2004;65:1238-47.
- [3] Lind C, Cadenas E, Hochstein P, Ernster, L. DT-diaphorase: purification, properties, and function. *Methods Enzymol* 1990;186:287-301.
- [4] Faig M, Bianchet MA, Talalay P, Chen S, Winski S, Ross D, et al. Structures of recombinant human and mouse NAD(P)H:quinone oxidoreductases: species comparison and structural changes with substrate binding and release. *Proc Natl Acad Sci USA* 2000;97:3177-82.

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6  
7 [5] Asher G, Reuven N, Shaul Y. 20S proteasomes and protein degradation "by  
8 default". *Bioessays* 2006;28:844-9.  
9  
10  
11 [6] Asher G, Dym O, Tsvetkov P, Adler J, Shaul Y. The crystal structure of NAD(P)H  
12 quinone oxidoreductase 1 in complex with its potent inhibitor dicoumarol.  
13 *Biochemistry* 2006; 45:6372-8.  
14  
15  
16 [7] Alard A, Fabre B, Anesia R., Marboeuf C, Pierre P, Susini C, et al. NAD(P)H  
17 Quinone-Oxydoreductase 1 Protects Eukaryotic Translation Initiation Factor 4GI. *Mol*  
18 *Cell Biol* 2010;30:1097-1105.  
19  
20  
21  
22 [8] Asher G, Tsvetkov P, Kahana C, Shaul Y. A mechanism of ubiquitin-  
23 independent proteasomal degradation of the tumor suppressors p53 and p73. *Genes*  
24 *Dev* 2005;19:316-21.  
25  
26  
27 [9] Ernster L, Ljunggren M, Danielson L. Purification and some properties of a highly  
28 dicoumarol-sensitive liver diaphorase. *Biochemical and Biophysical Research*  
29 *Communications* 1960;2:88-92.  
30  
31  
32  
33  
34  
35  
36 [10] Ernster L. DT diaphorase. *Methods in Enzymology* 1967;10:309-17.  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46 [11] Asher G, Lotem J, Cohen B, Sachs L, Shaul Y. Regulation of p53 stability and p53-  
47 dependent apoptosis by NADH quinone oxidoreductase 1. *Proc Natl Acad Sci USA*  
48  
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64  
65
- [12] Tsvetkov P, Reuven N, Shaul Y. Ubiquitin-independent p53 proteasomal degradation. *Cell Death Differ* 2010;17:103-8.
- [13] Schlager JJ, Powis G. Cytosolic NAD(P)H:(quinone-acceptor) oxidoreductase in human normal and tumor tissue: effects of cigarette smoking and alcohol. *Int J Cancer* 1990;45:403-9.
- [14] Workman P, Stratford IJ. The experimental development of bioreductive drugs and their role in cancer therapy. *Cancer Metastasis Rev* 1993;12:73-82.
- [15] Workman P. Enzyme-directed bioreductive drug development revisited: a commentary on recent progress and future prospects with emphasis on quinone anticancer agents and quinone metabolizing enzymes, particularly DT-diaphorase. *Oncol Res* 1994;6:461-75.
- [16] Cummings J, Spanswick VJ, Tomasz M, Smyth JF. Enzymology of mitomycin C metabolic activation in tumour tissue: implications for enzyme-directed bioreductive drug development. *Biochem Pharmacol* 1994;56:405-14.
- [17] Robertson N, Stratford IJ, Houlbrook S, Carmichael J, Adams GE. The sensitivity of human tumour cells to quinone bioreductive drugs: what role for DT-diaphorase? *Biochem Pharmacol* 1992;44:409-12.

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- [18] Gustafson DL, Beall HD, Bolton EM, Ross D, Waldren CA. Expression of human NAD(P)H: quinone oxidoreductase (DT-diaphorase) in Chinese hamster ovary cells: effect on the toxicity of antitumor quinones. *Mol Pharmacol* 1996;50:728-35.
- [19] Winski SL, Hargreaves RH, Butler J, Ross D. A new screening system for NAD(P)H:quinone oxidoreductase (NQO1)-directed antitumor quinones: identification of a new aziridinybenzoquinone, RH1, as a NQO1-directed antitumor agent. *Clin Cancer Res* 1998;4:3083-8.
- [20] Ough M, Lewis A, Bey EA, Gao J, Ritchie JM, Bornmann W et al. Efficacy of beta-lapachone in pancreatic cancer treatment: exploiting the novel, therapeutic target NQO1. *Cancer Biol Ther* 2005;4:95-102.
- [21] Robertson N, Haigh A, Adams GE, Stratford IJ. Factors affecting sensitivity to EO9 in rodent and human tumour cells in vitro: DT-diaphorase activity and hypoxia. *Eur J Cancer* 1994;30A:1013-9.
- [22] Plumb JA, Gerritsen M, Workman P. DT-diaphorase protects cells from the hypoxic cytotoxicity of indoloquinone EO9. *Br J Cancer* 1994;70:1136-43.
- [23] Asher G, Lotem J, Tsvetkov P, Reiss V, Sachs L, Shaul Y. P53 hot-spot mutants are resistant to ubiquitin-independent degradation by increased binding to NAD(P)H:quinone oxidoreductase 1. *Proc Natl Acad Sci U S A* 2003;100:15065-70.

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65
- [24] Garten S, Wosilait WD. Comparative study of the binding of coumarin anticoagulants and serum albumins. *Biochemical Pharmacology* 1971;20:1661-8.
- [25] Collier AC, Pritsos CA. The mitochondrial uncoupler dicumarol disrupts the MTT assay. *Biochem Pharmacol* 2003;66:281-7.
- [26] Gonzalez-Aragon D, Ariza J, Villalba JM. Dicoumarol impairs mitochondrial electron transport and pyrimidine biosynthesis in human myeloid leukemia HL-60 cells. *Biochem Pharmacol* 2007;73:427-39.
- [27] Du J, Daniels DH, Asbury C, Venkataraman S, Liu J, Spitz DR, et al. Mitochondrial production of reactive oxygen species mediates dicumarol-induced cytotoxicity in cancer cells. *J Biol Chem* 2006;281:37416-26.
- [28] Nolan KA, Zhao H, Faulder PF, Frenkel AD, Timson DJ, Siegel D, et al. Coumarin-based inhibitors of human NAD(P)H:quinone oxidoreductase-1. Identification, structure-activity, off-target effects and in vitro human pancreatic cancer toxicity. *J Med Chem* 2007;50:6316-25.
- [29] Lewis A, Ough M, Li L, Hinkhouse MM, Ritchie JM, Spitz DR, et al. Treatment of pancreatic cancer cells with dicumarol induces cytotoxicity and oxidative stress. *Clin Cancer Res* 2004;10:4550-8.
- [30] Cullen JJ, Hinkhouse MM, Grady M, Gaut AW, Liu J, Zhang YP, Weydert CJ, et al. Dicumarol inhibition of NADPH:quinone oxidoreductase induces growth inhibition of

pancreatic cancer via a superoxide-mediated mechanism. *Cancer Res* 2003;63:5513-20.

- [31] Reigan P, Colucci MA, Siegel D, Chilloux A, Moody CJ, Ross D. Development of indolequinone mechanism-based inhibitors of NAD(P)H:quinone oxidoreductase 1 (NQO1), NQO1 inhibition and growth inhibitory activity in human pancreatic MIA PaCa-2 cancer cells. *Biochemistry* 2007;46:5941–50.
- [32] Dehn DL, Siegel D, Zafar KS, Reigan P, Swann E, Moody CJ, et al. 5-Methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]-indole-4,7-dione, a mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1, exhibits activity against human pancreatic cancer in vitro and in vivo. *Mol Cancer Ther* 2006;5:1702–9.
- [33] Nolan KA, Doncaster JR, Dunstan MS, Scott KA, Frenkel AD, Siegel D, et al. Synthesis and biological evaluation of coumarin-based inhibitors of NAD(P)H:quinone oxidoreductase-1 (NQO1). *J Med Chem* 2009;52:7142-56.
- [34] Nolan KA, Scott KA, Barnes J, Doncaster J, Whitehead RC, Stratford IJ. Pharmacological inhibitors of NAD(P)H quinone oxidoreductase, NQO1: Structure/activity relationships and functional activity in tumour cells. *Biochem Pharmacol* 2010;80:977-81.
- [35] Cowen RL, Patterson AV, Telfer BA, Airley RE, Hobbs S, Phillips RM, et al. Viral delivery of P450 reductase recapitulates the ability of constitutive overexpression of

1 reductase enzymes to potentiate the activity of mitomycin C in human breast cancer  
2 xenografts. *Mol Cancer Ther* 2003;2:901-9.  
3

4  
5  
6  
7 [36] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, et al, Measurement of  
8 protein using bicinchoninic acid. *Anal. Biochem* 1985;150:76–85.  
9

10  
11  
12  
13  
14 [37] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to  
15 proliferation and cytotoxicity assays. *J. Immunol Methods* 1983;65:55–63.  
16

17  
18  
19  
20  
21 [38] Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and  
22 superoxide anion detection in endothelial cells. *J Leukoc Biol* 1994;55:253-8.  
23

24  
25  
26  
27  
28  
29 [39] Fernandes DC, Wosniak J Jr, Pescatore LA, Bertoline MA, Liberman M, Laurindo FR,  
30 et al. Analysis of DHE-derived oxidation products by HPLC in the assessment of  
31 superoxide production and NADPH oxidase activity in vascular systems. *Am J*  
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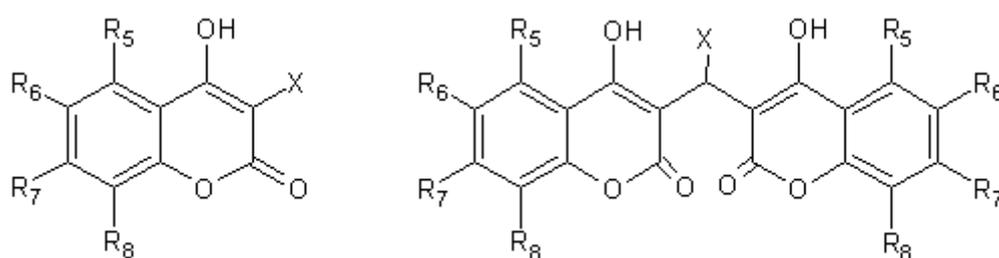
99 [41] Anwar A, Siegel D, Kepa JK, Ross D. Interaction of the Molecular Chaperone Hsp70  
100 with Human NAD(P)H:Quinone Oxidoreductase 1. *J Biol Chem* 2002;277:14060-7.  
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- [42] Dehn D, Winski SL, Ross D. Development of a New Isogenic Cell-Xenograft System for Evaluation of NAD(P)H:Quinone Oxidoreductase-Directed Antitumor Quinones: Evaluation of the Activity of RH1. *Clin Cancer Res* 2004;10: 3147-55.
- [43] Begleiter A, Leitha MK, Doherty GP, Digby TJ, Pand S. Factors influencing the induction of DT-diaphorase activity by 1,2-dithiole-3-thione in human tumor cell lines. *Biochem Pharmacol* 2001;61:955-64.
- [44] Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 1998;282:1497-501.
- [45] Kappus H, Sies H. Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. *Experientia* 1981;37:1233-41.
- [46] Bello RI, Go´mez-Dı´az C, Lo´pez-Lluch G, Forthoffer N, Co´rdoba-Pedregosa A, Pla´cido Navas B, et al. Dicoumarol relieves serum withdrawal-induced G0/1 blockade in HL-60 cells through a superoxide-dependent mechanism. *Biochem Pharmacol* 2005;69:1613-25.
- [47] Ormerod MG, Collins MK, Rodriguez-Tarduchy G, Robertson D. Apoptosis in interleukin-3-dependent haemopoietic cells. Quantification by two flow cytometric methods. *J Immunol Methods*. 1992;153:57-65.

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- [48] Gong X, Kole L, Iskander K, Jaiswal AK. NRH:quinone oxidoreductase 2 and NAD(P)H:quinone oxidoreductase 1 protect tumor suppressor p53 against 20s proteasomal degradation leading to stabilization and activation of p53. *Cancer Res* 2007;67:5380–8.
- [49] Iskander K, Gaikwad A, Paquet M, Long 2nd DJ, Brayton C, Barrios R, et al. Lower induction of p53 and decreased apoptosis in NQO1-null mice lead to increased sensitivity to chemical-induced skin carcinogenesis. *Cancer Res* 2005;65:2054–8.
- [50] Ahn KS, Sethi G, Jain AK, Jaiswal AK, Aggarwal BB. Genetic deletion of NAD(P)H:quinone oxidoreductase 1 abrogates activation of nuclear factor- kappaB, IkappaBalpha kinase, c-Jun N-terminal kinase, Akt, p38, and p44/42 mitogen-activated protein kinases and potentiates apoptosis. *J Biol Chem* 2006;281:19798–808.
- [51] Strom E, Sathe S, Komarov PG, Chernova OB, Pavlovska I, Shyshynova I, et al. Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation. *Nat Chem Biol* 2006;2:474-9.
- [52] Ghosh SP, Perkins MW, Hieber K, Kulkarni S, Kao TC, Reddy EP. Radiation protection by a new chemical entity, Ex-Rad: efficacy and mechanisms. *Radiat Res* 2009;171:173-9.

**Graphical abstract**

Differential effects of dicoumarol analogues as inhibitors of NQO1, production of superoxide and induction of apoptosis and toxicity in cancer cells.



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**Table 1.** Substituents in the **S** and **AS** series (Figure 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  $\mu$ M EO9. This concentration of EO9 alone will kill  $\geq 90\%$  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  $\pm$  standard deviation.

ID	R5	R6	R7	R8	X	$IC_{50}$ (nM) With BSA	Concentration needed to protect from 50% of EO9 toxicity ( $\mu$ M)		
							HT29	A549	MIA PaCa-2
<b>S1</b>	H	H	H	H	H	404 $\pm$ 184	211 $\pm$ 83	118 $\pm$ 5.6	141 $\pm$ 48
<b>S3</b>	OCH <sub>3</sub>	H	H	H	H	38 $\pm$ 2.1	18 $\pm$ 9.0	43 $\pm$ 25	54 $\pm$ 26
<b>S4</b>	H	OCH <sub>3</sub>	H	H	H	3300 $\pm$ 600	>1000	> 250	> 250
<b>S5</b>	H	H	OCH <sub>3</sub>	H	H	790 $\pm$ 355	875 $\pm$ 211	> 250	> 250
<b>S10</b>	H	CH <sub>3</sub>	CH <sub>3</sub>	H	H	233 $\pm$ 68	106 $\pm$ 29	165 $\pm$ 63	158 $\pm$ 72
<b>S11</b>	H	OCH <sub>3</sub>	OCH <sub>3</sub>	H	H	1497 $\pm$ 442	1954 $\pm$ 940	-	-
<b>S13</b>	H	H	CH <sub>3</sub>	CH <sub>3</sub>	H	149 $\pm$ 101	132 $\pm$ 9.8	68 $\pm$ 16	109 $\pm$ 21
<b>AS1</b>	H	CH <sub>3</sub>	CH <sub>3</sub>	H	1-naphthyl	1095 $\pm$ 290	105 $\pm$ 77	251 $\pm$ 78	148 $\pm$ 42
<b>AS2</b>	H	CH <sub>3</sub>	CH <sub>3</sub>	H	2-naphthyl	167 $\pm$ 83	50 $\pm$ 33	64 $\pm$ 38	89 $\pm$ 27
<b>AS3</b>	H	CH <sub>3</sub>	CH <sub>3</sub>	H	phenyl	660 $\pm$ 108	128 $\pm$ 69	114 $\pm$ 7.2	312 $\pm$ 55
<b>AS4</b>	H	H	7,8-C <sub>4</sub> H <sub>4</sub>	H	1-naphthyl	450 $\pm$ 325	58 $\pm$ 26	129 $\pm$ 29	359 $\pm$ 58
<b>AS5</b>	H	H	7,8-C <sub>4</sub> H <sub>4</sub>	H	2-naphthyl	225 $\pm$ 151	97 $\pm$ 52	94 $\pm$ 27	132 $\pm$ 56
<b>AS6</b>	H	H	7,8-C <sub>4</sub> H <sub>4</sub>	H	phenyl	880 $\pm$ 364	133 $\pm$ 41	> 250	205 $\pm$ 10
<b>AS14</b>	H	CH <sub>3</sub>	CH <sub>3</sub>	H	3,4 dimethylbenzyl	192 $\pm$ 41	62 $\pm$ 19	61 $\pm$ 7.8	114 $\pm$ 2.3

**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  $\pm$  standard deviation.

Cell Line	NQO1 Activity (nmol cytochrome c reduced / min / mg protein)
HT29	1691 $\pm$ 715
A549	3011 $\pm$ 532
HCT116 WT	545 $\pm$ 115
HCT116 BVP p53 $\neg$	357 $\pm$ 42
MIA PaCa-2	1252 $\pm$ 142
MDA-MB-231 WT	40 $\pm$ 28
MDA-MB-231 DTD	4171 $\pm$ 816
MDA-MB-468 WT	20 $\pm$ 17
MDA-MB-468 DTD	4180 $\pm$ 1589
T47D WT	141 $\pm$ 33
T47D DTD	5284 $\pm$ 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 hour IC <sub>50</sub> (μM)								
	MIA	HCT116	HCT116	MDA-MB-231		MDA-MB-468		T47D	
	PaCa-2	WT	p53 -/-	WT	DTD	WT	DTD	WT	DTD
<b>S1</b>	52 ± 26	19 ± 13	22 ± 13	50 ± 37	67 ± 31	30 ± 12	47 ± 23	51 ± 6.2	41 ± 8.3
<b>S3</b>	350 ± 142	80 ± 27	58 ± 33	133 ± 16	165 ± 37	174 ± 45	125 ± 5.2	98 ± 52	190 ± 77
<b>S4</b>	405 ± 22	48 ± 1.4	43 ± 13	77 ± 7.1	105 ± 60	58 ± 22	38 ± 4.2	283 ± 46	103 ± 84
<b>S5</b>	303 ± 32	187 ± 17	180 ± 7.1	202 ± 82	162 ± 78	148 ± 16	155 ± 35	109 ± 56	68 ± 31
<b>S10</b>	106 ± 33	35 ± 1.4	33 ± 5.6	66 ± 51	41 ± 2.6	51 ± 26	56 ± 30	66 ± 47	45 ± 16
<b>S11</b>	> 400	>400	>400	> 400	> 400	> 400	> 400	> 400	> 400
<b>S13</b>	119 ± 42	35 ± 16	41 ± 13	114 ± 36	173 ± 15	123 ± 31	141 ± 22	111 ± 38	103 ± 32
<b>AS1</b>	188 ± 2.9	56 ± 4.9	76 ± 48	32 ± 9.0	51 ± 64	107 ± 17	45 ± 29	124 ± 67	116 ± 71
<b>AS2</b>	162 ± 28	180 ± 21	165 ± 44	119 ± 48	115 ± 59	142 ± 68	75 ± 51	143 ± 57	95 ± 74
<b>AS3</b>	267 ± 31	178 ± 2.8	122 ± 65	102 ± 70	185 ± 18	231 ± 70	99 ± 46	145 ± 42	117 ± 69
<b>AS4</b>	163 ± 20	102 ± 18	111 ± 21	41 ± 5.2	60 ± 58	100 ± 21	67 ± 41	94 ± 22	88 ± 29
<b>AS5</b>	152 ± 16	39 ± 2.8	31 ± 4.7	41 ± 15	58 ± 15	102 ± 22	58 ± 34	58 ± 36	51 ± 17
<b>AS6</b>	222 ± 60	40 ± 45	68 ± 48	124 ± 40	94 ± 21	173 ± 27	100 ± 36	13 ± 61	89 ± 16
<b>AS14</b>	407 ± 15	176 ± 6.4	172 ± 43	152 ± 36	164 ± 25	248 ± 37	154 ± 21	170 ± 7.0	138 ± 3.5

**Figure captions**

**Figure 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.

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

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

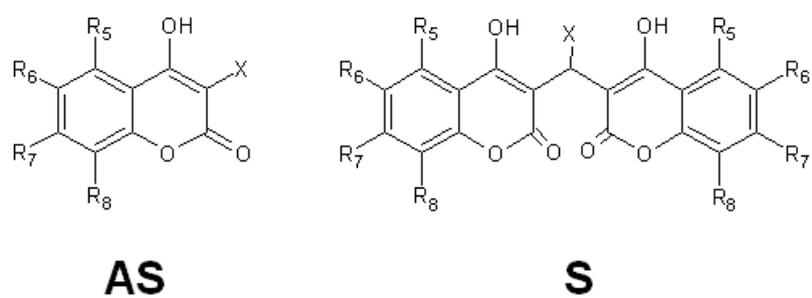
**Figure 4.** The relationship between superoxide production and toxicity in the MIA PaCa-2 cell line, **AS** compounds = ●; **S** compounds = ○; dicoumarol = **S1**.

**Figure 5.** Intracellular superoxide production is independent of the activity of NQO1 in MDA-MB-231 cells: Cells were treated for 4 hours with 100 $\mu$ M of dicoumarol (**S1**) and the other **S** or **AS** compounds. Each measurement was made on three separate occasions and values are given  $\pm$  standard deviation. (Black bars = WT cells; White bars = DTD cells).

**Figure 6.** Representative DNA histograms displaying cell distributions following 24 hour treatment with 200 $\mu$ M 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.

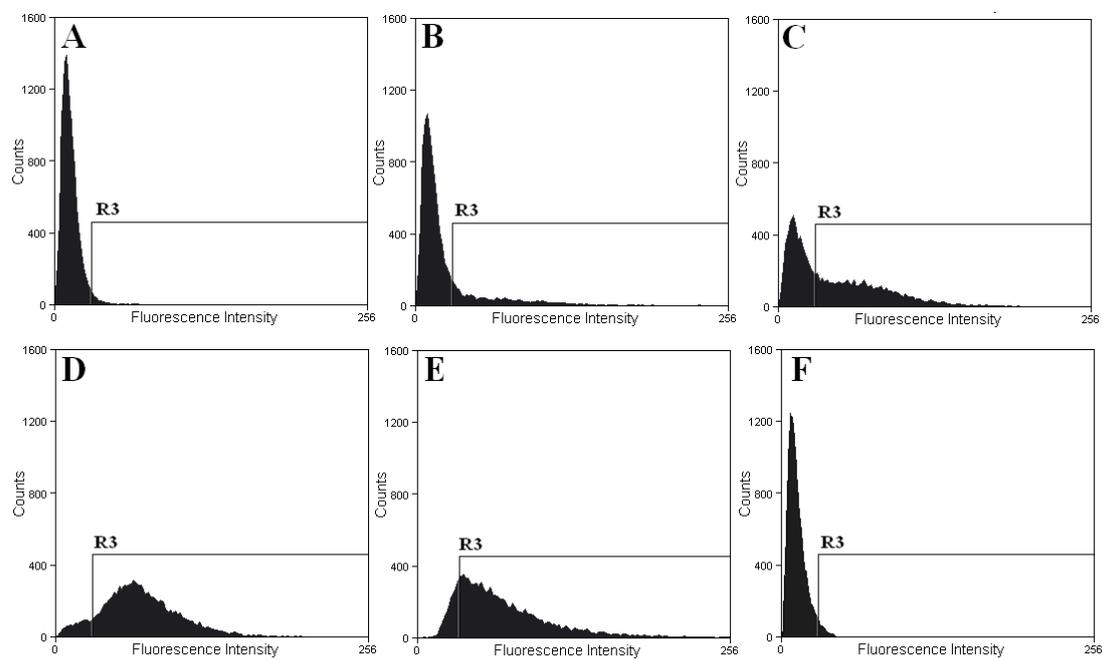
**Figure 7.** The percentage of cells in the sub-G1 region of the cell cycle compared with measurement of cleaved PARP by Western blot: MIA PaCa-2 cells were treated for 24 hours with vehicle or 200 $\mu$ M of dicoumarol (**S1**) or the other **S** or **AS** compounds then either stained with PI for 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 ( $\pm$  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.

Figure 1.



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Figure 2.



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Figure 3.

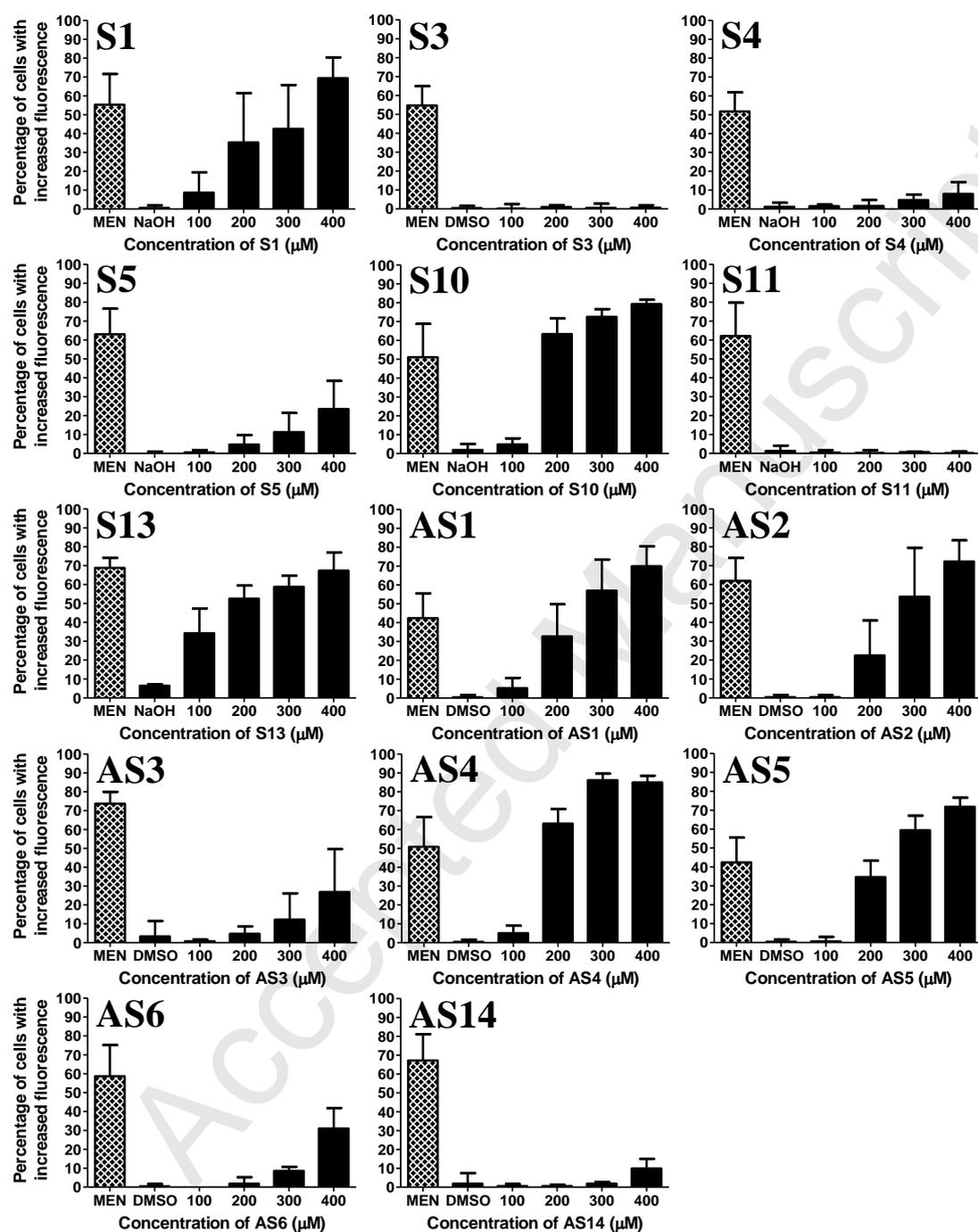


Figure 4.

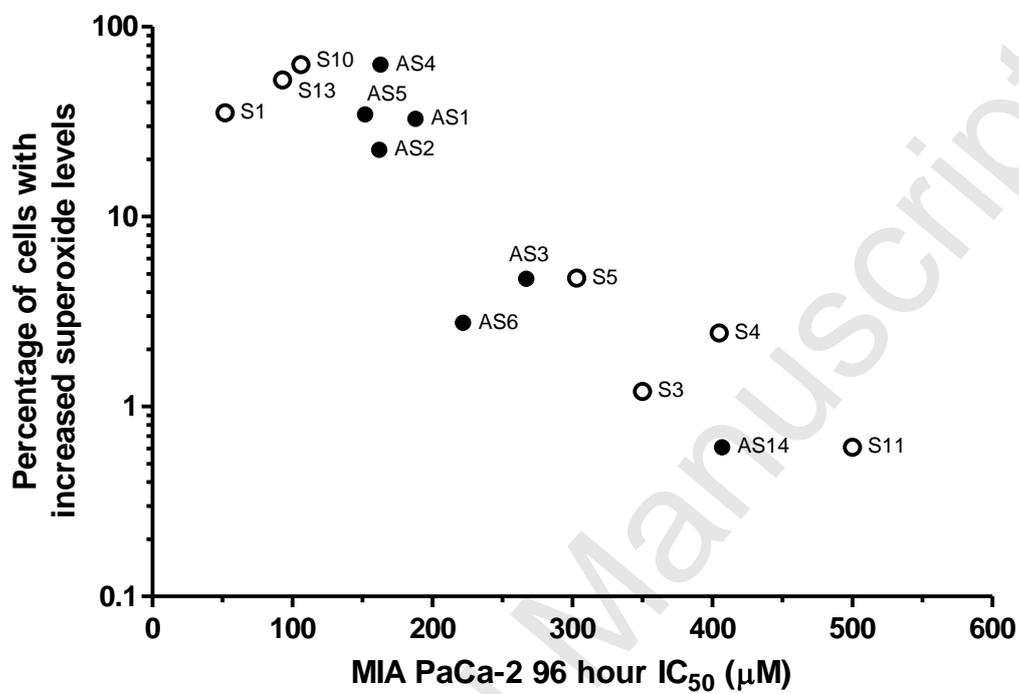


Figure 5.

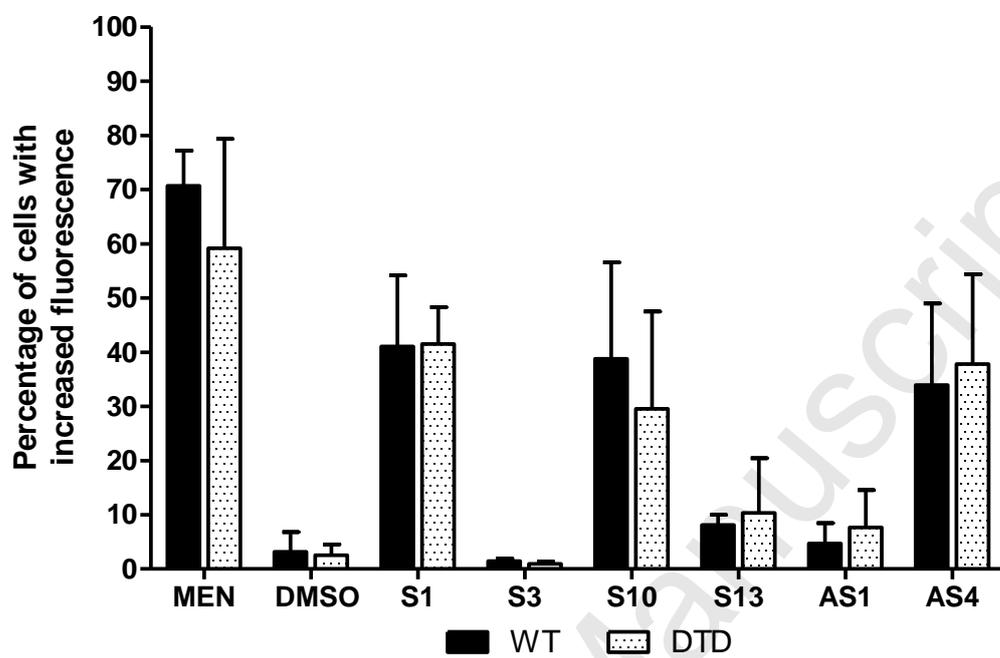
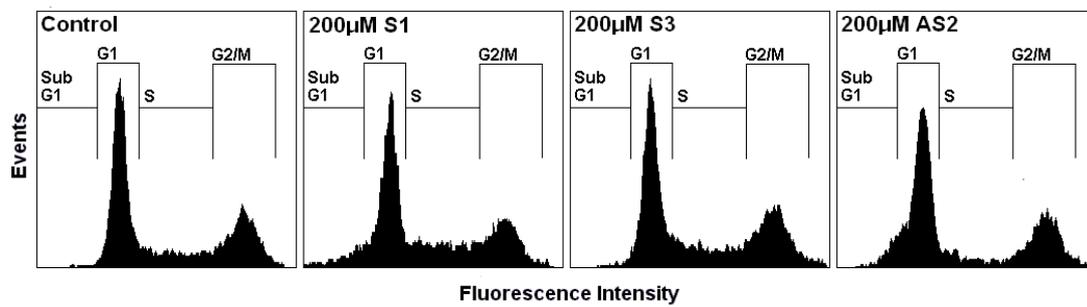
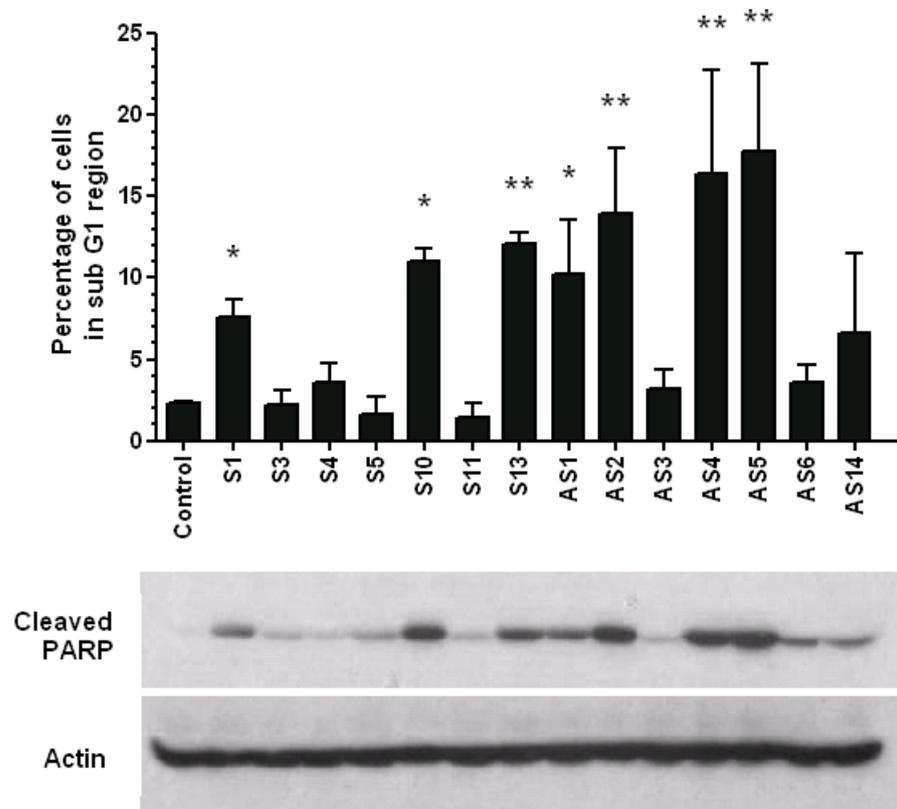


Figure 6.



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Figure 7.



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